CHRONIC PANCREATITIS:
ELECTROPHILIC STRESS TEMPLATE

Joan M Braganza DSc FRCP FRCPath

- **DEVELOPMENT**
- **RATIONALISING GEOGRAPHY**
- **PETROCHEMICAL CONNECTION**
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- **MICRONUTRIENT THERAPY**
- **MAST CELL PATHOLOGY**
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ELECTROPHILIC STRESS TEMPLATE

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Publishers Preface

This monograph presents a comprehensive review of the work of Professor Joan Braganza into the pathogenesis and treatment of chronic pancreatitis as a disease caused by oxidative and particularly electrophilic stress with an emphasis on environmental toxicants as one factor which is part of the balance of damaging and protective cellular forces in the pancreas, liver and other participating organs.

This is the second monograph of this type and follows “The Hamster Pancreas” written by Parviz Pour. Both books are published in open access eBook form under a Creative Commons license and are available on the Pancreapedia site. Pancreapedia will from time to time publish eBooks that are more substantive than a review article and where the author prepares the text and secures copyright approval for figures or tables published previously. Publishing can be done at no or low cost to the author. Authors should obtain approval from the Editor before preparing detailed content. As was carried out for this book, the Pancreapedia Editor and staff carry out light copy editing and format the book with final approval by the author. Our books are assigned an ISBN number and can be cited as any other book. We thank the American Pancreatic Association for their support and especially thank Melissa Wu and Juliana Lam, two students from the University of Michigan School of Information who served as Content/Community Manager for the Pancreapedia and were responsible for taking the edited content and preparing the book you see here.

John A. Williams

Editor-in-Chief, Pancreapedia

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Glossary & Conversion factors

Abbreviation	Form represented
α_1PI	alpha 1 proteinase inhibitor
α_2M	alpha 2 macroglobulin
AMP	adenosine monophosphate
AOT	antioxidant therapy
βNF	beta naphthoflavone
BDBF	brain- derived neurotrophic factor
BT PABA	N-benzoyl L-tyrosyl para-amino benzoic acid
BSP	bromosulphthalein
C18:2	linoleic acid
Ca^{++}	ionised calcium
CAPAP	carboxypeptide activartion peptide
CCK	cholecystokinin-pancreozymin
CDE	choline-deficient ethionine-supplemented diet
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CGRP	calcitonin gene related peptide
CH_3	methyl groups
CHRU	Crick Harper Raper Unit of Boots secretin
CI	confidence interval
Cl	clearance of probe
CRP	C-reactive protein
CT	computed tomography
CU	Clinical unit of highly purified or synthetic secretin
CV	coefficient of variation
CYP	cytochromes P450
DC	diene conjugation
DGA	D glucaric acid
ER	endoplasmic reticulum
ERCP	endoscopic retrograde choalngiopancreatography
FLLD	familial lipoprotein lipase deficiency
FRA	free radical activity
FROP	free radical oxidation products
GIH	highly purified secretin from the Gastrointestinal hormone laboratory
G-protein	guanine nucleotide-binding protein
G_s / G_i proteins	stimulatory / inhibitory variant G-proteins
GSH	glutathione in bioactive reduced form
GSSG	glutathione in reversibly oxidised form after binding to peroxides
GST	glutathione-S-transferases
GST-B	ligandin
HO	haem oxygenase
HOCl	hypochlorous acid
H_2O_2	hydrogen peroxide
H_2S	hydrogen sulphide
HPLC	high performance liquid chromatography
HPN	haemorrhagic pancreatic necrosis / necrotising pancreatitis
9,11 LA'	specific DC isomer of linoleic acid
MODS	multiple organ dysfunction syndrome
%MRLA	% molar ratio of specific DC isomer to parent linoleic acid
%MRVC	% molar ratio of oxidised forms to total vitamin C
MTA	mean trypsin acitivity in meal test
NAC	N-acetylcysteine

NGF	nerve growth factor
$NO^{\bullet}$	nitric oxide radical
NSAID	non-steroidal anti- inflammatory drug
O_2	molecular oxygen
$O_2^{-\bullet}$	superoxide anion free radical
$OH^{\bullet}$	hydroxyl free radical
PAH	polycyclic aromatic hydrocarbons
PMN	polymorphonuclear cells / neutrophils
PTA	peak trypsin activity after pancreozymin
PUFA	polyunsaturated fatty acids
RAP	recurrent/relapsing acute pancreatitis
RCT	randomised controlled trial
RER	rough endoplasmic reticulum
ROS	reactive oxygen species
RNI	recommended nutrient intake
RNS	reactive nitrogen species
RP	Raynauds phenomenon
RXS	reactive xenobiotic species
SAPE	sentinel acute pancreatitis event
SD	standard deviation
SE	standard error
SER	smooth endoplasmic reticulum
SH	thiol / sulphydryl groups
SIRS	systemic inflammatory response syndrome
SOD	superoxide dismutase
SP test	secretin pancreozymin test
SPINK1	specific pancreatic trypsin inhibitor Kazal type 1
S-S	disulphide linkages
$T_{1/2}$	half-life of probe
TAP	trypsinogen activation peptide
TBARS	thiobarbituric acid reactive substances
TNF-α	tumour necrosis factor alpha
TGF-β1	tumour necrosis factor beta 1
TRPV1	transient receptor vallinoid 1
UPR	unfolded protein response
UVF	ultraviolet fluorescence
V	volume of distribution
vWf	von Willibrand factor antigen
VF	visible fluorescence
ZG	zymogen granules

Conversion factors

serum/plasma	metric units	factor (multiply)	SI units
selenium	µg / l	0.0127	µmol / l
vitamin C / ascorbate	µg / ml, mg / l	5.678	µmol / l
vitamin E	mg / l	2.322	µmol / l
vitamin A	mg / l	3.490	µmol / l
β carotene	µg / l	1.863	nmol / l

Preface

This book is dedicated to the memory of two British pioneers from whom I learnt a great deal, Henry T Howat and Thomas L Dormandy.

Manchester University was the birthplace in 1943 of pancreozymin-cholecystokinin, as a result of studies by Alan A Harper and colleagues. After the second world war, Howat, who had served as a physician specialist with the army in the middle east, France and Germany, was appointed in 1948 as consultant physician at the adjacent Manchester Royal Infirmary. He soon set about assessing the new hormone, given in tandem with secretin, after duodenal intubation, documenting from analysis of timed aspirates not only valuable information on enzyme secretory capacity but also on hepatobiliary function. This diagnostic asset was well before the pancreas was brought into view by radioisotope scanning, ultrasonography or computed tomography. Thus, Howat attracted referrals of patients with possible chronic pancreatitis or cancer, and became Britain's first pancreatologist. Moreover, by forging links with like-minded people in the UK and Europe, he was a major force in creating the European Pancreatic Club. He argued persuasively that advances in molecular science and diagnostic imaging required that the management of pancreatic disease should pass from generalists to gastroenterologists, a position recognised by the award of CBE in 1971 and appointment as Professor of Gastroenterology the next year. Howat's legacy was cemented in 1979 by co-editorship, with Henri Sarles of Marseilles, of the first pancreatic tome.

Thomas Dormandy was a polymath who brought free radical science into the clinical domain, while also being a celebrated painter, musician and author of books far removed from his field of laboratory expertise. When the Nazis invaded Hungary in 1944, the family went into hiding in a convent before escaping to Geneva and then settling in London in 1948, adopting as their new surname that of a Hungarian village. Dormandy qualified as a doctor thrice - in Hungary, France and the UK, specialising as a surgeon but then finding his true calling as consultant in chemical pathology at London's Whittington Hospital, whence in due course he was instrumental in setting up the Society for Free Radical Research. An amusing aside is that in a letter to the Editor a reader damned The Lancet for publishing his 1969 article on 'biological rancidification'! Dormandy went on to author erudite papers on free radical pathology but also wrote 'in praise of peroxidation', emphasising the essentiality of reactive oxygen species, not least to ensure that cells have a finite lifespan. He was also the first to appreciate 'reductive stress' and to note that it could be the major route towards oxidative stress. In recognition, he was appointed Professor of Chemical Pathology at Brunel University; his mercurial mind and exquisite turn of phrase are legendary.

These 'giants' realised the importance of research funding and called for a multidisciplinary approach; stringency in designing and reporting studies; a focus on teaching; and support of young researchers.

I arrived in the UK in November 1966, having graduated as top student with several prizes at the final MBBS examinations in Bombay (Mumbai), newly married, and with the intention of a 'gap year' while my husband obtained a pediatric degree. That's not what transpired. Although I did not have the foresight to ask for references from my teachers, I secured a post as house officer in medicine at Oldham Royal Infirmary, a very busy unit where the appointee undertook alternate 24 hour shifts and alternate weekends as emergency officer. I was interviewed by Oscar Janus, a renowned general physician who engineered the next posting as house officer in surgery in William Ball's unit, following which the pairs' recommendations

enabled an interview for a senior house officer job in Howat's unit, to start in January 1968. It was a shock to be appointed. A self-imposed crash course on pancreatic disease was needed as I had not hitherto seen a patient with chronic pancreatitis!

Almost my first assignment was to perform and interpret secretin-pancreozymin tests. This entailed intubating the patient with a fluoroscopic double-lumen tube, then pushing the patient's wheelchair down long corridors to the radiology department, awaiting a slot to ensure correct tube placement, followed by a trek back for around 1.5 hours of testing. On the plus side, there was an incentive to find simpler tests of pancreatic function. Having gained the MRCP at the first attempt in 1969, I resigned the post soon thereafter when my young brother drowned and I returned to be with my parents in Mumbai. Howat had a grant from the Medical Research Council to investigate pancreatic and gastric secretion in anaesthetised cats. He co-opted me, thus enabling my return to Manchester with introduction to experimental and laboratory work, while continuing training as a medical registrar.

The research was utterly fascinating, but progress was interrupted when I developed tuberculosis of the cervical spine in June 1970 by post-partum contact in hospital, and in December 1971 after my husband's first stroke when our daughter was just 18 months old. Once again Howat came to the rescue, persuading me that taking over my husband's general practice was not the answer. I decided, despite Harper's advice that the research would easily gain a PhD, needing just a few more experiments, to settle for an MSc in order to rid the family of further strain. It was clear that I would not in the foreseeable future be able to take up a post away from Manchester.

Thus it was that 3 months after the birth of our son in 1974, I applied for and obtained the tenured post of University Lecturer in Gastroenterology attached to Howat's unit. I realised at once that the proliferation of diagnostic imaging tools was out-of-step with the singular lack of progress in treating exocrine pancreatic disease, not least chronic pancreatitis. The priority was to find a non- invasive test whereby to select the few patients with pain that might be pancreatic from the many with pain from other causes. Once this was done, the emphasis shifted to disease causation. In regard to chronic pancreatitis, so strong was the mindset that the disease equated to alcoholism that I was advised to see this as the explanation even if my mother was to deny it! Howat generously gave me access to his patients' clinical and laboratory records, an invaluable resource, backed up by his gift of a desk-top calculator to process the results of my deliberations.

His retirement in 1976 left me with 100 patients with chronic pancreatitis. The only options to treat severe pain were prescription of narcotic analgesics or piece-meal pancreatic surgery. Careful analysis of histories from patients who were clearly not 'alcoholic', eg. a woman with calcific disease who served in the royal air force guiding warplanes to and back from missions, made me wonder whether alcohol-like volatile chemicals might be involved in disease pathogenesis. Moreover, the annual increase in admissions and patients' youth pointed to some commonplace environmental factor that encouraged the disease. The steady increase in consumption of C18:2 fatty acids, eg. via corn oil, in western countries was striking.

The next fortuitous but inexplicable finding was an excess of copper in the first fraction of duodenal juice after Boots secretin in SP tests. This led me to seek out Thomas Dormandy in London after reading the proceedings of a CIBA foundation symposium on copper during the journey south to present the results at a meeting of the Physiology Society. Within minutes of seeing my slides, he phoned John Sorenson in the USA, advising that he had just met someone who knew more about copper metabolism than anyone for a lecture in a

forthcoming symposium at Arkansas and that I should be invited to speak. I sent my research registrar.

Progress thereafter was rapid. It was confirmed that the phenomenon was tied in with increased hepatic free radical activity; 1983 saw publication in The Lancet of my hypothesis that the disease is a casualty of xenobiotic detoxification reactions; five years of testing confirmed the concept; and, most importantly, the therapeutic corollary was realised by a trial of micronutrient antioxidant supplements in patients with chronic pancreatitis and relapsing acute pancreatitis.

When elected President of the Pancreatic Society of Great Britain and Ireland in 1990, I organised a symposium on 'The pathogenesis of pancreatitis', the proceedings of which, along with additional Chapters to round off the text, were published the following year by Manchester University Press. The contents page (shown in the big box) illustrates the range of topics covered. During the meeting I met Bent Henriksen OBE, managing director of 'Pharmanord UK', who advised me that his company could provide antioxidant tablets of far higher potency than I was using to treat patients, and also that methionine could be incorporated so as to lower the daily number of tablets to 4 (instead of 16). He was true to his word.

So successful was the new treatment for chronic pancreatitis, that by 1992 interventional endoscopy and pancreatic surgery were virtually redundant. The Manchester Royal Infirmary thus became the first NHS hospital to provide monitoring for oxidative stress and antioxidant status as part-and-parcel of clinical care, the initiative resourced by one-off payment from the referrer. In 1994 I was awarded the Doctor of Science degree by the University's faculty of science and engineering in recognition of a new template for pathogenesis of the disease; and my collected papers - including on diagnostic aspects - earned Fellowship of the Royal College of Pathologists in 1995.

The Pathogenesis of Pancreatitis symposium , 1990

In 1998 I was invited to organise a teaching session on chronic pancreatitis at the World Congress of Gastroenterology in Vienna. By now there were substantial advances in the field: laboratory studies had shown that whereas oxidative stress is associated with acute pancreatitis, electrophilic stress is prominent in chronic pancreatitis; clinical studies at Soweto were revelatory; a surgical audit verified the efficacy of micronutrient therapy at Manchester; and there was clear evidence for the involvement of oxidative stress in cystic fibrosis.

Papers based on the symposium proceedings (shown in the small box) were published under the title 'New developments on the aetiogenesis of chronic pancreatitis: implications for treatment and disease prophylaxis' (Digestion 1998; 59 supplement 4: 1-60).

Howat accepted the 'radical' path that I was pursuing, but warned of trouble ahead. Dormandy advised that it was important to share ideas because creative minds would always generate more: thus, I persuaded G Mann in London to investigate the effect of oxidative stress in the isolated perfused rat pancreas; A Borgstrom in Malmo to determine whether mast cell tryptase could activate trypsinogen, and gifted him ampoules of the enzyme; and, most recently, F Uboh in Nigeria to investigate the effect on the rat pancreas of inhalation exposure to kerosene fumes.

This monograph charts the 20-year road towards developing the electrophilic stress template, the only framework that accommodates all observations on chronic pancreatitis while offering specific medical therapy by way of micronutrient 'antioxidant' supplements. The exploration of many other ramifications - not least for first-line medical treatment of acute pancreatitis and the prospect for disease prevention in communities at risk of chronic pancreatitis - was cut short by my enforced resignation due to daily haemoptysis from aspergillus bilateral lung cavitation, consequent upon earlier tuberculosis. My thwarted priority was to investigate whether the rapid downward spiral to death in patients with acute pancreatitis might be aborted if the first medical respondent administers adrenaline by 'Epipen' to curb mast cells, as is standard treatment for anaphylaxis.

I admit being behind non-participation by the Manchester Royal Infirmary in national trials of an antagonist of the receptor for platelet activating factor in patients with acute pancreatitis, because it seemed unlikely that this agent was somehow unique within the toxic circulating brew; and in refusing to donate patients' samples for study of trypsin-control genes, because insufficiency of the cystic fibrosis transmembrane conductance regulator seemed far more relevant to the pathogenesis of chronic pancreatitis as was noted in our 1998 paper in the New England Journal of Medicine. This resistance did not gain friends.

Of course, the studies chronicled could not have materialised without input by experts from Manchester and London University, NHS hospitals, and the pharmaceutical industry: these were cited individually in a 'Meet the champions' interview by Martin Fernandez-Zapico, which is now available under the Pancreapedia enterprise. I am grateful to surgeons for allowing me into their theatres when my patients went under the knife; and I applaud the courage and generosity of Rory

Teaching Symposium at World Congress of Gastroenterology, 1998

McCloy who arrived at the hospital as a top pancreato-biliary surgeon in 1983 only to see that a decade later his skills were barely needed for chronic pancreatitis. I appreciate the expertise of Linda Hunt, superb statistitian who steered through the quagmire of data in various projects. It remains for me to thank the patients, many postgraduate students, secretaries and typists, above all to Jenny Parr without whose voluntary and painstaking input this monograph would not see the light of day.

Almoth Wright said that “a new idea in medicine has to pass 3 stages: when it is regarded as ridiculous; when doctors say ‘OK it is possible but where is the proof?’; and when everyone dismisses it as obvious”. Alan Read, professor of hepatology at Bristol, warned me after my lecture in 1989 that at least 20 years would elapse before my concept of cytochrome P450-mediated pancreatic injury would even be given a hearing. He was right, but the challenge has been exhilarating!

Chapter 1

Introduction

Although chronic pancreatitis (CP) is uncommon in the developed world, it exacts an inordinate toll in terms of morbidity, mortality and health economics. This predicament reflects the lack of agreement on specific medical treatment to control disabling pain and avoid pancreatitis relapses that will require hospitalization, leaving patients to face the further threats of narcotic addiction, job loss, dislocation of family, and social isolation. The therapeutic vacuum, in turn, points to the absence of consensus as to the pathogenesis of the disease and of pain, an irony that is highlighted by today's embarrassment of riches in relation to diagnostic tools[1]. It is harsh testimony that total pancreatectomy, the earliest operation for chronic pancreatitis in 1946[2], should still be on the treatment menu for a non-malignant disease that was identified at autopsy more than 200 years ago[3]. Clinicians meddle while the gland smoulders on (**Figure 1.1**), rather like the emperor who fiddled while Rome burnt down.

As in any field of science, myths that are promoted to dogma by public appeal stifle original thought: heretics run the risk of being lampooned, vilified, ignored or ousted[4]. In the context of chronic pancreatitis, the myth is that recurrent autodigestion episodes via 'prematurely activated trypsin' in acinar cells is the underlying problem. This has been elevated to doctrine by the discovery of trypsin-favoring gene mutations in patients with heredo-familial disease, notwithstanding experimental evidence against the gory interpretation[5, 6].

Any alternate philosophy must accommodate the predisposition conveyed by those mutations, as also by mutation(s) in the cystic fibrosis gene[7]. Moreover, it must rationalise demography, notably the following facets: the highest recorded prevalence of CP in Kerala province south India, despite a curious 6-fold decline between 1962 and 1987 in annual hospital admissions; the vulnerability of African Americans; the current endemicity in Soweto, South Africa[1, 8].

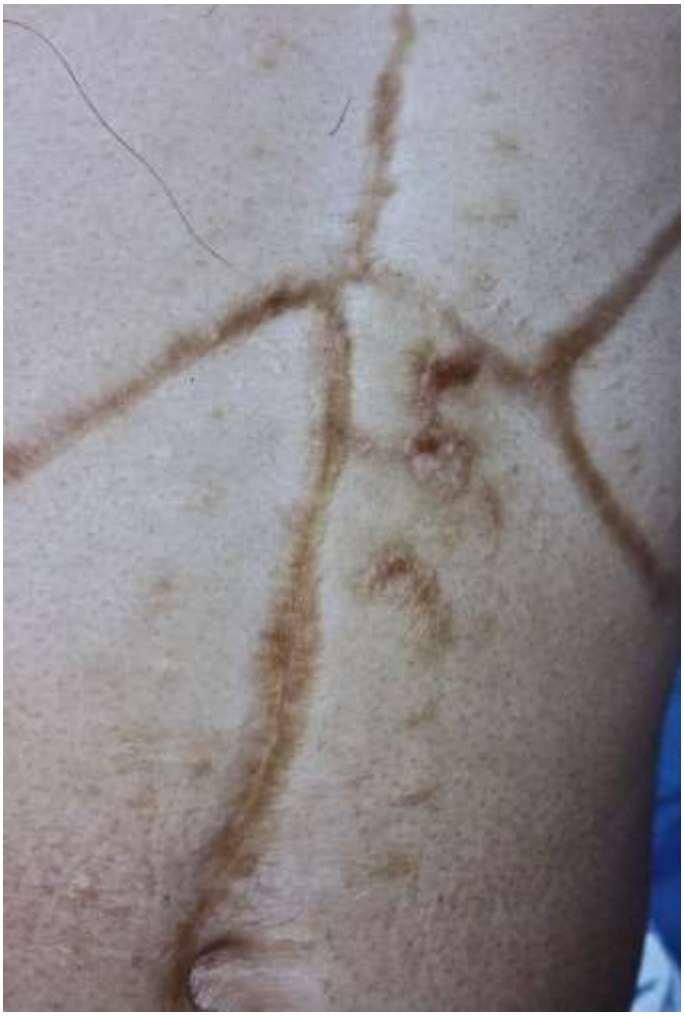

Figure 1.1 'Battle-scarred' abdomen of a woman with familial lipoprotein lipase deficiency and small-duct chronic pancreatitis. Surgical procedures included diagnostic laparotomy; cholecystectomy (no gallstones); distal pancreatectomy with splenectomy; gastroenterostomy; and finally, an attempt at total pancreatectomy which was abandoned after 9 hours. Other useless measures included pancreatic extracts; 2 coeliac plexus blocks; and splanchnicectomy. She was referred in 1995 on opiates, and with jaundice from secondary sclerosing cholangitis. Her subsequent progress is described in Chapter 15.

The monograph begins by describing chronic pancreatitis and relevant aspects of the normal gland as were known in the 1980s when, initially by chance, peculiarities were found in duodenal bile collected during routine secretin-pancreozymin (SP) tests. Many avenues for investigation were simultaneously opened up by the ensuing hypothesis that the disease is a casualty of 'detoxification' reactions that are generated via induced cytochrome P450 mono-oxygenases (CYP)[9,10]. Hence, the developments recorded herein are not strictly chronological. They culminated in the electrophilic stress template, ie. that injury is generally due to reactive xenobiotic species (RXS), over-and-above reactive oxygen species (ROS). This is the only philosophy that accommodates all aspects of the disease - from genetics and 'alcoholic' or 'tropical' aetiology through to pathophysiology. More importantly, the concept enables first-line micronutrient 'antioxidant' therapy[11].

Relapsing acute pancreatitis (RAP) is brought into the frame because of the overlap in aetiological factors and clinical identity of attacks. The likely role of electrophilic stress in lethal acute pancreatitis, by evoking the wholesale degranulation of mast cells, is examined too because of the potential for specific medical treatment. Finally, the scope for micronutrient prophylaxis against both chronic pancreatitis and acute pancreatitis is considered.

[Postscripts. (1) Unorthodox investigations had the approval of the ethical committee of the Manchester Royal Infirmary and, if germane, of institutions housing collaborating researchers, whereupon prior informed consent was obtained from participants - and parents when children were involved. Studies of a purely clinical nature were guided by judgement and experience. (2) The investigations were initiated by the author except when specified otherwise. (3) For the convenience of readers, a glossary of abbreviations, together with factors to enable conversion from metric to Systeme Internationale units for micronutrient antioxidants follows the contents pages in the preamble.]

Chapter 2

Chronic Pancreatitis: 1980s

The 1980s was a period of excitement for pancreatologists as the gland was brought into view by radio-isotope scanning, endoscopic retrograde cholangio-pancreatography (ERCP), ultrasonography and computed tomography (CT) raising the hope that tedious duodenal intubation tests might no longer be needed to diagnose pre-calcific chronic pancreatitis. It was also a time of frustration because trainees were force-fed the credo that the disease is synonymous with alcoholism and is due to primary ductal lithiasis[12]. How, one asked, could this theory rationalise the disease in non-alcoholics? It was also difficult to explain the co-existence of pancreatic, liver and kidney lesions with one or other of the triad showing the greatest damage, as was notable in the first documented case, the chequered medical history of the musical genius Beethoven, the earliest report to make the alcohol connection, and experimental injury?[13] There was a need, therefore, to re-visit the basics, as detailed in pancreatic tomes[14, 15], supplemented by individual papers.

2.1 Frame of reference

2.1.1 Embryology

Three points are potentially relevant. (i) The liver and pancreas originate in contiguous buds from the evolving foregut. (ii) Duct systems of the 2 pancreatic anlages then diverge. The smaller ventral channel (duct of Wirsung) opens into the origin of the hepatic duct, and this common channel will later be sheathed by Oddi's sphincter. The larger dorsal channel (duct of Santorini) opens into the gut directly, just cephalad to the common channel, and without a sphincter. Both pancreatic anlages fuse in the 7th week, the ventral duct now becoming the main pancreatic duct. In 5-10% of cases, fusion of the ducts is incomplete, such that the Santorini system remains dominant, so-called 'pancreas divisum'. (iii) Primitive pancreatic ductules are the source of cell clumps that are forerunners of acinar units as well as islets of Langerhans.

2.1.2 Anatomy and microanatomy

The following facets are notable in context. (i) A common ampullary channel could allow toxins in bile to reflux into the pancreatic duct, either directly or by way of the duodenum: in this event, as also when excess pressure is generated during ERCP, the single-layered epithelium of the smallest intralobular ducts would yield. However, the construction of many acini as anastomosing networks mitigates against intralobular obstruction. (ii) The rich blood supply to the gland is arranged in such a way that a portion initially perfuses islets, constituting a limited 'portal' circulation. Also of note[13], the pattern of branching leaves the periphery of acinar lobules vulnerable in ischemic states[16], in the same way as threatens zone 3 hepatocytes[17] (**Figure 2.1**). Electron microscopy shows that many of the capillaries are fenestrated, with pores around 100 nm diameter, facilitating the diffusion of substances such as hormones.

(iii) In an autopsy pancreatography study, ductal irregularities that would be classed as 'minimal-change-pancreatitis' (see below) were recorded in 37% of cases, without any histological evidence of disease[18]. (iv) The tight junctions between pyramidal acinar cells lie on the lateral aspect adjacent to the lumen, so that there is a wide surface area for communication with the interstitial space. A thin lamina at the basal aspect of each cell abuts on underlying connective tissue. (v) The pancreatic interstitium contains numerous adipocytes, including retinol-rich cells that would, many years later, be identified as precursors of fibrosis-promoting stellate cells[19]. (vi) Also herein are numerous mast cells, between basement membrane of capillaries and plasma membrane of acinar cells[20] - which, in time, would be shown

to be juxtaposed to membrane receptors for a tissue-type plasminogen activator[21], and also urokinase-plasminogen activator[22]. (vii) An extensive lymphatic drainage originates in the perilobular connective tissue and gains access to the gland's surface by channels that run alongside blood vessels. (ix) Ultratructural studies reveal nor-adrenergic nerve terminals in close association with perivascular smooth muscle cells; whereas cholinergic fibres and their terminal regions exist between secretory acini.

2.1.3 Physiology

A great deal was known about the regulation of pancreatic secretion by interaction between food-evoked neural and hormonal stimuli, the latter principally via secretin, cholecystokinin-pancreozymin (CCK) and gastrin, but information on stimulus-secretion coupling in acinar cells was scant. It was recognised that the pyramidal acinar cell is the epitome of a polarised unit, wherein synthesis of hydrolases occurs within the abundant rough endoplasmic reticulum (RER) at the basal pole and the bulk discharge of enzymes, exocytosis, at the apical pole. It was established that this is effected via a series of intracellular messengers. Occupation of the CCK-type receptor on the plasma membrane leads via a guanine nucleotide- binding protein (G-protein) to the activation of phospholipase C, producing inositol 1,4,5-trisphosphate and diacylglycerols. Such agents mobilize Ca^{2+}, probably from the RER and activate protein kinase C. The rise in cytosolic Ca^{2+} activates calmodulin and thereby protein kinase and phosphatase. A second low-affinity CCK receptor inhibits secretion. Signal transduction after occupation of the secretin-type receptor involves an increase in cyclic AMP which, in turn, activates protein kinase A. Both pathways alter the level of stimulatory and inhibitory G-proteins (G_s, G_i) and thus modulate protein phosphorylation[23, 24].

It was also appreciated that the actual exocytosis event involves interaction between G_s / G_i balance, actin, microtubules, and apical

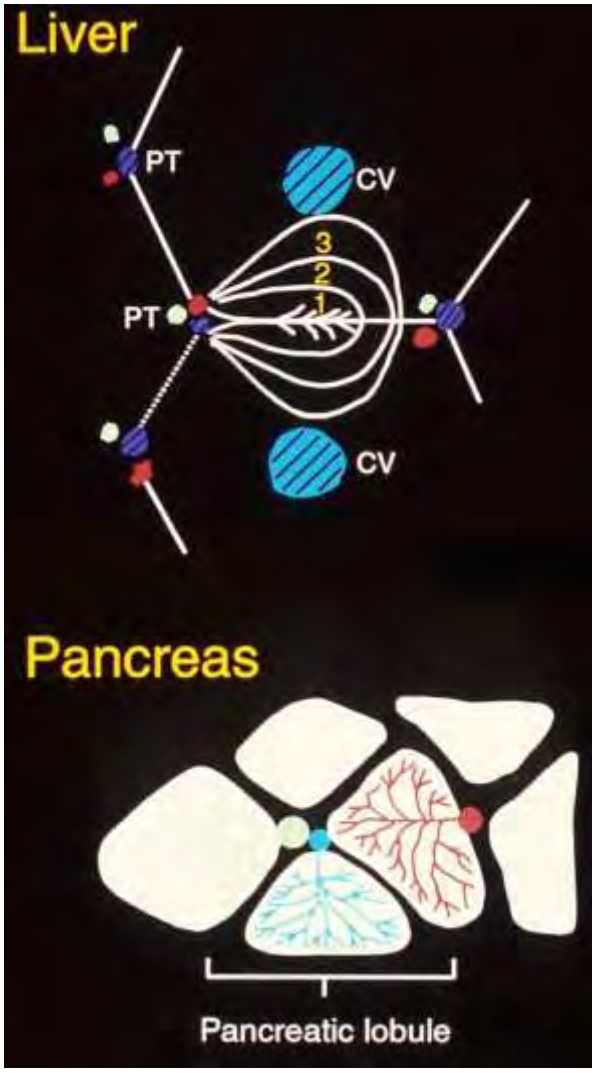

Figure 2.1 Microcirculatory arrangements in the liver with 3 functional zones determined by location of the central vein (CV) and portal tract (PT), contrasted with that in the pancreas. Composite figure generated for 1998 review in Digestion[13] from images redrawn with permission of authors [16,17]; now reproduced with permission of publisher, S Karger AG, Basel.

membrane fluidity[25]. Studies in a variety of cells pointed to the particular importance of the last factor by way of membrane phospholipid methylation[26], but were dismissed[23]. This was hasty, in that methionine lack had long been known to paralyse apical enzyme secretion[27]. In 1987 the presence of constitutive pathways for enzyme discharge from the acinar cell were documented, 2 at the apical pole and 1 at the basolateral pole[28].

Meanwhile, the multi-layered defence strategy against premature detonation of digestive enzyme

grenades during transit was being unravelled. (i) The potent proteases and phospholipase A_2 are produced as zymogens that are unleashed in cascade fashion when trypsinogen is activated to trypsin upon contact with enteropeptidase in the presence of bile salts within the duodenum. (ii) From the time of their production to discharge from the acinar cell, digestive hydrolases are held separate from lysosomal enzymes, in the later stages being packaged within zymogen granules (ZG) and stored at the apical pole. (iii) The serine protease inhibitor Kazal type 1 (SPINK 1), formerly called pancreatic secretory trypsin inhibitor, is co-packaged within ZG and can curb 20% of potential tryptic activity. However, perhaps because the enzyme-inhibitor complex tends to dissociate, a back-stop is in place, in that trypsin is soon destroyed by trace amounts of activated mesotrypsin and 'peptide Y'[15,29], the latter now identified as a form of chymotrypsin[7]. (iii) Once discharged by exocytosis, the cargo of released pro-enzymes, amylase and lipase in a small volume of chloride-rich juice is propelled downstream by secretin-stimulated flow of bicarbonate and water from centro-acinar and ductal cells.

2.2 The Disease

2.2.1 Definition

Chronic pancreatitis is a histological entity, characterised by continuing inflammation with progressive loss of acinar tissue and fibrosis. These are dynamic processes that generally manifest as attack-upon-attack of pancreatitis; their tempo is unpredictable; and the lesions are typically patchy. Each burst of inflammation may lead to foci of interstitial or peripancreatic fat necrosis[20], that are nowadays implicated in the genesis of pseudocysts and fibrosis[30]. Intraductal protein plugs (**Figure 2.2**) that sooner or later become impregnated with calcium carbonate (**Figure 2.3**) are a variable but dramatic feature; so too is nesidioblastosis. Thus, histology from the same pancreas at different time-points can be likened to ever-changing images in a kaleidescope. Enlarged nerve endings show breaching of perineurium in areas capped by inflammatory cells (**Figure 2.4**). As time goes by, acini re-differentiate into tubular complexes. Eventually all are obliterated within whorls of fibrous tissue while inflammatory cells disappear (**Figure 2.5**), an evolution that echoes the path from chronic active hepatitis to liver cirrhosis[31]. Islets of Langerhans succumb too.

Acute pancreatitis is a bout of pancreatic inflammation that usually manifests as excruciating abdominal pain accompanied by increased blood levels of pancreatic enzymes / pro-enzymes, and which, in the absence of complications, will be followed by a pancreatic reconstitution. This holds true even in patients who experience multiple attacks. In other words, acute pancreatitis and chronic pancreatitis are distinct diseases, although the clinical presentation as an 'attack' is indistinguishable.

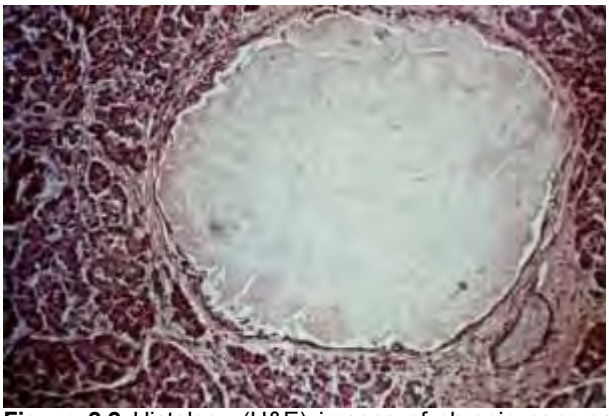

Figure 2.2 Histology (H&E) images of chronic pancreatitis: intraductal 'protein plug'.

Figure 2.3 Chronic pancreatitis: calcification within protein plug and fibrous strands (green)

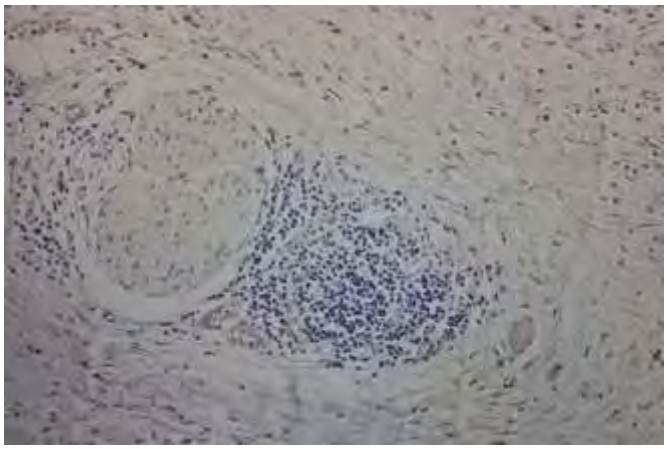

Figure 2.4 Chronic pancreatitis: inflammatory capping of nerve endings

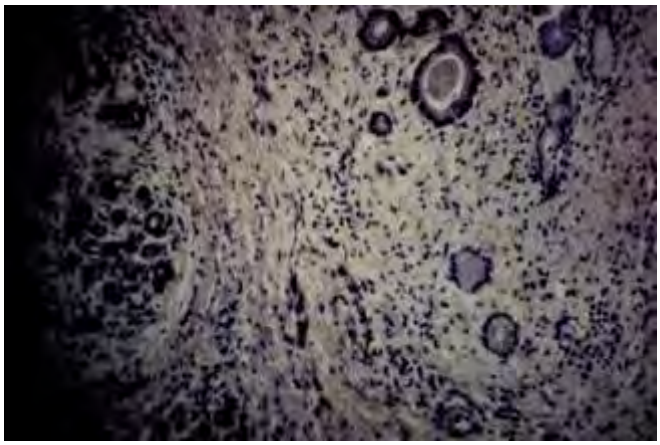

Figure 2.5 Chronic pancreatitis: histology of more advanced disease

Alongside this declaration from a symposium in 1984[32] was the recommendation that clinicians discard the useful adjective 'relapsing' which was adopted at the 1963 Marseilles symposium**.** Given the impracticality of pancreatic biopsy for the sole purpose of retrospective assignment, and that even in patients with chronic pancreatitis random needle biopsy is like searching for a needle in a haystack because the early lesions are patchy, clinicians were stymied unless pancreatic calculi were identified by admission plain X-ray of the abdomen or latterly by CT. The advice, although not foolproof, was to assess residual secretory capacity 6 weeks or more after the last attack, when a subnormal value(s) points to chronic pancreatitis[33].

2.2.2 Etiology

Alcoholism was regarded as the predominant cause of chronic pancreatitis in western societies, Brazil and South Africa[12]. However, studies from France showed that ethanol on its own is a weak agent, an average of 18 years elapsing before the heralding attack in men who drink > 80 gm ethanol daily (a decade shorter than presentation with liver problems), and that < 10% of alcoholics develop the disease. The paradox of no lower threshold for ethanol toxicity was attributed to the potentiating effect of diets that contain excessive amounts of fat and / or protein, unidentified environmental co-factors, and unknown disease-susceptibility genes.

Meanwhile studies in Kerala province in southern India, where residents did not consume alcohol, showed that patients with chronic pancreatitis accounted for around 1.5% of hospital admissions[34]. The disease typically presented as non-ketotic diabetes in young lean patients with extensive pancreatic calculi, parotid hyperplasia and a cyanotic hue of lips and mucous membranes. An attractive proposal was that hydrogen cyanide in dietary cassava (synonyms manioc, tapioca) might be responsible[35]. The theory was, however, discredited by many observations including that the same disease pattern was observed in countries such as Nigeria where cassava is not a staple food. Moreover, the hypothesis could not rationalise the 6-fold decline in hospital admissions by the mid 1980s[34].

As to other potential factors, gall stones were regarded as incidental. It was agreed that impedence to drainage of pancreatic juice, as by Vaterian stenosis, leads to uniform lesions of chronic pancreatitis upstream with smooth dilatation of the pancreatic duct; and also that in patients with pancreas divisum the ventral pancreas might be spared. A few cases could be ascribed to hyperparathyroidism, familial disease or drug treatment. Autoimmune pancreatitis was unknown at the time but 'primary inflammatory pancreatitis' was noted anecdotally[14].

Axiomatic in the 1984 guidelines was that pancreatic lesions of hemochromatosis or cystic fibrosis do not equate to chronic pancreatitis[32]. Yet, cystic fibrosis is the surest antecedent of non-calcific chronic pancreatitis: the diffuse lesions develop *in utero* and might display histological features of severe acute pancreatitis; they manifest at birth by hypertrypsinogenemia, and follow an accelerated course to exocrine pancreatic failure[33]. In 1989 the genetic basis for its transmission as an autosomal recessive trait was pinpointed to mutations in both alleles of the cystic fibrosis transmembrane conductance regulator gene (CFTR)[36]. The genetic basis for the rare hereditary form of chronic pancreatitis remained elusive, although known to be transmitted in autosomal dominant fashion with variable penetrance.

2.2.3 Pathogenesis / Pathophysiology

The party-line was that increased secretion of lactoferrin and calcium from acinar cells coupled with reduced secretion of a 'stone protein' (synonym lithostatin) within a milieu of falling bicarbonate production by ductal cells leads to protein precipitates in pancreatic ducts: these serve as niduses for calcium carbonate stones, which then ulcerate ductal mucosa to provoke periductal fibrosis[12]. The following were among other contemporary proposals: reflux of noxious bile; stagnation of pancreatic juice; recurrent fat necrosis perpetuating fibrosis; direct toxicity of ethanol; and slow cyanide poisoning[37].

All these hypotheses failed to explain why acinar cells appeared to be whipped into a state of hyperactivity[10]. As evidence, in chronic alcoholics without manifest disease, electron microscopy of pancreatic tissue showed expanded RER, microvesicular fat, areas of cytoplasmic sequestration and increased numbers of lysosomes. In symptomatic patients with early chronic pancreatitis, reports described large acinar cells, osmiophilic bodies resembling lipofuscin, and mitochondrial aberrations - in conjunction with dilated RER, and prominent lysosomes in ductal cells. Secretory patterns reflected these morphological changes. Thus, analysis of pure pancreatic juice from asymptomatic alcoholics disclosed increased amounts of protein and calcium, and an increased ratio of lysosomal to digestive hydrolases. Symptomatic patients with early disease displayed these abnormalities and many others: increased rate of protein synthesis; hyperscecretion of bicarbonate; and altered composition of pancreatic secretions, containing more anionic trypsinogen but less trypsin inhibitor than normal[10].

2.2.4 Experimental models

Whereas alcohol administration on its own did not produce the disease, the use of modified protocols that induce acute pancreatitis did so. Examples include repetitive hyperstimulating doses of caerulein (an analogue of gastrin and CCK), partial obstruction of the main pancreatic duct, or a choline-deficient DL-ethionine supplemented (CDE) diet - although damage is inflicted by utterly different routes, respectively microvasular, ductal, or intra-acinar[38]. Confusingly, a single subcutaneous dose of carbon tetrachloride (CCl_4) in rodents was shown to cause lesions of chronic pancreatitis in advance of liver lesions, and which could be "altered at will" to yield a spectrum from the patchy lesions of chronic pancreatitis with or without concretions, through to 'pancreatic cirrhosis' or 'cystic fibrosis'[31].

Moreover, an interesting parallel was emerging between the effects of certain toxins on the hepatocyte and acinar cell[39]. Thus, many of the indirect hepatotoxins which produce injury by interfering with a specific pathway or process - eg. DL ethionine, tetracycline, puromycin - are also associated with pancreatitis.

As in the case of liver damage by these substances (**Figure 2.6**), so is pancreatic damage by hyperstimulation and certain insecticides (**Figure 2.7**), products that are normally extruded

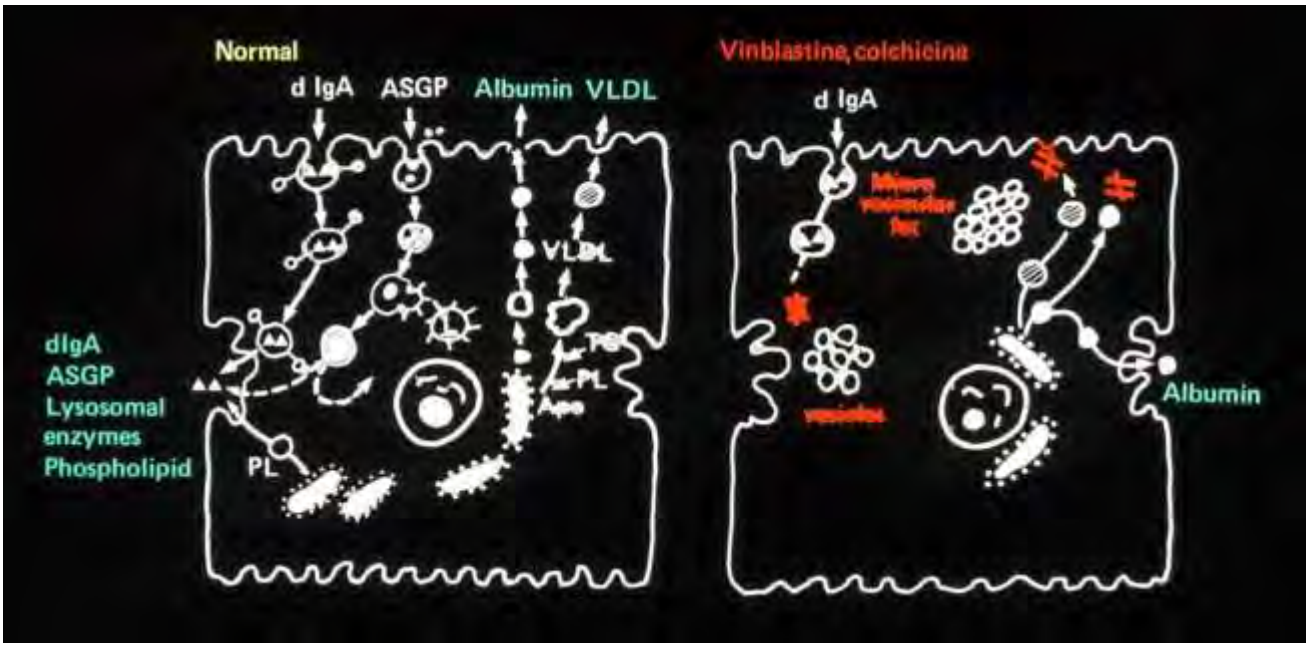

Figure 2.6 Schematic representation of macromolecular traffic in the normal hepatocyte (left) and consequences of interference to traffic routes by hepatotoxins (right). Abbreviations: dIgA, dimeric immunoglobulin A; ASGP, asialoglycoprotein; VLDL, very low density lipoprotein; PL, phospholipid; TG, triglycerides; L, lysosome. Reproduced from 1988 review in Int J Pancreatol[39]

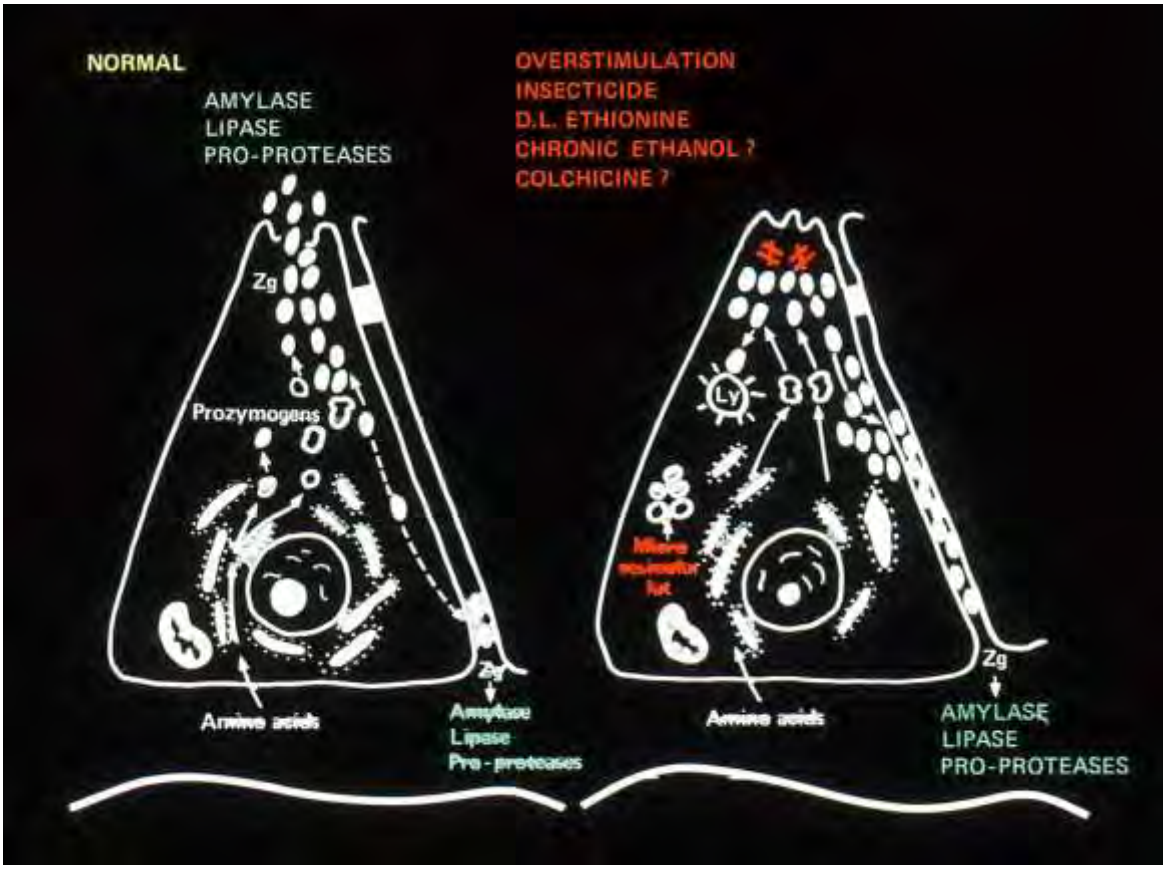

Figure 2.7 Schematic representation of the traffic of food digestive hydrolases in the normal acinar cell (left) and abnormal partitioning during experimental pancreatic injury as suggested in the 1980s (right); ZG, zymogen granules. Source as for Figure 2.6.

at the plasma membrane of the hepatocyte or luminal pole of acinar cell are seemingly forced to find egress through alternate routes, namely, the canalicular pole and basolateral membrane, respectively. In each instance the cytoplasm of affected cells contains vacuoles, representing secretory products that have accumulated because of microtubular damage (microvesicular fat in hepatocytes, nascent zymogens in acinar cells) and / or engulfment of redundant secretory material by lysosomes. By 1990, the term 'pancreastasis' was coined to describe the altered secretory polarity in the acinar cell, in line with intrahepatic 'cholestasis'[38].

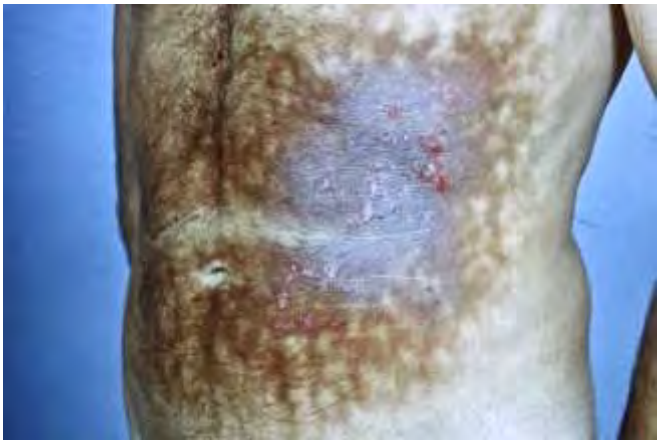

Figure 2.8 'Erythema ab igne' over the abdomen of a patient with unremitting pain.

2.2.5 Clinical features

Pain is the cardinal symptom. In many patients, it initially accompanies clear-cut attacks of pancreatitis and moves on to constant pain; in others it is constant from the start. It may be primarily in the epigastrium or dorsal spine, or bore through from front to back. A stooping posture might alleviate. The agonising intensity of pain may be evidenced by erythema ab igne (**Figure 2.8**) and / or improvised counter-irritant devices (**Figure 2.9**). The eventual loss of nearly all secretory parenchyma, as is recognised by steatorrhoea, often brings pain relief - supporting the notion that viable acini are a pre-requisite for pancreatic pain, provided that by then patients are not already addicted to narcotic analgesics. The progression to parenchymal eradication is more rapid in tropical pancreatitis, such that pain is less of a problem, but this path is not marked by steatorrhoea because fat intake is traditionally low. Irrespective of geography, end-stage disease is often accompanied by diabetes and pancreatic calculi. This classical pattern glosses over small-duct disease, which is as painful but difficult to detect[40]. The notion that pain is generally due to a plumbing problem (ie duct obstruction) now needed a re-think. Other modes of presentation include jaundice due to intra-pancreatic constriction of the common bile duct; symptoms due to space-occupying cysts / pseudocysts; effusions (pleural, ascites); and

Figure 2.9 Wooden blocks with inward-facing nails improvised by a patient with intractable back pain.

alimentary haemorrhage due to portal hypertension. A cautionary note was issued in regard to pancreatic cancer, ie. that proximal obstruction by a tumour might masquerade as chronic pancreatitis, which itself increases cancer risk.

2.3 Diagnosis

2.3.1. Secretin ± Pancreozymin test

The patient, fasted overnight, is intubated with a double-lumen tube which is advanced under fluoroscopic guidance into the duodenal loop until the furthermost ports are positioned adjacent to the assumed position of the ligament of Treitz. After a control period wherein gastric and duodenal secretions are aspirated separately with a mechanical intermittent-suction pump, secretin is given intravenously to stimulate the secretion of fluid and bicarbonate. Thereafter, timed duodenal aspirates are collected on ice, volumes and pH recorded, and aliquots retained for analysis. The results are expressed as peak bicarbonate concentration and bicarbonate output, and interpreted by reference to data in controls.

Many variations of the test have been described, involving impure secretin from the pig intestine prepared commercially by the Boots Company at Nottingham, UK, and marketed as Crick Harper Raper Units (CHRU); highly purified secretin from the Gastrointestinal Hormone Laboratory (GIH) at Karolinska, Sweden; or synthetic secretin (eg Schwarz-Mann), both marketed as Clinical Units (CU) - whether given as bolus intravenous injection, or by continuous infusion for variable periods.

In a further modification, the hormone pancreozymin (CCK) or an analogue such as CCK-octapeptide is administered to evoke enzyme secretion. It may follow or precede secretin or be given simultaneously by injection or infusion. Aliquots of aspirated fluid are frozen at -4ºC. Amylase and / or lipase and / or trypsin activity are measured and data expressed as peak enzyme concentration and output over a defined period. The aim is to stimulate the pancreas sub-maximally. The Manchester version of the test involves an intravenous injection of 2 CHRU of Boots secretin / kg body weight followed 30 minutes later by an intravenous injection of 2 CHRU of Boots pancreozymin / kg body weight. Duodenal aspirates are collected on ice every 10 minutes for 2 basal periods, and 3 periods after each hormone. Volumes of aspirates are recorded and aliquots analysed for bicarbonate and pancreatic enzymes. Secretory profiles in controls and patients with chronic pancreatitis or pancreatic cancer have been described - noting in particular the fall in post-secretin volume of aspirates in the latter but fall in peak bicarbonate concentration and especially bicarbonate output as being characteristic of chronic pancreatitis[41]. The fall in bicarbonate is best seen in the third period after secretin, by which time the hormone's choleretic effect is over and aspirates are water-clear.

The predictive value and efficiency of the hormone test varies widely, depending on whether the traditional mean-2SD cut-off value(s) or percentile estimate(s) is used to define normality; the number of parameters included in a logistic regression analysis; and, above all, whether the test is invalidated by spill-over of gastric juice or incomplete recovery of fluid[42]. The high cost of the SP test in terms of man-power is a further disincentive.

2.3.2 Meal test

The meal test is far simpler[43]. The patient, fasted overnight, is intubated with a single-lumen radio-opaque tube which is advanced under fluoroscopic guidance into the proximal jejunum, following which 500 ml of a standardised liquid test meal is given, fluid aspirated for 2 hours, and trypsin activity measured in an aliquot of the pooled aspirate. It seemed implausible that this single crude measure could be as sensitive as the multifaceted SP test in diagnosing chronic pancreatitis, as claimed.

A comparative study was carried out in 39 individuals, including 22 controls and 17 with chronic pancreatitis. Diagnosis of the disease was principally by histology of surgically resected specimens in 2 cases; radiological detection of pancreatic calculi in 8 cases; the combination of diabetes, steatorrhoea and previous pancreatitis attacks in 4 cases; or relapses of pancreatitis from an early age plus impaired glucose tolerance with abnormal ultrasonography and CT in 3 cases.

The peak concentration of bicarbonate after secretin was lower than the mean-2SD lower limit of normal in 14 patients (82%), as was post-secretin bicarbonate output in 13 (76%) and peak tryptic activity (PTA) after pancreozymin in 10 (59%). Mean tryptic activity (MTA) after the test meal was subnormal in 16 patients (94%). There was good correlation between the post-secretin bicarbonate output and MTA, especially in the patients (r=0.85, p < 0.001).

In the control group PTA was close to MTA (51.6 and 53.2 IU / ml, respectively), but in the chronic pancreatitis group MTA in response to the meal was less than PTA after pancreozymin (p < 0.001).Correlation and regression coefficients relating PTA and MTA were significant in both groups (**Figure 2.10**), and the slopes of the 2 regression lines were not significantly different (F test p > 0.05). Thus, for an assigned value of PTA, say 25 IU / ml, the corresponding MTA was similar at 30 IU / ml when the pancreas was normal but only a third of the expected result at 9.6 IU / ml in patients with chronic pancreatitis. The best approximation of the ratio of trypsin response to the test meal versus hormones in the patients was 30%, with 95% confidence intervals of 20-50%**.** This disproportionate reduction in tryptic response to endogenous stimulation accounts for the success of the meal test[44].

MTA in duodenal juice after a test meal depends not only on the amount of CCK released from the intestinal mucosa but also on dilution of pancreatic enzymes by gastric, biliary and intestinal secretions. In an attempt to unravel the factors, validated double-marker techniques were used to quantitate trypsin secretion in a separate study of 5 controls and 7 patients with chronic pancreatitis. In both groups the mean 10-minute output of trypsin (logged) over the 2-hour period after the test meal was very similar to the peak output (logged) in 10 minutes after pancreozymin: in fact, the components of the linear regression equation were nearly identical in controls and patients[45]. However, cumulative volumes of gastric contents entering the duodenum were twice as high in the chronic pancreatitis group, eg. at 120 minutes 710 ± 77 ml (mean ± SE) versus 353±48 ml in controls (2p < 0.05).

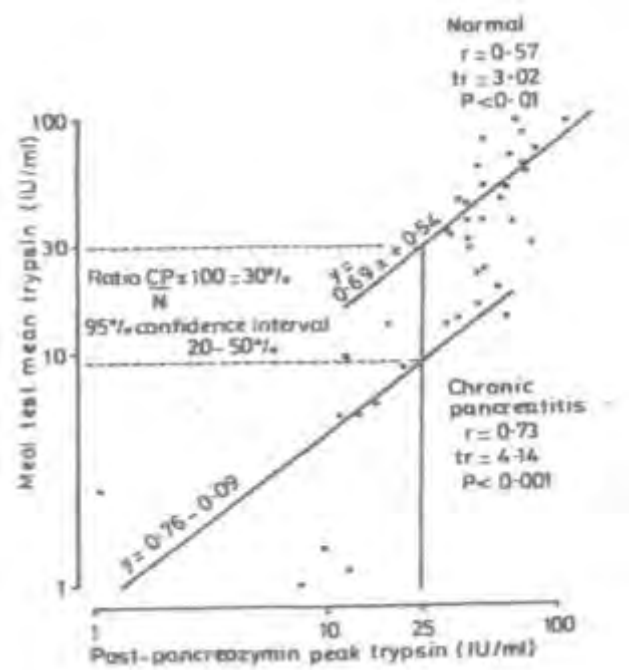

Figure 2.10 Extrapolated values for mean trypsin activity after a test meal in normal (N) and chronic pancreatitis (CP) groups for an assigned value of 25 for peak trypsin activity after pancreozymin. Reproduced from 1978 paper in Brit Med J[44] with permission of BMJ publishing group (© BMJ all rights reserved).

In 3 patients rapid gastric emptying was accompanied by hypersecretion of gastric acid. These data are not comparable to other studies because of differing selection criteria, ie. patients with end stage disease and steatorrhoea, alcoholics, or patients with diabetes. Although the

contribution of bile and intestinal secretions was not assessed, the inescapable conclusion is that the high sensitivity of the meal test is, in a sense, fortuitous: more surprising was its high specificity[46].

2.3.3 Non-invasive function tests

These were extensively researched in the 1980s, attempting to overcome the impracticality of methods involving duodenal intubation, the non-specificity of radio-isotope scanning, the operator-dependence of ultrasound scanning, and expense of CT. Among potential non-invasive avenues, a test involving the chymotyrpsin-hydrolysable substrate N-benzoyl L-tyrosyl para-aminobenzoic acid (BT-PABA) and a marker (^{14}C-PABA) to correct for problems of intermediate metabolism was the best. These substances were delivered in 500 ml of flavoured water along with 25 gm casein as a competitive substrate for chymotrypsin, followed by analysis of PABA and ^{14}C in a 6-hour collection of urine. The test could thus be done on an out-patient basis. Test sensitivity was just as good as for duodenal intubation tests and specificity > 90% when the mean-3SD value was used as cut-off, yielding a screening test with high efficiency and excellent predictive value of negatives[46]. Drawbacks were the imperative to exclude patients with compromised renal function or pregnant women; and invalidation of up to 15% of tests due to interference in the PABA assay by a variety of drugs and foodstuffs.

A persistently subnormal blood level of a pancreatic enzyme(s), eg. trypsinogen, indicates exocrine pancreatic failure as in cystic fibrosis, but lesser degrees of parenchymal destruction are not detected. The usefulness of chymotrypsin in a random sample of faeces was undermined by false positives when faecal pH is low. The value of faecal elastase measurement was not yet known.

2.3.4 ERCP.

The advent of ERCP afforded the opportunity to analyse the secretory abnormalities detected by SP tests in relation to alterations in ductal morphology. Accordingly, both tests were done within a month of each other in 45 patients with a compatible history. Diagnosis was by permutations and combinations among the following criteria: pancreatic histology; pancreatic calculi; persisting pancreatic damage ≥ 3 months after the last attack (ie. the combination of impaired glucose tolerance, abnormal meal test and abnormal ultrasound scan); and near-absence of trypsin in meal test aspirates of patients with painless steatorrhoea. Pancreatograms were numbered upwards from least to most abnormal, broadly conforming with the Cambridge criteria[15] : 1=normal in 8 cases; 2= 'minimal-change pancreatitis' in 8 cases (ie. normal main duct but abnormality in at least 3 side ducts); 3='moderate-change pancreatitis' in 6 (ie. main duct dilated too); or 'advanced-change pancreatitis' in 23 cases, arbitrarily sub-grouped as grade 4= gross alteration of the main duct in 9, grade 5= chain-of-lakes appearance due to multiple strictures in 5, and grade 6= truncated / obstructed main duct in 9 cases.

Correlation coefficients calculated on each of the secretory parameters showed significant trends of decreasing parameters with increased pancreatogram scores, - eg. bicarbonate output after secretin (**Figure 2.11**) . However, secretory parameters could not predict pancreatogram grade in an individual patient. Thus, the % correct allocation based on bicarbonate output (logged), peak bicarbonate concentration, volume of secretion and trypsin output were, respectively, only 24, 34, 24 and 33%. These data indicated that duct obstruction and parenchymal destruction are not separable factors in reducing secretion in chronic pancreatitis. The study showed, furthermore, that it is impossible in patients with chronic pancreatitis to predict accurately exocrine functional status on the basis of ductal structure and *vice versa*[47]: the same applies to correlation between functional or pancreatographic abnormalities and histology[48]. This position contrasts with that in pancreatic cancer where

secretory loss correlates with the length of duct obstructed.

It was not the intention of the investigation to evaluate the relative efficiency of ERCP and hormone tests in diagnosing chronic pancreatitis. However: (i) even using both tests a diagnosis before surgery was not made in 5 patients with small-duct disease; (ii) a fall in bicarbonate output in mechanically perfect tests permitted a confident diagnosis in 3 of 8 patients with normal ducts and 6 of 8 with minimal-change-pancreatitis, whereas the last finding in isolation is compatible with a normal gland[18]; and (iii) on rare occasions (1 of 45 in the study) an abnormal pancreatogram may provide the only diagnostic proof[10].

The combination of ultrasonography and ERCP was evaluated in a parallel study which showed the high attrition rate for each test (25% and 11%, respectively); low specificity of ERCP at 65%; and low sensitivity of ultrasound scanning at 55%[49].

2.3.5. Classification and misclassification

In the chapters to follow, a diagnosis of chronic pancreatitis is based on one or more of the following parameters in patients with a compatible history: histology of a previously resected specimen of the gland; calculi shown by plain X-ray of the abdomen or CT; reduced exocrine secretory capacity (< mean - 2 SD of control values in SP or meal tests; < mean - 3 SD for the PABA / ^{14}C excretion index); unequivocally abnormal pancreatogram (grade 3+); or borderline outcome of function test and ERCP but clear abnormality on ultrasonography or CT.

Some patients who present with one or more attacks of pancreatitis are wrongly classified as post-acute or RAP after the initial assessment because evidence of functional or structural damage is lacking. To increase the chance of detecting patients with early lesions of chronic pancreatitis, some authors suggested a scoring system whereby marginal abnormalities in each test can be summated. The aforesaid autopsy

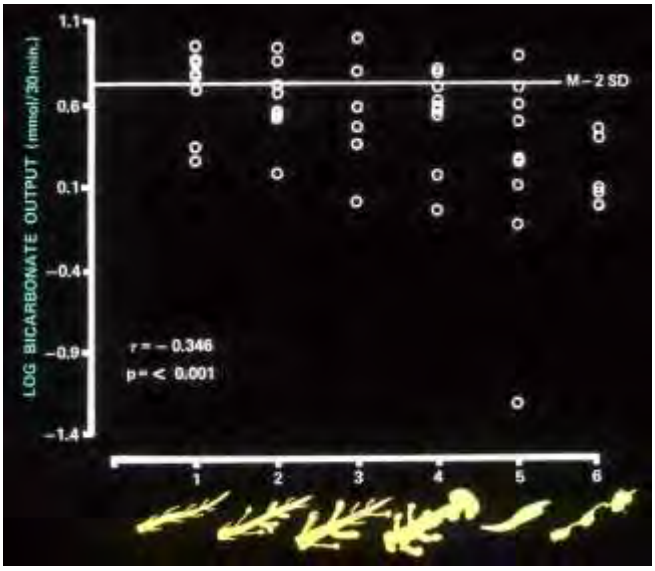

Figure 2.11 Pancreatographic appearances graded 1-6 and post-secretin outputs of bicarbonate in 45 patients with chronic pancreatitis. The horizontal line represents the lower limit of the reference range at mean-2SD of logged data in controls (5.8 mmol in 30 minutes, a value similar to the 10th percentile of 5.3 mmol). Figure from 1986 review in Recent Advances in Gastroenterology[10]

pancreatography study should sound a cautionary note. The recognition that heightened synthesis of pancreatic enzymes is an early feature of the disease offered a promising avenue forward, as also changes in the composition of pancreatic juice, but foiled by restriction to specialised centres[10]. Systematic histology of a surgically resected specimen of pancreas is difficult to justify. Hence it was deemed wise to temporise and repeat the tests at a later date when a decrement in function or alteration in the pancreatogram will eventually provide the diagnosis[10].

2.4 Treatment

The primary goal of treatment was - and remains - to control severe pain. In the 1980s the options for physicians were few : to insist on abstinence from alcohol, reinforced by behavioural / pain consultants and community groups; to 'rest the pancreas' by prescribing ordinary pancreatic extracts which were said to blunt the putative feedback loop that otherwise operates to increase CCK release from the upper small intestine; to enlist the help of nutritionists in

patients with malnutrition; to stent the common bile duct endoscopically in patients with an obstructive profile of liver function tests; and, above all, to desist from prescribing narcotic analgesics. When the last goal was threatened, surgical opinion was sought, while anaesthetists facilitated nerve block or ablation procedures - the efficacy of which was generally short-lived. Pancreatic surgery was the order of the day.

2.5 Summary

Whereas in the 1980s Bayesian philosophy enabled evaluation of tools wherewith to diagnose chronic pancreatitis[46], there was no rational philosophy for its causation. Hence, treatment options for relentless pain were grim, namely, narcotic analgesics or piece-meal ablation of the gland that not infrequently was preceded by duct drainage and / or nerve block procedures (**Figure 1.1**). Any advance clearly required a radical overhaul of thinking on disease pathogenesis.

Chapter 3

Serendipity!

In 1970, when reviewing the results of SP tests in Howat's laboratory, it became apparent that the volume of duodenal contents after secretin had fallen. Since the technique had varied little over the years, the decline suggested deterioration in the Boots secretin standard. This was confirmed by the company's Head of Bioassay, with assurance that batches from 119 onwards had been re-standardized. It was thus necessary to compare afresh the relative potency of the two brands of secretin that were in common use (Chapter 2). This was the start of a fortuitous series of events.

3.1 Boots versus GIH secretin

3.1.1 On feline pancreas

Like man, the cat secretes pancreatic juice only in response to meals, and is therefore the preferred species for the study of responses to secretin. Cats were fasted for 24 hours, anaesthetised, splanchnic nerves divided extraperitoneally, pancreatic duct cannulated, bile duct obliterated by the ligature retaining the pancreatic cannula, and gastric pylorus occluded by a tape ligature. Subsequently, vials from batch numbers 142 and 17421 of Boots and GIH products, respectively, were used to evaluate pancreatic fluid and bicarbonate responses. Preliminary experiments suggested that 1 CHRU of Boots secretin was around 4 times less potent than 1 CU of the GIH product (**Figure 3.1**).

This was borne out by a 4-point assay following 1 and 2 CHRU Boots secretin / kg and 0.25 and 0.5 CU / kg GIH secretin in each of 4 cats. It could thus be calculated that 1 CU was 3.8 times more potent than 1 re-standardised CHRU in increasing the flow rate of pancreatic juice and 4 times more

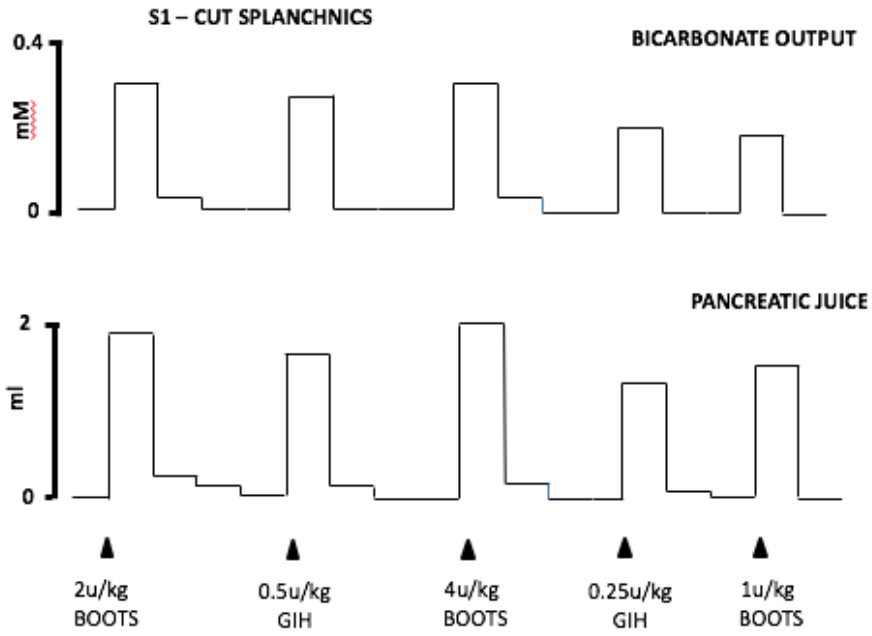

Figure 3.1 The volume of pancreatic juice and bicarbonate output per 15 minutes in response to intravenous injections of Boots and GIH secretin.in the anaesthetised cat. Reproduced from 1975 paper in Gut[50] with permission of BMJ publishing group (© BMJ all rights reserved).

potent in increasing bicarbonate[50]. This outcome was affirmed by a contemporaneous study of relative immunological potency[51].

The findings are of more than academic interest. From vast experience of secretin under the label Pancreotest (Astra, Sweden), the test's pioneer recommended a dose of 1 CU / kg body weight so as to elicit an adequate pancreatic response[52]. As argued with citations in the Manchester report[50], it was estimated that this CU was roughly twice as potent as the CHRU and, in broad agreement, volumes of aspirated fluid in 30 minutes after secretin were similar after corresponding doses of each product[41, 52]. The 4:1 relationship between CU and CHRU established by the secretory and immunological studies thus strongly suggests that the 4-fold increase in strength applied to GIH secretin from batch 16611 onwards has inadvertently resulted in a CU that is twice as potent as that of Pancreotest. This could mean that 1-2 CU / kg of GIH secretin, as in current usage, delivers inappropriately high stimulation to the exocrine pancreas, whereas the dose of 2 CHRU / kg in the Manchester SP test accords with the testing principle.

3.1.2 <u>On pepsin: 'Gastrozymin'</u>

It was known since the 1940s that acid in the duodenum elicits the secretion of pepsin. This effect was assigned as a physiological function of secretin, because it was detected at a dose submaximal for pancreatic bicarbonate secretion, 4 CU /kg / hr[53] - but at a time when the secretin standard was being reset, as described above. Studies in the dog[54] and man[55] suggested that the amount of secretin released during feeding is unlikely to exceed 0.5 CU /kg / hr. Accordingly, the question was examined afresh in the anesthetised cat preparation wherein pancreatic and gastric secretions can be obtained simultaneously.

Pancreatic secretion was initiated by 2 CHRU / kg / hr of Boots secretin and maximal flow rates were obtained when 16 or 32 CHRU / kg / hr were given. Pepsin secretion was evoked by 8 CHRU /

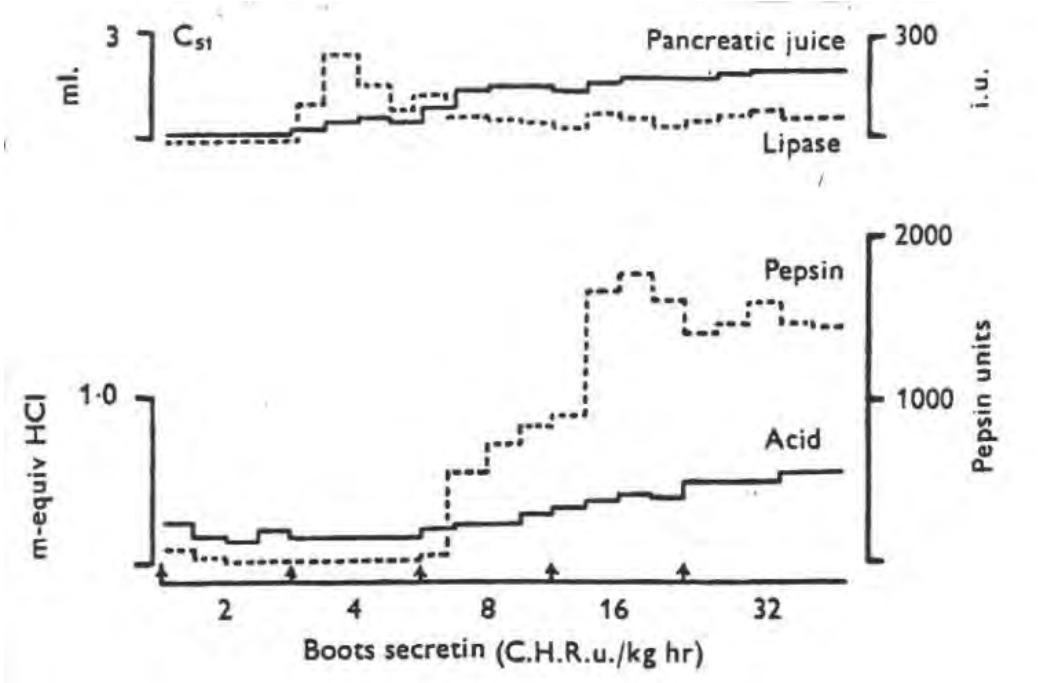

Figure 3.2 Gastric and pancreatic responses to a continuous intravenous infusion of Boots secretin in the anaesthetised cat. Reproduced from 1976 paper in J Physiol[56] (John Wiley).

kg / hr and doubled when the dose was doubled, but without a proportionate increase in acid secretion (**Figure 3.2**). By contrast, equipotent doses of GIH or synthetic secretin elicited no peptic response. A pharmacological dose of synthetic secretin, 16 CU /kg / hr, stimulated pepsin secretion but with blood-stained gastric aspirates, indicating mucosal damage[56].

The pepsigogic effect of Boots secretin could not be rationalised as an acid-sensitive cholinergic mechanism via the gastric antrum, because it was not inhibited by atropine. The addition of cholic acid in a concentration commonly present in Boots secretin did not stimulate pepsin secretion; neither did insulin or glucagon[57]. Prior and subsequent experiments showed that histamine, N-methyl histamine, pentagastrin, gastrin II and caerulein (decapeptide from the skin of the toad Hyla Caerulea, which shares the C-terminal tetrapeptide sequence Try.Met.Asp.Phe-NH_2 of gastrin and CCK) did not stimulate pepsin secretion in cats at doses that evoked near-maximal acid secretion[58]. The lack of pepsin stimulation by pentagastrin was confirmed in humans using a validated technique that involves infusion of the stomach with ^{14}C-labelled polyethylene glycol[59].

Intriguingly, further feline studies revealed that each acid secretagogue potentiated the peptic response to a dose of Boots secretin that was itself below the threshold for pepsin secretion[58, 60]. The possibility thus arose that a vasodilator substance in each acid stimulant increased the splanchnic circulation and thereby the concentration of co-administered secretin delivered to pepsin-secreting cells. This interpretation was supported by experiments wherein isopropylnoradrenaline was delivered retrogradely into the hepatic artery to increase gastric blood flow [57].

3.1.3 Comments

Boots secretin contains an additional substance which is a pepsigogue, possibly identical to the agent that had earlier been christened 'Gastrozymin'[61]. Moreover, the effect was seen at doses that are submaximal for pancreatic secretion. Although not directly relevant to pancreatic disease, these findings raised awareness that this or other substance in the impure Boots product accounted for the peculiar hepatobiliary responses noted in forthcoming SP tests.

3.2 Studies on Copper

3.2.1 Aspirates in SP tests

In 1977 the opportunity arose to analyse copper and zinc in aspirates of duodenal fluid during SP tests, as a fishing exercise. It seemed sensible to focus on copper, because there was no recognized link between copper and the pancreas. By contrast, zinc was known to participate in numerous enzymic processes connected to endocrine and exocrine pancreatic function.

Zinc output in post-secretin and post-pancreozymin fractions were found to be significantly lower in patients with chronic pancreatitis than in healthy controls, and correlated with enzyme output (JM Braganza & G Sturniolo, unpublished). This was not surprising given that 1-2 mg / day of zinc is now known to be secreted into the intestine via zymogen granules[62]. Reports since 2009 of subnormal zinc concentration in serum[63-65] or erythrocytes[66,67] of patients with chronic pancreatitis, in 1 report correlating with pancreatic hypofunction[67], are likely to represent loss of the normal facilitatory effect of pancreatic secretions on zinc absorption[68].

Copper in consecutive aliquots of duodenal aspirates was assayed by atomic absorption spectrometry[69]. Three groups were studied: 12 healthy volunteers, 12 patients with chronic pancreatitis and normal gall bladder function as confirmed by oral cholecystography; and 5 patients with end-stage disease and steatorrhoea who had been on pancreatic enzyme

supplements for 1-8 years, among whom 3 had undergone cholecystectomy for gallstones.

In the first subset of patients, diagnosis was principally by histology in 2 cases; pancreatic calculi in 4 cases; and reduced exocrine secretory capacity with (n=3) or without (n=3) abnormal pancreatograms in 6 patients who presented with relapsing pancreatitis and were investigated 10 weeks after the last relapse. Seven patients consumed excess alcohol (≥80 gm ethanol per day for at least 5 years). Two patients were on enzyme supplements, for 2 or 10 years.

Student's t test (2-tailed) was used to compare results between groups when variances were equal; otherwise, the approximate t test was used.

In controls, the high copper concentration in basal juice fell rapidly after secretin, whereas the output of copper was low in basal juice, increased sharply in the first 10 minutes after secretin, and then declined towards baseline before spiking again soon after pancreozymin (**Figure 3.3**). The pattern of copper secretion in the first subset of patients mimicked that in controls. However, in the post-secretin phase the volumes of aspirates were smaller but contained high concentrations of copper: the net result was an increase in copper output that was most obvious in the first period after secretin and only achieved significance in this sample. Copper outputs after pancreozymin did not differ significantly between patients and controls.

Further analysis of data for the first 10 minutes after secretin showed that outputs of bicarbonate and trypsin were lower in the patients ($p < 0.001$; $0.05 < p < 0.1$, respectively), who also displayed higher bile pigment concentration and output ($p < 0.005$; $0.05 < p < 0.1$, respectively). Although the profiles of copper and bilirubin after secretin were so similar, suggesting that bile was their common source, there was no correlation between these indices (r=0.11).

There were no differences in post-secretin outputs of copper among alcoholics and non-alcoholics, patients with or without diabetes, or those with calcific or non-calcific disease. It became apparent, however, that in the only 2 patients on long-term treatment with pancreatic extracts, the outputs of both copper and bilirubin were within normal limits. The subsidiary study of 5 patients on pancreatic supplements confirmed this impression: copper and bilirubin outputs were lower than in controls ($p < 0.005$, $p< 0.002$, respectively). The lack of copper and bilirubin responses to pancreozymin was explained by cholecystectomy in 3 cases[69].

3.2.2 Serum caeruloplasmin

The main carrier protein for blood copper is caeruloplasmin (synonyms copper oxidase; ferroxidase 1). This was measured in 43 patients with variable degrees of pancreatic hypofunction as gauged by the SP test (n=29), meal test (n=12), or the non-invasive BT-PABA / ^{14}C PABA test (n=2) when duodenal intubation was impossible (Chapter 2). Copper was analysed in a subset of 23 patients. C-reactive protein (CRP) was estimated to determine whether any elevation in caeruloplasmin might be a non-specific component of inflammation[70]. Serum samples were routinely assayed for alkaline phosphatase activity, protein, bilirubin, and aminotransferases.
The diagnosis of chronic pancreatitis was in the main by histology of resected specimens (n=17), pancreatic calculi on plain X-ray (n=12), unequivocal abnormalities in endoscopic pancreatograms (n=6), or clearly reduced exocrine secretory capacity in patients with compatible symptoms (n=8). Alcoholism was implicated in 21 patients; type IV hyperlipidaemia or pancreas divisum could account for 2 cases; but no explanation was forthcoming in the others.

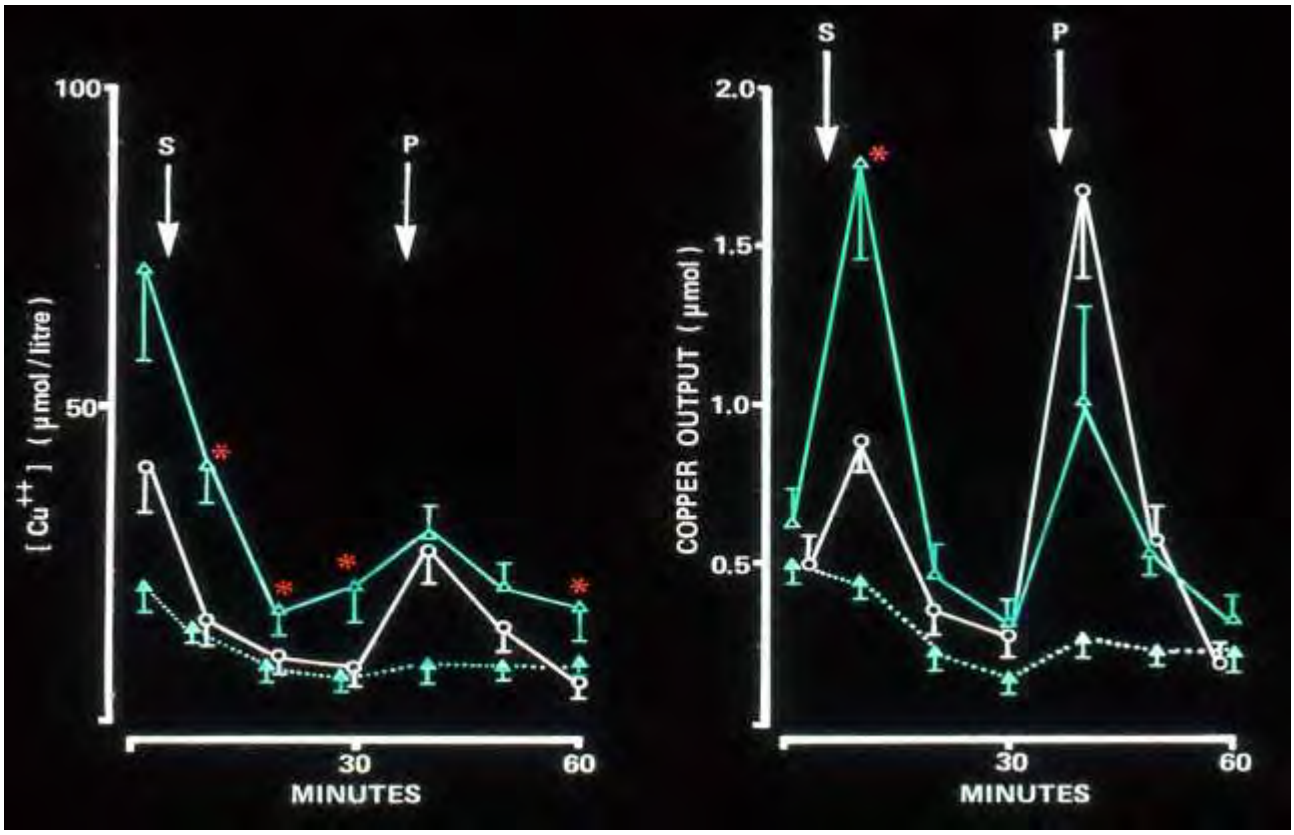

Figure 3.3 Concentration (left panel) and output (right panel) of copper in duodenal; juice during SP tests. Points are mean ± standard error for each group. Open circles = 12 volunteers; open triangles = 12 patients with chronic pancreatitis and normal gall bladder function; filled triangles = 5 patients on pancreatic supplements, of whom 3 had previous cholecystectomy. Asterisks indicate $p < 0.05$. Arrows show timing of secretin (S) and pancreozymin (P) injections. Reproduced from 1981 report in Clinical Science[69].

Cholangiography excluded biliary obstruction in 2 patients who were currently jaundiced. A previously jaundiced patient with distal constriction of the bile duct had undergone biliary by-pass surgery. Sclerosing cholangitis-like lesions were identified in a patient with ulcerative colitis. Normal ducts or subtle abnormalities were identified by ERCP in 16 of the remaining patients, all of whom had normal values for serum bilirubin and alkaline phosphatise. A detailed dietary history was obtained from each patient as also information on prescribed drugs such as oral contraceptives that might influence copper metabolism[71].

Thirty four patients were not acutely ill, had normal CRP and liver function profiles, and were on adequate diets. These were sub-grouped according to the degree of exocrine secretory impairment (**Table 3.1**). Serum caeruloplasmin activity was higher in patients with moderate dysfunction than in those with normal function, and higher still when pancreatic exocrine function was severely depressed (comparison by Student's t test, 2-tailed). However, values were low in patients taking pancreatic extracts.

A negative correlation was confirmed between the 30-minute output of bicarbonate after secretin or the 30-minute output of trypsin after pancreozymin and serum caeruloplasmin activity in patients who were not on pancreatic extracts (**Figure 3.4**), but not in 6 patients who received these long-term. In 3 patients with severe pancreatic dysfunction, serum caeruloplasmin activity was checked before and 6 months after supplemental extracts: the fall in serum enzyme activity after supplementation was significant

(mean ± SE: 0.66 ± 0.07 versus 0.48 ± 0.8, respectively, p< 0.05). There was a strong correlation between serum copper and caeruloplasmin activity in 23 samples where both items were measured (p<0.001)[72].

Serum CRP was elevated in 9 patients, including 3 with jaundice, of whom 1 also had arthritis and parotitis; a non-icteric patient with vitiligo and sclerosing cholangitis; another with a tumour-like mass in the head of the pancreas; 2 patients with infected pseudocysts; a patient tested soon after surgery; and a profoundly anorexic patient with a penetrating duodenal ulcer in whom serum caeruloplasmin was low at 0.28 units but CRP elevated at 6 units. Autoimmune pancreatitis was not known in the 1980s but in retrospect was a likely explanation in at least 3 of the group. There was no correlation between serum caeruloplasmin and CRP values.

3.2.3 Copper absorption

The possibility that pancreatic juice normally exerts a brake on copper absorption was investigated experimentally - with expert input from H Sharma of the Medical School's department of Medical Physics. Male Sprague-Dawley rats that had been maintained on a standard diet were denied food for 12 hours before the experiment. After anaesthesia by intraperitoneal urethane, a tracheostomy was performed, followed by laparotomy. The common

Table 3.1 Serum caeruloplasmin (copper oxidase) in chronic pancreatitis *

Pancreatic function	n	copper oxidase units		significance of differences	
No supplements	**26**				
A. Normal function	8	mean	0.423		
SP test	(5)	SD	0.058		
Meal test	(3)	SE	0.020		
				p<0.001	
B. Moderate dysfunction	10	mean	0.578		
SP test	(7)	SD	0.063		
Meal test	(3)	SE	0.020		
				p<0.005	0.05<p<0.10
C. Severe dysfunction	8	mean	0.680		
SP test	(7)	SD	0.084		
Meal test	(1)	SE	0.029		
Pancreatic supplements	**8**				
D. SP test	(6)	mean	0.355		
Meal test	(1)	SD	0.072		
PABA-^{14}C index	(1)	SE	0.025		

*Patients with normal values for serum C-reactive protein and alkaline phosphatase (ref 72).
Moderate dysfunction = preserved trypsin secretion after pancreozymin (peak value ≥ 26.4 IU / ml) but reduced bicarbonate output in 30 minutes after secretin (< 5.78 mmol) in SP test; or mean trypsin activity in meal test 10-24 IU / ml (normal ≥ 25 IU / ml). Severe dysfunction= reduced enzyme and bicarbonate in SP test; or mean trypsin activity in meal test < 10 IU / ml; or PABA excretion index < 0.30 (normal 0.76 at mean-3SD of control values).

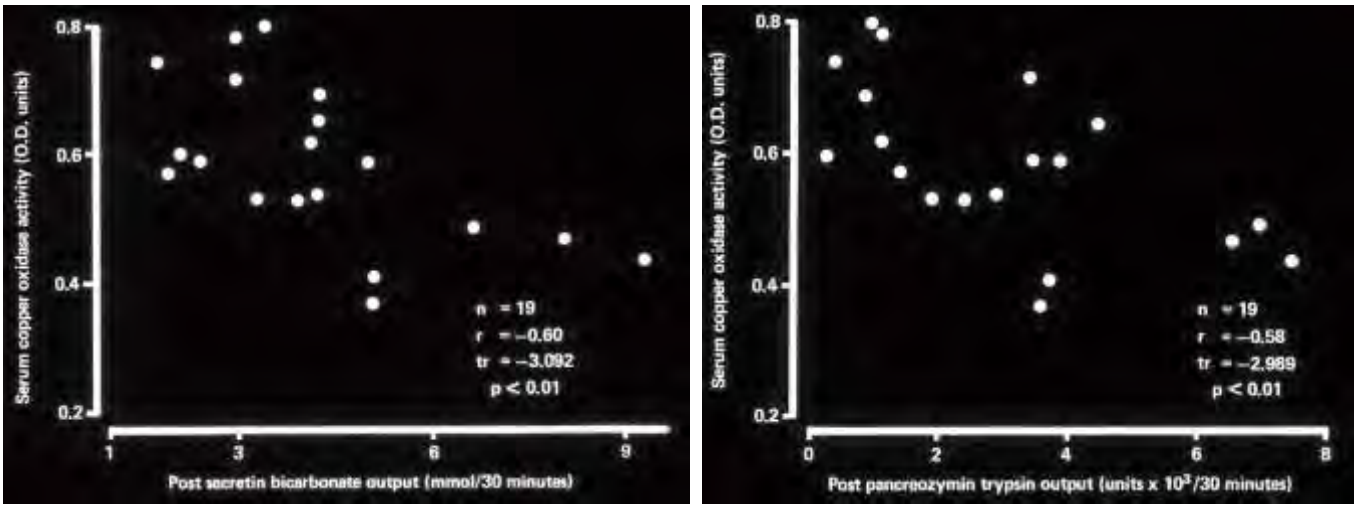

Figure 3.4 Relationship between post-secretin bicarbonate output or post-pancreozymin trypsin output and serum copper oxidase (caeruloplasmin) activity in 19 patients with chronic pancreatitis who were not taking enzyme supplements. Reproduced from 1981 paper in Clin Chim Acta[72]

bile-pancreatic duct was ligated adjacent to the duodenum, following which a closed duodeno-jejunal loop was constructed and flushed with 20 ml of warmed saline in order to purge it of pancreatic enzymes. Thereafter, a dose of 0.5 µmol (31.8 µg) of radioactive Cu (100 µg ^{64}Cu acetate with specific activity 0.5 mCi / mg in 0.25 ml saline) was mixed in a syringe with 0.25 ml saline or a test solution to simulate bicarbonate, pancreatic enzyme or bile-pancreatic fluid of rat pancreatico-biliary secretions. Pancreatic juice or bile (free of pancreatic juice, obtained by cannulating the common bile duct above the level of the pancreas) was collected from other rats and stored at -30º C until required. Pancreatic juice, bile and test solutions were analysed for electrolytes (sodium, potassium, chloride, bicarbonate), metals (magnesium, zinc, copper) and osmolality. The following additional measurements were carried out on pancreatic juice: total protein; activities of lipase, amylase, trypsin and chymotrypsin (the latter 2 after activation by enterokinase.

^{64}Copper activity in liver segments and blood were counted in a shielded gamma counter, while activities in the isolated duodeno-jejunal loop and carcass were measured by a scintillation probe. All counts were corrected for background activity by concurrent counting of appropriate standards. The sum of activities in liver, carcass and blood were then compared with the administered dose so as to estimate copper absorption for each experiment.

When ^{64}copper dissolved in saline was instilled into the loop, 4.92 ± 1.20 % (mean ± SD) of the dose was absorbed over 2 hours. This fell to 3.12 ± 0.77% when given in pancreatic juice ($p<0.005$), an inhibitory effect that was due to its protein rather than bicarbonate content, and was duplicated by bile (3.19 ± 0.46 %, $p<0.02$): the combination of bile and pancreatic juice did not further suppress copper absorption[73].

Next, clinical studies of copper absorption were mounted. This involved a computer-assisted deconvolution programme that generates net copper uptake from appearance and disappearance curves of ^{64}Cu in the non-caeruloplasmin fraction of serum after oral or intravenous doses on separate days. In 9 healthy volunteers who were not on any medication, net copper absorption within 10 hours of the oral dose in 350 ml water was 42.3 ± 9.7 % (mean ± SD). In 9 untreated patients with pancreatic hypofunction

due to chronic pancreatitis, the corresponding value was 35.9 ± 12.8%, not significantly different from controls. It was noted, however, that 3 patients on long-term treatment with pancreatic extracts had among the lowest absorption values[74]. Further studies in the rat duodenal-loop preparation showed, incongruously, that human pancreatic juice did not depress copper absorption, which was markedly inhibited by a 1:4 diluted sample of normal human bile[75].

3.2.4 Comments

An adult on a normal diet ingests 3-5 mg (47-79 µmol) of copper each day. A variable amount, on average 25%, is absorbed form the stomach and upper intestine. This fraction, loosely bound to albumin, is delivered to the liver where it is avidly taken up by hepatocytes and concentrated within cytosol and lysosomes. The molecular mechanisms involved in copper absorption, sequestration by hepatocytes, transfer to newly synthesised cuproenzymes therein, and copper disposal have now been characterised[76]. Two routes are available for copper discharge - bile which accounts for > 80%, and blood plasma where >90% is bound within caeruloplasmin. Biliary copper does not appear to be protein-bound: instead, in man, conjugated bilirubin is the identified ligand, for at least a portion. Since there was no reason to expect an increase in the unconjugated fraction as accompanies biliary infection, the lack of correlation between copper and bile pigment in the first clinical study (section 3.2.1) might indicate a non-synchronous effect of subclinical gall bladder dysfunction.

The high concentration of copper in basal aspirates during SP tests in patients with chronic pancreatitis, 18-189 µmol/l, could not be explained by spillage of gastric juice in that volumes were low, pH normal, and gastric juice has little copper, 1.57-6.29 µmol/l. It is equally unlikely that bile or pancreatic juice contributed, because concentrations of bile pigment and trypsin were low. Intestinal juice might have been the source.

The use of Boots secretin enabled recognition of increased biliary copper in untreated patients with chronic pancreatitis (**Figure 3.3**). Previous studies had shown that a contaminant in this product - rather than secretin *per se* - elicited bile acid, cholesterol and phospholipid secretion by a direct effect on hepatocytes[77, 78]. The effect could not be attributed to bile acids that contaminate some batches of Boots secretin[79], nor to CCK or glucagon that might be present[77].

There were 3 tentative conclusions. (i) As in the case of pepsin secretion, an unidentified agent in Boots secretin evokes the discharge from hepatocytes of copper into bile. (ii) Hepatocytes of patients with chronic pancreatitis are overloaded with copper. (iii) Inexplicably, long-term treatment with pancreatic extracts is associated with normalisation of copper data.

A quarter of a century later, studies from Poland[63], France[66], southern India[67] and South Africa[64] have confirmed the Manchester finding of high serum copper and caeruloplasmin levels in patients with chronic pancreatitis. More important is another study from India showing excessive levels of copper and iron but subnormal levels of zinc and selenium in pancreatic tissue of patients with chronic pancreatitis[65].

Both clinical studies at Manchester suggested that copper overload in the patients was driven by the degree of pancreatic secretory impairment, in the same manner that operates in the case of iron[80,81]. It was thus logical to view exocrine pancreatic insufficiency as the prime mover in any causal connection with copper overload; ie. that pancreatic secretions normally inhibit copper absorption. While this view was supported by rat experiments, it was discredited by clinical studies and later work using human pancreatic juice. In short, increased copper in bile, serum and pancreatic tissue of patients with untreated chronic pancreatitis could not be regarded as a homeostatic mechanism to compensate for increased absorption. A more sophisticated

explanation was needed for this finding and also for low levels of copper in bile and serum of patients on long-term treatment with pancreatic extracts.

3.3 Bilirubin hypersecretion

3.3.1 SP tests

Despite interest at Manchester in bile pigment behaviour during SP tests as a gauge of gall bladder function[82], an increase in post-secretin output of bilirubin in patients with chronic pancreatitis was not reported. A retrospective analysis of laboratory records was done to determine whether or not this was a new phenomenon. Tests were omitted at the outset if any of the following conditions applied: sub-standard potency of Boots secretin (Section 3.1); test technically unsatisfactory (eg. patient retching throughout, duodenal aspirate turbid); obstructive jaundice; severely restricted fat intake and / or prescription of pancreatic extracts for at least 6 months.

Sources and results of 87 tests are shown in **Table 3.2**. The distinction between acute and chronic pancreatitis was based on pre-set criteria (Chapter 2). Potential precipitants in the former group were gallstones (n=1), furosemide (n=1), hypercalcaemia (n=1), spree drinking (n=3), or type V hyperlipidaemia (n=1): the gall bladder was intact in each case. Among the latter group 25 drank excessive amounts of alcohol, whereas the disease was idiopathic in the others of whom 2 had ulcerative colitis but were not on steroids: 6 had undergone cholecystectomy. Nineteen healthy individuals had been enlisted in small groups over the years for quality control.

The icteric index (total bilirubin by colorometric assay) was used as a simple measure of bilirubin concentration in duodenal aspirates, having confirmed an excellent correlation between the two measurements in 175 random samples ($r=0.947$)[83]. Results were compared by Mann Whitney U tests (2-tailed), because numbers in most subgroups were too small to allow distributional assumptions. The pattern of changes in bilirubin secretion among controls and cholecystectomised patients after CCK conformed with expectation[82]. However, values in 30 minutes after secretin were higher in each group of patients with pancreatic disease (**Table 3.2**).

This change was evident in subsets with post-acute pancreatitis, chronic pancreatitis or pancreatic cancer. However, the pattern was different in that the bulk of the bilirubin response was in the first 10 minutes among patients in the first subset; whereas a lingering excess into the second and third collection periods after secretin in the other 2 groups indicated absence or dysfunction of the gall bladder (**Figure 3.5**).

Two other factors contributing to the wide variation in bile pigment responses to secretin were identified by consecutive secretory studies in the same patients under different circumstances. For example, in a young patient with idiopathic chronic pancreatitis, bile pigment in 30 minutes after secretin amounted to 10,664 units when assessed 6 weeks after a relapse, but 118,732 units 3 days after the next relapse: subnormal bicarbonate and trypsin outputs fell further in keeping with the pancreatitis flare-up (bicarbonate 3.86 and 2.25 mmol, trypsin 967 and 564 units, respectively). A similar pattern was found in 3 other patients. By contrast, in a middle-aged man with relapsing acute pancreatitis and type V hyperlipidaemia, bile pigment outputs were 32,744 and 6395 units / 30 minutes when assessed, respectively, 6 weeks after the last relapse and again in a quiescent phase following 12 months while off work and on a fat restricted diet with very little PUFA: bicarbonate and trypsin outputs now showed upward trends (bicarbonate 7.26, 9.38 mmol; trypsin 5125, 9614 units, respectively)[39, 83].

3.3.2 Comments

The unprecedented finding of increased bilirubin secretion after Boots secretin in patients with post-acute pancreatitis, chronic pancreatitis or

pancreatic cancer could not be dismissed as due to technical artefacts, CCK in the hormone preparation, variable 'washout', or spontaneous contraction of the gallbladder - because these considerations should apply equally to the group of volunteers and thus diminish rather than amplify differences between subgroups. Moreover, the uniformity of response argued against the phenomenon being driven by exocrine secretory capacity, which was preserved in patients with post-acute pancreatitis who displayed the highest bilirubin values in duodenal bile (**Table 3.2**). It seemed more likely that the finding reflected an increase in bilirubin production within hepatocytes, its release into bile yet another facet of Boots secretin's functional repertoire.

The metabolism of hemoglobin generates around 300 mg of bilirubin per day in adult humans. The unconjugated form, which has limited water solubility, is tightly bound to albumin within the

Table 3.2 Composition of duodenal fluid after Boots secretin

	Trypsin IU / 30 minutes	Bicarbonate mmol / 30 minutes	Bile pigment units / 30 minutes
Healthy controls (19)			
median	3220	8.27	5705
range	1764- 8622	4.45-17.00	483-14186
Cholecystectomy (10)			
median	4461	9.00	4084
range	3528-10759	5.87-20.92	1086-11896
significance	NS	NS	NS
Acute pancreatitis (7)			
median	↔ 4738	↔ 7.07	↑ 16722
range	2753-7081	3.07-11.25	4518-30324
significance	NS	NS	p=0.029
Chronic pancreatitis (44)			
median	↓ 2393	↓ 3.49	↑ 10707
range	0-9752	0.26-13.37	4902-27966
significance	p=0.021	p<0.001	p<0.001
Cancer of pancreas (7)			
median	↔ 3991	↓ 5.19	↑ 10189
range	396-7184	0.72-11.42	8288-30685
significance	NS	p=0.041	p=0.001

Outputs in duodenal aspirates from 87 individuals during 30 minutes after secretin. Significance of differences between test and control groups assessed by Mann Whiney U tests (2-tailed). Arrows emphasise direction of change from control values (ref 83).

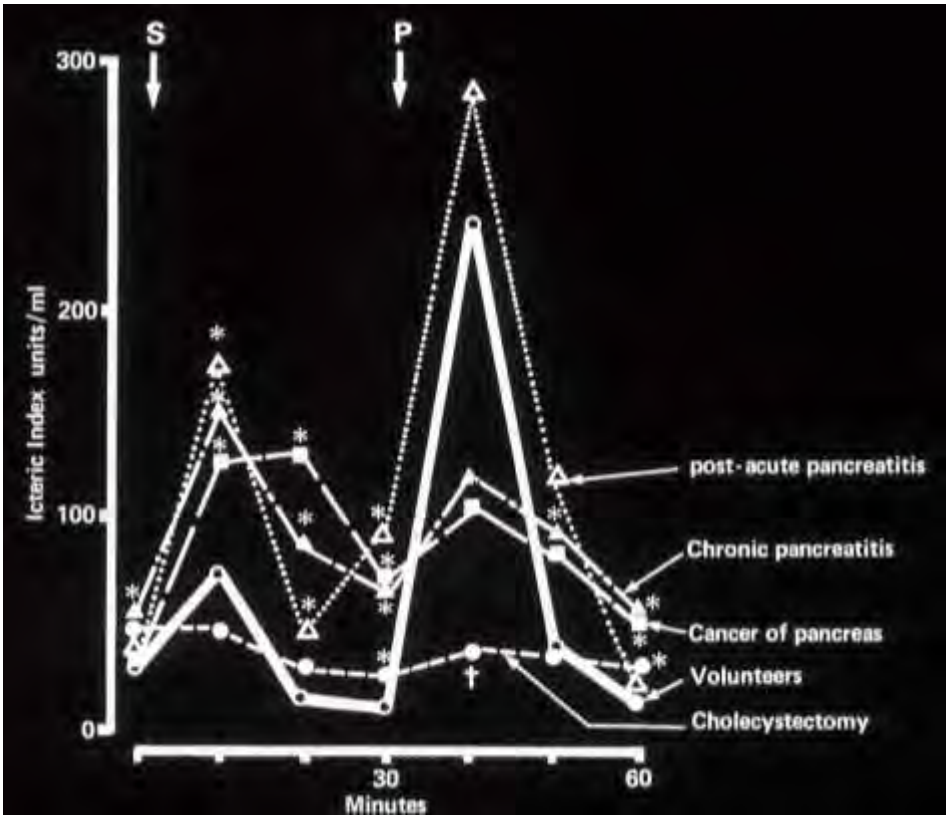

Figure 3.5 Icteric index of duodenal aspirates during SP tests. Arrows show secretin (S) and pancreozymin (P) injections. Asterisk or cross indicates significant increase or decrease, respectively, versus data in healthy controls. Numbers in groups as in Table 3.2. Reproduced from 1986 paper in Mt Sinai J Med[83]

bloodstream, and removed through uptake by hepatocytes. Once internalised, bilirubin undergoes biotransformation to facilitate a further increase in polarity by conjugation with glucuronic acid, glucose or xylose. Under physiological conditions the bulk of bilirubin in bile is in the diglucuronide form, which upon arriving in the intestine, is converted to urobilinogens and urobilins before excretion in faeces.

Thus in theory, an increase of bilirubin in hepatocytes could result from any of the following factors: increase in bilirubin load presented, as in haemolytic states; increased bilirubin uptake by hepatocytes due to increased blood flow; increased accumulation due to induction of glutathione-S-transferase B (GST-B, ligandin) and other binding proteins; or an increase in the fraction of bilirubin that is derived from hepatic heme, so called 'early bilirubin' and variously estimated at 23-37% of total.

In no patient was there any clinical or laboratory evidence of hemolysis (normal values for serum total and unconjugated bilirubin, urobiliin, reticulocyte count, haptoglobins), or any feature to suggest increased hepatic blood flow. Induction of hepatic proteins that are involved in the production and binding of bilirubin was a plausible explanation for several reasons. For example, the enhanced net uptake of bilirubin by the isolated perfused liver in phenobarbitone-treated rats is clearly due to induction of ligandin, which also binds other organic anions[84]. Moreover, it is well known that stimulation of the heme synthetic pathway is one of the earliest events following administration of this prototype inducer of liver proteins[85]. Unfortunately, applications for research support to investigate 'early bilirubin' were unsuccessful.

What might be the reason for the hitherto unreported increase in post-secretin output of both bilirubin and copper in patients with chronic

pancreatitis (while noting that patients with post-acute pancreatitis and pancreatic cancer also showed bilirubin hypersecretion and hence might therefore also have had increased copper outputs)? The answer was clearly not straightforward.

3.4. Introduction to free radical pathology

3.4.1 Pilot clinical study

In 1981, while reading the proceedings of a symposium on copper[86], during a train journey to present the inexplicable copper data at a London meeting of the Physiology Society, there was a 'Eureka moment' inspired by one of the discussants, TL Dormandy. Could the abnormal copper excretion and elevated serum caeruloplasmin in patients with chronic pancreatitis represent an excess of electron transfer reactions, popularly termed free radical activity (FRA), in the liver? Thus came about the seminal collaborative work described herein[87, 88], followed by independent studies at Manchester[89-91] and London[92-94] that further elucidated the findings.

Serial samples were obtained of duodenal aspirates (5 control patients, 4 chronic pancreatitis, 2 cancer), pure bile (3 controls, 4 chronic pancreatitis, 1 cancer, 1 recurrent acute pancreatitis with type 1 hyperlipidaemia), or pure pancreatic juice (2 controls, 3 chronic pancreatitis, 2 cancer) after consecutive intravenous injections of 2CHRU / kg of Boots secretin and 2CHRU / kg of Boots pancreozmin (Chapter 2). Pancreatic disease was excluded by normal ultrasound scan coupled with normal exocrine function test, endoscopic pancreatography or both studies in 5 control patients; and in 5 by laparotomy findings.

The diagnosis of chronic pancreatic was principally based on histological evidence (n=2), pancreatic calculi (n=6), or unequivocally abnormal pancreatogram (n=3) (Chapter 2). Among 7 patients with alcoholic disease, 3 had been teetotal for at least 3 months and were asymptomatic, while the other 4 as well as 4 with idiopathic disease were studied 1-12 weeks after admission with a relapse. Type 1 hyperlipidaemia caused recurrent pancreatitis in a patient with normal secretory test and pancreatogram. Of 5 patients with pancreatic cancer, 4 were well except for steatorrhoea and weight loss, but 1 was profoundly anorexic for 2 months: none drank alcohol excessively, and none had jaundice. Three controls and 2 patients with chronic pancreatitis had previously undergone cholecystectomy. None of the controls had any food fads; some of the patients with chronic pancreatitis and the patient with type 1 hyperlipidaemia were on low fat diets; and it remains possible that every patient with cancer ingested less food than normally.

Duodenal juice was aspirated in 10-minute fractions for 30 minutes after each hormone (Chapter 2). Bile or pancreatic juice was obtained by endoscopic cannulation of the ampulla of Vater: secretions were collected at timed intervals for 15 minutes after secretin, and 15 minutes after pancreozymin, weighed, divided into portions and snap frozen to -20ºC. All fluids were analysed within 4 weeks. In addition to the standard measurements (pH, bicarbonate, trypsin, icteric index), free radical oxidation products (FROPs) of an attack on PUFA were analysed. Three classes of products were identified as diene conjugates (DC), visible fluorescence (VF) and ultraviolet fluorescence (UVF). After exploratory work, analysis for thiobarbituric acid reactive substances (TBARs) to detect malondialdehyde, a product of lipid peroxidation, was abandoned because of the highly coloured complex formed between bile pigments and the acid.

Results in controls and patients were compared by Student's t test (2-tailed); associations among constituents of aspirates were examined by deriving correlation coefficients.

In duodenal juice FROPs were detected by all techniques but the high bile pigment

concentration interfered with the interpretation of VF. The pattern of change in DC (**Figure 3.6**) and UVF was broadly similar but not identical. FROPs declined progressively after secretin in controls, but increased in patients with chronic pancreatitis, although they secreted far less bicarbonate and trypsin ($p < 0.001$ for each). This finding and the higher icteric index of aspirates in the patients pointed to bile as the source of increased FROPs. The variable post-pancreozymin response in both groups reflected prior cholecystectomy in some. In 2 patients with pancreatic cancer that completely blocked the main pancreatic duct, irregular increases in FROPs greatly exceeded any level recorded in other participants, the spikes coinciding with spikes in icteric index. There was good correlation between UVF products and icteric index across the board ($r=0.798$, $p<0.001$).

In endoscopically collected bile both DC and UVF increased sharply after secretin in patients with chronic pancreatitis but not in controls (**Figure 3.7**). However, because of wide variations between individuals (concentrations much higher in 2 with idiopathic disease than 2 with alcohol-related disease), the difference in mean values did not attain statistical significance.

The lack of post-pancreozymin increase in these FROPs in the control group was unsurprising as 2 of the 4 had a previous cholecystectomy. In the anorexic patient with profound weight loss and a tumour in the neck of the pancreas the concentration of FROPs always exceeded the mean control value. Post-secretin samples in the patient with type 1 hyperlipidaemia contained high concentrations of FROPs: increased duodenal motility, as follows injection of pancreozymin (CCK), caused the cannula to fall back into the duodenum, and upon re-cannulation clear pancreatic juice was obtained in which no FROPs were found.

In pancreatic juice, low concentrations of FROPs were detected in a patient who was studied within a week of an alcohol-precipitated relapse: DC values ranged between 0.05 and 0.20 units throughout the test whereas UVF, usually 10-20 units, peaked sporadically to values between 50 and 80 units.

3.4.2 In vitro studies

High performance liquid chromatography (HPLC) centred on the identification of FROPs that cause DC in standard lipid extracts, with the following conclusions. In bile from control patients DC was low (< 0.25 absorbance units) and associated with several lipid fractions. In bile from patients with pancreatic disease total DC was 3-12 times higher after secretin, and was entirely accounted for by the extremely high DC in the phospholipid

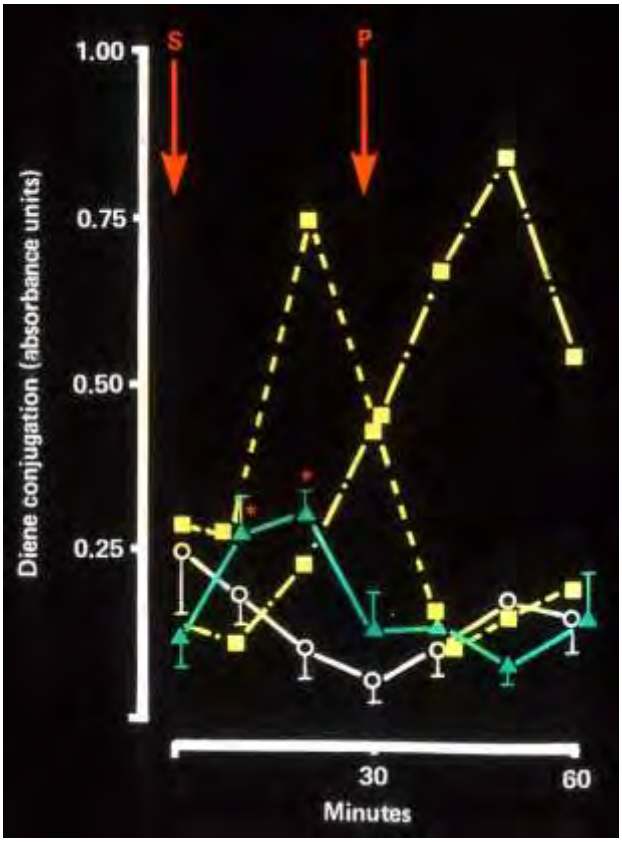

Figure 3.6 Diene conjugate products in duodenal aspirates during SP tests (mean ± SE) in 5 controls (open circles) and 4 patients with chronic pancreatitis (filled triangles). Asterisks indicate $p<0.05$. Also shown are the responses of a patient with cancer in the head of pancreas (hatched line and filled squares) and another with a tumour that constricted the bile duct (hatched with dots line and filled squares). Arrows indicate secretin (S) and pancreozymin (P) injections. UVF peroxidation products showed a broadly similar pattern Reproduced from 1983 Lancet publication [87]

(lecithin) fraction. Enzymic hydrolysis of this fraction yielded free fatty acids with the characteristic DC absorption. An extensive series of experiments established that these were indeed triggered by free radical oxidation - provided that linoleic acid was irradiated in the presence of albumin. The DC products then closely resembled the chromatographic pattern of biological material, whether bile or duodenal juice or serum[87, 88]. The predominant DC fatty acid *in vivo* proved to be an isomerised C18:2 compound, tentatively assigned as C18:2, *9 cis, 11 trans* linoleic acid (9,11 LA ') [88]. An automated HPLC method for bulk analysis of samples was developed in London[92].

A subsequent comprehensive analysis was done at Manchester on a sample of bile-laden duodenal juice after secretin stimulation from a patient with idiopathic chronic pancreatitis and exocrine pancreatic failure. This was shown by a combination of HPLC and capillary gas chromatography-mass spectrometry to be due mainly to 9Z, 11E-octadecanonoic acid, ie the *9cis, 11 trans* configuration[91].

A second approach of the *in vitro* work probed the activity of superoxide dismutase (SOD) in duodenal aspirates. In the pilot study this used the classical assay that involves xanthine / xanthine-oxidase / nitrotetrazolium[87]. The addition of either duodenal fluid or bile to the assay system increased the rate of generation of the superoxide anion free radical ($O_2^{-\bullet}$). In other words, the effect in the system was the reverse of the inhibition that is conventionally interpreted as SOD activity. Detailed studies confirmed that the effect was mediated by $O_2^{-\bullet}$, and that a similar increase could be obtained by adding pure bile acids or common detergents such as triton-x-100. Pancreatic juice did not stimulate $O_2^{-\bullet}$ generation, nor did it show SOD-like activity in this investigation[87], but in a later study was shown to possess a unique reoxidizing enzyme that requires the presence of hydrogen peroxide (H_2O_2) and is potentiated by bile[89].

3.4.3 Second clinical study

This focussed on total DC products, the specific DC isomer of linoleic acid, and UVF products in duodenal aspirates and / or sera from healthy volunteers (n=36) and patients who had sustained an attack of pancreatitis ≥ 6 weeks earlier and were subsequently classified as post-acute / RAP (n=20) or chronic pancreatitis (n=29). In the duodenal limb there were 11 volunteers, 15 patients with chronic pancreatitis and 10 with relapsing acute pancreatitis - among whom serum was also analyzed in 7 patients (chronic 3, acute 4). The serum-only limb involved 25 volunteers,10 with acute and 14 with chronic pancreatitis.

The diagnosis of chronic pancreatitis was based principally on histological evidence (n=5), pancreatic calculi (n=12), unequivocally abnormal pancreatograms (n=8) or impaired secretory function (n=4). Chronic alcoholism was documented in 9 patients, all of whom claimed to be drinking substantially less ethanol daily since the first attack. In the acute pancreatitis subgroup, 8 patients had previously undergone cholecystectomy for gallstones. In 2 patients (acute 1, chronic 1) attacks of pancreatitis continued despite restoration of normocalcaemia by parathyroidectomy. Detailed dietary histories established that most patients with acute pancreatitis but few with chronic pancreatitis had been on low fat diets for at least 6 months. Eleven healthy individuals participated in the duodenal intubation studies. Another group of 18 healthy subjects and 7 patients attending a clinic for minor surgery donated a sample for the serum study.

Assay methods for FROPs were as described in earlier reports[87, 92]. Data from analysis of duodenal juice were expressed as concentrations and outputs of FROPs in the first 10 minutes after secretin. In serum the concentration of linoleic acid (C18:2 9cis,12cis) and its specific DC isomer (9,11 LA ') were measured, and the molar ratio of

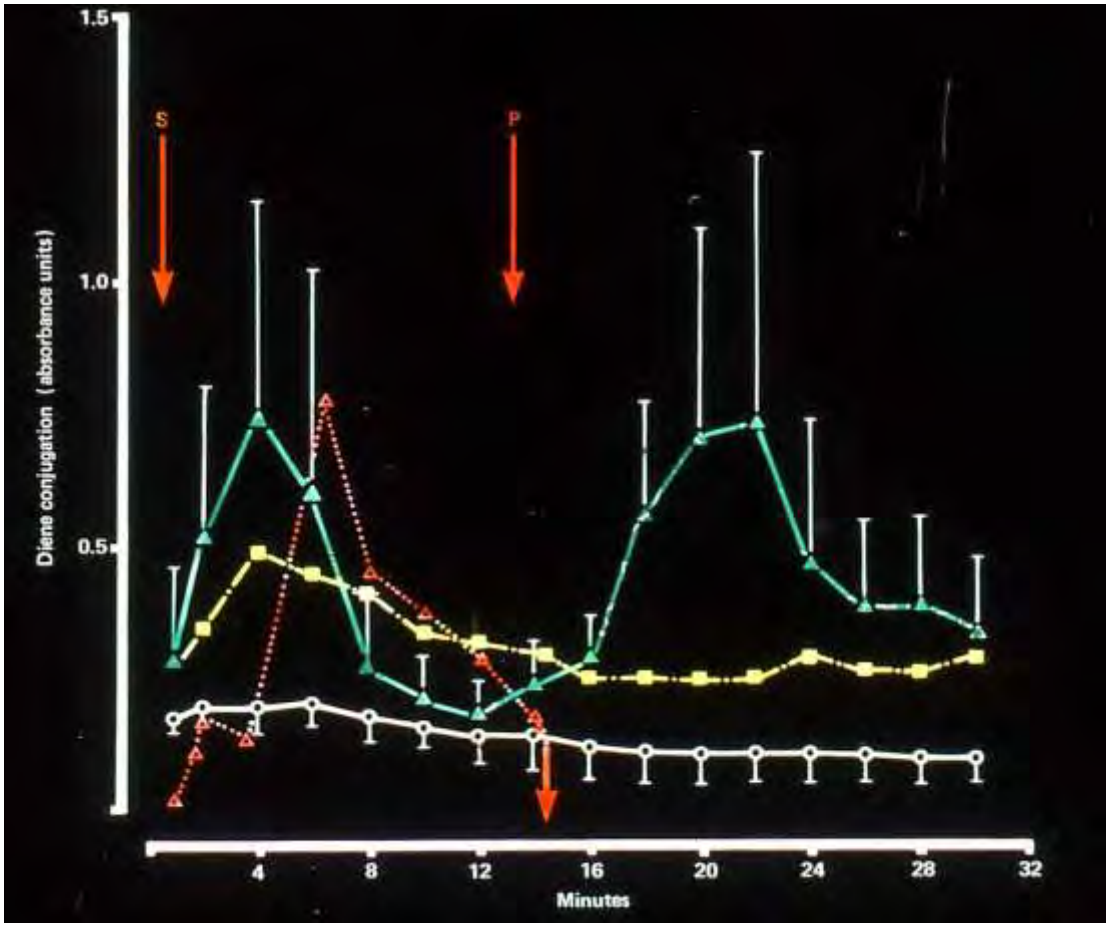

Figure 3.7 Diene conjugates in bile collected by endoscopic cannulation of the bile duct in 3 controls (open circles), 4 patients with chronic pancreatitis (filled triangles), a patient with hyperlipidaemia and recurrent acute pancreatitis (open triangles) in whom the cannula fell out at arrowed point, and an anorexic patient with cancer at the neck of pancreas (filled squares). Data as mean ± SE in the first 2 sets. Publication details as for Figure 3.6.

the isomer to parent fatty acid was derived (%MRLA').

Differences between values in different subgroups were compared by Student's t test (2-tailed), having confirmed that parameters were normally distributed. Differences between data on serum and duodenal juice from the same individuals were assessed by paired t tests (2-tailed). The Pearson correlation coefficient was used to examine relationships between different items.

In duodenal aspirates, standard analysis showed high bilirubin concentration and output in both sets of patients, in accordance with data in Section 3.3. Data on concentrations and outputs of FROPs are presented in **Table 3.3** and **Figure 3.8**, respectively. Expression of data as outputs increased discrimination between patients and controls. This was particularly true for the specific DC product, the output of which in 64% of patients exceeded the highest value in controls, versus 36% and 40%, respectively, for outputs of total DC and UVF substances.

Serum analyses showed that higher %MRLA' values in patients with acute pancreatitis than controls resulted from lower linoleic acid values (p= - 0.003), whereas higher %MRLA' in chronic pancreatitis reflected increase in the DC isomer (p=0.001) (**Table 3.4**).

Duodenal juice as well as serum was available in 7 patients with relapsing pancreatitis. Concentrations of the specific DC isomer and parent fatty acid were around 3 times lower in

Table 3.3 Lipid oxidation markers in duodenal fluid after Boots secretin

	C (n=10) Group1	AP (n=11) Group 2	CP (n=15) Group 3	Significance of difference 1vs2	1vs3	2vs3
9,11 LA' µmol / l	39.2±9.95 (9.40-111)	84.6±14.9 (31.5-152)	89.9±17.2 (34.8-237)	0.023	0.020	NS
9,12 LA µmol / l	839±146 (296-1526)	1550±175 (578-2354)	2492±435 (812-5920)	0.006	0.003	0.062
Molar ratio %	4.66±0.58 (2.43-8.20)	3.58±0.76 (2.06-9.07)	5.58±0.35 (1.80-5.97)	NS	NS	0.041
DC units / ml	0.17±0.03 (0.07-0.42)	0.28±0.04 (0.12-0.45)	0.37±0.06 (0.12-0.95)	0.060	0.008	NS
UVF units / ml	16.7±4.10 (1.05-39.0)	21.2±2.55 (10.0-35.0)	40.0±8.02 (5.60-112)	NS	0.016	0.036

Data as mean ± standard error (ranges in parentheses) of values in the first 10 minutes after secretin injection. C=controls; AP=acute pancreatitis; CP=chronic pancreatitis (From ref 90)

Table 3.4 Linoleic acid and its main oxidation product in serum

	C (n=25) Group 1	AP (n=10) Group 2	CP(n=14) Group 3	Significance of difference 1 vs 2	1 vs3	2 vs3
9,11 LA' µmol/l	23.9±2.21 (8.00-51.8)	24.2±2.19 (8.80-42.2)	35.5±3.75 (16.5-74.9)	NS	0.013	0.015
9,12 LA µmol/l	1103±71.2 (407-1823)	798±61.7 (392-1244)	935±57.6 (420-1448)	0.003	0.074	NS
Molar ratio %	2.21±0.16 (0.81-3.90)	3.22±0.37 (1.98-6.89)	3.89±0.34 (1.63-6.29)	0.02	0.001	NS

9,11 LA' = free radical-mediated isomer from attack on linoleic acid (9, 12 LA). Data as mean± standard error with ranges in parenthesis. C=controls. AP= acute pancreatitis, CP=chronic pancreatitis (From ref 90).

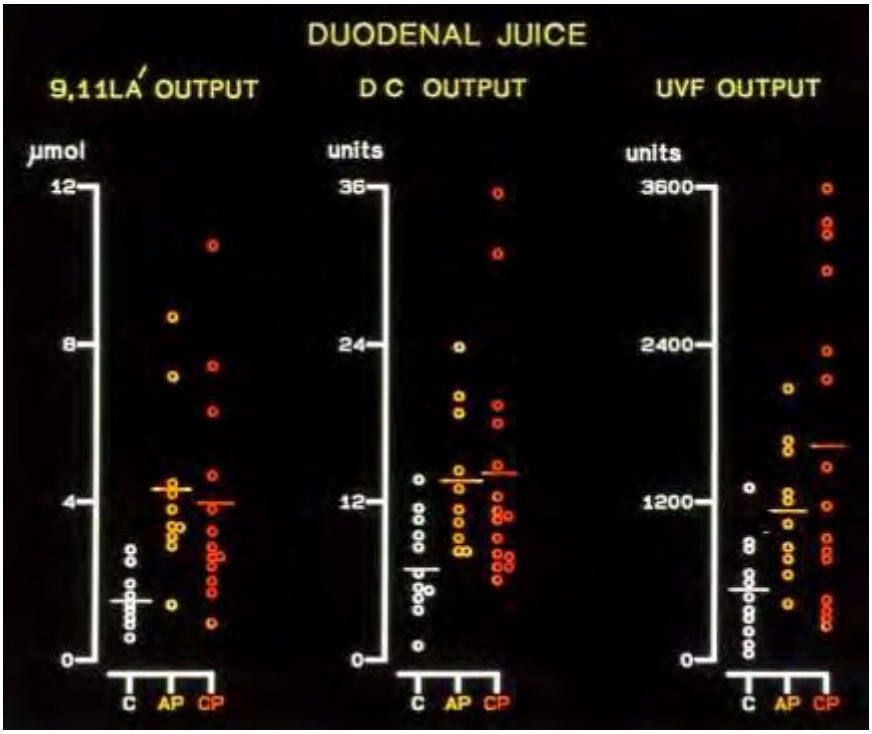

Figure 3.8 Outputs of free radical oxidation markers in duodenal aspirates collected in the first 10 minutes after injection of Boots secretin. Horizontal bars indicate mean value in control (C), post-acute pancreatitis (AP) or chronic pancreatitis (CP) sets. See text for details. Reproduced from 1990 paper in Free Radical Biol Med[90].

serum but these differences were ironed out when data were expressed as %MRLA'. There was good correlation between levels of the isomer in both fluids but this was less impressive for linoleic acid and non-existent for %MRLA', the serum value of which bore no relationship to DC or UVF in duodenal aspirates[90].

3.3.4 Comments

Free radicals (denoted $^{\bullet}$) are chemical species that are capable of independent existence and characterised by one or more unpaired electrons in their chemical structure, which imparts inherent instability[95, 96]. Molecular oxygen is itself an example by virtue of 2 unpaired electrons in its outer orbitals. Hence it is a good oxidizing agent, readily absorbing electrons from the molecule that it oxidizes (eg. chlorine) whereas a reducing agent such as sodium is an electron donor.

The bulk of oxygen undergoes single-step quadrivalent reduction, ie. electron gain, catalysed by cytochrome oxidase, but up to 10% is deliberately passaged via univalent step-wise reduction to yield ROS, including oxygen free radicals. The pathway generates successively $O_2^{-\bullet}$, then H_2O_2 which is not a free radical but the source of the next and highly reactive product, $OH^{\bullet}$, before dissipation to water. Moreover, these primary ROS interact with halides (eg. to generate hypochlorous acid, HOCl, the equivalent of household bleach that is essential for phagocytosis); nitrogen, to spawn a range of reactive nitrogen species (RNS) that participate in the inflammatory response[97]; sulphur; carbon; nucleic acids, and other chemical species before dissipation to water.

Arguably, every intracellular organelle has the capacity to generate ROS because they play physiological roles in vital processes as diverse as signal transduction in polarised cells; mitochondrial respiration; hydroxylation of lipophilic substrates, both endogenous (eg. cholesterol , bile acids, bilirubin) and exogenous (xenobiotics) by microsomal CYP; the production of disulphide bonds (S-S) that are indispensable for protein folding in the ER[98]; and perhaps above

all, in assuring that cells have a finite lifespan (as opposed to immortality, the hallmark of neoplasia)[99]. Transition metals in the extracellular milieu are very important because their variable valency allows them to undergo one-electron changes in oxidizable state. Thus iron can exist in the ferrous (Fe^{2+}) or ferric (Fe^{3+}) state. Similarly, copper has 2 common valencies, cuprous (Cu^{+}), and cupric (Cu^{2+}), such that under appropriate conditions copper salts can accept electrons from or donate electrons to $O_2^{-\bullet}$ .By contrast, zinc with only one valency, and manganese with its stable valency state do not promote free radical reactions[96].

Checks and counterchecks are built into biological systems to prevent an excess of electron transfer reactions which can wreak havoc. Within cells structural compartmentation is the key deterrent as it keeps apart the ingredients necessary for free radical generation[95]. So too is the enzyme SOD which enhances production of $O_2^{-\bullet}$ and thence H_2O_2, so as to deliver $OH^{\bullet}$ into the jaws of glutathione peroxidase and catalase. These preventive devices are buttressed by micronutrients that break the chain of free radical reactions. In the extracellular space, iron-binding (eg. transferrin, ferritin, lactoferrin) and iron-oxidising (eg. caeruloplasmin) proteins curb ROS production, while many substances (eg. bilirubin, mucin, urate, albumin, glucose, ascorbic acid) scavenge $OH^{\bullet}$ and / or other potent radicals[100].

A pathological burst of electron transfer reactions in experimental studies is readily detected, eg. by electron spin trapping or chemiluminescence. This is not generally the position in clinical practice where one looks for the tell-tale footprints ('markers','fingerprints')[101]. The best characterized of these are FROPs of an attack on lipids. When PUFA are subjected to free radical oxidation, a complex, often fast-changing constellation of FROPs is generated. Some of these have absorption or fluorescence characteristics that are products of well-defined molecular configurations (**Figure 3.9**)[90], albeit not defining chemical structures. However, numerous attempts to mimic the in vivo situation by subjecting model substrates to a free radical-generating source, typically ultraviolet irradiation, were wholly unsuccessful – not least in relation to DC products with the molecular re-arrangement that conveys stability.

HPLC data from the initial study described herein explains this failure and justifies a number of firm conclusions. (i) After stimulation with secretin, patients with chronic pancreatitis secrete into bile large amounts of an oxidized phospholipid that is virtually absent from the bile of control patients. (ii) This phospholipid is hydrolysed in the duodenum, the free fatty acids retaining the particular DC absorption of the parent compound. (Iii) The pattern can be replicated by ultraviolet irradiation of PUFA in vitro, but only in the presence of protein, albumin in particular. From subsequent exhaustive analysis, the following schema could be advanced for free radical attack on linoleic acid (C18:2 9cis,12cis), DC products in italic.

(1) Free radical initiation	C18:2 (9 cis,12 cis)	→ C18:2$^{\bullet}$ + H$^{\bullet}$
(2) DC rearrangement	C18:2 (9 cis,12 cis)	→ *C 18:2*$^{\bullet}$
(3) Protein absent; classical peroxidation	*C 18:2*$^{\bullet}$ + O_2	→ *C 18:2-00*$^{\bullet}$
(4) Protein present: stabilised non-DC isomerisation		
(a)	C18:2$^{\bullet}$ + protein	→ C18:2 (9 cis,12 trans) + protein$^{\bullet}$
(b)	C18:2$^{\bullet}$ + protein	→ C18:2 (9 trans,12 cis) + protein$^{\bullet}$
(5) Protein present: stabilised DC isomerisation		

(a) C 18:2• + protein → C 18:2• (9 cis ,11 cis) + protein•
(b) C 18:2• + protein → C 18:2• (9 cis, 11 trans) + protein•
(c) C 18:2• + protein → C 18:2• (10 cis,12 cis) + protein•
(d) C 18:2• + protein → C 18:2• (10 trans, 12 cis) + protein•

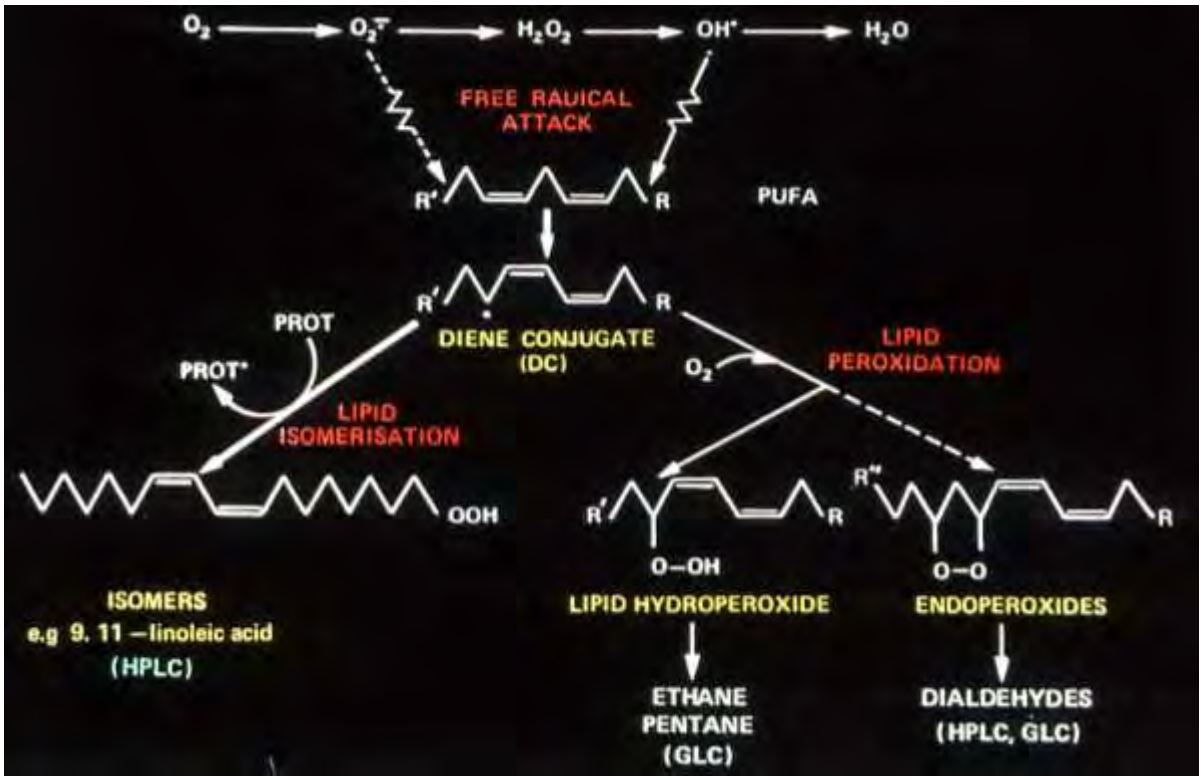

Figure 3.9 Schematic representation of the pathways of free radical attack on polyunsaturated fatty acids (PUFA). See text for explanation. Publication details as for Figure 3.8.

It could thus be concluded that the interaction between free radical-attacked linoleic acid and albumin results in isomerisation of linoleic acid without oxygen addition, and not in classical lipid peroxidation. The preference for a single isomer in duodenal bile and serum (5b above) - corroborated by a study using gas liquid chromatography with mass spectroscopy [91] - strongly suggests enzymic involvement, such as by linoleate isomerase of Butyrivibrio fibrisolvens in the rumen[102]. It is possible that unidentified bacteria in human bile / duodenal juice might be so endowed, such that this factor coupled with excessive ingestion of foods rich in the isomer rather than a pathological excess of tissue FRA accounted for the clinical observations[103]. This seems highly unlikely as controls would be expected to imbibe more calories in general, and that no pathogen was identified on bacteriological analysis of samples in the second clinical study. Moreover a different class of FROPs, as detected by UVF (**Figure 3.8**) was also higher in the patients. Around 15 years after these UK studies, excessive FRA in bile from patients with chronic pancreatitis was noted in an investigation from the USA which used the far better peroxidation markers of 4-hydroxynonenal and F_2-isoprostanes[104]; and also in a study from India which used the crude TBARs assay on endoscopically-aspirated samples of duodenal juice[105].

3.5 Overview and Summary

Collectively, data presented in this chapter highlight the huge impact of chance in biological

research. Of note in the present context was the fortunate use of Boots secretin which exhibited extra-pancreatic activities that are not inherent in the pure hormone; thereafter the opportunity to discover excess copper in Boots secretin-stimulated bile from patients with chronic pancreatitis; then the meticulous SP test register which showed that bilirubin hypersecretion after Boots secretin was a long-standing but overlooked feature of chronic pancreatitis, post-acute pancreatitis and pancreatic cancer; and finally, a meeting of minds with the pioneer of free radical pathology in clinical medicine.

A degree of lipid peroxidation associated with a low level of FRA is now recognised as a normal concomitant of ageing and cell turnover[96, 99, 106] . A more specific explanation, however, must account for the substantially higher concentration of a peroxidized phospholipid in bile of patients with pancreatic disease. Allowing for the possibility of as yet unknown free radical processes in the human liver, CYP and more generally systems involved in oxidative detoxification could be regarded as the likeliest source[107, 108]. The absence of overt liver disease does not detract from this assumption. Provided that the damaged lipids can be eliminated and the lipids replaced, the potential problem need not be clinically manifest. Of note in this regard, Boots secretin had been shown to increase the activity of microsomal CYP and also bilirubin UDP-glycosyl-transferase enzyme systems in rat liver[109].

Not known at the time of these studies, elegant *in vitro* work using linoleic acid-hydroperoxide as substrate has revealed the hydroperoxide-degrading effect of copper-conjugated bilirubin complexes - quite possibly a physiological function[110].

In summary, the traditional use of Boots secretin at Manchester unmasked excessive biliary copper, bilirubin and FROPs in patients with chronic pancreatitis while demonstrating abnormalities too in patients with post-acute pancreatitis and pancreatic cancer. It is sobering to reflect that this progression could not have occurred after 1990 when the Boots product was withdrawn.

Chapter 4

Pancreatic Disease: Casualty of Hepatic 'Detoxification' Reactions?

The challenge now was to design a framework that could integrate the shared excess of bilirubin and FROPs in bile from patients with chronic pancreatitis, post-acute pancreatitis and pancreatic cancer - and to accommodate the additional aberration of biliary copper excess in chronic pancreatitis (not investigated in acute pancreatitis or pancreatic cancer).

4.1 Clues

4.1.1 Shared rising trend

Records disclosed that 3-times more patients with chronic pancreatitis were admitted to the Manchester Royal Infirmary in 1975 compared to 1955, the trend amplified in the next decade (**Figure 4.1**); whereas age at presentation had fallen markedly (HT Howat & JM Braganza unpublished). The pattern suggested the promoting effect of some commonplace environmental factor(s), which might also underlie the rising trend of acute pancreatitis[111] and pancreatic cancer in the UK[112].

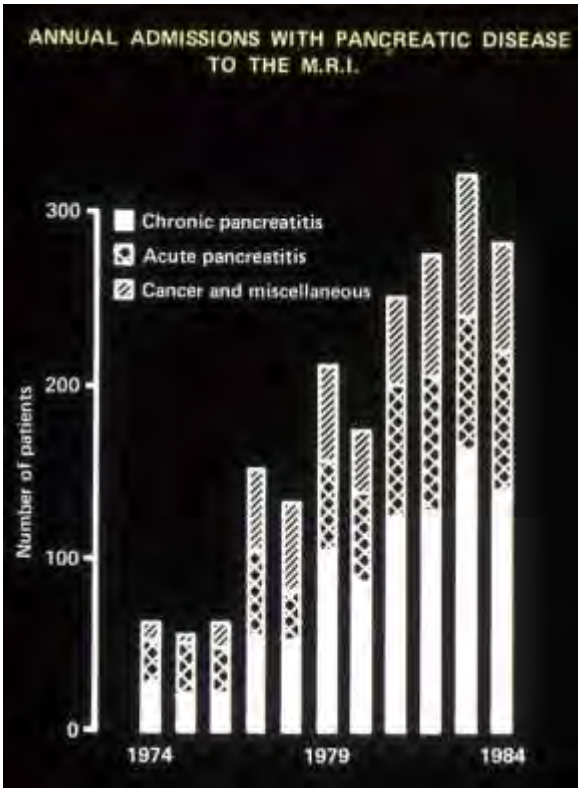

Figure 4.1 New admissions of patients with pancreatic disease between 1974 and 1984.

Studies from London[113] suggested that increased per capita consumption of alcohol was the explanation in chronic pancreatitis, a view supported by a later study from Southampton[114]. However, around 50% of Manchester patients in the 1980s were not 'alcoholic', a substantial number teetotal. Moreover, a focus on alcohol in isolation could not explain why < 10% of alcoholics develop the disease; the 18-year delay to the first symptom in alcoholics who do; the absence of a lower threshold for the pancreatic toxicity of ethanol in comprehensive studies from Marseilles; and the lack of an animal model based on alcohol alone (Chapter 2). An excess of dietary fat had been viewed as a potential promoter in both chronic pancreatitis and pancreatic cancer and thought to act at least in part via excessive release of CCK[12, 112].

4.1.2 Shared xenobiotic factors

Environmental lipophilic compounds (xenobiotics) were implicated to greater or lesser extent in the etiology of chronic pancreatitis (alcohol, cigarette smoke constituents, dietary nitriles, drugs such as azathioprine), pancreatic cancer (cigarette smoke constituents, coffee, industrial volatile chemicals, alcohol), and acute pancreatitis (industrial chemicals, many prescribed drugs, alcohol)[9].

4.1.3 Shared bile reflux

The strong association between gallstones and acute pancreatitis was interpreted as a mechanical phenomenon, namely, that temporary occlusion of Oddi's sphincter during a stone's migration allows reflux of abnormal bile into the pancreatic duct directly, or indirectly from the duodenum when stone passage causes the sphincter of Oddi to malfunction.

The implication that the pancreatic duct system is the primary target in acute pancreatitis resonated with the prevailing ductal-origin theories for chronic pancreatitis[12] and pancreatic cancer[112]. Thus, reflux of a biliary carcinogen could explain why most cancers are located in the head of the gland, as also its predilection to inflammatory enlargement in chronic pancreatitis should a biliary toxin gain access. In the latter context, the presence of bile pigment throughout the duct system had been noted in surgical specimens[115] (also HB Torrance & JM Braganza unpublished).

4.2 Cytochromes P450

Lipophilic substrates, whether endobiotics (eg. bile acids, bilirubin) or xenobiotics, are processed by CYP hemoproteins - formerly called mixed function oxidases. The enzyme system is ubiquitous in foetal life, but in adults is largely concentrated in the liver and organs such as the adrenal gland that have specialised roles in lipid metabolism. Numerous CYP families and subfamilies are recognised today, depending on preferred substrate[116]. Several cellular organelles display CYP activity, but microsomes are especially endowed within hepatocytes.

Many facets of CYP function are noteworthy. (i) CYP capture and utilise molecular oxygen to hydroxylate the substrate, in the process generating small amounts of ROS that mediate low-grade lipid peroxidation which is needed for vital biological processes[95, 107]. (ii) The yield of ROS increases upon 'enzyme induction', a phenomenon that might be accompanied by an increase in microsomal mass - leading to non-specific increase in manufacture of the cell's normal products. (iii) CYP induction is facilitated by an increase in dietary linoleic acid (C18:2)[117], or more highly unsaturated ω_3 fatty acids: these are incorporated into microsomal membranes where they are thought to exert a permissive effect by holding CYP-substrate complexes in active conformation[118]. (iv) The inducibility of certain CYP is under genetic control[119]. (v) Importantly, although the primary role of CYP in metabolising xenobiotics is to ensure their 'detoxification', certain compounds inadvertently undergo bioactivation, ie. the CYP-engendered RXS is more toxic than the parent compound. (vi) The products of hepatic xenobiotic metabolism are excreted in bile and / or discharged into the bloodstream, depending on polarity, charge and other factors[120]. (vii) CYP induction involves a mechanism to dispose of haem that is in excess of that required for augmented CYP synthesis, namely, up-regulation of haem oxygenase, a heat shock protein which increases the production of hepatic bilrubin (via biliverdin) as also of ferritin and carbon monoxide: the enzyme is transported in plasma and is a potent antioxidant, as also are the 3 heme degradation products.

Theoretical considerations and experimental findings exposed 2 potential threats from chronic CYP induction. The first is the increased yield of lipid peroxidation products, which are not only among the most cytotoxic of substances, but also known to generate powerful bioactive agents, including key regulators of the inflammatory response and carcinogenic compounds. The second relates to the increased yield of RXS - paracetamol hepatotoxicity being a case in point[121-123].

4.3 Hypothesis: Pancreatic disease is a casualty of liver 'detoxification reactions'

- Alcohol is implicated in chronic pancreatitis: it is also a potent inducer of CYP.
- Cigarette smoke is implicated in pancreatic cancer: it also generates RXS.
- Industrial fumes yield RXS and are linked to cancer and acute pancreatitis.
- Corn oil facilitates pancreatic cancer from CYP-activated nitrosamines [15, 124].
- Inheritance influences chronic pancreatitis / cancer, as also CYP inducibility.

It seemed highly unlikely that these parallels could represent coincidence, a consequence of pancreatic disease, or epiphenomena. Instead,

the overlap between factors that impact on CYP inducibility and those that were known or suspected in the etiology of exocrine pancreatic disease suggested a cause-and-effect relationship. Hence it was proposed that acquired disease of the exocrine pancreas (excluding acute pancreatitis associated with gall stones) represents hepatic 'detoxification' reactions that have gone awry - mediated by way of reflux into the pancreatic duct of RXS in bile[9]. This concept would also rationalise the biliary aberrations documented in Chapter 3.

4.4 Testing the concept

Many avenues for exploration were opened up, with emphasis on patients with chronic pancreatitis in the first instance. These included: use of drug probes to assess the status of CYP and related pathways; detailed drug, occupational and social histories; study of patients with epilepsy on anticonvulsant CYP inducers; investigation of 'Bantu' and 'tropical' chronic pancreatitis; animal models based on CYP induction and priming by PUFA; and so on.

Early encouragement came from a report in 1986 concerning the decline in peptic ulcer disease - not directly related, but relevant[125]. The increased frequency of chronic pancreatitis in the 25-year period from around 1955 in the UK could now be seen to follow by about 10 years a marked increase in the consumption of linoleic acid as documented in the USA: when intake between 1909-1913 was considered as 100%, values were around 125%, 150%, 215% and 280% in 1940, 1950, 1970 and 1980, respectively.

Chapter 5

Drug Metabolism and Ancillary Studies

Once in the bloodstream, a xenobiotic traverses the plasma membrane of the hepatocyte along the same pathway as does the endobiotic bilirubin, and then binds to cytosolic glutathione S transferase B (GST-B, synonym ligandin) **(Figure 5.1**)[126], whereby it is chaperoned to microsomes to be processed if necessary by the CYP isoform to which it is best matched. Metabolism by CYP typically occurs in 2 phases. In phase 1, which generates ROS, the xenobiotic is hydroxylated to yield a more polar intermediate metabolite, which is then rendered even more hydrophilic by phase II conjugation reactions that, in the main, involve glutathione (GSH), glucuronic acid or amino acids. The combined operation is designed to yield a detoxified water-soluble product that can be excreted into bile or returned to blood for renal excretion. The prior administration of a host of substances results in CYP induction, ie. an increase in the specific activity of the enzyme relative to microsomal protein. This phenomenon, when evoked by agents such as phenobarbital or ethanol, may be accompanied by expansion of the smooth endoplasmic reticulum (SER), resulting in augmented manufacture of normal products, eg. triglycerides and phospholipids that are discharged, respectively, into the bloodstream or bile. These facts governed the choice of investigations reported in this Chapter.

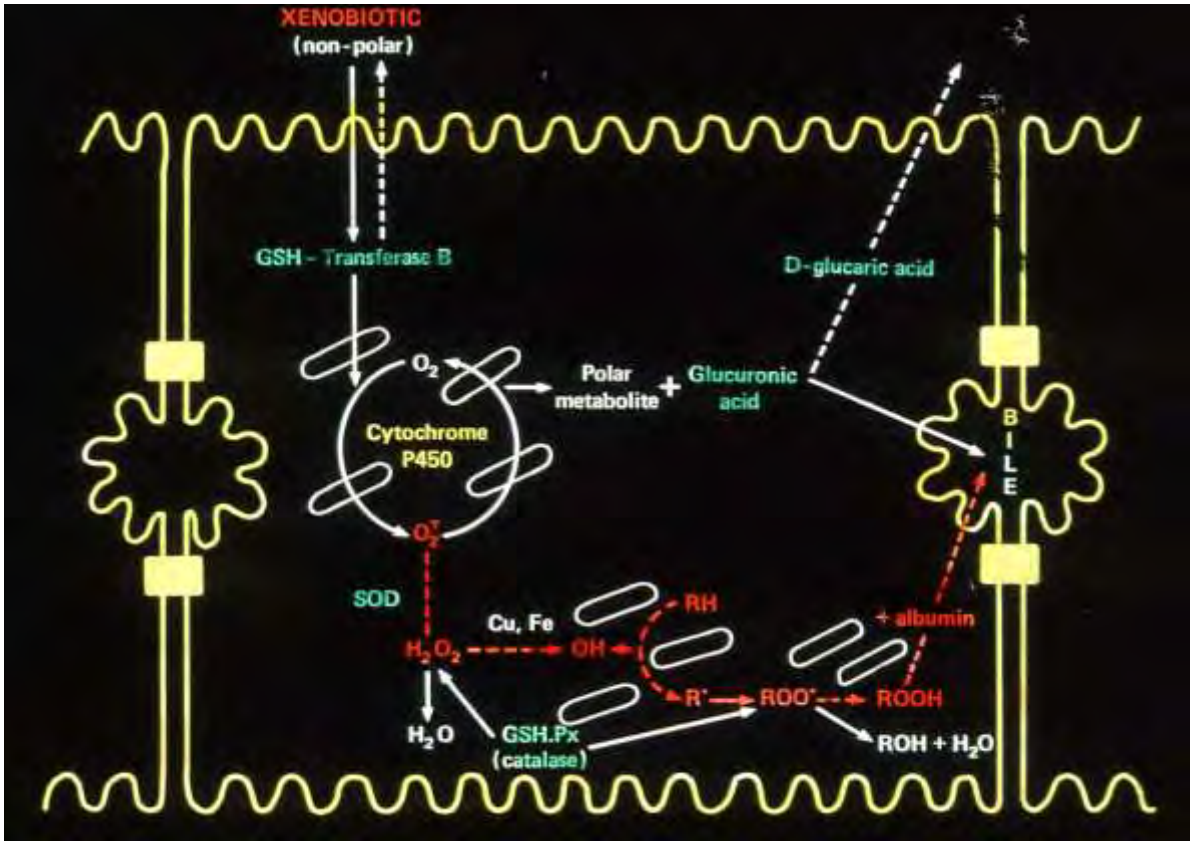

Figure 5.1 Schematic representation of the metabolism of a xenobiotic within the hepatocyte. O_2 =molecular oxygen; $O_2^{\cdot}$ = superoxide radical; H_2O_2 hydrogen peroxide, $OH^{\cdot}$ hydroxyl radical, R-H unsaturated fatty acid in cell membranes, ROOH lipid peroxides, GSH glutathione. Reproduced from 1987 paper in Clin Chim Acta [126]

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5.1 Routine tests

5.1.1 Bromosulphthalein

Organic anions such as bromosulphthalein (BSP) follow the same general path as outlined above. Hence disappearance kinetics following an intravenous dose of BSP have long been used as a surrogate to probe hepatic dysfunction: loss of hepatocytes as in advanced cirrhosis delays the early-phase clearance of BSP, whereas intrahepatic cholestasis impedes late-phase clearance.

Thirty patients with exocrine pancreatic disease were studied, but the results of 4 tests were later omitted, because of interference in the BSP assay (n=1) or erratic data (n=3). The diagnosis of chronic pancreatitis in 14 patients was mainly based on the presence of pancreatic calculi (n=4), unequivocally abnormal endoscopic pancreatograms (n=9), or grossly reduced bicarbonate output after secretin (n=1). When ductal morphology and exocrine secretory capacity were normal, patients who had experienced ≥ 1 attack of pancreatitis were classified as post-acute / RAP (n= 9). Fifteen of the 23 patients with either type of pancreatitis were on normal diets; 8 adhered to low fat diets for at least 3 months. Three patients had pancreatic cancer. Of these, one was fit and asymptomatic 18 months after a Whipple operation and was prescribed a supplement of 30 gm medium-chain triglycerides daily with reduction in long -chain fat. Two patients with cancer were anorexic for 3 months. Comprehensive information is given in the study report[127].

The liver was palpable in several patients (**Table 5.1**), but none had the stigmata of chronic liver disease, 1 was icteric, and 3 had undergone biliary bypass surgery to relieve constriction of the distal bile duct. Endoscopic cholangiography revealed subtle abnormalities in 11 cases (Chapter 6). Medical conditions that may be linked to pancreatitis were found in a few patients; several were heavy smokers; and some consumed excessive amounts of alcohol and/or caffeine -containing beverages.

The test was done exactly as described by its pioneers and interpreted by reference to their control data, as was routine hospital practice[128]. After an overnight fast, the recumbent patient was given an intravenous injection of 5 mg BSP / kg body weight over the course of 30 seconds. Blood samples were collected from the opposite cubital vein via an in-dwelling catheter at 3, 5, 7, 10, 15, 20, 25, 30, 35, 40 and 45 minutes after start of the injection which constituted time zero. Plasma BSP was measured by a standard spectrophotometric method for which, as was long recognised, measurements were adequately reproducible at plasma values > 3 mg BSP / ml plasma, but unreliable at lower concentration.

BSP disappearance curves were analysed by the peeling-off technique (**Figure 5.2**), wherein measured BSP concentrations in each test were plotted against time on a semi-logarithmic scale. The uncorrected initial disappearance constant, K_i, was derived by fitting a straight line through at least 3 of the 4 points between 3 and 10 minutes after the injection. The second exponential, K_2, was obtained in the same way, using measurements between the 30 and 45- minute time-points. If a nearly perfect fit of at least 3 points was unobtainable, the test was omitted (n=1).The corrected initial disappearance rate constant, K_1, was derived by plotting the numerical differences between the early plasma concentrations and the extrapolated straight line used to determine K_2. The corresponding points were re-fitted by a straight line through at least 3 of the first 4 points. The half-life ($T_{1/2}$) for uncorrected and corrected disappearance rates and for K_2 were read off the graph and the respective constants calculated by the formula K (% / min) = (0.693 x 100) ÷$T_{1/2}$. Student's t test (2-tailed) was used to compare results with those of 26 controls in the reference publication[128].

Table 5.1 BSP study: clinical information

ID	Age	Sex	Presentation	Possibly relevant factors	Liver	Bile duct	Albumin
	Bil	AP	ALT yGT	TG			
	(years)						g/l
	µmol/l	u/l	u/l u/l	mmol/l			
							(38-48)
	(22)	(100)	(40) (65)	(1.8)			
PB*	20	F	RP	?	-	N	38
	10	71	17 7	0.7			
AB	35	M	RP (C)	alcohol/cigarettes	+	A[1]	42
	11	128	59 135	4.9			
MB	54	F	RP(C)	cigarettes/tea	+	N	36
	10	48	38 29	1.6			
AC	57	M	RP (C)	alcohol/cigarettes	+	A[1]	33
	10	29	59 316	1.4			
NE	64	M	steatorrhoea	cigarettes /caffeine	+	N	32
	10	116	35 450	2.0			
HG*	78	M	RP	printing works/cigarettes/tea	-	N	40
	11	50	22 30	1.6			
BH	21	M	RP	?	-	N	39
	15	70	21 19	1.7			
LM	31	F	RP	Crohn's/steroids/azathioprine	+	A[2]	32
	10	50	86 91	2.0			
MP*	15	M	RP	?	-	N	38
	14	105	127 8	1.5			
MR*	54	F	pain	renal transplant/steroids/azathioprine	+	A[3]	31
	13	110	20 32	3.7			
ER*	47	M	pain (C)	alcohol/cigarettes/caffeine/pethidine	+(S)	A[2]	35
	8	165	88 71	1.1			
PT	35	F	diarrhoea	ulcerative colitis/steroids	++(S	A[3]	34
	20	485	111 180	2.1			
JH	49	M	steatorrhoea	cigarettes/phenobarbitone/caffeine	++	A[2]	29
	19	297	400 1290	2.7			
GH*	30	M	RP/jaundice	alcohol/pethidine	+	A[1]	31
	40	159	137 523	1.4			
AH	72	F	RP	gallstones/caffeine	-	N	42
	19	76	14 110	1.9			
AF	19	F	pseudocyst	alcohol	+	N	40
	17	94	28 30	1.8			
EH*	42	F	RP	?	-	A[2]	38
	10	74	21 6	2.0			
AN	59	M	pancreatitis	diabetes	++	N	41
	16	34	39 26	4.6			
MB*	51	F	RP	?	-	N	38
	13	62	23 10	1.5			
GJ	26	M	RP	?	-	N	38
	15	50	58 23	3.5			

GK	46	F	RP		?	-	N	37
	8	83	16	18	1.3			
MC	29	M	RP		diabetes	+	Caroli	38
	10	96	74	21	2.1			
DL	54	M	RP,arthralgia		cigarettes/caffeine	++(S)	A^3	40
	12	202	68	365	3.6			
JH*	48	M	metastases		chemical works/cigarettes/caffeine	++	N	32
	17	141	195	255	2.9			
EW*	37	F	pain,weight ↓		caffeine	+	N	30
	13	49	71	200	2.6			
DW*	64	M	steatorrhoea		caffeine	+	N	38
	8	72	25	9	1.5			

Top set= chronic pancreatitis; middle set=acute pancreatitis; bottom set=cancer of pancreas. *low fat diet for >3 months; RP relapsing pancreatitis; C calculi; +/- liver palpable or not; (S) spleen palpable; cholangiogram : N normal; A^1 bile duct constricted in head of pancreas, A^2 attenuated intrahepatic biliary ducts, A^3 irregular calibre of intrahepatic ducts; AP alkaline phosphatase; ALT alanine transferase; yGT gamma glutamytranspeptidase; TG triglycerides. Figures in brackets are the reference range for albumin;, upper limits of normal for other serum constituents (From ref 127).

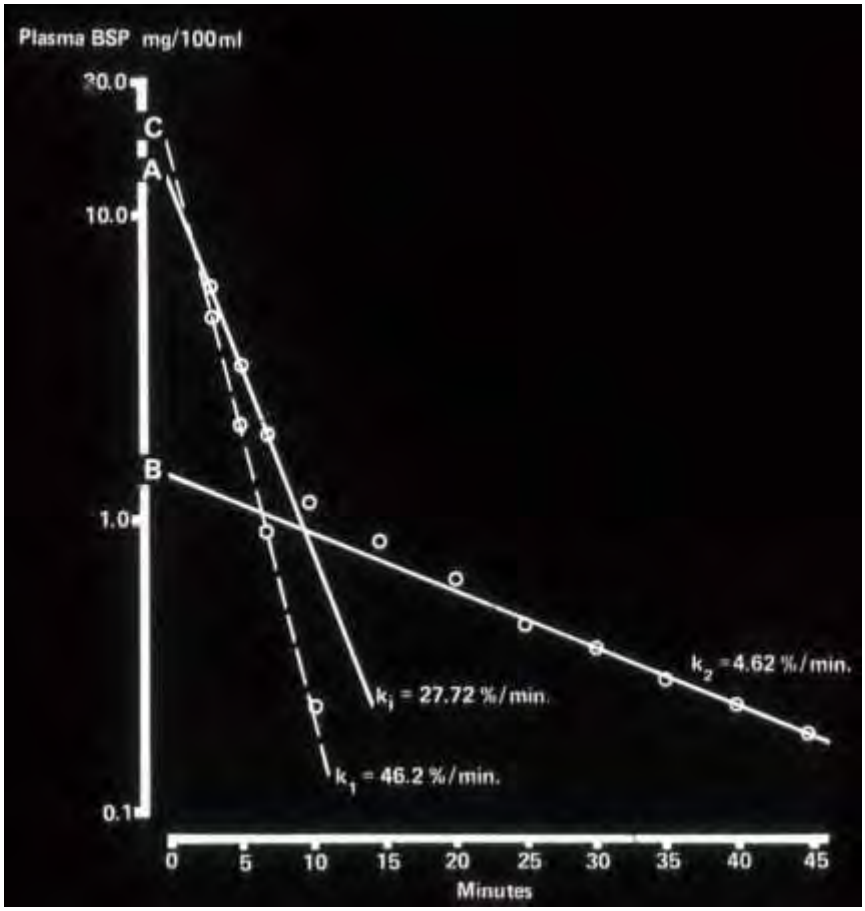

Figure 5.2 A representative plasma disappearance curve after BSP injection. A, B and C are extrapolated zero time intercepts of lines that describe early and late constants. Further details in text. Reproduced from 1984 paper in Clin Chim Acta[127].

Reference values (mean ± SD) with number of observations in their derivation were as follows: K_i, 12.6 ± 1.6 % / min (n= 26); K_1, 14.3 ± 1.5 % / min (n=16); K_2, 5.3 ± 1.9 % / min (n=16). By comparison, respective values in patients with pancreatic disease were 15.48 ± 1.7% ($0.05 < p < 0.10$), 26.06 ± 12.78 ($p<0.001$), and 3.12 ± 2.43 % / min ($p < 0.005$), ie. the net early- phase disappearance of BSP from plasma was higher but late-phase disappearance lower in patients than controls. There was no difference in subgroups with chronic or post-acute / RAP.

The corrected initial disappearance rate constant K_1 was above the 95% upper limit of the reference range, 117.3% / min, in 19 of the 26 patients studied (**Figure 5.3**). Among 7 patients with normal values, 6 were on low fat diets and a patient with cancer was profoundly anorexic (**Table 5.1**). The uncorrected constant K_i was below normal in 5 patients (**Figure 5.3**): there was evidence of liver disease in 3 of them (tender hepatomegaly in a chronic alcoholic, hepatomegaly plus jaundice, multiple liver metastases) but not in the other 2 cases. K_2 was reduced in 8 patients (**Figure 5.3**) including 2 with questionable data (ie. BSP concentration between 30 and 45 minutes < 2 mg / l). Primary biliary cirrhosis was identified in a patient with elevated serum IgM and antimitochondrial antibody. Liver biopsy was done in 3 of the other 5 cases: changes of biliary cirrhosis were found in 2, and sclerosing cholangitis-like changes in the third. The 2 patients who did not have a liver biopsy were chronic alcoholics with disturbed liver function tests (**Table 5.1**).

5.1.2 Serum triglycerides

This ancillary study was prompted by findings of hypertriglyceridaemia and / or elevated gamma-glutamyl transpeptidase (γGT) activity in several patients (**Table 5.1**) - given that both changes have been reported in association with hepatic 'enzyme induction'[129].

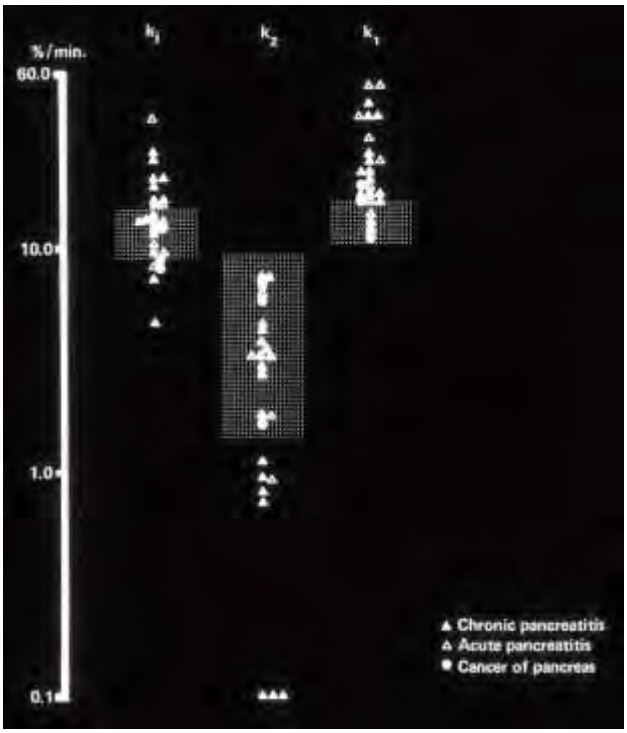

Figure 5.3 BSP clearance constants in patients with exocrine pancreatic disease. Dotted areas represent the published reference ranges (2SD on either side of the mean). Publication details as for Figure 5.2.

Accordingly in a separate investigation, 33 consecutive patients who were referred after an attack or relapse of pancreatitis ≥ 6 weeks earlier (chronic 23, acute 10) had an intravenous fat tolerance test to determine capacity to metabolise triglycerides[130]. Fasting hypertriglyceridaemia was present in 14 patients (42 %), including 9 with chronic and 5 with acute pancreatitis; the highest value was 6.2 mmol / l; and no patient had hyperchylomicronemia at the time of testing. In this subgroup, 4 displayed impaired triglyceride clearance (29 % of those with hyper-triglyceridaemia, representing 12 % of the whole cohort), and hence might be vulnerable to a further attack of pancreatitis due to massive hypertriglyceridaemia under certain conditions[131]. Although average triglyceride clearance in the other 10 patients was less than in patients with normal serum triglycerides ($p < 0.05$), values were within the reference range and did not correlate with serum triglyceride levels.

5.1.3 Comments

BSP in plasma, tightly bound to albumin, traverses the space of Disse and is internalised by a carrier mechanism that probably involves albumin receptors on the plasma membrane of hepatocytes. Once in the cytosol, it binds preferentially to GST-B, which also binds hormones, drugs and azo-dye carcinogens[132]. The concentration of GST-B within hepatocytes increases in response to chemicals that cause SER proliferation. Binding of an anion reduces its reflux into plasma, which in the case of bilirubin amounts to around 30 % of that initially taken up into hepatocytes. Hence, although the amount of ligandin seems to have no direct effect on influx rate, the higher its concentration the lower the rate of efflux, such that the net uptake of an anion is enhanced[84].

After GSH conjugation, BSP is actively excreted across canalicular membranes, so that about 60-100% of an injected dose is recoverable in bile within 2 hours[133, 134]. Extrahepatic elimination is negligible when the liver is functioning normally, but increasingly important in cholestatic states or when large doses of the dye are given. Reduced hepatic clearance of BSP has been recorded in obese, pregnant or elderly people, those with pyrexia, or when serum albumin is very low[135, 136]. With 1 exception (HG in **Table 5.1**), the study patients did not fall into those categories.

It is agreed that K_2 in the BSP disappearance curve (**Figure 5.2**) reflects maximal canalicular transport capacity, and is thus reduced in patients with primary biliary cirrhosis[128]. Judging by K_2 data, canalicular transport mechanisms were intact in the majority of patients with pancreatic disease (**Figure 5.3**), which accords with increased concentrations in bile of substances generated in hepatocytes (Chapter 3).

The finding of increased K_1 in patients with pancreatic disease was both new and not easily explained. There was no clinical feature to suggest increased hepatic blood flow. Induction of ligandin, and hence reduced efflux of BSP seemed the best explanation, based on experimental evidence[137] and also on clinical studies using another anion, indocyanine green, in patients on enzyme-inducing drugs[138].

An increase in hepatic microsomal mass in response to enzyme induction could also rationalise hypertriglyceridaemia and increased serum γGT (over-and-above that due to hepatocyte dysfunction) in both studies**.** Thus, in the first study potential enzyme inducers included cigarette smoke, alcohol, theophylline-containing beverages, steroids, azathioprine, phenobarbitone, and occupational chemicals (**Table 5.1**): in a few patients an inducer was not identified, but an excess of dietary PUFA might have played a part, in that 4 patients with normal K_1 values were on low fat diets for 6 months or more, and also because PUFA-enriched diets facilitate enzyme induction in experimental studies[117].

There is thus a 2-way relationship between hypertriglyceridaemia and pancreatitis, ie. as a consequence, or potentially causal. In regard to the latter, the ancillary study of lipid clearance suggested that a connection is indirect - an epiphenomenon of some other process[130]. Hepatic enzyme induction fits the bill.

5.2 Antipyrine, theophylline, debrisoquine

5.2.1 Pilot study 1983 / 1984

Analysis of the microsomal fraction in liver biopsy material from animals provides a simple way to detect CYP induction and characterise the sub-type involved, but this approach is clearly inapplicable as a routine measure in man. The disposal of the drug antipyrine was known to afford a broad gauge of CYP induction in humans[139]; moreover, it was accepted that antipyrine pharmacokinetics derived from blood or salivary sampling are effectively identical[140], and also that a genetic influence could be exposed, as in relation to CYP inducibility by phenobarbital[141]. The usefulness of theophylline to detect induction

by polycyclic aromatic hydrocarbons (PAH) of a narrow CYP sub-family - nowadays classified as CYP1A[116] - was under exploration[142, 143]. The disposal of debrisoquine was emerging as a marker of genetic influence on drug metabolism[119]. However, whereas cigarette usage or anticonvulsant drugs were known to augment antipyrine disposal[139, 144], the effect of anti-convulsants on theophylline disposition was debated[145, 146], and the combined effect of smoking and anticonvulsants had not been examined in man. Specialist knowledge of this background by JB Houston of the University Department of Pharmacy guided the studies[147].

Thirty two consecutive patients with pancreatic disease who required admission during a 9-month period were investigated unless any of the following criteria applied: age > 75 years; a prescribed drug that was known to inhibit CYP; overt liver disease; asthma; cardiac failure; renal failure. The aim was to detect hepatic CYP induction, whether across all isoforms (antipyrine test) or the specific isoform induced by PAH (theophylline test).

The diagnosis of chronic pancreatitis in 22 cases was mainly based on pancreatic calculi (n=7), unequivocally abnormal pancreatogram (n=12) or reduced exocrine function (n=3) (Chapter 2). Most of these patients presented with relapsing pancreatitis, and were assessed between 6 weeks and 14 months after the last episode: 4 were dependent on narcotic analgesics at the time of referral, and 1 had painless steatorrhoea. Distal bile duct constriction was excluded by endoscopic cholangiography in all patients. Post-acute / RAP was diagnosed in 6 patients with normal pancreatogram and pancreatic function test at least 6 weeks after the most recent attack: none of these patients had gallstones. Four patients had pancreatic cancer that did not obstruct bile drainage.

A questionnaire was devised to facilitate recognition of known or suspected risk factors for acute pancreatitis, chronic pancreatitis and pancreatic cancer (Chapter 2). Often, the patient's social circumstances had changed on medical advice, redundancy or redeployment. In order to facilitate interpretation of pharmacokinetic data, an *a priori* search was made for recognised CYP inducers in each patient, to cover the 6-month period preceding testing, because it was known that the clearance of theophylline may remain elevated for some time after cessation of exposure to inducers in cigarette smoke[148]. To this end, detailed dietary histories were taken with special emphasis on intake of fat, charcoal-broiled beef, caffeine, and Brassica vegetables: the information was supplemented by a 7-day weighed dietary record (completed by the patient or immediate relative) at home in the week following the tests. An arbitrary scoring system was implemented to quantify these factors, as also alcohol and cigarettes. Patients were questioned about exposure to workplace chemicals, and all drugs taken on a regular basis were recorded.

Patients were admitted to the hospital's programmed investigation unit for antipyrine, theophylline and debrisoquine tests, together with any pancreatic investigation that was deemed necessary, and dietary assessment. These investigations required 2 consecutive weeks, the drug studies done in the same week and commencing around 6 am. For the antipyrine test, the patient voided urine, and then drank an aqueous solution of the drug (10 mg / kg body weight): peripheral venous blood was drawn pre-dose and 3, 6, 9, 12, 27, 30 and 36 hours thereafter; the volume of the 48 hour urine collection was measured and an aliquot retained for analysis. All caffeine containing foods and beverages were withdrawn at least 48 hours before the theophylline study. After voiding urine, the patient received theopylline elixir (2.4 mg / kg) and blood was sampled at 90 minute intervals for 10.5 hours. For the debrisoquine test, a 10 mg tablet was administered, urine collected for 8 hr thereafter, and the 'debrisoquine metabolic ratio'

determined, ie. % dose excreted unchanged / % excreted as 4-hydroxy-debrisoquine, while recognising that the 'extensive metaboliser' phenotype (ratios 0.2-8.0) is overwhelmingly represented in healthy Britons[119]. Samples were stored at -18 º C until analysed by HPLC.

Full details of the biochemical methods have been published[149, 150]. After linearity of response and coefficients of variation were deemed satisfactory, the biological half-life ($T_{1/2}$) for antipyrine or theophylline was calculated from the slope of the log-concentration / time curve, following linear regression. To calculate drug clearance (Cl), the dose was divided by the area under the concentration curve which was determined by the log-trapezoidal rule with appropriate extrapolation to infinity. Volume of distribution (V) was calculated from dose divided by extrapolated drug Cl at time zero.

It was decided at the outset that controls would be kept to a minimum for the pilot study, expanding the number should a definitive investigation be warranted at a later date. Having confirmed that there was no difference between antipyrine data from 14 healthy volunteers studied by serial saliva sampling and 7 studied by plasma analysis, the information was amalgamated. This gave the following antipyrine referent values for the group of 21 healthy controls, aged 20-41 years: $T_{1/2}$ mean 11.6, median 11.5, range 8.3-16 hr; Cl mean 45 ml, median 49 ml, range 23-64 ml / kg / hr. Theophylline tests were done in only 7 controls, age 23-41 years, yielding $T_{1/2}$ mean 6.2, median 6.5, range 4.9-8.2 hr, and Cl mean 72, median 76, range 50-106 ml / kg / hr. None of the volunteers was on any drug; most drank alcohol socially (up to 8 pints of beer or 8 short drinks per week); all drank tea and coffee (up to 6 cups altogether per day); and the majority did not smoke while a few used < 5 cigarettes / day.

Data in patients and controls were compared using Mann Whitney U tests: differences were considered significant when $2p < 0.05$. Relationships between various measurements were examined by Kendall's method.

There was good correlation between $T_{1/2}$ and Cl of each drug (antipyrine $\tau = 0.50$, $p<0.01$; theophylline $\tau = 0.64$, $p < 0.01$). However, for both probes, $T_{1/2}$ was lower and Cl correspondingly higher in each subgroup of patients than in controls (**Table 5.2**). This 'induction' pattern was most striking in the group with acute pancreatitis, who had not altered their lifestyles. Data were much more variable in chronic pancreatitis and cancer subgroups, in keeping with the drastic change of environment in several patients, through illness (eg. anorexia, job loss) or medical advice (eg. reduced usage of alcohol, cigarettes, coffee).

Dietary and social histories enabled derivation of an 'induction' score for each patient, based on alcohol, cigarette, caffeine and brassica usage. Among the entire group of 32 patients, 17 scored > 50% of the maximum score of 12, but there was no correlation between scores and $T_{1/2}$ or Cl of either drug probe. The majority of patients habitually ingested > 100 gm fat / day but several had reduced fat intake on medical advice. The influence of other xenobiotics could not be quantified, but the more obvious agents were noted: among these were drugs such as phenytoin, prednisolone, azathioprine or analgesics in 10 patients; and occupational chemicals such as paints, dyes, solvents, or diesel fumes in another 10 cases. There was no correlation between pancreatic exocrine function as assessed by SP tests and the $T_{1/2}$ or Cl of either drug[149]. There was no difference in debrisoquine clearance kinetics among patients and controls[150].

5.2.2 Extended study to 1986

A consecutive series of 110 patients with pancreatic disease admitted between April 1983 and January 1986 was studied (71 male, 59 female; age-range 15-85 years) - now including patients on prescribed drugs that might inhibit

Table 5.2 Results: pilot study of antipyrine and theophylline disposition

	Antipyrine				Theophylline			
	$T_{1/2}$ (hr)		Cl (ml/kg/hr)		$T_{1/2}$ (hr)		Cl (ml/kg/hr)	
	Median	p	Median	p	Median	p	Median	p
Acute pancreatitis (6)	6.6	<0.001	112	<0.001	3.2	<0.01	185	<0.001
Chronic pancreatitis (22)	8.3	<0.01	74	<0.01	4.5	=0.025	141	<0.01
Cancer of pancreas (4)	8.3	<0.05	77	<0.01	4.6	=0.055	146	<0.01
Total (32)	**8.2**	**<0.001**	**86**	**<0.001**	**4.2**	**<0.01**	**161**	**<0.001**
Control *	11.5		49		6.5		76	

*Antipyrine test in 21 controls, theophylline test in 7 controls. Comparisons by Mann Whitney U tests (ref 149).

CYP, but excluding 8 with a complication needing surgery, or with asthma, renal failure or cardiac failure. After full investigation and social histories, patients with ≥ 1 pancreatitis attack were classed according to standard criteria (Chapter 2) as chronic pancreatitis (n=71; alcoholic 24, idiopathic 47 including 2 with hyperparathyroidism) or post-acute / RAP (n= 28; gallstones 10, idiopathic 18 including an alcoholic). Eleven patients had pancreatic cancer. Two control groups were studied. The first set comprised 15 healthy volunteers, including 7 participants in the pilot study, who were selected to cover an age range similar to the patients, and recruited from hospital staff or by advertisement in a local general practice. No volunteer had dietary fads, drank alcohol or caffeine-containing beverages in excess, or was on medication. The second set consisted of 5 patients with gallstones awaiting elective cholecystectomy, but with no symptoms or signs of co-existing pancreatic disease. The protocol was as described in the pilot study, but debrisoquine tests were omitted. After the first 18 months it was clear that antipyrine and theophylline tests yielded similar data. Considering that the theophylline test could be completed within a day, the antipyrine test was abandoned thereafter.

Several quantifiable host and environmental factors were known to influence CYP. These could be grouped as dietary or miscellaneous. Accordingly, intakes of fat / protein / carbohydrate / methyl xanthines (as in tea and coffee) / brassica vegetables were assessed by 7-day home food inventories, supervised by the hospital's senior dietitian, and then decoded using a Microdiet programme (Salford University, UK). Alcohol and cigarette usage contributed to the non-food inducers, and were registered as whether- or -not operating at the time of the study: in the case of current smokers, 4 subgroups were defined according to number of cigarettes smoked per day.

Statistical analysis was as described in the pilot study. In addition, a series of multivariate linear regression analyses examined the impact of various host and environmental factors on clearance of antipyrine or theophylline. A stepwise analysis with backward elimination was employed in the first instance, ie. variables were eliminated consecutively while their removal had no impact on the regression

Validation of the pharmacokinetic methods was accomplished in a number of ways. The

coefficients of variation (CV) from analysis of aqueous standards of each drug was obtained by quintuplicate analysis, to cover concentrations that should yield plasma levels found in the pilot study: derived CV 3.6-5.1% for antipyrine; 1.5-4.5% for theophylline. The CV inherent in analysis of plasma samples was derived by analysing 10 sets of samples in triplicate, yielding average CV for each drug < 10%. Since drug retention in containers for oral delivery might account for some of this variation, 14 containers were taken at random, rinsed with 50 ml water and drug concentration measured: mean loss was 1.72% of the original dose, range 0.9-2.6%. Paired theophylline tests were done in 7 individuals, following oral or intravenous delivery of the drug. The average bioavailability of theophylline, F_1, proved to be 1.01, and there was a significant correlation between drug Cl or $T_{1/2}$ after oral and intravenous dosing: this held true for 2 patients with exocrine pancreatic failure[151].

Figures 5.4 and **5.5** show the wide range of drug clearances in patients compared to healthy controls. The expected inverse correlation between $T_{1/2}$ and Cl of theophylline was realised in cigarette smokers (n=58) or non-smokers (n=48)[151]. There was fairly good correlation between antipyrine and theophylline Cl in a subset of 91 individuals (15 controls, 76 patients) who had both tests.

Theophylline Cl was faster in the entire cohort of 110 patients with pancreatic disease than in healthy volunteers (medians 104 and 68 ml / kg / hr, respectively, $p<0.05$), but there was no difference when antipyrine Cl in 76 patients was considered (medians 57 and 50 ml /kg /hr, respectively) - suggesting preferential involvement of the PAH-inducible subfamily of CYP. The theophylline result was largely influenced by very significant increases in theophylline Cl among patients with chronic pancreatitis, both in the alcoholic subset of 24 (median 132 ml / kg / hr, $p<0.001$) and the idiopathic subset of 47 (median 93 ml / kg / hr, $p<0.010$). In contrast to the pilot study (**Table 5.2**), theophylline Cl values in subgroups of 28 patients with post- acute pancreatitis and 11 with pancreatic cancer (medians 86 and 104 ml / kg / hr, respectively) were not significantly different from control values. Nonetheless, inspection of **Figure 5.6** shows that theophylline Cl exceeded the highest control value in around half the patients in these groups[151]. Results in 'gallstone

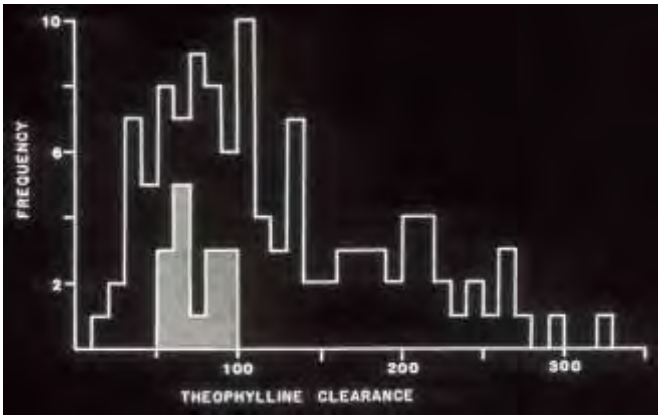

Figure 5.4 Frequency distribution of theophylline clearance, C_T, in 110 patients with pancreatic disease. Shaded areas indicate data from 15 healthy volunteers. Clearance is in ml/kg/hr Reproduced from 1989 paper in Clinical Science[151]

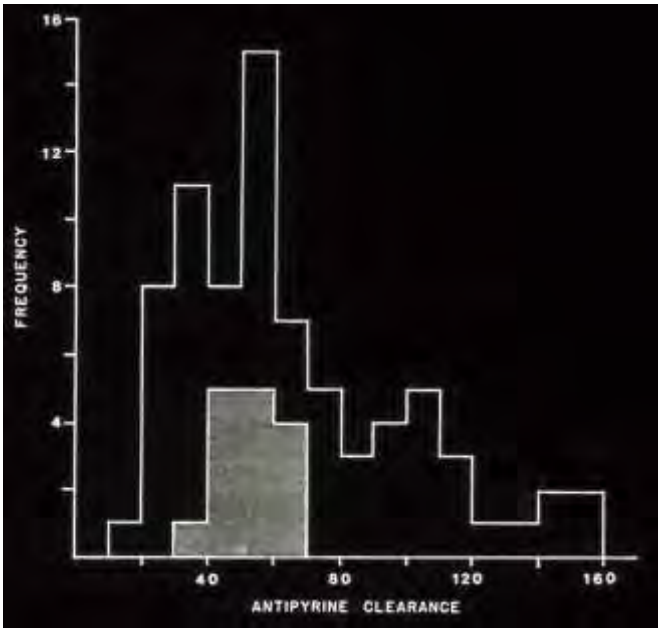

Figure 5.5 Frequency distribution of antipyrine clearance, C_A, in 76 patients with pancreatic disease. Clearance is in ml/kg/hr. Publishing details as in Figure 5.4.

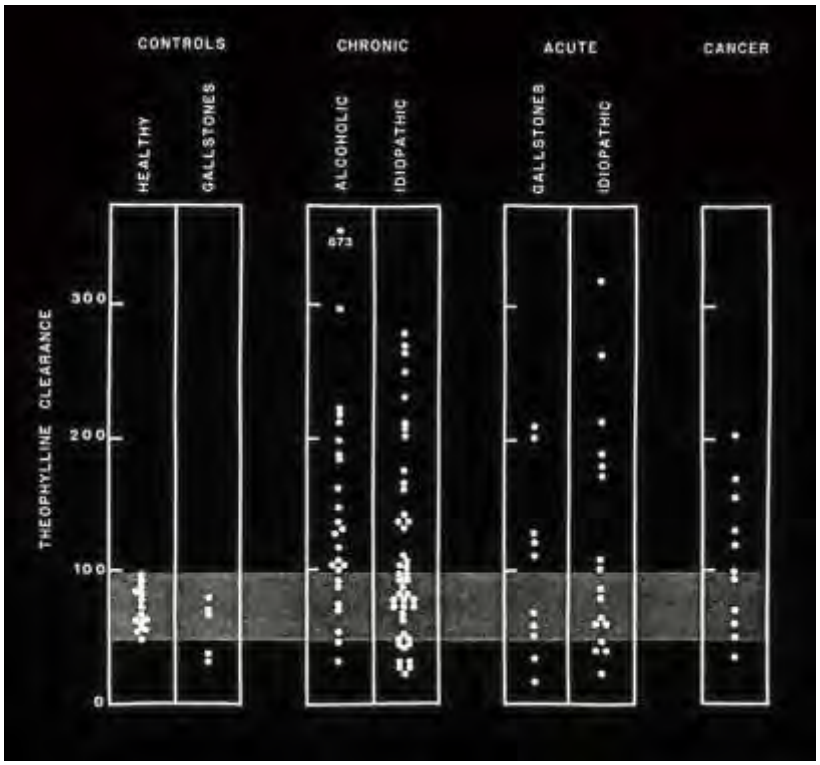

Figure 5.6 Theophylline clearance (C_T) in controls and groups with gallstones, chronic pancreatitis (CP), acute pancreatitis (AP) and pancreatic cancer (CA). Shaded area represents reference range. Publication details as for Figure 5.4.

Table 5.3 Non-smokers with high theophylline and/ or antipyrine clearance*

Sex	Age	Current job	Drugs	Theophylline ml/kh/hr	Antipyrine
M	49	oil processing	bextazxol, fenoterol	122	81
M	31	loading bay work	phenytoin	133	not tested
M	23	welder	none	178	53
M	44	technical officer	insulin	191	102
F	58	cleaner	noine	279	72
F	57	guesthouse keeper	none	104	116
M	70	civil servant	insulin	284	not tested
F	65	housewife	aspirin as needed	134	34
M	54	lorry driver	insulin	121	59
M	16	schoolboy	none	93	93
F	73	book keeper	tolbutamide, amitryptiline	107	51
F	23	housewife	none	100	55
M	26	carpenter	penicillamine	100	48
F	33	social worker	rheumox, salazopyrine	68	68

*Highest control values: theophylline clearance 99 ml/kg/hr; antipyrine clearance 66 ml/kg/hr. (From ref 151).

controls ' were of the same order as in healthy controls and gallstone-acute pancreatitis subgroup with either probe.

An evaluation of data from 56 patients who had a SP test within a month of the drug metabolism studies showed no correlation between bicarbonate or trypsin secretory capacity and Cl of either drug. Multivariate regression analysis identified around 50% of the variability in Cl of each probe. For theophylline, smoking and dietary protein emerged as inducers, whereas certain prescribed drugs, and methylyxanthines were inhibitory: the highest values for theophylline Cl were shared equally between smokers and non-smokers. For antipyrine, smoking and dietary protein had a positive influence, while age impacted negatively.

Among 48 non-smokers in the study, 14 (29%) had theophylline and / or antipyrine Cl values higher than the upper limit of the reference range: their occupations and current drug therapy were recorded, in search of a CYP inducer (**Table 5.3**).

5.2.3 Patients with epilepsy

Eighteen patients with epilepsy who were on maintenance anticonvulsant therapy within a dedicated care home were studied. There were 13 males and 5 females, of mean age 33 years (range 20-51), and mean weight 65 kg (range 41-88). All were otherwise well and without clinical or biochemical evidence of liver disease. Among the group, 10 smoked cigarettes, 10-30 / day. Almost all were receiving multiple anticonvulsants - including phenytoin (150-475 mg / day) in 11; valproate (1-2 gm / day) in 9; ethosuximide (1 gm / day) in 1, and primidone (375 mg / day) in another. Ten healthy volunteers from study 5.2.2 were chosen so as to match the smoker and non-smoker epilepsy patients by age and gender. Data from controls, epilepsy smokers, and epilepsy non-smokers were compared by one-way analysis of variance. Correlations were examined by parametric methods.

The results are summarised in **Table 5.4**. Theophylline and antipyrine disposal kinetics demonstrated CYP induction of a similar degree

Table 5.4 Drug metabolism in patients with epilepsy

	Controls	Patients with epilepsy	
		Non-smokers	Smokers
Theophylline			
n	10	8	10
Cl (ml/kg/hr)	70 (15)	121 (48)**	129 (47) **
$T_{1/2}$ (hr)	6.6 (1.1)	3.9 (0.9)***	3.5 (0.9) ***
V (l/kg)	0.61 (0.13)	0.65 (0.15)	0.60 (0.10)
Antipyrine			
n	10	4	4
Cl (ml/kg/hr)	53 (8)	92 (24)**	106 (17) ***
T ½ (hr)	11.5 (1.6)	5.3 (1.5) ***	4.5 (0.8) ***
V (l/kg)	0.75 (0.21)	0.53 (0.15)	o.52 (0.16)

Data as mean with standard deviation in parenthesis. Significance of differences from control values: ** p<0.01, *** p<0.001. There were no differences between smokers and non-smokers in the epilepsy group. Abbreviations: n number, Cl clearance, T ½ half life, V volume of distribution.

in smoker and non-smoker subgroups of patients with epilepsy; there was good correlation between disposal parameters for the probes[152].

5.2.4 Comments

By 1985 there were numerous publications on the pharmacokinetics of antipyrine and theophylline in controls[129, 139, 149], and it was recognised that the wide inter-individual variation in drug disposal probably reflects complex interactions between genetic and environmental factors that influence CYP function. Because of this scatter, the use of a single antipyrine or theophylline test in a group of patients with a specific disease was deprecated[153]. Nonetheless, it had been observed that long-term exposure to a CYP inducer such as phenobarbital narrows inter-individual variation in antipyrine elimination[141]. Moreover, it was suggested that, whereas antipyrine Cl rapidly reverts to normal upon removal of a CYP inducer[153], theophylline Cl remains augmented for some time thereafter[154].

The first prerequisite in the Manchester studies was to ascertain that any difference in drug disposal between patients and controls was not artefactual. This was done by the measures described in Section 5.2.2. The risk of anaphylactic reaction after intravenous antipyrine prevented paired oral and intravenous studies to confirm the excellent bioavailability that is accepted for this probe[153].

The wide ranges for $T_{1/2}$ and Cl of both drugs in controls of the pilot study - although smokers, chronic alcoholics and drug-takers were excluded - conformed with published data. It was thus remarkable to find significantly shorter biological $T_{1/2}$ and more rapid Cl of each probe in subgroups with exocrine pancreatic disease (**Table 5.2**), comprising a nearly consecutive series of 32 patients, irrespective of age, gender, disease type or duration, putative aetiological factor or exocrine functional status. While this finding cannot distinguish between enzyme induction as strictly defined and the general increase in CYP activity that may accompany increases in cell size and protein content, there is no reason why the pathophysiological effects should be any different.

The results clearly needed to be validated or refuted by a larger investigation, to include also patients with gallstone-related acute pancreatitis. The choice of controls posed a dilemma. Ideally, patients and controls should be matched for relevant variables but these could not be pinpointed from epidemiological surveys of patients with pancreatic disease, nor after analysis of data from the pilot study. For example: alcoholism was thought to have a variable influence on CYP[155] and was clearly associated with chronic pancreatitis but its role in acute pancreatitis and cancer was debated; cigarette smoke was established as a potent CYP inducer and linked to pancreatic cancer but evidence in regard to pancreatitis was conflicting; a high-fat diet was known to facilitate CYP induction and implicated in pancreatic cancer but not unequivocally with pancreatitis. Moreover, the demanding nature of the extended investigation, requiring 2 consecutive weeks for dietary and drug kinetic aspects, would make it impossible to enlist sufficient volunteers to cover each of these associations. Hence it was decided to expand the group of volunteers with a healthy lifestyle as used in the pilot study, and to use computer-assisted multivariate analysis to dissect out items contributing to CYP induction in patients, if that conclusion was borne out. A group with gallstones but without a history of pancreatitis would serve as controls for the gallstone-pancreatitis group: data from this subset were not amalgamated with data from healthy volunteers because it had been suggested that aberrant CYP activity might be involved in the development of gallstones[156].

The outcome of both studies left no doubt that CYP induction is a feature of chronic pancreatitis. In this regard it is of interest that multivariate analysis in the extended study picked out items that had been demonstrated individually to alter drug-metabolizing capacity in healthy subjects.

For example, it identified smoking followed by dietary protein as key contributors to accelerated theophylline Cl and the same factors in reverse order as contributors to accelerated antipyrine Cl. The magnitude of the positive effect was similar to that reported by others for both smoking and protein. The highest values for theophylline Cl were in patients with 'alcoholic' chronic pancreatitis, but the label was clearly misleading in that the multivariate analysis showed 'alcohol-ever' usage *per se* to exert an inhibitory effect on theophylline Cl[151]. There was no guarantee that the result would hold true if the patients were studied before they curtailed alcohol usage on medical advice. However, the discovery that ethanol induces a specific CYP subfamily[157], presently classed as CYP2E1[108], underlines the principle of selectivity in CYP induction. Not until 1999 did it become known that chlorzoxazone disposal provides an index of CYP2E1 activity[158].

The further finding, that theophylline Cl is a better indicator than antipyrine Cl of CYP induction in patients with chronic pancreatitis, underscores the point. Preliminary analysis of urinary metabolites after antipyrine tests suggested that the 3-hydroxymethylation pathway was induced (accounting for the correlation with theophylline Cl), but not other recognised pathways[150]. Nonetheless, the study in patients with epilepsy showed that there is a ceiling for overall CYP1A2 induction which is reached by treatment with anticonvulsant drugs, such that any further effect as from cigarette smoke is indiscernible[152].

There are several possible explanations for the Manchester findings. (i) The altered drug kinetics might have been iatrogenic, as was possible though not necessarily the sole explanation nor detracting from the potential pathological significance, in a quarter of patients in the pilot study. (ii) Pancreatic exocrine insufficiency might have indirectly altered drug disposal by evoking a rise in circulating CCK[159]: the lack of correlation between pancreatic enzyme secretory capacity after administration of the hormone and drug disposal parameters argued against this interpretation. (ii) Experimental biliary obstruction leads to induction of CYP3A1 so that accumulating bile acids can be detoxified. However, patency of the biliary tree was demonstrated by ERCP in every patient of the pilot study. (iii) CYP induction may simply be coincidental - most unlikely because the wide reference range should reduce rather than increase the chance of detecting significant differences in patients with specific diseases. (iv) Finally, given the many parallels between factors that are known or suspected to cause or promote exocrine pancreatic disease and those that are known or suspected to cause or promote CYP induction[9], the findings strengthened the case for CYP induction as a causative factor. If so, it was intriguing that the highest values for theophylline Cl were similar in smokers and non-smokers, emphasising the impact of other xenobiotic inducers that the study did not or could not quantify. In this regard it is now known that CYP1A is induced not only by PAH but also by chlorinated biphenyls[160], halogenated hydro-carbons, and C18:2 fatty acids as in corn, peanut and linseed oils[161].

Many CYP isoforms display polymorphism. CYP2D6 is an example, and its genetic status can be probed by use of the drug debrisoquine. At least in the case of this isoenzyme, genetic endowment was excluded as an explanation for increased CYP activity in patients with pancreatic disease[150].

5.3 D-glucaric acid

5.3.1 Study description and outcome

Glucuronic acid, derived via the hexose monophosphate shunt, is involved in the phase II metabolism of endobiotics such as bilirubin, and several xenobiotics. D-glucaric acid (DGA) is a minor offshoot, which exits across sinusoidal membranes (**Figure 5.1**)[126]. Measurement of DGA in random samples of urine was touted as a non-invasive mirror of phase I metabolism via CYP[162-164].

Accordingly, an 8-hour collection of urine between 9 am and 5 pm, was obtained for DGA analysis in a consecutive series of patients with pancreatic disease during a 9-month period[126]. Each patient had antipyrine and theophylline tests in the same week. Exclusion criteria were as follows: age under 20 years or over 75 years, congestive cardiac failure, asthma, renal failure. Chronic pancreatitis in 19 patients was diagnosed principally by histology of resected specimens (n=3), pancreatic calculi (n=6), unequivocally abnormal pancreatogram (n=9), or clearly reduced exocrine secretory capacity (n=1). Of these patients, 10 could be labelled as 'alcoholic' (Chapter 2) and there was no explanation in the others. Of 7 patients who were classified as post-acute / RAP, 1 drank excess alcohol on a regular basis, another had diabetes and was on phenformin for many years, and 2 patients including a young man with Caroli's disease had a previous cholecystectomy (no gall stones). Three patients had pancreatic cancer.

In 5 patients liver biopsy was done on clinical grounds. Histology identified chronic liver disease in 4 patients with idiopathic chronic pancreatitis (chronic active hepatitis-like picture in a woman with ulcerative colitis, secondary biliary cirrhosis in 2 patients who had previously undergone bile diversion, severe steatosis in a patient with long-standing diabetes) and metastases in a patient with cancer. In 2 other patients the presence of liver disease was strongly suspected on clinical examination coupled with abnormal liver function tests.

The control group consisted of 22 healthy volunteers, aged 21-70 years, with a healthy lifestyle and on no medication. A colorimetric method was used to measure DGA: full details and precision analysis have been published[126]. Data were expressed in 3 ways: DGA concentration, molar concentration ratio of DGA to creatinine, and 8-hour output of DGA. Results in patients and controls were compared by the Wilcoxon Rank Sum test. Kendall's correlation coefficient was used to examine reproducibility of DGA / creatinine ratios in 11 paired studies, and to assess the relationship between ratios and DGA outputs.

From the DGA standard curve, the predicted useful range of the assay was 10-200 μmol / l. Standard laboratory procedures showed good within-batch and between-batch precision at DGA concentrations towards the top end of this range, but only around 50% at intermediate level, and <10% when DGA concentration was < 20 μmol / l. The total working time for each run, including the preparation of fresh reagents was estimated at about 3 hours.

The results of the investigation can be summarised as follows. DGA concentration in controls and patients were similar; DGA / creatinine molar ratios were higher in the patients (medians 4.6 and 2.9 x 10^{-3}, respectively, $p< 0.005$), as was DGA output (14.0 and 8.8 μmol / 8 hours, respectively, $p< 0.005$); and there was good correlation between the last 2 measurements overall ($r = 0.75$, $p<0.001$). Although mean urinary DGA / creatinine ratios were nearly identical in urine samples from 11 patients who were studied on two separate occasions (5.0 and 5.1 x 10^{-3}), ratios were markedly different in some individuals.

In 29 patients who also had antipyrine and theophylline tests, urinary DGA proved to be a poor index of CYP induction. Thus, antipyrine Cl exceeded the highest control value in 17 patients (59%) and theophylline Cl in 21 patients, (72%), even though patients with liver disease were included, By comparison, figures for urinary DGA output and DGA / creatinine ratios were 21% and 45%, respectively. There was no correlation between antipyrine or theophylline pharmacokinetics and urinary DGA, howsoever expressed. Surprisingly, in patients with liver disease, in whom theophylline Cl was impaired, urinary DGA values were among the highest encountered [126].

5.3.2 Comments

Particular attractions of the urinary DGA test were its analytical simplicity, and reputed applicability to 'spot sample' analysis, such as in the out-patient setting. Moreover, DGA excretion, although a marker of a phase II pathway of xenobiotic metabolism, was said to match assessments of phase I reactions as gauged by drug kinetics. For example, a study in children with kwashiorkor showed that drug hydroxylation recovered alongside glucuronidation upon nutritional rehabilitation[166]. However, the Manchester investigation revealed an unacceptably poor performance of urinary DGA as an index of microsomal enzyme induction in patients with pancreatic disease.

This is disappointing because, as noted with citations in the paper reporting the study[126], previous investigators found increased urinary DGA in such disparate circumstances as pregnancy (presumably due to induction by endogenous hormones), cigarette smokers, patients on anticonvulsant drugs, or exposure to pesticides. Thus, the implication was that urinary DGA provides a global index of phase II reactions in the same way that antipyrine disposition was thought to provide of phase I reactions. It was suggested that discrepant findings might be due to inherent problems with the DGA assay, but this seems unlikely in view of excellent recovery of glucarate in the Manchester study. Gas liquid chromatography would increase the test's specificity, but detract from its simplicity and applicability.

Although DGA data reported here accord with the general pattern of enzyme induction in patients with exocrine disease, the yield was considerably lower than from antipyrine and, especially, theophylline test. The best performance of the last method might involve 2 main factors, namely, that theophylline Cl remains high for a considerable period after withdrawing an inducer; and that it specifically represents the activity of CYP1A2. Moreover, urinary DGA cannot of itself reflect the total hepatic pool of glucuronic acid because it is only one of several metabolites, and also because the proportion diverted towards conjugating bilirubin and also RXS for excretion into bile is unknown (**Figure 5.1**). The last aspect might be particularly relevant, considering increased amounts of bilirubin in secretin-stimulated bile from patients with pancreatic disease (Chapter 3).

The paradoxical increase of DGA excretion in patients with known or suspected chronic liver disease is reminiscent of a study in patients with acute viral hepatitis[167]. It could be that interference with bile canalicular function - as indicated by subnormal K_2 of BSP elimination in some patients (**Figure 5.3**) - reduces the apportioning of glucuronide into bile, resulting in a corresponding increase in urinary DGA excretion (**Figure 5.1**). Thus, as was concluded by the test's pioneer, "enhanced DGA excretion in mammals is an indicator of a hepatic effect which, although not necessarily due to enzyme induction, might nevertheless accompany it"[162].

For completion, it is necessary to mention a Scandinavian study of 1999 that purported to show decreased phase I metabolism and liver blood flow but normal phase II conjugation in patients with chronic pancreatitis[165]. The results are not comparable with the Manchester work for several reasons: it involved only 7 patients; all were on opiates; 2 patients were on pancreatic extracts and 1 on diazepam; and a cocktail of antipyrine, indocyanine green, and oxazepam was delivered in a single sitting.

5.4 Biliary fatty acids

5.4.1 Analysis of duodenal bile

Three observations prompted an investigation of fatty acids in duodenal bile collected during the first 10 minutes after secretin[168]. (i) It was known that expansion of the SER in hepatocytes accompanies experimental CYP induction by some drugs[121]. (ii) It was evident that an unidentified agent(s) in the intestinal mucosa, which is present in Boots secretin, stimulates the

hepatocyte directly, evoking the transfer into bile of phospholipid, bile acids and cholesterol (Chapter 3). (iii) It was of interest to learn whether the increased amount of linoleic acid (and its oxidation product) in bile (Chapter 3) extends to other PUFA.

Patients with pancreatic disease for this study represented a consecutive series - excluding any with evidence of biliary tract infection or bile duct obstruction or when the secretory test was technically unsatisfactory. The control group included 11 healthy volunteers, and 8 patients with miscellaneous disorders (spastic colon 6, reflux oesophagitis 1, lymphangiectasia1) in whom secretory test and pancreatogram were normal at initial assessment, and there was no further reason to consider pancreatic disease in a 2-year period of follow-up on appropriate treatment. The group with pancreatic disease included 27 on habitual diets (chronic pancreatitis n=16, post-acute pancreatitis n=8, pancreatic cancer n=3), and 11 whose fat intake in the preceding 6 months was substantially curtailed on medical advice (chronic pancreatitis n=6, post-acute pancreatitis n=3, or cancer n=2).

The main criterion for a diagnosis of chronic pancreatitis was pancreatic calculi (n=5), histology of the resected tail of pancreas (n=4), abnormal pancreatogram (n=10) or reduced secretory capacity (n=3). Chronic alcoholism was identified in 12 patients of whom 5 had mild type IV hyperliidaemia, which was also recorded in 4 non-alcoholics, while the disease was idiopathic in the remainder. Three patients had a previous cholecystectomy. In the subgroup with post-acute pancreatitis, ultrasound scanning showed multiple gallstones in 3 patients, including 2 who drank at least 60 gm alcohol daily.

SP tests were done as previously described, and aliquots of the timed collections of duodenal juice were routinely analysed for bicarbonate, trypsin, and bilirubin (Chapter 3). Attention was focussed on the first 10 minutes after the injection of secretin. In 2 patients the response to secretin was studied by analysis of duodenal juice and pure bile obtained endoscopically on the same day. One of these was an anorexic patient in whom it was impossible to distinguish between chronic pancreatitis and pancreatic cancer by history and comprehensive investigation. The ampulla of Vater was cannulated to a distance of 3 cm without X-ray guidance and secretin injected so as to obtain pancreatic juice for cytology and tumour markers: in fact, pure bile emerged, indicating inadvertent cannulation of the common bile duct. Two hours later the patient had a second secretin injection as part of a routine secretory study. Although cytology was negative, a tumour with diameter of 1 cm was found at laparotomy to completely obstruct the main pancreatic duct in mid-pancreas. In the second patient who was on treatment with the NSAID sulindac for arthritis, endoscopic cholangiography suggested sclerosing cholangitis but without biliary obstruction. She underwent a pure bile collection for bacteriology after an injection of secretin, having had the standard secretory test a few hours earlier.

The hospital's senior dietitian obtained detailed dietary histories from each patient, the questionnaire geared to revealing habitual diets before the first symptom, and, in particular, any change in the 6 month period before the secretory test. Latterly, in 7 cases the information could be supplemented by a weighed home-dietary record, with information processed using the 'Microdiet programme' of Salford University, so that the daily intake of each fatty acid could be extrapolated.

The procedure for extraction of free fatty acids from duodenal juice and analysis by gas liquid chromatography is detailed in the study report[168]: GN Smith from the University department of Organic Chemistry was the expert involved. The concentration of each fatty acid - C16.0 to C22.5 - was determined by comparison with known concentrations of margaric acid (C17:0). The output of each fatty acid could then be

derived and total output of free fatty acids calculated (designated A). The accuracy of the analytical technique was assessed by repeated analysis of the same sample, with assurance by variation level < 1%. For determination of total fatty acids (B), an initial saponifiaction step was carried. The ratio B/A provided an index of the relative amounts of total to free fatty acids in each sample of duodenal juice.

Log transformation (log 10(1+x) if data included any zero value) was used to normalise data on fatty acids and to stabilise the variables: group means were then compared by one-way analysis of variance, and multiple comparisons made by the modified least significance procedure. For concomitant bilirubin data, skewness was not fully eliminated by log transformation, and the Mann Whitney U test was applied. The Pearson correlation coefficient was used to examine the relationship between B/A and log trypsin (in the knowledge that its response runs parallel with that of other pancreatic enzymes after hormone injections); and between dietary fatty acid intakes and log fatty acid outputs. The Kendall rank correlation coefficient was used to examine the relationship between logs of bilirubin and fatty acid outputs.

A gas chromatogram of the unsaponified duodenal juice sample from a healthy volunteer and another from a patient with alcoholic chronic pancreatitis on unchanged diet are shown in **Figures 5.7** and **5.8**, respectively. In the control specimen, total saturated and unsaturated acids in duodenal bile were approximately equal. Among the former C16:0 (palmitic acid) predominated, the mean value after back transformation 11.3 mg in the first 10 minutes after secretin. Linoleic acd (C18:2) was the major unsaturated fatty acid (mean 7.94 mg) whereas very small amounts were detected of the highly unsaturated forms (C20:4 - C22:5). Fatty acid outputs in the group of 8 patients without pancreatic disease were similar to those in healthy volunteers. In both subgroups, fatty acids were largely in the free form, so that group values for B/A were close to unity (means of back-transformed data not significantly different at 1.09 and 1.16, respectively).

The profile from the patient was typical of the group with chronic pancreatitis on habitual diets, irrespective of whether the disease was alcoholic or idiopathic. Fatty acid outputs were significantly higher than in healthy volunteers and generally also higher than in patients without pancreatic

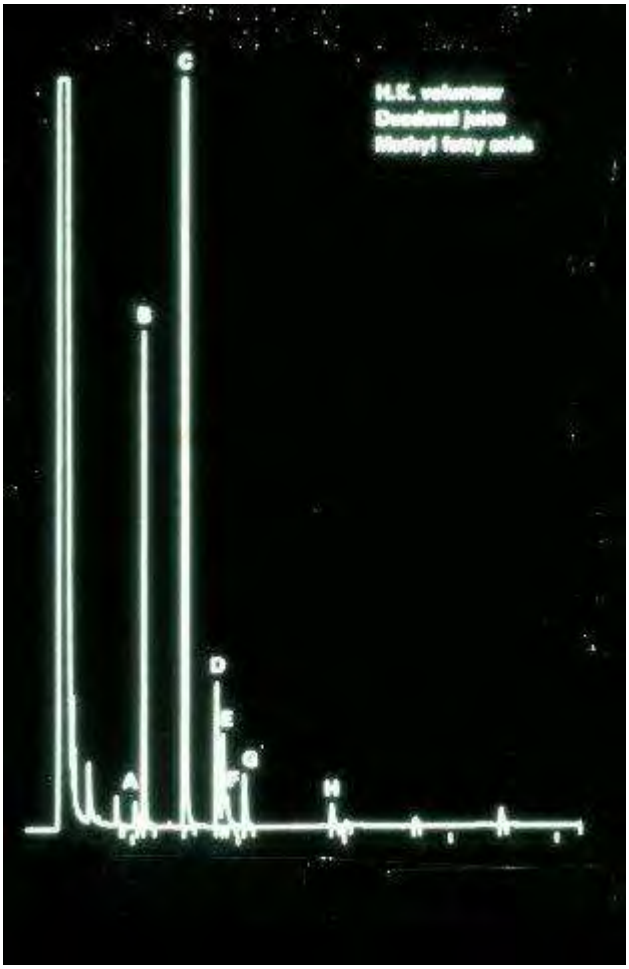

Figure 5.7 Typical gas liquid chromatography profile of fatty acids in duodenal juice within 10 minutes of Boots secretin from a healthy volunteer. The peaks starting from the origin are labelled A-J as follows: A = C16:1 (palmitoleic acid), B= C16:0 (palmitic acid) , C= C:17= margaric acid (internal standard), D=C18:2 (linoleic acid), E=C18:1cis (oleic acid isomer) , F=C18:1trans (another oleic acid isomer), G= C18:0 (stearic acid), H=C20:4 (arachidonic acid), I=C20:0 (arachidic acid), J=C22:5 (docosapentanoic acid). Reproduced from 1986 paper in Int J Pancreatol[168].

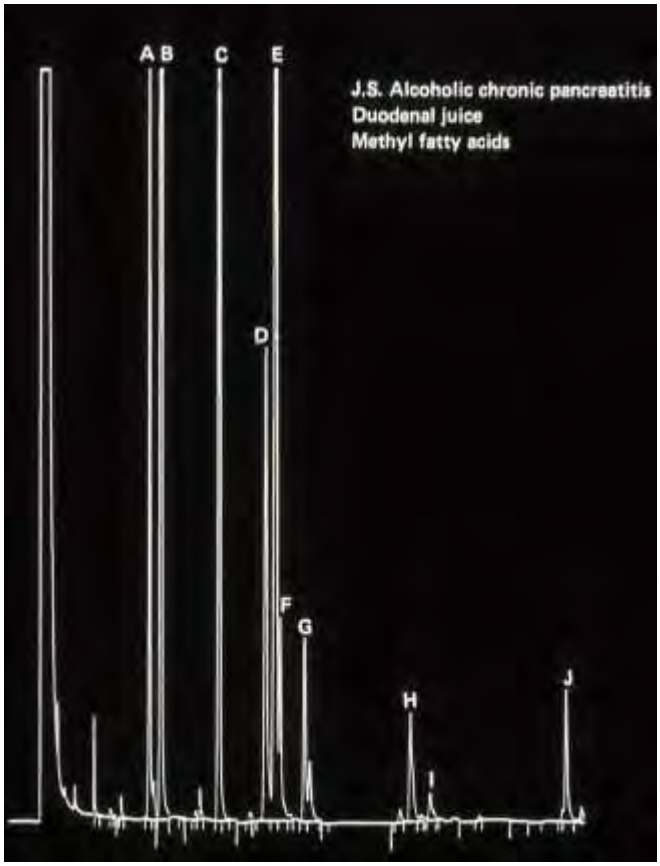

Figure 5.8 Fatty acid profile in a patient with alcoholic chronic pancreatitis on habitual diet. Information as for Figure 5.7.

disease. A similar pattern was found in subgroups with post-acute pancreatitis or cancer.

As might be expected, fatty acids in patients with post-acute pancreatitis were largely in the free form, so that the B/A ratio (1.22 after back-transformation) did not differ significantly from that in controls. A fall in pancreatic secretory capacity, as in several patients with advanced chronic pancreatitis and in two of 3 with cancer, led to a corresponding increase in B/A. The inverse relationship between trypsin outputs (logged) and B/A ratios (logged) in the patients as a whole was linear, with a highly significant correlation coefficient (r= -0.729, p< 0.0001).

Comparison of fatty acids in saponified samples of bile and duodenal juice from 2 patients showed that there was virtually no free fatty acid in bile and that outputs of fatty acids in each pair of samples were very similar. The order of collection was bile followed by duodenal juice in the patient with pancreatic cancer, and the reverse in the patient with relapsing acute pancreatitis on sulindac. The gallbladder was intact in the former patient but previously removed in the second. Profiles of fatty acids from the patient with cancer are shown in **Figure 5.9** and **Figure 5.10** for bile and duodenal juice, respectively.

The results of duodenal juice analysis in the study cohort are summarised in **Figure 5.11**. Patients with post-acute or chronic pancreatitis displayed increased outputs of every fatty acid.

Because total fatty acid outputs in patients on low fat diets were less than in patients on habitual diets, the computed intakes of fatty acids in 7 patients were examined in relation to biliary fatty acid composition. Significant correlations emerged in the case of total fatty acids and C16:0, C18:0 and C18:1.

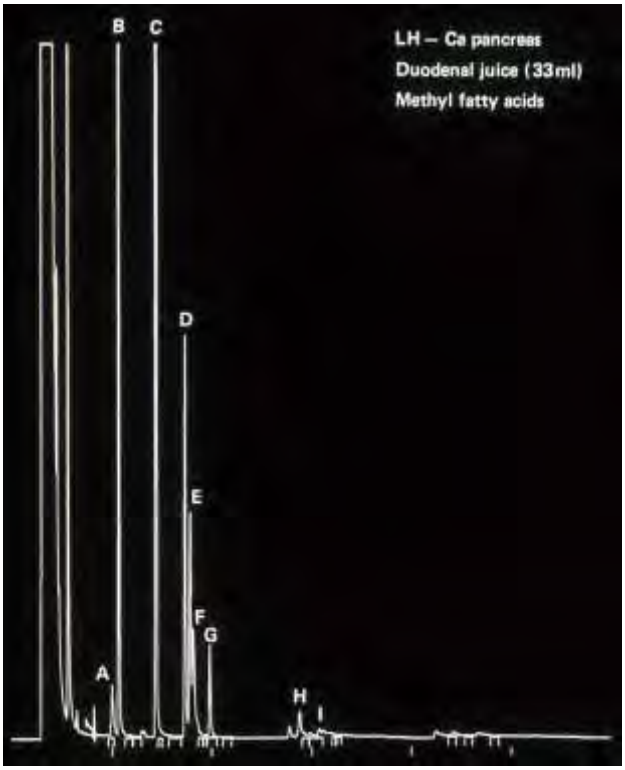

Figure 5.9 Fatty acid profile in saponified sample of duodenal juice in the first 10 minutes after Boots secretin in a patient with pancreatic cancer. Information as for Figure 5.7.

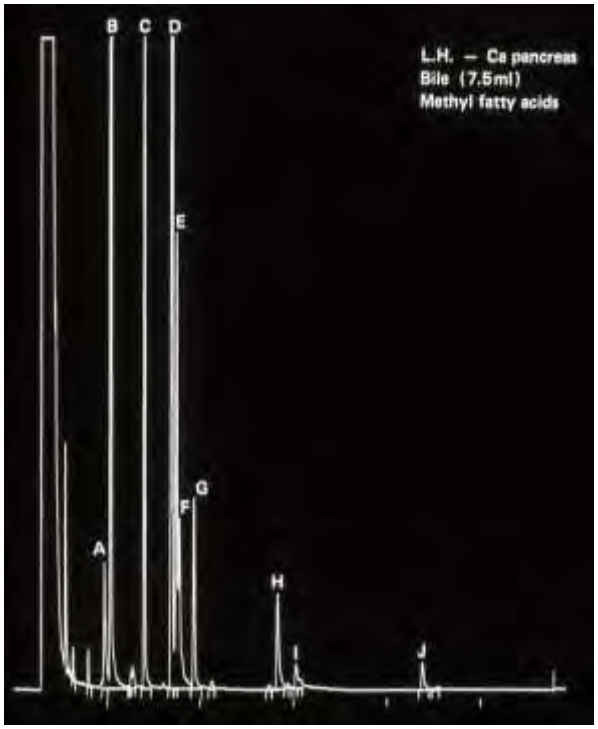

Figure 5.10 Fatty acid profile of saponified bile sample from same patient as in Figure 5.9, collected endoscopically 2 hours later after another secretin injection. Information as for Figure 5.7.

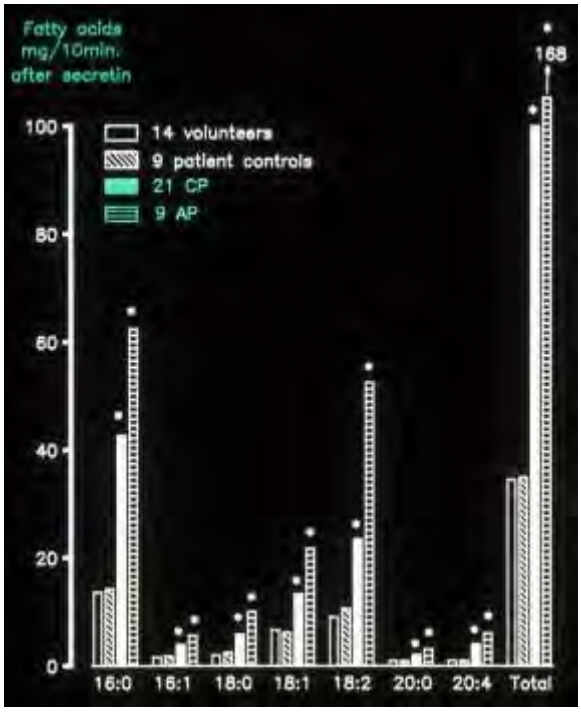

Figure 5.11 Outputs of fatty acids in duodenal juice within 10 minutes after injection of Boots secretin. Reproduced from 1988 review in Int J pancreatol[39]

In keeping with earlier observations (Chapter 3), the output of bilirubin was higher in duodenal juice from patients with than controls, There was good correlation between the output of bilirubin and total fatty acids in healthy controls (p=0.024) and in patients with pancreatic disease on habitual diets (p=0.039), but not in the group on reduced fat intake.

5.4.2 Comments

Some researchers recommend the use of non-absorbable markers during the hormone test to correct for fluid losses into the jejunum, but the technique is cumbersome[45]. It is probably valid to say that if performed carefully, the simple test may underestimate secretory rate but cannot overestimate it; whereas the reverse is true for marker studies. As an added precaution against spurious results due to variation in the amount of the elusive hepatogogue in Boots secretin, volunteers were enlisted at 6-monthly intervals during the study's 3-year span.

A previous study of fatty acids in bile from 13 individuals found no difference in results from controls and patients with a variety of biliary pathology[169]. However, that study is not strictly comparable for many reasons: aliquots of unstimulated hepatic or gallbladder bile were obtained randomly at laparotomy; concentrations of fatty acids were reported rather than outputs in a defined period; patients with peptic ulcer served as controls; 2 patients with 'white bile' were included; and fat intake was not considered. Data in **Figure 5.11** show higher outputs of phospholipid-embedded fatty acids in the bile-rich fraction of duodenal aspirates from patients with pancreatic disease (who had not altered their lifestyles) than in controls. The increase clearly represented hypersecretion and not merely 'wash-out' as evidenced by data from paired studies (**Figures 5.9, 5.10**). These observations confirm the validity of duodenal juice analysis after Boots secretin in providing information on the composition of hepatic bile.

A review of the prevailing literature revealed the following points. Phospholipids in bile originate in a hepatic pool that is functionally distinct from that which supplies plasma phospholipids. This precursor pool resides in the SER. Biliary lecithin constitutes 96% of biliary lipids, representing phospholipid that is newly synthesised in microsomes. Among several factors which govern this component - eg. enzyme induction, dietary lecithin, rate of bile acid synthesis - the first might be the most important, judging by studies in several animal species. It was also evident that determinants of lipid secretion into bile include those that influence synthesis as well as the rate of bile salt secretion, and perhaps a component of bile salt-independent secretion.

Against this background - and studies indicating increased uptake, biotransformation and conjugation phases of xenobiotic metabolism in patients with exocrine pancreatic disease (Sections 5.1-5.3) - the findings are in keeping with expansion of the SER in hepatocytes. The phenomenon apparently represents the sum of influences, including diet, prescribed drugs, alcohol, cigarette smoke, and other environmental chemicals. As to the first factor, there was little information in regard to the impact of diet on biliary phospholipd composition, in contrast to its recognised effect on the enzyme composition of hepatocyte SER.

Preliminary data relating dietary intake and biliary output of fatty acids suggest that the increased biliary output of highly unsaturated fatty acids in patients on normal lifestyles, although influenced by diet, is principally due to microsomal enzyme induction: linoleic acid, (C18:2), the main unsaturated fatty acid, is converted to arachidonic acid (C22:4) by microsomal enzymes. The corollary seems to be that dietary fat restriction in the patients normalises biliary phospholipid.

5.5. Overview and Summary

The first step in hypothesis testing (Chapter 4), ie. to demonstrate induction of hepatic CYP, was fulfilled by the clinical investigations. In fact, the dividends exceeded expectation. Thus, expansion of the hepatocyte SER was suggested by the results of BSP tests and bile analysis for fatty acids, although data from antipyrine and theophylline tests suggested preferential induction of CYP1A2 which is not associated with SER expansion in experimental studies. In other words, hepatic CYP induction as is associated with chronic pancreatitis is not the stereotype, but instead is attributable to a complex mix of xenobiotic exposures[13]. The potential role of ethanol was especially intriguing, in that a small dose had been shown by experimental studies to potentiate hepatic injury from volatile chemicals that are co-processed by CYP2E1, eg. toluene, xylene and trichloroethylene[170, 171].

If CYP induction is indeed the unifying factor in the pathogenesis of (non- gallstone) exocrine pancreatic disease, especially chronic pancreatitis, many dilemmas arose.

- Why does an overworked liver escape injury - or does it?
- Could poor defence against FRA rationalise normal CYP status in several patients?
- What is the CYP1A2 inducer in non-smokers?
- Above all, how might CYP induction relate to pancreatic disease?

Chapter 6

Hepatobiliary Aberrations: Reflux Link to Pancreatitis?

Three types of liver abnormality have long been recognised in patients with chronic pancreatitis: steatosis, as frequently accompanies alcoholic disease; alcoholic liver cirrhosis, a risk that is expected to post-date pancreatic disease by about 10 years (Chapter 2); and secondary biliary cirrhosis, when intra-pancreatic constriction of the bile duct is not relieved. There was, however, no information on liver histology in cohorts of patients such as those reported in Chapters 3 and 5, ie. with induction of drug-metabolising enzymes and increased hepatic FRA as evidenced by analysis of duodenal or pure bile. The potential for treatment or prevention of injury, as exhibited by experimental studies[122], was the particular incentive for the investigations reported herein.

6.1 Liver histology

6.1.1 Study description and outcome

Consecutive patients with a history of pancreatitis who required admission during a 6 month period in 1984 had a percutaneous liver biopsy if any of the following criteria applied, and provided that abdominal surgery was not warranted (eg. for gallstones, pseudocyst drainage, peptic ulcer): hepatomegaly, distorted serum liver function profile, abnormal cholangiogram. One of the patients was on the waiting list for surgical bile diversion to treat bile duct constriction; and another had already undergone that procedure. In patients who required surgery, a wedge biopsy of the liver was obtained at the start of the operation. A post-mortem liver biopsy was obtained from a woman who died of fulminating septicaemia despite aggressive antibiotic therapy 36 hours after ERCP to investigate recurrent pancreatitis: tight strictures were found in the intrahepatic ducts.

Of 38 patients, 29 were classified as chronic pancreatitis and 8 as acute pancreatitis, using standard criteria (Chapter 2). Several patients had undergone BSP and /or theophylline tests (Chapter 5). The majority with chronic pancreatitis had sustained an attack 6 weeks-14 months earlier; 2 had continual pain; and 5 had painless steatorrhoea. Alcoholism was recorded in 8 of these cases; 2 patients had ulcerative colitis and were on long-term treatment with salazopyrin; 2 were on treatment with azathioprine (Crohn's colitis, post renal transplant also on steroids); 1 was prescribed phenobarbitone for myelopathy; 3 had type IV hyperlipidaemia (including an alcoholic, and the renal transplant recipient); and an elderly woman had a positive antimitochondrial antibody test: the disease was idiopathic in the others. Nine patients, including the patient with presumed primary biliary cirrhosis, had pancreatic calculi. In the acute pancreatitis subgroup, potential risk factors included gallstones in 2 cases, choledochal cyst in 1, type IV hyper-lipidaemia in another, and prescribed drugs in 2 patients (oral contraceptive, sulindac plus indomethacin). Macronutrient intake - as gauged by weight, clinical features, morphometry, and rough-and-ready dietary assessment - was adjudged normal in all but 2 clearly undernourished patients, and an overweight woman with maturity onset diabetes,

Among patients with chronic pancreatitis, serum albumin was subnormal in 8 cases, while alanine transferase, alkaline phosphatase, bilirubin or γGT were elevated in 12, 6, 2 and 14 cases, respectively: at least 1 abnormality was recorded in 17 patients, and multiple abnormalities in several. In the acute pancreatitis set, 2 patients had hypoalbuminaemia; whereas alanine transferase, alkaline phosphatase or γGT were elevated in 3, 2 or 5 cases, respectively: the majority of patients had at least one abnormal parameter.

Percutaneous liver biopsies were obtained from 28 patients and operative wedge specimens from 10 cases. Small portions from each end of the sample were immediately fixed in glutaraldehyde, post-fixed in osmium tetroxide, dehydrated in alcohol, and embedded in epon. Semi-thin sections were stained by toluidine blue and examined by light microscopy and ultra-thin sections were cut for later ultrastructural examination. The rest of the specimen was fixed in buffered formalin for routine histological assessment and a battery of special stains - PAS before and after diastase, Perl's stain for iron, untoned reticulum, Shikata stain for Hepatitus B and copper-binding protein (HBsAg), hematoxylin picro sirius for collagen - was sandwiched between 2 sections stained with hematoxylin and eosin. Two pathologists without knowledge of the clinical details commented independently, and later arrived at a consensus decision on each case.

Hepatocytic changes were found in 18 patients with chronic pancreatitis (60%) and 5 with acute pancreatitis (63%). Three types of cells were distinguished, arbitrarily designated Types I, II, and III. The first refers to large cells with central or peripheral nucleus and clear cytoplasm (16 biopsies, 42% of total), closely resembling those in glycogen storage disease (**Figure 6.1**): in 3 cases this striking appearance was clearly due to extensive microvesicular fat that was unmasked by toluidine blue staining (**Figure 6.2**). Type III refers to typical ground glass hepatocytes (8 biopsies, 21%): large cells, usually with eccentric nucleus, granular eosinophilic cytoplasm, and granular basophilic condensation at the periphery (**Figure 6.3**), the latter shown by electron microscopy to represent mitochondria (**Figure 6.4**). Type II cells seemed to be an intermediate phenotype (12 cases, 32%). Excess lipofuscin was a feature in some biopsies (**Figure 6.5**).

Shikata stain for HBsAg was negative in all specimens. There was wide variation in the type and frequency of altered hepatocytes in the

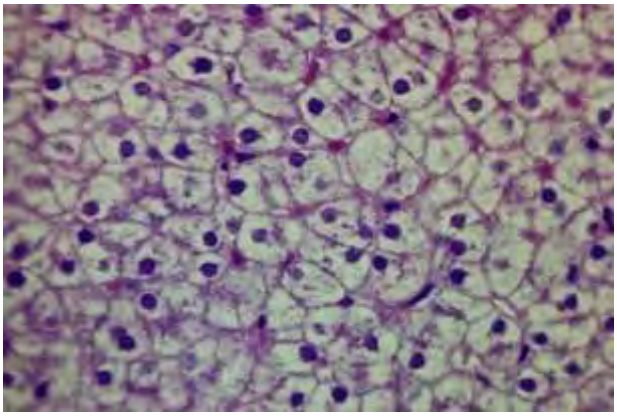

Figure 6.1 Water-clear type-I hepatocytes shown by H&E staining: see text for details. Reproduced from 1986 report in Mt Sinai J Med[172]

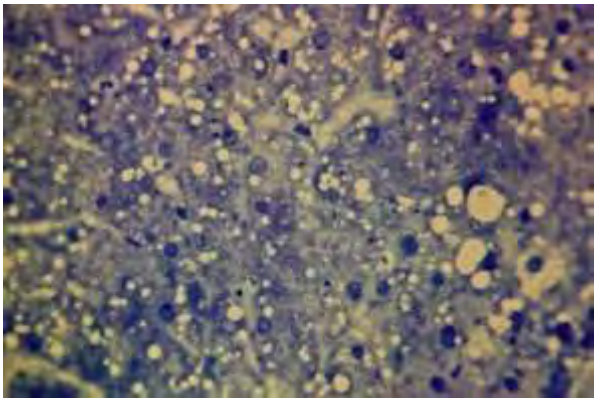

Figure 6.2 Toluidine blue stained semithin sections of a biopsy with prominent type-I hepatocytes shows extensive microvesicular steatosis. Publication details as for Figure 6.1.

biopsies. An attempt was made to define the extent of biopsy field occupied by altered hepatocytes in each case. For this purpose, both pathologists examined each specimen simultaneously and made a combined decision (**Table 6.1**). The spectrum of other parenchymal changes was similar in chronic and acute pancreatitis subsets. Portal tracts were normal in 10 patients (26%). Subtle changes included diffuse mild lymphocytic infiltration, usually accompanied by slight expansion of the tracts by fibrosis (11 cases, 19%) (**Table 6.1**). The tracts were clearly abnormal in the remainder (45%): bile duct proliferation in 5 patients (**Figure 6.6**) with (2 cases) or without (3 cases) focal ductopenia against a background of diffuse mild lymphocytic

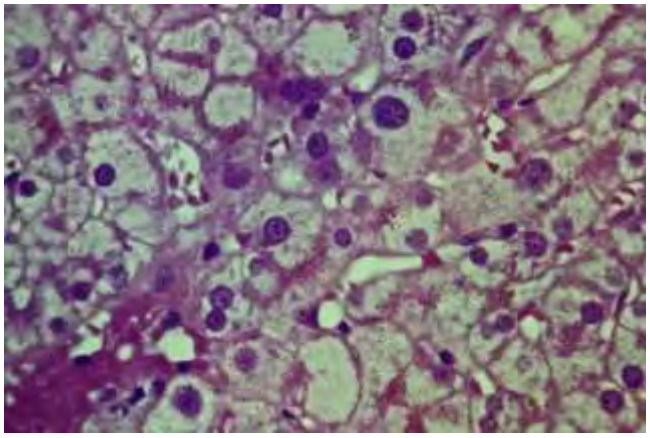

Figure 6.3 Ground-glass Type III hepatocytes shown by H&E staining. . Publication details as for Figure 6.1

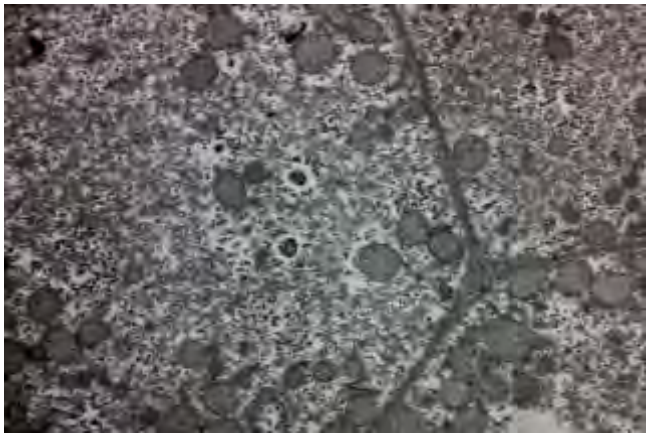

Figure 6.4 Electron microscopy of a biopsy with ground glass cells shows extensive margination of mitochondria to periphery of hepatocytes due to central proliferation of the smooth endoplasmic reticulum. (uranyl acetate and lead citrate x 2750). Publication details as for Figure 6.

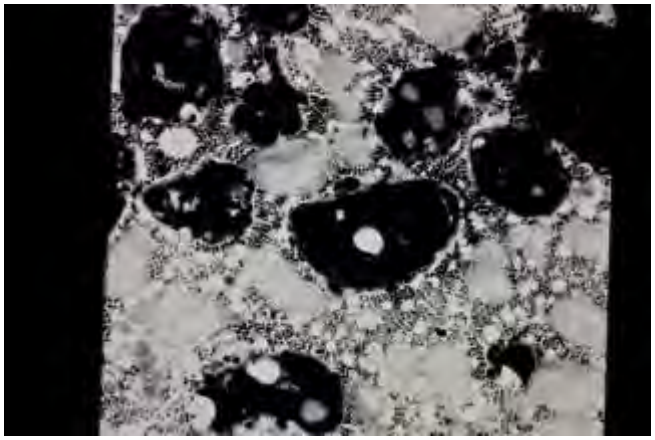

Figure 6.5 Electron microscopy shows **large** lipofuscin deposits (black) in hepatocytes indicating excess lipid peroxidation.

infiltration; features highly suggestive of primary sclerosing cholangitis in 3 cases (**Figure 6.7**); stage 3 primary biliary cirrhosis in 4 cases (**Figure 6.8**); an overlap between these entities in 2 cases; and miscellaneous changes in 3 patients (**Table 6.1**). Again, the spectrum was similar in patients with chronic or acute pancreatitis; there was no obvious difference between biopsies from patients with alcoholic or idiopathic disease; and the primary biliary cirrhosis-like changes were independent of antimitochondrial antibody status.

Overall, 95% of biopsies displayed hepatocytic and / or portal tract abnormalities. However, in a few instances altered heptatocytes occupied the entire field and yet portal tracts were normal; or vice versa (**Table 6.1**). To explore the connection further, portal tract changes were assigned a score of 1 when normal, 2 with diffuse lymphocytic infiltration, 3 when this was accompanied by focal bile duct proliferation with or without ductopenia, and 4 when the changes were highly suggestive of primary biliary cirrhosis or sclerosing cholangitis. The individual scores were compared to approximate percentage of altered hepatocytes in the respective biopsies using the Kendall rank correlation coefficient: a negative correlation emerged ($r = -0.359$, $p<0.001$)[172]

6.1.1 Comments

The study differs from other reports in several ways: the liver biopsies were obtained at a considerable interval after a pancreatitis attack or relapse; non-alcoholic patients accounted for the majority; the common bile duct was patent in most; and clinical or serological evidence of liver dysfunction was modest. These differences explain why the findings continue to be unique.

Large hepatocytes, by description very similar to cell type III, have been documented in patients on long-term treatment with drugs such as phenobarbitone: the ground glass appearance is due to proliferation of the SER[173], as was confirmed by electron microscopy in a patient with

.

Table 6.1 Liver histology in patients with pancreatitis

	Parenchyma					Portal tracts
	I (%)	II (%)	III(%)	Total(%)*	Miscellaneous†	
Chronic						
AB	0	0	0	0	iron +	lipogranulomatosis
ER	0	0	0	0	lipofuscin++,fat+, FN+	lymphocytes, FP
GH	0	0	0	0	cholestasis	bile duct obstruction
DW	30	0	10	40	INV+, fat+, lipofuscin+	lymphocytes
JP	0	20	30	50	_	normal
HB	0	0	40	40	lipofuscin++	normal
AS	0	10	0	10	fat+, FN+	PBC(3)
RS	10	0	0	10	_	lymphocytes
PB	0	0	40	40	iron+	normal
MB	30	20	0	50	INV+	lymphocytes
AP	50	10	0	60	INV+	normal
PM	0	0	0	0	iron+, fat+	lymphocytes, FP
FP	0	20	30	50	iron+	lymphocytes
GH	0	0	0	0	FN++, fat+	lymphocytes, FP, FD
JH	0	0	0	0	copper+	?SC ?PBC
BH	0	0	0	0	_	normal
MR	50	10	0	60	_	harmartoma
NE	0	0	30	30	INV+	lymphocytes
LM	0	0	0	0	_	lymphocytes
MP	100	0	0	100	microvesicular fat+++	lymphocytes
CA	60	40	0	100	microvesicular fat+++ lipofuscin+	normal
JJ	10	0	50	60	lipofuscin+	lymphocytes
JC	20	0	0	20	lipofuscin++	lymphocytes, FP,FD
AL	30	40	0	70	microcesicular fat+++	lymphocytes
JS	0	0	0	0	copper+	PBC(3)
GK	10	20	0	30	lipofuscin+++	normal
PT	0	0	0	0	cholestasis, INV	PBC(3)
JJ	0	0	0	0	cholestasis, INV	?SC ?PBC
TL	0	0	0	0	lipofuscin++	normal
AH	30	30	0	60	cholestasis, FN+, hyaline	PBC(3)
Acute						
MC	0	20	50	70	_	normal
MC	80	0	0	80	_	lymphocytes
AN	20	0	0	20	fat+, FN+	lymphocytes
AM	0	0	0	0	fat++, INV+	lymphocytes
EH	70	20	0	90	fat+++,INV+	normal
AH	0	0	0	0	fat+,INV+	SC
LH	60	0	0	60	_	SC
JS	0	0	0	0	cholestasis	SC

*Hepatocytes: type I=water-clear cells, type III=ground glass cells, type II=intermediate. †grade and type of parenchymal changes: - = normal, + = mild, ++ = moderate, +++ = severe; INV= intranuclear vacuoles; FN=fat necrosis. Portal tracts: FP=focal proliferation of bile ductules; FD=focal ductopenia; PBC=primary biliary cirrhosis-like changes as usually graded; SC=suggestive of sclerosing cholangitis; ?PBC ?SC= crossover pattern (ref 172).

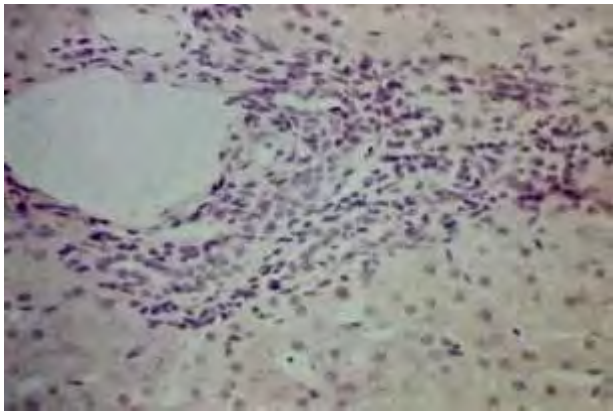

Figure 6.6 H&E stained section of biopsy from a patient with alcoholic calcific pancreatitis showing lymphocytic infiltration and bile ductular proliferation in portal tract. Publication details as for Figure 6.1.

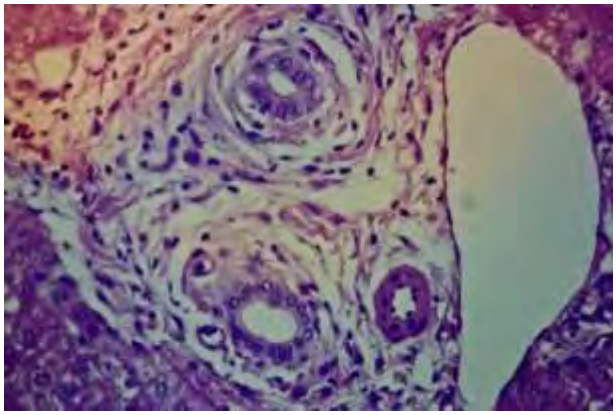

Figure 6.7 H& E stained section of biopsy from another patient with alcoholic disease showing the onion-skin appearance of portal tract, as in primary sclerosing cholangitis. Publication details as for Figure 6.1

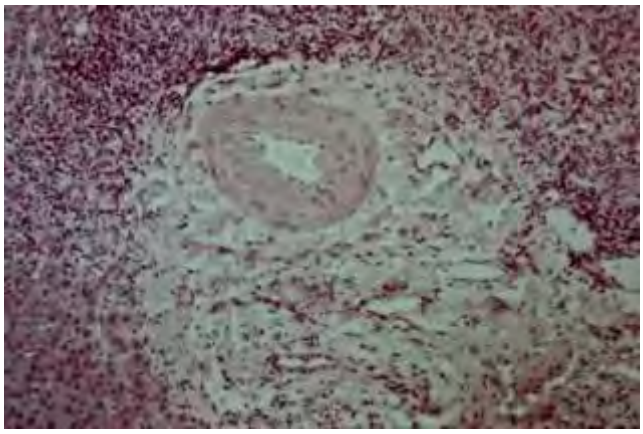

Figure 6.8 Portal tract appearance in H&E stained section is strongly suggestive of primary biliary cirrhosis (stage III); anti-mitochondrial antibody negative. Publication details as for Figure 6.1.

idiopathic chronic pancreatitis who presented with steatorrhoea (**Figure 6.4**). The occasional concurrence of cell types (**Table 6.1**) suggests that they may represent a continuum that evolves upon regular exposure to xenobiotics, whether or not identified. Indeed, considering the accelerated clearance of drug probes by many patients (Chapter 5), including several who had a liver biopsy, it may be that that hepatocytes which appeared 'normal' would display ultrastructural aberrations. Thus, extensive microvesicular steatosis in 3 patients was only revealed when semi-thin sections were stained by toluidine blue (**Figure 6.2**). The high frequency of portal tract lesions is discussed after the next section.

6.2 Endoscopic Cholangiograms

6.2.1 Study description and outcome

A list of patients with chronic pancreatitis, post-acute pancreatitis, pancreatic cancer, or chronic pain that was later diagnosed as spastic colon syndrome was drawn up from the ERCP register for 1981-1984 inclusive. Thereafter the clinical records were retrieved and examined. Cases were excluded in the following circumstances: biliary tree filled inadequately; follow-up information incomplete; diagnosis still in doubt; established liver disease; previous sphincterotomy or bile duct exploration; prior history of ascending cholangitis. Radiographs for detailed study came from 47 patients with chronic pancreatitis, 12 with post-acute pancreatitis, 7 with pancreatic cancer and 7 with spastic colon. Categorization into pancreatitis subgroups was by standard criteria (Chapter 2).

Alcoholism was recorded in 19 patients with chronic pancreatitis, of whom 9 had pancreatic calculi and 1 had gallstones. The non-alcoholic group of 28 patients, of whom 6 had pancreatic calculi, included 2 who had undergone renal transplantation and were on prednisolone and azathioprine, a patient with primary biliary cirrhosis (and pancreatic calculi), 2 with ulcerative colitis on salazopyrine, and a patient with Crohn's colitis on azathioprine. The group of 12 with post-

acute pancreatitis included 3 with gallstones; 3 whose attacks were not aborted by surgery (cholecystectomy 2, resection of choledochal cyst 1); a patient on immunosuppressive therapy for chronic renal failure; and another in whom the anti-mitochondrial antibody test was positive. Four of the group, including the patient with primary biliary cirrhosis, had hypertriglyceridaemia. The diagnosis of pancreatic cancer was confirmed by histology: the tumour was primarily in the neck, body or tail of the gland (n=2, 2, 1, respectively), or diffuse (n=2). The 'spastic colon' group presented with constant abdominal discomfort, usually accompanied by altered bowel habit: pancreatic tests were normal and patients remained well during a follow-up period of 2+ years.

Apart from hepatomegaly in the cancer group, there were no clinical signs of liver disease. Serum transaminases were generally normal, as was prothrombin time; whereas alkaline phosphatase was elevated in 35% of patients overall.

Radiographs from each patient were in the first instance reviewed independently by 2 observers who had no access to the clinical information: where there was disagreement, a final judgement was reached after a combined review. Every cholangiogram was systematically assessed with regard to the common bile duct and intrahepatic biliary tree, each according to 3 pre-set criteria, as follows: (a) 'irregularity', defined as calibre variation, either locally or over a longer segment; (b) 'roughening' implying an uneven duct outline, varying from a fine brush border to multiple mamillations extending over 1 cm or more; (c) 'stricturing', which indicated reduction in duct calibre by at least 50% and could be accompanied by beading (presumably due to focal pre-stenotic dilatation) or, rarely, sacculation[174]. Added features recorded for the intrahepatic element were 'nipping' (ie narrowing at the point of ductal origin) and 'pruning' (ie reduced number and crowding of second-order ducts). At least 3 different changes had to be present for a cholangiogram to be declared abnormal (**Table 6.2**). Where the changes in extra- or intra-hepatic biliary ducts were borderline, the results were regarded as normal. Results in the various groups were compared by Fisher's exact test.

Cholangiograms were classified as normal in all patients with the spastic colon syndrome, whereas they were regarded as abnormal in 55 of 66 patients overall with pancreatic disease (83%) (**Figures 6.9, 6.10**). When cases with intrapancreatic constriction of the bile duct were excluded from the analysis, 70% of the remaining patients with chronic pancreatitis had abnormal intrahepatic cholangiograms, as did 60% with acute pancreatitis and 100% with pancreatic cancer. In the chronic pancreatitis subset the frequency of abnormal cholangiograms was higher in patients with alcoholic compared to non-alcoholic disease (p=0.018).

6.2.2 Comments

With the advent of ERCP in the 1980s, associations between liver and pancreatic disease were increasingly recognised. Thus, patients with primary biliary cirrhosis or primary sclerosing cholangitis were noted to have ERCP evidence of asymptomatic chronic pancreatitis[176], with 1 report quoting a 77% incidence of abnormal pancreatograms in patients with the latter disease[177]. On the reverse side of the coin, the Manchester study showed abnormal cholangiograms in the majority of patients with exocrine pancreatic disease[175].

The outcome was in keeping with the liver biopsy findings (Section 6.1; cancer patients not biopsied). The range of portal tract changes noted was reminiscent of findings in patients with primary sclerosing cholangitis[178, 179]. Others have alluded to the covert nature of sclerosing cholangitis in the setting of ulcerative colitis[178, 180]: as in that condition so in pancreatitis, the presenting feature of rectal bleeding or agonizing

Table 6.2 Cholangiographic abnormalities in patients with pancreatic disease

	No.	Extrahepatic			Intrahepatic					Total abnormal	
		Irregularity	roughening	stricture	irregularity	roughening	stricture	pruning	nipping	No.	%
Chronic pancreatitis											
Alcoholic											
Calcific	9	3	1	4	6	0	1	0	2	8	89
Non-calcific	10	4	2	4	4	1	1	0	2	6	60
Non-alcoholic											
Calcific	6	0	0	3	6	0	0	1	2	6	100
Non-calcific	22	8	4	4	16	0	2	1	6	19	86
Total	47	15	7	15	32	1	4	2	12	39	83
Acute pancreatitis	12	2	1	2	6	0	3	0	4	9	75
Cancer of pancreas	7	2	1	4	4	0	0	0	2	7	100
Total pancreatic disease	66	19	9	21	42	1	7	2	18	55	83

See text for further details (from ref 175).

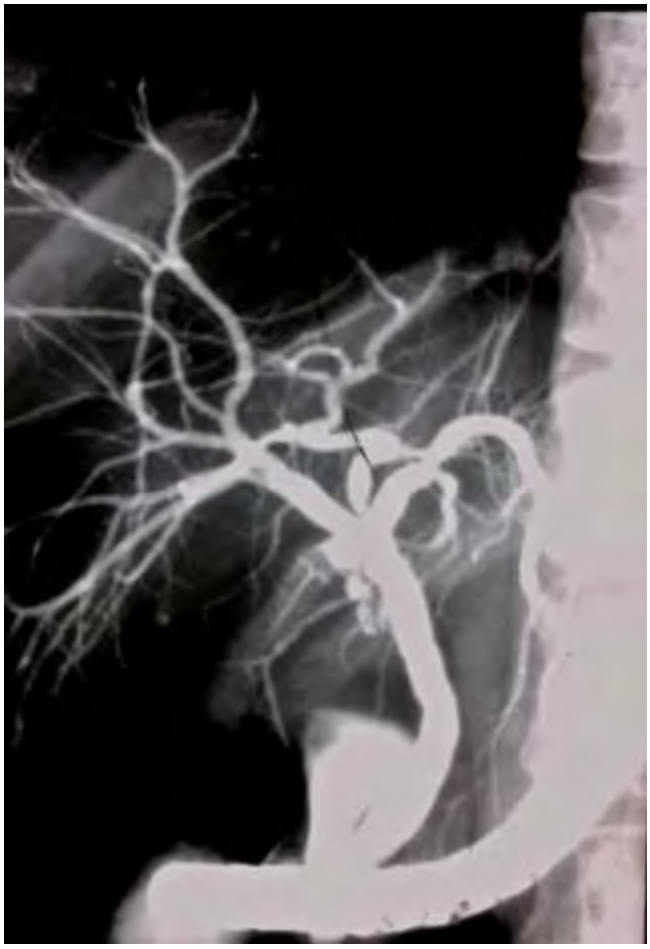

Figure 6.9 Cholangiogram of a patient with pancreatitis attacks despite cholecystectomy (no stones), on NSAIDs for arthritis. Note dilatation of the common bile duct, consistent with cholecystectomy; strictures in both main hepatic ducts, with calculi upstream. The pancreatic duct appeared normal. Reproduced from Clin Radiol[175].

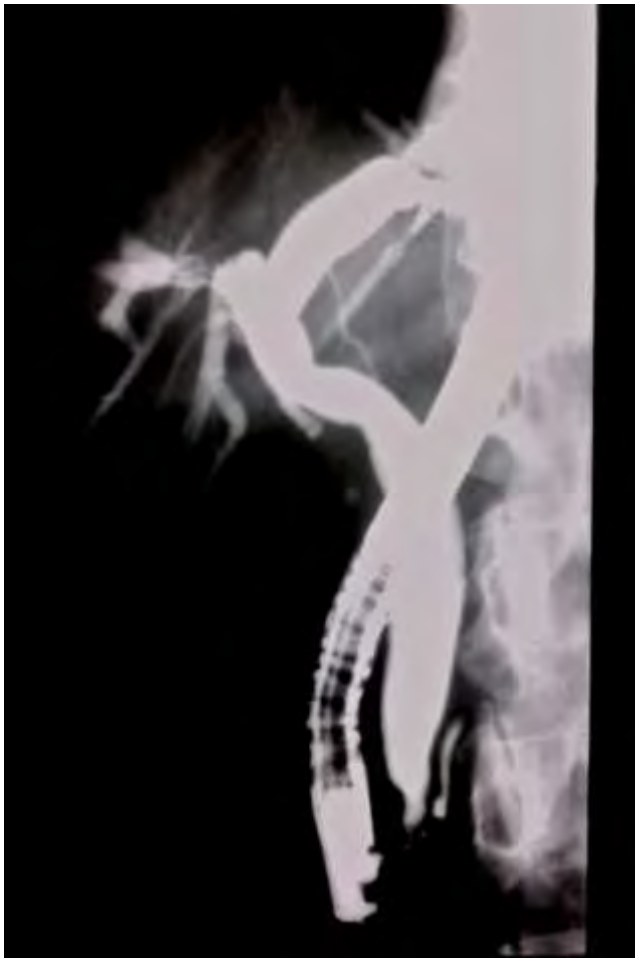

Figure 6.10 Retrograde cholangiogram of an alcoholic patient with relapsing acute pancreatitis. Note irregularity of the common bile duct, nipping of a main intrahepatic duct and calibre variation with beading in several third-order ductules. Publication details as for Figure 6.8.

abdominal pain, respectively, forces the patient to seek medical help at a time when the liver problem has not advanced sufficiently to cause symptoms or to substantially alter the serum liver function profile.

6.3 Chronic pancreatitis-type artificial bile and experimental pancreatitis

6.3.1 Study description and outcome

An experimental investigation was designed to test the possibility that pancreatitis might be initiated by reflux of bile laden with FROP[181], as found in clinical studies[87].

The procedure in rats was as follows. After anaesthesia the distal bile duct was tied off and a cannula inserted into the pancreatic duct at the duodenal entry point, following which 100 µl of a test solution was infused retrogradely into the duct over 2 minutes using a syringe pump. Observations on the animal's general condition, appearance of pancreas and peritoneal cavity were made at 3-hour intervals during the next 12 hours while the animal was still anaesthetised. Findings in the head and tail of the gland were graded separately on an arbitrary scale from 0-3 for oedema, fat necrosis, glandular necrosis, and haemorrhage. Peritoneal fat necrosis distant from the pancreas was scored from 0-3. A maximum score of 27 would indicate an advanced stage of haemorrhagic pancreatic necrosis (HPN, synonym necrotising pancreatitis). After termination of the experiment, the pancreas was removed, fixed and embedded in paraplast. Three 5 µ sections, each approximately 150 µ apart, were taken from the head region and 3 from the tail, and stained with haematoxylin and eosin. The summated extent of abnormality in head or tail sections section was graded on an arbitrary scale from 0-3 for oedema, inflammatory cell infiltrate, necrosis, fat necrosis and haemorrhage. The scores for head and tail were then added: rising scores, up to a maximum of 30, denoted increased disease severity.

The following test solutions were prepared: (i) isotonic saline, 150 mmol/l; (ii) bile salt solution with 2.09% bile salts of pH 8.4 and osmolality 258 mosmol/kg, comprising sodium glycocholate, sodium glycodeoxycholate, and sodium deoxycholate in a weight / weight ratio of 2:2:1 (also NaCl 85 mmol/l, NaHCO3 25 mmol/l and KCl 5 mmol/l); (iii) linoleic acid / bile salt in which the fatty acid was dissolved in the bile salt mixture to a concentration of 3.6 mmol/l or 25 mmol/l immediately before use; (iv) ultraviolet light-irradiated linoleic acid at both concentrations made up with bile salts and albumin 10 g/l to simulate secretin-stimulated bile in controls and pancreatitis patients, respectively (Chapter 3); and (v) lipoxidase-peroxidised linoleic acid / bile salt mixture without albumin.

The surgeon who did the experiments and the pathologist were unaware of the solutions under test, which were formulated by a chemist who ensured that more than 1 test solution was tested during a day's work and that studies in a given series were spread over the course of several days. The Mann Whitney U test was used to analyse data.

Ductal pressures were monitored by a transducer coupled to a polygraph. In a preliminary study of 5 rats, pressures were remarkably similar: peak 41 ± 0.5, plateau 24.2 ± 0.8, end 20 ± 0.95 cm water (mean ± SE). By incorporating methylene blue it was evident that 100 µl passed up to the ligature on the common bile duct and entered pancreatic duct branches in the head of the gland, whereas little entered the distal duct.

The results are summarised in **Table 6.3**. In the saline-treated group, transient oedema of the pancreatic head was seen soon after the infusion, and mild interstitial oedema was shown on histology. Treatment with bile salts increased oedema, caused foci of acinar necrosis, and elicited a moderate inflammatory exudate. The addition of linoleic acid in low or high dose further increased macroscopic injury at 3 hours, but not

thereafter, and microscopic damage scores were similar for both doses. The 'biological' DC product (solution iv) in a dose of 25 mmol/l caused a greater degree of damage than bile salts alone or with linoleic acid: areas of pancreatic necrosis and peritoneal fat necrosis were visible, and extensive areas of acinar necrosis by histology. The damage was not increased further by lipoxidase-peroxidise linoleic acid (solution v).

Most animals survived for the 12 hours of the experiment. In those that succumbed earlier, death seemed to be primarily from lung injury, in that respiratory difficulty was manifest and at post-mortem segmental consolidation and /or punctuate haemorrhages were apparent on the lung surface. There were significant positive correlations between macroscopic and microscopic assessments of pancreatic damage at 12 hours in rats that survived.

6.3.2 Comments

Two previous reports were germane to the Manchester study. (i) Bile from 'chronic alcoholic' rats had been shown to inflict greater damage when injected retrogradely into the pancreatic duct of other rats, whether or not recipients were 'alcoholic'. The authors speculated that free bile acids rather than biliary ethanol was responsible[182], but this explanation is only tenable for infected bile. (ii) More interesting and relevant was a report on the effect of retrograde injection of 200 µl sunflower oil, oleic acid or linoleic acid (concentration not specified): only the last acid caused extensive acinar necrosis, which could be attributed to its peroxidation, as indicated by measurement of malondialdehyde[183].

Retrograde injection models of pancreatitis are fraught with problems. That is why it was necessary to ascertain that unphysiological pressures were not produced, and also that osmolality and pH of test solutions were within the physiological ranges for hepatic bile. Furthermore, the independent effect of bile salts had to be factored in, which involved a painstaking gradation system to assess macroscopic and microscopic injury. Increments in serum level of pancreatic enzymes are no guide to severity, and their quantitation in the gland is meaningless in the presence of variable amounts of necrotic destruction. The high degree of significance as regards the difference in tissue damage in the group treated with ultraviolet light-irradiated linoleic acid compared to that treated with bile salts alone, despite small numbers (**Table 6.3**), indicates that the result was genuine. Neither in this study nor in the earlier investigation of

Table 6.3 Rat 'reflux' experiments: summary of results

Test solutions	n	Microscopic score 3-hr median (range)	12-hr median (range)	Histology score median (range)	Time of early death (hr)
(i) saline	10	2 (1 -5)	2.5 (1 -5)	4 (2 - 6)	10, 11.8
(ii) bile salts	10	2.3 (2 -5)	5.0* (3 -5)	11*** (9 – 15)	6, 10, 11.9
(iii) linoleic acid (25 mmol/l) + bile salts	6	4.2* (3-6)	5.3* (5-6)	11.5*** (9 – 15)	6, 11.5, 11.9
(iv) UV-peroxidised linoleic acid (25mmol/l) + bile salts + albumin	6	7.0***(5- 8)	8.0***(6 - 9)	16.9*** (12 -17)	11.5, 11.6
(v) lipoxidase - peroxidised linoleic acid + bile salts	5	5.3* (4 -7)	6.5*** (6 - 9)	16.0*** (11 – 25)	9

*p < 0.05, ** p<0.01, *** p<0.005, compared to saline-treated group or between groups linked by vertical brackets (ref 181).

unsaturated fatty acids[183] was pancreatic haemorrhage a feature.

The results cannot be extrapolated to a situation of repeated duodeno-pancreatic reflux[184], which is expected to also involve enteropeptidase-activated pancreatic proteases and phospholipase A_2. Moreover, although a long- term experiment in goats showed that perfusion of the pancreatic duct with normal bile via an intestinal loop at physiological pressure is innocuous[185], the effect of perfusing abnormal bile containing 25 mmol/l of oxidised linoleic acid in this way is unknown, while the potentially toxic effects of other substances in bile, such as RXS, adds a further unquantifiable dimension.

6.4 Overview and Summary

Today it is agreed not only that an increase in liver oxidative stress underlies most hepatopathies, but also that cholestasis ensues when the increase is acute, so as to disorganise the actin cytoskeleton of hepatocytes and cause internalisation of canalicular transporters that facilitate bile formation. In contrast, chronic lower-grade stress evokes an adaptive response such that FROP can be extruded[186].

Collectively, the investigations reported in this Chapter allow 3 broad conclusions. (i) Chronic induction of hepatic xenobiotic-metabolising pathways - and the inevitable increase thereby of ROS / RXS load - in patients with pancreatic disease is usually silent clinically but changes in hepatocytes and portal tracts are often evident microscopically, and in bile ducts by cholangiography. (ii) There is an inverse correlation between the degree of hepatocyte and portal tract damage. (iii) Artificial bile to simulate that which is found in Boots secretin-stimulated bile from patients with pancreatic disease causes substantial pancreatic necrosis when injected retrogradely in rats.

As regards the first point, alcohol and drugs such as azathioprine are implicated in pancreatitis, and are associated with SER proliferation in hepatocytes. Moreover, microvesicular steatosis is typical of liver damage that may accompany pregnancy, oral contraceptives, tetracycline or sodium valproate - conditions that increase RXS load and are also associated with pancreatitis. The phenomenon also follows experimental treatment with vinblastine or colchicine, which interfere with microtubular transport and secretory polarity in hepatocytes (**Figure 2.6**). The finding of excessive amounts of lipofuscin in liver biopsies is in keeping with this general concept, in that the pigment is a condensation product of malondialdehyde[187], indicating excess lipid peroxidation.

As regards the second point, chronic induction of xenobiotic-metabolising enzymes in hepatocytes, but without functional impairment, rationalises increases in bile content of bilirubin, copper, fatty acids and FROP (Chapters 3 & 5).). A review in 2002 argues for a key role of oxidative stress in the pathogenesis of sclerosing cholangitis, coupled interdependently with anticytoplasmic and anticatalase antibodies[188].

The third point supported the proposal that reflux of FROP-laden bile might underlie the connection between chronic exposure to xenobiotics and pancreatic disease. Nonetheless as early as 1984, a year after the Manchester ‘detoxification’ hypothesis was published[9] (Chapter 4), reports began to trickle in from animal studies which suggested that a dormant drug- metabolising capability in the pancreas might be awakened under particular circumstances[10, 189]

Chapter 7

Probing the Defence Arc: Dietary Antioxidants

Induction of CYP is primarily a defence mechanism, but increases the yield of ROS. Tissues possess a multilayered defence strategy against these: intra-cellular compartmentation; metal- binding and oxidising proteins that together with the bulk antioxidants (urate, bilirubin, albumin, glucose) operate mainly in the extracellular environment; antioxidant enzymes that spearhead intracellular defence; and micronutrients that act intracellularly in the native state and / or as a component of antioxidant enzymes and / or of GSH (**Figure 7.1**), while also contributing exracelullarly[100]. Could it be that habitual micronutrient supply determines the threshold of CYP-mediated toxicity from ROS in patients with chronic pancreatitis?

7.1 Versus intake in healthy controls

7.1.1 Study description and outcome

Patients with idiopathic chronic pancreatitis(n=15) were deliberately chosen to cover a range of theophylline clearance, as marker of CYP1A induction, varying from normal to accelerated. When compared to results from studies in non-smoker, non-alcoholic adults (Chapter 5). These patients were adjudged to be reliable witnesses, who were not dependent on narcotic analgesics and had not altered their diets substantially,

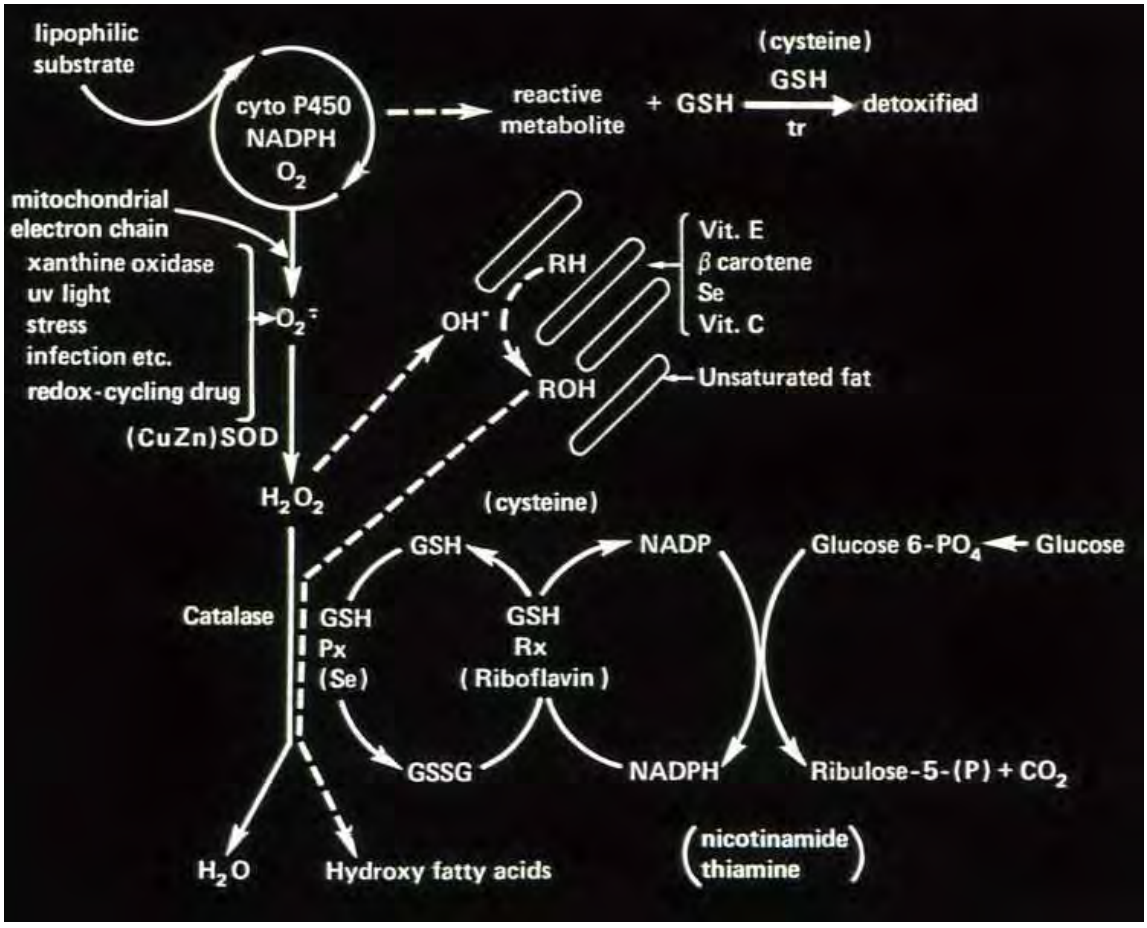

Figure 7.1 Antioxidant roles of some micronutrients. SOD=superoxide dismutase, RH=unsaturated fatty acid, ROH= oxidation product of fatty acid, GSH=glutathione in its bioactive reduced form, GSSG=oxidised glutathione upon quenching reactive oxygen species, GSH-Px= glutathione peroxidase, GSH-Rx=glutathione reductase, GSH-tr =glutathione transferase. Note that the essential amino acid methionine is a source of cysteine.

and in whom there was no clinical or biochemical evidence of liver disease. The pre-test duration of symptoms varied widely as did the interval since the last exacerbation, alcohol and cigarette usage, ERCP and secretory data (**Table 7.1)**. Among the male patients 2 had retired from work (HG for 2 years, NE for 1 year) and 3 were kept away by illness for several months (CA, AL, CR). Two of the female patients had experienced an attack of pancreatitis during pregnancy (PB, AP). A drug that was suspected of triggering an attack had been discontinued after the latest relapse in 3 patients.

Age and gender-matched healthy volunteers were enlisted from the hospital's ancillary staff and by advertisement through a local general practice. None was on a prescribed drug or smoked cigarettes; most drank alcohol socially (80-160 gm per week); all drank up to 8 cups of tea or coffee daily (which contain methyl xanthine inducers of CYP). A theophylline test was done exactly as described in Chapter 5. Habitual diet was assessed by a 7-day weighed inventory in the patient's home during the week following the theophylline test, with the cooperation of the patient's wife or mother as appropriate. Similar information was obtained from each volunteer. From these records, the foods were totalled and coded for analysis on a microcomputer using the Microdiet programme (Salford University, UK) which is based on published food tables, but incorporated additional information on selenium[190].

Paired t tests (2-tailed) were used in the main to compare data in controls and patients. Stepwise linear regression discriminant analysis was used to gauge the relative importance of various micronutrient antioxidants in distinguishing patients from controls. Thereafter, any contribution to the discrimination that might be afforded by theophylline kinetics was investigated.

The results are comprehensively described in the study report[190]. When assessed by national guidelines, the following points emerged in relation to energy and macronutrient intake: two-thirds of patients and a third of controls acquired < 85% of the recommended nutrient intake (RNI) of energy; a third of patients consumed < 85% of recommended protein; all participants obtained 25-50% of energy as fat, as is typical for British diets; and the calorific yield from fat was higher in the patients. **Figure 7.2** displays the main findings in relation to micronutrient antioxidants.

Table 7.1 Diet study 1: Clinical details

	Age[1] (years)	Age[2] (years)	Last attack (months)	Theophylline CL (ml /kg / hr)	Alcohol (gm / wk)	Cigarettes (no. /d)	Drugs	Pancreatogram [3]	Secretory impairment[4]
Female									
PB*	20	20	9	96	100	10	—	MIP	mild
CW	21	18	3	38	0	0	—	MIP	mild
AP*	23	15	10	107	50	0	—	ADP(D)	mild
KC	27	27	3	34	42	0	—	MIP	mild
FJ	30	26	2	52	50	5	—	MOP	mild
LM	35	33	5	215	0	15	prednisolone azathioprine	MOP	normal
BJ	43	38	18	141	280	20	—	ADP(D)	moderate
DM	74	73	steatorrhea	108	0	0	tolbutamide	failed	moderate
Male									
DB	18	18	0.5	177	160	10	—	MOP	normal
CA	24	15	7	179	140	10	—	MOP	normal
CR	26	25	steatorrhea	100	0	0	nsaid	MIP	severe
GM	28	19	2	215	0	20	phenytoin	normal	moderate
AL	41	40	3	70	140	0	—	ADP(D)	moderate
NE	66	48	steatorrhea	213	140	25	pancreatic extracts	normal	severe
HG	78	72	3	76	0	0	—	ADP(O)	moderate

[1] age at study, [2] age at 1st symptom, [3] pancreatogram and [4] pancreatic function testing as described in in Chapter 2.
*patients with relapse during pregnancy, nsaid =non-steroidal anti-inflammatory drug (ref 190).

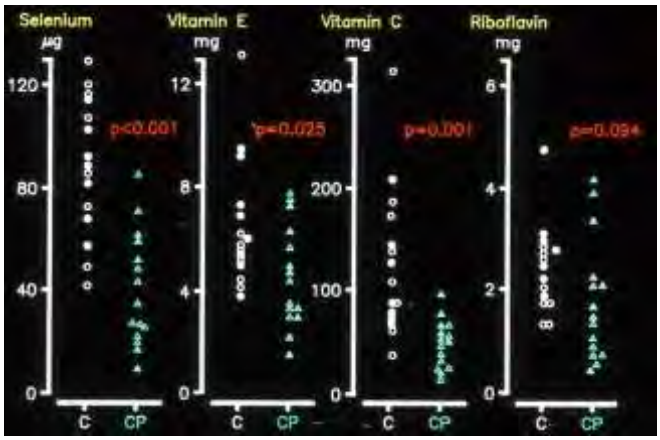

Figure 7.2 Main outcome of investigation into habitual intake of micronutrient antioxidants in patients with chronic pancreatitis. Circles indicate females and triangles indicate males among C=control and CP=chronic pancreatitis groups. Results are given for paired t tests: results by Student's t test were very similar. Reproduced from Hum Nutr Clin Nutr [190]

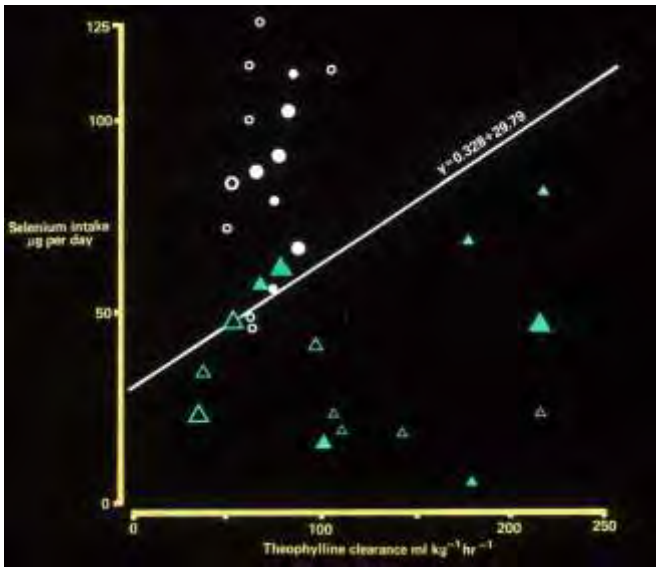

Figure 7.3 Relationship between theophylline clearance (as index of oxidant load via CYP1A2) and habitual daily intake of selenium in patients with chronic pancreatitis (triangles) and age / gender -matched controls (circles): open symbols represent females, closed symbols represent males. The size of the symbols denotes intakes of highly unsaturated fatty acids, C20:4-C24:6: smallest <500 mg, intermediate 500-1000mg, largest >1000mg per day. Publication information as for Figure 7.2.

Stepwise linear discriminant analysis identified lower selenium intake by the patients as the most important discriminator, the separation from control data was not improved by addition of other micronutrients suggested by one-dimensional tests. However, when theophylline Cl was entered into the analysis, a line of discrimination separated data from the majority of patients and the majority of controls (**Figure 7.3**). Although intakes of highly unsaturated fatty acids (C20:4 - C24:6) did not differ between the groups as a whole, among 6 participants in the overlap zone the 3 controls ingested 329, 320 and 82 mg/day, compared to 1970, 1049 and 750 mg/day by 3 patients.

7.1.2 Comments

Dietary studies are tedious and difficult to execute but the individual weighed inventory, when successful, provides a wealth of information that is difficult to match by other methods. Safeguards were put in place to circumvent potential pitfalls. (i) The dietitians who implemented the study were unaware of its precise goals. (ii) Participants were interviewed separately by a doctor and dietitian so as to arrive at a consensus on reliability. (iii) Family members were involved from the outset to improve compliance. (iv) Dietitians undertook spot checks by home visits or telephone contact. (v) The Microdiet database was modified to provide more accurate information on selenium in raw and cooked foods. (vi) Patients and controls were age and gender-matched to reduce bias.

The study identified lower intakes by the patients than controls of selenium, vitamin E, vitamin C and riboflavin. Poor intake was due to generally inadequate diet or low intake of specific foods. Since the identified items are derived from very different foodstuffs, it is unlikely that the result was spurious. Instead, their recognised roles in defending cells against excessive free radical activity (**Figure 7.1**), the close interaction between selenium and vitamin E in membrane protection against ROS[191] and, in particular, the nomogram identified by discriminant analysis (**Figure 7.3**) strongly supported the notion that chronic pancreatitis is a casualty of unmitigated oxidative detoxification reactions (Chapter 4). The intake of PUFA could tip the balance in favour of injury (**Figure 7.4**), because not only do they facilitate

CYP induction[117,118], but also their incorporation into membrane phospholipids renders membranes more vulnerable to peroxidation[192-194].

In any future study the altered circumstances by the time the patient is investigated would need to be borne in mind. For example, 2 patients in the current study suffered an attack during pregnancy (PB, AP in **Table 6.1**): when tested some months later, both had theophylline clearances at the upper limit of the reference range and extrapolated selenium intakes low at 41 and 23 µg/day. Increased free radical activity in normal pregnancy has been ascribed to increased cell turnover or decreased availability of antioxidants[195].

It was clearly necessary to test these assumptions by studying a set of CYP-induced individuals without chronic pancreatitis. Patients with epilepsy on long-term treatment with anticonvulsant inducers of CYP1A2 were the obvious choice (Chapter 5).

7.2 Versus intake in controls with epilepsy

7.2.1 Study description and outcome

Institutionalised patients with epilepsy were used for convenience, while realising that they were likely to be better nourished than otherwise. They were chosen after discussion with staff of the centre, so as to ensure that they were capable of giving informed consent. Exclusion criteria were as follows: history suggestive of pancreatic or liver disease; non-standard diet (because of refusal to eat, anorexia, nausea, psychiatric problem, etc); medication with a drug that was known to inhibit CYP (eg oral contraceptive). Their reliability as participants was not crucial because the nursing staff undertook to supervise the investigations. The age range of the group was similar to the healthy controls and patients with chronic pancreatitis studied earlier, but had an excess of men as several women were on an oral contraceptive (**Table 7.2**).

Dietary studies and theophylline tests were done

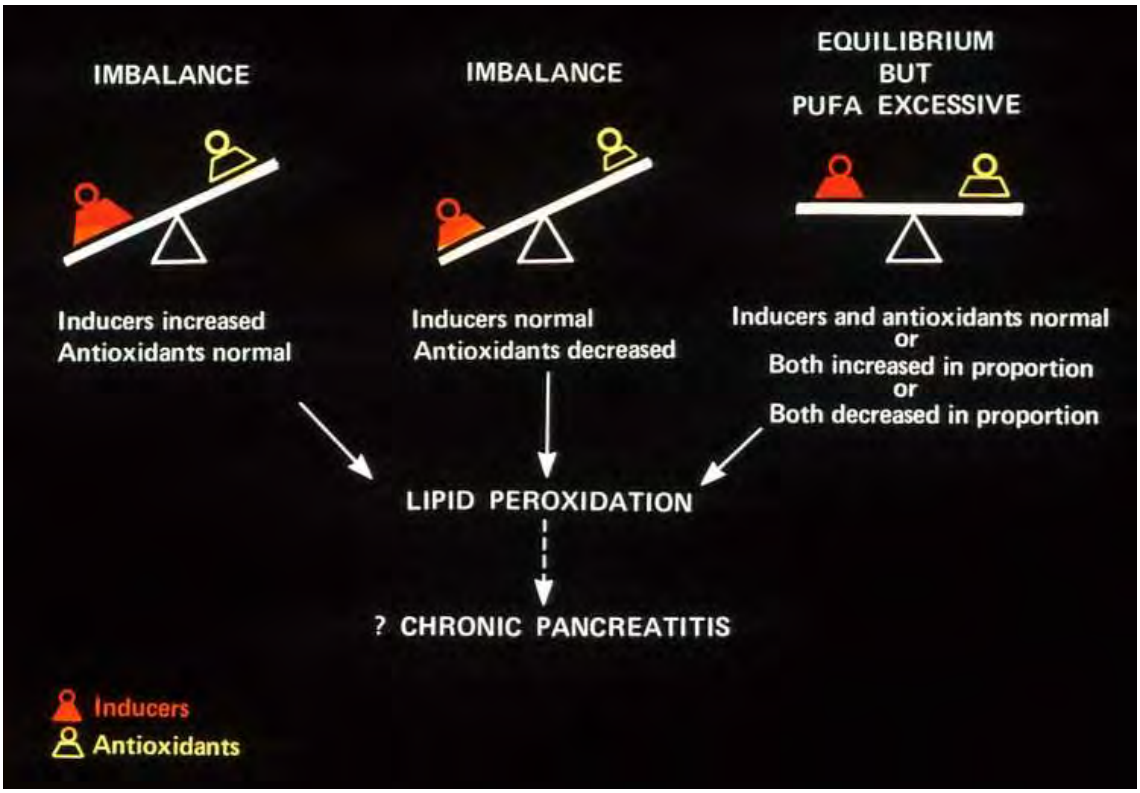

Figure 7.4 Hypothesis suggested by dietary and pharmacokinetic studies. PUFA= polyunsaturated fatty acids. Publication information as for Figure 7.2.

Table 7.2 Diet study 2: Social details of control group with epilepsy

ID	Age (yrs)	Gender	Anticonvulsants	Cigarettes (no. / day)	Occupation
JS	38	M	phenytoin, valproate	0	parcel packer
PU	26	F	carbamezapine, valproate, ethosuxamide	1-10	envelope maker
NW	33	M	phenytoin, carbamezapine	10-20	woodworker
JB	33	F	carbamezapine	0	laundry worker
KG	33	M	carbamezapine, ethosuxamide	0	workshop
FM	51	M	phenytoin, carbamezapine	10-15	farmer
SF	31	M	phenytoin, valproate	15-20	gardener
CS	34	F	phenytoin, carbamezapine	0	paper hat maker
JA	24	M	valptoate, carbamezapine	20-25	parcel packer
TR	23	M	valproate, carbamezapine	0	porter
SB	27	M	phenytoin, carbamezapine	20-30	farmer
PC	32	M	valproate	0	farmer
JH	31	M	phenytoin, valproate, primidone	0	porter
JMC	32	F	phenytoin, valproate	20-30	kitchen worker
DD	40	M	valproate, primidone	20-30	parcel packer

(ref 196)

as described earlier. Student's t test (2-tailed) was used to compare results in healthy controls, diseased controls with epilepsy and patients with chronic pancreatitis: when variances were significantly different, an approximate t test was substituted. Stepwise canonical variate analysis was used to determine the variables which maximally separated the 3 groups. In this method discrimination is achieved from weighted composites derived from the variables. Two such composites may be needed to separate 3 groups, and examination of the weight applied to standardised variables (ie expressed as the number of standard deviations from the mean) indicates which variables make the biggest contribution to the discrimination[196].

Theophylline Cl values were similar in epilepsy and chronic pancreatitis groups - 123 ± 59 and 120 ± 62 ml / kg / hr, respectively - each significantly higher than the value of 74 ± 16 ml / kg / hr in healthy controls (p=0.008, p=0.014, respectively), and indicating a similar degree of CYP1A induction (as was noted in Chapter 5).

The results of dietary studies are summarized in **Table 7.3**. By contrast to findings in the chronic pancreatitis group, diets of the epilepsy group provided higher energy and fat intakes than in controls. Intakes of antioxidants by the epilepsy group were generally excellent with the exception of selenium, the intake of which was similar to that in the chronic pancreatitis set, on average half that in controls. The only difference between the two sets of patients by one-dimensional analysis lay in the substantially higher intake of vitamin C by the group with epilepsy.

Upon stepwise canonical variate analysis of theophylline and antioxidant data, selenium, methionine and vitamin C emerged as the important variables. Two composites were derived and both were statistically significant. The weights applied to the standardised variables resulted in two scores:

	Score 1	Score 2
Selenium	-1.00	-0.41
Vitamin C	-0.27	0.80
Methionine	0.50	0.71

The first score was highly weighted on selenium; whereas vitamin C and methionine made equal contributions to the second score. **Figure 7.5** illustrates the discrimination achieved when the two scores for each participant were plotted against each other. The first score separated the controls with normal selenium intakes from both groups of patients whose intakes of selenium were lower. The second score separated the chronic pancreatitis and epilepsy sets on the basis of lower methionine and vitamin C intakes by the former. Assuming equal prior probabilities, the participants were allocated to a most probable group according to territorial boundaries: 36 of 45 participants (80%) were assigned correctly[196].

7.2.2 Comments

In this extended study, it was fortunate to have the wholehearted cooperation of nursing and kitchen staff at the epilepsy centre. Two differences from the first study were that the methionine content of food was probed more thoroughly[196]; and that the epilepsy group had an excess of men.

The question posed at the outset was: 'If long-term CYP induction is all-important in the of chronic pancreatitis by generating increased amounts of ROS, why doesn't every patient with epilepsy on anticonvulsant CYP inducers develop the disease'? Their better intakes of methionine and vitamin C now appeared to be the explanation. It was thus necessary to consider why these items were glossed over in the original study, and what their particular relevance might be.

In regard to the first question, the one-dimensional analysis indicated that the average intake of vitamin C in the chronic pancreatitis set was only a third of the value in controls (p= 0.001, **Table 7.3**). Yet in the original stepwise discriminant analysis it was overshadowed to the point of extinction by their low selenium intake and high theophylline Cl. Only by studying a group of epilepsy patients was the CYP induction

Table 7.3 Comparison of habitual diet in 3 sets (Controls, CP, and epileptics)

		C (n=15)	CP (n=15)	Epileptics (n=15)	Significance of differences (P) C vs CP	C vs EP	CP vs EP
energy	kcal/d	2200±480	1770±675	2780±74	0.054	0.017*	0.001
	% of NRI	93±15	74±22	100±24	0.009	ns	0.004
protein	gm/d	81±16	70±38	93±21	ns	ns	ns
	% of NRI	144±24	146±83	168±38	ns	0.043*	ns
fat	gm/d	83±24	72±27	129±39	ns	0.001*	<0.001
	% of energy	34±6	38±5	41±4	ns	0.003*	ns
PUFA	gm/d	9.0±3.9	7.4±5.6	9.0±4.5	ns	ns	ns
	% of energy	3.4±1.2	3.7±1.7	3.9±2.5	ns	ns	ns
selenium	µg/d	85±26	38±22	43±15	<0.001	<0.001	ns
vitamin E	mg/d	6.3±2.5	4.3±2.0	6.0±2.5	0.025	ns	ns
vitamin C	mg/d	121±73	43±23	95±23	0.001	ns	0.001
β carotene	mg/d	2.5±1.8	2.4±1.3	3.4±2.2	ns	ns	ns
riboflavin	mg/d	2.4±0.8	1.7±1.2	2.3±0.6	0.05<p<0.10	ns	ns
cysteine	g/d	1.2±0.3	1.1±0.8	1.2±0.4	ns	ns	ns
methionine	g/d	1.8±0.4	1.6±0.9	2.3±0.7	ns	ns	ns

PUFA=polyunsaturated fatty acids; adapted from ref 196. asterisk* indicates value significantly greater than in controls. Note that student's *t* test as used here gave a non-significant decrease in riboflavin from controls; whereas P<0.05 using paired *t* tests in earlier study (From ref 190)

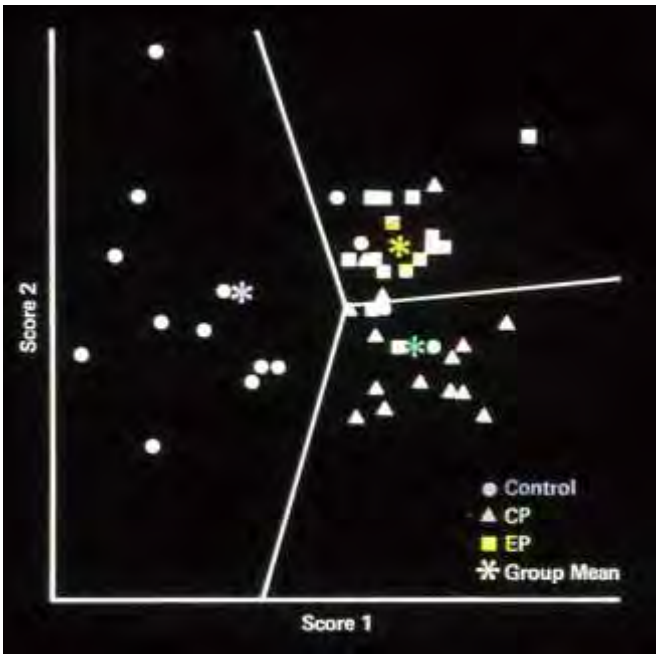

Figure 7.5. Composite scores derived by canonical variate analysis in the second dietary study. See text for details. Reproduced from Eur J Clin Nutr[196]

effect neutralised, to expose the substantially lower intakes of vitamin C in the chronic pancreatitis group. The emergence of methionine intake as an equally important item in distinguishing the two groups of CYP-induced patients was more surprising, although the earlier study showed that the subgroup of 8 female patients with chronic pancreatitis ingested lower amounts of sulphur amino acids than did matched controls ($p< 0.001$) - a difference that was not apparent in males, and was lost when data were amalgamated[190]. It should be emphasised that for both vitamin C and methionine, intakes by patients with chronic pancreatitis generally exceeded national recommendations, ie. for vitamin C 30 mg/day; for cysteine plus methionine 1.1 gm/day in males and 0.70 gm/day in females.

The answer to the second question pointed towards the complementary roles of methionine and vitamin C in removing non-biological free radicals that are generated when certain xenobiotics, such as paracetamol, halogenated hydrocarbons, and sodium valproate are processed by CYP. This synergism is well shown by in vitro studies[197]. Moreover, it is known that the yield of RXS is higher and tissue injury greater if CYP are induced to start with[170, 171, 198] . These issues are highlighted by experimental studies using carbon tetrachloride (CCl_4) which generates RXS upon processing by CYP. Thus, a PUFA-enriched diet, a prior dose of alcohol or phenobarbitone to induce CYP, or prior restriction of selenium and vitamin E intake accelerates and increases peroxidative damage; conversely, a dietary antioxidant supplement retards and reduces injury[171, 194, 199].

Methionine is incorporated via cysteine into GSH which, in its native state and/or via enzymes that utilise GSH, plays key roles in the removal of RXS[200, 201]. Within cells, ascorbic acid - the bioactive from of vitamin C - interacts with GSH via redox and non-redox mechanisms[202, 203]. Moreover, it is a potent scavenger of electrophiles[204, 205]; acts as a 'Michael donor' in toxicity studies of acrolein which is a derivative of acetaldehyde, as well as in reactions with genotoxic lipid peroxides[205]; and interacts with GSH and vitamin E in protecting lipid membranes[206]. The efficacy of GSH precursors such as methionine or N-acetylcysteine (NAC) in the treatment of paracetamol poisoning is known, and has been described in CCl_4 toxicity[207, 208]. It is less appreciated that mega-dose vitamin C abrogates injury too[209, 210].

For this interpretation to be plausible, a greater degree and range of exposure to potentially damaging xenobiotics should have been identified in patients with chronic pancreatitis than in those with epilepsy. This was not the case (**Tables 7.1, 7.2**). In fact, valproate treatment in the latter group was apparently innocuous, although it yields RXS and is prominent in the list of drugs linked to pancreatitis[15]. Also of note, cigarette smokers were equally represented among patients with epilepsy or chronic pancreatitis. The clue came from scrutiny of patients with both diseases.

7.3 Epilepsy plus chronic pancreatitis

7.3.1 Observations

Of 4 patients studied, 3 with epilepsy developed chronic pancreatitis at various intervals after anticonvulsant treatment (WH, SS, WS). The last (GM), with long-standing chronic pancreatitis suffered an exacerbation within 6 weeks of starting phenobarbitone to curb seizures due to a brain cyst (**Table 7.4**).

The cumulative load of exposure to volatile chemicals was substantial in each patient. Support for a causal connection with chronic pancreatitis was adduced from a concurrent pilot study of occupational chemicals (Chapter 8); as also the disappearance of symptoms when patient WS was forced to resign (although he remained on anticonvulsants); and similarly in patient WH when anticonvulsant CYP inducers were discontinued although his lifestyle was unchanged.

7.3.2 Comments

The possibility that regular exposure to volatile hydrocarbons might be the true culprit in the path towards chronic pancreatitis among CYP1A2-induced individuals was revealed by the study.

7.4 Overview and Summary

Overall, the investigations reported in this Chapter allowed - and continues to allow - 2 main conclusions. (i) The exocrine pancreas can withstand regular exposure to CYP inducers provided that micronutrient antioxidant supply, especially of selenium, is sufficient to meet the increased load of ROS (**Figure 7.6**). (ii) It succumbs when vitamin C / methionine intake falls short in the face of concurrent exposure to volatile sources of RXS.

Whereas an orally-administered source, eg. valproate, would arrive at the liver with its generous quota of defusing GSH / GST, parenteral exposure bypasses this protective mechanism. Silent liver damage in these circumstances is exposed by microvesicular steatosis in hepatocytes and / or changes in portal tracts and bile ductules (Chapter 6).

Table 7.4 Patients with epilepsy plus chronic pancreatitis

ID	Age (yrs)	Drugs (age)	Cigarettes no./day (age)	Alcohol gm/day(age)	Theophylline (Cl: ml/kg/hr)	Diet (/d)	Pancreatitis	Chemicals (age)
WH	80	mysoline (62-63) phenytoin (63-79) acetozolamide (80)	10 (62-78)	40 (30-study)	65	not done	1st attack age 78 last age 79 MOP	GA x 4 (62-64) spray weed killers in greenhouse (42-study)
SS	27	phenytoin (24-study) valproate (") carbamezapine (")	20(16-study)	60-80(16-study)	109	selenium 38µg vitamin C 33mg methionine 1 g	1st attack age 25 MOP	GA x 9 (24-25) hairdressing sprays/ dyes/lotions (24-study)
WS	34	phenytoin (21-study)	nil	180 (18-28) 40 (29-study)	133	selenium 44µg vitamin C 74mg methionine 2.4g	1st attack age 27 GB out for stones age 30 more attacks MOP no pains from age 32	GA x 1 (30) diesel exhaust fumes± solvents via engineering works, buses, chemical factories (28-31) jobless at study
GM	32	phenytoin (27-28) carbamezapine (29)	30-40 (16-21) 20 (22-26)	40 (17-20) spree (20-21) nil (21-study)	215	selenium 92µg vitamin C 46mg methionine 3.8g	1st attack age 20, GB out (no stones) more attacks to age 24 quiescent 25-28 attacks ++ age 29 onwards ERP normal, PFT ↓ cimetidine for DU age 25→	GA x 4 (17-20) diesel exhaust ± paint solvents± weedkillers ± trichloroethylene) via loading bays, lorry driving, horticulture

Top 3 patients had epilepsy followed by chronic pancreatitis; in the last patient pancreatitis attacks occurred within 3 weeks of starting anticonvulsants. Abbreviations: Cl=clearance, MOP=moderate changes on pancreatogram (Chapter 2). GA=general anaesthestic, GB=gall bladder, PFT=pancreatic function test, DU=duodenal ulcer, downward arrow =decreased, horizontal arrow=on-going (From ref 196)

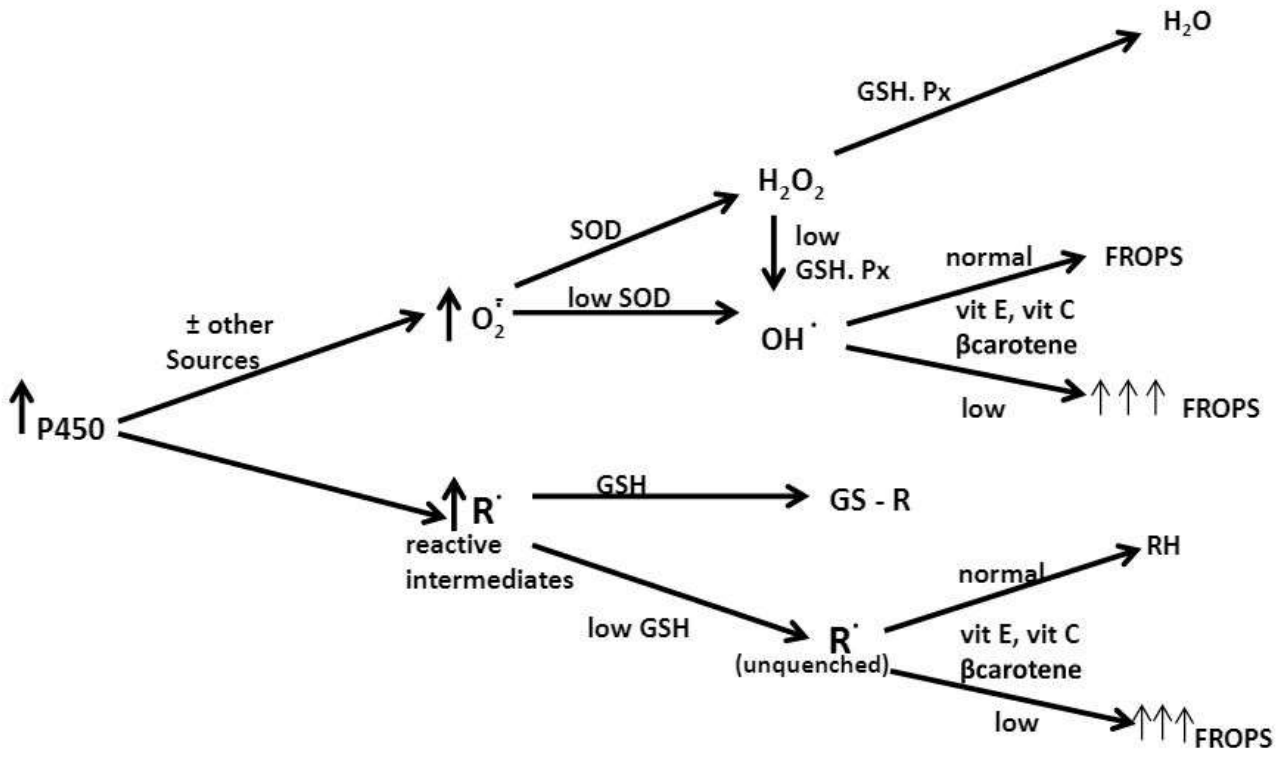

Figure 7.6 Explanation suggested for the increased levels of lipid peroxidation proiducts in duodenal juice from patients with chronic pancreatitis but normal values in those with relapsing acute pancreatitis. Abbreviations as in legends to earlier figures and in glossary. From 1990 paper in Free Radical Biol Med[90]

The further corollary was, - and continues to be - that rectification of distorted axes towards the position in health might facilitate treatment of chronic pancreatitis. This could be accomplished by reducing the intake of PUFA[194] and dietary CYP inducers[211]; and / or inhibiting CYP by a drug such as cimetidine[212]; and / or supplementing the diet with antioxidants[194] - whichever manoeuvres are appropriate after full investigation of each case. This interpretation could rationalise the common prescription of a low fat diet, and isolated reports suggesting that high dose cimetidine[213] or vitamin E [214] may be useful.

The conclusion regarding risk of chronic pancreatitis from anticonvulsant drugs seems to be that such therapy is only hazardous when antioxidant supply falters in the face of inhalation exposure to volatile hydrocarbons - an unlikely situation considering the protective environment of most patients with epilepsy - or when diets have been poor in antioxidants over many years. Once the disease is initiated, progressive parenchymal destruction would perpetuate the problem by compromising the absorption of lipophilic antioxidant vitamins.

Chapter 8.

Occupational volatile chemicals

Investigations of exposure to polycyclic aromatic hydrocarbons (PAH) in the workplace were prompted by findings that smokers and non-smokers with pancreatic disease displayed a similar degree of CYP1A2 induction, and that at least 4 non-smokers with high theophylline Cl reported jobs that might incur regular exposure to such xenobiotics (Chapter 5). A pilot study involved patients with recurrent pancreatitis who were seen between February 1984 and January 1985[215]. The results led to a case-control study to cover patients with chronic pancreatitis registered in all 7 health districts of Greater Manchester during the 5-year period from April 1985[216]. The studies were enabled by successive heads of the Occupational Health department in the Medical School - WR Lee, I Leck and N Cherry.

8.1 Pilot study

8.1.1 Description and outcome

Initially, 12 consecutive patients with idiopathic pancreatitis who were admitted with an attack or seen in the follow-up clinic were enlisted. The surprising outcome led to exploration of occupational histories in the next 7 patients who drank alcohol on a daily basis for several years before the first symptom, but whose attacks continued although they had become teetotal. Patients were classified as chronic pancreatitis (series 1, n=10; series 2, n=3) or post-acute / RAP (n=2, n= 4, respectively) by standard criteria (Chapter 2). In addition to a search for the accepted risk factors, detailed dietary and social histories were taken to reveal potential promoting factors such as diets rich in PUFA or other CYP inducer, and usage of cigarettes (Chapter 5).

The work done by each patient since leaving school was examined in detail by an occupational physician. This included: description of the work activity, particularly of exposures to volatile substances; type of workplace and provision if any of local and general ventilation; activities in adjacent working areas; and the relationship of symptoms - onset, severity, remission - to weekends, holidays or other periods away from work. Questions were directed at specific chemicals or fumes, rather than trying to extrapolate or assume such exposure from jobs or occupations. Manufacturers were contacted when the patient could only give proprietary names of substances. Finally, exposures from hobbies / 'do-it-yourself' activities were noted.

Two examples highlight the problems inherent in getting a comprehensive account.

(i) A man with calcific chronic pancreatitis that had become painless once the end-stage with steatorrhoea and diabetes was reached gave a long history of exposure to occupational chemicals. Between the ages of 30 and 52 years he worked in a stripping shed, cleaning metal parts in a long bath containing warm sodium hydroxide that was set against an outside wall. Vapours from the bath were properly discharged by an exhaust fan. However, behind him was a trichloroethylene degreasing bath, over which cleaned parts were allowed to drip, such that trichloroethylene vapour wafted past. (ii) A man aged 20 years at interview did not smoke cigarettes and drank alcohol socially. He had experienced recurrent pancreatitis attacks for 2 years and tests established a diagnosis of non-calcific chronic pancreatitis. From aged 17 years he worked as a storeman, hand-trucking goods into road delivery vans in a partly covered loading bay, where lorries were frequently backing, starting and shunting. In adverse wind conditions, the bay filled with diesel exhaust for hours on end.

Histories of the oldest patient with chronic pancreatitis, a young patient with this disease and a patient with relapsing acute pancreatitis are depicted in **Figure 8.1-8.3** so as to show how

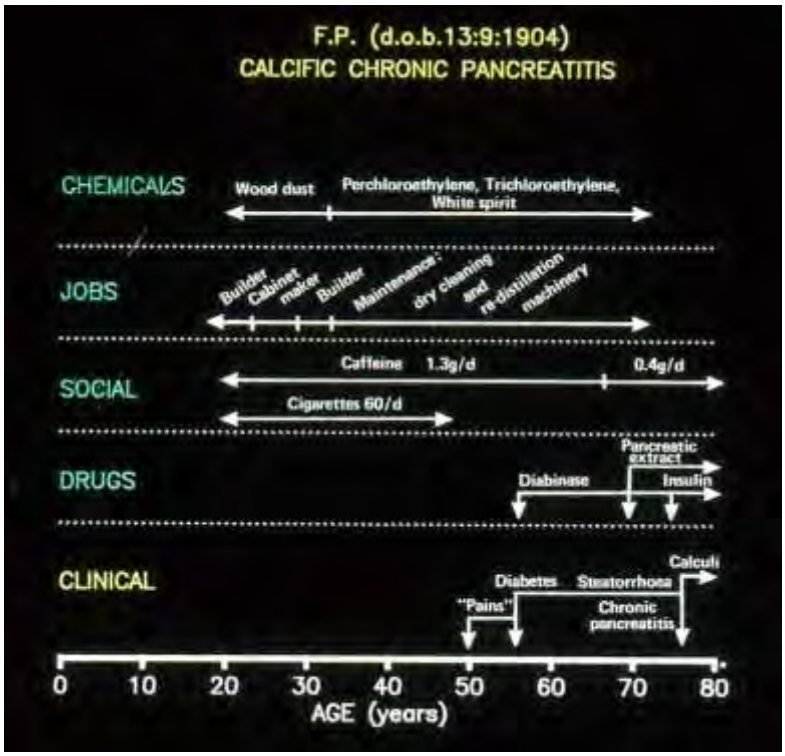

Figure 8.1 Clinical, social and jobs history of the oldest patient in the pilot study of occupational chemicals. Reproduced from 1986 paper in Int J Pancreatol [215]

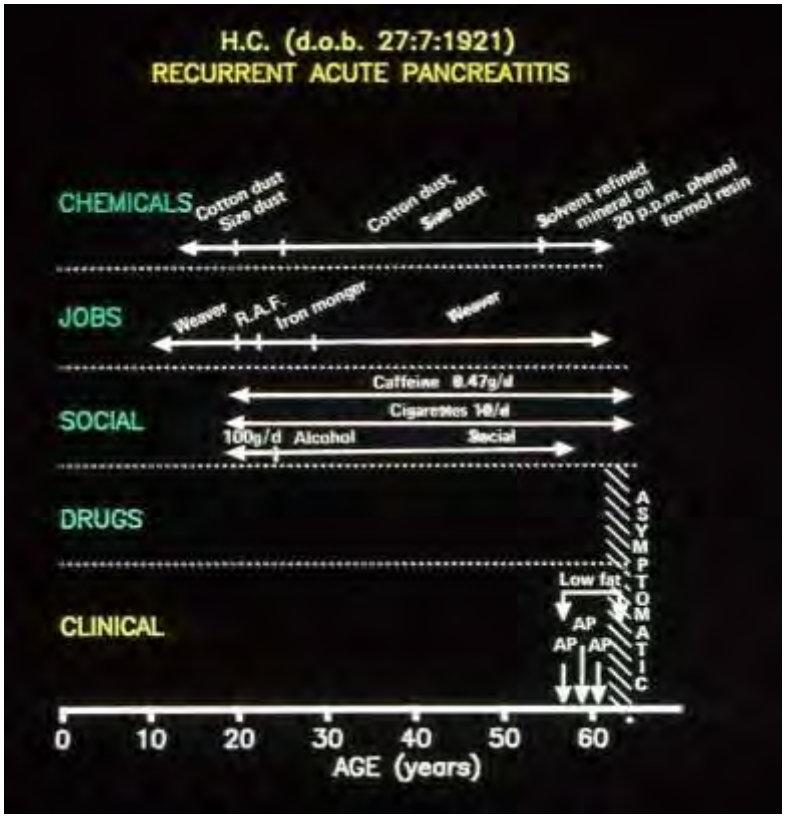

Figure 8.2 Clinical, social and occupational history of a patient with relapsing acute pancreatitis from the same study.

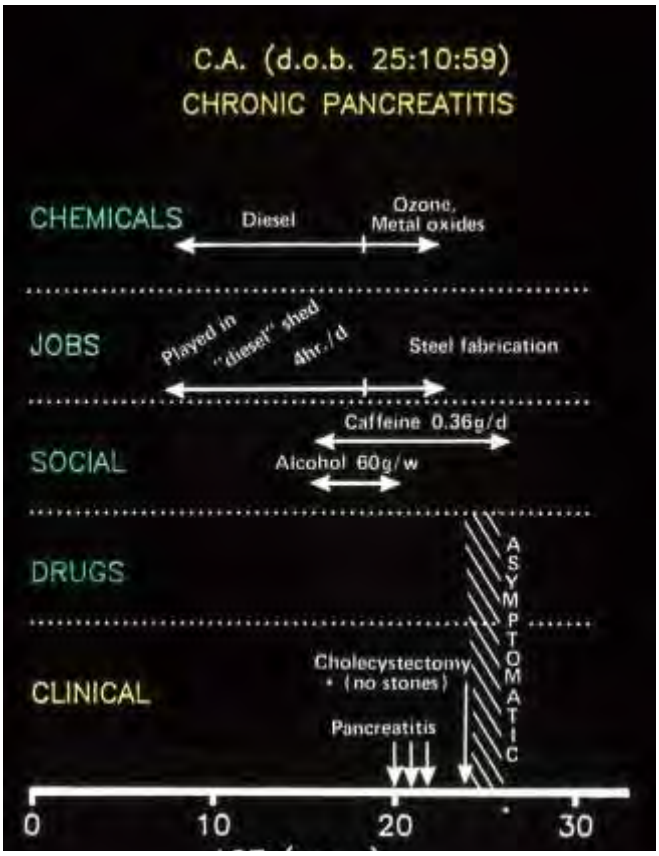

Figure 8.3 Clinical, social and occupational history of a young patient with chronic pancreatitis from the same study.

job descriptions by patients were translated into exposures to specific chemicals, while relating these to the time-course of illness. Details are tabulated in the study report[215].

In the group with idiopathic disease, 6 of the 12 patients had been regularly exposed to diesel exhaust fumes, while the remainder were exposed to a variety of degreasers and / or solvents. The age at first symptom in the former set tended to be lower than in the others (mean 26, range 14-41 years, versus mean 48, range 40-57 years), and the interval between first exposure to diesel exhaust fumes and first symptom was < 2 years in two patients. Several patients smoked cigarettes regularly, and some also drank large amounts of caffeine-rich tea and coffee, which contain methyl xanthine inducers of CYP1A.

Six of 7 patients in the second series had been regularly exposed to diesel fumes, and 2 were also exposed to other volatile chemicals. The last patient was regularly exposed to paint solvents. Most also smoked and / or drank large amounts of caffeine-containing beverages.

The various modes of presentation in patients exposed to the same volatile chemical was revealed as the study progressed, Thus, among the 12 patients exposed to diesel fumes, 9 had chronic pancreatitis, 2 had post-acute pancreatitis, and 1 presented with maturity-onset diabetes around 15 years before the first attack. The intensity of exposure to diesel exhaust was generally higher in patients with chronic pancreatitis, lower but over longer periods in patients with acute pancreatitis, and least in the man with maturity onset diabetes. Inter-relationships between endocrine and exocrine pancreatic dysfunction was highlighted by the histories of 5 patients: 3 presented with diabetes and subsequently developed exocrine pancreatic disease; 1 with chronic pancreatitis developed a non-functional apudoma; while 'striking hyperplasia' of pancreatic islets in the resected pancreatic specimen from the last patient led to an unsubstantiated diagnosis of insulinoma, until symptoms of exocrine pancreatic disease supervened.

None of the patients in the group with idiopathic pancreatitis had an attack of pancreatitis while on holiday. In fact, some became symptom-free when kept away from work by other illness, only to experience a return of symptoms upon resumption of work - such that 2 resigned and remained symptom-free. In 2 patients of the second series, pain stopped when relocated to a fume-free environment, or when holidays or sick leave kept them away from work[215].

8.1.2 Comments

The path towards establishing a new causal connection between occupational exposure to chemicals and a specific disease is fraught with

difficulties. The temporal association between exposure and initial presentation, exacerbation or remission is generally apparent when full medical and occupational histories are compared, but the disease process, once begun, might continue after the patient is removed from the suspected work environment. Moreover, the body does not distinguish between harmful substances encountered at work, through hobbies, or drug prescription. In the case of chronic pancreatitis the position is particularly difficult because its diagnosis can be elusive (Chapter 2), and attacks are unpredictable in patients with idiopathic disease. The association with alcoholism is established, but ethanol on its own is a weak agent in that an average of 15 years elapses before the first symptom in 'alcoholics'. Yet there are patients who suffer after just a year of consuming just 20 gm per day. This paradox has been interpreted as evidence of linearity between the mean daily consumption of alcohol and the logarithm of the risk of developing the disease[12]. Another proposal is that susceptibility to ethanol reflects the sum of concurrent influences, as by cigarettes and / or diets that are rich in fat and / or protein[10].

Experimental evidence instead suggests the CYP2E1-inducing effect of small doses of ethanol, so increasing the yield of RXS from co-processed chemicals such as thrichloroethylene, toluene, xylene, and carbon tetrachloride[170,171,199] - with evidence that hepatic hepatotoxicity reaches a peak when ethanol pretreatment precedes the chemical challenge by 18 hours [217]. It is unlikely to be a coincidence that patients often report this time-frame, eg. by the patient with RAP in **Figure 8.2** who noted that social drinking at the weekend of as little as a pint of ale or glass of sherry was invariably followed by an attack at the start of the working week. It should be stated, however, that whereas there is no doubt about CYP1A2 induction by chemicals in cigarette smoke, the inductive effect of volatile occupational chemicals is not clear-cut - except insofar that the particulate phase of diesel exhaust fumes is laden with PAH inducers [218].

Considering that the same range of chemicals was associated with a significant increase in pancreatic cancer in an epidemiological study[219], and interaction between exocrine and endocrine elements of the gland during experimental carcinogenesis, it is perhaps unsurprising that diabetes preceded pancreatitis in some patients of the present study, or that that 1 developed an apudoma and another nesidioblastosis.

The study findings gave credence to a contemporaneous case report[220] and other anecdotal experience included in earlier and later reviews[221-223]. Nonetheless**,** a formal investigation was clearly necessary.

8.2 Case-referent study: chronic pancreatitis

8.2.1 Description and outcome

Potential cases were identified retrospectively in 3 stages (**Figure 8.4**). First, the Korner Episode System (KES), which records all hospital in-patient episodes in England and Wales, was used to identify patients in the 7 heath districts in Greater Manchester who were discharged to an address within the 'study area' during the period 1.4.1985-31.3.1990, and whose stay in hospital was given any international classification of disease 577 code, ie. diseases of the pancreas. Next, hospital notes were inspected for evidence that the standard diagnostic criteria for chronic pancreatitis were fulfilled. Finally, any patient who first satisfied the diagnostic criteria was considered further provided that the following criteria applied: age > 18 years; an address within the study area; illness not regarded as secondary to pancreatic cancer, cystic fibrosis or trauma; no record of death. Efforts were made to contact these patients, once permission had been obtained from hospital physicians and general practitioners. Only patients who were still alive were eligible. There was no patient from the pilot study.

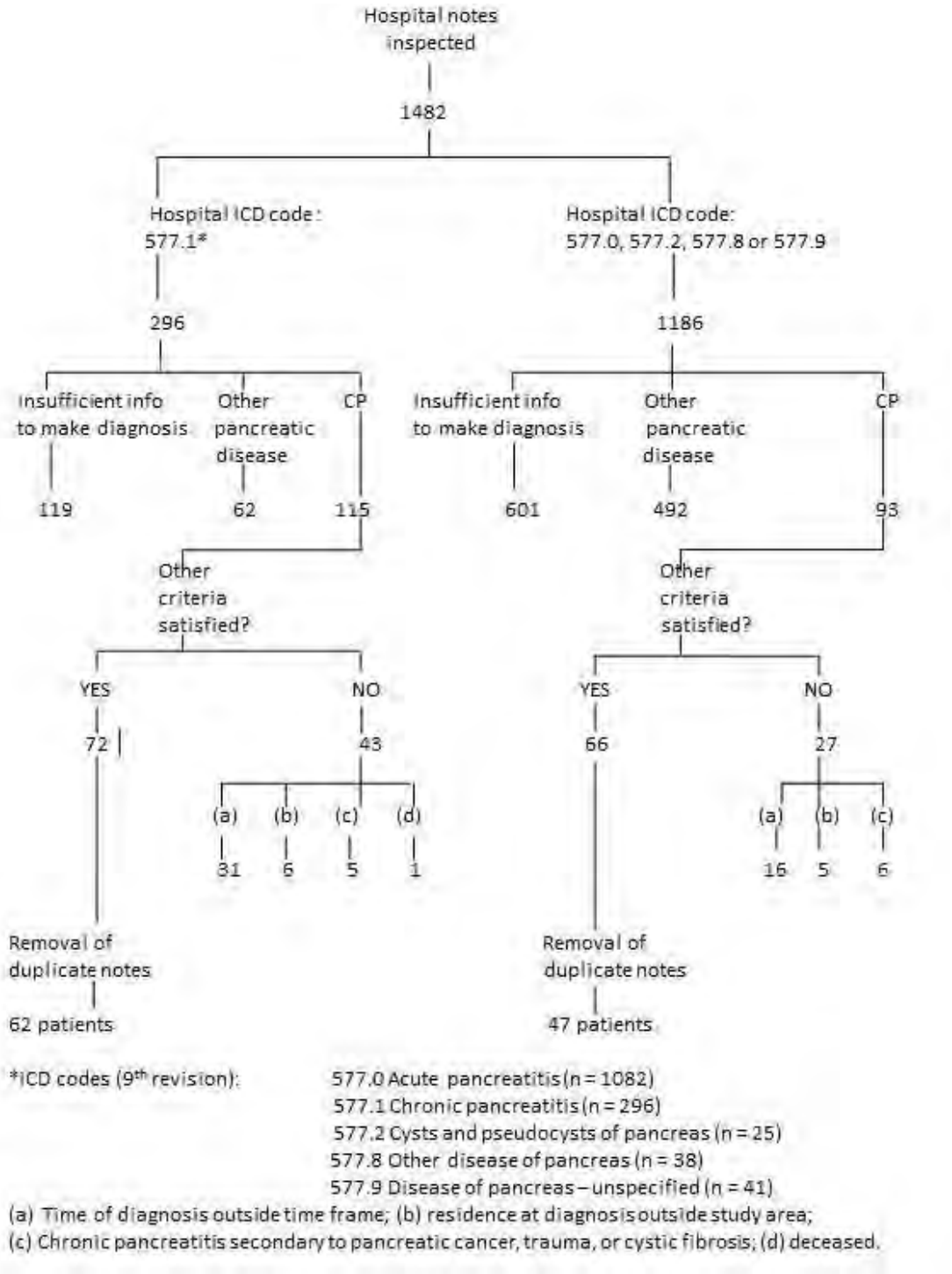

Figure 8.4 Flow chart depicting the process of recruitment in the case-control study of exposure to volatile hydrocarbons . Reproduced from 1994 paper in Occup Environ Med [216,] with permission of BMJ publishing group (© BMJ all rights reserved).

Potential referents were identified from the computerised records of the 5 family health services authorities (FHSA) which provide primary health care to residents of the 7 health districts. When an eligible patient had agreed to participate, 2 potential referents of the same gender and birth date were picked at random from the FHSA list that included the case. General practitioners of potential referents were approached for permission to contact their

patients, except in one district where the FHSA preferred to make the initial approach. If permission was refused by an individual or family doctor, he / she was replaced by another, selected in the same way as the original, from the FHSA list.

Structured interviews to ascertain occupational exposure and confounding variables were conducted in the homes of cases and referents. As the interviewer was also involved in identifying study subjects, it was not possible for interviews to be conducted blind to disease status. A list of jobs thought to involve potential hydrocarbon exposure was drawn up at the study's inception: advice on these was given by a panel of experts. At interview, all jobs held since leaving school were recorded. Thereafter, a further questionnaire focussed on workplace ventilation, respiratory protection and work practices. Copies of the occupational questionnaire were sent to 2 occupational hygienists and 2 occupational physicians who were not told about an individual's disease status. These 4 assessors independently rated each job, both for likelihood (definitely no, probably no, probably yes, definitely yes) and intensity (low, medium, high) of exposure to each of 12 chemical groups. In guidelines for rating exposure, it was suggested that the labels low, medium and high be regarded as < 30%, 30-80% and > 80% respectively of occupational exposure limits where these existed.

'Cumulative hydrocarbon exposure' score (CHE), independent of data, was calculated by a devised method. Only ratings of probable or definite exposure of medium or high intensity were taken further, with all such medium or high intensity scores given scores of 1 or 2, respectively. These scores were combined in 3 consecutive steps: across chemical groups, across assessors, and across jobs. In the first step, a single score for each combination of job and assessor (assessor job score) was calculated by adding chemical-specific scores. Next, a single score for each job was found by taking the median of the 4 assessors score (median job score). Finally, a CHE score up to the date of diagnosis in cases (or the same date for age-matched referents) was obtained as a weighted sum of the scores for each job, the weights reflecting time spent per job.

Three categories of lifetime exposure were defined from CHE score - 0 (zero), 0<CHE<10 (low), CHE ≥ 10 (high) - and used to examine the relationship between exposure and risk. Cumulative exposure scores (CE) were also compiled for each of the 12 chemical groups separately by combining ratings across assessors and jobs only. Information on exposures outside work was collected but, as the intensity was never more than the equivalent of low occupational exposure, was not considered further.

Ancillary information was ascertained as regards alcohol, cigarettes and diet to cover the time of interview, and 2 years prior to initial symptom. Weekly alcohol intake was expressed as a multiple of the recommended 'safe' upper limits for males and females (20 and 14 standard units, respectively). Cigarette usage prior to symptom onset was labelled as 'ever' or 'never'. To assess pre-symptom diet, a weekly food frequency questionnaire was administered, and data expressed as percentages of recommended daily intake. For complete documentation, social class was assessed. Further details are given in the study report [216] and a full account in the interviewer's research thesis[224].

Conditional logistic regression techniques were used to derive odds ratios (OR) and to control for confounding. Where appropriate, 90% confidence intervals (CI) were calculated, which may also be interpreted as one-tailed tests ($\alpha = 0.05$) of the hypothesis under study. To test for trend with level of exposure, a regression coefficient was estimated from the data on those with non-zero exposure scores: this coefficient divided by its standard error was used to give an approximate one-tailed test. Assuming that 15% of referents

would be exposed to hydrocarbons, it was estimated that the study would have 70% power to detect an increased risk corresponding to an OR of 2, and 90% power for an OR of 2.5, using a 1-tailed test with a significance level of 5%. Relationships between individual chemical groups and risk were expected to have low power.

Ascertainment was only 109 cases of chronic pancreatitis from a starting base of 1482 cases so coded by KES (**Figure 8.4**): of these, 4 had died, 1 declined to participate, and 2 could not be contacted, leaving 102 cases. Of 204 individuals who were initially chosen as referents from FHSA files, only 129 (63%) were included - because the doctor refused permission (7.4%), the subject refused (16.7%), or did not reply (11.8%). To replace the 75 refusals / non-responders, a further 110 names were extracted from FHSA lists, giving a total of 314 referents.

The median age of cases at diagnosis was 44 years (range 19-81 years) and 77 (75%) were males. Pre-symptom alcohol intake exceeded 'safe' levels in 70% of cases and 45% of referents. Cases left school at an earlier age than referents; they were more likely to have smoked cigarettes; they more often belong to social class IV or V; and a larger proportion of them had a diet which was deficient in ascorbic acid and / or selenium [216].

Occupational exposure profiles are summarised in **Table 8.1**, where a participant was regarded as having had a particular exposure if registering a non-zero CE score (individual chemical groups) or a non-zero CHE score (any chemical group). The most common exposure was to 'paint solvents' which includes paint, paint thinners, varnishes, and so on (n=59); followed by diesel exhaust fumes (n=33); fossil fuels, ie. paraffin, kerosene, petrol or diesel (n=28); and chlorinated solvents

Table 8.1 Exposure to hydrocarbons of a series of patients with chronic pancreatitis

Type of exposure	Cases	Referents	Total
1. Dyes	5 (5%)	4 (2%)	9 (3%)
2. Petrol exhaust fumes	8 (8%)	10 (5%)	18 (6%)
3. Diesel exhaust fumes	15 (15%)	18 (9%)	33 (11%)
4. Glue, adhesives	5 (5%)	6 (3%)	11 (4%)
5. Weedkillers, pesticides	2 (2%)	1 (0.5%)	3 (1%)
6. Oil mists	4 (4%)	10 (5%)	14 (5%)
7. 'Paint solvents'	23 (23%)	36 (18%)	59 (19%)
8. Paraffin, kerosene, petrol, diesel	15 (15%)	13 (6%)	28 (9%)
9. Disinfectants	2 (2%)	2 (2%)	4 (1%)
10. Chlorinated solvents	10 (10%)	11 (5%)	21 (7%)
11. Printing inks	3 (3%)	6 (3%)	9 (3%)
12. Rubber fume	2 (2%)	2 (1%)	4 (1%)
Any of the above	56 (55%)	81 (40%)	137 (45%)

Numbers (%) of cases & referents with non-zero scores. 102 cases of CP and 129 as reference patients (From ref 216)

(n=21). In the cohort as a whole, 56 cases (55%) and 81 referents (40%) had a non-zero CHE score, which was considerably more exposure in both groups than anticipated.

Crude and adjusted ORs for hydrocarbon exposures are given in **Table 8.2**. The unadjusted OR was 2.21 (90% CI 1.38 - 3.53) overall, incorporating ORs for low and high CHE exposure categories. Among those with non-zero scores, a significant trend emerged of increasing ORs with increasing CHE score (p=0.05). The risk of chronic pancreatitis showed a roughly exponential relationship with pre-symptom alcohol consumption, which also correlated with hydrocarbon exposure in referents. To control this confounding influence, alcohol consumption was included in a conditional logistic regression model, as also were smoking status, measures of social class, ascorbic acid and selenium intake each of which showed an independent association with disease risk. Four subjects were excluded from this analysis because of missing information. As shown in the table, ORs were lowered when adjusted for the ancillary factors listed above, but the adjusted OR of 2.67 for cases with high CHE still represented a significant increase (90% CI 1.22-5.87): the test for trend now gave p=0.09.

Next, relationships between CHE and disease risk were examined separately for those who drank less than harmful levels of alcohol as defined above, and those who drank more than this amount. To reduce confounding by level of consumption within each category, the continuous variable measuring alcohol intake was included as a covariate in these analyses. In the low alcohol group, ORs for none, low and high CHE scores were 1.00, 1.75 (90% CI 0.77-3.98) and 4.20 (90% CI 1.41-12.57), respectively. In the high alcohol group the corresponding ORs relative to the no exposure, low alcohol group were 3.80 (90% CI 0.97-14.86), 2.29 (90% CI 0.49-10.74) and 5.51 (90% CI 1.11-27.36). Thus the association with hydrocarbon exposure appeared weaker for 'alcoholic' compared to 'non-alcoholic' chronic pancreatitis, but the numbers were too small for a firm conclusion.

The association between disease risk and 4 chemical groups in which there were at least 20 exposed subjects is shown in **Table 8.3**. The ORs were adjusted for other variables, as before, but not for other occupational exposures. Because of the small number with scores more than 10, a cut-off point of 5 was used to define 'high' exposure to

Table 8.2 Crude & adjusted odds ratios for cumulative hydrocarbon exposure

CHE SCORE	Cases No (%)	Referents No (%)	Crude OR (90% CI)	Adjusted OR (90% CI)
0	46 (45%)	124 (60%)	1.00	1.00
LOW	32 (31%)	60 (29%)	1.61 (0.93-2.77)	1.20 (0.61-2.35)
HIGH	24 (24%)	21 (10%)	3.61 (1.91-6.82)	2.67 (1.22-5.87)
>0	56 (55%)	56 (55%)	2.21 (1.38-3.53)	1.64 (0.92-2.93)

See text for details (ref 216)

Table 8.3 Crude and adjusted odds ratios for exposure to selected chemicals

CE score	Cases (n)	Referents (n)	Crude OR	Adjusted OR (90% CI)
Paint solvents				
None	79	168	1.00	1.00
Low*	14	25	1.22	1.02 (0.43-2.44)
High*	9	11	1.79	0.87 (0.31-2.52)
>0	23	36	1.41	0.96 (0.48-1.93)
Diesel exhaust fumes				
None	87	186	1.00	1.00
Low*	10	12	1.96	1.96 (0.63-6.09)
High*	5	6	2.08	4.18 (1.09-16.06)
>0	15	18	2.04	2.66 (1.05-6.73)
Paraffin, kerosene, petrol, diesel				
None	87	191	1.00	1.00
Low*	11	8	3.09	2.70 (0.99-7.31)
High*	4	5	1.86	0.92 (0.26-3.30)
>0	15	13	2.62	1.82 (0.80-4.11)
Chlorinated solvents				
None	92	193	1.00	1.00
Low*	7	9	1.62	0.96 (0.30-3.04)
High*	3	2	3.17	4.41 (0.69-28.19)
>0	10	11	1.88	1.49 (0.58-3.81)

Asterisk Low: 0<score<5; High: score ≥ 5. Adjustments and exclusions as described in text (ref 186)

these confounding variables. Although there is a suggestion of a relationship between 'paint solvents' and risk in the crude data, this disappears after adjustment. The adjusted OR for diesel exhaust exposure was significant at the 5% level, OR 2.66 (90% CI 1.05-6.73), with suggestion of a dose-response relationship.

8.2.3 Comments

There are several potential criticisms of the investigation. (i) Some cases were undoubtedly missed because of inadequate testing, and the study was restricted to cases still alive: these exclusions are not a source of bias, however, as they were not exposure-related. (ii) Ideally referents should have been from residents in the study area at the time of diagnosis in the cases, but this was impossible and would only lead to bias if the exposure profile of the population had changed within the period to the time of study. (iii) Replacement of new referents to compensate for non-participants from the original list could lead to a false positive result if the new group had less hydrocarbon exposure, eg. if participation was related to social class. To check on this, a comparison was made of the class composition of study referents and area-specific data, classified by age and gender, from the 1981 census of

Great Britain: representation of manual classes was broadly similar (62% and 57%, respectively). (iv) Some misclassification with respect to hydrocarbon exposure is inevitable in retrospective assessments, but should not produce a false positive association when the ascertainment procedure operates equally in cases and referents. (v) The possibility of information bias must be acknowledged since the interviewer knew to which group a participant belonged. However the translation of job description into exposures was by a team unaware of assignment.

The CHE measure of exposure was one of several ways to summarise the data, but importantly it was chosen a priori. The method of classifying jobs as 'exposed' might have erred on the side of sensitivity at the expense of specificity, in that to receive a non-zero score, only 2 of the 4 assessors needed to rate the job as exposed. In fact, had a stricter criterion been used, that 3 of 4 needed to mark a job exposed, the strength of the association with risk would increase - adjusted ORs for low and high CHE scores 1.32 (90% CI 0.62-2.80) and 3.17 (1.41-7.13), respectively.

Methods for measurement and analytical control of alcohol consumption in the investigation were critically important. It was the interviewer's impression that cases were willing to admit to heavy drinking in the past. This, and the heavy consumption of alcohol by cases and referents suggests that previous consumption may be less prone to under-reporting than is said to affect current habits. The inclusion of alcohol as a continuous variable assumed an exponential relationship with disease risk, in line with large-scale studies from France[12]. It is thus most unlikely that the association between hydrocarbon exposure and disease risk is simply due to a confounding effect of alcohol.

There is not a 1-to-1 correspondence between job types and exposure. For example, employment as motor mechanics or auto engineers was associated with high exposure that involved both paints and petrochemicals. Moreover, In the subsidiary analysis of particular chemicals, the highest risk was from diesel exhaust fumes which are loaded with PAH inducers, particulates, nitrogen dioxide and a host of other toxic substances[218,225].

8.3 Overview and Summary

The combined results of pilot and formal investigations implicate occupational volatile chemicals as a risk factor in chronic pancreatitis. However, the phrase 'hydrocarbon exposure' to describe the association is an over-simplification, in the same way as is the descriptor 'alcoholic' for chronic pancreatitis.

The key point is that damaging chemicals arrive by the inhalation route, bypassing the protective liver sieve that is available for ingested xenobiotics. This begged the question whether injury might be initiated within the pancreas itself, via induced pancreatic CYP, over-and-above any damage inflicted by reflux of bile laden with FROP and RXS[10, 39].

Chapter 9

Casualty of Pancreatic 'Detoxification' Reactions!

Against the backdrop of information in Chapters 3-8, there was a quandary when faced with controlling recurrent attacks in young patients with idiopathic chronic pancreatitis, diffuse glandular involvement as shown by ERCP, and bile laden with FROP. Once all medical treatment options were exhausted, the grim alternatives were total pancreatectomy or addictive opiates. The finding of bilirubin within the head of pancreas in another patient who underwent an expeditious Whipple operation on suspicion of cancer suggested that bile diversion might be useful. This was undertaken after full consultation with surgeon, patients and family members. The outcome[226] and, consequently, an investigation of pancreatic CYP[227] are reported herein.

9.1 Futility of clinical bile diversion

9.1.1 Case reports

AL worked in the motor industry and as a sheet metal welder from aged 15 to 30 years, thus regularly exposed to ozone, metal oxides, petrochemicals and solvent vapours. Later, as foreman on a motor shop floor, he was in daily contact with trichloroethylene, perchloroethylene and paint solvents. He drank 140 gm ethanol per week between the ages of 19 and 41 years, and did not smoke cigarettes. He has been under a sunlamp for most of the day preceding the first pancreatitis attack in 1982, when aged 41 years. A second episode 2 months later precipitated referral. ERCP / CT strongly suggested a tumour in the head of pancreas. The following week at the time of pancreaticoduodenectomy a wedge biopsy of the liver was obtained. Pancreatic histology showed advanced chronic pancreatitis but no malignant cells. There were foci of bile pigment and cholesterol clefts deep within the gland substance (**Figure 9.1**) and also in adjacent fat: some foci were encircled by granulation tissue, others by foreign body giant cells. Toluidine blue-stained sections showed extensive cytoplasmic microvesiculation in surviving acinar cells (**Figure 9.2**). He stayed at home and had no attacks in the next 18 months although drinking alcohol as previously. However, within 3 months of returning to work he had another attack.

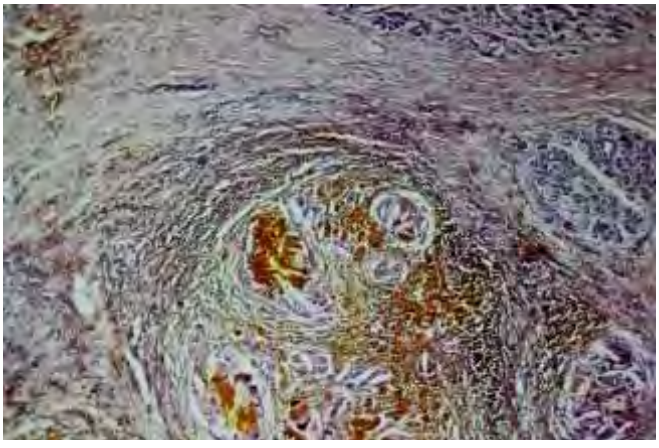

Figure 9.1 Histology of the resected head of pancreas (H & E original magnification x 60) in patient AL showing the intense fibrotic reaction and loss of pancreatic parenchyma, with bile pigment and cholesterol clefts deep within the gland substance. Reproduced from 1990 report in Gastroenterology[226]

CA experienced intermittent abdominal pain

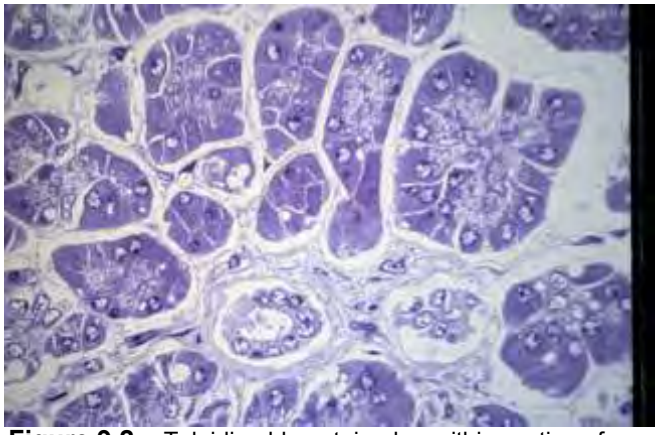

Figure 9.2 Toluidine blue-stained semithin section of the pancreas specimen (original magnification X 750). from the same patient,, showing cytoplasmic microvesiculation in surviving acinar cells Publication details as for Figure 9.1.

from the age of 16 years, and had a pancreatitis attack 2 years later. These continued every 2-months or so, despite cholecystectomy (no gallstones) when aged 20 years. He drank alcohol on a social basis (60 gm / wk, aged 17-20 years) and did not smoke cigarettes. On direct questioning at referral 3 years later because attacks continued although he had been teetotal, he mentioned that between the ages of 9 and 15 years he used to play for about 4 hours each evening in a warehouse loading bay where diesel trucks had been fuelled and shunted all day. From aged 18 years onwards his work as a welder exposed him daily to ozone, metal oxides and solvent vapours. ERCP showed moderate-change disease affecting the whole gland (**Figure 9.3**), and pancreatic secretory capacity was moderately compromised. At the time of choledocho-jejunostomy soon thereafter, the head of pancreas was stony whereas the body and tail were less abnormal. A wedge biopsy of the liver was obtained. He resumed work after 6 weeks, but 3 weeks later had another attack.

MP was a schoolboy aged 15 years when referred following 2 pancreatitis attacks in the previous 8 weeks. These followed the Xmas gift of a pushbike which he used to get to and from school for 5 miles each way in very heavy traffic. ERCP, secretory and hepatobiliary abnormalities were the same as in CA and he underwent the same operative procedure when aged 17 years after failure of intensive conventional therapy. He returned to school after 6 weeks but was felled 3 weeks later by another attack.

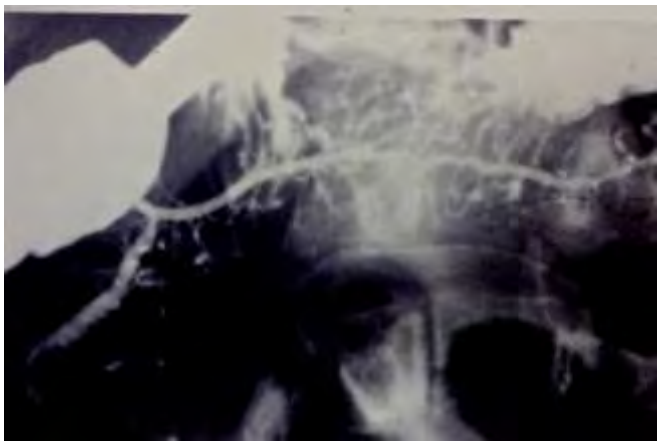

Figure 9.3 Endoscopic pancreatogram showing moderate-change pancreatitis in patient GM: similar changes were found in MP and CA. Publication details as in Figure 9.1.

GM has already been described in the context of patients with both epilepsy and chronic pancreatitis (**Table 7.4)**. He began to drink alcohol when aged 16 years such that between the ages of 17-20 years consumption reached 280 gm per week, while he smoked 30-40 cigarettes daily. He had a pancreatitis attack when 19 years old. The elevated serum level of alkaline phosphatase suggested he had gallstones but none were found in the resected gallbladder: operative cholangiography showed unimpeded passage of dye into the duodenum with free reflux into the pancreatic duct. On referral when aged 21 years, it was noted that he was teetotal but continued to smoke 20 cigarettes daily, and also that his work in the preceding years involved regular close exposure to diesel exhaust fumes and spray weed killers, due to working in a warehouse loading bay or as a gardener, respectively. Specialised test results were as described for CA and MP. A wait-and-watch policy was adopted while reinforcing the need to stay teetotal, cut cigarette usage and eat healthily. He was well for the next 2 years while unemployed, and also for the following 2 years when he checked in as a long distance lorry driver. Unfortunately, he developed epilepsy when aged 28 years due to a brain cyst and was started on phenytoin, later substituted by carbamezapine: 2 attacks in quick succession occurred 10 months later by which time he was re-employed as a painter, now exposed to trichloroethylene and paint thinners. Percutaneous liver biopsy was done to investigate persistently elevated alkaline phosphatase, with increased γGT.

Table 9.1 Hepatobiliary aberrations

	Antipyrine Cl ml/kg/hr (50, 37-66)	Theophylline Cl ml/kg/hr (68, 50-97)	BSP K1 %/min (14.3, 11.3-17.3)	DGA /creatinine mmol/mol (2.85, 0.4-4.8)	DC in bile units (6.24, 1.00-13.5)	UVF in bile units (494, 111-1330)	Hepatocytes type I (%)	II (%)	III (%)
AL	80	70	18	7.5	ND	ND	30	40	0
CA	53	178	22.5	3.8	13.7	1875	60	40	0
MP	93	92	23.1	6.0	14.8	2363	100	0	0
GM	150	209	39.6	5.4	14.5	2400	70	0	30

See text of Chapter 5 for details of metabolic studies, and Chapter 6 for hepatocyte grading. Values in parenthesis are referent ranges, with mean for bromosulpthalein test or median for other tests . ND=not done (From ref 226).

9.1.2 Hepatobiliary aberrations

These are summarised in **Table 9.1**. Although each patient had altered his lifestyle for variable periods due to illness or upon medical advice, xenobiotic-processing enzymes were induced (Chapter 5), FROP in secretin-stimulated aspirates of duodenal juice were elevated(Chapter 3), and liver stress further evidenced by water-clear hepatocytes indicating microvesicular steatosis (Chapter 6).

9.1.3 Micronutrient antioxidant therapy / outcome

Considering earlier dietary evidence of poor micronutrient antioxidant intake in patients with chronic pancreatitis (Chapter 7), each patient was prescribed a commercially-available compound-antioxidant supplement (Selenium-ACE, Wassen International, Leatherhead, UK). Since there was no guidance on dosage and also that free radical load was likely to differ between patients, a cautious approach was to advise just a tablet daily at first, increasing the dose at intervals to a maximum of 6 tablets daily. The plan was, if attacks continued, to add methionine tablets (Evans Ltd, Horsham, Essex, UK), beginning with a daily tablet and increasing the dose as required to a maximum of 12 per day. Amounts of individual antioxidants provided in this way are detailed in Chapter 17. The success of the novel strategy was demonstrated by the absence of painful attacks during a surveillance period of 5+ years wherein patients increased their intake of antioxidant-rich foods via dietary advice. Follow-up information in 2 patients is worth noting.

(i). After 2 years on treatment, **GM** had a vagotomy and pyloroplasty for recurrent peptic ulcer. A liver biopsy and transduodenal biopsy of the pancreatic head were obtained. Pancreatic histology showed interlobular fibrosis but no significant intralobular fibrosis or inflammation: surviving acinar cells appeared normal in semithin sections. Of particular note, microvesicular steatosis in hepatocytes was no longer present.

(ii). **MP** remained attack-free during his course through University for a biology degree and felt confident enough to stop antioxidant supplements as his diet was good. However an attack occurred while on holiday: he volunteered that he'd travelled by diesel-powered ferry to the Isle of Wight and lived in the vicinity of a a major oil refinery which spewed fumes. He was advised to resume treatment starting at the lowest dose. Several years later after his marriage, his mother-in-law who was a family doctor expressed concern that the treatment might account for the couple's failure to conceive. At the time an investigation was under way on the frequency of mutation in the cystic fibrosis transmembrane conductor regulator gene (CFTR) in patients with chronic pancreatitis. He was found to be a carrier; oligospermia was confirmed; but sweat and nasal potential difference studies were normal (Chapter 12). Assisted reproductive technology facilitated the birth of 2 healthy children. He retrained in Medicine thereafter, and is now a successful primary care physician.

9.1.4 Comments

Experience with these patients showed that, notwithstanding experimental evidence of the pancreatic toxicity of FROP-laden artificial bile when gently infused into the pancreatic duct (Chapter 6.3), ligating the bile duct and diverting abnormal bile away from the gland does not abolish pancreatitis attacks. The corollary is that pancreatic damage proceeds independently - not forgetting the operative cholangiogram of **GM** which showed free bilio-pancreatic reflux; or the intense fibrotic reaction in the vicinity of bile islands in the pancreatic specimen from **AL**. The finding of cytoplasmic microvesiculation in hepatocytes as well as surviving acinar cells from the latter patient is in keeping with free radical-mediated disturbance in secretory polarity (**Figures 2.6 & 2.7**). Follow-up observations in **MP** were prescient, hinting at a greater vulnerability to the problem when the quota of CFTR protein in acinar cells is half of what it should be. Also of note, the chemicals to which these patients were exposed are known to undergo metabolic activation via CYP, and RXS load would be higher upon CYP induction by alcohol, cigarette smoke or PUFA-rich diet.

9.2 Investigation of pancreatic CYP

9.2.1 Description and outcome

This study was made possible by JR Foster from Astra Zeneca in Cheshire. Samples of pancreas and liver were obtained from organ donor controls (n=7), and surgically from patients with chronic pancreatitis (n=6) or pancreatic cancer (n=10) by co-operation of surgeons from neighbouring hospitals. There was little or no social information on donor and cancer groups, and occupational histories were lacking in patients with chronic pancreatitis from collaborating centres. With a single exception, patients in the last group were not 'alcoholic' but all smoked cigarettes - including a young woman who worked as a forecourt attendant at a garage that serviced heavy goods vehicles.

Specimens were fixed in ethanol-acetic acid and embedded in paraffin. Rabbit polyclonal antibodies were obtained against the following phase I detoxification enzymes which were purified to homogeneity from rat liver: pregnenelone carbonitrile (CYP3A1), 3-methyl cholanthrene (CYP1A2), alcohol-inducible isoforms (CYP2E), and NADPH-CYP oxidoreductase. Also used was an antibody to the phase-II enzyme GST 5-5 that was raised in sheep. Further details as also of Western blots to ascertain reactivity and specificity of the antibodies against human tissues are given in the study report[227].

Because the antibodies were used at various dilutions and little was known about the relative epitope resistance of the proteins involved in fixation, comparison of enzyme amounts between clinical groups was adjudged invalid. Instead immunostaining intensity for each antibody on each tissue was scored qualitatively using an arbitrary system from zero (no stain) to 4 (intense stain). In liver biopsies, the lobular staining pattern and intensity in hepatocytes and bile duct epithelium were scored separately. In the pancreas, these features were scored independently for acini, duct and islets of Langerhans. 'Tubular complexes' were frequently seen and recorded as ductal structures, while recognising the debate on their precise cell of origin. In order to evaluate individual samples as groups, individual staining intensities were averaged and expressed for each tissue, each enzyme, and each group. Changes from control values of >50% were taken to be significant.

Reactions between antibodies to CYP oxidoreductase, CYP1A2 or CYP3A1 and human hepatic microsomes resulted in single bands corresponding to molecular weights of approximately 870, 60 or 55 kD in Western blot: for CYP2E a band of around 60 kD was accompanied by a fainter band 55 kD band in the

microsomal fraction containing the highest protein concentration. No reaction was seen in cytostolic fraction for any of these antibodies. By contrast the GST antibody reacted strongly with a single 20 kD band in hepatocyte cytosol, but not with microsomes.

Group scores for each enzyme in components of liver and pancreas are summarised in **Figure 9.4**. Using CYP3A1 as an example,

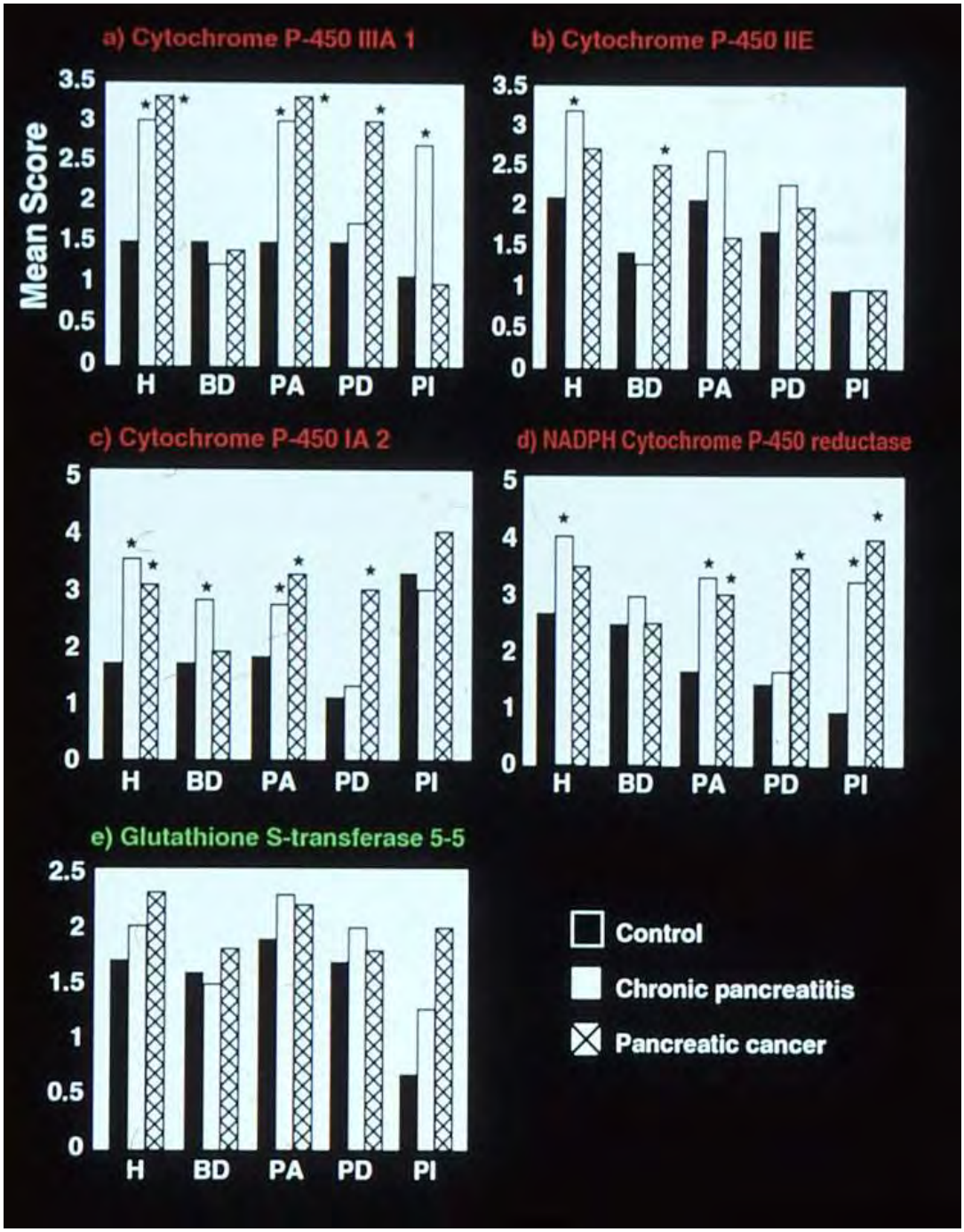

Figure 9.4 Profiles of drug-metabolising enzymes in liver and pancreas from donor, chronic pancreatitis and pancreatic cancer groups. H=hepatocytes; BD=bile ducts; PA=pancreatic acini; PD=pancreatic ducts; PI=pancreatic islets of Langerhans. Tubular structures in the pancreas were taken to represent ductal elements. Asterisks indicate mean value> 50% of appropriate value in donors. Filled squares=control; open squares=chronic pancreatitis; hatched squares=pancreatic cancer. From J Pathol[227].

immunolocalization in the exocrine pancreas and liver are shown in **Figures 9.5** and **9.6**, respectively.

This isoenzyme was expressed equally in all cell types of pancreas and liver from organ donors (**Figures 9.4a, 9.5 a, 9.6b**). In chronic pancreatitis the isoenzyme was induced in pancreatic acinar cells, islets of Langerhans, and hepatocytes (**Figures 9.4a, 9.5 c, 9.6d**). In pancreatic cancer induction of the isoenzyme was evident in acinar cells, tubular complexes that were taken to represent ductal elements, and hepatocytes but not in pancreatic islets (**Figures 9.4a, 9.5 e, 9.6f**).

The other results can be condensed as follows (i) There was enzyme zonality in the normal liver - CYP3A1 and the oxidoreductase showing centrilobular preference, CYP1A2 periportal preference, and CYPE1 evenly distributed - but none in the pancreas where ductal cells generally showed lower enzyme levels than did acini. (ii) Liver enzyme induction in patients with pancreatic disease was associated with loss of zonality, the order of enzyme increase broadly similar, ie. CYP3A1 > CYP1A2 > CYP2E=oxidoreductase, and a maximum score of 4 was attained for at least one enzyme in each

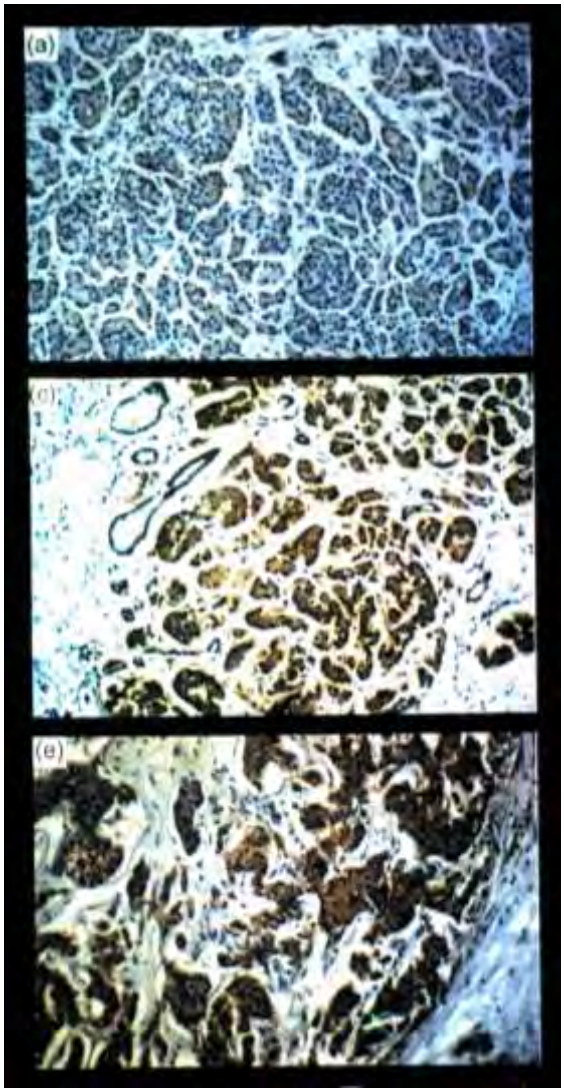

Figure 9.5 Immunolocalization of CYP3A1 in exocrine pancreas: (a) control, (c) chronic pancreatitis, (e) pancreatic cancer. Modified from Figure in J Pathol[227].

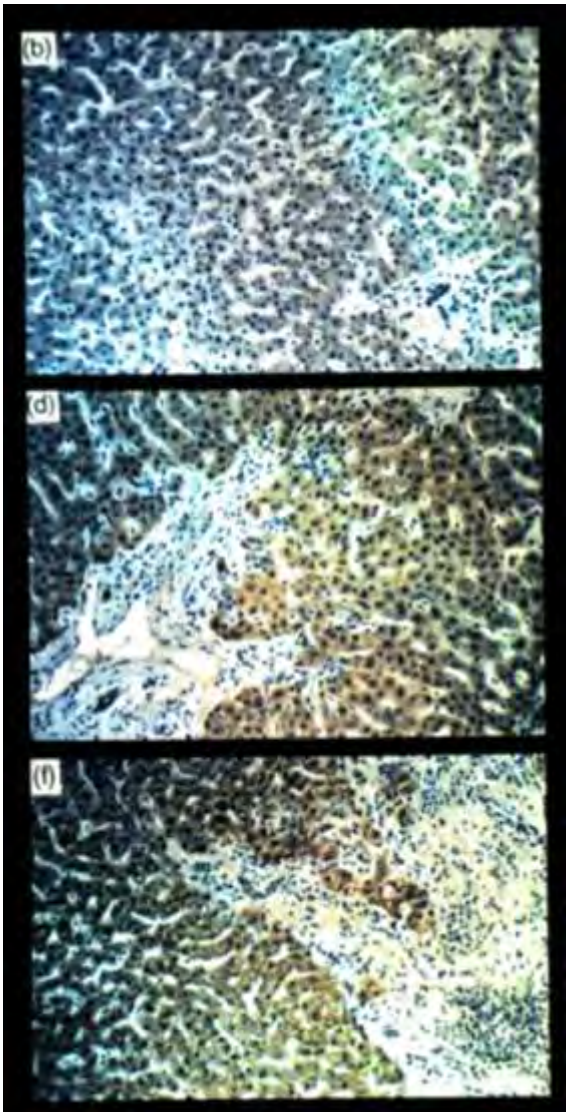

Figure 9.6 Immunolocalization of CYP3A1 in liver: (b) control, (d) chronic pancreatitis, (f) pancreatic cancer. Modified from Figure in J Pathol [227].

patient, whereas GST 5-5 was not altered substantially. (iii) Pancreatic acinar but not ductal cells displayed clear enzyme induction in chronic pancreatitis, the order of change slightly different from that in the liver, ie. CYP3A1 = oxidoreductase > CYP1A2 > CYP2E. A similar pattern was noted in pancreatic cancer, irrespective of whether the tumour had acinar, ductal or intermediate phenotype. A maximum score was obtained for one or more phase-I enzymes in every patient from both groups, but GST 5-5 was not induced. (iv) Certain unidentified cells of pancreatic islets from donor adults contained very high levels of CYP1A2, such that there was no further increase in pancreatic disease. Levels of the oxidoreductase and GST 5-5 were low in islets from donors, but clearly increased in pancreatic disease.

9.2.2 Comments

Whereas induction of hepatic drug-metabolising enzymes in patients with chronic pancreatitis was anticipated given earlier pharmacokinetic data (Chapter 5), the finding of equally potent induction in pancreatic acinar cells was not, although the futility of bile diversion raised the possibility (Section 9.1) as also the small amount of data from laboratory studies[10]. The strong presence of CYP in islets of Langerhans of controls was unexpected. All these findings have since been confirmed, and extended insofar that pancreatic CYP2E1 was found to be strongly induced in patients with alcoholic chronic pancreatitis[228, 229]. The combination of increased CYP activity in acinar cells but without a concomitant increase in activity of the phase II conjugating enzyme GST 5-5, or other GST isoforms[230], is both conducive to and consistent with electrophilic stress.

The information in **Table 9.2** is a distillate from studies itemised in previous reviews[11, 37]. It underlines the parallel changes in hepatocytes and pancreatic acinar cells, that can now be

Table 9.2 parallel changes in liver & pancreas in chronic pancreatitis

	Hepatocyte	Acinar cell
Compatible with CYP induction	↑ Cell size	↑ Cell size
	Ground glass hepatocytes	
	↑SER mass	↑ RER mass
	↑ Phospholipids in bile	↑ Protein in pancreatic juice
		Calcium in pancreatic juice
	↑ Drug metabolism	
	BSP-κ_1 [= ↑ligandin]	
	Antipyrine clearance [= ↑ CYP overall]	Pancreatic contribution
	Theophylline clearance [=↑ CYP1A]	
	Urinary D-glucaric acid [=↑ phase-2]	
	↑ CYP by immunocytochemistry	↑CYP by immunochemistry
Compatible with electrophilic / oxidative stress	Microvesicular steatosis	Microvesiculation
		Pancreastasis episodes
		Tubular complexes
	Dilated SER	Dilated RER
	↑ Lipofuscin in tissue [= ↑lipid peroxidation]	↑ Lipofuscin in tissue
	↑ FROP in bile	↑ FROP in pancreatic juice
		↑ Lysosomal enzymes in pancreatic juice
	↑ Bilirubin in bile [= ↑ haem oxygenase in liver]	↑ Mucin and PAP/*regIII* in pancreatic juice
	↑ Copper in bile	↑ Lactoferrin in pancreatic juice
		↑ Albumin in pancreatic juice
	↑ serum caerulopalmin [= ↑ ferroxidase 1]	↑ serum PAP
	↑ serum ferritin	
	Sclerosing cholangitis-like lesions	Sclerosing ductal lesions

Abbreviations: SER = smooth endoplasmic reticulum; RER = rough endoplasmic reticulum; BSP-κ_1 = first -phase corrected disappearance curve after injection of sulphobromophthalein; CYP =cytochrome P450 ; CYP1A= the isoform inducible by polycyclic and other hydrocarbons; FROP = free radical oxidation products; PAP/*reg111* = secretory stress, pancreatitis- associated protein subtype. Upward arrows indicate increases. Updated from ref 37 for Chapter in Pancreapedia (From ref 11).

confidently interpreted as evidence that xenobiotic metabolizing enzymes are induced in each cell type. It shows too that enzyme induction is injurious despite, in the case of acinar cells, mobilisation of antioxidant defences such as lactoferrin, mucin and pancreatitis associated proteins (PAP)[231]. The functional equivalent is an increase in pancreatic juice of FROP[232], and in the ratio of lysosomal to digestive hydrolases: as noted in an early review, the latter is unsurprising because lysosomes are known to be peculiarly susceptible to injury from excess FRA and lipid-based FROP[10].

Investigations on surgically-resected pancreatic specimens from patients with chronic pancreatitis have, over the years, afforded evidence of on-going oxidative / electrophilic stress: structural anomalies by electron microscopy[10]; FRA signals[233]; increased FROP with decreased GSH[65, 234]; increased levels of pro-oxidant copper / iron but decreased levels of antioxidant zinc / selenium[65]; and markers of the endoplasmic stress-unfolded protein response (ER stress-UPR)[235].

A better balance between pro- oxidant CYP and antioxidant GST is exhibited by islets of Langerhans [227, 230], with potential sequele. (i) CYP induction would rationalise nesidioblastosis. (ii) Since a proportion of arterial blood first perfuses islets (Chapter 2), it is likely that RXS generated therein are delivered to some acini by the insulo-portal conduit, so adding to their burden of toxicants[13, 229], and thus potentially rationalising the patchy nature of lesions in early chronic pancreatitis[13,31] . (iii) The inducibility of GST in islets [227,230] should protect against RXS-mediated injury to β cells and hence diabetes, except when the toxic load via induced CYP1A2 is overwhelming. Tropical chronic pancreatitis might be a pointer to that scenario[201, 236] . A more subtle finding from the study concerns zonality of hepatic CYP expression in healthy individuals and patients with chronic pancreatic disease, underlining the influence of blood supply (**Figure 2.1**). In the pancreas, a reductive mode of CYP action might be favoured within acini that are furthermost from the arterial input (**Figure 2.1**): this mode is known for CCl_4 toxicity[147], to which the gland is prone[31].

9.3 Overview and Summary

There are standard lines of reasoning when liver and pancreatic disturbances coexist[39, 237]: liver damage is secondary to pancreatic disease (eg. when the distal bile duct is constricted by disease in the head of pancreas); liver dysfunction causes pancreatic disease (eg. gallstone pancreatitis); or both are attacked independently by the same agent (eg. alcohol); or an autoimmune process affects each.

The pattern of hepatic involvement in patients with pancreatic disease as revealed by the Manchester studies is different, and linked to electrophilic stress. Two factors help to explain why the liver escapes overt injury whereas the pancreas bears the brunt of the clinical assault: its abundance of GSH[238] / GST[239]; and parenteral entry of xenobiotics, whether volatile petrochemicals in human chronic pancreatitis (Chapter 8) or experimentally injected CCl_4[31] or dibutyltin[240]. It should be underlined that ordinary amounts of ethanol are processed by dehydrogenases, with CYP2E1 brought into play when over-consumption is protracted in which case ROS are released[108], and oxidative stress precedes pancreatic injury[241]. Moreover, a small dose of ethanol is a potent inducer of CYP2E1, thus increasing the yield of RXS that are generated when certain xenobiotics are metabolised by this enzyme[170,171].

Two further puzzles might now be resolved. These concern liver and pancreatic copper overload without increased copper absorption in chronic pancreatitis; and high biliary concentration of bilirubin in the absence of haemolysis (Chapter 3).The answer to both could lie in long-term assimilation by the inhalation route of copper along with many other metals via cigarette

smoke[242-244] and / or industrial fumes[245]. The biological influence of such sources is shown by induction of CYP1A2 (Chapter 5). Moreover, exposure to copper and other metals increases the activity of the potent antioxidant heme oxygenase[246, 247], which degrades excess heme to generate other antioxidants, notably bilirubin.

Finally, the finding of CYP induction in pancreatic cancer specimens offers an explanation for the predisposition conveyed by chronic pancreatitis, and the susceptibility of African Americans to both diseases. In regard to the second association, high intrinsic activity of CYP1A is reported[248], which would amplify the risk from RXS. In regard to the first connection, there are established links between ROS / RXS and carcinogenesis in general[249]. Moreover, low methyl status is conducive to both chronic pancreatitis (Chapter 7) and pancreatic cancer[250].

In summary, chronic pancreatitis is a casualty of 'detoxification' reactions within the gland, over and-above injury that might be caused by entry of RXS and FROP carried in bile or the bloodstream (**Figure 9.7**). Obviously, inhaled xenobiotics would pose a far greater threat than orally ingested compounds.

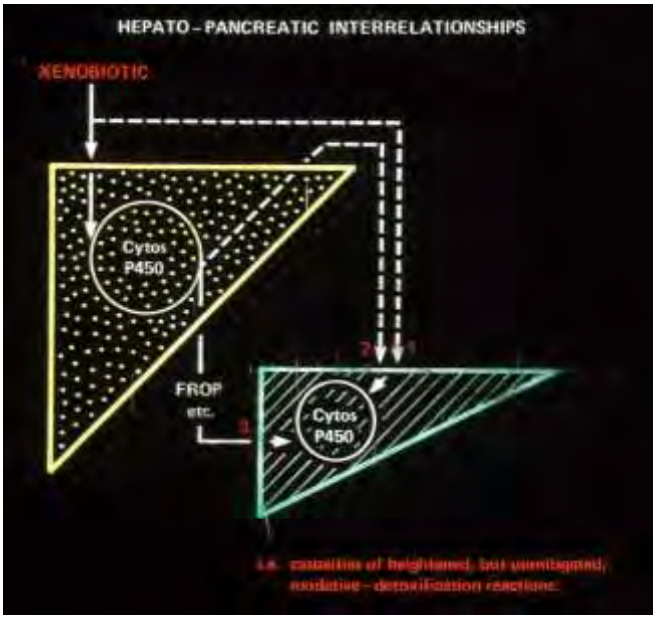

Figure 9.7 Depiction of modified concept in relation to CYP involvement in pancreatic disease, reproduced from 1988 review in Int J Pancreatol on the role of the liver[39] .

Chapter 10

Taking Stock

Within the short time since publication of the 'detoxification' hypothesis[9,10], progress was rapid and allowed some firm conclusions, while raising ever-more questions.

10.1 Premises of 1983 confirmed

Results of the first raft of investigations supported the original hypothesis as set out in Chapter 4. Moreover, subclinical damage to hepatocytes and cholangioles was identified in patients with chronic pancreatitis, alongside high levels of FROP, bilirubin and copper in secretin-stimulated bile (Chapter 9).

10.2 Modification of 1986 confirmed

Confirmation that CYP in pancreatic acinar and islet cells are induced in patients with chronic pancreatitis and pancreatic cancer should not have come as a surprise. In phylogenetic terms the human arrangement of the hepatico-pancreatico-duodenal complex is a relatively recent development compared to the primitive nature of drug-metabolising enzymes (**Figure 10.1**)[222, 251]. Of related interest, an autopsy report of acute pancreatitis affecting entopic and ectopic pancreas in a patient taking methyldopa could only be rationalised on the basis of pancreatic CYP involvement: the drug undergoes

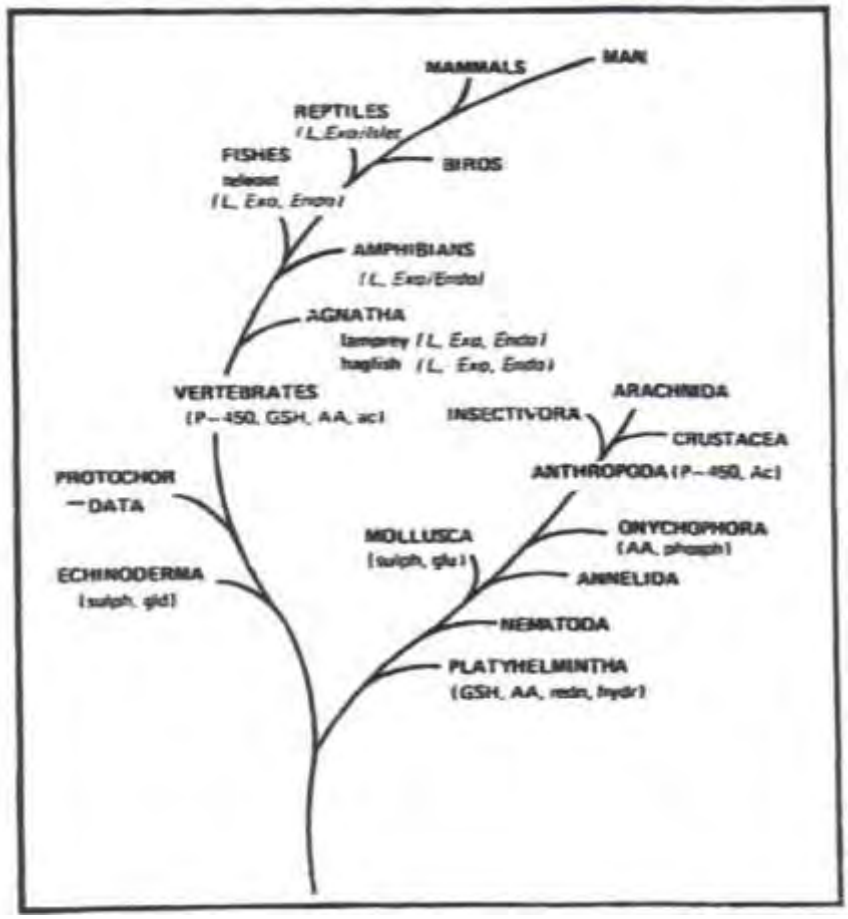

Figure 10.1 Phylogenetic tree showing that xenobiotic metabolising enzymes were present in animals well before the different components of the hepato-exocrine-endocrine pancreas separated. Conjugating reactions: with GSH (glutathione), AA (amino acids), Sulph (sulphate), gld (glucuronic acid). Primitive degradation reactions: redn (reduction), hydr (hydrolysis). Drug metabolising enzymes: P-450 (cytochrome P450-dependent), Ac (acetylation). L=liver, Exo=exocrine pancreas, Endo=endocrine pancreas, Islet=islet arrangement of endocrine pancreas. Figure generated for ref [222] (Manchester University Press).

bioactivation to yield RXS[252].

10.3 Electrophilic stress: component clauses

Cell damage in chronic pancreatitis could now be envisaged as due to 3 interacting factors: chronic CYP induction (in particular of CYP1A2), due to a complex and variable mix of xenobiotics; concurrent exposure to a toxicant(s), especially a volatile petrochemical, that undergoes bioactivation; and insufficiency of micronutrients that help to sustain SH and CH_3 moieties[13].

10.4 Hepatisation of the pancreas

Experimental work demonstrates the plasticity of acinar cells, a reflection of their origin from ductal elements in the developing gland (Chapter 2). Thus, acini revert to a ductal phenotype under conditions of oxidative stress[10] (**Figure 10.2**).

Even more interesting and a reminder that pancreas and liver evolve from the same duodenal bud in fetal life, is the metamorphosis of acinar cells to 'pancreatic hepatocytes' upon experimental exposure to a range of chemicals[253]. It seems that chronic pancreatitis reflects this position in that acinar cells are clearly able to manufacture heme for incorporation into CYP, and they produce albumin such that its level in pancreatic juice represents a 5-fold increase over that in health[254] . It is thus not inconceivable that they can produce bilirubin too, given that it is the natural pathway for disposal of excess heme - a better explanation than bile reflux for the finding of bilirubin and cholesterol clefts deep within the parenchyma in the resected head of pancreas from the patient described earlier (**Figure 9.1**), as too for the anecdotal operative finding of bile throughout the main duct [115] . The histological similarity between chronic active hepatitis and chronic pancreatitis is obvious[1]; moreover, the phrase 'pancreatic cirrhosis' was already introduced in1950 to describe the end stage of the latter[31].

10.5 More questions

- How might a burst of FRA trigger a pancreatitis attack?
- Does the Manchester philosophy rationalise disease geography?
- How are associated gene mutations accommodated?
- Could long-term CYP induction provide a model for chronic pancreatitis?
- Crucially, can micronutrient supplements afford first-line treatment?

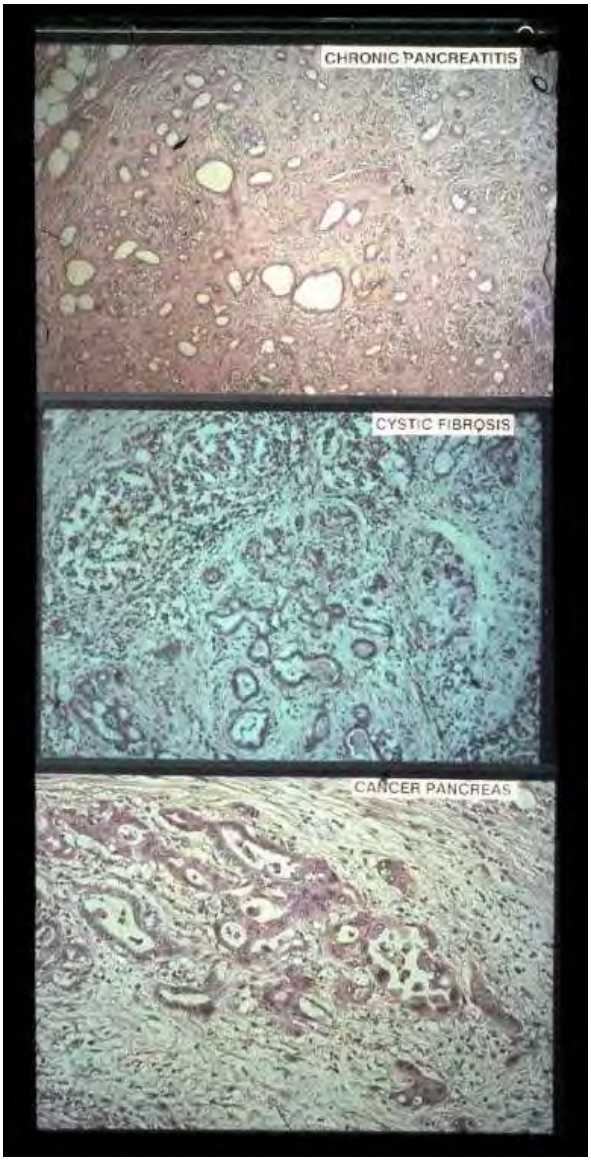

Figure 10.2 Tubular complexes in (a) chronic pancreatitis, (b) cystic fibrosis, and (c) pancreatic cancer. Note that the complexes in (a) lie within pancreatic lobules and are lined by regular cuboidal epithelium, whereas in (c) irregular tubular structures are lined with frankly atypical epithelium.

Chapter 11

Free Radical Pathology of a Pancreatitis Attack

It was difficult to see how bursts of CYP-mediated electron transfer reactions in acinar cells - against a background of methionine / vitamin C / selenium insufficiency - might trigger pancreatitis attacks[255]. These typically punctuate the course of chronic pancreatitis, are clinically indistinguishable from acute pancreatitis, accompanied by agonizing pain, and diagnosed by elevated blood amylase. At least 3 sets of clues were already available[33, 256, 257].

(i)Time-course studies of acute pancreatitis in children with Reye's or haemolytic uraemic syndrome showed that the increase in circulating enzymes initially involves trypsinogen; that only the zymogen form is seen throughout the course of mild pancreatitis; but that in severe disease, trypsin appears from around 48 hours in complex with α_1 proteinase inhibitor (α_1Pi) and is then offloaded to α_2 macroglubulin (α_2M)[258]. Prospective studies of ERCP-induced pancreatitis generally concurred showing increased circulating trypsinogen by the first sampling point of 1 hour, preceding the onset of pain by around 4.5 hours and followed at around 24 hours by the appearance of trypsin-inhibitor complexes, of similar magnitude in mild and severe disease, and at peak amounting to <10% of trypsinogen load[259]. In the same setting, rises in interleukin (IL)-1 and IL-6 were noted from 2 and 4 hours, respectively, in advance by about 24 hours of increments in CRP, amyloid A, procalcitonin and tumour necrosis factor-alpha (TNF-α)[257]. Similarly, in experimental pancreatitis the presence of extracellular trypsin was little and late, appearing at around 4 hours and seemingly originating in the pancreatic interstitium[260].

(ii) Experimental work in the 1950s showed that a fall in fluid and enzyme discharge via the duct system is a very early event in lethal CDE-dietary pancreatitis, which cuts off supply of the essential amino acid methionine[261] - a model that is easily modified to produce inflammatory fibrosis. Not only has pancreastasis since been confirmed as the initial event in every animal model of acute pancreatitis[38], but also the pattern was later demonstrated in human graft pancreatitis[262]. In the clinical setting a paradoxical increase was noted in pancreatic juice of secretory stress proteins, ie. PAP / reg III, and PSP / reg - of which the latter is equivalent to the so- called pancreatic stone protein / lithostatin[231] - that are likely delivered via constitutive pathways at the apical pole of the acinar cell[28].

(iii) It had been recorded that pancreatic and peritoneal mast cells degranulate early in the course of acute pancreatitis, and that the gland has a large population of these - in the paraduodenal area and around blood vessels, ductal elements and nerve endings[20]. ROS are their natural activator, evoking piece-meal degranulation with release of an array of chemicals that account for the cardinal features of inflammation[263]. Any involvement of mast cells in the pathophysiology of acute pancreatitis would be expected to apply equally to chronic pancreatitis in that the first attack carries the same risks[264]. Moreover, mast cell pathology could explain why the distinctive and potentially lethal lesion of HPN bears the hallmarks of tissue infarction, not of digestion by hydrolases[265].

Thus it seemed that a pancreatitis attack represents a reversal in secretory polarity in the acinar cell; that methionine lack is somehow involved at inception whereas trypsin is not; and that the mast cell plays a pivotal role in the transition from 'pancreastasis' to pancreatitis[1,263]. These deductions have now been validated. However, it was some time before a link between

increased FRA and any of these events was established[266,267].

11.1 On secretory polarity

11.1.1 Manchester studies on the isolated gland

In a joint venture with RM Case of the Physiology department in the medical school, studies were carried out on the isolated feline pancreas. An infusion of secretin was given to initiate and maintain a flow of pancreatic juice. It was observed that under steady state conditions a surprisingly large amount of amylase (and other proteins) was discharged, the bulk in venous effluent (**Figure 11.1**), suggesting that it may represent endocrine secretion of acinar products that are manufactured continually and exit basolaterally by the constitutive, non-regulated vesicular pathway[28]. The rate of protein discharge into the venous outflow was unaffected by superimposed bolus injections of acetylcholine in physiological dose. Each evoked a 4-fold increase in amylase output, redistributed such that pancreatic juice and exudate contained, respectively, 11 times and 4.5 times that in venous blood[268].

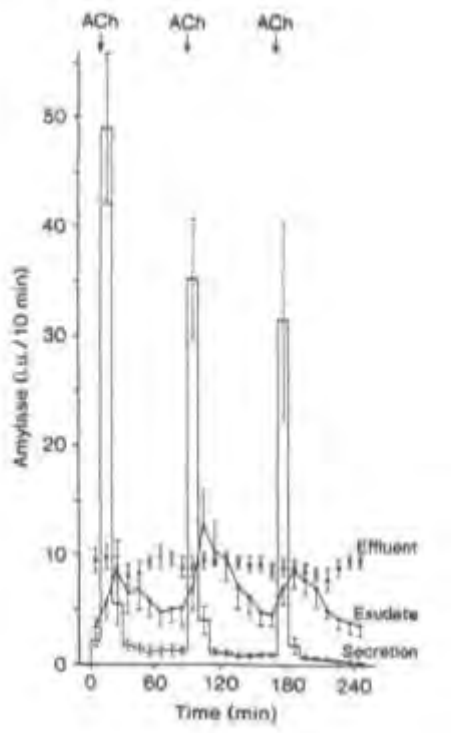

Figure 11.1 Amylase output in pancreatic juice, exudate and venous effluent during continuous stimulation of the perfused cat pancreas by secretin, with superimposed bolus doses of acetylcholine at the arrows marked ACh. Data represent mean ± SE of observations in 9 glands. From Pancreas [268] with permission of the publisher, Walters Kluwer Health.

Subsequent independent work by the physiology group were revelatory. On the assumption that newly synthesised enzymes are cleared more quickly from the cytoplasmic vesicular pool than from zymogen granules in the acinar cell, enzymes were radio-labelled preferentially in either pool and their appearance in pancreatic juice and 'blood' (ie. venous effluent plus exudate) monitored by pulse-chase experiments in rats. Animals were injected subcutaneously with a mixture of tritiated amino acids. After 48 hours, when it could be deduced that any labelled protein in the pancreas would be within zymogen granules, animals were anaesthetised, and samples of pancreatic juice and blood collected at 30 minute intervals before and after an optimal dose of CCK, and then after a 10-times higher dose. Optimal dosing trebled the content of labelled proteins in pancreatic juice, without altering the amount (or amylase activity) in blood. Following hyperstimulation, secretion into the pancreatic duct was inhibited and blood amylase activity doubled but without an increase in content of labelled proteins[269]. Later studies from elsewhere on the same type of preparation identified the blood enzyme rise by 5 minutes[270].

The findings suggested that zymogen granules are not the source of circulating enzymes and, hence, that any pathological exocytosis across the basolateral membrane or transport across the ductal membrane, although of scientific interest, might be insignificant in pathogenetic terms. The inference was that circulating enzymes are derived from an alternate enzyme pool.

In further experiments the bolus of titrated amino acids was delivered into the arterial line of the isolated perfused gland 5 minutes before injection

of an optimal or supramaximal dose of CCK: 2 hours later pieces of the gland were treated to extract the labelled proteins. On optimal dosing, very little (0.02%) of newly synthesised protein appeared in pancreatic juice in the first 30 minutes, 17% in exudate and 4.3 % in venous effluent, each component decreasing substantially within the next 30 minutes. During hyperstimulation, when secretion into the duct system stops, the amount of newly synthesised enzyme in exudate and venous outflow in the first 30 minutes increased to 23.5% and 23.2%, respectively, and then declined.

Thus it could be deduced that a rapidly transported pool of enzymes contributes substantially to the non-polarised secretion of exocrine proteins by the pancreatic acinar cell and that discharge from this pool increases during hyperstimulation, which indicates that constitutive secretion is sensitive to regulation[269]. In yet another series of elegant *in vitro* experiments, dissipation of the normally acidic gradient across the *trans*- Golgi network was shown to inhibit the process by which newly synthesised proteins reach zymogen granules, but not to increase secretion via constitutive-like routes[271].

11.1.2 Basolateral exocytosis

Studies of hyperstimulation pancreatitis in the 1970s revealed fusion images of granule and basolateral membrane in some acinar cells after several hours[15]. Sophisticated work since the turn of the century has identified the molecular basis of physiological apical exocytosis, and also of pathological basolateral exocytosis as is associated with hyperstimulation, exposure to alcohol, its acetaldehyde metabolite, and fatty acids. These experiments demonstrate the exquisite interplay between SNAP proteins (soluble N-ethylmaleimide-sensitive proteins); their SNARE receptors on donor vesicles and target membranes, which include isoforms of VAMPs (vesicle associated membrane proteins) and syntaxins; and fusion regulatory proteins, notably Munc18b and Munc18c. In essence, the basolateral membrane has the machinery for exocytosis but this is constrained by binding of Munc18b to Syn-4. Displacement of Munc18b during hyperstimulation pancreatitis allows fusion of VAMP8 in zymogen granule membrane and SNAP23 in the basolateral membrane, unlocking this exit route[272]: such displacement was recently noted in the resected specimen of a patient with chronic pancreatitis[273]. Whereas VAMP2 was regarded as the major granule v-SNARE, evidence has been adduced for the involvement of VAMP8 in granule-granule fusion, as opposed to granule-apical membrane fusion. In fact its genetic deletion abrogates basolateral exocytosis[272].

11.1.3 Comments

Provided that the human pancreas behaves as does that of the cat and rat, the presence of pancreatic enzymes in the bloodstream of healthy individuals could now be taken to represent endocrine secretion via a constitutive pathway in the basolateral membrane of acinar cells. It seems that increased mobilization of this route, which leads directly off the RER production site, accounts for the sharp increase in circulating enzymes at the inception of acute pancreatitis, including at the outset the zymogens (not activated forms) of trypsin, elastase and phospholipase A_2[256, 266] (**Figure 11.2**).

11.2. Free radicals as detonator

11.2.1 Baltimore studies and beyond

Seminal experiments from Baltimore in the 1980s used the isolated canine pancreas that is retrogradely perfused with autologous blood through splenic and superior mesenteric arteries. The preparation is stable for up to 4 hours, such that it does not gain weight, utilizes glucose, extracts oxygen and releases carbon dioxide throughout. Three models were developed to simulate clinical pancreatitis, as in alcoholics with hyperlipidaemia (free fatty acid infusion), or from a migrating gallstone (partial duct obstruction plus secretin stimulation), or under ischaemic conditions[274].

In each case an early rise in blood amylase was noted alongside a fall in pancreatic juice recovered from the cannulated pancreatic duct, disturbances that were ameliorated by pre-treatment with scavengers of ROS, ie. SOD and catalase (Chapter 3). The former was sufficient in ischaemia-provoked injury, indicating the noxious effect of $O_2^{-\bullet}$, but both were needed in the other two models, indicating the additional toxic effect of $OH^{\bullet}$ derived from H_2O_2. Another series of experiments showed that allopurinol also ameliorated in each instance[275]. It is difficult to know for certain whether this effect signifies inhibition of xanthine oxidase - converted from its normal dehydrogenase form by sulphydryl (SH, thiol) depletion and / or transiently activated chymotrypsin - or additional non-selective actions. That repertoire includes free radical scavenging, copper chelation, $O_2^{-\bullet}$ removal, inhibition of lipid peroxidation, down-regulating expression of heat shock proteins, defusing oxidants released extracellularly during frustrated phagocytosis, and effects on the antioxidant status of cells[276] .

Later experiments investigated whether antioxidants could ameliorate after pancreatitis is under way. Ischaemic injury could not be alleviated, possibly because of the downstream

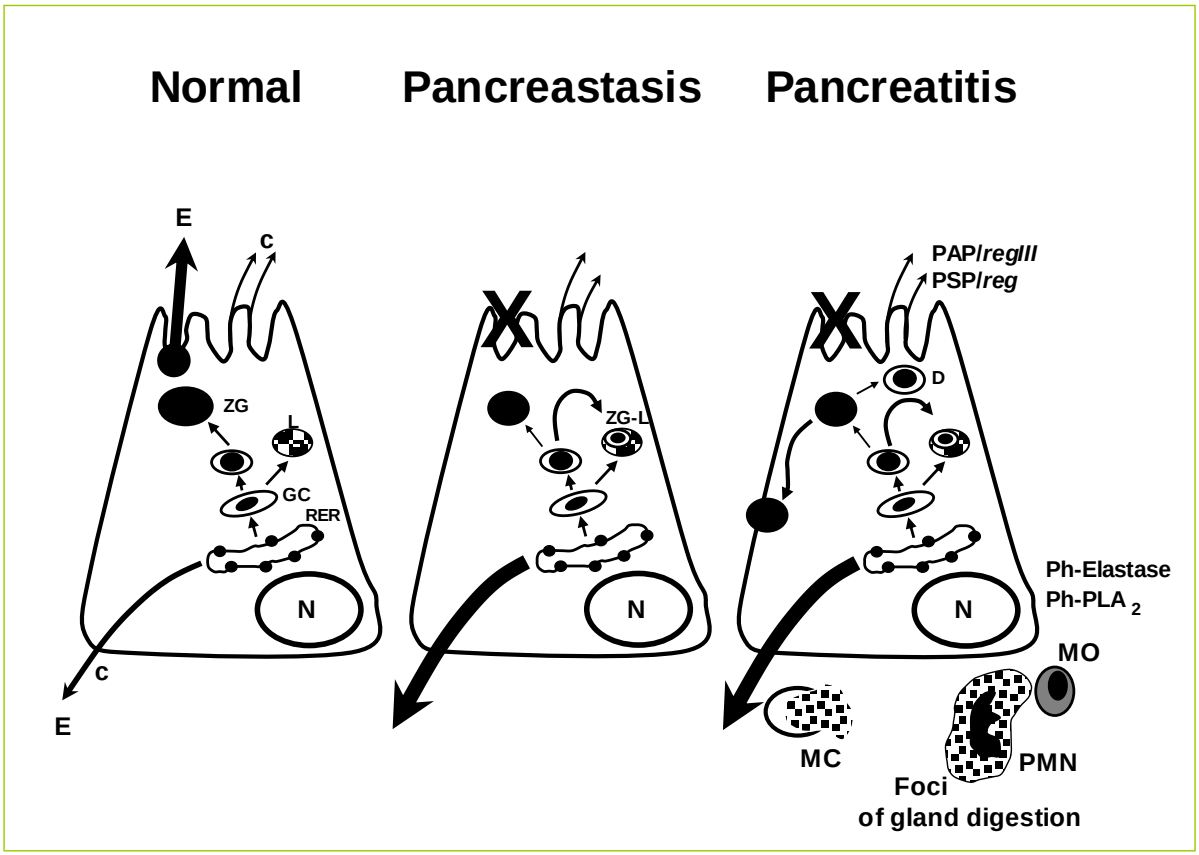

Figure 1

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ar
ZC [illegible] centripetal dissolution of granules; PAP/regIII = pancreatitis associated protein; PSP/reg = pancreatic stone protein / islet regenerating protein; MC = mast cell; PMN = ploymorphonuclear cell; MO = monocyte; Ph-PLA$_2$ phospholipase A$_2$ from phagocytes and mast cell. Adapted from 1990 article [33] for 2011 seminar in Lancet[1] (Elsevier imprint).

injurious effects of peroxynitrate derived from interaction between $O_2^{-\bullet}$ and nitric oxide ($NO^{\bullet}$). There was debate as to whether the offensive cycle could be broken in the model of mild oedematous pancreatitis provoked by caerulein hyperstimulation, while recognising that, as time goes by, activated phagocytes add to oxidant load.

The early reports prompted a spate of generally affirmative studies from elsewhere, using different animals and experimental protocols, including the arginine model which reflects injury from reactive nitrogen species (RNS). These have been reviewed several times over, most recently in 2013[277]. The outcome in the lethal CDE dietary model is of special interest: the combination of allopurinol and dimethylsulphoxide (which traps $OH^{\bullet}$) was found to reduce peri-pancreatic edema but not hyperamylasemia or mortality; both disturbances were reduced by catalase and also by the heat-denatured enzyme, which suggested that the protective effect was due to sulphur amino acids contained in the enzyme rather than to inhibition of free radical activity *per se*[278]. A synthetic analogue of vitamin C proved to be highly effective in curbing lethality in this model[279], as also in ameliorating injury in the caerulein hyperstimulation model of mild pancreatitis[280]. Fifteen years later, dramatic protection of subcellular organelles against oxidative stress in the hyperstimulation model was shown when ascorbate - the bioactive form of vitamin C - was given together with NAC[281], in keeping with evidence that the protocol depletes pancreatic sulphydryls (thiols, SH group)[282]. Selenium, a co-factor for GSH-peroxidase which helps to refurbish GSH that is depleted by peroxide removal, also ameliorated in several models.

Unsurprisingly, direct exposure of the pancreas to pro-oxidants (eg. xanthine / xanthine oxidase, tert-butyl hydroperoxide, or H_2O_2) - whether delivered by close arterial injection or intraductally in the isolated rat pancreas, or to perifused acini - was shown to be highly toxic [283, 284].

11.2.2 Direct evidence of electron transfer burst

This has been forthcoming in several experimental models. (i) In the CDE dietary model a signal suggestive of an $OH^{\bullet}$ adduct was detectable at 6-12 hours, using electron spin resonance spectroscopy[285]. (ii) An increase in ROS during caerulein pancreatitis was demonstrated by chemiluminescence of pancreatic fragments taken at various time-points[286]: this was evident within 5 minutes - as also was an increase in stress activated protein kinase (SAPK)[287] - peaking at 20 minutes. The increase in ROS and attenuation by allopurinol in isolated acinar cells was shown by digital imaging microscopic fluorography using a fluorescent H_2O_2-sensitive probe[288]. (iii) In the bile salt model chemiluminescence depicted increased FRA within 5 minutes, peaking at 15 minutes[286]. This was also demonstrated by cerium-based histochemistry[289]. (iv) Flow cytometry using a fluorescent dye showed an early increase in ROS together with a fall in pancreatic GSH in rats following pancreatic duct obstruction: at 6 hours there was evidence of increased lipid peroxidation, the effect was over by 48 hours, allowing GSH level to recover[290].

In relation to clinical evidence, analysis of peripheral blood by electron spin resonance spectroscopy identified a free radical burst soon after ERCP[291]. Moreover cerium-histochemistry demonstrated pancreatic oxidative stress in human acute-on-chronic pancreatitis[233].

11.2.3 Free radicals and secretory polarity

Low grade oxidative stress in cells is physiological, but an abrupt increase in free radical load can quickly interfere with secretory polarity. Ischaemia-reperfusion injury to renal tubules[292] or pancreas grafts[293] are examples: it is now known that sensors within plasma membranes of secretory cells detect a redox shift initiated by xanthine oxidase in the bloodstream, such that the resultant oxidative stress is

transferred intracellulary to step up the synthesis of antioxidant enzymes[294].

Theoretically, there are many ways in which a free radical burst can interfere with signal transduction in the polarised acinar cell: jeopardising agonist-receptor coupling; altering the balance between G_i and G_s proteins; wounding membranes; disorganising the actin cytoskeleton and / or microtubules; reducing membrane fluidity; interfering in $[Ca^{2+}]_i$ oscillation; inactivating CFTR in the apical membrane[222, 266, 295]. It is possible that the main cellular target varies in different animal models of pancreatitis and the clinical situations that they mimic. However, the absolute dependence on methionine for acinar cell integrity makes it necessary to focus on disrupted methionine metabolism as a potential common denominator[27, 31, 37, 222, 261].

11.2.4 Comments

The cited literature leaves no doubt that a burst of electron transfer reactions in the acinar cell is the trigger for acute pancreatitis, by imposing a blockade on apical exocytosis. There is experimental evidence that, whereas an excess of ROS is incriminated in acute pancreatitis[201,277], RXS tend to be involved in the progression to inflammatory fibrosis[201]. Of related interest, oxidative stress is now also firmly implicated in the pathogenesis of cholestasis [186].

11.3 Methyl and thiol homeostasis

11.3.1 Methionine metabolism

The essential amino acid is avidly taken up by pancreatic acinar cells and incorporated into the 'universal methyl donor', S-adenosylmethionine (SAM / SAMe)[37, 255,256]. (**Figure 11.3**) There is a minor route back to methionine via methyl-thioadenosine, but the bulk of SAM is passaged via a folate-dependent enzyme to S-adenosylhomocysteine (SAH), and thereafter to homocysteine. This is a watershed, whence metabolism proceeds via transmethylation or transsulphuration pathways. The former involves interaction with choline-betaine and vitamin B12-folate shuttles that are facilitated by ascorbic acid in a limited route back to methionine. Both steps in the onward transmission along the transsulphuration route are powered by vitamin B_6-dependant enzymes. Pyridoxal-5´-phosphate is also a co-factor for 2 other enzymes involved in the synthesis of the gaseous mediator hydrogen sulphide (H_2S) from homocysteine and cysteine[296], seemingly provoked when the progression to GSH is impeded.

Methyl (CH_3)[297] and SH (essentially GSH) moieties[282] are essential for apical exocytosis, as is folate which acts as a methyl donor[298]. Whereas GSH is apparently needed for successful docking between corresponding domains of granule and plasma membranes, CH_3 groups seem to be especially important because they enable membrane phospholipid methylation which is a prerequisite for signal transduction in cells such as platelets[26], and also considering that co-denial of choline - which provides a route back to methionine from homocysteine - in the CDE dietary model is lethal: here adenine is trapped, depleting cells of ATP, while jeopardising the synthesis of protein and inositol trisphosphate[17]. The pathway of methionine metabolism also impacts on the correction of reductive stress by biomolecules with electrophilic methyl groups. Such stress and its causation are addressed in later Chapters.

11.3.2 Glutathione cycle

GSH is a tripeptide (L-γ glutamyl-L-cysteinenylglycine) that is synthesised and metabolised by the reactions of the γ-glutamyl cycle, with γGT at its hub. Cellular turnover of GSH is accompanied by its transport as GSH out of cells, yielding - via plasma membrane γGT - γ-glutamyl amino acids which are transported back into cells for reassembly to GSH by the concerted action of 3 enzymes[201] (**Figure 11.3**).

GSH is pluripotent. It facilitates redox control; ensures cytoskeleton integrity; buttresses the signal transduction apparatus; and maintains

normal mitochondrial function. In the acinar cell with its huge turnover of digestive hydrolases that contain numerous S-S linkages, the role of GSH as a reservoir and transport form of cysteine / cystine is hugely important. Moreover, it mops up hydrogen and lipid peroxides; detoxifies ROS and RXS; protects vulnerable enzymes in the methionine metabolic pathway; and contributes to the extracellular antioxidant shield.

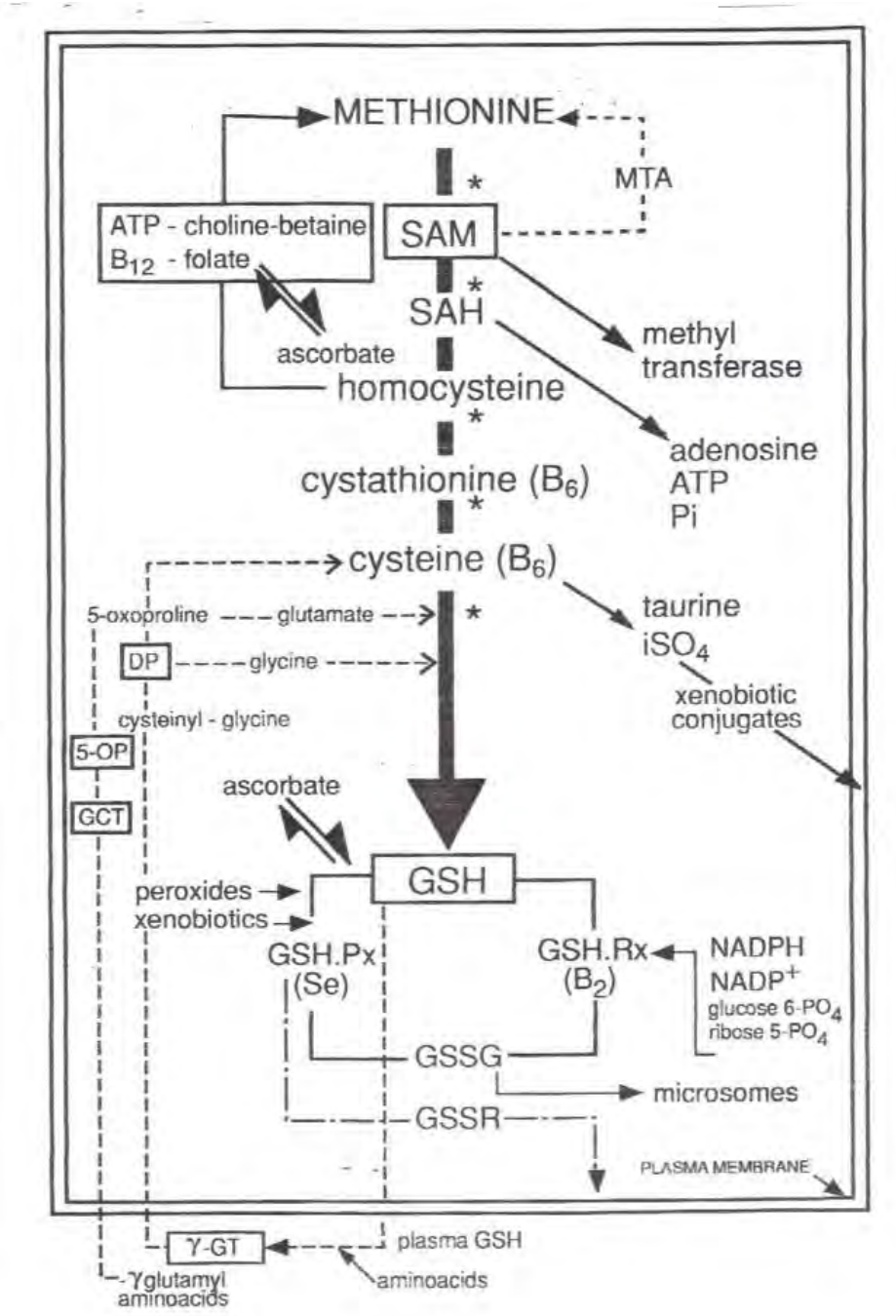

Figure 11.3 Methionine metabolism. Key metabolites: SAM, sulphadenosylmethionine; SAH, sulphadenosyl homocysteine; GSH, glutathione. Other abbreviations: MTA, methylthioadenosine; Pi, activated phosphate groups; iSO4, inorganic sulphate; B2, B6, B12, respectively, riboflavin, pyridoxine and cobalamin; GSH.Px, glutathione peroxidase; GSSR, conjugates of glutrathione with electrophiles from xenobiotics; NADPH and NADP, reduced and oxidised forms, resprectively, of nicotinamide adenosine phosphatase; DP, 5-OP and GCT, enzymes in the synthesis of glutathione . Asterisks indicate enzymes that are known to be vulnerable to electrophilic / excessive oxidative stress, From 2000 review in Digestion [256] with permission of the publisher, S.Karger.

In regard to redox, the GSH / GSSG ratio is far higher in the cytosol (30:1 to 100:1) than within the ER (1:1 to 3:1) where disulphide (S-S) bond formation is a prerequisite for protein folding. Whereas the utilisation of GSH in control of peroxides (GSSG) is soon made good via interlocking GSH peroxidase-GSH reductase / NADPH-NADP / glucose 6 phosphate-ribose 5 phosphate shuttles, it is permanently excreted from cells in conjugates with RXS (**Figure 11.3**)[201,222]. In these circumstances, the ability of ascorbic acid to substitute for GSH via redox and non-redox routes is invaluable[202,203]: moreover, they interact to protect CFTR from free radical attack[255].

Cysteine and ascorbic acid are pluripotent too. The former is the rate-limiting component in GSH synthesis; source of taurine and inorganic sulphate that facilitate the removal of RXS; key to proper protein folding in the ER[98]; and seemingly even more important than GSH for redox control[299]. Ascorbic acid fuels regeneration of CH_3 moieties; substitutes for GSH; acts as a 'Michael donor' in reactions with acrolein and genotoxic FROP; protects CFTR; is a key contributor to the extracellular antioxidant shield; and defuses histamine from mast cells (see below).

As regards selenium, lower habitual intake of which was the main factor distinguishing patients with idiopathic chronic pancreatitis and matched controls (Chapter 8), its antioxidant role is generally linked to presence at the active centre of enzymes that are redox catalysts, of which the best known are GSH peroxidase which removes H_2O_2 and lipid peroxides, and thioredoxin reductase which is homologous to GSH reductase and critical for redox regulation of protein function and signalling. However, there is evidence that the element serves other important roles in the detoxification of xenobiotics[300].

The pancreas has the fourth highest concentration of GSH among body organs, around 2 μmol/g, representing about half that in the kidney and a quarter of that in the liver[238]. A unique feature is the very low level of the crucial first enzyme in GSH synthesis, but compensated for by the second highest level of γGT on both basolateral and granule membranes. Another peculiarity is that, despite the high need for cysteine, acinar cells have a low complement of enzymes in the transsulphuration pathway relative to hepatocytes - made good by high levels of sulphydryl oxidase which catalyses the formation of S-S bonds, albeit with H_2O_2 as a by-product. They also possess little GST and virtually no copper-SOD[255]. This precarious position overall is underlined by the finding that a physiological dose of caerulein results in substantial GSH depletion[201] and evokes a rise in SAPK[287], emphasising the role of GSH in signal transduction but also showing that a slight increase in oxidative stress is a normal component of stimulated apical exocytosis. Despite this evidence, GSH depletion does not of its own cause acute pancreatitis, whereas methionine depletion does. In this regard, it has been noted that pancreatic GSH depletion is not due to excessive oxidation, but is perhaps explained by the presence of activated proteases, as is addressed below[299].

11.3.3 Redox signalling / disulphide stress

The importance of a transient fall in GSH for redox signalling in the acinar cell was recognised by 2010[255]. This aspect has been advanced immeasurably by more recent studies[299]. In particular, redox status has been shown to affect signal transduction through covalent modification of redox sensors: sulphur switches off sensitive targets that include cysteine and methionine residues, so allowing 'transient oxidation of proteins to enable transmission of a signal and subsequent enzymic reduction to their basal oxidation state'[299]. Redox imbalance causes oxidative damage, as a result up-regulating pro-inflammatory genes to elicit increases in a range of chemokines and cytokines.

11.3.4 Comments

The essentiality of methionine in relation to pancreatic integrity can now be traced to several factors: provider of CH_3 groups that are essential for apical exocytosis in acinar cells; source of cysteine which is the rate-limiting step in the synthesis of GSH, with both interacting to maintain cellular redox; and as an antioxidant. Given that selenium is a co-factor for enzymes that facilitate redox control, its lack has been implicated in vulnerability to oxidative stress, not least in chronic pancreatitis[300].

11.4 What role for trypsin?

11.4.1 Party-line

The belief that autodigestion underlies acute pancreatitis continues (**Figure 11.4**)[301], conflating 3 facts from studies of experimental pancreatitis

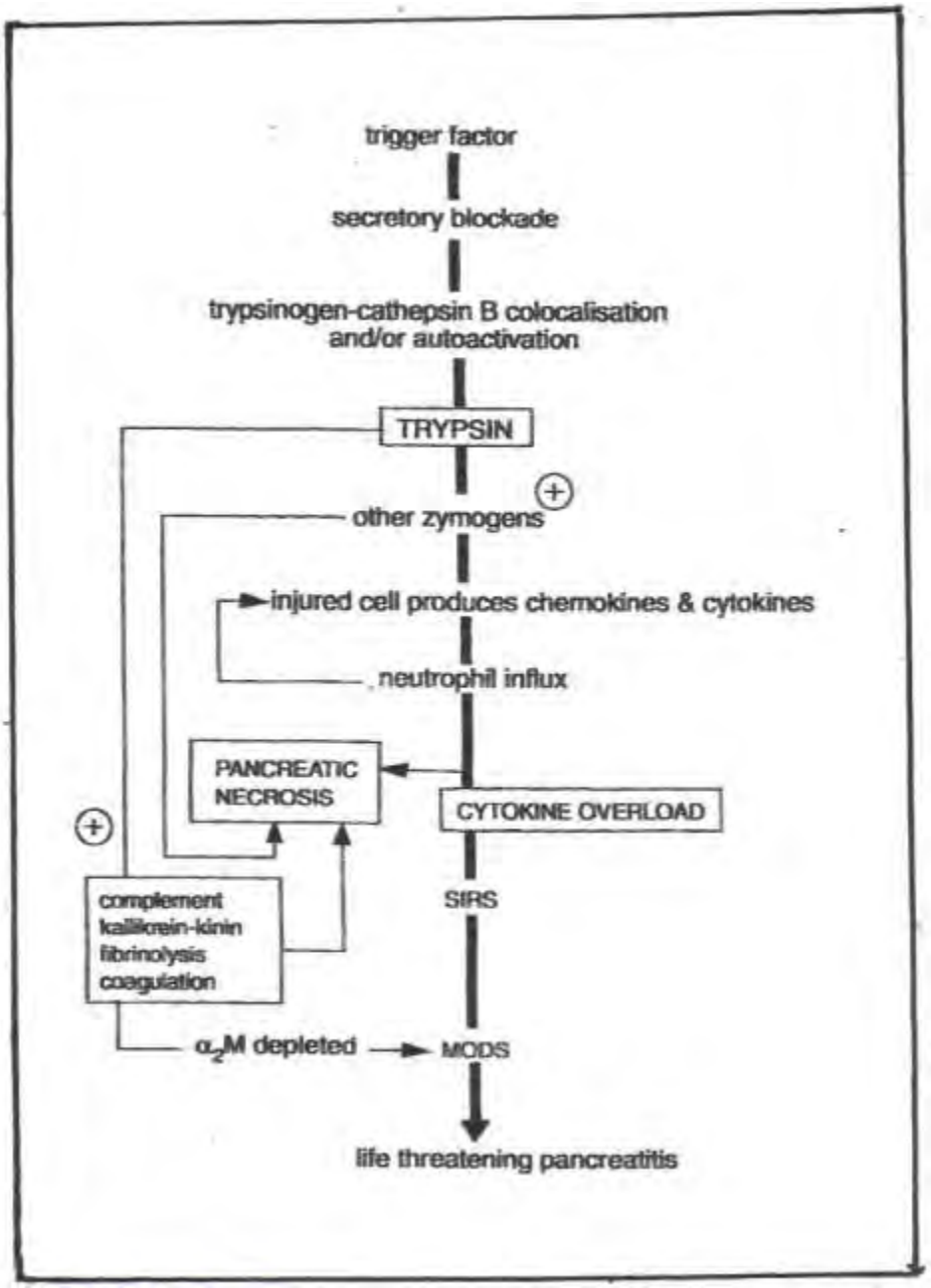

Figure 11.4 Schema of the prevailing hypothesis for acute pancreatitis pathogenesis. SIRS, systemic inflammatory response syndrome; MODS, multiple organ dysfunction syndrome; α_2 M, alpha$_2$ macroglobulin. From ref 256 with permission.

into a framework that is buoyed by the discovery of trypsin-favouring gene mutations (Chapter 13). The facts are these. (i) Apical exocytosis in the acinar cell is hindered at the outset, while minimal basolateral exocytosis occurs rather late. (ii) The cell contains active trypsin within crinophagic and / or autophagic vacuoles. (iii) It is later converted into a pro-inflammatory unit **(Figure 11.2**).

The many objections to the autodigestion philosophy have been enumerated previously[5, 256, 266] , and are not affected by the genetic discoveries. (i) In spontaneous pancreatic insufficiency in animals, the degree of trypsinogen 'activation' seems to exceed that in haemorrhagic pancreatitis. (ii) Pancreatic proteases are not cyotoxic *per se*. (iii) Pancreatic extracts from afflicted glands of several species including humans are virtually devoid of active pancreatic enzymes. (iv) Increases in blood levels of elastase and phospholipase A_2 during human pancreatitis are not of pancreatic origin. (iv) Specific trypsin inhibitors have been singularly impotent in treatment. (v) Trypsin involvement is excluded beyond doubt in foreign serum-induced pancreatitis.

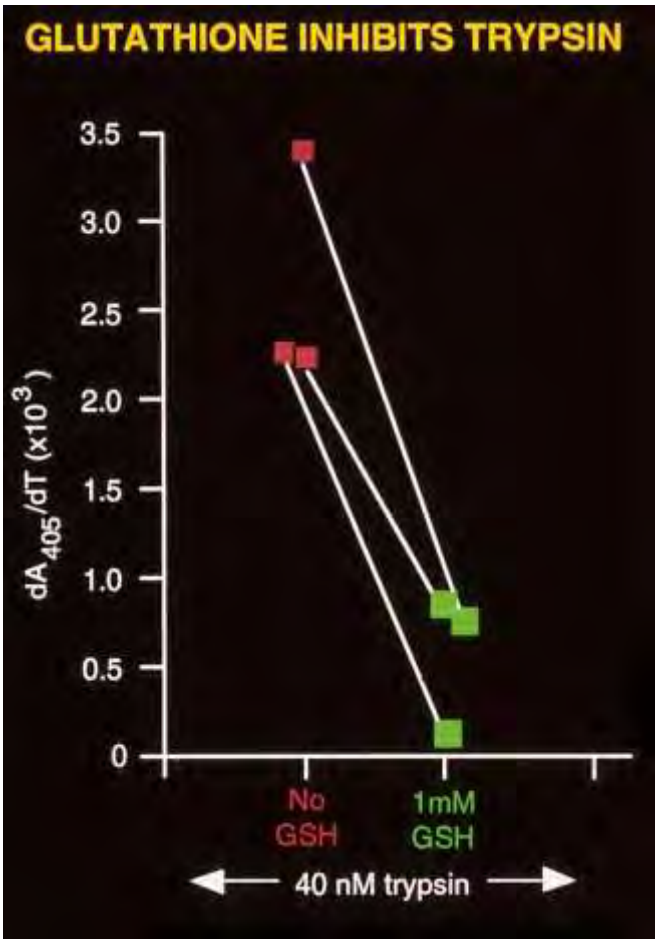

Figure 11.5 Data showing the potent inhibitory effect of glutathione in controlling trypsin *in vitro*

11.4.2 Alternate interpretation

A teleological perspective is that the activation by cathepsin B of trypsinogen within crinophagic vacuoles is a physiological route to the destruction of pent-up pro-enzyme by trypsin-activated mesotrypsin and chymotrypsin[29,302].

11.4.3. GSH on trypsin

It has been known for some time that thiols are potent inhibitors of trypsin and other proteases[302], but this attribute has been ignored by protagonists of the autodigestion hypothesis. Should leaky lysosomal membranes, in the face of heightened oxidative stress allow into the cytoplasm trypsin that escapes SPINK1 protection, it would be immediately inhibited by GSH in a non-stoichometric reversible reaction involving SH-SS exchange. **Figure 11.5** shows that 1mM of GSH, which is the lower limit of the concentration expected in the cytosol of acinar cells, immediately inhibits 20 μM trypsin which is in excess of that expected in zymogen granules (C Chaloner & JM Braganza, unpublished).

In relation to experimental pancreatitis, an *in vitro* study demonstrated that pre-treatment of acini with either of the antioxidants pyrorolidine dithiocarbamate or NAC prevents the co-localization phenomenon as otherwise follows supramaximal stimulation by CCK; also showing the prophylactic value of the combination of ascorbic acid and NAC[256]. Since oxidants do not activate trypsinogen or pro-phospholipase A_2 *in vitro* [303, 304], the corollary is that these antioxidants do not act directly but rather by protecting enzymes in the methionine metabolic route, as well as CFTR at the apical pole of the acinar cell (Chapter 13).

11.4.3 Comments

The role if any for trypsin in the pathogenesis of acute or chronic pancreatitis is now questioned by former proponents of the autodigestion theory[6], while other scientists perceive a housekeeping role for the co-localization phenomenon[287]. The conclusion from investigations using genetically-modified rodents is that daily exposure to unrealistically high levels of trypsin is sufficient to overwhelm defences and cause acute but not chronic pancreatitis. Of late the trypsin paradigm has been watered down within a multifaceted concept that includes simultaneous activation of the NF-κB pathway, Ras signalling and ER stress[305].

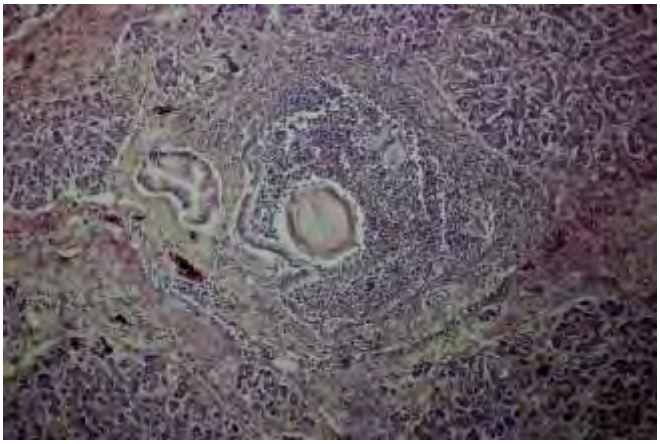

Figure 11.6 Hordes of leucocytes engaged in frustrated phagocytosis as indicated by the halo surrounding debris : histology of ectopic pancreas in a patient with methyldopa-associated fatal pancreatitis[252]. The same changes were noted in the entopic gland. Microphotograph by courtesy of the author, E. Bembow.

11.5 What initiates inflammation?

11.5.1 Acinar cell metamorphosis

The switch from pancreastasis to pancreatitis reflects the release into the pancreatic interstitium of chemokines and cytokines, later aided-and-abetted by hordes of invading leucocytes that engage in frustrated phagocytosis (**Figure 11.6**) - as is shown by high concentrations in peripheral plasma of neutrophil elastase-α_1PI complexes. It is presumed that the first wave of chemoattractants originates in the injured acinar cell: in this regard, not only do ROS participate in the physiological activation of NFkB but also an excess of FRA activates stress response genes. In the particular context of hyperstimulation pancreatitis, SAPK in the gland increased by 57-fold within just 5 minutes[287], in parallel with the reported ROS burst[286], but well in advance of the reported co-localization phenomenon that generates trypsin, or the fall in GSH[306]. The synthesis of PAP is also up-regulated[256].

11.5.2 Mast cell

The immediacy of the inflammatory response, intense pain, ileus, and pulmonary reaction instead suggest a pivotal role of mast cells in the transition: ROS are their natural activator[1,256,257,263]. However the downside is this: bile salts, linoleic acid oxidation products, radiocontrast media, and RXS including from opiates elicit an anaphylactoid (non IgE-dependant) reaction, with abrupt wholesale degranulation of the cells[263].

Mast cell mediators would account for the characteristic features of a severe attack: shock-like state; hyperpermeability of splanchnic capillaries; haemoconcentration; extensive foci of fat necrosis; profound depletion of plasma ascorbic acid (Chapter 17); early activation of complement, kallekrein-kinin and fibrinolytic cascades (Chapter 17); and leucocyte chemokinesis[307]. The activation by these mediators of mast cells in the gastroduodenum, mesentery and peritoneum - and, thereby of proteinase activated receptor-2 (PAR-2) - potentially explains ileus, ascites, and loss of intestinal integrity with endotoxaemia: the transfer of mediators across the diaphragm could account for the pleural reaction in severe disease, and transfer via lymphatics would rationalise hypoxaemia and its association with fibrin degradation products [256, 257, 263] (**Figure 11.7**).

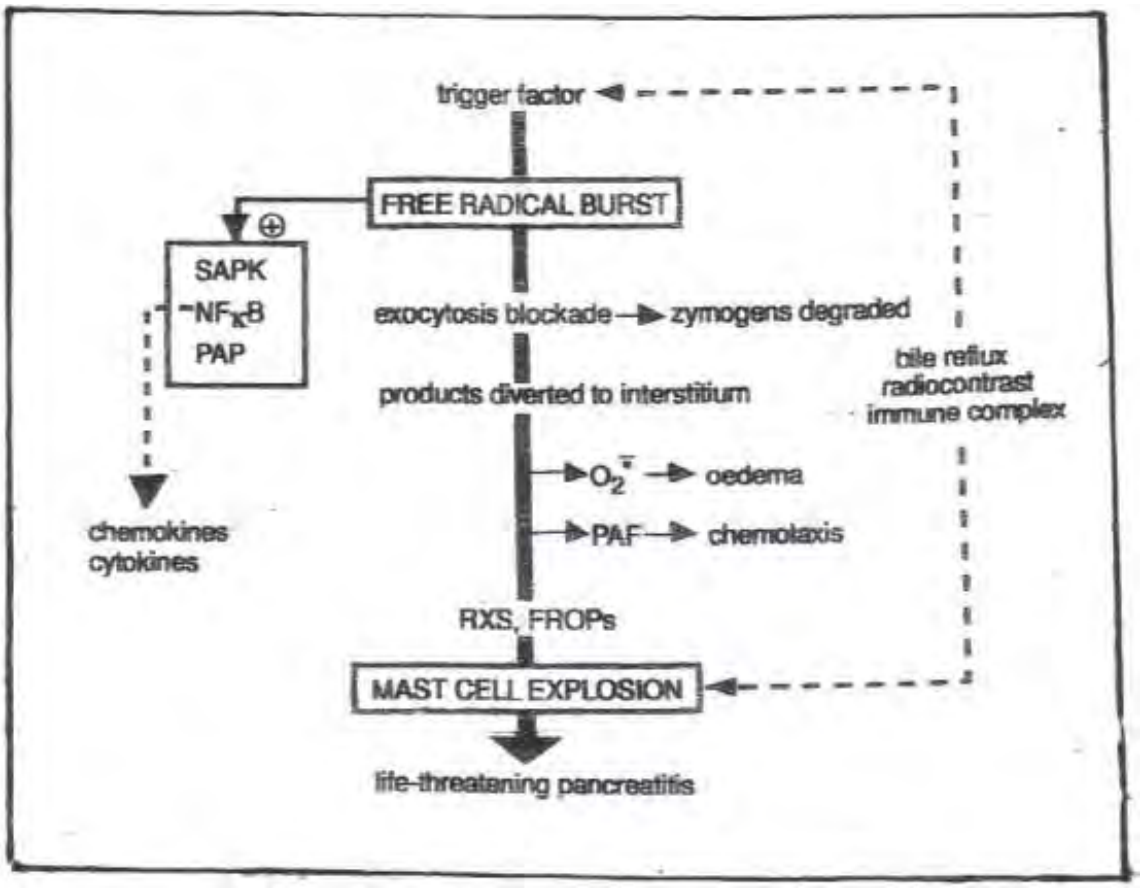

Figure 11.7 Proposal for pancreatic mast cell as pivotal player in life-threatening acute pancreatitis. Abbreviations: SAPK, stress activated protein kinase; NFκB, ubiquitous inducible transcription factor; PAP, pancreatitis associated protein; $O_2^{\cdot}$ superoxide; PAF platelet activating factor.

This view might also explain why the median interval to death is shorter, < 48 hours, and the degree of initial shock greater from interstitial pancreatitis, which accounts for a third of the toll in the first week and a quarter of that overall, than from wholesale necrosis [256].

There is ample support for this concept by way of old papers[263], and more recent material included in reviews[256,257,308]: the topic is revisited in the penultimate chapter. Here it is worth noting that the juxtaposition of mast cells, adipocytes including precursor stellate cells, and 2 classes of plasminogen activator in the interstitium close to the plasma membrane of acinar cells is poised to favour both nociceptive and pro-fibrosis pathways under conditions of excessive oxidative stress in acinar cells[13]. Studies of specimens from patients with chronic pancreatitis support these connections[309-311].

11.5.3 Comments

There is a wealth of evidence implicating mast cells in the evolution of both acute pancreatitis and chronic pancreatitis.

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11.6 Acute / RAP link to chronic pancreatitis

11.6.1 SAPE hypothesis

Currently, there is huge interest in this progression, driven by the so-called sentinel acute pancreatitis event (SAPE), '2-hit' hypothesis. In this proposal, the first 'hit' is defined as an attack of acute pancreatitis that is sufficiently severe to activate resident macrophages and stellate cells. The second 'hit' is envisaged as being delivered by a further attack 'of pancreatitis or oxidative stress that propagates this inflammatory response resulting in the deposition of collagen' [312]. An

epidemiology study identified alcoholism as having the biggest impact on the transition, biliary factors the least impact, with genetic / idiopathic factors displaying intermediate influence[313]. A recent retrospective investigation concluded that 10% of patients with a first episode of acute pancreatitis and 36% of those with RAP develop chronic pancreatitis; the risk higher in smokers, alcoholics, and men[314].

11.6.2 Focus on oxidative / electrophilic stress

An alternate explanation revolves around the balance between pro-oxidant and antioxidant forces, and class of reactive species. (i) Whereas the habitual intake of selenium in Manchester patients with chronic pancreatitis generally fell short in relation to oxidant load via induced CYP1A2 (**Figure 7. 3**), selenium intake of patients with RAP was normal or lay in the borderline zone[315]. (ii) An independent biochemical study from Cardiff in the UK confirmed and extended these findings by comparing blood antioxidant profiles - selenium, copper, zinc, vitamins A and E, and 5 carotenoids - in sets with chronic pancreatitis, RAP and controls. The first set showed multiple deficiencies in both alcoholic and idiopathic categories. By contrast profiles were normal in the RAP set[316]. (iii) Later observations at Manchester identified persisting deficiency of erythrocyte GSH - which seems to reflect methionine lack and / or dysfunction of enzymes in its metabolic pathway (**Figure 11.3**) - in patients with RAP or chronic pancreatitis: the difference lay in plasma vitamin C levels which were in the lower quartile of the reference range in most patients with chronic pancreatitis and negligible in calcific disease, but normal (between attacks) in those with RAP[37]. (iv) Analysis of duodenal fluid collected during SP tests identified excess lipid peroxidation associated with chronic pancreatitis, but not with RAP (**Figure 3.8**). (v) Studies of patients with congenital lipoprotein lipase deficiency (LLPD) and numerous pancreatitis attacks but without progression to chronic pancreatitis (Chapter 15)

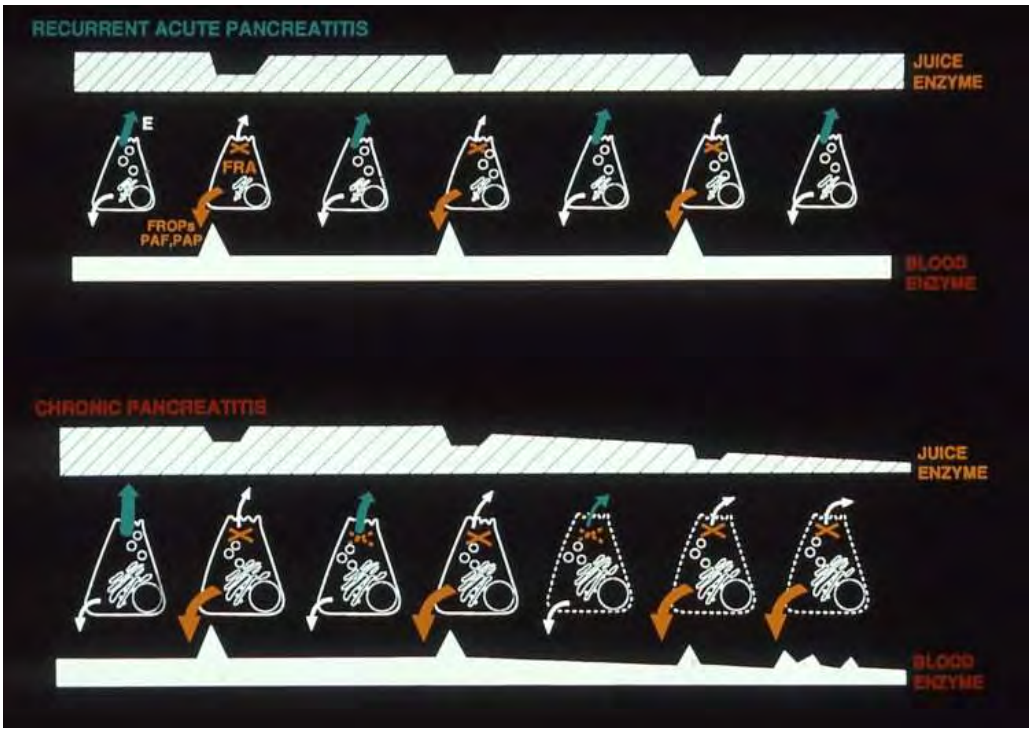

Figure 11.8 Schematic representation of the evolution of recurrent acute pancreatitis and chronic pancreatitis characterised, respectively, by full recovery after an attack(s) or loss of acinar mass (hatched cell outlines). E amylase, lipase, zymogens; FRA burst of free radical activity jeopardizing apical exocytosis; FROP free radical oxidation products; PAF platelet activating factor; PAP pancreatitis associated protein. From QJMed[37]

belie the SAPE theory[317]. (vi) Oxidative stress is involved in animal models of acute pancreatitis and the clinical conditions that they simulate[277], but electrophilic stress from RXS is the hallmark of clinical chronic pancreatitis and several animal models such as via CCL_4 or dibutyltin[201]. **Figure 11.8** depicts the Manchester interpretation for these different outcomes.

11.7 Overview and summary

A burst of electron transfer reactions in acinar cells triggers a domino effect wherein oxidative / electrophilic stress is the linking thread: pancreastasis by one or other means, especially by prejudicing the supply of CH_3 and SH moieties; alteration of redox, which activates stress response genes that effect transformation of the acinar cell into a pro-inflammatory unit; disulphide stress; recruitment of interstitial mast cells via basolateral discharge of FROP; and frenzied behaviour of neutrophils with extracellular discharge of their chemical cargo of enzymes (eg. phospholipase A_2, elastase) together with HOCl which immobilises antiproteases[5, 266, 267]. A toxic brew of ROS, RNS, RXS, mast cell products, cytokines, FROP, substance P and so on contributes to death from acute lung injury[318]. It is a wonder that any patient survives!

Chapter 12

Rationalising Disease Geography

The prevalence of chronic pancreatitis in Europe is around 10 times lower than in parts of southern India and South Africa, traditionally idiopathic in the former area and alcoholic in the latter, and with a tendency to large pancreatic calculi. A shared genetic predisposition is not the explanation in that far fewer gene mutations are associated with alcoholic disease (Chapter 13). Protein-calorie malnutrition due to poverty is untenable too, because its most extreme form of kwashiorkor is not associated with the disease[319].

In the classical description of 'tropical pancreatitis' as reported in the 1960s from Kerala province in southern India, malnourished patients presented in the first or second decade of life with ketone-resistant diabetes, pancreatic calculi detected on plain abdominal X-ray, and pancreatic exocrine failure although not steatorrhoea as fat intake is low[34]. Other idiosyncrasies included parotid gland prominence, a cyanotic hue (that possibly represented methaemoglobinaemia) and, occasionally, endomyocardial fibrosis. The attractive hypothesis of hydrogen cyanide toxicity from the dietary staple, cassava (synonyms tapioca, manioc), could also explain the familial pattern[35], but was undermined by absence of the disease in other countries with similar dietary reliance.

The evolution of the disease in South Africa was equally intriguing. Historically, African Blacks consumed home-brewed beer of low alcohol content, around 3%, which was associated with 'Bantu siderosis' due to excess iron that leached out of utensils during the fermentation process. After the repeal in 1962 of legislation that forbade the sale of Western-type alcohol to Blacks, the pattern of liver injury evolved over time to that of alcoholic disease in developed countries, while liver iron content decreased. Hospital admission statistics at Baragwanath hospital which serves the township of Soweto disclosed a trickle of patients with calcific chronic pancreatitis from the 1970s, but 55 new cases between 1981 and 1983 - of whom two-thirds still drank mainly home brews. Over the next 4.5 years, 90 new cases were identified with a male:female ratio of 6:1, mean age 40 years, and mean alcohol intake of 180 gm / day for an average of 15 years. The later surge in disease frequency by the 1990s, such that at least 5 new cases of alcoholic disease were registered each month, was observed to parallel urbanisation and industrialisation[8].

The opportunity to examine a cohort in each region along the lines of the Manchester studies generated interesting parallels despite ethnic differences (**Figure 12.1**). The research sabbaticals in southern India and South Africa were enabled by invitations from V Mohan and I Segal respectively.

12.1 Chennai, South India: 1988

12.1.1 Social histories

These were ascertained from 79 patients attending 2 hospitals in Chennai (formerly Madras) in the province of Andhra Pradesh. - the excellent privately run, MV Hospital, and the Government Peripheral Hospital[320]. The presence

Figure 12.1 Examples of patients investigated at Chennai (left frame) and Soweto (right frame):

of pancreatic calculi confirmed the diagnosis in most patients. In the others, small-duct disease was diagnosed when faecal chymotrypsin concentration was below the reference range and pancreatic ultrasound scan was unequivocally abnormal in patients with a compatible history.

Every patient was interviewed by the same team, consisting of a gastroenterologist and 2 nutritionists who traced lifestyle and symptomatology since childhood. An aide-memoire was drawn up to facilitate the enquiry into domestic, aetiological, social, cultural, and occupational aspects. Particular attention was paid to the 5-year period preceding the first episode of abdominal pain or, when this was denied, to the period 10-15 years before symptoms of diabetes. Note was made of birthplace and residence thereafter, religion, income group, literacy, travelling distance to school or workplace, environment (rural, semiurban, urban), and mode of transport - whether by foot, pushbike, motorcycle, car, or public transport.

An arbitrary scoring system, from 0 to 3 was used to quantify exposure to the more commonly mentioned xenobiotics, the score arrived at by consensus where zero implied negligible exposure and 3 indicated heavy exposure. For cigarettes and alcohol the system was the same as used at Manchester. Exposures to smoke from a firewood cooker or vehicle emissions, and to fumes from kerosene, diesel or petrol were graded according to hours per day of close contact. It was realised that vehicle emissions deliver smoke as well as chemical fumes. In regard to dietary xenobiotics, attention was focused on cyanogens, and whether-or-not cooking oil was largely composed of unsaturated fatty acids which facilitate CYP induction, and its source, whether tinned or 'loose' in that the latter is subject to alteration by rubber seed oil.

Many micronutrient antioxidants are derived from meat, fish and eggs. Hence, an arbitrary score of 0 was assigned to vegans, and higher scores depending on days per week when these items were consumed, a score of 0 indicating negligible intake and 3 implying a good supply.

Among the cohort of 79 patients, there were 53 sporadic cases with calcific disease and diabetes; 6 sporadic cases with non-calcific disease, usually with diabetes; 4 pairs of first-degree relatives with calcific or non-calcific disease, with or without diabetes; and 2 families in which several members had either variant of the disease.

Full details for each patient are tabulated in the study report[320]. The prominent findings were as follows. (i) A pancreatitis episode was the presenting feature in 45% of sporadic cases with calcific disease plus diabetes but in only 2 was pain the current dominant problem. In the other subsets diabetes was the reason for presentation.(ii) In the survey overall, weight loss from uncontrolled diabetes and / or maldigestion was often profound in the 6 month period before presentation, a BMI < 18 was taken as indicating malnutrition. However, patients frequently recalled their good pre-morbid weight, indicating that malnutrition was a consequence of the disease. (iii) Prominent parotid glands were noted in 4 cases. (iv) Daily, modest alcohol intake was documented in 5 patients (9%), and 27 smoked cigarettes or were in close contact with a family member who did (51%). (v) Exposure to smoke from a firewood cooker in a small ill-ventilated area was mentioned by 17 patients (32%): it was not unusual for family members to congregate therein at mealtimes when the cooker was still alight (assigned a score of 1, an additional 34% of cases), such that all but 4 cases were exposed daily to smoke by one or other means. (vi) The frequency of close exposure to kerosene fumes via cooker and/ or lamp and / or occupation was 75%. Close exposure to diesel / petrol fumes emerged in 21%. Overall, regular exposure to petrochemical products - whether fumes from kerosene, diesel, petrol, or via vehicle emissions was recorded in 92% of cases. (vii) There were 18

patients who were regularly exposed to other volatile chemicals in the work environment or through hobbies in the decade preceding symptoms, and of these 3 had received CYP-inducing drugs. (viii) The range of xenobiotic exposure was similar in patients with calcific or non-calcific disease.

As an amusing aside, whereas the initial enquiry on volatile chemicals was largely directed towards occupational exposure, based on the Manchester experience (Chapter 8), the youngest member of the largest afflicted family piped up with the question, "and what about kerosene smell from our cooker?" For this illuminating comment, which prompted recall to the clinic or letters to all patients with specific questions on kerosene cookers or lamps, the boy asked for and received the author's camera! In that family of 8 members (**Figure 12.1**), there was unequivocal evidence of exocrine pancreatic disease in 6 (ie. pancreatic calculi or low faecal chymotrypsin with abnormal ultrasound scan); the last 2 had virtually no chymotrypsin in faeces but the scan did not show clear evidence of fibrosis . The largest pancreatic calculi were found in the mother and sister who had the closest contact with kerosene fumes for the longest periods.

Dietary enquiry revealed that most patients in the series used a cooking oil that was largely composed of C18:2 fatty acids - eg. peanut, gingelly - which was generally purchased 'loose'. Regular consumption of foods containing cyanogenic glycosides was recorded in 58% of cases, but regular ingestion of cassava in only 17%. Dietary antioxidant intake was adjudged grossly inadequate in 15 vegans (28%) and suboptimal in 22 others (2%) who ate animal products less than twice a week: this pattern was found in cases of calcific or non-calcific disease.

12.1.2 Drug metabolism studies

Theophylline metabolism as an in vivo probe for the potentially toxic CYP1A2 pathway of xenobiotic disposal was studied in 11 healthy volunteers and 11 sporadic cases of calcific chronic pancreatitis: malnutrition in the latter set was shown by subnormal BMI and expected to compromise drug handling. In fact, theophylline clearance was faster in patients than controls (median 69, range 39-114 versus median 45, 33-56 ml /kg / hr, p=0.003). In keeping with these findings, social histories identified a higher exposure level in patients than controls to xenobiotics that are CYP inducers and/or yield RXS upon metabolism (score 7, 4-11 versus 3, 2-9, p=0.002). However, the concentration of D-glucaric acid in urine, as marker of a phase-II conjugating pathway that facilitates the removal of RXS (Chapter 5.3), was similar in patients and controls. The combination is conducive to electrophilic / oxidative stress[321].

12.1.3 Selenium status

Serum selenium, measured by atomic absorption spectroscopy - through the expertise of JP Day from the University Department of Inorganic Chemistry - had been extensively researched at Manchester (**Figure 12.2**), noting markedly lower levels in patients with chronic pancreatitis, painful disease in particular. Moreover, repeated exposures of healthy volunteers to CYP substrates resulted in a drastic lowering of serum selenium concentration (**Figure 12.3**).

At Chennai studies in 20 healthy controls and 38 patients yielded data that were very similar to the Manchester findings. In addition, selenium concentrations in groups with primary insulin-dependant (n=9) and non insulin-dependant diabetes (n=11) were found to conform to the reference range [322].

It could be concluded that: (i) the bioavailablity of the element is equally high in south India and north west England; (ii) the decrement in selenium among patients with chronic pancreatitis is of a similar order in each geographic area ($p<0.001$), thus denying a connection with calculi or pancreatic exocrine failure in that the frequency of

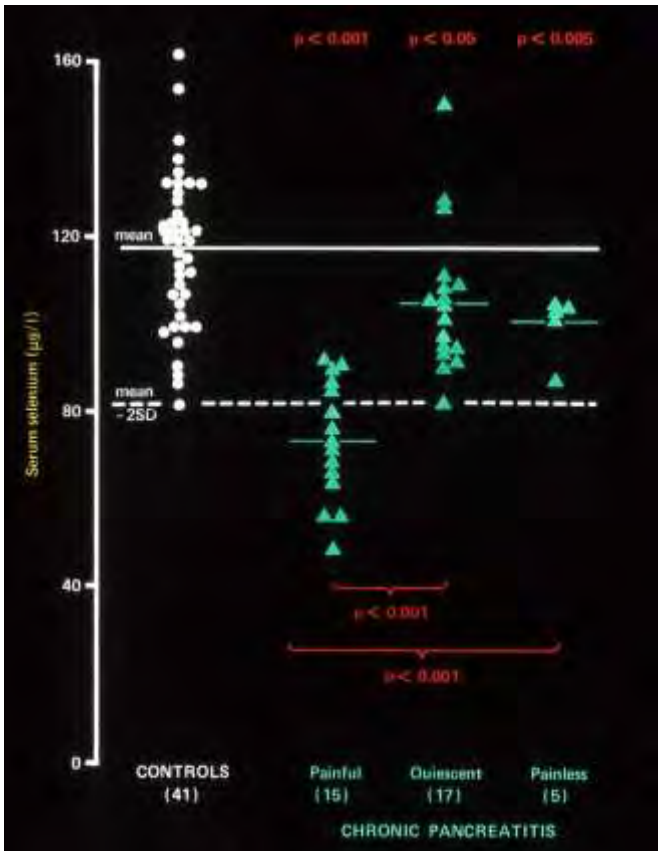

Figure 12.2 Serum selenium concentrations: Manchester 1988 paper in Trace Elements Med [300] reproduced with permission.

each characteristic was substantially higher in the Chennai series; (iii) selenium levels do not account for the accelerated course to diabetes in tropical chronic pancreatitis.

12.1.4 Micronutrient antioxidant profiles

More general profiles of micronutrient antioxidant status in Chennai and Manchester were compared in a subsequent study[323]. Individual datapoints in controls and patients with chronic pancreatitis are shown in **Figure 12.4** and **Figure 12.5**, respectively. As in the case of selenium, the bioavailablity of vitamin E (expressed as the molar ratio of α-tocopherol to cholesterol) proved to be equally high in each geographic zone, but that of β-carotene and ascorbic acid was severely compromised in the tropical area ($p<0.001$): of particular note in regard to the last factor, plasma vitamin C level was similar in Manchester and Chennai controls. Moreover, the concentration in urine of inorganic sulphate, which reflects habitual intake of sulphur amino acids, was higher at Chennai ($p<0.02$). Antioxidant profiles of chronic pancreatitis groups reflected these indigenous

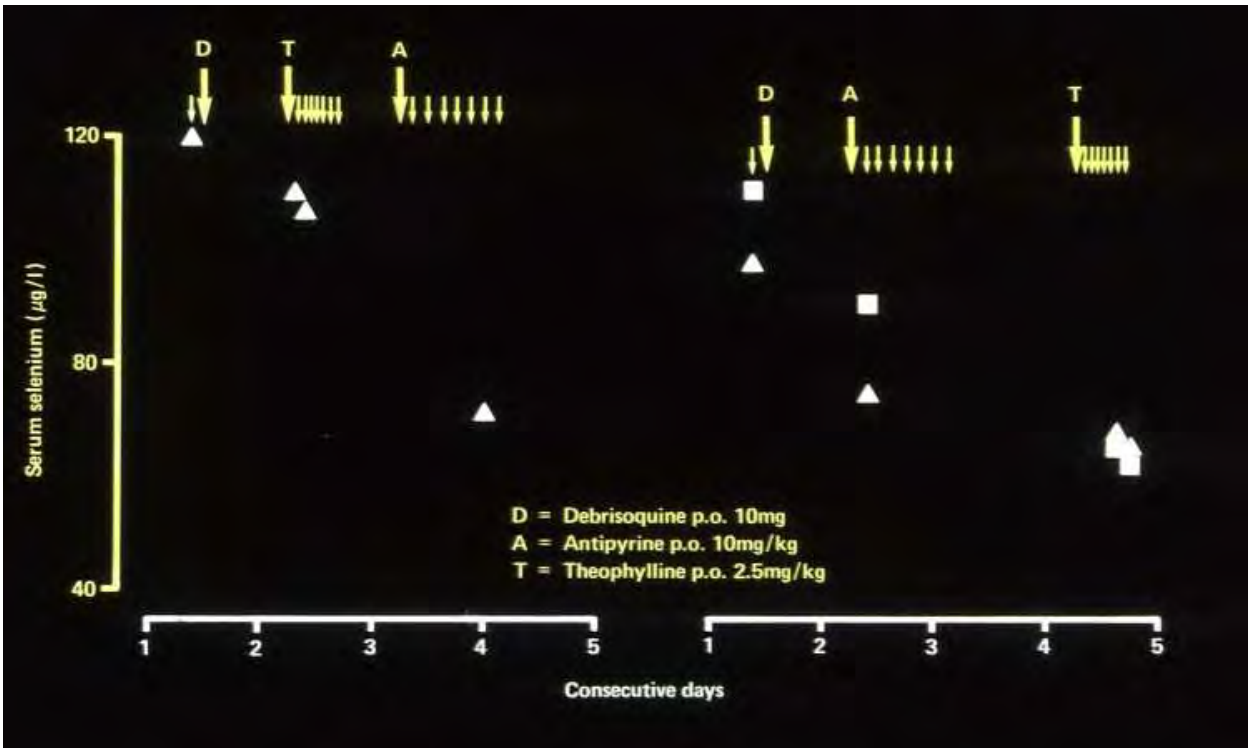

Figure 12.3 The detrimental impact on serum selenium of repetitive exposure to cytochrome P450 substrates in Manchester volunteers. Publication details as in Figure 12.2.

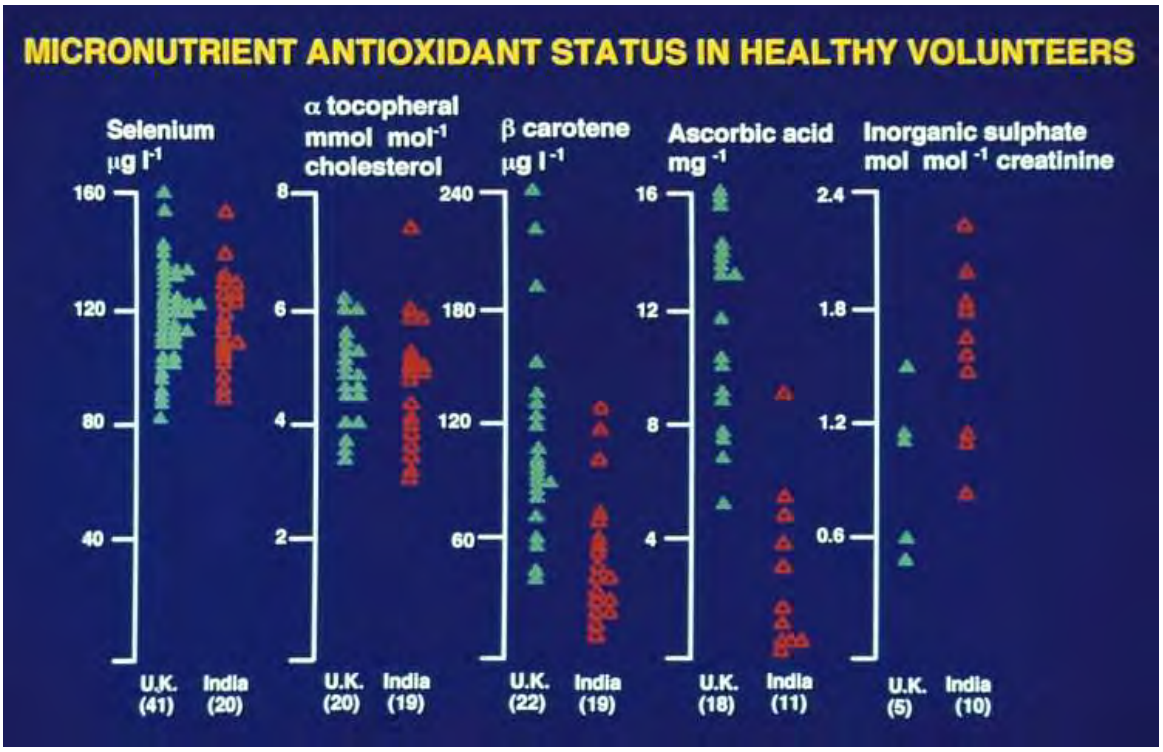

Figure 12.4 Individual data points in controls at Manchester (closed symbols) and Chennai (open symbols). Reproduced from 1993 paper in Scand J Gastroenterol [323].

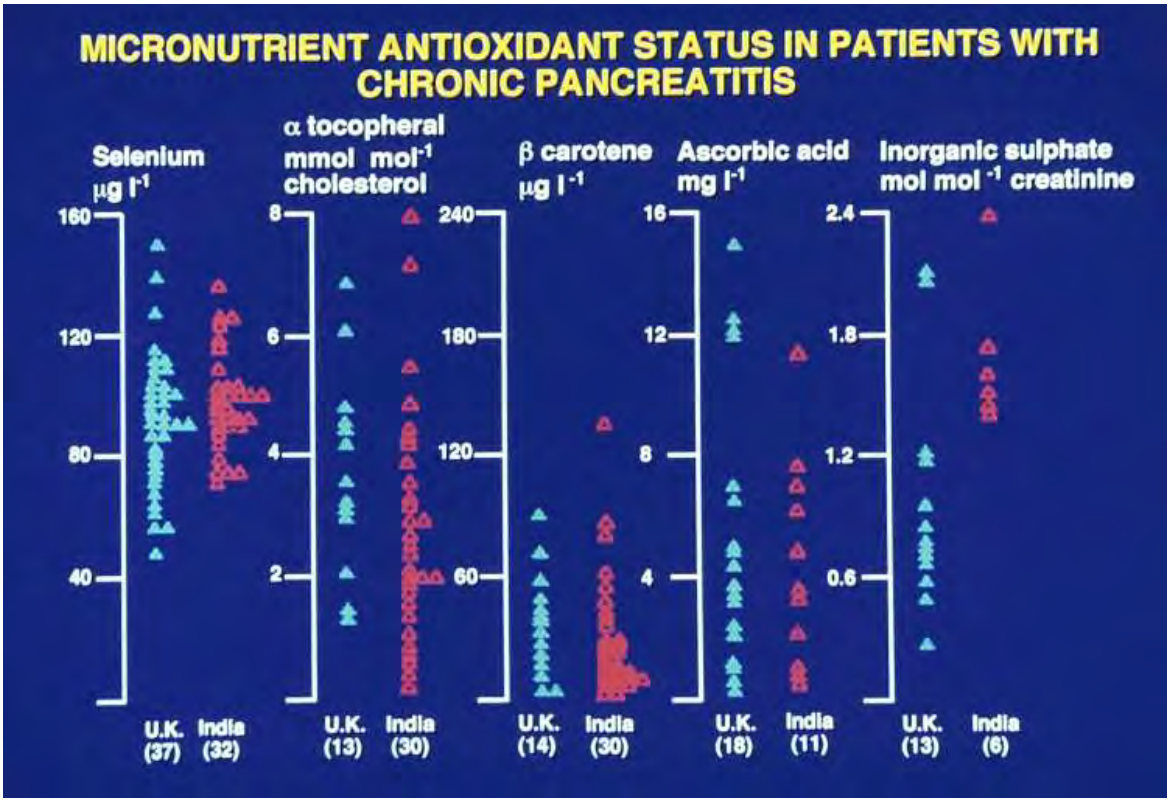

Figure 12.5 Individual data points in patients with chronic pancreatitis at Manchester (closed symbols) and Chennai (open symbols). Publication details as for Figure 12.4.

differences - a decrement in selenium and α-tocopherol from respective control values evident in each zone; β-carotene and ascorbic acid subnormal only in the Manchester cohort; and urinary sulphate higher in the Chennai set ($p<0.001$).

12.1.5 Comments

These studies in patients with chronic pancreatitis at Chennai identified regular exposure to volatile chemicals that induce CYP and / or yield RXS upon processing thereby, against a background of habitually poor bioavailability of ascorbic acid and β-carotene, likely due to hostile culinary practices. The implication of pancreatic electrophilic / oxidative stress was not directly confirmed because the requisite biochemical methods had not yet been developed in the Manchester laboratory. However, later investigations from elsewhere in India support this view[105, 324, 325].

Of note, macronutrient malnutrition and low BMI were adjudged to be post hoc, as was more recently concluded from observations at Delhi[326]. Similarly, reports on micronutrient status from dietary and / or blood analysis reflect the position after the disease is established and patients have altered their diets - showing subnormal intake of several micronutrients including choline in patients from Delhi[327], and subnormal serum concentration of zinc and folate in patients from Cochin[67].

A connection with cassava or other dietary source of hydrogen cyanide was made less likely by urinary levels of inorganic sulphate, which indicated excellent intake of sulphur amino acids that detoxify hydrogen cyanide. This does not, however, negate the possibility that the cassava threat is from its non-nitrile fraction which, as in most if not all instances of potential toxicity from CYP-generated RXS, depends on neutralisation by GSH[201, 236].

Thus in theory, the toxicity of cyanogenic glycosides may be due to liberated cyanide, toxicity of the parent molecule, a non-cyanide moiety that is produced upon metabolism via CYP, or a combination of these. The paradox that long-term feeding of cassava produces chronic pancreatitis-like lesions in rat and rabbit pancreas, whereas hydrogen cyanide does not, implicates the non-cyanide moiety. This deduction is supported by painstaking experimental work with a series of nitriles, notably crambene (1–cyano-2-hydroxy-3-butene). Acute administration in rats caused an early phase of bile and pancreatic juice hypersecretion, akin to the changes documented in human chronic pancreatitis. Morphologically there was profound edematous pancreatitis with widespread acinar cell apoptosis, preceded several hours earlier by an 80% depletion in GSH. Moreover, repeated dosing led to a picture of early chronic pancreatitis. Subsequent studies revealed that crambene not only oxidises GSH to GSSG, but also cleaves GSH into its building blocks and modulates GSH synthesis[201].

The destructive effect on micronutrient antioxidants of frying vegetables at high temperature was exposed by the preliminary finding of low plasma ascorbic acid despite ample total vitamin C in Chennai controls - as is the only plausible interpretation of low values for the 'free radical trapping ability of plasma' (FRAP) in the later Delhi trial of micronutrient therapy (Chapter 16). This is also the likely explanation for the low serum level of β-carotene in Chennai controls, and low folic acid in a study from Cochin which, as in Delhi and Chennai, recorded normal blood levels of vitamin C but unfortunately failed to measure the bioactive form of ascorbic acid.

The 6 fold drop in annual hospital admissions between 1962 and 1987 in Kerala[34], the adjacent coastal province which has the highest literacy rate in India, has been something of a mystery because dietary intake of cassava was unchanged during this period. The kerosene connection provides the best answer, in that the decline coincides with the introduction of electricity, lowering the dependence on kerosene

cookers and lamps[1]. In regard to the latter, 2 patients who had a theophylline test at Trivandrum were college students who worked late into the night under light from kerosene lamps: high clearance values of 100 and 110 ml /kg /hr indicated induction of CYP1A2 (V Balakrishnan & JM Braganza unpublished).

As noted with citations in an earlier review of the tropical disease[236], pancreatic electrophilic stress beginning in childhood can explain high values of the antioxidant lactoferrin in pancreatic juice observed in Kerala - the values far higher than in France - as also large amounts of another antioxidant, mucin, in pancreatic ducts. Steady exposure to kerosene fumes could also underlie parotid gland prominence, cyanotic hue, and endomyocardial fibrosis. The first is due to gland hyperplasia[328], another example of extra-hepatic enzyme induction: in support of this interpretation, inducible CYP isoforms have been identified in human salivary glands[329]. In regard to the cardiac lesion, exposure of dogs to kerosene smoke induced similar changes[330].

12.2 Soweto, South Africa: 1993

12.2.1 On oxidative stress

These studies were done at the Gastro Intestinal Unit of Baragwanath Hospital in Soweto, the township on the outskirts of Johannesburg. Their basis was an appreciation of the roles of ROS and RXS in tissue damage from alcoholism (**Figure 12.6**)[64]. The investigations were undertaken at a time when the Manchester laboratory had refined techniques to detect oxidative / electrophilic stress and micronutrient status[331-333].

Fourteen consecutive patients attending the out-patient clinic were investigated. Blood samples were analysed for free radical markers and

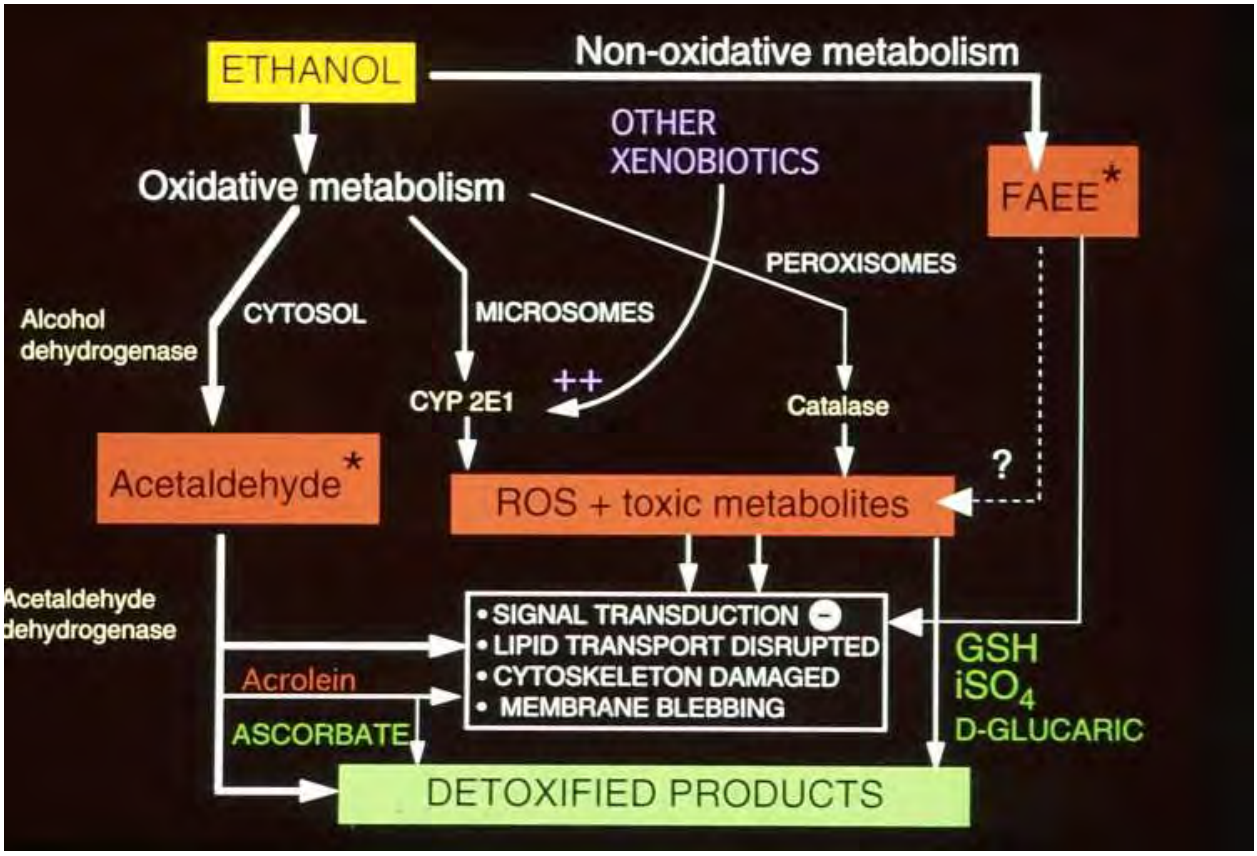

Figure 12.6 Schema for the metabolism of ethanol, asterisks indicating main routes. FAEE fatty acid ethyl esters, a transient product; CYP2E1 ethanol-inducible isoform of cytochrome P450; ROS reactive oxygen species; iSO_4 inorganic sulphate. From Pancreas[64] with permission of publisher.

micronutrient antioxidants; a urine sample was obtained for analysis of sulphate. The diagnosis of calcific chronic pancreatitis was made in the previous fortnight in 2 cases but up to 4 years earlier in the others. There were 13 men and a woman, of mean age 40 years (range 27-53) and average BMI 20 (range 15-27). Twelve patients had consumed 80-120 gm / day of alcohol for 1-24 years before the first symptom; the majority also smoked cigarettes, 2-20 daily, for 1-17 years; and most had been industrial workers until enforced retirement due to ill health. The PABA excretion index (Chapter 2) identified pancreatic secretory impairment in 10 patients, and 3 with secretory failure were already prescribed enzyme supplements but compliance was poor. Diabetes had developed in those 3 cases, and overt liver disease from secondary sclerosing cholangitis in another patient. Substantial liver dysfunction was shown by routine blood tests in 6 cases, a predominantly cholestatic picture and preserved serum albumin.

Among 15 asymptomatic hospital workers who served as controls, the male:female ratio was 13:2; mean age was 34 years (range 19-47); and average BMI 23 (19-31).Subsequent questioning showed that they drank on average 20 gm/day of ethanol except for one who had consumed >120 gm/day for 10 years. It also turned out that most smoked cigarettes, up to 10/day.

The results provided evidence in the patients of augmented oxidative stress (**Figure 12.7**) and poor micronutrient antioxidant status, involving every item measured[331, 332] (**Figure 12.8**). Perhaps more importantly, they disclosed the involvement of RXS, ie. electrophilic stress - by way of the lower ratio of inorganic to ester sulphate but higher ratio of D-glucaric acid to creatinine in

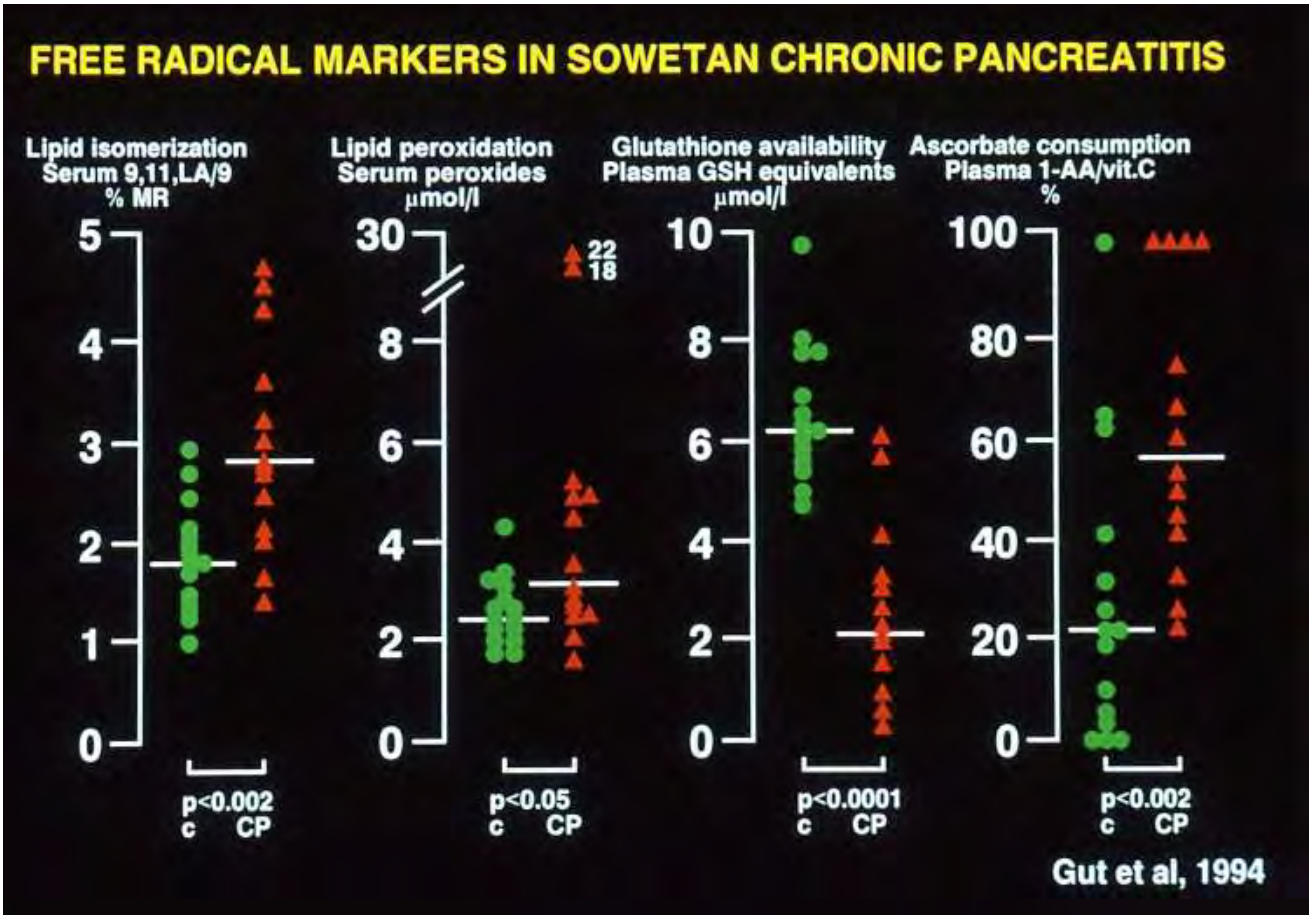

Figure 12.7 Individual datapoints on markers of excess oxidative stress in patients with chronic pancreatitis at Soweto. C controls; CP chronic pancreatitis; significance values from 2-tailed Mann-Whitney U tests. Reproduced from 1994 paper in Clin Chim Acta [331].

urine, and subnormal plasma GSH[333].

12.2.2 Tests for Bantu siderosis

In order to assess whether iron overload might be contributing to oxidative stress, serum samples were analysed for iron and copper-binding proteins[334]. The results were examined alongside data on ascorbic acid which, although generally a potent antioxidant, promotes FRA in states of iron overload (Chapter 15). The outcome is summarised in **Figure 12. 9**.

When compared to information from corresponding groups at Manchester, African samples had less ascorbic acid ($p<0.0001$), but more caeruloplasmin ($p<0.0001$) which normally reduces the toxicity of free iron: a significant inverse correlation was found between these variables in the African material[334].

African and British controls had similar values for serum iron, transferrin, iron binding capacity, % iron saturation and ferritin: the same was true in patients[334]. However, in 6 African patients the concentration of ferritin exceeded 300 µg/l (677 pmol/l) . The absence of low molecular mass iron or copper that can drive free radical reactions confirmed that excess ferritin did not reflect Bantu siderosis. Instead, by reference to a published nomogram[335] (**Figure 12.10**), it can be speculated that the finding represented subclinical liver disease or inflammation[335], or perhaps oxidative stress.

12.2.3 Genesis of alcoholic chronic pancreatitis

The key question was which of the many potential factors - social status, amount of alcohol, concurrent cigarette usage, micronutrient lack, oxidative / electrophilic stress, iron overload - was most relevant to the development of chronic pancreatitis at Soweto[64].

The idea was to investigate alcoholic controls, ie. patients admitted with acute alcoholic psychosis along lines used in patients with chronic pancreatitis - after ascertaining upon recovery that the latter diagnosis was excluded by the combination of normal ERCP, ultrasound scan and faecal chymotrypsin concentration. There was no prior information upon which to base

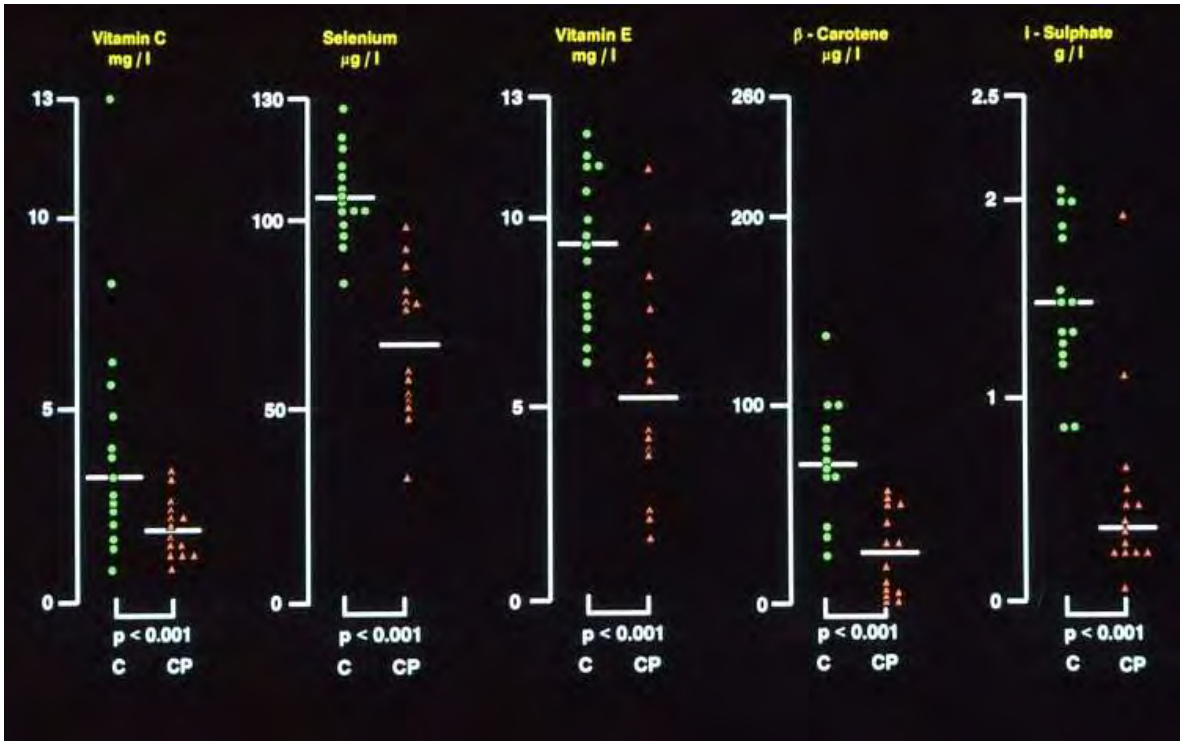

Figure 12.8 Individual datapoints on micronutrient status in controls (C) and patients with chronic pancreatitis (CP) at Soweto. Horizontal bars represent median values. Reproduced from 1995 paper in Clin Chim Acta [332] .

power calculation / study number. Hence the number was arbitrary, a third more than had revealed significant differences between Soweto controls and patients with chronic pancreatitis (see above).This was regarded as a cross-sectional study that would be controlled in the sense that the 3 groups - healthy controls, alcoholic controls, alcoholic chronic pancreatitis - were broadly homogeneous.

The new study involved 21 consecutive patients with acute psychosis against a background of heavy alcohol intake (>80 gm/day), provided that they gave informed consent upon recovery, with no prior symptom to suggest chronic pancreatitis and that pancreatic tests were normal. There were 18 men and 3 women, with characteristics very similar to the group of 14 with chronic pancreatitis described earlier (Section 12.2). Whereas both sets of patients came from a working-class background, the control set of 14 (now excluding the man who drank >120 gm/day of ethanol) were hospital clerks, nurses or porters.

A structured questionnaire was administered to ascertain alcohol consumption, cigarette usage, exposure to volatile xenobiotics, and intake of fresh fruit / vegetables - each aspect graded on a scale of 1 to 4, where 4 represents highest exposure to each xenobiotic or best diet. Alcoholic controls drank more alcohol than did patients with chronic pancreatitis (medians 105 and 76 gm/day, respectively, p=0.003), a mixture of home brews and Western-style spirits in both sets: the duration of consumption was known in the chronic pancreatitis set but not precisely in alcoholic controls. There was no difference in cigarette

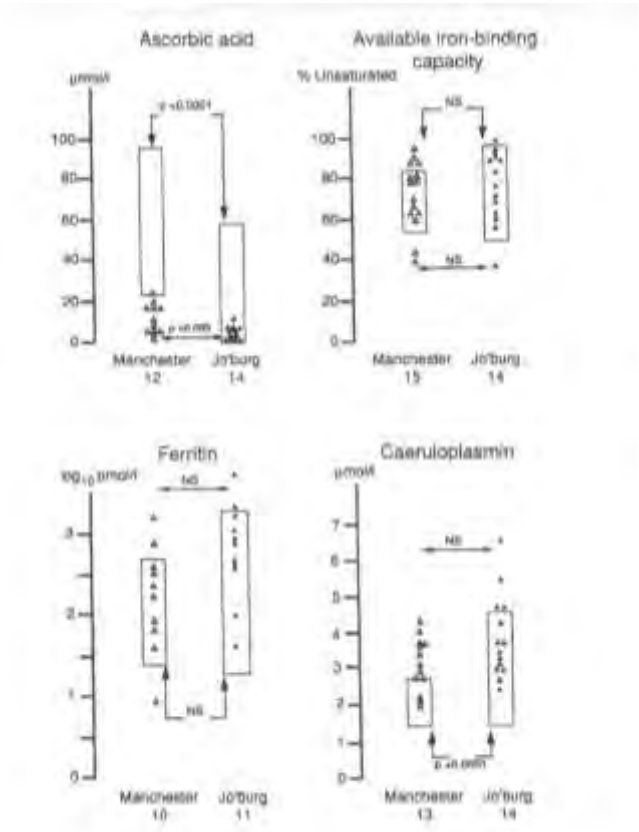

Figure 12.9 Salient findings in eliminating Bantu siderosis. Blocks represent control data; open triangles indicate patients with chronic pancreatitis at Manchseter, filled triangles patients at Soweto (Johannesburg); comparisons by Student's t test. From 1996 paper in Q J Med [334]

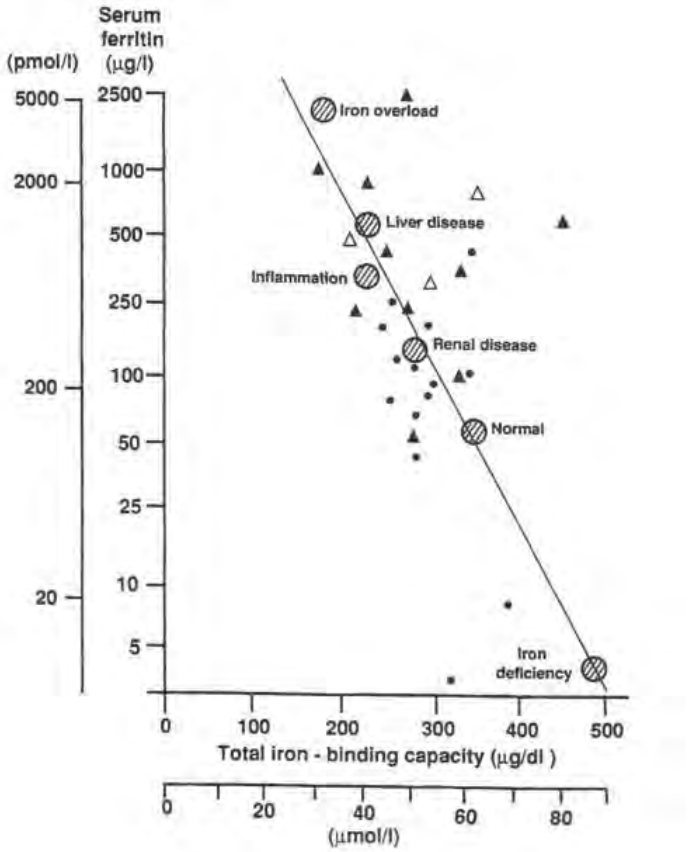

Figure 12.10 Diagram constructed from a published nomogram [335] using data from African controls (filled circles), African patients (filled triangles) and 3 Manchester patients with calcific chronic pancreatitis and serum ferritin >300 μg/l (open triangles). Publication details as for Figure 12.9.

usage between the 3 study groups; intake of fresh fruit / vegetables was equally poor in both alcoholic groups (median score =1) compared to healthy controls (median score =4); and there was significantly more exposure to occupational volatile chemicals in the chronic pancreatitis set than in alcoholic or healthy controls (median scores 4, 1 and 1 respectively). As to the last finding, petrochemicals, paints, solvents, dyes and glues were commonly mentioned: a woman who developed the disease within a year of drinking 30 gm/day of ethanol worked from 10 years earlier with furniture polish strippers and varnishes. Nine patients were also exposed to paraffin in cookers or lamps because, although electricity was available, paraffin appliances, candles, firewood, and coal were cheaper. This information was not ascertained from alcoholic controls.

Routine blood tests disclosed severe megaloblastic anaemia due to folate deficiency in a woman from the alcoholic psychosis set. Excluding this patient, group haemoglobin values in healthy and alcoholic control groups were similar and higher than in the chronic pancreatitis group. Mean corpuscular volume and fasting serum triglycerides were higher than control values in both alcoholic sets. Elevation in one or more enzymes in the serum liver function profile was noted in 11 of 21 alcoholic controls, but none had overt liver disease or increased serum bilirubin. In the chronic pancreatitis set, elevated γGT was a feature in 10 of 12 patients in whom it was measured. Across all groups, the enzyme correlated positively with bilirubin and more strongly with alkaline phosphatase.

Micronutrients that were measured in serum or plasma included selenium, vitamin C, vitamin E (absolute and relative to cholesterol), vitamin A and its precursor β-carotene, and zinc: urinary inorganic sulphate (absolute and relative to creatinine) informed on the intake of sulphur amino acids. Oxidative stress markers in serum or plasma included GSH, γGT, ascorbic acid (as % molar ratio of oxidised forms of vitamin C relative to the total, %MRVC), and %MRLA'. Transferrin, caeruloplasmin and ferritin gave information wherewith to implicate or exclude iron overload - while recognizing the antioxidant function of the latter 2 proteins. Urinary D-glucaric acid gave insight on a phase-2 pathway of xenobiotic metabolism (Chapter 5).

Detailed results are tabulated for each of the 3 study groups in the published report[64]. The following is a summary of the outcome. (i) Bantu siderosis was excluded by analysis of iron binding capacity. (ii) Both alcoholic sets displayed elevated levels of the metal-sequestering proteins, likely a non-specific response to increased FRA. (iii) Both alcoholic sets also had poor micronutrient status. Serum zinc level was especially low in alcoholic controls who were recovering from an acute episode of psychosis. (iv) The oxidative attack affected only vitamin C in alcoholic controls but extended to linoleic acid in patients with chronic pancreatitis. (v) The distinction between alcoholic groups was made by 4 measurements (**Figure 12.11**), which together pointed to electrophilic stress in chronic pancreatitis. (vi) A significant inverse correlation emerged between plasma GSH and γGT (**Figure 12.12**), suggesting that GSH lack underlies hepatic cholestasis - even taking into account the positive correlation of γGT with bilirubin and alkaline phosphatase. (vii) Moreover, the inverse correlation between caeruloplasmin and ascorbic acid was upheld.

The possibility that a deficiency of additional micronutrients that safeguard against increased circulating homocysteine might render Sowetan alcoholics susceptible to thrombosis led to an ancillary study using surplus serum samples. These were analysed for homocysteine, folate, vitamin B_{12} and vitamin B_6: the first 2 vitamins facilitate the re-synthesis of methionine from homocysteine, whereas the third vitamin is a co-factor for 2 enzymes that ensure passage of homocysteine down the transsulphuration pathway towards cysteine and thence GSH (**Figure 11.2**). Fifteen non-alcoholic hospital workers donated a blood sample in 2000 to enable interpretation of the results. The concentration of homocysteine was higher in alcoholic than healthy controls ($p<0.001$), accompanied by a lower level of vitamin B_{12} ($p<0.001$), normal vitamin B_6 and increased folate ($p=0.003$) - the last likely due to yeast used for home brews [64].

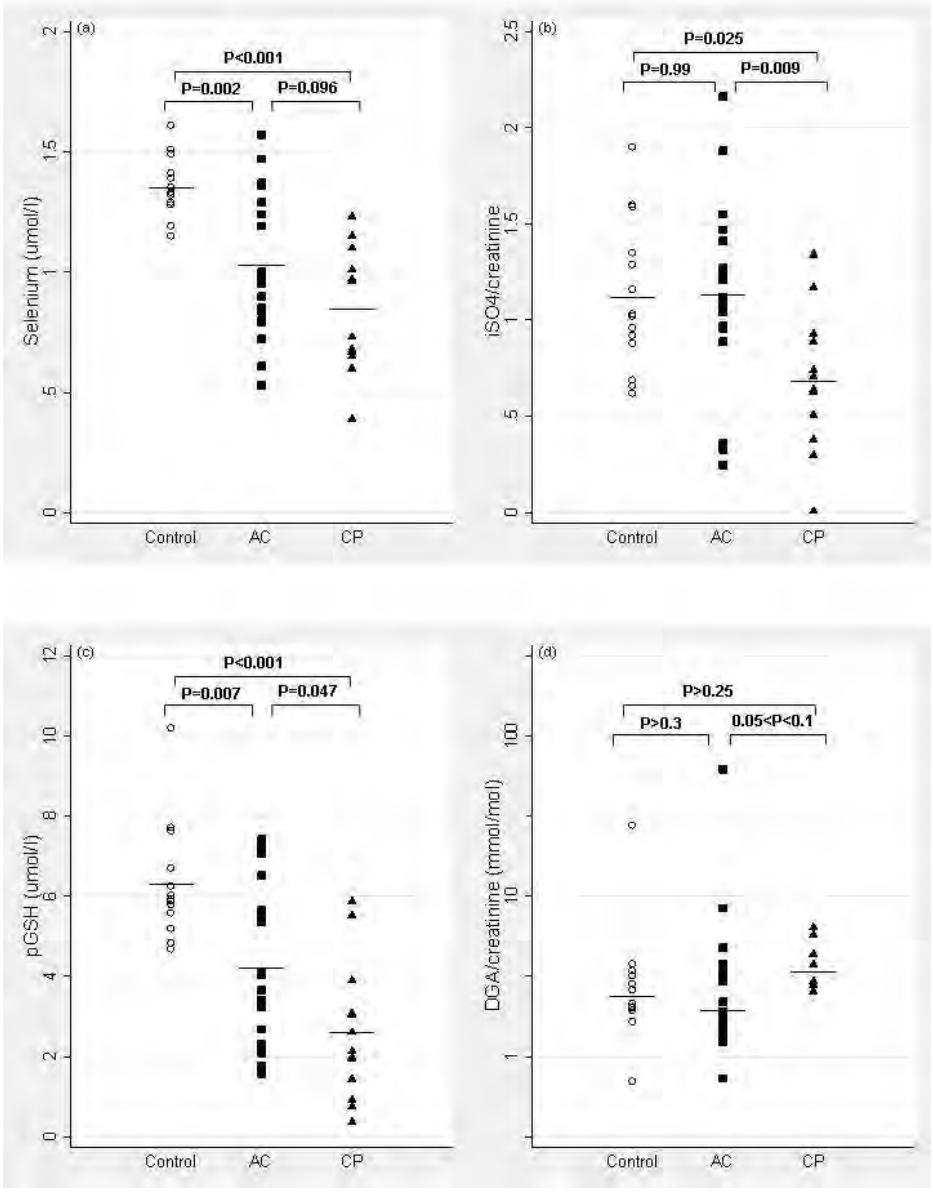

Figure 12.11 Distribution of data that facilitated the distinction between alcoholic controls (AC) and chronic pancreatitis (CP) sets. iSO_4 =inorganic sulphate; pGSH = plasma glutathione in immediately bioactive form; DGA =D-glucaric acid (plotted on log scale). A to C show data means and D shows medians: comparisons by appropriate tests. From 2011 report in Pancreas[64] with permission.

12.2.4 Comments

The first run of investigations in 1993 identified excess oxidative stress in patients with 'alcoholic' chronic pancreatitis, excluded iron overload as the explanation, showed low micronutrient antioxidant status, and hinted at the importance of RXS (ie.electrophilic stress) in disease pathogenesis. The role of CYP induction could not be gauged because the use of chlorzoxazone as a probe of CYP2E1 induction by alcohol had not yet been recognised[158]. CYP1A2 induction was detected by theophylline test in only 2 of the 14 cases examined, but as was noted in the first documentation of pancreatic CYP induction in man[227], alcohol is known to increase the yield of RXS via this isoenzyme too.

The later study involving alcoholic controls had as its main strength the homogeneity of participants: the combination of similar ethnicity, near-identical living conditions and stereotyped diet is seldom achieved outside the laboratory. Despite the

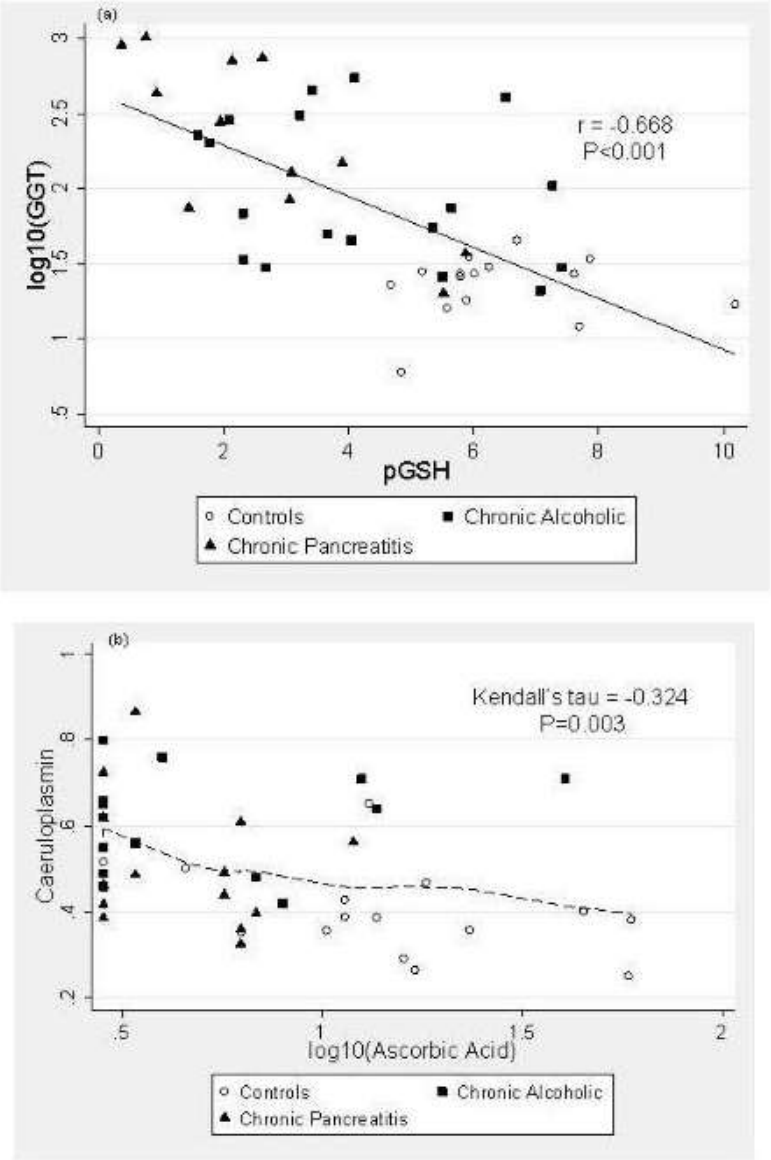

Figure 12.12 Relationships between plasma glutathione concentration (pGSH) and γ-glutamyl transpeptidase activity (GGT); or plasma ascorbic acid and caeruloplasmin concentrations in Soweto studies. Publication details as in Figure 12.11.

middle-class status with higher income and education of healthy controls, their cigarette usage was the same as by their compatriots with alcoholic psychosis or chronic pancreatitis. Moreover, maize porridge was still the dietary staple, as was evidenced by similar values as in alcoholic controls of vitamin E, vitamin A and sulphur amino acids - the last as gleaned by data on urinary inorganic sulphate.

The 'drink till drop' culture of working class Sowetans made it impossible to be accurate on alcohol intake, and it could not be determined whether or not any of the alcoholic controls progressed to chronic pancreatitis because Sowetans are notoriously reluctant to attend follow-up clinics, the more so when the taboo diagnosis is 'alcoholic psychosis'(I Segal, personal communication).

There are no comparable studies in alcoholics without chronic pancreatitis. Yet, each of the main outcomes resonates with published information. (i) Recent data from Italy and the USA, cited in a recent review[1], show alcohol to be the major cause in only 34% and 44% of cases, respectively. (ii) The involvement of volatile petrochemicals in causation, suggested by xenobiotic exposure scores, is echoed in an independent study from Soweto which noted that regular exposure to such fumes emanates not only in the workplace but also in the home from paraffin lamps and heaters[336]. (iii) The inverse correlation between plasma GSH and γGT is consistent with what is known about GSH metabolism: the enzyme located on the plasma membrane of hepatocytes is the key to import from the plasma GSH pool of constituent amino acids for GSH re-synthesis[201]. (iv) The inverse correlation between caeruloplasmin and ascorbic acid, which is normal in premature babies, suggests that sustained lack of ascorbic acid causes compensatory mobilisation of the iron-sequestering / oxidising protein in adults too. (v) Finally, elevated blood homocysteine in alcoholic controls at Soweto - as also shown in patients with chronic pancreatitis from south India[337] and north west England[338] but not Holland[339] - suggests the need for micronutrient supplements to protect against thrombosis. This point is re-addressed in Chapter 18.

12.3 Overview and summary

In the period 1988-1993 the etiological mix and phenotype were dissimilar among patients with chronic pancreatitis in north west England (Manchester), South Africa (Soweto), and southern India (Chennai). Thus, the alcoholic: idiopathic ratio was roughly 50:50 in Manchester, 95:5 in Soweto and 5:95 in Chennai. Moreover, the mode of presentation and disease phenotype were very different. At Manchester this was generally as a pancreatitis attack or unremitting pain in healthy-looking individuals in whom pancreatic calculi, or diabetes at presentation was rare, as also a family history of the disease. At Soweto an attack of pancreatitis was the frequent harbinger, but in clearly malnourished patients in whom pancreatic calculi but not diabetes was frequently noted at first assessment, and there was no family history of the disease. At Chennai the classical features of end-stage disease - pancreatic calculi, diabetes, exocrine pancreatic failure - were usually evident at presentation which was often precipitated by symptoms of diabetes, and the involvement of more than one family member was not infrequent.

Despite this incongruity, the same triad of factors could be identified in each cohort:
(i) regular exposure to a CYP inducer (eg. smoke, C18:2 oil, ethanol);
(ii) concurrent exposure to a volatile toxicant that yields RXS (eg. petrochemicals);
(iii) poor status of micronutrient antioxidants (eg. ascorbate).

Together these factors promote electrophilic stress, as is identified in the pancreas and liver of patients with chronic pancreatitis (**Table 9.2**). Thus, each puff of cigarette smoke delivers 10^{15}

free radicals in the gas phase (eg. nitrogen oxides) and 10^{14} in the tar phase (eg. from bezopyrene). The fractionation of petroleum yields the following products: natural gas (C_{1-2}) used for fuel and in the chemical industry; liquefied or bottled gas(C_{3-4}) used as fuel, or for synthesis of rubber compounds, or in the petrochemical industry; petroleum ether (C_{4-5}), for solvents or to anaesthetise small animals; gasolines (C_{6-10}), in cleaning fluids, solvents and for refining stock; kerosenes (C_{5-16}), used as jet, tractor and gas turbo-fuels as well as in lamps and stoves in under developed countries; gas oil (C9-16), used as diesel and furnace oil; lubricating stocks ($C_{>17}$), for white oils, lubricating oils and greases; waxes ($C_{>20}$), for celing wax; and bottoms ($C_{>20}$), used as heavy fuel oil, road oil and asphalt[222].

Experimental studies show that the pancreas is highly susceptible to damage from a huge range of chemicals[222], but the field of inhalation toxicology to the gland has been lacking until relatively recent work on cigarette smoke[1]. Some time ago, it was shown that exposure of rats to motorcycle exhaust and organic extracts thereof caused a dose and time-dependent increase in CYP and GST content of liver, kidney and lung microsomes[340]. More recent studies from Nigeria indicate inhalation injury to the rat liver from kerosene and petrol fumes[341]: There is currently a flurry of interest in the health hazards to humans of petrochemical fumes. For example, a study from Nigeria on attendants at refuelling stations showed electrophilic stress, in that blood malondialdehyde was significantly elevated and GSH depressed[342].

In contrast to the dearth of information on the metabolism of petrochemicals by the pancreas, a great deal is known about its ability to metabolise ethanol (**Figure 12.6**). In vitro studies show how easily acinar cells are injured by each metabolite, ie. acetaldehyde, fatty acid ethyl esters and ROS. Yet, long-term feeding with ethanol alone does not induce the disease experimentally; less than 10% of alcoholics fall prey; and among those who do, the interval to first symptom is around 15 years. This paradox suggests that in vivo the gland is well protected against injury from ethanol, as also that other factors increase its toxicity. Cigarette usage is a major co-factor; occupational volatile chemicals are identified as an independent threat (Chapter 8); whereas endotoxaemia and CCK hyperstimulation have been speculatively implicated. Of note too, links between gene mutations and alcoholic disease are modest relative to connections with idiopathic disease (Chapter 13).

Clinicians are familiar with the concept that prior CYP induction as by ethanol increases paracetamol or CCl_4 hepatotoxicity. However, it is not commonly known that the enzyme involved, CYP2E1 - and / or other CYP isoenzymes that generate RXS - are induced in pancreatic acinar cells of patients with chronic pancreatitis but without a parallel increase in GST and antioxidant enzymes [255]; or that a small dose of ethanol increases the yield of RXS from many xenobiotics [170,171]; or that chronic exposure to ethanol results in its preferential processing via CYP with increase in ROS generation[108].

Against this background, data from Soweto are illuminating. The progressive lowering in plasma GSH level from healthy individuals to alcoholic controls to patients with alcoholic chronic pancreatitis - accompanied by stepwise increments in γGT activity - is compatible with pressure from ROS in alcoholic controls, compounded by RXS from inhaled petrochemicals in chronic pancreatitis (**Figure 12.11**). A similar pattern in serum selenium values is consistent with this interpretation, because repetitive exposure to CYP substrates seems to divert selenium from blood into tissues[300]. Moreover RXS draw on inorganic sulphate for their removal as esters, and they mobilise the glucuronic acid route that generates D-glucaric acid for detoxification (Chapter 5). It might hence be argued that the adjective ‘alcoholic’ is a misnomer which diverts attention away from the shared path

to pancreatic damage by RXS in patients with chronic pancreatitis, irrespective of geography.

Table 12.1 summarises micronutrient profiles in the study participants from Manchester, Soweto and Chennai. It is probably wrong to regard Manchester controls as the ideal norm, but needs be as there is no comparable data from elsewhere.

Table 12.1 Micronutrient profiles by geography

		Manchester		Soweto		Chenai	
		C	CP	C	CP	C	CP
Selenium	µmol/l	1.5	1.2 *	1.3 ↓	0.8 *	1.5	1.3 *
	(µg/l)	(117)	(95)	(105)	(67)	(117)	(99)
Vitamin C	µmol/l	74	40 *	23 ↓	11 *	63	27 *
	(mg/l)	(13)	(7.0)	(4.0)	(1.9)	(11)	(4.8)
Ascorbic acid	(µmol/l)	62	31 *	21 ↓	5.5 *	16 ↓	25
	(mg/l)	(11)	(5.5)	(3.7)	(0.9)	(2.8)	(4.4)
β-carotene	(nmol/l)	205	67 *	156	47 *	86 ↓	56
	(µg/l)	(110)	(36)	(84)	(25)	(46)	(30)
α-tocopherol	(mmol/mol cholesterol)	4.8	3.8 *	5.2	3.0 *	4.7	2.8 *
i-sulphate	(mol/mol creatinine)	0.9	1.0	1.1	0.7 *	1.5 ↑	1.7

C=controls, CP=chronic pancreatitis sets. Data in SI and (metric) units for mean values upon analysis of serum (selenium, β-carotene, α-tocopherol), plasma (vitamin C and its bioactive form of ascorbic acid), or urine (inorganic sulphate). Asterisks indicate a significant decrease relative to local controls. Downward or upward arrows show values significantly less or greater than in Manchester controls. Relevant publications are given in the text. Conversion factors for SI-metric units are given after the glossary on page v.

Chapter 13

Accommodating Gene Mutations

Today there is no doubt that a variety of gene mutation(s) increases the risk of chronic pancreatitis (**Table 13.1**). The key question is this: how might these different mutations result in one and the same disease? The following section is a broad-brush interpretation of current opinion, glossing over the myriad mutation subtypes[7,312,343,344].

13.1 Received wisdom

- Many mutations can, in theory, generate unbridled trypsin in the gland - hence seemingly pointing to autodigestion as the common denominator.
- The PRSS1 mutation causes classical hereditary pancreatitis (HP) via an autosomal dominant mode of inheritance. Other gene mutations are disease modifiers but may combine in complex genotypes - not uncommonly with environmental overtones - that might underlie familial disease.
- Biallelic pathogenic variants in SPINK1 or CFTR can result in hereditary pancreatitis via an autosomal recessive mode of inheritance.
- Biallelic severe mutations in CFTR cause cystic fibrosis, whereas mutation in both bicarbonate-specific alleles or in one such allele plus a non-specific severe mutation in the other allele results in chronic pancreatitis without lung involvement.
- SPINK1 mutation is particularly common in 'tropical' disease as seen in India.
- Alcoholic chronic pancreatitis in males is strongly linked to mutation in CLDN2 which is located on the X chromosome.
- ER stress, independent of trypsin activation, is induced by CPA1 mutation, and sometimes also by mutation in CTRC

Table 13.1 Chronic pancreatitis-associated genes

Gene	Encoded protein	Mutation	Pathogenecity
PRSS1 (protease, serine 1)	cationic trypsinogen	gain-of-function	trypsin is prematurely activated and/or resists degradation and/or reduces secretion and/or causes protein misfolding & ER stress
PRSS2 (protease, serine 2)	anionic trypsinogen	loss-of function	loss of normally protective action in mitigating trypsin activity
SPINK1 (serine protease inhibitor Kazal-type 1)	pancreatic secretory trypsin inhibitor / acute phase protein	loss-of-function	disease modifier ? altered interaction with trypsin
CTRC	chymotrysinogen C	loss-of-function	loss of normal 2nd-line defence to trypsin; ?damaging when also mutated *SPINK1/CFTR*; might induce ER stress
CASR	calcium sensing receptor	loss-of- function gain-of-function	injurious if combined with *CFTR* mutation; Ca^{++} toxicity when combined with alcohol
CTSB	cathepsin B	affects pro-peptide region	speculative; ? modifier by altering trafficking and/or altering capacity to degrade PSTI
CFTR	cystic fibrosis transmembrane conductance regulator	loss-of-function	impaired HCO_3^- secretion fosters intraductal trypsin by autoactivation
CLDN2 *	claudin 2	atypical localization	high risk variant impairs H_2O and Na^+ secretion encouraging intraductal trypsinogen auto-activation; ? other non protease-dependant
CPA1	carboxypeptidase 1	misfolding	ER stress

Abbreviations:ER = endoplasmic reticulum; Ca^{++} ionic calcium. * see text for discussion.

or PRSS1 .

- In children with pancreatitis, PRSS1 and/or SPINK1 mutations are particularly associated with chronic disease and CFTR mutation with relapsing acute disease[345].

Notwithstanding these developments and despite data dredging by north American and European consortia, the alcohol paradox remains unexplained. Current advice is that mutation at a particular locus involving PRSS1 -PRSS2 (rs10273639) or CLDN-RIPPLY1-MORC4 (rs12688220) decreases disease risk, whereas mutation in Ripply transcriptional repressor of CLDN2 is highly represented in alcohol-related disorders, especially in males. The outcome of studies on genes connected with alcohol metabolism has not been conclusive. All that can be said is based on oriental studies which suggest that the presence of even a single mitochondrial ALDH2*2 allele - an enzymatically inert nearly-dominant variant of the gene whose product is designed to remove acetaldehyde - is protective against alcohol dependence. More recent data reveal that polymorphisms in genes encoding fucosyltransferase 2 non-secretor status and blood group B inexplicably double the risk of alcoholic chronic pancreatitis[344], bringing to mind a Manchester study from 1988 in which an increased frequency was found of HLA-B21 in 52 patients with alcoholic disease versus 344 controls (p=0.002), but no difference in ABO secretor status[346].

13.2 Against a central role for trypsin in HP

As observed previously[13, 255] (Chapter 11), the potent inhibitory effect of thiols (essentially GSH in biology) on trypsin and other proteases by way of SH-SS exchange is not acknowledged by proponents of the theory that mutation(s) in trypsin-control genes is synonymous with pancreatic autodigestion. A landmark study from Manchester, masterminded by FS Steven (**Figure 13.1**) showed that thiol-inhibited trypsin is easily re-activated by a variety of oxidants such as sodium periodate, mercury compounds or cystine (oxidised form of cysteine); but also that high concentrations of the oxidant resulted in irreversible cleavage of the significant S-S bond with loss of trypsin activity[302]. GSH that is diverted for trypsin control from an early age in kindreds with HP would compromise its availability for other vital functions eg. control of oxidative stress, and protection of enzymes that ensure the delivery via the methionine-metabolic pathway of CH_3 and SH moieties which are essential for apical exocytosis.

In keeping with this philosophy, oxidative stress in HP kindreds has been noted in studies from the USA[347] and France[,348]. Both found elevated SOD in erythrocytes of patients as well as unaffected family members. Erythrocyte GSH-Px was depressed to the same extent in each subset in the first study, but in the second study unaffected members had increased levels, such that only in patients was the SOD: GSH-Px ratio elevated, indicating bruising oxidative stress. Patients had low selenium and in the USA study also low vitamin E but elevated GST. Thus, as could be anticipated, micronutrient therapy ameliorated

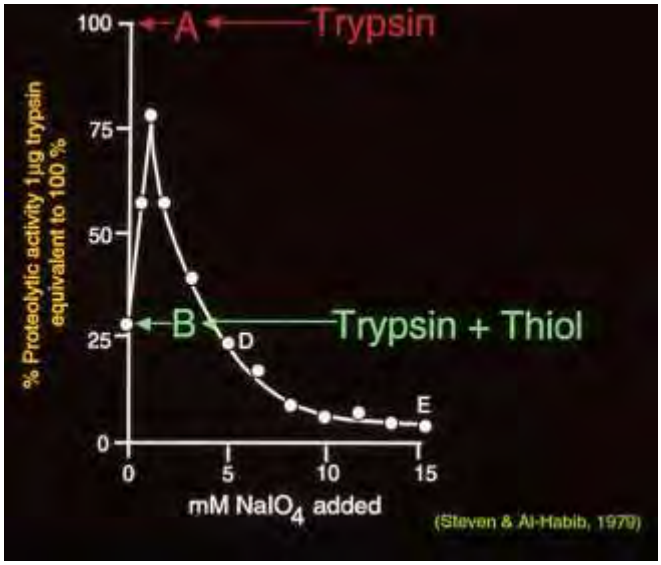

Figure 13.1 Redrawn graph with authors' permission[302] for a 1998 review in Digestion[13] and reproduced with permission of the publisher, S Karger AG, Basel. The results show inhibition of trypsin by dithiothreitol followed by its biphasic reactivation (line B) but subsequent loss (curve DE) upon incremental additions of a potent oxidant, sodium periodate ($NaIO_4$).

symptoms in 3 children with HP[349] (Chapter 16).

Older arguments against a pathogenic role for intra-acinar trypsin in patients with pancreatitis have already been cited[5, 256, 266]. More recent arguments are crystallised in editorials[6, 305], which now expound that intra-acinar trypsin is neither a prerequisite for chronic pancreatitis nor involved in pancreatic inflammation (Chapter 11).

As to the latest discovery in relation to the strong association between a particular mutation in *CLDN2* and alcoholic chronic pancreatitis in men, it is inconceivable that 21 consecutive alcoholic controls in Soweto escaped the mutation: instead disease development in 14 compatriots was associated with regular exposure to petrochemical fumes[64, 336]. Moreover, the intraglandular autodigestion concept and linked SAPE hypothesis for the development of chronic pancreatitis does not rationalise the findings in **Table 9.2**, which indicate induction of drug metabolising enzymes and electrophilic stress. Increased frequencies of gene mutations that influence xenobiotic metabolism are in line with this thinking, eg. of *GSTT-1*A* which tends to yield RXS[350], and of the *PON1-192Q* allele of paraoxanase which compromises its xenobiotic detoxification capability[351].

The expectation that recurrent episodes of acute pancreatitis will lead to chronic pancreatitis, based on the SAPE notion (Chapter 11), is undermined by observations in patients with FLLD (Chapter 15). Recurrent attacks in the setting of hereditary or alcoholic disease would be expected to cause chronic pancreatitis eventually, by way of steady CH_3 / SH loss with continual redirection of FROP into the interstitium and activation of pro-fibrotic pathways (Chapter 19).

13.3 On *CFTR* mutation

The pancreatic lesion in classical cystic fibrosis (CF) is a diffuse form of chronic pancreatitis that begins *in utero* when it may be accompanied by haemorrhages, and is invariably identified by hypertrypsinogenemia in neonates - the enzyme level falling exponentially in line with loss of acini until all are eradicated within the first decade. This pattern suggests a permanent blockade to apical exocytosis in acinar cells in CF, whereas that is an isolated event in acute pancreatitis as after ERCP, but returns sporadically in RAP and with greater frequency and duration in chronic pancreatitis (**Figure 13.2**)[295].

Moreover, considering the role of oxidants in the acquired forms of pancreatitis, it was hypothesised in 1996 that CFTR is a free radical target, whether at the luminal pole of pancreatic acinar or ductal cells, or in other organs[37]. As to the acinar cell, this interpretation requires the presence of CFTR at the apical pole; evidence that *CFTR* mutation hinders exocytosis; and that the CFTR protein is vulnerable to oxidative / electrophilic stress. The first prerequisite is satisfied by a largely ignored study[352].

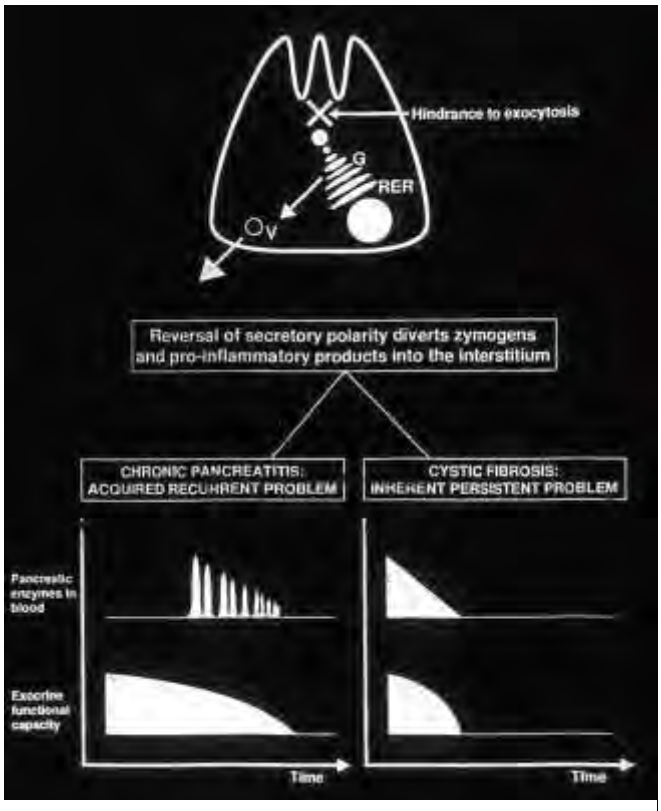

Figure 13.2 Schema that rationalises the overlapping pathophysiology of cystic fibrosis and chronic pancreatitis.

Evidence for the involvement of CFTR in exocytosis has come from Manchester investigations that were mounted along standard lines that are used to examine bacterial resistance to antibiotics - prompted by the finding of lower blood concentration and / or increased clearance of several antibiotics in patients with CF[353, 354]. LB Quesnel was the expert here. Nasal epithelial cells were studied because this tissue is a target organ in the disease. The material was from polyps of CF patients with the severe biallelic Δ508 mutation and polyps from non-CF controls. Cells were examined for uptake, accumulation, and exocytosis of cloxacillin or gentamycin. The main outcomes were as follows.

(i) Two antibiotics accumulated excessively in CF cells (**Figure 13.3**). (ii) This was not due to enhanced antibiotic uptake, which was a slow process, not energy-driven: instead it was due to significantly reduced exocytosis of antibiotic as shown by confocal microscopic analysis using a fluorescent dye with or without gentamycin tagging (**Figure 13.4**). (iii) The mutated CFTR protein was not subject to normal regulation by activated protein kinase A or ATP. (iv) CFTR dysfunction could be linked to a breakdown in membrane trafficking. (iv) CF cells had high levels of ATP and energy charge, in keeping with a study of erythrocytes[355].

Mutation in the related multidrug resistance gene (MDR1) differs in that the phenotype reflects interference in the ability of this P-glycoprotein to pump drugs out of cells, for which function ATP hydrolysis is indispensable; whereas binding of the nucleotide without ATP hydrolysis is sufficient for CFTR function. Of note, the distinction between exocytosis and basolateral discharge is difficult to make by using secretagogues on acinar suspensions[356] - as opposed to confluent /

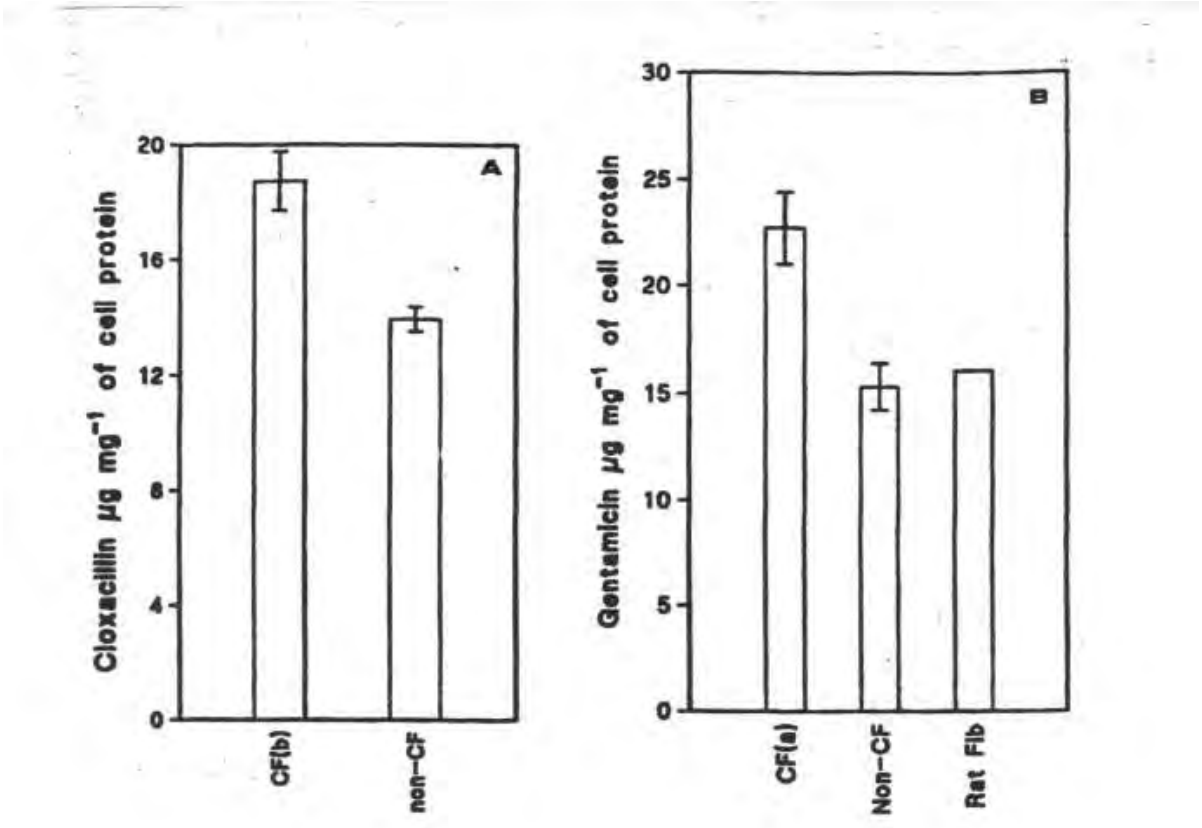

Figure 13.3 Greater accumulation in cystic fibrosis (CF) than non-cystic fibrosis (non-CF) cells over a 95-hour period of exposure to (A) cloxacillin (26% increase, $p<0.01$) and (B) gentamicin (33%, $p<0.01$). Studies in 6 different flask cultures of CF cells and 3 of non-CF cells. From 1993 paper in Med Sci Res[353] (Elsevier).

semi-confluent cells that retain their secretory polarity - because basolateral channels are sensitive to regulation too[271].

The findings of hypertrysinogenemia in neonate CF carriers, and of the increased severity of hyperstimulation pancreatitis in CFTR heterozygous mice (but with redundant channels for chloride transport), underline the need of a full complement of CFTR protein for apical exocytosis[255]. They suggest, furthermore, that the CFTR deficit is exposed by oxidative strain in that neonatal antioxidant systems are precarious and, as noted previously (Chapter 11), experimental acute pancreatitis is detonated by a burst of FRA.

As to chronic pancreatitis, a 1998 report on 134 patients from Manchester revealed an increased frequency of CFTR mutations compared to controls[357]. Studies from elsewhere have confirmed and extended these findings[326, 358]. It is hardly surprising that the 90% fall in CFTR protein in compound heterozygotes with idiopathic chronic pancreatitis results in abnormal nasal potential difference and sweat test[255, 357]. The key point is that in these sites, high levels of CYP expression persist into adult life[359], such that an increase in toxic electrophiles would impair CFTR function in the absence of CFTR mutations. This view rationalises abnormal sweat tests in the following disparate groups: African patients with alcoholic chronic pancreatitis; Indian patients with trisomy 21 (wherein an extra copy of SOD increases the yield of ROS); patients with kwashiorkor-marasmus who have an absolute lack of defence to ROS and RXS. Germane to these arguments, both abnormal sweat test and elevated serum trypsinogen have been documented in malnourished Canadian children. Additionally of note, nasal potential difference studies indicate CFTR hypofunction in patients with RAP, as is associated with pancreas divisum, in keeping with oxidative strain. Citations for all these studies were given in an earlier review[255].

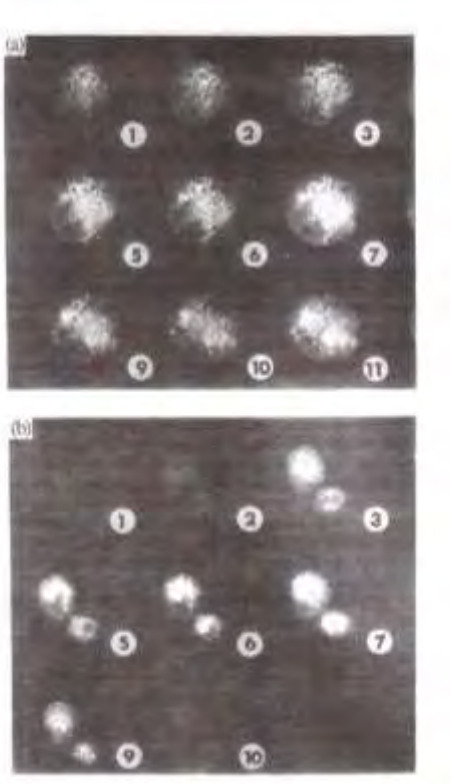

Figure 13.4 Confocal microscopic optical sections (1.5µm) of single cells with accumulated fluorescent-tagged gentamicin sulphate (10 mg/l/48 hr): (a) non-cystic fibrosis and (b) cystic fibrosis cells at equivalent illumination and magnification. From 1998 paper in J Antimicrob Chemother [354]

The final piece of the jigsaw in chronic pancreatitis pathogenesis, ie. intraductal calcifying precipitates in patients with large duct disease, is provided by evidence that CFTR in the luminal membrane of centro-acinar and proximal ductal cells is a channel not only for Cl^-, but also for HCO_3^- and the potent antioxidants GSH[360] and thiocyanate[361]. A reduced quota of CFTR in these cells - whether due to pancreas-selective mutations in CFTR[343], or oxidative / electrophilic stress by whatever route - would result in less HCO_3^- and antioxidants in pancreatic juice at a time when (between attacks) it contains increased amounts of protein, mucus and lactoferrin. Ways in which HCO_3^- lack might promote lithogenicity are now understood[362], as also the key role of GSH in lysing disulphide bonds in mucus[360].

The suggested role of GSH lack in lithogenesis is supported by unpublished preliminary work from Manchester, which showed that its concentrations in pancreatic juice (obtained by endoscopic cannulation in the first 10 minutes after an intravenous injection of Boots secretin) were 1.11, 1.67, 2.02 and 3.02 µmol/l in 4 healthy controls, but 0.029 µmol/l in a patient with idiopathic calcific chronic pancreatitis, increasing to 2.5 µmol/l after 8 months on micronutrient therapy. Moreover, in regard to cytoplasmic mislocalization of CFTR observed in alcoholic, idiopathic and autoimmune pancreatitis[362], the ability of the antioxidant curcumin to rescue DF508-CFTR localization in cell lines suggests that oxidants are responsible for the phenomenon [363].

Over-and-above these pathogenicity pathways, are jeopardised endocytosis of shed granule membranes and impaired solubilisation of secreted (pro)enzymes upon experimental acidification of acinar and ductal lumina - leading to the histological picture of both cystic fibrosis and large-duct chronic pancreatitis[364]. Indeed, the importance of HCO_3^- lack is underlined by a study of mice with disrupted cilia function[365].

A number of papers now confirm the Manchester hypothesis of 1996 that CFTR is a free radical target[37]**:** citations until 2010 were covered in a previous review[255]. (i) Sublethal oxidant stress by exposure of cells to tert-butylhydroquinin suppresses CFTR expression despite increased GSH synthesis; while stress from pyocyanin, as is released by certain bacterial pathogens, impairs Cl^- transport. (ii) Not only do in vitro studies show this to be true for oxidants in cigarette smoke - including metals[366] - but also nasal potential difference data indicate compromised CFTR function in vivo. Moreover, cigarette smoke exposure induces CFTR internalisation and insolubility[367]. (iii) Alcohol disrupts the expression, function and localisation of CFTR [368], while free fatty acids impair CFTR function[368].

For all these reasons 'a new horizon' has very recently been proclaimed for the pathogenesis and potential treatment of chronic pancreatitis, revolving around CFTR insufficiency[368]. In fact, CFTR dysfunction was identified some time ago by Manchester workers as the likely factor in the overlap between aspects of cystic fibrosis and chronic pancreatitis, as also its role in precipitating a pancreatitis episode should its complement in the apical membrane of acinar cells be immobilised by a burst of FRA[11, 37, 255, 295]. To reiterate, electrophilic stress is a unifying mechanism for CFTR malfunction in the context of chronic pancreatitis: moreover, CFTR can be protected by ascorbic acid or thiols[255].

The Manchester group was among the first to identify oxidative stress in patients with CF, initially in adults by analysing nasal epithelial cells (**Figure 13.5**)[369], and later in children using a validated gas chromatography method[370] to measure pentane in expired air[371]. Furthermore,

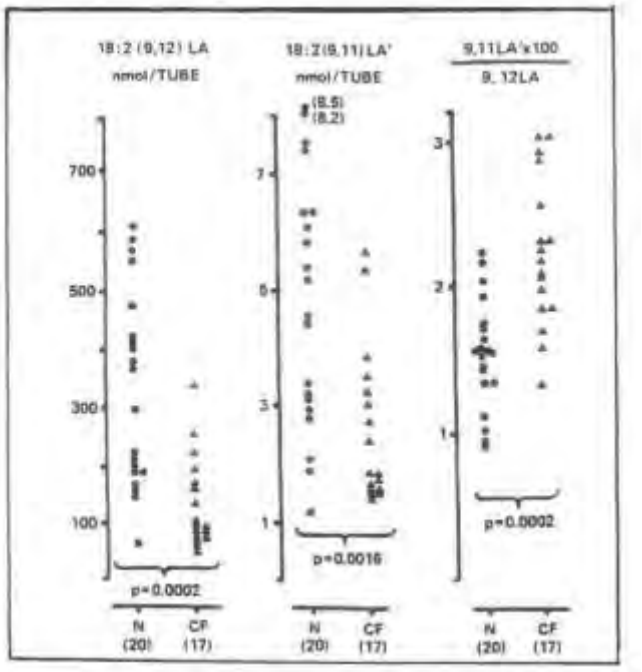

Figure 13.5 Analysis of nasal epithelial cells from normal individuals (N) and patients with cystic fibrosis (CF) shows a higher percentage of the 9 cis 11 trans isomer of linoleic acid relative to the parent fatty acid (18:2, 9 cis 12 cis) in the latter set despite subnormal absolute values of each. Reproduced from 1989 paper in Clin Chim Acta[369].

from analysis of serum samples in adults, it emerged that low selenium levels in the patients was associated with increased %MRLA' [369] - as was later found too in chronic pancreatitis (Chapter16). Today oxidative stress and inflammation are seen as integral features of the disease, driven by unfolded CFTR via ER stress-UPR [372, 373].

13.4 Miscellaneous

Hypertriglyceridaemia and hyperparathyroidism are associated with pancreatitis. In a study from Taiwan, CFTR mutation rate was 26% in a group with the former condition and pancreatitis compared to 1.3% in a group without pancreatitis. As to the latter problem, a study of 826 patients showed that only the subset with a history of pancreatitis had a mutation in SPINK1 or CFTR or both genes. RAP as associated with pancreas divisum showed a similar pattern. This information has been reviewed[255].

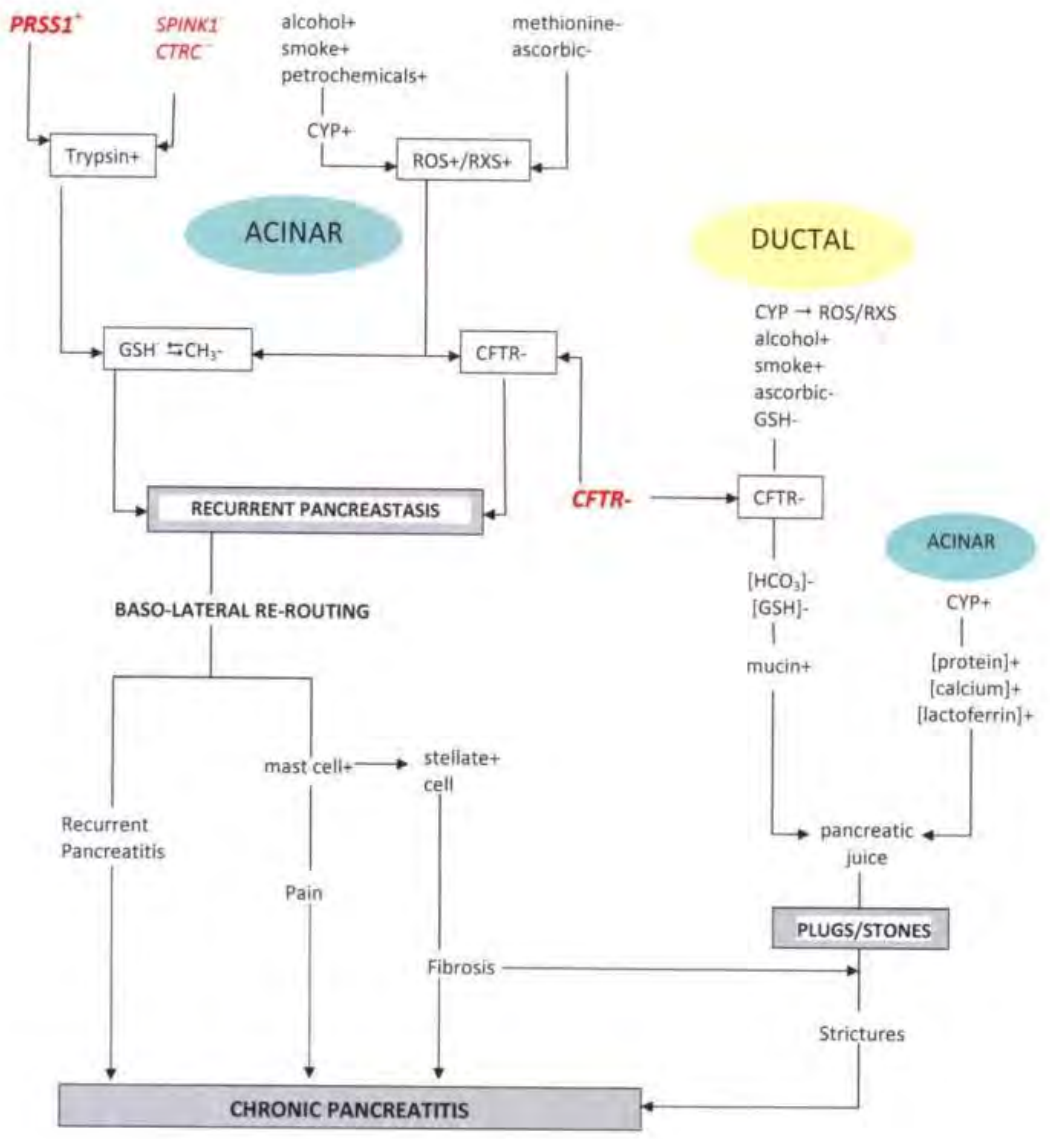

Figure 13.6 Schema for the pathogenesis of chronic pancreatitis to accommodate genetic and environmental agents. Gene mutations in red italics; plus or minus symbols indicate increase or decrease, respectively; items in square brackets signify concentration. Simplified from Figure in 2010 review in JOP [255].

13.5 Tweak to template

Figure 13.6 is a construct based on the aforesaid deliberations until 2010[255], which has been adopted with minimal modification by others who, however, have not considered oxidant attack on CFTR[374]. The construct makes it possible to see that when environmental and genetic factors combine to cause CH_3 / SH / ascorbate lack, as in tropical chronic pancreatitis, the disease begins at an early age, runs an accelerated course and might be familial. Thus, recent work identified a genetic predisposition, mainly via mutation in SPINK1, in 50% of Indian children with chronic or RAP, and 33% with acute pancreatitis[375]: an earlier report described the mutation in every member of the Indian family shown in **Figure 12.1**, and also in unaffected first-degree relatives of both parents[376]. Permutations and combinations among CYP induction, dietary antioxidant insufficiency, trypsin-favouring mutations, and acinar with or without ductal CFTR involvement would determine outcome - whether large or small-duct chronic pancreatitis, or RAP.

13.6 Conclusion

Despite the salutary lesson that there is no specific treatment to avert the course of CF although the defective gene was discovered a decade earlier than that for HP, pancreatologists pin their hopes for treatment of chronic pancreatitis on advances in that field[368]. In so doing we miss the fundamental point, ie. that CFTR dysfunction in chronic pancreatitis is easily brought about by electrophilic / oxidative stress, and that the impact of HP mutations is best explained by depletion in GSH. It would be a step too far to say that genetic testing is irrelevant, given the increased risk of cancer in the HP setting, but having identified a potentially predisposing mutation, the question today is what to do about it.

Chapter 14

Towards an Animal Model Based on CYP Induction

There is no animal model that replicates the full spectrum of disturbances in human chronic pancreatitis: biphasic pattern of pancreatic protein secretion; lithogenicity; hypersecretion of bile laden with FROP; CYP induction; hepatisation of the pancreas; mobilisation of natural antioxidants, and so on (Chapters 5, 9, 10). These aberrations are rationalised by electrophilic stress as a result of regular exposure to several xenobiotics simultaneously - alongside the nullifying effect of oxidants on CFTR when micronutrient antioxidant supply falls short [255].

The Syrian golden hamster (*mesocricetos auratus*) has been extensively studied in relation to pancreatic cancer[124]. Moreover, CYP that have been implicated in human chronic pancreatitis are highly inducible in hamster pancreas[189]. Hence this was the obvious species for study.

Male hamsters were bred and reared by Intersimian (now Shamrock Farms), Essex, UK. They were transferred to the Animal Unit of Manchester Medical School at 8 weeks.

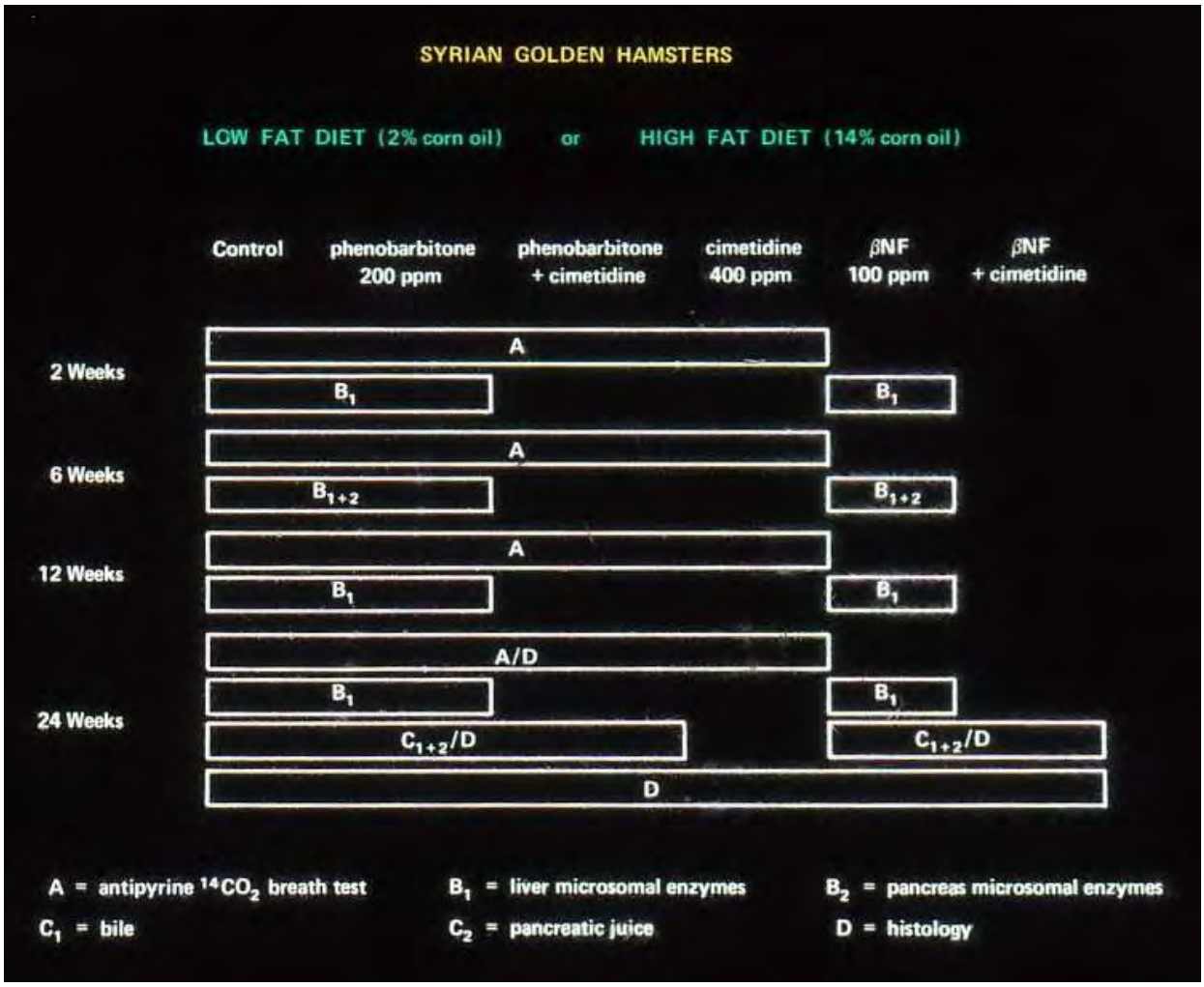

Figure 14.1 Plan of 24-week study to determine the effect of prototype inducers of cytochrome P450 (phenobarbitone, β-naphthoflavone) ± an inhibitor (cimetidine) administered via diets that contained low (2% corn oil) or high (14% corn oil) amounts of fat (LF, HF).

Subsequently, one or other test diet was fed for 6 months. The diets were prepared by Special Diets Services, Essex, UK. The manufacturer was asked to provide low fat diets with 2% corn oil (LF), or high fat diets with 14% corn oil (HF), without or with phenobarbitone (PB, 200 ppm), β-naphthoflavone (βNF, 100 ppm), cimetidine (400 ppm), or a combination of cimetidine and each CYP inducer - while ensuring that LF and HF diets were isocaloric and contained normal amounts of antioxidants.

The aims were to determine whether high intake of PUFA might be sufficient to injure the pancreas; whether the 'broad' CYP induction pattern of alcohol might be simulated by PB treatment; whether βNF addition could mimic the preferential CYP1A induction as is associated with idiopathic disease in man; and, last but not least, whether the recognised inhibitory effect of cimetidine on CYP activity might have therapeutic potential, bearing in mind an anecdotal report[213]. The design of the rather ambitious project is shown in **Figure 14.1**. Its fruition required close co-operation between senior members of 4 University Departments - Pharmacy, Physiology, Pathology, Medicine - as also research associates, and staff of the Animal Unit.

14.1 Drug metabolism studies

14.1.1 Description and outcome

The expert adviser was JB Houston of the Pharmacy department in Manchester University. The hope was that ^{14}C-antipyrine breath tests would enable non-invasive monitoring of 'broad' CYP induction (Chapter 5.2). Selectivity in CYP isoenzyme induction should in theory be detected by using a panel of marker substrates to analyse microsomal pellets from liver and pancreas: 7-ethoxycoumarin-O-demethylase (ECOD) , ethoxyresorufin-O-deethylase (EROD), 7-methoxy coumarin O-deethylase (MCOD), laurate hydroxylase (LH) and aldrin epoxidase (AE) to report, respectively, on non-specific CYP increase, CYP1A, mainly CYP2B with a touch of CYP3A, CYP4, and CYP3A with a touch of CYP2B.

A series of preliminary experiments generated support for the plan. (i) Accelerated antipyrine Cl with reduced T ½ was evident from breath tests within 3 days of intra-peritoneal PB injections, whereas 2 days' of βNF injections (in arachis oil) had no impact. (ii) In these circumstances, total CYP content of liver microsomal pellets showed the expected big increase upon PB treatment irrespective of dietary fat content, but values were around 50% less upon βNF treatment.(iii) ECOD activity mirrored CYP content. (iv) By contrast, the HF diet alone led to doubling of EROD activity, a value not increased by PB but about half that generated by βNF, whether with LF or HF diet. (v) MCOD activity mimicked that of EROD.

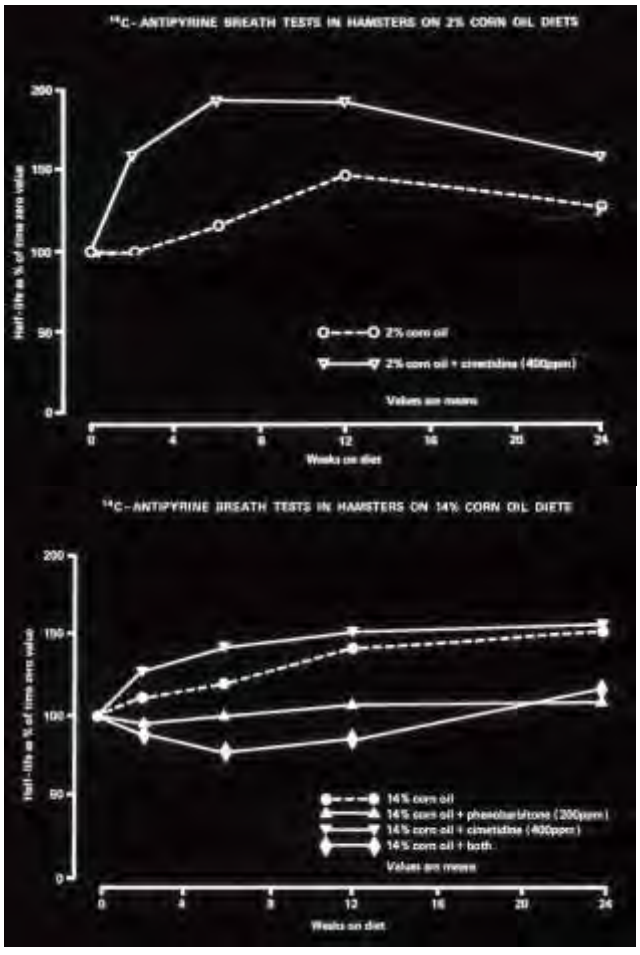

Figure 14.2 Monitoring by repeated antipyrine breath tests in low fat group ± cimetidine (**Figure 14.2 top**); and high fat groups ± drugs (**Figure 14.2 bottom**).

For a variety of reasons, these studies have barely been reported[377]: hence the results are given in some detail herein (JB Houston, J Parker, JM Braganza unpublished). ^{14}C-antipyrine breath tests confirmed that cimetidine is a potent inhibitor of CYP in hamsters reared on a LF diet alone, but not in those on a HF diet, whether alone or plus drugs (**Figure 14.2**).

Profiles of liver CYP content in animals of the 4 main subgroups were instructive (**Figure 14.3**). The HF diet-drug combination resulted in the highest values, the response evident by just 2 weeks in the case of βNF. The best separation of PB and βNF effects accrued from measurement of EROD activity (**Figure 14.4**), which again highlighted the effect of HF diet alone. **Table 14.1** summarises data on liver isoenzyme probes at 6 weeks. The pancreatic effect was miniscule by comparison (**Table 14.2**). Hence pancreatic analysis was abandoned thereafter.

14.2 Secretory studies

14.2.1 Description and outcome

Full details of anaesthesia, surgical procedures, experimental protocols and results have been published[378, 379], with SC Rutishauser as expert adviser. In brief, the cystic duct and gastric pylorus were ligated; separate cannulae were inserted into the upper and lower portions of the bile duct so as to collect bile and pancreatic juice, respectively; body temperature was recorded continually and maintained at 37-38° C by means of a thermistor with heating pad; and rehydrating

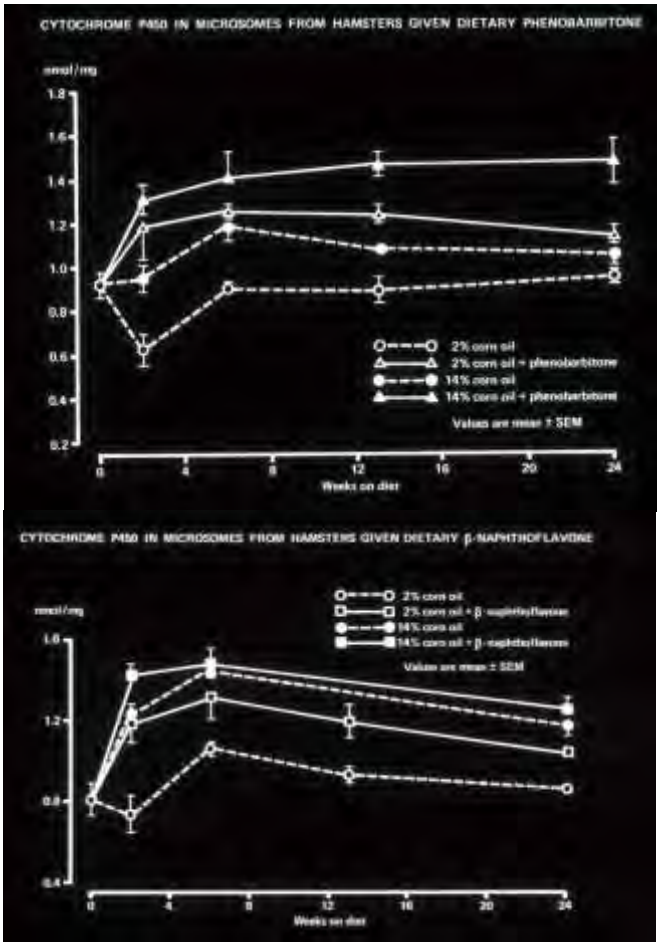

Figure 14.3 Time-course of changes in liver cytochrome P450 in phenobarbitone- treated (**Figure 14.3 top**) and β-naphthofavone-treated groups (**Figure 14.3 bottom**).

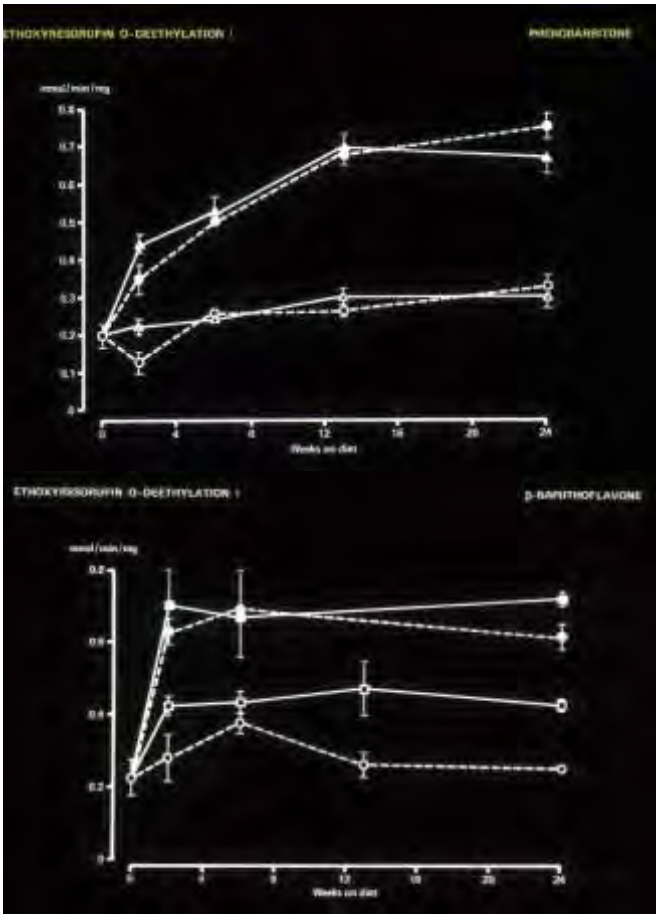

Figure 14.4 Ethoxyresorufin activity in liver mcrosomes from phenobarbitone-treated (**Figure 14.4top**) and β-naphthoflavone series (**Figure 14.4bottom**).

Table 14.1 Liver microsomal cytochromes P450 at 6 weeks

	---------- 2% corn oil----------		---------- 14% corn oil----------	
	control	phenobarbitone	control	phenobarbitone
cytochrome P450 (nmol/mg)	0.91±0.02	1.26±0.03***	1.18±0.06††	1.51±0.12*
ECOD (nmol/min/mg)	4.22±0.26	5.28±0.18*	5.86±0.34††	7.10†0.24*
EROD (nmol/min/mg)	0.259±0.007	0.245±0.011	0.504±0.005††	0.524±0.092
MCOD (nmol/min/mg)	2.42±0.11	3.05±0.10**	3.70±0.29††	4.55±0.47*
LH (nmol/min/mg)	12.30±0.50	18.80±1.10**	11.30±0.80	11.60±0.50
AE (nmol/min/mg)	2.96±0.12	5.94±0.15*	3.45±0.22	7.40±0.40*

Data as mean ± standard errorin 4 experiments. Asterisks=difference from corresponding control group *p<0.05, **p<0.01, ***p<0.001. Crosses=differences from control low fat diet †p<0.05, ††p<0.01

fluid was infused throughout.

A priming bolus dose of Boots secretin, 2CHRU / kg (batch no.93454), was administered to ensure that there was no obstruction to flow of pancreatic juice and bile. This was followed by an infusion of the hormone, 5 mCHRU / min, between 120 and 180 minutes. Secretions were collected at timed intervals during the infusion and for 2 hours thereafter. Since the Boots product contains a substantial amount of CCK (10-25 CHRU per 100 CHRU secretin) and also bile acids[79], it was judged that near-maximal secretion of pancreatic enzymes should ensue. The typical secretory pattern in a pilot experiment is shown in **Figure 14.5**.

At the end of the experiment a sample of blood was obtained by cardiac puncture; the liver and pancreas were dissected free and weighed, before being processed as described in Section 14.3. Bile and pancreatic juice samples were weighed and the CO_2 content of the latter estimated at once, as a measure of HCO_3 concentration. Sample tubes were then purged with nitrogen, capped and frozen at -70° C for later analysis of FROP by methods described in Chapter 3. Consecutive pairs of 30-minute bile samples were pooled for sufficient material. Statistical comparisons were by the Mann-Whitney U test or Student's t test as appropriate, with secretory data expressed as mean ± standard error (SE).

In relation to bile secretion the main findings from the study as a whole are shown in **Table 14.3**, and can be summarised as follows. (i) When the results for 'all xenobiotic groups' were pooled, spontaneous and secretin-evoked flow-rates of bile were higher in hamsters fed HF than LF diets (spontaneous 1.56 ± 0.05 vs 1.40 ± 0.05 µl / minute / gm liver, $p<0.05$; secretin-stimulated 1.98 ± 0.05 vs 1.70 ± 0.05 µl / min / gm liver, $p<0.05$). (ii) Hamsters fed on unmodified HF diets had higher biliary outputs of linoleic acid (9,12 LA) than those on LF diets, but lower outputs of 9,11 LA' - the trend carried through upon addition of drugs. (iii) HF diets resulted in increased UVF products in bile, indication lipid peroxidation, especially on co-treatment with βNF. (iv) Cimetidine did not abrogate, instead increasing UVF products over the levels on the HF-PB combination.

Table 14.2 ECOD activity at 6 weeks (nmol/min/mg)

	Liver	Pancreas
2% corn oil	4.22±0.26	0.0011±0.0005
2% corn oil plus phenobarbitone	5.28±0.18	0.0024±0.001
14% corn oil	5.86±0.34	0.0023±0.001
14% corn oil plus phenobarbitone	7.10±0.24	0.0030±0.001
2% corn oil	4.60±0.84	0.0006±0
2% corn oil plus β-napthoflavone	4.42±0.84	0.0018±0.006
14% corn oil	7.10±1.00	0.0009±0.0002
14% corn oil plus β-napthoflavone	7.53±1.00	0.0007±0.0015

Data as mean ± standard error

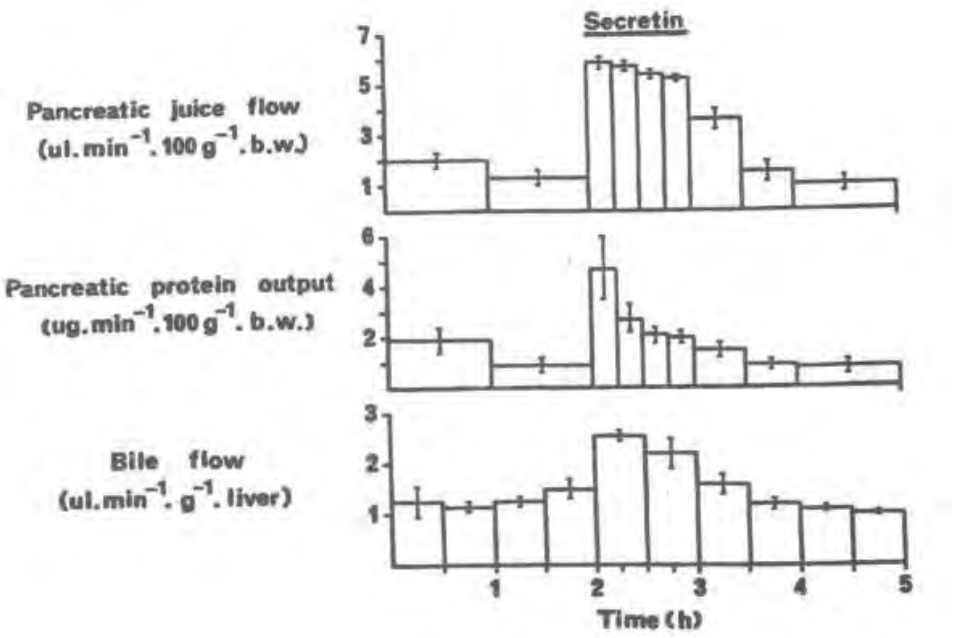

Figure 14.5 Secretory data obtainable, as shown by preliminary experiments in 5 hamsters aged 6-7 months and fed on a conventional laboratory diet. From 1991 paper in Eur J Gastroenterol Hepatol [378] with permission of publisher, Walters Kluver Health.

Table 14.3 Hamster studies: outputs in bile of products of lipid metabolism

	UVF, mU /min / gm liver		*9,11* LA', pmol / min / gm liver		9,12 LA, nmol / min / gm liver	
	LF	HF	LF	HF	LF	HF
Control	**134±19**	**367±36[a]**	**2.93±0.47**	**1.14±0.21[a]**	**1.86±0.23**	**2.92±0.21[a]**
Phenobarbitone	140±25	294±37[a]	2.90±0.72	0.87±0.22[a]	2.20±0.27	2.54±0.28
Ditto + cimetidine	297±24[b,c]	837±79[a,b,c]	3.26±0.33	0.45±0.18[a]	2.15±0.11	3.04±0.21[a]
β naphthoflavone	221±25[b]	883±142[a,b]	3.79±0.43	1.19±0.43[a]	2.30±0.23	4.01±0.45[a]
Ditto + cimetidine	194±8[b]	635±42[a,b,c]	4.04±0.47	1.01±0.43[a]	2.30±0.23	4.01±0.45[a]
All xenobiotic groups	**215±14[b]**	**698±60[a,b]**	**3.44±0.26**	**1.09±0.16[a,**	**2.20±0.10**	**3.26±0.18[a]**

Data as mean ± standard error. LF low fat diet, HF high fat diet. Control data from series 1 with hamsters on low or high fat diet alone (ref 378). [a] significantly different from corresponding low fat group; [b] significantly different from low fat or high fat control group; [c] significantly different from equivalent group without cimetidine (ref 379).

Table 14.4 Pancreatic secretion in hamster experiments

	Flow rate, µl/min/ 100 gm body wt		Protein output, mg/hr/ 100 gm body wt		Bicarbonate concentration, mM	
Diet	LF	HF	LF	HF	LF	HF
Control	**3.9±0.3**	**2.9±0.4[a]**	**1.2±0.1**	**0.8±0.1[a]**	**117±4**	**112±3[a]**
Phenobarbitone	3.7±0.5	3.3±0.6	1.0±0.2	1.1±0.2	123±3	110±3[a]
Ditto + cimetidine	4.0±0.4	2.5±0.6[a]	1.2±0.1	1.0±0.3	125±3	99±3[a,b]
β-naphthoflavone	3.7±0.5	2.1±0.4[a]	1.4±0.2	1.1±0.3	126±6	110±5[a]
Ditto + cimetidine	4.4±0.2	3.0±0.5[a]	1.2±0.1	1.0±0.3	119±3	100±4[a,b]
All xenobiotic groups	**4.0±0.2**	**2.7±0.3[a]**	**1.2±0.1**	**1.1±0.1**	**123±2**	**107±3[a]**

Data as mean ± standard error. Control data from ref 378. LF low fat diet; HF high fat diet. [a] significantly different from corresponding LF group; [b] significantly different from control LF or HF groups (ref 379).

As to pancreatic secretion, HF diets suppressed flow-rate and bicarbonate concentration of pancreatic juice, irrespective of drug added (**Table 14.4**). However, the lowering of protein output by the HF diet alone compared to LF diet alone was not seen upon drug supplementation.

Scrutiny of individual data-points revealed the likely explanation for the last finding (**Figure 14. 6**). Thus, there was a higher frequency of protein hypersecretion, outputs >1.75 mg/ hr/ 100 gm body weight, in animals on the drug-supplemented HF diets (7/31) compared to HF diet alone (1/19), $p<0.001$. By contrast low protein outputs , <0.75 mg/hr/100 gm body weight, were equally represented at around 50% among animals on HF diets with or without additional xenobiotics. The inclusion of cimetidine did not influence flow rate or protein content of pancreatic juice, but bicarbonate concentration was lowered by about 10 mM in the presence of the HF diet-PB group.

14.3 Histology studies

14.3.1 Description and outcome

These studies, as directed by IJM Jeffrey, have not yet been comprehensively reported but important facets were described in the papers on secretion [378, 379]. For liver examination, a block was taken from the central portion and processed: selected portions were stained with Sudan black

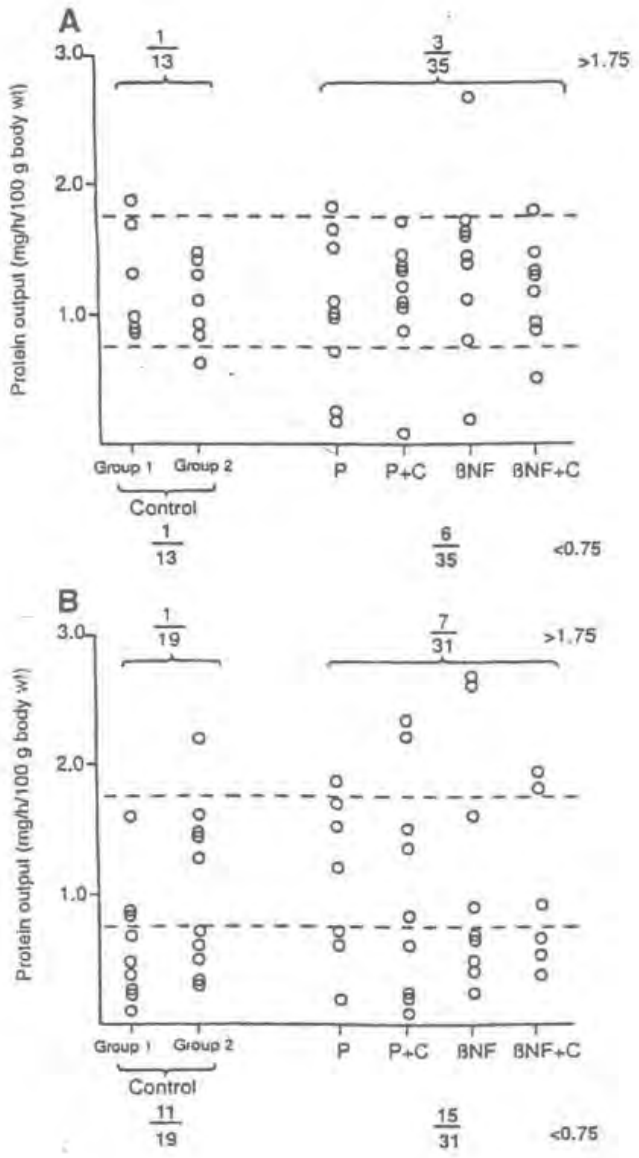

Figure 14.6 Protein output during 1 hr of the secretin infusion in hamster studies. Hatched horizontal bars represent upper and lower limits of reference range (respectively > 1.75 or <0.75 mg/hr/100gm body wt) by reference to hamsters on standard chow. From 1995 paper in Int J Pancreatol[379] (Elsevier**).**

and the Ziehl Neelson method to identify micro-vesicular fat or lipofuscin, respectively. The intensity of lipofuscin staining and severity of microvesicular steatosis were graded according to arbitrary scales as mild (grade 1), moderate (grade 2) or severe (grade 3). The pancreas was divided into 3 portions of approximately similar size which were fixed in 10% neutral buffered formalin, dehydrated and then embedded in paraffin. Three sections from each portion were prepared and stained with haematoxylin and eosin. After mounting, all slides were coded by laboratory staff and later examined by the same pathologist who had no knowledge of the animal's diet. Fatty change, acinar cell loss, inflammatory atrophy, and chronic pancreatitis (implying a periductal location of inflammation) were assessed along an arbitrary scale from 1-5 in

each lobe, a subjective score to reflect severity plus extent of abnormality. An overall score for each pancreas was obtained by taking the average of grades in each lobe.

The frequency of each pancreatic abnormality in subgroups on LF versus HF diet, inducer drug or no drug, and cimetidine versus no cimetidine were initially examined by univariate analysis (Chi squared). Relationships between secretory and histology changes were examined by Kendall's rank correlation coefficients (tau). For all these tests, differences were regarded as significant when $p<0.05$ (2-tailed).

Hepatocytes of hamsters on the LF diet looked no different from those in animals on standard chow (**Figure 14.7a**), but typical ground-glass cells were noted in several animals on the HF-PB diet (**Figure 14.7b**), as also but with more obvious microvesicular steatosis on βNF co-treatment (**Figure 14.7c**). Liver lipofuscin content was increased by all HF diets, especially upon drug co-treatment (**Figure 14.7d**): there was a positive correlation between lipofuscin score and UVF products in bile [378].

The frequency and degree of pancreatic acinar loss was greater in hamsters fed HF than LF diets. This was usually associated with replacement by adipose tissue (lipoatrophy) ; but sometimes with inflammatory atrophy; and occasionally with the typical picture of chronic pancreatitis, including tubular complexes. Further analysis suggested that a 50% loss of acinar cells was associated with a 67% decrease in flow rate and 58% decrement in protein output. Not surprisingly, a correlation matrix confirmed positive associations between each of the 4 histological descriptors of pancreatic damage, and negative correlations between each of these and indices of pancreatic functional damage (**Table 14.5**).

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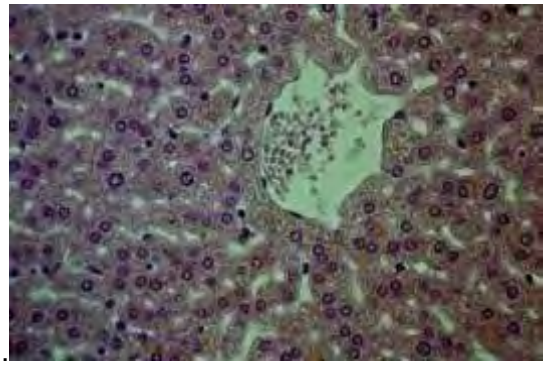
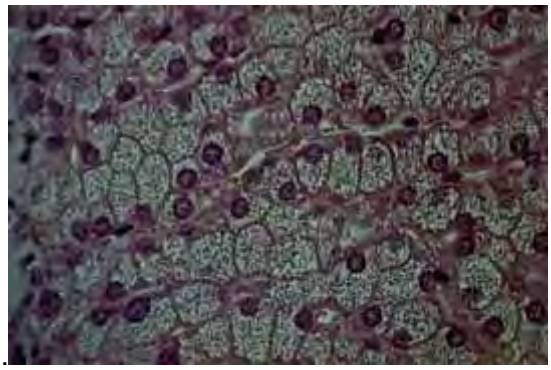


b.


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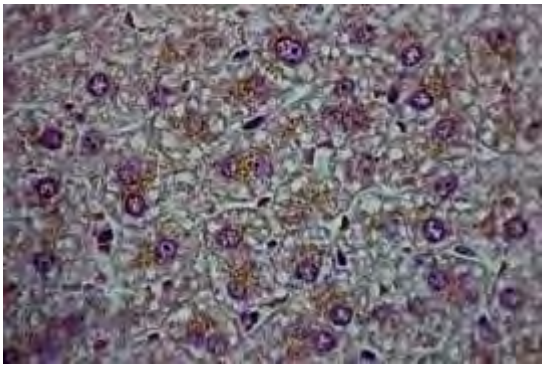

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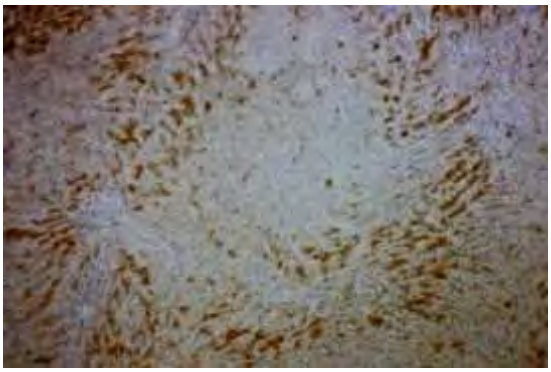

Figure 14.7 Sample liver histology (H & E) in hamster studies: from top to bottom a) normal hepatocytes; b) ground glass cells in animals fed high fat + phenobarbitone diet; c) microvesicular fat in animals fed high fat + β-naphthoflavone diet; d) excess lipofuscin shown as brown staining in all groups fed a high- fat diet, here upon supplementation with β-naphthoflavone.

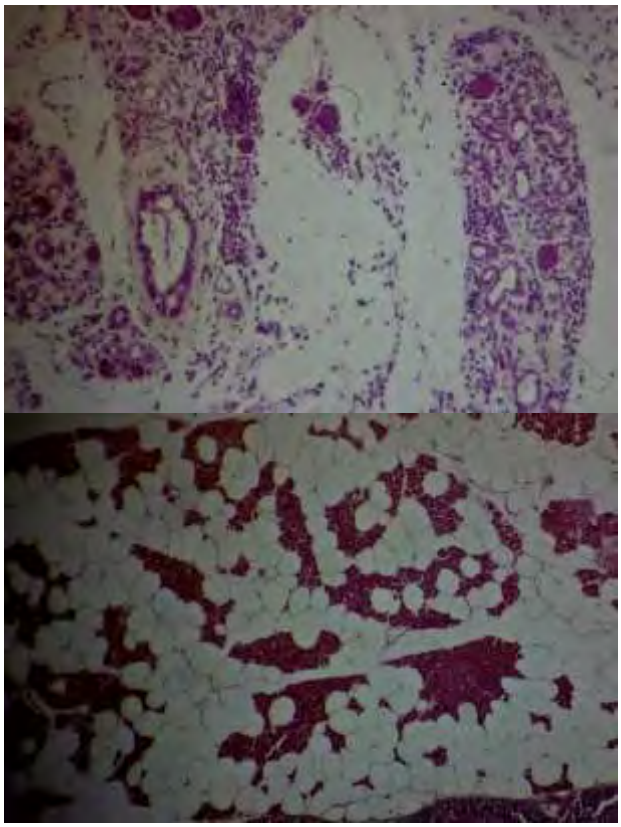

Figure 14.8 Examples of pancreatic pathology at 24 weeks in hamster studies: chronic pancreatitis with tubular complexes (**14.8 top**); extensive lipoatrophy (**Figure 14.8 bottom**).

14.4 Overview and summary

It was hoped that the study would provide a platform from which to explore the critical roles of concurrent micronutrient antioxidant lack and exposure to volatile petrochemicals in the pathogenesis of chronic pancreatitis[13, 255], as were identified by clinical data (Chapters 7 & 8). In the event, the plan was foiled as a result of unfortunate but well-intentioned action by the diets' manufacturer.

Preliminary antipyrine breath tests and secretory tests raised optimism that the project would deliver the goods. It was particularly satisfying to find that, as envisaged (Chapters 4 &10), long-term increase in linoleic acid consumption is sufficient of itself to injure the pancreas (Table 14.4). Here it is worth noting that damage was almost certainly wrought by heightened FRA consequent upon CYP1A induction, as shown by parallel liver studies (Table 14.3), despite pancreatic CYP levels being miniscule in comparison. The last finding is reminiscent of a human study from France [228].

The main differences from the anticipated results were that the isomerisation pathway of free radical attack on biliary PUFA was spared, and that hamsters appeared to be pain-free. Both anomalies pointed to selenium sufficiency, as opposed to insufficiency in patients with chronic pancreatitis (Chapters 7 & 12). In regard to the first difference, clinical studies showed increases in both %MRLA' (indicating free radical-mediated isomerisation of linoleic acid[87, 88]) and UVF products (indicating lipid peroxidation[87, 90]), the former route apparently reflecting the influence of

Table 14.5 Hamster studies: correlation matrix on pancreatic histology and secretory data

	Acinar loss	Fatty change	Inflammatory atrophy	Chronic pancreatitis	Flow rate	Protein output
Fatty change	0.6447 (<0.0001)					
Inflammatory atrophy	0.6327 (<0.0001)	0.2638 (0.006)				
Chronic pancreatitis	0.6203 (<0.0001)	0.2650 (0.004)	0.9026 (0.0001)			
Flow rate	-0.5467 (<0.0001)	-0.2761 (<0.0001)	-0.5870 (<0.0001)	-0.5425 (<0.0001)		
Protein output	-0.4303 (<0.0001)	-0.2135 (0.012)	-0.4645 (<0.0001)	-0.4517 (<0.0001)	0.5528 (<0.0001)	
Bicarbonate output	-0.2492 (<0.0001)	-0.304 (0.754)	-0.3691 (<0.0001)	-0.3545 (<0.0001)	0.6135 (<0.0001)	0.3839 (<0.0001)

Kendall Tau Coefficients with 2-tailed significance values in parenthesis

selenium lack (Chapters 13 &16). Moreover, pancreatic pain seemed blunted in Chennai patients compared to their Manchester counterparts, *pari passu* with their better selenium status (Chapter 12). Hence the diet manufacturer was specifically asked about the selenium content of diets. It was thus discovered that selenium-rich fish extract was unwittingly added to all diets in order to disguise the bitter taste of βNF-supplemented diets. It was a 'bitter pill' for the investigators!

Nonetheless, the Manchester research remains unique. Thus, there appears to be no other 24-week experimental study on the potential pathological effect of a prototype CYP inducer or even of a corn oil-enriched diet in isolation on any organ. In this context, it is generally assumed that CYP induction is protective except when there is co-exposure to a xenobiotic that undergoes bioactivation. However, a study in rats showed that high dose βNF via the diet for 6 weeks enhanced liver oxidative stress which contributed to its carcinogenic potential: the compound, an agonist of the aryl hydrocarbon receptor, induced not only the CYP gene battery but also genes that are regulated by the transcription factor NF-E2-related factor 2, presumably an adaptive response to oxidative stress [380].

The investigation in hamsters showed that chronic CYP induction causes hepatic peroxidative stress and related injury (UVF products in bile, lipofuscin-laden hepatocytes) even though dietary antioxidant content was good, and excellent in the case of selenium[378]. As in the clinical setting so too in hamsters, the exocrine pancreas succumbed under the oxidative burden, with hypofunction and abnormal histology. Intriguingly, the frequent finding of pancreatic lipoatrophy bore a striking resemblance to that described in patients at Kerala in south India, albeit then often with pancreatic calculi[381]: high consumption of fish in this coastal region should, as in the hamster study, ensure high selenium intake. Inflammatory atrophy or typical chronic pancreatitis (non-calcific) occurred less often but it is worth noting that all these disturbances were brought about non-invasively, exploiting incontrovertible evidence of induced xenobiotic-metabolising pathways in Manchester patients with chronic pancreatitis, whether 'alcoholic' or idiopathic (Chapter 5). Ethanol is a potent inducer of CYP2E1 (Chapter 12) but its long-term addition via the diet in laboratory experiments only produces chronic pancreatitis when accompanied by some other manoeuvre such as dietary corn oil enrichment or hyperstimulating doses of caerulein which increase free radical load.

A previous excellent review should be acknowledged[201], as also other individual papers. (i) A single injection of CCl_4 is sufficient to initiate chronic pancreatitis-like changes in rats, the lesions in advance of liver injury[31]. (ii) Certain nitriles evoke hypersecretion of bile, as is the case in the human disease[201]. (iii) An injection of dibutyltin leads to biliary and pancreatic lesions, seemingly by causing epithelial shedding that obstructs flow of secretions: a modified protocol involving repeated injection and alcohol feeding results in chronic pancreatitis[240]. The common denominator is that each of these xenobiotics undergoes metabolic activation via CYP.

In summary, the hamster experiments generated CYP and secretory data that are close to the human non-calcific disease. A protocol involving genetically engineered rodents that are CFTR carriers and / or CYP induction in guinea pigs, which like man cannot synthesise vitamin C, might well be successful in generating a model of calcific disease. The undeniable conclusion from the evidence at hand is that the exocrine pancreas is a versatile but also vulnerable xenobiotic metabolising organ[222].

Chapter 15.

Antioxidant Therapy for Relapsing Pancreatitis: Exploratory

When researching material for the 'detoxification' hypothesis (Chapter 4), the potential usefulness of micronutrient antioxidant therapy (AOT) for patients with chronic pancreatitis and (non-gallstone) relapsing acute pancreatitis was foreseen, but since this was uncharted territory in any field of medicine progress had to be by trial and error. There was no commercially available tablet that would deliver only the 3 items that were shown to be lacking in the habitual diets of CYP-induced patients with idiopathic chronic pancreatitis, ie. selenium, vitamin C and methionine (Chapter 7). SeACE tablets were available (Wassen International, Leatherhead, UK), each containing 100 µg selenium derived from enriched yeast, 1500 U vitamin A (as retinol equivalents), 90 mg vitamin C, and 45 U vitamin E. It seemed likely that the organic source of selenium would deliver sulphur amino acids, but in any event tablets of methionine, 0.25g, were available too (Evans Medical Ltd, Horsham, UK). The anecdotal experience of AOT recorded herein allowed selection of an optimal prescription wherewith to mount a placebo-controlled trial (Chapter 16). The inclusion of cases due to metabolic disease is intended to show the new treatment's scope.

15.1 Idiopathic relapsing pancreatitis

15.1.1 Woman with recalcitrant pancreatitis

EF was on numerous medications at the time of referral in March 1984, when aged 63 years. These were for seronegative arthritis (started in 1970), hyperthyroidism, pernicious anaemia, large hiatus hernia, diverticular disease, anxiety and insomnia - and included drugs that have been implicated in pancreatitis. She did not smoke cigarettes or drink alcohol. After the first attack of pancreatitis in 1982, she underwent cholecystectomy: several small stones were found in the gall bladder but none in the bile duct. Attacks recurred every few months despite a low fat diet and withdrawal of NSAIDs.

Initial assessment showed the following: increased serum triglycerides (2.4 mmol/l, normal 0.8-1.8); normal serum immunoglobulin profile; large periampullary duodenal diverticulum; mild dilatation of the biliary tree and apparent pancreas divisum on ERCP (minor papilla could not be cannulated); calcification in the main abdominal arteries with an atrophic pancreas and prominent main pancreatic duct on CT; normal SP test. The CT appearances were compatible with ageing but an iatrogenic cause of relapsing pancreatitis seemed more likely, while bearing in mind potential anatomical explanations. Hence, drugs except flurazepam and carbimazole were discontinued, and ordinary pancreatic extracts were started as these were reputedly beneficial in controlling pain in chronic pancreatitis (Chapter 16).

Two further attacks in the next few weeks led local doctors to prescribe daily buprenorphine. Therefore, after full discussion with patient and relatives, a pylorus-preserving pancreatico-duodenectomy was done. This should remove precipitating anatomical factors, and address the possibility of an undisclosed tumour obstructing the pancreatic duct in the neck of the gland. A pancreatogram through the pancreatic stent showed slight dilatation of the Santorini system with a stenotic lesion at the junction of body and tail of the gland. After a stormy post-operative course with sudden heart failure, she made a slow but steady recovery and was discharged in September 1984 on treatment with digoxin, diuretics, potassium supplements and cimetidine. Carbimazole was inadvertently discontinued, such that she was frankly hyperthyroid a month later and treated with radioiodine.

In December 1984, she had a further severe attack of pancreatitis. By then dietary studies had begun to show low intakes of several micronutrients in patients with idiopathic chronic pancreatitis[190] (Chapter 7). The computer-assisted analysis of the patient's diet revealed a similar pattern. Her daily intakes of vitamin C (11mg), vitamin E (1.9 mg), β-carotene (0.7mg), riboflavin (1.1mg) and selenium (22µg) were less than the RNI of 30mg, 6mg, 1.5mg, 1.6mg, and 60 µg, respectively, whereas intakes of sulphur amino acids seemed adequate at 2.3gm (RNI 1.4gm for women). Therefore, she was started on 2 tablets of SeACE daily, while advice on antioxidant-rich foods was given and the low fat diet discontinued. There were no further attacks during the next 2 years of follow up before discharge to the care of the referring consultant.

The operative specimen confirmed pancreas divisum. Pancreatic histology showed changes compatible with early chronic pancreatitis: on ultrastructural inspection prominent collections of lipofuscin were seen in pancreatic acinar and ductal cells together with extensive microvesiculation in the former (**Figure 15.1**).

Histology of a wedge biopsy specimen of the liver specimen was normal except that the amount of lipofuscin, the bulk in zone I hepatocytes, was far more than acceptable for the patient's age. Frozen sections were processed for CYP1A, using a monoclonal antibody derived from hepatic microsomes of a rat treated with βNF - the first time this immunolocalization technique was used on human pancreas. The levels were markedly increased in the patient's liver, across the lobule although mostly in zone 1 hepatocytes (**Figure 2.1**), and broadly correlating with lipofuscin deposition. Two sites within the pancreas exhibited activity, each containing 2-3 cells but in the absence of control material artefactual activity could not be excluded[382].

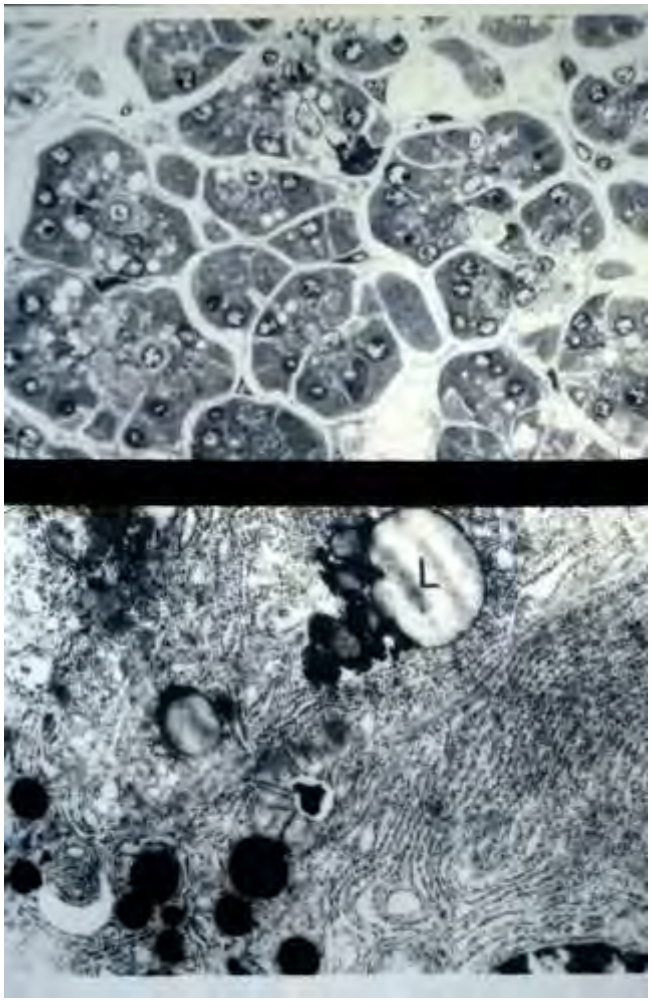

Figure 15.1 Cytoplasmic microvesiculation in acinar cells (top frame) and excess glandular lipofuscin (black deposits, bottom frame) in patient EF with recaclcitrant pancreatitis.

15.1.2 Boy with large-duct calcific chronic pancreatitis

SA was 7.5 years old when he had his first attack of colicky abdominal pain. Over the next 2 years episodes lasted about a week, the pain was worse after meals, and eased by curling up with knees to chin. His schooling was disrupted. The diagnosis of chronic pancreatitis was confirmed when increased serum amylase was noted in a painful episode, followed by ERCP evidence of a hugely dilated main pancreatic duct with many calculi (**Figure 15.2**), and he was referred in April 1985.

Figure 15.2 Hugely dilated pancreatic duct system with multiple calculi shown by ERCP in the young patient, SA.

There was no family history of pancreatitis or diabetes, and no clinical feature to suggest cystic fibrosis. The boy's mother was closely questioned about her son's environment with particular reference to recreational drugs; hobbies (eg. involving paint or glue); surreptitious practices (eg. cigarettes, alcohol); and dietary fads. Nothing unusual emerged. However, it turned out that the family home was at the perimeter of Manchester's very busy airport, and that the boy's passion for plane-spotting drew him to the airport's open observation deck very close to the take-off and landing bays, after school on most days and all day at weekends. The potential relevance of kerosene fumes was not appreciated until later (Chapter 12).

The following results were within normal limits: fasting serum triglycerides; serum calcium; sodium concentration in pilocarpine-stimulated sweat; pulmonary function tests; urinary chromatography for branched chain amino acids; random blood sugar measurements. Despite the grossly abnormal pancreatogram, there was substantial pancreatic secretory capacity as gauged by the PABA excretion index of 0.63 (lower limit at mean-3SD, 0.76) (Chapter 2).

At around this time an investigation of theophylline pharmacokinetics to probe CYP1A2 activity was under way (Chapter 5). SA had the test, using theophylline elixir, 2.5mg/kg body weight: T ½ of 1.9 hours was orders of magnitude shorter, and Cl higher, 450 ml/kg/hr, than the values in adults with chronic pancreatitis (Chapter 5). Lest the cause was innate, a pharmacogenetic study was done on the patient and immediate family members using debrisoquine. This showed that the boy was the product of an 'extensive metaboliser phenotype', as in 94% of the British population[119], but that the grossly increased activity of CYP1A2 was not reflected in the way he metabolised debrisoquine, which is processed by CYP2D6.

Analysis of the boy's habitual diet showed normal calorie and macronutrient intake: unsaturated fat intake was 26.8 gm/day, of which linoleic acid contributed 3.5gm (reference ranges in 15 healthy adults 15-45 and 3.3-15 gm/day, respectively)[190]. Among micronutrients, only selenium intake of 38 µg/day was less than the RNI for adults. After full discussion with the boy's mother, 4 tablets of the compound antioxidant preparation were prescribed per day, using a formulation in which 1500U of β-carotene was substituted for vitamin A in order to circumvent potential toxicity from high doses of the latter. It was also advised that cimetidine (800 mg in divided doses) should be added to inhibit CYP if an exacerbation occurred while on AOT, considering earlier positive reports[212, 213]. Treatment started on 19 / 6/ '85, after which no further attacks of pancreatitis were recorded during 2 years of follow up: cimetidine was not used; he attended school regularly and gained 8 kg. When the facility to measure selenium became available, the baseline serum sample registered 72 µg/l , lower than in 41 adult controls (median 118 µg/l, range 81-161), rising to 116 µg/l after 4 weeks of treatment and 144 µg/l when the dose was increased after 8 weeks to 6 tablets daily. This could be lowered after dietary advice on antioxidant-rich foods[383]. Methods to measure blood levels of the other antioxidants had not been developed.

15.1.3 Open study of patients with recurrent pancreatitis

Patients with recurrent attacks, whether RAP or chronic pancreatitis, were invited to participate in the study provided that they did not currently drink excess alcohol, and did not have gallstones or a metabolic disorder conducive to pancreatitis. They were asked to start treatment with 1 SeACE tablet per day, increasing by a tablet at fortnightly intervals until a daily dose of 3 tablets was reached. Thereafter the advice was to add a methionine tablet per day, again increasing the dose at intervals to a maximum of 4 gm daily, following which, if symptoms persisted, the SeACE was to be increased to a maximum of 6 tablets daily. The first 23 patients with idiopathic pancreatitis (chronic 18, relapsing acute 5) who were treated in this way are listed in **Table 15.1**: the list excludes the afore-mentioned frail woman with multiple pathology described above (EF), but includes the young boy (SA) as also 4 patients described in Chapter 9 whose histories illustrated the futility of bile diversion(MPi, CA, GM AL) - among whom MPi re-presented many years later

Table 15.1 Exploratory study of micronutrient antioxidant therapy

Name	Sex	Rx Age	Onset Age	ERCP	Xenobiotics	Tablets per day SeACE	Methioinine
Chronic pancreatitis							
SA	M	13	9	ADPD(C)	? jet fumes	2	0
MPi	M	22	14	MOP	? vehicle exhaust	6	12
BH	M	23	17	MOP (C)	cigarettes, vehicle exhaust	6	8
DB	M	24	19	MOP	cigarettes (? vehicle exhaust)	2	0
CA	M	30	16	MOP (C)	diesel exhaust, solvents	6	8
CT	F	30	23	MIP	nil specific	3	0
KC	F	33	27	MIP	contraceptive pill	4	8
GM	M	34	19	MOP (C)	cigarettes, anticonvulsants diesel exhaust	6	8
HG	F	37	32	MIP	contraceptive pill	1	0
LM	F	39	33	MOP	cigarettes, azathioprine,steroids	3	2
PM	M	40	32	ADPO (C)	cigarettes, diesel exhaust	6	12
AL	M	47	40	ADPD	spray paints	3	0
MH	F	49	35	normal	nil specific	3	0
JL	F	50	45	divisum	general anaesthetic x 5	3	8
EHa	F	58	49	ADPD (C)	photocopier inks/solvents	6	8
BM	F	68	68	ADPO	NSAID	3	8
WH	F	80	78	ADPD	anticonvulsants, weed killers	6	8
Recurrent acute pancreatitis							
DC	F	29	`27	normal	NSAID, frusemide	3	8
AH	F	46	36	normal `	cigarettes, NSAID, frusemide	6	8
MPh	F	47	42	normal	NSAID, cyclosporine	3	0
EHu	F	57	42	normal	cigarettes, diesel exhaust furniture spray polishes	6	8
AN	M	65	60	normal	diesel exhaust	2	0

ERCP changes: MIP-minimal changes; MOP=moderate changes; ADP= advanced changes with main duct dilatation (D) or obstruction (O) , sometimes with calculi (C). Diagnosis of chronic pancreatitis was by abnormal secretin pancreozymin test when pancreatogram was normal or showed minimal changes. Xenobiotic histories were usually by occupational physicians. NSAID= non steroidal anti-inflammatory drug. Rx=antioxidant start. Adapted from ref 384.

with infertility and was found to have a CFTR mutation [357].

The findings suggested that a combination of 6 compound antioxidant tablets plus 8 methionine tablets in divided doses each day was a suitable prescription for formal testing. These doses would be in excess of requirement for several patients, but occasionally insufficient[384]. It was considered prudent not to increase the dose of methionine above 2 gm/day because a patient with a strong family history of schizophrenia became psychotic just 8 weeks after the dose was doubled, although a previous report indicated that this risk only applies when the dose exceeds 10 gm/day[385].

15.2 Metabolic predisposition

15.2.1. Familial lipoprotein lipase deficiency(FLLD)

Relapsing pancreatitis in patients with this condition is notoriously difficult to treat. They were a good test for the therapeutic efficacy of AOT[317].

In patient **TS** recurrent episodes of abdominal pain led to a diagnosis of FLLD when 6 years old. After laparotomy for severe abdominal pain when aged 18 years, acute pancreatitis was diagnosed and she needed intensive care followed by surgical drainage of a pancreatic pseudocyst. She had 93 attacks of pancreatitis in the next decade such that she used opiates daily, despite standard measures to control hyperchylomicronemia. She was labelled as small-duct, diffuse, non-calcific chronic pancreatitis. Further surgery included cholecystectomy, partial pancreatectomy with splenectomy, and gastroenterostomy. Finally, total pancreatectomy was attempted but abandoned after 9 hr: a photograph of her battle-scarred abdomen is shown in **Figure 1.1**. A percutaneous transhepatic cholangiogram identified intrahepatic duct dilatation caused by biliary stricture. Other measures for pain control were tried without success - ie. high doses of ordinary pancreatic extracts, 2 coeliac plexus blocks, and splanchnicectomy. As no more surgery was possible, she was referred in 1995 to the metabolic team at the Manchester Royal

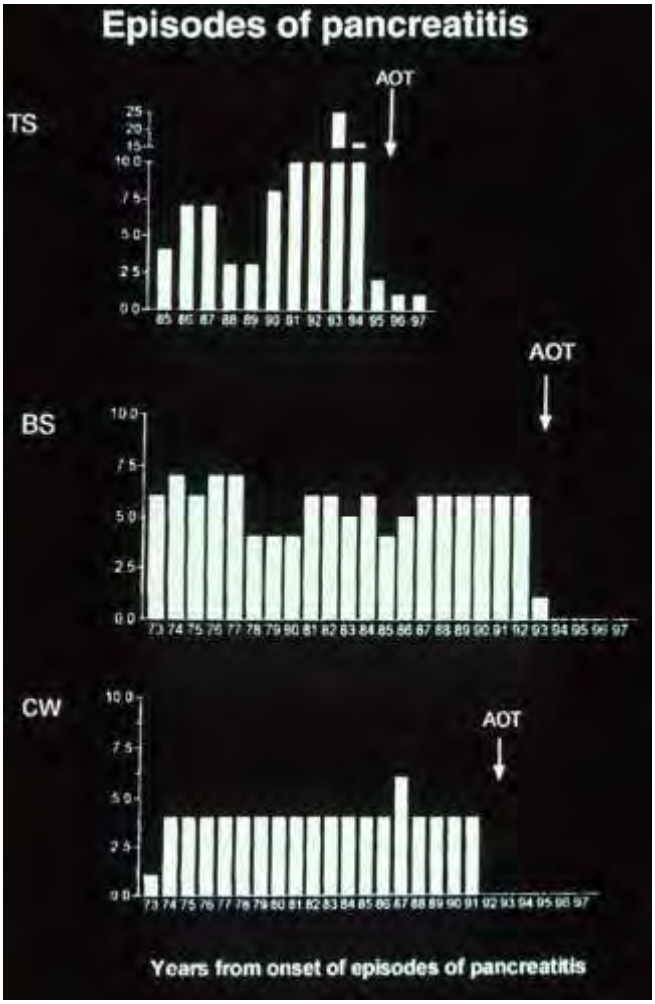

Figure 15.3 Number of pancreatitis episodes documented by hospital admissions each year in 3 patients with familial lipoprotein lipase deficiency before and after micronutrient antioxidant therapy (AOT). Reproduced from 1989 paper in J Clin Endocrinol Metab[317]

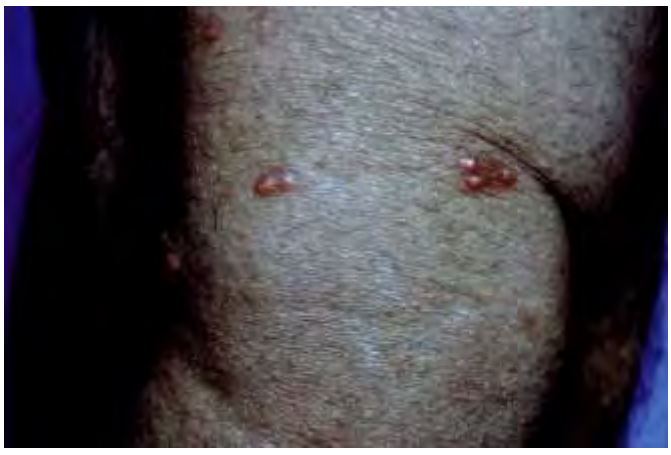

Figure 15.4 Eruptive xanthomata over the shoulder area in patient CW; these were also seen in BS.

Infirmary, and thence to the Pancreatobiliary unit. Transjugular liver biopsy showed features of suppurative cholangiolitis. The PABA excretion index was moderately reduced (Chapter 2). Micronutrient antioxidant therapy was started, after which there were only 2 mild episodes of pancreatitis (**Figure 15.3**), associated with acknowledged temporary non-compliance with treatment. Serum alkaline phosphatase activity decreased to 250 U/l from the pre-treatment value of 2000-4000 U/l (upper limit of normal 330 U/l), probably due to decreased inflammation in the head of the gland.

The initial diagnosis of FLLD in patient **BS** was after biopsy of eruptive xanthomata at the age of 7 years. She spent a significant part of her adolescence in hospital with abdominal pain despite strict adherence to a low fat diet and other measures. Laparotomy was done on 2 occasions, the second when severe abdominal pain followed spontaneous delivery of a still-born infant. Acute pancreatitis was confirmed and acute renal failure delayed recovery. She was referred in 1989 with ever-more attacks despite fibric acid derivatives and fish oil. Micronutrient therapy started in 1993 when she was 41 years old, since when no further attacks occurred in a follow up of 5 years.

Eruptive xanthomata were an early and recurrent feature of FLLD in **CW** too (**Figure 15.4**). Despite dietary fat restriction and clofibrate from age 15 years, he was admitted 4-5 times per year with pancreatitis attacks. These were not controlled by a range if lipid-lowering manoeuvres. In 1991 when he was aged 41 years, AOT was started, since when there were only 3 minor episodes of abdominal pain in the next 5+ years.

Table 15.2 Blood antioxidant profiles in 3 patients with familial lipoprotein deficiency *

	Reference ranges †	TS		BS		CW	
		Before	After	Before	After	Before	After
Vitamin C (mg/l)	4-20	14.5	18.3	14.6	19.7	10.4	20.4
Selenium (µg/l)	83-152	47	161	66	127	60	142
Glutathione (µmol/gm Hb)	7.5-12.2	7.2	12.4	5.7	8.6	7.5	8.5
Vitamin E (mg/l)	5.7-14.9	43	246	70	134	28	141
(mmol/mol cholesterol)	3.5-6.2	19.2	31.3	10.7	22.8	6.6	24.3
β-carotene (µg/l)	38-254	53	1504	60	306	67	151

*Data at baseline and after 10 weeks on treatment with a 10-week supplement of micronutrient antioxidants. †Plasma for vitamin C, serum for selenium, vitamin E and β-carotene; whole blood for glutathione. Adapted from ref 317.

Table 15.3 Serum cholesterol and triglycerides in 3 patients with FLLD in exploratory antioxidant trials

	TS		BS		CW	
	Triglycerides mmol/l	Cholesterol mmol/l	Triglycerides mmol/l	Cholesterol mmol/l	Triglycerides mmol/l	Cholesterol mmol/l
Range pre-treatment	14.2-51.8	9.4-15.2	9.3-57	8.5-12.9	6.2-31	8-20
Median value pre-treatment	23.8	10.3	25.8	10.1	11.5	12.5
Range 10 weeks post-treatment	19.2-62	6-18.4	15-40.2	6.8-12.9	6.2-74.8	6.8-17.4
Median value post-treatment	27	10.0	23.8	10.1	19.4	13.5

Measurements were made at 3-4 monthly intervals to coincide with out-patient visits. For each patient at least 8 measurements were available before and after micronutrient antioxidant therapy, excluding samples during a pancreatitis episode. Reference ranges were as follows: fasting serum triglycerides, 0.4-2.2 mmol/l; fasting serum cholesterol, 3.0-6.5 mmol/l. Adapted from ref 317.

Laboratory studies showed subnormal serum level of selenium in each patient (**Table 15.2**), as also lowered or borderline values for whole blood GSH (which seems to be a rough-and-ready measure of methionine assimilation). Moreover, abolition of pancreatitis attacks in each patient occurred without change in serum lipid profiles (**Table 15.3**).

15.2.2 Patient with primary haemochromatosis

SW was born and reared in a heavily industrialised area of Lancashire. She left school at 16 years, and then worked in the catering business for 14 years which included a 3-year stint in a factory which made domestic gas appliances. She smoked 25 cigarettes daily from aged 17 years, but did not drink excessive amounts of alcohol. In July 1992, when aged 37 years, she presented to the local hospital with a 10-month history of constant severe abdominal pain, weight loss of 25 kg and inability to eat. These symptoms had not been helped by a cholecystectomy for gallstones 5 months earlier. Extensive pancreatic calculi on plain abdominal X-ray along with hyperamylasaemia made the diagnosis of acute-on-chronic pancreatitis. A trial of pancreatic extracts failed, such that opiate analgesics were unavoidable. The records showed that 4 years earlier she had been admitted with severe macrocytic anaemia and low folate. Her grandmother was known to have pernicious anaemia, but relevant tests excluded this diagnosis and she responded well to blood transfusion followed by folate supplements. Two years later she had a Caesarean section for pre-eclampsia.

Admission assessments at referral in October 1992 showed a cachectic woman, BMI 13.5. There was a large tender mass in the epigastrium, and serum amylase was elevated at 934U/l (upper limit 317). On the morning after admission, parenteral treatment was started of micronutrient antioxidants after baseline blood samples were taken for assessment of oxidative stress markers and antioxidant status. The regimen included 2 gm ascorbate in divided doses by bolus intravenous injections together with 1 mg sodium selenite and 300 mg/kg of NAC by continuous intravenous infusion for the first 24 hours. The same dose of ascorbate was given over the next 24 hours, but doses of selenium and NAC were halved.

An urgent CT scan showed extensive pancreatic calculi and an inflammatory mass in the head of the gland with a dilated duct system, gastric outlet obstruction and, unexpectedly, excess liver iron (**Figure 15.5**). Standard tests soon confirmed primary hemochromatosis: serum iron 40 µmol/l with iron binding capacity 41µmol/l, yielding a saturation index of 98%; serum ferritin 3220 µmol/l; 116µmol iron/gm dry weight in percutaneous liver biopsy specimen; gross iron deposition in hepatocytes with large numbers of siderophages but without significant necrosis, fibrosis or cirrhosis; HLA typing A3,9; B7,14; CW7.

The patient and her relatives were informed of the potential danger in continuing ascorbate treatment in the presence of excess circulating iron, but elected in favour, because pain had already decreased substantially such that she needed very little pethidine and was able to sip high-calorie fluids. Therefore, after an echocardiogram confirmed normal ventricular size and function, and full neurological assessments including tests for colour vision were normal, treatment was continued. Over the next 4 days she received 1 gm ascorbate daily in divided doses while doses of selenium and NAC were progressively lowered. By this stage abdominal pain had disappeared and the patient was switched to the oral regimen described in the first placebo-controlled trial. A week later the patient was discharged on this treatment and advised to continue high-calorie drinks.

At the first out-patient review in March 1993, she was asymptomatic but the concentration of pancreatic isoamylase in serum was still elevated.

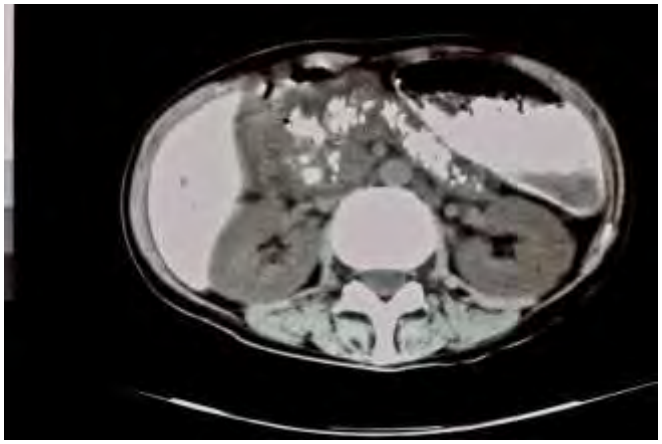

Figure 15.5 Computed tomography scan of the upper abdomen in patient SW with unsuspected primary hemochromatosis before treatment, October 1992. Reproduced from 1995 paper in Clin Drug Invest[386] (Springer).

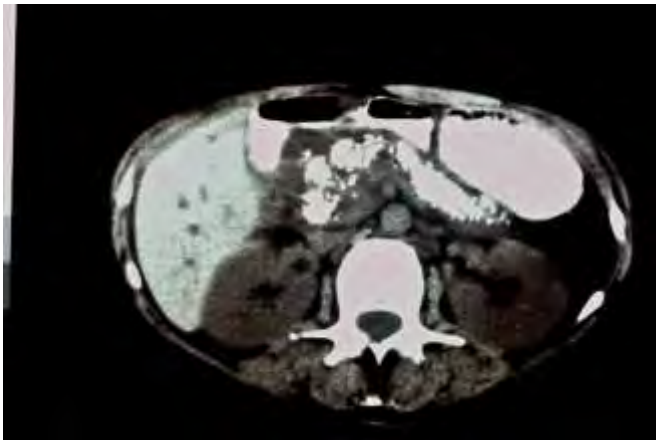

Figure 15.6 Computed tomography scan in patient SW 11 months after micronutrient antioxidant therapy. Publication details as for Figure 15.5.

After the blood antioxidant profile was analysed (see below) the dose of SeβCE was lowered to 3 tablets daily. By the next visit in October 1993, the serum isoamylase value was normal, in keeping with resolution of the acute inflammatory changes surrounding the pancreatic head mass (**Figure 15.6**). The PABA excretion index was subnormal but above the level for steatorrhoea (Chapter 2): hence pancreatic extracts were withheld.

A venesection programme began in November 1992, while relatives were screened. By March 1994 the patient was back to her pre-morbid weight, had not used analgesics for over a year, was fully rehabilitated socially, and ran the pancreatic diet section with appetising recipes on the 'pancreatic patients support group' website ! Further annual checks until 1998 showed that this excellent state of affairs was maintained, and she was returned to the care of the local consultant.

The baseline level of vitamin C in plasma was well below the lower limit of the reference range, with virtually no ascorbic acid, so that 100% of the vitamin was in oxidised forms: by October 1993 all these indices had normalised on micronutrient therapy. The same pattern was found for selenium, vitamin E and β-carotene. Surprisingly, the concentration of GSH in plasma was normal throughout. The serum concentration of %MRLA' was excessive at admission but had normalised by October 1993.

Among the specialised studies to detect low molecular mass iron that is capable of participating in free radical reactions, the bleomycin assay detects iron that is free of transferrin such that it is chelated by bleomycin and redox-recycled to degrade DNA *in vitro*: a modification, the iron-binding antioxidant assay, gives further information in terms of percentage inhibition or stimulation of DNA damage. The admission serum sample showed 4.80 μmol/l of bleomycin iron (normally nil) and the subsidiary assay showed 46% stimulation (normally 26% inhibition): both indices had improved 6 months after micronutrient therapy coupled with venesection, and were normal after a further 6 months[386].

15.3 Overview and summary

This subjective experience of AOT in patients with relapsing pancreatitis showed that success was independent of pancreatitis type (relapsing acute, large or small-duct chronic pancreatitis, presence or not of intraductal calculi); attack route of inciting agent (ductal via reflux of abnormal bile, microvascular via increased chylomicra, intra-acinar as from prescribed drug or inhalation exposure to occupational chemical); or putative source of heightened free radical activity (CYP1A,

xanthine oxidase, ultraviolet light irradiation, iron overload).

The congruity pointed to protection of a shared metabolic pathway that is crucial for apical exocytosis in the acinar cell, given that pancreastasis underlies a pancreatitis attack. Laboratory studies had already identified the methionine metabolic pathway as the 'Achilles heel' (Chapter 11). However, theoretical considerations indicated another potentially important role of AOT, ie. protecting CFTR, both at the apical pole of acinar cells where it seems to facilitates exocytosis, and in ductal elements where it facilitates secretion of HCO_3^-, GSH and thiocyanate (Chapter 13). Moreover, the observation that pain control in patients with chronic pancreatitis was achieved in patients without overt attacks seemed to indicate suppression of nociceptive agents that are activated even when the secretory aberration is subclinical. The success of micronutrient therapy - without recourse to lithotripsy or other procedure to clear huge intraductal calculi - in the young boy (SA) was especially gratifying, and repeated in 20 other children (G Des las Heras Castano, NM Sharer, JM Braganza, unpublished).

The precise sequence of events leading to relapsing pancreatitis in patients with FLLD is not established, but studies in the isolated perfused pancreas suggest that repeated bouts of oxidative stress associated with ischaemia-reperfusion injury are relevant[274]. Furthermore, chylomicra and very low density lipoproteins are the source not only of pro-inflammatory non-esterified fatty acids, but also of polyunsaturated fatty acyl groups that are highly susceptible to lipid peroxidation. Of note in the patients described herein, attacks were abolished by AOT. It is likely that lipid-phase agents contributed to success, although serum lipid profiles were unchanged. The conundrum is why the first patient went on to develop chronic pancreatitis. Speculatively in light of earlier observations (Chapter 12), her very poor selenium status might have been a factor in that this has been experimentally linked to pancreatic fibrosis[300]; and also that she lived in a congested, traffic fume- polluted city in north west England.

In relation to primary haemochromatosis and Wilson's disease, painless damage to pancreatic acinar cells along with interstitial fibrosis are recognised features. However, clinicians have been exhorted to disregard these metal-storage conditions - as also kwashiorkor and cystic fibrosis in which the exocrine pancreas is invariably affected - in any discussion of pancreatitis[32]. There appears to be no previous or subsequent report of calcific chronic pancreatitis in association with primary haemochromatosis. The following arguments reflect on the danger from prescribing ascorbic acid and why it was averted in the patient at Manchester: citations are given in the case report[386].

Many of the physiological pathways that yield ROS involve the controlled release of low molecular mass iron (Fe^{++}). An excess is prevented by an efficient system of iron-binding and iron-oxidising proteins, buttressed by micronutrients that act synergistically and display metabolic redundancy. The synthesis of iron-sequestering proteins is stepped up to counter excess FRA. Should they fall short, then - and only then - ascorbate may switch from antioxidant to pro-oxidant because it can lead to iron mobilisation and the reduction / redox-cycling of iron, augmenting $OH^{\bullet}$ production. Primary hemochromatosis is known to exemplify this scenario, the main reactions proceeding as follows.

$Fe^{++} + Cu^{++}\text{Ferroxidase1} \rightarrow Fe^{+++} + Cu^{+}\text{Ferroxidase 1}$

$O_2 + 4H^+ + 4Cu^{+}\text{Ferroxidase 1} \rightarrow 2H_2O + 4Cu^{++}\text{Ferroxidase 1}$

$Fe^{+++} + \text{Ascorbate} \rightarrow Fe^{++} + \text{Dehydroascorbate}$

$Fe^{++} + H_2O_2 \rightarrow Fe^{+++} + OH^{\bullet} + OH^-$

Because of this threat, the use of vitamin C is contraindicated in iron-overload states, not surprising after reports of myocardial depression, encephalopathy and even death. The co-administration of GSH precursors likely mitigated against this potential catastrophe in the Manchester patient[197], while conceding that treatment would probably have been withheld had the iron-storage disease been identified beforehand! The previous history of folic acid deficiency and family history of pernicious anaemia were of interest, in that ascorbate facilitates the resynthesis of methionine from homocysteine by the enzyme methionine synthase, for which folate and vitamin B_{12} are cofactors. Also of interest was the history of pre-eclampsia because oxidative stress is implicated in its pathogenesis[387].

The low serum selenium level at admission in the patient was typical of findings in patients with painful chronic pancreatitis[300], as also negligible ascorbate which is typical in patients with extensive pancreatic calculi, or cysts / pseudocysts[37]. Pain control within just 4 days of parenteral micronutrient therapy was remarkable, as was resolution of the inflammatory mass in the head of the gland, such that the patient was able to eat and drink normally by 10 days.

The latter feature was not unique, as shown by disappearance of the 'reverse 3' sign upon resolution via AOT of the non-calcific head mass in a patient with idiopathic disease (**Figure 15.7, Figure 15.8**) (JM Braganza unpublished), and shrinkage of the head mass in a patient with alcoholic calcific disease[257].

In summary, AOT was a promising new approach to management of patients with chronic pancreatitis and RAP, but needed validation or debunking by randomised controlled trials (RCT).

Figure 15.7 'Reverse-3' sign by barium meal examination of the descending duodenum, suggesting pancreatic head cancer in a patient with non-calcific chronic pancreatitis

Figure 15.8 Normal duodenal contour after micronutrient antioxidant therapy.

Chapter 16.

Clinical trials of AOT: Manchester and beyond

Placebo-controlled trials of methionine or SAM-incorporating AOT at Manchester in the 1980s were followed in the decade 2000-2010 by positive reports from Spain, Italy, northern Ireland, the Czech republic, and India. **Figure 16.1** is a reminder of the underlying philosophy, ie. to buttress the pathway of methionine metabolism which was known to be crucial for pancreatic integrity[27].

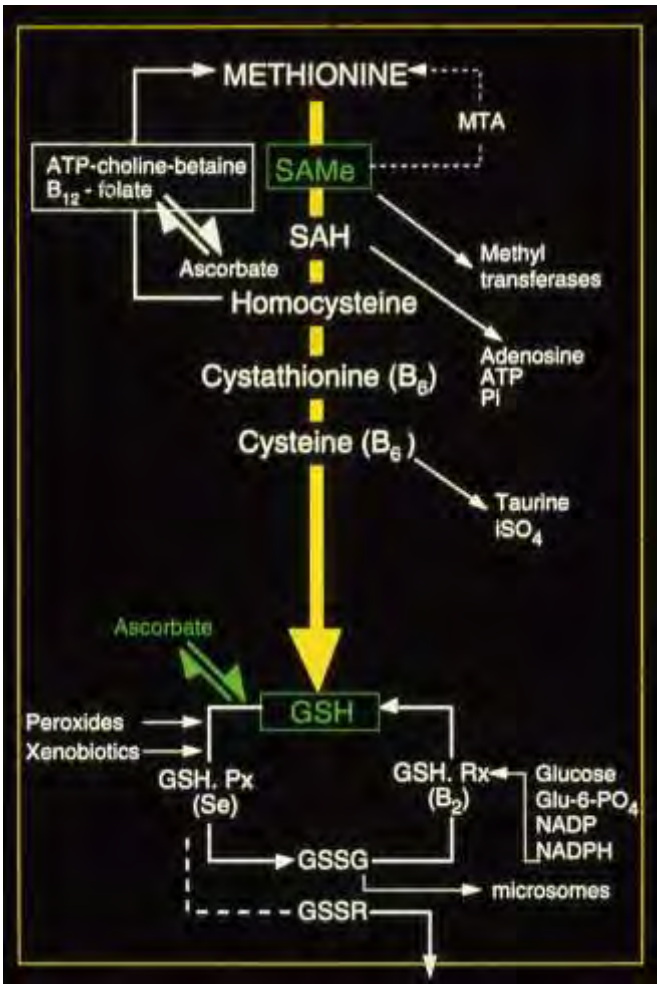

Figure 16.1 Simplified schema of methionine metabolism to facilitate an understanding of the rationale and outcomes of methionine-based trials of antioxidant therapy. Micronutrient co-factors for enzyme action are shown in parenthesis. See Glossary for abbreviations. Figure reproduced from 1998 review in Digestion[13] with permission of S Karger AG,

16.1 RCTs: Manchester late 1980s

16.1.1 SeβCE plus methionine

Recruitment. From July 1985 consecutive patents were considered if there were at least 2 documented attacks of pancreatitis in the previous year and / or constant pain that suggested a pancreatic origin. The diagnosis of chronic pancreatitis in 18 patients was based upon detection of pancreatic calculi, ERCP evidence of an unequivocally abnormal duct system, or clearly reduced pancreatic secretory capacity upon assessment 6-8 weeks after recovery from the last attack. When these tests were normal, as in 5 patients, a diagnosis of RAP was retained (Chapter 2) (**Table 16.1**).

Patients were excluded in the following circumstances: previous or current antioxidant therapy; stones in gall bladder or common bile duct; addiction to narcotic analgesics; unreliable witnesses (eg. children or mentally-retarded patients); large pseudocyst or obstructed bile duct requiring surgery; suspected pancreatic cancer; pain highly suggestive of non-pancreatic origin (eg. spastic colon, costochondritis); end-stage disease with steatorrhoea and little or no pancreatic isoamylase in serum, because pancreatic pain may then disappear to be replaced by colonic pain from uncontrolled steatorrhoea; chronic renal failure. Patients were grouped into 3 categories: idiopathic chronic pancreatitis, alcoholic disease (> 60 gm ethanol daily in women or > 80 gm daily in men for at least a year before the first attack), and recurrent acute pancreatitis. Recruitment stopped in November 1987 because the supervising clinician's appointment was due to end the following April and it seemed desirable for the same person to conduct all the interviews.

Table 16.1 Placebo-controlled trial of SeβCE plus methionine

Name ‡	Sex	Trial Age	Onset Age	ERCP	Xenobiotics	Group†	First Rx
Chronic pancreatitis							
TH	M	21	15	ADPO(C)	cigarettes, dry cleaning solvents	1	P
BH	M	22	20	normal	cigarettes, paints, degreasers	1	P
KH	M	25	21	ADPO	alcohol , cigarettes	2	A
SR	M	26	17	MOP	cigarettes, degreasers	1	A
AP*	F	29	16	ADPD (C)	nil specific	1	A
MO*	M	33	28	MOP	cigarettes, diesel exhaust	1	P
GP	M	34	22	ADPS	alcohol, cigarettes	2	A
MH	M	35	31	ADPO	alcohol, cigarettes	2	A
TR	M	37	21	calculi ++	alcohol, cigarettes, diesel exhaust/oils	2	P
DS	F	38	28	MIP	cigarettes	1	P
JGi	M	42	39	MIP	alcohol, solvents	2	A
JS	M	47	36	failed	cigarettes, solvents, chemicals +++	1	P
BR	M	49	38	divisum	diesel exhaust	1	A
KS	M	49	36	calculi ++	alcohol, cigarettes, solvents	2	P
MC*	M	51	40	MOP	cigarettes	1	P
JC	M	51	31	failed	solvents, inks	1	P
RT	M	52	44	ADPD	alcohol, cigarettes, solvents	2	P
JGa	M	54	35	normal	solvents, degreasers	1	P
Recurrent acute pancreatitis							
NE	F	17	7	normal	nil specific	3	P
SS	F	27	24	normal	cigarettes, anticonvulsants,dyes	3	A
CS	M	29	18	normal	cigarettes	3	A
MA	F	62	54	normal	steroids, NSAID	3	P
JF	F	72	69	normal	general anaesthetics x 10	3	A

See text for details. *Patients omitted from final analysis. ERCP changes classified as in footnote to Table 15.1. with a further category of advanced change disease ADPS=main duct segmented,chain-of-lakes. † group 1= idiopathic chronic pancreatitis; 2=alcoholic chronic pancreatitis; 3=recurrent acute pancreatitis. ‡ first treatment:P=placebo, A=active. Adapted from ref 389

Trial design. A 20-week double-blind double-dummy crossover trial was regarded as being the most appropriate, given the wide age-range and circumstances of patients in the pilot study (**Table 15.1**). Moreover, it was realised that this duration exceeded any previous trial, and that an extension might prejudice compliance. The trial was co-ordinated by a senior pharmacist, the decision as to whether active or placebo treatment was given in the first phase determined by random number tables that were applied separately to each diagnostic subgroup. Active treatment consisted of 6 tablets per day of selenium-βcarotene-vitamins C and E (Wassen International); and 8 tablets per day of methionine (Evans Ltd) in divided doses. Placebos for the compound antioxidant tablet were provided by its manufacturer and for methionine by the Boots company (Nottingham, UK): these matched their active counterparts by appearance, but the methionine placebo lacked the distinctive garlic-like odour of the true substance.

Protocol. Prospective patients were interviewed at length to explain the nature and purpose of the study and to obtain their informed consent. They were told that either or both treatments might control painful attacks: several had been referred by surgeons and thus knew that pancreatic surgery was being considered. Once entered into the trial, each patient listened to a pre-recorded tape giving details instructions. The patient then completed a pain vocabulary score-sheet to allow assessment of background pain in the 10 week period preceding the trial, and a series of questionnaires to assess psychological aspects - based on validated measures in the context of chronic back pain, as there was no instrument to gauge quality of life in patients with chronic pancreatic pain. These proforma were completed in the clinician's presence. Blood samples were taken, following which the patient was given a trial diary and asked as far as possible to avoid major lifestyle changes for the trial duration (eg. cigarettes, alcohol, job, hobbies, diet). They returned at 10 weeks when they were questioned about clear-cut pancreatitis attacks (and whether or not reported for a blood amylase check), and also regarding treatment side-effects and major lifestyle changes. An ultrasound scan was done in patients who had an attack. Thereafter, blood samples were taken, questionnaires administered, and new diaries issued. Patients reported again at 20 weeks when the same procedure was followed, after which an ultrasound scan was arranged if needed, and all patients placed on active treatment.

Clinical assessments. The following aspects were probed: (i) frequency of attacks; (ii) pain vocabulary score-sheets; (iii) questionnaires to inform on pain psychology; and (iv) pain diaries.

As to the first aspect, patients were asked to report to the general practitioner or nearest hospital for a blood amylase test if they had a severe attack of pain. Letters of intimation were sent to the relevant authorities and a sealed letter given to the patient to hand to the assessing officer.

In regard to the second, an exploratory study involving 59 previous patients with relapsing pancreatitis who were not addicted to narcotic analgesics at referral gave an idea of pain vocabulary. A McGill Standard Pain Questionnaire was circulated and responders received the same questionnaire some weeks later. Scrutiny of the responses identified 36 words that were used frequently and were reproducible. A principal component analysis was carried out on these words, from which emerged a subset of 11 words that were highly weighted on the general component, ie. the most representative words to describe pancreatic pain in the north west of England. A scoresheet was constructed including all 36 words with a 10-cm visual analogue scale alongside each. This was the first questionnaire presented to patients at a time when concentration was likely to be highest.

Questionnaires in relation to pain psychology were based on a local in-depth study of severe back pain, not ideal but the best at the time. The following components were covered: somatic anxiety; depressive symptomatology; pain experience, which assesses current psychological status on 'emotionality' and 'worry' scales; and pain locus which probes patients' beliefs on pain, its treatment, cognitive control, and pain responsibility.

Finally, the pain diary incorporated a single 10 cm scale for each day to gauge overall distress - of necessity incorporating somatic and psychological aspects of pain. Full details are given in the trial report[388].

Chemical analyses. Serum levels were monitored of selenium, β-carotene, vitamin E, and % MRLA' (Chapter 3). Plasma was analysed for GSH-(Se)-peroxidase and SAM (SAMe). Inorganic sulphate was measured in urine, as an index of sulphur amino acid intake. Methods to

measure vitamin C, ascorbic acid and GSH had not yet been developed. Full methodological details have been published[389].

Statistics. Attack frequency during active and placebo phases were compared by the McNemar test for change. Scores generated by various questionnaires at 3 time-points were compared by the Wilcoxon matched pairs signed-rank test: 95% confidence intervals were derived for median change. For both aspects, analyses were first done on data from all patients who completed the trial and then re-applied after excluding patients in whom one or other circumstance would have biased the results (see below). Diaries were examined in 2 ways. A conventional method compares cumulative or average scores by a non-parametric technique such as the Wilcoxon matched pairs test. Time-series analysis allows for serial dependency and is especially useful for analysis of background pain with superimposed peaks. Further refinements allowing for carry-over effects could be introduced if warranted. Baseline blood levels of analytes were compared with reference ranges by the Mann-Whitney U test, and effects of treatment gauged by the Wilcoxon test.

Parametric methods (Student's t-test with correction for multiple comparisons, paired t-test, ANOVA, discriminant analysis) were generally suitable for analysing biochemical data. Non-parametric methods (Mann-Whitney U-test, Wilcoxon signed rank test) were also used when appropriate. Two-tailed test of significance were applied throughout.

Results: Numbers. Of 28 patients who enrolled, a man with idiopathic chronic pancreatitis required urgent surgery within a fortnight of trial start to control haematemesis from unsuspected portal hypertension. Four patients - 2 with idiopathic chronic pancreatitis and 2 with alcohol-related disease - failed to keep the 10-week appointment without explanation, and were lost to follow up; each of them lived >50 miles away. Among 23 patients who completed the course, there were major lifestyle changes during one or other arm of the trial in 2 patients. The start of pregnancy, half-way into the first phase in AP could have magnified symptoms, considering the increased need of antioxidants, and that she had an attack in both previous pregnancies. Again, MO's change in job from diesel-truck driver to office-based salesman, which coincided with treatment switchover, could result in improvement from withdrawal of volatile xenobiotics alone (Chapter 8). A third patient, MC, was admitted with a pancreatitis attack 5 weeks into the first phase which turned out to be placebo, but at the time of discharge from hospital was mistakenly given active treatment for the remaining 5 weeks - the error uncovered when the pharmacist later decoded the trial, and subsequently confirmed by tablet analysis and measurement of β-carotene in pre-trial, admission and crossover blood samples.

When these 3 patients were omitted, there were 20 who qualified on all counts, of whom 8 had idiopathic chronic pancreatitis, 7 had alcoholic chronic pancreatitis, and 5 had RAP. (The overall loss of 6 patients from the first subgroup explains the disparity in numbers receiving active or placebo treatment in the first phase). No specific side-effects were reported.

Results: Positive impact of active treatment. This was evident from analysis of attack frequency (**Table 16.2**) and pain vocabulary score sheets (**Tables 16.2, 16.3**). As to the latter, a strong placebo-effect was apparent, but active treatment - whether given in the first or second phase - resulted in the lowest scores.

Pain diaries analysed by the Wilcoxon method showed that average daily scores registered in the second 5-week period on active treatment were lower than on placebo (**Table 16.4**). A suitable model emerged on time-series analysis in patients with constant pain but not when this was interrupted by attacks. A significant reduction in pain score on active treatment was detected in 4

Table 16.2 Summary of trial results: (i) Attacks & background pain

	Intention to treat n=23	Final analysis n=20
Attack of pancreatitis	n=23	n=20 †
Placebo	8 (35%)	6 (30%)
Active	1 (4%)	0 (0%)
p (2-tailed)	0. 040	0.032
Background pain: 11 best words	n=22 *	n=20 †
Placebo mean score	21.8	34.1
Active mean score	18.3	18.3
Median difference	5.9	8.6
95% confidence interval	-2.55, 17.0	0.05, 18.8
p (2-tailed)	0.10	0.049

*omitting MC who had placebo for 5 weeks and active treatment for 15 weeks; † omitting MC,AP and MO. See text for details. Adapted from ref 388.

Table 16.3 Summary of results: (ii) Further analysis of pain vocabulary scoresheets

Word set *	Baseline	After Placebo	After Active	B vs P	B vs A	P vs A †
Total of 11 best words	44.4 2.9-71.6	34.1 0-100	18.3 0-65.8	10.2 (-1.4, 22.8) p = 0.073	22.5 (12.3, 30.9) p < 0.001	8.6 (0.05,18.8) p = 0.049
Total of 36 words	152 2.9-200	94.6 4.0-337	51.1 0-224	40.4 (2.6, 75.9) p = 0.031	70.4 (39.2, 100) p < 0.001	23.7(-7.9, 59.2) NS
Average of words						
≥ 2.5 cm at baseline (n=20)	6.35 2.77-8.24	3.34 0-10.0)	2.12 0-7.20	3.03 (1.69, 4.13) p = 0.001	3.97 (2.54, 4.95) p < 0.001	0.64(-0.57, 1.79) NS
≥ 5 cm at baseline (n=18)	7.58 6.33-9.63	3.92 0.09-10.0	2.48 0-7.82	3.99 (2,31, 5.15) p < 0.001	5.31 (4.21, 6.23) p < 0.001	1.04 (-0.00, 2.26) p = 0.055
≥7.5 cm at baseline (n= 18)	8.59 7.75-9.63	4.08 0-10.0	2.64 0-8.72	4.54 (2.88, 6.23) p < 0.001	6.14 (4.92, 7.24) p < 0.001	1.3 (-0.02, 2.62) p = 0.055

*Median and range of total or average scores: in the latter assessment the number of qualifying respondents are given in parenthesis. †Median difference, with confidence intervals in parenthesis, comparing scores at baseline (B), after placebo (P) or active treatment (A). Adapted from ref 388.

Table 16.4 Results summary: analysis of diaries by conventional method

	Placebo*	Active*	Median difference†
First 5 weeks	1.39 0.06-5.84	1.19 0.17-4.66	0.23 (-0.13, 0.80) NS
Second 5 weeks	1.01 0.12-6.26	0.90 0.14-3.80	0.30 (0.04, 0.74) p=0.03
10 weeks	1.88 0.22-5.76	1.01 0.16-4.26	0.26 (-0.06, 0.84) p=0.01

*average daily scores with ranges; † 95% confidence intervals in parentheses (ref 389)

cases (TH, BR, TR, KS) by this method: overall, the median of mean change in pain scores on active treatment was -0.44 (95% CI, -0.03, 1.29, P=0.093). As to psychological assessments, baseline scores were typical of people with recurrent medical problems but less extreme than in those with musculoskeletal disorders such as back pain. Active treatment was associated with fewer symptoms of general distress - including emotionality and worry aspects, but numbers were too small to be confident about interpreting the other indices.

Results: Antioxidants. After the biochemical assays were completed, several months after the clinical paper was published, it became apparent that a patient with RAP had very high levels of vitamin E at baseline. Questioning revealed that he had inadvertently taken a food supplement containing the vitamin, prior to but not during the trial: samples from this patient were omitted from further consideration. Compliance with active treatment was shown by a clear increase in blood concentration of at least one of the prescribed items in the other 19 patients. The results are summarised in **Table 16.5**. The subnormal baseline level of selenium was not reflected in GSH-(Se) peroxidase activity, and supplementation with selenium had no impact on enzyme activity. There was no substantial carry-over effect of active treatment.

Results: Paradoxical behaviour of SAM. Subnormal baseline levels of SAM drifted upwards after placebo, when this was given in the first phase but not when preceded by active treatment. . Scrutiny of the results revealed that the highest values in the former set were in 3 patients who had sustained a pancreatitis attack in the preceding 10 weeks; additional blood samples available at the time of the attack in 2 of them showed surges over pre-treatment levels.

Table 16.5 Micronutrient antioxidant data from placebo-controlled trial of selenium-βcarotene-CE plus methionine

		Baseline †			After placebo†			After Active †		
	Controls	P	A	T	P	A	T	P	A	T
Selenium (µg/l)	n 41	11	8	19	11	8	19	11	8	19
	M 117	84	86	85	84	83	83	103 ‡,#	120 ‡,#	110 ‡,#
	SD 17	14	12	13	16	15	14	13	17	16
	r 81-161	51-104	69-101	51-104	44-104	69-108	44-108	84-124	101-151	84-151
	p	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.01	NS	NS
β-carotene (µg/l)	n 22	10	8	18	11	8	19	11	8	19
	M 110	46	32	40	42	41	42	154 ‡,#	236 ‡,#	188 ‡,#
	SD 55	24	29	26	27	65	45	133	293	218
	r 38-254	10-91	4-93	4-93	9-102	0-186	0-186	40-455	22-903	22-903
	p	<0.001	<0.001	<,0.001	<0.001	<0.001	<0.001	NS	NS	NS
Vitamin E (mg/l)	n 20	10	8	18	11	8	19	11	8	19
	M 11	10	10	10	10	11	11	17 ‡,#	23 ‡,#	20 ‡,#
	SD 2.9	4.9	3.9	4.4	5.4	6.3	5.7	5.4	9.4	7.7
	r 6.7-15	2.7-17	4.1-15	2.7-17	2.9-23	5.2-26	2.9-26	10-26	15-41	10-41
	p	NS	NS	NS	NS	NS	NS	<0.001	<0.001	<0.001
Vitamin E (mmol/mol cholesterol)	n 20	9	8	17	11	8	19	11	8	19
	M 4.8	4.1	3.5	3.8	4.1	3.9	4.0	6.8 ‡,#	7.6 ‡,#	7.2 ‡,#
	SD 0.8	1.8	1.0	1.5	1.8	1.2	1.5	1.7	1.5	1.6
	r 3.1-6.2	1.4-6.9	1.5-4.5	1.5-6.9	1.3-6.9	1.9-5.6	1.3-6.9	3.2-9.7	5.5-9.8	5.2-9.8
	p	NS	<0.005	<0.02	NS	<0.001	<0.05	<0.001	<0.001	<0.001
SAM (nmol/l)	n 16	11	8	19	11	6	17	11	8	19
	M 74	51	45	49	64	52	60	48	38 ‡,#	44 #
	SD 28	20	21	20	21	14	20	17	10	15
	r 34-120	21-99	18-78	18-99	48-106	34-68	34-106	34-85	14-60	14-85
	p	<0.02	<0.005	<0.005	NS	<0.02	NS	<0.005	<0.001	<0.001

NB. Plasma glutathione peroxidase and urinary inorganic sulphate values were similar in controls and patients throughout the trial. † Patients grouped according to P=placebo first, A=active first, or T=total. ‡ significantly different from corresponding baseline value; # significantly different from corresponding placebo value. SAM=S-adenosyl methionine. Adapted from ref 389.

This was not seen with the other antioxidants. Moreover, whereas active treatment consistently increased blood levels of selenium and the vitamins, it resulted in a downward drift in SAM concentration. Levels of inorganic sulphate were similar in controls and patients at every stage.

Results: Free radical activity / oxidative stress. Data on %MRLA' identified increased FRA at baseline, an inexplicable fall after placebo but a further substantial fall after active treatment (**Table 16.6**).

The overlap in % MRLA' and also selenium values between control sera and baseline trial samples was eliminated by discriminant analysis. The same discriminant function separated datapoints from controls and patients after placebo treatment, but not after active treatment (**Figure 16.2**). In other words, the selenium component of the prescription corrected oxidative stress as reported by the lipid isomerisation pathway of attack on linoleic acid (Chapter 3).

16.1.2 S-adenosylmethionine

This key donor of CH_3 groups was proving successful in a parallel Manchester study of rats with allograft pancreatitis (Chapter 17).Hence the possibility arose that replenishment of SAM might be solely responsible for the success of the clinical trial. The main procedural differences in the second trial were the use of only the 11 validated pain descriptors, and omission of psychological assessments. Patients were randomised to receive in the first phase either 2 x 400 mg tablets 8-hourly of SAM (as S-adenosylmethionine sulphate-p-toluenesulfonate, BioResearch SpA, Milan, Italy), or placebo tablets from the same source. Biochemical methods varied in that now fluorimetry was used to measure selenium (after confirming congruity of results with former analysis by atomic absorption spectrometry), and that an additional 'marker' of excess free radical activity had been developed, ie. the percentage of vitamin C that is in oxidised forms, rather than as ascorbic acid (%MRVC) (Chapter 17.4).

Of 30 patients who enrolled, 6 withdrew because of gastrointestinal intolerance to active treatment, 3 needed urgent medical attention (bowel obstruction in a patient with Crohn's disease, variceal haemorrhage in a patient with cirrhosis, myocardial infarction), and 1 patient defaulted. Of the 20 who completed the trial, 10 received SAM and 10 placebo in the first phase. There were 8

Table 16.6 Linoleic acid oxidation marker in placebo-controlled trial of selenium-βcarotene-CE plus methionine

		Baseline†			After placebo†			After Active †		
	Controls	P	A	T	P	A	T	P	A	T
All analytes	n 28	11	8	19	11	8	19	11	8	19
Linoleic acid (umol/l)	M 1160 SD 317 r 407-1823 p	857 312 426-1162 <0.001	965 206 604-1244 <0.001	903 271 426-1244 <0.001	934 252 648-1309 <0.001	882 230 611-1265 <0.001	913 231 611-1309 <0.001	919 191 673-1164 <0.001	1020 130 847-1187 <0.001	962 172 673-1187 <0.001
9, 11 isomer (µmol/l)	M 10 SD 22 r 8-46 p	35 14 16-61 <0.001	34 18 17-75 <0.001	34 15 16-75 <0.001	28 9 16-43 <0.001	30 20 13-76 <0.001	29 14 16-76 <0.001	25 8 11-37 <0.001	29 9 16-41 <0.001	27 9 11-41 <0.001
Molar ratio (%)	M 1.91 SD 0.79 r 0.81-3.90 p	4.34 1.66 1.86-6.92 <0.001	3.52 1.53 1.63-6.29 <0.02	3.99 1.62 1.63-6.29 <0.002	3.15 ‡ 1.07 1.28-4.46 <0.02	3.20 ‡ 1.30 1.28-5.99 <0.05	3.17 ‡ 1.14 1.28-5.99 <0.02	2.81 # 1.01 1.23-4.46 NS	2.77 # 0.69 1.90-3.83 NS	2.80 ‡# 0.87 1.23-4.46 <0.05

NB In practice only the percentage molar ratio of the isomer to parent fatty acid is used † Patients grouped as P = placebo first, A= active treatment first, or T=total ‡ significantly different from corresponding baseline value; # significantly different from corresponding placebo value. Adapted from ref 389

patients with RAP and 12 with chronic pancreatitis, including 2 with 'alcoholic' disease and 10 with idiopathic disease.

There was no benefit from active treatment by any clinical measure. The new biochemical information concerned vitamin C. Its baseline concentration in plasma was less than in healthy controls (mean±SD, 9.1±5.3 versus 14±3.8 mg/l, $p<0.05$), as was its bioactive fraction, ascorbic acid (5.7±3.5 versus 11±3.2 mg/l, $p<0.05$): moreover, the % of vitamin C in oxidised forms was higher in the patients (37.9±9.7 versus 19±8.5%, $p< 0.05$), indicating excess FRA. Subnormal baseline values for selenium, vitamins C, E and β-carotene were unchanged by placebo or active treatment. As regards SAM concentration, active treatment resulted in trebling over baseline. The percentages of oxidatively altered linoleic acid and vitamin C exceeded control values at the end of both placebo and active phases of treatment, indicating that oxidative stress was not alleviated by SAM treatment[390].

16.1.3. SAM plus selenium and β-carotene

This prescription resulted from a manufacturing error whereby β-carotene was inadvertently substituted for vitamin C, a mistake only identified after the study was over. The intention was to enrol 30 patients but the trial was abandoned when 3 fully compliant patients had an attack of pancreatitis while on subsequent treatment with the active prescription. Of the 14 who had been enlisted, there were 6 withdrawals: intolerable gastrointestinal symptoms in 3 cases; unpredictable medical problems in 2 cases (retinal detachment, prostate cancer), default by the last. When the trial was decoded, it transpired that a patient who completed the study and 2 who dropped out had a pancreatitis attack during the active phase, while 1 had an attack during the placebo phase. Pain scoresheets and diaries generated similar values during active and placebo treatment. The biochemical data mimicked those in the SAM trial, again showing that oxidative stress was not corrected by active treatment, although blood levels of selenium and β-carotene had normalised.

16.1.4. Comments on pioneering RCTs at Manchester

In setting up the first trial of micronutrient therapy, particular attention was paid to potential toxicity. Sodium selenite in a dose > 2000 μg/day was reported to cause side effects, the safety margin

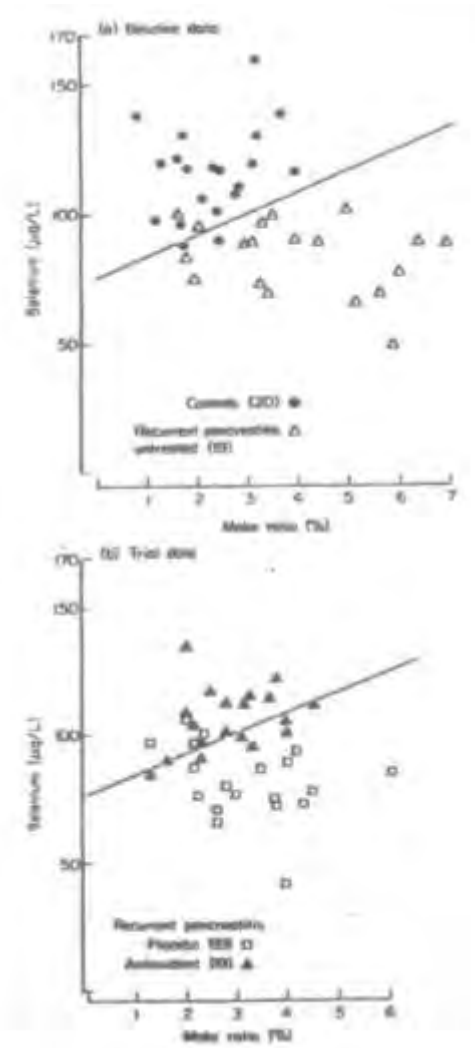

Figure 16.2 Discriminant analysis of serum selenium and the % molar ratio (MR) of the 9 cis 11 trans isomer relative to parent linoleic acid in controls and patients with recurrent pancreatitis. Discriminant function: selenium=8.76 x % MR + 75.74. Note that this function also applies to data from placebo-treated patients but fails to distinguish between controls and antioxidant-treated patients. Reproduced from 1992 paper in Aliment Pharmacol Ther[389]

increased when it is delivered as enriched yeast. Doses of vitamin A >200,000 U daily for 2 months or 40,000 U daily for 8 months reputedly ran risks of psychosis and hepatotoxicity, whereas β-carotene was seemingly non-toxic. A gm of vitamin C and up to 1200 U per day of vitamin E were regarded as safe. Organic psychosis was a threat from >10 gm methionine per day[385]: however, in the exploratory study a patient with a family history of schizophrenia developed such symptoms after 4 gm methionine per day for 6 weeks.

Daily doses of micronutrients in the first trial were 2-18 times the maximum RNI but well within the safety margin for each. The 20-week duration - compared with 2, 4 or 12 weeks in trials of pancreatic extracts - might have contributed to the 14% drop-out rate. Active treatment was associated with a significant reduction in attack rate and background pain, the latter over-and-above a strong placebo effect. The wide confidence intervals reflected the small number of patients and possibly also the decision to restrict the dose of methionine to 2 gm / day, although patients in the exploratory study occasionally needed twice as much (**Table 15.1**).

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Biochemical data confirmed that adequate exposure to antioxidants occurred in the active phase with no residual effect 10 weeks after switching to placebo, validating the crossover trial design. They also showed that doses were appropriate with regard to selenium and β-carotene but perhaps excessive for vitamin E: assays for vitamin C and its bioactive form of ascorbic acid had not been set up. Data on plasma SAM hinted at the likely modus operandi of the successful prescription. Given the suboptimal intake of methionine in CYP-induced patients with chronic pancreatitis (Chapter 7), the subnormal baseline plasma level of its first metabolite SAM was perhaps predictable. However, its downward drift upon active treatment in the first trial was not, as also the upward drift during placebo treatment and very high values at the time of an attack. These findings suggest impedance to the onward intracellular metabolism of SAM under conditions of oxidative stress, an interpretation reinforced by significantly increased plasma levels of SAM in the second trial when oxidative stress was uncorrected. They also are in accord with a report that, at the time of an alcohol-induced relapse, neutrophil methionine and cysteine levels exceeded those in acute cholecystitis controls, GSH was largely oxidised, and levels of urinary inorganic sulphur were subnormal[391].

Collectively these findings indicate the extension of free radical pathology from the pancreas to peripheral blood during a pancreatitis attack (Chapter 17). They suggest that oxidative / electrophilic stress in acinar cells interferes with methionine metabolism by attacking one or more enzymes in the methionine metabolic pathway, causing proximate metabolites to reflux into blood. The vulnerability of several enzymes in the route is recognised in regard to liver injury from paracetamol[222], and is underlined by the observation that GSH mitigates CCl_4 hepatotoxicity by protecting a critical cysteine moiety in SAM synthetase[392].

The concept of oxidative / electrophilic stress is dynamic and fluid, ie. the shortfall in antioxidant defence varies depending on an ever-changing load of FRA. This reasoning led to search for a nomogram that might be meaningful in clinical practice. After examining relationships between %MRLA' and each of the measured micronutrients, a 'selenium vs %MRLA' nomogram was identified (**Figure 16.2**). The identity of the discriminant line with that which separated data from controls and patients with cystic fibrosis[369] supports the notion that a selenium-dependant enzyme dictates partitioning between isomerisation and peroxidation pathways of free radical attack on PUFA, low selenium status favouring the former. Any ambition to pinpoint micronutrient influence on the

peroxidation route is foiled by the multiplicity and ephemeral nature of generated FROP.

The inefficacy of SAM alone in the second study shows that some other agent accounted for success in the original trial. Vitamin E was dismissed by its normal values in this and the next trial, which also discredited selenium and β-carotene. By a process of elimination, persisting lack of vitamin C with excess oxidation of its bioactive form of ascorbic acid could be implicated[390]. This conclusion is in line with reports indicating the ability of ascorbic acid to substitute for GSH and vice versa, by non-enzymic and enzymic interaction between their redox shuttles[202, 203]. Accordingly, Manchester chemists strove to validate assays for these key antioxidants[393, 394]. In the process a kit method which was touted as a simple gauge of 'total antioxidant capacity' was rejected, because the value reflects bulk antioxidants that are unaffected by treatment, ie. uric acid, glucose, albumin and bilirubin[395].

The twin problems of hindrance to methionine metabolism within an oxidative environment and methionine insufficiency are shown in reviewed later investigations of patients with quiescent chronic pancreatitis, whether alcoholic or idiopathic and irrespective of geography[11, 255]. (i) Peripheral blood displays a strong tendency to produce ROS. (ii) Plasma / serum contains excessive amounts of protein carbonyls and - it seems invariably in patients with active disease - lipid-based FROP as detected by measuring F_2 isoprostanes, lipid peroxides, thiobarbituric acid reacting products, the 9,11 isomer of linoleic acid and so on. (iii) Erythrocytes have subnormal levels of certain antioxidant enzymes, and GSH. (iv) Transmethylation and transsulphuration pathways remain fractured[337]. (v) ^{11}C methionine scanning demonstrates good pancreatic uptake of the amino acid but then its regurgitation coupled with impaired enzyme secretion into the duodenum [396,397].(vi) Subnormal plasma concentrations are reported of sulphur amino acids and thiols derived via the transsulphuration route, including GSH. (vii) H_2S, which is linked to pancreatic pain[1], appears in exhaled air[398]. It is not known whether any of these aberrations has a bearing on displacement of Munc18c into the cytosol of intact acinar cells, as was noted in the resected specimen of a patient with stable disease[273]: this 'SM protein' is involved in pathological basolateral exocytosis (Chapter 11).

16.2 Subsequent trials

16.2.1 Not relevant

Two studies tested treatments that did not address the fundamental problem in patients with chronic pancreatitis, ie. that underlying pancreastasis is linked to insufficiency of CH_3 and SH moieties in acinar cells in the face of electrophilic stress (Chapter 11). Thus, as only to be expected, a trial of allopurinol to inhibit xanthine oxidase was negative[399]. So too was a trial of curcumin[400] although the micronutrient is a potent antioxidant and inhibits the anaphylactoid response of mast cells[401]. Two other reports focused on treatment of a pancreatitis flare-up and found dramatic benefit from intra-rectal allopurinol or dimethylsulfoxide[402], or from intravenous chlorophyll-A[403]: the control of neutrophil-derived ROS is the likely explanation[404].

16.2.2 Manchester prescription

Table 16.7 summarises information on 8 completed trials that revolve around restoring methionine metabolism - whether or not these were placebo-controlled, but excluding any trial reported only in abstract[388 324, 348, 389, 405-409].

Almost as many meta-analyses have been published in the past 3 years[374, 410-414] ! Despite the inclusion in most assessments of RCTs adjudged satisfactory on mechanistic grounds, without considering the basis for treatment or legitimacy - eg. irrelevant measures cited in 16.2.1 above - all but the report with most infringements[414] concluded that active treatment reduces pain, and that although side-effects in up

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Table 16.7 Summary of clinical trials aimed at restoring methione metabolism

Author	Clinical	Trial type/Active treatment	Outcome	Biochemistry
Uden [388,389] Manchester 1990 / 1992	n=20 ACP 7, ICP 8 RAP 5 Opiates none Steatorrhoea none	20 week switchover double-dummy double-blind Rx: 6/day Se-βcar-C-E (Wassen,UK) 8/day methionine (Evans,UK) Dose/day: Se 600µg, βcar 9000 IU (= 5.4mg), C 540mg, E 270 IU (=270mg), methionine 2g	Attacks ↓ 11 pain words VAS ↓ Pain diaries 2nd 5-wk ↓ Pain psychology ↔ Placebo effect	Baseline Se, βcar < controls; E, A ↔; post-Rx all ↑↑ Baseline SAM < controls; downward drift post-Rx Baseline MRLA > controls; post-Rx ↓ Baseline pGSH.Px , uSO$_4$ = controls; post-Rx ↔ pSAM in attack ↑; Se vs MRLA nomogram useful No carry-over at 10 week
De las Heras Castano [405] Santander 2000	n=19 ACP 11, ICP 5, RAP3 Opiates? Steatorrhoea ?	Open trial for 12 months Rx: 4/day (hospital preparation) Dose/day: Se 300µg, βcar 12mg, C 600mg, E 188mg, methionine 1.6g	Admissions vs previous year ↓ Pain word VAS vs previous year ↓ Pancreatic function vs previous year ↔	Not done
Uomo [349] Naples 2001	n=3 children with HP	2 year open in 4 blocks of 6 months, Rx 2nd & 4th Rx: 2/day SAM, 3/day multivitamin (sources ?) Dose/day: Se 75µg, A 2.4g, C 180mg, Mg 300mg, E 30mg, SAM 800mg	Painful days ↓ Analgesics ↓	Not done
Kirk [406] Belfast 2006	n=19 all ACP ? Opiates ? Steatorrhoea ?	Trial type: as Manchester 1990 Rx: 4/day Antox (Pharmanord, UK) Dose/day: Se 300µg, βcar 12mg, C 600mg, *E 152mg, methionine 1.6g	SF-36 Quality of life score ↑ Pain ↓ Physical/social well-being ↑ (Nausea)	No control data; post-Rx Se,βcar,C & E > placebo Post-Rx A,βcar & TAC ↔ Post-Rx Fox1,MDA, & pGSH ↔ Carry-over at 10 week
Bhardwaj [324] Delhi 2009	n=127 study power 80% ACP 35, ICP 92 Calculi ? Opiates ? Steatorrhoea 20%	6 month parallel placebo or active Rx: ?/day Belamore G (Osper, India) Dose/day: as Manchester 1990 Plus all on pancreatic enzyme supplements	Admissions ↓ Pain free: active > placebo Pain days/month ↓ Analgesics ↓, man-days lost ↓ Improvement by 3 months (Headache, constipation)	Baseline A,E < controls, C = controls Baseline FRAP,E-TGSH & E-SOD < controls, Post-Rx all these ↑, vitamins by 1 month Baseline S-SOD & TBARS > controls, post-Rx ↓ 3-month data not given
Shah [407] Manchester 2010	n=137 ACP 84 Antox 68, non 69 28 disease-duration matched pairs	Prospective cross-sectional study of consecutive patients already on Antox (Pharmanord,UK) versus standard Rx. Antox dose not stated, likely variable	VAS: Antox vs Non (p=0.01) EORTC QLC C-30 &QLQ PAN-28 Quality of life: Antox group better on all counts (p<0.001-<0.0001) Opiates Antox vs Non (p<0.01)	Not done
Siriwardena [408] Manchester 2012	n=70 study power 80% ACP 51, ICP 19 CT calculi 66 Opiates? mean 88mg/d Marked EPI in many Prior intervention 54%	6 month parallel placebo or active Intended 4-block design ie +/- intervention Rx: 6/day Antox v1.2 (Pharmanord, UK) Dose/day: Se 300 µg, C 720 mg *E 228 mg, methionine 2.88g *βcar from coating 2.5mg	Clinic & Diary numeric pain score↔ Brief pain inventory ↔ 4 Quality of life scores↔ In-patient days↔Out-patient visits↔ 4-block data not given (Bad taste, heartburn, diarrhea, nausea)	No baseline data; no raw data post-active or placebo All data as change from baseline No evidence of micronutrient lack at baseline No evidence of oxidative stress by MRLA at baseline GSH and MRLA change post-active =post-placebo Post-active Se, βcar, E, C >> post-placebo Increments post-active suggest major reporting errors
Dhingra [409] Delhi 2013	n=61 ACP 21, ICP 40 strict exclusion criteria Steatorrhoea 15	3 month parallel placebo or active Rx 8/day Belamore G (Osper, India) Dose/day: as Manchester 1990, Delhi 2000	Pain days/month Pain diary, Analgesic use Pain less on active, data sparse	Baseline TBARS > controls, FRAP < controls Baseline TGF-β1 & PDGF-AA > controls (p 0.07) Post-active ↓drift in TBARS & ↑FRAP Post-active PDGF-AA ↓, TGF-β1↔

Abbreviations alphabetically. Clinical ACP: alcoholic; HP: hereditary; ICP: idiopathic; RAP: recurrent acute; EPI: exocrine pancreatic insufficiency by faecal elastase assay. Trial type βcar: β carotene; C, E: vitamins C & E; Mg: magnesium; Rx: active treatment; SAM: sulphadenosylmethionine; Se: selenium. Outcome EORTC QLC-30: European Organization for Research and Treatment of Cancer Quality of Life Questionnaire Core questions 30, and Pancreatic Modification questions 28; VAS: visual analogue score; vs: versus. Biochemistry A: retinol; AA: ascorbic acid; αcar: α carotene; E-TGSH, E-SOD: erythrocyte total glutathione and superoxide dismutase respectively; FRAP :free radical trapping ability of plasma; Fox1: ferrous oxidation; MDA: malondialdehyde; MRLA molar ratio of linoleic acid isomer to parent acid; pGSH: plasma glutathionine; ROS: reactive oxygen species; S-SOD: serum superoxide dismutase; TAC total antioxidant capacity; TBARS thiobarbituric acid reacting products of lipid peroxidation; TGF-β1: transforming growth factor-beta1; PDGF-AA: platelet-derived growth factor-AA. Arrows signify direction & degree of change. * doses from senior technical officer at Pharmanord, Denmark. Adapted from ref 11.

to 19% were usually mild (eg.headache, nausea, allergy, constipation, diarrhoea), they did cause some patients to withdraw from the trial. The first trial, published in 1990[388], was the only RCT to tick all the boxes in the report under the Cochrane banner[411]: an attempt to gauge quality-of-life used questionnaires devised for patients with chronic backache and was inappropriate in retrospect but the best available at the time. Interestingly, reports that were deemed fit for inclusion were not the same in these meta-analyses. All authors called for further large-scale studies.

Subjective assessments during this period concluded that micronutrient antioxidant therapy was convincing[415, 416]; had potential[417, 418]; could be useful as adjuvant therapy[419]; was poor, based on the authors' limited experience[420]; or useless[421].

In the original cross-over trial clinical improvement was accompanied by migration of datapoints towards the control zone in the nomogram relating serum levels of a lipid oxidation marker and selenium (**Figure 16.2**)[389]. Amelioration of oxidative stress concurrently with clinical improvement was shown in 2 other trials[324, 409], of which the second also documented a reduction in markers of fibrosis[409]. Clear benefit from active methionine / SAM -incorporating treatment accrued in all but the 2014 'Anticipate' trial[408], although its authors argued strongly in favour 2 years earlier[407], in a cross-sectional study of patients already on micronutrient supplements versus no supplements under a later policy (**Table 16.7**). Unfortunately, serious flaws render the second report invalid, while diluting the value of micronutrient therapy in each meta-analysis (see below). .

Of special note in **Table 16.7** is the observation that the combination of SAM, in lieu of methionine (**Figure 16.1**), plus vitamins A,C,E and magnesium was beneficial in 3 children with hereditary pancreatitis[349]. Moreover, as described in Chapter 15, combination therapy was also highly successful in abolishing attacks in patients with FLLD, and in a patient with primary haemochromatosis.

16.2.3 Other prescriptions

(i) As observed above, SAM alone or with selenium and β-carotene, was ineffective in studies from the UK. (ii) This was also true for selenium and vitamin C in a study from India, the report of which gave no diagnostic criteria, stated gall stones / common bile duct stones as the commonest etiological factor, did not specify if these problems were on-going, and administered proton pump inhibitors plus pancreatic extracts - both with antioxidant potential - to treatment and no-treatment groups[422]. (iii) By contrast, in an open study from Brno in the Czech Republic, vitamin C (0.5 gm/day) plus vitamin E (100 mg/day) for 12 months resulted in substantial pain reduction, to the point of abolition in 44% of 70 patients with mainly alcoholic disease. This was largely attributable to vitamin C, in that vitamin E levels were inexplicably unchanged by treatment, and with the greatest decrements in ROS generation within blood and in its concentration of lipid peroxides among patients with the most functional parenchyma, judging by grade of pancreatogram abnormality[423] (iv) A study reported in Polish[424] is cited in 2 meta-analyses. The prescription (vitamin C 0.4 gm/day, vitamin E 300 mg/day) or no treatment was administered in an open RCT for 6 months in 91 patients with alcoholic disease, while blood antioxidant levels were monitored. The English abstract reports that 68% of the group of 46 on active treatment became pain-free versus 31% of 45 untreated (p=0.002). The treated group also had fewer pancreatitis relapses (p=0.03) and their weight improved (p= 0.001), as did pancreatic exocrine and endocrine function (p=0.001, p=0.015 respectively). (v) An anecdotal report suggested protection from pancreatitis attacks by a grape seed extract with potent antioxidant qualities[425].

A trial of the Manchester prescription plus pregabalin, a presynaptic voltage-gated blocker of the calcium channel, has just been reported (NCT01528540) and is discussed in Chapter 19. The results are awaited of EUROPAC-2, a long-running RCT involving a potent commercial preparation of the antioxidant combination ('Antox version 1.2', Pharmanord, UK), versus magnesium, versus placebo in patients with hereditary or idiopathic disease.

16.2.4 Comments on studies bar 'Anticipate' trial

(i) Methionine / SAM-based treatment curbs background pain in chronic pancreatitis pari passu with a fall in markers of oxidative stress and restoration of erythrocyte GSH. This holds true irrespective of geography, putative aetiology, pancreatogram appearance and whether or not intraductal calculi are present.

(ii) The Delhi study shows that blood micronutrient profiles improve within a month and pain is controlled by 3 months, in keeping with the Manchester and Belfast experience at 10 weeks.

(iii) The Brno study indicates that the degree of electrophilic stress is highest in patients with the most functional parenchyma, ie. with near-normal pancreatograms, and this subgroup experiences the greatest improvement on treatment. Moreover, the study shows that it is illogical to expect relief from oxidative stress-induced inflammatory pain in patients with little functional parenchyma.

(iv) Misleading information from measures of total antioxidant capacity is underlined by the studies from Belfast and Delhi. In the former case, a kit method failed to detect increased antioxidant status following micronutrient therapy, unsurprising as it reflects the bulk antioxidants of plasma[395]. As to the FRAP assay, as used in Delhi, vitamin C via its bioactive component of ascorbic acid normally contributes 20% to the reading, whereas the contribution from other items in the standard micronutrient prescription is negligible. As argued in an earlier review[255], the assay value in Delhi controls is much less than elsewhere although the endogenous component was normal and plasma 'total vitamin C' as high as at Manchester, on average 12 mg/l[390]: furthermore, the lower reading in patients than controls improved on treatment although their baseline 'total vitamin C' level was normal. The first anomaly may be methodological. The second strongly suggests low values in Delhi controls of ascorbic acid with a further fall in chronic pancreatitis, but masked in the 'total vitamin C' assay. Loss of ascorbic acid due to harsh culinary practices is the likely explanation (Chapter 12).

(v) Studies of acute pancreatitis in cyclosporin-treated pancreatic allografts[426] suggested that SAM may be enough (Chapter 17). However, as shown by the aborted clinical trial at Manchester, it was not[390]; whereas combined treatment attenuated damage in a cyclosporin-hyperstimulation model of pancreatic fibrosis[427].

(vi) It seemed unlikely that vitamin C alone could be effective - as in reports from the Czech Republic and Poland. Clinical improvement in these studies, as also in the report on grape seed extract, suggest that methionine intake was adequate, such that ascorbic acid protected enzymes in the methionine metabolic route. There are experimental precedents. Thus: a synthetic analogue of ascorbic acid improved survival in the virulent CDE dietary model of acute pancreatitis, and was very helpful in the caerulein hyperstimulation model (Chapter 11); ascorbic acid ameliorated in dibutyltin pancreatitis[428] ; and it attenuated the secretory blockade caused by the potent oxidant tertbutylhydroperoxide[283]. Worth noting too, is a study of patients admitted with acute pancreatitis in whom mega-dose treatment with vitamin C alone, 10 gm/day intravenously for 5 days compared to 1 gm/day in a parallel set, proved beneficial clinically as also in correcting oxidative stress and immune dysregulation[429] (Chapter 17). The multifarious properties of ascorbic acid in affording protection

against ROS / RXS and especially in curbing mast cells have already been described.

(vii) Later longer-term studies showed that the positive effect of treatment transcends gene mutation and ethnicity, over-and-above HP-associated PRSS1 mutation[348]: eg. idiopathic disease with CFTR mutation in the UK[357]; and idiopathic disease in India, associated with mutation in both SPINK1 and CFTR[326].

16.2.5 Critique on 'Anticipate' trial

This study is invalidated on many fronts. (i)There are no baseline data on micronutrients or oxidative stress markers. (ii) Relevant information is presented in a befuddling manner, as change from baseline. (iii) The authors failed to cite their earlier positive report[407]. (iv) It is assumed that the lack of clinical improvement despite increases in blood levels of prescribed micronutrients - to highly toxic levels if the data are to be believed - implies treatment failure, when in the absence of any evidence that oxidative / electrophilic stress is corrected, merely shows that patients with chronic pancreatitis can absorb micronutrients. (v) Pain in the majority of patients was almost certainly not of pancreatic origin.

Arguments In support of the last deduction have been published[401]. The diagnosis was uncertain in 9 patients with near-normal pancreatograms but no secretory study. Faecal elastase concentration < 100 µg/gm showed severe exocrine insufficiency in several patients (Pancreatic Elastase 1™ Stool Test, Schebo® , Biotech AG, Giessen, Germany): gut pain due to undigested fat, whether or not accompanied by steatorrhoea, is a major contributor to poor life quality in patients with chronic pancreatitis. Narcotic addiction, as shown by an average morphine dose of 88 mg /day, causes gut dysmotility and also intractable pain that is indistinguishable from that of chronic pancreatitis - quite apart from ulterior motives of drug addicts in bemoaning unremitting pain. Readers are not told how many, if any, of the trial participants were in employment - salient in that occupational chemicals are connected to disease development (Chapter 8). It is also not divulged how many of the 50% of patients with prior intervention had a duct-drainage procedure - which should blunt pancreatic oxidative stress and mast cell-activated pain circuits.

In a later publication is tacit admission - after analysis of blood cytokine profiles in a subset - that in these opiate-dependant patients, active pancreatic inflammation was not the cause of pain[430].

Thus, the authors' and commentators' assertion of treatment failure exposes misapprehension[401]. It is tantamount to pronouncing penicillin ineffective in bacterial infection when fever is due to, say, collagen vascular disease. In other words the 'Anticipate' trial compared placebo with a cosmetic substance - a fatal fissure that cannot be plastered over by a bewildering plethora of life-quality questionnaires administered to narcotic addicts. The argument that a preponderance of alcoholics (51 of 70) accounted for the negative result is thus unwarranted - and in any case ignored results from Brno (59 0f 70)[423] and misquoted data from Delhi (40 of 127)[324].

16.3 Pancreatic extracts: micronutrient therapy by proxy

Pancreatic extracts have found their way into clinical practice to treat painful chronic pancreatitis despite consensus from analysis of RCT that there is no clear evidence of benefit. Proponents argue that success depends upon the use of non-enteric coated material to ensure delivery of proteases into the duodenum[432, 433] - rather than enteric coated preparations that deliver the enzymes further downstream[434]. It is posited that intraduodenal delivery 'puts the pancreas to rest' by dampening the feedback loop that otherwise leads, via high circulating levels of CCK, to pain from pancreatic ductal hypertension. Clinicians are informed that patients with small-duct disease and mild to moderately impaired

pancreatic secretory capacity will benefit, whereas those with advanced disease / steatorrhoea will not. A proton pump inhibitor is advised, to safeguard the extracts in transit through the stomach. Treatment is advocated for 4 weeks, to be continued for 6 months in responders and indefinitely should pain resurface upon attempted withdrawal of treatment.

Each aspect of the argument and advice is questionable[435]. (i) In the original successful trial active treatment ameliorated pain within just a week. (ii)The apical exocytosis apparatus in acinar cells is paralysed in a pancreatitis attack, and remains hindered thereafter in patients with chronic pancreatitis[1]: hence the gland needs to be coaxed into activity, not lulled into further inertia. (iii)There is evidence against the proposal that pancreatic proteases in the duodenum inhibit pancreatic secretion in man. (iv) The advice on gastric acid inhibitors speaks from hindsight, as none was used in either trial. (v) Apparently so too is the restriction to patients with small-duct disease, which seems odd in that their big complement of secretory parenchyma, relative to that in patients with large-duct disease, should ensure sufficient intra-duodenal protease to maintain physiological levels of CCK release.

A more plausible explanation is that pancreatic enzymes in non-enteric coated formulas are irrelevant, and that what matters is the fortuitous delivery of other anti-nociceptive substances. Micronutrients with antioxidant potential are the prime candidate because of strong evidence implicating electrophilic / oxidative stress in disease pathogenesis (Chapters 3 & 5), and the success of micronutrient therapy shown by meta-analyses. Moreover, the pancreas actively assimilates selenium, zinc, and methionine.

Selenium, zinc and other trace metals that contribute to the antioxidant repertoire of tissues should survive procedures to extract pancreatic enzymes from porcine glands, and the sulphur amino acids might, whereas at risk of denaturation are the vitamin antioxidants including choline which interacts with methionine to guarantee pancreatic integrity. Attempts to settle the question - upon shrinkage of the productive pancreatic laboratory at Manchester Royal Infirmary - were unfruitful (requests to IS Young at Belfast in 2010, PM Garg at Delhi in 2013). However, repeated interrogation of the literature unearthed 2 papers, both in the context of cystic fibrosis, that support the principle.

The first was from Holland in 1992, documenting the presence of selenium, zinc and copper in 4 pancreatic enzyme preparations among which 'pancreatic grain' - probably non-enteric coated - scored highest[436]. The second report from Switzerland in 1998 went further[437]. Analysis of 5 enteric-coated preparations confirmed the presence of selenium. More importantly, a longitudinal study showed that, as a result of increased selenium intake from increasing doses of pancreatic enzymes to control steatorrhoea, plasma selenium and selenium-glutathione peroxidase activity increased significantly and in parallel, peaking by the eighth month but evident from the first sampling point at four months. The authors calculated that extracts provided the children with a selenium supplement amounting to 50% or higher of the recommended daily allowance of 1 µg/kg/day.

A subnormal concentration of selenium in serum / plasma has been reported in patients with chronic pancreatitis, irrespective of geography[255]: in studies from Manchester the lowest values were in patients with painful disease[300], in contrast to relatively high values in patients with painless disease in south India[322]. A subnormal level of zinc has been noted in plasma or erythrocytes of patients with the disease[64, 66,67]. The information on copper is dichotomous: pancreatic acinar cells have virtually no copper-superoxide dismutase[255], but an increase in serum copper as caeruloplasmin is recorded in patients and does not represent an acute phase reaction (Chapter 3). Like selenium, zinc stabilizes mast cells[438].

Selenium is maximally absorbed from the duodenum. The increment in circulating levels is more rapid when the element is delivered in organic form, whereupon an increase in plasma selenium can be expected in 1-2 weeks of supplementation at doses of 50-200 µg/day in selenium-deficient individuals[437]. Almost certainly, selenium in pancreatic extracts is in organic form, as selenomethionine, selenodiglutathione, selenocysteine and other compounds - which implies increased availability of sulphur amino acids that are critical for pancreatic viability. Unfortunately however, it is impossible to estimate selenium intake in trials of pancreatic extracts because neither the weight nor enzyme composition was specified of the material.

Provision of the extracts as a liquid in the first successful study might explain the rapidity of pain relief if from absorbed selenium, especially in that Scandinavia is a low selenium area: however, this deduction is predicated on the assumption that heat treatment of granules to destroy pancreatic enzymes in the placebo somehow curbed selenium absorption too[432]. The western states of the USA constitute a high-selenium area. It is unclear if this extends to Florida in the east, from where the second trial originated[433]. A gradual increase over time in assimilation of selenium, zinc and sulphur amino acids could rationalize the reported improvement in life quality and pain in 2 long-term observational studies - of coated or unspecified material[434]. Co-prescription of a gastric proton pump inhibitor, as advised, would enhance antioxidant activity in many ways[439]. In light of all this evidence, the long-term administration of pancreatic extracts to patients with painful chronic pancreatitis can be regarded as AOT by proxy, irrespective of whether any additional benefit accrues[435].

16.4 Value of long-term treatment

16.4.1 Surgical audit: Manchester 1983-1992

After the positive results of micronutrient AOT in 49 patients (Chapters 15 & 16. 1) - elderly patient with recalcitrant pancreatitis; child with large-duct calcific disease; 23 patients with recurrent attacks in the dose-seeking study; 3 patients with FLLD; a patient with haeomochromatosis; and 20 who qualified in the first RCT trial of SeβCE plus methionine - the prescription was issued to registered patients with chronic pancreatitis, relapsing or post-acute pancreatitis, and from the outset to new referrals with these conditions.

In June 1992 a cross-sectional audit was undertaken by a senior surgical registrar of patients with chronic pancreatitis on standard AOT attending the Pancreato-Biliary Unit[440]. The case notes of patients were retrieved and patients omitted if they fell into the following categories: painless disease throughout; known exocrine pancreatic failure; addicted to narcotic analgesics; chronic renal failure; pregnancy; current pain likely due to non-pancreatic origin; pancreatic cyst / pseudocyst >5cm; pancreatic cancer so strongly suspected that surgical resection for diagnosis was called for; follow-up < 2 months because laboratory data indicated that normalisation of selenium and vitamin C levels could not be guaranteed before then.

The final analysis included 94 patients, 60 males and 34 females, of average age 45 years (median 45, range 8-83)[431]. Follow-up was for an average of 30 months (median 19.5, range 2-131), with 22% of cases monitored for 5+ years. Comprehensive assessments of pain and its psychological impact were clearly impractical in the out-patient setting. Hence, crude and subjective impressions of pain were graded as ‘no pain, ‘clear improvement’ or ‘no change’, together with information on analgesic usage. Also recorded were weight pre-treatment and at last attendance, ability to work and engage in social activities, number of days in hospital in the year pre-AOT and total number of days during the period of follow- up. Resources for blood assays to assess assimilation of the prescribed micronutrients were not available until some

months after the audit date. The only gauge of concordance with treatment was a strong signal for vitamin C on urine dipstick testing at clinic visits. Non-compliance was admitted by 4 patients, including 2 who consumed alcohol in excess.

Exposure to xenobiotics was recorded in all-or-none terms to cover the 2-year period before the first symptom. Occupational exposure was present in 68% of cases; 67% smoked >10 cigarettes daily, and 45% drank excess alcohol (≥80 gm/day in men, ≥60 gm/day in women). Hypertriglyceridaemia was found in 9% of cases, a past history of hyperparathyroidism in 2%, multiple factors in 35% and no factor in 10%. At the time of the survey, biliary tract disease was regarded as coincidental or secondary to bile duct constriction in the head of the pancreas: 25% of patients had undergone cholecystectomy prior to referral, but only 2% for gallstones, which were found at initial referral in a further 21 cases (22%). Overall, pre-referral surgery on the biliary tract, pancreas, stomach or duodenum had been done in 32 patients (34%).

After imaging studies, 'large-duct' disease' (moderate or advanced-change pancreatitis on ERCP and/or calculi) was identified in 85% of patients, and 'small-duct' disease by secretory studies in 15; an inflammatory mass in 10% of cases; and a pseudocyst in 14%. Before treatment 16% had diabetes mellitus; none was on pancreatic enzyme supplements.

Statistical comparisons were made by confidence limits (95%), student's t test (2-tailed), χ^2 (2-tailed), Wilcoxon matched-pairs test and Spearman rank correlation coefficient. Differences were regarded as significant when $p \leq 0.05$.

No patient needed operative ERCP or any form of duct decompression or resective pancreatic surgery during 248 patient-years of follow up. The total number of days spent in hospital while on treatment was significantly lower than in the preceding year (median 4 days, range 0-82 with 95% CI 2.2% versus 18, 0-150 days, CI 5.5%, $p<0.05$). The percentage of patients who were rendered pain free was 78% and a further 7 % had a substantial reduction in pain. Of 19 patients who were still in pain, including 2 who failed to take the tablets as prescribed, 7 (7% of the total) had intermittent pain compared to pre-treatment continuous pain. Two patients had continuous pain compared to 29 before micronutrient therapy ($p<0.001$), and treatment had no impact on pain in 12 cases of whom 6 had cysts/pseudocysts (including 2 non-compliant patients), and 4 cases with fluid collection that did not resolve.

Among 69 patients in whom body weights pre- and post-treatment were available, AOT was associated with significant weight gain. Social rehabilitation, as gauged by the ability of patients to get back to pre-morbid activities, was achieved in 88% of cases. Of the 76 patients previously in employment, 88% were back at work and 80 % of them were doing the same job. Of the 42 patients who drank alcohol excessively, a third continued to drink as previously, half had abstained altogether, and the others had reduced their intake to 'safe' limits. Inexplicably, there was no significant change in ordinary analgesic usage, but no way of knowing whether this was due to residual pain or the fear of it, or for a concomitant unrelated problem. There were no consistent side effects that could reasonably be attributed to AOT.

16.4.2 Extended observations: Manchester 1998

The positive outcome of the surgical audit continued through until 1998, such that pancreatic surgery for painful chronic pancreatitis was virtually obsolete (RF McCloy & JM Braganza, unpublished). This excellent result was facilitated by strict guidelines: patient-controlled devices to deliver morphine forbidden; psychiatric help sought early when dependence on narcotic analgesics or despair at social upheaval loomed; input of primary care practitioners solicited; nutritionist, social worker and pharmacist engaged

from the outset. The morphine prescription in patients already dependent at referral was devolved to the pain team. A weekly medical-surgical clinic was preceded by an inter-disciplinary discussion on patients to be seen that day, co-ordinated by a medical registrar, nurse specialist, and biochemist. Each doctor had a printout of previous antioxidant and % MRLA' data, so that selenium dosage could be adjusted with reference to the nomogram (**Figure 16.2**), while whole blood GSH helped to assess methionine adequacy, and that of vitamin C by reference to % MRVC. The concentration of pancreatic isoamylase in serum was also measured at each visit, because an increase above normal could reflect continuing subclinical pancreastasis.

The full micronutrient prescription was usually needed for 6 months while dietary advice was given on antioxidant-rich foodstuffs. Negotiation with executives from Pharmanord, UK, resulted in a combination tablet that incorporated methionine, so reducing the number to an average of 4 per day compared to 14 per day in the original trial. Further improvements were made by the company in order to increase the daily dose of methionine, while limiting β-carotene to the shell, because of cosmetic distress from a yellow hue, resulting in 'Antox version 1.2'. However, a switchover trial design without a wash-out phase, as in the seminal Manchester study[388], is now inappropriate because of a substantial carry-over effect (Chapter 18.3).

Treatment failure in around 10% of approximately 300 patients with chronic pancreatitis was associated with large cysts / pseudocysts, non-compliance by unreformed alcoholics, and undiagnosed neoplasia in 2 patients (adenocarcinoma, papillary mucinous).

Treatment also failed in 3 patients with RAP (ie. with the highest amount of functional parenchyma) who were referred after the audit. The addition of folate to provide more CH_3 groups did not help, but a choline supplement aborted further attacks (JM Braganza unpublished). This benefit concurs with a shift in thinking, ie. that 'reductive stress' is the common cause of ROS generation in human pathology[255]. The problem is considered alongside other 'stresses' in Chapter 19, but noteworthy now is its correction by biomolecules with electrophilic methyl groups, ie. SAM, phosphatidylcholine, betaine and carnitine[441-443].

The need to address reductive stress in patients with chronic pancreatitis is in tune with the experimental observation that polyenylphosphatidyl choline protects against pancreatic oxidative stress in alcohol-treated rats[444]. When choline intake falls short, the phospholipid can be synthesised from phosphatidyl ethanolamine, but incurs severe pressure on 'the universal methyl donor', SAM (**Figure 16.1**). Today there is a convergence of thought on the mechanisms of liver and pancreatic damage from a protracted excess of alcohol. Thus, the combination of 3 methyl donors - SAM, folate, betaine - alleviated alcoholic liver injury, while at the same time rectifying the elevated SAM / SAH ratio and homocysteine level[445]. As noted earlier, elevated plasma homocysteine concentration has been recorded in patients with chronic pancreatitis from south India, in conjunction with subnormal folic acid[337]; north west England, alongside decreased vitamin B_6[338]; and south Africa, in conjunction with low vitamin B_{12}[64] - albeit not in Dutch patients[339].

All this evidence notwithstanding, Manchester clinical studies illustrate the critical importance of ascorbic acid in protecting the exocrine pancreas, as is highlighted by its ameliorating effect in animal models of mild and severe pancreatitis[255]. Choline intake and status were unfortunately not assessed, but are needed urgently. In the meantime, the addition of a choline supplement to the successful micronutrient cocktail is judicious - rather than relying on dietary advice alone - and should probably take precedence over calls

to prescribe zinc[325], folate[337] or magnesium[446]. The inescapable conclusion is that the CDE dietary model of acute pancreatitis, which is easily modified to cause inflammatory fibrosis, is highly relevant to clinical pancreatitis.

Unfortunately, after 1998 new consultants brought their training experience to bear, such that the micronutrient prescription ceased, patient-controlled pumps to deliver morphine were purchased from monies raised by the patients support group, and morphine usage soared as did interventional ERCP - practices exposed by the 'Anticipate' trial (Section 16.2.5)[408].

16.4.3 Prospective follow-up study: Delhi 2016

A report of this investigation in 313 patients has recently appeared on-line[447]. While generally affirming the long-term benefit of AOT at Manchester, the report is questionable on several fronts. (i) It is difficult to see how a diagnosis of small-duct disease was made at the outset without a secretory study in around 15% of patients. (ii) If the entry CT finding of an 'atrophic pancreas' without a pancreatic function test in 214 patients is taken to imply end-stage disease, by which point natural resolution of pancreatic pain is anticipated, it is difficult to see why AOT was administered at all to this subgroup with likely gut pain. (iii) Presciption / withdrawal of A0T was based on intuition, not hard evidence as to whether-or-not oxidative stress was present at recruitment and if corrected-or-not. (iv) Opiate-dependant patients were included and apparently allowed on-demand self-treatment. (v) Also included were patients in whom pain was more likely to reflect a non-pancreatic problem such as peptic ulcer. Telephonic questioning would not recognise these subtleties. (vi) Readers are informed that 'young age and less advanced chronic pancreatitis with adequate pancreatic reserve' accounted for AOT failure in 15% of patients - but in the absence of any test of exocrine pancreatic function the statement is tenuous. (vi) The authors failed to hark back to first principles when the treatment was devised at Manchester in the late 1980s (Chapter 15): some patients needed a daily dose of 4 gm methionine to control symptoms.

All these differences from the Manchester experience might explain why a substantial number of patients at Delhi were judged to be failures of AOT and went on to have endoscopic procedures, lithotripsy and finally surgery - although the presence of pancreatic calculi *per se* does not equate to painful disease. Whereas the positive outcome of AOT overall merits its adoption as first-line treatment by medical practitioners, should a patient not respond within 10 weeks it is imperative that there is access to a tertiary referral centre where the focus should be as follows. Is the pain really pancreatic? Is there an underlying tumour? What is the potential xenobiotic load via alcohol / smoke / occupational volatile chemicals? Is there evidence for on-going oxidative / electrophilic stress? Is micronutrient antioxidant status adequate? Biochemical assessments are not cheap, but should be considered against the expense of the traditional approach to painful chronic pancreatitis - in-patient days, endoscopy and lithotripsy costs, surgery, and unquantifiable effects of narcotic addiction, job loss and social upheaval. This is not to detract from the importance of counselling on an antioxidant-rich diet, as also on withdrawal of alcohol and cigarettes.

16.5 Why does the prescription work?

In relation to lowering of pancreastasis-pancreatitis episodes, the likely explanation is the restoration of the apical exocytosis machinery in acinar cells, by protection of CFTR and enzymes in the methionine metabolic route. In regard to amelioration of background pain, the blunting role of the prescription on mast cell-induced activation of central sensitisation nociceptive pathways might be more important than the expected reduction in interstitial fluid pressure consequent upon correction of subclinical pancreastasis. Removal of H_2S-provoked pain might be relevant too[1]. Oxidative / electrophilic stress that

permeates the interstitium is expected to provoke fibrosis, but this is a long way from disease-initiating mechanisms and hence, although muted by AOT, is unlikely to be a factor in pain control. These aspects are re-addressed in Chapter 19.

16.6 Overview and summary

So impressive was the outcome of micronutrient therapy for chronic pancreatitis at Manchester that by 1998 not only was operative ERCP or surgery to treat pain virtually redundant [37, 431], but also a course of treatment emerged as a useful therapeutic test[1]. (i) If a comprehensively investigated patient with unexplained chronic abdominal pain fails to gain relief after 10 weeks of the micronutrient prescription, the possibility of small-duct chronic pancreatitis is excluded. (ii) If after this time-frame a patient with chronic pancreatitis fails to gain full relief from the treatment and does not have a large cyst / pseudocyst, the residual pain is likely to be non-pancreatic: eg. gastric ulcer, Tietze's syndrome, spastic colon. (iii) If a treatment-compliant patient with the disease responds well to start with but then has an unexplained setback, the strong possibility of an undisclosed pancreatic tumour warrants diagnostic laparotomy even though every imaging test is negative - as in 2 cases in the series (tiny adenocarcinoma in the neck of the gland in the first case; papillary mucinous tumour in the other, revealed 2 years after freedom from recurrent attacks with weight gain of 10 kg): this was also the experience of clinicians in the recent report from Delhi[447].

Success was less consistent in patients with idiopathic RAP in whom the highest safe doses of micronutrients sometimes proved insufficient to protect the large amount of functional parenchyma against excess FRA. In this group, in particular, the value of choline supplements needs to be probed.

Finally, experts in the free radical field offered the following advice. (i) The phrase 'micronutrient therapy' is less emotive than 'antioxidant therapy' and in the context of chronic pancreatitis is germane in that non-antioxidant roles of micronutrients and / or key metabolites are vital (eg. trypsin control by GSH). (ii) It is important to identify electrophilic stress from RXS, as opposed to stress from an excess of ROS alone, because natural rectifying measures against the former are substantially fewer.

Chapter 17

Towards first-line treatment for Acute Pancreatitis

Bearing in mind experimental evidence that a burst of FRA is the detonator of acute pancreatitis, it seemed logical that AOT should be the lynchpin in management, while recognising that by the time of admission a toxic brew is in the circulation (Chapter 11), the inflammatory response is in disarray and frustrated phagocytosis is driving on towards multiple organ dysfunction syndrome (MODS) - not least shock lung (**Figure 17.1 top**) and ileus (**Figure 17.1 bottom**). Eclectic studies described in this Chapter generated data that were novel at the time of publication and which still hold clues for specific medical treatment of this potentially fatal disease.

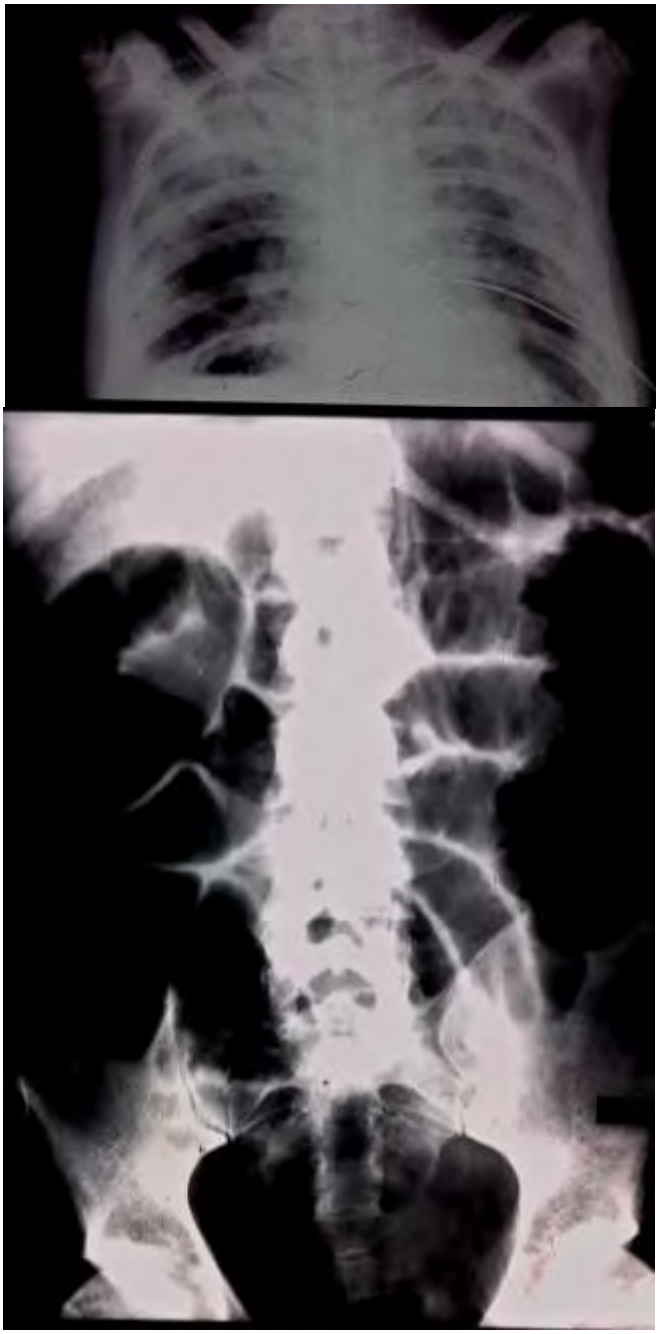

Figure 17.1 Xrays of patients with a fatal pancreatitis: shock lung (**top**); ileus (**bottom**).

17.1 Anecdotal clinical experience: positive impact of N-acetylcysteine

In 1986 the opportunity arose to administer NAC to a patient who developed acute lung injury and also anuric renal failure within hours of laparotomy under general anaesthesia to drain a pancreatic abscess. The decision was based on the following observations. (i) Benefit from this GSH precursor is established in clinical paracetamol poisoning which results from RXS produced via CYP. (ii) SH groups in cysteine and GSH protect against the potent oxidant HOCl - which is released extracellularly and immobilises $\alpha_1$protease inhibitor during frustrated phagocytosis - and thus against extracellular neutrophil elastase[256].

The improvement was dramatic[448], and prompted a randomized trial in consecutive patients with MODS, of whom half had acute pancreatitis (**Figure 17.2**). APACHE II scores were compiled at admission to the intensive care unit and at intervals thereafter. The intention was to match patients by age and admission scores, so that half had optimal standard treatment including intermittent haemodialysis, and the others had additional NAC. Seven of 8 patients in the former group died, conforming with the expected mortality of 84% for admission scores >35. However, in the latter group only 1 of 7 patients

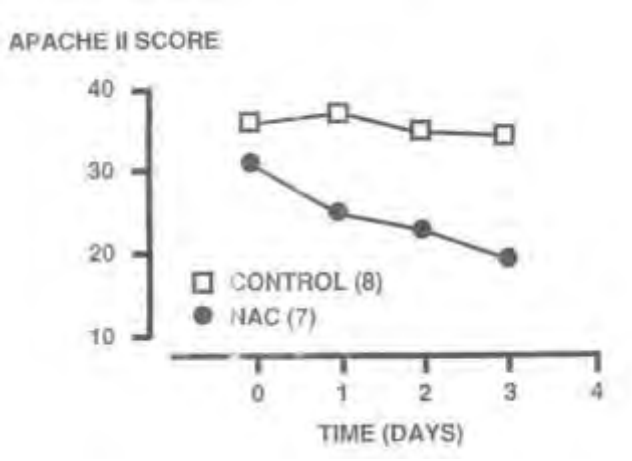

Figure 17.2 Median APACHE II scores of consecutive patients with multiple organ failure. Values significantly lower on 2nd and 3rd days in subgroup receiving N-acetylcysteine (NAC) over-and above optimal treatment including haemodialysis. (A Holmes, AR Moreton & JM Braganza report to North West Kidney Club, UK 1987).

died, whereas 73% mortality is expected for the registered scores of 30-34[384].

17.2 Allograft pancreatitis: protection by S-adenosylmethionine

17.2.1 First study

This investigation was proposed and directed by IV Hutchinson of the Immunology department in the medical school. Rats of Pebold Virol Glaxol (PVG).RT1^c strain, aged 2-3 months, were bred and maintained in the animal unit. Male PVG.RT1^u recipients were rendered diabetic by a single intravenous injection of streptozotocin, 60 mg/kg. The pancrcreaticoduodenal method of grafting was used, in which exocrine secretions continue to drain into the duodenum[449].

Study groups are listed below, data later compared by paired or Student's t tests.

Series A: to establish surgical technique and study the course of rejection (daily inspection, abdomen palpation, blood glucose)
A1, n=8 PVG.RT1^u-to-PVG.RT1^u isografted
A2, n=10 PVG.RT1^c-to-RVG.RT1^u allografted

Series B: for graft histology (paraffin- fixed and stained with haematoxylin-eosin)
B1, n=8 isografts, 2 sacrificed on days 2,4,6,8
B2, n=16 allografts, 2 sacrificed daily to day 8

Series C: for blood amylase (tail vein blood sample for 8 days analysed by blue starch method)
C1, n=4 sham-operated for 45 minutes
C2, n=4 isografted
C3, n=4 allogarfted

By 24 hours of streptozotocin treatment, blood glucose exceeded 28 mmol/l (normal < 8 mmol/l). Normoglycaemia was restored in each isografted animal, the early increase in serum amylase at day 2 was waning (**Figure 17.3**), and all animals survived >100 days. By contrast persisting hyperglycaemia in allografted animals was accompanied by a second peak in blood amylase on day 6. Seven of 10 rats died by day 8.

Histology of isografts showed mild oedema on day 2, which had vanished along with inflammatory cells by day 8. In allografts both features were far more striking at day 2 when

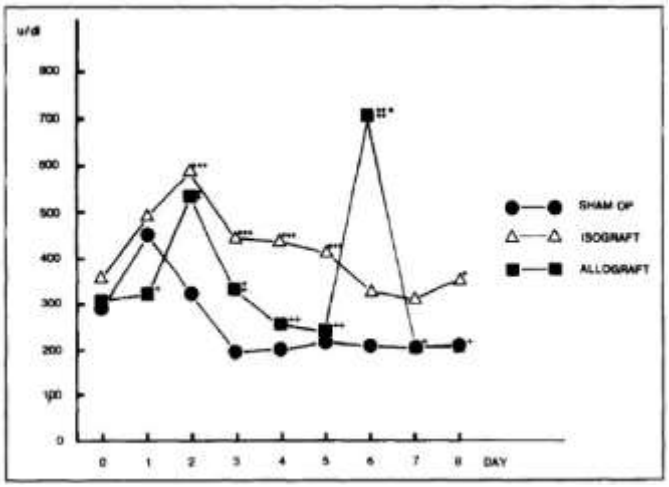

Figure 17.3 Blood amylase concentrations (mean ± SD) in pancreas-grafted rats. Small closed circles indicate higher values than in sham-operated group (• p,0.05, •• p<0.02, ••• p<0.01, •••• p<0.001). Plus sign shows lower values in allografted vs isografted group (+ p<0.05, ++ p< 0.02). Asterisk signifies higher level in allografted than isografted animals (*p<0.01). Reproduced from 1989 paper in Am J Surg [449]

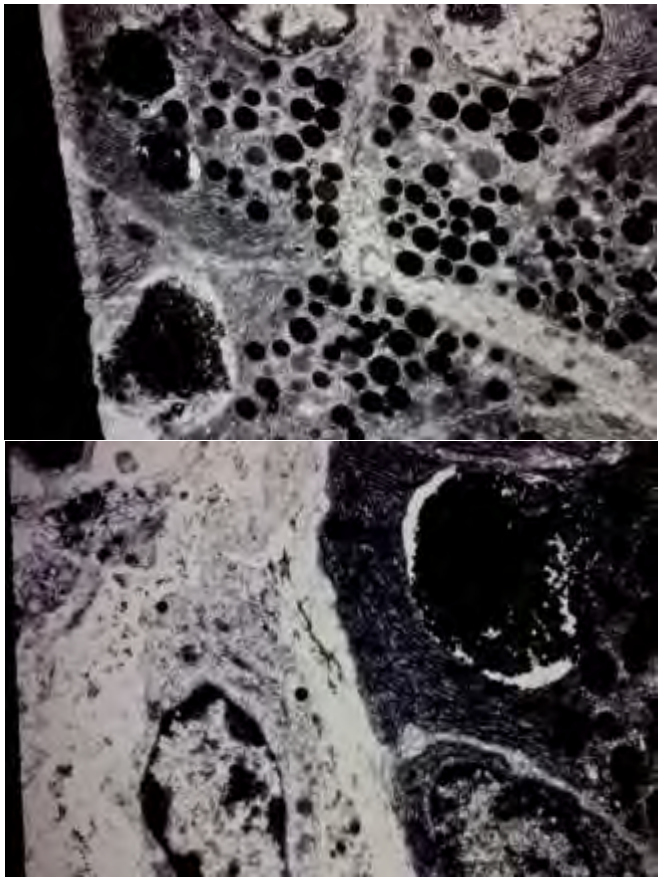

Figure 17.4 Electron microscopy images of allograft pancreatitis on day 5. Surviving acinar cells are packed with zymogen granules with no extrusion images (**top**). Necrotic debris and ghosts of acini are notable (**bottom**). Publication details as for Figure 17.3.

electron microscopy showed that acinar cells were stuffed with zymogen granules, their nuclei appearing dense and pyknotic. These appearances were evident to day 5, but now alongside necrotic debris and ghosts of perished acini (**Figure 17.4**). Striking features at day 8 were the virtual wipe-out of acini, tubular complexes, and punctuate haemorrhages without thrombosis in grafted vessels

Tracking of phagocyte subtypes identified an early PMN infiltrate, macrophages by day 3 and lymphocytes. - particularly T cells - from day 4 onwards as PMN numbers declined. All structures (acinar cells, ducts, islets) became strongly positive for the expression of class 1 MHC antigens from day 3-4, probably evoked by cytokines from indigenous leucocytes. Class II antigens were detected only on resident macrophages and infiltrating leucocytes. Islets of Langerhans looked normal until day 6 or 7 and apparently not a target for infiltrating cells, but were not seen beyond day 8, coincident with rising blood glucose.

The investigation indicated that there are 2 waves of damage in pancreas allografts. The first occurs soon after surgery, is also seen in isografts, and is accompanied by hyperamylaseaemia and interstitial edema with a mild interstitial infiltrate of neutrophils. It is self-limiting in syngeneic grafts which present no antigenic stimulus to the recipient, but a second damaging wave begins on day 5 in allografts and is clearly due to the alloimmune response. The main histological features are apoptosis and redifferentiation of acini into tubular complexes, followed by necrosis and interstitial hemorrhage with a coincident second rise in serum amylase. By contrast islets appear normal and normoglycaemia is usual for the first week. In other words, exocrine and endocrine elements are differentially affected.

Ischaemia-reperfusion injury is the likely explanation for the first wave, via free radical-initiated pancreastasis in acinar cells, causing pro-inflammatory FROP to be diverted into the pancreatic interstitium. The second wave is clearly due to the allograft immune response, with release of lymphokines from activated T cells and thereby increased MHC antigen expression and vascular permeability. In turn, extravasated erythrocytes attract and activate macrophages, resulting in apoptosis. The potent rejection-driven response and presence of effector cells is set to provoke frustrated phagocytosis, such that extracellular elastase is free to breach capillaries.

17.2.2 <u>Second study:</u>

Table 17.1 Survival of rat pancreaticduodenal allografts

Group	Treatment	Number	Graft survival (days)	Median survival (days)
1	None	10	7 (n=2)*, 8 (n=6)†, 9 (n=1)#, 10 (n=2)‡	8
2	Carrier	4	8 (n=2)*, 10 (n=2)*	9
3	Cyclosporin A (20mg/kg/day)	8	8 (n=1)*, 10 (n=2)*, 11 (n=3)*, >100 (n=2)	11
4	S-adenosylmethionine (25 mg/kg tid)	7	7 (n=1),8 (n=2), 9 (n=3), 10 (n=1)	◊
5	S-adenosylmethionine (25 mg/kg bid) plus Cyclosporin A (20mg/kg/day)	8	31 (n=1), >100 (n=7)	>100 (p=0.007)Δ

*died while normoglycaemic; ◊ sacrificed because of chronic diabetes. Δ significance of difference between group 5 and groups 1 to 4 (Mann Whitney rank sum non-parametric test). Abbreviations: tid=3 times daily; bid= twice daily Adapted from ref 449.

Table 17.2 Leucocyte profiles in pancreatic duodenalgrafts

Group	Days after transplantation					
	2	4	6	8	10	12
Syngeneic untreated	++ P	+++ P plus M	++ M > P	+ M > L	± M plus L	± M plus L
Allogeneic untreated	+++ P	+++ M plus L	+++ M plus L	++ M plus L	NT P > M plus L	NT
Allogeneic + Cyclosporin A	++ P	+ M plus L > P	++ M plus L	+ M plus L > P	NT	NT
Allogeneic + S-adenosylmethionine	+ P	++ M	+++ P, M plus L	++++ P> M plus L	NT	NT
Allogeneic + Cyclosporin + S-adenosylmethionine	+ P	+ M plus L	± M plus L	± M plus L		± M plus L

Adapted from ref 426; information on syngeneic and untreated allogeneic grafts from preceding study. Data indicate type and proportion of cells within infiltrate: P= polymorphonuclear cells, M =monocytes, L=lymphocytes, NT=no identifiable acinar tissue; ± =minimal to ++++ severe/dense infiltration.

Only allografted animals were used to test the effect of cyclosporin in isolation or in combination with SAM. Cyclosporin A was administered by gavage in a dose of 20 mg/kg/day for 14 days post-operatively. SAM was given intramuscularly in a dose of 25 mg/kg thrice daily at 8-hourly intervals for 10 postoperative days[426]. Graft rejection was defined by persistently elevated blood glucose. Survival, blood glucose and serum amylase data were compared with data on isografted animals[449]. The Mann Whitney rank sum non-parametric method was used to compare survival (**Table 17.1**).

Cyclosporin treatment alone did not alter survival of allografted animals. Treatment with SAM alone did not prevent graft failure or haemorrhagic pancreatitis but the animals did not die, albeit developed diabetes, and were sacrificed at 3 weeks. Treatment with the cyclosporine- SAM combination resulted in abolition of both the second amylase peak (**Figure 17.3**) and diabetes; no animal developed severe pancreatitis and all survived >100 days.

Graft leucocyte profiles are summarised in **Table 17.2**. The infiltrate in the combined treatment group was always minimal: neutophils had vanished by day 8 and lymphocytes by day 12. Ducts, acinar cells and islets were perfect for at least the first 2 days, although some did display

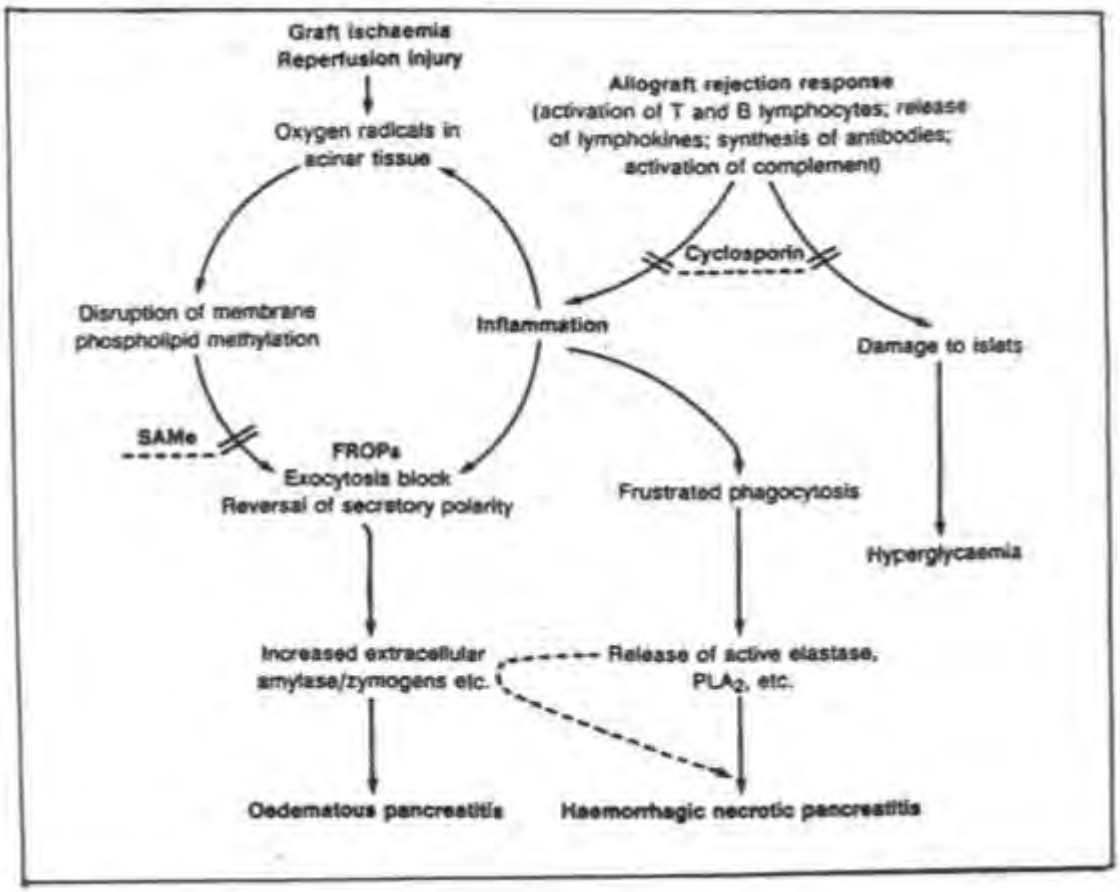

Figure 17.5 An integrated scheme to rationalise allograft pancreatitis and its protection by the combination of S-adenosyl-L-methionine (SAMe) and cyclosporin. FROPs=free radical poxidation products; PLA_2 =phospholipase A_2. Reproduced from 1992 paper in Drug Invest [426]

The ability of SAM treatment to abort the vicious cycle, when cyclosporin alone could not, underlines the importance of CH_3 and SH moieties in restoring apical exocytosis and thus in lifting the 'road block' trigger (**Figure 17.5**). It is impossible to say which of the drug's many attributes most accounted for success: key CH_3 donor facilitating membrane phospholipid methylation; replacer of high energy phosphate groups that are depleted by ischaemia-reperfusion injury; provider via homocysteine of methionine, which is itself vulnerable to free radical attack; progenitor via the transsulphuration route of GSH which protects α_1PI from attack by phagocyte-derived oxidants; or a combination of effects.

Perhaps transcending all these is correction of reductive stress (Chapter 19). The question now was if NAC plus SAM might accelerate recovery and prevent fatality in human acute pancreatitis.

17.3 Clinical trial of SAM plus NAC for first 24 hours

17.3.1 Study description and outcome

The studies described in sections 17.1 and 17.2 suggested that parenteral administration of both SAM and NAC for the first 24 hours from admission might restore apical exocytosis in acinar cells and thus abort the downward spiral towards death when inflammatory responses go awry.

It was calculated that 80 patients should be enrolled in the clinical trial to achieve a study power of 0.9, assuming that active treatment would reduce mortality by 50% as in the pilot study of gravely ill patients (**Figure 17.2**). The

plan was to enrol consecutive patients with a first attack of acute pancreatitis from 3 hospitals, unless informed consent was denied or unobtainable[450].

The diagnosis was accepted when serum amylase was ≥1000 U/l (Phadebas method, normal < 300) in patients with a compatible clinical picture: in all but 1 patient, an ultrasound scan was done within the next 48 hours and revealed a swollen pancreas. After obtaining baseline blood samples for assay of FROP and micronutrient antioxidants, patients were randomised to receive optimal supportive care or additional treatment for the next 24 hours with GSH precursors, ie. NAC (Evans Medical, Surrey, England) by constant infusion into a peripheral vein at a dose of 300 mg/kg in 500 ml of 5% dextrose; plus SAM (BioResearch, Milan) concurrently but via a separate peripheral vein at a dose of 43 mg/kg in 500 ml of normal saline.

The randomisation procedure was applied separately to groups predicted at admission to follow a mild or severe course, based on admission APACHE II scores < or ≥ 8, respectively. Routine CT to assess disease severity was not available. As planned at the outset, the impact of treatment would be gauged from the change in APACHE II scores at 48 hours compared to baseline, days needed in hospital, complication rate, and mortality attributable to the disease. Primary comparison of results in control and treatment groups would be by Chi squared or Wilcoxon rank test, but data would then be re-analysed to take account of the time-lag to treatment, whether 'early' (<15 hours) or 'late' (>15 hours), by analogy with paracetamol poisoning where it is recognised that NAC treatment is more effective if given early. The study began in 1990.

Of 80 cases enrolled, the only patient in whom ultrasound verification of the diagnosis was not obtained turned out to have a perforated duodenal ulcer, and was omitted. There were 42 males and 37 females, of average age 55 years (range 22-84), of whom 43 and 36 were categorised, respectively, as mild or severe disease. The first subset included 21 on supportive care (11 males and 10 females, average age 49 years, range 27-72) and 22 on active treatment (13 males and 9 females, mean age 42 years, range 26-78). The second subset included 19 in the supportive arm (9 males and 10 females, mean age 64 years, range 42-84) and 17 on active treatment (9 males and 8 females, mean age 65 years, range 22-78).

Risk factors could be assigned retrospectively as follows: gallstones (n=39); alcohol (n=19); iatrogenic (n=12): the last subset included prescribed drugs (n=5), cardiopulmonary bypass (n=4), diagnostic ERCP (n=1), or endoscopic sphincterotomy for ascending cholangitis (n=2). Hypertriglyceridaemia was identified in 12 patients. Two or more risk factors were found in 9 cases, whereas the disease was idiopathic in 14 cases.

Active treatment did not facilitate recovery, whether the pre-set criteria were considered individually or by logistic regression analysis. Of 8 deaths attributable to acute pancreatitis, 4 were in the supportive limb and 4 in the active limb of the trial: all but 1 death involved patients in whom treatment started late, >15 hours, but of the utmost importance, the last succumbed although treatment started within 4 hours.

17.3.2 Comments

Of the many factors that might have contributed to this disappointing outcome, a flawed philosophy seems the least likely because a raft of later studies reinforce rather than discredit it[267, 451, 452] . Any or all of the following factors could be relevant.

- The time-lag to treatment was usually long, occasionally >240 hours. Of note ERCP studies show that at least 4 hours elapse before the disease produces symptoms[259].

- Benefit from NAC in the pilot study (**Figure 17.2**) was associated with a longer duration of treatment. Well after the pancreatitis trial was under way, an extended duration of NAC treatment was advocated too for paracetamol hepatotoxicity.
- Deficiency of micronutrient antioxidants could prejudice recovery of the methionine metabolic pathway.
- The high proportion of patients with severe disease, because of the hospital's status as a tertiary referral centre, meant that disequilibrium was likely in relation to proteases/anti-proteases and immune /anti-immune systems - over-and-above oxidant/anti-oxidant imbalance.
- Intermittent haemodialysis, which would debulk circulating cytokines and other noxious agents was routinely used in the successful pilot study of NAC (**Figure 17.2**). An internet search shows that this approach is now supported in the management of MODS in general, as also in ameliorating severe experimental pancreatitis.
- Animal models reveal how quickly pancreatitis ensues after pancreastasis. The rapidity was also invoked as the critical factor in the failed phase III trial of an inhibitor of the PAF receptor [256,257].

17.4 Why treatment failure: micronutrient lack/persisting oxidative stress?

17.4.1 Ascorbic acid

This bioactive form of vitamin C is a key antioxidant in blood plasma, with particular activity against the powerful oxidants released by activated neutrophils and macrophages[453], as are implicated in the transformation from oedematous pancreatitis to HPN[266] as well as the progression to MODS[5]:moreover it can substitute for GSH [202,203]. Hence an HPLC method was researched to dissect out the components of total vitamin C reported by a standard spectrophotometric method[393] (**Figure 17.6**).

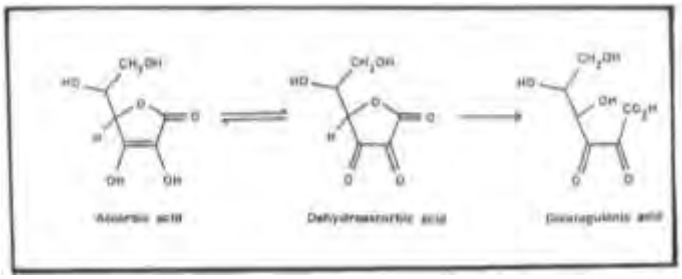

Figure 17.6 Components of vitamin C. The irreversibly denatured product 2,3-diketo-1-gulonic acid disintegrates into oxalic acid and L-threonic acid. Reproduced from 1993 paper in Br J Surg [454]

Thereafter, vitamin C and ascorbic acid levels were measured in admission plasma samples from consecutive patients with a first attack of pancreatitis admitted to 3 hospitals in a 6-month period (n=29): these patients were then entered into the aforesaid trial of GSH precursors. Contemporaneous samples were obtained after an overnight fast from 30 healthy volunteers who did not smoke cigarettes, and drank modest amounts of alcohol on a social basis. Samples were also obtained at admission from 27 patients with other abdominal crises[454].

Diagnostic criteria for acute pancreatitis, time from first symptom, and predicted disease severity were recorded exactly as in Section 17.3: the patients were part of the NAC plus SAM trial but the outcome of vitamin C analysis was not known when the trial was launched. Of 29 patients, 11 and 9 were admitted $< \text{or} \geq 15$ hours from the first symptom, respectively; 24 were graded as mild disease and 5 as severe disease on the basis of admission APACHE II scores. Because the practice at the Manchester Royal Infirmary was to administer AOT as soon as patients could eat and continued until blood levels of micronutrients had normalised, information on vitamin C for days 2, 7 and 14 was obtained from patients in the other 2 centers.

Relationships between vitamin C and ascorbic acid were investigated by parametric methods (Student's t test, paired t test, Kendall's

correlation coefficient). Ascorbic acid concentration <0.5 μg/ml, the lower limit of detection, was regarded as zero in deriving the percentage of the vitamin in bioactive form. Non-parametric methods were used for intergroup comparisons (Chi squared test, Wilcoxon matched pairs signed rank test, Kruskal-Wallis one-way analysis of variance, Mann-Whitney U test). Differences were considered significant when 2p<0.05.

The study outcome is summarised in **Figure 17.7**. Reference ranges, medians and ranges, were as follows; total vitamin C 15, 6.3-19 μg/ml (n=30); ascorbic acid 12, 4.5-18 μg ml (n=26 omitting 4 samples that showed interference in the assay, p=0.002 vs total vitamin C); % molar ratio of oxidised forms relative to total vitamin C (%MRVC) 13, 0-34%. The shortfall in ascorbic acid could not be attributed to the irreversibly denatured form of the vitamin.

Compared with control values, admission samples from patients with acute pancreatitis had far lower levels of vitamin C (2.8, 2-10 μg/ml, p<0.001) and ascorbic acid (<0.5, <0.5-6 μg/ml, p<0.001): as in controls, there was a shortfall in ascorbic acid, but its degree was greater (p<0.001) such that the median %MRVC was 100% - indicating severe oxidative / electrophilic stress. The concentration of vitamin C was below the lowest control value in 24 of 29 patients overall (83%) - 91% and 78% in patients presenting within or after 15 hours, respectively; 79% or 100% in subgroups with mild or severe disease, respectively. The distribution of vitamin C data did not differ between subgroups classified by etiology. Ascorbic acid could not be measured in 4 samples because of interference in the assay. Of the remaining 25 samples, 23 (92%) had a level below the lowest control value and 14 (56%) had values lower than the detectable cut-off. Ascorbic acid and %MRVC values were similar in patients with mild and severe pancreatitis.

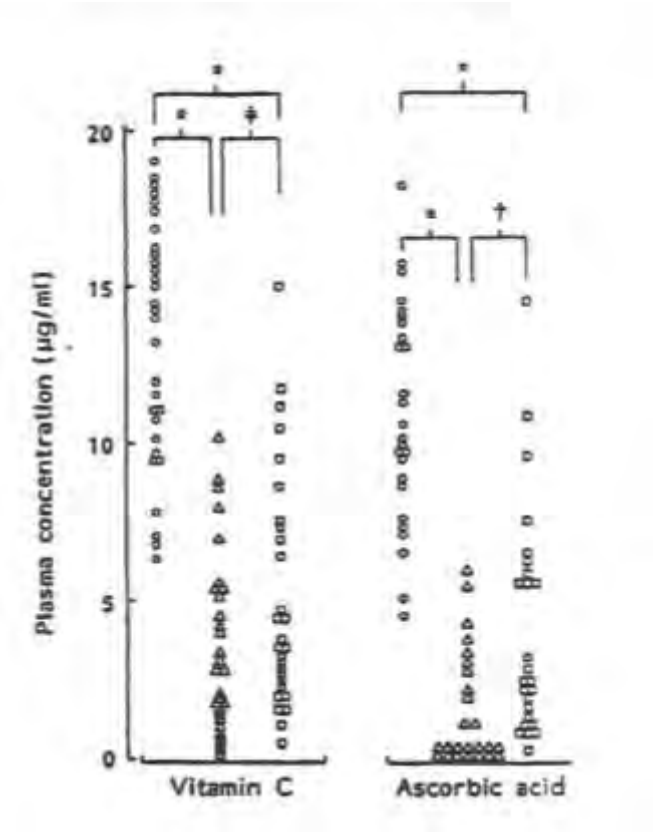

Figure 17.7 Concentrations in plasma of vitamin C and ascorbic acid in healthy controls (o) and at admission of patients with acute pancreatitis (∆) or other causes of the 'acute abdomen' (□). * p<0.001; † p<0.005, ‡ p >0.05 (Chi squared and Mann-Whitney U tests). Publication details as for Figure 17.6.

Admission samples from patients with other abdominal emergencies had vitamin C levels as low as in the acute pancreatitis set (**Figure 17.7**), 17 (63%) with values less than the lowest value in controls. However, in only 1 of 27 patients was ascorbic acid undetectable (p<0.001 versus acute pancreatitis set), and group levels were higher than in acute pancreatitis (p<0.001), such that %MRVC generally conformed with control data. There was no correlation between parameters of disease severity and indices of vitamin C status.

Regression analysis showed a significant linear relationship between vitamin C and ascorbic acid concentrations in healthy or disease controls. Too many patients with acute pancreatitis had ascorbic acid values below the detection limit to permit a similar analysis.

Follow-up data from the acute pancreatitis set showed that plasma vitamin C level had not normalised 2 weeks after the attack (**Figure 17.8**). The median concentration was of the order associated with scurvy, although none of the patients had this condition and there is no evidence linking acute pancreatitis with scurvy. A literature survey suggested that permutations and combinations of the following factors could be responsible: inadequate pre-morbid diet; sequestration of the vitamin from plasma into blood cells; its diversion via interstitial fluid into the pancreas; enhanced renal excretion. Follow-up data from the group with other abdominal crises established that no patient went on to develop sign or symptom of pancreatic disease.

Corroborative evidence came some years later in a study from New Zealand in which plasma ascorbic acid but not total vitamin C was measured at admission, days 2 and 5: very low baseline values fell further by day 2, and further still by day 5 in patients with severe disease. This was not due to loss in urine[455].

17.4.2 Selenium, β-carotene, vitamin E

This investigation was an extension of the vitamin C study, but now with more patients who were then enrolled into the on-going clinical trial of GSH precursors, and involving many more assays. The study was done in 2 phases. The first was exploratory, seeking further evidence for oxidative stress and clues for its correction. The assay methods for micronutrient antioxidants were being developed, and hence the number of analyses on each sample differed. Once all the assays were up and running, a phase II investigation was mounted to determine whether or not the findings were a non-specific result of an acute abdominal crisis[456].

Healthy hospital personnel and patients with minor surgical disorders, age 19-70 years, donated blood samples as and when each assay was developed: they did not smoke, drank alcohol on a social basis, were not on any medication and were on their habitual diets. Diagnosis, time-classification and severity grading in patients were as in Section 17.3.

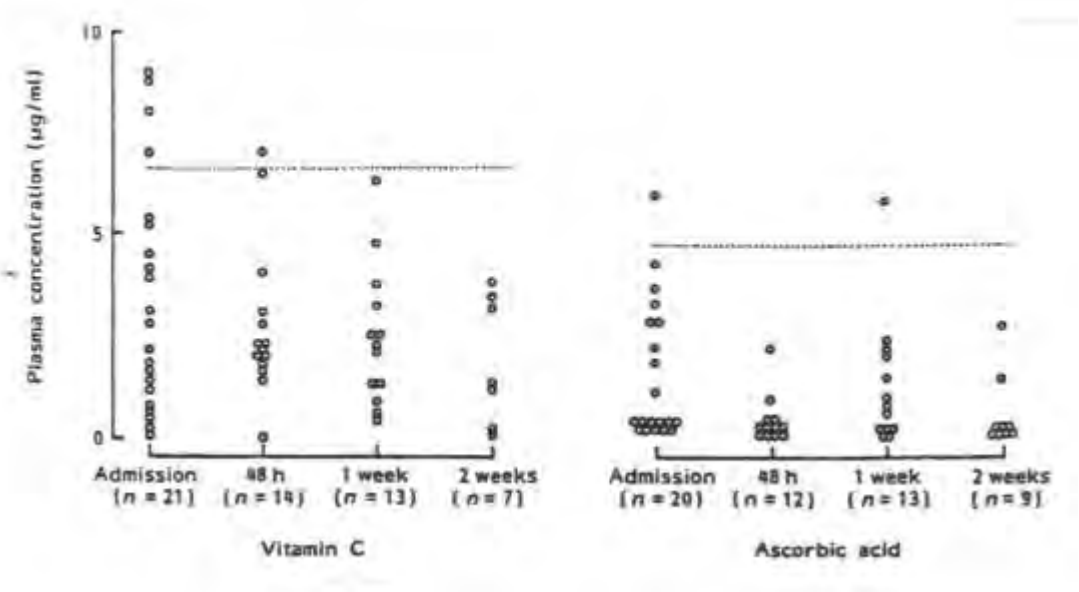

Figure 17.8 Follow-up data on vitamin C and ascorbic acid levels in plasma from patients with acute pancreatitis. Dotted lines indicate lower limits of reference ranges. Publication details as for Figure 17.6.

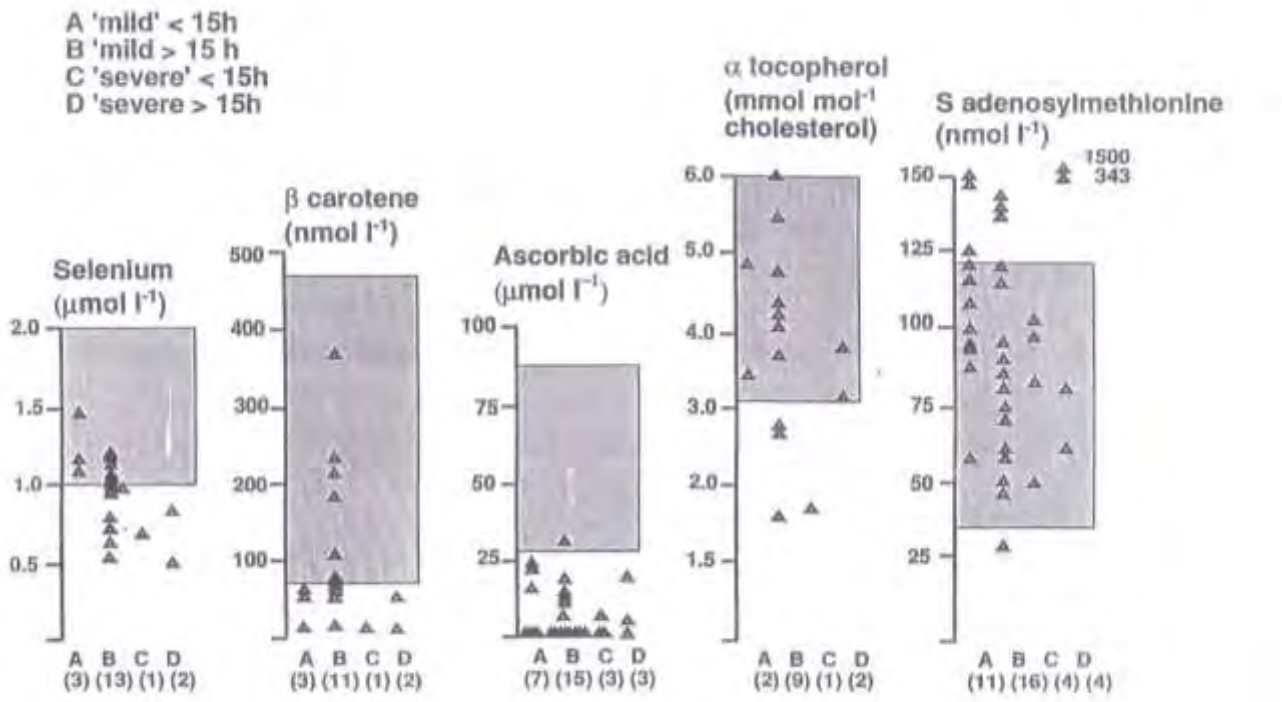

Figure 17.9 Distribution of data relating to micronutrient status in subgroups of patients with acute pancreatitis classified according to interval from first symptom and predicted severity. Shaded blocks represent reference ranges; numbers of measurements are given in parenthesis. Reproduced from 1995 paper in Int J Pancreatol [456]

Individual values for micronutrients - and also for SAM - in the first study are shown in **Figure 17.9**, data now expressed in SI units for conformity with SAM data (conversion factors given after Glossary). For selenium, β-carotene, ascorbic acid and α-tocopherol relative to cholesterol, values were very significantly less in patients than controls: the converse was true for plasma SAM for which higher values were found in patients (p=0.021). Homogeneity across subgroups was shown by one-way ANOVA for all items except selenium which tended to be lower in patients with severe disease, but noting that the 2 severe categories had to be fused because numbers were small.

The phase II study compared full biochemical profiles in 19 controls, 17 patients with acute pancreatitis and 17 consecutive patients with other abdominal crises (acute appendicitis n=3; acute cholecystitis, acute biliary colic acute diverticulitis, n=2 for each; empyema of the gall bladder, acute Crohn's ileitis, small intestinal obstruction due to adhesions, colonic obstruction by cancer, n=1 of each; no clear diagnosis, n=4). Using an Apache II score < or ≥ 8 to predict severity, 14 of 17 patients with acute pancreatitis were assigned as mild disease and 3 as severe disease. The same cut-off in the 'acute abdomen' group, legitimised by a report on intra-abdominal sepsis[457], assigned 14 and 17 patients, respectively as mild or severe disease - the latter set including a diabetic patient with gall bladder empyema, an elderly patient with diverticular abscess, and the patient with active Crohn's ileitis.

These 3 groups were compared in regard to micronutrient profiles, initially by one-way ANOVA, and then by multiple comparison procedures via the conservative Scheffé test (**Table 17.3**).

Next, stepwise linear discriminant analysis was applied to determine the variables that best separated data from controls and pancreatitis patients. Thereafter scores were calculated for the group with other abdominal crises. This analysis picked out ascorbate, selenium and %MRLA' in forward stepwise order as affording optimal separation between blood biochemical profiles of control and acute pancreatitis groups: addition of the other items did not improve discrimination. The discrimination function was: 0.056 x (ascorbic acid) + 2.157 x (selenium) - 2.064 x (log_{10} MRLA) - 3.126 (**Figure 17.10**). Mean scores for controls and acute pancreatitis were 1.98 and -1.98, respectively, higher concentrations of selenium and ascorbic acid but lower %MRLA associated

Table 17.3 Outcome of phase II study: Components of oxidative stress at admission in patients with acute pancreatitis

	Oxidatively altered substrates		Micronutrient antioxidants			
	linoleic acid % MRLA	vitamin C % MRVC	selenium µmol/l	β-carotene nmol/l	ascorbic acid µmol/l	α-tocopherol µmol/l
Group 1 Controls (n=19)	1.98, 0.86-3.81	14.6 ± 13.7	1.23 ± 0.17	219, 67-1396	60.5 ± 20.7	24.4 ± 6.85
Group 2 Acute pancreatitis (n=17)	4.05, 1.08-9.44	65.7 ± 40.5	0.89 ± 0.29	51.0, 6.0-365	9.45 ± 12.3	16.0 ± 6.77
Group 3 Other acute abdomen (n=17)	2.43, 1.25-3.97	24.8 ± 18.2	1.04 ± 0.14	133, 22-503	26.0 ± 20.7	24.8 ± 9.97
Overall differences, one-way ANOVA	p < 0.001	p < 0.001	p < 0.001	p < 0.001	p < 0.001	p = 0.002
Significance of differences by Scheffé 5%	Group 1 vs 2 Group 2 vs 3	Group 1 vs 2 Group 2 vs 3	Group 1 vs 2 Group 1 vs 3 Group 2 vs 3	Group 1 vs 2 Group2 vs 3	Group 1 vs 2 Group 1 vs 3	Group 1 vs 2 Group 2 vs 3

Data as mean ± SD except for %MRLA and β-carotene where data as medians with ranges. Adapted from ref 456.

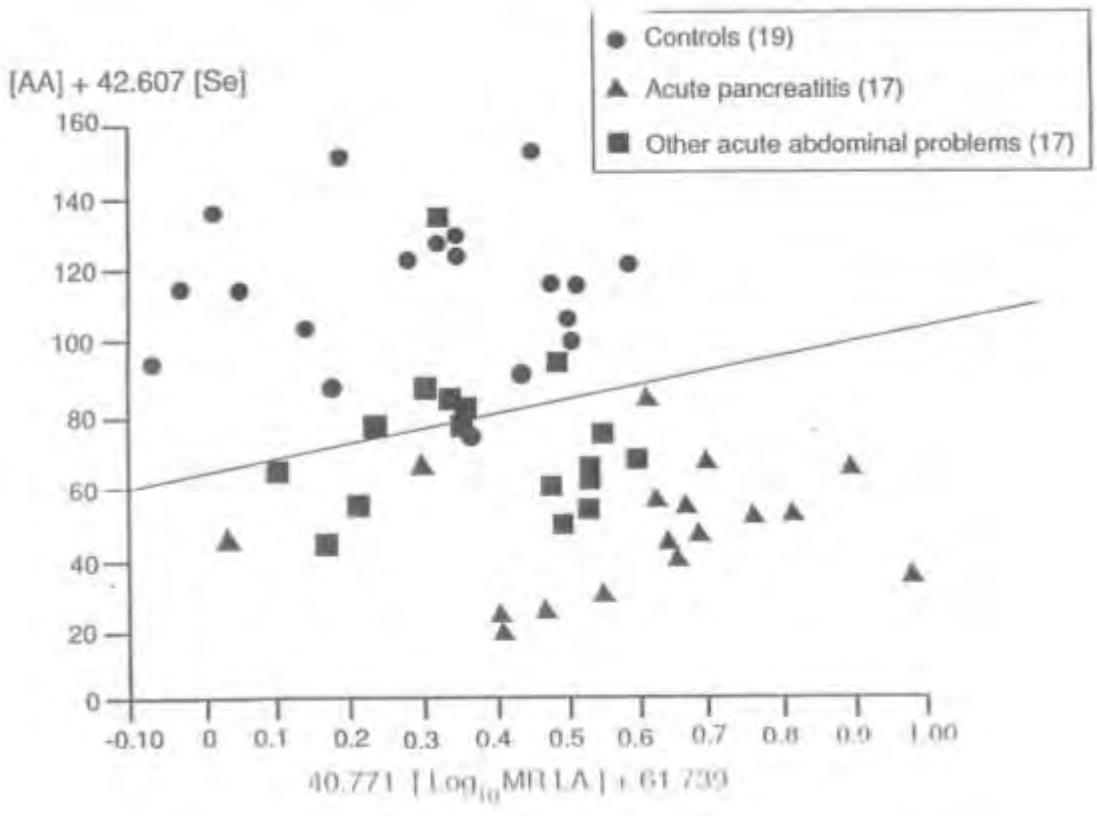

Figure 17.10 Nomogram identifying excess oxidative stress in admission blood samples from patients with acute pancreatitis. AA= ascorbic acid; MRLA= molar ratio of a specific linoleic isomer to the parent acid. Publication details as for Figure 17.9.

with controls and the reverse with pancreatitis. Assuming equal prior probabilities, cases with scores >0 were more likely to be controls and scores <0 to be patients. Using this cut-off, 18 of 19 controls were correctly allocated as were all 17 pancreatitis patients (97%). The mean score in the other abdominal crises group was -0.37 - 6 allocated as controls and the others as acute pancreatitis.

17.4.3 Folic acid

Work published in abstract identified subnormal values for blood folic acid in admission blood samples of patients with acute pancreatitis, albeit in a later set[458]. (**Table 17.4**)

17.4.4 Impact on methionine metabolic pathway

Subnormal GSH levels in blood were noted in the folic acid study, but an increase in plasma SAM ensued in the earlier investigation (**Figure 17.9**) - the combination indicating disruption of the methionine metabolic pathway wherein several enzymes are vulnerable to oxidative / electrophilic stress (Chapter 16). Curiously, and similar to the position in cystic fibrosis (Chapter 13), erythrocytes showed an increase in ATP and energy charge, presumably to counter the oxidative threat[459].

!7.4.5 Impact on linoleic acid and vitamin C

The oxidative attack on dissimilar substrates by the time of admission in patients with acute pancreatitis is evident from data in **Table 17.3**: there was homogeneity across disease subgroups. More importantly, the degree of attack was higher than in patients with other causes of the 'acute abdomen'.

17.4.6 Impact on inflammatory response

An imbalance between fired-up innate but passive immune arcs of the inflammatory response was revealed, respectively, by data on plasma von-Willibrand factor antigen (vWf)[460] and urinary neopterin levels[461](**Figure 17.11**). Antioxidant lack is among the recognised factors that might cause the detrimental imbalance.

17.4.7 Implications of new findings

The rea son d'etre for these studies was to devise first-line treatment for acute pancreatitis, considering the disappointing failure of SAM plus NAC. Collectively, the investigations confirmed that oxidative stress extending to the vascular compartment is a shared feature of acute pancreatitis and other abdominal crises, but that its degree is higher in the former, and accompanied by a greater decrement in selenium, β-carotene and α-tocopherol. The elevation in plasma SAM concentration in the acute

Table 17.4 Folate and glutathione lack in acute pancreatitis

	Acute Pancreatitis (n=24)	Controls (n=30[a], n=18[b])	Difference* Wilcoxon Rank Sum
Red cell folate (µg/l erythrocytes)	353 151-573	500[a] 220-894	p<0.001
Serum folate (µg/l)	4.4 <1.1-15.0	8.1[a] 1.8-18.1	p<0.001
Plasma vitamin C (mg/l)	4.4 1.1-11.6	14.2[a] 6.3-19.0	p<0.001
Whole blood GSH (µmol/l erythrocytes)	2749 2215-3895	3469[b] 2567-4179	p<0.01
Plasma total glutathione (µmol/l)	2.56 0.95-5.61	5.75[b] 3.90-8.30	p<0.001

(from ref 458)

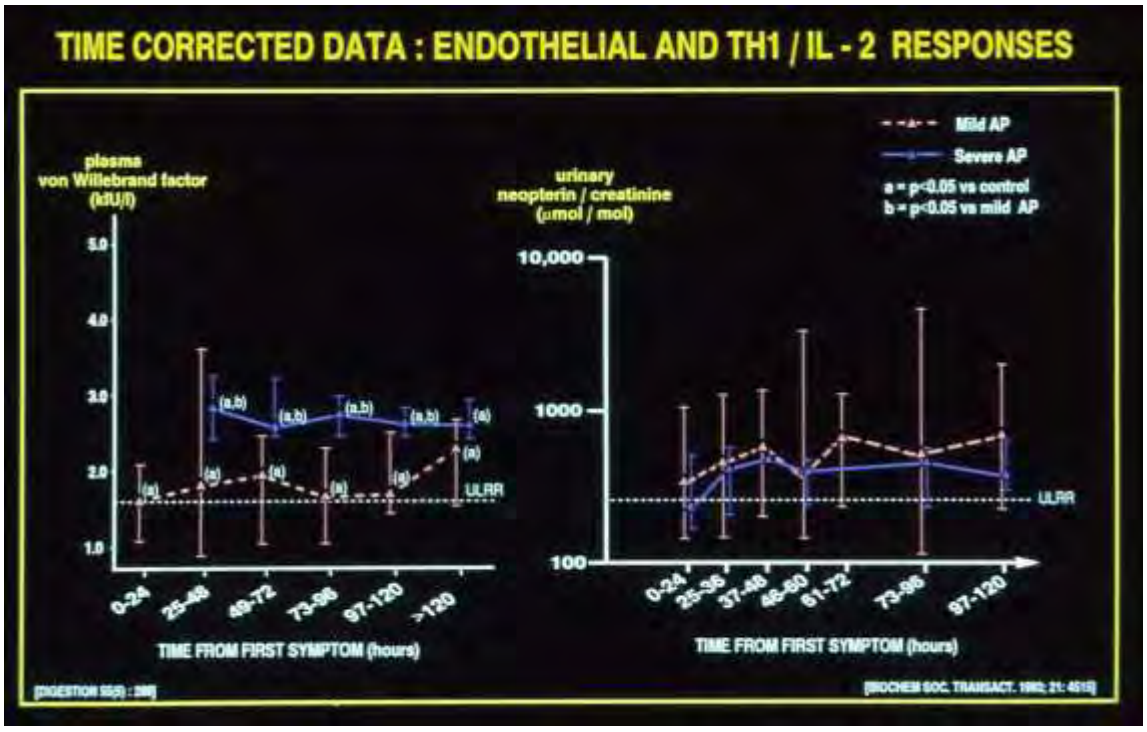

Figure 17.11 Early activation of the innate inflammatory response in acute pancreatitis as shown by increase in serum level of von Willibrand factor antigen, the more so in severe disease, contrasts with a blunted immune arc as reported by urinary neopterin. Data from studies published in abstract [460, 461].

pancreatitis set, as in patients with acute-on-chronic pancreatitis (Chapter 16), affirms the notion that a breakdown in the methionine metabolic pathway in acinar cells is integral to a pancreatitis attack (Chapter 11). This concept was later endorsed by others[452], and today excess oxidative stress is accepted as being critically important in the pathogenesis of acute pancreatitis[277]. Evidence of disrupted folate and GSH homeostasis underlines the diminished capacity to regenerate CH_3 moieties, as also the vulnerability to cysteine insufficiency and any further burst of electron transfer reactions, eg. upon exposure to radio-contrast media for ERCP or CT.

Given all this information and success of the multi-antioxidant approach to treatment of chronic pancreatitis and RAP (Chapters 15 & 16), it might be expected that a similar regimen administered by the parenteral route would be successful. It was not[462]. As confusingly, a study from the selenium-poor region of Rostock in Germany, where higher concentrations of pesticides are found in pancreatic juice than bile, announced dramatic improvement and decreased mortality upon mega-dose treatment with selenium alone in patients who always arrived within a short time of symptom onset [463]. Also of interest is a report from China showing impressive recovery with normalisation of immune responses by treatment with mega-dose vitamin C, 10 gm / day for a week[429].

The glaring clue to these incongruities was the virtual wipe-out of ascorbic acid in admission plasma samples of patients with acute pancreatitis - glossed over as another manifestation of oxidative stress. So it is, but in quite another context that was appreciated only after a further study of patients at Soweto.

17.5 Why treatment failure : fibrinolysis precedes hypercoagulability

17.5.1 Soweto study: description and outcome

An aggressive course of acute pancreatitis has been recorded at Soweto[264], against a backdrop of poor micronutrient intake and oxidative stress

in healthy compatriots (Chapter 12). Reports from developed countries link severe disease with the following disturbances: increase in plasma fibrinogen-fibrin degradation products; trypsinogen activation; huge load of trypsinogen in blood and urine; depletion of α_2 macroglobulin; exaggerated inflammatory reaction; antioxidant depletion.

It is assumed that heightened plasmin activity is a response to activation of prothrombin by prematurely activated trypsin, but in none of the published studies were trypsinogen load, markers of trypsinogen activation and plasmin-thrombin balance assessed concurrently by analysis of the same set of samples. Trypsinogen load is important because it seems to reflect basolateral redirection of acinar cell secretions to compensate for pancreastasis, and also because it could have a bearing on trypsinogen autoactivation[29]. Moreover, in relatively few patients have any of these aberrations been assessed during the critical first 24 hours and in no report has the potential impact of antioxidant lack / oxidative stress been considered. Most studies describe profiles at or beyond 48 hours, by which time the worst affected patients may have perished[256].

Prospective studies in at-risk groups indicate that whereas blood trypsinogen shows an early presymptomatic surge, 24-48 hours elapse before a significant elevation of trypsin markers in blood and / or urine. This delay shows that, as in experimental acute pancreatitis, the intra-acinar phase of 'trypsinogen activation' is not propagated extracellularly, and might be just a housekeeping function[287] (Chapter 11). Thus, it could be that the coagulation disturbance is part-and-parcel of the hyper-inflammatory state, which tends to immobilise proteinase inhibitors. In fact, plasmin and thrombin have been proposed as potential activators of trypsinogen because they have overlapping substrate specificities. In vitro studies show that plasmin activates trypsinogen, albeit less well than trypsin. There is debate as to whether thrombin can do so, but the evidence suggests that it has ~~has~~ a physiological role in inhibiting fibrinolysis by activating procarboxypeptidase B (**Figure 17.12**). Individual citations for all these facts are given in the Soweto study report[464].

The overall aim of the new research was to seek clues to the virulent nature of acute pancreatitis in Sowetans by measuring markers of oxidant-antioxidant, innate-immune, trypsin-trypsinogen and plasmin-thrombin status in admission blood samples - taking advantage of the proximity of Soweto to Baragwanath hospital which should ensure that patients arrive by 24 hours of symptom onset.

In a 6-month period from August 1994, consecutive patients with a first attack of acute pancreatitis consented to the study (n=25). Omitting a man who was later shown to have a perforated duodenal ulcer, the group had 21 males and 3 females of median age 37 years (range 24-88). Excessive alcohol consumption was documented in 17 patients (median and range 1400, 700-3675 gm / week for 18, 7- 35 years) and strongly suspected in 4 cases. An alcoholic binge preceded admission in 1 of the first set and 3 of the second. The remaining 3 patients comprised an ex-alcoholic, a patient with gallstones, and the last with idiopathic disease.

Two control groups of Soweto residents were assessed. The first involved 12 outwardly healthy hospital workers (10 men, 2 women, age 41, 28-49 years). The second comprised 9 patients (7 men, 2 women, 41, 27-73 years) with other abdominal crises: 2 with appendicitis; I each with perforated duodenal ulcer, incarcerated umbilical hernia, colonic obstruction, sigmoid volvulus, perforated colon cancer, or severe pelvic inflammatory disease; and the last unexplained. Of the first control set, 11 drank 20-30 units of alcohol per week, as did 7 of the 9 disease controls. Studies in 14 healthy hospital workers at Manchester (8 men and 6 women, age 44, 22-58

years) provided a reference frame: of these 10 drank around 30 units of alcohol per week.

Approximately 30 ml of peripheral blood and a urine sample were obtained at admission from patients, who had eaten very little for at least 12 hours, and after an overnight fast in controls. The blood sample was divided between plain tubes for serum studies and acidified citrate tubes for plasma studies. The prepared material was snap-frozen in suitable fractions, stored at – 70 °C and batch-transferred to Manchester for analysis or onward destination.

Trypsinogens were measured by solid-phase double-antibody ELISA, whereas radioimmunoassy was used to measure the surrogate marker of trypsinogen activation, carboxypeptidase B activation peptide (CAPAP). As noted by the laboratory in Sweden, urine is a convenient medium for assay of anionic trypsinogen because it contains little of the cationic isoform, and it is also the preferred medium for assay of CAPAP because high levels

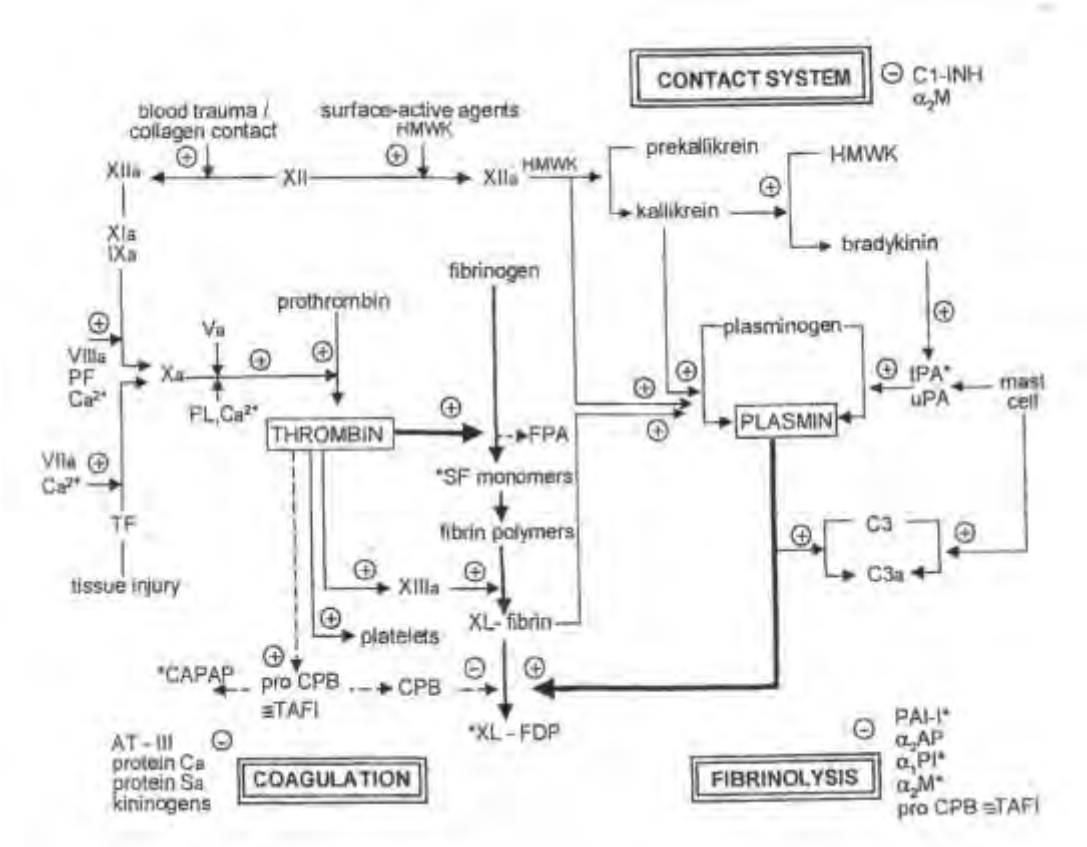

Figure 17.12 Schematic representation of the main operational links between the contact system of plasma proteolysis, coagulation and fibrinolysis pathways. The suffix 'a' shows the activated form of a component; encircled plus symbols indicate activation of a reaction or system; minus symbols indicate inhibition; asterisks signify items that were measured in the Soweto investigation. Abbreviations arranged alphabetically: α_1PI = alpha 1 proteinase inhibitor, with main function the inhibition of neutrophil elastase and extracellular trypsin; α_2AP = alpha 2-antiplasmin; α_2M = alpha 2 macroglobulin which inhibits all proteases; AT-III = antithrombin III which inhibits thrombin, factors XIIa, Xia, IXa, Xa, Va and kallikrein; Ca^{2+} =ionized calcium; C1-INH = C1 esterase inhibitor which also inhibits factor XIIa, kallikrein and bradykinin; C3, C3a = complement factor , the activated form said to indicate recruitment by plasmin; FPA = fibrinopeptide A which splits off fibrinogen to allow monomer formation; FDP = fibrin degradation products of which all but fragment E are reflected in the XL-FDP assay; KMWK = high molecular weight kininogen; proteins Ca, Sa = activated froms of proteins that inhibit factors Va and VIIa; PAI-1 = plasminogen activator inhibitor; PL = phospholipid; pro-CPB = procarboxypeptidase B which is nowadays seen as synonymous with TAFI (thrombin activatable fibrinolysis inhibitor), providing an explicit molecular link between activation of coagulation and fibrinolytic systems; SF = soluble fibrin; TF = tissue factor; tPA , uPA = tissue-type and urokinase plasminogen activators; XL = cross linked. Reproduced from 2002 paper in Am J Gastroenterol [464].

of its zymogen show immunoreactivity in the serum assay but the zymogen does not enter urine. Markers of coagulation status were assayed in the specialist haematology laboratory at Manchester: soluble fibrin as a surrogate marker of thrombin activity by ELISA; so too for cross-linked fibrin degradation products (XL-FDP) as a surrogate marker of plasmin activity; commercially available methods for tissue-type plasminogen activator (tPA) and its inhibitor (PAI-1) (**Figure 17.12**). The protease inhibitors α_1PI and $\alpha_2$macrogiobulin (α_2M) were measured in the routine hospital laboratory by standard methods. Data on micronutrient antioxidant levels, markers of oxidative stress (plasma GSH, %MRLA', %MRVC), membrane damage (lactate dehydrogenase, LDH) and trackers of inflammation (vWf, CRP, neutrophil elastase, neopterin) were made possible by the initiative of pancreatic laboratory personnel or kind collaboration with local experts.

Statistical analysis was done by parametric and non-parametric methods with significance set at 2p<0.05, but non-parametric tests were preferred (Mann Whitney U, paired signed rank, Kruskal Wallis, Dunn's correction for multiple comparison, Pearson correlation coefficient) because several variables in the acute pancreatitis set were highly skewed. Hence, results in the text are given as medians with ranges, while a 'box-and-whisker' format is used for the Figures.

Healthy controls at Soweto had higher levels than Manchester controls of anionic trypsinogen in serum (20, 9-53 µg/l vs 10, 7-17 µg/l, p=0.004) and of the tPA: PAI-1 ratio (0.14, 0.07-0.38 vs 0.07, 0.02-0.30, p=0.047). Their very low levels of vitamin C and β-carotene coupled with greater oxidation of ascorbic acid has been noted previously (Chapter 12).

Patients with acute pancreatitis reported a delay of 12-18 hours from symptom onset but serum CRP was elevated at admission - indicating, by reference to ERCP-induced pancreatitis[259], an actual time-lag of around 24 hours. The disease was graded as mild in 17 patients of whom 8 showed abnormalities on admission x-rays: 'sentinel loop' in 3, 'colon cut-off' in 2, pleural effusion in 2, basal atelectasis in the last. Despite prompt treatment in the intensive care unit, 7 patients became gravely ill and 3 died - aged 25, 34 and 38 years. Two deaths occurred on the day after admission from multiple organ failure, and the third on day 17 from ARDS and ketoacidosis. These 3 patients presented after an alcoholic binge against a background of known or suspected alcoholism. The 4 surviving patients spent 9-26 days in the unit for acute lung injury coupled in 2 cases with renal injury and in the other 2 with ketoacidosis.

Routine blood tests at admission showed similar values in subgroups with mild or severe disease, each with elevated aspartate transferase and γGT. Group values for amylase, glucose, calcium, creatinine were not significantly different.

Blood antioxidant profiles showed negligible values for ascorbate, associated with increased oxidation; and also subnormal levels of β-carotene, selenium and plasma GSH - but the magnitude of each disturbance was of the same order in mild and severe disease subsets. As expected, the concentration of each inflammation marker was significantly elevated in patients with acute pancreatitis as a whole, the increments of vWf, neutrophil elastase and LDH-5 greater in severe than mild disease (K-D John, A Blann, L Sandle, C Chaloner, I Segal & JM Braganza unpublished).

Reference values for trypsinogen and related data were as follows: total trypsinogens, 37 (20-80) µg/l; anionic, 20 (9-53) µg/l; cationic, 18 (11-27) µg/l; CAPAP, 1.30 (0.09-2.22) nmol/l; α_1PI, 1.41 (1.11-1.80) gm/l; α_2M, 2.05 (1.63-3.01) gm/l. Results in patients with acute pancreatitis are shown in **Figure 17.13**. There was no increase in urinary CAPAP in 5 patients with mild disease, whereas a clear signal was present in all with

severe disease. For the group as a whole, α_1PI values represented a significant increase above control levels (2p<0.001), but there was no difference between mild and severe pancreatitis subgroups. The concentration of α_2M was of the same order as in controls (p=0.594)[464].

After all these analyses, sufficient plasma was available for the haemostasis marker studies in 17 patients - 12 with mild disease including the 5 with no increase in urinary CAPAP, and 5 with a severe outcome. Reference values were as follows: tPA, 7.8 (5.1-20) µg/l; PAI-1, 54 (22-113) µg/l; tPA:PAI-1 ratio, 0.14 (0.07-0.38); SF, 0.98 (0.30-4.5) mg/l; XL-FDP, 26 (10-100) µg/l. +Platelet counts were normal at admission in every patient. The results are shown in **Figure 17.14**.

In the subset with mild pancreatitis a surge in XL-FDP (p<0.001 vs controls) was seen without deflection in the other measurements. In the subset with severe disease, a markedly higher concentration of XL-FDP was accompanied by an increase in soluble fibrin (SF) (p<0.001 vs controls), and doubling of the tPA:PAI-1 ratio (p=0.031 vs controls, p=0.082 vs mild disease) because a 1.5 fold increase in the inhibitor fell short of the 3-fold increase in the activator. In retrospect it was observed that an arbitrary value

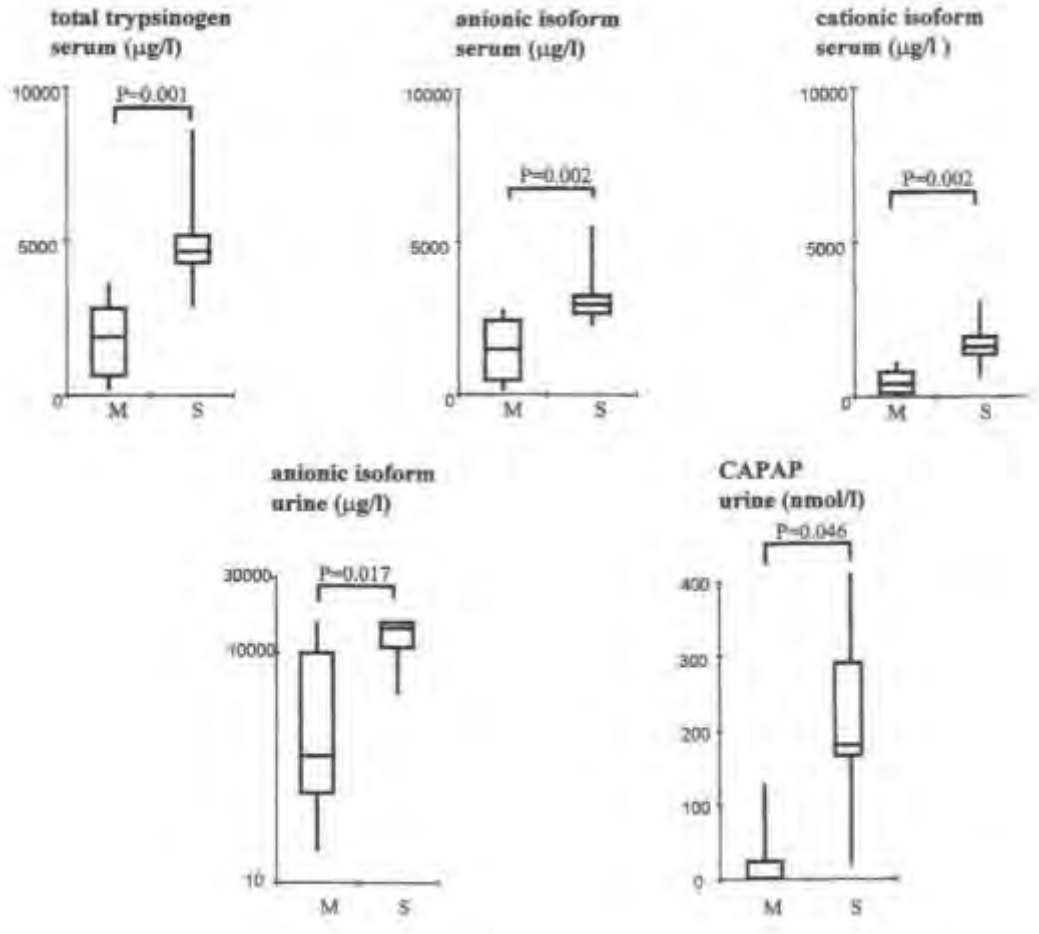

Figure 17.13 Interquartile ranges, median values (boxed and horizontal bars, respectively), and observed ranges of serum trypsinogen and its isoforms, urinary anionic trypsinogen and carboxypeptidase activating peptide (CAPAP) in subsets with mild (M) or severe (S acute pancreatitis as gauged by actual outcome. Comparisons by Mann Whitney U test (2-tailed). When corrected for multiple comparisons, the significant differences between mild and severe disease were retained by parametric tests but lost for urinary trypsinogen and CAPAP by non-parametric tests. Publication details as for Figure 17.12.

for an admission XL-FDP reading ≥ 200 µg/l would have identified each patient with a severe outcome, and 6 of 7 with assumed mild disease in whom plasma was available from among 8 with x-ray evidence of pulmonary or intestinal reaction. Of interest, higher concentrations of XL-FDP were associated with lower concentrations of ascorbate, selenium and β-carotene[464].

Trypsinogen, CAPAP and hemostasis profiles in 8 of the 9 Sowetans with non-pancreatitis causes of acute abdominal pain conformed with local controls. The profile of a patient with a perforated duodenal ulcer and serum amylase 800 u/l (normal 10-220 u/l by Boehringer Mannheim kit) was the exception: modest increase in serum and urine levels of anionic trypsinogen, normal levels of serum cationic trypsinogen and CAPAP, and an identical pattern of haemostais disturbance as in patients with mild pancreatitis.

A correlation matrix on data from patients with acute pancreatitis did not support a link between trypsinogen activation and that of plasminogen or prothrombin , or visa versa . Instead, it revealed significant positive influences on trypsinogen activation - as judged by urinary CAPAP - of trypsinogen load, PAI-1 and CRP (**Figure**

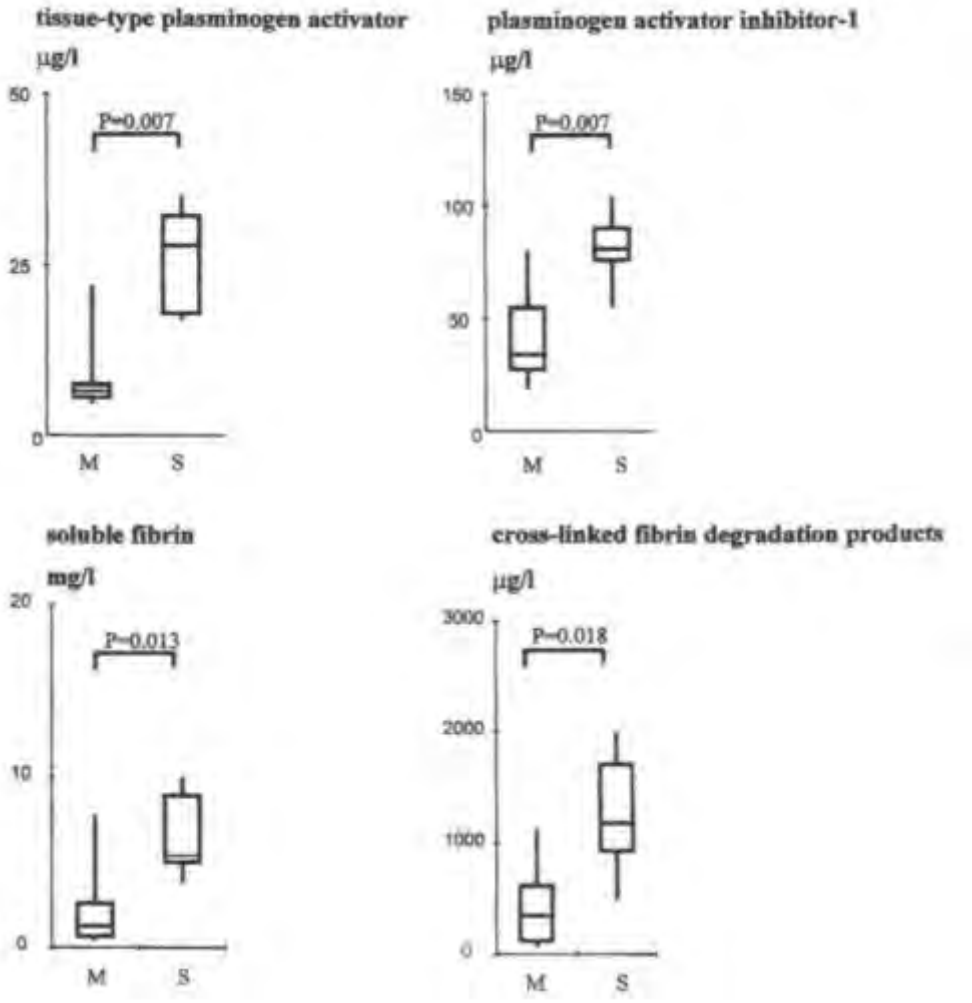

Figure 17.14 Interquartile ranges, median values (boxes and horizontal lines, respectively) and observed ranges of haemostasis markers that showed significant differences between subsets of 12 patients with mild (M) and 5 with severe (S) acute pancreatitis. When corrected for multiple comparisons, the difference was only retained for cross-linked fibrin degradation products irrespective of statistical method used. Publication details as for Figure 17.12.

17.15). Further exploration by sum-of-squares analysis showed that clear increases in the latter 2 indices not infrequently co-existed with an absent or insubstantial CAPAP signal, whereas there was a striking exponential increase in CAPAP above a serum total trypsinogen threshold of around 3000 µg/l. This curvilinear relationship held true when serum anionic or cationic isoforms were considered separately. The precise relationship between CAPAP and urinary anionic trypsinogen could not be ascertained because zymogen values >25,000 µg/l were not resolved further.

17.5.2 Comments

Compared to Manchester controls, those at Soweto had higher serum levels of FROP and anionic trypsinogen but lower levels of certain antioxidants (Chapter 12): this combination suggests subclinical oxidant-mediated pressure on the apical exocytosis machinery in acinar cells with compensatory redirection of zymogens via the endocrine constitutive route within the basolateral membrane (Chapter 11). The higher tPA:PAI-1 ratio in non-alcoholic non-diabetic Sowetans exposed their profibrinolytic state, a tendency that would be amplified under conditions of profound oxidant stress as is associated with acute pancreatitis (Sections 17.1, 17.2), because the inhibitor is vulnerable to oxidant attack, but the catalytic activity of plasminogen activator and its binding affinity for plasminogen are not.

In assessing trypsinogen activation during acute pancreatitis, urinary CAPAP is superior to the trypsinogen activation peptide (TAP) because serum / urine has little or no CAPAP but readily measurable amounts of TAP. However, after the analyses were completed, an article appeared on the activation *in vitro* by thrombin of procarboxypeptidase B. This finding delayed publication of the Soweto investigation until reassurance from a study in patients with myocardial infarction that CAPAP is not released into the systemic circulation under conditions of increased thrombin activity *in vivo* [465].

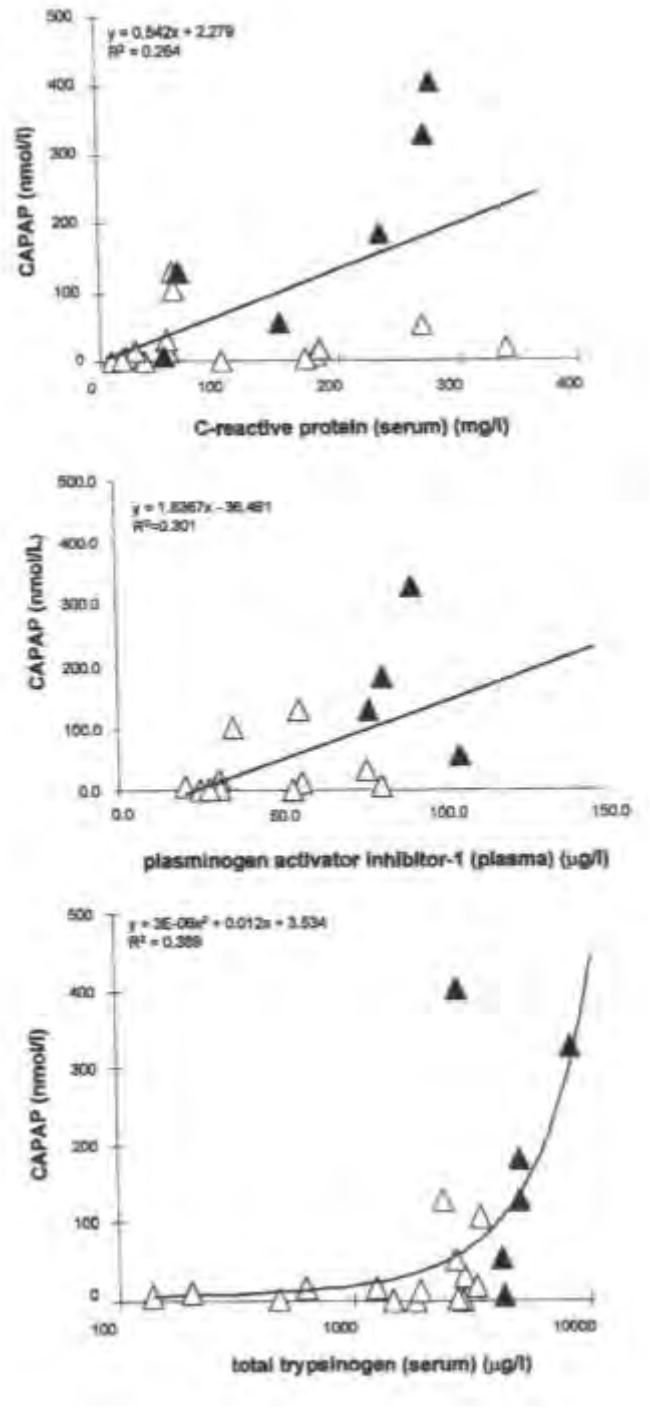

Figure 17.15 Computer-assisted sum-of-squares analysis of relationships involving trypsinogen activation that were positive in a correlation matrix. There was no urine sample in a patient with severe pancreatitis in whom serum CAPAP was elevated at 12 nmol/l, as also from 2 with mild disease and serum CAPAP values increased at 3 and 10 nmol/l. Of these missing samples, 2 were from the subset of17 patients in whom haemostasis markers were measured: hence there are only 15 data points in the middle frame. Publication details as for Figure 17.12.

Whereas data on the measured parameters in patients with acute pancreatitis as a whole broadly conformed with data from other countries, subgroup analysis was revealing in regard to the role of trypsinogen activation in fibrinolysis. Thus, in mild pancreatitis within 24 hours of symptom onset, which equates to 27-31 hours from disease onset[259], there was a median 12-fold increase in plasma XL-FDP such that the concentration was as high as after streptokinase treatment of myocardial infarction. Moreover, the surge occurred without increase in soluble fibrin, about 12 hours before a rise in fibrinogen is expected, and in 5 patients without an increase in urinary CAPAP. Technical artefacts could not be implicated.

Two previous clinical studies noted this initial hypocoagulable phase in acute pancreatitis: (i) an investigation in 55 patients of whom some were seen within 3 hours of symptom onset; (ii) analysis of venous effluents from pancreatic transplants. The deduction thus seems to be that, early in the course of acute pancreatitis, preformed soluble fibrin quickly polymerises to serve as a substrate for plasmin and later also for trypsin. This dissociation between enhanced plasma fibrinolytic activity and unchanged coagulation is similar to the pattern in experimental endotoxemia and also when the systemic inflammatory response syndrome (SIRS) is provoked by sepsis. The pattern suggests early and preferential activation of the 'contact system' (**Figure 17.12**), with its profibrinolytic, anticoagulant (via thrombin inhibition), antiadhesive and proinflammatory properties. The Soweto work suggests that contact system activation might be identifiable by an admission concentration of XL-FDP ≥ 200 µg/l, as in the majority of patients in whom lung and / or intestinal reaction was shown by admission x-rays. It was also evident that the phenomenon applies equally to patients with perforated duodenal ulcer. Citations for all these assertions are available in the study report[464].

Mast cell pathology is the only viable explanation (Chapter 11). Not only has early degranulation of pancreatic mast cells been confirmed in experimental pancreatitis, but also micronutrient lack - as in Sowetans - removes a stabilising effect on the cells[257]. The virtual wipe-out of ascorbic acid in admission blood samples irrespective of disease severity - as noted previously in studies from the UK (**Figure 17.7**)[454] and New Zealand[455] - can now be confidently attributed to its utilisation in mopping up histamine[466], which generates H_2O_2[467]. Moreover the benefit claimed for mega-dose selenium[468] or vitamin C[429] now becomes explicable.

The coagulation marker profiles in patients with severe pancreatitis indicated further activation of fibrinolysis but accompanied by enhanced coagulation - an evolution in tune with the natural progression of contact system activation, the continuing influence of which was suggested by comparing data on XL-FDP and soluble fibrin with corresponding data in patients with complicated myocardial infarction. Levels of XL-FDP were 4 times higher in acute pancreatitis although levels of soluble fibrin were 4 times lower. This interpretation is also in line with prolonged depletion of α_2M in acute pancreatitis and after fibrinolysis treatment of myocardial infarction, but with the critical difference that plasminogen levels remain low for some time in the former but recover rapidly on the latter setting. It is in keeping too with the far higher concentration in peritoneal fluid than plasma of several proteinases, including those that indicate contact system activation, but a lower proportion of functional antiproteases[464].

The correlation matrix seemed to deny a role for trypsin in the activation of plasminogen during the first 24 hours of alcoholic acute pancreatitis[464]. However, increasing trypsin release is expected thereafter and would fuel both plasminogen and prothrombin activation. The striking relationship between an exponential increase in serum

trypsinogen load and a linear increase in urinary CAPAP (**Figure 17.15**) brings to mind that between serum anionic trypsinogen and its complexes with α_1PI, using a double log plot, in an investigation of post-ERCP pancreatitis[259]. These findings strongly suggest autoactivation or a process with similar kinetics that operates within the pancreatic interstitium where zymogens accumulate in experimental pancreatitis and which has been identified as the site of pathologically significant activation of trypsinogen[260]. In contrast, the positive influence of CRP or PAI-1 could be dismissed as spurious in that near-zero values of urinary CAPAP not infrequently co-existed with clear increase in one or other inflammatory marker.

The possibility that tryptase released from mast cells in the pancreatic interstitium might ignite the load of trypsinogen therein as acute pancreatitis evolves led to donation of tryptase ampoules to Swedish collaborators, who have now concluded that this does not happen *in vitro*[469]: the exquisite dependance of tryptase activity on pH, heparin level to maintain quadrivalent structure, etc , might yet question whether the position is resolved for conditions *in vivo*.

Finally, hyperhomocysteinemia during acute pancreatitis is a worrying finding because it increases the risk of ischaemic injury to the gland[451]. Once again the evidence points to vulnerability of the methionine metabolic pathway and its dependency on vitamin co-factors, such as folic acid and choline.

17.6 Overview and summary

The Manchester group was, of course, not the first in attempting to plough the minefield of acute pancreatitis! As long ago as 1925, a surgical pioneer deliberated over “the suddenness of its onset, the illimitable agony which accompanies it, and the mortality attendant upon it”[470]. Moreover, among numerous clinical trials, perhaps the most promising of a PAF receptor antagonist failed at the last hurdle, the authors concluding that treatment is likely to be effective only if given at time zero. The latter is akin to the axiom in treating myocardial infarction, although acute pancreatitis does not have anything like the same degree of public awareness[257]. Immediate hemofiltration coupled with GSH precursor therapy is hardly justifiable when the majority of patients recover without specific intervention. This theme is explored further in the penultimate Chapter. Among other questions raised by the studies described herein is whether premorbid antioxidant lack predisposes to acute pancreatitis: that facet is examined next.

Chapter 18

More on micronutrient lack and pancreatitis risk

Increased FRA, micronutrient insufficiency, and disrupted methionine homeostasis are features of both acute pancreatitis and chronic pancreatitis (Chapters 7, 17). In the latter case, induced CYP are the likely source of increased electron transfer reactions and micronutrient lack is premorbid (Chapter 12). This begged the question whether pre-morbid lack of micronutrient antioxidants might also be involved in susceptibility to acute pancreatitis, eg. in patients with cholelithiasis[471] or coronary artery bypass grafting (CABG)[472]. Moreover, it was of interest to find out what impact AOT along the successful lines in chronic pancreatitis might have in oxidative stress-prone diseases that are not known to be associated with aberrant methionine metabolism. Systemic sclerosis with Raynaud's phenomenon (RP) is a case in point. Not only does this disease exemplify the dire effect of recurrent ischemia-reperfusion episodes, but also it can impair exocrine pancreatic function[473].

18.1 Gallstones

18.1.1 Pathogenesis: conventional view

Acute pancreatitis is triggered by way of a migrating stone that transiently obstructs Oddi's sphincter en route to the duodenum: the stones are generally small, most are mulberry-like and contain bile pigment[471], and cholecystectomy is expected to prevent further attacks. Traditionally, cholelithiasis in a patient with chronic pancreatitis is viewed as secondary to constriction of the intra-pancreatic portion of the common bile duct.

More recent studies indicate that the relationship is not so clear-cut. Thus: pancreatogram abnormalities have been recorded in nearly 50% of patients with gallstones but no history of pancreatitis; impaired pancreatic function in around 25% and scarring consistent with chronic pancreatitis in several patients as revealed by MRCP 5 years after acute biliary pancreatitis. Moreover, cholelithiasis is now cited as the main 'cause' of chronic pancreatitis in China[474], and in a third of cases in Italy[475].

Most stones are cholesterol-rich. The classical view on their pathogenesis runs as follows. The hepatocyte produces bile that is supersaturated in cholesterol; the carriage of lipids in bile is deranged; the gall bladder crystallises these defects; intestinal factors compound them; and each aspect involves several sub-clauses such that numerous permutations and combinations are possible[476].

Among pigment-rich stones, the black variety indicates excess of the acid calcium salt of unconjugated bilirubin as is typified by hemolytic states, but also found in patients with cirrhosis, those receiving total parenteral nutrition, and occasional patients with chronic pancreatitis (**Figure 18.1**). It is not clear why their frequency has increased in developed countries, to at least 30% by 1995. Brown pigment stones are ascribed to biliary infection. For both varieties a multifactorial model for pathogenesis is favoured: the hepatocyte produces bile that is rich in bilirubin conjugates, notably mono-unconjugates that are readily hydrolysed by hepatobiliary β-glucuronidases; the acid calcium salt forms at the normal pH of gall bladder contents; mucin hypersecretion prevents the desirable increase in acidification and serves to gel bilirubinate. Animal models are confusing because different species fed with the same lithogenic diet produce cholesterol-rich or pigment-rich stones or a mixture.

Economic ($ 5 billion per annum in the USA by 1995), morbidity, and mortality (3000 deaths per annum by 1995) statistics highlight the need for

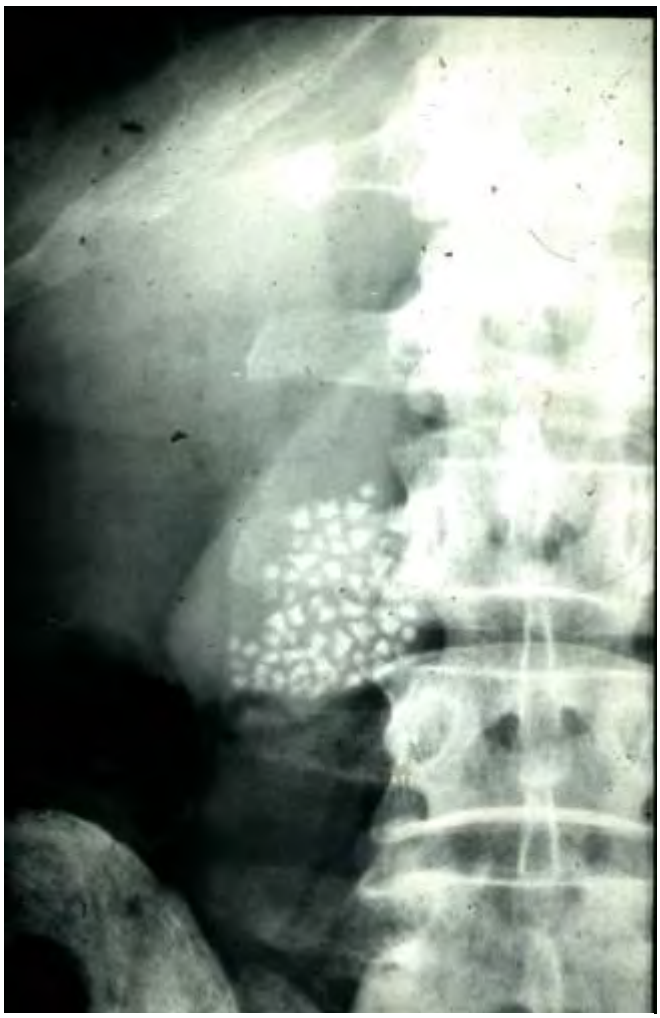

Figure 18.1 Radio-opaque pigment gallstones in a young patient with idiopathic chronic pancreatitis, poor antioxidant intake and excessive amounts of bilirubin and lipid oxidation products in secretin-stimulated bile

prevention - but how? Ursodeoxycholic acid and NSAID have been proposed but are prohibited by cost or gastrointestinal side effects, respectively.

18.1.2 Hypothesis: casualty of oxidative stress ?

By analogy with the evolution of calcifying chronic pancreatitis (Chapters 3, 5, 6), it seemed possible that insufficiency of micronutrient antioxidants relative to oxidant load in hepatocytes is important in cholelthiasis too, due to mobilisation of other antioxidant resources - bilirubin, mucin, lactoferrin - that inadvertently serve as fusogens / pro-nucleating agents / facilitators of crystallization. Aberrant activities of CYP and HO (heme oxygenase) are integral to this schema because their differential inhibition or activation helps to rationalise the varied composition of human gallstones as well as inter-species variation in outcome when the same lithogenic diet is given. The proposal takes into account the strong influence of inheritance, gender, age, species and environmental factors on CYP function, but the primary consideration is wastage of hepatic heme and / or increased HO synthesis when micronutrients such as selenium, ascorbate, or methionine are deficient[476].

Several observations support the hypothesis in relation to cholesterol-rich stones. (i) Their development in 2 men during estrogen treatment for prostate cancer suggests a link to inhibition of CYP cholesterol 7-α-hydoxylase, the rate-limiting enzyme for conversion of cholesterol to bile acids. (ii) An isoform of the same CYP family is involved in the metabolism of nifedipine and shows polymorphism: patients with cystic fibrosis, who have a predilection to gallstones, showed poor disposal of this probe[477]. (iii) Experimental gallstones are promoted by a variety of dietary manipulations: increased sucrose, cholesterol or PUFA; low fibre; decreased vitamin C, vitamin E or methionine (in the presence of a diet that delivers 0.05% cholic acid and 10% cholesterol). Sucrose-rich diets dampen the function of certain CYP, while deficiencies of vitamin C and E down-regulate CYP cholesterol 7-α-hydroxylase - changes conducive to increased HO activity and thus increased bilirubin in bile but with lowered GSH. These disturbances would rationalise the striking increase in unconjugated bilirubin and mucin in gallbladder bile from patients with cholesterol gallstones. Moreover, guinea pigs which, like man, cannot synthesise vitamin C, rapidly develop cholesterol gallstones when lithogenic diets are modified by vitamin C deprivation, but not otherwise. (iv) The favourable effect of phenobarbitone on cholesterol supersaturated bile in monkeys, as also of small amounts of ethanol in protecting against human gallstones, is rationalised by expansion of

hepatocyte SER with increased synthesis and secretion of solubilising phospholipids[476].

The evidence is even stronger for pigment-rich stones. Thus, a strong free radical signal has been recorded from human stones; and black stones are easily produced in dogs that are reared on cholesterol-rich diets lacking methionine and cysteine, especially when the diet also lacks iron. Deficiency of sulphur amino acids, and hence of GSH, is expected to compromise CYP cholesterol 7- α-hydroxylase activity while increasing that of HO, resulting in a net increase of bilirubin in bile: experimental iron deficiency results in induction of certain CYP and augmented bile lactoferrin. The increased frequency of black stones nowadays could reflect increased exposure to xenobiotics, but always against a backdrop of suboptimal antioxidant intake[476].

18.1.3 Dietary study 1991

The plan was to organise 7-day weighed food inventories over a 6-month period from June 1991 in equal numbers of patients who had not consciously changed their diets, patients on low-fat diets, and age / gender-matched controls. Food tables would be used to derive intake of certain macronutrients, essential amino acids, essential fatty acids and, above all, antioxidants. Although today it is realised that numerous micronutrients, including perhaps all vitamins have antioxidant potential[478,479] (**Table 18.1**), this was not the position in 1991: moreover, food tables were incomplete for several items such as ubiquinone (Co-enzyme Q10).

Recruitment of cases was via waiting lists for cholecystectomy. Consecutive symptomatic patients with cholesterol gallstones were identified, provided that they did not live more than 20 miles from the hospital because that would make home visits difficult, or if perusal of records identified another reason for a special diet or a problem that might influence food intake. Contact was made by personal interview or introductory letter. Patients were excluded if taking an over-the-counter vitamin, antioxidant or other food supplement. The presence of gallstones was verified by ultrasonography: transluscence on plain x-ray of the abdomen was taken to imply cholesterol-rich stones, as was confirmed in every patient who subsequently had a cholecystectomy. A register was drawn up of potential controls following advertisement in the hospital. The list had a preponderance of hospital cleaners with a few laboratory technicians who persuaded their friends and family members to participate. Acceptance for the study required that there was no history of abdominal surgery, ischaemic heart disease or diabetes; no symptoms to suggest gallstones; no dietary fad; and no previous or current use of a food supplement. As each suitable patient was identified, a control subject was selected to be age-(within 3 years) and gender-matched and studied within the same fortnight as the patient[480].

Patients and healthy volunteers were interviewed and instructed on the dietary method by a dietitian who collaborated in the chronic pancreatitis studies (Chapter 7). Participants were visited before, during and after the recording. Thereafter the intake of macronutrients (items consumed in gm or greater amounts) and micronutrients (mg or lower amount) were derived using the Microdiet programme of Salford University, which had been modified to take better account of selenium and sulphur amino acids. Only when the dietitian was satisfied that the protocol had been followed satisfactorily, was the dietary record accepted.

After the dietary studies were complete, came warning of a systematic tendency to under-reporting in 7-day inventories and how to detect the problem. Researchers were advised to calculate the ratio of energy intake (EI) to estimated basal metabolic rate (BMR) from consideration of body mass, age, gender, physical activity, sample size and duration of data collection - giving a lower limit of 1.35 for acceptance of an individual's reliability in reporting

Table 18.1 Micronutrients with direct or indirect function as antioxidant

ascorbic acid≡ bioactive vitamin C	key plasma antioxidant; substitutes for GSH in cells; defuses histamine from mast cells; acts as 'Michael donor'
α-tocopherol≡ vitamin E	major scavenger of peroxyl radicals in lipid substrates and membranes
retinol ≡ vitamin A	protects lipids especially in conjunction with taurine
β-carotene≡vitamin A precursor	quenches singlet oxygen in lipid substrates in a manner that does not need a regeneration reaction
riboflavin ≡ vitamin B_2	co-factor for GSH reductase
thiamine ≡ vitamin B_1	co-factor for transketolase in hexose monophosphate shunt
nicotinic acid ≡ vitamin B_5	essential for NADPH which is integral to function of GSH reductase
pyridoxine ≡ vitamin B_6	co-factor for 2 enzymes that ensure homocysteine transsulphuration towards cysteine and GSH
cyanocobalamin ≡ vitamin B_{12}	essential for vitamin B_{12}-folate route back to methionine from homocysteine
folic acid	ditto
choline	essential for alternative route back to methionine from homocysteine
betaine	ditto
vitamin D	membrane antioxidant by inhibiting iron-dependent lipid peroxidation
selenium	co-factor for GSH peroxidase; protects thiols; facilitates haem incorporation into CYP
methionine	essential for methyl group and GSH synthesis
cysteine	detoxifies xenobiotics via synthesis of GSH and taurine, inorganic sulphate, mercapturic acids
sulphur	detoxifier via inorganic and ester sulphate; as sulphides helps eliminate chain-initiating hydroperoxides
copper*	co-factor for superoxide dismutase; traps excess copper via caeruloplasmin which has ferroxidase1 activity
zinc	co-factor for superoxidase dismutase; protects via metallothionein; stabilizes membranes
manganese	co-factor for mitochondrial superoxide dismutase
iron*	co-factor for catalase which protects peroxisomes
magnesium	essential for hexose monophosphate shunt
phosphorus	essential for hexose monophosphate shunt
coenzyme Q10	inhibits membrane lipid peroxidation; vital for mitochondrial energy

*pro-oxidant in excess. ≡ equivalent to. Abbreviations:CYP, cytochrome P450 mono-oxygenases; GSH, glutathione; NADPH, nicotine adenine dinucleotide phosphate in its reduced form

habitual food intake[481]. Data were re-examined retrospectively in accordance with this advice.

Students t test (2-tailed) was used to compare macronutrient and micronutrient intakes in the current group of 18 current controls and the group of 15 studied a decade earlier[190]. For most nutrients mean± SD was derived for each group; mean differences between matched pairs then calculated by paired Students t tests with 95% confidence intervals (CI), in general accepting a 5% significance level, but also noting differences where $0.05<2p<0.10$. If $\log_{10}$ transformation was needed to render the distribution more Gaussian, geometric means and ranges were derived for inter-group comparison, followed after statistical analysis by back-transformation to yield the geometric mean of the ratios with approximate 95% CI. Where a suitable transformation could not be found, data were expressed as medians and ranges, with differences between groups assessed by the Wilcoxon matched-pairs signed-rank test. Power calculations were done when the intake of a nutrient of interest in patients on unchanged diets was less than in matched controls ($2p < 0.05$).

Only 9 among 22 consenting patients had not changed their diets: these together with the first 9 patients on low-fat diets formed the test group (**Table 18.2**).

Information from 18 matched controls indicated broadly similar social backgrounds, jobs, BMI, current alcohol and cigarette usage. Fourteen of the 18 pairs were female. An average physical activity level of 1.55 was assigned for the patients who had sedentary lifestyles, and 1.70 for controls who were more active.

There was no difference in macronutrient intake of the current and earlier set of volunteers (Chapter 7). Comparison of data from 18 current controls and all 18 patients with gallstones showed no difference in intake of carbohydrate or its components (sugars, starch / dextrins, fibre).

Table 18.2 Characteristics of participants in study of antioxidant intake and gallstones

			Controls				Gallstones				
Pairs	Gender		Job	Age yr	BMI kg/m²	Alcohol gm/d	Cigarettes no /d	Job	Age yr	BMI kg/m²	Alcohol gm/d	Cigarettes no /d
1	M		retired electrician	49	26	19	35	retired manager	50	21	0	never
2	M		textiles worker	66	25	3	ex-	engineer	67	28	20	ex-
3	M		retired porter	70	23	0	never	retired painter	69	25	0	ex-
4	M		retired porter	75	21	15	ex-	retired metalworker	72	28	65	never
5	F		dietitian	26	23	11	never	care assistant	26	30	0	8
6	F		nurse	47	26	7	never	financial consultant	44	25	11	ex-
7	F		ward cleaner	26	21	18	never	laboratory technician	26	19	2	never
8	F		ward cleaner	39	27	0	7	care assistant	40	33	13	never
9	F		ward cleaner	51	26	21	ex-	housewife	51	30	21	10
		Mean		50	24	11*			49	26	11*	
		SD		18	2.5	(0-21)			17	4.6	(0-65)	
10	F		secretary	48	26	12	5	office clerk	45	20	16	never
11	F		ward cleaner	52	25	0	ex-	tabulator	53	27	0	ex-
12	F		retired cleaner	70	27	0	ex-	retired factory worker	71	18	0	never
13	F		secretary	53	22	0	never	receptionist	56	26	0	never
14	F		ward cleaner	56	27	0	ex-	domestic cleaner	59	22	0	ex-
15	F		physiotherapist	42	20	19	never	sales person	44	29	3	ex-
16	F		nurse	31	22	0	never	receptionist	34	20	9	ex-
17	F		ward cleaner	51	29	5	never	teacher	51	24	12	ex-
18	F		nurse	53	20	7	30	child worker	50	31	0	ex-
		Mean		51	24	0*			51	24	0*	
		SD		11	3.3	(0-19)			10	4.4	(0-16)	
	Overall	Mean		50	24	4*			50	25	2.5*	
		SD		14	2.8	(0-21)			14	4.6	(0-65)	

*alcohol data as median and range. BMI=body mass index. Pairs 1-9 =patients on habitual diet; pairs 10-18 =patients on low fat diet (ref480)

Patients on a low fat diet ingested less total fat, the saturated palmitic and stearic fatty acids, and unsaturated olieic and linolenic acid, but not of cholesterol or arachidonic acid. The markedly lower intake of linoleic acid by the group as a whole ($p<0.001$) was notably influenced by lower intakes in patients who had not changed their diets ($p=0.009$) rather than a reflection of low-fat diets ($p=0.026$) - a conclusion affirmed when fatty acid intake was expressed relative to that of total fat. Both subgroups of patients contributed to the lower intake of protein by the group as a whole ($p=0.004$). Overall, patients with gallstones had a lower energy intake than controls, due to lower intake of protein (17 kJ/gm), compounded by advice to reduce intake of fat (37 kJ/gm).

As to micronutrient antioxidant intake, attention was focussed on 16 established items including vitamin D which had been identified as a protector of lipid membranes[479]. The only difference in micronutrient intake by the current compared to earlier set of volunteers was their lower intake of vitamin C, probably due to a preponderance of unskilled workers whereas there were a number of dietitians previously. Matched-pairs analysis of data from patients who claimed not to have changed their diets showed lower intakes than

Table 18.3 Matched pairs analysis involving patients with gallstones on unchanged diets

Intake/day	Controls	Patients	Mean/Median difference and 95% CI	p
methionine (gm)	1.9 ± 0.4	1.5 ± 0.3	0.3, 0.04 to 0.6	0.031
cysteine (gm)	1.2 ± 0.3	1.0 ± 0.2	0.2, -0.02 to 0.5	0.063
α-tocopherol (mg)	7.4 ± 3.2	3.8 ± 2.1	3.6, 0.5 to 6.7	0.029
vitamin D (µg)	2.5 (0.8-8.6)	1.2 (0.5-4.5)	2.1, 1,1 to 4.0	0.033
β-carotene (µg)	2382 (561-5063)	723 (66-3196)	3.3, 0.8 to 14.5	0.10
vitamin C (mg)	84 (21-204)	37 (9-109)	2.2, 0.8 to 6.1	0.10
thiamine (mg)	1.4 ± 0.7	1.1 ± 0.5	0.3, -0.4 to 1.0	NS
riboflavin (mg)	2.1 (1.0-4.7)	2.1 (1.1-2.8)	0.3, -0.6 ti 1.8	NS
niacin (mg)	40 (26-82)	31 (15-54)	1.3, 0.9 TO 1.9	NS
retinol (µg)	433 (148-3940)	365 (69-2246)	1.2, 0.5 to 3.0	NS
selenium (µg)	75 ± 34	43 ± 29	32, -7 to 70	0.092
zinc (mg)	11 ± 3	8 ±2	4, -0.2 to 7	0.059
manganese (mg)	3.5 (1.7-9.2)	2.0 (1.0-3.9)	1.7, 1.1 to 2.7	0.018
phosphorus (mg)	1493 ± 509	1103 ± 328	390, -83 to 862	0.094
sulphur (mg)	629 ± 170	555 ± 170	74, -70 to 218	NS
magnesium (mg)	311 (160-638)	223 (115-480)	45, -74 to 274	NS

Daily intake as mean ± SD, geometric mean (range) or, for riboflavin and magnesium, median (range). CI = confidence intervals for mean differences, or geometric mean of ratios after back transformation, or CI for median difference in the case of riboflavin and magnesium. NS not significant. Copper and iron omitted as can act as pro-oxidant (ref 480).

corresponding controls of 4 antioxidants (methionine, α-tocopherol, vitamin D, manganese) at the conventional 5% significance level, and of 6 more at the 10% level (cysteine, β-carotene, vitamin C, selenium, zinc, phosphorous) - ie. 10 of 16 potential items originating in very dissimilar dietary sources, when at most 2 differences might be expected by chance (**Table 18.3**). Imposition of a low fat diet amplified the deficit in α-tocopherol but caused a fortuitous increase in vitamin C, β-carotene, manganese and phosphorus because these patients compensated by eating more fruit and vegetables.

Intakes of essential amino acids and other trace substances were similar in current controls and controls of 1986. Patients on unchanged diets ingested less linoleic acid and amino acids than matched controls. Power calculation on nutrient differences that were most striking gave the following assignments at a 5% significance level: linoleic acid 88%; methionine 60%; vitamin E 63%; vitamin D 53%; phenylalanine 52%. Retrospective analysis for under-reporting[481] showed that 10 of 18 controls, and 11 of 18 patients under-reported food intake, but there was no significant difference in the mean energy intake: estimated BMR ratio in the subgroup of patients on habitual diets and their matched controls. In accordance with further advice that individual records should be scrutinised and values below the 99.7% percentile excluded, data from 2 patients on habitual diets were retrospectively substituted by results from 2 patients who were studied at a later date and with healthier energy intake: estimated BMR ratios of 1.28 and 2.2. This adjustment did not alter the earlier outcome in regard to lower intake of antioxidants by patients on regular diets. Tabulated results are available in the study report[480].

In conclusion, notwithstanding that under-reporting appears to be inherent in 7-day home dietary inventories, the hypothesis that suboptimal intake of micronutrient antioxidants might be germane to the pathogenesis of cholesterol gallstones was borne out by the investigation. It was difficult to dismiss these results as epiphenomena in light of earlier arguments (Section 18.1.2) but there was clearly a need for biochemical clarification.

18.1.4 Biochemical study

The plan was to analyse fasting plasma / serum samples from patients with cholesterol gallstones and unchanged lifestyles for the 4 main

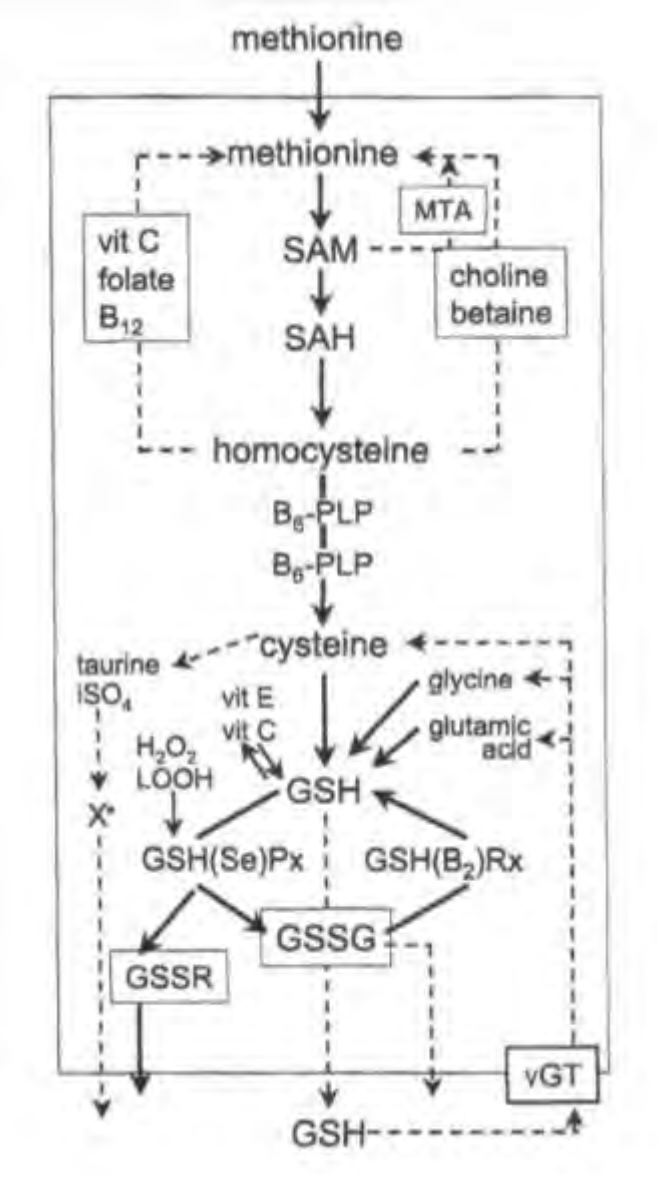

Figure 18.2 Diagram of methionine utilization to facilitate interpretation of dietary and biuochemial studies in relation to cholelithiasis. Abbreviations explained in Glossary. From 2004 paper in Clin Chim Acta [482]

micronutrient antioxidants, GSH, and factors that impact or report upon GSH homeostasis; comparing the results against laboratory referent ranges. **Figure 18.2** is a reminder of influences on GSH metabolism, so as to facilitate interpretation of results.

Recruitment was slow, such that only 24 patients could be investigated in the scheduled 18- month period. This was for 2 main reasons. First, most patients had already switched to a low fat diet at referral. Second, the study was restricted to patients with uncomplicated gallstone disease (normal CRP, bilirubin and alkaline phosphatase), without a prior medical problem (eg. eating disorder, diabetes), and who were not taking food supplements. **Table 18.4** gives social information including exposure to xenobiotics because these are potentially relevant to gallstone development via challenge to enzymes that are also involved in cholesterol and bilirubin metabolism[476].

The control database was derived from studies of hospital workers, their relatives, and patients with minor surgical problems: they did not smoke, drank little or no alcohol, had no dietary fads, were not taking food supplements, and had no previous or current medical problem.

Full details of procedures and laboratory assays are given in the study report [482]. Assays for methionine, homocysteine and vitamin co-factors for enzymes that influence its metabolism (vitamin B_6, B_{12}, folate) were done at Pretoria, south Africa, on residual deep frozen material from patients and age/gender-matched controls.

Independent sample t tests were used to compare data in controls and patients. Where variances differed significantly, separate unspooled variance t tests were used instead. When age influenced a variable (as for retinol, vitamin E and its ratio to cholesterol), age-adjusted differences in means were calculated by analysis of covariance. Log transformation for data on γGT was required prior to assessment as raw data were positively skewed. When no satisfactory transformation could be found - as for plasma hemoglobin (measured to safeguard against spurious GSH readings from erythrocyte lysis invisible to the naked eye), β-carotene, and % MRVC - non-parametric Mann Whitney U tests were employed.

Table 18.4 Characteristics of patients with gallstones in biochemical study

Patient	Gender	Age yr	BMI kg/m^2	Alcohol gm/d	Cigarettes number/d	Job at diagnosis	Duration yr	Chemicals
1	F	31	30.1	0	15	housewife	10	none specific
2	F	60	29.1	7	0 (ex 20)	cleaner / bus conductor	30 / 6	bleaches, solvents / vehicle emissions
3	F	31	22.9	22	20	lampshade maker	1	glues, resins
4	F	55	35.1	30	0	housewife	30	none specific
5	F	36	29.9	0	20	laundress / industrial packer	8 /1.5	solvents / machine grease
6	F	47	28.9	0	12	laundress	2	none specific
7	M	65	26.2	10	10	textile worker	14	dyes, bleaches
8	F	57	24.7	10	0	factory cleaner	20	smoke, bleaches, solvents
9	F	56	3o.3	0	6 (ex 40)	factory worker	20	solvents
10	M	80	24.6	0	0	insurance agent	45	none specific
11	M	67	27.7	20	30	engineer	51	machine coolants
12	F	26	18.7	2	0	laboratory technician	9	solvents
13	F	37	31.2	27	0	marketing consultant	15	none specific
14	F	47	31.9	0	0	housewife	27	none specific
15	F	44	24.8	8	0	dry cleaner	7	solvents
16	F	61	33.6	0	0	office worker	25	none specific
17	F	31	32.6	0	0	hairdresser	2	bleaches, solvents, lotions
18	F	24	35.4	64	30	housewife	6	none specific
19	F	57	26.5	30	0	cleaner	6	bleaches, solvents, disinfectants
20	F	63	21.2	8	0	music teacher	22	none specific
21	F	44	24.6	11	0	photograph processor	20	glues, solvents
22	M	59	28.6	23	0	colourist	46	dyes, paints, solvents, paraffin
23	F	53	26.8	0	0	tabulator	8	machine oil
24	M	26	21.3	7	0	motorbike restorer / drain engineer	5 /10	petrol, diesel / resins, thinners, solvents

Alcohol and cigarette usage extrapolated from amounts in a typical week. Occupations and chemical exposures were as stated by patients. Modified from ref 482.

Two-tailed test for significance were used throughout.

Table 18.5 gives the salient results. Not included is the information that among other markers of oxidative stress, %MRLA' conformed to control data but lipid peroxides were higher in the patients ($p<0.05$).

18.1.5 Comments

The investigations generated 3 largely novel findings in patients with cholesterol gallstones. (i) Micronutrient antioxidant lack is a feature. (ii) This is accompanied by a fall in plasma GSH coupled with elevated γGT activity, but preservation of GSH level in erythrocytes which facilitate the inter-organ transport of GSH. (iii) Poor status of sulphur amino acids, folate and vitamin B_6 is revealed.

At the time of the study, the only precedent was the finding of an inverted U-shaped relationship between serum ascorbic acid and gall bladder disease in women[483]: total vitamin C was not measured. While it is impossible to be certain that low intake of several antioxidants explains the poor serum / plasma profiles of micronutrient antioxidants, the dietary analysis made this likely (Section 17.1.3). When the biochemical information is examined alongside dietary data, it becomes clear that whereas the outcome in regard to vitamin E status relative to lipid is similar ($p=0.021$ and $p=0.032$, respectively), the biochemical study shows vitamin C, β-carotene and vitamin B_6 levels to be disproportionately lower than anticipated. Since these items are highly susceptible to oxidative attack, the inference seems to be that heightened oxidation contributes to the subnormal levels in patients with cholesterol gallstones. There was no suggestion from the dietary survey that *in vitro* loss during food preparation was responsible. Speculatively, oxidising agents in cigarette smoke, occupational chemicals or alcohol might be responsible.

The corollary of *in vivo* oxidative stress was supported by GSH measurements, with the liver implicated as the site of the problem. Thus, it is known that surplus GSH generated in organs of its active synthesise is the source of plasma GSH, and that the liver is the predominant donor: when oxidative stress in hepatocytes compromises GSH delivery, constituent amino acids from the existing plasma pool are made re-available to hepatocytes and erythrocytes via increased

Table 18.5 Summary biochemical profiles in patients with gallstones

	Controls n	mean ± SD	Patients n	mean ± SD	Difference in means [95% confidence interval]	Significance of difference≡ 2P
Main micronutrient antioxidants						
vitamin C (μmol/l)*	54	68.80 ± 26.86	24	45.48 ± 29.93	23.32 [9.72 to 36.92]	0.001
α-tocopherol (mmol/mol cholesterol)	47	5.24 ± 0.98	18	4.41 ±1.30	0.92 [0.36 to 1.48]	0.002
β-carotene (nmol/l)	47	279, 67-1153 †	18	104, 7 - 672 †	160 [76 to 268] ‡	0.001
selenium (μmol/l)	45	1.14 ± 0.20	24	1.07 ± 0.29	0.07 [-0.06 to 0.21]	0.280 §
Glutathione status						
whole blood (μmol/l)	29	1221 ± 245	18	1244 ± 270	-23 [-177 to 131]	0.763
plasma (μmol/l)	30	6.08 ± 1.46	17	4.62 ± 1.28	1.47 [0.61 to 2.32]	0.001
Y glutamyl transpeptidase (u/l)	26	14.3, 3 - 75 †	20	41.6, 10 - 478 †	-17.5 [-45 to -9]	<0.001
Homocysteine status and influences						
homocysteine (μmol/l)	14	7.20 ± 1.33 ‡	14	6.53 ± 2.50	-1.33 [-2.86 to 0.23]	0.092
homocysteine : methionine ratio	13	0.29, 0.133-0.450 †	12	0.459, 0.283-1.080 †	-0.148 [-0.287 to -0.034] ‡	0.005
methionine (μmol/l)	14	23.42,17.06-50.70 †	12	17.61, 12.10-33.68 †	5.79 [2.34 to 9.97] ‡	0.003
cysteine (μmol/l)	14	280.8, 236-721.5 †	12	250.1, 219.8-297.0 †	20.6 [0.40 to 43.7] ‡	0.401
vitamin B_6-pyridoxal-5-phosphate (nmol/l)	14	40.55 ± 14.57	14	21.95 ± 9.91	18.60 [8.92 to 28.28]	<0.001
folate (μg/l)	14	24.86, 16.67-38.66 †	14	18.07, 13.61-66.50 †	5.97 [1.18 to 10.28] ‡	0.012
vitamin B_{12} (ng/l)	14	205.1 ± 39.4	14	192.4 ± 35.0	12.7 [-16.0 to 41.6]	0.377

* ascorbic acid values reflected these. † medians and ranges, with groups compared by Mann-Whitney *U* test. ‡ geometric means and ranges compared by *t* test on logged values. § median of pairwise differences with approximate 95% confidence intervals .Fuller' information in ref 482.

activity of γGT (**Figure 18.2**). This was vividly shown in a study of chronic alcoholics at Soweto (**Figure 12.2**). .Against this background, low total plasma glutathione (mainly GSH but a contribution from the reversibly oxidised form GSSG) together with increased γGT activity reinforces the notion that the methionine metabolic pathway in hepatocytes is under oxidative strain in patients with cholesterol gallstones.

The Pretoria arm of the study revealed subnormal levels of folate and vitamin B_6 (as gauged by activity of the B_6-dependent enzyme pyridoxal-5-phosphate). Yet the increase in plasma homocysteine was modest (p=0.092), a paradox rationalised by the subnormal level of methionine (**Table 18.5**), in keeping with its lower intake (Section 18.1). Hence as might be expected, the homocysteie:methionine ratio was elevated in the patients.

Together, these dietary and biochemical investigations bring antioxidant lack, deranged hepatic GSH status, and disturbed homocysteine metabolism into the field of cholesterol gallstones: although direct evidence for oxidative stress in the organ was not adduced, the alterations in GSH and γGT are highly suggestive of its presence. The free radical pathology of black pigment gallstones has been recognised for some time, and underlined by a cited report on the preventive value of melatonin in the experimental setting [476]. Considering the very high levels of bilirubin in secretin-stimulated bile from patients with chronic pancreatitis and acute pancreatitis (Chapter 3), it is surprising that black pigment stones are not reported more often.

The finding of antioxidant lack and inferred hepatic oxidative stress in patients with gallstones does not help to say which particular stage in cholelthiasis might be facilitated thereby. It would also be premature to conclude that antioxidant deficiency lays an individual prone - under conditions of increased hepatic FRA - to the full spectrum of gallstones, although other strands of evidence raise this intriguing possibility. For now the conclusion seems to be that prior antioxidant lack leaves individuals susceptible to the abrupt increase in pancreatic FRA, and hence pancreatitis, evoked by a migrating stone (Chapter 11).

18.2 Ischaemic heart disease

18.2.1 Patients awaiting CABG

The plan was to investigate patients with >75% stenosis in ≥ 3 coronary vessels in the 12-month period from June 1992. They would be identified from the waiting list for CABG, and would exclude those with a history of diabetes, Raynauds phenomenon (RP), lung or pancreatic disease, as also anyone who had a systemic infection in the previous month or was taking over-the counter food supplements.. Once informed consent was obtained, drugs that might introduce artefacts into the study would be discontinued, while accepting that treatment with allopurinol in the build-up to surgery was adopted by some surgeons to safeguard against xanthine oxidase-medaited injury.

Sixteen male patients were studied (median age 63, range 35-70 years). Each was on a low fat diet for at least 6 months and on optimal treatment with a β-blocker, calcium antagonist and nitrate. Eight patients received allopurinol, 300 mg twice daily for 2 days before scheduled surgery: their clinical characteristics were no different from the other 8 patients who did not. Social histories showed that 2 patients had never smoked cigarettes and the others had stopped 1-20 years earlier. Six drank little or no alcohol, 9 drank alcohol on a social basis, and 1 consumed 80 gm per day. The control group consisted of 8 men of broadly similar age (56, 50-60 years) with no significant medical or surgical history and a normal exercise cardiogram. This age-matching was done to prevent bias, in that laboratory referent ranges were based on studies with a preponderance of younger individuals. None of the group smoked cigarettes; most drank alcohol

on a social basis; all were on habitual diets; and none was on a food supplement.

After an overnight fast, 30 ml venous blood were processed so as to obtain serum and plasma for measurement of the main antioxidants and certain FROP. In addition, heparinised whole blood was processed so as to enable assessment of erythrocyte lipid peroxidisability, ATP and energy charge. An appropriate time-scale of analysis was implemented so as to take account of potential degradation of samples. Spare deep-frozen material was sent to Pretoria for assay of homocysteine and vitamins that are involved in its metabolism[338].

Laboratory methods, including for ATP and energy charge in erythrocytes, are given in the study report[484]. Retinol (vitamin A) is not generally regarded as an antioxidant, but studies just prior to the investigation suggested that it could indirectly facilitate antioxidant defence. Four markers of FRA were used, as validated in previous studies[331]: % of oxidised to total glutathione in plasma; % MRVC in plasma; lipid peroxides and %MRLA' in serum. More specific methods to detect free radical attack on lipids were at the time either only applicable to tissue samples or not described.

An SPSS/PC package facilitated comparison of data from controls and patients using the Wilcoxon rank sum test. The same approach was used to compare levels of analytes in patients who had or had not received allopurinol, and to assess whether values in controls conformed with laboratory referent ranges. Differences were considered to be significant when $2p<0.05$.

The investigation showed that patients who were not taking allopurinol had similar levels as controls of serum uric acid, an important endogenous antioxidant. Although its level was lower in the allopurinol-treated group (mean 283 μM vs 383 μM in non-treated group $p<0.05$), antioxidant and free radical marker profiles were very similar in the 2 subgroups. Compared to controls, the patients had lower serum / plasma levels of ascorbate, β-carotene, α-tocopherol and, particularly GSH (1.83 versus 6.53 μmol/l,

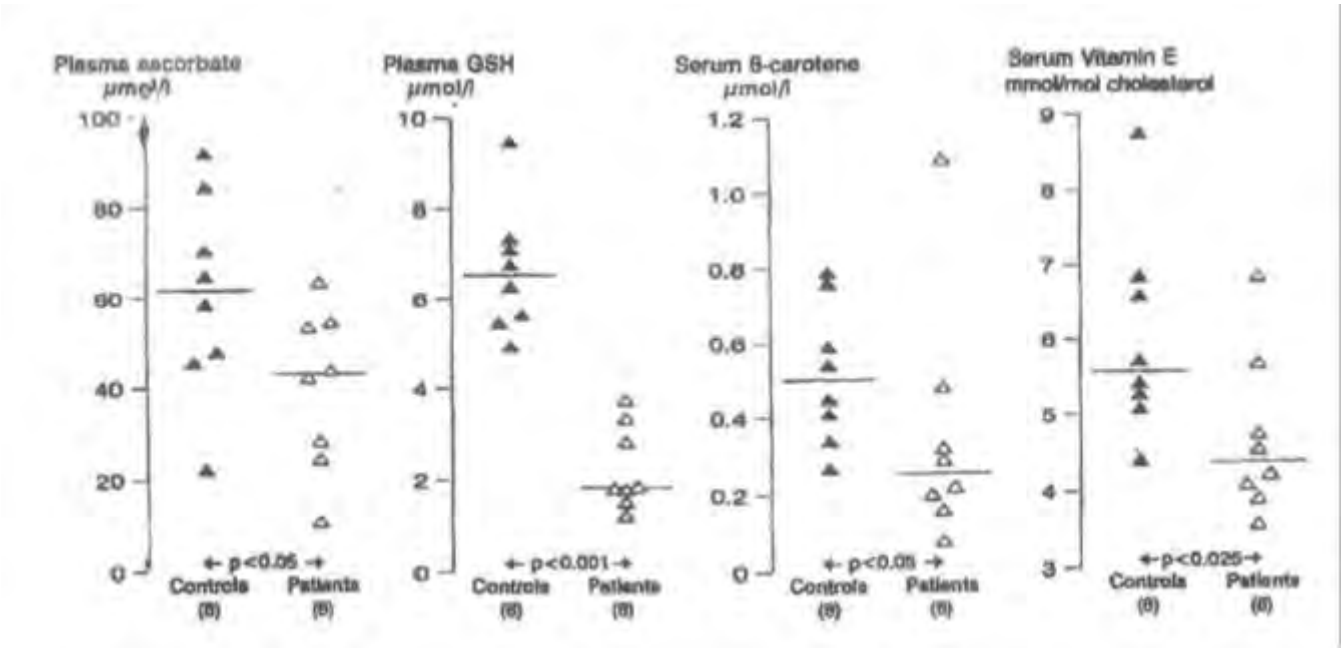

Figure 18.3 Plasma levels of 2 aqueous-phase antioxidants, ascorbate and GSH (glutathione) and serum levels of 2 lipid-phase agents, β-carotene and α-tocopherol.in patients awaiting coronary artery bypass grafting. Serum selenium values were similar in controls and patients. Reproduced from 1996 paper in Clin Chim Acta [484]

p<0.001) (**Figure 18.3**). Serum selenium levels conformed with those in controls but, of note, control values represented a significant decrement compared to the laboratory reference range derived from a preponderance of younger individuals. The concentration of lipid peroxides was higher in patients than controls (allopurinol treated p<0.05; amalgamated p <0.01), as was the proportion of oxidised to total glutathione (allopurinol treated 0.05<p<0.10; amalgamated p<0.025). In contrast, there was no increase in %MRVC or %MRLA', the latter in keeping with preserved selenium status (Chapter 16). Erythrocytes showed similar values as controls of GSH, ATP and energy charge, while their membranes showed no more than the expected rate of peroxidisability *in vitro*[484] .

Results of the ancillary study on homocysteine showed that the degree of hyperhomocysteinaemia was very similar in Manchester patients awaiting CABG and those with chronic pancreatitis, but also that vitamin B_6 lack was more pronounced in the latter set[338].

18.2.2 Comments

It is generally accepted that oxidative modification of low density lipoprotein (LDL) within extracellular pockets in the arterial intima, followed by uptake of the modified LDL by macrophages and formation of cholesterol-laden foam cells, are initiating events in the pathogenesis of atherosclerosis[485]. It is also recognised that hyperhomocysteinaemia is a detrimental later accompaniment[486]. Current advice is to increase the intake of fruit and vegetables which are sources of ascorbate and carotenoids, but this action could inadvertently lower the intake of vitamin E which is the arch protector against oxidation of lipid substrates.

The lack of contemporaneous data on blood micronutrients and free radical markers in patients with severe ischaemic heart disease was an added incentive - over-and above the aforesaid goal - to the Manchester investigation in 1992. It identified oxidative stress by the marked GSH deficit in plasma; showed that erythrocytes are robust, and that allopurinol as prescribed does not safeguard against plasma GSH oxidation or lipid peroxidation in serum.

The first finding on antioxidants in serum / plasma went beyond existing data. The observation of extremely low plasma GSH concentration with excess GSH oxidation strongly suggests poor prior intake of sulphur amino acids and insufficient protection of this key metabolite. It also identified lack of vitamin C, which not only substitutes for GSH via redox and non-redox interaction[202,203], but postpones lipid peroxidation within plasma until its biologically active component, ascorbate, is consumed. Thus, physiological levels of ascorbate have been shown to be as potent as the drug probucol in protecting LDL against oxidative modification, and only the vitamin spared α-tocopherol and β-carotene from oxidation within this lipid fraction. The second finding of robust erythrocytes is akin to that noted in patients with gallstones (**Table 18.5**). It is compatible with the belief that available antioxidants are avidly sequestered by red cells. As to the third outcome, the reputed benefit of allopurinol in reducing cardio-pulmonary complications after CABG might reflect its restraining effect on oxidants produced by activated phagocytes[276].

Thus, prior antioxidant lack would set the stage for acute pancreatitis following any fall in blood pressure and possibly from exposure to halogenated anaesthetics during CABG.

18.3 Raynaud's phenomenon (RP)

18.3.1 Identification of oxidative stress

The plan was to measure the 4 main micronutrient antioxidants (ascorbic acid, α-tocopherol, selenium, β-carotene) and 3 markers of oxidative stress (%MRLA', %MRVC, lipid peroxides) in serum / plasma from patients with RP. The aim was to ascertain if antioxidant status might influence clinical outcome, ie. primary disease

(PRP), limited cutaneous systemic sclerosis (ICSS) or diffuse systemic sclerosis (dSSc). The investigation in 1993 was proposed by MIV Jayson and AL Herrick of the University department of Rheumatology.

Among 28 patients who were studied, 10, 9, and 9, respectively, had PRP, ICSS or dSSc, respectively. The main findings are illustrated in **Figure 18.4**. Serum selenium was lower in patients with RP as a whole than in 15 non-smoker non-alcoholic controls (median and range, 83, 36-134 vs 100, 79-131 µg/l, respectively, p< 0.05), and this was most pronounced in the dSSc subgroup. The lower level of ascorbic acid in the patients overall than in controls (4.5, 0-11.7 vs 10.6, 6.0-18.5 mg/l, respectively, p<0.01) was evident in all categories of patients, and was not due to excessive oxidation.

The combination of deficiencies was associated with an increase in % MRLA' (3.40, 1.21-10.72 % vs control 1.91, 0.86-3.81 %, p <0.01), such that discriminant analysis using these 3 items afforded 91% sensitivity and 75% specificity in distinguishing between controls and patients[487].

Retrospective analysis identified cigarette smoking as an important factor in the lower ascorbic acid levels of the patients. However, even when smokers were excluded, ascorbic acid levels were less than and %MRLA' levels greater than respective values in controls. The lipid-phase antioxidants were unaltered in the patients and, in keeping, serum concentrations of lipid peroxides were within normal limits. Further information is provided in the study report[487].

18.3.2 Antioxidant plus allopurinol trial

Given that ischaemia-reperfusion injury is linked to the production of ROS via xanthine oxidase, it was logical to administer allopurinol - which inhibits the enzyme and is a free radical scavenger[276] - alongside the micronutrient combination to buttress ascorbic acid, selenium and thiols. The study would focus on patients with lSSC because they tend to have the most severe

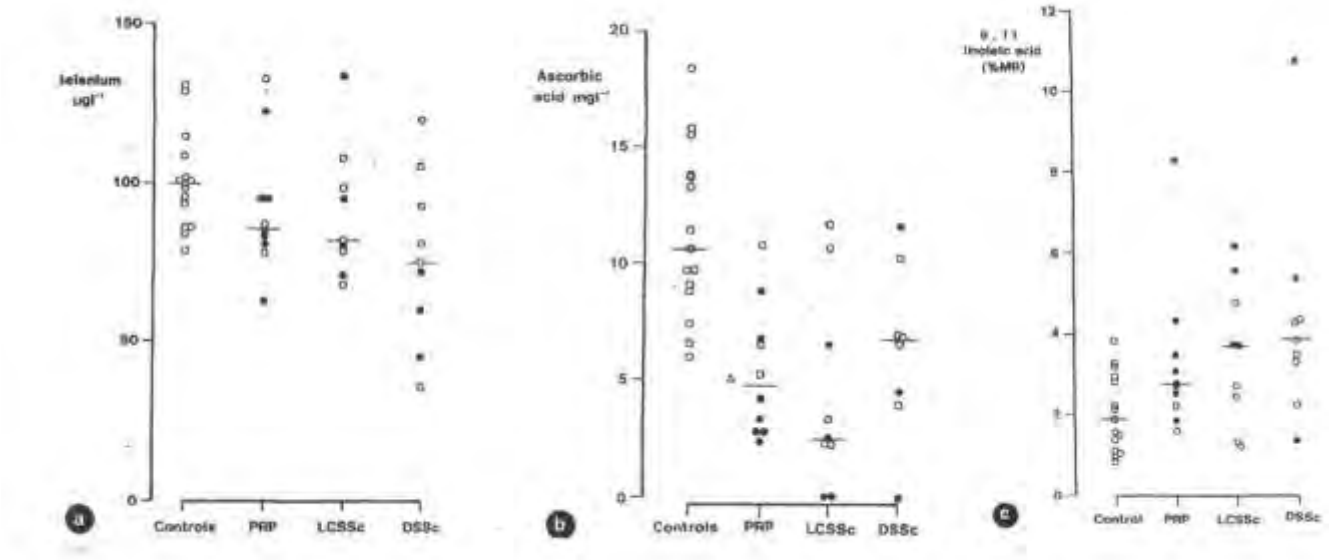

Figure 18.4 Excess oxidative stress in patients with Raynaud's phenomenon, shown by increase in the concentration of the 9,11 isomer of linoleic acid in serum and coupled with deficiencies of selenium and ascorbate. Solid symbols represent smokers. Categories of patients as defined in the text. Adapted from 1994 paper in J Rheumatol [487] and reprinted with the Journal's permission.

peripheral ischaemia, often in association with the anticentromere antibody. The double-blind placebo-controlled switch-over trial was mounted along the lines of the successful 20-week trial in patients with chronic pancreatitis (Chapter 16).

Thirty three non-smoker patients participated in the investigation. The median duration of RP was 10 years (range 2-50) in the active-first group and 10 years (4-53) in the placebo-first set. In the initial 10-weeks of study, active treatment made no impact on symptoms, re-warming curve, or vWF which signals endothelial injury / activation and was the primary end-point of the study - despite a fall in uric acid signifying the efficacy of allopurinol, and clear increases in micronutrients but without normalization of %MRLA'. Full results of the exemplary but disappointing trial have been published[488].

18.3.3 Comments

Patients in both studies had established disease, with RP for several years in the majority. Even today, the precise pathogenesis of the condition is unclear[489], nor is it known why it is reversible in some individuals but progresses relentlessly to digital loss and internal organ damage in others. Agreed aspects of pathophysiology include disturbance of the microvasculature, abnormal neural control mechanisms, and platelet activation - aided and abetted by enhanced collagen synthesis and immune dysregulation during the transition to SSc. The collaborative work at Manchester was the first to explore the possibility that a greater degree of micronutrient antioxidant lack might underlie progression to SSc, via persistently increased oxidative stress; and thereafter to test the therapeutic potential of allopurinol plus micronutrient supplements - but alas, to no avail. The irony is that alteration in cellular redox due to persistent oxidative strain is now assigned a central role in the downward spiral to dSSc: cited mechanisms include ' an autoamplification circuit linking ROS, Ras and ERK 1-2 which in turn amplifies and maintains the autocrine loop made up of cytokines, growth factors and their cognate receptors' [490]. Ras and ERK are components of the protein chain that enables transmission of a signal from specific cell-surface receptors to DNA in the cell's nucleus.

18.4 Overview and summary

Absolute deficiency of specific micronutrients for long periods results in clearly defined diseases. Scurvy (vitamin C), rickets (vitamin D) and beri-beri (vitamin B_1) are among many examples. The pathophysiology of each condition might include but does not depend upon increased FRA.

Environmental toxicology / free radical pathology has introduced the rather different perspective of 'suboptimal' antioxidant status, ie. relative to demand from the prevailing load of reactive chemicals. Among nutrients that are now known to feed into the protective package against an excess of ROS / RXS (**Table 18.1**), some fulfil the definition of an antioxidant as 'any substance that when present at low concentration relative to that of an oxidisable substrate, considerably delays or inhibits oxidation'[100]. Others do not, but act indirectly to ensure sufficiency of the key agent that does, ie. GSH.

The essentials of GSH metabolism are worth recalling. Cysteine, derived directly from the diet or manufactured from precursor methionine, is the rate-limiting component in GSH synthesis. The utilisation of GSH in the removal via GSH (selenium)-peroxidase of ROS and lipid peroxides is soon made good by interlinking shuttles involving GSH-(riboflavin)-reductase / NADPH / G6PD (**Figure 7.1**). In contrast GSH in conjugates with RXS, via GST, is actively excreted and cannot be recycled[201, 222]. Plasma GSH represents the surplus from organs of active synthesis, notably the liver, minus any lost thereafter due to excessive oxidation - not least by reacting with cystine, which is the oxidised form of cysteine as exists in plasma. Component amino acids are made re-available to cells via membrane γGT, which can also transfer the γ- glutamyl moiety of GSH to extracellular

cystine, enabling its uptake into cells for GSH re-synthesis. Hence, decrements in plasma GSH are a sensitive gauge of GSH status, as apparently are increments in γGT (in the absence of cholestasis)[64, 491].

There is no easy way to assess GSH status of internal organs, say pancreatic acinar cells. Erythrocytes are not a faithful surrogate for several reasons: they do not possess CYP which could increase reactive load; they do not have the wherewithal to dispose of GSH conjugates with RXS; and they are committed to GSH control of aldehydic products of lipid peroxidation which would otherwise oxidise hemoglobin. On the plus side, their vulnerability to untransformed xenobiotics and ROS in the bloodstream[492] coupled with the rapid increase in erythrocyte GSH upon treatment with NAC or SAM[493, 494] suggest that erythrocyte GSH might be a useful index of electrophilic / oxidative stress when inhaled xenobiotics and methionine lack are implicated in pathogenesis, eg. in chronic pancreatitis[324] (Chapters 7 & 8).

Kwashiorkor-marasmus exemplifies the scenario wherein a very low quota of ingested antioxidants is soon extinguished by the huge burden of pro-oxidant forces. Studies from Jamaica show that both categories of malnourished children exhibit chronic adaptation, in the form of increased activities of GST and GSH-refurbishing systems in erythrocytes. The difference seems to lie in the inability to maintain GSH level in kwashiorkor, seemingly due to ultra-poor intake of selenium in the immediately antecedent period of intense xenobiotic challenge, plus iron overload due to insufficient up-regulation of binding proteins[319]. The preferential loss of pancreatic acinar cells attests to their vulnerability to electrophilic / oxidative strain, as witnessed by experimental studies using CCl_4[31], nitriles or dibutyltin[201].

The investigations described in this Chapter show that antioxidant lack is common to disparate chronic conditions that are risk factors for acute and / or chronic pancreatitis. Hence any role is as disease facilitator, not initiator. The flip-side is that progression to a particular disease is dictated by unique circumstances which induce bursts of electron transfer reactions such as to overwhelm protection of critical substrates within critical locations[222].

Among several factors that might be detrimental, the following are noteworthy: the production rate, ie. acuteness of the upsurge in reactive load; the oxidisable substrate in relation to tissue-specific biochemistry; the quota of co-factors required for free radical production; whether the cell or extracellular environment is the primary locus; the route of attack, whether vascular, interstitial or intracellular; if ROS or RXS are involved; and the complement of immune systems to amplify and perpetuate damage[222, 319]. Moreover, ROS are just one of many potentially damaging agents. Notable among the others are proteases, cytotoxic protein and lipid fragments, cytolytic properties of the complement cascade, reactive nitrogen species, and injurious chemicals from macrophages, platelets, neutrophils and mast cells[13, 222, 257, 384].

Tissue-specific biochemistry might be the most important factor. In general, cellular processes that have a housekeeping or protective function, eg, GSH and linked systems, seem to display considerable reserve. In contrast, a pathway may be rate-limiting because of some unusual demand of a tissue, eg. glucose metabolism by the brain or methionine metabolism by pancreatic acinar cells. The 'Jekyll and Hyde' behaviour of certain micronutrients amplifies the conundrum. Nicotinic acid is a good example[319]. It functions as NAD and NADP. The latter in its reduced form of NADPH is essential for the reduction of GSSG by GSH-reductase, so ensuring an adequate pool of GSH to allow detoxification of ROS / RXS via GSH-peroxidase and GST (**Figure 7.1**). In contrast, NADPH furnishes reducing equivalents for CYP function, and thereby potentially increases the load of RXS! In the kwashiorkor scenario CYP activity is generally subnormal[319],

whereas it is increased in chronic pancreatitis (Chapter 5).

Table 18.6 summarises information on the main micronutrient antioxidants, oxidative stress markers, GSH and homocysteine status in chronic pancreatitis, cholelithiasis, atherosclerosis as manifested by severe ischaemic heart disease, and RP. For chronic pancreatitis it represents the findings overall while recognising that there are geographical differences (Chapter 12). For gallstones, the information is mainly from Manchester, whereas reports from elsewhere buttress that on atherosclerosis and RP[495-497].

Lack of ascorbic acid emerges as the common denominator, in keeping with its important roles as the main antioxidant of plasma, sponge for histamine from mast cells, intracellular adjuvant in homocysteine removal via the folate-vitamin B_{12} shuttle, and substitute for GSH. The shared fall in plasma GSH - not directly measured in Manchester studies of ISSc but strongly inferred in reports elsewhere[498] - might reflect ascorbate lack but also hints at an inadequate supply of methionine for production of GSH in the liver, which is both the main factory for GSH synthesis and main source of plasma GSH[201]. Selenium lack is accompanied by increased %MRLA' [369, 389], the tendency enhanced when coupled with lack of ascorbic acid[456] (Chapter 17). By contrast and as is only to be expected, deficiency of lipid-phase antioxidants is associated with an increase in products of lipid peroxidation.

The widest span of micronutrient antioxidant deficiencies emerges in patients with chronic pancreatitis, and is reflected in an increase of aqueous and lipid-phase markers of oxidative stress: however it is recognised that any pre-morbid lack of vitamin E is amplified post hoc, ie when a substantial amount of pancreatic parenchyma is lost and compromises lipid absorption. Profiles in cholelithiasis and severe ischaemic heart disease are broadly similar, with substantial lack of lipid-phase antioxidants. In patients with RP as a whole, ascorbate and selenium lack are prominent, the latter most pronounced in patients with the most fibrosis, the

Table 18.6 Summary of observations in disparate diseases *

	Chronic pancreatitis[1]	Gallstones	Ischaemic heart disease	Raynaud's phenomenon
Micronutrient antioxidants				
ascorbic acid	↓[2]	↓	↓	↓
selenium	↓	↔	↔	↓
α-tocopherol	↓	↓	↓	↔
β-carotene	↓	↓	↓	↔
red cell glutathione	↓	↔	↔	ND
Oxidative stress markers				
%oxidation within vitamin C	↑	↔	↔	↔
plasma glutathione	↓	↓	↓	?↓[6]
γ glutamyl transpeptidase	↑	↑	ND	ND
% isomerisation of linoleic acid	↑	↔	↔	↑
lipid peroxides	↑	↑	↑	↔
On homocysteine status				
homocysteine	↑[3]	↑	↑	↑
methionine	↓	↓	ND	ND
vitamin B_6	↓	↓	↓	ND
folic acid	variable[4]	↓	↔	↔
vitamin B_{12}	variable[5]	↔	↔	↔

*Evaluations versus controls in respective studies. Upward arrows indicate significant increase, downward arrows decrease, horizontal arrows no difference.
1 mainly from studies at Manchester, Soweto, and Madras but also Delhi for erythrocyte glutathione and Cochin for folate and vitamin B_{12} (Chapters 12, 16).
2 Madras control values for ascorbic acid and β-carotene were significantly lower than at Manchester or Soweto, likely due to loss by frying vegetables at high temperature; hence no further fall in chronic pancreatitis and no increase in oxidation within vitamin C
3 consensus from studies at Cochin, Manchester and Soweto
4 subnormal in Cochin, normal in Manchester , elevated in alcoholics of Soweto
5 subnormal in alcoholics of Soweto, normal in Cochin and Manchester
6 suggested by improvement on intravenous treatment with GSH precursors - see text

set with dSSc (**Figure 18.4**).

The strong association between cholelithiasis and atherosclerosis is generally ascribed to the metabolic syndrome of altered body habitus and insulin resistance[499]. The studies described herein offer an alternative explanation, ie. a shared pattern of disease facilitators - antioxidant lack, oxidative stress, and compromised GSH status. This is unlikely to be mere coincidence in that the deficiency of antioxidants involved protectors of lipid substrates which are primary targets in both diseases, albeit at very different loci. Moreover, each disease is associated with low plasma GSH but preserved erythrocyte GSH - pointing to precarious GSH reserve in organs of active GSH synthesis. In turn, this could implicate insufficiency of precursor methionine / cysteine, which is in tune with the afore-mentioned effect of experimental methionine lack on cholelithiasis, and also with the observation that early augmentation of sulphur amino acid intake attenuates the development of atherosclerosis in stroke-prone hypertensive rats[500]. Thus, an antioxidant supplement might have prophylactic value in at-risk groups but is not expected to be beneficial once disease is under way[501].

The investigation of patients with RP identified ascorbate and selenium lack coupled with increase in %MRLA' but not lipid peroxides: if this disproportionation carries through to cell membranes, an increase in membrane rigidity is expected, as is known to occur in dSSc and may increase blood viscosity with exacerbation of digital ischaemia[487]. Yet, the combination of allopurinol and micronutrients did not rein in FRA in a meticulous clinical trial. The obvious conclusion is that treatment was too-little, too-late. This deduction is supported by the positive effect of a 5-day course of intravenous NAC in patients with RP due to SSc[498]**.** The increase in blood homocysteine - another shared feature of the diseases examined herein, as also of chronic pancreatitis - is poised to compromise organ perfusion.

In summary:

- Micronutrient lack adds a further dimension, over-and-above the mechanistic, to the association between cholelithiasis or ischaemic states and acute pancreatitis.
- The relevance of deficiency of a particular micronutrient antioxidant to the disease under question can only be assessed by reference to an appropriate biochemical marker, eg. linoleic acid isomer in relation to selenium.
- A trial of AOT can be declared a failure only if clinical / biochemical indices of disease activity declared a priori are unchanged once oxidative stress is corrected. In this context the failure of treatment to correct excessive %MRLA' values in the RP trial - despite clear increases in blood levels of prescribed micronutrients - is noteworthy.
- Administration of a daily pill containing a small dose of methionine, folic acid, vitamin C or combinations to the population at large might have a significant impact in decreasing the economic burden not only from pancreatitis but also from atherosclerosis and gallstones.
- Clearly, manufacturers of nutraceuticals must lower the cost of tablets, if the health benefit of a prophylactic approach is to be realised.

Chapter 19.

Electrophilic stress and pancreatitis: 2016

The enigma of chronic pancreatitis, wherein episodes of pancreastasis punctuate a lithogenic fibro-inflammatory course, is resolved by perception of electrophilic stress as both the detonator of the apical exocytosis blockade in acinar cells, and inflammatory motor when reactive metabolites are shunted into the pancreatic interstitium and activate mast cells (**Figure 11.2**). Likewise, the dilemma of early death from acute pancreatitis is rationalised in terms of mast cell pathology, as a result of their wholesale abrupt degranulation upon contact with RXS, linoleic acid oxidation products, or detergents (**Figure 11.7**, Chapters 11, 17). These concepts have paved the way for first-line medical treatment incorporating micronutrients that have more than 'antioxidant' properties.

19.1 Stresses and stressors

19.1.1 Electrophilic stress

This phrase indicates the threat when electrophilic compounds (ie. with a relative electron deficit) steal electrons from nucleophiles, as are most biological macromolecules. Xenobiotics are the major pathological source, by way of RXS that are inadvertently generated upon processing by CYP. Highly reactive carbonyl products derived from oxidation of PUFA in cell membranes are the most relevant endogenous source[222].

19.1.2 Oxidative stress

This descriptor points to the threat from an unusually high concentration of ROS, of which many are free radicals (ie. with an unpaired electron)[96, 101]. The best known are $O_2^{-\bullet}$ which is quenched by SOD, H_2O_2 which is removed by catalase and GSH peroxidase, and the highly reactive $OH^{\bullet}$ - leaving aside products of interaction with nitric oxide. As was noted in Chapter 3, about 10% of molecular oxygen undergoes ROS-yielding stepwise reduction during a variety of physiological processes. Evidently cells can tolerate the burden, deliberately allowing low-grade oxidative stress for such vital roles as signal transduction and calcium homeostasis[502], membrane turnover, redox control, and genomic stability. A pathological excess of ROS - as is associated with CYP induction[108], ultraviolet irradiation, xanthine oxidase activity under conditions of ischaemia-reperfusion, and so on - threatens cell viability by jettisoning just those homeostatic mechanisms that physiological oxidative stress secures[222]. Transition metals, i.e. iron and copper, promote electron transfer reactions[100] (Chapter 15.3). Insofar that ROS are integral to CYP function, the degree of electrophilic stress might be thought to mirror oxidative stress, but studies in the context of ageing show that the level of electrophilic stress can be disproportionately greater than that of its oxidative drive[106].

19.1.3 Reductive stress

This idiom describes abnormally high electron (reducing) pressure behind a blockade of an enzymic step in the ATP energy production staircase. The blockade may be due to absence of an enzyme, or to malfunction. When electron pressure is sufficiently high some of the electrons may react with O_2 directly and generate ROS. Swings in electron pressure (redox potential) mimic and are reciprocally linked to swings in pH (proton pressure)[255, 441, 442]. In fact, just as alkalosis is rarely if ever a problem unless deliberately induced because all metabolic processes tend to be acid-generating, so too reducing pressure / reducing stress seems to be the main route to oxidative stress, at least in the long term. The problem is epitomised by alcoholism, hypoxia, redox cycling compounds such as doxorubicin (that cause electron dislocation), and uncouplers of electron flow, such as NSAIDs, cyclosporin, and cytokines.

19.1.4 Endoplasmic reticulum stress

If not quickly rectified, any of the above stresses activates the ER stress-UPR which, in turn, exacerbates oxidative stress and elicits inflammation by activating stress response genes such as NF-kB[503, 504]. The exocrine pancreas with its huge rate of protein synthesis is particularly vulnerable when subjected to congestion in the busy protein-trafficking lanes - an inevitable consequence of pancreastasis episodes, despite the acinar cell's best efforts to compensate by endocrine re-routing of newly synthesised enzymes; removal of zymogen granules via the three-pronged strategy of centripetal dissolution, crinophagy and basolateral redirection; and down-regulation of enzyme synthesis (**Figure 11.2**). The close integration between oxidative, electrophilic, ER, and inflammation stress is now regarded as the basis for many chronic diseases[373], and increasingly, for chronic pancreatitis[7, 235, 505].

19.2 Electrophilic stress template for chronic pancreatitis

19.2.1 Component clauses

Since it was first proposed 30+ years ago[9], this disease model has evolved in line with new observations[10, 13, 37]: implicit is acceptance that the disease transcends geography, putative environmental factor, or genetic make-up[1]. The 1998 version views the acinar cell as the site of mounting electrophilic stress that steadily erodes

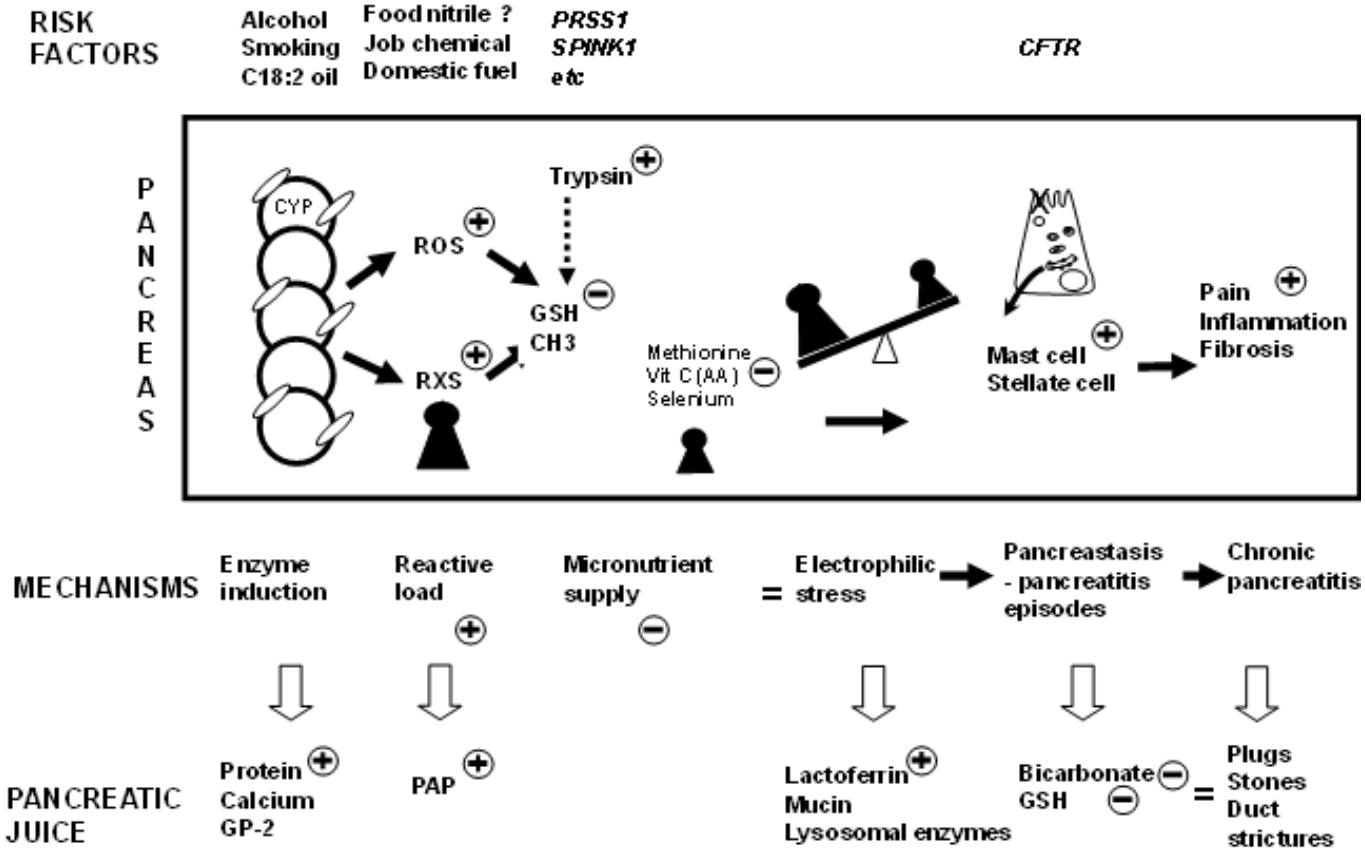

Figure 19.1 A framework for the pathogenesis and pathophysiology of chronic pancreatitis. Gene mutations are shown in italics. ROS=reactive oxygen species; RXS = reactive xenobiotic species; AA = ascorbic acid; GSH = glutathione; PAP = pancreatitis associated protein, activated by electrophilic stress; GP-2 = secreted component of zymogen granule membranes, analogous to the renal cast protein. Encircles plus or minus symbols represent increases or decreases, respectively. Figure from Pancreapedia[11].

CH_3 and SH (principally GSH) moieties, as a result of CYP induction, concurrent exposure to a toxicant that yields RXS, and insufficiency of refurbishing micronutrients (**Figure 19.1**). The need to build a fourth factor into the equation was anticipated[13], so as to accommodate gene mutations that might favour the cytoplasmic presence of trypsin[7]: the enzyme, as also chymotrypsin, is readily inhibited by GSH via SH-SS exchange[13, 302] should it break loose of constraint by SPINK1[7,15,29] (**Figures 11.5** , **13.1**), but less GSH is then available for control of electrophilic / oxidative stress and other vital roles[255,349].

Today yet another factor should be formally included in the schema, ie. CFTR lack, whether due to *CFTR* mutation(s) or oxidant attack on the protein via alcohol, cigarette smoke or other noxae[255, 368]. Either way, a reduced quota of functional CFTR in ductal elements would compromise the delivery of chloride/ bicarbonate / fluid, as also in theory that of the antioxidants GSH and thioredoxin[360, 361, 372]. This combination in the presence of protein / calcium hypersecretion is poised towards intraductal mucus plugs and lithogenesis[362]. The tendency to focal ductal occlusion is amplified by neutrophil extracellular traps, a phenomenon driven by IL-17, whereby the cells utilise ROS and histone citrullination via peptidyl arginine deaminase-4 to cause extrusion of decondensed chromatin. This facilitates aggregation of viable, necrotic and apoptotic cells as also of microbes and crystals: bicarbonate ions and calcium carbonate crystals in pancreatic juice have been implicated in the pancreatitis setting[506].

The first 3 clauses help to explain why patients on CYP-inducing anticonvulsant drugs rarely develop chronic pancreatitis (Chapter 7), or why profound electrophilic / oxidative stress but with low CYP activity in kwashiorkor results in painless loss of acini, not chronic pancreatitis[319]. The concept does allow for a steady build-up of ROS alone, as in elderly people [507], and patients with HP[347-349].

Within this framework, each burst of electron transfer reactions (FRA in popular parlance) hinders apical exocytosis to trigger an attack of pancreatitis by interfering with the methionine-to-GSH metabolic pathway, which interacts closely with ascorbate and selenium (**Figure 11.3**). The diversion of FROP into the interstitium unleashes mast cells - thus triggering inflammation[256], the activation of nociceptive mechanisms that promote a chronic pain syndrome[13,311,508], pro-fibrotic interactions[13,310,509] and, in principle, also mast cell extracellular traps[510]. Meanwhile, the acinar cell generates its own pro-inflammatory mediators under the influence of redox-sensitive signalling cascades[97], but pancreatitis is said not to ensue when basolateral exocytosis is prevented[273].

Cystic fibrosis, which is usually due to severe mutation in both alleles of the *CFTR* gene, causes an accelerated non-calcific form of chronic pancreatitis that begins *in utero*: oxidative stress and inflammation are now regarded as integral features of the disease, driven by unfolded CFTR via the ER stress-UPR system[372, 373]. This is not the position depicted in **Figure 19.1**, which instead seeks to understand the increased frequency of *CFTR* mutation, with or without mutation in *SPINK1*, among patients with idiopathic chronic pancreatitis - the tropical variant in particular (Chapter 13).

Thus, neonatal hypertrypsinogenaemia in *CFTR* carriers, and the enhanced susceptibility to experimental pancreatitis so conveyed [511], suggest hindrance to CFTR-facilitated apical exocytosis in the acinar cell under conditions of excessive FRA[13]; an interpretation supported by *in vitro* studies of antibiotic exocytosis (Chapter 13.3). Two other points are noteworthy in connection with ductal CFTR function. First and as was predicted[13], CFTR is easily inactivated by oxidants, which would have the same impact as pancreas-selective mutations in *CFTR*, causing the aforementioned changes towards intraductal

lithiasis. Second, cytoplasmic displacement of CFTR has been noted in alcoholic, idiopathic and autoimmune pancreatitis specimens[362]: that oxidants might be responsible is suggested by curcumin rescue of DF508-CFTR localization in cell lines[363].

The template envisages permutations and combinations among the aforesaid factors including oxidant attack on CFTR in ductular epithelium as determining outcome - whether RAP, small-duct chronic pancreatitis, or large-duct disease with or without calculi; age at onset; and rate of progression. The worst combination is in patients with tropical pancreatitis[236,255,326,376].

The popular notion of pancreatic autodigestion by prematurely activated trypsin in acinar cells has no part in the philosophy[5,256,265-267] and is now increasingly questioned[6, 305]. Recent confirmation of the 1980s observation that the concentration of lysosomal enzymes is increased in pancreatic juice[512], simply highlights the vulnerability of lysosomes to free radical attack[10]. As to the finding of ER stress in chronic pancreatitis, many xenobiotics have been shown to influence the UPR signalling route, with either pro-survival or pro-death features. This is unsurprising given that resident CYP straddle ER membranes[513].

Although not in the schema, it is conceivable that ROS / RXS via CYP might be involved in the genesis of autoimmune pancreatitis, as in autoimmune polyendocrine syndrome type I, Addison's disease and autoimmune hepatitis[514,515]: ROS can alter the structure of cellular antigens to produce a 'neo-antigen' which could then initiate a detrimental reaction to the original antigen by molecular mimicry. The concept becomes plausible with the finding from studies in hepatocytes that newly synthesised CYP enters the secretory pathway to arrive at the outer surface of the plasma membrane[516]. It is not yet known whether RXS that enter bile[120] or pancreatic juice[463] can elicit neutrophil extracellular traps, but that would explain early occlusion of bile and pancreatic ducts in dibutyltin pancreatitis[201], as also leucocyte hordes and proteinaceous matter in pancreatic ducts of ectopic and entopic pancreas of a patient with methyldopa-induced pancreatitis[252](**Figure 11.6**).

19.2.2 Evidence in support

This has been examined in depth in previous Chapters, to cover each building block of the template. The information in **Table 9.2** shows that pancreatic and liver damage proceed along similar lines, by way of heightened but unmitigated oxidative-detoxification reactions via CYP. The key point is that the pancreas falls clinical victim while liver injury is generally silent; the best explanation is that xenobiotics hit the gland via the arterial route (**Figure 19.2**). This would explain why cigarette smoking is now perceived to be a greater threat than alcoholism: in addition to the huge burden of free radicals so delivered (Chapter 12.3), are nicotine and pro-oxidant metals such as copper[242-245,517]. Nicotine and pancreas-specific nitrosamines, which undergo bioactivation via CYP, are regarded as posing the main threat[518], but nicotine does not affect CYP1A2[519] , induction of which is a prominent feature in patients with chronic pancreatitis (Chapter 5). Hence it is more likely that PAH in smoke are relevant in this context, as also C18:2 oils and polychlorinated biphenyls[160,161], such that the yield of RXS from nicotine increases.

The alcohol paradox is rationalised if co-exposure to volatile petrochemicals is involved in disease pathogenesis (Chapters 7-9, 12) This interpretation came about from a time-course study of Manchester patients with idiopathic chronic pancreatitis or RAP[215], and was later confirmed in relation to chronic pancreatitis by a large-scale case-referent investigation[216]. Endorsement has been forthcoming from subsequent studies of patients with idiopathic disease in southern India and alcoholic disease in South Africa[64, 320, 336].

The exquisite sensitivity of the exocrine pancreas to CYP1A induction and RXS is evidenced by experimental work using a high corn oil diet, CCl_4, dibutyltin, nitriles and a host of other chemicals[222] (Chapter 14). However, the field of inhalation toxicology to the pancreas has been a vacuum until fairly recent evidence of injury from cigarette smoke in rodents[1,520,521].This should soon be rectified, scepticism notwithstanding[522], because the health risk from volatile petrochemicals is currently under intense scrutiny[341, 342, 523]. As already observed, acinar damage would be aggravated by RXS that are generated in CYP-induced islets and delivered by the insulo-portal conduit, and also by relatively long-lived FROP and RXS that are produced within the CYP-induced liver should they find their way into the gland via refluxed bile or the bloodstream (**Figure 9.7**).

Of all the findings in **Table 9.2**, the increase in bilirubin is most revealing because it indicates induction of heme oxygenase to combat severe oxidative stress. Anecdotally, a further surge was found to accompany a pancreatitis relapse in the immediate aftermath of exposure to petrochemical fumes[39], mimicking the abrupt enzyme rise when phenobarbitone-treated rats are exposed to RXS from halothane gas[524]. The combination of induced CYP1A, increased copper, and induced heme oxygenase is a unique exposé of environmental toxicology in humans. The findings cannot be dismissed as a consequence of impaired pancreatic function because there was no correlation with its degree as gauged by SP tests. Copper excess, as shown by increase in its concentration in bile / serum of patients in north west England and in erythrocytes / pancreatic tissue of patients in southern India (Chapter 5), is best explained by inhalation exposure via cigarette and / or petrochemical smoke[517,525], underlining the importance of volatile xenobiotics in disease causation. Normal levels of serum caeruloplasmin (**Table 3.1**), biliary copper and bilirubin in Manchester patients on long-term treatment with pancreatic extracts (

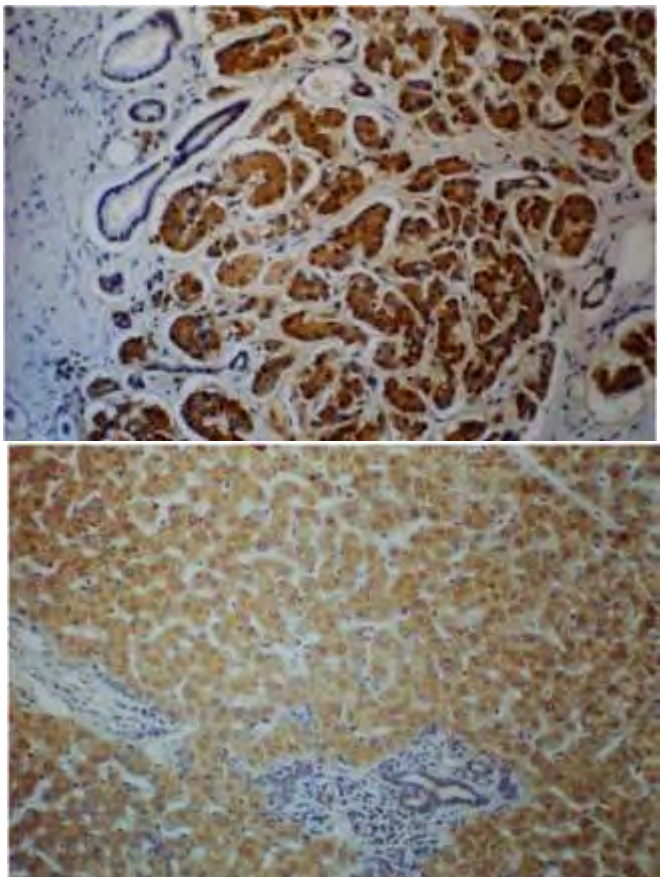

Figure 19.2 . Immunolocalisation of cytochrome P450 isoform in surgical specimens from a 27-year old woman with calcific chronic pancreatitis. She drank little alcohol, smoked 40 cigarettes a day, and worked as a forecourt attendant at a car and lorry-fuelling station. (top) Pancreas fragment shows that the enzyme (brown stain) is strongly expressed in acinar cells but absent from epithelium of dilated ducts or expanded stoma .(bottom) The needle biopsy fragment of the liver shows that the enzyme is strongly expressed across the liver lobule and weakly expressed in bile duct epithelium .Reproduced from Lancet [1].

Figure 3.3) can, at long last, be rationalised by the selenium and zinc content of extracts[435-437]: zinc induces metallothionen, which sequesters dietary copper and thus limits its bioavailablity[525]. Moreover, patients tend to have retired by that stage, eliminating occupational exposure to volatile xenobiotics. In other words, copper overload is the driver of pancreatic hypofunction (**Figures 3.4a & 3.4b**), rather than the other way round! There are 2 important inferences, ie. that chronic pancreatitis is a copper-overload disease, and that it represents hepatisation of the gland.

As to how a burst of electron transfer reactions might paralyse apical exocytosis in acinar cells to trigger a pancreatitis attack, clinical evidence points to a breakdown in the delivery of CH_3 and SH moieties via the methionine metabolic pathway (Chapter 11). The route also impacts on the correction of reductive stress by biomolecules with electrophilic methyl groups. These include SAM, phosphatidylcholine, betaine and carnitine. They appear to act by binding to positively charged nitrogen or sulphur moieties, a poising mechanism that is demonstrable *in vitro* when the reaction mix includes catalytically active iron, H_2O_2 and ascorbic acid: CO_2 and CO are formed from the ascorbate molecule in parallel with generation of methane gas[442]. It is now recognized that albumin acts as a sacrificial anti-reductive protein which when modified by $OH^{\bullet}$ radicals emits a signal to proteolytic degradation and elimination[443].

Studies of habitual diets in Manchester patients identified a deficit relative to demand via induced CYP1A2 of selenium, ascorbic acid and methionine (Chapter 7) - the outcome verified by biochemical investigation (**Table 12.1, 16.5**). The corollary that supplements of these micronutrients should be beneficial was borne out by a placebo-controlled study at Manchester in the late 1980s, and is now confirmed by meta-analyses of similar studies, even when a flawed RCT is included (Chapter 16). As to that inclusion, the editorial board of 'Gastroenterology' should redact the paper, not only because of its many shortcomings[401] including non-reference to the authors' previous positive findings[407], but especially in view of their subsequent tacit admission that pain in a representative subsection of trial patients was not due to pancreatic inflammation[430]! Of note, the 'Lancet' withdrew a similarly retrogressive paper on childhood vaccination.

The need for micronutrient supplements is underscored by recent experimental work, showing that chronic exposure to alcohol inhibits the uptake of ascorbic acid by the pancreas *in vivo* and by acinar cells *in vitro* [526]. Previous studies from the same group, cited in the study report, indicate a negative impact too on uptake of biotin and thiamine: the last effect also brought about by exposure to nicotine.

19.2.3 Detecting oxidative / electrophilic stress

One or other direct method to detect a burst of FRA has been used in experimental studies of pancreatitis (Chapter 11) and occasionally in clinical studies[233,291,423]. In the main, however, the detection of oxidative stress in human disease relies upon biochemical 'fingerprints'. The choice from the immense library must be guided by the perceived target of attack - whether lipid, protein or DNA. There is no perfect 'marker' but when the threat is foremost on lipids, the best today is F_2 isoprostane, and 'thiobarbituric acid reacting substances (TBARS)' the least specific [101,401].

The detonating attack in pancreatitis is on enzymes and receptors that are protected by GSH interacting with ascorbic acid[255]. Hence, telling measures from serum / plasma analysis might include %MRVC, GSH coupled with γGT; protein carbonyls; and allantoin which signifies oxidation of uric acid: tests of 'total antioxidant capacity' in blood reflect mainly uric acid and other bulk antioxidants[401]. A nomogram is advantageous, as when the %MRLA' is examined alongside selenium concentration in the same sample (**Figure 16.2**).

Since dysregulated methionine metabolism due to RXS seems to underlie chronic pancreatitis, an index of its repair by AOT would be most helpful, eg. by analysing a metabolite(s) generated by the pathway, and / or ^{11}C methionine isotope scanning[396,397]. These resources are scarce, but an increase in erythrocyte GSH upon micronutrient therapy seems to be is an indirect pointer[324]. It is much more difficult to conclude that stress is electrophilic, ie. due to RXS. Studies in patients at Soweto suggest that the following

indices are useful: decrement in plasma GSH and serum selenium associated with an increase in γGT activity (in the absence of cholestasis); urinalysis showing an increase in D-glucaric acid and the ratio of ester to total inorganic sulphate (**Figure 12.11**).

19.2.4. On Pain

The sensitization of pancreatic nociceptors within a milieu of sustained inflammation is now regarded as the critical initiating event in pain genesis - the afferent barrage leading to sensitization of higher- order neurons and thence central sensitization[401, 512, 527]. Resected pancreatic specimens from patients with chronic pancreatitis identify 'neuroplasticity' (ie. increased neural density with hypertrophy) and 'hyperinflammation', the former correlating with pain severity, the latter with neuropathic pain syndromes in general.

As has been documented with citations[401], the molecular agents that influence nociception are now established: nerve growth factor (NGF); transforming growth factor beta (TGF-ß, which is also a potent activator of stellate cells); NGF-responsive gene products including the transient receptor potential vallinoid 1 (TRPV1), substance P and calcitonin gene-related peptide (CGRP); and the crucial mediator of central pain, brain-derived neurotrophic factor (BDNF). As time goes by this pervading neurogenic assault renders insignificant the contribution to pain from dwindling flares of pancreatitis or compromised flow through tubular structures.

The Manchester proposal of 1998 that mast cells mediate intense pain is now amply supported[13, 311, 508] : pancreatic neuritis, which also accompanies pancreatic cancer, displays enrichment with perineural mast cells, the only component of the infiltrate to correlate with pain sensation[508]. It is worth reiterating that whereas the activation of mast cells by ROS or cytokines during ordinary inflammation involves piece-meal degranulation, an anaphylactoid (non-IgE) response occurs upon exposure to certain substances[256, 263] . Among these are RXS (including from opiates); lipid oxidation fragments; detergents (eg. bile salts); radiocontrast media; and a high dose of arginine, probably due to its cationic charge[537].

It has long been known that histamine from mast cells evokes an axon reflex with release of substance P, and also that mast cells form neuro-effector junctions[528], but the mast cell-nociception link now goes much further[401]. Mast cells synthesise, store and release NGF which protects indirectly against RXS. They express TRPV1, which responds to stressors including hydrostatic pressure. Tryptase, which unlike trypsin is resistant to α_1PI, awakens PAR-2 on the basolateral membrane of acinar cells, which might help to overcome the secretory blockade: unfortunately PAR-2 also increases the expression and release of BDNF in microglia. Mast cells co-secrete TGF-ß1 and its chymase activator. A vicious cycle is set in motion because substance P and NGF reactivate the cells. These findings explain not only the phenomenon of opiod-induced hyperalgesia[512], but also why mast cell control is proposed for treatment of complex regional pain syndromes[529].

This approach is not of itself applicable to chronic pancreatitis, however, as shown by the inefficacy of curcumin in a clinical trial[400], although it is a potent antioxidant, inhibits the anaphylactoid response of mast cells [530], and corrects CFTR displacement in cell lines[363]. Heme oxygenase shares the first 2 attributes[307], and is already recruited as is revealed by large amounts of bilirubin in patients' bile[83] (**Table 3.2**). The inference is that these substances do not protect the critical intra-acinar target of oxidant attack. That target, the pathway of methionine metabolism, was pinpointed in the 1950s by prescient observations in experiments using halogenated hydrocarbons or the CDE diet[27,31].

In short, the treatment imperative in chronic pancreatitis is to prevent peripheral pain sensitisation by speedy removal of the primary pro-inflammatory factors. That is precisely what micronutrient therapy strives to achieve by restoring apical exocytosis in acinar cells while also curbing mast cells[401] (**Figure 11.2**). It makes sense for treatment to begin at the first opportunity in every patient with acute pancreatitis, RAP, or chronic pancreatitis and to be continued through until micronutrient intake improves sufficiently via advice from a nutritionist (Chapter 16). If peripheral sensitisation has already set in, adjuvant therapy with pregabalin is logical in order to intercept the noxious upward spiral towards central sensitization at spinal cord and brain level[527], but it should be remembered that the drug also ameliorates visceral pain, as from the gut in patients with occult fat maldigestion or opiate-induced gut dysmotility[531].

Success of the Manchester antioxidant prescription plus pregabalin in a RCT has recently been reported[532]. 'Narcotic-naïve' patients with pain despite clearance of ductal calculi received either the combination (n=42) or placebos (n=45) for the first 2 months whereupon pregabalin was stopped and all patients had open AOT. Active treatment ameliorated pain as assessed by a variety of measures, and was associated with a greater frequency of pain abolition than among patients on placebo in the first phase (47.6 vs 26.7%, p=0.04). Pain was lowered further by 6 months in the set that initially received active treatment. At 6 months the mitigating effect of AOT became evident too in the set that received placebos for the first 2 months.

This AOT-pregabalin protocol makes sense and seems to convey a substantial improvement over the AOT-alone approach. Prior clearance of ductal calculi in the cited RCT deflects potential criticism that a lesser degree of compromised outflow explains improvement in the group on active treatment. However, experience at Manchester shows that the manouevre is both unnecessary and unduly meddlesome (eg. **Figure 12.2**). In corroboration, patients at Chennai with multiple pancreatic calculi tended to have little or no pain even before the stage of exocrine pancreatic failure was reached.

19.2.5 On fibrosis

The proposal from Manchester in 1998, that excess oxidative stress favours pancreatic fibrosis by activating inflammatory and stellate cells[13], is now buttressed by direct studies[310,509], and numerous investigations in relation to fibrosis in other organs[490,533,534]. A role for 'alternatively activated macrophages' has recently been suggested[535] but, surprisingly, the potential importance of mast cells as intermediary is barely mentioned although they secrete prodigious amounts of TGF-β, a pro-fibrogenic cytokine. Pancreatic fibrosis *per se* is unlikely to play any major role in the genesis of pancreatic pain. Nonetheless it is of interest that thiol / vitamin supplementation and dietary advice to improve antioxidant intake are now recommended in chronic obstructive airways disease which, like chronic pancreatitis, is associated with inhaled xenobiotics, on-going inflammation, mucin hypersecretion and a fibrotic tendency[533]. A similar but not identical strategy has been proposed for autoimmune rheumatic disease[534].

19.2.6 Transition from RAP to chronic pancreatitis

Among factors that have been implicated in this transition - which has been recently estimated at 36%[314] - are cigarette smoking, alcoholism, male gender and *SPINK1* mutation[314, 518]. Observations in patients with FLLD (**Figure 15.3**) should dispel the SAPE hypothesis (Chapter 11.6). Instead, studies from the UK suggest that a persistent shortfall in micronutrient antioxidant status relative to demand from the prevailing oxidant load is an important consideration, ie. that the balance is better in patients with RAP[37,316, 317]. Also of note, RXS rather than ROS tend to be involved in most clinical settings of chronic pancreatitis and simulating animal models[201].

19.2.7 The new Koch's postulates

It has been advised that any schema for a causal connection in a polygenic disease should fulfil a set of postulates derived from Koch's classical work on tuberculosis[536]. The Manchester proposal that oxidative / electrophilic stress in pancreatic acinar cells is the 'obligate intermediate phenotype' in chronic pancreatitis fulfils the criteria[13,37].

- The relationship is mechanistically plausible. The template depicted in **Figure 19.1** represents the evolution of large-duct disease. CYP-mediated oxidant strain without prior enzyme induction or prior micronutrient lack leads to small duct disease, as is shown by animal studies (Chapter 14).
- Oxidative / electrophilic stress precedes the disease. Studies of outwardly healthy individuals from Soweto, where the disease may now be endemic, fulfil this clause (Chapter 12).
- Conventional treatment does not ameliorate oxidative strain. Placebo arms of micronutrient therapy trials bear witness to this requirement (Chapter 16).
- Methionine-based therapy ameliorates symptoms while at the same time correcting oxidative / electrophilic stress. Meta-analyses attest to the first statement; 3 trials offer biochemical evidence for the second (Chapter 16).
- Disease genes co-segregate with oxidant stress-facilitating genes. The co-segregation of CFTR mutations with idiopathic chronic pancreatitis is strong evidence. The identification of oxidative stress in affected members among HP kindreds is further evidence in support (Chapter 13).
- It should be possible to produce an animal model by inducing chronic oxidative / electrophilic stress. Although this integral aspect of Koch's work has been dropped from the modified recommendations, it can hardly be coincidental that experimental protocols which generate RXS or prejudice their removal cause small-duct chronic pancreatitis. A CYP-inducing regimen (eg. high corn oil diet) in CFTR carrier rodents or animals on diets restricted in selenium / vitamin C should provide a model of large-duct disease.

Of note, these postulates are not fulfilled by any other theory on pathogenesis - whether duct-first, acinar-first, 2-hit, or multiple-cause hypotheses[1].

19.3 Electrophilic stress and fatal acute pancreatitis

19.3.1 Anaphylactoid reaction of mast cells

By 2001 there was a strong case for a burst of ROS as the detonator of acute pancreatitis[201, 256, 266], as is now accepted[277]. However, the failure of clinical therapy with GSH precursors and / or micronutrient antioxidants when delivered post hoc, except when combined with hemofiltration (Chapter 17), indicates that oxidant stress is only part of the picture.

The following are prominent among experimental ways to cause life-threatening disease: feeding a CDE diet, mega-dose arginine by intra-peritoneal injection, or retrograde instillation of bile salts into the pancreatic duct. Each of these protocols elicits an increase in pancreatic FRA plus a potentially anaphylactoid reaction of mast cells - the latter provoked, respectively, by estrogen-derived RXS[256], cationic charge[537], or detergent action[263]. Thus it seems likely that mast cells are the stealth bomber in lethal acute pancreatitis, operating under the autodigestion smokescreen[263].

This philosophy goes against the grain of the accepted concept that acute pancreatitis in the first week exemplifies the (non- infection) SIRS. The lack of change in blood levels of inflammatory markers despite dramatic clinical benefit from an antagonist ('Lexipafant') of the receptor for platelet activating factor (PAF) in a UK phase-II

trial suggests otherwise[538]. This clinical efficacy, albeit finally discredited by a phase-III study[539], is in stark contrast to its singular inefficacy when SIRS occurs in the sepsis setting[257]. PAF is generated from membrane phospholipid or manufactured de novo - in the pancreas too, in that PAF soon appears in portal blood during experimental pancreatitis, and its inhibition abrogates pancreatic edema[256]. PAF production is activated by a transient rise in intracellular Ca^{2+} and is accompanied by release of arachidonic acid, which spawns leucotrienes and a range of FROP. All these substances have powerful effects on platelets, inflammation and allergic reactions.

PAF does not activate mast cells, but is discharged in prodigious amounts by mast cells once activated, eg. by ROS / FROP, and it is recognised as a key mediator of the anaphylactic / anaphylactoid reaction which is diagnosed by cardiovascular collapse and respiratory difficulty. It is recognised that the delivery of mast cell products into the bloodstream as in the anaphylactic reaction to bee sting manifests preferentially as cardiovascular collapse, whereas that provoked by an oral antigen such as peanuts leads to rapid asphyxiation. Moreover, studies of urticaria show that although the reaction is initially contained, it involves all the mediators associated with a systemic response[257].

Profound shock is a striking feature of acute pancreatitis as caused by the CDE diet, whether in young female mice or male animals pre-treated with estrogen[540]. It is thus probable that the early burst of FRA in acinar cells, as shown by electron spin resonance[285], is due to reactive metabolites that are generated during estrogen processing by CYP and which cannot be removed because of methionine-choline lack (Chapter 11). Not only does the mast cell possess estrogen receptors, whereas macrophages and other immune cells do not, but also the connection has been proposed as an explanation for the female predilection to other allergic conditions, eg. airways inflammation[541]. These arguments, and acceptance that shock at admission is an ominous sign in clinical acute pancreatitis, suggest that inflammatory derangement and organ dysfunction associated with acute pancreatitis represent gradations of the anaphylactoid response - whether provoked by radio-contrast media during ERCP, refluxed bile salts, or a host of prescribed drugs (including morphine) and xenobiotics that undergo metabolic activation[263]. In support of this view, whereas hemoconcentration and thrombocytopenia in experimental anaphylactic shock are mediated by histamine and PAF respectively, neutrophilia is not explained by these mediators, TNF-α or IL-1 but rather by dopaminergic mechanisms. These would be recruited by the axon reflex elicited by histamine, resulting in release of substance P and other nociceptive transmitters, as also of noradrenaline (norepinephrine), adrenaline (epinephrinre), dopamine and acetylcholine[257].

Perusal of the literature in 2001 revealed that mast cell control is the common denominator among beneficial measures in acute pancreatitis, not infrequently after the disease is under way[257]. Successful agents / actions in experimental studies up to that time included the following: methionine, an ascorbate analog; β-adrenegic agonists; PAF inhibitors; fibrinolysin; dextran; heparin; hexamethonium; splanchnic block and post-ganglionic sympathectomy. To these can now be added hematin[307]; hydrogen gas[542,,543]; the monoterpine carvacrol[544]; and epoxyeicosatrienoic acids (EETs), as indicated by improvement from inhibition of soluble epoxide hydrolase which otherwise would convert EETs into less active metabolites[545]. EETs are generated from arachidonic acid via cylooxygenases, lipoxygenases and, in particular CYP2C / 2J: not only are they potent inhibitors of mast cells but they also down-regulate NFκB and curb ER stress, both of which are implicated in experimental acute pancreatitis.

In clinical studies the only successful outcomes after disease inception (other than when coupled with haemofiltration) have been brought about by mega-dose selenium in a study from Germany where patients often arrived within 2 hours of symptom onset[463]; and mega-dose vitamin C in a study from China[429]. These micronutrients stabilise the mast cell, as does GSH[263]. Moreover, the virtual wipe-out of ascorbic acid in admission plasma samples from all patients with acute pancreatitis becomes understandable by the release of histamine from mast cells (Chapter 17). In similar vein is the protective value against ERCP-induced pancreatitis of adrenaline sprayed

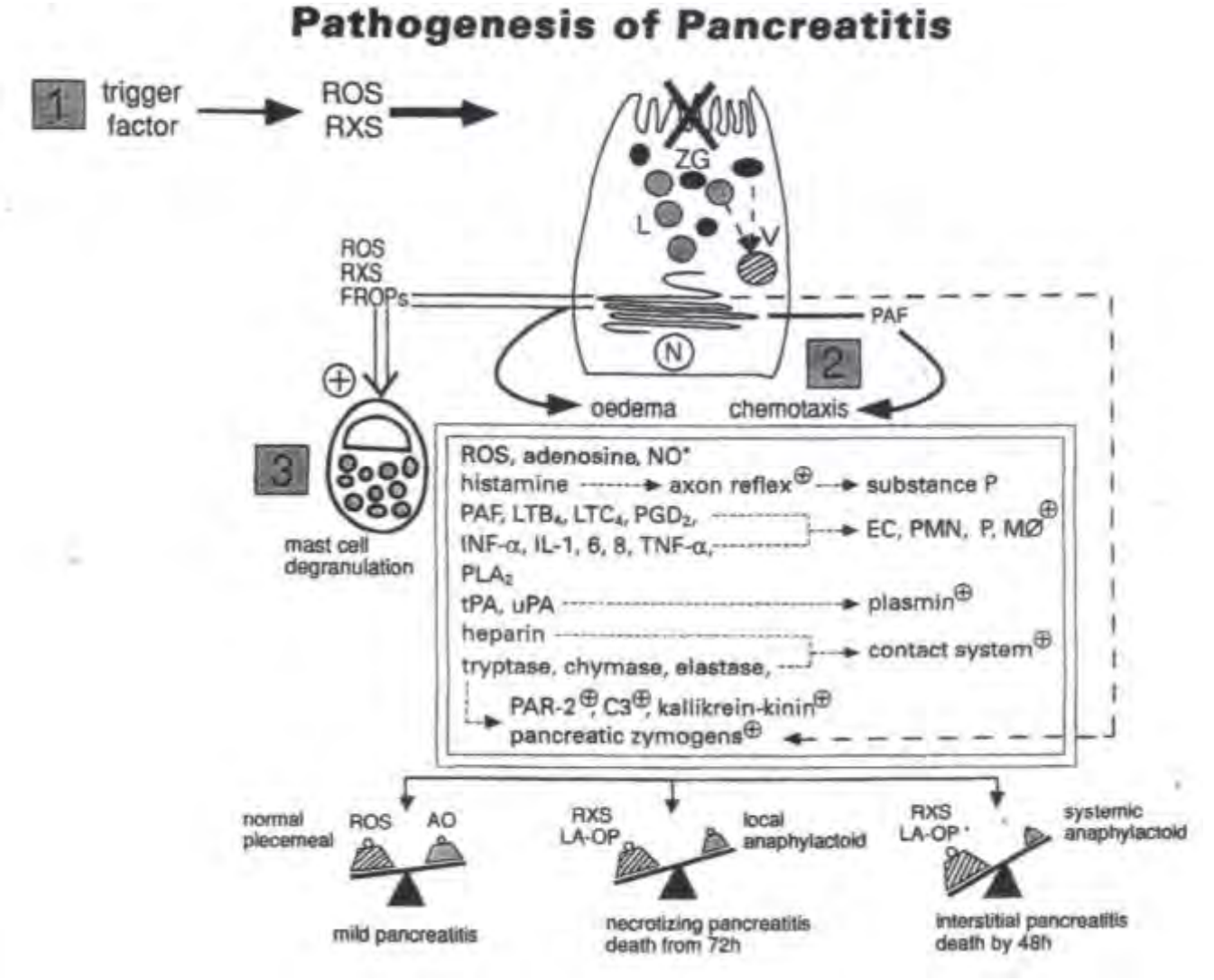

Figure 19.3 Schema to show how the degree of antioxidant lack determines the outcome of wholesale degranulation of mast cells when exposed to reactive xenobiotic species (RXS), linoleic acid oxidation products (LA-OP), radiocontrast media or bile salts. Plus symbol indicates activation; that of trypsin by tryptase is hypothetical. LTB_4, LTC_4 NB text fused here= leukotrienes; PGD_2 = prostaglandin $D_{2.}$; INF-α =interferon series; TNF-α = tumour necrosis factor–α; EC=endothelial cell; PMN = neutrophil; M) = macrophage; PLA_2 = cell-membrane lysing from of phospholipase A_2 which could hydrolyse adiposyte membranes to allow lipase entry and thus fat necrosis; tPa, uPA = tiisue-type and urokinase plasminogen activators; PAR-2 = proteinase activated receptor-2; C3 = complement subtype electrophilic stress and antioxidant protection might dictate the outcome when wholesale degranulation of pancreatic mast cells occurs in a pancreatitis attack. Note that a high degree of electrophilic stress (denoted by scales at the bottom) favours an anaphylactoid reaction. Reproduced with permission from 2001 paper in Digestion [257]

on the duodenal papillae[546]. The proferred explanation of vasoconstriction is against the ethos of treatment which is to maintain the microcirculation. Much more likely is protection against a local anaphylactoid reaction to ERCP contrast media by the huge population of mast cells that resides in the para-duodenal area.

Considering all these observations, a scenario is suggested wherein prior micronutrient status determines outcome - whether death by 24 hours from a systemic anaphylactoid syndrome when the antioxidant deficit is extreme but the gland shows only interstitial pancreatitis, or after 72 hours from a local anaphylactoid response when the deficit is moderate but the gland shows necrotising pancreatitis[257](**Figure 19.3**). Studies of impoverished patients in the heavily polluted city of Soweto support the interpretation (Chapter 17). Another relevant observation is that serum tryptase levels were higher on the day after admission in patients with compared to without organ dysfunction[547].

Against this background (and information in Chapters 11, 15 and 18) it becomes possible to appreciate that the modified Koch's postulates are fulfilled for the pathogenesis of, acute pancreatitis when mast cells are factored into the equation[256, 257, 263].

Of course, as time goes by the transformed acinar cell, misfiring leucocytes and immune disarray would contribute increasingly to the toxic brew in the circulation, such that no single antagonist can be expected to abort the downward spiral to death from acute lung injury[318]. Fortunately, removal of the offending trigger - whether spontaneously as by passage of a gallstone into the duodenum, or by hospital admission which removes a patient from occupational volatile chemicals - allows a self-limiting course of most attacks.

The real problem is the time interval that elapses before aggressive supportive therapy can be instituted in patients who might develop life-threatening disease. This has been compared to the detrimental delay in treating myocardial infarction. In fact, acute pancreatitis might be viewed as a natural concomitant for several reasons: antioxidant protection of the myocardium is already poor in patients with advanced atherosclerosis who would be at risk should perfusion pressure fall (Chapter 18.2); a pro-coagulative state is imminent via contact system activation (Chapter 17.5); and an increase in circulating noradrenaline would increase myocardial workload. Elevated levels of troponin in admission serum samples are recorded in around a third of patients, but are currently interpreted as evidence of rhabdomyolysis rather than cardiac strain[548].

19.3.2 Proposal for first-line treatment

The principle is this. Whereas the primary goal of treatment ought to be the restoration of normal secretory channels in the acinar cell (**Figure 11.2**), it is the behaviour of mast cells that determines outcome in an individual case (Chapter 11.5.2). In other words death or spontaneous recovery is determined at the outset, as was also implied by the conclusion that 'Lexipafant' would have been successful of given at time zero[539]. Given the inevitable time-lag to treatment, it is logical that the first medical attendant injects subcutaneous adrenaline via an 'Epipen' to stabilise the mast cell, as is standard practice in patients with, say, known allergy to peanuts[257]. That action requires immediate confirmation of the diagnosis, as is enabled by the 'Actim' urinary dipstick test which identifies the excess of circulating trypsinogen consequent upon pancreastasis: importantly, no dire problem is likely if the drug is given to a patient who falsely tests positive[257]. Once in hospital, treatment might proceed along the lines suggested in 2001[257], but modified by provision of vitamin C in a far higher dose[429].

19.4 Summary

Given all the information in this monograph, it is difficult to justify denial of micronutrient 'antioxidant' supplements to patients with pancreatitis, irrespective of disease type or putative aetiology. There is surely room for improvement in the prescription for patients with RAP or chronic pancreatitis, not least by incorporation of choline, and possibly of magnesium, folic acid or zinc[11]. The addition of pregabalin for the first 10 weeks in patients with chronic pancreatitis, so as to forestall opiate prescription while blood antioxidant levels build up, is logical and worthwhile. Preliminary evidence suggests that a cannabinoid receptor agonist will not be beneficial in this regard[549]. As to the proposal that a pharmacological chaperone (eg.lumacaftor which partially corrects the folding / processing defect in d508 CFTR) or CFTR gating activator (ivacaftor) might find a place in the management of chronic pancreatitis[368], it is worth noting that pulmonologists emphasise the need for a multi-targeted approach, incorporating antioxidants[533]. More promising is an ER chaperone such as phenylbutyric acid to encourage apical enzyme secretion in acinar cells, suppress trypsinogen 'activation', and reduce ER stress[550].

Chapter 20.

Coda

In 1991 the therapeutic horizon for patients with pancreatitis was aglow with optimism: there was every reason to believe that pharmacological doses of micronutrient 'antioxidants' would afford pain relief in chronic pancreatitis; that a small daily dose would be useful as prophylaxis in communities where the disease is endemic; and that parenteral treatment should facilitate recovery from acute pancreatitis[551]. Yet today, 25 years later, the therapeutic horizon is bleak again, with opiates and invasive procedures (lithotripsy, pancreatic duct stenting, surgery including total pancreatectomy) or intensive supportive therapy all that is on offer for chronic pancreatitis or acute pancreatitis, respectively[301,518].

The major stumbling block is the reluctance of seasoned pancreatologists to abandon the macabre notion that 'autodigestion' commencing in the acinar cell underlies pancreatitis, although it is apparently rubber-stamped by genetic studies showing an association with mutation in trypsin-favouring genes[7], and also by an investigation in genetically-modified rodents showing that an unfeasibly high concentration of trypsin for an unfeasibly long period results in acute (but not chronic) pancreatitis[552]. In fact, both sets of observations are explained by the utilisation of GSH to control proteases, thereby leaving enzymes in the methionine metabolic pathway vulnerable to oxidative stress and hence unable to deliver CH_3 groups that are indispensable for apical exocytosis (Chapter 11). The absence of chronic pancreatitis in the second setting is in keeping with evidence that RXS, rather than an excess of ROS alone, steer towards that outcome.

The phrase 'prematurely activated trypsin' is generally used to describe the basis for pancreatitis, but it is both emotive and misrepresentative. There is no evidence that the phenomenon via co-localization with lysosomal enzymes in acinar cells is anything more than a sophisticated natural device whereby cathepsin B ensures the controlled activation and thereby safe degradation of trypsin by enzymes such as mesotrypsin and chymotrypsin, thus fulfilling a housekeeping role[5,6,29,256,266,287,305]. This interpretation rationalises the therapeutic inefficacy of specific trypsin inhibitors, in contrast to broad-spectrum inhibitors which also curb mast cells[256]. In fact, by 1998 observations on caerulein pancreatitis showed that hyperamylasaemia and pancreatic edema were detectable within 30-60 minutes, but an increase in cytoplasmic trypsin, as opposed to within vacuoles incorporating lysosomal cathepsin B, was not found before 60-120 minutes[553]. In other words, 'prematurely activated trypsin' was not responsible for pancreatitis. Later evidence from experimental and clinical studies indicates that the appearance of trypsin is late and small in relation to total pancreatic load - another epiphenomenon that, nevertheless, could aggravate injury (Chapter 17).

Provided that this psychological barrier can be overcome and the flawed dogma abandoned, there are numerous research opportunities for budding pancreatologists:

- Exploring the prophylactic value of micronutrient 'antioxidant' supplements in groups at risk of acute pancreatitis, eg. in patients awaiting CABG, as has been shown in patients with FLLD (Chapter 15);
- Ditto in relation to chronic pancreatitis for population prophylaxis in areas where the disease is endemic - eg. by a supplement of vitamin C plus selenium in Soweto; or vitamin C with β-carotene and folic acid in Kerala [255, 325];
- Investigating habitual choline intake by studies akin to those at Manchester which

identified insufficiency of methionine / vitamin C / selenium in patients with idiopathic chronic pancreatitis;

- Ditto in relation to vitamin D, in that the metabolism of this lipid antioxidant is dysregulated in pancreatic diseases[554];
- Investigating CYP involvement by immunolocalization in biopsy specimens from patients with autoimmune pancreatitis;
- Probing the effect of volatile petrochemicals, especially kerosene, on the exocrine pancreas of rodents - recalling the protection conferred by mega-dose vitamins C or E against experimental hepatotoxicity from gasoline fumes[555];
- Generating a non-invasive animal model for calcific chronic pancreatitis that involves such exposure alongside CYP1 induction by a high-corn oil diet plus sub-standard micronutrient 'antioxidant' intake;
- Further investigation of the above in rodents with genetically engineered CFTR insufficiency;
- Refining the Manchester prescription for first-line treatment of chronic pancreatitis and RAP by incorporation of choline and / or magnesium and / or folate and / or zinc;
- Testing a combination of micronutrient 'antioxidants' and conventional mast cell stabiliser in settings of chronic and recurrent acute pancreatitis;
- Comprehensive analysis of ordinary pancreatic extracts for 'antioxidant' content;
- Above all, ascertaining whether an injection of adrenaline at various time-points in various animal models can abort or blunt the course of acute pancreatitis.

The list is not intended to be comprehensive, but rather to convey a flavour of the avenues that the author would have pursued, the last in particular, if still in a practice that was unpredictably cut short.

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