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GENETIC AND MOLECULAR FACTORS IN THE AETIO-PATHOGENESIS OF PANCREATITIS IN HUMANS

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GENETIC AND MOLECULAR FACTORS IN THE AETIO-PATHOGENESIS OF PANCREATITIS IN HUMANS

by

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Molecular and Genetic Factors in the
Aetio-pathogenesis of Pancreatitis in Humans

ABSTRACT

Both acute (AP) and chronic (CP) pancreatitis are complex diseases, with a number of aetiologies and complex pathogeneses. A number of contributing factors are assessed here.

Genetic studies were performed looking at a high activity polymorphism of the alcohol metabolising enzyme cytochrome P450 2E1. Assessing a role in alcohol abuse and end organ disease; alcohol abusers (n= 239) and controls (n= 208) were studied. A significantly lower number of alcohol consumers (2.1%) had the polymorphism than controls (5.8%); p= 0.049, Fisher's exact test. Any association with end organ disease could not be further elucidated due to the rarity of the polymorphism in this population. In another genetic study, looking at a polymorphism in interleukin-1a, no associations were found with CP; of note no associations were found with genotypes implicated in AP.

A double-blind, placebo controlled crossover trial of a leukotriene receptor antagonist in chronic pancreatitis revealed no benefit.

Studies of production of arachidonic acid metabolites leukotriene E_4 (LTE_4), prostaglandin E_2 (PGE_2) and (a known marker of mast cell activation) prostaglandin D_2 ($11\beta\text{-}PGF_{2\alpha}$) were performed. Analysis looked at both acute (n= 19) and chronic pancreatitis (n= 19), employing age and sex matched controls. The LTE_4 studies did not reveal any significant difference in levels. PGE_2 levels were not different between CP patients and controls while they were significantly higher in AP than controls; p= 0.006, independent samples t-test. The variation appeared most marked for mild disease; one way ANOVA p= 0.024 and direct comparison of patients with mild disease and their matched controls; p= 0.011. The $11\beta\text{-}PGF_{2\alpha}$ study conversely showed no difference in AP but significantly higher levels in CP in comparison to their matched controls; p= 0.001, Mann Whitney U test.

Based on a previous pilot study in CP and a difference in variance of LTE_4 in AP in the above study, a genetic study of the known functional polymorphism in the gene of leukotriene C_4 synthase (the first dedicated enzyme in the formation of the cysteinyl leukotrienes) was performed. Controls totalled 108 subjects; AP 238 (mild= 169 patients; severe= 69) and CP 57 subjects; no difference in the genotype or allele frequencies were found.

In summary: A possible role for a functional polymorphism in cytochrome P450 2E1 (not previously examined in patient groups) in protection against alcoholism has been identified. Perhaps analogous to the protection associated with high activity forms of alcohol dehydrogenase and low activity forms of aldehyde dehydrogenase.

PGE_2 is elevated in acute pancreatitis in humans consistent with the majority of the data in animals. Again consistent with the bulk of animal data this appears to be most marked in mild disease, possibly indicating a protective, and therefore potentially therapeutic, role.

$11\beta\text{-}PGF_{2\alpha}$, a metabolite of PGD_2 and marker of mast cell activation, is elevated in chronic but not acute pancreatitis. This implicates mast cells in chronic pancreatitis and would be consistent with their known role in fibrosis and tissue remodelling and suggests a possible therapeutic target.

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ABBREVIATIONS used in this text:

AA	Arachidonic Acid
AC	Alcoholic Controls (those without evidence of end organ disease)
AChE	Acetyl Choline Esterase
AICP	Alcohol Induced Chronic Pancreatitis
aka	also known as
ALC	'Alcoholics' fulfilling the criteria for ICD 10.1 and/or 10.2
ALD	Alcoholic Liver Disease
ANOVA	Analysis of Variance
AP	Acute Pancreatitis
APACHE II	Acute Physiolgy and Chronic Health Evaluation II
APD	Accessory Pancreatic Duct
bp	Base Pairs
CBD	Common Bile Duct
CCK	Cholecystokinin
COX	Cyclo-oxygenase
CFTR	Cystic Fibrosis Transmembrane Regulator
CP	Chronic Pancreatitis
CRP	C-Reactive Protein
CT	Computed Tomography
CYP2E1	Cytochrome P450 2E1
Cys-LT	Cysteinyl Leukotriene
DNA	Deoxyribonucleic Acid
dNTP	2' deoxyriboNucleotide 5'-triphosphate
EDTA	Ethylene Diamine Tetra-acetic Acid
EIA	Enzyme Immuno-Assay
ELISA	Enzyme Linked Immuno-Sorbent Assay
ERCP	Endoscopic Retrograde Cholangio-Pancreatography
FAEE	Fatty Acid Ethyl Esters
FLAP	Five Lipoxygenase Activating Protein
γ^{32P}-ATP	gamma radiation labelled deoxyridoadenosine triphosphate
GC/MS	Gas Chromotography/ Mass Spectroscopy
HC	Healthy Controls
HP	Hereditary Pancreatitis
HPLC	High Performance Liquid Chromotography
ICD	International Classification of Diseases
ICP	Idiopathic Chronic Pancreatitis
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
kb	kilobase
5-LO	5-lipoxygenase
LC/MS	Liquid Chromotography Mass Spectroscopy
LT	Leukotriene
LTC_4S	Leukotriene C_4 Synthase
µg	microgram
µl	microlitre
MAP	Mild Acute Pancreatitis
$MgCl_2$	Magnesium Chloride
MOF	Multi-Organ Failure

MPD	Main Pancreatic Duct
n	number
NC	Normal Controls
ng	nanogram
NFκB	Nuclear Factor Kappa B
ns	Non-Significant
NTP	deoxy-Nucleotide Tri-Phosphate
PAGE	Poly Acrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PC	Prostacyclin (aka PGI_2)
PCR	Polymerase Chain Reaction
PG	Prostaglandin
PNK	Polynucleotide Kinase
PSTI	Pancreatic Secretory Trypsin Inhibitor (aka SPINK 1)
RFLP	Restriction Fragment Length Polymorphism
RIA	Radio-Immuno-Assay
mRNA	messenger Ribo-Nucleic Acid
ROS	Reactive Oxygen Species
RXS	Reactive Xenobiotics
SAP	Severe Acute Pancreatitis
SPINK 1	Serine Protease Trypsin Inhibitor Kazal Type 1 (aka PSTI)
SNP	Single Nucleotide Polymorphism
sTNF-R	secretory Tumour Necrosis Factor Receptor
TAE	Tris-Acetate Ethylene diamine tetra-acetic acid (Tris Acetate buffer)
Taq	Taq (thermostable) DNA polymerase
TBE	Tris-Boric acid Ethylene diamine tetra-acetic acid (Tris Borate buffer)
TCP	Tropical Chronic Pancreatitis
TGF	Transforming Growth Factor
Th Cell	T Helper Cell
T_m	Melting Temperature
TNF	Tumour Necrosis Factor
TX	Thromboxane
USS	Ultrasound Scan
UV	Ultra-Violet
VNTR	Variable Number Tandem Repeat polymorphism

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My wife for her continuing love and forbearance throughout, despite spending most of her married life married to someone researching, and writing, a thesis. My family and friends who wonder if I'll ever say 'I have no writing to do tonight'.

AUTHORS DECLARATION

At no time during the registration for the degree of Doctor of Medicine has the author been registered for any other University award.

This trial aspect of this study was financed in part by Merck, Sharpe and Dohme, U.K.

A programme of advanced study was undertaken including the laboratory work discussed here, a clinical trial and supervised statistical and information technology instruction.

Relevant scientific seminars and conferences were regularly attended at which work was often presented; other institutions were visited for consultation and practical purposes and papers prepared for publication (see Publications section).

Conferences Attended and [Presentations] made:

National/International:

Pancreatic Society of Great Britain and Ireland. Leeds, November 2002.
[Arachidonic Acid Metabolites in Acute and Chronic Pancreatitis in Humans.]

Pancreatic Society of Great Britain and Ireland. Southampton, October 2001.
[Antibiotic Use and Fungal Infections in Acute Pancreatitis in a large District General Hospital in the United Kingdom.]

European Pancreatic Club. Toulouse, June 2001.
[A High Activity Polymorphism in the Promoter Region of the Alcohol Inducible Enzyme Cytochrome P450 2E1 in Alcoholism and End-Organ Disease.]
[Report of a Trial of a Leukotriene Receptor Antagonist in Chronic Pancreatitis in Humans.]
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Pancreas Club Inc. (USA). Chicago, May 2001.
[A High Activity Polymorphism in the Promoter Region of the Alcohol Inducible Enzyme Cytochrome P450 2E1 in Alcoholism and End-Organ Disease.]
[Report of a Trial of a Leukotriene Receptor Antagonist in Chronic Pancreatitis in Humans.]

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[Evidence of a Role for Leukotrienes in Chronic Pancreatitis in Humans. Winner of Best Oral Presentation of selected posters]
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American Pancreatic Association/International Association of Pancreatology Combined Meeting. Chicago, November 2000.
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Surgical Research Society. Cork, October 2000.
[Evidence of a Role for Leukotrienes in Chronic Pancreatitis in Humans.]

PANCON 2000 Coimbatore, India July 2000
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Regional:

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[Faecal Pancreatic Elastase 1: An Early Marker of Pancreatic Exocrine Insufficiency in Chronic Pancreatitis.]

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British Society of Gastroenterology (South West Branch). Torbay, June 2000.
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Signed Mark Cartwell

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INTRODUCTION

Pancreatitis can be broadly divided into two groups, they are Chronic Pancreatitis (CP) and Acute Pancreatitis (AP). Classification both within and between these forms has been debated for many years, the most recent meetings being in Cambridge 1983 (Sarner, M. & Cotton, P. B., 1984) and Marseille 1984 (Singer, M. V. et al., 1985); with its more recent Rome update 1988 (Sarles, H. et al., 1989).

CP is generally viewed with respect to its aetiology, which is addressed, in part, within these systems. AP may be subdivided by aetiology but also with respect to its' severity (Bradley, E. L., III, 1993).

This Thesis will address both Acute and Chronic Pancreatitis, although Chronic Pancreatitis predominates and features in all areas studied.

1.1 Pancreatic Anatomy:

The pancreas, one of the butchers 'sweet breads', is a macroscopically lobulated gland with a fine capsule which lies retroperitoneally in the abdomen, roughly at the level of the first lumbar vertebra. It is often described as the shape of a broad walking stick (figure 1.1). It is separated for ease of description into a head, neck, body and tail passing from right to left and sloping gently upwards. The handle of the walking stick is as the head gives off, inferiorly, the uncinate process which passes infero-posteriorly then towards the left and behind the superior mesenteric vessels which were, in turn, behind the head (figure 1.2).

Some clinically relevant anatomical features of the pancreas are (figure 1.2): Its anterior surface is crossed in its lower part by the transverse mesocolon and thus it lies predominantly in the supracolic compartment and behind the lesser peritoneal sac (omental bursa). Thus the predominant site of a pancreatic pseudo-cyst (a fluid collection with a defined wall) complicating pancreatitis is the lesser sac and is often amenable to drainage into the stomach or duodenum. The head of the pancreas is 'held' in the concavity of the 'C' shaped duodenal curve and can therefore be involved in duodenal ulceration.

The pancreas is directly related to the superior mesenteric vessels (as above), the splenic artery, which courses posterior to it's upper border, and the pancreatico-duodenal arteries, which run in the grooves between the pancreatic head and duodenum. Thus, with gross pancreatic necrosis these vessels can be involved and eroded thus leading to pseudo-aneurysm formation and haemorrhage.

Pancreatic juice is drained to the duodenum via the main pancreatic duct (MPD), of Wirsung, and the accessory pancreatic duct (APD), of Santorini (figure 1.1). Their anatomy is best understood in light of pancreatic embryology. The pancreas is formed by

the fusion of dorsal and ventral buds from the duodenum (figure 1.3). The smaller ventral bud migrating posterior to the duodenum to join the dorsal bud at is lower margin. The duct systems anastomose then separate leaving the proximal part of the lower, ventral duct to drain the main bulk of the pancreas (MPD) whilst the proximal part of the superior, dorsal duct drains part of the head and the uncinate process (APD). The relevance of this is that failed migration can lead to annular pancreas (whereby the pancreas encircles, and may obstruct, the duodenum) or the ducts (and pancreas) may fail to fuse normally, in the condition called pancreas divisium. There has been debate as to whether this condition predisposes to pancreatitis, since the technique of ERCP allowed us to identify it in living patients (Rosch, W. et al., 1976), until the present (Quest, L. & Lombard, M., 2000). It is probably true to say that it does so in some circumstances, for example, when associated with insufficiency of the minor papillae (Varshney, S. & Johnson, C. D., 1999) and perhaps should not be taken as being the cause without further, e.g. anatomical (Burtin, P. et al., 1991) or functional (Varshney, S. & Johnson, C. D., 1999) evidence.

The MPD enters the duodenum in common with the common bile duct (CBD) at the hepato-pancreatic ampulla of Vater (figure 1.1); the common bile duct having arisen in common with the ventral pancreatic bud (figure 1.3). Also, secondary to the embryological rotation discussed the CBD lies (in its lower part) posterior to, indenting (sometimes embedded within) the pancreatic head. Thus it can be seen how passage of a gallstone can be associated with pancreatitis, although the exact mechanism is still an area of debate, see below. In addition, pancreatic enlargement or fibrosis can lead to obstruction of the CBD.

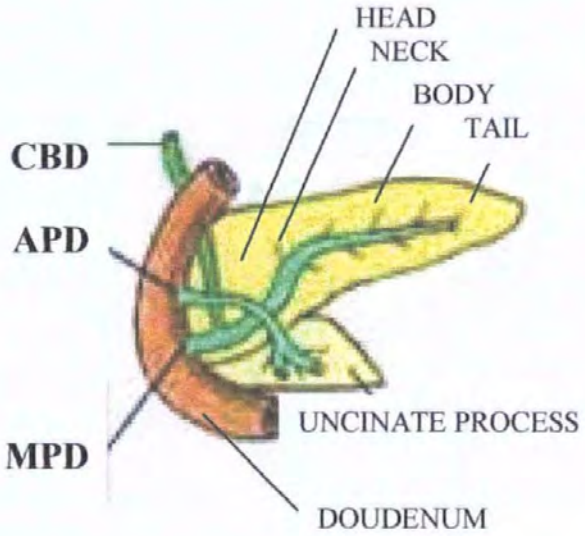


Figure 1.1. Diagrammatic Representation of Pancreatic Anatomy.
MPD = main pancreatic duct
APD = accessory pancreatic duct
CBD = common bile duct
Courtesy of Andrew Whitaker, www.instantanatomy.net

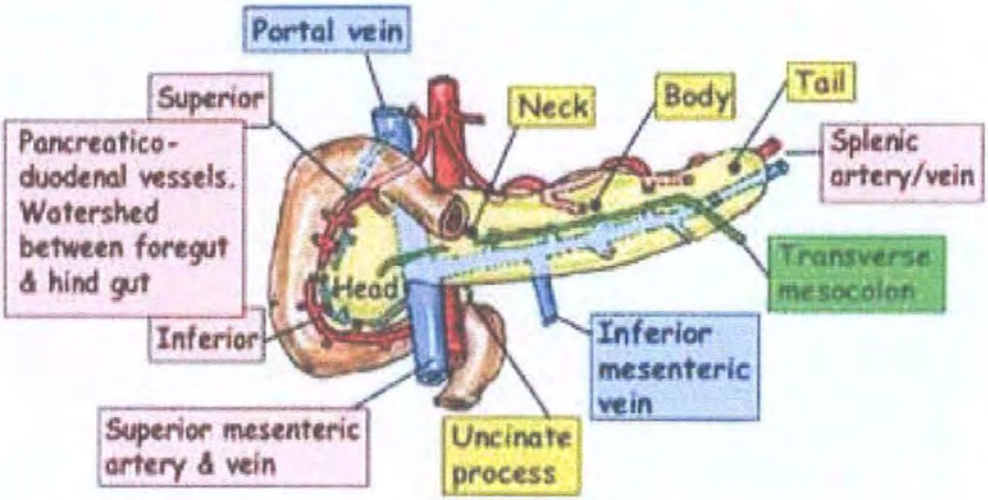


Figure 1.2 Detailed Pancreatic Anatomy.
Courtesy of Andrew Whitaker, www.instantanatomy.net

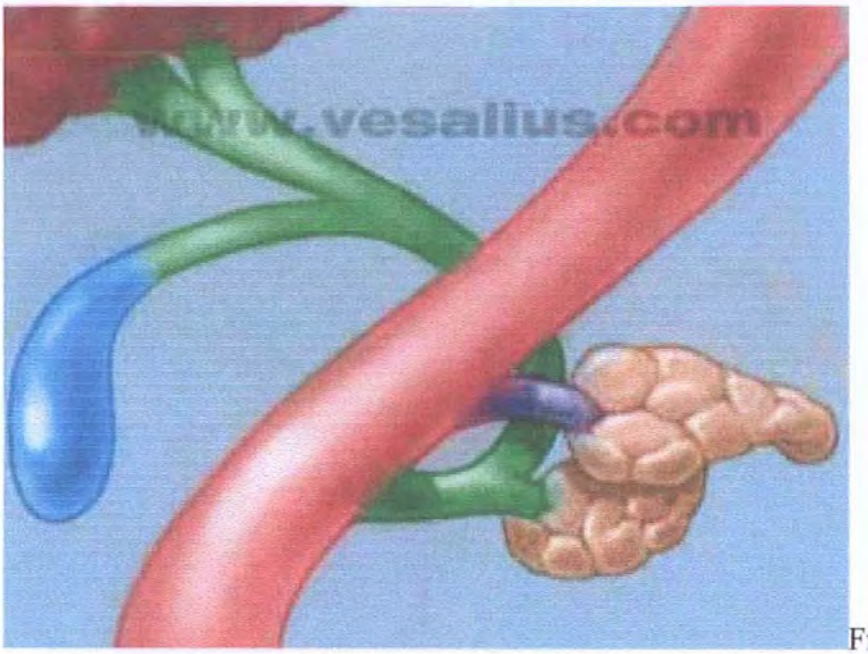


Figure 1.3a.

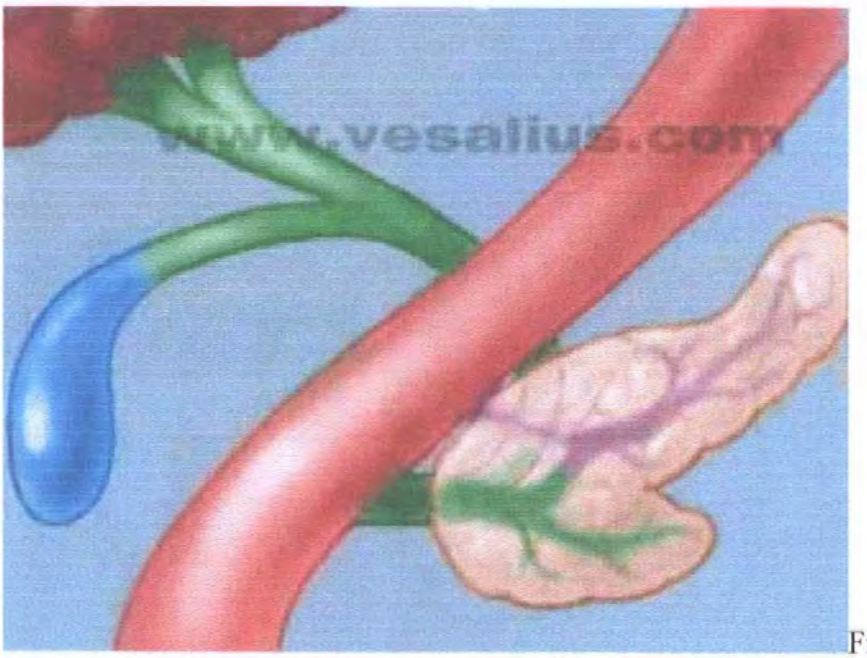


Figure 1.3b.

Figure 1.3. Pancreatic Embryology. Courtesy of www.vesalius.com.
Green represents the ventral duct arising in common with the common bile duct and purple the dorsal duct.

1.2 **Pancreatic Function:**

The pancreas is a composite gland having both exocrine and endocrine functions.

The exocrine function of the pancreas is secretion of digestive enzymes in a bicarbonate rich alkaline fluid via the pancreatic ducts into the duodenum. Water follows the osmotic gradient created by electrolyte transport. This constitutes a volume of some 2.5 litres per day and 6-20 grams of protein (Case R. M., 1998).

1.2.1 **Control of Secretion:**

Most evidence on the mechanisms involved in control of pancreatic secretion comes from rats and, at least in terms of control, they are not a good model for humans. However, the cellular mechanism involved for rats is probably one of two mechanisms which act together in humans (Case R. M., 1998).

Basal levels of electrolyte and water secretion are low in humans (as they are in the dog and cat, but not certain other species, especially the rabbit) which can lead to an accumulation of enzymes in the ductal tree, subsequent to increased flow 'washing' them out (Case R. M., 1998). This may partially explain some species resistance to naturally occurring pancreatitis.

Pancreatic duct cells are the major source of this bicarbonate rich fluid in which the pancreatic enzymes are secreted, these cells are primarily stimulated by the hormone secretin which stimulates increased volume and bicarbonate concentration at the expense of chloride. This cellular mechanism has an integral, rate limiting role for the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). Thus the existence of pancreatic fibrosis and insufficiency seen in Cystic Fibrosis and the apparent increased frequency of CFTR mutations found in CP (Cohn, J. A. et al., 1998; Sharer, N. et al., 1998) can be

explained. Secretin is produced by the S cells of duodenum and jejunem in response to acid (pH 4.5-5) in the duodenum and to a lesser extent protein and fat digestion products (Ganong, W. F., 1995; Guyton, A. C. & Hall, J. E., 2000).

Digestive enzymes are secreted by the pancreatic acinar cells, this being largely dependent on the hormone(s) CCK (cholecystokinin), which only has a weak effect on fluid volume except to potentiate the effect of secretin. The independent role in fluid volume control may also be enacted via the acinar cells (Case R. M., 1998). CCK is secreted by the I cells of duodenum and jejunum in response to long chain fatty acids, peptides and amino acids (Ganong, W. F., 1995; Guyton, A. C. & Hall, J. E., 2000).

Somatostatin is secreted by the delta (or D) cells of the pancreatic islets and the intestinal mucosa. It has an inhibitory effect on a number of gastro-intestinal hormones, most notably in this context secretin. It is secreted in response to a number of factors associated with intake of food, e.g. acid in the duodenal lumen and fatty acids, amino acids and glucose in the blood (Ganong, W. F., 1995; Guyton, A. C. & Hall, J. E., 2000).

Further controls of secretion include extrinsic nervous control: Sham feeding has been shown to increase enzyme secretion, but have little effect on volume (Anagnostides, A. et al., 1984). Gastric distension has been shown to have a similar effect (Cargill, J. M. & Wormsley, K. G., 1979).

The enzymes produced by the pancreas are:

PROTEOLYTIC

- Trypsin(ogen)s
  Chymotrypsin(ogen) } endopeptidases
  Elastases
  (Pro)Carboxypeptidases

LIPOLYTIC

- Lipase (+ colipase)
  Phospholipase A2
  Cholesterol esterase

CARBOHYDROLYTIC

- Amylase

They are secreted in excess to absolute requirements (presumably to enable maximal digestion) and show some adaptation to dietary intake (Metzger, A. & DiMagno, E. P., 1998).

The controls of pancreatic secretion and especially its increase in response to food is the basis of pancreatic rest as a central part of the management of AP. It is well known for recurrence of pain to occur on refeeding in patients recovering from AP. However, the relevance of pancreatic rest has been questioned (Ballinger, A., 1998), especially in light of evidence suggesting that during a bout of AP in mice and rats pancreatic secretion is maximally suppressed (Niederau, C. et al., 1990). Also studies suggest that naso-jejunal feeding (distal to the site of maximal CCK stimulation) is safe and superior (on balance of benefits and disbenefits) to parenteral feeding (Kalfarentzos, F. et al., 1997; McClave, S. A. et al., 1997; McGregor, C. S. & Marshall, J. C., 2001; Pupelis, G. et al., 2002). Nasogastric feeding is even thought by some now to be safe, this in a very small study (Eatock, F. C. et al., 2000).

Also influencing the control of secretion forms the basis of various treatments which have been tested or employed in AP and CP. Somatostatin analogues are of benefit in the treatment of pancreatic fistulae and are used to prevent the same in pancreatic surgery, 'though the extent of their benefit, if any, is not fully elucidated (Li-Ling, J. & Irving, M., 2001). They have been looked at in AP but not shown to be of proven benefit (Uhl, W. et al., 1999a), although a benefit was suggested by meta-analysis (Andriulli, A. et al., 1998) prior to the largest trial (Uhl, W. et al., 1999b).

1.2.2 **Control of digestive enzymes within the pancreas:**

Digestive enzymes need to be active in the gut but controlled within the pancreas (to avoid auto-digestion) both when intra-cellular and intra-ductal.

Secretion by exocytosis, from zymogen granules within the acinar cells, is as pro-enzymes. A series of control mechanisms maintain a basal, low level equilibrium of activation and inactivation. In zymogen granules and thence the duct system the pancreatic secretory trypsin inhibitor (PSTI/SPINK 1, serine protease inhibitor Kazal type 1) fulfils this role with an ability to 'control', by direct 1 to 1 binding, up to 10 –20% activation of trypsinogen (Braganza, J. M., 1998; Naruse, S. et al., 1999) at physiological ratios. Further control involves enzyme Y and mesotrypsin which when activated degrade trypsinogen and other zymogens (Gates, L. K. et al., 1999). Cytoplasmic control also includes glutathione in it's reduced form and a degree of activation can be reversed by antioxidants (Braganza, J. M., 1998). Interstitial control is by alpha1-protease inhibitor (Gates, L. K. et al., 1999).

The possibility of therapy to control pancreatic 'autodigestion' in acute pancreatitis has led to trials of Gabexate, a protease inhibitor, however there does not appear to be a mortality benefit and evidence for a decrease in complications is weak (Dervenis, C. et al., 1999).

1.2.3 **Endocrine Function:**

The endocrine function of the pancreas is involved primarily with the control of glucose metabolism; via the secretion, of insulin (islet β cells) and glucagon (islet α cells). In addition, somatostatin (discussed earlier) is secreted by the δ cells.

Insulin is an anabolic hormone promoting the storage of glucose, fatty acid and amino acids. It is secretion is regulated primarily in response to blood glucose levels; additional factors include blood levels of some amino acids and β-keto acids, autonomic nerves and some gut hormones.

1.3 **Acute Pancreatitis:**

Acute pancreatitis (AP) is a common disease, seen in both surgical and non-surgical practice, accounting for around 3% of admissions with acute abdominal pain (de Dombal, F., 1991). It carries a relatively high mortality of around 10% (Wilson, C. & Imrie C.W., 1990) and, at present, we have no specific non-operative therapy. Mortality seems to have plateaued in the last one to two decades (Glazer, G. & Mann, D. V., 1998). In the previous two decades a decrease in mortality had been seen due to a number of factors: increased diagnosis of mild cases; improved diagnosis, understanding of natural history, imaging and selective operative intervention.

The mortality of 5 to 10% can be sub-divided (approximately) into; mild cases 0%, severe ~20% (infected necrosis ~25%, sterile necrosis ~10%)(Dervenis, C. et al., 1999).

There is marked variability in progression and outcome from a brief bout of pain; through a long complicated course with recovery; to a rapid deterioration and death. This is one of the conundrums of pancreatitis.

Severe acute pancreatitis is generally regarded as acute pancreatitis associated with complications. These may be systemic complications, i.e. dysfunction of single or multiple organ systems, and/or local complications. The forms are most commonly discussed in terms of the 1993 Atlanta Consensus (Bradley, E. L., III, 1993), Table 1.1.

As an aid to comparing studies in AP various attempts to categorise the disease had been made. From Marseilles (Sarles, H., 1963; Singer, M. V. et al., 1985) and Cambridge (Sarner, M. & Cotton, P. B., 1984) to Atlanta these have given a basis from which to compare outcome of different management techniques and enabled discussions to be carried out using agreed and standardised terminology.

	Associated with:	Evidenced by:
Mild Acute Pancreatitis	Minimal organ dysfunction Uneventful Recovery No features of Severe AP	
Severe Acute Pancreatitis	Systemic/Organ failure or dysfunction	Shock (systolic BP <90mmHg)
		Pulmonary Insufficiency ($PaO_2 \leq 60$mmHg)
		Renal Failure (serum creatinine > 177μmol/l, after rehydration)
		Gastro-intestinal bleeding (>500ml/24 hours)
		Disseminated Intravascular Coagulation (platelets $\leq 10^5/mm^3$; fibrinogen <1g/l; fibrin split products> 80μg/ml)
		Severe metabolic disturbance (serum Calcium ≤1.87mmol/l)
	Local Complications	Necrosis (>3cm/30% nonenhancement CT; can be infected or sterile)
		Abscess (infected collection containing little or no necrotic tissue)
		Pseudocyst (collection enclosed by a wall of fibrous/granulation tissue)

Table 1.1. Summary of the Atlanta Classification System for Acute Pancreatitis, after (Bradley, E. L., III, 1993).

Local effects consist, in the mildest form, of oedema of the pancreas and/or surrounding fat. Acute fluid collections can occur, these will then often resolve or can go on to form walled off collections, termed pancreatic pseudocysts. Infected collections are termed pancreatic abscesses and carry an increased mortality. While pancreatic necrosis can occur in varying degrees, including total pancreatic necrosis (Figure 1.4). This also carries an increased mortality, however, the greatest mortality is associated with infected pancreatic necrosis.

Systemic complications, as outlined in Table 1.1, can occur in any of the bodys' organ systems. Classically ARDS, the adult respiratory distress, occurs but all degrees of respiratory dysfunction can occur as it can with all systems, defined levels being used for classification. How distant organ dysfunction occurs is a matter of debate. It is increasingly thought that those with local and systemic complications may represent two prognostic subgroups, however, with a large degree of overlap.

Death tends to occur in the first week from multiple organ failure or thereafter from sepsis, either local to the pancreas or systemically (Mann, D. V. et al., 1994). However, those patients who survive, even severe episodes (Fenton-Lee, D. & Imrie C.W., 1993), generally have low long-term morbidity and good quality of life (Broome, A. H. et al., 1996).

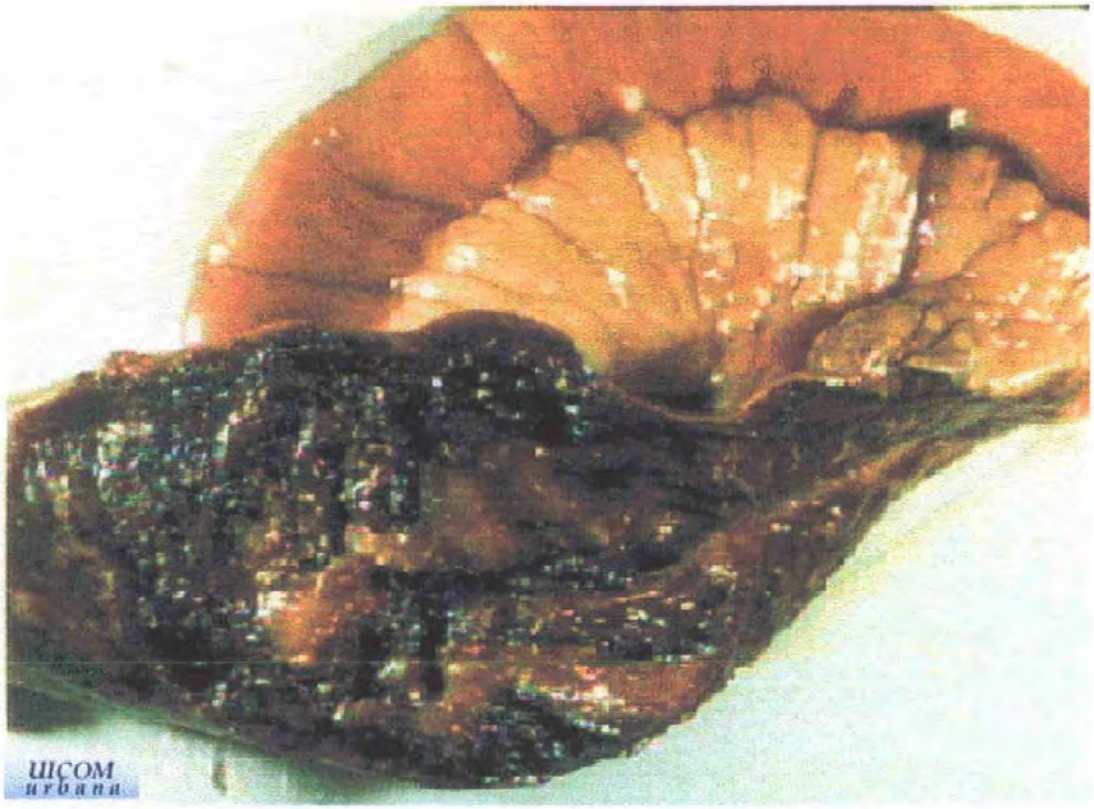


Figure 1.4. Total Pancreatic Necrosis.
Courtesy University of Illinois (www.med.uiuc.edu)

1.3.1 **Aetiology:**

The two common causes of AP remain gallstones, accounting for 30-50% of cases in Britain, and ethanol, accounting for around 8-38% of cases (Corfield, A. P. et al., 1985; Thomson, S. R. et al., 1987; Mann, D. V. et al., 1994; De Beaux, A. C. et al., 1995); the most extreme figures (low for alcohol and high for gall stones) from a study looking specifically at first episodes (Corfield, A. P. et al., 1985). The mechanisms by which the various causes lead to AP are, again, not fully understood.

If these causes are not identified other potential causes should be sought which include; hyperlipidaemia, tumours (especially periampullary tumours) and infective causes; including viral, e.g.. mumps and coxsackie, and parasitic, e.g. ascaria and clonorchis. 'Idiopathic' (or unidentified aetiology) remains a common diagnosis in reported series and appears to diminish with repeated investigation, the recent UK Guidelines suggest that this group should constitute no more than 20-25% of diagnoses of AP (Glazer, G. & Mann, D. V., 1998).

1.3.2 **Diagnosis:**

Abdominal pain, especially epigastric, +/- radiation to the back, associated with vomiting, are the common presenting features. Signs include abdominal tenderness (ranging from mild through rebound to rigidity) and, less commonly, Cullen's and Grey-Turners signs, which contribute to a more specific diagnosis. However, presentation can be varied and missed diagnosis remains a factor in the high mortality (Kingsnorth A.N., 1998).

A clinical diagnosis is supported by the finding of raised serum (or urinary) pancreatic enzymes; most commonly amylase. They are typically elevated (amylase >3-5x, lipase >2x normal) during an attack of AP. However, they must be sought to give a result and may not

be elevated even in a severe attack (lack of sensitivity) and can be non-specifically elevated in clinically similar conditions (lack of specificity). In addition, amylase is short lived in the blood (leading some commentators to recommend urine levels to be tested). Amylase also has other isoenzymes, which may, rarely, be the source of an abnormally high result. Lipase is marginally superior to amylase (Table 1.2). Combination of the two investigations improves sensitivity and specificity.

Further assays, which show promise, include: Trypsin2-alpha1antitrypsin complex, which also correlates with severity and Alpha2-macroglobulin-trypsin complex. Trypsinogen 2 is probably closest to a practical, accurate form and for which a simple urine test is available (Kylanpaa-Back, M. et al., 2000) Table 1.2. In addition, serum levels correlate with severity.

As many as 12-42% of cases of fatal AP go undiagnosed prior to death. In one analysis, of patients diagnosed post-mortem, Wilson and Imrie (Wilson, C. & Imrie, C. W., 1988) suggest that a timely diagnosis may have altered management, potentially allowing survival, in 45% of cases. In addition to a variable clinical presentation, the lack of a highly specific diagnostic test leads to difficulties.

	Sensitivity	Specificity
Amylase	52-95%	86-98%
Lipase	74-100%	34-100%
Trypsinogen-2 (serum or urine)	91-98%	89-95%

Table 1.2. Sensitivites of Diagnostic Tests in Acute Pancreatitis. Data from (Kemppainen, E. et al., 1998).

1.3.3 **Prognostication:**

As AP has such a range of clinical courses it is useful to be able to predict which patients are likely to have a severe (complicated) course. This enables most intensive observation of those at greatest risk, implementation of investigation and of management appropriately and opportunely. It also provides for standardised comparisons in and with studies. Attempts at predicting severity in AP have been made for over two decades, initially consisting of multi-factor scoring systems, such as the Ranson's (Ranson, J. H. et al., 1974) and the Glasgow (Blamey, S. L. et al., 1984) Criteria. Other indicators are now also known and a large number of potential serum markers are being evaluated. The details of all the potential predictors are extensive and a number of reviews exist (Dervenis, C. et al., 1999; Glazer, G. & Mann, D. V., 1998). In summary a combination of recognised predictors of severe outcome is, at present, the best approach:

- Simple Tests:

Clinical Assessment - not regularly reliable, still a vital component.

Obesity - Body Mass Index ≥30 (Lankisch, P. G. & Schirren, C. A., 1990; Funnell, I. C. et al., 1993; Martinez, J. et al., 1999).

Chest X-Ray - pleural effusions (especially left sided and bilateral) (Lankisch, P. G. et al., 1994; Maringhini, A. et al., 1996).

C-Reactive Protein (CRP) - >150mg/l (values from 100-210mg/l evaluated by various authors)(Leese, T. et al., 1988; Wilson, C. et al., 1989).

- Multi-factor scoring systems:

e.g. The Acute Physiology and Chronic Health Evaluation (APACHE)-II ≥8 (values between ≥6 - ≥9 recommended by various authors) (Larvin, M. & McMahon, M. J., 1989; Wilson, C. et al., 1990). Table 1.3.

- CT imaging: (Dynamic) contrast enhanced CT of the pancreas. Necrosis and fluid collections correlate with outcome, systems such as that of Balthazar et al (1990) are useful measures.

In addition, APACHE II and CRP can be used for daily monitoring of a patients progress

A – Acute Physiology Score

PHYSIOLOGICAL VARIABLE	HIGH ABNORMAL RANGE					LOW ABNORMAL RANGE			
	+4	+3	+2	+1	0	+1	+2	+3	+4
Temperature – Rectal (°C)	≥41	39-40.9		38.5-38.9	36-38.4	34-35.9	32-33.9	30-31.9	≤29.9
Mean arterial pressure (mmHg)	≥160	130-159	110-129		70-109		50-69		≤49
Heart Rate (ventricular response)	≥180	140-179	110-139		70-109		55-69	40-54	≤39
Respiratory Rate (non-ventilated or ventilated)	≥50	35-49		25-34	12-24	10-11	6-9		≤5
Oxygenation: A-aDO_2 or PaO_2 (mmHg) a: $FiO_2 \geq 0.5$ record A-aDO_2 b: $FiO_2 < 0.5$ record only PaO_2	≥500	350-499	200-349		<200 >70	61-70		55-60	<55
Arterial pH	≥7.7	7.6-7.69		7.5-7.59	7.33-7.49		7.25-7.32	7.15-7.24	<7.15
Serum Sodium (mmol/L)	≥180	160-179	155-159	150-154	130-149		120-129	111-119	≤110
Serum Potassium (mmol/L)	≥7	6-6.9		5.5-5.9	3.5-5.4	3-3.4	2.5-2.9		<2.5
Serum Creatinine (mg/100ml)- double poins for Acute Failure	≥3.5	2-3.4	1.5-1.9		0.6-1.4		<0.6		
Haematocrit (%)	≥60		50-59.9	46-49.9	30-45.9		20-29.9		<20
White blood count ($x10^3/mm^3$)	≥40		20-39.9	15-19.9	3-14.9		1-2.9		<1
Glasgow Coma Score (15 minus actual score)									
A = Total of 12 above variables (the Acute Physiology Score)									
Serum HCO_2 (venous mmol/L) - NOT preferred use if no ABGs	≥52	41-51.9		32-40.9	22-31.9		18-21.9	15-17.9	<15

B – Age Points

Age (years)	Points
≤44	0
45-54	2
55-64	3
65-74	5
≥75	6

C - Chronic Health Points

If the patient has a history of severe organ system insufficiency or is immunocompromised assign points as follows: for non-operative or emergency post-operative patients – 5 points; elective post-operative - 2 points.

Organ insufficiency or immunocompromised must have been evident prior to this hospital admission and conforms to the following criteria:

Liver: Biopsy proven cirrhosis and documented portal hypertension; or prior episodes of hepatic failure.

Cardiovascular: NY Heart Association Class VI

Respiratory: Chronic restrictive, obstructive or vascular disease resulting in severe exercise restriction, e.g. unable to climb stairs or perform household duties. Or documented chronic hypoxia, hypercapnia, secondary polycythaemia, severe pulmonary hypertension (>40mmHg) or respirator dependency.

Renal: Recurring chronic dialysis.

Immunocompromised: The patient has received therapy that suppresses resistance to infection (e.g. immuno-suppression, chemotherapy, radiation, long-term or recent high-dose steroids) or has disease that is sufficiently advanced to suppress resistance to infection (e.g. leukaemia, lymphoma, AIDS).

Table 1.3: The APACHE II (Acute Physiology and Chronic Health Evaluation) Score. Total of Sections A + B + C. (from Knaus, W. A. et al., 1985)

1.3.4 **Management:**

In AP the management focuses on symptom control, supportive treatment and prevention of complications. In mild acute pancreatitis intravenous fluid administration, analgesics and avoidance of oral fluid or food intake is sufficient in most cases. The treatment of severe pancreatitis involves adequate volume replacement, nutritional support and careful monitoring of vital functions with early treatment of organ failure. Identification of pancreatic and peripancreatic necrosis is best demonstrated and evaluated by contrast-enhanced computerized tomography (CT). Infection of such necrosis is associated with poor outcome and surgical intervention in certain specific situations, most notably that of infected pancreatic necrosis (McFadden, D. W. & Reber, H. A., 1994; Warshaw, A. L., 2000), is one of the few specific interventions available in AP.

Many additional aspects of management have developed and are developing. Although many of these are still contentious a number of consensus documents have been produced giving guidelines on the management of AP (Banks, P. A., 1997a; Glazer, G. & Mann, D. V., 1998; Dervenis, C. et al., 1999).

Areas of contention in its management include:

- Antibiotic Prophylaxis.

As infected pancreatic necrosis is associated with a much higher mortality than sterile necrosis the prevention of its development is an appealing avenue. A debate regarding antibiotic prophylaxis has been ongoing for many years, with the weight of evidence most often said, in the past, to fall against prophylaxis. However, much of the data was concerning ampicillin (Cameron et al., 1975; Finch et al., 1976) which we now know has limited pancreatic penetration (Trudel et al., 1994; Spicak et al., 1999), also studies included mild cases which may have masked a significant effect in severe cases.

A number of more recent trials have been performed, in addition to increasing evidence on which antibiotics attain appropriate tissue levels this debate continues (Barie, 1996). On balance it can now be argued that antibiotic prophylaxis is justified in patients with predicted severe disease or proven necrosis (Golub et al., 1998; Powell et al., 1998). This corresponds to present practice commonly found in the UK (Powell et al., 1999). However, much debate continues (Slavin & Neoptolemos, 2001) and certainly further studies are required to delineate the role, if any, of routine prophylaxis.

Even the recent studies have not consistently shown a survival benefit or reduced pancreatic infections. Cefuroxime has been shown to decrease total infectious complications, need for operative intervention and death, but not pancreatic infection, in a trial of 60 patients (Sainio et al., 1995). Ceftazidime (with amikacin, which is poorly penetrant, and metronidazole) reduced all infections but not mortality in a small trial (Delcenserie et al., 1996). Ofloxacin (with metronidazole) in a small trial was shown to decrease physiological disturbance and has good penetration and spectrum characteristics (Schwarz et al., 1997). Imipenem has been shown to decrease pancreatic and extrapancreatic infection, need for operation and systemic complications and be superior to perfloxacin without metronidazole, but has not to shown a survival benefit (Pederzoli et al., 1993; Bassi et al., 1998; Nordback et al., 2001).

In addition to which antibiotic, if any, is best other issues in antibiotic prophylaxis awaiting resolution include: Duration of administration and whether to commence antibiotics on purely prognostic grounds or only after necrosis is seen (as in most of the trials).
The role of gut decontamination, in an attempt to prevent bacterial translocation, either alone or in addition to systemic prophylaxis is a further issue. Evidence at present is limited, although animal studies suggest a possible benefit. A single trial in humans showed only a non-significant decrease in mortality (although significance was obtained

with mulitvariate analysis) though it did show decreased pancreatic infections (Luiten EJ and Bruining, 1999). This trial involved oral, rectal and intravenous antibiotics.

•Nutrition.

The role of the gut in the development of infected necroses is becoming increasingly apparent and this brings us to another area of contention in the management of AP: Nutrition. As part of the, afore mentioned, pancreatic rest it had been traditional to 'feed' patients (who were expected to not be able to take orally or who did not settle) parenterally. The advantages of enteral feeding, especially in a context where translocation of gut organisms may be an important factor, led people to explore the possibility of feeding enterally in AP. This done especially beyond the ligament of Treitz which should thereby cause less stimulus to pancreatic secretion. A number of trials have shown advantages or a least no greater complications (Kalfarentzos et al., 1997; McClave et al., 1997; Hamvas and Pap, 1998; Nakad et al., 1998; Berger and Papapietro, 1999; Powell et al., 2000). Although enteral feeding didn't have the hoped beneficial effect on inflammatory markers (Powell et al., 2000).

•Endoscopic Sphincterotomy.

Early (first one to three days of an attack) ERCP and endoscopic sphincterotomy (ES) in AP has a logical basis. CBD stones causing acute pancreatitis can be identified and removing a stone obstructing the CBD and/or pancreatic duct and decompressing the obstructed system should improve outcome.

Some retrospective studies and four controlled trials have been performed. The trials have not all had the same conclusion and the largest is only available in abstract form (i.e. not peer reviewed) even some 8 years hence. Early ERCP has been shown to have a decreased overall complication rate in 'all' gallstone AP patients, but especially those with severe

disease (Neoptolemos et al., 1988; Fan et al., 1993). Another study which did not show this in fact showed a (non-significantly) worse outcome in the treatment group (Folsch et al., 1997). This later study did not delineate mild and severe disease and excluded those with cholangitis or an elevated bilirubin, taking those to 'require' ERCP. The Hong Kong study (Fan et al., 1993) showed a significant reduction in biliary sepsis (presumably a number of these patients would have fallen into the automatic ERCP group in the Folsch study (1997)). Of interest the largest study, only ever published in abstract form, did show significantly lower complication and mortality rates in the treatment group (Nowak et al., 1995). This 'despite' excluding those with an impacted stone at the papilla, who routinely had ES. A meta-analysis which included all four studies, severely limited by the varied inclusion criteria and lack of details on the largest'trial, showed a significant reduction in both mortality and complications (Sharma & Howden, 1999).

Thus, evidence exists but the full role has not been delineated. It would seem reasonable to state that in AP with gallstones and evidence of biliary obstruction (obstructive liver function tests or biliary dilatation) early ERCP and ES is appropriate. As it is in a predicted severe bout (slightly more contentiously). There is likely to be less benefit of early ERCP, if any, in predicted mild disease without obstruction.

1.3.5 **Pathogenesis:**

Each aetiological cause of AP may initiate a bout by different (though overlapping) mechanisms. However, all theories of the pathogenesis suppose common pathways, although the exact point of the start of commonality is not fully understood.

Why gallstones cause AP is not fully understood. Obstruction of the pancreatic duct at/near its confluence with the CBD is the most obvious and some evidence exists for obstruction (Steer, M. L. & Meldolesi, J., 1987) and reflux of bile (Opie, E. L., 1970). These theories

are not fully accepted, and certainly events such as acinar cell damage can lead to enzyme activation by a number of methods.

In gallstone and other 'obstructive' causes of AP it is suggested that the back pressure resulting leads to increased ductal pressure, stassis of the pancreatic juice and/or reflux of bile thence abnormal activation of the pancreatic digestive enzymes. This activation could occur either intraductally, intracellularly (Scheele, G. A. et al., 1984; Saluja, A. et al., 1989) or in the interstitium (Kloppel, G. et al., 1986) leading to autodigestion. The cause of this activation could be intracellular activation by co-localization which has been shown to occur in models of duct obstruction (Saluja, A. et al., 1989). Autodigestion is also thought of as a central factor (and arguably the factor) in all forms (Kloppel, G. & Maillet, B., 1993).

This autodigestion leads pancreatic parenchymal and/or fat damage then to the activation and recruitment of inflammatory cells and inflammatory mediators (Brady, M. et al., 1999).

Alcohol induced AP is an area of contention. It is accepted by many that a truly acute form of alcohol induced pancreatitis exists (Sarner, M. & Cotton, P. B., 1984; Singh, M. & Simsek, H., 1990; Pelli, H. et al., 2000) although it appears the majority of cases of AP due to alcohol are in fact exacerbations of CP, if undiagnosed (Meier P.B., 1995; Robles-Diaz, G. & Gorelick, F.S., 1997). Thus the mechanism has much overlap with that discussed below for Alcohol Induced Chronic Pancreatitis, AICP. A summary of the effects implicated as having a role in acute pancreatitis includes: Alcohol has been shown to lead to increased fragility of zymogen granules (Haber, P.S. et al., 1994) which is associated with activation of digestive enzymes intracellularly. This can thus lead to cellular death, auto-digestion and instigation of inflammatory cascades. The induction of

enzymes involved in alcohol and other xenobiotic metabolism may lead to creation of reactive oxygen species (ROS) and the cellular dysfunction, thence inflammation +/- autodigestion and inflammatory cascades this may induce (Braganza, J. M., 2001a). Indeed acute ethanol has been shown to lead to oxidative changes (Altomare, E. et al., 1996). Similarly, direct toxic effects of alcohol, its metabolites and metabolites of other xenobiotics (reactive xenobiotic species, RXS) produced by these enzymes (Schenker, S. & Montalvo, R., 1998).

Thus a central feature and possibly the central pathogenic factor in the common forms of AP may be auto-digestion, this occurring in the acinar cells themselves, interstitium or ducts/acini. The autodigestion then leads to necrosis, initially of the interstitial then peripancreatic fat probably due to lipase (the one pancreatic enzyme not requiring activation) (Kloppel, G. & Maillet, B., 1993). The progression onto pancreatic parenchymal necrosis can then be ascribed to a spiralling of enzymatic activation and/or alternatively the induction of inflammatory cascades, possibly associated with microvascular changes.

However, auto-digestion as a central or initiating feature is not fully accepted (Braganza, J. M., 2001a). The argument here is dependent on oxidative stress as the initiating and central event, there is certainly evidence that oxidative stress is involved and as alluded to this is probably a central factor in alcohol induced pancreatic damage. Also the inflammatory pathways can lead to a build up of ROS further involving this pathway. Microcirculatory changes also occur in AP and may be involved in progression (Menger, M. D. et al., 2001).

We do know that various components of the inflammatory cascades appear to be activated in AP (Brady, M. et al., 1999; Osman, M. O. & Jensen, S. L., 1999), however, whether

they are primary or secondary factors is, again, unknown, as is the stage they become relevant. It is postulated that they could be the central factor in all AP and they certainly have a major part to play in the systemic effect of AP such as the systemic inflammatory response syndrome (SIRS) and multiple system organ dysfunction (MSOD) (Brady, M. et al., 1999). Although, prior evidence implicating proteases even in some systemic effects does exist (Ohlsson, K., 1990). Those cytokines known to be involved are predominantly those derived from activated macrophages, e.g. TNFα, IL-1β and also IL-6, and also various chemokines (Viedma, J. A. et al., 1992; McKay, C. J. et al., 1996; Norman, J., 1998) and are discussed at greater depth in section 1.5.1.

It is argued that an inflammatory pathway fundamental in possibly both local necrosis (fat and pancreatic) and also the development of systemic complications (often in advance of pancreatic necrosis) is activation of mast cells and releases of its mediators; histamine, PGD_2 and LTE_4 (Braganza, J. M., 2000). The argument is based on a number of points. They are consistent with pathological findings (which also support auto-digestion as a primary event). Wherein local fat necrosis is first seen, this is the site of a number of mast cells and these are seen to degranulate very early in the course of experimental AP, even prior to findings suggestive of activation of pancreatic enzymes.

Indeed, as early as 1969 the mast cell, with its combination of secretory mediators was proposed as a cell pivotal in forming a common pathway between 'biliary', lipolytic and proteolytic forms of experimental acute pancreatitis (Wanke, M., 1969). With mast cells plentiful in the normal pancreas (Goldowski, M. et al., 1959) and increased in some experimental forms of AP (Frick, G., 1969). Whilst, very recently, mast cell stabiliser pre-treatment has been shown to decrease local and systemic exudation and systemic neutrophil activity in a sodium taurodeoxycholate model in the rat (Dib, M. et al., 2002).

Evidence for mast cell derived mediators, especially the arachidonic acid metabolites is discussed in that section.

1.3.6 **Predisposition to Severe Disease:**

Thus it is seen that a number of postulated but no fully proven mechanisms of both the initiation and progression of AP exist.

The severity of an attck may be down to the severity of the insult, however, other factors implicated are genetic which could pertain to all the proposed (Braganza, J. M., 2001b). It is probably the fact that multiple factors are involved and a number of these may be genetic.

An improved understanding of factors involved in acute pancreatic inflammation and genetic components to severity stand to aid our overall understanding and therefore ability to treat/minimise the problems associated with this condition. Especially because therapies for these multi-factorial conditions probably requires a multi-therapy approach especially in systems where 'redundancy' exists, such as inflammatory cascades.

1.4 **Chronic Pancreatitis:**

Chronic Pancreatitis, as mentioned, has various aetiologies which may or may not cause disease through related pathological mechanisms. There is much overlap in clinical findings and natural history. At least in part, common mechanisms again appear to exist, especially in terms of some final common pathways and involvement of the inflammatory pathways.

It's incidence and prevalence are difficult to ascertain and vary dependent on the techniques by which the figures are obtained. In Western practice figures for incidence are around 2-10 new cases per 100,000 population per year and prevalence 15 per 100,000 population (Worning, H., 1998). The figures available (from an in depth study by Balaji et al, (1994)) for Southern India, where the Tropical sub-form of CP is common, gave a prevalence of 126-168 cases per 100 000 population.

The most recent consensus classification of pancreatitis from Marseille/Rome (Sarles, H. et al., 1989) divided CP thus:

- Chronic calcifying pancreatitis, which includes most of what is generally considered CP; that is Alcohol Induced CP (AICP), Idiopathic CP (ICP), Tropical CP (TCP) and Hereditary Pancreatitis (HP). Which generally present with recurrent bouts of, and/or continuous, pain and progresses to exocrine and/or endocrine insufficiency, see below.
- Chronic obstructive pancreatitis, where the changes occur proximal to an obstruction such as tumour or stricture.
- Chronic inflammatory pancreatitis, a separate entity where parenchyma is replaced by fibrosis infiltrated by mononuclear cells.

The clinical features of CP are fairly consistent throughout the subdivisions of chronic calcifying pancreatitis with variation in degree, rather than form.

Pain is typically the first feature, especially in Western forms, although it may only be elicited in the history when a 'later' feature presents. Pain may be continuous or intermittent; which may be in acute exacerbation's with or without a background of pain (Warshaw, A. L. et al., 1998). Indeed a bout of AP, or an acute exacerbation of CP, is commonly the initial presentation. The pain is classically upper abdominal with radiation to the back and may be found to diminish as the disease progresses, probably due to the decreasing amount of functional pancreatic tissue (Ammann, R. W. et al., 1984). Nausea +/- vomiting are also often features, especially associated with bouts of pain, as is anorexia.

As discussed the common forms of CP are classified as forms of chronic calcifying pancreatitis. As such pancreatic calcification is a finding in these forms of CP, however, it is not found invariably but is regarded as an inevitable consequence if the disease is of long enough duration. There is some debate as to whether non-calcifying forms of these diseases constitute different disease entities (Sarles, H. et al., 1992).

Pancreatic Carcinoma may complicate, and is probably predisposed to by, CP (Ekbom, A. et al., 1994). In TCP, studies have shown rates of 8.3% at 8 year follow up (Augustine, P. & Ramesh, H., 1992) and 3.25% at 4.5 years (Chari, S. T. et al., 1994), both studies showing pancreatic carcinoma to occur at a younger age than 'sporadic' cases with average ages found to be in the mid-forties. Hereditary Pancreatitis, HP, also has a higher than expected frequency of pancreatic cancer, an international study showing eight cases in 246 patients with HP, average age at diagnosis was 57 years and an estimated cumulative risk at 70 years of 40% (Lowenfels, A. B. et al., 1997). In CP (AICP and ICP, primarily) another international study showed 56 cancers in 2015 patients over an average 7.4 year follow up (Lowenfels, A. B. et al., 1993).

Other major clinical features are linked to the effect of CP on the function of the pancreas:

Loss of exocrine function: Malabsorption, evidenced by steatorrhoea and/or weight loss, is due to loss of functional exocrine pancreatic tissue, although as much as 90% functional loss can be sub-clinical (DiMagno, E. P. et al., 1973). Typically exocrine function is lost in advance of endocrine function. A study performed during these MD studies but not included in this thesis suggests that a commercially available measure of Pancreatic Elastase in the faeces may be an indicator of decreasing pancreatic function prior to it becoming clinically evident (Cartmell, M.T. et al., 2003). In agreement with a study in cystic fibrosis over 5 years (Walkowiak, J. & Nousia-Arvanitakis, S., 2001).

*Loss of Endocrine Function:*Diabetes mellitus is the result of endocrine insufficiency and generally occurs late in CP (Ammann, R. W. et al., 1987; Nauck, M. A., 1998).

The classification of 'calcifying' CP is not simple as its overlap with AP or, more specifically, recurrent bouts of AP is difficult to define. From the Cambridge Conference onward CP was considered as a continuing disease characterised by irreversible morphological change (Sarner and Cotton, 1984). However, tissue is rarely available for histological diagnosis. Thus surrogate markers must be employed, such as functional and structural tests. The latest concensus document is the Zurich International Workshop (Ammann, R.W., 1997). It defines Alcohol Induced Chronic Pancreatitis as a typical history and 'excessive alcohol intake' (≥80g/day for males, ≥60g/day for females). For definite CP one of the following is also required:

Pancreatic calcification.

Moderate to marked duct lesions (Cambridge Criteria (Axon, A.T.R. et al., 1984)).

Marked exocrine insufficiency (steatorrhoea markedly reduced by enzyme supplementation).

Typical Histology.

For probable CP one of:

Mild ductal changes.

Recurrent or persistent pseudocysts.

Pathological secretin test.

Endocrine insufficiency (e.g. abnormal glucose tolerance test).

For clinical classification late stage CP was defined as probable or definite CP (as above) and end-stage CP as exocrine insufficient. Though others may have typical history and recurrent problems their diagnosis is reserved until more objective evidence, as above, is found.

1.4.1 **Management**

Much management of CP is based around symptom control as it is characterised by irreversible changes. Thus pain management and treatment of exocrine and endocrine insufficiency are the major issues.

However, removal of aetiological factors may help, especially in obstructive CP. This is an area of debate for AICP as the disease may progress with abstinence whilst pain will eventually abate with disease progress ± continued alcohol consumption. Some evidence suggest abstinence improves progression of pain (Hayakawa, T. et al., 1989) whilst other evidence is against (Lankisch, P.G. et al., 1993). However, it would seem sensible to advise abstinence on the basis that it may decrease pain and/or progression (Gullo, L. et al., 1988) and that abstinence generally improves survival (Bullock, K.D. et al., 1992).

The evidence on treatments of pain in CP is variable, complicated by the varied disease progression. The subject is well reviewed by the American Gastroenterological Association (AGA) (Warshaw, A.L. et al., 1998) and an approach suggested (A.G.A., 1998) as laid out in figure 1.5.

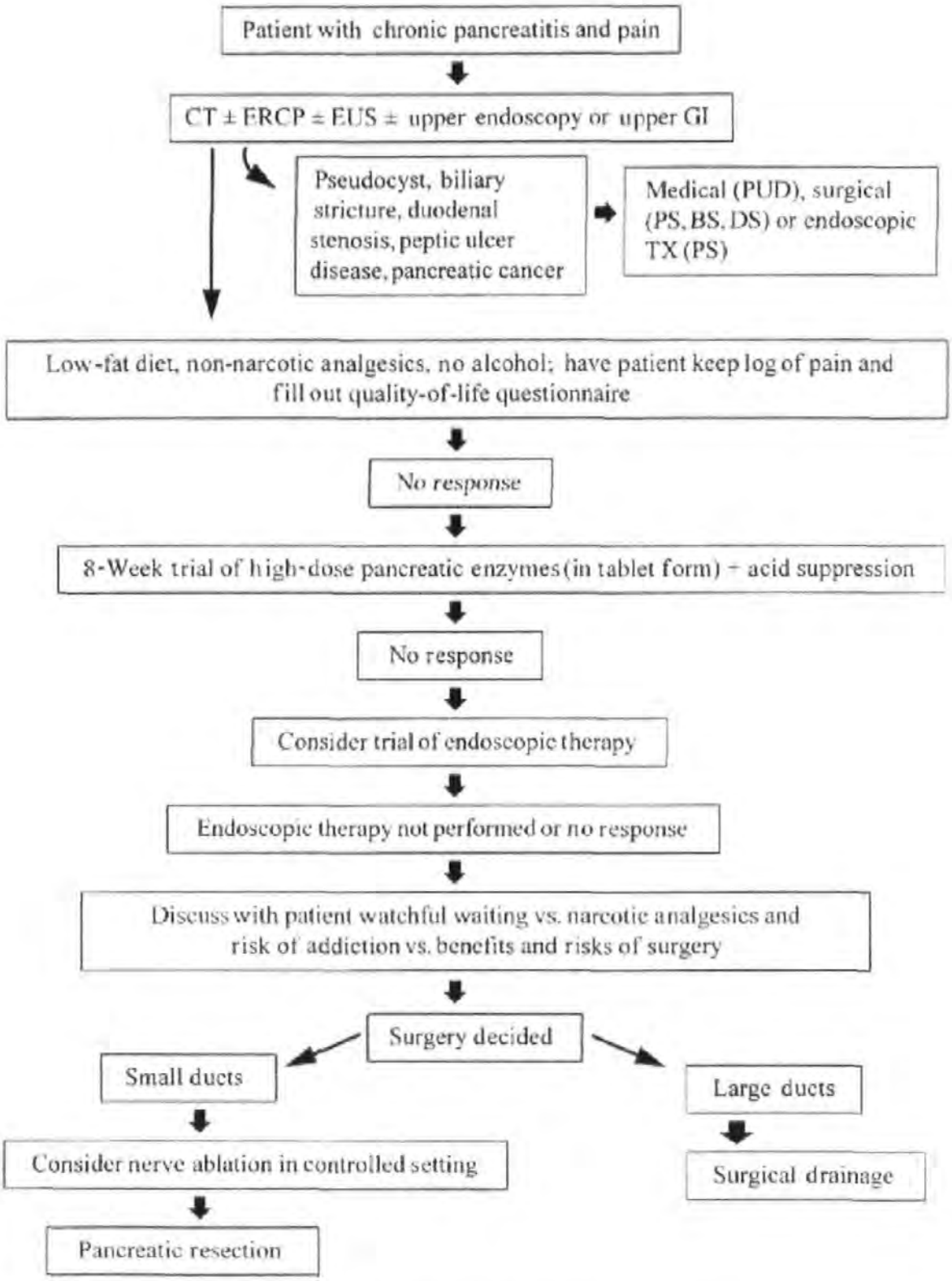


Figure 1.5 Algorithm for the management of chronic pancreatitis.
From the 'American Gastroenterological Associations medical position statement: Treatment of pain in chronic pancreatitis.' (1998)
Courtesy of Elsevier Health Sciences.

Management of exocrine insufficiency is with pancreatic enzyme supplements, normally porcine in origin, this is separate from the supplementation used as a strategy for pain relief. Endocrine insufficiency is managed as with other forms of diabetes mellitus.

1.4.2 **Aetiological sub-divisions:**

In western practice Alcohol Induced Chronic Pancreatitis (AICP) is the commonest form accounting for some 60 – 90% in the majority of studies (Worning, H., 1998). Its definitive diagnosis is as described above. The level of intake necessary to cause CP is not at a distinct level, indeed studies have shown a linear relationship between intake and the logarithm of the relative risk, without a lower 'safe' cut off (Durbec, J. P. & Sarles, H., 1978). Risk for a given alcohol intake undoubtedly varies between individuals, as evidenced by only a small proportion of even heavy consumers of alcohol suffering from this disease (Singh, M. & Simsek, H., 1990). Studies of asymptomatic alcoholics have shown changes consistent with CP, on differing investigations, in 0 – 30% of patients (Worning, H., 1998). AICP is more common in males than females (as with alcohol consumption and this ratio is decreasing in most studies, probably as women consume more alcohol) and appears to be increasing in frequency alongside increased alcohol intake (Pitchumoni, C. S., 1998); and mortality was also seen to decrease with decreased alcohol sales in Sweden (Romelsjo, A. & Agren, G., 1985). The explanation for the lack of consistency of development of AICP and other alcohol induced end organ diseases is probably multi-factorial, see below.

Idiopathic Chronic Pancreatitis (ICP), the second most common form in western practice has, as its name suggests, no known aetiology. Given the evidence regarding alcohol exposure above, making a definite of diagnosis in a social drinker can be difficult. Unlike AICP the sex distribution is approximately equal in the majority of studies (Chari, S. T. & Singer, M. V., 1998). Some commentators regard ICP has having two sub-divisions based

on age of onset; early onset/juvenile and late onset ICP (Layer, P. et al., 1994). A bimodal distribution of age of onset appears to exist, giving a peak in adolescence to early adulthood and another around the ages of 55-65 (Ammann, R. W., 1992). Why some people suffer ICP is again unknown and probably multi-factorial in nature. Some potential aetiological factors have been proposed whilst recent evidence has shown a probable role for candidate genes investigated in association studies, most notably CFTR (Cohn, J. A. et al., 1998; Sharer, N. et al., 1998) and SPINK 1 (Witt, H. et al., 2000; Truninger, K. et al., 2002).

Hereditary Pancreatitis (HP) is an inherited condition of autosomal dominant inheritance with a penetrance, from epidemiology studies , of around 80% (Sibert, J. R., 1978). Recently a number of mutations of the Cationic Trypsinogen (CaT) gene, accounting for over half of cases, have been described (Chen, J. M. et al., 1999; Ferec, C. et al., 1999; Whitcomb, D. C. et al., 1996a). HP has an early age of onset (normally childhood) however this does vary on the mutation responsible (Gorry, M. C. et al., 1997).

Tropical Chronic Pancreatitis (TCP) is the most common form of CP in some regions of the tropics, including some African and Asian countries, most notably Southern India. It was first described as a tropical form of pancreatitis in Indonesia by Zuidema in1955 (Zuidema, P. J., 1955). However, as early as 1937, Kini in India described operating on a man with pancreatic calculi complicating CP (Kini, M. G., 1937).

TCP was many years ago described clinically thus, 'recurrent abdominal pain in childhood, diabetes around the age of puberty and death at the prime of life'(Geevarghese, P. J., 1968). The sex ratio is around 1:1 (as with HP and ICP), hospital studies showing a slight male preponderance and field studies a similar female preponderance (Ramesh, H., 1997). Age of onset is normally childhood to early adulthood (along with HP and juvenille ICP)

but the age at presentation does appear to be increasing (Ramesh, H., 1997). Pain is less of a feature than in AICP and this is more closely mirrored in ICP, in western practice (Pitchumoni, C. S., 1998). Further clinical features compared with other forms include a high frequency of calcification at around 90%. Also the nature of the calcification differs, with larger intraductal stones (Chari, S. T. et al., 1992; Mohan, V. & Pitchumoni, C. S., 1998). Steatorrhoea is seen less frequently and is probably because of the low fat diet consumed in the areas where TCP is prevalent. Diabetes mellitus is common in TCP (it is even said to be inevitable (Mohan, V. & Pitchumoni, C. S., 1998)) and in its form known as FCPD (fibrocalculus pancreatic diabetes) accounts for around 12-16% of diabetes in Kerala, South India (Thomas, P. G. et al., 1990).

1.4.3 **Pathology and Cellular Involvement in CP:**

The pathology of CP shows much consistency across types but also some variation. Generally the changes, in the forms we are discussing, are initially patchy with adjacent lobules affected to differing degrees (Kloppel, G. & Maillet, B., 1998). Typified by fibrotic changes, with infiltration of leukocytes (Kloppel, G. & Maillet, B., 1998). There is irreversible degeneration of acinar cells which are replaced by fibrous tissue. The pancreatic duct can be variously narrow, of normal calibre or dilated. Dilatation with areas of stenosis associated with scarring is the most common finding, but is not necessary for 'upstream' pathology to exist. Ductal lithiasis is a nearly universal finding with so-called protein plugs in the smaller ductules (found less commonly in TCP (Sarles, H. et al., 1994)). Calcification of the stones is not invariable and some commentators describe two forms of CP (Sarles, H. et al., 1992).

Macrophage and T-cell infiltrates predominate and at increased levels in CP (Emmrich, J. et al., 1998; Gansauge, F. et al., 2001; Ockenga, J. et al., 2000). Lymphocytes are found at the margins of parenchyma and fibrosis and increase in greater numbers than do

macrophages (Emmrich, J. et al., 1998), with a lesser proportion of memory cells than normal tissue or tissue adjacent to tumour.

Systemic increases in T-cells (both $CD4^+$ and $CD8^+$ subtypes) have been found in CP, apparently returning to normal after resection of an inflammatory mass in the pancreatic head (Gansauge, F. et al., 2001). In a study of peripheral leukocytes in CP although no increase in total number of leukocytes or lymphocytes was found an increased ratio of $CD4^+$:$CD8^+$ cells were found (Ockenga, J. et al., 2000). This is in contrast to, but not incompatible with the above study of pancreatic tissue (Emmrich, J. et al., 1998), where $CD8^+$ cells just predominated. However, further studies of pancreatic tissue in CP did find a predominance of $CD4^+$ over $CD8^+$ cells in the pancreas in CP (Hunger, R. E. et al., 1997; Ebert, M. P. et al., 1998).

According to the necrosis-fibrosis theory of chronic pancreatitis circulating leukocytes responding to cytokine chemotaxis infiltrate the pancreas and become resident cytotoxic T-cells (this is also compatible with other theories of pathogenesis). Recurrent acinar cell injury is caused by cytokine release from activated cytotoxic T-cells and macrophages, which also release TGF beta. TGF beta (a group of five cytokines) increases infiltration, differentiation and proliferation of pancreatic stellate cells. The perpetuation of the antigenic stimulus and cytokine production results in destruction of pancreatic parenchyma and fibrosis.

Mast cells have again been implicated in CP, in addition to AP, in that they have been found in increased numbers in CP (Okolo CN et al., 2000). Another study confirmed this (Esposito, I. et al., 2001), also showing that numbers correlated with fibrosis and inflammation; in addition, this study showed that IgE-activation of mast cells increased in CP.

Eosinophils, stimulated by mast cells mediators and producers of cysteinyl leukotrienes, have been found in the perineural inflammation (Keith, R. G. et al., 1985). The interaction between mast cells and eosinophils is seen in that mast cell mediators have been shown to activate eosinophils (Raible, D. G. et al., 1992). Whilst the cysLTs (produced by both) are chemotactic for mast cells.

1.4.4 **Aetio-pathogenesis:**

The underlying 'primary' pathology in CP is a matter of much debate. Many in-depth reviews, often reaching different conclusions, exist. The debate is often said to come down to either the 'protein plug' hypothesis which proposes that protein plugs obstruct the duct and damage the ductal epithelium leading to atrophy and fibrosis (Scheele, G. A. et al., 1996) while the 'necrosis fibrosis' hypothesis suggests recurrent bouts of acute inflammation cause necrosis thence fibrosis (Kloppel, G. & Maillet, B., 1992). They can, in part, be brought together in the 'two hit hypothesis', however, the debate as to which 'hit' comes first persists (Ammann, R. W. et al., 1999). This debate can be said in part to be a matter of semantics (Longnecker, D. S., 1996). Both mechanisms can be associated with increased oxidative stress but this most directly can be associated with the hypothesis of acinar cells as the site of primary dysfunction (Braganza, J. M., 1998). Indeed reviews concluding a number of features as primary exist: Oxidative stress (Braganza, J. M., 1998); recurrent AP, probably autodigestive, leading to necrosis then fibrosis (Kloppel, G. & Maillet, B., 1991); protein plug, then stone obstruction of ducts leading to fibrosis (Sarles, H. et al., 1989).

It should be borne in mind that the various sites and mechanisms are not mutually exclusive (Longnecker, D. S., 1996). It could be considered that the pathogenesis is a variable 'hit' disease dependent on the significance of each insult; the sequence, if not unimportant, certainly unknowable for most aetiologies at present.

If abnormal activation of the digestive enzymes is seen as a fundamental 'fault', this would be closest to the necrosis-fibrosis view but with minor duct/acinar events as possible primary events in some forms. This is favoured because HP in which we know the defect (at least in some cases) (Whitcomb, D. C. et al., 1996b) is pathologically indistinguishable from other forms, in any given case. HP is due to abnormal activation, or more strictly speaking abnormal inactivation of the enzyme Cationic Trypsinogen, here only one 'knock-out' hit is required. Further associations implicate abnormal enzyme activation as having a role (Witt, H. et al., 1999). It should be noted that the necrosis-fibrosis theory was delineated based primarily on pathological observations (Kloppel, G. & Maillet, B., 1991) long before the defect in HP was understood.

Acinar cell damage secondary to a number of insults can lead to abnormal cellular transport and enzyme activation, alternatively direct damage could lead to inflammation and/or fibrosis without autodigestion. Considering the acinar cell as a site of primary insult (Freedman, S. D., 1998) can thus in part bring together the necrosis fibrosis and oxidative stress theories; whilst denying necrosis fibrosis (certainly due to autodigestion) as the sole primary event.

Increased oxidative stress leads to tissue damage by a number of mechanisms, most notably lipid peroxidation (Slater, T. F., 1984). Evidence for which exists in CP (Schoenberg, M. H. & Birk, D., 1998). These compounded by diminished micronutrients (Rose, P. et al., 1986) which function as antioxidants and sustain GSH (the active form of glutathione) stores for the cytochrome P450 pathway of detoxification.

Ductal obstruction (consistent with the 'protein plug' hypothesis) certainly is implicated in the association of CP with mutations of CFTR (Sharer, N. et al., 1998; Cohn, J. A. et al., 1998) and pancreatic fibrosis and insufficiency in Cystic Fibrosis (CF). Here a defective

CFTR decreases bicarbonate and fluid secretion increasing concentration/decreasing solubility of protein . This could then promote protein plug and thence stone formation. Stones/strictures which can also obstruct larger ducts (indisputably the cause of obstructive CP (Gyr, K. et al., 1984)) are unable to explain the patchy pattern found in common forms of CP. Also, obstruction has been shown to lead to co-localisation of acinar zymogens and lysosomes (Saluja, A. et al., 1989, Hirano, T. et al., 1991) which is a cause of premature enzyme activation; again revealing the lack of a true distinction between theories in such a complex multi-factorial disease.

In CP of other aetiologies, and indeed HP due to mutations which have a lower penetrance, a variable number of hits at various site(s) is required. Whereas in high penetrance mutations only the one 'knock-out punch' is required.

Looking specifically at AICP the mechanism is again unknown. However, evidence exists for mechanisms implicating all of the pathways discussed, again implicating a multi-factorial aetio-pathogenesis. It may be through direct toxic, or indirect, effects. In addition, induction of enzymes by ethanol may lead to injurous bioactivation of oxygen free radicals, limited evidence exists for a number of the mechanisms (Braganza, J. M., 1996; Schenker, S. & Montalvo, R., 1998). Further, this effect may be due to alcohol or its metabolites, in all cases functional or productive variations in metabolising enzymes may be relevant.

In Wistar rats ethanol administration has been shown to increase pancreatic lysosomal and zymogen fragility (Apte, M. V. et al., 1994). Chronic ethanol administration has also been shown to increase the protein and trypsinogen concentration of pancreatic juice in rats and, with longer duration, also to effect acinar cell ultrastructure (Gronroos, J. M. et al., 1988). These factors are of particular relevance when taken along side the ability of lysosomal

enzymes to activate the digestive enzymes contained in the zymogen granules and thus premature activation of pancreatic enzymes, leading to pancreatic autodigestion.

Similarly, in TCP evidence for a contribution of a number of factors exists. From dietary toxins, oxidative stress (as evidenced by increased lipid peroxidation (Ganesh Pai, C. et al., 1999)), possibly due to lack of anti-oxidant micronutrients (Braganza, J. M. et al., 1993). To recurrent gastro-enteritis leading to bouts of dehydration with the possible associated decrease in pancreatic secretary volume (which as discussed above is normally around 2.5 litres). Thus both digestive enzyme concentration and potential for protein precipitation would increase. This coincides with the theory of pathogenesis (seemingly ignored in the majority of both western and 'tropical' reviews of the pathogenesis of CP and TCP) proposed in 1980 by Nwokolo and Oli in Nigeria (1980).

Inflammatory mediators and cells are no doubt involved in the pathogenesis but evidence as to whether they are primary or secondary factors is limited and an area of debate (Friess, H. et al., 1998).

To account for these factors not leading to CP more widely genetic components (or hits) in one or more of the pathways could be present

1.4.5 **Evidence for a genetic component in Chronic Pancreatitis:**

One form of pancreatitis has a definite genetic basis and that is Hereditary Pancreatitis. As discussed above, before the genetic basis of HP was understood epidemiological studies showed it was due to dominant inheritance with a penetration of 80% (Sibert, 1978). Since that time a number of mutations in the gene for cationic trypsinogen have been found in families with HP. The relevant region of chromosome 7 was originally implicated in quick succession by Le Bodic, L. et al. (1996), Whitcomb, D.C. et al. (1996b) and Pandya, A., et

al. (1996). Then the first mutation discovered later that year (Whitcomb, D.C. et al., 1996a). Mutations in this gene have been shown to be strongly associated with HP and are found in more than 50% of HP families (Ferec, C. et al., 1999). The various mutations have slightly differing phenotypic forms.

In other forms the role genetic predisposition plays is less distinct, however, various factors in the diseases epidemiology implicate genetic factors, and indeed some genetic associations have been found:

In AlCP only a proportion of alcoholics get CP (around 5% (Dreiling, D.A., 1985)) (Singh, M. & Simsek, H., 1990; Haber, P. et al., 1995). A larger subgroup develop alcoholic liver disease, there is some overlap between these groups (Singh, M. & Simsek, H., 1990). Studies of asymptomatic alcoholics have shown changes consistent with CP, on differing investigations, in only 0 – 30% of patients (Worning, H., 1998). A number also develop asymptomatic pancreatic fibrosis (Meier, P.B., 1995), however, even then, at post-mortem studies at least 32% of pancreata of alcoholics are found to have no or minimal fibrosis (Pitchumoni, C.S. et al., 1984). In addition, as discussed, the level of alcohol intake necessary to cause CP is not at a distinct level, without a lower 'safe' cut off (Durbec, J.P. and Sarles, H., 1978).

Risk for a given alcohol intake undoubtedly varies between individuals.

Diet has been implicated (Durbec, J.P. & Sarles, H., 1978) and smoking may have a role (Bourliere, M. et al., 1991; Haber, P. et al., 1995). However, perhaps the best controlled study on smoking failed to confirm this (Haber, P.S. et al., 1993a) and it remains a matter of debate (Haber, P. et al., 1995).

A genetic predisposition could explain these findings and indeed an increased frequency of CFTR (cystic fibrosis transmembrane conductance regulator) mutations have been found in AICP, although less commonly than in ICP (Cohn, J.A. et al., 1998; Sharer, N. et al., 1998), see below. The same is true for SPINK 1 mutations (Witt, H. et al., 2001). Alcohol dehydrogenase polymorphisms have been analysed in two small studies, larger appropriately controlled studies are required (Haber, P. et al., 1995). A number of commentators (Adler, G. & Schmid, R.M., 1997; Singh, M. & Simsek, H., 1990) have proposed a genetic component or at least believed it worthy of analysis (Meier, P.B., 1995).

With no identified aetiological factor, but no obvious family associations in most cases ICP may have a genetic component in parallel with unknown factors. CFTR mutations have been found in up to 11 times the expected number of ICP patients (Cohn, J.A. et al., 1998; Sharer, N. et al., 1998). There is also an association with SPINK 1 mutations (Witt, H. et al., 2000; Truninger, K. et al., 2002).

TCP has long been known to have a family tendency. Indeed, one of Geevarghese and Pitchumoni's early essays (Pitchumoni, C.S. & Geevarghese, P.J., 1966), and an early study (Pitchumoni, 1970), were entitled Familial Pancreatitis. More recent studies (Mohan, V. et al., 1989; Thomas, P.G. et al., 1990) in southern India have again shown familial aggregation. Of the proposed aetiological factors in TCP all occur outside the regions where TCP is common and can be found where TCP does not occur (Mohan, V. & Pitchumoni, C.S., 1998). In addition, many of the proposed factors are associated with poverty and Kerala, where TCP is most common, has some of the best socio-economic markers in India. However, some of these factors are likely to have a role in its pathogenesis but it is believed that a genetic factor would be a reasonable, or even probable, explanation for the geographic variation, especially in relatively static

populations (Pitchumoni, C.S. & Geevarghese, P.J., 1966; Ramesh, H., 1997; Mohan, V. & Pitchumoni, C.S., 1998).

Genetic mapping using genetic variation can be used to investigate traits with Mendelian inheritance (as with HP). However, to identify possible genetic associations in multifactorial disease is more complex; a number of techniques can be employed. A known candidate gene can be studied. If that gene has genetic variability (as most do) these can then be studied to see if they are associated with the disease. The variation, known as polymorphism (or mutation, especially if is has a functional effect) can be of various forms. Single nucleotide polymorhisms (SNP, also known as point mutations), variable number tandem repeats (VNTR, which are polymorphic microsatellites) and insertion/deletion polymorphisms.

A polymorphism can be studied in isolation and its association to a given disease studied by comparing the frequency of each allele (polymorphic variant) in patient groups compared to controls. Obviously it is important to have as few other differences between the patient groups and controls. Another weakness is that in multifactorial disease the 'association' may be relevant but not numerically strong because of other factors. Association studies are best performed when the polymorphism (mutation) has a functional role. This can be true of all forms of polymorphism: SNPs which can affect transcription, even when outside the coding region, (e.g. that discussed later in leukotriene C_4 synthase, where it creates a transcription factor binding site) or translation (e.g. by changing the amino acid for which a codon codes, as in the R122H mutation in HP). Insertion/deletion polymorphisms can equally affect transcription or translation (e.g. the CYP2E1 insertion studied here which is associated with increased activity; though the mechanism here is not yet understood).

Non-functional polymorphisms can be linked to function variation as above but they are also useful in themselves, though their use in simple association studies is very limited. Their variability and distribution through out the genome are useful for mapping with both microsatellites (VNTRs) and SNPs used thus, especially as many thousands are identified as part of the Human Genome Project.

1.5 **Cytokines:**

Cytokines are small peptides involved in cell-cell signalling in a predominantly paracrine but also autocrine and even endocrine manner. They are secreted by leukocytes and other cell types and act through specific receptors. They are integral to the inflammatory and post-inflammatory cascades.

They are a disparate group and individual cytokines are often pleiotropic (having multiple actions). They signal a number of 'messages' ranging from chemotaxis (the 'chemokines') (Rossi, D. & Zlotnik, A., 2000), to cellular activation/proliferation (Dinarello, C. A., 1998) to fibrosis (Apte, M. V. et al., 1999). Not all are proinflammatory (e.g. interleukin(IL)-10), and a balance between, for example, a secreted receptor antagonist and agonist (e.g. IL (interleukin)-1a, IL-1b and IL-1 receptor antagonist) further enables regulation (Dinarello, C. A., 1998). They are sometimes regarded as being grouped by the cellular subtypes which secrete them, such as T helper 1 versus T helper 2 cells, there is, however, a great deal of overlap in their actions, some which remain to be fully elucidated (Dinarello, C. A. & Mier, A. W., 1987; Haddad, J. J., 2002).

Tumour necrosis factor alpha (TNF-alpha) and IL-1 are the archetypal Th1 cytokines. The interleukin 1 family includes the proinflammatory cytokines (IL-1 alpha and IL-1beta). They are integrally involved in inflammation, primarily through stimulation of macrophages, neutrophils, lymphocytes and endothelial cells for production of cytokines (Dinarello, C. A., 1998).

1.5.1 **Evidence on Cytokines in Acute Pancreatitis:**

- TNF-alpha is elevated in the pancreas in experimental AP (Norman, J.G. et al., 1995). In humans it is elevated, especially in severe disease (Pooran, N. et al., 2003). High levels have been found in serum and lymph in AP patients with ARDS (Montravers, P. et al.,

1995), and in around two thirds of ICU patients with AP (Brivet, F.G. et al., 1999). TNF-alpha is not easily measured as it is rapidly metabolised in the liver and some studies have shown elevated levels only in small percentages of patients (Dugernier, T.L. et al., 2003). TNF-alpha has been found to be produced at higher levels, in vitro, by monocytes from patients with systemic complications (McKay, C.J. et al., 1996) implicating it in progression to systemic disease. Though one study of 16 patients did not show elevation from mononuclear cells (De Beaux, A.C. et al., 1996).

Soluble TNF receptors are found to be elevated when septic complications occur (Heresbach, D. et al., 1998) and more so in more severe disease (Kaufmann, P. et al., 1997). Where they may be predictive of severe disease (Hirota, M. et al., 2000).

•Interleukin-1 has been shown to be of importance in a number of animal studies, with elevated mRNA levels(also for IL-1ra) and IL-1 itself in the pancreas and serum (Fink, G.W. & Norman, J.G., 1997; Mozo et al., 2002). While the IL-1 receptor is not required for pancreatitis per se but is required for its 'full progression' (Norman, J.G. et al., 1996).

However, evidence from humans is not so clear. Though high levels have been found in lymph and serum of AP patients with ARDS (Montravers, P. et al., 1995), in the study by McKay, C.J. et al. (1996) IL-1beta did not appear elevated in complicated disease (whilst TNF-alpha, IL-6 and IL-8 did). Another study did not find IL-1beta in a number of the patients with post ERCP pancreatitis, but did show elevated IL-1ra in comparison to patients who did not develop pancreatitis post procedure (Messmann, H. et al., 1998). Also IL-1 levels and ratio to IL-1ra were lower where septic complications occurred (Heresbach, D. et al., 1998).

Thus TNF-alpha has an important role as IL-1 probably does, though evidence in humans for the later is inconclusive. But neither IL-1beta nor TNF-alpha would appear to be causal, as perfusion of the human pancreas with either does not induce pancreatitis (Denham, W. et al., 1998).

•Interleukin-6 is a cytokine produced early in the pathway of acute phase protein production and since being shown to be elevated early in the course of human AP (Viedma, J.A. et al., 1992) has been studied of interest not just from the point of view of aetiopathogenesis but as an early marker and predictor of disease (Heath et al., 1993;Leser et al., 1991).

•Interleukin-10 is an anti-inflammatory cytokine, has been shown to be elevated in AP in humans (Brivet, F.G. et al., 1999) and provides a possible therapeutic avenue. Pre-treatment with IL-10 has been shown to decrease post-ERCP pancreatitis (Deviere, J. et al., 2001), but this not universally (Dumot, J.A. et al., 2001).

•The chemokines are cytokines involved in chemotaxis. Interleukin 8 is the 'prototypical' CXC chemokine (these are chemotactic for neutrophils), whilst monocyte chemotactic protein 1 (MCP-1) is the prototypical CC chemokine (which predominantly affect monocytes). Both are produced in response to IL-1beta and TNF-alpha (Andoh, A. et al., 2000).

IL-8 has been seen to be higher in complicated than uncomplicated AP (Gross, V. et al., 1992) and in severe AP than controls and mild AP (Pooran, N. et al., 2003) and to rise over the first days of an attack, more rapidly so in severe disease (Berney, T. et al., 1999) and decrease with clinical improvement (Gross, V. et al., 1992). Higher levels in severe AP have also been found for other CXC chemokines (Shokuhi, S. et al., 2002). This would be

in agreement with the findings of neutrophil involvement in the pancreas in AP (Folch, E. et al., 1998) and systemic complications (Mora, A. et al., 1997). MCP-1 (along with other, but not all, CC chemokines) increases in AP patients who develop complications (Rau, B. et al., 2003).

•Worthy of discussion, though data is limited (especially in humans), is the nuclear factor kappaB (NF-κB) which is a transcription factor for many cytokines. NF-κB increases with morphological damage and cytokine mRNA in experimental AP (Vaquero, E. et al., 2001). Transfection of pancreas with a viral vector containing NF-κB cause pancreatic and lung infiltration by neutrophils (Chen, X. et al., 2002). Indeed NF-κB seems to have a central role being involved in the production of chemokines in response to TNFalpha and IL-1beta and of cytokines in response to neutrophils (Kim, H. et al., 2000), also being early in the cascade it offers a potential therapeutic avenue. However, its role is not simple as it has a number of actions (Grisham, M.B., 1999) and further details await elucidation.

Thus the cytokine response in AP is both anti- and pro-inflammatory and is certainly involved with the progression, if not initiation, of AP, with imbalance of response having a role in outcome. Especially involved is 'Th1 type' response and neutrophils in the systemic complications.

1.5.2 **Evidence on Cytokines in Chronic Pancreatitis:**

Cytokines involved in the T-cell and macrophage responses, such as TNF-alpha, interferon-gamma and IL-1alpha and beta and IL-6 warrant consideration in CP as T-cells and macrophages are major cell types involved, as discussed above.

In a study by Bamba et al (1994) serum levels of both IL-1beta and IL-6 were found to be elevated in patients with CP. Il-1b was confirmed as elevated in serum in CP (Szuster-

Ciesielska, A. et al., 2000). Also TNF-alpha was found at increased serum levels in the study by Szuster-Ciesielska et al (2000). Interestingly, this study did not show increased levels of either IL-10 or TGF-beta (see below). In addition, mRNA (in peripheral blood mononuclear cells) of TNF-alpha and both soluble TNF-Receptors (p55 and p75) are significantly elevated in CP compared to healthy controls (Hanck, C. et al., 1999a). From these observations it is clear that the T-cell response in the CP includes a Th-1 type with secretion of TNF and related mediators. Pancreatic cell lines have been shown to produce IL-6 and the chemokine IL-8 in response to TNF-alpha and to endotoxin (Blanchard, J. A. et al., 2000), implicating the Th-1 pathway further. That said, evidence on Th-2 mediators is lacking.

Chemokines IL-8 and MCP-1 are also secreted by human periacinar myofibroblasts in response to IL-1beta and TNF-alpha and RANTES to TNF-alpha, although a further chemokine (MIP-1alpha) was not elevated in this study (Andoh, A. et al., 2000). Chemokines are also found in the pancreata of patients with CP, which are not found in normal pancreata, including MCP-1 (Saurer, L. et al., 2000).

A study, arguably, showing conflicting results with the above findings, but looking at the pancreatic juice of patients with CP did not detect IL-6, IL-10 or TNF alpha, although TGF-beta was found in a greater number of patients than controls (Kazbay, K. et al., 2001). TGF-beta has, on account of its role in the induction of fibrosis, been one of the most studied cytokines in chronic pancreatitis, although as stated above serum levels were not increased in one study (Szuster-Ciesielska, A. et al., 2000).

TGF-beta1 mRNA expression is increased in AICP, with transcripts found in several cell types including pancreatic stellate cells, acinar, and ductal cells (Casini, A. et al., 2000). Immunohistochemical studies showed TGF-beta maximal in ductal and acinar cells (Korc,

M. et al., 1994) and perifibrotic acinar cells and spindle cells (Haber, P. S. et al., 1999), with similar findings in human CP and a rat model. In a further study TGF-beta1 was found in CP throughout the ducts, rather than isolated acinar and distal ductular cells in normal controls (Slater, S. D. et al., 1995). Analogous findings were shown with enhanced mRNA expression of TGF-beta1, TGF beta Receptor-II, connective tissue growth factor (which is regulated by TGF-beta), and collagen type I in CP compared with normal controls (di Mola, F. F. et al., 1999).

Functional animal studies investigating TGF-beta also suggest a role in CP. A rat model indicated a peak of TGF-beta1 activity occurring before the peak of fibrosis (Su, S. B. et al., 2000). The pancreatic morphology of transgenic mice that overexpress TGF-beta1 in the pancreas partially resembles morphological features of chronic pancreatitis, (such as progressive accumulation of extracellular matrix). Also increased mRNA levels of TGF-beta1 occur early, coincidentally with collagen types I and III, and later with increasing numbers of pancreatic stellate cells (Vogelmann, R. et al., 2001). Another study of a transgenic mouse overexpressing TGF-beta1 in pancreatic beta cells revealed massive fibrosis of the pancreas; in adult mice, most of the acini were replaced by fibrotic and adipose tissues (Sanvito, F. et al., 1995). In a series of studies by van Laetham et al. TGF-beta was not only being secreted in ductal cells and mononuclear cells (Van Laethem, J. L. et al., 1995) in humans, but was shown to promote fibrosis following repeated bouts of experimental acute pancreatitis (Van Laethem, J. L. et al., 1996).

1.5.3 **Evidence for a genetic component to cytokine responses in CP:**

In any condition with an inflammatory or fibrotic basis, or even a component, this process will be subject to genetic variation. The variation in response may predispose the individual to a more proinflammatory/fibrotic response, or an imbalanced response to a given (relevant) stimulus. As such, the influence of genetic variability of either the

inflammatory response or fibrosis may affect predisposition to disease or progression of disease and therefore its clinical presentation. Cytokines are key mediators in both pathways.

With the study of cytokines in chronic pancreatitis in it's infancy, the evidence regarding a genetic component in the responses of different cytokines is also early in its development. The 'Th1 cytokines' involved with recruitment and activation of T cells and macrophages are implicated by the evidence above and are the first to have been studied with regard to genetic predisposition.

In a study, available in abstract form only, an association between CP and genetic variation in the interferon gamma gene (a microsatellite in intron 1) was found in a study of 54 patients with CP and 104 controls. Increased frequencies of the 12 (high production) and 14 alleles were associated with CP (O'Reilly, D. A. et al., 2000a) . This is in keeping with a Th-1 response in the pancreas.

Studies from two groups, again available only in abstract form, have looked at a possible role for genetic predisposition in relation to TNF-alpha. It must be born in mind that a difficulty, as with any genetic study, is with the surrounding area of the chromosome, and the TNF-alpha gene lies within the MHC (class III) region. This is proposed as the reason for an apparent functional association of TNF levels associated with the TNF-alpha –238 polymorphism in anti-phospho lipid syndrome (Bertolaccini, M. L. et al., 2001).

There are polymorphisms at the -238 and -308 positions in the promoter region of the TNF-alpha gene; the frequency of the variant allele at -308 is associated with increased transcription (in a reporter gene study) (Wilson, A.G. et al., 1997) and increased production of TNF-alpha (Louis, E. et al., 1998). In the first of the two studies the high

production variant allele was found at significantly higher frequency in patients with AICP; whilst for the -238 polymorphism, there was no difference in the frequency (Abdulrazeg, E-S. M. et al., 2000).

The second study also indicated a possible genetic role in TNF-alpha studied with regard to microsatellite haplotypes. However, in this study those haplotypes associated with intermediate function, notably the TNFa6b5c1d3e3 haplotype, were found significantly more frequently in CP than normal controls, with a suggestion of an association of the higher secreting haplotypes with alcoholic liver disease (O'Reilly, D. A. et al., 2000b).

1.5.3 **Genetics of IL-1a in Acute Pancreatitis:**

The Interleukin-1 gene cluster, located on the long arm of chromosome 2 (Nicklin, M. J. et al., 1994), consists of: IL-1a (encoding IL-1α). IL-1b (encoding IL-1β). IL1-RN which encodes the IL1-receptor antagonist. The IL-1 receptors IL-1RI and, the 'decoy' IL-1RII. IL-1α and IL-1β share a range of activities acting via both receptors; IL-1α generally remaining cytosolic, acting in an autocrine manner, although some becomes membrane bound and is active after cell death (Dinarello, C. A., 1998).

A number of polymorphisms exist within IL-1a. Specifically the $(AC)_n$ repeat VNTR polymorphism within intron 5 (Todd, S. & Naylor, S. L., 1991; Epplen, C. et al., 1994). In a previous study of this polymorphism, in acute pancreatitis, the 2,4 genotype was associated with alcoholic acute pancreatitis and the 1,2 genotype with worse organ failure score (Smithies, A. M., 2001). This polymorphism has also been looked at in Rheumatoid (Gomolka, M. et al., 1995) and Juvenille Chronic (Donn, R.P. et al., 1999) Arthritis with no positive associations detected. Also no association was found with Tuberculosis in a study by Bellamy et al. (1998). Smithies also looked at a VNTR polymorphism in IL1a (no association), a SNP in IL-1b finding no associations and a VNTR in IL1-rn showing weak

associations with AP and severity for the 1 allele and 1,1 genotype (Smithies et al., 2000). Significant differences were not confirmed in IL-1rn (or IL-1b) in a further study (Powell, J. J. et al., 2001).

1.6 **Alcohol Metabolism:**

As discussed above, alcohol is the primary aetiological factor for chronic pancreatitis in Western countries accounting for around 70% of cases (Worning, H., 1998). In addition, it is one of the two major causes of the acute form of the disease, along with gallstones. Whether these cases in fact represent acute exacerbations of either previously known or unknown chronic pancreatitis (CP) in all, or some, cases is a matter of debate (Singh, M. & Simsek, H., 1990).

The genetic predisposition to both alcoholism and alcohol induced end-organ damage is an area of debate. Family and twin studies suggest a genetic component to alcoholism (Reed, T. et al., 1996; National Institute of Alcohol Abuse and Alcoholism, 2000). As discussed alcohol-induced pancreatitis occurs in approximately 5% of alcoholics (Dreiling DA, 1985); alcoholic cirrhosis in around 10% and hepatitis in 10-35% (Grant, B. F. et al., 1988). No, or minimal, fibrosis is found in 32% of pancreata of alcoholics (Pitchumoni, C. S. et al., 1984). The heterogeneity of the response to alcohol implicates genetic factors. Some evidence suggests that the majority of genetic predisposition to psychosis and liver disease may be accounted for by disposition to alcoholism (Reed, T. et al., 1996).

One area of possible relevance is the role of mutations and polymorphisms of the alcohol metabolising enzymes. In humans, three main pathways are involved. Two of these are oxidative pathways (Figure 1.6) and one is non-oxidative, metabolising to fatty acid ethyl esters. The primary mechanism, at low alcohol levels, is metabolism of ethanol to ethylaldehyde by ADH (alcohol dehydrogenase) and the metabolism of ethylaldehyde to acetic acid by ALDH (aldehyde dehydrogense) (Parkinson, A., 1996). The other oxidative pathway via cytochrome P450 2E1 is a minor pathway at low alcohol levels, accounting for around 10% at low alcohol levels. CYP2E1 is however inducible by alcohol and may

therefore account for a higher percentage of function in alcoholic patients (Lieber, C. S., 1997a), Figure 1.6.

The relevance of the enzymes is obvious though not simple. As discussed above the mechanism of alcohol induced end organ damage is incompletely understood. It may be through directly toxic, or indirect, effects. In addition, induction of enzymes by ethanol may lead to injurous bioactivation of oxygen free radicals, limited evidence exists for a number of the mechanisms (Braganza, J. M., 1996; Schenker, S. & Montalvo, R., 1998). Further, this effect may be due to alcohol or its metabolites. In all cases functional or productive variations in metabolising enzymes may be relevant. Thus variability in all or any of the three pathways could be implicated in genetic predisposition.

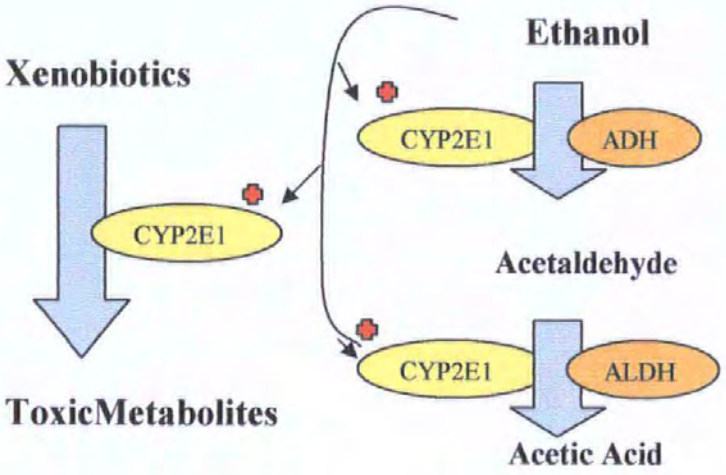


Figure 1.6. Oxidative Metabolism of Ethanol.
CYP2E1 = Cytochrome P450 2E1
ADH = Alcohol Dehydrogenase
ALDH = Aldehyde Dehydrogenase

1.6.1 **Alcohol and Aldehyde Dehydrogenase:**

ADH forms the primary phase 1 metabolising enzyme of ethanol at low levels. The genes coding for the enzyme are ADH1 to ADH5 (ADH6 and 7 are more recently identified) each encoding a different subunit (α, β, γ, π, χ). In addition, there are three alleilic variants of the β subunit and two of the γ subunit. The functional protein is a hetero- or homodimer of two subunits. The enzymes are divided into three major classes: Class I contains ADH1 ($\alpha\alpha$, $\alpha\beta$, $\alpha\gamma$), ADH2 ($\beta\beta$, $\beta\gamma$) and ADH3 ($\gamma\gamma$). Class II contains ADH4 ($\pi\pi$) and Class III ADH5 ($\chi\chi$). Class I is primarily involved with the oxidation of small, aliphatic alcohols, including ethanol. Class II and III have little role in metabolism of ethanol. Class I isoenzymes containing a β2 subunit (coded for by ADH2*2) are especially active in oxidising ethanol at physiological pH (termed atypical ADH), these are present in 85% of the Japanese and Chinese population, but are present at very low frequencies in Caucasians. ADH3*1 also has more rapid metabolism of ethanol to acetaldehyde (Bosron, W. F. et al., 1988) and is the predominant form in the Japanese and Chinese, whilst the two alleles are approximately equal in Caucasians. The risk of alcoholism appears to be diminished by possesion of a rapidly metabolising allele (Chao, Y. C. et al., 1994) probably due to higher concentrations of aldehydes and the concomitant unwanted effects.

In a small study looking at ADH3*1 when results for the higher activity allele were pooled for alcoholics with end organ disease (either liver disease (59 patients) or chronic pancreatitis (13 patients)) this allele was significantly more frequent in patients than controls (Day, C. P. et al., 1991); possibly indicating that the higher aldehyde levels are related to end organ damage. ADH3 polymorphisms were not different between controls and AICP patients (n= 15) in France (Couzigou, P. et al., 1991). Chao et al (1995) looked at alcohol induced AP, AAP (n= 48), alcoholic cirrhosis (n= 75), 19 'well' alcoholics and 235 healthy controls. They found ADH2*1 and ALDH2*1 increased in ALD compared

with normal controls; ALDH2*1 in alcohol induced AP compared to controls, while ADH2*1 was lower in alcohol induced AP compared to ALD; this was in Chinese patients who have a high rate of ADH2*2 and ALDH2*1, unlike Caucasians. In a Japanese population ADH2*2/2*2 genotype was increased in AICP v alcoholic controls (no significancant difference was found for, ALDH2, and CYP2E1) (Maruyama, K. et al., 1999).

Interestingly, although the class I ADH isoenzymes are said to be most closely associated with ethanol metabolism (Holmes, R. S., 1994) a recent study found significant levels of ADH ethanol metabolism in rat pancreas blocked by a class I-III inhibitor but not a class I-II inhibitor (nor interestingly a CYP450 inhibitor carbonmonoxide, 'though these rats had not been prefed with alcohol to induce p450 activity) (Haber, P. S. et al., 1998).

The ALDH enzymes are again divided into three classes: ALDH1 are cytosolic enzymes that oxidise a wide variety of xenobiotic aldehydes. ALDH2 are sited in the mitochondria and (with their low Km) are primarily responsible for oxidising simple aldehydes, including acetaldehyde. ALDH3 for which acetaldehyde is not a substrate.

A polymorphism (ALDH2*2) of the gene coding ALDH2 (in fact a homotetramer of the gene product) again common in Japanese and Chinese populations, causes a significantly decreased rate of metabolism of acetaldehyde and in combination with atypical ADH leads to a flushing syndrome secondary to a build up of acetaldehyde (Mizoi, Y. et al., 1983) (similar to the mechanism of action of disulfiram (Antabuse) an ALDH inhibitor). The ALDH2*2 allele has not been described in Caucasian populations. A novel polymorphism in the ALDH2 gene occurring in Caucasians has been described recently (Chou, W-Y. et al., 1999; Harada, S. et al., 1999). Aldehydes can also be oxidised by aldehyde oxidase and xanthine oxidase.

1.6.2 **Fatty Acid Ethyl Esters:**

Evidence is also now emerging that Fatty Acid Ethyl Esters (FAEEs) may be implicated in end organ damage, including in the pancreas. FAEEs are found at increased levels in ethanol fed rats (Hamamoto, T. et al., 1990), FAEEs can lead to uncoupling of mitochondrial phosphorylation (Lange, L. G. & Sobel, B. E., 1983) and increased lysosomal fragility in acinar cells (Haber, P. S. et al., 1993b). FAEEs have also been shown to induce a pancreatitis-like injury in rats (Werner, J. et al., 1997). In addition, FAEE synthase activity is highest in the pancreas (Hamamoto, T. et al., 1990). The gene for one enzyme responsible for FAEE synthesis have been cloned and localised to chromosome 11 (Bora, P. S. et al., 1997).

Study of FAEE synthase molecules and genes are in their infancy, FAEE synthase III is a newly identified gene in which polymorphic variation has been described. This was not in a peer-reviewed article but is referred to as unpublished data in the peer reviewed article (Beckemeier, M. E. & Bora, P. S., 1998).

1.6.3 **Cytochrome P450 2E1:**

Cytochrome P450 2E1 (CYP2E1) is the major component of the microsomal enzyme oxidising system, which is one of the two major pathways of oxidative metabolism of ethanol (Lieber CS & DeCarli LM, 1970; Lieber, C. S., 1997b). It also metabolises a large number of xenobiotics (Parkinson. A., 1996). CYP2E1 is induced to greater activity by its substrate ethanol, probably via a number of mechanisms, including transcriptional, post-transcriptional and postranslational (Ohnishi K & Lieber CS, 1977; Takahashi T et al., 1993; Lieber, C. S., 1997b).

CYP2E1 activity is expressed in the liver, at sites of maximal alcohol induced damage (Tsutsumi, M. et al., 1989), and the pancreas, where it is also induced by chronic alcohol

consumption (Kessova, I. G. et al., 1998; Norton, I. D. et al., 1998); two of the major sites of damage following chronic consumption of ethanol. In addition, it is found in the brain (Upadhya, S.C., et al., 2000), also a site of ethanol induced damage.

Genetic variations in CYP2E1 have been looked at in a number of studies. Their association with alcoholism has been studied, with no association found for the c1/c2 alleles (Iwahashi, K. et al., 1995; Maezawa, Y. et al., 1995) but a positive association for the D form of the C/D polymorphism in Japanese subjects (Iwahashi, K. et al., 1998).

Association with alcohol induced end-organ disease for these polymorphisms is also conflicting, showing association with the c1 allele (Maezawa, Y. et al., 1994), c2 allele (Tsutsumi, M. et al., 1994; Tanaka F et al., 1997; Grove, J. et al., 1998) or no association at all (Carr, L. G. et al., 1995; Pirmohamed, M. et al., 1995; Carr, L. G. et al., 1996; Matsumoto, M. et al., 1996; Frenzer A et al., 1997; Itoga, S. et al., 1999; Yang, B-M et al., 2001). Many of the polymorphisms of CYP2E1 occur at a low frequency in certain populations, especially Caucasoids, and thus an association may be difficult to identify.

Recently, an insertion polymorphism in the promoter region of the gene coding for the enzyme CYP2E1 has been described; sequencing has shown a 96 base pair (bp) insertion as a series of eight repeats, as opposed to six in the wild type (Hu, Y. et al., 1999; Fritsche, E. et al., 2000). This corresponds to a restriction fragment length polymorphism, between positions –2270 and –1672. Presence of which is associated with higher CYP2E1 metabolic activity (employing an in vivo chlorazoxazone 6-hydroxylation test) in the presence of recently consumed alcohol or obesity (McCarver, D. G. et al., 1998). The Hu et al (1999) study, a luciferase reporter study, did not show a difference in constitutive expression, this is consistent with McCarver's findings of increased activity only in the induced state.

The 96 bp insertion, previously described, is a 729 base pair fragment employing the PCR based analysis of Fritsche et al. (2000). The wild type allele is 633 bp in length. In addition, a GenBank record also exists for a 48 base pair deletion (Accession No. J02843), corresponding to 585 base pairs, which has net been identified in any other studies and is of no known functional significance.

1.7 **Arachidonic Acid Metabolites:**

Arachidonic Acid (AA) is a free fatty acid liberated from cell membrane phospholipids by the action of phospholipases A_2 in response to certain hormonal, neuronal or immunological stimuli. The eicosanoids are fatty acid mediators derived, via various pathways, from the polyunsaturated (four double bonds) arachidonic acid (Serhan, C. N. et al., 1996). Their name derives from their chemical composition wherein they have twenty carbon atoms (Greek: eikosi, twenty).

AA is metabolised down two primary pathways: By the enzyme 5-lipoxygenase (5-LO) to the leukotrienes (Samuelsson, B. & Funk, C. D., 1989) and the cyclo-oxygenases (COX-1 and COX-2) to the prostaglandins and thromboxanes (Smith, W. L. et al., 1996) (Figure 1.7). A further product of the breakdown of membrane phospholipids in response to these stimuli is PAF (platelet activating factor) trials of an antagonist of which (lexipafant) initially showed great promise in management of severe AP (Kingsnorth, A. N. et al., 1995) although a more recent, larger, trial suggests a benefit only in subgroup analysis (Johnson, C. D. et al., 2001). Formed directly from the phospholipids these lipid mediators are released on formation and not stored as, say, cytokines are.

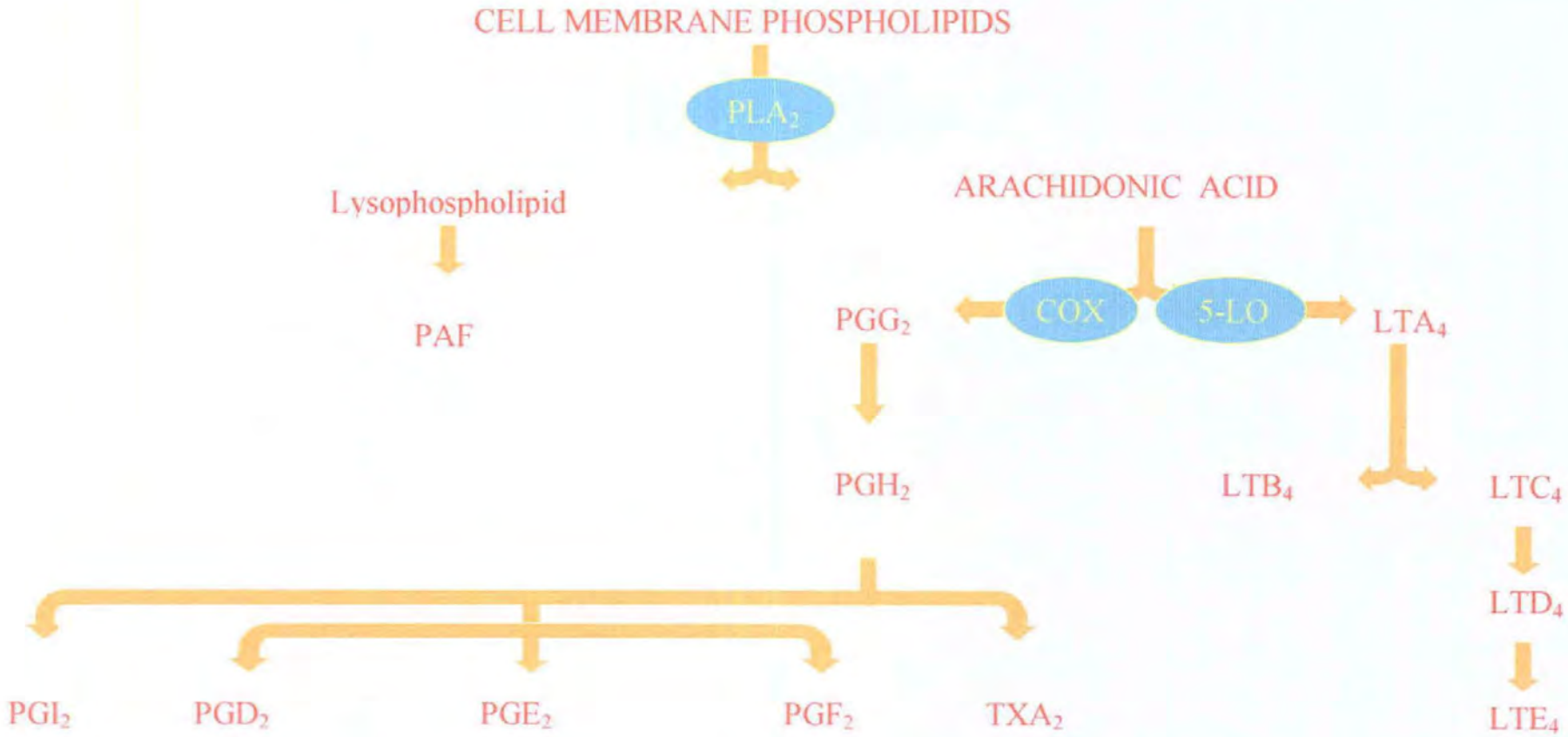


Figure 1.7. Arachidonic Acid pathway.

LT = leukotriene	PLA_2 = phospholipase A_2	PAF = platelet activating factor
PG = prostaglandin	COX = cyclo-oxygenase	
TX = thromboxane	5-LO = 5-lipoxygenase	

1.7.1 **Leukotrienes:**

Leukotrienes (LTs) are inflammatory mediators formed from arachidonic Acid by 5-lipoxygenase (5-LO) via 5-HPETE (hydroperoxyeicosatetraenoic acid), figure 1.8. 5-LO is found predominantly in leukocytes, where it requires calcium influx for activation (Samuelsson, B. & Funk, C. D., 1989). It requires FLAP (five lipoxygenase activating protein), probably for AA binding and presentation (Mancini, J. A. et al., 1993a).

LTs are grouped A-E based on their structure, with a suffix indicating the number of double bonds. The first in the pathway is the unstable LTA_4, which is hydrolysed to LTB_4 or converted to LTC_4 by addition of a glutathione group by the enzyme LTC_4 synthase (Fig 1.8). LTC_4 and its subsequent metabolites LTD_4 and LTE_4 are collectively known as the cysteinyl (or peptido-) LTs (Samuelsson, B. et al., 1987). The cysteinyl LTs (cys LTs) constitute the slow reacting substance of anaphylaxis (SRS-A) (Bremm, K. D. et al., 1983) and they are known to act though via the cysteinyl-LT1-Receptor (Figueroa, D. J. et al., 2001).

Cysteinyl LTs are formed by a number of cell types, most notably basophils (Schleimer, R. P. et al., 1985; Mita, H. et al., 1993), mast cells (MacGlashanJr, D. W. et al., 1982; Schleimer, R. P. et al., 1985; Colamorea, T. et al., 1999; Hsieh, F. H. et al., 2001) and eosinophils (Weller, P. F. et al., 1983) in response to a number of stimuli (Weller, P. F. et al., 1983; Verhagen, J. et al., 1984; Shaw, R. J. et al., 1984; Mahauthaman, R. et al., 1988). Whilst neutrophils are more responsible for production of LTB_4 (Weller, P. F. et al., 1983; Shaw, R. J. et al., 1984; Verhagen, J. et al., 1984). The chemotactic action both LTB_4 and cys-LTs have for eosinophils have been suggested to indicate a possible role for neutrophils in initiation of eosinophil recruitment and thus cys-LT formation (Shaw, R. J. et al., 1984). The interaction with mast cell products must also be noted in that PGD_2 and

histamine from mast cells are activators of eosinophils (Raible, D. G. et al., 1992). As well as the mast cells ability to produce LTB_4 and cysteinyl LTs (Schleimer, R. P. et al., 1985). Both LTB_4 and LTD_4 have a chemotactic function for neutrophils whilst for eosinophils LTD_4 is far more potent than LTB_4 (Spada, C. S. et al., 1994). In addition, the cysLTs cause vasoconstriction and increase vascular permeability (Dahlen, S-E. et al., 1981; Mayatepek, E. & Hoffmann, G. F., 1995).

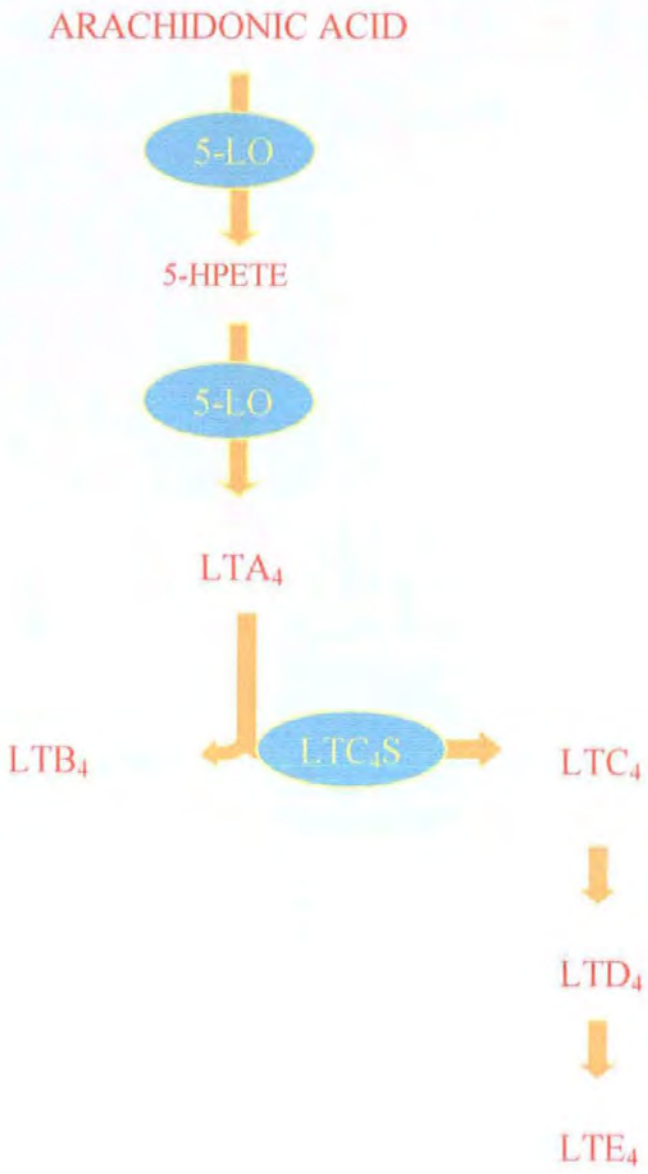


Figure 1.8. The 5-lipoxygenase Pathway of Arachadonic Acid Metabolism.
LT = leukotriene
5-HPETE = 5-hydroperoxyeicosatetraenoic acid
LTC_4S = leukotriene C_4 synthase

1.7.2 **Leukotriene C_4 Synthase:**

This is the first dedicated enzyme in the formation of the cysLTs and functions as a specific glutathione-S-tranferase. LTC_4 synthase is over-expressed in bronchial biopsies of patients with aspirin intolerant asthmatics (Sampson, A. P. et al., 1997).

A polymorphism exists in the promoter region of LTC_4 synthase. The A to C base pair change creates a novel binding site for the transcription factor AP-2, figure 1.9. This single nucleotide polymorphism (SNP) has been shown to be linked to aspirin induced asthma (Sanak, M. et al., 1997). It was postulated that the SNP may alter expression of LTC_4 synthase and now has been associated with functional significance. The (-444)C variant is associated with increased transcription in a reporter gene study (Sanak, M. et al., 2000) and increased production of LTC_4 by stimulated eosinophils with the A/C and C/C genotypes (Sampson, A. P. et al., 2000). It also may predict those who would most benefit from leukotriene receptor antagonists (Sampson, A. P. et al., 2000).

This SNP creates a novel cutting site for the enzyme *Msp I*, allowing identification by PCR and restriction endonuclease digestion (Sanak, M. et al., 1997). Figure 1.9.

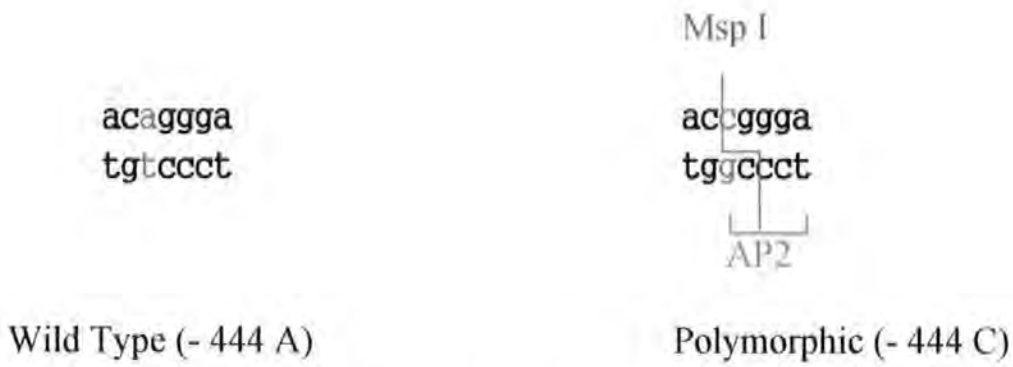


Figure 1.9. Nucleotide Sequence surrounding --
Promoter region A to C polymorphism (blue) of LTC_4 synthase
Showing the transcription factor AP 2 binding site (CCCG, red)
and the Msp I cutting site (c/c g g, green) created.

1.7.3 **Leukotrienes in the Pancreas and Pancreatitis:**

Cysteinyl LTs are implicated in a number of conditions. Most notably asthma (Mayatepek, E. & Hoffmann, G. F., 1995). In asthma LT production is most closely associated with, and increases with aspirin challenge in, AIA patients (Christie, P. E. et al., 1991). Cys-LTs are also elevated in other conditions associated such as: Active Crohn's disease, elevated urinary levels (Kim, J. H. et al., 1995). Interstitial cystitis, elevated urinary levels (Bouchelouche, K. et al., 2001). Juvenile rheumatoid arthritis, elevated urinary levels (Fauler, J. et al., 1994). Chronic urticaria, successful treatment with cys-LTR1 antagonist (Pacor, M. L. et al., 2001). Atopic dermatitis, elevated urinary levels (Hishinuma, T. et al., 2001; Sansom, J. E. et al., 1997). Kawasaki disease, increased urinary levels (Mayatepek, E. & Lehmann, W. D., 1995). Eosinophilic cellulitis, elevated in affected skin (Wong, E. et al., 1984).

5-lipoxygenase activity has been shown in the human pancreas, along with FLAP (Natsui, K. et al., 1991; Mancini, J. A. et al., 1993b).

Cysteinyl LTs have been shown to be elevated in pancreatic lymph and ascites in pigs with experimental AP (Vollmar, B. et al., 1989). Interestingly, in taurcholate induced, experimental AP in rats LTB_4 was seen to be elevated without elevation of PGs (Zhou, W. et al., 1994), the reverse true in CP. However, in another rat study LTB_4 was again elevated but here not as early as the prostaglandins (Closa, D. et al., 1993), cysteinyl LTs were not evaluated in these studies. In a study by Folch et al (1998b) both cysLTs and LTB_4 were elevated in the pancreas in a sodium taurcholate (necro-haemorrhagic) rat model concomitant with neutrophil infiltration. In the lung neutrophil infiltration was more delayed at the final measurement point, at this stage the LTs were not elevated in the lung; indicating LT are not involved at the earliest stages in the lung in this model. Also LTC_4

has been shown to increase rat acinar cell enzyme secretion (Jaworek, J. & Konturek, S. J., 1993).

Hirano et al. showed that in a rat model pre-treatment with a cysLT receptor antagonist decreased pancreatic oedema and microvascular leakage (Hirano, T., 1997).

A study by Coelle et al. (1983) looking at protective effects of PGE_2 in a diet-induced model of AP found decreased mortality with PGE_2 but increased with, the COX inhibitor, indomethacin. However, in a dog model of AP indomethacin lessened haemodynamic effects (Kiviniemi, H. et al., 1988). These indicate one or all of; the complexity in the pathogenesis of pancreatic inflammation, the limited benefits from animal models but also that possibly the indomethacin, by blocking PG production, lead to increased LT production in a mechanism analogous to aspirin induced asthma. It is interesting to postulate a link in ICP with the proposed role of analgesic use in its aetiology.

1.7.4 **Measurement of Leukotrienes:**

The cysteinyl LTs, as discussed, are LTC_4, D_4 and E_4. Levels of cysLTs have been assessed in broncho-alveolar lavage fluid (Wenzel, S. E. et al., 1995), joint fluid (Koshihara, Y. et al., 1988), tears and blister fluid (Bisgaard, H. et al., 1985) representing local production. To represent whole body production in blood, plasma or serum measurements are limited by levels being too low to measure (Heavey, D.J. et al., 1987; Sampson, A.P. et al., 1995) and the risk of ex-vivo production. This is not a problem for urinary levels where LTE_4 represents whole body cysLTs as demonstrated that radiolabelled LTC_4 is excreted, as LTE_4 representing around 5-7% of total cysLTs (Kumlin, M., 1997) and proportionate to administration/production (Christie, P. E. et al., 1994). Measurement of LTE_4 in the urine correlates with response to aspirin challenge in

sensitive asthmatics (Christie, P. E. et al., 1991) and also is proportionate to infused dose of LTC_4 and LTE_4 (Smith, C. M. et al., 1992).

The technique of measurement has varied over time. Initially high performance liquid chromatography (HPLC was used), more recently techniques employing gas chromatography/ mass spectroscopy (GC/MS) (Kishi, N. et al., 2001) and liquid chromatography/ mass spectroscopy (LC/MS)(Mizugaki, M. et al., 1999) were used the latter two being highly accurate but laborious and requiring expensive equipment. More recently immuno-assays have become available. Notably radio-immunoassays (RIAs) (Granstrom, E. et al., 1987) and enzyme immunoassays (EIAs). The monoclonal antibody based EIA from Cayman chemicals has been validated (Kumlin, M. et al.,1995) and employed in a number of studies. The debate as to the need for purification is undecided, it is argued that contaminants mean results may be unreliable (Westcott, J. Y., 1999). However, at least in paired samples, direct measurement has been validated (Kumlin, M. et al., 1995). If purification is required the methods available include HPLC and the more recently available immuno-affinity resin (Cayman Chemicals) which has been validated (Westcott, J. Y. et al., 1998).

LTB_4 metabolism is less well understood and therefore at present although an EIA for LTB_4 is available measurement in urine is not reliable, although local measurement would be reasonable.

1.7.5 **Leukotriene Receptor Antagonists:**

Study of cysteinyl leukotriene receptors are in their infancy. Two have been identified on smooth muscle cells. Their role in the inflammatory actions are just coming to light. They have been identified on lung macrophages cells, peripheral eosinophils and other inflammatory cells (Figueroa, D. J. et al., 2001), including mast cells (Mellor, E. A. et al.,

2001). In addition, effects of the receptor antagonists on inflammatory cells, most notably eosinophils, implicates direct (though cannot exclude indirect (Moqbel, R., 1999)) anti-inflammatory effects. They suppress eosinophil numbers both in blood (Reiss, T. F. et al., 1998) and airways (Calhoun, W. J. et al., 1998).

The cysteinyl-LT1-receptor antagonist group of drugs are of proven benefit and licensed in the treatment of asthma. Probably acting to block mast cell derived cysLTs to diminish the early response and the late response possibly secondary to blockage of eosinophil derived cysLTs (Sampson, A. P. & Holgate, S. T., 1998). Cys LT-Receptor antagonists have also proved of benefit in atopic dermatitis (Capella, G. L. et al., 2001).

In addition, as noted, pre-treatment with one cysteinyl LT1-receptor antagonist, Pranulokast, has been shown to decrease some of the inflammatory changes associated with experimental AP (Hirano, T., 1997).

1.7.6 **Prostaglandins:**

As discussed above, the prostaglandins (PGs), along with thromboxanes, are the products of the cyclo-oxygenase metabolism of arachidonic acid, figure 1.6. There are two cyclo-oxgenases (isoforms) termed COX-1 and COX-2 (Xie, W. L. et al., 1991). The two appear to have distinct roles one being constitutive in nearly all cells (COX-1) and the other induced (COX-2), being undetectable in cells at rest. This pattern has been confirmed in pancreatic acinar cells (Zabel-Langhennig, A. et al., 1999). The induction of COX-2 being, most commonly, inflammatory stimuli. The COX enzymes in fact also possess (in a spatially distinct active site) peroxidase activity responsible for the conversion of PGG_2 to PGH_2, see figure 1.7.

The prostaglandins are divided into groups A-J based on the substituent groups on the cyclopentane ring, the ring distinguishing them from the other eicosanoids. A suffix is then generally applied (as with LTs) according to the number of double bonds in the side chains. Which metabolites are formed is somewhat dependent on the cell type concerned. Platelets form predominantly thromboxanes (TXs), vascular endothelium prostacyclin (PGI_2 aka PC). These are both formed enzymatically whilst the major prostaglandins (D,E,F) are formed non-enzymatically with enzymatic conversion also occurring in the case of E_2 and D_2.

The active TX (TXA_2) stimulates platelet aggregation whilst this is inhibited by PC. Similarly TXA_2 is a vasoconstrictor whilst PGs are vasodilators most notably PC and PGEs (Crofford, L.J., 2001)

In pancreatic disease $PGF_{2\alpha}$ increases in haemorrhagic but not oedematous AP whereas the opposite was found for PC (Berger, Z. et al., 1989)); whilst Closa et al (Closa, D. et al., 1994a; Closa, D. et al., 1994b) found elevation of PGs D_2, E_2, 6-keto $F_{1\alpha}$ and $F_{2\alpha}$ but not E_1 and TXB_2 (the breakdown product of TXA_2). Here indomethacin has been seen to decrease a number of these effects whilst increasing mortality(Closa, D. et al., 1994c). In contrast one study examining arachidonic acid metabolites in AP and CP in rat models found that although a number of PGs (E_2, D_2, 6-keto $F_{1\alpha}$), TXB_2 and PAF increased in a duct ligation model of CP, in their AP model PGE_2, along with 6-keto $PGF_{1\alpha}$ and TXB_2 decreased significantly (Zhou, W. et al., 1994).

It is interesting to note the study by Victorov & Hoek (1995) in which PGs E_2, D_2, $F_{2\alpha}$ and 6 keto- $F_{1\alpha}$ were produced by cultured rat Kupffer cells in response to lipo-polysaccharide (after a lag) and ethanol (gradually), the former having a greater effect and PGE_2 more markedly raised than the other PGs and TXB_2.

1.7.7 **Prostaglandin E_2 in the Pancreas and Pancreatitis:**

PGE_2 was initially found to be elevated in AP in a dog model in 1976 (Glazer, G. & Bennett, A., 1976) with, on bioassays, elevated prostaglandin like activity in blood and PGE_2-like material in peritoneal exudate. Closa et al also found increased PGE_2 early in a taurcholate model in rats (Closa, D. et al., 1993), and this earlier than the LTs; the same group have repeated this finding a number of times in studies looking at a number of AA metabolites (Closa, D. et al., 1994a; Closa, D. et al., 1994b; Closa, D. et al., 1994c). PGE_2 was again elevated (in a necrotising, 'though not oedematous model) of AP in rats (Gloor, B. et al., 2001) along with TXB_2 and LTB_4, reduced by steroids. A study by Jaworek and colleagues (2001) found increased PGE_2 in experimental AP in a caerulein rat model, enhanced with pre treatment with LPS, which corresponded to decreased inflammation.

In light of the 'protective' effects of PGE_2 on gut mucosa a possible protective role in AP was investigated. In a mouse model Manabe et al. (Manabe, T. & Steer, M. L., 1980) found prior and concurrent administration of PGE_2 decreased mortality and markers of the pancreatic damage. A study over a longer time period in rats did not show significant differences and questioned the longer term relevance of the previously seen short term effect (Martin, D. M. et al., 1981), 'though in a different animal and model.

PGE_2 again appeared to have a protective effect, in rats over 7 days, improving mortality and inflammation (Coelle, E. F. et al., 1983); interestingly in this study indomethacin (a non-selective COX inhibitor) significantly worsened outcome implying the protective effects of PGE2 +/- other COX products outweighed other 'pro-inflammatory' COX products. This effect of indomethacin has been shown again (Closa, D. et al., 1994c), associated with decreased levels of PGE_2, as well as other prostaglandins. A study not showing a benefit for PGE_2 (given intraductally, intraperitoneally or subcutaneously), in

1983, was performed on a rat model with the pancreatitis induced by retrograde pancreatic duct injection of sodium taurcholate (Lankisch, P. G. et al., 1983). The original group then looked again (Taylor, I. L. et al., 1985) and found the protective effect of PGE_2 (4 day mortality) still occurred with 'post' treatment, however, this did not persist to 7 day mortality or if the inducing diet was continued for longer. They, quite rightly, concluded that the effect can be inferred to depend on duration of inflammatory stimulus, time of treatment and type and dose of PG.

Further positive evidence for a protective effect came in 1983 (Standfield, N. J. & Kakkar, V. V., 1983) again with a survival benefit, with both pre- and post-induction treatment. This same study showed a beneficial effect for membrane stabilisation effect for PGE_2 in humans. Lysosomal stabilisation was also seen in rats (Manabe, T. et al., 1993). Lysosomal stabilisation in the liver (Dlugosz, J. et al., 1982), kidney(Triebling, A. T. et al., 1984) and pancreas (Gabryelewicz, A. et al., 1983) has also been shown for prostacyclin. PGE_2 treatment has also been shown to decrease the hyperamylasaemia and oedema in pancreata of opossums with common bile duct ligation (Ramirez, R. et al., 1984). In a caerulain model in rats again decreased findings of AP (e.g. oedema and necrosis) were seen with PGE_2 (Robert, A. et al., 1989). Van Ooijen et al found a decrease in mortality with PGE_2 although this only became significant when combined with thromboxane inhibition in a rat model (van Ooijen, B. et al., 1989).

As discussed the majority, but not all, studies have shown a beneficial or 'protective' effect for PGE_2. Further contradictory studies warranting mention are that Reber required PGE_2 to cause pancreatitis with enterokinse and glycodeoxycholate (Reber, H. A., 1985). The first study to show a significant negative effect of PGE_2 treatment was in 1986 (Olazabal, A., 1986). Another 1986 study (Wedgwood, K. R. et al., 1986) found an increase in haemorrhagic, as opposed to oedematous, AP in cats with PGE_2 in a high dose; the same

effect also with histamine, but not isoproterenol, implying increased permeability as the mechanism.

The effect of PGE_2 on pancreatic function has also been investigated with contradictory effect seen: An inhibitory effect on volume and bicarbonate whilst stimulating enzyme secretion (Rosenberg, V. et al., 1976). PGE_2, PC and PGF1alpha inhibited secretagogue induced enzyme secretion from isolated rat acini (Jaworek, J. & Konturek, S. J., 1993), although this was much less marked than that from a stable PGE_2 analogue.

In summary, PGE_2 appears to be elevated in most models of AP, and in the one model of CP studied; although the time scale is variable. As before this is associated with the weakness of animal models as a whole. Whether the role of PGE_2 is generally 'pro' or 'anti' inflammatory or 'protective' you could not definitively conclude; certainly to the point of suggesting therapy. However, the balance of the animal evidence would suggest that its effects are more beneficial than detrimental, perhaps in a complex balance with the other lipid, and non-lipid, mediators.

1.7.8 **Measurement of PGE_2:**

In vivo PGE_2 is converted to 13,14-dihydro, 15-keto PGE_2 then some on to 13,14-dihydro, 15-keto PGA_2 both of which can be converted to bicyclo PGE_2 by reaction with Na_2CO_3 which is stable. Thus to measure urinary PGE_2 an initial 'derivitisation' step is performed converting the metabolites to a single, stable, measurable form.

1.7.9 **Prostaglandin D_2 in the Pancreas and Pancreatitis:**

As discussed above PGD_2 has been shown to be elevated in pancreatitis in a small number of studies in AP (Closa, D. et al., 1994a) and CP (Zhou, W. et al., 1994). Mast cells produce PGD_2 (Schleimer, R. P. et al., 1985), by both COX-1 (early) and COX-2

(delayed) dependent mechansims (Reddy, S. T. et al., 1999). The value of the measurement of PGD_2 and its metabolites is therefore not just of itself but because it is a marker of mast cell activation (O'Sullivan, S. et al., 1996; O'Sullivan, S. et al., 1997; O'Sullivan, S., 1999a).

As discussed in section 1.3.5 there is evidence and a theoretical argument for a role for mast cells in both AP and CP.

1.7.10 **Measurement of PGD_2:**

Measurement of PGD_2 can be simplified by that of it's urinary, stable metabolite 9-alpha, 11-beta $PGF_{2\alpha}$ (Liston, T. E. & Roberts, L. J., 1985; O'Sullivan, S., 1999b). Which corresponds to mast cell activation (O'Sullivan, S. et al., 1996; O'Sullivan, S. et al., 1997). An EIA, which recognises 9-alpha, 11-beta $PGF_{2\alpha}$ (and 2 closely related PGD_2 metabolites) has been validated against the 'gold standard' of gas chromatography mass-spectroscopy GC-MS (O'Sullivan, S. et al., 1999b).

AIMS

To identify factors which are associated with a person's predisposition to Acute and Chronic Pancreatitis and the role of Arachidonic Acid metabolites in these conditions.

1) To look for association between the known, functional 96 base pair Insertion/Deletion Polymorphism in the Cytochrome P450 2E1 gene and alcoholism and/or alcohol induced end organ disease (Alcohol Induced Chronic Pancreatitis and Alcoholic Liver Disease).

2) To assess if the exon 5 dinucleotide repeat polymorphism of IL-1a is associated with disposition to Chronic Pancreatitis, especially those genotypes implicated in Acute Pancreatitis

3) To see if use of a cysteinyl LT receptor antagonist is beneficial to patients with painful Chronic Pancreatitis.

4) To assess if elevated levels of the Arachidonic Acid metabolites PGE_2, PGD_2 and the cysteinyl LTs are found in Acute and/or Chronic Pancreatitis and whether a functional single nucleotide polymorphism in the promoter region of the gene for the enzyme LTC4 synthase has a role in predisposition to either condition or severity in Acute Pancreatitis.

MATERIALS AND METHODS:

GENERAL

In this chapter the general reagents and methods used during this work are discussed. Specific reaction conditions, subjects and techniques are addressed in the relevant Materials and Methods chapters.

3.1 General Materials:

3.1.1 Water

Double distilled water was used to make up all stock, general purpose and specialist solutions. Sterile water (Baxter Healthcare, Newberry, UK) was used to make primer solutions and used for PCR and in preparation of EIA reagents.

3.1.2 Reagents

All reagents used were analytical grade or equivalent.

Acetic Acid, Disodium ethylene diamine tetra-acetic acid, Glycerol, Hydrochloric acid, Magnesium chloride, Orthoboric acid, Sodium hydroxide, Sucrose and Tris (hydroxymethyl) aminomethane were purchased from (BDH Laboratory Supplies-Merck Limited, Leicester, UK). Ethanol and methanol purchased from (Rathburns Limited,Tweedale, UK). Ammonium persulphate, Ethidium Bromide, Tetra-methyl-ethylene-diamine (TMED), Triton –X-100 and Xylene cyanol purchased from (Sigma Chemicals, Poole, UK).

3.1.3 Specialised reagents, enzymes and materials

Agarose, the DIG luminescent detection system and the restriction enzymes were purchased from (Boehringer Mannheim GmBH, Germany). Taq polymerase, PCR buffer

and deoxynucleotide 5'-triphosphates (dNTP's) were purchased from Gibco (Life Technologies, Paisley, UK).

3.1.4 **Stock solutions**

i. Tris/borate electrophoresis buffer (TBE)

   10x solution: 0.89mM Tris base, 0.89M Boric acid, 2mM EDTA (pH8)

ii. Ethidium bromide

   10mg ml^{-1} in H_20

iii. Tris-acetate (TAE) buffer

   50x solution: 242g Tris base, 57.1ml Glacial acetic acid, 0.5M EDTA (pH8)

iv. Phosphate Buffered Saline (PBS)

   100mM potassium phosphate buffer (pH 7.4) in 0.9%NaCl

3.1.5 **Autoclaving**

All solutions and all glassware and plastic ware used in the techniques of DNA analysis were autoclaved at a temperature of 121^{0}C and pressure of 15 p.s.i for 30 minutes in a steam autoclave (Rodwell Instruments, UK).

3.2 **Extraction of Genomic DNA**

5-10ml of peripheral venous blood was taken from all subjects into 5% EDTA (Becton Dickenson,Oxford, UK) and used to extract that subjects genomic DNA.

DNA extraction of UK samples employed the commercially available Nucleon BACC2 kit (Tepnel Life Sciences plc, Manchester, UK). In summary, 5-10ml sample of frozen blood was thawed at room temperature and transferred to a 50ml falcon tube (Philip Harris, Cardiff, UK). 40mls of Nucleon A (10mM Tris-HCL, 320mM sucrose, 5mM MgCl2, 1% Triton X-100, pH 8.0 using 40% NaOH) (Tepnel Life Sciences plc, Manchester, UK) was

added and the sample shaken for 4 minutes at room temperature and then centrifuged at 1300g for a further 4 minutes at room temperature in a MSE Mistral 1000 centrifuge (MSE, UK). The supernatant containing the lysed red cells was discarded and 2ml of Nucleon B (400mM Tris-HCL pH 8.0 using 40% NaOH, 60mM EDTA, 150mM NaCl, 1% SDS) was added to the pellet to disrupt nuclear membranes. The pellet was resuspended by vortexing (Vortex Genie 2, Scientific Instruments Inc., USA) and then incubated at 37^0C for 30 minutes (Y14 Waterbath, Cambridge, UK) to ensure that the nuclear membranes were disrupted and the protein denatured. The suspension was transferred to a 15ml falcon tube and 500µl of 5M sodium perchlorate was added, to deproteinise, and the mixture was inverted 7 times. 2ml of chloroform (Sigma, Poole, UK) at -20^0C was then added and the tubes inverted a further 7 times. 300µl of resuspended Nucleon silica (Tepnel Life Sciences plc, Manchester, UK) was added without re-mixing the phases and then centrifuged at 1300g for 3 minutes at room temperature. The upper, aqueous, phase containing the DNA was decanted to a fresh tube. The DNA was precipitated by addition of ice-cold 100% ethanol (Rathburns Ltd, Tweedale, UK) and gentle inversion. The DNA was extracted into an Eppendorf tube (Fisher, Loughborough, UK) containing 70% ethanol using a hooking pasteur pipette, it was then air dried and finally resuspended in an Eppendorf tube containing 0.5ml of sterile water.

The DNA sample was quantified visually and by measuring the optical density using a Cecil 5500 Spectrophotometer scanning 240nm to 280nm (Cecil, UK) and compared to a blank containing double distilled water at 260nm. DNA samples were then stored at -20^0C.

DNA from some peripheral blood samples employed in the CYP 2E1 study were extracted employing the commercially available QIAmp DNA Blood Minikits (Qiagen, Hilden, Germany).

3.3 **DNA polymorphism analysis:**

All polymorphism analyses employed PCR (polymerase chain reaction) based techniques. The insertion/deletion polymorphism of CYP 2E1 employs PCR and is of sufficient size to be visualised with agarose gel electrophoresis, chapter7.

The dinucleotide repeat polymorphism in intron 5 of IL-1a was analysed with polyacrylamide gel electrophoresis (PAGE) following PCR, chapter 8.

Restriction fragment length polymorphism (RFLP) analysis was used to investigate the LTC_4 synthase polymorphism. This consists of a Single Nucleotide Polymorphism (SNP) which creates a novel cutting site for the enzyme MspI (Roche, Lewes, UK) within the PCR product, chapter 9.

3.3.1 **Polymerase Chain Reaction:**

This technique employs a DNA polymerase enzyme to extend complimentary DNA to the relevant portion of DNA. A primer is designed which is complimentary to a sequence of around 20 base pairs (bp) on the 'sense' strand 5' of the polymorphism of interest. The equivalent is done for the anti-sense strand.

These primers (or amplimers) bind during the appropriate annealing temperature to the complimentary site on the native DNA. The polymerase then adds nucleotides to the primers complimentary to the next matching base of the native DNA. During recurrent rounds the strands amplified are thus the area between the two primers (Figure 3.1).

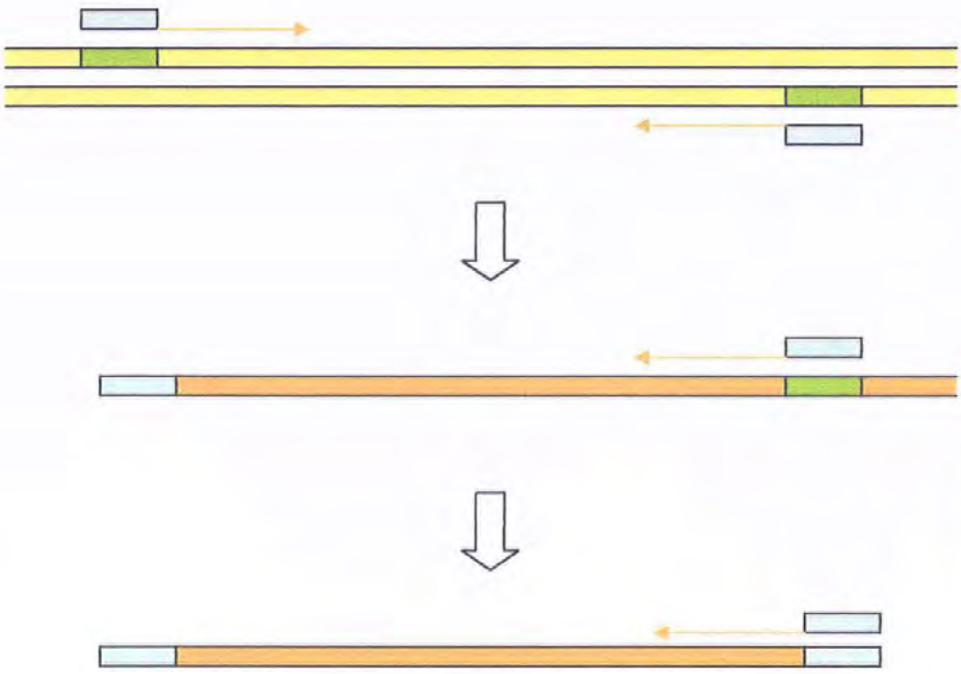


Figure 3.1. DNA Replication in Polymerase Chain Reaction.
The replication proceeds on consecutive cycles 'focusing' on and amplifying the portion of DNA sited between the primers.

Primer

Sequence of DNA to which the primer is complimentary

Native DNA

DNA copy of the native DNA formed from the nucleotides contained in the reaction mixture; the polymerase catalysing the reaction.

The reaction thus requires native DNA, Primers (sense and anti-sense), nucleotides (NTPs), DNA polymerase (Taq), Magnesium ($MgCl_2$), PCR buffer and water. The Taq polymerase warrants further brief comment. This enzyme (derived from Thermus aquaticus) is heat (thermo-) stable and can thus remain intact whilst the PCR reaction is cycled through temperatures of up to 94^0c. The commercial preparation is from Eschericia coli cloned with the T. aquaticus DNA polymerase gene.

PCR consists of cycles of denaturation (of the genomic and newly formed double stranded DNA to single strands), annealing of the primers (to their complimentary sites) and extension (of the primers by the Taq). As each cycle builds on the previous cycle copies of the exact length multiply (the PCR 'product').

3.3.2 **Oligonucleotide Primer design and production**

Oligonucleotide primers are used in both sense and antisense directions. Sense primers were complementary to the 5' - 3' strand of DNA and antisense were complementary to the 3' - 5' strand of DNA. Primers were synthesised commercially by MWG-Biotech (Germany) at a scale of 0.2μmol.

Primers employed in these studies had been previously described, these were then checked against the GeneBank record to confirm their sequence and ordered. Primers are designed to be complimentary to sequences either side of the polymorphic area, however, this is within certain limits. Primers function best if approximately 20 bases in length and approximately 50% made up of guanosine and cytidine (G+C) bases. Sequences are checked on DNAstar software (Lasergene, USA) to avoid the possibility of forming 'primer dimers' or 'hairpin-loops', when complimentary to other sequences, which may result in the formation of anomalies or artefacts. Thus primers must not identify an area of similar length elsewhere in the genomic DNA as this may confuse the analysis; this may be

a particular problem if analysing genes with considerable sequence homology. The target amplification lengths ranged from approximately 200-800 base pairs. The primer sequences for each analysis are detailed in the following chapters.

3.3.3 **Performance of the Polymerase Chain Reaction:**

All PCR reactions were prepared on ice in 0.2ml thin walled PCR tube strips (Anachem, Luton, UK).

The reaction mixtures consisted of 100-200ng of genomic DNA, 10 pmoles of both sense and antisense primers, 2.5µl 10x PCR reaction buffer (50mM Tris-HCL, pH9, 250mM KCl, 1% Triton X-100, 0.1% [w/v] gelatin), variable amounts of $MgCl_2$, 400mM of each NTP and 2 units of Taq polymerase. The final volume was made up to 25µl with distilled water (in the case of the IL-1a $(AC)_n$ dinucleotide repeat polymorphism a total volume of 30µl was used). 30 cycles of amplification were carried out in either a iCycler (Bio-Rad Laboratories Inc., Hemel Hempstead, UK) or a 48 well DNA engine (Genetic Research Instrumentation, USA).

Details of exact proportions of reagents, especially $MgCl_2$ concentration, were optimised by trial and error aiming to produce a prominent defined band representing the 'true' product with a minimum of accessory bands. Higher magnesium concentration favours more product but also (by promoting the reaction) more accessory bands, whilst higher annealing temperature favours less accessory bands at the expense of the amount of product. The initial annealing temperature, with a battery of Mg concentrations, was the mean of the melting temperatures (T_m) of the two primers plus two degrees.

The standard reaction settings for PCR were: A hot start of 94^0c for 4 minutes was employed to promote denaturing. Then 30 cycles of: Denaturation at 94^0C for 30 seconds,

annealing for 2 minutes (at a temperature optimised for each reaction), and extension at 72^0c for 2 minute. A further extension of 10 minutes (at 72^0c) was performed following the 30 cycles. Samples were then held at 4^0C until analysed.

3.4 **Separation of DNA fragments by agarose gel electrophoresis**

All PCR products were checked by electrophoresis on horizontal agarose gels, this was also the means of formal analysis for the LTC_4 synthase and CYP 2E1 genetic studies. The IL-1a study employed PAGE to analyses frequencies of the alleles.

The percentage agarose employed dependent on the fragments size to be viewed, see individual chapters. To make a 1% gel: 1 gram of agarose (Boehringer Mannheim, Germany) was added to 100ml of 0.5x TBE buffer in a 400ml glass bottle, and heated in a microwave for 2-3 minutes at 650 watts to dissolve. The agarose solution was cooled to 60^0C-65^0C and 10µl ethidium bromide (10mg/ml solution) was added and mixed by swirling. The agarose solution was poured into a 10cm ultraviolet light-transparent perspex tray (Flowgen, Leicester, UK), which was sealed at both ends with masking tape and contained moulds to generate the sample wells (14 and 20 tooth combs). After the gel was set it was placed at room temperature to harden the agarose, after removing the tape the gel was submerged in 0.5-1% TBE buffer in an electrophoresis tank (Flowgen, Leicester, UK), and the comb moulds were removed. The gel was run for variable times and voltage dependent on fragment size. Also known standards were run on each gel and/or DNA ladders, of known base pair lengths (Roche, Lewes, UK).

3.4.1 **Result assessment:**

To minimise error all results were duplicated as follows. If a result considered probable or definite this was repeated to obtain at least one definite result confirmed with a probable or

second definite result. Unreadable results were considered no result or unreadable. Those 'no results' were repeated once and if the result unreadable these were not further analysed. An unreadable but present result if on repeating was readable it was further repeated for confirmation.

3.5 **Statistical Analysis of data:**

All the data collected was entered into a computerised spreadsheet (Microsoft® Excel 7.0). This allowed statistical analysis to be performed within the SPSS for Windows 9.0 (SPSS Inc., USA) statistical package.

Details in each study are contained in the relevant chapters.

3.6 **Ethical Approval:**

Ethical approval was gained from the Local Research Ethics Committee for collection of samples and all studies performed.

MATERIALS AND METHODS:

CYTOCHROME P450 2E1

Based on the knowledge of the previously described (Fritsche, E. et al., 2000; Hu, Y. et al., 1999) functional (McCarver, D. G. et al., 1998) insertion polymorphism in the promoter region of CYP 2E1 a possible relevance in alcohol consumption and alcohol induced end-organ disease was assessed.

4.1 **Subjects**:

Venous blood samples were drawn with patients giving informed consent and Local Research Ethics Committee Approval was obtained.

Samples were collected on 239 Caucasoids who chronically consumed alcohol, ALC, with a history of 'excessive alcohol intake', based on the Zurich Criteria for Alcohol Induced Chronic Pancreatitis (Ammann, R. W., 1997), of consumption of greater than 80 grams of pure alcohol per day for males and 60g for females, for at least two years. They all fulfilled the criteria for ICD (the International Classification of Diseases) 10.1 and/or 10.2; that is harmful use and/or alcohol dependence syndrome. As stated alcohol consumption was minimal of 80/60 grams for all ALC, the actual ranges (available on United Kingdom patients only) were 56 to 400 units per week (median 106) for a duration of 2 to 45 years (median 10). Ages 25 to 73 years (median 47). Males constituted 85% of the patient group.

This study was in collaboration with the University of Magdeburg, specifically the Department of Surgery and Outpatient Clinic for Addiction Diseases, who kindly collected samples on patients with AICP and 'alcoholics' without evidence of end-organ disease.

These sub-divided as follows (also see Table 4.1):

Sixty seven (36 local British and 31 German) consumers of alcohol without known end-organ disease, AC.

One hundred and seventy two with alcohol related end-organ disease (AEOD), of which:

One hundred and forty four (39 local British, Plymouth, and 105 German, Magdeburg) patients had alcohol-induced chronic pancreatitis (AICP); all fulfilled the criteria for late- or end-stage (exocrine insufficient) AICP, as defined by the Zurich criteria (Ammann, R. W., 1997). That is they had a history consistent with CP, typically of pain and recurrent bouts of clinical AP. In addition, one or more of pancreatic calcification; moderate to marked pancreatic ductal changes (based on the Cambridge classification (Axon, ATR et al., 1984)); steatorrhoea corrected with pancreatic enzyme supplementation or histological for 'definite' CP (37 Plymouth AICP and 105 Magdeburg AICP). Or a history, for 'late stage' as above plus one or more of mild changes (Axon, ATR et al., 1984); recurrent/ persistent pseudocysts or diabetes mellitus (2 Plymouth and none of the German patients).

Twenty eight patients with alcoholic liver disease (ALD), having biopsy proven cirrhosis or a history, consistent with alcoholic hepatitis, including jaundice associated with excess alcohol consumption without other evident cause. Patients with evidence of both AICP and ALD were excluded to allow for sub-group analysis.

Two hundred and eight samples of cord blood from Caucasoids with a normal, healthy delivery taken in Derriford Hospital, Plymouth, UK were employed as normal controls. Male constituted 47% (n= 98) and females 53% (n= 110).

4.2 **Polymerase Chain Reaction:**

DNA amplification was performed for the promoter region of CYP2E1 as previously described (Fritsche, E. et al., 2000), employing amplimers 5'-GTG ATG GAA GCC TGA AGA ACA- and 5'-CTT TGG TGG GGT GAG AAC AG-. A standard reaction mixture was used as described above in section 6.3.3. A temperature of 66^0C was found to be optimal as the annealing temperature, with a $MgCl_2$ concentration of 3mM.

Products were analysed on a 1.5% agarose gel stained with 0.01%v/v ethidium bromide, viewed under Ultraviolet light and compared to 50 and 100 bp molecular weight ladders (Roche Diagnostics, Lewes, UK).

4.3 **Statistics:**

Two sided Fishers' Exact tests were used throughout. A p value of <0.05 was taken as significant. Where appropriate multisided contingency tables were employed initially for comparisons with sub-group analysis only when a statistically significant result was seen in the initial comparison. Individual sub-group analyses for end-organ disease(s) involved comparison with alcoholic controls only. The χ^2 result, where expected cell numbers are greater than 5, are shown in brackets in table legends.

Patient Group			Total
Normal Controls (UK)			208
ALC			239
Of ALC:	Total	Total UK	Total German
AC	67	36	31
AEOD	172	67	105
Of AEOD:			
AICP	144	39	105
ALD	28	28	0

Table 4.1.
Subdivision of subject groups studied for polymorphism of CYP 2E1.
UK = Subjects from Plymouth.
German = Subjects from Magdeburg.
ALC = Fulfilling criteria for ICD 10.1 and/or 10.2
AC = Without evidence of ALD or AICP.
AEOD = Alcohol induce end organ disease.
AICP = Alcohol Induced Chronic Pancreatitis.

MATERIALS AND METHODS:

INTERLEUKIN 1 ALPHA STUDY

5.1 **Subjects**:

Venous blood samples were drawn with patients giving informed consent. Samples were collected from 54 patients with a diagnosis of 'end stage' CP, based on the Zurich Criteria for Alcohol Induced Chronic Pancreatitis (Ammann, R. W., 1997) and cord blood from 150 Caucasoids with a normal, healthy delivery taken in Derriford Hospital, Plymouth, UK were employed as normal controls. The patients subdivided on aetiology of AICP 38 and ICP 16. ICP patients had no history of 'excessive alcohol consumption' as defined above, additionally they had no history of borderline alcohol consumption but no or minimal to moderate 'social' drinking. There were 19 females (35%); age range 27 to 87 years (median 55 years). One hundred fifty cord blood samples from normal deliveries were employed as normal controls (NC), females constituting 53% (n= 80). These forming the same control group as in the AP study cited.

5.2 **Analysis:**

Products were analysed on a polyacrylamide gel, viewed by autoradiography and compared to known standards, after Smithies (2001). PAG electrophoresis (PAGE) is a gel electrophoresis technique enabling more precise separation of fragments than agarose gel electrophoresis. It enables separation of fragments differing in length by only one or two base pairs. Thus allowing analysis of the $(AC)_n$ dinucleotide repeat discussed in the introduction. The technique uses autoradiograhy to reveal the fragment separation on the gel. Wherein primers used in the PCR reaction are 'labelled' with a beta radiation source. The gel, after drying, is placed on an X-ray film which is thus marked at the sites of the fragments. The beta source employed is phosphorous ($\gamma^{32}P$) incorporated in the phosphate

of a nucleotide (in this case deoxyriboadenosine triphosphate) giving γ^{32}P-ATP. This nucleotide is then incorporated onto the 5' end of a primer thus the PCR product is labelled.

5.3 5' End Labelling of Oligonucleotide Primers:

The 5' end of the sense primer was radio-labelled using "Ready-To-Go" T4 Polyucleotide Kinase (Pharmacia Biotech, Sweden).

In the "Ready-To-Go" T4 Polynucleotide Kinase (PNK) reaction each individual reaction tube contained, reconstituted in a final volume of 50µl, a solution containing 8-10 units of "FPLC pure" T4 PNK, 50mM Tris-HCL (pH7.6), 10mM $MgCl_2$, 5mM DTT, 0.1mM spermidine, 0.1mM EDTA (pH8), 0.2µM ATP and stabilisers. 25µl of distilled water was added to the tube that contained the "Ready-To-Go" T4 PNK, the tube was then incubated at room temperature for 2-5 minutes. The contents of the tube were then mixed by gently pipetting up and down. 5-10 pmoles of the desired oligonucleotide primer was added as well as a sufficient volume of distilled water to bring the total reaction volume to 48µl. 2µl of γ ^{32P}dATP (10µCi/µl) was added and mixed gently. The sample was centrifuged briefly at 13,000 rpm for 30 seconds in a benchtop Microfuge (Hereus Septech Germany) to collect the contents at the bottom of the tube, and then incubated for 30-45 minutes in a water bath at 37^0C. The reaction was terminated by placing on ice. The sample was then precipitated to remove unincorporated nucleotides using 2µl of Quick Precip^{TM} (Advanced Biotech Corps, USA) a biologically inert product that is used as a carrier for the rapid precipitation of DNA and oligomers >16 bases. To the sample 0.1 volumes of 5M NaCl and 2-3 volumes of -20^0C 100% ethanol was added. The sample was briefly vortexed and then centrifuged for 2-3 minutes at 13,000 rpm at room temperature. After centrifugation the sample was vortexed vigorously and centrifuged again for another 30 seconds. The

supernatant was decanted and the tube was rinsed with 70% ethanol. The precipitated DNA was resuspended in 50µl of distilled water and the side of the tube was rinsed with the water to ensure complete recovery, the tube was then vortexed.

5.4 **Polymerase Chain Reaction:**

To assess the frequency of the (AC)n tandem repeat polymorphism previously discussed DNA amplification was performed for the relevant region of intron 5 IL 1a as previously described (Smithies, A. M., 2001), the amplimers used were 5'-GGT-ATT-TAC-ACC-ATA-GGT-GGG-GAC- and 5'-GCA-CCC-ATG-TCA-AAT-TTC-ACT-GG- (MWG Biotechnology, Germany), these were initially derived from the published sequence Genbank Accession number X03833.

Standard reaction conditions were used. The optimum annealing temperatures were found to be 56^0C. The optimum magnesium chloride concentration was found to be 1mM. In addition to the normal PCR reagent mixture was included 1µl of diluted sense amplimer labelled with γ^{32}P-ATP.

5.5 **Preparation and Performance of the PAGE:**

All PCR reactions were checked by running a 10µl aliquot of the sample and 1-1.5µl of loading buffer on a 1% horizontal agarose gel for 1 hour at 150-200 volts, as described in Chapter 6. The gel was visualised under UV light to confirm an appropriately sized product band. If the PCR was successful the PCR products were electrophoresed on a vertical denaturing polyacrylamide gel.

A Biorad Sequigen GT 30x50cm electrophoresis system (Bio-Rad Laboratories Inc., Hemel Hempstead, UK) was used with a 49 well vinyl sharkstooth comb with a 0.4mm

spacer set. Before use, all aspects of the rig were washed in warm soapy water (glass plates, spacers and comb and clamp binders). After a thorough rinse in water, plates were dried and washed in 70% IMS solution (Rathburns, Tweedale, UK). The inner surface of the back plate were siliconised with a thin layer of Repelcote (a 2% solution of Dimethydichlorosilane in Octamethycyclotetra-siloxane) (BDH Lab. Supplies-Merck, Leicester, UK), this was to allow the gel to be removed from the plates after running. The plates were allowed to dry and then a small amount of petroleum jelly ran down each edge of the base plate to grip the vinyl spacers. The top plate was then placed on top and sandwiched together using two arm clamps. The assembled plate unit was then stood in a casting gel tray and laid in a horizontal position. The denaturing polyacrylamide gel was prepared using a series of solutions (National Diagnostics, U.S.A.): Ultra Pure Sequagel. Sequencing ready made concentrate, diluent and buffer. 36 mls of concentrate (237.5g acrylamide, 12.5g methylene bisacrylamide and 500g urea), 15mls of buffer (50% 8.3M urea in 1M Tris-Borate, 20mM EDTA buffer pH 8.3), 99mls of diluent (500g 8.3M urea), and 12mls of 10% formamide (Sigma Chemicals, Poole, UK) were mixed in a 250ml conical flask. 70µl TEMED (Sigma chemicals, Poole, UK) and 1.5ml of 10% Ammonium Persulphate (Sigma chemicals, Poole, UK) were used to polymerise the gel.

The plate assembly was filled with the gel solution using a 200ml syringe connected to a delivery tube (part of the gel system). When the polyacrylamide reached the top, the vinyl sharkstooth comb was inserted between the plates in an inverted position to give a straight line. The gel was allowed to polymerise for at least one hour at room temperature. When the gel was set, the casting tray was removed and the gel unit placed in the base or lower buffer chamber of the gel system. This contained 1x TBE buffer, the buffer chamber at the back of the base plate was also filled with 1x TBE buffer to the top of the plate in the reservoir at the top of the system.

The system was connected to a Biorad PC3000 power pack (Bio-Rad Laboratories Inc., Hemel Hempstead, UK) and pre-warmed at 1,800V, for approximately 45 minutes, until the temperature indicator read 45-50^{0}c. The gel was then disconnected from the power supply and the comb reinserted to generate the 40+ wells. 6µl of the amplified DNA was mixed with 3µl of 10x Stop solution (Gibco, Life Technologies, Paisley, UK). The stop solution contained formamide and was based on a standard sequencing gel-loading buffer (98% deionized formamide, 10mM EDTA [pH8], 0.025% xylene cyanol FF, 0.025% bromophenol blue). The samples were loaded into the wells using a 10µl Drummond sequencing pipette (Drummond Laboratories, USA). Electrophoresis was carried out for 3-4 hours at 1,700-2000V (until the marker dye had migrated to the desired distance) with the gel around the optimum temperature of 50°c. The gel was removed from the plate and transferred onto 3MM filter paper (Heto Laboratory Equipment, Camberry, UK) and dried on a vacuum/heated gel drying system (Heto Laboratory Equipment, Camberry, UK) at 80^{0}C for approximately 1-2 hours.

5.6 **Autoradiography:**

The gels were autoradiographed following drying the drying proceedure. The exposure was performed in a Cronex cassette between Cronex lightening plus intensifying screens. The filters were covered with a sheet of X-ray film, Kodak XLS5, whilst in a dark room and the cassette firmly closed. The film was exposed at -80^{0}C for 12-72 hours. Usually after 16 hours the film was developed using X-ray developer, indicator stop-bath solution and liquid fixer (Kodak Ltd, Exograph Image Systems, Jetbury, UK). This confirmed the specific activity of the labelled probe giving a readable result or predicting the length of total exposure to X-ray film required. If necessary a second sheet of X-ray film was placed in the cassette and left for a further period. The polymorphic DNA bands were identified and sized according to the distance they travelled during electrophoresis.

Genotypes for each subject were assessed by comparison to known standards previously elucidated and allele frequencies for each group calculated.

5.7 **Statistics:**

Fisher's exact tests were employed, other than in the multi-sided tables for genotypes in some analyses where the Chi squared (χ^2) test was employed, see results chapter. As the 2,4 genotype had previously been associated with AP of alcoholic aetiology a direct comparison for this genotype in AICP versus Controls was made. Similarly we performed a direct comparison of CP versus controls (i.e. predisposition to disease) for allele frequency for 1,2 genotype as it was associated with severity (organ failure score >2) previously (Smithies, A. M., 2001).

Multisided contingency tables were employed for other initial comparisons between groups for all alleles and genotypes, sub-group analysis only employed if a statistically significant result was seen in the initial comparison. A p value of <0.05 was taken as significant.

MATERIALS AND METHODS:

CYSTEINYL LEUKOTRIENE RECEPTOR ANTAGONIST TRIAL IN CHRONIC PANCREATITIS

Medical treatment of chronic pancreatitis is an area of contention. Some reasonable and widely used therapies lack evidence, e.g. acid suppression and enzyme supplementation (Warshaw, A. L. et al., 1998). Whilst other potentially useful therapies remain to be more fully evaluated (Warshaw, A. L. et al., 1998).

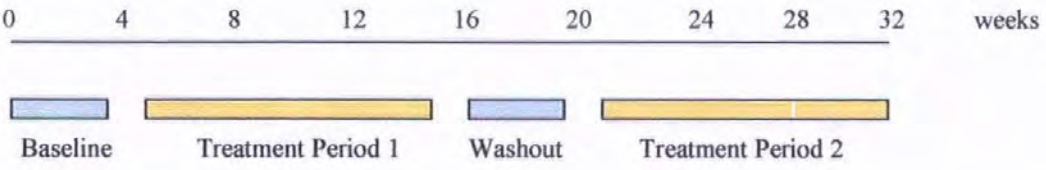
This study was performed in collaboration with not only my supervisor Professor Andrew Kingsnorth but also my colleagues Derek O'Reilly and Christine Porter; in addition considerable statistical input was made by Dr David Wright of the Mathematics and Statistics Department of the University of Plymouth.

6.1 **Trial Design and Subjects:**

A placebo-controlled, double-blind, cross-over trial of 8 months duration. (Figure 6.1) was designed. This constituted a one month baseline, followed by three months in treatment arm A, a month of 'wash out' and three months in treatment arm B. Patients with CP, late or end stage by the Zurich criteria (Ammann, R. W., 1997), and suffering regular bouts of, or continuous, pancreatic pain, were recruited.

Analysis suggested a sample of 30 patients would give a power of 90% in a placebo controlled, double blind cross-over study to detect a decrease in VAS of pain of 20%. After 6-8 months of recruitment it became evident that at our single centre it would not be possible to recruit this number who would remain in trial, 30 of our patients attending our

clinic replied positively to recruitment letters, of these 23 attended and were recruited. We therefore recalculated the power based on the VAS variability found in those patients thus far recruited. This suggested a power of 80% for 16 patients, we thus persisted to analyse the data which would be available from our centre. Five dropped out of the trial within the first 1 to 2 months, failing to attend after repeated call for follow up, that is without completion of a month of therapy. Of the remaining 18 three withdrew; one due to a new abdominal pain on placebo, one due to rash on active treatment and a further participant for personal reasons whilst on placebo. Fifteen remained in the study throughout. Of the 15 who completed the trial 7 had definite alcohol induced chronic pancreatitis (on the Zurich Criteria), 6 idiopathic chronic pancreatitis (although one of these had had moderate alcohol consumption) and 2 hereditary pancreatitis.



Treatment Arm: AB received active treatment in Period 1.
BA received placebo in Period 1.

Figure 6.1. Study Design.

6.2 **Outcome Measures:**

Primary outcome measure was pain as measured by visual analogue pain scores (VAS). These were recorded daily for one month after enrolment and prior to medication; three months of treatment, the one month wash out period and a further three month treatment arm. VAS scores were from an unmarked linear scale of 10 cm. Also, daily analgesics diaries (AD) were completed throughout the trial.

In addition, Quality of Life Questionnaires (QLQs) were completed monthly. It has been suggested that all clinical trials in CP should include Quality of Life assessment (Warshaw, A. L. et al., 1998) and we employed the QLQ advocated by Glasbrenner and Adler (Glasbrenner, B. & Adler, G., 1997) based on the European Organisation for Research and Treatment of Cancer core questionnaire of 30 questions with a disease specific supplement of 20 questions (Figure 6.2), which was originally employed in cancer but has been employed and validated in CP (Bloechle, C. et al., 1995; Buchler, M. et al., 1995).

Inflammatory markers employed were baseline C-reactive protein (CRP) and plasma secretory Tumour Necrosis Factor-Receptors (sTNF-R) were taken immediately prior to and following each arm of treatment, following unblinding of the study these were then measured for pre- and post- the active treatment arm. CRP was serum values processed routinely in Derriford Hospital, Department of Microbiology. CRP is a standard marker of inflammation and has been shown to elevated in AP and to be indicative of the severity of such an attack (Buchler, M. et al., 1986; Mayer, A. D. et al., 1984). Soluble TNF-Receptors (sTNF-R) have been shown to be elevated in AP (Kaufmann, P. et al., 1997) and also in CP (Hanck, C. et al., 1999a) where they have also found at increased levels in peripheral blood mononuclear cells (Hanck, C. et al., 1999b). The sTNF-Rs are soluble forms of the two known TNF receptors (respectively 55 and 75 kilo Daltons in molecular weight, (Tartaglia, L. A. & Goeddel, D. V., 1992)) detectable in the serum of healthy

individuals and consistent over time (Aderka, D. et al., 1992). They may be independently regulated (Spinas, G. A. et al., 1992). However, they are of themselves anti-inflammatory in nature, circulating and 'mopping up' TNF (Porteu, F. & Nathan, C., 1990).

		No	Yes
1.	Do you have any trouble doing strenuous activities, like carrying a heavy shopping bag or a suitcase?	1	2
2.	Do you have any trouble taking a *long* walk?	1	2
3.	Do you have any trouble taking a *short* walk outside the house?	1	2
4.	Do you have to stay in bed or a chair for most of the day?	1	2
5.	Do you need help with eating, dressing, washing yourself, or using the toilet?	1	2
6.	Are you limited in any way in either doing your work or doing household jobs?	1	2
7.	Are you completely unable to work at a job or to do household jobs?	1	2

During the last week:		**Not at all**	**A little**	**Quite a bit**	**Very much**
8.	Were you short of breath?	1	2	3	4
9.	Have you had pain?	1	2	3	4
10.	Did you need to rest?	1	2	3	4
11.	Have you had trouble sleeping?	1	2	3	4
12.	Have you felt weak?	1	2	3	4
13.	Have you lacked appetite?	1	2	3	4
14.	Have you felt nauseated?	1	2	3	4
15.	Have you vomited?	1	2	3	4
16.	Have you been constipated?	1	2	3	4
17.	Have you had diarrhea?	1	2	3	4
18.	Were you tired?	1	2	3	4
19.	Did pain interfere with your daily activities?	1	2	3	4
20.	Have you had difficulties in concentrating on things, like reading a newspaper or watching television?	1	2	3	4
21.	Did you feel tense?	1	2	3	4
22.	Did you worry?	1	2	3	4
23.	Did you feel irritable?	1	2	3	4
24.	Did you feel depressed?	1	2	3	4
25.	Have you had difficulty remembering things?	1	2	3	4
26.	Has your physical condition or medical treatment interfered with your *family* life?	1	2	3	4
27.	Has your physical condition or medical treatment interfered with your *social* activities?	1	2	3	4
28.	Has your physical condition or medical treatment caused you financial difficulties?	1	2	3	4

For the following questions please circle the number between 1 and 7 that best applies to you.							
29.	How would you rate your overall physical condition during the past week?						
	1	2	3	4	5	6	7
	Very poor						Excellent
30.	How would you rate your overall quality of life during the past week?						
	1	2	3	4	5	6	7
	Very poor						Excellent

During the last week:		Not at all	A little	Quite a bit	Very much
31.	Was your physical power decreased?	1	2	3	4
32.	Did you have to change your eating habits?	1	2	3	4
33.	Did you lose weight?	1	2	3	4
34.	Did you have fever or shivering?	1	2	3	4
35.	Did you notice yellow staining of your eyes or skin?	1	2	3	4
36.	Did you feel bloated?	1	2	3	4
37.	Did you feel thirsty more often than usual?	1	2	3	4
38.	Did you feel sudden colicky pain?	1	2	3	4
39.	Did you feel a lack of appetite?	1	2	3	4
40.	Have you felt itching?	1	2	3	4
	Did you feel pain				
41.	before or after eating?	1	2	3	4
42.	in straight body position?	1	2	3	4
43.	in your belly, radiating to the back?	1	2	3	4
44.	at other sites?	1	2	3	4
	If yes,				
45.	Did you take analgesics?	1	2	3	4
46.	If yes, did they help you?	1	2	3	4
47.	How much has the medical treatment burdened you?				
	1 2 3 4 5 6 7 Not at all Very much				
48.	In what way has the medical treatment burdened you?				
49.	Besides the medical treatment, what has helped you the most so far?				
50.	How confident are you regarding your health for the near future?				
	1 2 3 4 5 6 7 Hardly confident Very confident				

Figure 6.2: Quality of Life Questionnaire.

6.3 **Laboratory analysis of samples:**

Plasma samples were taken for sTNF-R values; whole blood was diluted one in 2, with phosphate buffered saline, prior to centrifugation for removal of lymphocytes (for an additional study) and plasma extraction. This dilution factor, dependent on packed cell volume, should be normalized as paired samples were used and paired statistical analysis. This is a slight weakness dependent on the initial collection of samples for additional studies with no plain serum stored. Analysis employed a commercially available Enzyme Linked Immuno-Sorbent Assay (ELISA) (Quantikine, R and D Syatems,Abingdon, UK), separate antibodies are employed for sTNF-Rs 1 and 2.

6.3.1 **Biochemistry of the Assay:**

This is a sandwich based ELISA with antibody to the antigen being measured (sTNF-RI or sTNF-RII) coated on the wells. After fixing the sample sTNF-R to the well an enzyme linked polyclonal antibody for the antigen is added and then the unbound reagent washed off. The 'colour reagents' added contain the substrates (stabilised hydrogen peroxide and tetramethylbenzidine) for the enzyme and the catalysed reaction produces colour change proportional to the amount of enzyme present.

50 µl of assay diluent RD1M is added to each well (coated with murine monoclonal antibody to sTNF-RI and –RII respectively) then 200 µl of standard or sample and incubated for 2 hours at room temperature. The standard is a serial dilution of sTNF-RI and -RII respectively. The sample is a 10 fold dilution of the above plasma. All samples and standards are performed in duplicate.

After incubation each well was aspirated and washed (for a total of three washes) with 400µl of wash buffer. 200 µl of sTNF –RI/II conjugate was then added to each well and incubated for a further 2 hours at room temperature. The aspiration/wash step then

repeated. 200 µl of substrate solution was added to each well and incubated for 20 minutes at room temperature, protected from light. The reaction was then stopped with 50 µl of 'stop solution' (sulphuric acid).

The optical density was then determined (within 30 minutes) using a microplate reader (Dynatech Immunoassay System, Dynatech Laboratories) set to 450 nm, with wavelength correction set to 550 nm.

6.4 **Statistics:**

Mean VAS scores were taken as the primary outcome measure. Statistical comparison of VAS for analysis of cross-over employed t-tests, as did comparison of scores to baseline. Again crossover analysis for the QLQ employed t-test after analysis in the fashion validated for the QLQ. Paired t-tests were used to analyse the plasma sTNF-Receptor results (differences being normally distributed).

MATERIALS AND METHODS:

ARACHIDONIC ACID METABOLITES

7.1 Urinary levels of Arachidonic Acid metabolites:

For this work sample collection was at Derriford Hospital. Due to our laboratory not being licensed for tritiated (^3H) compounds the laboratory work was performed in the Department of Clinical Immunology, Royal Free Hospital, London under the guidance of Siobhan O'Sullivan.

7.1.1 Subjects:

Urine was collected from two groups of patients, each with age, sex and ethnically matched healthy controls. The matched controls age was within 1 year for those under 35; 2 years for 35-50 years and 5 years over 50.

Those with AP had a clinical diagnosis of AP and a serum amylase level >3x the upper limit of the normal range and totalled 19. Four had severe disease and 15 mild (Bradley, E.L.III, 1993); APACHE II scores 0 to 12 and Acute Physiology Scores 0 to 7. Table 7.1. Urine was collected within 72 hours of onset of pain and 48 hours of admission to hospital.

Those with CP totalled 19. Each having late or end stage disease by the Zurich criteria (Ammann, R. W., 1997a). The aetiology was alcoholic in 12 (all denied recent alcohol consumption) and idiopathic in 7.

The urine was collected, stored temporarily at 4^0c, then centrifuged at 2000rpm for 3 minutes, to remove particulate matter, it was then stored at -80^0c until thawed for the analysis.

Age (years)	Sex	Aetiology	Severity	APS	APACHE II
22	F	GS	M	0	0
27	M	GS	M	4	4
34	F	GS	M	5	5
35	M	Ethanol	M	3	3
41	F	GS	M	1	1
50	F	GS	M	1	3
51	F	GS	M	0	2
52	M	GS	M	3	5
57	F	GS	M	2	5
59	F	GS	M	1	4
64	F	I	M	3	6
70	M	I	S	4	12
72	F	I	S	4	9
72	F	GS	S	4	9
73	F	GS	M	1	6
75	M	GS	M	3	9
80	M	I	S	7	12
80	M	I	M	6	11
92	F	GS	M	1	7

Table 7.1. Subjects in urine studies with Acute Pancreatitis.
Sex- F = female; M = male.
Aetiology- GS = gallstones; I = idiopathic.
APS- Acute Physiology Score
APACHE II- Acute Physiology and Chronic Health Evaluation

7.1.2 **Sample Purification:**

Prior to analysis of the samples, using Enzyme Immuno-Assays (EIA), purification is performed to remove contaminants which might interfere with the assay.

For the two PG studies to measure the percentage loss during purification the sample was first spiked with a tritiated PG (in this case $PGF_{2\alpha}$ was used). 5ml of urine was aliquoted into falcon tubes into each was added approximately 10,000 cpm of tritium-labelled $PGF_{2\alpha}$ which is then mixed thoroughly by vortexing. A sample of this is then removed and spotted onto blotting paper and read for scintigraphy count. Further scintillation counting is then performed on the eluted sample and the fraction of loss calculated.

For the PGs a 6 ml C-18 SPE cartridge (Cayman Chemicals, USA) was used for the purification. It was first activated by rinsing with 5 ml methanol and then with 5 ml water. The tritiated urine sample was then passed through the SPE cartridge. Which was then rinsed with a further 5 ml of water and 5ml of HPLC grade heptane. The eicosanoids are then eluted from the column with methanol. Each step was performed under a gentle vacuum. The methanol was then 'blown off' by evaporation under a stream of dry nitrogen and the PGs resuspended in 500 µl of EIA Buffer by vortexing.

Whether LTE_4 analysis involves significant crossover interaction with other molecules in the urine is an area of debate. It has been shown that although immunologically based techniques may detect a lesser proportion of urinary LTE_4 this is consistent (Kumlin, M., 1997). Therefore purification is arguably unnecessary for paired samples on the same patient, it can also be argued that purification is unnecessary if serial dilutions give consistent results. As samples were unpaired the urine here was purified and a single serial dilution performed on all samples to confirm lack of gross contamination.

This employed the commercially available cysteinyl-LT affinity sorbent resin (Cayman Chemicals, USA) (Westcott, J.Y. et al., 1998). Following the standard protocol: 25µl of resin was added to 1 ml of urine and mixed gently for 30-60 minutes. Briefly centrifuged at 500 x g to sediment the resin. The supernatant was decanted and the pelleted resin washed once with 1 ml of PBS. The resin was resuspended in 0.5 ml of cold methanol and vortex briefly for 5 minutes then centrifuged to sediment the resin and the methanol collected; this wash step is repeated. The methanol washes will displace the adsorbed cys-LTs from the resin. The methanol is again 'blown off' under nitrogen and the LTs resuspended in 500µl EIA buffer.

7.1.3 **Molecules measured in the EIAs:**

All three EIAs employ the same standard protocol with the exception that an initial step of derivitisation is required for the preparation of bicyclo-PGE_2.

LTE_4 is the final cysteinyl-LT in that pathway, is stable in the urine and is measured as representative of the bodies production of cysteinyl-LTs.

In order to measure production of PGD_2 9-alpha,11-dehydro-PGF2 ($PGF2\alpha$) was measured, as discussed this is the primary breakdown product of PGD_2 in the urine and has been shown to be a measure of both PGD_2 and of mast cell activation.

Bicyclo-PGE_2 is not a naturally occurring PG, however it is a stable product of the conversion of all PGE_2 metabolites. PGE_2 is rapidly converted in vivo to it's 13,14-dihydro-15-keto metabolite of which 90% is cleared by the lungs in its first pass; this product is not stable. It and its product 13,14-dihydro-15-keto PGA_2 (both excreted in the urine) are both converted to the stable bicyclo-PGE_2 by reaction with Na_2CO_3. Thus an initial step in the preparation for this EIA is the 'derivatisation'. Both the bicyclo-PGE_2

standard and samples are incubated with carbonate buffer over night, then phosphate buffer and bicyclo-EIA buffer (for dilution).

7.1.4 **Enzyme Immuno-Assay:**

Three Enzyme-Immuno Assays (EIA) were employed, they are commercially available kits from Cayman Chemicals (USA).

Each employs the same basic concept of competition, between the relevant eicosanoid and an acetylcholinesterase (AChE) conjugate of that eicosanoid, for a limited number of rabbit antiserum binding sites, specific for the molecule being measured. Because the concentration of conjugate is held constant while the concentration tested varies, the amount of conjugate that is able to bind to the rabbit antiserum will be inversely proportional to the concentration of eicosanoid in the well, for a portion of its reaction curve. This rabbit antiserum-eicosanoid (either free or conjugate) complex binds to the rabbit IgG mouse monoclonal antibody that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's Reagent (which contains the substrate to AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow colour and absorbs strongly at 412 nanometres (nm). The intensity of this colour, determined spectrophotometrically, is proportional to the amount of conjugate bound to the well, which is (as stated) inversely proportional to the amount of free eicosanoid present in the sample well during the incubation.

7.1.5 **Biochemistry of AChE EIAs:**

The electric organ of the electric eel, *Electrophorus electricus*, contains an avid AChE capable of massive catalytic turnover during the generation of its electrochemical discharges. The electric eel AChE has a clover leaf-shaped tertiary structure consisting of a triad of tetramers attached to a collagen-like structural fibril. This stable enzyme is capable

of high turnover (64,000 per second) for the hydrolysis of acetylthiocholine. A molecule of the analyte (eicosanoid) covalently attached to a molecule of AChE serves as the tracer in these AChE based enzyme immunoassays.

Ellman's reagent consists of acetylthiocholine and 5,5'-dithio-*bis*-(2-nitrobenzoic acid). Hydrolysis of acetylthiocholine by AChE produces thiocholine. The non-enzymatic reaction of thiocholine with 5,5'-dithio-*bis*-(2-nitrobenzoic acid) produces 5-thio-2-nitrobenzoic acid, which has a strong absorbance at 412 nm. AChE has several advantages over other enzymes commonly used for enzyme immunoassays: Unlike horseradish peroxidase, AChE does not self-inactivate during turnover. This property of AChE also allows multiple development of the assay if it is accidentally splashed or spilled. In addition, the enzyme is highly stable under the assay conditions, has a wide pH range (pH 5-10), and is not inhibited by common buffer salts and preservatives. Since AChE is stable during the development step, it is unnecessary to use a "stop" reagent, and the plate may be read whenever it is convenient. Plates are pre-coated with mouse monoclonal antibody and blocked with a proprietary formulation of proteins.

50 µl samples are added to the test well (each in duplicate) with a 1 in 10 dilution performed in addition to the main (purified) sample. This enables identification of contamination and allows a result, which falls in the linear and reliable portion of the concentration absorbance curve, to be more easily obtained at 'first run'.

EIA Buffer was added to Non-Specific Binding wells (100µl) and to Maximum Binding wells (50µl). Two fold serial dilutions of the PG standard, were added to duplicate standard wells (50µl). 50 µl of AChE labelled eicosanoid, was then added to all wells

except the Total Activity and the Blank wells and 50 µl of eicosanoid anti-serum to each well except the Total Activity, the Non-Specific Binding and the Blank wells.

The plate is then covered with a plastic film and incubated at room temperature in the dark for 18 hours prior to developing. The wells are emptied and washed five times with wash buffer, then 200µl of Ellman's reagent is added to each well and, in addition, 5 µl of AChE labelled eicosanoid.

This is then allowed to develop for 60 minutes (with a maximum binding reading of approximately 0.2 A.U. with blank subtracted). The plate was read at a single wavelength between 405 and 420 nm.

Thus the blank wells have and measure only the Ellman's reagent absorbance. The non specific binding wells have AChE labelled eicosanoid, no sample activity to compete but also no anti-sera to bind the eicosanoid and thus represent only non-specific binding of AChE labelled eicosanoid to the well. The maximum binding wells have AChE labelled eicosanoid, no sample to compete with that and anti-sera such that all anti-sera sites should bind AChE labelled eicosanoid. The total activity wells have AChE labelled eicosanoid that has not been washed and therefore represent the total activity of that enzyme in the development.

7.1.6 **Analysis of Results:**

All results are analysed as PG/LT per unit creatinine to normalise for urine dilution. The urine creatinines were evaluated by the Derriford Hospital Combined Laboratory.

7.1.7 **Statistics:**

Unpaired t-tests were used to analyse the data if the data did not significantly differ from a normal distribution (Shapiro-Wilk test). If the data was not normally distributed a non-parametric test (Mann-Whitney U) was used. The results were also assessed for equality of variance.

7.2 **Genetic Studies:**

Two studies were performed looking at the A(– 444)C LTC_4 synthase polymorphism. One in AP and one in CP. The difference in urinary LTE_4 levels discussed in Results Chapter 10, suggested a possible genetic component to levels in AP and thus we studied this known functional polymorphism in the first dedicated enzyme in the cysteinyl LT pathway. In CP a pilot study showed a significant difference in ICP and a, non-significant, difference in AICP; thus the analysis was also performed in CP.

7.2.1 **Subjects:**

Acute pancreatitis samples were taken from patients with a clinical diagnosis of AP and an amylase level greater than 3x the upper limit of the normal level. Aetiology was based on history, examination and investigations. For the purposes of this studied aetiologies were gall stones, alcohol (a history of ethanol consumption of greater than 80g per day for 6 months or more) or idiopathic (which also includes other minor causes such as hyperlipidaemia). ERCP induced AP was excluded. AP patients were further subdivided by severity; based on organ failure or local complications (Bradley, E. L., III, 1993). One hundred and sixty nine patients had mild disease (MAP, 71%) and sixty nine had severe disease (SAP, 29%). One hundred and forty were females (59%). Aetiologically 141 had gallstones (60%); 50 (21%) alcohol and 48 (20%) idiopathic. Age range was 19 to 91 years; with a median of 60 years.

In CP, samples on 57 Caucasoid patients, with late- or end-staged CP based on the Zurich Criteria for Alcohol Induced Chronic Pancreatitis (Ammann, R. W., 1997b) were collected. Aetiologically sub-divided as alcohol induced (41 patients) or idiopathic (16 patients). Females were 19 in number (33%). Age range 27 to 87 years, median 53 years.

Controls were cord blood samples and totalled 103, 60 (58%) of whom were females.

7.2.2 **Polymerase Chain Reaction:**

DNA amplification was performed for the promoter region of LTC_4 synthase, employing amplimers 5'-TCC ATT CTG AAG CCA AAG GC- and 5'-GTG ACA GCA GCC AGT AGA GC- (after Sanak et al.(1997)). The standard reaction settings were again used with the optimised annealing temperature found to be 59^0C, with a magnesium concentration of 2mM.

7.2.3 **Restriction Endonuclease digestion:**

The PCR products were subjected to digestion by the restriction endonuclease Msp I (Roche Diagnostics, Lewes, UK) in a total volume of 25µl. The polymorphism removes a cutting site for the enzyme MspI and thus the PCR product can be subjected to digestion and the frequency of the polymorphism analysed (Figure 7.1).

The digestion mixture mix included: 18µl PCR product, 3.5µl water, 2.5µl endonuclease buffer and 1µl (5 units) of enzyme Msp I at 5000unitsml^{-1}. The digest was performed over 4 hours at 37^0c. To confirm complete digestion analysed on a 1.5% agarose gel stained with 0.01%v/v ethidium bromide, viewed under Ultraviolet light and compared to 50 and 100 bp molecular weight ladders (Roche Diagnostics, Lewes, UK). Digestion was optimised to take 4 hours with samples run on gels after 1 and 16 hours also, complete digestion was present in the majority of samples tested at 1 hour and all samples at 4 and

16 hours. A consistent cutting site for Msp I in the PCR product is also present, this acted as an internal control showing complete digestion as no 'full length' 563 bp product should remain.

7.2.4 **Statistics**

Two sided Fishers' Exact tests were used throughout (SPSS 9.0, SPSS Inc., Chicago, Ill., USA). A p value of <0.05 was taken as significant. Where appropriate multisided contingency tables were employed initially for comparisons with sub-group analysis only when a statistically significant result was seen in the initial comparison.

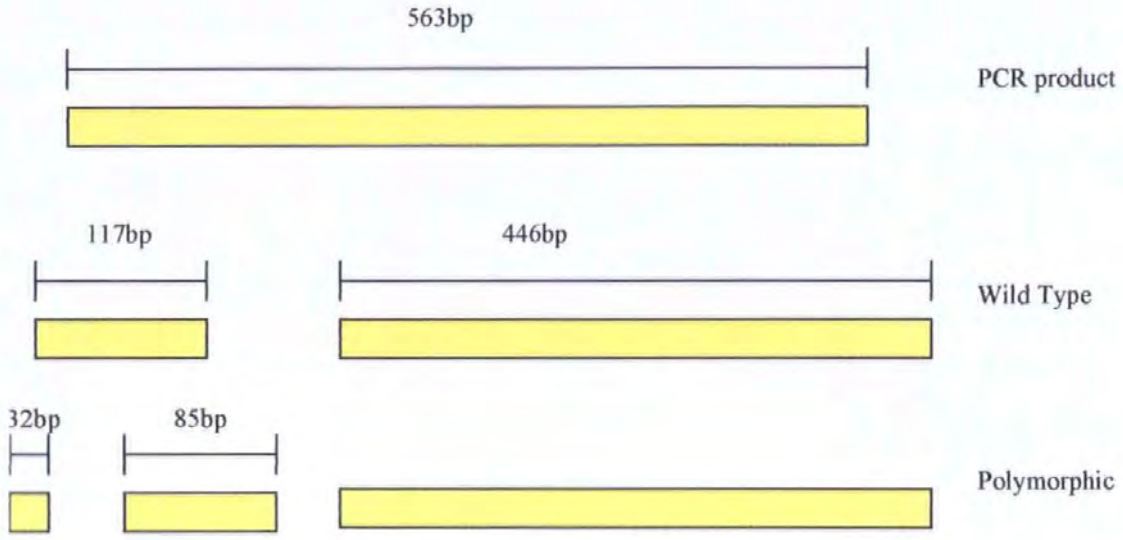


Figure 7.1. PCR product of LTC_4 synthase promoter region with separation of fragments at Msp I cutting sites in the Wild Type and Polymorphic forms.

RESULTS:

CYTOCHROME P450 2E1

Results are displayed in Tables 8.1 to 8.3. Of 208 normal controls 12 (5.8%) had the insertion polymorphism (Figure 8.1), and all were heterozygotes. In contrast, 5 of 239 ALC subjects (2.1%) had the insertion. Of these, in the AICP group two subjects had the insertion and one of these was homozygous for the insertion. In the AC group three subjects had the insertion. In addition, one AICP and one AC had a band which would correspond to the 48 base pair deletion found in GenBank (Figure 8.2), as did two normal controls.

Results from the British and German groups, all European Caucasoids, were similar: Three of the 138 German subjects (2.2%) and 2 of the 102 British (2%); two of the 32 German AC, 1 of the 35 British AC, 1 of the 106 German AICP and 1 of the 39 British AICP had the insertion.

Analysis in the first instance is with regard to presence or absence of the 96bp insertion. This is used as the primary outcome as the paper demonstrating a functional effect of the polymorphism was that the effect was associated with presence of the polymorphism (McCarver, D. G. et al., 1998). Further analysis is of the genotypes for the insertion. Analysis for the deletion, not known to be associated with a functional effect, is not performed.

Comparing ALC to normal controls for presence of the insertion polymorphism, showed that it was less abundant in the former than the latter ($p=0.049$), table 8.2. The same comparison for genotype of the insertion was comparable ($p=0.030$), table 8.3.

Employing Fisher's exact test for presence of the insertion a three by two contingency table of normal controls, AC and AEOD again revealed a statistically significant difference ($p=0.045$), table 8.2. Again, analysis for insertion genotypes was comparable ($p=0.011$), table 8.3.

To delineate whether the difference was for alcoholism per se or end organ disease we further analysed sub-groups. Comparing AC with NC and comparisons within ALC subgroups analysis did not reveal any significant differences, as might be expected with such a low frequency in the patient groups. Although genotype analysis for those with end-organ disease compared with alcoholic controls approached significance with $p=0.068$.

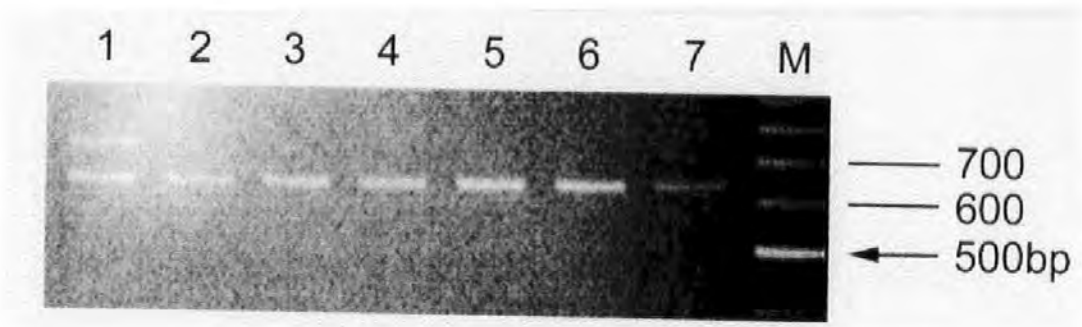


Figure 8.1: Agarose gel showing wild type homozygotes (633 b.p., lanes 2-7) and a heterozygote for the insertion polymorphism (729 b.p., lane 1). Run alongside a molecular weight marker (M).

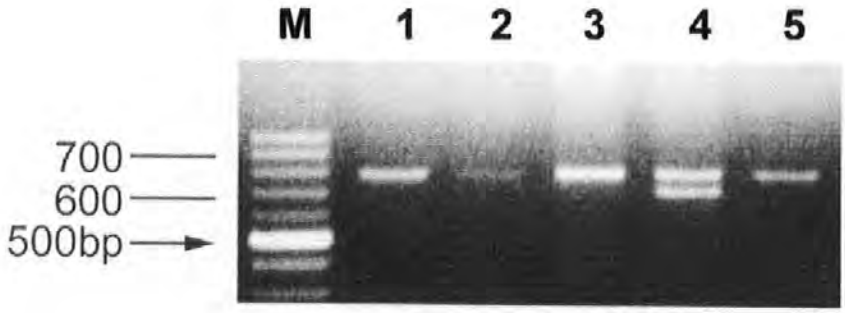


Figure 8.2: Agarose gel showing wild type homozygotes (633 b.p., lanes 1-3 and 5) and a heterozygote for the deletion polymorphism (585 b.p., lane 4). Run alongside a molecular weight marker (M).

Patient Group	Total	Homozygote wild type (wt/wt)	Heterozygote for insertion (wt/Ins)	Homozygote for insertion (Ins/Ins)	Heterozygote for deletion (wt/Del)
Normal Controls	208	194 (69%)	12 (5.8%)	0 (0.0%)	2 (1.0%)
ALC	239	232 (97%)	4 (1.7%)	1 (0.4%)	2 (0.8%)
Of ALC:					
AC	67	63 (94%)	3 (4.5%)	0 (0.0%)	1 (1.5%)
AEOD	172	169 (98%)	1 (0.6%)	1 (0.6%)	1 (0.6%)
Of AEOD:					
AICP	144	141 (98%)	1 (0.7%)	1 (0.7%)	1 (0.7%)
ALD	28	28 (100%)	0 (0.0%)	0 (0.0%)	0 (0.0%)

Table 8.1. Full genotype data on all subjects.
ALC = ICD 10.1/10.2
AC = alcoholic controls
AEOD = alcoholic end organ disease
AICP = alcohol induced chronic pancreatitis
ALD = alcoholic liver disease

Patient Group	Total	Presence of insertion polymorphism (wt/Ins and Ins/Ins)
Normal Controls[a,b]	208	12 (5.8%)
ALC[a]	239	5 (2.1%)
Of ALD:		
AC[b]	67	3 (4.5%)
AEOD[b]	172	2 (1.2%)
Of AEOD:		
AICP	144	2 (1.4%)
ALD	28	0 (0.0%)

Table 8.2. Subjects with the (high activity) insertion polymorphism.

Fisher's Exact Test: $p = 0.049$[a] $p = 0.045$[b] (χ^2 4.110 $p = 0.043$)

ALC = ICD10.1/10.2
AC = alcoholic controls
AEOD = alcoholic end organ disease
AICP = alcohol induced chronic pancreatitis
ALD = alcoholic liver disease

Patient Group	Total	*Genotype of Insertion Polymorphism*	
		Heterozygote (wt/Ins)	Homozygote (Ins/Ins)
Normal Controls[a,b]	208	12 (5.8%)	0(0.0%)
ALC[a]	239	4 (1.7%)	1(0.4%)
Of ALC:			
AC[b,c]	67	3 (4.5%)	0(0.0%)
AEOD[b,c]	172	1 (0.6%)	1(0.6%)
Of AEOD:			
AICP	144	1 (0.7%)	1(0.7%)
ALD	28	0 (0.0%)	0(0.0%)

Table 8.3. Insertion Polymorphism Genotypes.

Fisher's Exact Test: $p = 0.030^a$
$p = 0.011^b$
$p = 0.068^c$

ALC = ICD 10.1/10.2
AC = alcoholic controls
AEOD = alcoholic end organ disease
AICP = alcohol induced chronic pancreatitis
ALD = alcoholic liver disease

RESULTS:

INTERLEUKIN 1 ALPHA

The full results, in terms of both alleles and genotypes, are displayed in Tables 9.1 and 9.2, respectively. Whilst a PAGE picture representing genotypes is seen in Figure 9.1.

9.1 **Direct Comparisons based on Acute Pancreatitis Results:**

The specific comparison of NC versus all CP for the 1,2 genotype (Figure 9.2), which Smithies study (Smithies, A. M., 2001) implicated in worse organ severity score in AP, did not show a significant difference (Fisher's exact test). The 1,2 genotype occurring in 23% and 17%, respectively. While the comparison of AICP, ICP and NC (18%, 13%, 23% respectively) was again not significant. Thus the weak suggestion in the AP study of an association with severity does not translate to a predisposition to CP.

The other specific question addressed was whether the significant association between the 2,4 genotype and alcohol induced AP was also seen in AICP, Figure 9.3. This genotype was found in 12% of controls and 18% of subjects with AICP which is not a statistically significant difference (Fisher's exact test).

9.2 **Alleles:**

The common alleles 1,2 and 4 occurred in control (NC) and patient (AIPC, ICP) groups in 37%, 36% and 38% for allele 1 (respectively); 33%, 29% and 34% for allele 2 and 17%, 24% and 16% for allele 4. There are no significant differences for the multi-sided table of NC versus CP. Comparison with aetiological subdivisions and all alleles was again non-significant (χ^2 test). Thus simple direct comparisons were not performed further.

9.3 **Genotypes:**

The genotypes also showed no overall statistically significant difference, χ^2 test p= 0.372. With aetiological subdivision of the patient groups χ^2 test p= 0.038. On this basis it is statistically valid to look at the subdivision. AICP v NC for genotypes χ^2 test p= 0.434. ICP v NC Fisher's exact p=0.084; there was no significant difference.

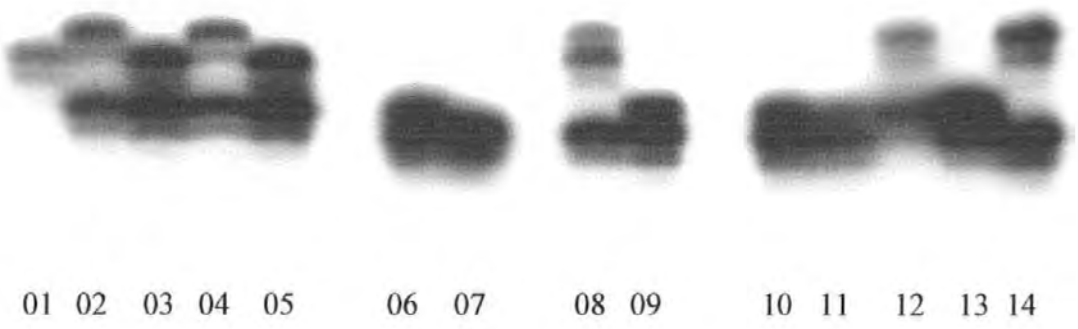


Figure 9.1. PAG autoradiograph showing a number of IL-1a polymorphism genotypes.

Allele	Normal Controls [N=300]	CP [N=108]	Of which:	
			ACIP [N=76]	ICP [N=32]
-1	0 (0.0%)	1 (0.9%)	1 (1.3%)	0 (0.0%)
0	7 (2.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
1	111 (37%)	39 (36%)	27 (36%)	12 (38%)
2	100 (33%)	33 (31%)	22 (29%)	11 (34%)
3	13 (4.3%)	3 (2.8%)	3 (3.9%)	0 (0.0%)
4	51 (17%)	23 (21%)	18 (24%)	5 (16%)
5	11 (3.7%)	7 (6.5%)	3 (3.9%)	4 (13%)
6	1 (0.3%)	1 (0.9%)	1 (1.3%)	0 (0.0%)
7	2 (0.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
8	4 (1.3%)	1 (0.9%)	1 (1.3%)	0 (0.0%)

Table 9.1. Interleukin 1a alleles in control and patients groups.

Genotype	Normal Controls [N=150]	CP [N=54]	Of which:	
			ACIP [N=38]	ICP [N=16]
1,1	18 (12%)	6 (11%)	2 (5.2%)	4 (25%)
1,2	35 (23%)	9 (17%)	7 (18%)	2 (13%)
1,4	22 (15%)	10 (19%)	9 (24%)	1 (6.3%)
2,2	19 (13%)	6 (11%)	3 (7.9%)	3 (19%)
2,4	18 (12%)	7 (13%)	7 (18%)	0 (0.0%)
4,4	2 (1.3%)	3 (5.6%)	1 (2.6%)	2 (13%)
Less common genotypes:	36 (24%%)	13 (24%)	9 (24%)	4 (25%)
-1,1	0 (0.0%)	1 (1.9%)	1 (2.6%)	0 (0.0%)
0,1	1 (0.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
0,2	6 (4.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
1,3	3 (2.0%)	2 (3.7%)	2 (5.2%)	0 (0.0%)
1,5	4 (2.7%)	4 (7.4%)	3 (7.9%)	1 (6.3%)
1,6	1 (0.7%)	1 (1.9%)	1 (2.6%)	0 (0.0%)
1,7	1 (0.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
1,8	3 (2.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
2,3	4 (2.7%)	1 (1.9%)	1 (2.6%)	0 (0.0%)
2,5	2 (1.3%)	3 (5.6%)	0 (0.0%)	3 (19%)
2,7	1 (0.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
2,8	1 (0.7%)	1 (1.9%)	1 (2.6%)	0 (0.0%)
3,4	4 (2.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
3,5	2 (1.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
4,5	3 (2.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)

Table 9.2. Interleukin 1a genotypes in control and patients groups.

RESULTS:

CYSTEINYL LEUKOTRIENE RECEPTOR ANTAGONIST TRIAL IN CHRONIC PANCREATITIS

Twenty three patients were recruited. Of these five dropped out of the within the first 1 to 2 months, failing to attend after repeated call for follow up without completion of a month of therapy. Of the remaining 18 three withdrew; one due to a new abdominal pain on placebo, one due to rash on active treatment and a further participant for personal reasons whilst on placebo.

Of these 15, seven had definite alcohol induced chronic pancreatitis (on the Zurich Criteria), 6 idiopathic chronic pancreatitis (although one of these had had moderate alcohol consumption) and 2 hereditary pancreatitis.

10.1 Visual Analogue Score Results:

In VAS crossover analysis, the primary outcome measure, a non-significant difference was seen between the groups with the marginally better outcome in the placebo arm (Figure 10.1); $t=1.51$, $p=0.156$.

In addition, when compared to baseline month neither first arm was significantly different, although these were not stated outcome measures. A non-significant decrease is seen in the mean VAS during Singulair treatment in comparison to baseline and washout for both the AB and BA groups respectively (Figure 10.2, Tables 10.1). In the crossover analysis there is a non-significant benefit for placebo. Taken with the findings of improvement in the AB and BA arms for active drug this crossover difference appears to be due to the fact that the randomisation allotted a disproportionate number of patients with the higher mean VAS to

the AB arm and the Period 2 placebo effect was thus, possibly, more exaggerated in this group. This is also seen as a decrease in the active second arm but less dramatically from a lower baseline. This is a possible explanation for the non-significantly lower scores on placebo.

Thus in the primary outcome measure there is no significant effect for active drug (or placebo).

10.1.1 **Analgesic Consumption:**

Analgesic use, as one might expect, was seen to mirror VAS. This is demonstrated when comparing graphs of analgesic consumption and VAS as in figure 10.3. With the scoring system employed those patients using opiates as first line for pain, as in patient 12, are seen to have zero or scores of 5 plus when their pain scores are anything above zero.

10.2 **Inflammatory markers:**

All baseline CRP results were ≤13.

Soluble TNF-Receptor results are laid out in table 10.2. Levels are those in the diluted plasma and thus levels are approximately half those in plasma. Analysis employing paired t-tests revealed no significant effect for treatment for sTNF-R1, sTNF-R2 and total sTNF-Rs, figure 10.4.

10.3 **Quality of Life Questionnaires:**

In view of the smaller numbers and risk of introducing type 1 error in this secondary outcome measure a simple crossover comparison was made, without sub-division to analyse the more discrete areas. Only six patients in each arm sufficient data for full analysis. The data was non-normally distributed (Shapiro-Wilks test 0.625 ($p<0.01$) and

0.5 1.1 ($p<0.01$), respectively) for both arms. Mann Whitney U test failed to show a significant difference in difference of physical scores.

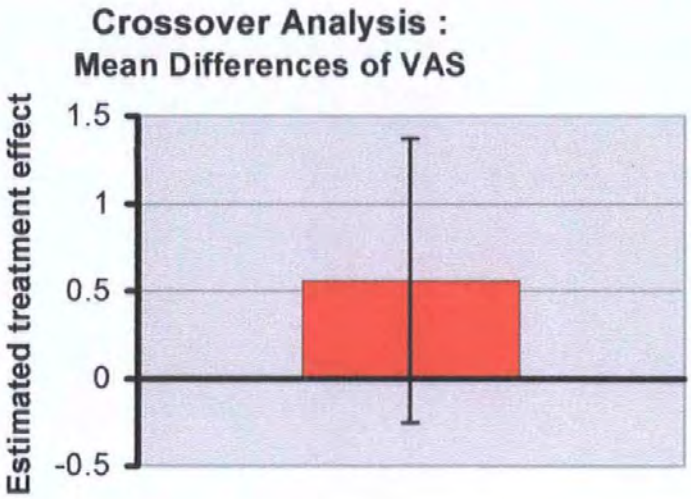


Figure 10.1. Crossover Analysis.
Error bar shows 95% confidence intervals. The error bar showing no effect. The error bar is narrow as the difference in means is around means of 1.7 and 4.3 in the two treatment arms.

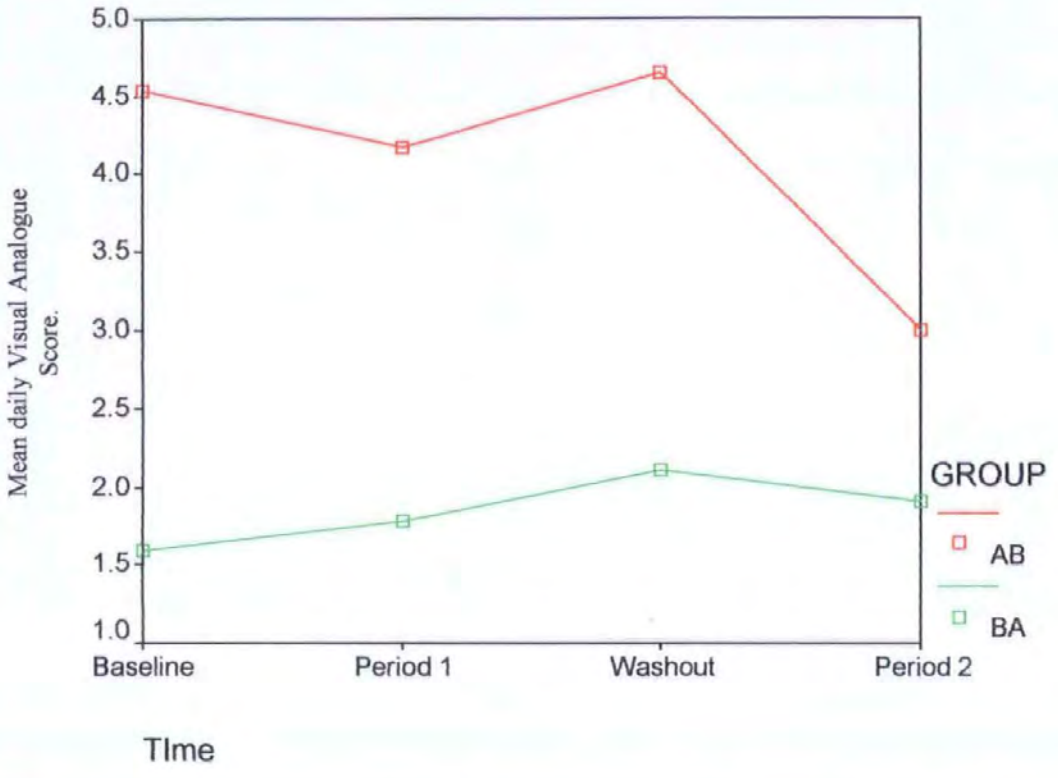


Figure 10.2. Graph of mean daily Visual Analogue Scores (y-axis) for the four periods studied. Period A is active, B placebo.

Group Statistics

	Treatment group	N	Mean	Std. Deviation	Std. Error Mean
BASE	AB	9	3.8023	2.6822	.8941
	BA	10	2.2365	2.0710	.6549
Base-Period 1	AB	8	-.2732	1.6444	.5814
	BA	8	.1829	.7737	.2735
Washout-period 2	AB	8	-1.4152	2.3045	.8148
	BA	7	-.2028	.6368	.2407
DIFF	AB	8	.9947	1.6598	.5868
	BA	7	-.1293	1.1338	.4286

Table 10.1a

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
BASE	Equal variances assumed	.341	.567	1.433	17	.170	1.5658	1.0927	-.7397	3.8713
	Equal variances not assumed			1.413	15.039	.178	1.5658	1.1083	-.7959	3.9275
Base-Period 1	Equal variances assumed	3.887	.069	-.710	14	.489	-.4561	.6425	-1.8342	.9220
	Equal variances not assumed			-.710	9.955	.494	-.4561	.6425	-1.8886	.9765
Washout-period 2	Equal variances assumed	8.401	.012	-1.342	13	.203	-1.2124	.9034	-3.1640	7392
	Equal variances not assumed			-1.427	8.202	.190	-1.2124	.8496	-3.1631	7383
DIFF	Equal variances assumed	1.304	.274	1.507	13	.156	1.1240	.7458	-.4873	2.7353
	Equal variances not assumed			1.547	12.357	.147	1.1240	.7267	-.4542	2.7022

Table 10.1b

Tables 10.1: Showing mean VAS data for each period and the t-test results of the analysis.

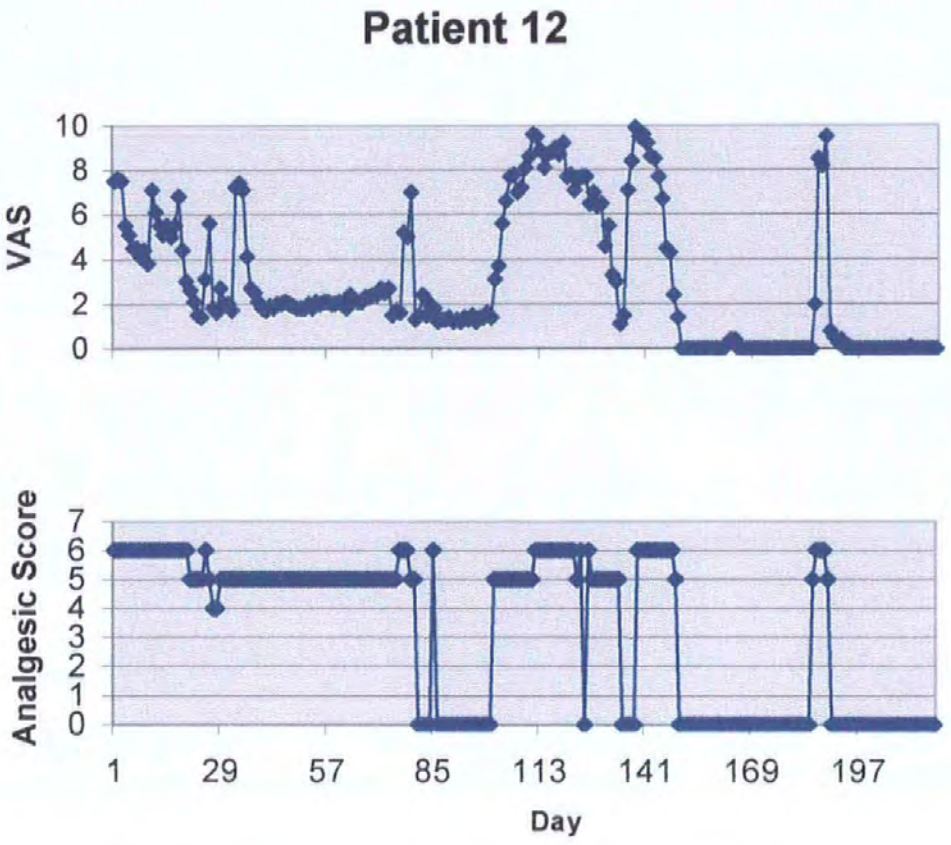


Figure 10.3. Correlation of analgesic consumption and pain typified by Patient 12, who was in arm AB (active drug in Period 1).

	sTNF-R1		sTNF-R2		Total sTNF-Rs	
Patient Number	Pre	Post	Pre	Post	Pre	Post
1	18.5	19.8	49.9	38.4	68.3	58.2
2	29.2	29.9	66.0	78.5	95.2	108.4
3	32.2	36.9	63.1	73.1	95.3	110.1
4	25.8	13.6	59.4	37.7	85.2	51.2
5	50.0	28.9	108.6	57.8	158.6	86.7
6	31.3	55.5	71.0	109.9	102.2	165.4
7	63.7	71.4	173.9	221.3	237.6	292.7
8	20.0	23.0	57.7	61.2	77.8	84.2
9	43.6	41.2	81.1	82.1	124.7	123.3
10	23.4	33.3	65.9	67.1	89.3	100.4
11	32.2	56.5	65.4	111.7	97.6	168.1
12	32.5	34.9	107.3	66.9	139.8	101.8
13	54.6	39.0	106.0	97.5	160.7	136.5
14	49.0	63.3	127.5	214.6	176.5	277.9

Table 10.2 Patients mean soluble TNF-R levels, in pg/ml.

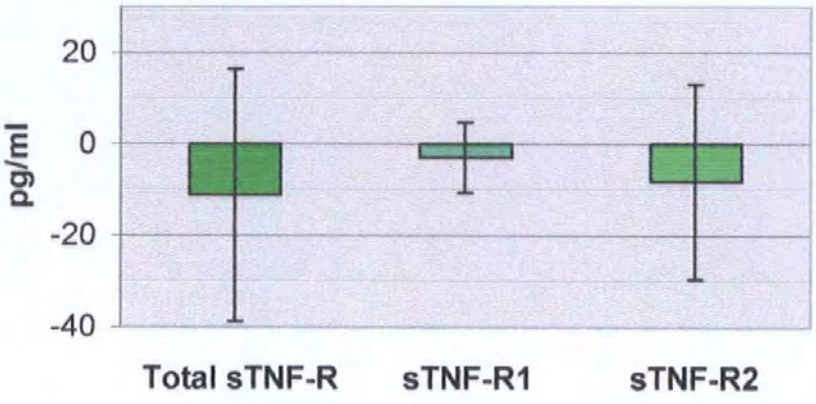


Figure 10.4. Mean paired differences in soluble TNF receptors, separately and in total. Error bars show 95% confidence intervals.

RESULTS:

ARACHIDONIC ACID METABOLITES

A pre-study power calculation revealed a power of 98% to identify a doubling of urinary metabolite level (given a normal distribution); if 12 patients and 12 controls were recruited. This based on the mean levels and standard deviation found previously in healthy volunteers (O'Sullivan, S. et al., 1996). The level of 2 fold increase was chosen as, in response to allergen, in the study cited asthmatics had a 3.5 fold increase whilst another study showed healthy volunteers have a 3 fold increase to methacholine (O'Sullivan, S. et al., 1998).

11.1 Urinary LTE_4 levels:

11.1.1 Acute Pancreatitis:

Analyses were performed based on the prospectively designed pairings this study included 15 patients and controls. The Mann-Whitney U test was employed as the data was non-normally distributed. This did not reveal any significant difference. The distribution can be seen in Box-Whisker plot in Figure 11.1. In addition, normal distribution (of the larger groups, mild and their controls) was obtained on log transformation, independent samples t-tests on this transformed data again did not attain significance.

However, Levene's test for equality of variance did show a significant difference ($p=0.003$). In figure 11.2 this can be seen graphically with higher levels seen especially a higher and longer 'tail' to the patient graph. The explanation for this could have been a variation in data as the study was not specifically designed or powered to assess such variation. However, as one explanation could have been a genetic predisposition to increased levels in the setting of an appropriate stressor this issue was addressed; on the

basis of a known genetic variation responsible for elevated LTE_4 responses in asthma, as discussed.

I felt it appropriate to confirm the result excluding smokers, as smoking has been associated with raised urinary levels (Fauler, J. & Frolich, J. C., 1997), although Cys LTs have also been shown to be lower in smokers in saliva (Wu-Wang, C. Y. et al., 1992) and no different in broncho-alveolar lavage fluid (Zijlstra, F.J. et al., 1992). In the AP group smokers constituted two of the three high 'tail' values (figure 11.2) though this was not the case for the two smoking controls. This would thus if anything weaken the possibility of a minor importance of cysteinyl LTs in AP in humans. The result in CP was again no different on excluding smokers, see below.

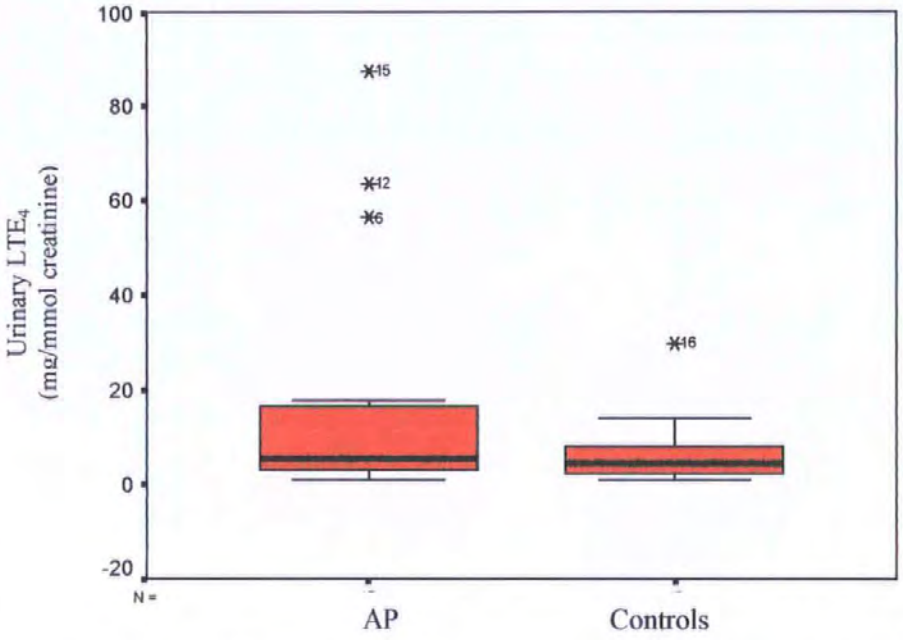


Figure 11.1: Urinary LTE_4 levels (ng/mmol creatinine) in AP. Box-Whisker plot showing median (bold line), interquartile range (box), range (whiskers) and outliers.

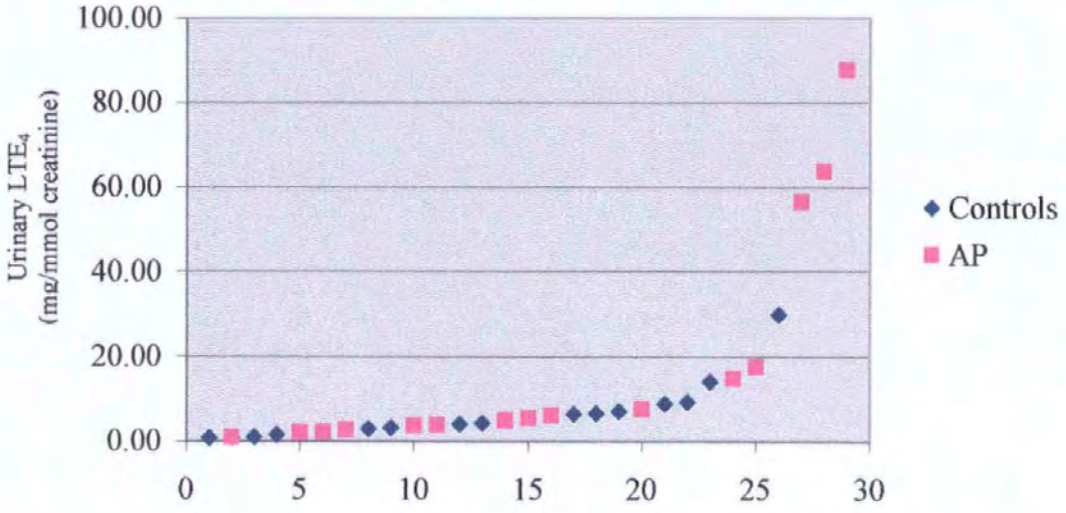


Figure 11.2: Urinary LTE_4 levels (ng/mmol creatinine) for AP.

11.1.2 **Chronic Pancreatitis:**

There was no significant difference between the 19 CP patients studied and their age and sex matched controls. This despite a pilot study of ours of 19 CP patients with unmatched healthy, young controls (numbering 16) which suggested a possible difference in ICP (5 patients) ($p= 0.04$; unpaired t-test) and non-significantly higher levels in AICP.

Interestingly, one control patient had levels considerably higher than all other subjects; on review of the control questionnaire this person had a degree of what was believed to be stress incontinence and may possibly have been interstitial cystitis which has been associated with raised LTE_4 (Bouchelouche, K. et al., 2001).

In light of this gross outlier we analysed the data both including and excluding this result (and the relevant matched CP patient). Employing the Mann-Whitney U test neither analysis showed a significant difference. In this group Levene's test did not show a significant difference of variance. Figure 11.3.

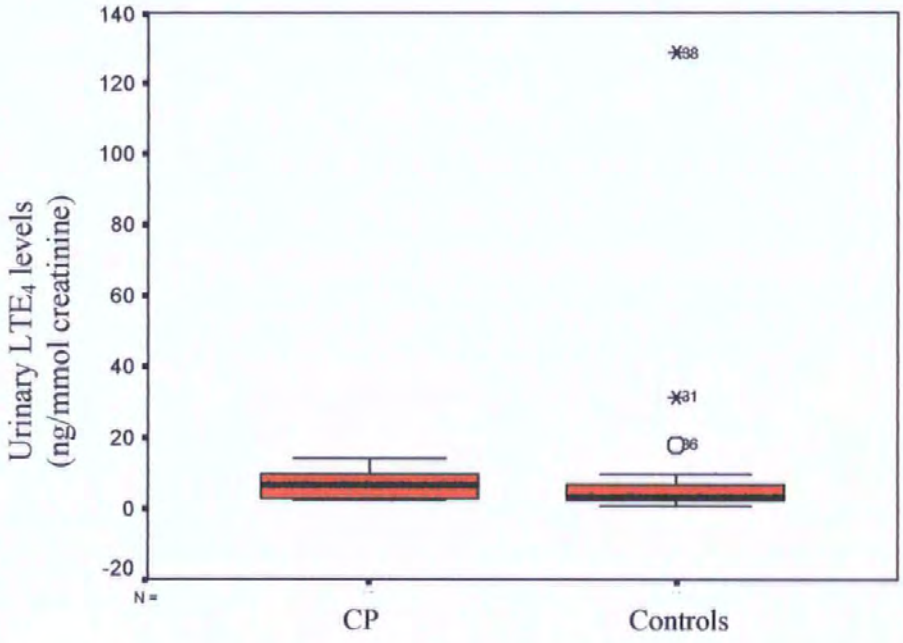


Figure 11.3: Box whisker plot of urinary LTE_4 levels (ng/mmol creatinine) in CP.

11.2 LTC_4 Synthase Genetic Studies:

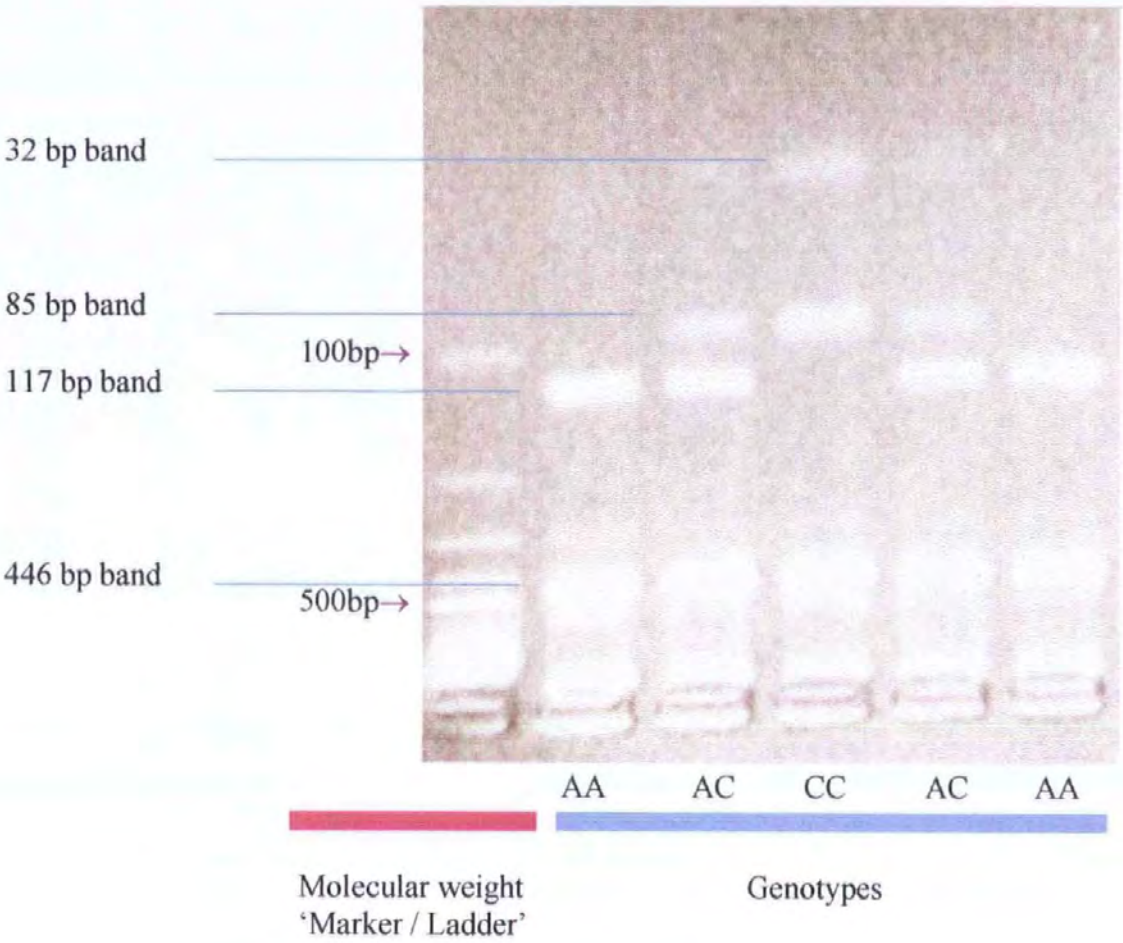


Figure 11.4: An agarose gel showing the Msp I restriction endonuclease digestion products of the LTC_4 synthase promoter region. The relevant products for each genotype are seen (see also figure 7.1).

11.2.1 **Acute Pancreatitis:**

The variation in skew in urinary LTE_4 levels implied a possible genetic component and we have studied the - 444 functional polymorphism in LTC_4 synthase, which is associated with increased cysteinyl LT production and asthma. The results are laid out in Table 11.1, showing genotypes and Table 11.2, showing alleles. As can be seen in the same proportion bars in the bar charts for the severe and mild groups there were no significant differences in distribution of either genotypes or alleles. In comparing this group to controls again no difference was seen, for genotype or allele.

This implies that the observed variation in variance and the group with high LTE_4 levels in AP is not due to genetic predisposition. This despite the known association of increased production of the cysteinyl LT with the 'C' allele in the promoter region of the enzyme LTC_4 synthase.

	Genotype			
	AA	AC	CC	Total
Mild	75 (44%)	76 (45%)	18 (11%)	169
Severe	32 (46%)	30 (44%)	7 (10%)	69
Total AP	107 (45%)	106 (45%)	25 (11%)	238
Controls	47 (46%)	47 (46%)	9 (9%)	103

Table 11.1 AP patients with Leukotriene C_4 Synthase Genotype by Severity.

	Allele		
	A	C	Total
Mild	226 (67%)	112 (33%)	338
Severe	94 (68%)	44 (32%)	138
Total AP	320 (67%)	156 (33%)	476
Controls	141 (69%)	65 (31%)	206

Table 11.2 The Distribution of Leukotriene C_4 Synthase Allele in AP by Severity.

11.2.2 **Chronic Pancreatitis:**

We similarly assessed the frequency of the above genotypes and alleles in 57 patients with CP (41 AICP and 16 ICP) and assessed it in relation 103 samples from a geographically similar normal control group. This based on the pilot study data referred to above for urinary LTE_4 levels.

Again there were no significant differences, in the genotype study (Table 11.3) and in the allele study (Table 11.4).

	Genotype			Total
	AA	AC	CC	
CP	32 (56%)	19 (33%)	6 (11%)	57
Controls	47 (46%)	47 (46%)	9 (9%)	103

Table 11.3 CP patients and controls with Leukotriene C_4 Synthase Genotype

	Alleles		Total
	A	C	
CP	83 (73%)	31 (27%)	114
Controls	141 (69%)	65 (31%)	206

Table 11.4 The Distribution of Leukotriene C_4 Synthase Alleles in patients with Chronic Pancreatitis (CP) and Controls.

11.3 Urinary PGE_2 Levels:

11.3.1 Acute Pancreatitis:

The study looked at PGE_2 levels in 18 patients with AP and their matched controls revealed a statistically significant difference. The AP patients had a higher level in comparison to controls; t = 3.078, p=0.006; employing an unpaired t-test. The data was broken down to analyse whether this difference was maintained for just the mild or severe patients. Comparing the mild AP patients to their matched controls using an unpaired t-test gave a p value of 0.011; t = 2.902. In addition, this significance was maintained when comparing mild AP (n= 14) to the grouped controls; p = 0.012. One way analysis on variance for the groups mild AP, severe AP (n=4) and controls gave an F value 3.613; p = 0.024. For the severe group it can be seen (Figure 11.5) that the PGE_2 levels and means are higher than the control groups, however this did not reach statistical significance in light of the small numbers, and the study was not powered to detect such a difference. Comparing the mild to severe groups, though with small numbers, did not attain statistical significance (p=0.188; t=1.390).

These results show that PGE_2 is elevated in AP and that in light of the ANOVA and subgroup analysis that this is most marked in mild disease.

Evidence on smoking or nicotine and PGE_2 is variable with a number of studies showing decreases especially in gastric mucosa (Quimby, G.F. et al., 1986) but also saliva (Wu-Wang, C.Y. et al., 1992); a few increases (Badawi, A.F. et al, 2002) and some no change (Zijlstra, F.J. et al., 1992; Hawkey, C.J. 1986). For completeness sakes the analysis was performed excluding smokers and this confirmed the significant difference in AP and also the CP result below.

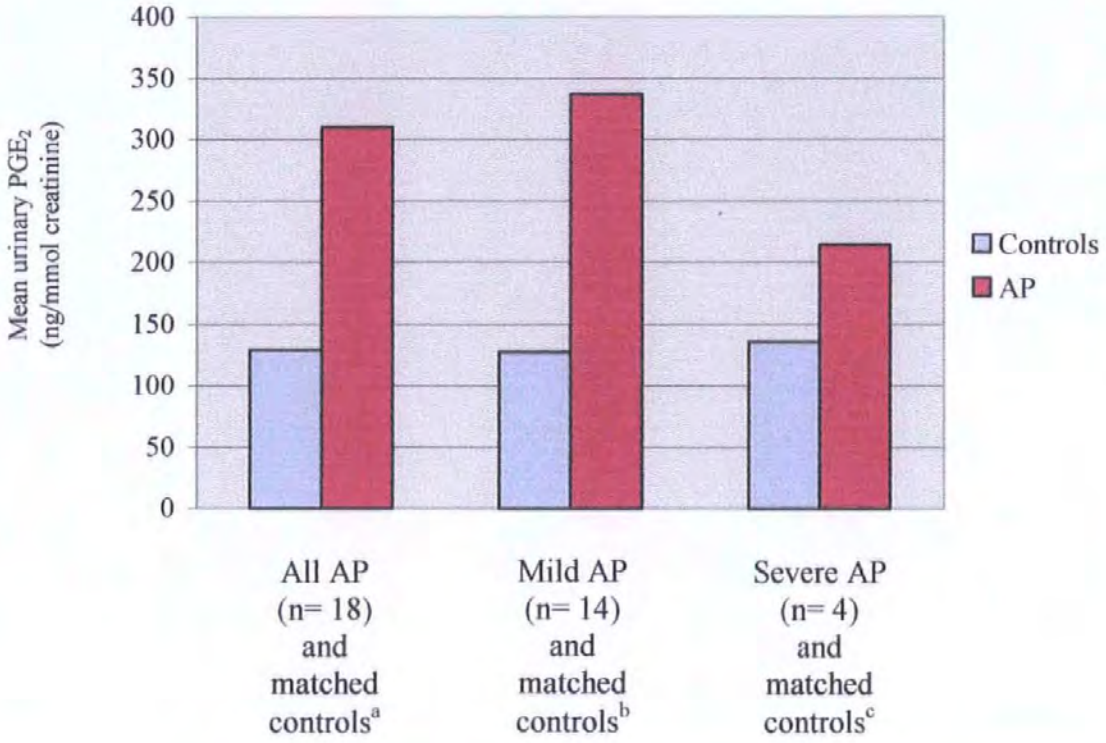


Figure 11.5: Mean urinary PGE_2 (ng/mmol creatinine) for all AP patients and also the mild and severe groups separately.
Analysed by unpaired t-tests: $p = 0.006^a$
$p = 0.011^b$
$p = 0.291^c$
Comparing the higher levels in mild disease with severe AP did not attain significance with these numbers, $p = 0.188$.

11.3.2 **Chronic Pancreatitis:**

The study of PGE_2 urinary levels in CP (n= 18) did not show any significant difference (Mann Whitney-U test). In fact the controls gave slightly higher values than the patients, Figure 11.6. The non-significant result was confirmed when excluding smokers.

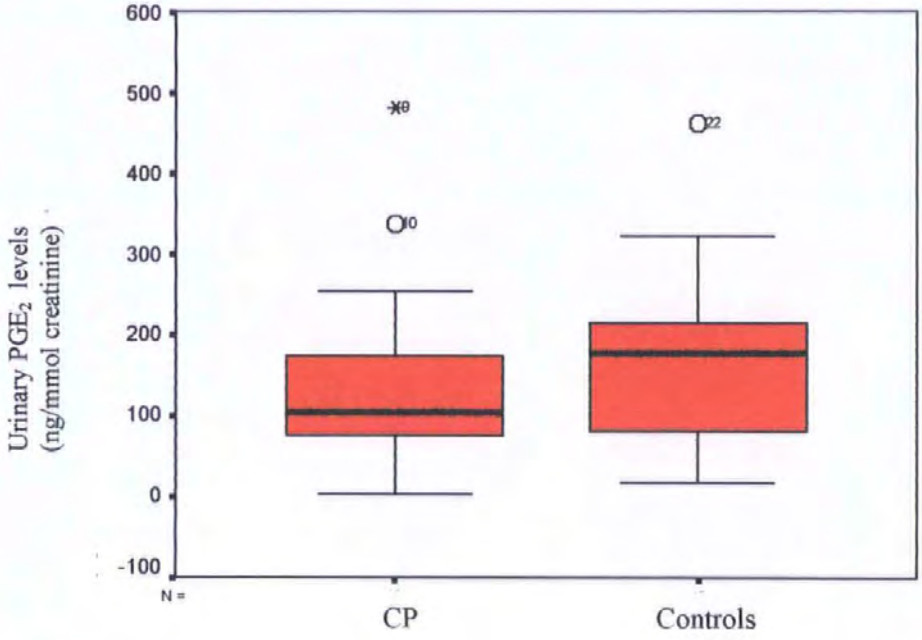


Figure 11.6: Box whisker plot of urinary PGE_2 levels (ng/mmol creatinine) in CP patients (n= 18) and matched controls.

11.4 11β-$PGF_{2\alpha}$ Urinary Levels:

The PGD_2 stable metabolite which is also a marker of mast cell activation.

11.4.1 Acute Pancreatitis:

The analysis in AP of 14 patients revealed no significant difference (means 29 and 26.1 ng/mmol creatinine) on independent samples t-testing for this normal data ($t = 0.416$; $p = 0.681$). The data is shown in figures 11.7 and 11.8. The inclusion of a 'mean' for severe and matched controls is somewhat dubious as there were only two in each group. The actual values were 72.2 ng/mmol creatinine and 5.2 (severe AP) and 39.9 and 2.7 (controls).

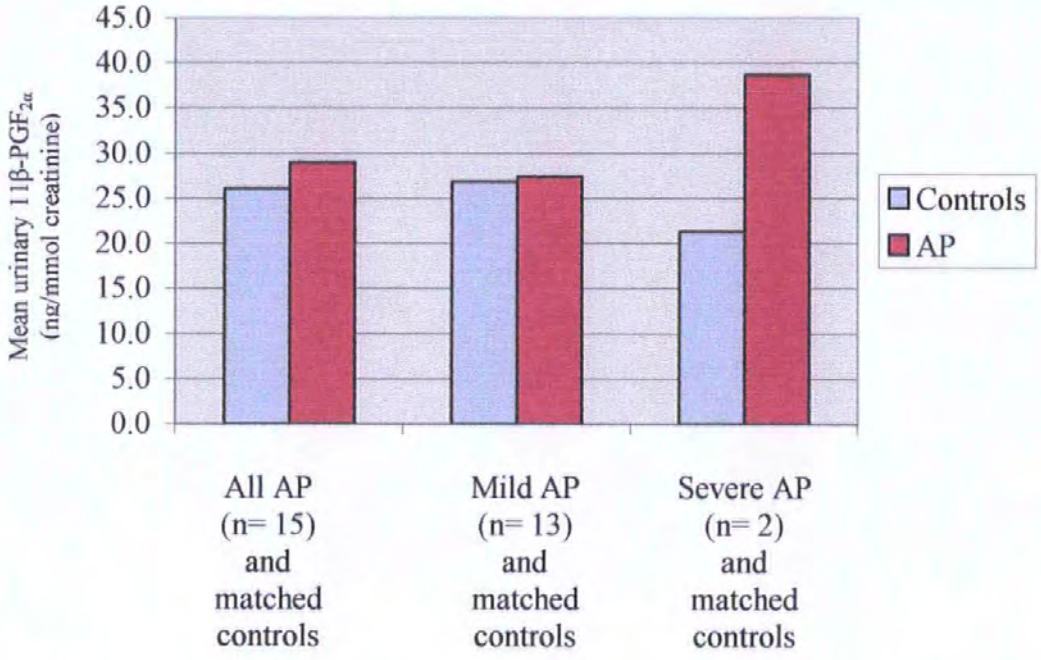


Figure 11.7: Mean Urinary 11β-$PGF_{2\alpha}$ (ng/mmol creatinine) in AP groups and matched controls.

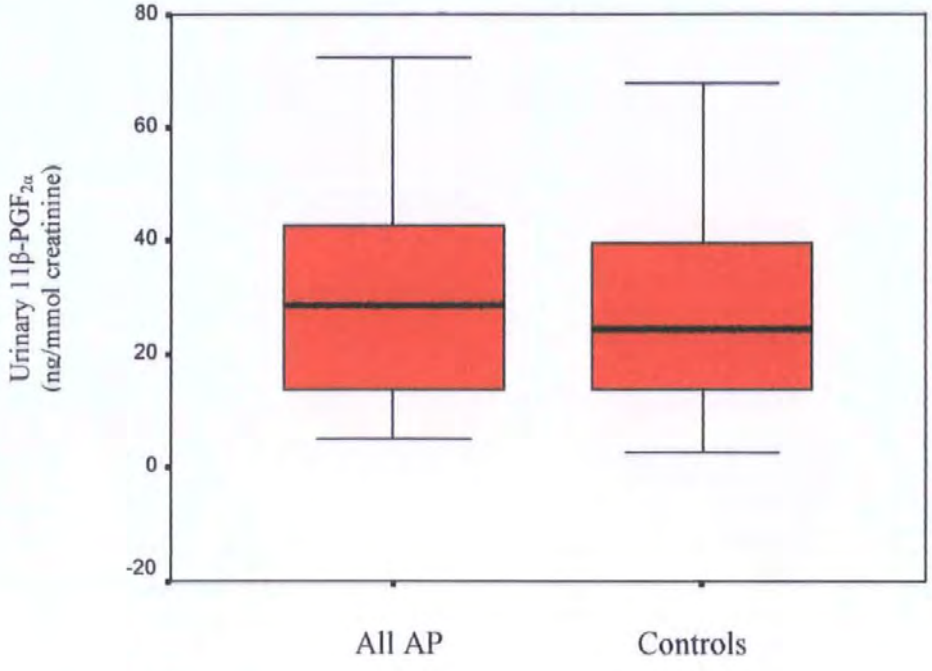


Figure 11.8: Urinary 11β-$PGF_{2\alpha}$ (ng/mmol creatinine) in AP and controls, in Box-Whisker plot showing interquartile range (box) and range (whiskers).

11.4.2 **Chronic Pancreatitis:**

In chronic pancreatitis for 11β-$PGF_{2\alpha}$ we studied 17 patients and their matched controls, Figure 11.9. In the comparison of CP with controls a highly significant difference was seen (Mann Whitney U test, 45.0), $p = 0.001$.

Analysed with sub-groups employing Kruskal Wallis was again highly significant ($p < 0.0009$). Thus individual subgroup analyses were performed to assess if the difference was consistent or reserved to particular groups (Mann Whitney U). The difference was found to hold for both AICP (n=10), $p = 0.001$ and ICP (n=7), $p = 0.038$ (each group compared to their matched controls).

Evidence on smoking and PGD_2 and metabolites is minimal, the one study show no difference (Zijlstra, F.J. et al., 1992). However, again for completeness sake the study was confirmed excluding smokers and again the negative result in AP and the significant difference seen in CP was confirmed.

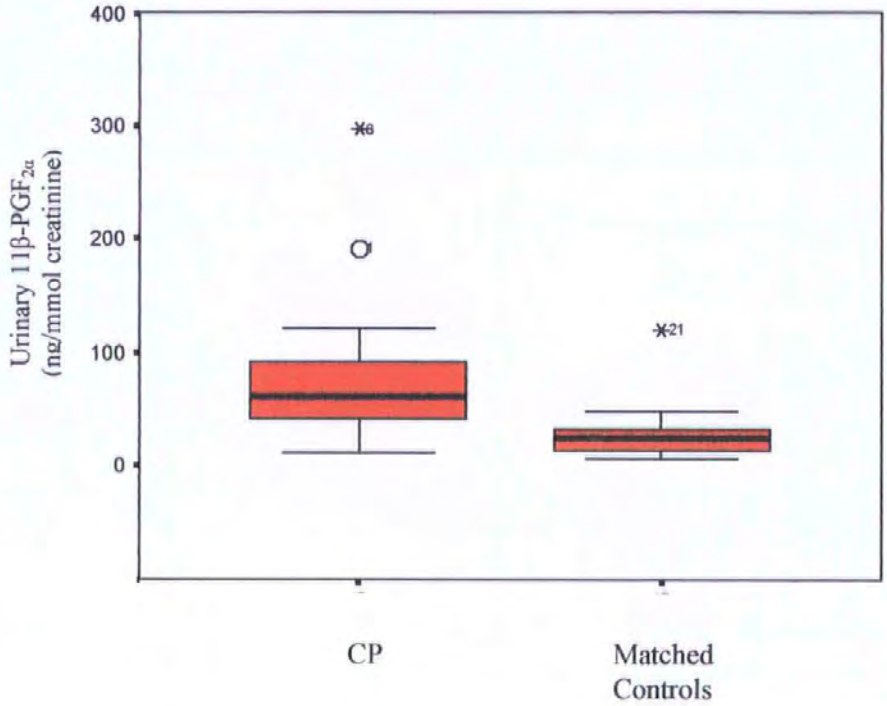


Figure 11.9: Urinary 11β-$PGF_{2\alpha}$ (ng/mmol creatinine) in CP and matched controls. Analysed by Mann Whitney U test: $p = 0.001$

DISCUSSION

This thesis has addressed a number of genetic and molecular factors in the initiation and progression of both acute and chronic pancreatitis.

The first study looked at a functional polymorphism in the gene encoding a major enzyme (cytochrome P450 2E1) in the metabolism of ethanol, the most well recognised aetiological factor in chronic pancreatitis. There was then a small study looking at polymorphic variation of the gene of a cytokine (IL-1a) which itself is implicated in, at least the progression of, chronic pancreatitis. The studies then moved to the major body of work looking at arachidonic acid metabolites: A trial of a receptor antagonist of the cysteinyl leukotrienes (Montelukast Sodium) was performed. Leukotriene E_4, Prostaglandin E_2, Prostaglandin D_2 levels (directly or via stable metabolites) were then assessed in both acute and chronic pancreatitis and as was a functional polymorphism in the gene encoding the first committed enzyme (LTC_4S) in the pathway of one of these (LTE_4).

Ethanol is a major cause of both acute and chronic pancreatitis. However, the mechanism is poorly understood. Whether it is via direct toxic effects, effects of its metabolites, effects of other metabolites of enzymes induced by ethanol, oxidative stress induced by these factors or a combination is far from fully delineated (Braganza, J. M., 1996). Which ever one (or more) of these prove to be the case in the fullness of time a possible role for genetic factors in ethanol metabolising enzymes may well be relevant. As discussed, studies looking at genetic predisposition to alcoholism and alcohol induced liver and cerebral disease initially showed a genetic factor to both with a separate (additional) genetic component to end-organ disease. However, the continuation of that study with further numbers concluded that the majority if not all of genetic predisposition to these end-organ diseases was conferred within the predisposition to alcoholism itself (Reed, T. et

al., 1996). However, this study only addressed alcohol induced liver and cerebral disease, not pancreatic disease.

The study performed here looking at CYP 2E1 indicates a decreased frequency of the high production (in the induced state) form in those ICD 10.1/10.2 alcohol consumers. This is the first study to look at this polymorphism in patient groups. In previous studies of healthy groups of American Caucasoids frequencies of 6.9% (McCarver, D. G. et al., 1998) and 4.2% (Fritsche, E. et al., 2000) are seen. Another previous study found the insertion in 2.1% of healthy Swedish subjects (Hu, Y. et al., 1999).

At low frequencies these results on Caucasoid subjects could all be consistent with the frequencies found in my patient, as well as my control, groups; 2.1% of total ALC; 2.2% of German ALC and 2% of British ALC; 5.8% of controls (British). However, in the large numbers in this study the results do indicate a statistically significant difference, which remains when isolating the smaller numbers of only local, British subjects for insertion genotype between NC and ALC ($p = 0.03$). Population stratification is a confounding factor in all genetic association studies and the possibility of this is recognised.

The original study describing the polymorphism studied showed greater CYP2E1 metabolic activity associated with the insertion. In that study chlorazoxone hydroxylation was higher in the patients with presence of the polymorphism and who were obese or recent consumers of alcohol (McCarver, D. G. et al., 1998); both circumstances when CYP2E1 would be induced. The two later descriptions delineate the pattern of 8 repeats of 42-60 bp, as opposed to 6 in the wild type (Hu, Y. et al., 1999; Fritsche, E. et al., 2000). Hu et al (1999) did not find an increased constitutive expression in luciferase transfection experiments, which would agree with McCarver et als' (1998) findings of increased enzymatic activity only in the induced state (obese subjects and recent alcohol consumers).

The finding of lower frequency in ICD 10.1 and 10.2 implies that if you more rapidly metabolise ethanol the increased levels of toxic/ unpleasant metabolites would decrease the chances of your consuming larger quantities of ethanol. This is synonymous with the findings that high activity forms of ADH and low activity forms of ALDH are associated with protection against alcoholism in a number of studies, as previously reviewed (Bosron, W. F. et al., 1993). This is due, it is believed, to the increased production/ decreased metabolism of the ethanol metabolite acetaldehyde, to which associated unpleasant side effects such as flushing are ascribed (Bosron, W. F. et al., 1993). The slight caveat to this is that CYP2E1 can also metabolise acetaldehyde (Terelius, Y. et al., 1991), however analogous effects and conversion of substrates to alternative (toxic) metabolites may still be increased.

Due to the low frequency in my patient populations it was not possible to delineate whether there was only a relationship to alcohol misuse per se, or a relationship to end-organ disease. Especially, as my subject group has a disproportionately large number of individuals with end-organ disease when compared to the proportions in alcoholics in the wider community, as described earlier. If a lower frequency existed in alcoholics it could then be expected to be increased in those with end-organ disease compared to alcoholic controls, if a toxic metabolite were involved. The sole homozygote was a patient with AICP and genotype analysis of AEOD versus AC approached significance, with $p=0.068$. However, to infer a finding from such a result would not be justified. Although, it was only in isolating the homozygotes of another functional polymorphism in CYP2E1 that an association with ALD was found in a previous study (Maezawa, Y. et al., 1994).
This study thus indicates a possible novel genetic factor in predisposition to alcoholism though no definitive claims could be made. A further line of investigation is would be to confirm or refute the association found in alcoholism and also assess any contribution of this genetic factor to alcohol induced end-organ disease. Thus, further analysis in ethnic

populations with a higher frequency (e.g. Chinese (Hu, Y. et al., 1999), Taiwanese or African American (Fritsche, E. et al., 2000) populations) of this polymorphism would be useful.

Another interesting finding was the smaller band identified (figure 8.2). The very first sequencing of this region showed a run of 5 repeats (Umeno, M. et al., 1988)(Assession No. J02843), which had not been seen in the two further studies (Fritsche, E. et al., 2000; Hu, Y. et al., 1999), this form would correspond to the smaller band seen in four of my 447 samples, corresponding to 585 bp. Initial attempts were made to sequence this (and the other) bands by PCR, running of products on TAE agarose gel, band excision and repeated PCR. However, commercial sequencing failed to succeed probably due to the repeats present in the sequence. Further PCR of a smaller area (nested PCR) would have been the next line but time constraints did not allow this.

With regard to IL-1a the results did not show a significant result in the pre-planned (primary outcomes). These being comparing the genotypes previously associated with severity in AP (1,2) for predisposition to CP and that associated with alcohol induced AP (2,4) for association with AICP. There were no significant differences in allele or genotype distribution. These negative data are only strengthened in light of this as a continuation of that study employing the same control group.

With regard to the results of the Singulair trial it was adequately powered and although some drop out occurred the results hold suggesting it is not of value in the treatment, especially with regard to pain, of Chronic Pancreatitis. This is perhaps less surprising when taken in light of the urinary LTE_4 results in CP patients which showed no increase in levels; despite the fact that this was in contrast to the unmatched control pilot study performed by our group. Other therapies do hold out hope e.g. reduction of oxidant stress,

where small studies showing benefit (las Heras, Castano G. et al., 2000; Banerjee, B. & Bagchi, D., 2001; Uomo, G. et al., 2001); though of only two controlled trials one showed benefit (with anti-oxidant therapy) (Uden, S. et al., 1990) and one, employing indirect suppression of oxidation with allopurinol, no benefit (Banks, P.A. et al., 1997b). This situation is well reviewed (Warshaw, A.L. et al., 1998). Potentially, larger studies of these and pilot studies of further novel therapies could improve our ability to manage chronic pancreatitis.

The bulk of evidence on cysteinyl LTs is in acute pancreatitis. They are elevated in pancreatic lymph and ascites in pigs with experimental AP (Vollmar, B. et al., 1989) and in the pancreas in a sodium taurcholate (necro-haemorrhagic) rat model (Folch, E. et al., 1998). Hirano et al. showed that in a rat model pretreatment with a cysLT receptor antagonist decreased pancreatic oedema and microvascular leakage (Hirano, T., 1997). Though my data on urinary LTE_4 levels showed no significant difference overall the difference in variance led me to address this further. The genetic studies in both AP and CP do not show a genetic component, at least in relation to LTC_4 synthase. The conclusions must be from these studies that there is no relevance for cysteinyl LTs (either functionally or genetically) in pancreatitis in humans.

With regards to the results in the PGE_2 study. These show that PGE_2 is elevated in AP and, although it cannot be categorically stated, that in light of the ANOVA and subgroup analysis this is most marked in mild disease. This is the first study of PGE_2 in humans in AP and would be in agreement with the bulk, though by no means all, of the animal data.

As addressed in the introduction PGE_2 has been seen to be elevated in a number of animal models of AP (Glazer, G. & Bennett, A., 1976; Closa, D. et al., 1993; Jaworek et al., 2001) and in a necrotising, 'though not oedematous model, in rats (Gloor, B. et al., 2001).

In contrast one study examining arachidonic acid metabolites in AP and CP in rat models found that although PGE_2 increased in a duct ligation model of CP it decreased in their AP model (Zhou, W. et al., 1994). Of course my CP study would again be contradictory to this single study. Of note Victorov & Hoek (1995) found that PGE_2 was produced by cultured rat Kupffer cells this occurred in response to lipo-polysaccharide (after a lag) and ethanol (gradually).

The suggestion of higher levels in mild disease would again concur with the majority, though not all, animal data. Administration of PGE_2 has decreased mortality and markers of the pancreatic damage mortality and inflammation (Manabe, T. & Steer, M.L., 1980; Coelle, E. F. et al., 1983; Standfield, N. J. & Kakkar, V. V., 1983; Ramirez, R. et al., 1984; Robert, A. et al., 1989). Possibly via membrane and lysosomal stabilisation (Standfield, N. J. & Kakkar, V. V., 1983; Manabe, T. et al., 1993).

Some of this effect is dependent on model and time scale (Taylor, I. L. et al., 1985). Some studies have shown no significant benefit (Lankisch, P. G. et al., 1983) or questioned the longer term relevance of the previously seen short term effect (Martin, D. M. et al., 1981). Of concern with regard to potential therapy, and showing the need for confirmatory studies, is that Reber required PGE_2 to cause pancreatitis with enterokinse and glycodeoxycholate (Reber, H. A., 1985). Whilst a negative effect of PGE_2 treatment has also been seen (Olazabal, A., 1986).

Despite these few negative studies with regard to a potential benefit associated with PGE_2, the majority of positive studies and the data presented here, the only study in humans, suggest that, as thought previously, this avenue warrants pursuing and perhaps indicates a potential therapeutic option. Though confirmation in appropriately powered studies, assessing the difference between mild and severe disease, is required.

The results on 11β-$PGF_{2\alpha}$ as a product of PGD_2 and a marker of mast cell activation (Schleimer, R. P. et al., 1985) are interesting. In AP there was no association, but it was marked in CP. As discussed earlier PGD_2 has been shown to be elevated in pancreatitis in a small number of studies in AP (Closa, D. et al., 1994a) and CP (Zhou, W. et al., 1994), this second study finding no significant increase in AP, agreeing with the results of this thesis with regard to PGD_2.

Three studies have shown mast cells in increased numbers in CP, two as abstracts, (Okolo CN et al., 2000; Emmrich, J. et al., 2000) and one paper which showed numbers correlating with fibrosis and inflammation (Esposito, I. et al., 2001).

The mast cell is well positioned to be a major player in chronic pancreatitis since it is increasingly being found to have a crucial role in fibrosis and tissue remodelling.

Firstly, mediators involved in CP (as outlined in the introduction) are integral in the function of mast cells and mast cells in their formation:

•TNF-alpha. Mast cells produce TNF-alpha (Gordon, J.R. & Galli, S.J., 1990; Bischoff, S.C. et al., 1999; Gibbs, B.F. et al., 2001; Coward, W.R. et al., 2002).

•Chemokines. The CXC chemokine IL-8, involved in CP, is also produced by mast cells (Moller, A. et al., 1998; Gibbs, B.F. et al., 2001), as is MCP-1 (also a player in CP), the CC chemokine (Baghestanian, M. et al., 1997). Also deletion of the receptor for this in mice (CCR2) shows protection from pulmonary fibrosis (Moore, B.B. et al., 2001).

In addition, in one study mast cell tryptase leads to IL-8 production by human umbilical vein endothelial cells (Compton, S.J. et al., 1998).

•IL-6 is also produced by mast cells (Moller, A. et al., 1998; Lorentz, A. et al., 2000).

•TGFbeta. Mast cells are integrally involved with TGFbeta which as discussed is believed to have a major role in CP.

TGFbeta1 causes mast cell adhesion (in mice) (Rosbottom, A. et al., 2002). It also promotes their migration while inhibiting growth (Olsson, N. et al., 2001), this is true for isoforms TGFbeta1, 2 and 3 (Olsson, N. et al., 2000).

In addition to being chemotactic for mast cells, mast cells have been shown to secrete TGFbeta1 (Kanbe, N. et al., 1999; Lindstedt, K.A. et al., 2001) which is also activated by the mast cell product chymase (Lindstedt, K.A. et al., 2001).

Both TNFalpha and TGFbeta from mast cells are the cause of type 1 collagen production by fibroblasts in mice (Gordon, J.R. & Galli, S.J., 1994).

This discussion brings us on to the evidence of mast cells directly in fibrosis and tissue remodelling.

Mast cell products have been shown to cause fibroblast proliferation and collagen synthesis (Garbuzenko, E. et al., 2002) via not only the cytokines mentioned but tryptase (Gruber, B.L. et al., 1997). TGFbeta and tryptase cause fibroblast collagen IV and VIII production (Xu, X. et al., 2002) though in this study (with human mast cells) TNFalpha and chymase decreased collagen production. Such actions are not only via mediators but direct contact with fibroblasts leading to contraction collagen lattice (Yamamoto, T. et al., 2000). Mast cells can produce, indeed, type VIII collagen (Ruger, B. et al., 1994). 'In return' fibroblasts produce factors chemotactic for mast cells (Kiener, H.P. et al., 2000)

Mast cell products chymase and tryptase are also involved in these actions. Chymase releases TGFbeta1 from extracellular matrix (Taipale, J. et al., 1995) and causes a mitogenic response in fibroblasts, also true of tryptase. Tryptase causes fibroblast chemotaxis and collagen type 1 mRNA (Gruber, B.L. et al., 1997). Tryptase has again been shown to cause proliferation of, and also collagen type production by, fibroblasts (Abe, M. et al., 1998).

The complexity of this remodelling role and the fact that it is not simply fibrotic can be seen. In a study by Cho et al. (2000) there was production by stimulated mast cells of plasminogen activator inhibitor 1 but, not only its absence, indeed the production of tissue type plasminogen activator and heparin in others (Valent, P. et al., 1998; Sillaber, C. et al., 1999). In these later studies production was constitutive rather than from stimulated mast cells, this could imply 'abnormal' continued stimulation of mast cells in fibrotic conditions, such as CP, may disturb the vital balance of mast cell control of remodelling, as discussed in vascular disease by Wojta et al. (2002).

Mast cells are not simply a single homogenous lineage but have varied role and characteristics based on site and products. The primary division employed is tryptase positive (all mast cells) and chymase positive or negative. Also delineation on cytokine profile has been proposed, different cytokine roles for different mast cells (Bradding, P. et al., 1995). These divisions are early in their delineation, however, not all characteristics identified may be attributable to all subdivisions. That said the known actions of mast cells fit with the cytokines found in CP, the cell types found and histological findings. Thus a conclusion can be made of an active, indeed central, role for mast cells in CP. Playing a part in healing, fibrosis and remodelling, as in other conditions (Miyachi, Y. & Kurosawa, M., 1998; Chiappara, G. et al., 2001; Li, C.Y. & Baek, J.Y., 2002). This central remodelling, but especially fibrotic, role would fit with Espisito et al.'s study (2001) and as

such they present a potential therapeutic target. As all proposed ideas of pathogenesis involve inflammatory cascades, and as discussed they are not mutually exclusive, these findings would not exclude any one theory of aetiopathogenesis of chronic pancreatitis in humans. However, they could fit most closely with the 'fibrosis' aspect of the 'necrosis fibrosis' theory.

In summary, these studies have addressed various issues of interest in Acute and Chronic Pancreatitis assessing areas not previously addressed in Humans.

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Acute pancreatitis

MT Cartmell, AN Kingsnorth

Acute pancreatitis is a common disease. As knowledge of its pathophysiology improves, evidence is found to confirm and refute present management and also to suggest new approaches. This article addresses some of these areas in the context of the management of acute pancreatitis.

Acute pancreatitis (AP) is a common disease, seen in both surgical and non-surgical practice, accounting for around 3% of admissions with acute abdominal pain. It carries a relatively high mortality of 5–10% and, at present, there is no specific non-operative therapy. Mortality seems to have plateaued in the last decade. In the previous two decades a decrease in mortality has resulted from a number of factors: increased diagnosis of mild cases, improved diagnosis, understanding of natural history, imaging and selective operative intervention. Unsurprisingly, mortality varies with the severity of cases: the mortality for severe cases is ≈20%, for cases of infected necrosis is ≈25%, and for cases of sterile necrosis is ≈10%.

Death tends to occur in the first week from multiple organ failure or thereafter from sepsis, either local to the pancreas or systemic. Patients who survive, even severe episodes, generally have low long-term morbidity and good quality of life.

DEFINITIONS

Attempts to categorize AP have been made in recent years in light of improved knowledge of the pathology and natural history of the disease. From Marseilles and Cambridge to Atlanta (Bradley, 1993) these have given a basis from which to compare the outcomes of different management techniques and enabled discussions to be carried out using agreed and standardized terminology.

Many aspects of management have developed and are developing. Although many of these are still contentious a number of consensus documents have been produced giving guidelines on the management of AP (Banks, 1997; Glazer and Mann, 1998; Dervenis et al, 1999). This article discusses some recent developments and contentious areas, within a framework of the management of AP.

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ASSESSMENT

Aetiology

The two common causes of AP remain gallstones, accounting for around 40–50% of cases in Britain, and ethanol, accounting for around 20–30% of cases. The mechanisms by which the various causes lead to AP are not fully understood. If these causes are not identified other potential causes should be sought which include:

- Hyperlipidaemia
- Tumours (especially periampullary tumours)
- Infective causes, including viral, e.g. mumps and coxsackie, and parasitic, e.g. ascaria and clonorchis.

'Idiopathic' (or unidentified aetiology) remains a common diagnosis in reported series and appears to diminish with repeated investigation. The UK guidelines (Glazer and Mann, 1998) suggest that this group should constitute no more than 20–25% of diagnoses of AP.

Diagnosis

Common presenting features are abdominal pain, especially epigastric, with or without radiation to the back, associated with vomiting. There may be signs of abdominal tenderness (ranging from mild through rebound to rigidity) and, less commonly, Cullen's and Grey-Turner's signs, which contribute to a more specific diagnosis. However, presentation can be varied and missed diagnosis remains a factor in the high mortality (Kingsnorth, 1998).

From 12–42% of cases of fatal AP go undiagnosed before death. In one analysis of patients diagnosed post-mortem, Wilson and Imrie (1988) suggest that a timely diagnosis may have altered management, potentially allowing survival, in 45% of cases. In addition to a variable clinical presentation, the lack of a highly specific diagnostic test leads to difficulties.

Diagnostic tests

At present the mainstay of diagnosis, beyond clinical assessment, is the biochemical tests of amylase (blood or urine) and lipase. These pancreatic enzymes are typically elevated (amylase >3–5x normal, lipase >2x normal) during an attack of AP. However, they must be sought to give a result. Levels may not be elevated even in a severe attack (lack of sensitivity) and can be non-specifically elevated in clinically similar conditions (lack of specificity). In addition, amylase is short lived in the blood (leading some commentators to recommend urine levels to be tested). Amylase has other isoenzymes which may, rarely, be the source of an abnormally high result. Lipase is marginally superior to amylase (*Table 1*). Combination of the two investigations improves sensitivity and specificity.

Assays which show promise include:

- Trypsin 2-alpha 1 antitrypsin complex, which also correlates with severity
- Alpha 2 macroglobulin-trypsin complex
- Trypsinogen 2. This is probably closest to a practical, accurate form and a simple urine test is available (Kylanpaa-Back et al, 2000) (*Table 1*). Serum levels also correlate with severity.

Prognostic tests, indicators, systems and imaging

As AP has such a range of clinical courses it is useful to be able to predict which patients are likely to have a severe (complicated) course. This allows intensive observation of those at greatest risk, implementation of investigation and appropriate and opportune management. It also provides for standardized comparisons in studies.

Attempts at predicting severity in AP have been made for over two decades, initially consisting of multi-factor scoring systems, such as Ranson's (Ranson et al, 1974) and Glasgow criteria (Blamey et al, 1984). Other simple indicators are now also known and a large number of potential serum markers are being evaluated. The details of all the potential predictors are extensive and a number of reviews exist. A combination of recognized predictors of severe outcome is, at present, the best approach:

- Simple tests:
  a. Clinical assessment — not regularly reliable, still a vital component
  b. Obesity — body mass index ≥30 kg/m^2
  c. Chest X-ray — pleural effusions (especially left sided and bilateral)
  d. C-reactive protein level >150 mg/litre.
- Multi-factor scoring systems: e.g. APACHE-II score ≥8 (values between ≥6 and ≥9 recommended by various authors), which is equal or superior to other systems
- Computed tomography (CT) imaging: (dynamic) contrast-enhanced CT of the pancreas. Necrosis and fluid collections correlate with outcome. Systems such as that of Balthazar et al (1990) are useful measures.

TREATMENT

The mainstay for the patient with a predicted severe or severe episode of AP is supportive care and appropriate fluid resuscitation in a high dependency or intensive care setting. There are also medical and interventional options available.

Antibiotic prophylaxis

As discussed above, mortality in AP is primarily the result of septic complications beyond the first week, this accounting for the majority of deaths. In addition infected pancreatic necrosis is associated with a much higher mortality than sterile necrosis. A debate regarding antibiotic prophylaxis has been ongoing for many years, with the weight of evidence most often said, in the past, to fall against prophylaxis. However, much of the data covered ampicillin which we now know has limited pancreatic penetration, and studies included mild cases which may have masked a significant effect in severe cases.

With more recent trials and increasing evidence on which antibiotics attain appropriate tissue levels this debate continues (Barie, 1996), but on balance probably favours antibiotic prophylaxis in patients with predicted severe disease (Golub et al, 1998; Powell et al, 1998). This corresponds to current practice in the UK (Powell et al, 1999). Comparisons of penetration in different degrees of inflammation, into pancreatic juice and even into peripancreatic fat (Barie, 1996) may be of significance in the clinical setting.

However, even recent studies have not consistently shown a survival benefit or reduced pancreatic infections with antibiotic prophylaxis. The question of 'which antibiotic?' leads to even less widespread evidence; with clinical trials, animal studies and penetration studies not consistently testing the same antibiotics and variation also seen within antibiotic 'families'. Although further studies are required, it would be reasonable to recommend one of:

TABLE 1.
Sensitivity and specificity of some tests for acute pancreatitis

	Sensitivity (%)	Specificity (%)
Amylase	52–95	86–98
Lipase	74–100	34–100
Trypsinogen-2 (serum or urine)	91–8	89–95

Data from Kemppainen et al (1998)

Cefuroxime, which has been shown to decrease total infectious complications, need for operative intervention and death, but not pancreatic infection, in a trial of 60 patients (Sainio et al, 1998)

Ceftazidime, which (with amikacin, which is poorly penetrant, and metronidazole) reduced all infections but not mortality in a small trial (Delcenserie et al, 1996)

Ofloxacin, which (with metronidazole) in a small trial was shown to decrease physiological disturbance (Schwarz et al, 1997) and has good penetration and spectrum characteristics

Imipenem, which has been shown to decrease pancreatic and extrapancreatic infection (Pederzoli et al, 1993) and be superior to perfloxacin without metronidazole (Bassi et al, 1998), but not to show survival benefit. It has good pancreatic penetration and is the most consistently tested; however, its cost may exclude it as first-line prophylaxis, especially in light of its lack of comparison with some of the viable alternatives.

Any of the above (except imipenem) should probably be combined with metronidazole.

Other issues in antibiotic prophylaxis awaiting resolution include the role of gut decontamination in an attempt to prevent bacterial translocation, either alone or with systemic prophylaxis, the duration of administration, and whether to commence antibiotics on purely prognostic grounds or only after necrosis is seen (as in most of the trials).

Pancreatic rest and suppression

A mainstay of AP management has been to maintain the patient nil by mouth. This can avoid worsening pain and nausea and rests the pancreas from stimulation by food, with the aim of decreasing pancreatic secretion and thus autodigestion, although basal secretion may already be suppressed in AP. Other avenues to rest the pancreas seem to lack any significant benefit:

Nasogastric aspiration has not been shown to benefit outcome; however, it has a role in selected cases to rest an ileus and prevent vomiting and aspiration

Cimetidine has not consistently been shown to benefit patients

Evidence for ranitidine in patients is limited although it probably plays a worthwhile role in gastritis and ulcer prophylaxis

Somatostatin and its analogue octreotide are known to decrease pancreatic secretion but have failed to consistently show benefits in patients with AP (Uhl et al, 1999a). Although a meta-analysis suggested a benefit (Andriulli et al, 1998) this was before the largest study (Uhl et al, 1999b), a randomized, double-blind trial which failed to show any significant benefit.

Suppression of the inflammatory response

Blockade or suppression of the inflammatory pathways involved in AP is an appealing therapeutic avenue. As our rapidly developing understanding of this area is in its early days, there are many potential but not yet fully evaluated therapies.

The most studied to date is the platelet-activating factor antagonist lexipafant. After two phase II trials and a multicentre UK phase III trial there appeared to be a significant improvement in organ failure incidence and severity, and reduction in pseudocyst formation and mortality in selected patients (Kingsnorth, 1997). However, a second larger, international phase III trial failed to show any benefit for lexipafant (unpublished data, British Biotech, Oxford, 1999).

Nutrition

Patients unable to eat for a prolonged period, in the catabolic state of AP, require nutritional supplementation. This is most commonly achieved with total parenteral nutrition (TPN). With the apparent paradox of feeding enterally a patient whose gut and pancreas is being rested, recent evidence (in the form of three small, randomized trials) would suggest that enteral nutrition (jejunal feeding, distal to the cholecystokinin cells) is safe and possibly superior to TPN (Dervenis et al, 1999).

The potential benefits are improved nutritional status, protection of the gut's mucosal barrier against bacterial translocation and fewer septic complications of the means of administration.

Analgesia

Many AP patients suffer from severe abdominal pain. Prescription of pethidine, rather than morphine, in acute biliary pain (including pancreatitis) is often advised because of the potential for morphine to cause the sphincter of Oddi to spasm, thus increasing intraductal pressure. However, concern over the addictive potential of pethidine and possible inferiority to morphine confounds the issue. Anecdotal evidence abounds on both sides of this debate, but objective clinical data are lacking.

Morphine causes increased rate and amplitude of sphincter of Oddi contractions and increased basal pressure (Helm et al, 1988) while pethidine can decrease the frequency of contractions (Thune et al, 1990). However, findings are inconsistent and the clinical significance is untested. Patient-controlled analgesia with pethidine can be useful for systemic analgesia in AP. Alternatives include epidural analgesia, transcutaneous electrical nerve stimulation and coeliac plexus block.

Interventions

CT-guided aspiration of pancreatic necrosis is a useful technique for distinguishing between infected and sterile pancreatic necrosis (Banks, 1997; Dervenis et al, 1999), which are often clinically indistinguishable.

Endoscopic retrograde cholangiopancreatography (ERCP) definitely has a role to play in management of AP. However, the exact circumstances in which it should be used have not been fully evaluated. A number of trials have shown a benefit of early ERCP in biliary pancreatitis, although those showing survival benefit did not exclude patients with cholangitis or biliary obstruction (Banks, 1997; Dervenis et al, 1999). A study which did so failed to show survival benefit (Folsch et al, 1997). It can be said that ERCP should be used in biliary pancreatitis with evidence of cholangitis or biliary obstruction and may benefit patients with severe AP caused by gallstones. These groups of patients may be indistinguishable, so ERCP is reasonable in all patients with prognostically severe AP and gallstones.

Surgical intervention is indicated in patients with infected pancreatic necrosis, but its role in sterile necrosis is uncertain and should probably only be employed in patients who are deteriorating or failing to settle after attempts at conservative management have been exhausted (Banks, 1997; Dervenis et al, 1999). A further indication, which is not uncommon, is the patient in whom imaging is unavailable or unable to exclude an intra-abdominal catastrophe for which an operation would be necessary, e.g. mesenteric infarction.

CONCLUSIONS

Many aspects of our knowledge of AP and its management have been and are being advanced. In many areas further evaluation is required. The end-point of such work will hopefully be improved outcome for our patients. Debate and study to this end can be facilitated by use of standardized terminology and assessment against accepted guidelines and practices. HM

Conflict of interest: none.

More details and comments on the references and some points raised are available at: www.cartmellsreferences.freeserve.co.uk

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KEY POINTS

- Acute pancreatitis is a common disease with relatively high mortality.
- Standardized terminology is now available and should be employed to aid debate and comparison.
- Diagnosis is still hampered by lack of a highly sensitive and specific test; however, many potential tests are being developed.
- On balance, antibiotic prophylaxis is advisable in severe acute pancreatitis.
- Suppression of inflammation may, in the future, be a therapeutic option.
- Enteral nutrition is probably as, or more, safe than total parenteral nutrition.
- Infected necrosis is an absolute indication for operation.

Letter In Press: *Clinical Chemistry and Laboratory Medicine.*

Faecal Elastase 1 Measurement in Chronic Pancreatitis.

Further to the review article by Messr's Dominici and Franzini we concur with the importance of considering faecal pancreatic elastase 1 as a good test of pancreatic function, the role of which is yet to be fully defined in Chronic Pancreatitis. Comparing it with other tests for chronic pancreatitis is more about defining this role for pancreatic function testing. A small study we conducted would indicate faecal pancreatic elastase 1 testing is not only reliable over a few days but over several months (mean co-efficient of variation 16%, median 6%) whilst decreasing levels pre-date clinically evident insufficiency, potentially allowing earlier treatment in this nutritionally vulnerable group.

Keywords: Elastase 1; Pancreatic function tests; Chronic pancreatitis.

Abbreviations: CP, chronic pancreatitis; E1, faecal pancreatic elastase 1;

We are writing further to the review article on faecal elastase by Mssr's Dominici and Franzini(1). We concur with the authors that the commercially available faecal pancreatic elastase 1 test (E1) is a simple and valid test of pancreatic exocrine function. We also agree with the important distinction discussed between studies showing good correlation with 'gold standard' tests of pancreatic function, such as Loser et al., 1996 (2) and those studies showing reasonable, although poorer, correlation in comparison with a combination of structural and functional tests(3). It is unsurprising that E1 lacks some correlation in 'mild' cases in comparison with the very complex secretin (caerulein)-cholecystokinin test given this measures a number of digestive enzymes (as well as volume and bicarbonate) and the definition of mild includes a decrease in 1 to 3 of the enzymes tested. Thus although elastase correlates with the duodenal enzymes measured and faecal elastase with its duodenal levels(4)

it will not always decrease when the secretin-cholecystokinin test reveals mild insufficiency. The lack of correlation in some cases of CP is also unsurprising as pancreatic insufficiency is not required for a definitive diagnosis of CP. Thus a patient suffering from CP, which is diagnosed on a combination of findings (5), may demonstrate no insufficiency. This raises the issue of the role of pancreatic function tests, including the uncomplicated E1, in CP.

To address our concern with regard to the tests reproducibility in an individual over time and to look at possible roles for E1 we looked at 13 patients with CP (5) with 3 to 4 tests over 7 months. Only three patients varied between diagnostic categories: One had a single reading in the mild to moderate category then 3 normal readings, this patient was on oral enzyme supplements and although, as discussed in the review article, these do not interact immunologically with this test there is some evidence that they may affect secretion via a negative feedback loop (6,7), which might explain this variation.

The other two patients both showed gradual decreases in function, figure 1. One of these has (within a year) developed steatorrhoea and commenced on enzyme supplements. The other has been unavailable for follow up. On this basis, excluding these two patients, intra-individual variation revealed a mean (median) co-efficient of variation of 16% (6%).

This would indicate that the test is reproducible overtime and that decreasing levels may predate clinical deterioration (as also seen by one study, available in abstract form, in cystic fibrosis over 5 years (8)). This would indicate one role for E1 in CP where earlier detection of pancreatic exocrine insufficiency would enable supplementation in a nutritionally vulnerable group; especially given that 90% of pancreatic exocrine function can be lost prior to its being clinically evident (9).

Yours sincerely,

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Book Chapter (In Press):

Cytokines in Chronic Pancreatitis.

Mark T. Cartmell and Andrew N. Kingsnorth.

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An additional paper, written during though not directly related to this thesis:

Use of the Internet and Information Technology for Surgeons and Surgical Research.

Mark T. Cartmell and Andrew N. Kingsnorth.

Annals of the Royal College of Surgeons of England 84; 352-356.

Three additional papers, as yet unpublished:

A Double-Blind Placebo Controlled Trail of Leukotriene Receptor Antagonist in Chronic Pancreatitis in Humans

Mark T. Cartmell, Derek A. O'Reilly, Christine Porter, Andrew N. Kingsnorth.

[Re-submitted to '*Journal of Hepato-Biliary-Pancreatic Surgery*' with requested changes]

A High Activity Polymorphism of Alcohol Inducible Cytochrome P450 2E1 in Alcohol Abuse and End Organ Disease

Mark T. Cartmell, Hans-Ulrich Schulz, Derek A. O'Reilly, Bing-Mei Yang, Volker Kielstein, Simon P. Dunlop, Walter Halangk, Andrew G. Demaine, Andrew N. Kingsnorth.

[Completed, for Submission to '*Alcohol*']

Arachidonic Acid Metabolites in Pancreatitis in Humans: PGE_2 in the acute and PGD_2 and mast cells in the chronic form.

Mark T. Cartmell, Siobhan O'Sullivan, Derek A. O'Reilly, Zak H. Rahman, Andrew G. Demaine, Andrew N. Kingsnorth.

[In Preparation]