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A STUDY OF THE ROLE OF CYTOKINES IN ACUTE PANCREATITIS IN MAN

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A STUDY OF THE ROLE OF CYTOKINES IN ACUTE PANCREATITIS IN MAN

by

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For

Mum and Dad

A study of the role of cytokines in acute pancreatitis in man

Alison Marie Smithies

Abstract

Introduction: Acute pancreatitis is an inflammatory disease with a diverse aetiology and variable clinical course. The IL-1 gene cluster has been implicated in this disease.

Aims: The aims of the study were to investigate polymorphisms of the genes encoded within the IL-1 gene cluster in patients with acute pancreatitis and normal controls and to determine the relationship between the polymorphisms and protein levels.

Methods: Genotype and allele frequencies were determined in controls (n=217) and patients with acute pancreatitis (n=137) using the polymerase chain reaction (PCR) followed by digestion with restriction endonucleases where applicable. Protein levels were determined using *in vitro* stimulation of PBMCs followed by Enzyme Linked Immunosorbent Assay (ELISA). Patients were categorised according to severity, organ failure scores and aetiology.

Results: Allele 1 of the VNTR^{86} polymorphism in the IL-1RN gene was significantly increased in the severe group of patients compared to controls (81.9% vs 63.0%, $\chi 2$=9.38, p=0.002, Pc=0.004) and in the idiopathic group compared to controls (82.4% vs 63.0%, $\chi 2$=9.33, p=0.002, Pc=0.004). The polymorphisms within the genes and between the genes were strongly linked. Significantly more of the *MspI*-VLP-VNTR^{86}-*SspI* 2-3-2-2 haplotype was observed in the control (15.7% vs 0.1%, $\chi 2$ =2528.11, p<0.0000001, Pc<0.0000001) and patient (14.0% vs 0.1%, $\chi 2$ =4368.10, p<0.0000001, Pc<0.0000004) populations than expected. Significantly more of the *PstI-AvaI-AluI-TaqI* 2-2-2-1 haplotype was observed in controls (27.7% vs 9.7%, $\chi 2$ =31.39, p<0.0000001, Pc<0.0000005) and patients (12.5% vs 2.0%, $\chi 2$=53.69, p<0.0000001, Pc=0.0000007) than expected. Preferential combinations of the genotypes existed within controls and patients. The median IL-1α and IL-1β protein levels from unstimulated PBMCs were significantly increased in patients compared to controls: median values (interquartile range). In the IL-1α study, significant differences were found at 24 hours: 193.5 (127.5-363.5) pg/ml vs 1.0 (0.0-3.0) pg/ml, p=0.005, 48 hours: 256.5 (171.5-417.0) pg/ml vs 6.5 (2.0-16.0) pg/ml, p=0.006 and at 72 hours: 210.5 (138-427) pg/ml vs 0.5 (0-7) pg/ml, p=0.005. In the IL-1β study, significant differences were found at 24 hours: 663 (507-782) pg/ml vs 12 (5-53) pg/ml, p=0.004, 48 hours: 620 (570-1080) pg/ml vs 14.5 (11-36) pg/ml, p=0.004 and at 72 hours: 545.5 (442-771) pg/ml vs 12.5 (2-43) pg/ml, p=0.006.

Conclusion: Polymorphisms of the IL-1 gene cluster are associated with susceptibility to and /or severity of the acute pancreatitis. Polymorphisms within the IL-1 gene cluster are in linkage disequilibrium. Unstimulated PBMCs from patients with acute pancreatitis secrete significantly more IL-1α and IL-1β protein levels compared to those from controls. The (AC)n, *Alu I* and VNTR^{86} polymorphisms do not correspond to differences in functional protein levels.

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AUTHOR'S DECLARATION

All of the studies presented in this thesis were performed by the author. At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

Signed: A Smithies

Date: 12/9/01

PUBLICATIONS AND CONFERENCES

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LIST OF ABBREVIATIONS

$A1^0$	Unstimulated subjects with allele 1
$A1^5$	Stimulated subjects with allele 1
$A2^0$	Unstimulated subjects with allele 2
$A2^5$	Stimulated subjects with allele 2
A AP	Alcoholic acute pancreatitis
ACTH	Adrenocorticotropin hormone
AIDS	Acquired immunodeficiency syndrome
AP	Acute pancreatitis
APACHE II	Acute Physiology and Chronic Health Evaluation II
APP	Acute phase proteins
ARDS	Adult respiratory distress syndrome
bp	Base pairs
C^0	Unstimulated controls
C^5	Stimulated controls
CARS	Compensatory anti-inflammatory response syndrome
CAT	Cationic trypsinogen gene
CCK	Cholecystokinin
CD	Cluster of differentiation
CDE	Choline-deficient ethionine-supplemented (diet)
cDNA	Complimentary deoxyribonucleic acid
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CMV	Cytomegalovirus
CO_2	Carbon dioxide
CRF	Corticotropin releasing factor
CRP	C-reactive protein
CSF	Colony stimulating factor
CT	Computerised Tomography
DFX	Desferrioxamine
DIC	Disseminated Intravascular Coagulation
DNA	Deoxyribonucleic acid
dNTPs	2' Deoxyribonucleotide 5'-triphosphates
EBV	Epstein-Barr Virus
E. coli	Escherichia coli

EDTA	Ethylene diamine tetra-acetic acid
ELAM	Endothelial cell adhesion molecule
ELISA	Enzyme Linked Immunosorbent Assay
ERCP	Endoscopic retrograde cholangio-pancreatography
ES	Endoscopic sphincterotomy
ESRD	End stage renal disease
ET	Endothelin
FAEE	Fatty acid ethyl ester
FCS	Foetal calf serum
γ^{32P}	dATP gamma phosphate 32 deoxyadenosine
G AP	Gallstone acute pancreatitis
GB	Ginkgo Biloba
GRE	Glucocorticoid responsive element
GRO	Growth related oncogene
G/T	Genotype
GTP	Glycerine tri-phosphate
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HP	Hereditary pancreatitis
HUVECs	Human umbilical vein endothelial cells
I AP	Idiopathic acute pancreatitis
IBD	Inflammatory bowel disease
ICAM	Intercellular adhesion molecule
ICE	Interleukin-1 β converting enzyme
IDDM	Insulin dependent diabetes mellitus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-1R	Interleukin-1 receptor
IL-1ra	Interleukin-1 receptor antagonist
IL-1R-AcP	Interleukin-1 receptor accessory protein
i.q.r.	Interquartile range
kb	Kilobase
kDa	Kilodalton
kpa	Kilopascals

LAK	Lymphocyte activated killer cell
L-NAME	N^G-nitro-L-arginine methyl ester
L-NNA	N^G-nitro-L-arginine
LPO	Lipid peroxide
LPS	Lipopolysaccharide
LT	Leukotriene
MAC	Membrane attack complex
MARS	Mixed antagonist response syndrome
MCP	Monocyte chemoattractant protein
MHC	Major histocompatibility complex
MIG	Monokine induced by interferon-γ
MIP	Macrophage inflammatory protein
mmHg	Millimetres of mercury
MODS	Multiple organ dysfunction syndrome
mRNA	Messenger ribonucleic acid
n	Number
NFκB	Nuclear factor kappa beta
ng	Nanogram
NK	Natural killer cell
NO	Nitric oxide
NOS	Nitric oxide synthase
ns	Non-significant
OFR	Oxygen free radical
OFS	Organ failure score
P^0	Unstimulated patients
P^5	Stimulated patients
PAF	Platelet activating factor
PAF-AH	Platelet activating factor acetylhydrolase
PAG	Peridontitis-associated genotype
PAGE	Polyacrylamide gel electrophoresis
pANCA	Perinuclear antineutrophil cytoplasmic antibodies
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
Pc	Corrected p value
PCR	Polymerase chain reaction

pg	Picogram
PG	Prostaglandin
PHA	Phytohemaglutinin
PLA_2	Phospholipase A_2
PNK	Polynucleotide kinase
PST	Periodontitis susceptibility trait
PSTI	Pancreatic secretory trypsin inhibitor
RANTES	Regulated upon activation normal T cell expressed and secreted
RER	Rough endoplasmic reticulum
RFLP	Restriction fragment length polymorphism
RIA	Radioimmunoassay
R/R MS	Relapsing/remitting multiple sclerosis
sCR1	Soluble complement receptor type 1
sIL-2R	Soluble interleukin-2 receptor
SIRS	Systemic inflammatory response syndrome
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
TAP	Trypsinogen activation peptide
TBE	Tris-boric acid- ethylene diamine tetra-acetic acid
TGF	Transforming growth factor
Th	T helper cell
TNF	Tumour necrosis factor
TX	Thromboxane
µg	Microgram
µl	Microlitre
UTR	Untranslated region
UV	Ultraviolet
VCAM	Vascular cellular adhesion molecule
VIP	Vasoactive intestinal polypeptide
VLP	Variable length polymorphism
VNTR	Variable number of tandem repeats
WBC	White blood cell
XOD	Xanthine oxidase

CHAPTER 1: THE PANCREAS AND ACUTE PANCREATITIS

Despite its first description more than 100 years ago (Fitz, 1889), acute pancreatitis (AP), an acute inflammatory disease of the pancreas remains a potentially fatal disease. It is characterized by diverse aetiologies, poorly understood pathophysiology, variable clinical course and managed largely by supportive treatment regimes.

1.1 The Normal Pancreas

1.1.1 Anatomy

The pancreas (Gk. *pan*-all, *kreas*-flesh) is a soft, lobulated gland which is located in the epigastric and left hypochondriac regions. It is 12-15cm in length and extends obliquely across the posterior abdominal wall behind the peritoneum from the curve of the duodenum to the hilum of the spleen. For purposes of description, the gland is divided into 4 parts: the head, neck, body and tail (Moore, 1996).

The pancreas develops from 2 endodermal buds, a small ventral bud and large dorsal bud (Sadler, 1990). These buds arise from the 2nd part of the duodenum in the 4th week of gestation and their resulting parenchyma and ducts later fuse to form a single gland. The main pancreatic duct (of Wirsung) is formed from the distal part of the dorsal pancreatic duct and the entire ventral pancreatic duct. It begins in the tail of the pancreas and runs through the substance of the gland near its posterior surface receiving exocrine secretions from interlobular ducts all along its length. The main pancreatic duct unites with the common bile duct ("the common channel") to form a small chamber, the ampulla of Vater that opens into the second part of the duodenum on the major duodenal papilla. A layer of circular muscle surrounds the ampulla of Vater to form the sphincter of Oddi. This sphincter reacts to neuronal and hormonal stimuli at the time of food ingestion and regulates the amount of pancreatic juice that enters the duodenum. The accessory duct (of Santorini) is formed from the proximal part of the dorsal pancreatic duct and drains the upper part of the head. It communicates with the main pancreatic duct to open on the major

duodenal papilla but also opens separately into the duodenum on the minor duodenal papilla, superior to the major duodenal papilla.

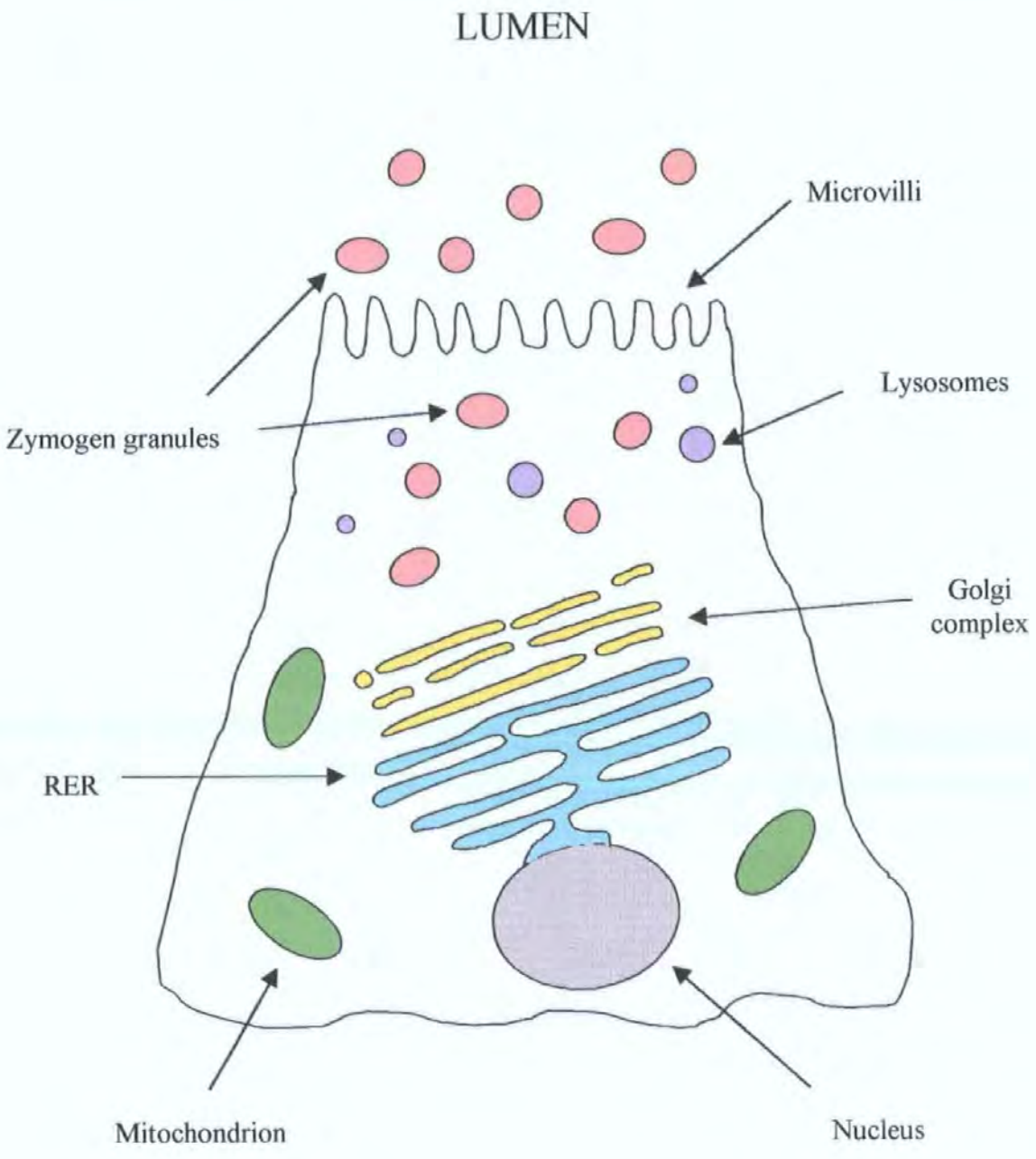
1.1.2 Histology and Physiology

Septa extend from the fibrous capsule that surrounds the pancreas and subdivide the parenchyma into lobules. Each lobule contains both exocrine and endocrine tissue. The exocrine portion consists of clusters of acini and secretory ducts and is concerned with the production of enzymes used in the digestion of protein, fat and carbohydrate. The endocrine portion consists of islets of Langerhans with specialist cells that produce hormones such as insulin and glucagon. Acute pancreatitis is a disease that affects the exocrine pancreas and therefore only the histology and physiology of this part of the gland will be discussed.

The acini (singular: acinus) are composed of spherical groups of acinar cells that secrete digestive proenzymes into a network of ducts that meet to form the main pancreatic duct. Acinar cells have a broad base and narrow apical surface covered by short microvilli. The basal half of the cell contains basophilic rough endoplasmic reticulum (RER), while the apical half close to the lumen contains eosinophilic zymogen granules that store the proenzymes synthesised by the cell. Acinar cells also have many mitochondria indicating that they are highly active cells. The proenzymes are synthesized by the RER and transferred to the Golgi complex that packages them into granules (Figure 1.1) (Stevens and Lowe, 1992).

Each acinus is drained by a small ductule called an intraacinar duct that is formed by centroacinar cells that are located at the centre of each acinus. These intraacinar ducts empty into intercalated ducts, then intralobular ducts and finally into interlobular ducts, which run in the septa between the pancreatic lobules. The interlobular ducts join the main pancreatic duct that runs longitudinally from the tail of the pancreas to its head and empty into the duodenal lumen at the ampulla of Vater.

Figure 1.1 The Acinar Cell



Pancreatic acinar cells have a broad base and narrow apical surface covered by short microvilli. The basal half of the cell contains basophilic RER, while the apical half close to the lumen contains eosinophilic zymogen granules that store the proenzymes synthesized by the cell. Acinar cells also have many mitochondria indicating that they are highly active cells. The proenzymes are synthesized by the RER and transferred to the Golgi complex that packages them into granules. The enzymes then migrate towards the apical cell membrane where they are secreted from the cell by exocytosis.

The pancreatic juice produced by the exocrine gland consists of 2 main components: digestive enzymes released by acinar cells and bicarbonate secreted by the ductal cells. The premature activation of the digestive enzymes is important in the pathogenesis of acute pancreatitis.

1.1.2.1 Pancreatic enzymes

Acinar cells secrete many different digestive enzymes including proteases, lipases, amylases, nucleases and trypsin inhibitors. With the exception of amylase and lipase, these enzymes are capable of attacking pancreatic cell membranes and are therefore secreted as the inactive proenzymes trypsinogen, chymotrypsinogen, proelastase 2, proprotease E, procarboxypeptidase A and B, prophospholipase A2 and procolipase. Amylase and lipase are not harmful to the pancreas and are therefore secreted in their active forms.

In order for the proenzymes to be able to cleave their substrates they first require activation. This is carried out by trypsin which itself has to be activated from its own proenzyme trypsinogen. Activation of trypsinogen to trypsin is usually performed by enterokinase, an enzyme located in the brush border of the duodenum, however, trypsin also has the ability to activate trypsinogen. Enterokinase activates trypsinogen by the cleavage of the trypsinogen activation peptide (TAP). Once in its active form, trypsin can activate the other proenzymes during a process known as the "pancreatic enzyme cascade" (Rinderknecht, 1988).

1.1.2.2 Enzyme synthesis and secretion

As with all eukaryote proteins, digestive enzymes and lysosomal hydrolases are synthesized on ribosomes attached to the RER. They then migrate within membrane-enclosed compartments to the Golgi complex, where lysosomal hydrolases are packaged into lysosomes and digestive enzymes into zymogen granules.

The zymogen granules migrate towards and fuse with the apical plasma membrane where the enzymes are released into the lumen during a process known as exocytosis. This is a complex process that is dependent on the hydrolysis of membrane polyphosphoinositides by phospholipase C to inositol 1,4,5 trisphosphate and diacylglycerol and subsequent activation of protein kinase C (Steer and Meldolesi, 1987).

1.1.2.3 Control of enzyme secretion

Pancreatic enzyme secretion is controlled through hormonal and neuronal pathways. The thought, sight, smell and taste of food during the cephalic phase of digestion and distension of the stomach during the gastric phase control pancreatic secretion via the parasympathetic vagal nerve. During the intestinal phase, fat and amino acids present in the duodenum cause the release of cholecystokinin (CCK) from cells in the mucosa of the duodenum. This CCK binds to its receptor on the membrane of the acinar cell and also interacts with the parasympathetic vagal nerve that releases acetylcholine and leads to the discharge of zymogen granules from the acinar cell by exocytosis. Acinar cells also have receptors for gastrin, VIP and secretin on their cell membranes, however, these are only weak stimulants of pancreatic enzyme secretion (Vander et al, 1990).

1.1.2.4 Protective mechanisms

The pancreas is protected from autodigestion by a variety of safety mechanisms that occur within the acinar cell, the lumen of the pancreatic duct and duodenum (Lankisch and Banks, 1998a).

In the acinar cell:

1. Digestive enzymes capable of attacking pancreatic cell membranes are synthesized and stored as inactive zymogens
2. Lysosomal hydrolases and digestive enzymes travel within membrane-bound vesicles and mature along different pathways

3. Trypsin inhibitor within zymogens (Pancreatic Secretory Trypsin Inhibitor) prevents premature activation of trypsinogen from trypsin
4. Acid pH within the zymogens inactivates any trypsin that might be prematurely activated from trypsinogen

In the pancreatic duct:

1. Low Ca^{2+} concentrations in duct lumen discourage trypsinogen activation
2. Any activated trypsin is inhibited by trypsin inhibitor in alkaline pH of duct lumen

In the duodenum:

1. Enterokinase, which activates trypsinogen is located in the duodenum rather than the pancreas

1.2 Pancreatitis

Pancreatitis is an inflammatory disease of the pancreas that exists in acute or chronic forms. Acute pancreatitis is distinguished from chronic pancreatitis by the absence of continuing inflammation, irreversible structural changes and permanent impairment of exocrine and endocrine function (Mergener and Baillie, 1998). For the purpose of this thesis, only acute pancreatitis will be considered.

1.2.1 Classification of acute pancreatitis

The heterogeneity of acute pancreatitis has made it difficult to classify. A number of international symposia were held between 1963 and 1988 in an attempt to categorize the disease. However, due to a lack of clinical information and morphological description, these were of limited use to the clinician attempting to diagnose and manage the condition (Banks, 1994). To provide a more comprehensive classification system, a symposium was held in Atlanta in 1992 which was based on information obtained by CT scanning (Bradley, 1993). The definitions that were derived from this symposium were:

"*Acute pancreatitis* is an acute inflammatory process of the pancreas with variable involvement of other regional tissues or remote organ systems."

"*Severe acute pancreatitis* is associated with organ failure and or local complications such as necrosis, abscess or pseudocyst."

"*Mild acute pancreatitis* is associated with minimal organ dysfunction and an uneventful recovery and it lacks the features of severe acute pancreatitis".

"*Acute fluid collections* occur early in the course of acute pancreatitis, are located in or near the pancreas and always lack a wall of granulation or fibrous tissue."

" *Pseudocyst* is a collection of pancreatic juice enclosed by a wall of fibrous or granulation tissue, which arises as a consequence of acute pancreatitis, trauma or chronic pancreatitis."

"*Pancreatic abscess* is a circumscribed intra-abdominal collection of pus usually in proximity top the pancreas, containing little or no pancreatic necrosis, which arises as a consequence of acute pancreatitis or pancreatic trauma."

"*Pancreatic necrosis* is a diffuse or focal area (s) of non viable pancreatic parenchyma which is typically associated with peripancreatic fat necrosis."

1.3 Epidemiology

1.3.1 Incidence

The incidence of acute pancreatitis varies considerably between different countries and between different regions within countries. The incidence is low in England and the Netherlands with figures of 11.7 and 10.2 per 100,000 population respectively (Giggs et al, 1988; Tran and Van Schilfgaarde, 1994); intermediate in Scotland and Germany with figures of 24.2 and 15.6 per 100,000 population respectively (Thomson et al, 1987; Assmus et al, 1996); and high in the United States of America and Finland where reported figures are as high as 79.8 and 73.4 per 100,000 population (Go, 1994; Jaakkola and Nordback, 1993). Many of these studies were performed over a long period of time and show the incidence of acute pancreatitis is increasing. In Britain alone there are as many as

10,000 new cases each year (Kingsnorth, 1997). The increase maybe due to improved diagnosis and increased awareness rather than due to a true increase in the number of cases (Larvin, 1996). An increase in males has been strongly correlated with increased alcohol consumption (Tran and Van Schilfgaarde, 1994; Jaakkola and Nordback, 1993).

1.3.2 Mortality rates

There is also a wide variation in mortality rates ranging from 6.1% to 19.6% (De Beaux et al, 1995; Corfield et al, 1985). In addition, there is disagreement regarding trends in mortality rates. Whilst some have suggested the mortality rates have remained constant despite the changing incidence of acute pancreatitis (Larvin, 1996), others suggest mortality rates are decreasing (De Beaux et al, 1995; Wilson and Imrie, 1990; McKay et al, 1999). The latter may be due to a number of factors including increased diagnosis of mild cases, a better understanding of the clinical course and improved diagnostic and management strategies (Cartmell and Kingsnorth, 2000).

1.4 Aetiology

A number of aetiological factors are known to cause acute pancreatitis and these can be divided into 4 main groups: gallstones (40%), alcohol (30%), idiopathic (20%) and 'other' causes (10%). 'Other' causes include trauma, drugs, infection, metabolic disorders, obstruction, ischemia, cystic fibrosis and hereditary pancreatitis.

1.4.1 Gallstones

Acute pancreatitis associated with gallstones is the most common form of the disease accounting for 40% of all cases (Lankisch et al, 1997), however, the risk of an individual with gallstones developing acute pancreatitis is low (3.4%) (Moreau et al, 1988). The diameter, number, shape and weight of the gallstone are important risk factors. (Diehl et al, 1997; Houssin et al, 1983).

1.4.1.1 Theories explaining the pathogenetic mechanism of gallstone-induced acute pancreatitis

The association between gallstones and acute pancreatitis was first reported by Opie in 1901 (Opie, 1901). He suggested 2 theories for the pathogenesis of acute pancreatitis based on his findings at two post-mortems: Theory 1: "The impaired flow hypothesis": Impaired pancreatic secretion due to an impacted gallstone at the duodenal papilla caused acute pancreatitis. Theory 2: "The common channel with bile reflux hypothesis": A common channel was created between the pancreatic duct and the common bile duct when a gallstone became impacted at the duodenal papilla. This common channel allowed bile to reflux from the biliary duct into the pancreatic duct to initiate acute pancreatitis.

"The duodenal reflux hypothesis" was suggested by Seidel (Seidel, 1910) and later by Pfeffer et al (Pfeffer et al, 1957). It was based on the principle that gallstones passing through the sphincter of Oddi caused sphincter insufficiency allowing duodenal juice containing activated enzymes to reflux back through the sphincter into the pancreatic duct to initiate acute pancreatitis. This was demonstrated in dogs using a closed duodenal loop model of acute pancreatitis created by ligating the duodenum above and below the ampulla of Vater. Duodenal juice containing activated enzymes was forced through the sphincter of Oddi and was found to induce acute pancreatitis. Although the duodenal reflux hypothesis was successfully demonstrated in animal models, passage of gallstones through the duodenal papilla in humans has been shown to produce stenosis of the sphincter and not sphincter insufficiency (Lankisch and Banks, 1998b).

Another theory that was suggested was "the combined bile and pancreatic duct obstruction hypothesis". To determine whether bile duct obstruction or bile reflux were important factors in gallstone-induced acute pancreatitis, Senninger et al, ligated the common bile duct (bile reflux possible), separate pancreatic and bile duct (bile reflux impossible), pancreatic duct (bile reflux possible) and bile duct (controls) of American opossums. They found that bile obstruction alone did not cause acute pancreatitis,

pancreatic duct obstruction alone caused mild acute pancreatitis while obstruction of both pancreatic duct and bile duct caused severe acute pancreatitis. From these results they concluded that pancreatic duct obstruction and bile duct obstruction but not bile reflux were important in severe acute pancreatitis (Senninger et al, 1986). Lerch et al, also used the opossum model to evaluate the severity of acute pancreatitis after ligation of the pancreatic duct, pancreatic and bile ducts and common bile duct. In contrast to findings by Senninger et al, they found that severe acute pancreatitis developed in all models regardless of whether bile reflux occurred. They concluded that neither bile duct obstruction or bile reflux caused acute pancreatitis or increased severity of the disease, but that acute pancreatitis was caused by pancreatic duct obstruction alone (Lerch et al, 1993).

It is now widely accepted that gallstone-induced acute pancreatitis is due to a transient blockage of the ampulla by migrating gallstones which causes hypertension in the pancreatobiliary duct system. This hypothesis is called "the transient obstruction hypothesis" and is based largely on findings by Acosta et al, who carried out a study on 78 patients with gallstone-induced acute pancreatitis admitted to hospital within 48 hours of the onset of symptoms. Sixty-three percent of patients were found to have gallstones at the ampulla of Vater implying that impaired pancreatic outflow due to obstruction was important in gallstone-induced acute pancreatitis. It was suggested that the absence of ampullary obstruction in some patients was due to the early migration of the gallstone into the duodenum. Furthermore, the decrease in the incidence of ampullary obstruction correlated with the presence of gallstones in faeces. Acosta et al, also found that the degree of pancreatic damage was directly related to the duration of ampullary obstruction. The longer the impaction time, the more severe the disease. They suggested that the gallstones in patients with mild acute pancreatitis migrated within 48 hours of the onset of symptoms and caused minimal pancreatic damage, however, in patients with severe disease migration occurred later resulting in irreversible pancreatic lesions. This study demonstrated that

early removal of the gallstones with the restoration of pancreatic outflow was important in preventing severe acute pancreatitis (Acosta et al, 1980).

1.4.2 Alcohol

Alcohol-induced acute pancreatitis is the second most common form of the disease and accounts for 30% of all cases (Lankisch et al, 1997). Since only 5% of alcoholics develop alcoholic acute pancreatitis, other factors related to the host must be important in susceptibility to the disease (Meier, 1995). The risk of developing the disease increases with the duration and amount of alcohol consumed (Mergener and Baillie, 1998; Lankisch and Banks, 1998c). Although binge drinking in the chronic alcoholic can cause acute pancreatitis, episodes of acute pancreatitis in the non-alcoholic binge drinker are rare (Meier, 1995; Robles-Diaz and Gorelick, 1997). Young males tend to be most at risk of developing alcohol-induced acute pancreatitis due to the social pressures placed upon them to consume excessive amounts of alcohol.

It remains controversial as to whether alcoholic acute and chronic pancreatitis are separate disease entities or whether acute pancreatitis reflects underlying chronic pancreatitis. Ammann and Muellhaupt have suggested that "true" alcoholic acute pancreatitis does exist in a small number of patients, however, in the majority of cases it reflects underlying alcoholic chronic pancreatitis (Ammann and Muellhaupt, 1994). Diagnosis of acute pancreatitis presumes that the pancreas is structurally and functionally normal prior to the attack, however, in most cases the condition of the pancreas is largely unknown. Generally, the disease is considered to be alcoholic acute pancreatitis unless there is evidence of chronic pancreatitis e.g. calcifications. The mechanism by which alcohol initiates acute pancreatitis remains unknown.

1.4.2.1 Theories explaining the pathogenetic mechanism of alcohol-induced acute pancreatitis

"The large duct obstruction hypothesis" postulates that alcohol causes contraction of the sphincter of Oddi that obstructs the pancreatic duct in a similar manner to Opie's first hypothesis for gallstone-induced acute pancreatitis. A variation of this theory is that contraction of the sphincter of Oddi caused by alcohol creates "a common channel" in much the same way as Opie's second theory for gallstone-induced acute pancreatitis. An alternative hypothesis based on Pfeffer's closed loop model and demonstrated by Goff implies that instead of causing contraction of the sphincter of Oddi, alcohol causes it to relax allowing duodenal contents with activated enzymes to reflux back into the pancreas to initiate acute pancreatitis (Goff, 1993). In addition to its effects on the sphincter of Oddi, studies on cats have shown that alcohol increases the pancreatic duct permeability allowing large molecules including activated pancreatic enzymes to enter the parenchyma to initiate acute pancreatitis (Wedgwood et al, 1986).

"The small duct obstruction hypothesis" suggests that alcohol causes the development of "protein plugs" in the small pancreatic ducts. These plugs cause obstruction resulting in increased pancreatic pressure and the extravasation of activated pancreatic enzymes into the pancreatic tissue. This has been supported by findings from Guy et al, who demonstrated elevated levels of protein plugs in the pancreatic juice of patients with alcoholic pancreatitis compared to controls (Guy et al, 1983). These protein plugs may form as a result of decreased lithostathine levels (pancreatic stone protein) that inhibit protein plug formation (Multigener et al, 1985; Schenker and Montalvo, 1998). However, it has been shown that mRNA levels of lithostathine in the rat pancreas are actually increased after 4 weeks of ethanol exposure (Apte et al, 1996).

The third theory is "the toxic-metabolic hypothesis". Alcohol and its metabolites, acetaldehyde and fatty acid ethyl esters (FAEEs) may have direct toxic effects on pancreatic acinar cells. Xanthine oxidase oxidizes ethanol and acetaldehyde with the release of oxygen free radicals (OFRs). Increased OFR levels and reduced antioxidants in

animal models of acute pancreatitis results in oxidative stress which causes lipid peroxidation and increased acinar cell membrane permeability (Braganza and Rinderknecht, 1988; Iimuro et al, 1996; Nordback et al, 1991). Ethanol administered to rats has also been shown to increase the mRNA and protein levels of digestive (trypsinogen, chymotrypsinogen and lipase) and lysosomal enzymes (cathepsin B) (Apte et al, 1998). Moreover, the pancreas of a rabbit incubated with a 3% concentration of ethanol over a 2 hour period was found to have increased concentrations of α amylase, trypsinogen and chymotrypsinogen but decreased levels of the protective trypsin inhibitor compared to controls (Steer et al, 1979). FAEEs extracted from the pancreas of intoxicated humans and infused into rats at concentrations comparative to those found in human plasma caused pancreatic edema, trypsinogen activation and vacuolization of acinar cells (Werner et al, 1997a). In addition, FAEEs added directly to rat pancreatic lysosomes were found to increase their fragility (Haber et al, 1993).

It is likely that alcohol-induced acute pancreatitis is mediated at least in part by increased acinar cell membrane permeability, increased pancreatic enzyme synthesis and their release from fragile zymogen and lysosomal compartments. Furthermore, ethanol may reduce pancreatic blood flow suggesting a role for ischemia-reperfusion injury in alcoholic acute pancreatitis (Toyama et al, 1997).

1.4.3 Idiopathic

Idiopathic aetiology accounts for 20% of cases (Lankisch et al, 1997). It has been suggested that the majority of these cases are caused by biliary sludge and microlithiasis (Lee et al, 1992; Ros et al, 1991).

1.4.4 Other causes

The remaining 10% of cases of acute pancreatitis are due to rare causes such as trauma of the pancreas due to accidental abdominal injuries (Ryan et al, 1994) or following

medical/surgical procedures such as endoscopic retrograde cholangiopancreatography (ERCP), endoscopic sphincterotomy (ES) (Gottlieb and Sherman, 1998), biliary tract surgery (Vernava et al, 1987) and cardiopulmonary bypass (Ohri et al, 1991). Drugs may also be associated with the development of acute pancreatitis. (McArthur, 1996).

Acute pancreatitis may also be caused by a number of infectious agents such as parasites (Toxoplasma, Cryptosporidium, Ascaris), bacteria (Mycoplasma, Legionella, Leptospira, Salmonella), fungi (Aspergillus) and viruses (Mumps, Coxsackie, Hepatitis B, Cytomegalovirus, Varicella-zoster virus, Herpes simplex virus) (Parenti et al, 1996). Patients infected with the human immunodeficiency virus (HIV) are particularly at risk of developing the disease (Dassopoulos and Ehrenpreis 1999; Manocha et al, 1999). It has been suggested however, that this may be due to opportunistic infections such as cytomegalovirus, drugs or neoplasm associated with AIDS rather than the HIV itself (Lankisch and Banks, 1998c).

Metabolic disorders such as hyperlipidemia (Fortson et al, 1995) and hypercalcemia (Frick et al, 1995), and obstructive disorders such as by pancreatic tumors (Lin and Feller, 1990), duodenal diverticula (Griffen et al, 1968) and pancreas divisum (Bernard et al, 1990) have also been shown to be causative. Furthermore, since the pancreas has a very abundant blood supply it is particularly susceptible to diseases that cause ischemia (Sakorafas et al, 2000).

Recent research in the field of pancreatitis has focussed on the role of cystic fibrosis and hereditary pancreatitis as possible causes. Although both have been implicated in acute pancreatitis, most studies have been performed in patients with chronic pancreatitis.

1.4.4.1 Cystic fibrosis (CF)

Cystic fibrosis is the most common inherited disease of the exocrine pancreas and is caused by a defective cystic fibrosis transmembrane conductance regulator (CFTR)

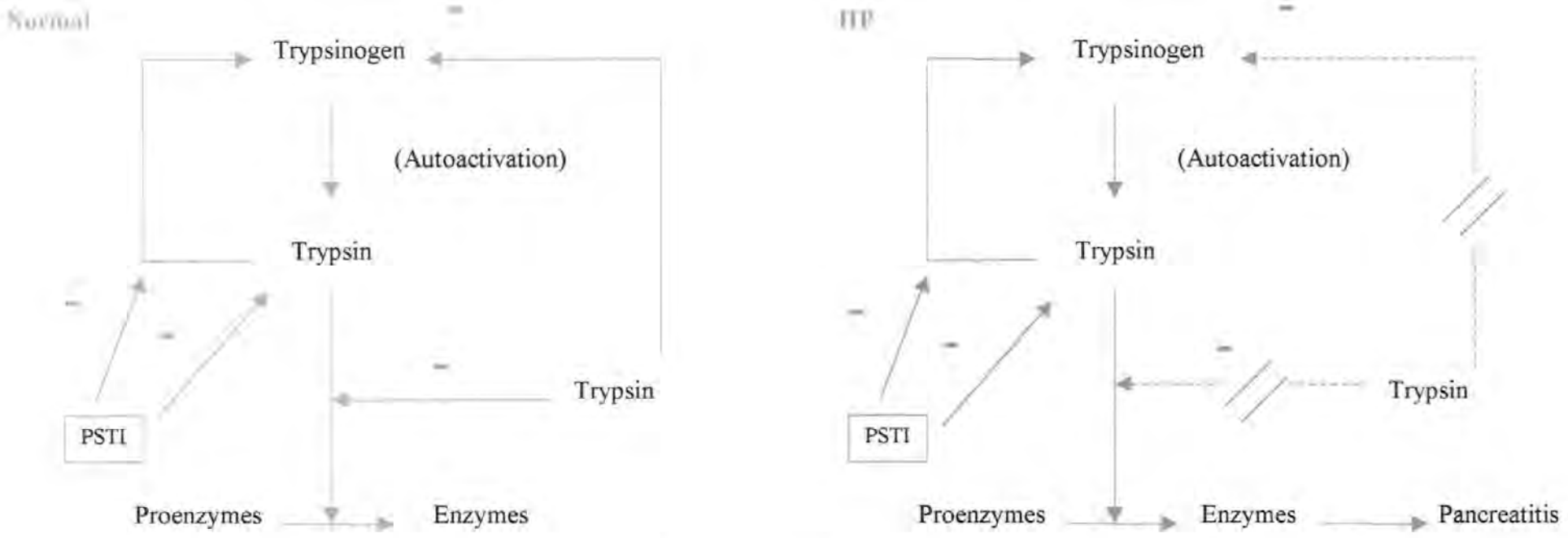
protein. When the CFTR is defective there is reduced water and ion transport across the apical membrane of ductal cells resulting in the production of highly viscous secretions that obstruct the ducts of the pancreas. Mutations of the CFTR gene have been associated with chronic idiopathic pancreatitis (Cohn et al, 1998; Sharer et al, 1998), however, to date there have been no reports of an association between CFTR gene mutations and acute pancreatitis.

1.4.4.2 Hereditary Pancreatitis (HP)

Hereditary pancreatitis is a rare form of the disease and since its description in 1952 by Comfort and Steinberg, only 100 kindreds have been reported worldwide. HP is an autosomal dominant disorder with 80% penetrance characterized by recurrent attacks of acute pancreatitis with onset typically at 5-10 years of age. It is caused by specific mutations in the cationic trypsinogen (CAT) gene which has been mapped to chromosome 7q35 (Whitcomb et al, 1996a). Two important mutations known as R117H and N21I have been identified in the gene which define 2 types of HP: type I hereditary pancreatitis (R117H) and type II hereditary pancreatitis (N21I) (Gates et al, 1999).

Whitcomb et al, identified a G to A mutation in exon 3 of the CAT gene which involved the substitution of arginine for histidine at codon 117 (R117H). The arginine acts as a hydrolysis site (cleavage site) whereby trypsin is degraded and inactivated (Whitcomb et al, 1996b). Overactivation of trypsin is prevented by trypsin hydrolysis and the trypsin inhibitor, PSTI. The R117H mutation prevents hydrolysis of the trypsin and this leads to the second protective mechanism (PSTI) becoming overwhelmed leading to uncontrolled trypsin production and subsequent activation of other proenzymes (Whitcomb et al, 1996b)(Figure 1.2). This intermittent overwhelming of PSTI in the presence of R117H explains why these individuals experience attacks of acute pancreatitis.

Figure 1.2: Mechanism for the development of Hereditary Pancreatitis



Normally inactive trypsinogen is converted to active trypsin with the cleavage of TAP by two mechanisms: 1) enterokinase in the duodenum 2) autoactivation by trypsin itself. The pancreas has 2 protective mechanisms to prevent trypsin from being overactivated: 1) trypsin hydrolysis 2) trypsin inhibitor, PSTI.

HP is caused by a G to A mutation in the cationic trypsinogen gene that causes the substitution of arginine for histidine at codon 117 (R117H). The arginine acts as a hydrolysis site (cleavage site) whereby trypsin is degraded and inactivated. When the R117H mutation is present the arginine hydrolysis site is replaced with histidine and trypsin hydrolysis is prevented. The second protective mechanism, PSTI can only inactivate 20% of trypsin and becomes overwhelmed at times leading to uncontrolled trypsin production and subsequent activation of other proenzymes. This intermittent overwhelming of PSTI in the presence of R117H explains why individuals experience attacks of acute pancreatitis.

(Adapted from Whitcomb et al, 1996b)

Gorry et al, have described a N21I substitution in exon 2 in HP patients without the R117H mutation. The molecular basis for HP in these patients is unknown although the presence of isoleucine may inhibit access to the hydrolysis site of trypsin (Gorry et al, 1997). Studies have shown that patients with type I hereditary pancreatitis (R117H mutation) have a more severe form of the disease in terms of age of onset and requirement for surgery than those with type II hereditary pancreatitis (N21I mutation)(Creighton et al 2000; Gorry et al, 1997).

Other mutations have been identified in the CAT gene. An A16V substitution in exon 2 has been found to affect the first amino acid of the trypsinogen molecule and therefore the cleavage site of the signal peptide (Witt et al, 1999). A K23R substitution occurs in exon 2 that has been found to alter the structure of PSTI binding site (Ferec et al, 1999) and a -28delTCC has been shown to increase transcription of trypsinogen (Ferec et al, 1999).

The recurrent attacks of acute pancreatitis caused by a mutated CAT gene and overwhelmed PSTI subsequently lead to chronic pancreatitis. This further substantiates reports by Ammann and Muellhaupt, which suggested a progression of acute to chronic pancreatitis.

The pathogenetic mechanism involved in HP suggests a role for excess trypsin activation in the pathogenesis of other forms of acute pancreatitis. Furthermore, a study by Creighton et al, demonstrated that 19% of patients referred to a clinic with idiopathic chronic pancreatitis showed mutations in the CAT gene suggesting that HP could be an undetected cause of idiopathic chronic pancreatitis (Creighton et al, 1999). This may also be the case for patients with idiopathic acute pancreatitis.

The pathophysiology of acute pancreatitis will be discussed in Chapter 3.

1.5 Pathology

Two main forms of acute pancreatitis are recognized pathologically: acute edematous pancreatitis (80%) and acute necrotizing pancreatitis (20%)(Banks, 1994). Acute edematous pancreatitis, which corresponds clinically to mild acute pancreatitis, is characterised by interstitial edema, limited peripancreatic fat necrosis and an absence of organ dysfunction. Acute necrotizing pancreatitis, which corresponds clinically to severe acute pancreatitis, is characterised by pancreatic parenchymal necrosis, extensive peripancreatic and intrapancreatic fat necrosis, and the development of organ failure/local complications. This form of the disease may also be associated with hemorrhage in which case it is called acute necrotizing hemorrhagic pancreatitis (Krumberger, 1993).

1.6 Clinical Presentation

Patients with acute pancreatitis generally present with a sudden onset of severe epigastric pain, nausea and vomiting. In 50% of patients, the pain radiates through to the back and is frequently confused with the pain of myocardial infarction (Mergener and Baillie, 1998). Clinical signs of acute pancreatitis include fever with temperatures reaching 38.5°C, dehydration due to episodes of vomiting, confusion due to hypoxia, abdominal tenderness and guarding. There may also be signs of hypovolemic shock and jaundice in gallstone-induced acute pancreatitis. In less than 5% of severe cases bruising occurs around the umbilicus (Cullen's sign) or flanks (Grey Turner's sign) and represents the spread of the retroperitoneal inflammatory infiltrate (Sargen and Kingsnorth, 1998). These skin associated features of acute pancreatitis have been associated with poor prognosis and fatal outcome in 35% of cases (Dickson and Imrie, 1984).

1.7 Natural History

The natural history of acute pancreatitis is variable. The majority of patients (80%) suffer from mild acute pancreatitis, a self-limiting disease that responds to conservative

treatment. Up to 20% of patients with acute pancreatitis, however, proceed to a clinically severe form with the systemic inflammatory response syndrome (SIRS) and organ complications (Mergener and Baillie, 1998). Prognosis following an attack of acute pancreatitis depends largely on whether the necrotic pancreas becomes infected with bacteria. 40-70% of patients with severe disease will develop infection and up to 50% will die (Lemaire et al, 1997).

1.8 Complications

Acute pancreatitis is often associated with local and systemic complications. Most early deaths from acute pancreatitis occur within the first week and are due to systemic complications, in particular respiratory complications (Neoptolemos et al, 1998). Deaths that occur after the first week are largely due to septic complications.

1.8.1 Local complications

Local complications that affect the pancreas or peripancreatic tissue include: acute fluid collections, pseudocyst, pancreatic necrosis and pancreatic abscess (as previously defined in Section 1.2.1).

1.8.2 Systemic complications

Systemic complications are those which involve failure of organ systems distant from the pancreas. The most common systemic complications are respiratory, renal, cardiovascular, haematologic and metabolic complications (Gross et al, 1993). Failure of more than 1 organ system results in a potentially fatal condition known as multiple organ dysfunction syndrome (MODS).

MODS is defined as "a syndrome of progressive but potentially reversible organ failure, involving two or more systems remote from the original insult" (Neoptolemos et al, 1998).

A common sequence of events is respiratory failure, followed by renal and then cardiovascular and finally coagulation failure. The mortality rate is directly related to the number of organ systems involved with 1, 2, 3 and 4 organ system failures corresponding to 18%, 54%, 90%, 100% mortality respectively (Larvin, 1996).

Evidence obtained from animal models has shown that acute pancreatitis is an inflammatory process and these experiments are reviewed in Chapter 3.

CHAPTER 2: IMMUNOLOGY AND THE ACUTE INFLAMMATORY PROCESS

Acute pancreatitis is an example of an acute inflammatory response. This response forms part of the non-specific natural immune system.

2.1 Components of the acute inflammatory response

The acute inflammatory response is a complex biological process that involves the interaction of many different components including plasma mediator systems (coagulation, fibrinolytic, kinin and complement), specific cells (neutrophils, monocytes/macrophages, endothelial cells) and cell-derived mediators (PAF, arachidonic acid metabolites, oxygen free radicals, nitric oxide, cytokines). The role of each of these components in acute pancreatitis is discussed in Chapter 3.

2.1.1 Plasma mediator systems

The coagulation, fibrinolytic, kinin and complement systems are a series of plasma enzyme cascades that co-activate and interact with each other during inflammation. Activation of Hageman factor (Factor XII) by foreign agents such as bacteria is central to the activation of these plasma mediator systems. Activated Hageman factor results in the activation of thrombin and conversion of fibrinogen to fibrin and the conversion of plasminogen to plasmin. Plasmin degrades fibrin and these products are chemotactic for neutrophils and cause activation of the complement system. In addition, activated Hageman factor induces the conversion of kininogen to bradykinin by kallikrein in the kinin system. Kallikrein amplifies Hageman factor production and therefore acts as a positive feedback loop. Bradykinin and other kinins are vasoactive peptides that cause increased vascular permeability, vasodilatation and the release of arachidonic acid metabolites. The complement system is a group of plasma proteins that are involved in the control of inflammation, the activation of phagocytes and the lytic attack on cell membranes. The products of complement component C3 cleavage have important roles in

inflammation. C3a and C5a cause the degranulation of mast cells with the release of histamine. C5a is chemotactic for phagocytes and causes the release of prostaglandins and leukotrienes. C3b is an opsonin which binds to the antigen surface and facilitates phagocytosis while C5b-C9 are important in cell lysis by the formation of membrane attack complexes (MAC) that form pores in target cell membranes and cause cell death (Burnett, 1997).

2.1.2 Cell mediators

The main cells that are involved in inflammation are neutrophils, monocytes/macrophages and endothelial cells.

2.1.2.1 Neutrophils

Neutrophils are large cells (diameter: 12-14μm) with a multi-lobed nucleus whose neutral cytoplasmic granules are poorly stained with haematoxylin and eosin (neutrophilic). They are derived from the myeloid cell lineage and account for 40-75% of leucocytes. Their function is to carry out phagocytosis (Section 2.2.5).

2.1.2.2 Monocytes/macrophages

Monocytes are large cells (diameter: 20μm) with a kidney bean shaped nucleus that circulate briefly in the bloodstream before migrating to the tissues to differentiate into macrophages. They are derived from the myeloid cell lineage and account for 1-5% of leucocytes. Their function is to perform phagocytosis and to act as antigen presenting cells.

2.1.2.3 Endothelial cells

Endothelial cells are elongated cells whose function during the acute inflammatory response is to allow the migration of leucocytes from the blood to the site of tissue injury. This process is mediated by adhesion molecules that are present on the surface of both

leucocytes and endothelial cells. There are 3 types of adhesion molecules that are sequentially activated during a process known as "the adhesion cascade" (Adams and Nash, 1996). First, selectins such as E (endothelium) selectin, P (platelet) selectin and L (leucocyte) selectin; second, integrins such as CD18 and CD11 and third, intercellular adhesion molecules such as ICAM-1 and VCAM-1. The expression of adhesion molecules is upregulated on endothelial cells in response to inflammatory mediators such as cytokines (IL-1, IL-6, IL-8, TNFα) during a process known as "endothelial cell activation" (Hunt and Jurd, 1998). Because of their central role in mediating leucocyte migration into damaged tissues, adhesion molecule interactions represent a therapeutic target for inflammatory diseases (Serafini and Rosemurgy, 2000). The role of adhesion molecules in inflammation is explained in more detail in Section 2.2.3.

2.1.3 Cell-derived mediators

2.1.3.1 Platelet activating factor (PAF)

PAF (1-0-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a biologically active phospholipid that is released from cell membranes by the action of phospholipase A_2 (Imaizumi et al, 1995). It is produced by a variety of cells including endothelial cells, monocytes, macrophages and neutrophils and may be synthesised by 2 pathways: the *de novo* pathway and the remodeling pathway. The *de novo* pathway maintains normal physiological intracellular PAF levels in organs such as the brain and kidney, while the remodeling pathway occurs in neutrophils, monocytes and endothelial cells that are stimulated during pathological conditions by other inflammatory mediators such as cytokines or OFRs (Johnson, 1999). PAF has many biological effects that are mediated through its binding to its specific PAF receptor on target cells. The receptor is G protein-coupled and subsequent binding of PAF leads to elevated intracellular calcium levels followed by production of inflammatory mediators such as IL-1 and TNFα and to changes in vascular permeability. The main pathological effect of PAF during inflammation is to

cause the migration of activated leucocytes from the blood vessels into the interstitium of organs and the release of substances such as proteolytic enzymes, elastase, cathepsin B and superoxide ions that cause tissue damage (Kingsnorth, 1996). Blockade of the PAF receptor using PAF antagonists such as Lexipafant™ has been shown to have beneficial effects in acute pancreatitis (Kingsnorth et al, 1995).

2.1.3.2 Arachidonic acid metabolites

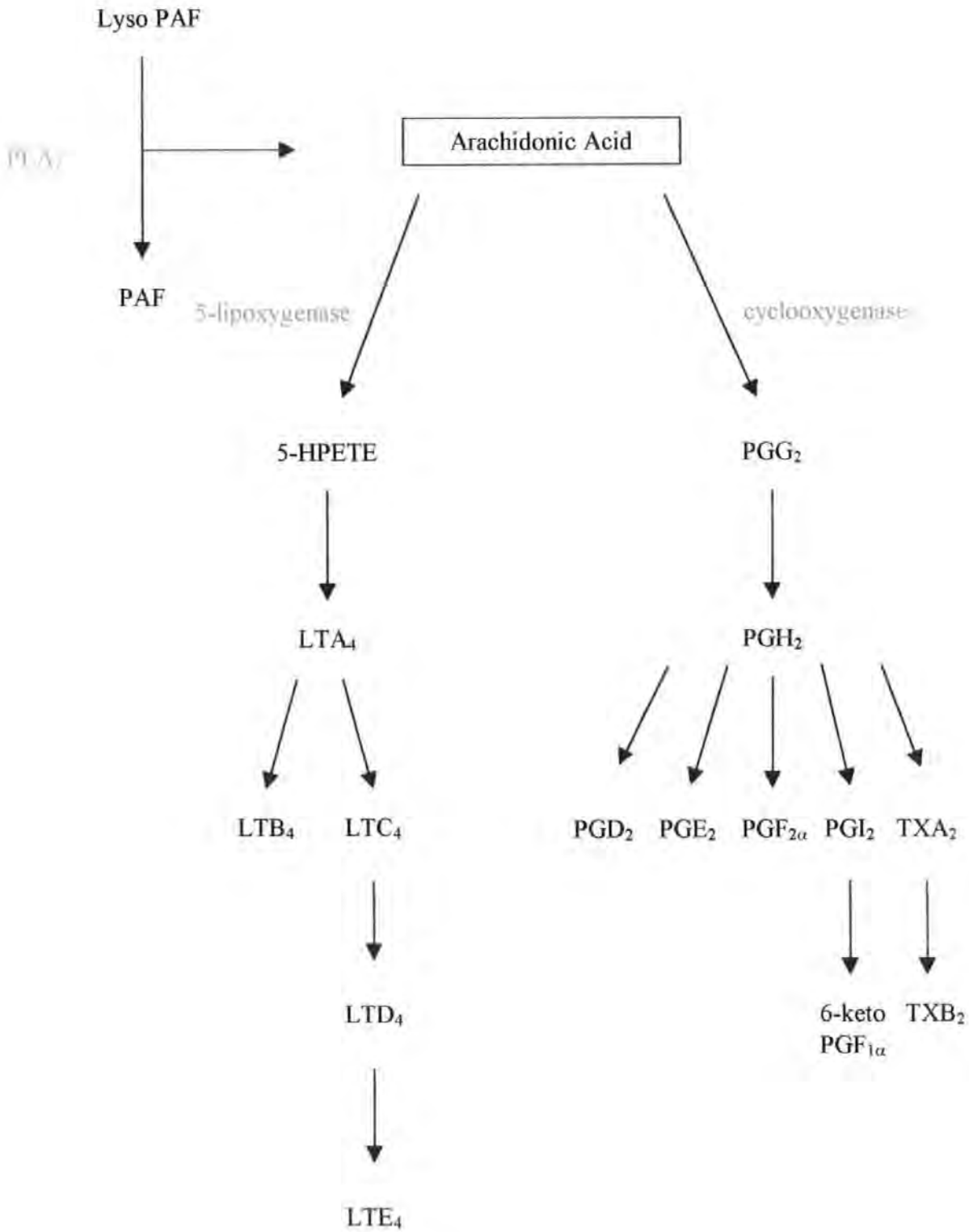
Arachidonic acid is released as a side product of PAF production by the action of phospholipase A_2 during the remodeling pathway (Johnson, 1999). Arachidonic acid is oxidized further by 5-lipoxygenase to produce leukotrienes e.g. LTB_4, whilst oxidation by cyclooxygenase produces prostaglandins e.g. PGI_2 and thromboxanes e.g.TXA_2 (Figure 2.1). These metabolites have different effects in inflammation. Leukotrienes cause increased vascular permeability, whilst prostaglandins cause vasodilatation and pain, and thromboxanes cause vasoconstriction and platelet aggregation. LTB_4 is an important leukotriene since it attracts leucocytes particularly neutrophils to sites of inflammation (Burnett, 1997).

2.1.3.3 Oxygen free radicals (OFRs)

OFRs (e.g $O_2^{-\bullet}$, $OH^{\bullet}$, $O^{\bullet}$) are a group of highly reactive oxygen species that are produced as a side product of normal oxidative metabolism and by phagocytes during the respiratory burst of phagocytosis. OFRs have an unpaired electron and cause damage to other molecules when they transfer the unpaired electron to them. Cells have antioxidant mechanisms to protect themselves against the harmful effects of the OFRs and these include enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase and endogenous free radical scavengers such as vitamins E, C and β carotene (Sweiry and Mann, 1996).

Figure 2.1: Production of PAF and arachidonic acid from cell membranes

Remodeling pathway



During the remodeling pathway Phospholipase A_2 (PLA_2) acts on cell membranes with the release of PAF. Arachidonic acid is formed as a side product. Further oxidation by 5 lipoxygenase produces leukotrienes whereas further oxidation by cyclooxygenase produces prostaglandins and thromboxanes.

Excess production of OFRs or failure of the protective antioxidant mechanisms results in oxidative stress. The OFRs attack lipids in cell membranes (lipid peroxidation), causing increased membrane permeability and subsequent cell death. In addition to their direct toxic effect on cells, OFRs also cause the production of chemotactic factors and the production of PAF and arachidonic acid metabolites.

2.1.3.4 Nitric Oxide (NO)

Nitric oxide is produced by endothelial cells and macrophages. Its function is to regulate blood vessel tone and microvascular permeability. NO is synthesized from the amino acid L-arginine by the family of enzymes known as nitric oxide synthase (NOS). There are 2 main types of NOS known as constitutive cNOS (calcium dependent) and inducible iNOS (non-calcium dependent). Synthesis of NO by endothelial cells cNOS has a protective and anti-inflammatory function, whilst production of NO by iNOS can lead to the sustained production of high concentrations of NO leading to vasodilatation, tissue edema, and stimulation of immune cells to produce cytokines (Anggard 1994).

2.1.3.5 Cytokines

Cytokines are a diverse group of low molecular weight signalling proteins that are produced by a wide variety of stimulated cells. They control the amplitude and duration of immune and inflammatory responses by affecting the activity, proliferation and differentiation of immune cells and therefore represent potential therapeutic targets (Cohen and Cohen, 1996). They act at low concentrations (picomolar concentrations) in an autocrine, paracrine or endocrine manner by binding to specific cell surface receptors on target cells to cause altered gene expression. Cytokines also exhibit pleiotropy (a cytokine may have multiple effects on a target cell) and redundancy (different cytokines can induce the same effect in a particular target cell). Furthermore, they do not act alone but interact in a "cytokine network". A particular cytokine may increase/decrease the production of

another cytokine (cytokine cascade), block (antagonism) or amplify (synergism) its effect or increase/decrease the expression of its cytokine cell surface receptors (receptor transmodulation)(Vilcek, 1998).

There are many different types of cytokine and the number continues to rise. For the purpose of this study, cytokines will be classified into interleukins, TNFα, interferons, colony stimulating factors and TGFβ. The cell origin and immunological function of selective cytokines are shown in Table 2.1.

2.1.3.5.1 Chemokines

Chemokines are a group of chemotactic cytokines that are involved in the activation and migration of inflammatory cells from the blood into damaged tissues. They are structurally related proteins each containing 4 cysteine residues in the mature protein (with the exception of lymphotactin) and are subdivided into 4 families (α, β, δ and γ chemokines) on the basis of the relative position of the cysteine residues (Luster, 1998). α chemokines (CXC) contain a single amino acid between the first two cysteine residues. These are further subdivided into ELR and non-ELR α chemokines according to the presence or absence of a glutamic acid-leucine-arginine (ELR) sequence immediately preceding the first cysteine residue. α chemokines attract neutrophils and lymphocytes and examples include IL-8, GROα, GROβ, GROγ and MIG. β chemokines (CC) contain adjacent cysteine residues. They are also subdivided into 2 families: the monocyte chemoattractant protein-eotaxin family and "other β chemokines". The monocyte chemoattractant protein-eotaxin family includes the MCP1-5 and eotaxin and examples of "other β chemokines" include MIP-1α, MIP-1β and RANTES. β chemokines attract lymphocytes, monocytes, eosinophils and basophils. δ chemokines (CXXXC) contain 3 amino acids between the first two cysteines and examples include neurotactin and fractalkine. The only γ chemokine (C) identified to date is lymphotactin (IL-16) which only contains 2 cysteines in the mature protein.

Table 2.1: Selected cytokines, cell origin and immunological functions

Cytokine	Immune cells	Other cells	Immunological function
IL-1α/β	Monocytes/macrophages	Endothelial, epithelial and neuronal cells, fibroblasts	Activation of T and B cells, macrophages and endothelium Stimulation of acute phase response
IL-2	T cells	-	Proliferation and/or activation of T, B and NK cells
IL-3	T cells, mast cells, thymic epithelium	Keratinocytes, neuronal cells	Proliferation of pluripotent stem cells Production of various blood cell types
IL-4	T and B cells, macrophages, mast cells and basophils, bone marrow stroma	-	Activation of B cells. Differentiation of Th2 cells and suppression of Th1 cells
IL-5	T cells, mast cells	-	Development, activation and chemoattraction of eosinophils
IL-6	T cells, monocytes or macrophages	Fibroblasts, hepatocytes, endothelial and neuronal cells	Activation of haematopoietic stem cells. Differentiation of B and T cells. Production of acute phase response
IL-7	Bone marrow stroma	-	Growth of B cell precursors. Proliferation and cytotoxic activity of T cells
IL-8*	T cells, monocytes, neutrophils	Endothelial and epithelial cells, fibroblasts	Chemoattraction of neutrophils, T cells, basophils Activation of neutrophils
IL-9	T cells	-	Development of erythroid precursors

IL- Interleukin * also classed as a chemokine

Table 2.1 continued

Cytokine	Immune cells	Other cells	Immunological function
IL-10	T and B cells, macrophages	Keratinocytes	Suppression of macrophage functions and Th1 cells Activation of B cells
IL-11	Bone marrow stroma	Trophoblasts	Stimulation of haemopoietic precursors Production of actute phase proteins
IL-12	B cells, macrophages	-	Differentiation of Th1 cells. Activation of NK cells and T cells
IL-13	T cells	-	Activation of B cells. Inhibition of monocytes or macrophages
IL-14	T cells	-	Proliferation of activated B cells but inhibition of immunoglobulin secretion
IL-15	T cells	-	Proliferation of T cells
IL-16*	T cells	-	Chemotaxis Increased expression of class II MHC cytokine synthesis, suppress antigen induced proliferation
IL-17	T cells	-	Activation of macrophages and neutrophils
IL-18	Macrophages	Keratinocytes, adrenal cortical cells, osteoblasts	Induces IFNγ production in T cells and NK cells

IL-Interleukin * also classed as a chemokine

Table 2.1 continued

Cytokine	Immune cells	Other cells	Immunological function
TNFα	Macrophages, lymphocytes, neutrophils	Astrocytes, endothelium, smooth muscle	Activation of macrophages, granulocytes, cytotoxic cells and endothelium. Enhanced HLA class I expression Stimulation of acute phase response Antitumour effects
IFNα,β	T and B cells, monocyte or macrophages	Fibroblasts	Antiviral activity Stimulation of macrophages and NK cells Enhanced HLA class I expression
IFNγ	T and NK cells	-	Antiviral activity Stimulation of macrophages and endothelium Enhanced HLA class I and class II expression Suppression of Th2 cells
G-CSF	T cells, macrophages, neutrophils	Fibroblasts, endothelium	Development and activation of neutrophils
M-CSF	T cells, macrophages, neutrophils	Fibroblasts, endothelium	Development and activation of monocytes/macrophages
GM-CSF	T cells,macrophages, mast cells, neutrophils, eosinophils	Fibroblasts, endothelium	Differentiation of pluripotent stem cells Development of neutrophils, eosinophils and macrophages
TGFβ	T cells, monocytes	Chondrocytes, osteoblasts, osteoclasts, platelets, fibroblasts	Inhibition of T and B cell proliferation and NK cell activity

TNF Tumour necrosis factor; IFN Interferon; CSF Colony stimulating factor; TGF Transforming growth factor

(Adapted from Reeves and Todd, 1996)

The action of chemokines is mediated through their binding to specific G-protein coupled cell surface receptors on target cells. Four families of human chemokine receptor have been identified: CXC (CXCR1-CXCR4), CC (CCR1-CCR8), CXXXC (CX_3CR1) and C (XCR1)(Rossi and Zlotnik, 2000).

Chemokine production is stimulated by a variety of factors including the pro-inflammatory cytokines IL-1 and TNFα. During inflammation they provide signals to convert low affinity selectin mediated interactions into higher affinity integrin-mediated interactions that leads to extravasation of leucocytes. Furthermore, once inside the tissue leucocytes migrate along a chemotactic gradient created by chemokines to the exact site of injury by a process known as chemotaxis (Luster, 1998). Because of their central role in pathological processes such as inflammation, chemokines and their receptors represent a target for therapeutic intervention. A recent example of this has been demonstrated in the field of AIDS research. NSC 651016, a ditamycin analog has been found to inhibit HIV-1 replication by downregulating CCR5 and CXCR4 chemokine receptor expression and preventing HIV cell entry (Howard et al, 1998). Cytokines that are particularly important in the pathophysiology of acute pancreatitis include: IL-1, IL-1ra, IL-2, IL-6, IL-8, IL-10, IL-12 and TNFα and their involvement in the disease is reviewed in Chapters 3 and 4.

Having described the different components of the immune system that are involved in the acute inflammatory response, the sequence of events that occur during the process will now be explained.

2.2 Events in the acute inflammatory response

The function of the acute inflammatory response is to contain and control infection or injury, to eliminate pathogens and to initiate healing and tissue repair. Acute inflammation is

characterised by 5 main events: vasodilatation, increased vascular permeability, leucocyte migration, chemotaxis and phagocytosis.

2.2.1 Vasodilatation

This is caused by a variety of inflammatory mediators including histamine, serotonin, prostaglandins e.g. PGE_2, NO and bradykinin and leads to increased blood pressure and blood flow to the area.

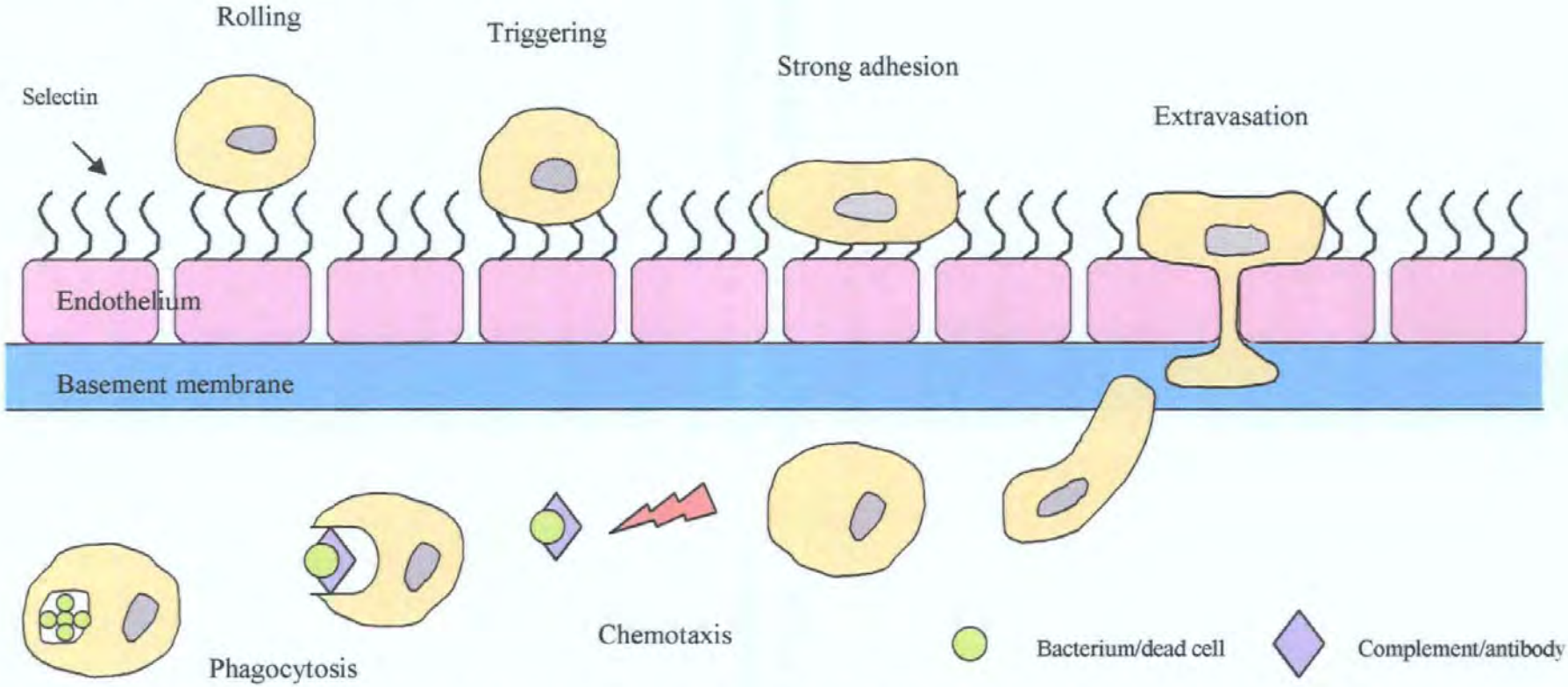
2.2.2 Increased vascular permeability

Histamine, serotonin, leukotrienes e.g. LTD_4, NO, PAF, cytokines e.g. IL-1, bradykinin and complement C3a and C5a (via histamine) are responsible for the increased permeability of blood vessel walls that occurs during inflammation. This allows inflammatory exudate rich in neutrophils and plasma proteins (antibodies, fibrinogen and other components of the plasma mediator systems) to enter the tissue. The exudate is responsible for the swelling and in part, the pain and has several important functions in the inflammatory process.

2.2.3 Leucocyte migration

Leucocyte migration involves the adherence of leucocytes to the endothelium (margination/pavementing) which then "crawl" between the endothelial cells into the tissue. This process has 4 stages collectively known as "the adhesion cascade" and is mediated by adhesion molecules present on the surface of the leucocytes and endothelial cells (Adams and Nash, 1996)(Figure 2.2). The first stage of the adhesion cascade is known as "rolling". This involves the leucocytes rolling along the blood vessel wall which become slowly tethered by low affinity selectins.

Figure 2.2: Leucocyte migration, chemotaxis and phagocytosis



Leucocyte migration has 4 stages collectively known as "the adhesion cascade". "Rolling" involves the leucocytes rolling along the blood vessel wall which become slowly tethered by low affinity selectins. "Triggering" involves the upregulation of integrins on the surface of leucocytes following their activation by PAF, cytokines and chemoattractants. "Strong adhesion" is where integrins on the surface of leucocytes bind strongly to intercellular adhesion molecules on the surface of the endothelial cells causing the leucocytes to be fixed to the endothelium. "Extravasation" involves the migration of the leucocytes through the intercellular spaces between the endothelial cells into the tissue. Once inside the tissue, the leucocytes migrate towards the exact site of injury by following an increasing concentration gradient of chemotactic factor (chemotaxis). When the leucocytes reach the exact site of injury, they engulf microorganisms, damaged cells or tissue debris during phagocytosis. This process is enhanced if the particle is opsonised. The particle is destroyed by lysosomal enzymes and/or OFRs formed during the respiratory burst.

(Adapted from Adams and Nash, 1996)

"Triggering" is the second stage and involves the upregulation of integrins on the surface of leucocytes following their activation by PAF, cytokines e.g. IL-1 and chemoattractants e.g. IL-8, LTB_4, C5a. The third stage is called "strong adhesion" whereby integrins on the surface of leucocytes bind strongly to intercellular adhesion molecules on the surface of the endothelial cells causing the leucocytes to be fixed to the endothelium. "Extravasation" (diapedesis/ transendothelial migration) is the final stage of the adhesion cascade and involves the migration of the leucocytes through the intercellular spaces between the endothelial cells into the tissue.

2.2.4 Chemotaxis

Once inside the tissue, the leucocytes migrate towards the exact site of injury by following an increasing concentration gradient of chemotactic factor e.g LTB_4, C5a, PAF and IL-8 during a process called chemotaxis.

2.2.5 Phagocytosis

When the leucocytes for example, neutrophils reach the exact site of injury, they engulf microorganisms, damaged cells or tissue debris during a process known as phagocytosis. This process is enhanced if the particle e.g. microorganism is coated with either antibody (IgG) or complement C3b during a process called opsonisation. The opsonised microorganism becomes attached to the neutrophil membrane receptors for the opsonin and engulfment occurs. The opsonised particle is internalized into the cell within a membrane bound vesicle called a phagosome. The phagosome fuses with lysosomes to form a phagolysosome. Fusion of the lysosomes with the phagosome allows the discharge of lysosomal enzymes into the phagolysosome, which degrade the dead microorganism. Activation of the phagocyte also triggers the respiratory (oxidative) burst in which the

membrane-bound NADPH-oxidase produces oxygen intermediates such as superoxide radicals, hydrogen peroxide, hypochlorous acid and cholamines that are toxic to microorganisms.

2.3 Polymorphisms and the acute inflammatory response

It is well known that different allelic forms or polymorphisms are known to exist in the genes coding for proteins. These are normal variations within the human genome, which occur more frequently in a given population than abnormal mutations. Polymorphisms within the exons of a gene can affect the structure of expressed protein, whilst variation within the introns and promoter region can modify the expression of the gene. These variations may predispose an individual to an inflammatory disease or a more severe form of the disease. Even if a polymorphism does not have a functional effect, it maybe in linkage disequilibrium with a functional polymorphism and may still act as a marker for disease susceptibility or severity.

The IL-1 gene cluster has a central role in a number of inflammatory diseases as discussed in Chapter 4. Polymorphisms in members of this gene complex may affect the amplitude and duration of an inflammatory response. Since acute pancreatitis is an inflammatory disease, they may also be involved in this disease.

CHAPTER 3: PATHOPHYSIOLOGY OF ACUTE PANCREATITIS

Information regarding the pathophysiology of acute pancreatitis is limited since clinical and post-mortem studies of the early stages of the disease are almost impossible to obtain. The current understanding is therefore based on experimental animal models, the relevance of which is questionable.

3.1 Experimental models of acute pancreatitis

There are a variety of *in vivo* and *in vitro* experimental models used to study acute pancreatitis, however, none of these models exactly mimics the human disease and they all have limitations (Banerjee et al, 1994). The 5 most commonly used *in vivo* models are shown in Table 3.1.

3.2 Pathophysiology of acute pancreatitis

Following initiation of the disease by for example gallstones or alcohol, the pathophysiological process is thought to occur in 3 phases: a local inflammatory response of the pancreas, a systemic inflammatory response that can result in organ failure or multiple organ failure and sepsis with the translocation of bacteria from the gut (Kingsnorth, 1997).

3.2.1 The local inflammatory response

3.2.1.1 Normal pancreas

Normally, digestive enzymes and lysosomal hydrolases are synthesized on ribosomes attached to the RER and then migrate to the Golgi complex, where lysosomal hydrolases for example, cathepsin B are packaged into lysosomes and separated from digestive enzymes which are packaged as zymogen granules. During exocytosis, the zymogen granules migrate towards and fuse with the apical membrane where the enzymes are released into the duct lumen and pass to the duodenum to be activated by enterokinase (Figure 3.1).

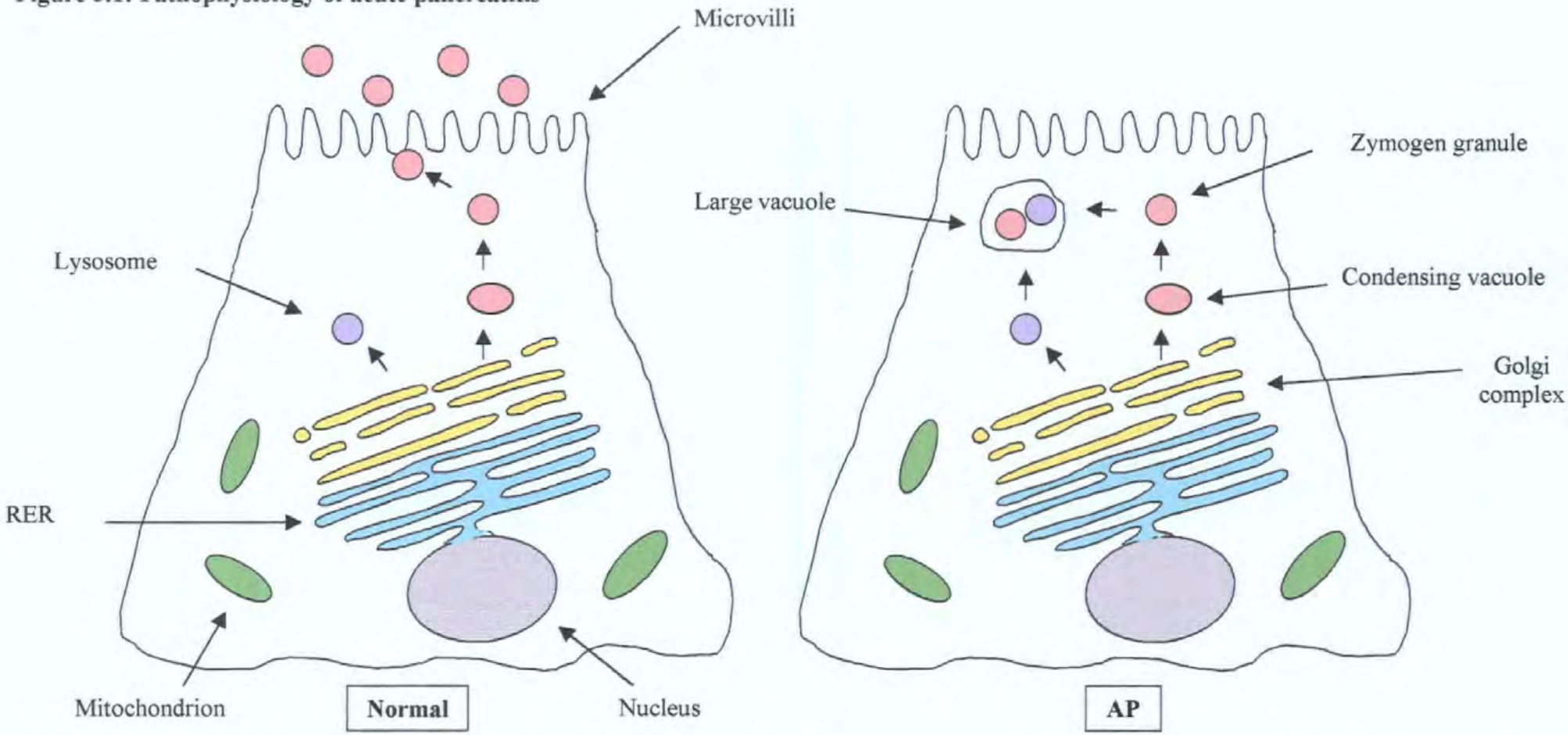
Table 3.1: Experimental *in vivo* models of AP

Model	Agent	Administration	Animal	Type of pancreatitis
Secretagogue	Cerulein*	Intraperitoneal, Intravenous, Subcutaneous	Rat, Dog Mouse	Edematous Necrotizing
Diet	CDE Diet	Oral	Mouse (Female)	Necrotizing /Hemorrhagic
Duct injection	Bile/Taurocholate	Pancreatic duct	Rat, Dog	Edematous/Necrotizing
Duct ligation	-	-	Rat, Opossum, Dog	Necrotizing/Hemorrhagic
Microvascular	Microspheres	Pancreatic artery	Rat	Edematous/Necrotizing/Hemorrhagic

* Cerulein is a cholecystokinin (CCK) analogue
CDE Choline-deficient ethionine-supplemented
Edematous pancreatitis corresponds clinically to mild acute pancreatitis while necrotizing pancreatitis corresponds clinically to severe acute pancreatitis

(Adapted from Banerjee et al, 1994)

Figure 3.1: Pathophysiology of acute pancreatitis



Blockage of zymogen secretion by for example gallstones or alcohol leads to the accumulation of zymogen granules within the acinar cell. This allows the colocalisation of zymogen granules and lysosomes within large vacuoles to occur ("crinophagy"). The lysosomal hydrolases activate the digestive enzyme precursor trypsinogen to trypsin, which in turn activates the pancreatic enzyme cascade and leads to autodigestion of the gland.

(Adapted from Lankisch and Banks, 1998a and 1998b)

A number of safety mechanisms protect the pancreas from autodigestion (Section 1.1.2.4), however, during acute pancreatitis these protective mechanisms fail and there is premature activation of pancreatic enzymes within the acinar cell that leads to autodigestion of the gland.

3.2.1.2 Acute pancreatitis

The cellular injury that occurs during acute pancreatitis is the result of a blockage in the secretion of zymogen granules from acinar cells and their subsequent accumulation within the acinar cell. Once this blockage occurs, the zymogen granules undergo abnormal fusion with lysosomes a process known as "crinophagy". This results in the formation of large vacuoles that contain both digestive enzyme and lysosomal hydrolase. The lysosomal hydrolase then activates the digestive enzyme precursor trypsinogen to trypsin, which in turn activates the pancreatic enzyme cascade and leads to autodigestion of the gland (Figure 3.1)(Watanabe et al, 1984; Saito et al, 1987; Steer and Meldolesi, 1987; Saluja et al, 1989).

An alternative theory has been proposed whereby in the presence of calcium and low pH trypsinogen is autoactivated to trypsin with the subsequent activation of other zymogens within the acinar cell (Whitcomb, 1999; Leach et al, 1992, Bettinger and Grendell, 1991). The recent identification of two mutations in the CAT gene that renders trypsin resistant to degradation in patients with HP supports a role for the premature activation of trypsinogen in acute pancreatitis (Gorry et al, 1997; Whitcomb et al, 1996b) (Section 1.4.4.2).

Whichever mechanism of trypsinogen activation is involved, the result is that protease secretion occurs at the basolateral membrane of the acinar cell into the interstitium of the pancreas, rather than at the apical membrane (Fernandez del Castillo et al, 1993). The activated enzymes damage the cells and cause the release of cytokines and activation of the complement system (Karne and Gorelick, 1999). These molecules cause

the migration of leucocytes (neutrophils and macrophages) from the blood into the pancreatic interstitium due to an upregulation of receptors on both leucocytes and endothelium. The leucocytes release inflammatory mediators including cytokines and OFRs that induce the local pancreatic inflammation (Kingsnorth, 1997).

Despite the central role of enzymes in the pathogenesis of acute pancreatitis, clinical trials using protease inhibitors such as Aprotonin (Cox, 1977; Imrie et al, 1978) and Gabexate mesilate (Buchler et al, 1993; Andrulli et al, 1998) and antisecretory agents such as Somatostatin (Gjorup et al, 1992) and Ocreotide (Uhl et al, 1999) have been disappointing.

3.2.2 The systemic inflammatory response syndrome (SIRS)

Loss of control of local pancreatic inflammation results in the systemic inflammatory response syndrome (SIRS). As defined at the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference of 1991:

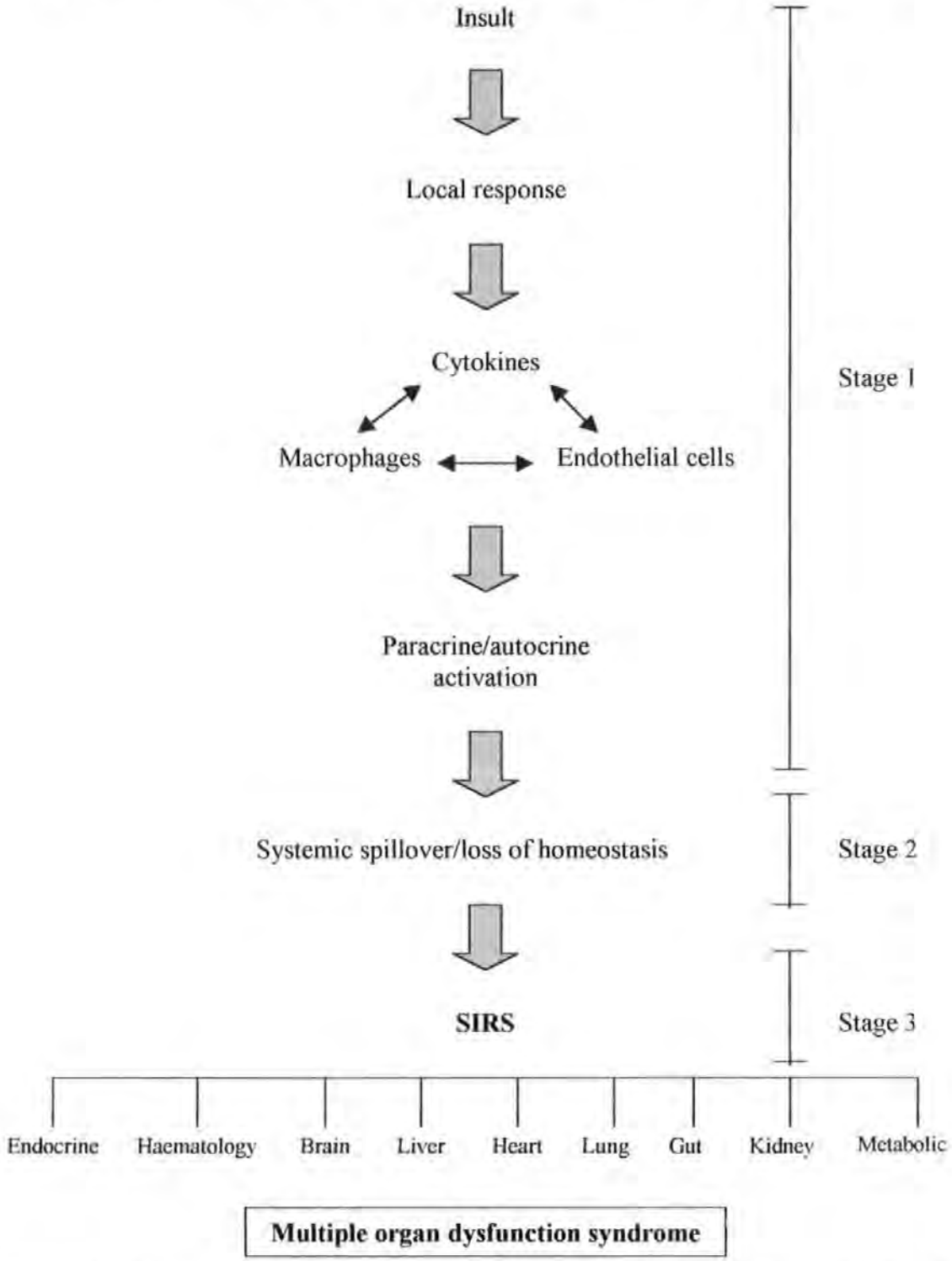
"*SIRS* is a systemic inflammatory response to a variety of severe clinical insults. The response is manifest by two or more of the following criteria: Temperature > 38°C or < 36°C; Heart rate > 90 beats/minute; Respiratory rate > 20 breaths/minute or $PaCO_2$ < 4.3kPa and WBC count >12000 cells/mm^3, < 4000 cells/mm^3 or >10% immature cells" (Bone et al, 1992).

High levels of macrophage-derived pro-inflammatory cytokines are released from the damaged pancreas into the general circulation. These cytokines activate systemic leucocytes and cause the upregulation adhesion molecule expression on neutrophils and endothelial cells. This increases cell adhesion and allows leucocytes to migrate from the blood into the parenchyma of distant organs in particular lungs, heart and kidneys where they release more inflammatory mediators such as cytokines, PAF, proteases and OFRs that cause tissue damage (Kingsnorth, 1997).

The systemic inflammatory response syndrome (SIRS) is one of 2 main components that are activated following tissue injury. The other component is the compensatory anti-inflammatory response syndrome (CARS). (An intermediate mixed antagonist response syndrome (MARS) also exists). SIRS that occurs when there is a systemic spillover of pro-inflammatory mediators and CARS that occurs when there is a systemic spillover of anti-inflammatory mediators act as opposing forces in a balance (Bone, 1996a). SIRS (and CARS) occurs in 3 stages (Figure 3.2)(Bone, 1996b). Stage 1 (Local response): The pancreas produces pro-inflammatory mediators whose function is to cause an inflammatory response, promote wound repair and recruit phagocytes. Anti-inflammatory mediators are also released to downregulate this process. If the pro-inflammatory and anti-inflammatory mediators balance locally homeostasis is restored. Stage 2 (Systemic spillover): If homeostasis is not restored in stage 1, small quantities of pro-inflammatory and anti-inflammatory mediators are released into the systemic circulation to recruit cells to the pancreas. This continues until the pancreatic damage is resolved and homeostasis is restored. Stage 3: (SIRS or CARS): If the balance between the pro-inflammatory and anti-inflammatory mediators is not restored and homeostasis is not reestablished after stage 2, SIRS due to a massive release of pro-inflammatory mediators or CARS due to massive release of anti-inflammatory mediators will develop.

When SIRS and CARS are unbalanced a variety of clinical sequelae occur depending on whether SIRS or CARS predominates. These have been termed **CHAOS** (**C**ardiovascular shock (SIRS predominates), **H**omeostasis (SIRS and CARS balance), **A**poptosis (SIRS predominates), **O**rgan dysfunction (SIRS predominates) and immune **S**uppression (CARS predominates) (Bone, 1996c). The systemic inflammatory state of an individual patient must be determined in order to administer the correct treatment. If the pro-inflammatory SIRS predominates, anti-inflammatory therapy will be required, however, if the anti-inflammatory CARS predominates agents that stimulate the immune system such as G-CSF and IL-2 will be beneficial (Davies and Hagen, 1997).

Figure 3.2: Development of the systemic inflammatory response syndrome (SIRS)



Stage 1 (Local response): The pancreas produces pro-inflammatory mediators whose function is to cause an inflammatory response. Anti-inflammatory mediators are also released to downregulate this process. If the pro-inflammatory and anti-inflammatory mediators balance locally homeostasis is restored.
Stage 2 (Systemic spillover): If homeostasis is not restored in stage 1, small quantities of pro-inflammatory and anti-inflammatory mediators are released into the systemic circulation to recruit cells to the pancreas. This continues until the pancreatic damage is resolved and homeostasis is restored.
Stage 3 (SIRS or CARS): If the balance between the pro-inflammatory and anti-inflammatory mediators is not restored and homeostasis is not reestablished after stage 2, SIRS due to a massive release of pro-inflammatory mediators or CARS due to massive release of anti-inflammatory mediators will develop. One of the sequelae that occurs when SIRS predominates is organ dysfunction. Failure of more than one organ results in a condition known as multiple organ dysfunction syndrome (MODS).

(Adapted from Davies and Hagen, 1997)

One of the sequelae that occurs when SIRS predominates is organ dysfunction. Failure of more than one organ results in a condition known as multiple organ dysfunction syndrome (MODS). As defined by the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference of 1991:

"*Multiple organ dysfunction syndrome* is the presence of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention" (Bone et al, 1992).

A common sequence of organ failure is found to be respiratory failure, then renal failure, then cardiovascular failure and finally coagulation failure (Larvin, 1996). The mortality rate is directly related to the number of organ systems involved with 1, 2, 3 and 4 organ system failures corresponding to 18%, 54%, 90%, 100% mortality respectively (Larvin, 1996). The outcome of MODS can be explained by the "two hit" hypothesis. The "first hit" in severe acute pancreatitis is the initial pancreatic insult that leads to SIRS and MODS. Resolution occurs in most patients with MODS provided there is no "second hit" such as a chest infection. A "second hit" superimposed on MODS, however, often results in death (Brady et al, 1999).

3.2.3 Sepsis

As discussed above, SIRS can occur as a result of massive activation of pro-inflammatory mediators by severe tissue trauma. SIRS can also occur or be enhanced by bacterial infection of the pancreatic necrosis, a condition known as sepsis. As defined at American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference of 1991:

"*Sepsis* is SIRS in response to infection. This systemic response is manifested by two or more of the criteria for SIRS as a result of infection" (Bone et al, 1992).

The extent of bacterial infection is related to the degree of pancreatic necrosis which inturn correlates with disease severity (Schmid et al, 1999). Once the necrotic

pancreas is infected, cytokines and bacterial toxins are released into the blood circulation and pass to distant organs for example the lungs, where they initiate inflammatory reactions and cause the SIRS which can lead to MODS and death (Lemaire et al, 1997). The bacteria that infect the necrotic pancreas originate from the gut by a process known as "bacterial translocation". They include gram-negative bacteria such as *Escherichia coli*, *Pseudomonas* spp, *Proteus* spp, *Klebsiella* spp and gram-positive bacteria such as *Staphylococcus aureus*, *Streptococcus faecalis*, *Enterococcus*. Occasionally fungi may also be present in the pancreas (Schmid et al, 1999).

3.2.3.1 Bacterial translocation

"*Bacterial translocation* is defined as the passage of microbes and endotoxin across the intestinal barrier" (Lemaire et al, 1997) and has demonstrated in different animal models of acute pancreatitis. These include opposums (Runkel et al, 1990, Runkel, et al, 1995), dogs (Kazantsev et al, 1994), rats (Medich et al, 1993; Runkel, et al, 1991) and cats (Widdison et al, 1994). Bacterial translocation from the intestinal lumen to extraintestinal sites including pancreatic necrosis may occur via the lymphatic, blood circulation or direct transperitoneal route. The route of bacterial translocation is dependent on the animal model. In opposums with duct ligation-induced acute pancreatitis and dogs with taurocholate-induced acute pancreatitis bacteria spread via lymphatics (Runkel et al, 1990, Runkel et al, 1995; Kazantsev et al, 1994). In rats with cerulein-induced acute pancreatitis the bacteria spread transperitoneally (Medich et al, 1993) while in cats with duct perfusion-induced acute pancreatitis translocation occurred via the blood circulation and transperitoneally (Widdison et al, 1994). Bacterial translocation is caused by 3 factors: 1) disruption of the intestinal microflora, 2) loss of intestine wall barrier function and 3) impaired host defence (Runkel, 1996).

3.2.3.1.1 Disruption of intestinal microflora

During acute pancreatitis there is an imbalance in the gram-negative and gram-positive bacteria in the intestine skewed towards the gram-negative bacteria. This shift in intestinal microflora is believed to be due to reduced gut motility. The link between reduced gut motility, disrupted intestinal microflora and bacterial translocation has been demonstrated in rats using morphine as a depressant of gut motility (Runkel et al, 1993) and in a time course study in rats with duct ligation-induced acute pancreatitis (Muncy et al, 1993).

3.2.3.1.2 Loss of intestine wall barrier function

Loss of mucosal barrier function of the intestine wall results in leakage of bacteria and endotoxin from the gut lumen. Increased intestinal permeability in acute pancreatitis has been shown in dogs using plasmid-labelled *E. coli* (Kazantsev et al, 1994), a clinical strain of *E. coli* in cats (Widdison et al, 1994) and by fluorescent beads (Medich et al, 1993). The mechanism of increased intestinal permeability remains unknown, however, it may be caused by reduced gut motility or intestinal ischemia (Runkel, 1996).

3.2.3.1.3 Impaired host defense

There is evidence to suggest that the host defense system is impaired during acute pancreatitis. Curley et al, found that the proportion of circulating levels of $CD4^+$ cells was reduced during the disease and correlated inversely with translocating endotoxin (Curley et al, 1993). Widdison et al, showed that the local clearance of *E. coli* from the pancreas was impaired in cats with duct perfusion-induced acute pancreatitis indicating a loss of phagocytic function. Furthermore, administration of the immunostimulant drug, Levimasole™ was found to improve phagocytic function and reduce pancreatic infection (Widdison et al, 1992).

3.3 Plasma mediator systems of inflammation

The most extensively studied plasma mediator systems in acute pancreatitis are the kinin and complement systems.

3.3.1 The kinin system

Elevated levels of bradykinin have been found in dogs with severe acute pancreatitis (Satake et al, 1985), whilst bradykinin administered to rabbits with cerulein-induced acute pancreatitis was found to significantly reduce pancreatic blood flow and increase disease severity compared to controls (Yotsumoto et al, 1993).

Experiments using bradykinin antagonists have provided conflicting results. Hoffmann et al, demonstrated that the bradykinin antagonists, HOE140 and CP-0597 improved pancreatic microcirculation in an ischemia-reperfusion model of acute pancreatitis in rats compared to untreated controls (Hoffmann et al, 1996a; Hoffmann et al, 1996b). HOE140 also improved survival in taurocholate-induced acute pancreatitis in rats (Kanbe et al, 1996) and prevented edema formation in cerulein-induced acute pancreatitis in rats (Griesbacher et al, 1993). In addition, Closa et al, found that HOE140 inhibited bradykinin-mediated NO generation and thromboxane release and improved the disease severity in taurocholate-induced acute pancreatitis in rats (Closa et al, 1995).

In contrast, Lerch et al, found no beneficial effect of HOE140 on the survival rates in 4 different models of acute pancreatitis (Lerch et al, 1995). Furthermore, in one study HOE140 actually increased the severity of disease (Weidenbach et al, 1995). Cicardi et al, have demonstrated that pretreatment with C1 inhibitor plasma concentrate, a kallikrein inhibitor may offer benefit to patients at high risk of developing acute pancreatitis following ERCP. Patients pretreated with C1 inhibitor showed reduced levels of amylase compared to the untreated patients. Since the C1 inhibitor has to be administered before acute pancreatitis is induced, it is probably only of benefit to patients with ERCP-induced acute pancreatitis (Cicardi et al, 1994).

3.3.2 The complement system

Decreased levels of serum C3 and increased levels of complement products implicate the complement system in acute pancreatitis (Seelig et al, 1975; Horn et al, 1982; Roxvall et al, 1990). Acute pancreatitis was induced in C5 sufficient mice (Hc^1Hc^1) and in mice with a mutant Hc gene that were genetically deficient in circulating C5 (Hc^0Hc^0). All mice developed the disease, however, the mice deficient in C5 had significantly reduced pancreatic inflammation compared to those with C5 (Merriam et al, 1997).

Vesentini et al, have shown that rats with taurocholate-induced acute pancreatitis pretreated with C1 esterase inhibitor, an inhibitor of both kallikrein and complement had improved survival compared to untreated rats (Vesentini et al, 1993). Combined treatment of C1 esterase inhibitor and antithrombin III, an inhibitor of the coagulation system given either prophylactically or therapeutically was also shown to improve the survival of rats with taurocholate-induced but not cerulein-induced acute pancreatitis (Yamaguchi et al, 1997). In contrast, Niederau et al, showed no beneficial effect of C1 esterase in 3 different models of the disease (Niederau et al, 1995). In a clinical study, C1 esterase inhibitor administered therapeutically to 2 children with severe acute pancreatitis following allogeneic hematopoietic stem cell transplantation was found to improve pancreatic inflammation and prevented MODS (Schneider et al, 1999).

In other studies, soluble complement receptor type 1 (sCR1), another complement inhibitor administered to rats with cerulein-induced acute pancreatitis failed to reduce local or systemic complications (Weiser et al, 1996; Acioli et al, 1997).

Guice et al, demonstrated that cerulein-induced acute pancreatitis in rats produced lung injury with neutrophil sequestration and increased lung permeability. The lung injury was ameliorated by the induction of neutropenia, complement depletion or by scavenging OFRs. They suggested that the local and systemic injury that occurs during acute pancreatitis is the result of complement activation with the production of C5a and C3a leading to neutrophil activation and the production of OFRs (Guice et al, 1989).

3.4 Cell mediators of inflammation

3.4.1 Neutrophils

Overactivation of neutrophils occurs during acute pancreatitis (Widdison and Cunningham, 1996). Serum levels of neutrophil elastase were found to be elevated during the disease and correlated with severity (Gross et al, 1990; Uhl et al, 1991; Mora et al, 1997). Administration of ONO-5046, a neutrophil elastase inhibitor prevented lung injury in 2 animal models, but had no effect on pancreatic inflammation (Imamura et al, 1998; Guo et al, 1995). The neutrophil elastase inhibitor R-020 also prevented sepsis-induced lung and liver damage in rats (Murata et al, 1994). Combined treatment of a neutrophil elastase inhibitor and pancreatic elastase inhibitor in a rat model of acute pancreatitis with septic challenge significantly reduced mortality, however, individual administration of either inhibitor did not offer any benefit. This suggests that neutrophil and pancreatic elastase may have a synergistic action in acute pancreatitis (Yamano et al, 1998).

Neutrophil depletion studies have also implicated neutrophils in acute pancreatitis. Methotrexate, a neutropenia-inducing drug administered to a rat model caused a reduction in pancreatic injury (Fujimoto et al, 1997). Also Urge-8, an anti-neutrophil monoclonal antibody given after pancreatitis induction prolonged survival in rats (Han et al, 1996). Neutrophil depletion has been found to promote apoptosis not necrosis of pancreatic acinar cells during acute pancreatitis. This shift towards apoptosis may be the mechanism by which neutrophil depletion reduces the severity of acute pancreatitis (Sandoval et al, 1996).

Neutrophils activated during acute pancreatitis are also involved with the associated lung injury. Pretreatment with anti-neutrophil serum was found to prevent the development of lung injury in animal models (Bhatia et al, 1998; Guice et al, 1989). In addition, prophylactic treatment of rats with a polyclonal anti-rat neutrophil antibody to cause neutropenia and therapeutic treatment with an anti-rat monoclonal antibody to prevent neutrophil adherence to the endothelium prolonged survival and prevented lung injury associated with the disease (Inoue et al, 1995).

3.4.2 Monocytes/macrophages

Serum neopterin levels, a marker of macrophage activation were found to be increased in acute pancreatitis and correlated with severity (Uomo et al, 1996; Mora et al, 1997).

A recent study has demonstrated that Zymosan, a macrophage stimulant caused pancreatic barrier dysfunction in a dose and time dependent manner. Pretreatment with NAC, an OFR scavenger showed that the pancreatic barrier damage was mediated at least in part by OFRs released from the macrophages (Andersson et al, 1997). Furthermore, administration of CN1493, a macrophage-pacification compound to rats with acute pancreatitis decreased pancreatic disease severity via a reduction in TNFα levels (Yang et al, 1998a) and reduced hepatocellular injury by down regulating TNFα and IL-1β expression (Yang et al, 1998b).

Monocyte phagocyte function may determine the outcome of acute pancreatitis. Patients with severe disease showed impaired monocyte phagocyte function, which lead to a decreased rate of clearance of protease-antiprotease complexes (Larvin et al, 1993; Liras and Carballo, 1996). In addition, mice with CDE-induced acute pancreatitis fed glucan, a chemical that enhances macrophage function had reduced disease severity and improved survival rates (Browder et al, 1987). HLA-DR expression, an indicator of monocyte function was also found to be reduced in patients with acute pancreatitis and correlated with outcome (Richter et al, 1999).

Widdison and Cunningham have suggested that monocyte function is overwhelmed not impaired during acute pancreatitis. They implied that phagocytes have only limited capacity to phagocytose bacteria and protease-antiprotease complexes and that systemic release of proteases during acute pancreatitis saturates the phagocytes causing the reduced rate of clearance (Widdison and Cunningham, 1996).

3.4.3 Endothelial cells and adhesion molecules

3.4.3.1 Endothelial cells

Endothelin is released from vascular endothelial cells in response to a variety of stimuli and causes vasoconstriction, increased permeability and increased neutrophil chemotaxis (Todd et al, 1997). It mediates its action via 2 surface receptors known as endothelin ETa and ETb that have both been located on pancreatic acinar cells (Hildebrand et al, 1993). Administration of endothelin to rats with acute pancreatitis was found to increase capillary permeability in the pancreas, whilst blockade of the endothelin receptor reduced capillary permeability (Eibl et al, 2000). Endothelin antagonism using PD145065 also reduced the severity of both local and systemic manifestations of acute pancreatitis in mice with CDE-induced disease (Todd et al, 1997). In contrast, an earlier experiment showed that endothelin protected rats against cerulein-induced acute pancreatitis, while an endothelin antagonist BQ-123 increased disease severity (Kogire et al, 1995).

3.4.3.2 Adhesion molecules

Circulating levels of ICAM-1 were found to be elevated in patients with severe acute pancreatitis and levels correlated with disease severity (Kaufmann et al, 1996). This association between ICAM-1 levels and disease severity was also demonstrated in a rat model. Furthermore, treatment with a monoclonal antibody against ICAM-1 at the time of acute pancreatitis induction was found to reduce the local pancreatic injury and systemic lung injury (Werner et al, 1999). It has also been demonstrated that gut endothelial dysfunction associated with severe acute pancreatitis in rats was decreased by the injection of monoclonal antibodies against ICAM-1 and a PAF antagonist reducing the systemic inflammatory response (Wang et al, 1999). In another study, ascitic fluid from rats with acute pancreatitis caused an increase in the expression of ICAM-1 and VCAM-1 molecules in human umbilical vein endothelial cells (HUVECs) suggesting a possible role for ICAM-1 in the systemic manifestations of acute pancreatitis (Masamune et al, 1999).

In another study, pancreatitis was induced in mice that were either deficient in ICAM-1, deficient in neutrophils or both. The severity of pancreatitis and pancreatitis-associated lung injury was significantly reduced in mice deficient in ICAM-1 and mice deficient in neutrophils compared to controls, however, there was no additive protective effect of mice deficient in ICAM-1 and neutrophils (Frossard et al, 1999).

Some studies have shown that events that lead to inflammation occur in a particular sequence. Lundberg et al, showed in mice with CDE-induced acute pancreatitis that the disease is associated initially with systemic release of inflammatory cytokines, followed by increased expression of pulmonary ICAM-1 and VCAM-1, followed by neutrophil infiltration which subsequently lead to lung injury (Lundberg et al, 2000a). The same group also demonstrated this sequence of events with selectins whereby there was an increase in inflammatory cytokines, followed by an increase in P and E selectin, followed by increased infiltration of leucocytes and lung tissue damage (Lundberg et al, 2000b). Folch et al, also demonstrated that in the lungs of rats with taurocholate-induced acute pancreatitis there was increased P selectin which caused increased leucocyte infiltration. They expanded the study to show that P selectin or ICAM-1 neutralisation prevented infiltration of neutrophils into the lung. In addition, they found that xanthine oxidase levels increased during acute pancreatitis and that inhibition of XOD prevented the increase of P selectin in the lung and neutrophil infiltration (Folch et al, 1999).

Pretreatment with IS-741, a carboxamide derivative has been shown to reduce neutrophil infiltration into the pancreas in rats with cerulein-induced acute pancreatitis by inhibiting the upregulation of integrin CD11b (Yamauchi et al, 1999). The same drug has been shown to prevent pancreatitis-associated lung injury following septic challenge in rats with cerulein-induced acute pancreatitis (Yamaguchi et al, 1999a).

3.5 Cell-derived mediators of inflammation

3.5.1 Platelet activating factor (PAF)

Isolated pancreatic lobules have been found to synthesize PAF (Soling and Fest, 1986) and pancreatic PAF levels were elevated in both cerulein-induced rat models (Konturek et al, 1992; Zhou et al, 1993; Zhou et al, 1994) and in human acute pancreatitis (Kingsnorth, 1996). In addition, the injection of PAF has been found to induce acute pancreatitis in animal models (Emanuelli et al, 1989; Jancar et al, 1988). Yotsumoto et al, also showed that the simultaneous administration of PAF with cerulein to rabbits exacerbated the detrimental effects of this secretagogue model (Yotsumoto et al, 1994). PAF given intraperitoneally caused similar changes in pancreatic histopathology to acute pancreatitis induced by cerulein (Konturek et al, 1992). PAF levels were also increased in lung tissue and were associated with the lung injury seen in acute pancreatitis (Zhou et al, 1992). PAF is inactivated by the enzyme platelet activating factor acetylhydrolase (PAF-AH). Therapeutic administration of this enzyme to an animal model has been found to protect against acute pancreatitis and its associated lung injury (Hofbauer et al, 1998).

PAF antagonists have been found to reduce the severity of acute pancreatitis and its complications. There are a variety of PAF antagonists: BN-52021, CV6209, WEB2170, TCV309 and BB-882. BN-52021 administered prophylactically to rats with immune complex-induced (Jancar et al, 1988), cerulein-induced (Jancar et al, 1995), bile-induced (Zhou et al, 1992) and taucholate-induced acute pancreatitis (Dabrowski et al, 1995) was found to reduce severity of the disease. A similar reduction in severity was also achieved with CV6209 pretreatment in a cerulein-induced rat model (Fujimura et al, 1992).

The role of PAF in the systemic complications of acute pancreatitis was demonstrated in a bile-induced rat model using pretreatment with WEB2170. There was a reduction in pulmonary inflammatory mediators, vascular permeability and leucocyte infiltration compared to controls (Zhou et al, 1992). Similarly, pretreatment with TCV309 reduced the severity of cerulein-induced and PAF injection-induced acute pancreatitis in

rats (Tomaszewska et al, 1992) and was found to prevent the activation of bronchoalveolar macrophages in cerulein-induced rats with septic challenge (Yamaguchi et al, 1999b). Furthermore, BB-882, also known as Lexipafant™, administered therapeutically in a microembolisation-induced rat model caused significant reduction in pancreatic inflammation (Formela et al, 1994) and pulmonary capillary permeability (Galloway and Kingsnorth, 1996). In contrast, a study performed by Rivera et al, demonstrated that BB-882 did not improve the survival or decrease disease severity in rats with cerulein plus bile-induced acute pancreatitis (Rivera et al, 1998). BB-882 administered prior to acute pancreatitis induction has also been found to prevent intestinal barrier dysfunction and reduce bacterial translocation in taurocholate-induced rat model and further proves that PAF plays a role in systemic manifestations of the disease (Andersson et al, 1998; Wang et al, 1999; Liu et al, 1999).

Lexipafant™ is the only PAF antagonist to have been studied in clinical trials. Two randomised double-blind placebo-controlled phase II trials have been conducted to assess the efficacy of Lexipafant™ as a therapy for human acute pancreatitis (Kingsnorth et al, 1995; McKay et al, 1997). In both studies, patients who received Lexipafant™ had improved survival and decreased organ failure score compared to those receiving placebo. A subsequent phase III multicentre double-blind placebo-controlled clinical trial showed that patients treated with Lexipafant™ had fewer local complications, a more rapid resolution of systemic complications and reduced mortality (Kingsnorth, 1998). Furthermore, patients who had received the treatment within 48 hours of onset of symptoms showed significantly reduced mortality, suggesting that Lexipafant™ is particularly effective if administered early. Although these clinical trials showed that Lexipafant™ has therapeutic potential, this maybe due to the relatively small numbers of patients involved in the studies. This theory is supported by a larger multicentre study involving both Europe and the USA which has shown that Lexipafant™ does not reduce organ failure or mortality in severe acute pancreatitis (Bhatia et al, 2000).

3.5.2 Arachidonic acid metabolites

Studies implicating arachidonic acid metabolites in acute pancreatitis are limited and often conflicting.

3.5.2.1 Leukotrienes

Leukotrienes are the 5-lipoxygenase metabolites of arachidonic acid. LTB_4 levels were found to be elevated in rats with taurocholate-induced acute pancreatitis (Zhou et al, 1994; Folch et al, 1998a), while LTC_4 and LTD_4 levels were increased in two acute pancreatitis models in pigs (Vollmar et al, 1989). A study using AA861, a 5-lipoxygenase inhibitor provided evidence that leukotrienes may cause acute pancreatitis in rats by enhancing amylase secretion from pancreatic acinar cells (Sato et al, 1988). In addition, pretreatment with the leukotriene receptor antagonist, pranlukast hydrate was found to protect against the histological features which occur in the pancreas during cerulein-induced acute pancreatitis in rats (Hirano, 1997).

3.5.2.2 Thromboxanes

Thromboxanes and prostaglandins are the cycloxygenase metabolites of arachidonic acid. Zhou et al, found that TXB_2 (metabolite of TXA_2) was significantly decreased in rats with taurocholate-induced acute pancreatitis (Zhou et al, 1994), however, other studies using the same animal model have demonstrated that levels of TXB_2 were significantly increased (Van Ooijen et al, 1988; Closa et al, 1993). Pretreatment of rats with taurocholate-induced acute pancreatitis using a TXA_2 synthesis inhibitor called OKY-046 reduced disease severity by decreasing thromboxane levels and significantly improved survival compared to untreated controls (Iida et al, 1998). Other studies involving experimental acute pancreatitis have demonstrated that TXB_2 levels were reduced by pretreatment with HOE, a bradykinin inhibitor (Closa et al, 1995) or pretreatment with Verapamil, a calcium channel blocker (Closa et al, 1996).

3.5.2.3 Prostaglandins

Zhou et al, found that the levels of PGD_2, PGE_2 and 6 keto $PGF_{1\alpha}$ (a metabolite of PGI_2) were decreased in a rat model of acute pancreatitis (Zhou et al, 1994), whereas it had previously been demonstrated that 6 keto $PGF_{1\alpha}$ levels were significantly increased in dogs with the disease (Kiviniemi et al, 1987).

PGI_2, PGE_1 and PGE_2 have been shown to have therapeutic potential in acute pancreatitis. Treatment with the PGI_2 analogue, OP2507 was found to have no effect in rats with taurocholate-induced acute pancreatitis (Iida et al, 1998), however, iloprost, another PGI_2 analogue was beneficial in rats with cerulein-induced acute pancreatitis, particularly in pancreas regeneration (Jurkowska et al, 1996). Treatment with the PGE_1 analogues, Misoprostol and MR356 was also found to be therapeutic in rats with acute pancreatitis (Buscail et al, 1990; Sakai et al, 1992). Interestingly, Poszar et al, found that optimum PGE_1 ameliorated the disease in rats with closed loop duodenal acute pancreatitis, however, higher doses increased disease severity (Pozsar et al, 1996). Other studies have shown that PGE_2 and not PGE_1 had a beneficial effect on rats with acute pancreatitis (Manabe et al, 1993; Hirano et al, 1993) and the effect was more pronounced when combined with ONO-3307 a protease inhibitor (Hirano et al, 1992).

The protective effect of prostaglandins in acute pancreatitis maybe due to their ability to stabilise cellular and subcellular organelles such as zymogen granules, lysosomes and mitochondrial membranes thereby preventing the colocalisation of lysosomal hydrolases and zymogens that is known to cause acute pancreatitis (Manabe et al, 1993).

3.5.3 Oxygen Free Radicals

Superoxide and hydrogen peroxide free radicals directly injure isolated rat pancreatic acini, whilst SOD and catalase suppress the damage caused by them. OFRs released from the damaged pancreas into the bloodstream also damage vascular

endothelium at distant sites contributing to the complications associated with acute pancreatitis (Tamura et al, 1992).

Levels of the oxidants, xanthine oxidase (XOD) and lipid peroxides (LPO) were significantly increased in animal models of acute pancreatitis, whilst levels of SOD were decreased suggesting that oxidative stress occurs in acute pancreatitis (Nonaka et al, 1989; Dabrowski and Gabryelewicz, 1992). This oxidative stress has also been shown in human acute pancreatitis with increased superoxide and LPO levels and decreased levels of an OFR scavenger in patients compared to controls (Braganza et al, 1995; Tsai et al, 1998). The oxidative stress was found to correlate with severity of the disease (Tsai et al, 1998) and it has been suggested that oxidative stress can be used as a prognostic marker in disease (Wereszczynska-Siemiatkowska et al, 1998).

Oxidative stress can be improved by either decreasing the OFR level or increasing the antioxidants/OFR scavengers levels to restore the imbalance.

3.5.3.1 Decreasing OFR levels

Since the main source of OFRs in acute pancreatitis is from the XOD system (Sanfey et al, 1985) and neutrophils (Gough et al, 1990), methods that inhibit the XOD system or neutrophil function/infiltration may be therapeutic. Studies using allopurinol, a XOD inhibitor, have produced conflicting results. Pretreatment with allopurinol was found to reduce the severity of acute pancreatitis in some animal models (Sanfey et al, 1984; Wisner and Renner, 1988), but not in others (Lankisch et al, 1989; Creagh et al, 1993). Neutropenia induced by nitrogen mustard was found to reduce the level of superoxide anion and improve the severity in a cerulein-induced rat model (Ito et al, 1996). Pretreatment with catalase, SOD and monoclonal antibody anti-ICAM-1 in experimental rat models was found to be effective in reducing tissue damage, neutrophil infiltration and OFR production (Inoue et al, 1996; Poch et al, 1999). Furthermore, oxypurinol, a XOD inhibitor administered to rats with acute pancreatitis was found to prevent neutrophil

infiltration in the lungs (Folch et al, 1998b). Also, the inhibition of neutrophils and neutrophil superoxide anion generation using a polyclonal antineutrophil antibody was found to diminish pulmonary neutrophil accumulation in rats (Murakami et al, 1995).

3.5.3.2 Increasing antioxidant/OFR scavenger levels

The most common studies of antioxidants in acute pancreatitis involve SOD and catalase. Pretreatment with SOD and catalase has shown to be beneficial in some animal models (Sanfey et al, 1984; Schoenberg et al, 1994; Schoenberg et al, 1990; Steer et al, 1991; Niederau et al, 1992; Furukawa et al, 1994), but not in others (Niederau et al, 1992; Furukawa et al, 1994), suggesting that different models of acute pancreatitis have different degrees and mechanisms of OFRs. A recent study has shown that when transgenic Cu/Zn SOD mice and control mice were given cerulein to induce acute pancreatitis, the transgenic mice which over express SOD had reduced inflammation compared to control mice (Kikuchi et al, 1997).

Other studies using the free radical scavengers, melatonin (Qi et al, 1999), desferrioxamine (DFX) alone/in combination with a PAF antagonist ginkgo biloba (GB) (Soybir et al, 1999) and CV3611 (Nonaka et al, 1991), and the antioxidant N-acetyl-L-cysteine were all found to improve severity of acute pancreatitis in animal models (Wang et al, 1995).

3.5.4 Nitric Oxide

The role of NO in diseases such as acute pancreatitis can be determined by manipulating NO levels using NO donors and NO inhibitors. The role of NO in the pathophysiology of acute pancreatitis is uncertain since studies have produced conflicting results. Some show a protective effect for NO donors whilst others show a protective effect for NO inhibitors.

3.5.4.1 Protective effects of NO donors

NO has a protective role in the pancreas and distant organs that are involved in the systemic complications of acute pancreatitis. Endogenous NO is involved in the regulation of exocrine pancreatic secretion in rats (Konturek et al 1994; Molero et al, 1995) and pancreatic blood flow (Satoh et al, 1994). Furthermore, inhibition of NOS was found to reduce exocrine and endocrine pancreatic secretion in humans (Konturek et al, 1997).

NO donors have been shown to reduce pancreatic inflammation in rat models of acute pancreatitis (Werner et al, 1997b; Werner et al, 1998; Molero et al, 1995). In addition, blocking the action of NOS using the NOS inhibitor, L-NAME was found to increase the inflammation (Molero et al, 1995).

Pancreatic microcirculation was improved following the administration of the NO donor, L-arginine in a rat model (Dobosz et al, 1996). Furthermore, inhibition of NOS was found to aggravate rat acute pancreatitis by reducing pancreatic capillary blood flow (Dobosz et al, 1998; Dobosz et al, 1999).

Exogenous administration of NO by sodium nitroprusside, a NO donor inhibited lung injury associated with a rat model. Furthermore, inhibition of NO using L-NAME actually increased the pulmonary injury suggesting that NO has a protective role against pancreatitis-induced lung injury possibly by inhibiting neutrophil influx (O' Donovan et al, 1995). This is supported by another study which showed that NO decreased neutrophil accumulation in the pancreas and liver in cerulein rats, while rats pretreated with the NO synthase inhibitor (L-NNA) had increased levels of neutrophils in the pancreas and liver (Inagaki et al, 1997a).

Endothelial-leucocyte adherence is an important aspect of inflammatory diseases such as acute pancreatitis. The presence of endogenous NO reduces endothelial-leucocyte adherence during acute pancreatitis and impairment of NO production leads to leucocyte adhesion and emigration (Kubes et al, 1991; Masamune et al, 2000).

3.5.4.2 Protective effects of NO inhibitors

In contrast to the studies by Kubes et al, and Masamune et al, Chen et al, have shown that pancreatic NO levels directly correlate with the amount of leucocytes adherent to the endothelium suggesting that NO could have an adverse role in the microcirculation (Chen et al, 1998). This is supported by a study which showed that pancreatic edema was reduced by L-NAME in rats with acute pancreatitis (Abe et al, 1995).

A recent study using a rat model showed that during acute pancreatitis there was induction of iNOS, raised NO levels and increased inflammation (Al-Mufti et al, 1998). Furthermore, Satoh et al, have shown that iNOS expression occurred in a severe model but not in a mild model of rat acute pancreatitis. They suggested that in severe disease excess NO is produced because iNOS is expressed and that this does not occur in mild forms of the disease (Satoh et al, 1998).

NO levels were found to be increased in cerulein-induced pancreatitis in mice pretreated with LPS but not in those without LPS. This rise in NO was subsequently inhibited by the NOS inhibitor (L-NNA), suggesting that NO maybe an important mediator of systemic effects of endotoxemia in acute pancreatitis (Kikuchi et al, 1996). In another study, NO released from alveolar macrophages was found to contribute to lung injury associated with the disease and administration of the NOS inhibitor, L-NMMA protected against it (Tsukahara et al, 1996).

3.5.5 Cytokines

The first association between cytokines and the pathophysiology of acute pancreatitis was suggested in 1988 by Rinderknecht who hypothesized that acute pancreatitis occurred as a result of excessive stimulation of leucocytes (Rinderknecht, 1988). Since then much evidence has accumulated to support the role of these proteins in the disease. The role of the IL-1 gene cluster in acute pancreatitis is reviewed in Chapter 4.

3.5.5.1 Interleukin-2 (IL-2)

IL-2 is an immunomodulatory cytokine that is produced by $CD4^+$ T helper cells. Decreased IL-2 production or a decrease in the number of $CD4^+$ T helper cells is associated with impaired cellular immunity and increased susceptibility to infection. Curley et al found that IL-2 production was decreased in a CDE diet-induced murine model of acute pancreatitis and that the decrease was exacerbated by a subsequent endotoxin challenge resulting in high levels of mortality. Furthermore recombinant IL-2 administered to the mice soon after the endotoxin challenge improved IL-2 secretion and was shown to be protective, suggesting a potential role for IL-2 as therapy in acute pancreatitis and sepsis (Curley et al, 1996).

Levels of $CD4^+$ T helper cells have also been found to be decreased during acute pancreatitis reflecting an impaired cellular immune function (Curley et al, 1993; Widdison and Cunningham, 1996). In addition, serum soluble interleukin-2 receptor (sIL-2R) levels which are a marker of the activation of lymphocytes are increased in acute pancreatitis and are useful predictors of disease severity (Pezzilli et al, 1994; Salomone et al, 1996).

3.5.5.2 Interleukin-6 (IL-6)

IL-6 is a pro-inflammatory cytokine that is produced by monocytes, macrophages and endothelial cells which is involved in the synthesis of acute phase proteins such as C-reactive protein (CRP) from the liver. Recent studies have shown that serum IL-6 levels are increased in patients with acute pancreatitis and that the levels correlate with severity of the disease (Leser et al, 1991; McKay et al, 1996; Inagaki et al, 1997b). Furthermore, IL-6 concentrations appear to be more useful early predictors of disease severity and mortality than CRP due to the fact that circulating levels of IL-6 peak 24-48 hours before those of CRP (De Beaux et al, 1996; Ikei et al, 1998; Pezzilli et al, 1998; Chen et al, 1999). Determination of serum IL-6 levels therefore allows the selection of appropriate treatment

in the early stages of disease to prevent the progression from mild to severe acute pancreatitis.

3.5.5.3 Interleukin-8 (IL-8)

IL-8 is an α chemokine (CXC) that has the ability to attract and activate neutrophils and to upregulate cell adhesion molecule expression. It is secreted by a variety of cells including monocytes/macrophages, neutrophils and endothelial cells (Baggiolini et al, 1989). Serum IL-8 levels are elevated in patients with acute pancreatitis and correlate with levels of neutrophil elastase, a marker of neutrophil activation (Gross et al, 1992; McKay et al, 1996). IL-8 is the earliest cytokine to appear in the serum of patients with AP (Kingsnorth et al, 1995) and is believed to be responsible for attracting neutrophils into the parenchyma of the pancreas and lung early in the course of the disease. Similar to IL-6 levels, IL-8 levels have been found to correlate with severity of the disease (Gross et al, 1992; McKay et al, 1996; Berney et al, 1999).

Further evidence supporting a role of IL-8 in acute pancreatitis comes from experiments involving the administration of sodium fusidate (Osman et al, 1998a), and hydrocortisone (Osman et al, 1999) to rabbit models of the disease. These studies showed a reduction in IL-8 levels and mortality rates. Similarly, phase II clinical trials with Lexipafant™, a PAF antagonist in human acute pancreatitis have shown a reduction in the levels of IL-8 with beneficial effects on disease severity and mortality (Kingsnorth et al, 1995). Furthermore, studies using a neutralising monoclonal antibody (WS-4) against IL-8 activity have reduced disease severity and mortality in a rabbit model of bile-induced pancreatitis. Serum concentrations of IL-8 were found to be decreased, adhesion molecule expression was down regulated and lung injury was reduced (Osman et al, 1998b). A similar reduction in lung injury was observed following the administration of an anti-human IL-8 antibody to rats (Mulligan et al, 1993).

3.5.5.4 Interleukin 10 (IL-10)

IL-10 is a macrophage-derived anti-inflammatory cytokine which has a protective role in acute pancreatitis by inhibiting the secretion of pro-inflammatory cytokines such as IL-1, IL-6 and TNFα from monocytes, macrophages and Th1 cells (Fiorentino et al, 1991). Normally IL-10 is not detectable in the serum of healthy individuals, however, during acute pancreatitis levels are significantly elevated on the first day of illness and progressively decrease in the following days. Furthermore, IL-10 levels inversely correlate with severity of the disease being significantly higher in patients with mild disease compared to those with severe disease on the first day of illness (Pezzilli et al, 1997; Galloway and Kingsnorth, 1996). This secretory profile differs to that of the proinflammatory cytokines that are significantly higher in severe acute pancreatitis patients compared to mild acute pancreatitis patients. The low levels of serum IL-10 in severe acute pancreatitis may reflect an inadequate anti-inflammatory mechanism to counteract and down regulate the inflammatory response initiated by pro-inflammatory cytokines in patients with severe disease.

It has recently been demonstrated that administration of IL-10 before and after the induction of experimental acute pancreatitis is able to decrease the severity of the disease. Van Laethem et al, administered recombinant IL-10 intraperitoneally before and during cerulein-induced acute pancreatitis in mice and found that in treated animals there was a decrease in disease severity which was probably due to reduced serum IL-6, IL-1β and TNFα levels (Van Laethem et al, 1995). Administration of IL-10 to a cerulein-induced rat model (Rongione et al, 1995) and a CDE diet-induced mouse model (Kusske et al, 1996) have also shown that IL-10 reduced the severity of the pancreatitis. Similar protective effects of IL-10 were seen in animal models of sepsis whereby its administration protected against lethal endotoxemia in mice (Howard et al, 1993) and sublethal endotoxin in primates (Van der Poll, et al, 1997).

Experiments using IL-10 knockout mice have shown that IL-10 is protective against lung injury in acute pancreatitis. Lung injury was more severe in the IL-10 knockout mice compared to controls (Gloor et al, 1998). Moreover, pretreatment with IT9302, a synthetic IL-10 agonist has been found to reduce lung injury in rabbits with acute necrotising pancreatitis and to improve mortality rates (Osman et al, 1998c). Furthermore, Denham et al, have successfully transfected the human IL-10 gene into the mouse pancreas using a plasmid/liposome vector. The gene was functional and was found to improve mortality and severity of cerulein-induced acute pancreatitis (Denham et al, 1998a).

3.5.5.5 Interleukin 12 (IL-12)

IL-12 is a heterodimeric cytokine composed of 2 subunits known as IL-12 p35 and p40 that is produced by macrophages and is responsible for the differentiation of a naïve $CD4^+$ T cell into a Th1 cell rather than a Th2 cell (Adorini, 1999). Pezzilli et al, 1999 investigated the levels of total IL-12 heterodimer and the two subunits p70 and p40 in the serum of patients with acute pancreatitis. The levels of total IL-12 heterodimer and the p40 subunit were found to be significantly increased during days 1 to 6 following admission, while the level of the p70 subunit was significantly increased on day 1 but subsequently decreased on days 2, 3 and 4 of illness. Increased levels of the p40 subunit were also associated with increased susceptibility to infection (Pezzilli et al, 1999).

Experiments involving cerulein-induced acute pancreatitis in control, nude (athymic) and $CD4^+$ T cell depleted mice have indicated a role of $CD4^+$ T cells in experimental acute pancreatitis. In the control mice, $CD4^+$ T cells were present in the pancreas during acute pancreatitis and correlated with severity, however, in the nude and $CD4^+$ T cell depleted mice the characteristic pancreatic features of severe acute pancreatitis were absent. Subsequent transfer of T cells into the nude mice partially restored the severity of acute pancreatitis and demonstrated that $CD4^+$ T cells are involved in the

development of tissue injury during acute experimental pancreatitis in mice (Demols et al, 2000).

3.5.5.6 Tumour Necrosis Factor Alpha (TNFα)

TNFα is a macrophage derived pro-inflammatory cytokine that is capable of inducing the secretion of other cytokines including IL-1, IL-6, IL-8 and IL-10 (Tracey and Lowry, 1990). Serum TNFα levels are elevated in patients with sepsis and correspond to disease severity (Damas et al, 1989). Since acute pancreatitis shares many clinical characteristics with sepsis, several clinical studies have been carried out to demonstrate a correlation between serum TNFα levels and disease severity in acute pancreatitis, however, the results are conflicting. Early studies showed that serum TNFα levels were not a reliable indicator of disease severity (Banks et al, 1991; Paajanen et al, 1995; De Beaux et al, 1996). TNFα is not always detectable in the serum of pancreatitis patients because it is rapidly removed from the circulation by the liver (Grewal et al, 1994a). In contrast, a more recent attempt to correlate serum TNFα levels with severity has found that they were significantly elevated in severe patients compared to mild patients on days 1-3 following admission, but this trend did not continue on days 4-7 (Chen et al, 1999).

Isolated peripheral blood monocytes from 26 patients with moderate to severe acute pancreatitis and stimulated with LPS showed that TNFα secretion was significantly increased in patients with systemic complications compared to patients with an uncomplicated course (McKay et al, 1996). De Beaux et al, however, showed that in a similar experiment with a mixed PBMCs culture containing both monocytes and lymphocytes taken from 58 patients, TNFα levels were similar to those of controls following stimulation with the same mitogen (De Beaux et al, 1996).

TNFα mediates its actions through its binding to 2 membrane bound surface receptors on the target cells known as p55 and p75. Following exposure to TNFα, the target cells downregulate their response to TNFα by shedding these receptors into the

circulation (Van der Poll and Lowry, 1995). Studies have shown that the soluble receptors appear to be better indicators of disease severity and increased levels predict organ failure in patients with acute pancreatitis (De Beaux et al, 1996; Heresbach et al, 1998; Kaufman et al, 1997). Furthermore, Spinas et al, demonstrated a significant correlation between peak serum TNFα and sTNFR55 in human volunteers after intravenous administration of *E. coli* endotoxin suggesting that sTNFR55 may reflect the degree of TNFα-induced inflammation and may reflect severity of disease (Spinas et al, 1992). Experiments using germ-free rats have shown that TNFα levels rise in the pancreas despite the absence of endotoxin indicating a primary role of TNFα in the disease (Hughes et al, 1995). Vaccaro et al, have recently shown that acinar cells that are stressed also produce TNFα during acute pancreatitis (Vaccaro et al, 2000).

Although TNFα does not induce acute pancreatitis, TNFα levels are toxic to acinar cells and induce apoptosis in cerulein-induced model in rats suggesting that acinar cells do not only die from necrosis or autodigestion during acute pancreatitis (Gukovskaya et al, 1997; Norman et al, 1997a). Furthermore TNFα receptor gene knockout animals have shown that acinar cells unable to respond to TNFα do not undergo apoptosis during acute pancreatitis (Norman et al, 1997a). The TNFα-induced apoptosis correlates with severity of the disease (Gukovskaya et al, 1997; Norman et al, 1997a).

Neutralization of TNFα with an anti-TNFα polyclonal antibody in a rat model of bile-induced acute pancreatitis showed a decrease in pancreatic severity, improved pulmonary complications and survival (Grewal et al, 1994b; Hughes et al, 1996a). In contrast, an earlier experiment carried out by Guice et al, showed that the administration of an anti-TNFα antibody to a cerulein-induced rat model actually increased edema in both the pancreas and lung (Guice et al, 1991).

Norman et al, used a recombinant type I TNFα receptor to antagonise TNFα both prophylactically and therapeutically in a CDE-induced mouse model of acute pancreatitis.

Both methods of treatment resulted in improved survival and a decrease in severity with reduced levels of IL-1 and IL-6, however, the effect was more pronounced in mice that had received the treatment therapeutically demonstrating that the timing of treatment is important in outcome (Norman et al, 1996a).

Methods that interfere with the synthesis and release of TNFα may have therapeutic potential. As with many genes, TNFα expression is controlled by transcription factors such as nuclear factor κB (NFκB). Pretreatment with amobarbital, a drug which blocks the activation of the transcriptional factor showed that both NFκB activation and TNFα expression were prevented with a decreased severity in a pancreatic duct-ligation acute pancreatitis model in rats (Dunn et al, 1997). In addition an inhibitor of p38 map kinase which is also involved in TNFα production has prevented pancreatitis-associated lung injury in bile-induced model in rats (Yang et al, 1999). Calcium has a role in TNFα release and pretreatment with a calcium channel blocker called diltiazem was found to inhibit its release in acute pancreatitis and ameliorate severity of the disease in a bile-induced rat model (Hughes et al, 1996b)

Another method of inhibiting TNFα and IL-1 production is by macrophage inhibition using CNI-1493, a small organic molecule that inhibits the translation of mRNA to protein. IL-1 and TNFα production in the pancreas and lungs were found to be decreased in animals treated with CNI-1493 and there was a decrease in severity (Denham et al, 1997a). A later study has shown that CNI-1493 is capable of preventing many of the sequelae of experimental pancreatitis. Hepatocellular production of IL-1 and TNFα and hepatocellular injury during acute pancreatitis are also attenuated in animals treated with CNI-1493 (Yang et al 1998b).

Other methods of reducing TNFα levels that have been shown to improve the severity of acute pancreatitis and its complications include: pretreatment with propentoxyfylline, a xanthine derivative in rats with cerulein-induced acute pancreatitis and endotoxemia (Sugita et al, 1997); octreotide treatment administered simultaneously

with taucholate induction in rats (Marton et al, 1998) and administration of anti-inflammatory cytokines such as IL-10 and IL-1ra (Denham et al, 1998a; Kusske et al, 1996; Rongione et al 1997; Tanaka et al, 1995).

CHAPTER 4: THE INTERLEUKIN-1 GENE CLUSTER AND ITS ROLE IN ACUTE PANCREATITIS

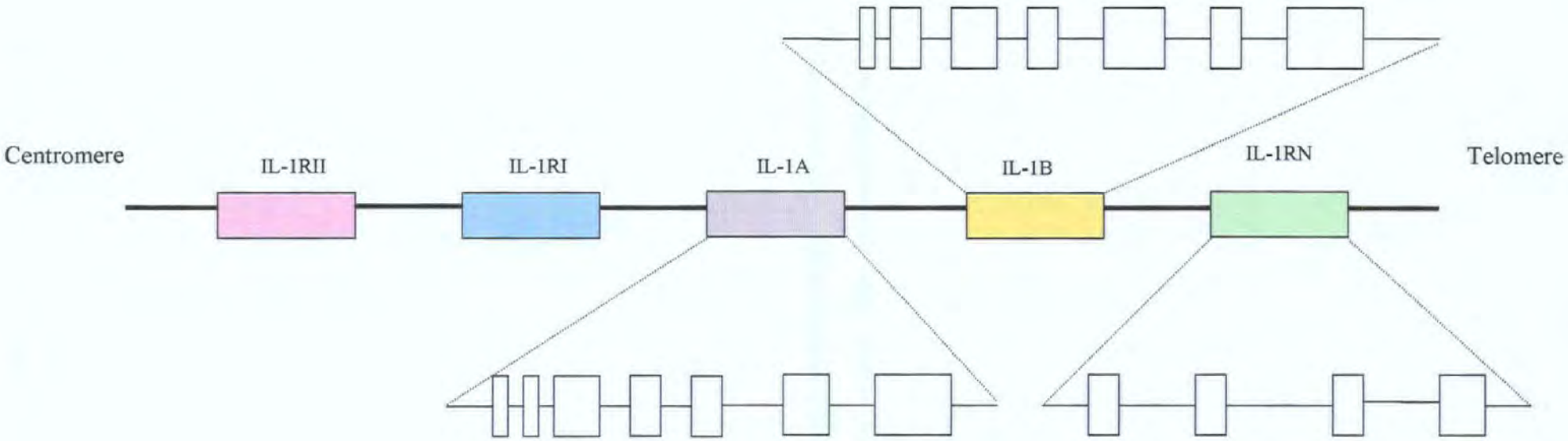
4.1 Introduction to the Interleukin-1 Gene Cluster

The interleukin-1 gene cluster describes a collection of genes that are located on the long arm of chromosome 2 (2q13-2q21) which are concerned with the synthesis and regulation of the pro-inflammatory cytokine, interleukin-1 (IL-1) (Webb et al, 1986; Lafage et al, 1989; Lennard et al, 1992; Nicklin et al, 1994). IL-1A and IL-1B code for the 2 forms of the agonist interleukin-1 alpha (IL-1α) and interleukin-1 beta (IL-1β) respectively, while IL-1RN codes for the receptor antagonist (IL-1ra). The intron-exon organisation of these genes suggests duplication of a common gene more than 350 million years ago (Dinarello, 1994). IL-1 has many biological actions that are mediated through its binding to the two IL-1 receptors known as Type I IL-1 receptor (IL-1RI) and Type II IL-1 receptor (IL-1RII). The genes for these receptors have also been mapped to the same region of chromosome 2 (Sims et al, 1995; Dale and Nicklin, 1999)(Figure 4.1).

4.2 Interleukin-1 alpha and Interleukin-1 beta

The first form of human IL-1 to be identified was IL-1β. Its cDNA sequence was cloned in 1984 from peripheral blood monocytes by Auron et al. This cDNA sequence was translated to form a 269 amino acid precursor which could be cleaved into a mature protein comprising 153 amino acids with a molecular weight of 17kDa (Auron et al, 1984). The cDNA sequence for human IL-1α was cloned in 1986 from a library of human macrophage cDNA (March et al, 1985) and was found to be homologous to the murine IL-1α cDNA sequence (Lomedico et al, 1984). Translation of the human IL-1α cDNA sequence produced a 271 amino acids precursor, which could be cleaved into a mature form of 159 amino acids with a molecular weight of 17kDa (March et al, 1985).

Figure 4.1: Map of the Interleukin-1 gene cluster and Interleukin-1 receptor genes



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(Adapted from Nickin et al, 1994 and Dale and Nicklin, 1999)

The complete genomic sequences of human IL-1A and IL-1B have also been determined (Furutani et al, 1986; Clark et al, 1986). These genes each contain 7 exons and share 45% homology in their nucleotide sequences (Gubler et al, 1986). IL-1A is 7.8kb long, while IL-1B is 10.5kb long. Both genes give rise to proteins that form a β-pleated sheet tertiary structure (Murzin et al, 1992) and which exhibit 26% homology at the amino acid level (Gubler et al, 1986).

Many different cell types, in particular monocytes and macrophages produce IL-1 (Dinarello, 1991). Transcription can be induced by a wide variety of microbial (endotoxin) and non-microbial stimulants. Non-microbial stimulants include stress factors (e.g. ischemia-reperfusion), neuroactive substances (e.g. Substance P), inflammatory substances (e.g. complement components), cell matrix components (e.g. collagen), clotting factors (e.g. thrombin), lipids (e.g. PAF) and cytokines (e.g. IL-1, TNFα, IL-2, IL-3, IL-12, GM-CSF) (Dinarello, 1996). Despite the similarity in gene and protein structure, the two forms of IL-1 are synthesized in different ways.

4.2.1 Synthesis of Interleukin-1 alpha

Following stimulation by an inducing agent such as endotoxin, the monocyte starts to synthesize the 31kDa proIL-1α precursor in association with microtubules (Stevenson et al, 1992; Rubartelli et al, 1990). This precursor comprises 2 subunits, a 16kDa propiece and a 17kDa unit that eventually forms the mature IL-1α protein. Because the 31kDa proIL-1α lacks a leader peptide, it is not secreted and remains in the cytosol. This intracellular proIL-1α is fully active and exerts a direct autocrine effect on the cell by binding to the nuclear DNA (Mosley et al, 1987). Approximately 10% to 15% of the 31kDa proIL-1α, however, undergoes a post-translational modification known as myristoylation (addition of fatty acids) that facilitates its passage to the cell membrane (Stevenson et al, 1993). This myristoylated proIL-1α is then transported to the cell membrane to which it becomes attached. This membrane bound IL-1α is biologically

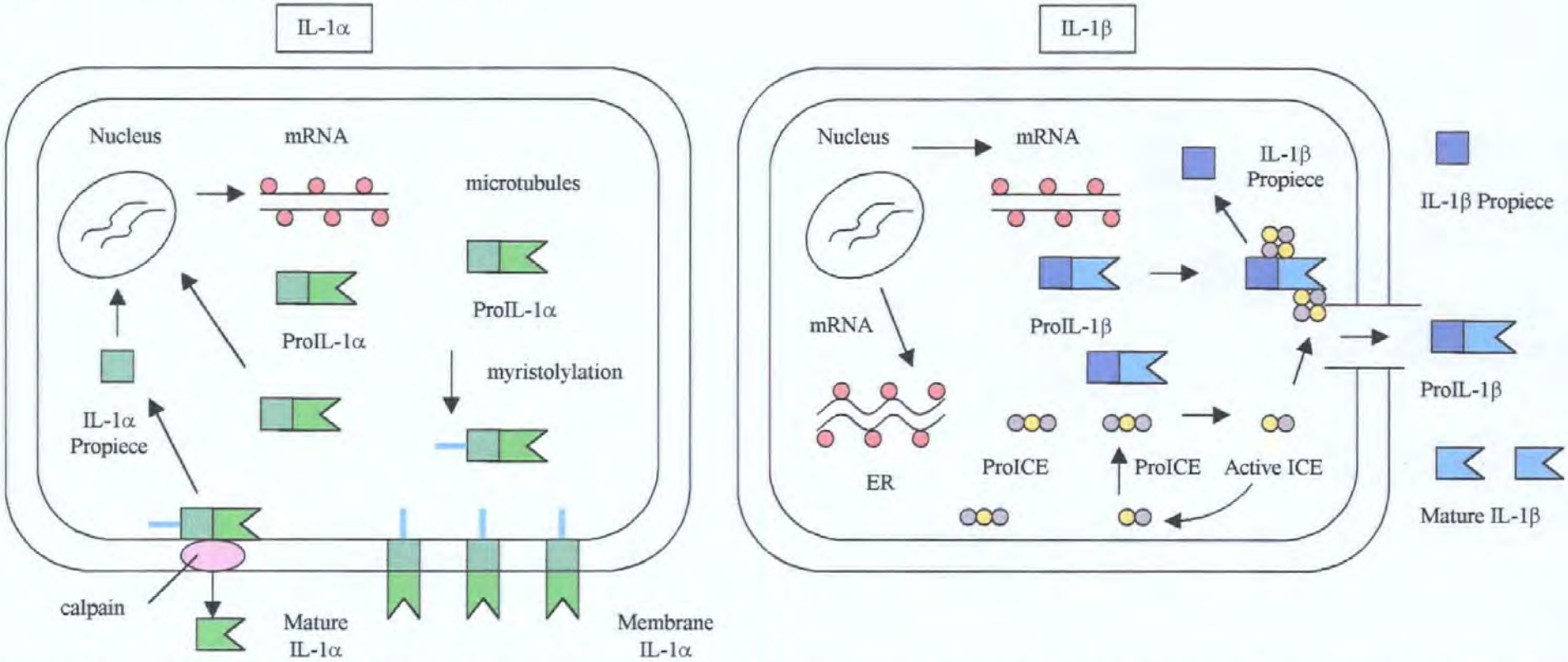
active and serves as a paracrine messenger to adjacent cells. Alternatively, the myristoylated proIL-1α may be cleaved into the 17kDa mature IL-1α by the calcium-dependent membrane-associated cysteine proteases called calpains with the release of the 16kDa IL-1α propiece (Kobayashi et al, 1990).

After cleavage, this 17kDa mature IL-1α is released into the extracellular compartment while the 16kDa IL-1α propiece, like the intracellular 31kDa proIL-1α binds to the nuclear DNA and acts as an autocrine messenger (Stevenson et al, 1992) (Figure 4.2).

4.2.2 Synthesis of Interleukin-1 beta

Following stimulation by an inducing agent, the inactive 31kDa proIL-1β is synthesized by polysomes in the cytosol and associated with microtubules (Rubartelli et al, 1990). Like the proIL-1α, the proIL-1β is comprised of a 16kDa IL-1β propiece and a 17kDa unit that eventually forms the mature IL-1β protein. Simultaneously, interleukin-1β converting enzyme (ICE) is translated in the rough endoplasmic reticulum as an inactive precursor (proICE). This proICE is converted to its active heterodimer form by active ICE present in the cytosol. After activation, the active ICE heterodimer molecules accumulate at the inner surface of the cell membrane where they combine with each other to form ICE tetramers. Each ICE tetramer associates with 2 molecules of the 31kDa proIL-1β and causes cleavage that results in the formation of the 17kDa mature IL-1β and 16kDa IL-1β propiece (Wilson et al, 1994; Walker et al, 1994). The 17kDa mature IL-1β is secreted into the extracellular compartment through a membrane channel while the 16kDa IL-1β propiece becomes distributed both inside and outside the cell (Higgins et al, 1994). A small amount of 31kDa proIL-1β may be transported into the extracellular space via the membrane channel.

Figure 4.2: Synthesis of IL-1α and IL-1β by monocytes



Stimulated monocytes synthesize the 31kDa proIL-1α and proIL-1β precursors that comprise a 16kDa propiece and a 17kDa mature protein. ProIL-1α binds to the nuclear DNA and acts as an autocrine messenger. 10%-15% of the proIL-1α becomes myristoylated and transported to the cell membrane to which it becomes attached and acts as a paracrine messenger. This myristoylated proIL-1α may also be cleaved into the 17kDa mature IL-1α by calpain with the release of the 16kDa IL-1α propiece. The 17kDa mature IL-1α is secreted while the 16kDa IL-1α propiece acts as an autocrine messenger. During IL-1β production, inactive ProICE is simultaneously produced and is converted to its active heterodimer form by active ICE. Two ICE heterodimers combine to form ICE tetramers. Each ICE tetramer associates with 2 molecules of the 31kDa proIL-1β to cause cleavage that results in the formation of the 17kDa mature IL-1β and 16kDa IL-1β propiece. The 17kDa mature IL-1β is secreted through a membrane channel while the 16kDa IL-1β propiece becomes distributed both inside and outside the cell. A small amount of 31kDa proIL-1β may also be secreted from the cell via the membrane channel.

(Adapted from Dinarello, 1996)

When ICE activity within the cell is inhibited, increased proIL-1β is found in the extracellular compartment where it can bind to soluble type II IL-1R (Sims et al, 1994; Symons et al, 1991)(Figure 4.2).

4.2.2.1 Interleukin-1 beta converting enzyme (ICE)

ICE also known as Caspase 1, is an intracellular cysteine protease that cleaves inactive proIL-1β at aspartic acid position 116 to its mature active IL-1β form. A second ICE cleavage site in proIL-1β is located at aspartic acid position 27 and accounts for the 22kDa form of IL-1β found in monocyte cell supernatants. The importance of ICE in the processing of IL-1β is demonstrated in ICE-deficient mice whose macrophages do not release mature IL-1β upon stimulation *in vivo* (Kuida et al, 1995). ICE is specific for proIL-1β and does not cleave proIL-1α (Howard et al, 1991), however, it has also been found to cleave proIL-18 into mature IL-18, a new member of the IL-1 family (Gu et al, 1997).

The cDNA sequence for ICE was isolated in 1992 and was found to encode a 45kDa precursor form of ICE known as ProICE (Ceretti et al, 1992; Thornberry et al, 1992). The gene for ICE designated IL1BC has also been identified. It is 10.5kb long, consists of 9 introns and 10 exons and is located on chromosome 11 (q22.2-q22.3) (Cerretti et al, 1994). ICE is synthesized in a 45kDa precursor form (proICE) which is cleaved internally twice by itself to its mature heterodimer form which consists of a 10kDa and a 20kDa chain containing the active site (Wilson et al, 1994; Gu et al 1995). Two molecules of the ICE heterodimer are required to form a tetramer with 2 molecules of proIL-1β in order for cleavage to occur (Wilson et al, 1994). The activity of ICE is regulated by a competitive inhibitor that reduces maturation and secretion of IL-1β and encourages the accumulation of proIL-1β within the cell (Thornberry et al, 1992). Furthermore, ICE has 5 isoforms resulting from alternative splicing of mRNA. ICEα is the

most active whereas ICEε is inactive and can have an inhibitory effect on the enzyme activity (Wu et al, 1995).

The ICE gene is homologous to the *ced*-3 gene of the nematode *Caenorhabditis elegans* that has a role in apoptosis (Yuan et al, 1993). Overexpression of the ICE gene leads to increased apoptosis in cells transfected with the gene suggesting a role for IL-1 and ICE in apoptosis (Miura et al, 1993; Faucheu et al, 1995). It is important to know whether ICE and IL-1 play a role in apoptosis since therapies aimed at inhibiting ICE and hence reducing IL-1 production are being investigated at present and could have a secondary effect on inhibiting apoptosis and prolonging the survival of malignant cells.

4.3 Interleukin-1 receptor antagonist

Interleukin-1 receptor antagonist (IL-1ra) is a naturally occurring inhibitor of IL-1 that competes for occupancy of the IL-1 receptors but does not induce a signal (Dripps et al 1991; Granowitz et al, 1991a). The gene that encodes IL-1ra known as IL-1RN consists of 4 exons with 2 alternative first exons that give rise to the 2 main forms of IL-1ra: a secreted form (sIL-1ra) and an intracellular form icIL-1ra (Lennard et al, 1992). When the secretory promoter (exon1s) is used, the protein possesses a signal peptide and is secreted (sIL-1ra), however, when the intracellular promoter (exon1ic) is used there is no signal peptide and the protein remains intracellular (icIL-1ra)(Haskill et al, 1991; Butcher et al, 1994).

4.3.1 Secretory Interleukin-1receptor antagonist (sIL-1ra)

The secreted form of IL-1ra was first reported as an IL-1 inhibitor binding activity of 22kDa in the supernatant of human monocytes cultured on adherent IgG (Arend et al, 1985). The cDNA for this form of the antagonist sequence was subsequently isolated from a monocyte library and was translated to form a 17kDa unglycosylated protein consisting of 177 amino acids. Cleavage of a 25 amino acid leader peptide followed by a variable

degree of glycosylation resulted in the mature protein comprising of 152 amino acids with a molecular weight of 22-25kDa (Hannum et al, 1990; Eisenberg et al, 1990). IL-1ra shares 26% amino acid homology with IL-1β and 19% homology with IL-1α (Eisenberg et al, 1990; Carter et al, 1990). Despite having a similar β pleated sheet tertiary structure to the agonists (Vigers et al, 1994), IL-1ra does not initiate a signal on binding to the IL-1 receptors. Cells known to produce the secretory form of the antagonist include monocytes, macrophages and neutrophils (Arend et al, 1998). sIL-1ra can also be produced by the liver following stimulation by IL-1 or IL-1 and IL-6 in much the same way as C reactive protein (CRP) and therefore it has been suggested that IL-1ra is an acute phase protein (Gabay et al, 1996).

4.3.2 Intracellular Interleukin-1receptor antagonist (icIL-1ra)

The cDNA for the intracellular form (icIL-1ra) was first isolated from keratinocytes and digestive epithelial cells and was translated to form a 159 amino acid non-glycosylated protein with a molecular weight of 18kDa (Haskill et al, 1991; Hammerberg et al, 1992). icIL-1ra is expressed constitutively by these cells and in a large excess over IL-1α. Unlike the secreted form of IL-1ra, the intracellular form is not normally found in plasma but may be released from cells when they are damaged to counteract the co-released IL-1. Epithelial cells such as keratinocytes are exposed to the external environment and are therefore particularly at risk from insults. In addition, icIL-1ra may also have a role in inhibiting the intracellular binding of IL-1α to nuclear receptors and hence preventing its action as an autocrine messenger (Haskill et al, 1991; Arend, 1993). icIL-1ra production can also be induced in monocytes and fibroblasts (Arend et al, 1998). More recently, a second form of icIL-1ra known as 16kDa non-glycosylated icIL-1ra type II that is generated by alternative translation initiation of the mRNA has been described in neutrophils, monocytes and hepatic cells (Malyak et al, 1998). The function of this form of icIL-1ra remains unknown

but it may act as an IL-1ra reservoir that is released upon cell death to help the secreted form to limit inflammation.

Transcription of IL-1ra can be stimulated by a wide variety of cytokines (IL-1, IL-2, IL-3, IL-4, IL-6, IL-10, IL-13, IFNγ, GM-CSF, TGFβ) and other stimuli including LPS, adherent IgG, acute phase proteins (APP) and immune complexes (Arend et al, 1998).

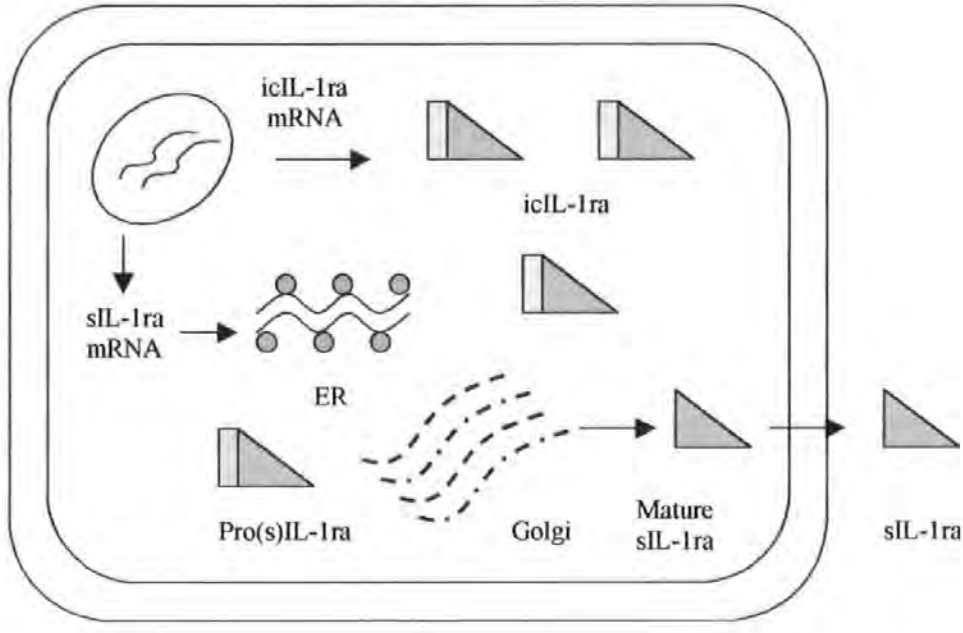
4.3.3 Synthesis of Interleukin-1 receptor antagonist

During the first 24 hours following stimulation the monocyte uses the sIL-1ra promoter. The primary transcript is pro(s)IL-1ra that is translated in the endoplasmic reticulum and transported to the Golgi. Following cleavage of the leader sequence, sIL-1ra is secreted from the cell (Arend, 1993). After 48 hours, the monocyte exhibits macrophage characteristics and uses the icIL-1ra promoter to transcribe icIL-1ra. Since icIL-1ra lacks a leader sequence, it is not secreted but remains intracellular (Andersson et al, 1992) (Figure 4.3).

4.4 The Interleukin-1 receptor family

The IL-1 receptor family consists of 3 molecules: 2 true receptors called IL-1 type I (IL-1RI) and IL-1 receptor type II (IL-1RII) and IL-1 receptor accessory protein (IL-1R-AcP). The receptors belong to the immunoglobulin superfamily and exist in membrane and soluble forms. They have 3 domains, an extracellular domain, a transmembrane domain and a cytoplasmic domain however, the lengths of these domains varies between the 2 types of receptors.

Figure 4.3: IL-1ra synthesis by monocytes



During the first 24 hours following stimulation the monocyte uses the sIL-1ra promoter. The primary transcript is pro(s)IL-1ra that is translated in the endoplasmic reticulum and transported to the Golgi. Following cleavage of the leader sequence, sIL-1ra is secreted from the cell. After 48 hours, the monocyte exhibits macrophage characteristics and uses the icIL-1ra promoter to transcribe icIL-1ra. Since icIL-1ra lacks a leader sequence, it is not secreted but remains intracellular.

(Adapted from Dinarello, 1996)

4.4.1 Membrane receptors

4.4.1.1 Type I receptor

IL-1RI is an 80kDa glycoprotein expressed at the surface of numerous types of cells including endothelial cells, fibroblasts, chondrocytes smooth muscle cells and T lymphocytes. The extracellular, transmembrane and cytoplasmic domains of this receptor contain 316, 20 and 213 amino acids, respectively (Sims et al, 1988). IL-1RI is the only one of the 2 receptors that is capable of transmitting a signal when it is occupied by IL-1 (Stylianou et al, 1992; Sims et al, 1993). IL-1 is a very potent cytokine and requires only 50-200 receptors per target cell to transmit a signal (Shirakawa et al, 1987). Because IL-1RI is involved in signal transduction, its expression can affect the activity of IL-1.

Increased expression of this receptor causes increased IL-1 activity. Agents known to increase the expression of IL-1RI include phorbol esters, PGE_2, dexamethasone, vitamin D3, IL-2 and IL-4 (Dinarello, 1994). IL-1 itself can also upregulate or downregulate IL-1RI expression depending on whether the cell produces PGE_2. In cells producing PGE_2 it increases IL-1RI expression (Takii et al, 1992), but in cells that do not produce PGE_2 it reduces IL-1RI expression (Takii et al, 1994).

4.4.1.2 The interleukin-1 receptor accessory protein

The interleukin-1 receptor accessory protein (IL-1R-AcP) is required for signal transduction following the binding of IL-1 to IL-1RI. The process of signal transduction is described in Section 4.5. Unlike the genes for the "true" IL-1 receptors which are located on chromosome 2 (Sims et al, 1995), the gene which codes for IL-1R-AcP has been mapped to 3q38 (Dale et al, 1998).

4.4.1.3 Type II receptor

The second receptor is a 68kDa glycoprotein that is found on B lymphocytes, neutrophils and bone marrow cells. The extracellular, transmembrane and cytoplasmic

domains are 336, 26 and 29 amino acids long respectively (McMahan et al, 1991). Due to the short cytoplasmic domain, IL-1RII does not transmit a signal but acts as a "decoy receptor" and is a negative regulatory factor for IL-1 which remains trapped on the receptor which prevents it from inducing a signal (Colotta et al, 1993). It has been suggested that the function of the membrane bound type II receptor is to serve as the precursor for a soluble IL-1 binding factor that can be shed under appropriate circumstances to antagonize and modulate IL-1 activity (Sims et al, 1993). Because IL-1RII is a "decoy receptor", increased expression inhibits IL-1 activity. Agents known to increase the expression of IL-1RII include dexamethasone, IL-4 and IL-13 (Colotta et al, 1993; Re et al, 1994; Colotta et al, 1996) and IL-1 itself (Dinarello, 1996). IL-1RI has a higher affinity for IL-1α and IL-1ra whereas the IL-1RII has a higher affinity for IL-1β.

4.4.2 Soluble receptors

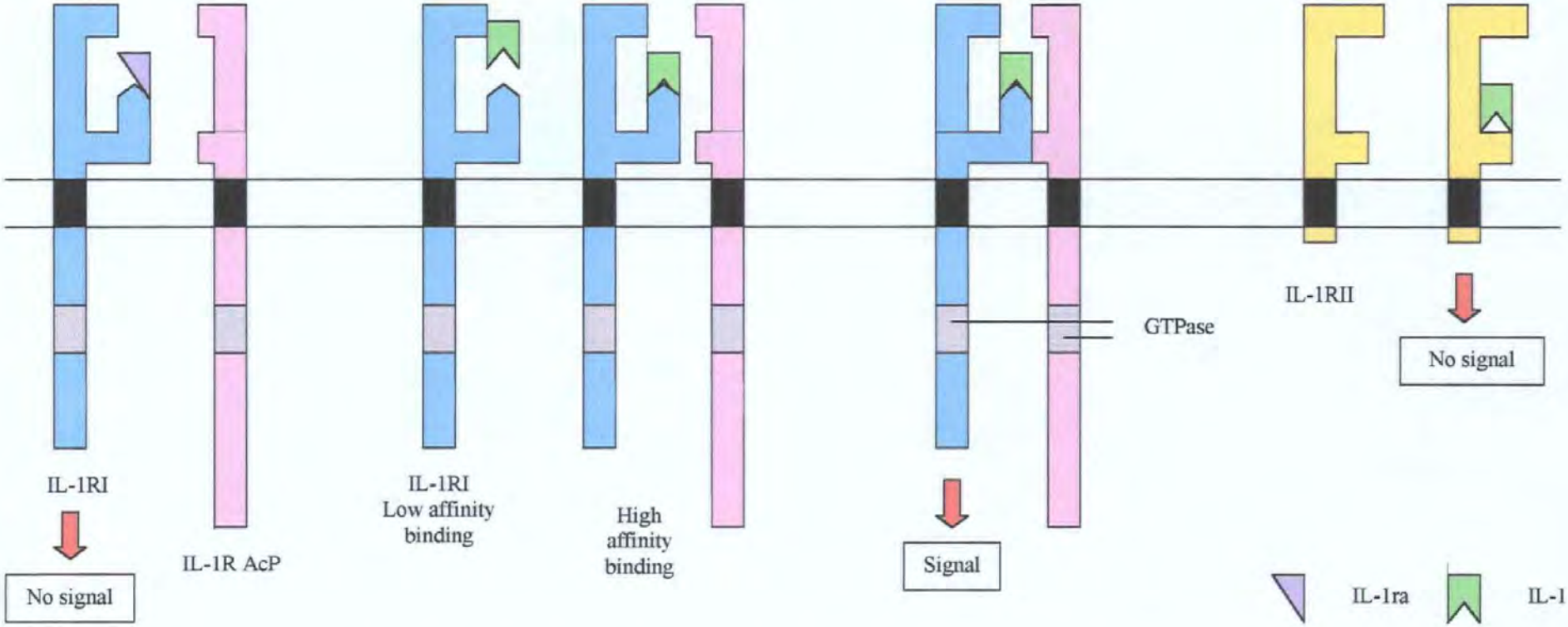
The soluble forms of the 2 receptors result from the cleavage of the extracellular portion of the membrane receptors and exhibit 28% amino acid homology. These receptors are found in both healthy (Sims et al, 1994; Symons et al, 1991) and diseased subjects (Arend et al, 1994). Like the membrane IL-1 receptors, the soluble receptors exhibit preferential binding. The soluble extracellular domain of IL-1RI (sIL-1RI) binds IL-1ra>IL-1α>IL-1β, while the soluble extracellular domain of IL-1RII (sIL-1RII) binds IL-1β>proIL-1β>IL-1α>IL-1ra. Since the membrane receptors act as a source of soluble receptors, agents that increase the expression of the membrane forms also increase the levels of the soluble forms. Increased levels of sIL-1RI preferentially bind IL-1ra and hence increase IL-1 activity whereas increased sIL-1RII preferentially bind IL-1β and decrease IL-1 activity. Because of their inhibitory effect on IL-1 activity, sIL-1RII exhibit therapeutic potential.

4.5 Signal transduction

IL-1 binds to the extracellular domain of either type I (IL-1RI) or type II (IL-1RII) receptors on the target cell. IL-1RI has a higher affinity for IL-1α whereas IL-1RII has a higher affinity for IL-1β. IL-1 binds to IL-1RI with its two receptor binding sites to form a low affinity binding complex (IL-1/IL-1RI). This causes a structural change in the cytoplasmic domain of the receptor which allows the IL-1R accessory protein (IL-1R-AcP) to combine with the low affinity binding complex (IL-1/IL-1RI) to form a high affinity heterodimer complex (IL-1RI/IL-1/IL-1R-AcP) with the initiation of signal transduction (Greenfeder et al, 1995). The cytoplasmic domains of the IL-1RI and IL-1R-AcP contain areas of GTPase activity. When the IL-1/IL-1RI and IL-1R-AcP form the heterodimer complex they are in close proximity and there is hydrolysis of GTP by intrinsic GTPase activity of the cytoplasmic domains. This leads to a signal transduction phosphorylation cascade and to activation of NF-κB regulated transcription. Because IL-1RII lacks a cytoplasmic domain when IL-1 binds to it no signal is transduced. IL-1RII therefore acts as a decoy receptor for IL-1 (Dinarello, 1998). IL-1ra binds to IL-1RI with only one receptor binding site (Evans et al, 1995) and therefore does not result in a structural change of the receptor. This means that IL-1R-AcP does not form a high affinity complex with the IL-1/IL-1RI so no signal is transduced and there is no biological response (Dripps et al, 1991)(Figure 4.4).

The binding of IL-1 to its receptor on a target cell and the subsequent signal transduction, affects the expression of a wide range of genes within the cell. Genes whose expression is increased include those that encode enzymes such as cyclooxygenase (production of prostaglandins and thromboxanes), phospholipase A_2 (production of PAF and leukotrienes, prostaglandins and thromboxanes) and inducible nitric oxide synthase (production of NO). The expression of cytokines such as IL-1, TNFα, IL-6, and IL-8 and adhesion molecules such as ICAM-1, ELAM and VCAM-1 are also increased.

Figure 4.4: Cell receptors for IL-1



IL-1 binds to IL-1RI with its two receptor binding sites to form a low affinity binding complex. This causes a structural change in the cytoplasmic domain of the receptor which allows IL-1R-AcP to combine with the low affinity binding complex to form a high affinity heterodimer complex. The close proximity of the two GTPase leads to hydrolysis, a signal transduction phosphorylation cascade and to activation of NF-κB regulated transcription. Because IL-1RII lacks a cytoplasmic domain when IL-1 binds to it no signal is transduced. IL-1RII therefore acts as a decoy receptor for IL-1. IL-1ra binds to IL-1RI with only one receptor binding site and therefore does not result in a structural change of the receptor. This means that IL-1R-AcP does not form a high affinity complex with the IL-1/IL-1RI so no signal is transduced and there is no biological response.

(Adapted from Dinarello, 1996)

Genes whose expression is decreased following IL-1 receptor binding include genes for albumin and cytochrome p450 (Dinarello, 1996).

The administration of IL-1 to humans with certain solid tumours causes both local manifestations such as localised pain and edema and systemic manifestations such as hypotension, fever and systemic pain that are caused by the products of the upregulated gene expression. IL-1, TNFα, IL-6 and PAF activate leucocytes, IL-8 and LTB_4 are chemotactic for the activated leucocytes and ICAM-1, ELAM, and VCAM-1 are involved in leucocyte migration. Furthermore, leukotrienes and NO increase vascular permeability allowing the inflammatory exudate to accumulate in the damaged tissue causing the local edema, while prostaglandins are responsible for pain and fever. Prostaglandins and NO also cause vasodilatation leading to hypotension (Dinarello, 1997).

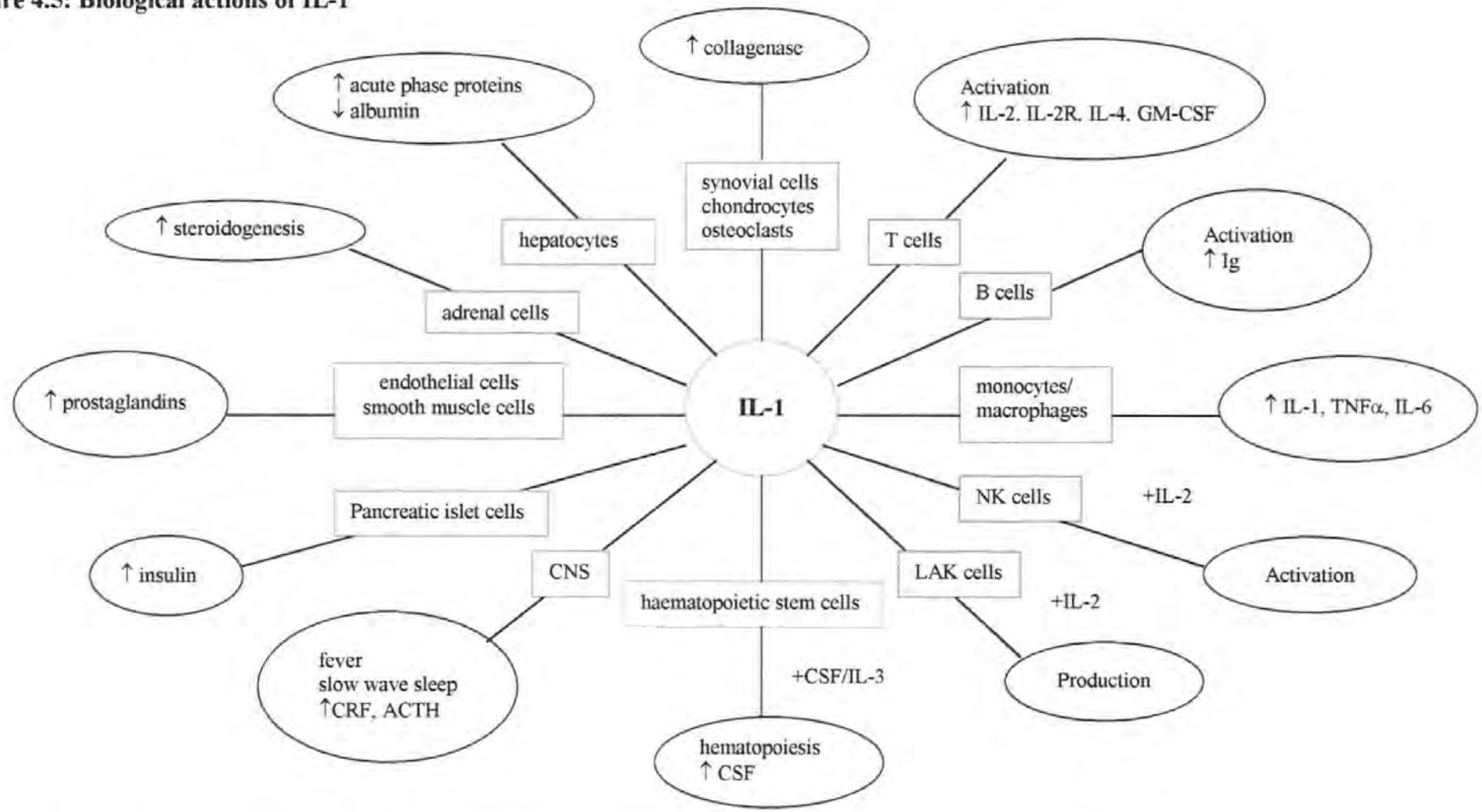
4.6 Biological actions of Interleukin-1

IL-1 is a pleiotropic cytokine that shares a wide variety of biological actions with TNFα. These actions can be classified as non-immunological, immunological and hematopoietic (Figure 4.5). IL-1 may act alone or in synergy with other cytokines.

4.6.1 Non-immunological actions

IL-1 acts on the central nervous system to induce fever (Dinarello and Woolf, 1982), slow wave sleep (Dinarello, 1988a) and the release of the hormones corticotropin-releasing factor (CRF) and adrenocorticotropin (ACTH) that cause steroidogenesis in the adrenal glands (Sapolsky et al, 1987). IL-1 also acts directly on the adrenal glands to induce steroidogenesis (Lee et al, 1988). Small doses of IL-1 have been shown to induce insulin production, whereas larger doses are cytotoxic to β cells of the pancreas and may therefore be implicated in the pathogenesis of diabetes (Mandrup-Poulsen et al, 1986).

Figure 4.5: Biological actions of IL-1



IL-1 has many biological actions that can be divided into non-immunological, immunological and hematopoietic.

Another important action of IL-1 is to cause the acute phase response in the liver resulting in an increase in hepatic protein synthesis and a decrease in albumin production (Ramadori et al, 1985). IL-1 also induces prostaglandin synthesis in endothelial cells and smooth muscle cells. These substances cause vasodilatation and are responsible for the hypotension associated with IL-1 (Dinarello, 1988b; Dejana et al, 1987). In addition, IL-1 exhibits catabolic effects that include the induction of collagenase production in synovial cells, chondrocytes and osteoclasts (Dayer et al, 1986; Eastgate et al, 1988). These catabolic properties of IL-1 in cartilage and bone are involved in the pathogenesis of diseases such as rheumatoid arthritis and osteoarthritis.

4.6.2 Immunological actions

IL-1 causes T cell activation resulting in the increased IL-2 production and expression of IL-2 receptors (Kaye and Janeway, 1984) and induces the production of IL-4 and GM-CSF from activated T cells (Herrmann et al, 1988). In B cells it causes activation and increased immunoglobulin synthesis (Abbas et al, 1987; Lipsky et al, 1983). IL-1 acts on monocytes and macrophages inducing its own synthesis and the production of TNFα and IL-6 (Lovett et al, 1986; Navarro et al, 1989). Furthermore, IL-1 synergizes with other cytokines such as IL-2 to activate NK cells (Ben Aribia et al, 1987) and produce LAK cells (Kovacs et al, 1989). Since these cells have tumoricidal effects, IL-1 indirectly promotes anti-tumour activity. *In vitro* studies using tumour cell lines have demonstrated that IL-1 is also directly toxic to tumour cells, however, the response of the tumours to IL-1 appears to be clonal dependent. Some cell lines such as human ovarian carcinoma are killed by IL-1 (Kilian et al, 1991), whereas others such as human astrocytoma are not susceptible to IL-1 and actually proliferate more in its presence (Lachman et al, 1987). Furthermore, it has been shown that mice with subcutaneous B16 melanoma tumours that are known to produce IL-1 benefited from IL-1ra treatment (McKenzie et al, 1996). IL-1 therapy would therefore not be applicable to this type of malignancy. IL-1 has been implicated in the

pathogenesis of leukemia (Rambaldi et al, 1991; Wetzler et al, 1994) and therefore it is important to emphasize that IL-1 treatment for solid tumours may increase the risk of developing leukemia.

4.6.3 Hematopoietic actions

IL-1 causes the production of hematopoietic growth factors such as CSF from a variety of cells. Furthermore, it synergizes with CSF or IL-3 to promote the growth of primitive hematopoeitic stem cells (Stanley et al, 1986). Levels of IL-1 are important in the pathogenesis of hematopoietic diseases such as leukaemia and bone marrow failure states. Increased levels of IL-1 have been described in patients with leukaemia (Rambaldi et al, 1991; Wetzler et al, 1994) whereas decreased levels have been found in bone marrow failure states such as aplastic anaemia (Nakao et al, 1989). Patients with leukaemia would therefore benefit from IL-1ra therapy, whereas patients with aplastic anaemia would benefit from IL-1 therapy. Furthermore, because of its role in promoting hematopoeisis, IL-1 can also be used in combination with chemotherapy and after bone marrow transplants to accelerate bone marrow recovery (Dinarello, 1996).

4.7 Interleukin-1 and Interleukin-1receptor antagonist in health and disease

With the exception of skin keratinocytes, other epithelial cells, ovarian granulosa cells and certain cells of the central nervous system, the cells of healthy individuals do not produce IL-1. During pathological conditions such as infection, however, the transcription of IL-1 is upregulated in a wide variety of cells particularly monocytes and macrophages. Increased IL-1 production has been reported in a number of diseases including: Alzheimer's disease (Griffin et al, 2000); AP (Chen et al, 1999); IBD (Nakamura et al, 1992; Ligumsky et al, 1990); sepsis (Cannon et al, 1990); rheumatoid arthritis (Eastgate et al, 1988); acute myelogenous leukaemia (Rambaldi et al, 1991); chronic myelogenous leukaemia (Wetzler et al, 1994); IDDM (Mandrup-Poulsen et al, 1986); tuberculosis

(Fujiwara et al, 1986); leprosy (Watson et al, 1984); meningococcemia (Girarddi et al, 1988) and atherosclerosis (Galea et al, 1996).

In contrast to IL-1, IL-1ra circulates at low levels in healthy individuals (Hurme and Santilla, 1998). During disease these IL-1ra levels increase dramatically in an attempt to counteract the rise in IL-1 levels. Despite the elevation of IL-1 during disease, levels often remain low and are difficult to detect. Consequently IL-1ra levels have proven to be a better indicator of disease than IL-1. Elevated levels of IL-1ra have been detected in a variety of human diseases (Table 4.1).

It has been suggested that the IL-1ra: IL-1 ratio is important in the pathogenesis of disease (Dinarello and Wolff, 1993). The amount of IL-1ra that is required to block the action of IL-1 is very high despite relatively low plasma levels of IL-1. Granowitz et al, found that a 100 fold excess *in vitro* and 1000 fold excess *in vivo* of IL-1ra relative to IL-1 was required to block the action of IL-1 in models of endotoxemia (Granowitz et al, 1991b). The reason why so much IL-1ra is required to block IL-1 activity when IL-1 levels are relatively low is not fully understood and a number of theories have been postulated. These include: an underestimation of IL-1 production during disease; high potency of IL-1/high sensitivity of IL-1 type I receptors to IL-1; binding of IL-1ra to the soluble type I receptor; poor tissue penetration of IL-1ra; short plasma half-life of IL-1ra and rapid clearance of IL-1ra from the body by the kidneys.

Diseases are thought to occur when the IL-1ra: IL-1 ratio is decreased. The increased production of IL-1 relative to IL-1ra disturbs this balance favouring the pro-inflammatory state and there is insufficient IL-1ra to counteract the action of IL-1. This was demonstrated in experimental endotoxemia in human volunteers where despite there being an 100 fold excess of IL-1ra relative to IL-1, the balance remained in favour of IL-1 activity resulting in shock (Granowitz et al, 1991b).

Table 4.1: Increased levels of IL-1ra in human disease

Disease	Reference
Inflammatory diseases	
Periodontitis	Ishihara et al, 1997
Idiopathic pulmonary fibrosis	Smith et al, 1995
Sarcoidosis	Rolfe et al, 1993; Chensue et al, 1992
Myocardial infarction	Shibata et al,1997
Subarachnoid hemorrhage	Mathiesen et al, 1997
Cerebral infarction	Beamer et al,1995
Hemophagic lymphohistiocytosis	Henter et al, 1996
Thermal injury	Mandrup-Poulsen et al 1995; Endo et al, 1996
Poliomyositis/dermatomyositis	Gabay et al,1994
Ulcerative colitis	Hyams et al 1994
Acute hepatitis	Sekiyama et al, 1994
Pars Planitis and Behcet Disease	Benezra et al, 1997
Infectious diseases	
HIV infection	Thea et al, 1996; Rimaniol et al, 1997
Sepsis	Fischer et al, 1992; Pruitt et al, 1996
Experimental endotoxemia	Granowitz et al, 1991b
Pediatric sepsis syndrome	Samson et al, 1997
Neonatal sepsis	DeBont et al, 1995
Autoimmune diseases	
SLE	Suzuki et al, 1996; Chang, 1997
Relapsing/remitting multiple sclerosis	Nicoletti et al,1996
Juvenile chronic arthritis	Prieur et al, 1987; De Benedetti et al, 1995; Muller et al, 1997
Rheumatoid arthritis	Malyak et al, 1993; Chikanza et al, 1995
Neurological diseases	
Depression	Maes et al, 1995
Schizophrenia	Maes et al,1996
Malignant diseases	
Hodgkins disease	Gruss et al,1992
Glioblastoma	Tada et al,1994
Pituitary adenoma	Sauer et al,1994
Gynaecological cancer	Fujiwaki et al,1997
Bronchogenic carcinoma	Smith et al, 1993
Other	
Pre-eclampsia	Kimya et al,1997
Asthma	Yoshida et al, 1996
Chronic renal failure	Pereira et al, 1994

Despite many studies detecting elevated levels of IL-1 or IL-1ra in disease as discussed above, relatively few have measured both proteins and demonstrated an imbalance between them. Low IL-1ra: IL-1 ratios have been associated with acute myelogenous leukaemia (Rambaldi et al, 1991), chronic myelogenous leukaemia (Wetzler et al, 1994), hairy cell leukaemia (Barak et al, 1994), Lyme arthritis (Miller et al, 1993), rheumatoid arthritis (Firestein et al, 1994; Chomarat et al, 1995, Chikanza et al, 1995), psoriasis (Kristensen et al, 1992; Corradi et al, 1995), smokers versus non-smokers (Janson et al, 1993), IBD (Casini-Raggi et al, 1995; Nishiyama et al, 1994; Hyams et al, 1995; Dionne et al, 1998; Ishizuka et al, 2001), severe acute pancreatitis (Mayer et al, 2000; Powell et al, 2001), chorionamnionitis (Baergen et al,1994), newly diagnosed IDDM (Netea et al, 1997), periapical lesions (Shimauchi et al,1998), sarcoidosis and idiopathic pulmonary fibrosis (Mikuniya et al, 1997), nonbacterial prostatitis (Nishimura et al, 1998), chronic hepatitis due to hepatitis C virus (Gramantieri et al, 1999), pediatric astrocytomas and ependymomas (Ilyin et al, 1998), head and neck squamous cell carcinoma (Von Biberstein et al, 1996) and increased risk of acute renal allograft rejection (Teppo et al, 1998).

The variation in IL-1 and IL-1ra protein levels may be genetically determined. A number of polymorphisms are known to exist in the genes that encode IL-1α, IL-1β and IL-1ra. It has been shown that polymorphisms in other cytokine genes such as IL-10, IFN-γ and TNFα determine functional protein levels (Eskdale et al, 1998, Pravica et al, 1999, Pociot et al, 1993).

4.7.1 The Interleukin-1 gene cluster polymorphisms

The members of the interleukin-1 gene cluster are highly polymorphic. There are several different types of polymorphisms, however, for the purpose of this study only 4 types will be considered.

Single Nucleotide Polymorphisms (SNP)

Single nucleotide polymorphisms involve the substitution of one nucleotide for another with the creation of 2 alleles. Restriction endonucleases that recognize and cleave specific sequences within the DNA strand can be used to detect these nucleotide changes provided the substitution creates or destroys the restriction endonuclease cleavage site. However, in the majority of cases SNPs cannot be detected by restriction endonucleases. An example of a single nucleotide polymorphism is:

Allele 1 ...CGA

↓

Allele 2 ...TCGA

Taq I restriction endonuclease recognizes and cleaves the sequence T↓CGA (↓ represents the position of cleavage). In the example above, the substitution of a Thymine for a Cytosine creates a *Taq I* site. If the polymorphism is present on both chromosomes, 2 bands are visible on the agarose gel (homozygous), if the polymorphism is present on neither chromosome, only 1 band is visible (homozygous) and if the polymorphism is present on 1 chromosome only, 3 bands are visible (heterozygous).

Dinucleotide repeat polymorphisms

These polymorphisms consist of continuous repeating units of a dinucleotide repeat sequence, for example (AC)n. The different number of dinucleotide repeats correspond to different alleles. An example of a dinucleotide polymorphism is:

Allele 1	ACAC	2 repeats
Allele 2	ACACAC	3 repeats
Allele 3	ACACACAC	4 repeats
Allele 4	ACACACACAC	5 repeats

Variable number of tandem repeat polymorphisms (VNTR)

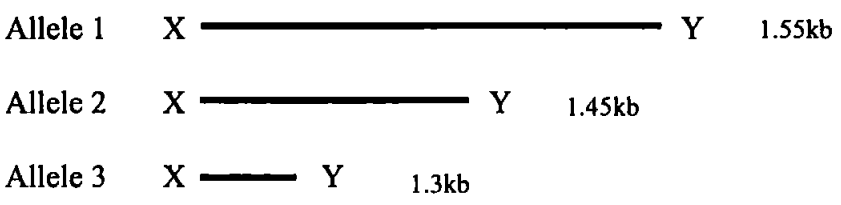
These polymorphisms are similar to dinucleotide repeat polymorphisms because they consist of repeating units of a given number of base pairs, however, the repeating sequences are longer e.g. 86 base pairs (bp) long and are interrupted by non-repeating sequences. An example of a variable number of tandem repeat polymorphism is:

Allele 1	—86bp—86bp—86bp—86bp—	4 repeats
Allele 2	—86bp—86bp—	2 repeats
Allele 3	—86bp—86bp—86bp—86bp—86bp—	5 repeats
Allele 4	—86bp—86bp—86bp—	3 repeats
Allele 5	—86bp—86bp—86bp—86bp—86bp—86bp—	6 repeats

86bp = a set number of base pairs — non-repeating sequence

Variable Length Polymorphisms (VLP)

A variable length polymorphism occurs when the distance between 2 points on the gene (X and Y) varies in length, each length variation creating a different allele. An example of a variable length polymorphism is:



The following polymorphisms of the IL-1 gene cluster are discussed in the order in which they appear on the long arm of chromosome 2 relative to the centromere.

4.7.1.1 IL-1RI Gene

At least six single nucleotide polymorphisms have been described in the 5' untranslated region of the IL-1RI gene. A T to C substitution has been identified at

position 131(T→C^{131}) in exon 1B which created a *Pst I* restriction endonuclease site, while a C to T substitution at position 157 (C→T^{157}) in the same region did not create or destroy a restriction endonuclease site (Bergholdt et al, 1995). Sitara et al, found an A to G substitution in intron 1B at position 52 (A→G^{52}) that created a *Msp I* restriction endonuclease site (Sitara et al, 1999) and a single nucleotide polymorphism was identified in exon 1C at position 97 (G→A^{97}) which created *Sty I* restriction site (Sitara et al, 2000). An A to T substitution was also reported at position 140 (A→T^{140}) in exon 1C, however, this did not create or destroy a restriction endonuclease site (Sitara et al, 1999). Furthermore, a *Hinfl* single nucleotide polymorphism has recently been characterised and found to be associated with IL-1RI plasma levels (Bergholdt et al, 2000).

4.7.1.2 IL-1A Gene

Single nucleotide polymorphisms also exist in the promoter region of the IL-1A gene at position -889 (C→T^{-889}) and in exon 5 at position +4845 (G→T^{+4845}) which create *Nco I* and *Fnu 4HI* endonuclease restriction sites respectively (McDowell et al, 1995; Van den Velden and Reitsma, 1993). The *Fnu 4HI* polymorphism, which causes the substitution of a serine for an alanine at position 114 is 100% linked with *Nco I* polymorphism (Jouvenne et al, 1999). An (AC)n repeat polymorphism has been identified at position 7695 within intron 5 which consists of 6 alleles (Todd and Naylor, 1991) or 7 alleles (Epplen et al, 1994). In addition, a variable number of tandem repeats (VNTR) polymorphism has been found at position 8912 in intron 6 which consists of 7 alleles corresponding to between 5 and 18 copies of a 46 base pair sequence ($VNTR^{46}$). Allele 1 (800bp, 9 repeats), allele 2 (1220bp, 18 repeats), allele 3 (760bp, 8 repeats), allele 4 (940bp, 12 repeats), allele 5 (620bp, 5 repeats), allele 6 (1080bp, 15 repeats) and allele 7 (660bp, 6 repeats). The repeat region is flanked at the 5' end by a poly (dA-dC) and at the 3' end by a poly (dG-dT) and the repeats are conserved except for a nucleotide change (A to G) at position 8925 and a 4-nucleotide deletion (TAGG) at position 9128. Each 46bp

repeat contains 3 potential transcription factor-binding sites: SP1, viral enhancer element and glucocorticoid-responsive element (GRE) (Bailly et al, 1993). The presence of these transcription factor-binding sites implies that the polymorphism may be involved in IL-1α gene regulation. A recent study has shown that there was an inverse relationship between the number of repeats and IL-1α production, suggesting that the polymorphism had a negative regulatory role on IL-1α gene expression (Bailly et al, 1996).

4.7.1.3 IL-1B Gene

Single nucleotide polymorphisms have been identified in the promoter of the IL-1B gene at positions 1423 ($C\rightarrow T^{1423}$) and 1903 ($T\rightarrow C^{1903}$) which destroy *Ava I* and *Alu I* restriction endonuclease sites respectively (Guasch et al, 1996). The C→T polymorphism at position 1423 is identical to the previously-described C to T transition polymorphism at position -511 ($C\rightarrow T^{-511}$) (Di Giovine et al, 1992). Allele 2 of this polymorphism has been associated with high IL-1β secretion (Nemetz et al, 1999). The T→C polymorphism at position 1903 is identical to the *Alu I* polymorphism described in the promoter region at position -31 (El-Omar et al, 2000). In exon 3 at position 3263 there is a C to T substitution ($C\rightarrow T^{3263}$) which does not create or destroy a restriction endonuclease site (Langdahl et al, 2000). Furthermore, single nucleotide polymorphisms at position 5810 ($G\rightarrow A^{5810}$) in intron 4 and at position 5887 ($C\rightarrow T^{5887}$) in exon 5 create *BsoFI* and *Taq I* restriction sites respectively (Guasch et al, 1996). The nucleotide substitution in exon 5 is identical to the previously described *Taq I* polymorphism at position +3953 ($C\rightarrow T^{+3953}$)(Pociot et al, 1992). Allele 2 of this polymorphism has been associated with high IL-1β secretion (Pociot et al, 1992). A recently described *Aci I* restriction site created by a G to A substitution at position 3877 ($G\rightarrow A^{3877}$) in intron 4 has been found to be identical to the *BsoFI* described by Guasch et al (Langdahl et al, 2000).

4.7.1.4 IL-1RN Gene

A G→A nucleotide substitution has been identified in the promoter of the IL-1RN gene at position 1731 ($G\rightarrow A^{1731}$)(Clay et al, 1996). As discussed in Section 4.3, the IL-1RN gene has 2 first exons: exon1ic codes for intracellular IL-1ra and exon1s codes for secretory IL-1ra. Single nucleotide polymorphisms have been described in exon1ic at positions 1812 ($G\rightarrow A^{1812}$), 1868 ($A\rightarrow G^{1868}$), 1887 ($G\rightarrow C^{1887}$) (Clay et al, 1996) and 1934 ($T\rightarrow C^{1934}$) (Langdahl et al, 2000). Nucleotide substitutions have also been found at positions 8006 ($T\rightarrow C^{8006}$) in exon 2 and 8061 ($C\rightarrow T^{8061}$) in intron 2 which create *Msp I* and destroy *Mwo I* restriction sites respectively (Guasch et al, 1996). A variable length polymorphism (VLP) has been identified in intron 2 at position 190 (Genbank: M63099) that consists of 3 alleles. Each allele corresponds to a different length of DNA: allele 1 (1.55kb), allele 2 (1.45kb) and allele 3 (1.3kb)(Steinkasserer et al, 1991). A variable number of tandem repeats (VNTR) polymorphism is also known to exist in intron 2 at position 8902 that consists of 5 alleles corresponding to between 2 and 6 copies of an 86 base pair sequence ($VNTR^{86}$). Allele 1 (410bp, 4 repeats), allele 2 (240bp, 2 repeats), allele 3 (500bp, 5 repeats), allele 4 (325bp, 3 repeats), and allele 5 (595bp, 6 repeats). Each repeat contains 3 potential protein-binding sites: an α-interferon silencer A, a β-interferon silencer B and an acute phase response element (Tarlow et al, 1993). Studies regarding an association between this polymorphism and functional IL-1ra protein levels are conflicting. One study suggests that allele 2 (2 x 86bp repeats) correlates with low IL-1ra protein level. (Tarlow et al, 1993), whilst another study has reported that allele 2 corresponds to high IL-1ra protein levels (Danis et al, 1995). Single nucleotide polymorphisms are also described at positions 9589 ($A\rightarrow T^{9589}$) in intron 3 and 11100 ($T\rightarrow C^{11100}$) in exon 4 which create *Ssp I* and *MspA1I* restriction sites respectively (Guasch et al, 1996). Tables 4.2A-C show positive and negative associations between these polymorphisms and human disease.

Table 4.2A: Positive and negative associations between polymorphisms in the IL-1 gene cluster and inflammatory diseases

Gene	Polymorphism	+/-	Population	Disease association	Reference
IL-1A	Nco I^{-889}	-	American	Periodontitis	Gore et al, 1998
IL-1A	Nco I^{-889}	-	British	Single and multivessel coronary artery disease	Francis et al, 1999
IL-1A	Nco I^{-889}	-	British	Ulcerative colitis	Mansfield et al, 1994
IL-1A	Nco I^{-889}	-	British	Crohn's disease	Mansfield et al, 1994
IL-1A	Fnu 4HI^{+4845}	-	American	Silicosis	Yucesoy et al, 2001
IL-1B	Ava I^{-511}	+	Hungarian	Ulcerative colitis	Nemetz et al, 1999
IL-1B	Ava I^{-511}	+	Hungarian	Crohn's disease	Nemetz et al, 1999
IL-1B	Ava I^{-511}	+	Japanese	Alcoholic liver disease	Takamatsu et al, 2000
IL-1B	Ava I^{-511}	-	French	Crohn's disease	Heresbach et al, 1997
IL-1B	Ava I^{-511}	-	French	Ulcerative colitis	Heresbach et al, 1997
IL-1B	Ava I^{-511}	-	American	Periodontitis	Gore et al, 1998
IL-1B	Ava I^{-511}	-	British	Single and multivessel coronary artery disease	Francis et al, 1999
IL-1B	Ava I^{-511}	-	Japanese	Chronic obstructive pulmonary disease	Ishii et al, 2000
IL-1B	Taq I^{+3953}	+	Japanese	Alcoholic liver disease	Takamatsu et al, 2000
IL-1B	Taq I^{+3953}	+	American	Periodontitis	Galbraith et al, 1999a
IL-1B	Taq I^{+3953}	+	American	Periodontitis	Gore et al, 1998
IL-1B	Taq I^{+3953}	+	British	Early-onset periodontitis	Parkhill et al, 2000
IL-1B	Taq I^{+3953}	-	Hungarian	Ulcerative colitis	Nemetz et al, 1999
IL-1B	Taq I^{+3953}	-	British	Ulcerative colitis	Mansfield et al, 1994
IL-1B	Taq I^{+3953}	-	French	Ulcerative colitis	Heresbach et al, 1997
IL-1B	Taq I^{+3953}	-	Hungarian	Crohn's disease	Nemetz et al, 1999
IL-1B	Taq I^{+3953}	-	British	Crohn's disease	Mansfield et al, 1994
IL-1B	Taq I^{+3953}	-	French	Crohn's disease	Heresbach et al, 1997
IL-1B	Taq I^{+3953}	-	British	Acute pancreatitis	Powell et al, 2001
IL-1B	Taq I^{+3953}	-	British	Single and multivessel coronary artery disease	Francis et al, 1999
IL-1B	Taq I^{+3953}	-	American	Silicosis	Yucesoy et al, 2001
IL-1RN	VNTR^{86}	+	Dutch	Ulcerative colitis	Bioque et al, 1996
IL-1RN	VNTR^{86}	+	Jewish	Ulcerative colitis	Duerr and Tran, 1995
IL-1RN	VNTR^{86}	+	British	Ulcerative colitis	Mansfield et al, 1994
IL-1RN	VNTR^{86}	+	Jewish	Ulcerative colitis	Tountas et al, 1999
IL-1RN	VNTR^{86}	+	Spanish	Ulcerative colitis (pANCA)	Papo et al, 1999
IL-1RN	VNTR^{86}	+	Japanese	Alcoholic liver disease (hepatic fibrosis)	Takamatsu et al, 1998
IL-1RN	VNTR^{86}	+	American	Vulvar vestibulitis	Jeremias et al, 2000
IL-1RN	VNTR^{86}	+	British	Single coronary artery disease	Francis et al, 1999
IL-1RN	VNTR^{86}	+	S. African	Asthma	Pillay et al, 2000
IL-1RN	VNTR^{86}(A1)-Taq I^{+3953}(A1)	+	British	Early-onset periodontitis	Parkhill et al, 2000
IL-1RN	VNTR^{86}	-	British	Ulcerative colitis	Louis et al, 1996
IL-1RN	VNTR^{86}	-	Spanish	Ulcerative colitis	Garcia-Parades et al, 1996
IL-1RN	VNTR^{86}	-	German	Ulcerative colitis	Hacker et al, 1997
IL-1RN	VNTR^{86}	-	French	Ulcerative colitis	Heresbach et al, 1997
IL-1RN	VNTR^{86}	-	Non-Jewish	Ulcerative colitis	Tountas et al, 1999
IL-1RN	VNTR^{86}	-	British	Crohn's disease	Louis et al, 1996
IL-1RN	VNTR^{86}	-	Spanish	Crohn's disease	Garcia-Paredes et al, 1996
IL-1RN	VNTR^{86}	-	Dutch	Crohn's disease	Bioque et al, 1996
IL-1RN	VNTR^{86}	-	British	Crohn's disease	Mansfield et al, 1994
IL-1RN	VNTR^{86}	-	French	Crohn's disease	Heresbach et al, 1997
IL-1RN	VNTR^{86}	-	Jewish	Crohn's disease	Tountas et al, 1999
IL-1RN	VNTR^{86}	-	Non-Jewish	Crohn's disease	Tountas et al, 1999
IL-1RN	VNTR^{86}	-	British	Acute pancreatitis	Powell et al, 2001
IL-1RN	VNTR^{86}	-	Italian	Ischemic heart disease	Manzoli et al, 1999

IL-1RN	VNTR^{86}	-	Japanese	Polymyositis	Son et al, 2000
IL-1RN	VNTR^{86}	-	Japanese	Dermatomyositis	Son et al, 2000
IL-1RN	VNTR^{86}	-	British	Multivessel coronary artery disease	Francis et al, 1999
IL-1RN	VNTR^{86}	-	Japanese	Chronic obstructive pulmonary disease	Ishii et al, 2000

Table 4.2B: Positive and negative associations between polymorphisms in the IL-1 gene cluster and autoimmune diseases

Gene	Polymorphism	+/-	Population	Disease association	Reference
IL-1RI	Pst I^{131}	+	Danish	IDDM	Pociot et al, 1994
IL-1RI	Pst I^{131}	+	Danish	IDDM	Bergholdt et al, 1995
IL-1RI	Pst I^{131}	+	British	IDDM	Metcalfe et al, 1996
IL-1RI	Pst I^{131}	-	Danish	Nephropathy (IDDM)	Tarnow et al, 1997
IL-1RI	Pst I^{131}	-	Irish	Nephropathy (IDDM)	Loughrey et al, 1998
IL-1A	Nco I^{-889}	+	Norwegian	Juvenile chronic arthritis	McDowell et al, 1995
IL-1A	Nco I^{-889}	+	French	Chronic polyarthritis	Jouvenne et al, 1999
IL-1A	Nco I^{-889}	-	Irish	Nephropathy (IDDM)	Loughrey et al, 1998
IL-1A	Nco I^{-889}	-	British	Juvenile chronic arthritis	Donn et al, 1999
IL-1A	Nco I^{-889}	-	French	Spondylarthropathies	Djouadi et al, 2001
IL-1A	Nco I^{-889}	-	British	Autoimmune thyroid disease	Hunt et al, 2000
IL-1A	Nco I^{-889}	-	Finnish	Multiple sclerosis	Luomala et al, 2001
IL-1A	Fnu $4HI^{+4845}$	+	French	Chronic polyarthritis	Jouvenne et al, 1999
IL-1A	Fnu $4HI^{+4845}$	-	American	Graves' disease	Cuddihy and Bahn, 1996
IL-1A	Fnu $4HI^{+4845}$	-	Italian	Polymyalgia rheumatica	Boiardi et al, 2000
IL-1A	$(AC)n^{7695}$	-	German	Rheumatoid arthritis	Gomolka et al, 1995
IL-1A	$(AC)n^{7695}$	-	British	Juvenile chronic arthritis	Donn et al, 1999
IL-1A	$VNTR^{46}$	-	French	Rheumatoid arthritis	Bailly et al, 1995
IL-1B	Ava I^{-511}	+	British	Rheumatoid arthritis	Di Giovine et al, 1994
IL-1B	Ava I^{-511}	-	British	Multiple sclerosis	Feakes et al, 2000
IL-1B	Ava I^{-511}	-	Finnish	Multiple sclerosis	Luomala et al, 2001
IL-1B	Ava I^{-511}	-	British	Autoimmune thyroid disease	Hunt et al, 2000
IL-1B	Ava I^{-511}	-	French	Rheumatoid arthritis	Cantagrel et al, 1999
IL-1B	Ava I^{-511}	-	Italian	Polymyalgia rheumatica	Boiardi et al, 2000
IL-1B	Taq I^{+3953}	+	Irish	Nephropathy (IDDM)	Loughrey et al, 1998
IL-1B	Taq I^{+3953} (with KM)	+	American	Alopecia areata	Galbraith et al, 1999b
IL-1B	Taq I^{+3953}	+	American	Multiple sclerosis	Kantarci et al, 2000
IL-1B	Taq I^{+3953}	+	Swedish	Myasthenia gravis	Huang et al, 1998
IL-1B	Taq I^{+3953}	+	French	Rheumatoid arthritis	Cantagrel et al, 1999
IL-1B	Taq I^{+3953}	+	British	Primary biliary cirrhosis	Donaldson et al, 2001
IL-1B	Taq I^{+3953}	-	Italian	Polymyalgia rheumatica	Boiardi et al, 2000
IL-1B	Taq I^{+3953}	-	French	Spondylarthropathies	Djouadi et al, 2001
IL-1B	Taq I^{+3953}	-	Dutch	Multiple sclerosis	Schrijver et al, 1999
IL-1B	Taq I^{+3953}	-	Finnish	Multiple sclerosis	Luomala et al, 2001
IL-1B	Taq I^{+3953}	-	Swedish	Wegeners granulomatosis	Huang et al, 2000
IL-1B	Taq I^{+3953}	-	British	Autoimmune thyroid disease	Hunt et al, 2000
IL-1B	Taq I^{+3953}	-	Danish	Nephropathy (IDDM)	Tarnow et al, 1997
IL-1B	Taq I^{+3953}	-	British/American	Type I autoimmune hepatitis	Cookson et al, 1999
IL-1B	Taq I^{+3953}	-	British	Primary sclerosing cholangitits	Donaldson et al, 2000
IL-1RN	$VNTR^{86}$	+	African American	Nephropathy (IDDM)	Freedman et al, 1997
IL-1RN	$VNTR^{86}$	+	British	Nephropathy (IDDM)	Blakemore et al, 1996
IL-1RN	$VNTR^{86}$	+	Chinese	IgA nephropathy (recurrent gross hematuria)	Liu et al, 1997
IL-1RN	$VNTR^{86}$	+	Chinese	IgA nephropathy	Shu et al, 2000
IL-1RN	$VNTR^{86}$	+	Chinese	Henoch-Schonlein purpura nephritis	Liu et al, 1997
IL-1RN	$VNTR^{86}$	+	British	Graves' disease	Blakemore et al, 1995
IL-1RN	$VNTR^{86}$	+	British	Alopecia areata	Tarlow et al, 1994
IL-1RN	$VNTR^{86}$	+	British/German	Psoriasis (early onset)	Tarlow et al, 1997

IL-1RN	VNTR^{86}	+	British	Lichen sclerosus	Clay et al, 1994
IL-1RN	VNTR^{86}	+	British	SLE	Blakemore et al, 1994
IL-1RN	VNTR^{86} (with HLA-DR17,DQ2)	+	Swedish	SLE	Tjernstrom et al, 1999
IL-1RN	VNTR^{86}	+	French	Sjogren's syndrome	Perrier et al, 1998
IL-1RN	VNTR^{86}	+	Italian	Polymyalgia rheumatica	Boiardi et al, 2000
IL-1RN	VNTR^{86}	+	Dutch	Multiple sclerosis	Crusius et al, 1995
IL-1RN	VNTR^{86}	+	American	Multiple sclerosis	Kantarci et al, 2000
IL-1RN	VNTR^{86}	+	Italian	Multiple sclerosis	Sciacca et al, 1999
IL-1RN	VNTR^{86}(with HLA-DRB1*1501)	+	Spanish	Multiple sclerosis (R/R)	De la concha et al, 1997
IL-1RN	VNTR^{86}	+	American	Juvenile idiopathic inflammatory myopathies	Rider et al, 2000
IL-1RN	VNTR^{86}	+	British	Primary biliary cirrhosis	Donaldson et al, 2001
IL-1RN	VNTR^{86}	-	African American	Non-diabetic ESRD	Freedman et al, 1997
IL-1RN	VNTR^{86}	-	Danish	Nephropathy (IDDM)	Tarnow et al, 1997
IL-1RN	VNTR^{86}	-	Irish	Nephropathy (IDDM)	Loughrey et al, 1998
IL-1RN	VNTR^{86}	-	Australian	SLE	Danis et al, 1995
IL-1RN	VNTR^{86}	-	Swedish	Multiple sclerosis	Huang et al, 1996
IL-1RN	VNTR^{86}	-	French	Multiple sclerosis	Semana et al, 1997
IL-1RN	VNTR^{86}	-	Dutch	Multiple sclerosis	Schrijver et al, 1999
IL-1RN	VNTR^{86}	-	British	Multiple sclerosis	Feakes et al, 2000
IL-1RN	VNTR^{86}	-	Finnish	Multiple sclerosis	Luomala et al, 2001
IL-1RN	VNTR^{86}	-	Swedish	Myasthenia gravis	Huang et al, 1998
IL-1RN	VNTR^{86}	-	British/American	Type I autoimmune hepatitis	Cookson et al, 1999
IL-1RN	VNTR^{86}	-	British	Primary sclerosing cholangitis	Donaldson et al, 2000
IL-1RN	VNTR^{86}	-	British	Corneal melting (systemic vasculitis)	McKibbin et al, 2000
IL-1RN	VNTR^{86}	-	French	Rheumatoid arthritis	Cantagrel et al, 1999
IL-1RN	VNTR^{86}	-	French	Rheumatoid arthritis	Perrier et al, 1998
IL-1RN	VNTR^{86}	-	French	Spondylarthropathies	Djouadi et al, 2001
IL-1RN	VNTR^{86}	-	American	Graves' disease	Cuddihy and Bahn, 1996
IL-1RN	VNTR^{86}	-	German	Graves' disease	Muhlberg et al, 1998
IL-1RN	MspA1I^{11100}	-	British	Autoimmune thyroid disease	Hunt et al, 2000

Table 4.2C: Positive and negative associations between polymorphisms in the IL-1 gene cluster and infectious, metabolic, malignant and neurological diseases

Gene	Polymorphism	+/-	Population	Disease association	Reference
				INFECTIOUS	
IL-1A	(AC)n	-	African	Tuberculosis	Bellamy et al, 1998
IL-1B	Ava I^{-511}	+	Finnish	EBV	Hurme and Helminen, 1998a
IL-1B	Ava I^{-511}	+	British	Meningococcal disease	Read et al, 2000
IL-1B	Ava I^{-511}	-	African	Tuberculosis	Wilkinson et al, 1999
IL-1B	Ava I^{-511}	-	African	Tuberculosis	Bellamy et al, 1998
IL-1B	Taq I^{+3953} $A1^{+}$ - $VNTR^{86}$ $A2^{-}$	+	African	Tuberculosis (Tuberculous pleurisy)	Wilkinson et al, 1999
IL-1B	Taq I^{+3953}	-	German	Severe sepsis	Fang et al, 1999
IL-1RN	$VNTR^{86}$	+	German	Severe sepsis	Fang et al, 1999
IL-1RN	$VNTR^{86}$	+	African	Tuberculosis	Bellamy et al, 1998
IL-1RN	$VNTR^{86}$	+	African	Tuberculosis (Manteux response)	Wilkinson et al, 1999
IL-1RN	$VNTR^{86}$	+	Finnish	EBV	Hurme and Helminen, 1998a
IL-1RN	$VNTR^{86}$	+	Finnish	CMV	Hurme and Helminen, 1998b
IL-1RN	$VNTR^{86}$	+	Brazilian	Ureaplasma urealyticum vaginal colonisation	Jeremias et al, 1999
IL-1RN	$VNTR^{86}$	-	African	Severe malaria	Bellamy et al, 1998
				METABOLIC	
IL-1B	Ava I^{-511}	-	Danish	Osteoporotic fractures	Langdahl et al, 2000
IL-1B	Aci I^{3887}	-	Danish	Osteoporotic fractures	Langdahl et al, 2000
IL-1B	Taq I^{+3953}	+	German	Osteoarthritis	Moos et al, 2000
IL-1B	Taq I^{+3953}	-	Danish	Osteoporotic fractures	Langdahl et al, 2000
IL-1RN	$VNTR^{86}$	+	British	Postmenopausal bone loss	Keen et al, 1998
IL-1RN	$VNTR^{86}$	+	Danish	Osteoporotic fractures	Langdahl et al, 2000
IL-1RN	$VNTR^{86}$	+	German	Osteoarthritis	Moos et al, 2000
IL-1RN	$VNTR^{86}$	-	Hungarian	Bone mineral density	Bajnok et al, 2000
IL-1RN	$MspA1I^{11100}$	-	Danish	Osteoporotic fractures	Langdahl et al, 2000
				MALIGNANT	
IL-1B	Ava I^{-511}	+	British/Polish	Gastric cancer	El-Omar et al, 2000
IL-1B	Alu I^{-31}	+	British/Polish	Gastric cancer	El-Omar et al, 2000
IL-1B	Taq I^{+3953}	+	British/Polish	Gastric cancer (protective)	El-Omar et al, 2000
IL-1B	Taq I^{+3953}	+	British	Pancreatic cancer	Barber et al, 2000
IL-1B	Taq I^{+3953}	-	Swedish	Multiple myeloma	Zheng et al, 2000
IL-1RN	$VNTR^{86}$	+	British/Polish	Gastric cancer	El-Omar et al, 2000
IL-1RN	$VNTR^{86}$	-	German	Hematopoietic malignancies	Demeter et al, 1996
IL-1RN	$VNTR^{86}$	-	Swedish	Multiple myeloma	Zheng et al, 2000
IL-1RN	$VNTR^{86}$	-	British	Secondary acute myeloid leukemia	Langabeer and Linch, 1998
				NEUROLOGICAL	
IL-1A	Nco I^{-889}	+	British/American	Alzheimer's disease	Nicoll et al, 2000
IL-1A	Nco I^{-889}	+	American	Alzheimer's disease	Du et al, 2000
IL-1A	Nco I^{-889}	+	Italian	Alzheimer's disease	Grimaldi et al, 2000
IL-1A	Nco I^{-889}	-	Japanese	Temporal lobe epilepsy	Kanemoto et al, 2000
IL-1B	Ava I^{-511}	+	Italian	Alzheimer's disease	Grimaldi et al, 2000
IL-1B	Ava I^{-511}	+	Japanese	Temporal lobe epilepsy	Kanemoto et al, 2000
IL-1B	Ava I^{-511}	-	Japanese	Schizophrenia	Tatsumi et al, 1997
IL-1B	Taq I^{+3953}	+	British/American	Alzheimer's disease	Nicoll et al, 2000
IL-1B	Taq I^{+3953}	-	French	Schizophrenia	Laurent et al, 1997
IL-1B	Taq I^{+3953}	-	Japanese	Temporal lobe epilepsy	Kanemoto et al, 2000
IL-1RN	$VNTR^{86}$	+	Italian	Alzheimer's disease	Grimaldi et al, 2000
IL-1RN	$VNTR^{86}$	-	Japanese	Temporal lobe epilepsy	Kanemoto et al, 2000
IL-1B-IL-1A-IL-1RN	AvaI(A1)-NcoI(A2)-$VNTR^{86}$(A1)	+	Finnish	Schizophrenia	Katila et al, 1999

4.7.2 Differential Regulation

Another explanation as to why there may be an imbalance in the IL-1ra/IL-1 system is that gene expression and synthesis of IL-1 and IL-1ra are differently regulated within the same cell (Poutsiaka et al, 1991; Arend et al, 1991). Some stimuli such as LPS, IL-1 and TNFα induce the transcription of both IL-1 and IL-1ra, while others induce the transcription of one but not the other. Stimuli inducing IL-1ra but not IL-1 include immune complexes and adherent IgG, while those that induce IL-1 but not IL-1ra include the complement component C5a and *Borrelia burgdorferi*. Furthermore, cytokines such as IL-4, IL-10, IL-13 and TGFβ increase the production of IL-1ra but simultaneously decrease the production of IL-1 (Dinarello, 1996). In addition IL-1 and IL-1ra have different expression kinetics. IL-1 is produced before IL-1ra both *in vivo* and *in vitro* peaking at 1 hour compared to 2 hours for IL-1ra *in vivo* (Granowitz et al, 1991b) and at 2 hours compared to 4 hours for IL-1ra *in vitro* (Vannier et al, 1992).

The IL-1 gene cluster has been implicated in many diseases as discussed above and shown in Tables 4.2 A-C and its role in acute pancreatitis will now be reviewed.

4.8 The Interleukin-1 gene cluster and acute pancreatitis

Despite several reports of elevated serum levels of IL-1, IL-6 and TNFα in experimental animal models (Norman et al 1994; Grewal et al 1994a), until recently there had been no reports of plasma IL-1β in patients with acute pancreatitis. Serum IL-1β levels are known to increase 1 hour after the initiation of experimental pancreatitis and it has been suggested that its absence in the serum of acute pancreatitis patients may be due to the fact that IL-1β levels have already peaked and declined before the patients have been admitted to hospital. IL-1β is however an inducer of other cytokines such as IL-6, and IL-8 which peak later and are therefore easier to measure in the serum of patients on admission. Chen et al, have recently measured serum IL-1β levels from 50 patients on days 1-7 following admission and found that they were significantly higher in patients with severe

compared to mild disease with a median peak occurring on day 1 (Chen et al, 1999). McKay et al, had failed to show this difference in IL-1 secretion in an *in vitro* model which involved the LPS stimulation of isolated monocytes taken from 26 patients with moderate to severe disease (McKay et al, 1996). More recently, 2 studies have shown that there is an imbalance between IL-1ra and IL-1 beta levels in patients with severe acute pancreatitis (Mayer et al, 2000; Powell et al, 2001).

Studies have demonstrated that IL-1 and TNFα are initially produced in the pancreas parenchyma within 30 minutes of induction of experimental pancreatitis and are produced later in distant organs in particular the lungs, liver and spleen (Norman et al, 1997b). The subsequent rise in inflammatory cytokines in these organs is linked to specific organ disorders. Elevations of IL-1 and TNFα in the lungs for example are associated with the development of ARDS (Parsons et al, 1992). The production of IL-1 and TNFα in the pancreas is due to the infiltrating macrophages and neutrophils and experiments that involve the depletion of specific leukocyte populations have demonstrated that macrophages are primarily responsible for production of IL-1 and TNFα during experimental acute pancreatitis (Fink and Norman, 1996).

There is evidence to suggest that although IL-1β and TNFα are produced in the pancreas during acute pancreatitis they do not cause the disease. Firstly, isolated human pancreas perfused with IL-1 and TNFα does not induce acute pancreatitis (Denham et al, 1998b) and secondly, the treatment of isolated acinar cells with IL-1 and TNFα does not cause colocalisation of zymogen granules and lysosomes or the activation of digestive enzymes (Fink et al, 1997).

The role of IL-1 in acute pancreatitis has also been demonstrated by experiments involving IL-1 antagonism. These include recombinant IL-1ra administration, IL-1 receptor gene knockout mice and inactivation of ICE. IL-1ra administered prophylactically or therapeutically to bile-induced rat model (Tanaka et al, 1995), cerulein-induced rat model (Norman et al, 1995a) and CDE-induced mouse model (Norman et al, 1995b) have

all shown a reduction in pancreatic damage, lung injury and mortality. Interestingly, therapeutic treatment proved to be more beneficial than prophylactic treatment (Norman et al, 1995a; Norman et al, 1995b). IL-1ra does not appear to act directly on acinar cells since adminstration does not directly reduce disease severity. Rather the effect appears to be due to an indirect mechanism that interrupts interactions between acini and leucocytes possibly by the downregulation of TNFα and IL-6 (Fink et al, 1997). In addition to recombinant IL-1ra, another cytokine suppressive agent (FR167653) has recently shown to improve the survival of rats with closed duodenal loop-induced acute pancreatitis (Hirano, 1999). Although the experimental studies show that IL-1ra could have potential therapeutic value in acute pancreatitis, clinical studies have been disappointing. Phase III trials on 893 patients with sepsis, a disease which shares many clinical features with acute pancreatitis have shown that treatment with recombinant human IL-1ra has no clinical benefits compared to placebo (Fisher et al, 1994).

IL-1 receptor gene 'knockout' mice are deficient in IL-1 receptor and cannot recognise or respond to IL-1. When cerulein is administered intraperitoneally to these mice, acute pancreatitis still occurs, however, it is less severe than in control mice. This indicates that IL-1 does not cause acute pancreatitis but is necessary for the progression and severity (Norman et al, 1996b). Furthermore, knockout mice deficient in TNF type I receptor, IL-1 type I receptor or both showed that an identical beneficial effect on severity and mortality was obtained with blocking either receptor but no additional beneficial effect was achieved by blocking both receptors together. This suggests that that IL-1β and TNFα make an equivalent contribution to the severity of acute pancreatitis (Denham et al, 1997b). As previously mentioned in Section 4.2.2, the Interleukin-1β converting enzyme (ICE) cleaves IL-1β into its active form before it leaves the cell. Any method of inhibiting or eliminating this enzyme will therefore prevent the secretion of this cytokine. Mice pretreated with VE-13045 (an ICE inhibitor) prior to induction of acute pancreatitis by bile injection and transgenic mice with a disrupted ICE gene fed a CDE-diet showed decreased

severity and mortality compared to controls. In these experiments there was no alteration in IL-1mRNA levels, however, IL-1 and TNFα protein levels were significantly reduced (Norman et al, 1997c).

4.9. Summary

It has been suggested that acute pancreatitis may have a genetic component since the natural history and aetiology of the disease are diverse and may be related to impaired host defense. The majority of patients develop only mild disease with minimum organ dysfunction and without complications, however, there is a significant proportion who develop severe disease with both local and systemic complications. Furthermore, individuals with gallstone disease or alcoholism do not inevitably develop acute pancreatitis. Only 3.4% of patients with gallstones (Moreau et al, 1988) and 5% of alcoholics develop the disease (Meier, 1995).

The interleukin-1 gene cluster has been implicated in the pathogenesis of acute pancreatitis. Serum IL-1 levels have been found to be elevated in both humans (Chen et al, 1999) and in experimental animals models with the disease (Norman et al, 1994). Furthermore, studies that reduce the level of IL-1 by preventing its production (e.g the ICE inhibitor, VE-13045) (Norman et al, 1997c), manipulating IL-1 receptor expression (e.g. IL-1 receptor gene "knockout" mice) (Norman et al, 1996b) or preventing the binding of IL-1 to its receptor (e.g. recombinant IL-1ra) (Tanaka et al, 1995; Norman et al, 1995a) have been shown to decrease the severity and mortality of acute pancreatitis and further implicate the cytokine in the disease. More recently an IL-1ra: IL-1 imbalance has been reported in patients with severe acute pancreatitis (Mayer et al, 2000; Powell et al, 2001). In conclusion, there is compelling evidence to suggest that the IL-1 gene cluster may contribute to the susceptibility to and/or severity of acute pancreatitis, and this may be determined by polymorphisms within the genes.

4.10 Aims of the study

The aims of this study were to:

1) investigate polymorphisms of the genes coding for the IL-1 gene cluster (IL-1A, IL-1B, IL-1RN) and IL-1 receptors (IL-1RI and IL-1RII) in patients with acute pancreatitis and normal healthy controls
2) determine any functional relationship between the IL-1 gene cluster polymorphisms and protein levels.

CHAPTER 5: MATERIALS AND METHODS

5.1 Subjects

The characteristics of the subjects used in the study are shown in Table 5.1.

5.1.1 Controls

Two hundred and seventeen Caucasian cord bloods obtained following healthy obstetric delivery at Derriford Hospital in Plymouth were used as healthy controls for the DNA study. In addition, ten healthy Caucasian adults were used as controls for the protein study. These controls were comprised of 4 males and 6 females with an age range from 24-48 years and a median age of 27 years. The controls were sex and ethnically matched with the acute pancreatitis patients.

5.1.2 Patients

The patient cohort consisted of one hundred and thirty seven patients with acute pancreatitis who were admitted to Derriford Hospital in Plymouth. This group comprised 66 males and 71 females with an age range from 21-86 years and a median age of 58 years. The criteria for diagnosis of acute pancreatitis were:

1) A clinical presentation consistent with the disease (Table 5.2)
2) Radiological evidence (Table 5.3)
3) Hyperamylasaemia >660units/l (Hitachi 911, Hitachi Corporation, Japan; normal level <220units)

Table 5.1: Characteristics of the controls and patients used in the genetic study

	Normal Controls n =217*	AP Patients n=137*	Mild AP n=99	Severe AP n=38	OFS=0 n=89	OFS≥1 n=48	OFS≥2 n=35	OFS≥3 n=19	OFS≥4 n=12	A AP n=21	I AP n=36	G AP n=80
Age Range (years)	N/A	21-86	21-86	26-78	21-81	26-86	26-84	26-78	26-76	28-62	25-84	21-86
Median Age (years)	N/A	58	56	65.5	54	64	66	66	65.5	43	60.5	60
Sex (M:F)	98:119	66:71	49:50	17:21	44:45	22:26	17:18	11:8	7:5	20:1	20:16	26:54

AP Acute pancreatitis
n represents the number of subjects in each group
M male
F female
Mild and **severe** disease severity groups are according to the Atlanta convention classification
OFS organ failure scores according to Kingsnorth et al, 1995
A Alcoholic pancreatitis - if daily consumption of ≥80g alcohol per day for 6 months
G Gallstone pancreatitis - if radiological or ERCP evidence of cholelithiasis
I Idiopathic pancreatitis - if no identifiable aetiology
N/A Age not applicable to controls as taken from a bank of cord bloods
* Although the controls and patients used in the different studies were taken from the same populations, the numbers differ in each study because results were not obtained for all subjects.

Table 5.2 Clinical presentation of acute pancreatitis

CLINICAL PRESENTATION
Symptoms
Abdominal pain +/- radiating through to the back
Nausea
Vomiting
Signs
Fever (38.5°C)
Dehydration
Confusion (secondary to hypoxia)
Abdominal tenderness
Hypovolemic shock
Jaundice (10-20%)
Grey-Turner/Cullen sign (<5%)

Table 5.3: Radiological evidence of acute pancreatitis

RADIOLOGICAL EVIDENCE
Plain X rays:
Gallstones
Intestinal Ileus
Absence of pancreatic calcification (chronic pancreatitis)
Absence of free sub-diaphragmatic air (perforated viscus)
Ultrasound Scan:
Pancreatic edema
Free intraperitoneal fluid
Fluid collection (pseudocyst, abscess)
CT Scan:
Pancreatic edema
Free intraperitoneal fluid
Fluid collection (pseudocyst, abscess)
Area of necrosis

CT= computerised tomography

The patient population was subdivided into groups according to severity, organ failure scores and aetiology.

5.1.2.1 Classification according to severity

Patients were classified as having mild disease (n=99) or severe disease (n=38) according to criteria defined by the Atlanta convention classification on the basis of physiological, biochemical and hematological measurements (Table 5.4).

5.1.2.2 Classification according to organ failure scores

Patients were classified according to the organ failure scores OFS=0 (n=89), OFS≥1 (n=48), OFS≥2 (n=35), OFS≥3 (n=19) and OFS≥4 (n=12) as described by Kingsnorth et al, 1995 (Table 5.5).

5.1.2.3 Classification according to aetiology

Patients were considered to have alcoholic pancreatitis if their daily admitted consumption was greater than 80g alcohol per day for a period of time exceeding 6 months (Wilson et al, 1985)(n=21); gallstone pancreatitis if they had radiological or ERCP evidence of cholelithiasis or gallstones (n=79); or idiopathic if no cause could be identified (n=37) (Table 5.6). Patients with chronic pancreatitis were excluded from the study and CT or ERCP was used to ensure that a case of alcoholic acute pancreatitis was not chronic pancreatitis.

Table 5.4 Classification of acute pancreatitis according to severity

CATEGORY	CRITERIA
Mild acute pancreatitis	Minimal organ dysfunction
Severe acute pancreatitis	Organ failure and /or local complications
Predicted severe disease	APACHE II Score ≥ 8
Organ Failure:	
Cardiovascular	Systolic blood pressure <90mmHg
DIC	Platelets ≤100,000 per mm^3 Fibrinogen <1g/l
Gastrointestinal	500ml of blood loss in 24 hours
Metabolic	Serum calcium ≤1.87mmol/l.
Renal	Serum Creatinine >177umol/l (after rehydration)
Respiratory	pO_2<60mmHg (8Kpa)
Local Complications:	
Acute fluid collection	Lacks a wall of granulation or fibrous tissue; Located in or near the pancreas
Pseudocyst	Collection of pancreatic secretion bound by fibrous or granulation tissue wall
Abscess	Circumscribed collections of pus
Necrosis	Diffuse or focal nonviable pancreatic parenchyma

(Adapted from Bradley, 1993).

DIC Disseminated Intravascular Coagulopathy
mmHg millimetres of mercury
Kpa Kilopascals

Table 5.5 Classification of acute pancreatitis according to organ failure scores

SYSTEM	0 Normal	1 Abnormal	2 Mild	3 Moderate	4 Severe
			← Clinically significant →		
Pulmonary					
PaO_2/FiO_2 (mmHg)	≥400	≥300 to <400	≥200 to <300 Acute lung injury	≥100 to <200 ARDS	<100 Severe ARDS
Renal					
Creatinine (mg/100ml)	≤1.5	>1.5 to ≤2.0	>2.0 to≥3.6	>3.6 to≤5.0	>5.0
Creatinine (umol/l)	≤133	>133 to≤169	>169 to≤310	>310 to≤440	>440
Cardiovascular					
SBP (mmHg)	≥90	<90	<90		
Lactate (mmol/l)		Responsive to fluid	Unresponsive to fluid	2-5	>5
CNS					
GCS	15	13-14	10-12	6-9	≤5
Coagulation					
Platelets ($x10^9$/l)	≥120	≥80 to <120	≥50 to <80	≥20 to <50	<20

Organ failure score was the sum of the score obtained in each system
PaO_2= arterial partial pressure of oxygen;
FiO_2 Fraction of inspired oxygen;
SBP= systemic blood pressure;
GCS= Glasgow Coma Score;
ARDS= Acute respiratory distress syndrome

(Adapted from Kingsnorth et al, 1995)

Table 5.6 Classification of acute pancreatitis according to aetiology

AETIOLOGY	CRITERIA
Alcohol	≥80g alcohol per day for 6 months
Gallstone	Radiological or ERCP evidence of gallstone or choliethiasis
Idiopathic	No identifiable aetiology

ERCP Endoscopic Retrograde Cholangiopancreatography

5.2 Collecting Samples

Following Local Ethical Committee approval, peripheral venous blood samples were collected by venepuncture from all patients. Blood for DNA extraction was collected in 5% disodium ethylene diamine tetra-acetic acid (Na_2EDTA) and stored at -20°C in sterile polypropylene Falcon tubes until use. Blood for cell culture was collected in sodium heparin tubes and was used immediately for protein analysis.

5.3 Materials

5.3.1 Water

All general purpose, specialized and stock solutions were reconstituted using distilled tap water obtained from a Millipore water dispenser (Millipore Ltd, Watford, UK). Sterile bottled water (Baxter Healthcare, Thetford, UK) was used for PCR and restriction digestion reactions.

5.3.2 Reagents

All reagents used were analytical grade or molecular biology grade or equivalent.

5.3.2.1 General purpose reagents

Acetic acid, hydrochloric acid, magnesium chloride 6 hydrate, orthoboric acid, sodium chloride and sodium hydroxide were purchased from BDH Laboratory Supplies-Merck Limited (Lutterworth, UK). Chloroform, ethanol, industrial methylated spirit and methanol were supplied by Rathburn Limited (Walkerburn, UK). Ammonium persulfate, disodium ethylene diamine tetra-acetic acid (Na_2-EDTA), ethidium bromide (2,7-diamino-10-ethyl-9-phenyl-pheanthridinium), formamide, glycerol, sucrose, Tris (hydroxymethyl) aminomethane, Triton X-100, trypan blue and xylene cyanol were obtained from Sigma Chemicals (Poole, UK) and orange G from Fischer Scientific (Loughborough, UK).

5.3.2.2 Specialised reagents, enzymes and materials

"Expand™" high-fidelity PCR system, molecular weight markers, multi-purpose agarose, restriction endonucleases and restriction endonuclease buffers were purchased from Roche Diagnostics (Germany). Deoxynucleoside 5'-triphosphates (dNTPs), radioactivity (γ^{32P}), stop solution, and T4 polynucleotide kinase (T4 PNK) were supplied by Amersham Pharmacia Biotech (Sweden). "Super Taq" polymerase and 10 X "Super Taq" buffer were provided by HT Biotechnology (Cambridge, UK). The oligonucleotide amplimers were obtained from MWG Biotechnology (Germany). Repelcote (2% solution of dimethyldichlorosilane in octamethylcyclotetrasiloxane), Sequagel® concentrate, diluent and buffer were provided by Flowgen Instruments Ltd (Lichfield, UK). "Lymphoprep™", phosphate buffered saline (PBS), RPMI 1640 without L-glutamine, penicillin/streptomycin and foetal calf serum were supplied by Gibco GRL (Paisley, UK). Temed, L-glutamine and Lipopolysaccharide 0127:B8 were supplied by Sigma (Poole, UK) and "Quick-Precip™" was purchased from Advanced Biotech Corps (USA). DNA extraction kits and IL-1A, IL-1B and IL-1ra ELISA kits were purchased from Scotlab (Coatbridge, UK) and R and D Systems Europe (Abingdon, UK) respectively.

5.3.3 Stock solutions

Tris/borate electrophoresis buffer (TBE)

10 X: 0.89mM Tris(hydroxymethyl)aminomethane base, 0.89M orthoboric acid

2mM EDTA (pH 8.0)

Ethidium bromide

10mg ml^{-1} in H_2O

Orange G loading buffer

0.25% orange G, 10% v/v glycerol in 10 X TBE

Xylene cyanol loading buffer

0.25% w/v xylene cyanol, 10% v/v glycerol in 10 X TBE

Reagent A (Nucleon DNA Extraction kit)

10mM Tris HCl, 320mM sucrose, 5mM $MgCl_2$, 1% Triton X-100, Adjusted to pH 8.0 using 40% NaOH

5.3.4 Autoclaving

All solutions, glassware and plastics used in the techniques of DNA and protein analysis were autoclaved at a temperature of 121°C and pressure of 15psi for 30 minutes in a PriorClave autoclave (PriorClave Ltd, Woolwich, UK).

5.4 Extraction of High Molecular Weight DNA

High molecular weight DNA was extracted from whole blood using a Nucleon® BACC (blood and cell culture) genomic DNA extraction kit (Scotlab, Coatbridge, UK) which consisted of Reagents A and B, sodium perchlorate, and Nucleon® resin. 10-20mls of peripheral venous blood were collected by venepuncture from all subjects in 5% ethylenediaminetetracetic acid (EDTA) vacutainer tubes (Becton Dickinson, Oxford, UK) and stored at -20°C in 50ml sterile polypropylene Falcon tubes (Phillip Harris Scientific, Cardiff, UK) prior to subsequent DNA extraction. The stored blood was thawed at room temperature and 10mls aliquoted into fresh 50ml Falcon tubes. Four times the amount by volume of Reagent A (10mM Tris-HCl, 320mM sucrose, 5mM $MgCl_2$, 1% Triton X-100, pH to 8.0) was then added to the blood and the mixture was shaken for 4 minutes using a Luckham R100/TW Rotatest shaker. Next the contents of the 50ml Falcon tube were centrifuged in a MSE Mistral 1000 centrifuge (MSE Scientific Instruments, Leicester, UK) at 1300g for 4 minutes, after which time the supernatant containing lysed red cells was discarded leaving a pellet containing PBMCs. 2 mls of Reagent B (400mM Tris HCl, pH to 8.0, 60mM EDTA, 150mM NaCl, 1% SDS) were then added to the pellet which was resuspended by vortexing briefly. Following this, the pellet suspension was incubated in a 37°C waterbath (Grant, Cambridge, UK) for 10-15 minutes enabling nuclear membrane

disruption to occur, after which time, it was transferred to a 15ml sterile polypropylene Falcon tube (Phillip Harris Scientific, Cardiff, UK). Next 500µl of 5M sodium perchlorate (Scotlab, Coatbridge, UK) were added to the 15ml Falcon tube and the tube was inverted 10-15 times. Then 2mls of chloroform (-20°C)(Rathburn Ltd, Walkerburn, UK) were added to the 15ml Falcon tube to emulsify the 2 phases which was inverted and centrifuged at 1300g for 3 minutes. Next 200µl of Nucleon® silica resin were added to the 15ml Falcon tube without disturbing the phases and the contents of the Falcon tube were centrifuged again at 1300g for 3 minutes. The resulting upper aqueous phase containing the DNA was transferred to a clean 15ml Falcon tube ensuring the interphase with silica resin and underlying organic phase were not disturbed. The aqueous phase was then centrifuged for 1 minute at 1300g to pellet any residual silica and after centrifugation, the supernatant was transferred to a clean 15ml Falcon tube and twice the amount by volume of 100% ethanol (-20°C) (Rathburn Ltd, Walkerburn, UK) was added to it to precipitate out the DNA. The 15ml Falcon tube was inverted 10 times and the resulting DNA 'strand' was hooked out using a sterilized glass rod. Finally, the DNA 'strand' was washed in 70% ethanol, dissolved in 500µl sterile bottled water and kept in the fridge at 2-8°C for 24 hours (Figure 5.1).

5.5 Quantification of High Molecular Weight DNA

The extracted DNA was diluted 1:5 using sterile, bottled water to give 100-500ng of DNA per microlitre of dilute solution. The concentration of DNA was confirmed on a sample of specimens using a Cecil 5500 spectrophotometer (Cecil Instruments, Cambridge, UK).

5.6 Detection of Polymorphisms

All polymorphisms studied in this thesis were analyzed using PCR followed by analysis of the PCR product by gel electrophoresis (Figure 5.2).

Figure 5.1: Summary of the technique for DNA Extraction

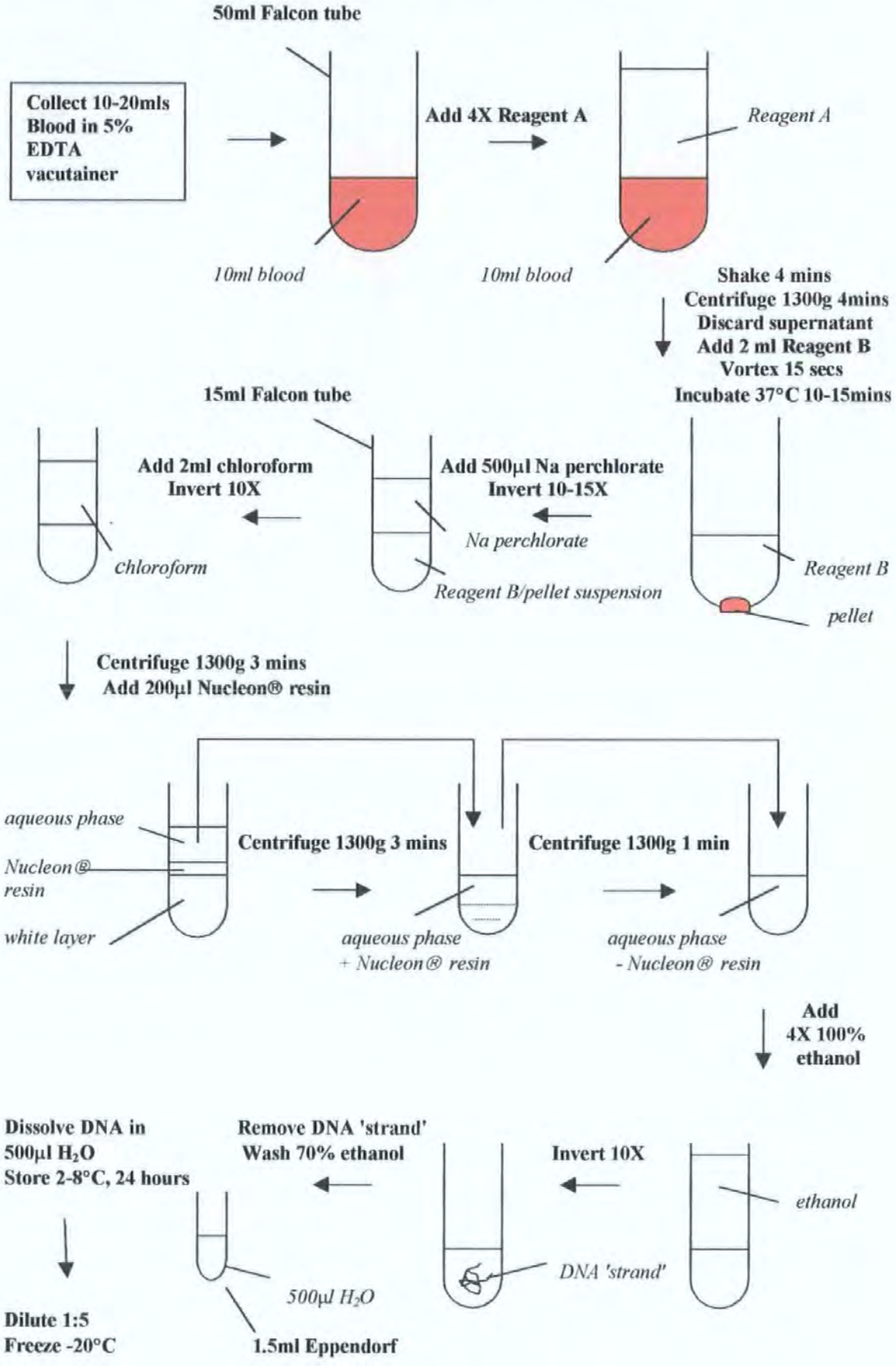
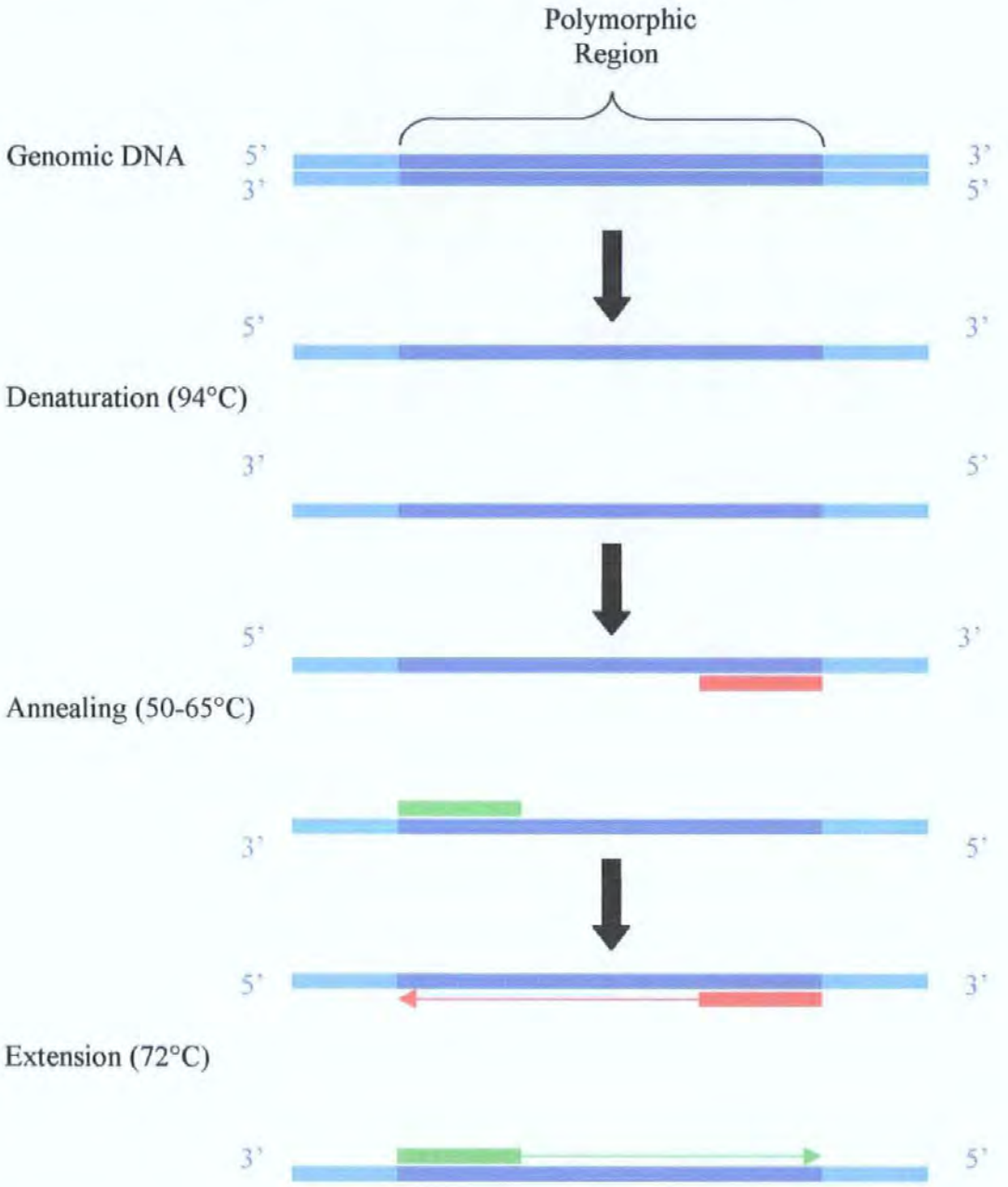


Figure 5.2: The Polymerase Chain Reaction (PCR) Process



PCR is a process during which the polymorphic region of a strand of DNA is amplified (replicated). It involves the use of amplimers that are designed to flank the polymorphic region and which in conjunction with Taq DNA polymerase enzyme, synthesize new DNA from dNTPs on the opposite strands of DNA. The process consists of repetitive cycles comprising denaturing, annealing and extension steps that take place in a thermal cycler. A typical reaction consists of 30 repetitions. The PCR product is then separated using gel electrophoresis.

(Adapted from Connor, 1995)

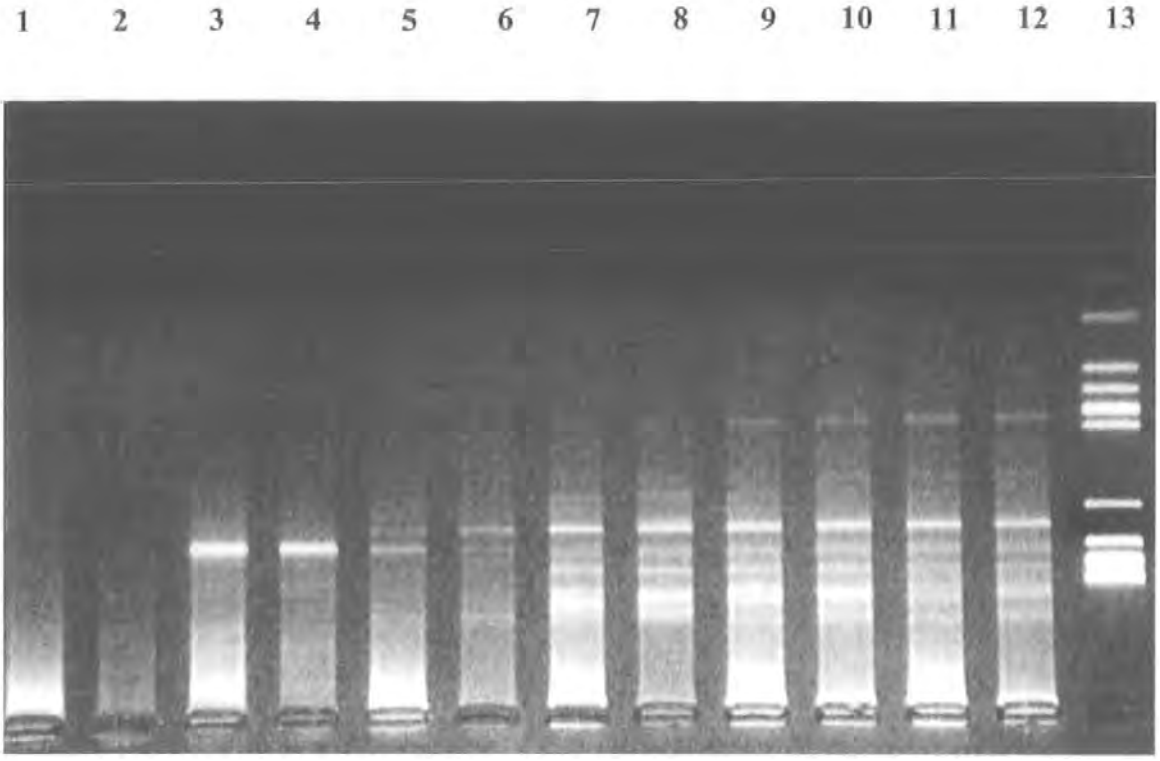
5.6.1 Amplimer Design and Production

In each study, the appropriate gene sequence was obtained from Genbank using the website address: http://www.ncbi.nlm.nih.gov/entrez/nucleotide. Amplimers were then designed for both strands of DNA. The forward amplimer was complementary to the DNA template, whereas the reverse amplimer was both complementary and reversed. All amplimers were designed so that they were approximately 24 bases long, with a GC content of at least 50%. Care was taken to avoid poly G, C, A and T sequences since these may result in primer dimers where the amplimers bind to each other rather than to the DNA template or hairpin loops which occur when the primers bind to themselves. In addition, dinucleotide repeat regions within the amplimer sequence were avoided since these may be polymorphic. All amplimers were manufactured at a scale of 0.01μmol and supplied in lyophilized form by MWG Biotechnology (Germany). The lyophilized amplimers were re-suspended initially in 1ml and then diluted to 10pmol/μl using sterile water (Baxter Healthcare, Thetford, UK).

5.6.2 PCR Optimisation

Following dilution to 10pmol/μl, the optimum conditions for each set of amplimers were determined. This was achieved using different magnesium chloride titrations (1.5-6.5mM) at different annealing temperatures (50°C-65°C). Figure 5.3 shows the effect of increasing magnesium chloride concentrations on 2 different genomic DNA samples A and B. The reaction volume was 30μl and PCR conditions consisted of 30 cycles of 94°C for 30 seconds, 62°C for 2 minutes and 72°C for 2 minutes.

Figure 5.3: Effect of increasing magnesium chloride concentration on PCR amplification.



PCR reactions were performed using various magnesium chloride concentrations (1.5-6.5mM) to demonstrate the effect on the amplification of an 860bp fragment. Each lane contains 10µl of PCR product.

Lane 1 - Subject A - 1.5mM $MgCl_2$
Lane 2 - Subject B - 1.5mM $MgCl_2$
Lane 3 - Subject A - 2.5mM $MgCl_2$
Lane 4 - Subject B - 2.5mM $MgCl_2$
Lane 5 - Subject A - 3.5mM $MgCl_2$
Lane 6 - Subject B - 3.5mM $MgCl_2$
Lane 7 - Subject A - 4.5mM $MgCl_2$
Lane 8 - Subject B - 4.5mM $MgCl_2$
Lane 9 - Subject A - 5.5mM $MgCl_2$
Lane 10 - Subject B - 5.5mM $MgCl_2$
Lane 11 - Subject A - 6.5mM $MgCl_2$
Lane 12 - Subject B - 6.5mM $MgCl_2$
Lane 13 - Molecular weight marker IX (72bp-1353bp)

The optimum magnesium chloride concentration for this reaction was 2.5mM (Lanes 3 and 4) since this concentration produced a single band on the gel, the size of which was determined using the molecular weight marker in lane 13.

5.6.3 PCR Reaction

Each PCR reaction mixture consisted of 100-500ng of genomic DNA, 10pmol of each amplimer pair, 2µl 10X "Super Taq" buffer (50mM Tris-HCl, pH 9, 1.5mM $MgCl_2$, 250mM KCl, 1% Triton X-100, 0.1% (w/v) gelatin), 1-2mM $MgCl_2$, 0.125mM of each dNTP and 0.8 units of Taq DNA polymerase. All PCR reactions were carried out in 30µl volumes in 0.2ml thin-walled microtubes (Advanced Biotechnology, Epsom, UK) in a Cyclogene thermocycler (Techne Ltd, Cambridge, UK). The samples were then stored at 4°C until analysis by gel electrophoresis.

5.7 Single Nucleotide Polymorphism Analysis

5.7.1 Restriction endonuclease digestion

Six nucleotide polymorphisms were analyzed using restriction endonucleases in this study. The amplimers used in each assay are shown in Table 5.7. The polymorphic region of DNA was amplified using the polymerase chain reaction and the PCR products were checked prior to digestion by running a 5µl sample with 1.5µl orange G loading buffer on a 1% agarose gel for 30 minutes hour at 200 volts. Following this, 5-10µg of DNA was digested using 10 units of the appropriate restriction endonuclease (Roche Diagnostics, Germany), and 3µl of endonuclease buffer (Roche Diagnostics, Germany). The reactions were carried out in 30µl volume in 0.5ml tubes. The contents of the tube were then centrifuged briefly in a Biofuge 13 microfuge (Heraeus Sepatech, Germany) and then incubated in a 37°C or 65°C waterbath (Grant, Cambridge, UK) for 1 hour, 3 hours or overnight depending on the endonuclease (Table 5.7). The digestion products were analyzed by agarose gel electrophoresis on a 3% gel run at 100 volts for 1 hour.

Table 5.7: Amplimer sequences, PCR conditions and digestion conditions used in the single nucleotide polymorphism studies

Restriction endonuclease	Gene	Amplimer Sequence	PCR Conditions*	Digestion Conditions
Pst I C↓TGCAG	IL-1RI	*Pst I*: 5'TTG GAG GAT GGC CCA TGA AGA CC3' *Pst II*: 5'CTG TTA CGC GCC CGG ATG AAA AA3'	Hot start: 94°C, 4 mins Denaturation: 94°C, 30 secs Annealing: 59°C, 2 mins Extension: 72°C, 2 mins	37°C, 1 hour
Ava I C↓T/CCGA/GG	IL-1B	*Ava I*: 5'GGC AGA GCT CAT CTG GCA TTG ATC3' *Ava II*: 5'GTA TCT GCC AGT TTC TCC CTC GC3'	Hot start: 94°C, 4 mins Denaturation: 94°C, 30 secs Annealing: 59°C, 2 mins Extension: 72°C, 2 mins	37°C, overnight
Alu I AG↓CT	IL-1B	*Alu I*: 5'GTG AAA TCA GGT ATT CAA CAG AG3' *Alu II*: 5'CTC CCT TAG CAC CTA GTT GTA AGG3'	Hot start: 94°C, 4 mins Denaturation: 94°C, 30 secs Annealing: 57°C, 2 mins Extension: 72°C, 2 mins	37°C, 3 hours
Taq I T↓CGA	IL-1B	*Taq I*: 5'GAC CTG AAG CTG GAA CCC ATG TC3' *Taq II*: 5'CGC TCC AGC ACT CTT GTT TCA GC3'	Hot start: 94°C, 4 min Denaturation: 94°C, 30secs Annealing: 60°C, 2 mins Extension: 72°C, 2 mins	65°C, 3 hours
Msp I C↓CGG	IL-1RN	*Msp I*: 5'CAT GTG GTT AGT GGC CAC CCT ATT G3' *Msp II*:5'CAT ATG TGC CCT TCA GTA GCC AGG C3'	Hot start: 94°C, 4 mins Denaturation: 94°C, 30 secs Annealing: 61°C, 2 mins Extension: 72°C, 2 mins	37°C, 1 hour
Ssp I AAT↓ATT	IL-1RN	*Ssp I*: 5'TTG TGG GGA CCA GGG GAG AT3' *Ssp II*: 5'AGC CTG GCA CTC TGC TGA AT3'	Hot start: 94°C, 4 mins Denaturation: 94°C, 30 secs Annealing: 58°C, 2 mins Extension: 72°C, 2 mins	37°C, 1 hour

*30 cycles of denaturation, annealing and extension. Amplimers supplied by MWG Biotechnology, Germany. Restriction endonucleases supplied by Roche Diagnostics, Germany (↓ cleavage site).

5.7.2 Agarose gel electrophoresis

To prepare a 1% agarose gel, 1g of multi-purpose agarose (Roche Diagnostics, Germany) was added to a 250ml Duran bottle and dissolved in 100ml 0.5 x TBE by boiling. In order to visualize the DNA, 10µl of a 10mg/ml ethidium bromide solution was then added to the gel to make a final concentration of 0.1% w/v. The contents of the Duran bottle were then poured into a 100ml (14 x 11cm) UV light transparent perspex tray sealed at both ends with masking tape (Gibco GRL, Paisley, UK) and two 14 plastic tooth combs were added to form the wells. The gel was allowed to set at room temperature for 30 minutes, after which time the gel was transferred to a horizontal gel electrophoresis system (Gibco GRL, Paisley, UK) which contained 0.5 x TBE buffer. 10µl of PCR products were then added to 1µl of orange G loading buffer (Fischer Scientic, Loughborough, UK) on a multiwell plate. Orange G dye unlike xylene cyanol migrates with the amplimer in front of the PCR fragments and therefore does not obscure the bands when visualized. Next using a Gilson Pipetman (Anachem Ltd, Luton, UK) the PCR product/orange G mixture was added to the wells in the agarose gel and the gel was run at 200 volts for 30 minutes to 1 hour using a E452 power pack (Flowgen Instruments Ltd, Lichfield, UK). Molecular weight markers (Roche Diagnostics, Germany) were added to the end well on each gel so the size of the DNA fragments could be determined. The choice of molecular weight marker depended on the size range of the PCR fragments. The gel was then visualized using a UVP computer software package (Vision Works 3.0) (UVP International, Cambridge, UK) and UVP UV transilluminator (UVP International, Cambridge, UK), and a photograph of the samples was taken using a Sony video graphic printer on UPP-110HA Sony photographic paper (Sony Corporation, Japan). For each 1% increase in the gel thickness, an additional 1g of multi-purpose agarose was added to the 100ml of 0.5 x TBE.

5.7.3 Automated sequencing

A single nucleotide polymorphism which destroyed an *Ava I* site was identified at nucleotide 1423 in the IL-1B DNA sequence by Clark *et al*, 1986 (Genbank accession number: X04500). Automated sequencing carried out on homozygotes for alleles 1 and 2 using a Li-Cor L-4200-L2 automated sequencer (MWG Biotechnology, Germany), confirmed the presence of a C to T substitution which destroyed the *Ava I* polymorphic site. This is identical to the previously described *Ava I* C→T transition polymorphism reported at position -511 in the IL-1B DNA sequence by (Di Giovine *et al*, 1992).

5.8 Dinucleotide repeat, VLP and VNTR Analysis

The polymorphic region of the gene was amplified using the polymerase chain reaction and the PCR products were separated using either agarose or polyacrylamide gel electrophoresis. Amplimers used for the assays are shown in Table 5.8. The forward amplimer used in the (AC)n repeat study was 5' end labelled with γ^{32P} ATP (Amersham Pharmacia Biotech, Sweden) using T4 polynucleotide kinase (T4 PNK).

5.8.1 5'-end labelling of the (AC)n amplimers

50pmol of the forward amplimer to be labelled was added to a tube containing 8-10 units of T4 PNK, which had been reconstituted in 25µl sterile water (Baxter Healthcare, Thetford, UK). 1µl of γ^{32P} ATP (10µCi/µl) (Amersham Pharmacia Biotech, Sweden) and 19µl of sterile water were then added to the tube to make the total volume 50µl. The tube was then centrifuged for 30 seconds in a Biofuge 13 microfuge (Heraeus Sepatech, Germany) before being incubated at 37°C for 30 minutes. Following incubation, 5.5µl of 5M sodium chloride (BDH laboratory supplies-Merck Ltd, Lutterworth, UK), 2µl of "Quick-Precip™" (4°C)(Advanced Biotech Corps, USA) and 165µl of 100% ethanol (-20°C) (Rathburn Ltd, Walkerburn, UK) were added to the tube to precipitate the DNA which was then vortexed to mix the contents.

Table 5.8: Amplimer sequences, PCR conditions and method of gel electrophoresis used in the dinucleotide repeat, VNTR and VLP polymorphism studies

Polymorphism	Gene	Amplimer Sequence	PCR conditions	Gel electrophoresis
(AC)n (2bp)	IL-1A	Forward: γ^{32}P -5' GGT ATT TAC ACC ATA GGT GGG GAC 3' Reverse: 5' GCA CCC ATG TCA AAT TTC ACT GG 3'	Hot start: 94°C, 4 mins Denaturation: 94°C, 30secs Annealing: 56°C, 2 mins Extension: 72°C, 2 mins	Polyacrylamide 6% gel
VNTR (46bp)	IL-1A	Forward: 5'GCC TCT AGT CTC ATA GAA CTT AGT C3' Reverse: 5'GTG AGG TCA GGC CAT TGC ACT G3'	Hot start: 94°C, 4 mins Denaturation: 94°C, 30 secs Annealing: 62°C, 2 mins Extension: 72°C, 2 mins Elongation: 72°C, 45 secs *	Agarose 2%
VLP	IL-1RN	Forward: 5'GTT GCT GGA TAC TTG CAA GGA CCA3' Reverse: 5'CCC TCC ATG GAT TCC CAA GAA CAG3'	Hot start: 94°C, 4 mins Denaturation: 94°C, 30 secs Annealing: 64°C, 2 mins Extension: 72°C, 2 mins	Agarose 1%
VNTR (86bp)	IL-1RN	Forward: 5'CCC TCA GCA ACA CTC CTA TTG AC3' Reverse: 5'TCA TCT TCC TGG TCT GCA GGT AA3'	Hot start: 94°C, 4 mins Denaturation: 94°C, 30 secs Annealing: 60°C, 2 mins Extension: 72°C, 2 mins	Agarose 2%

*Expand™ High Fidelity PCR System (Roche Diagnostics, Germany) was used to amplify the DNA. This method requires an additional elongation step of 72°C, 45secs (1 cycle)

Next the tube was centrifuged again at 13,000 rpm for 3 minutes in the microfuge to pellet out the DNA, after which time the supernatant was discarded. 165µl of 70% ethanol were then added and the contents of the tube were centrifuged again for 30 seconds. Following centrifugation, the supernatant was discarded leaving a pellet. Residual ethanol was allowed to evaporate and the pellet was re-suspended in 50µl of sterile water.

5.8.2 Polyacrylamide gel electrophoresis (PAGE)

A 30cm x 50cm Sequi-Gen™ GT electrophoresis cell (BioRad Laboratories, Hemel Hempstead, UK) consists of front and back plates*, 2 red spacers, 2 black electrodes clamps, a gel caster, a support, a buffer chamber, top and bottom electrode connectors, a syringe with rubber tubing and a 30cm comb. Whilst laying in a horizontal position, the front and back plates of the cell were cleaned with 70% industrial methylated spirit (Rathburn Ltd, Walkerburn, UK). Repelcote (BDH Laboratory supplies-Merck Ltd, Lutterworth, UK) was then applied to the back plate and allowed to dry. Following this, the two red spacers, which had been coated in a thin layer of petroleum jelly to help them adhere, were placed on the sides of the back plate. The front plate of the cell was then placed on the top of the back plate and was held in place using the 2 black electrode clamps which were attached either side of the cell so the 2 plates were flush with each other. Next the gel rig was positioned vertically in the gel caster and was held in place using clips attached to the gel caster. The cell was then positioned horizontally on the bench so the gel could be added. A 6% polyacrylamide gel was then used to analyze the (AC)n repeat polymorphism of the IL-1A gene. This consisted of: 36mls Sequagel® concentrate (237.5g acrylamide, 12.5g methylene bisacrylamide, 500g urea), 15mls Sequagel® buffer (50% urea in 1M Tris borate, 20mM EDTA buffer pH8.3), 99mls Sequagel® diluent (500g 8.3M urea) (National Diagnostics, Atlanta, USA), and 12mls 10% formamide (Sigma Chemicals, Poole, UK).

* The back plate comprises 2 glass plates separated by a well

70µl Temed (Sigma Chemicals, Poole UK) and 1500µl 10% ammonium persulfate were then added to polymerize the gel (4°C) (BDH laboratory supplies-Merck Ltd, Lutterworth, UK). Using the syringe and rubber tubing, the gel mixture was injected into the cell through the injection port in the gel caster between the front and back plates until it had completely covered the surface of the glass plates. Care was taken to avoid air bubbles and buffer leakage, since these may lead to sparking and short-circuiting when the voltage is applied. A 30cm 49-well vinyl sharkstooth comb with a 0.4mm spacer set (BioRad Laboratories, Hemel Hempstead, UK) was then positioned upside down at the top of the cell between the front and back plates to form a straight edge. The gel was allowed to set for 1 hour after which time the syringe and gel caster were removed. The cell was then positioned vertically in the buffer chamber which contained 1 X TBE to a depth of approximately 1 inch and was held in position with the support. 1 X TBE was then added to the cell into the well provided which separates the 2 layers of the back plate and the top and bottom electrode connectors were attached. The gel was pre-heated to 50°C by running it at 2000 volts for 45 minutes using a PC3000 powerpac 3000 power supply (BioRad Laboratories, Hemel Hempstead, UK). The PCR products were then prepared for loading. 6µl of each PCR product was added to 3µl of stop solution containing formamide (98% deionised formamide, 10mM EDTA pH8, 0.025 xylene cyanol, 0.025% bromophenol blue)(Amersham Pharmacia Biotech, Sweden) in a multi-well plate. When the cell had reached its optimum temperature, the comb was inverted so the teeth made wells in the gel, and the PCR product/stop solution mixture were added to each well using a 10µl Drummond sequencing pipette (Drummond Laboratories USA). The gel was then run at 1500-2000 volts for 4 hours maintaining the temperature at 50°C. The temperature was continually monitored using a temperature indicator attached to the front plate of the cell. After 4 hours, the electrodes were disconnected and the 1 X TBE drained out of the well between the 2 layers of the back plate via the drain port. The cell was laid horizontally on the bench and the black electrode clamps were removed. Using a wedge to break the seal

between the front and back plate, the back plate was slowly lifted off the front plate. Since the back plate was covered in Repelcote, the gel remained attached to the front plate. The front plate was then transferred to a tray and was covered with 10% methanol/acetic acid solution (Rathburn Ltd, Walkerburn, UK) to fix the gel. The front plate was then gently agitated for approximately 5 minutes to detach the gel. When it had become totally detached a piece of 3MM filter paper 25cm X 50cm (Phillip Harris Scientific, Cardiff, UK) was laid rough side down on top of the gel. The top left-hand corner of the filter paper had been cut so the gel could be orientated later. Light pressure was applied to the paper to encourage the gel to adhere to the filter paper and then the gel was carefully lifted off the front plate attached to the filter paper. The radioactive region of the gel was then determined using a radioactivity counter and the filter paper was reduced in size to aid drying. The gel attached to the filter paper was then covered with "Saran Wrap™" and dried using a Sue 300 gel dryer (Heto Laboratory Equipment, Germany) at 80°C for 1-2 hour. After the gel was dry, it was transferred to a Cronex photographic cassette between Cronex lighting and intensifying screens. In a dark room it was covered with a 25cm x 50cm piece of Kodak XLS5 X ray film (Scientific Imaging Systems Ltd, Cambridge, UK) and cassette firmly closed. The film was exposed overnight at a temperature of -80°C after which time it was defrosted and developed in Kodak X ray developer (Anachem, Luton, UK) for 2 minutes, then indicator stop bath solution (Anachem, Luton, UK) for 30 seconds and finally liquid fixer (Anachem, Luton, UK) for 2 minutes. The film was then rinsed with tap water and allowed to air dry. Allele scoring was then carried out independently by 2 observers.

5.9 Isolation of peripheral blood mononuclear cells (PBMCs) from whole blood

30mls of peripheral venous blood was collected from all subjects by venepuncture in sodium heparin vacutainer tubes (Becton Dickinson, Oxford, UK) and transferred to two 50ml sterile polypropylene Falcon tubes (Phillip Harris Scientific, Cardiff, UK). The blood

was then diluted 1:1 using Dulbecco's PBS (Gibco GRL, Paisley, UK) which had been warmed to 37°C. Next 7mls of "Lymphoprep™" (Gibco GRL, Paisley, UK) were added to eight different 15ml universal tubes and 7mls of the blood/PBS solution was carefully added to it dropwise using a Pasteur pipette (Richardsons of Leicester, Leicester, UK) being careful not to break the "Lymphoprep™" surface. The contents of the tubes were then centrifuged at 2000rpm for 30 minutes in a MSE Mistral centrifuge, producing a 4-layered mixture, a top layer of serum, followed by a layer of lymphocytes, then a layer of Ficoll and finally a bottom layer of red cells. The top layer of serum was discarded to allow access to the layer of lymphocytes that were removed using a Pasteur pipette and transferred to a clean 15ml universal tube. Four times the amount by volume of PBS was then added to the cells to wash them and they were centrifuged at 1400rpm for 10 minutes after which time the supernatant was removed. The cells were then washed again in 4 times the amount by volume of RPMI 1640 without L-glutamine (Gibco GRL, Paisley, UK) and centrifuged at 1400rpm for 10 minutes. Following centrifugation, the supernatant was removed, and the pellet was re-suspended in 1ml RPMI 1640 supplemented with 5% penicillin/streptomycin (Gibco GRL, Paisley, UK), 10% foetal calf serum (Gibco GRL, Paisley, UK) and 5% L-glutamine (Sigma Chemicals, Poole, UK) by gently sucking it up and down using a Pasteur pipette.

5.9.1 Calculating cell number

In order to standardize the cell culture experiments the number of cells present per ml of cell suspension was determined. 50µl of cell suspension was mixed with 50µl of trypan blue solution (Sigma Chemicals, Poole, UK) in an Eppendorf and a drop of this cell suspension/trypan blue solution was then placed on a Neubauer Haemocytometer. The number of cells present in 4 small squares was then determined on X 40 magnification.

If for example, the number of cells in 4 squares was 360, to calculate the number of cells per 1ml of cell suspension: n x 4 x 2 x 10^4= 10^6

(number of cells in 4 small squares x 4 x 1 in 2 dilution with trypan blue x 10^4)

360 x 4 x 2x 10^4 = 30 X 10^6 cells per ml

The 1ml of cell suspension was then transferred to a clean 50ml Falcon tube and 29ml of culture medium was added to it (1:30 dilution) to adjust the cell concentration to 1x10^6 cells per ml (Figure 5.4).

5.9.2 Culturing peripheral blood mononuclear cells

Optimum cell culture conditions for maximal protein secretion were found to be 1 x 10^6 cells per ml, stimulated with 5μg/ml LPS for 72 hours (Appendix 1). Four time points were used in this experiment to provide a more accurate representation of an individual's secretory profile. 1ml of cell suspension was added to 2 Eppendorfs labelled 0μg/ml LPS time 0 hours and 5μg/ml LPS time 0 hours. 5μg/ml LPS was added to the Eppendorf labelled 5μg/ml LPS, while the cell suspension in the Eppendorf labelled 0μg/ml remained unstimulated. The 2 Eppendorfs were then centrifuged in a Heraeus Sepatech microfuge for 30 seconds to pellet out the cells and the supernatant was transferred to a fresh Eppendorf and stored immediately at –20°C. 4 mls of the cell suspension was then added to each well on a 6-well flat-bottomed tissue culture plate (Fischer Scientific, Loughborough, UK) labelled 0μg/ml LPS (A1) 24 hours, 5μg/ml LPS 24 hours (A2), 0μg/ml LPS 48 hours (B1) 5μg/ml LPS 48 hours (B2), 0μg/ml LPS 72 hours (C1) and 5μg/ml LPS 72 hours (C2) seeding cells at 1 x 10^6 cells per ml. The cells in wells A2-C2 were stimulated with 5μg/ml LPS (*E.coli* 0127:B8) for 24, 48 and 72 hours respectively while cells in the wells A1-C1 remained unstimulated (Figure 5.5). The plates were then placed in a plastic box containing 100mls of sterile water and 4 drops of "Sigma clean™" water bath treatment (N-alkyldimethylbenzylammonium chloride, ethyl alcohol, methyl alcohol) (Sigma Chemicals, Poole, UK) to prevent evaporation from the plates.

Figure 5.4: Summary of the technique for isolating PBMCs

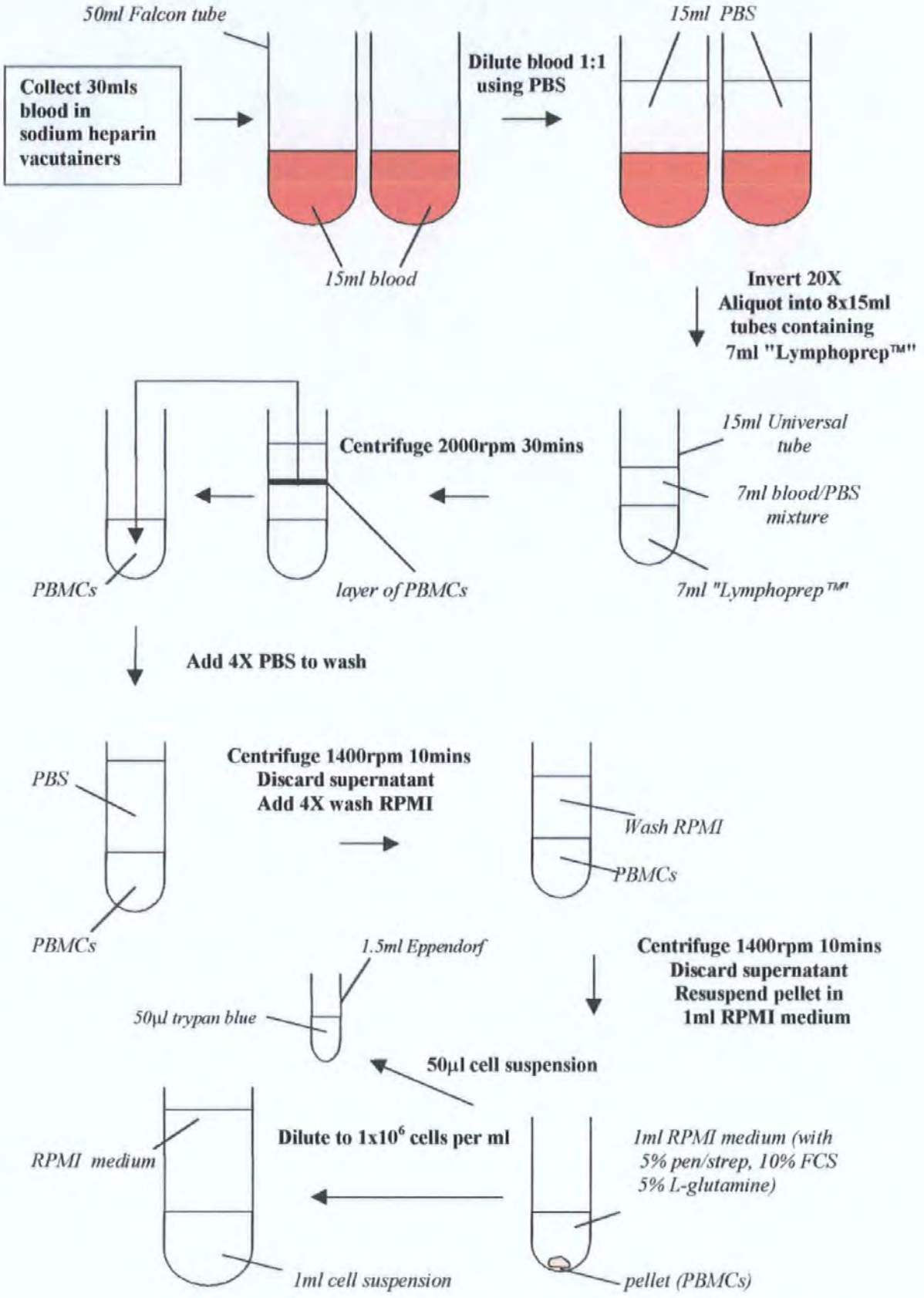
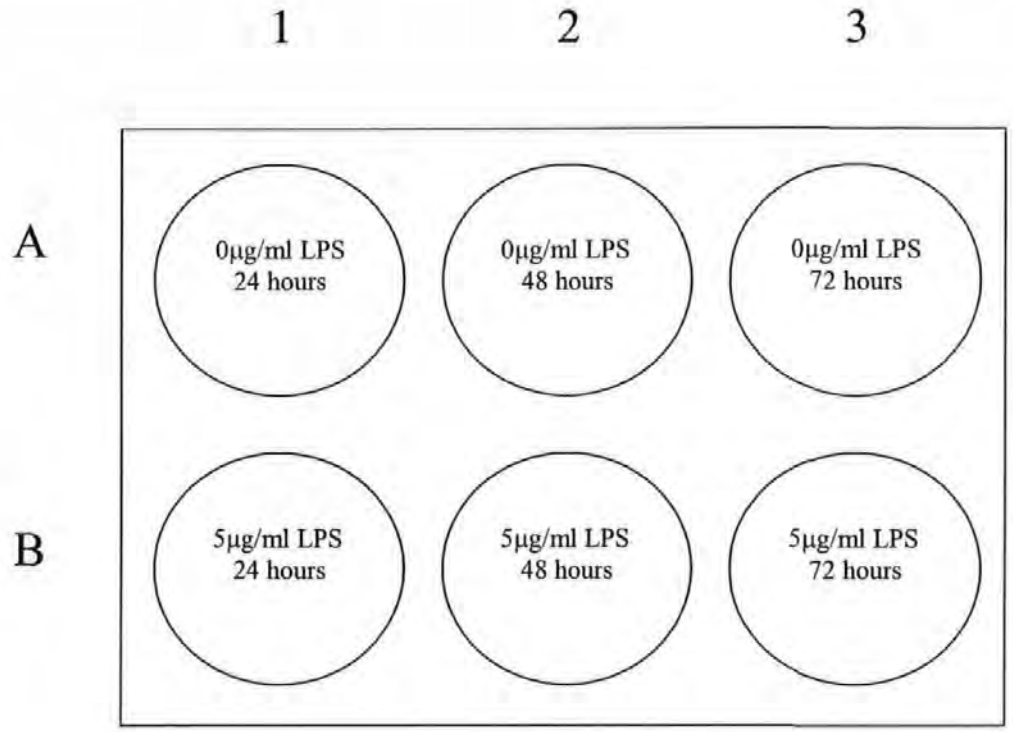


Figure 5.5: Layout of each 6 well flat-bottomed cell culture plate



Each well on the cell culture plate contains 4mls cell suspension at a concentration of $1x10^6$ cells per ml. The cells in row B are stimulated with 5μg/ml LPS *E. coli* 0127:B8, while those in row A remain unstimulated. All cells are then incubated for 24, 48 and 72 hours at 37°C, 5% CO_2.

The lid of the box was perforated to allow CO_2 to reach the cells, but also minimize evaporation. All cells were incubated at 37°C in 5% CO_2 in a Leec incubator (Jencons-PLS, Leighton Buzzard, UK). After 24, 48 and 72 hours, the supernatant from each well was removed, centrifuged to pellet out the cells and the supernatant was transferred to a clean Eppendorf and stored at -20°C for batch cytokine assay. The amount of protein present was determined using a Quantikine® ELISA kit (R & D Systems Europe, Abingdon, UK). Cell viability was confirmed at each time point by adding 20µl of trypan blue to the cells attached to the culture plate and then observing them under a microscope to ensure that the cell membranes were intact.

5.10 Quantification of protein levels

Each Quantikine® ELISA kit consisted of a microplate, conjugate, standard, calibrator diluent, wash buffer concentrate, colour reagent A, colour reagent B, stop solution and plate covers. All reagents were brought to room temperature before use. The optimisation experiment indicated that the stimulated supernatants needed to be diluted 1:10 using RPMI without L-glutamine to ensure the measurements would not exceed the absorbance-protein concentration graph plotted by the microplate reader while the unstimulated supernatants were used 1:1.

5.10.1 Preparation of the standards

To prepare the standards, 3ml of the calibrator diluent RD5 was added to the standard, inverted and allowed to stand for 15 minutes. 500µl of neat calibrator diluent RD5 was then added to 6 Eppendorfs labelled 1000, 500, 250, 125, 62.5 and 31.2pg/ml. Next 500µl of the undiluted standard (2000pg/ml) was added to the Eppendorf labelled 1000pg/ml to give a 1/2 dilution and the contents of the tube were mixed by inversion. 500µl of the 1/2 dilution was then added to the tube labelled 500pg/ml to give a 1/4 dilution. 500µl of the 1/4 dilution was then transferred to the tube labelled 250pg/ml to

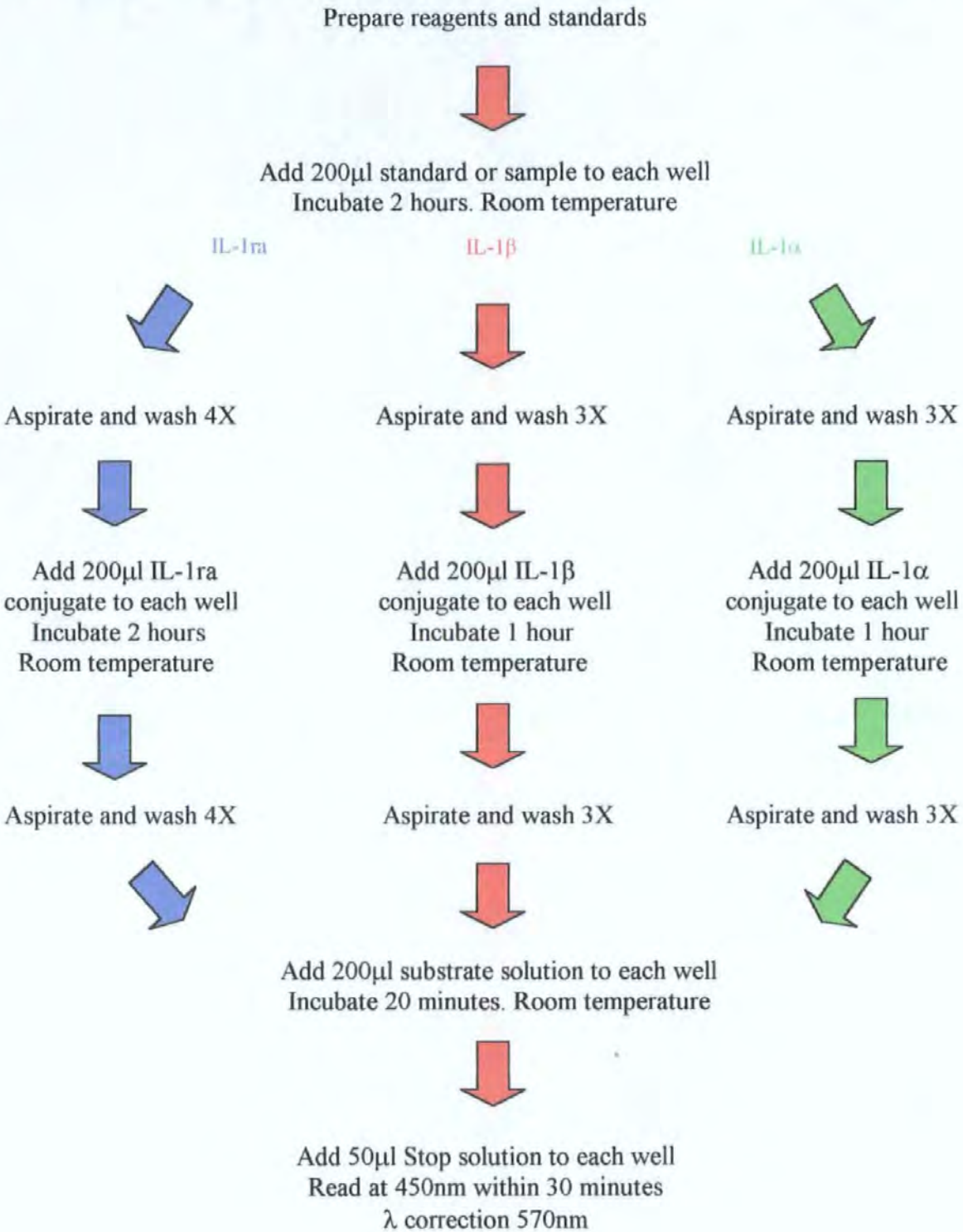
give a 1/8 dilution and so on. The undiluted standard served as the high standard (2000pg/ml) and the calibrator diluent RD5 served as the zero standard (0pg/ml).

5.10.2 Quantifying protein levels

200µl of the 8 standards (2000, 1000, 500, 250, 125, 62.5, 31.2 and 0pg/ml) were added in duplicate to columns 1 and 2 on the ELISA microplate. Next 200µl of neat calibrator diluent RD5 was added to column 3 to act as blanks. 200µl of the samples were then added in duplicate to columns 4-12 on the ELISA plate. The microplate was then covered with the plate cover and the samples were incubated at room temperature for 2 hours. After incubation, the samples were discarded, and the wells were washed with 200µl wash buffer three times*, blotting the plate onto paper after each wash. 200µl of conjugate was then added to each well and the microplate was covered again with a plate cover and incubated for 1 hour* at room temperature. After 1 hour, the conjugate was discarded and the wells were washed 3 times* with 200µl wash buffer blotting onto paper after each wash as before. Next equal volumes of the colour reagents A and B were mixed into a sterile bottle to make up the substrate solution. 200µl of the substrate solution was then added to each well and the microplate was covered with a plate cover and allowed to incubate for 20 minutes at room temperature. After 20 minutes, 50µl of stop solution was added to each well causing a colour change from blue to yellow. The optical density and hence the amount of protein in each well was then determined within 30 minutes using a Dias microplate reader (Dynal, UK) set to 450nm and corrected to 570nm to avoid optical imperfections in the plate (Figure 5.6). To determine the amount of protein in each well, a log-log graph was plotted of absorbance vs protein concentration by the microplate reader.

* 4 times (IL-1ra study); * 2 hours (IL-1ra study)

Figure 5.6: Summary of the Quantikine® ELISA protocols



(Adapted from the Quantikine® Human IL-1ra, IL-1β and IL-1α Immunoassay booklets)

5.11 Analysis of the data

The genotype data obtained for each study was recorded on Microsoft® Excel 7 spreadsheets so statistical analysis could be carried out within the same application or in applications into which Excel spreadsheets could be imported.

5.11.1 Allele and genotype frequencies

The occurrence of each allele was expressed as a percentage of the total number of alleles present in the population to give an allele frequency. Homozygotes represented 2 copies of a given allele whereas heterozygotes represented only 1 copy. The number of individuals carrying a particular genotype was also calculated as a proportion of the total number of genotypes in the population to give a genotype frequency. Comparison of allele and genotype frequencies between controls, patients and patient subgroups were made in 2 x 2 contingency tables using the χ^2 test. The statistics were performed on the *Statcalc* program of the computer software package Epi Info6 (World Health Organisation, Switzerland). P values were then corrected (Pc) for the number of comparisons made using the Bonferroni inequality method (Tiwari and Terasaki, 1985). Statistical significance was obtained when $Pc<0.05$. Correcting p values is necessary to show that the results are more likely to be significant and less likely to be due to chance.

5.11.2 Hardy-Weinberg equilibrium

This is a statistical test that shows whether a polymorphism conforms to normal distribution within a population. For a biallelic polymorphism the expected genotype frequencies are calculated using the equation $1= p^2 + q^2 + 2\ (pq)$ where p and q are the frequencies of the 2 alleles within the population. Hardy-Weinberg equilibrium occurs if the expected frequency does not differ significantly ($p<0.05$) from the observed frequency.

5.11.3 Haplotype analysis and linkage disequilibrium

In addition to analysing the genotypes for each gene, the genotypes of different genes were also combined to give haplotypes and analysed to determine whether the genes were linked and hence influence each other's expression. Individuals were considered for haplotype analysis provided they were not heterozygous at more than 1 locus. Double heterozygotes were omitted from the study since the 2 different haplotypes were possible on each chromosome. This subjective removal of the heterozygotes may introduce a bias if a large number of double heterozygotes are present, however, since the bias is likely to be consistent in both controls and patients the 'error' is not significant. The haplotype data were analysed using the *Epitable* program of the computer software package *EpiInfo6* (World Health Organisation, Switzerland). Linkage disequilibrium occurs when alleles at 2 loci appear more frequently than could be expected by random association. Expected frequency of an allelic association between 2 loci was calculated by multiplying the frequency of 1 of the alleles with the other. Expected and observed frequencies were compared using χ^2 test. Linkage was apparent if $p<0.05$.

5.11.4 Combined genotype analysis

The genotypes of the different genes were also combined for each subgroup of the patient population to determine whether the genotypes were preferentially expressed in a given group. Haplotype analysis could not be used in this case, since the number of subjects in each patient subgroup would become too small following elimination of the double heterozygotes that statistical significance could not be obtained. In this type of analysis double heterozygotes were included. The same analysis was carried out on the control population.

5.11.5 ELISA analysis

The ELISA data were analysed by the Mann-Whitney U test (*Statgraphics Plus2.1)*

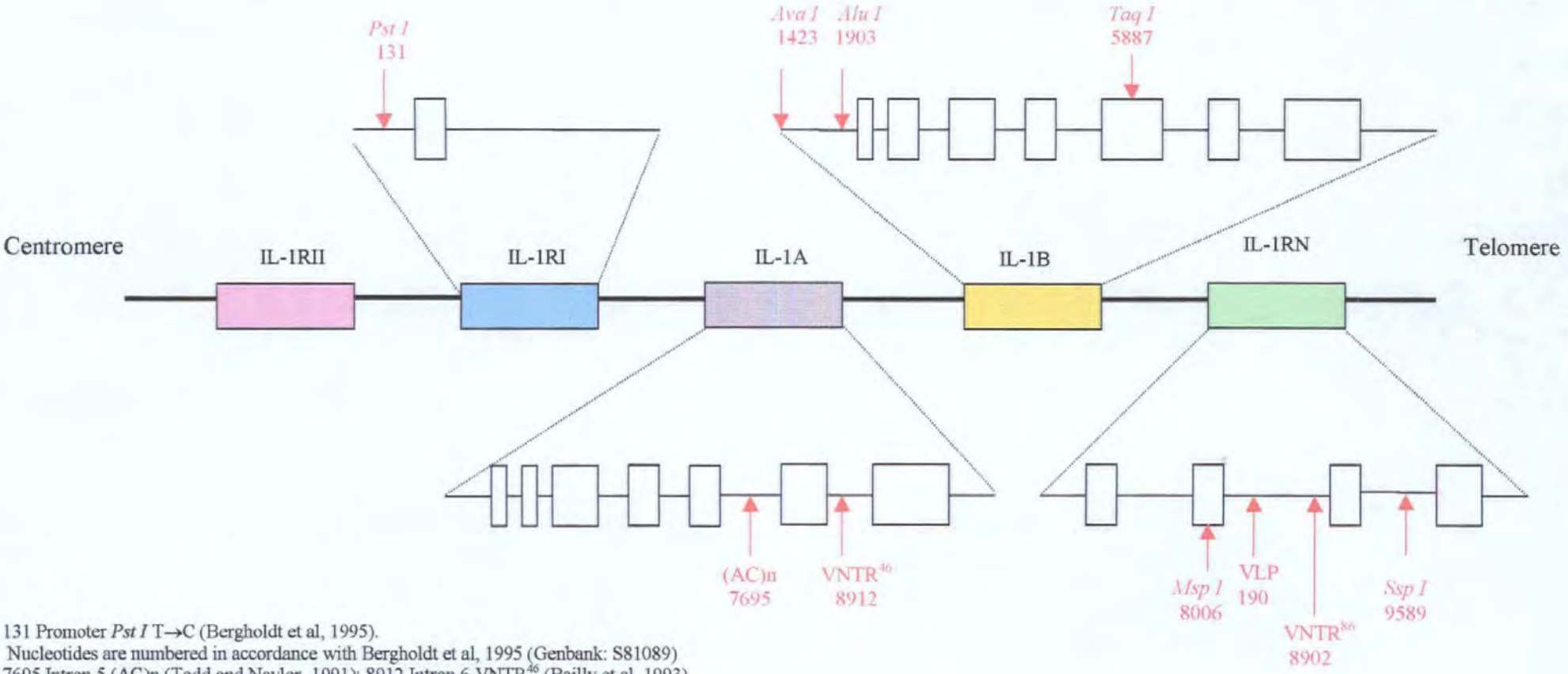
CHAPTER 6: RESULTS

The following results are discussed in the order in which the polymorphisms occur on the long arm of chromosome 2 relative to the centromere, beginning with *Pst I* polymorphism in the IL-1RI gene and ending with the *Ssp I* polymorphism in the IL-1RN gene. For each study, comparisons of the genotype and allele frequencies were made between the:

1) controls and patients
2) controls and subgroups of the patient population (mild, severe, OFS=0, OFS≥1,2,3,4, alcoholic, idiopathic, gallstone)
3) mild group and severe group
4) OFS=0 group and OFS≥1,2,3,4 groups
5) different combinations of the aetiological groups

using the $\chi 2$ test and 2 x 2 contingency tables. All of the significant data are discussed, however, only the data that are significant following correction for the number of comparisons involved are included in the tables. All polymorphisms conformed to the Hardy-Weinberg equilibrium. Figure 6.1 illustrates the interleukin-1 gene cluster and location of the polymorphisms studied in this thesis.

Figure 6.1: Map of the Interleukin-1 gene cluster and Interleukin-1 receptor genes illustrating the positions of polymorphisms investigated in this study



131 Promoter *Pst I* T→C (Bergholdt et al, 1995).
Nucleotides are numbered in accordance with Bergholdt et al, 1995 (Genbank: S81089)
7695 Intron 5 (AC)n (Todd and Naylor, 1991); 8912 Intron 6 VNTR[46] (Bailly et al, 1993)
Nucleotides are numbered in accordance with Furutani et al, 1986 (Genbank: X03833)
1423 5'UTR *Ava I* C→T; 1903 Promoter *Alu I* T→C (Guasch et al, 1996); 5887 Exon 5 *Taq I* C→T (Guasch et al, 1996).
Nucleotides are numbered in accordance with Clark et al, 1986 (Genbank: X04500)
8006 Exon 2 *Msp I* T→C (Guasch et al, 1996); 190 Intron 2 VLP (Steinkasserer et al, 1991); 8902 Intron 2 VNTR[86] (Tarlow et al, 1993); 9589 Intron 3 *Ssp I* A→T (Guasch et al, 1996)
Nucleotides are numbered in accordance with Lennard et al 1992 (Genbank: X64532) except for VLP which is numbered in accordance with Eisenberg et al 1991 (Genbank: M63099)

(Adapted from Nickin et al, 1994 and Dale and Nicklin, 1999)

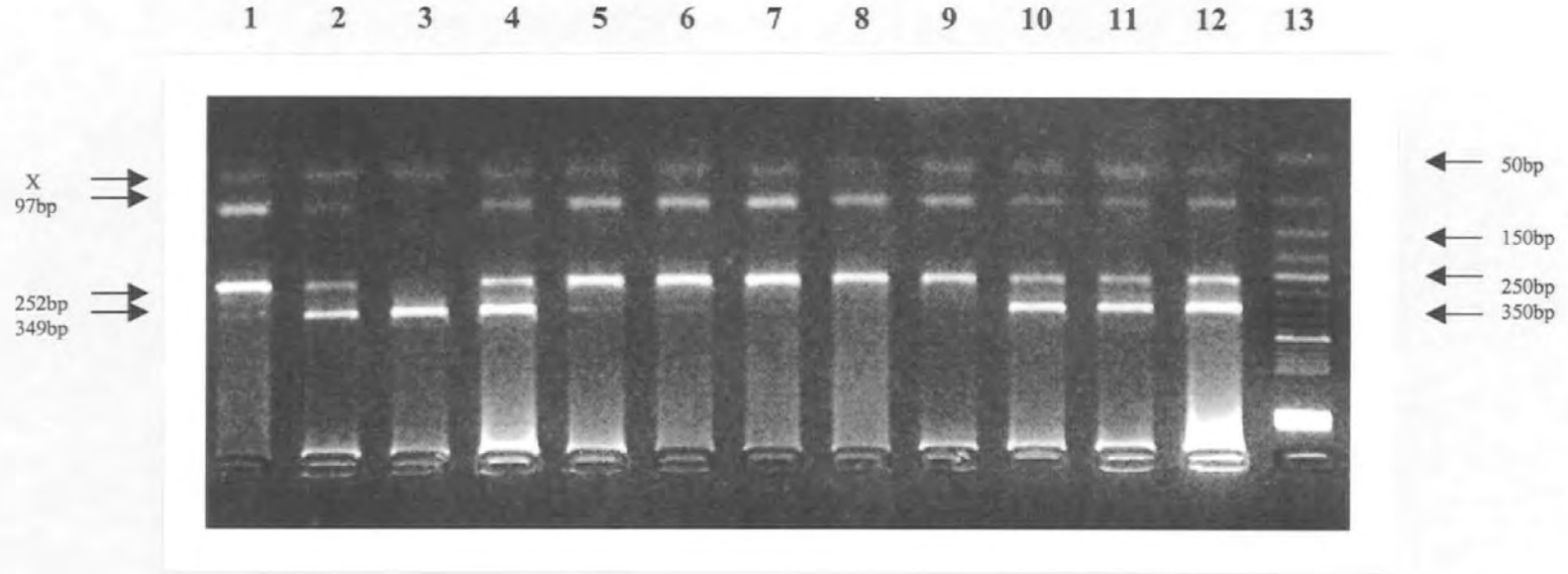
6.1 DNA Studies

6.1.1 IL-1RI

6.1.1.1 Pst I polymorphism

Figure 6.1 illustrates the location of the *Pst I* polymorphism in the IL-1 gene cluster. Digestion of PCR products amplified by *Pst I* and *II* amplimers with *Pst I* generated fragments of 349bp and 97bp, 252bp for alleles 1 and 2. These gave rise to three possible genotypes: 1,1; 1,2; 2,2. The $T \rightarrow C^{[131]}$ SNP creates a *Pst I* restriction site. Homozygotes for allele 1 (349bp) occur when the SNP is not present on either chromosome. Homozygotes for allele 2 (97bp, 252bp) occur when the SNP is present on both chromosomes. Heterozygotes (97bp, 252bp, 349bp) occur when the SNP is present on one chromosome only (Figure 6.2).

Figure 6.2: 3% agarose gel illustrating the genotypes detected at the *Pst I* locus



Two alleles were detected: allele 1 (349bp), allele 2 (97bp,252bp). Band X is an artefact.
Lanes 1,5,6,7,8 and 9 - 2,2
Lanes 2,4,10,11 and 12 - 1,2
Lane 3 - 1,1
Lane 13 - Molecular weight marker XIII (50bp ladder)

Table 6.1A: Frequency of IL-1RI *Pst I* genotypes in controls, patients and patient subgroups

Genotype	Controls %	Patients %	Mild AP %	Severe AP %	OFS=0 %	OFS≥1 %	OFS≥2 %	OFS≥3 %	OFS≥4 %	A AP %	I AP %	G AP %
1 , 1	**7.1**	**3.0**	**3.1**	**2.7**	**4.5**	**0.0**	**0.0**	**0.0**	**0.0**	**4.8**	**2.9**	**2.6**
	(12)	(4)	(3)	(1)	(4)	(0)	(0)	(0)	(0)	(1)	(1)	(2)
1 , 2	**43.2**	**36.6**	**38.1**	**32.4**	**37.5**	**34.8**	**36.4**	**36.8**	**33.3**	**33.3**	**45.7**	**33.3**
	(73)	(49)	(37)	(12)	(33)	(16)	(12)	(7)	(4)	(7)	(16)	(26)
2 , 2	**49.7**	**60.4**	**58.8**	**64.9**	**58.0**	**65.2**	**63.6**	**63.2**	**66.7**	**61.9**	**51.4**	**64.1**
	(84)	(81)	(57)	(24)	(51)	(30)	(21)	(12)	(8)	(13)	(18)	(50)
Total (n)	**169**	**134**	**97**	**37**	**88**	**46**	**33**	**19**	**12**	**21**	**35**	**78**

This table shows the frequency of the 3 genotypes of the *Pst I* polymorphism in the IL-1RI gene in controls and patient subgroups. n represents the number of genotypes in each group. The number in brackets is the actual number of subjects with a particular genotype. Comparisons were made between the control and patient group, control and patient subgroups, mild and severe groups, OFS=0 and OFS≥1,2,3,4 groups and different patient aetiologies for each genotype using the $\chi 2$ test and 2 x 2 contingency tables. No significant differences were found.

Table 6.1B: Frequency of IL-1RI *Pst I* alleles in controls, patients and patient subgroups

Allele	Controls %	Patients %	Mild AP %	Severe AP %	OFS=0 %	OFS≥1 %	OFS≥2 %	OFS≥3 %	OFS≥4 %	A AP %	I AP %	G AP %
1	28.7	21.3[a]	22.2	18.9	23.3	17.4[b]	18.2	18.4	16.7	21.4	25.7	19.2[c]
	(97)	(57)	(43)	(14)	(41)	(16)	(12)	(7)	(4)	(9)	(18)	(30)
2	71.3	78.7[a]	77.8	81.1	76.7	82.6[b]	81.8	81.6	83.3	78.6	74.3	80.8[c]
	(241)	(211)	(151)	(60)	(135)	(76)	(54)	(31)	(20)	(33)	(52)	(126)
Total (n)	338	268	194	74	176	92	66	38	24	42	70	156

This table shows the frequency of the 2 alleles of the *Pst I* polymorphism in the IL-1RI gene in controls and patient subgroups. n represents the number of alleles in each group. The number in brackets is the actual number of subjects with a particular allele. Comparisons were made between the control and patient group, control and patient subgroups, mild and severe groups, OFS=0 and OFS≥1,2,3,4 groups and different patient aetiologies for each allele using the $\chi 2$ test and 2 x 2 contingency tables.

[a] = patients vs controls (Allele 1:21.3% vs 28.7%, $\chi 2$=4.35, p=0.037 (1df), Pc=0.037, Allele 2:78.7% vs 71.3%,$\chi 2$=4.35, p=0.037 (1df), Pc=0.037)

[b]= OFS≥1 vs controls (Allele 1: 17.4% vs 28.7%, $\chi 2$=4.77, p=0.029 (1df), Pc=0.029; Allele 2: 82.6% vs 71.3%, $\chi 2$=4.77, p=0.029 (1df), Pc=0.029)

[c] = gallstones vs controls (Allele 1: 19.2% vs 28.7%, $\chi 2$=5.01, p=0.025 (1df), Pc=0.025; Allele 2: 80.8% vs 71.3% $\chi 2$=5.01, p=0.025 (1df), Pc=0.025)

Summary of the results obtained from the *Pst I* locus

Genotype frequency

Controls vs patients/ controls vs patient subgroups/ mild group vs severe group, OFS=0 group vs OFS≥1,2,3,4 groups/ different combinations of the aetiological groups

There were no significant differences in the *Pst I* genotype frequencies between the controls, patients or patient subgroups.

Allele frequency

Controls vs patients

Comparisons of the *Pst I* allele frequencies between the control group and patient group showed that allele 1 was significantly decreased in patients compared to controls (21.3% vs 28.7%, $\chi 2$=4.35 p=0.037, Pc=0.037), whereas allele 2 was significantly increased between the two groups (78.7% vs 71.3%, $\chi 2$= 4.35, p=0.037, Pc=0.037).

Controls vs patient subgroups

When the patient group was subdivided according to severity, organ failure scores and aetiology, and comparisons made between the different subgroups and controls, allele 1 was found to be significantly decreased in the OFS≥1 group compared to controls (17.4% vs 28.7%, $\chi 2$=4.77, p=0.029, Pc=0.029), whereas allele 2 was significantly increased between the same two groups (82.6% vs 71.3%, $\chi 2$=4.77, p=0.029, Pc=0.029). In addition, allele 1 was significantly decreased in the gallstone group compared to controls (19.2% vs 28.7%, $\chi 2$=5.01, p=0.025, Pc=0.025), while allele 2 was significantly increased between the two groups (80.8% vs 71.3%, $\chi 2$=5.01, p=0.025, Pc=0.025).

Mild group vs severe group, OFS=0 group vs OFS≥1,2,3,4 groups, different combinations of the aetiological groups

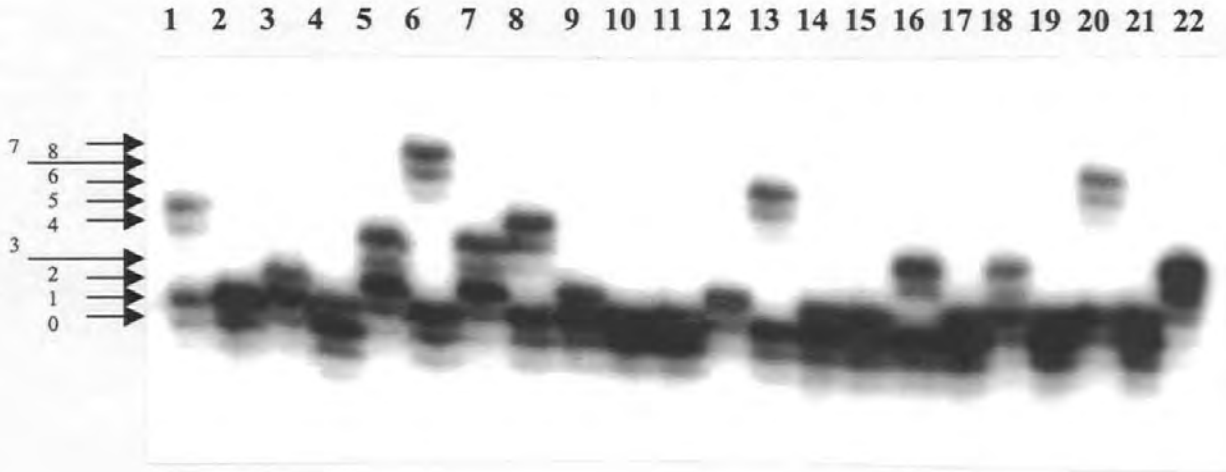
No significant differences in the *Pst I* allele frequencies were found when comparisons were made between the mild and severe groups, between the OFS=0 group and OFS≥1,2,3,4 groups or between different combinations of the aetiological groups.

6.1.2 IL-1A:

6.1.2.1 (AC)n repeat polymorphism

Figure 6.1 illustrates the location of the (AC)n repeat polymorphism in the IL-1 gene cluster. Nine different alleles (alleles 0-8) were detected each corresponding to a different number of copies of the AC dinucleotide repeat. Twenty two genotypes were observed (Figure 6.3).

Figure 6.3: 6% polyacrylamide gel illustrating the genotypes observed at the (AC)n repeat locus.



Nine alleles were detected: alleles 0-8. Allele 1 was the most common allele.
Lanes 1 and 8 - 1,5
Lanes 2,10 and 11 - 1,1
Lanes 3,9,14,15,17,19 and 21 - 1,2
Lane 4 - 0,1
Lanes 5,7 and 18 - 2,4
Lane 6 - 1,8
Lane 12 - 2,2
Lane 13 - 1,6
Lane 16 - 1,4
Lane 20 - 2,7
Lane 22 - 3,4

Table 6.2A: Frequency of IL-1A (AC)n repeat genotypes in controls, patients and patient subgroups

G/T	Controls %	Patients %	Mild AP %	Severe AP %	OFS=0 %	OFS≥1 %	OFS≥2 %	OFS≥3 %	OFS≥4 %	A AP %	I AP %	G AP %
0 , 1	**4.0**	**1.5**	**1.0**	**2.6**	**2.2**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**2.8**	**1.3**
	(6)	(2)	(1)	(1)	(2)	(0)	(0)	(0)	(0)	(0)	(1)	(1)
0 , 2	**0.7**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
	(1)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
1 , 1	**12.0**	**12.4**	**11.1**	**15.8**	**14.6**	**8.3**	**11.4**	**21.1**	**25.0**	**14.3**	**5.6**	**15.0**
	(18)	(17)	(11)	(6)	(13)	(4)	(4)	(4)	(3)	(3)	(2)	(12)
1 , 2	**23.3**	**28.5**	**25.3**	**36.8**	**21.3**	**41.7**	**45.7**[ac]	**42.1**	**41.7**	**19.0**	**30.6**	**30.0**
	(35)	(39)	(25)	(14)	(19)	(20)	(16)	(8)	(5)	(4)	(11)	(24)
1 , 3	**2.0**	**0.7**	**1.0**	**0.0**	**1.1**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**1.3**
	(3)	(1)	(1)	(0)	(1)	(0)	(0)	(0)	(0)	(0)	(0)	(1)
1 , 4	**14.7**	**13.1**	**16.2**	**5.3**	**15.7**	**8.3**	**5.7**	**5.3**	**8.3**	**9.5**	**13.9**	**13.8**
	(22)	(18)	(16)	(2)	(14)	(4)	(2)	(1)	(1)	(2)	(5)	(11)
1 , 5	**2.7**	**3.6**	**3.0**	**5.3**	**1.1**	**8.3**	**8.6**	**10.5**	**8.3**	**4.8**	**2.8**	**3.8**
	(4)	(5)	(3)	(2)	(1)	(4)	(3)	(2)	(1)	(1)	(1)	(3)
1 , 6	**0.7**	**0.7**	**0.0**	**2.6**	**0.0**	**2.1**	**2.9**	**0.0**	**0.0**	**0.0**	**0.0**	**1.3**
	(1)	(1)	(0)	(1)	(0)	(1)	(1)	(0)	(0)	(0)	(0)	(1)
1 , 7	**0.7**	**0.7**	**1.0**	**0.0**	**1.1**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**1.3**
	(1)	(1)	(1)	(0)	(1)	(0)	(0)	(0)	(0)	(0)	(0)	(1)
1 , 8	**2.0**	**0.7**	**1.0**	**0.0**	**1.1**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**1.3**
	(3)	(1)	(1)	(0)	(1)	(0)	(0)	(0)	(0)	(0)	(0)	(1)
2 , 2	**12.7**	**10.2**	**8.1**	**15.8**	**6.7**	**16.7**	**17.1**	**10.5**	**8.3**	**0.0**	**13.9**	**11.3**
	(19)	(14)	(8)	(6)	(6)	(8)	(6)	(2)	(1)	(0)	(5)	(9)
2 , 3	**2.7**	**1.5**	**1.0**	**2.6**	**2.2**	**0.0**	**0.0**	**0.0**	**0.0**	**4.8**	**0.0**	**1.3**
	(4)	(2)	(1)	(1)	(2)	(0)	(0)	(0)	(0)	(1)	(0)	(1)
2 , 4	**12.0**	**13.9**	**17.2**	**5.3**	**14.6**	**12.5**	**5.7**	**5.3**	**0.0**	**33.3**[b]	**16.7**	**7.5**[d]
	(18)	(19)	(17)	(2)	(13)	(6)	(2)	(1)	(0)	(7)	(6)	(6)
2 , 5	**1.3**	**3.6**	**4.0**	**2.6**	**5.6**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**2.8**	**5.0**
	(2)	(5)	(4)	(1)	(5)	(0)	(0)	(0)	(0)	(0)	(1)	(4)
2 , 6	**0.0**	**0.7**	**1.0**	**0.0**	**1.1**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**1.3**
	(0)	(1)	(1)	(0)	(1)	(0)	(0)	(0)	(0)	(0)	(0)	(1)
2 , 7	**0.7**	**1.5**	**2.0**	**0.0**	**2.2**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**5.6**	**0.0**
	(1)	(2)	(2)	(0)	(2)	(0)	(0)	(0)	(0)	(0)	(2)	(0)
2 , 8	**0.7**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
	(1)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
3 , 4	**2.7**	**3.6**	**4.0**	**2.6**	**5.6**	**0.0**	**0.0**	**0.0**	**0.0**	**9.5**	**2.8**	**2.5**
	(4)	(5)	(4)	(1)	(5)	(0)	(0)	(0)	(0)	(2)	(1)	(2)
3 , 5	**1.3**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
	(2)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
4 , 4	**1.3**	**0.7**	**1.0**	**0.0**	**1.1**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**1.3**
	(2)	(1)	(1)	(0)	(1)	(0)	(0)	(0)	(0)	(0)	(0)	(1)
4 , 5	**2.0**	**0.7**	**1.0**	**0.0**	**1.1**	**0.0**	**0.0**	**0.0**	**0.0**	**4.8**	**0.0**	**0.0**
	(3)	(1)	(1)	(0)	(1)	(0)	(0)	(0)	(0)	(1)	(0)	(0)
4 , 6	**0.0**	**1.5**	**1.0**	**2.6**	**1.1**	**2.1**	**2.9**	**5.3**	**8.3**	**0.0**	**2.8**	**1.3**
	(0)	(2)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(0)	(1)	(1)
Total (n)	**150**	**137**	**99**	**38**	**89**	**48**	**35**	**19**	**12**	**21**	**36**	**80**

This table shows the frequency of the 22 genotypes of the (AC)n polymorphism in the IL-1A gene in controls and patient subgroups. n represents the number of genotypes in each group. The number in brackets is the actual number of subjects with a particular genotype. Comparisons were made between the control and patient groups, controls and patient subgroups, mild and severe, OFS=0 and OFS≥1,2,3,4 groups and different patient aetiologies for each genotype using the $\chi 2$ test and 2 x 2 contingency tables.

[a] = OFS≥2 vs controls (45.7% vs 23.3%, $\chi 2$=7.12, p=0.008 (1df), Pc=0.04)

[b] = Alcoholics vs controls (33.3% vs 12.0%, $\chi 2$=6.72, p=0.01 (1df), Pc<0.05)

[c] = OFS≥2 vs OFS=0 (45.7% vs 21.3%, $\chi 2$=7.36, p=0.007 (1df), Pc=0.035)

[d] = Gallstone vs alcoholics (7.5% vs 33.3%, $\chi 2$=9.9, p=0.002 (1df), Pc=0.01)

Table 6.2B: Frequency of IL-1A (AC)n repeat alleles in controls, patients and patient subgroups

Allele	Controls %	Patients %	Mild AP %	Severe AP %	OFS=0 %	OFS≥1 %	OFS≥2 %	OFS≥3 %	OFS≥4 %	A AP %	I AP %	G AP %
0	**2.3**	**0.7**	**0.5**	**1.3**	**1.1**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**1.4**	**0.6**
	(7)	(2)	(1)	(1)	(2)	(0)	(0)	(0)	(0)	(0)	(1)	(1)
1	**37.0**	**37.2**	**35.4**	**42.1**	**36.5**	**38.5**	**42.9**	**50.0**	**54.2**	**31.0**	**30.6**	**41.9**
	(111)	(102)	(70)	(32)	(65)	(37)	(30)	(19)	(13)	(13)	(22)	(67)
2	**33.3**	**35.0**	**33.3**	**39.5**	**30.3**	**43.8**	**42.9**	**34.2**	**29.2**	**28.6**	**41.7**	**33.8**
	(100)	(96)	(66)	(30)	(54)	(42)	(30)	(13)	(7)	(12)	(30)	(54)
3	**4.3**	**2.9**	**3.0**	**2.6**	**4.5**	**0.0**	**0.0**	**0.0**	**0.0**	**7.1**	**1.4**	**2.5**
	(13)	(8)	(6)	(2)	(8)	(0)	(0)	(0)	(0)	(3)	(1)	(4)
4	**17.0**	**17.2**	**20.7**	**7.9**	**20.2**	**11.5**	**7.1**	**7.9**	**8.3**	**28.6**	**18.1**	**13.8**
	(51)	(47)	(41)	(6)	(36)	(11)	(5)	(3)	(2)	(12)	(13)	(22)
5	**3.7**	**4.0**	**4.0**	**3.9**	**3.9**	**4.2**	**4.3**	**5.3**	**4.2**	**4.8**	**2.8**	**4.4**
	(11)	(11)	(8)	(3)	(7)	(4)	(3)	(2)	(1)	(2)	(2)	(7)
6	**0.3**	**1.5**	**1.0**	**2.6**	**1.1**	**2.1**	**2.9**	**2.6**	**4.2**	**0.0**	**1.4**	**1.9**
	(1)	(4)	(2)	(2)	(2)	(2)	(2)	(1)	(1)	(0)	(1)	(3)
7	**0.7**	**1.1**	**1.5**	**0.0**	**1.7**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**2.8**	**0.6**
	(2)	(3)	(3)	(0)	(3)	(0)	(0)	(0)	(0)	(0)	(2)	(1)
8	**1.3**	**0.4**	**0.5**	**0.0**	**0.6**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0**	**0.6**
	(4)	(1)	(1)	(0)	(1)	(0)	(0)	(0)	(0)	(0)	(0)	(1)
Total (n)	**300**	**274**	**198**	**76**	**178**	**96**	**70**	**38**	**24**	**42**	**72**	**160**

This table shows the frequency of the 9 alleles of the (AC)n polymorphism in the IL-1A gene in controls and patient subgroups. n represents the number of alleles in each group. The number in brackets is the actual number of subjects with a particular allele. Comparisons were made between the control and patient group, control and patient subgroups, mild and severe groups, OFS=0 and OFS≥1,2,3,4 groups and different patient aetiologies for each allele using the $\chi 2$ test and 2 x 2 contingency tables. No significant differences were found.

Summary of the results obtained at the (AC)n repeat locus

All of the significant data are discussed, however, only the data that are significant following correction for the number of comparisons involved are included in the tables.

Genotype frequency:

To make the genotype analysis easier, genotypes were divided into 1,1; 1,2; 1,4; 2,2; 2,4 and "other".

Controls vs patients

There were no significant differences in genotype frequencies between the patient group compared to the control group.

Controls vs patient subgroups

When the patient group was subdivided according to severity, organ failure scores and aetiology and the subgroups compared with controls, the 1,2 genotype was significantly increased in the OFS≥1 group compared to controls (41.7% vs 23.3%, $\chi 2$ =6.09, p=0.014*). The 1,2 genotype was significantly increased in the OFS≥2 group compared to controls (45.7% vs 23.3%, $\chi 2$=7.12, p=0.008, Pc=0.04). The 2,4 genotype was significantly increased in the alcoholic group compared to controls (33.3% vs 12.0%, $\chi 2$=6.72, p=0.01, Pc<0.05).

Mild group vs severe group

No significant differences in genotype frequency were found between mild group and severe group.

OFS=0 group vs OFS≥1,2,3,4 groups

Analysis of the genotype data with respect to organ failure scores showed the 1,2 genotype was significantly increased in the OFS≥1 group compared to the OFS=0 group (41.7% vs 21.3%, $\chi 2$=6.32, p=0.012*). The 1,2 genotype was significantly increased in the OFS≥2 group compared to the OFS=0 group (45.7% vs 21.3%, $\chi 2$=7.36, p=0.007, Pc=0.035).

Different combinations of the aetiological groups

When the data was analysed with respect to aetiology, the 2,4 genotype was significantly decreased in the gallstone group compared to the alcoholic group (7.5% vs 33.3%, $\chi 2$= 9.9, p=0.002, Pc=0.01).

Allele frequency:

Controls vs patients

No significant differences in allele frequency were found between the control and patient groups.

Controls vs patient subgroups

When the patient group was subdivided according to severity, organ failure scores and aetiology and the subgroups compared with controls, allele 4 was significantly decreased in the severe group compared to controls (7.9% vs 17.0%, $\chi 2$ =3.91, p=0.048*) while allele 6 was significantly increased between the same two groups (2.6% vs 0.3% $\chi 2$ =4.05, p=0.044*). Allele 3 was significantly decreased in the OFS≥1 group compared to controls (0.0% vs 4.3%, $\chi 2$ =4.30, p=0.038*). Allele 4 was also significantly decreased in the OFS≥2 group compared to controls (7.1% vs 17.0%, $\chi 2$=4.29, p=0.038*) while allele 6 was significantly increased between the same groups (2.9% vs 0.3%,$\chi 2$=4.5, p=0.034*). Allele 6 was also significantly increased in the OFS≥4 group compared to controls (4.2% vs 0.3%, $\chi 2$ =5.32, p=0.021*).

Mild group vs severe group

Analysis of the data with respect to severity showed allele 4 was significantly decreased in the severe group compared to the mild group (7.9% vs 20.7%, $\chi 2$=6.34, p=0.012*).

OFS=0 group vs OFS≥1,2,3,4 groups

When the data was analysed with respect to organ failure scores allele 2 was significantly increased in the OFS≥1 group compared to the OFS=0 group (43.8% vs 30.3%, $\chi 2$=4.93,

p=0.026*) whereas allele 3 was significantly decreased between the same groups (0.0% vs 4.5%, $\chi 2$=4.44, p=0.035*). Allele 4 was significantly decreased in the OFS≥2 group compared to the OFS=0 group (7.1% vs 20.2%, $\chi 2$=6.23, p=0.013*).

Different combinations of the aetiological groups

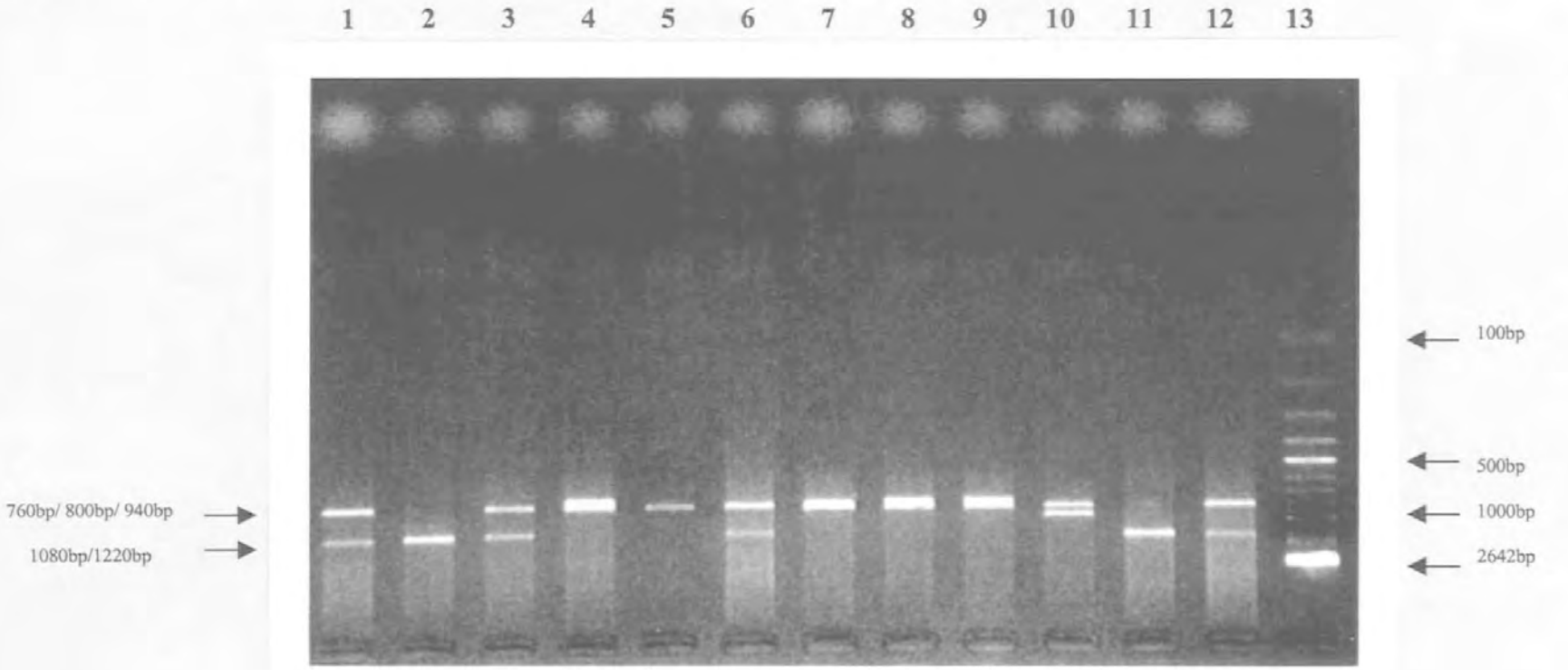
Comparisons of the allele frequencies between the different aetiological groups showed allele 4 was significantly decreased in the gallstone group compared to the alcoholic group (13.8% vs 28.6%, $\chi 2$=5.22, p=0.022*).

* p value became non-significant following correction for the number of comparisons made.

6.1.2.2 VNTR[46] polymorphism

Figure 6.1 illustrates the location of the VNTR[46] polymorphism in the IL-1 gene cluster. Five different alleles (alleles 1-4 and 6) were observed corresponding to 9 (800bp), 18 (1220bp), 8 (760bp), 12 (940bp) and 15 (1080bp) copies of the 46bp repeat sequence. These gave rise to 12 different genotypes. Alleles 5 and 7 corresponding to 5 (620bp) and 6 (660bp) copies of the repeat sequence were not detected (Figure 6.4).

Figure 6.4: 3% agarose gel showing the genotypes detected at the IL-1A VNTR[46] locus.



Five different alleles (alleles 1-4 and 6) were detected corresponding to: 9(800bp), 18(1220bp), 8(760bp), 12(940bp) and 15(1080bp) copies of the 46bp repeat sequence. Alleles 5 and 7 corresponding to 5 (620bp) and 6 (660bp) copies of the repeat sequence were not detected.
Lanes 1,6 and 12 - 1,2
Lanes 2 and 11 - 2,2
Lane 3 - 1,6
Lanes 4,7,8, and 9 - 1,3
Lane 5 - 1,1
Lane 10 - 1,4
Lane 13 - Molecular weight marker XIV (100bp ladder)

Table 6.3A: Frequency of IL-1A VNTR[46] genotypes in controls, patients and patient subgroups

G/T	Controls %	Patients %	Mild AP %	Severe AP %	OFS=0 %	OFS≥1 %	OFS≥2 %	OFS≥3 %	OFS≥4 %	A AP %	I AP %	G AP %
1,1	**31.6**	**25.0**	**24.2**	**27.0**	**29.2**	**17**	**20.6**	**26.3**	**25.0**	**28.6**	**19.4**	**26.6**
	(59)	(34)	(24)	(10)	(26)	(8)	(7)	(5)	(3)	(6)	(7)	(21)
1,2	**29.9**	**33.8**	**34.3**	**32.4**	**31.5**	**38.3**	**38.2**	**31.6**	**33.3**	**38.1**	**44.4**	**27.8**
	(56)	(46)	(34)	(12)	(28)	(18)	(13)	(6)	(4)	(8)	(16)	(22)
1,3	**12.8**	**14.7**	**15.2**	**13.5**	**15.7**	**12.8**	**14.7**	**15.8**	**16.7**	**23.8**	**5.6**	**16.5**
	(24)	(20)	(15)	(5)	(14)	(6)	(5)	(3)	(2)	(5)	(2)	(13)
1,4	**2.7**	**2.9**	**3.0**	**2.7**	**3.4**	**2.1**	**2.9**	**0.0**	**0.0**	**0.0**	**2.8**	**3.8**
	(5)	(4)	(3)	(1)	(3)	(1)	(1)	(0)	(0)	(0)	(1)	(3)
1,6	**3.7**	**3.7**	**5.1**	**0.0**	**2.2**	**6.4**	**2.9**	**0.0**	**0.0**	**0.0**	**2.8**	**5.1**
	(7)	(5)	(5)	(0)	(2)	(3)	(1)	(0)	(0)	(0)	(1)	(4)
2,2	**7.5**	**4.4**	**5.1**	**2.7**	**3.4**	**6.4**	**5.9**	**0.0**	**0.0**	**4.8**	**5.6**	**3.8**
	(14)	(6)	(5)	(1)	(3)	(3)	(2)	(0)	(0)	(1)	(2)	(3)
2,3	**6.4**	**11.8**	**11.1**	**13.5**	**11.2**	**12.8**	**8.8**	**15.8**	**16.7**	**0.0**	**16.7**	**12.7**
	(12)	(16)	(11)	(5)	(10)	(6)	(3)	(3)	(2)	(0)	(6)	(10)
2,4	**2.1**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
	(4)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
3,3	**1.6**	**2.9**	**2.0**	**5.4**	**3.4**	**2.1**	**2.9**	**5.3**	**8.3**	**4.8**	**2.8**	**2.5**
	(3)	(4)	(2)	(2)	(3)	(1)	(1)	(1)	(1)	(1)	(1)	(2)
3,4	**0.5**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
	(1)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
4,6	**0.5**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
	(1)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
6,6	**0.5**	**0.7**	**0.0**	**2.7**	**0.0**	**2.1**	**2.9**	**5.3**	**0.0**	**0.0**	**0.0**	**1.3**
	(1)	(1)	(0)	(1)	(0)	(1)	(1)	(1)	(0)	(0)	(0)	(1)
Total (n)	**187**	**136**	**99**	**37**	**89**	**47**	**34**	**19**	**12**	**21**	**36**	**79**

This table shows the frequency of the 12 genotypes of the VNTR[46] polymorphism in the IL-1A gene in controls and patient subgroups. n represents the number of genotypes in each group. The number in brackets is the actual number of subjects with a particular genotype. Comparisons were made between the control and patient groups, controls and patient subgroups, mild and severe, OFS=0 and OFS≥1,2,3,4 groups and different patient aetiologies for each genotype using the $\chi 2$ test and 2 x 2 contingency tables. No significant differences were found.

Table 6.3B: Frequency of IL-1A VNTR[46] alleles in controls, patients and patient subgroups

Allele	Controls %	Patients %	Mild AP %	Severe AP %	OFS=0 %	OFS≥1 %	OFS≥2 %	OFS≥3 %	OFS≥4 %	A AP %	I AP %	G AP %
1	**56.1**	**52.6**	**53.0**	**51.4**	**55.6**	**46.8**	**50.0**	**50.0**	**50.0**	**59.5**	**47.2**	**53.2**
	(210)	(143)	(105)	(38)	(99)	(44)	(34)	(19)	(12)	(25)	(34)	(84)
2	**26.7**	**27.2**	**27.8**	**25.7**	**24.7**	**31.9**	**29.4**	**23.7**	**25.0**	**23.8**	**36.1**	**24.1**
	(100)	(74)	(55)	(19)	(44)	(30)	(20)	(9)	(6)	(10)	(26)	(38)
3	**11.5**	**16.2**	**15.2**	**18.9**	**16.9**	**14.9**	**14.7**	**21.1**	**25.0**	**16.7**	**13.9**	**17.1**
	(43)	(44)	(30)	(14)	(30)	(14)	(10)	(8)	(6)	(7)	(10)	(27)
4	**2.9**	**1.5**	**1.5**	**1.4**	**1.7**	**1.1**	**1.5**	**0.0**	**0.0**	**0.0**	**1.4**	**1.9**
	(11)	(4)	(3)	(1)	(3)	(0)	(1)	(0)	(0)	(0)	(1)	(3)
5	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
6	**2.7**	**2.6**	**2.5**	**2.7**	**1.1**	**5.3**	**4.4**	**5.3**	**0.0**	**0.0**	**1.4**	**3.8**
	(10)	(7)	(5)	(2)	(2)	(5)	(3)	(2)	(0)	(0)	(1)	(6)
7	**0.0**	**0.0**	**0.0**	**0.0**	**0**	**0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
Total (n)	**374**	**272**	**198**	**74**	**178**	**94**	**68**	**38**	**24**	**42**	**72**	**158**

This table shows the frequency of the 7 alleles of the VNTR[46] polymorphism in the IL-1A gene in controls and patient subgroups. n represents the number of alleles in each group. The number in brackets is the actual number of subjects with a particular allele. Comparisons were made between the control and patient groups, controls and patient subgroups, mild and severe, OFS=0 and OFS≥1,2,3,4 groups and different patient aetiologies for each allele using the $\chi 2$ test and 2 x 2 contingency tables. No significant differences were found.

Summary of the results obtained at the IL-1A VNTR[46] locus

All of the significant data are discussed, however, only the data that are significant following correction for the number of comparisons involved are included in the tables.

Genotype frequency

To make the genotype analysis easier, genotypes were divided into 1,1; 1,2; 1,3; 2,2; 2,3 and "other".

Controls vs patients

No significant differences in the genotype frequencies were found between the control and patient groups.

Controls vs patient subgroups

When the patient group was subdivided according to severity, organ failure scores and aetiology and the subgroups compared with controls, the 1,1, genotype was significantly decreased in the OFS≥1 group compared to controls (17.0% vs 31.6%, $\chi 2=3.88$, p=0.049*). The 2,3 genotype was significantly increased in the idiopathic group compared to controls (16.7% vs 6.4%, $\chi 2=4.27$, p=0.039*).

Mild group vs severe group /OFS=0 group vs OFS≥1,2,3,4 groups

No significant differences in genotype frequency were found between mild group when compared with the severe group or between the OFS=0 group and OFS≥1,2,3,4 groups.

Different aetiological groups

Analysis of the data with respect to aetiology showed the 1,3 genotype was significantly decreased in the idiopathic group compared to the alcoholic group (5.6% vs 23.8%, $\chi 2=4.1$,p=0.043*), while the 2,3 genotype was significantly increased between the two groups (16.7% vs 0.0%, $\chi 2=3.91$, p=0.048*).

Allele frequency:

Controls vs patients/ Controls vs patient subgroups/ mild group vs severe group

No significant differences in allele frequency were found between these groups.

OFS=0 group vs OFS≥1,2,3,4 groups

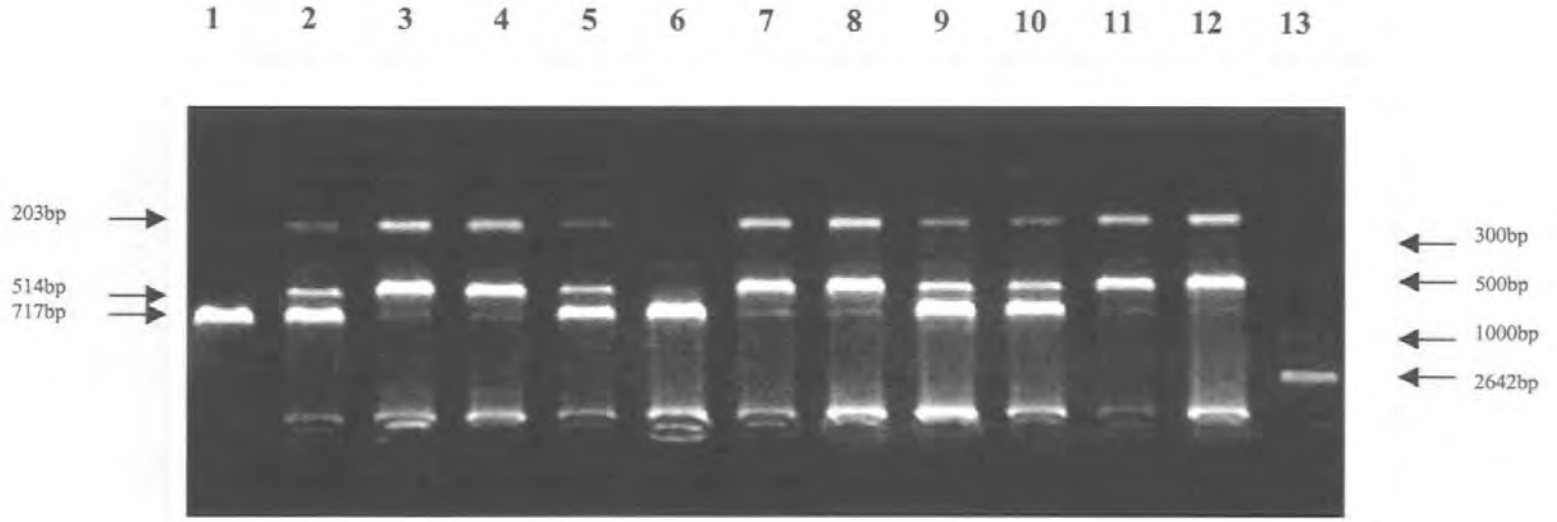
Analysis of the data with respect to organ failure scores showed allele 6 was significantly increased in the OFS≥1 group compared to the OFS=0 group (5.3% vs 1.1%, $\chi 2=4.32$, p=0.038*).

Different combinations of the aetiological groups

No significant differences were found in allele frequencies between the different aetiological groups.

* p value became non-significant following correction for the number of comparisons made.

Figure 6.5: 3% agarose gel displaying the genotypes observed at the *Ava I* locus.



Two alleles were detected: allele 1(203bp,514bp), allele 2 (717bp).
Lanes 1 and 6 - 2,2
Lanes 2,5,9 and 10 - 1,2
Lanes 3,4,7,8,11 and 12 - 1,1
Lane 13 - Molecular weight marker XIV (100bp ladder)

Table 6.4A: Frequency of IL-1B *Ava I* genotypes in controls, patients and patient subgroups

Genotype	Controls %	Patients %	Mild AP %	Severe AP %	OFS=0 %	OFS≥1 %	OFS≥2 %	OFS≥3 %	OFS≥4 %	A AP %	I AP %	G AP %
1 , 1	**42.4**	**43.4**	**42.4**	**45.9**	**47.2**	**36.2**	**38.2**	**36.8**	**41.7**	**42.9**	**50.0**	**40.5**
	(92)	(59)	(42)	(17)	(42)	(17)	(13)	(7)	(5)	(9)	(18)	(32)
1 , 2	**39.6**	**47.1**	**46.5**	**48.6**	**42.7**	**55.3**	**55.9**	**57.9**	**58.3**	**57.1**	**41.7**	**46.8**
	(86)	(64)	(46)	(18)	(38)	(26)	(19)	(11)	(7)	(12)	(15)	(37)
2 , 2	**18.0**	**9.6**	**11.1**	**5.4**	**10.1**	**8.5**	**5.9**	**5.3**	**0.0**	**0.0**	**8.3**	**12.7**
	(39)	(13)	(11)	(2)	(9)	(4)	(2)	(1)	(0)	(0)	(3)	(10)
Total (n)	**217**	**136**	**99**	**37**	**89**	**47**	**34**	**19**	**12**	**21**	**36**	**79**

This table shows the frequency of the 3 genotypes of the *Ava I* polymorphism in the IL-1B gene in controls and patient subgroups. n represents the number of genotypes in each group. The number in brackets is the actual number of subjects with a particular genotype. Comparisons were made between the control and patient group, control and patient subgroups, mild and severe groups, OFS=0 and OFS≥1,2,3,4 groups and different patient aetiologies for each genotype using the $\chi 2$ test and 2 x 2 contingency tables. No significant differences were found.

Table 6.4B: Frequency of IL-1B *Ava I* alleles in controls, patients and patient subgroups

Allele	Controls %	Patients %	Mild AP %	Severe AP %	OFS=0 %	OFS≥1 %	OFS≥2 %	OFS≥3 %	OFS≥4 %	A AP %	I AP %	G AP %
1	**62.2**	**66.9**	**65.7**	**70.3**	**68.5**	**63.8**	**66.2**	**65.8**	**70.8**	**71.4**	**70.8**	**63.9**
	(270)	(182)	(130)	(52)	(122)	(60)	(45)	(25)	(17)	(30)	(51)	(101)
2	**37.8**	**33.1**	**34.3**	**29.7**	**31.5**	**36.2**	**33.8**	**34.2**	**29.2**	**28.6**	**29.2**	**36.1**
	(164)	(90)	(68)	(22)	(56)	(34)	(23)	(13)	(7)	(12)	(21)	(57)
Total (n)	**434**	**272**	**198**	**74**	**178**	**94**	**68**	**38**	**24**	**42**	**72**	**158**

This table shows the frequency of the 2 alleles of the *Ava I* polymorphism in the IL-1B gene in controls and patient subgroups. n represents the number of alleles in each group. The number in brackets is the actual number of subjects with a particular allele. Comparisons were made between the control and patient group, control and patient subgroups, mild and severe groups, OFS=0 and OFS≥1,2,3,4 groups and different patient aetiologies for each allele using the $\chi 2$ test and 2 x 2 contingency tables. No significant differences were found.

Summary of the results obtained at the *Ava I* locus

All of the significant data are discussed, however, only the data that are significant following correction for the number of comparisons involved are included in the tables.

Genotype frequency

Controls vs patients

Comparisons of the *Ava I* genotype frequencies between patients and controls showed the 2,2 genotype was significantly decreased in the patient group compared to controls (9.6% vs 18.0%, $\chi 2=4.71$, p=0.03*).

Controls vs patient subgroups

When the patient group was subdivided according to severity, organ failure scores and aetiology, and the subgroups compared with controls, the 1,2 genotype was significantly increased in the OFS≥1 group compared to controls (55.3% vs 39.6%, $\chi 2=3.89$, p=0.049*). The 2,2 genotype was significantly decreased in the alcoholic group compared to controls (0.0% vs 18.0%, $\chi 2 = 4.51$, p=0.034*).

Mild group vs severe group/ OFS=0 group vs OFS≥1,2,3,4 groups/ different combinations of the aetiological groups

No significant differences in genotype frequency were found when the mild group was compared with the severe group, OFS=0 group compared with the OFS≥1,2,3,4 groups or between different combinations of the aetiological groups.

Allele frequency

Controls vs patients/controls vs patient subgroups/ mild group vs severe group/ OFS=0 group vs OFS≥1,2,3,4 groups/ different combinations of the aetiological groups

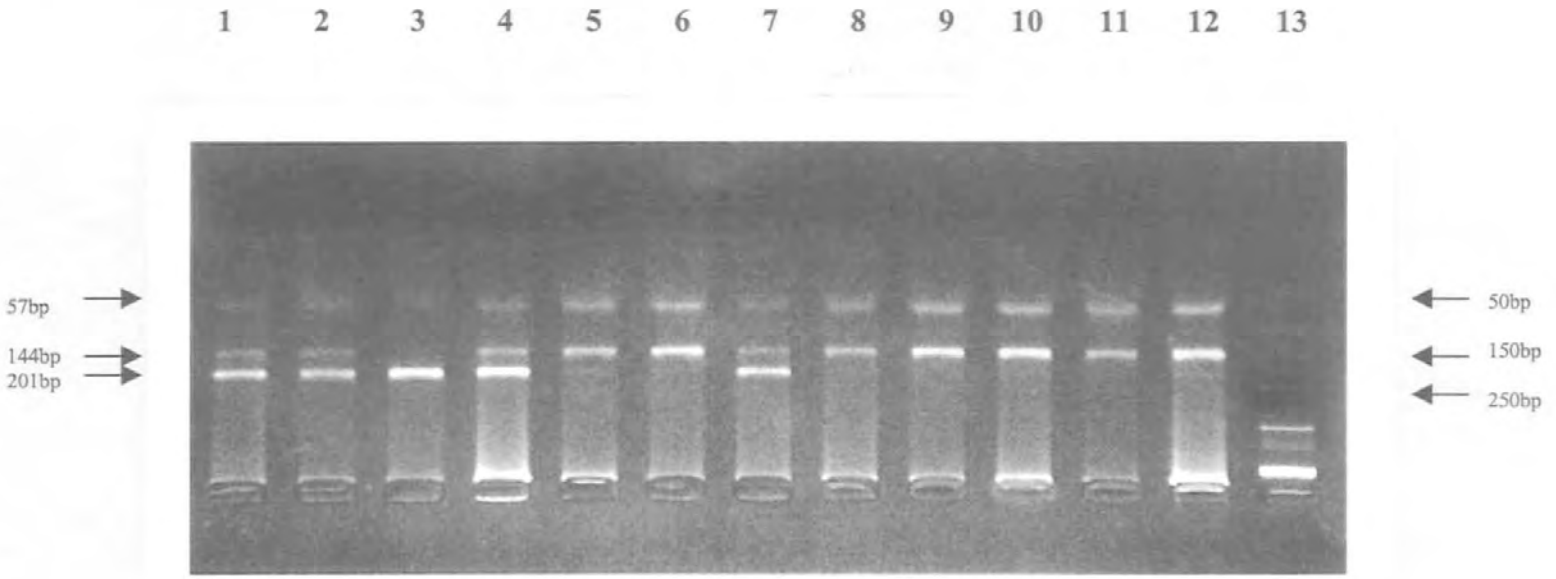
No significant differences were found between the *Ava I* allelic frequencies in controls and patients or between subdivisions of the patient group.

* p value became non-significant following correction for the number of comparisons made.

6.1.3.2 Alu I polymorphism

Figure 6.1 illustrates the location of the *Alu I* polymorphism in the IL-1 gene cluster. Digestion of PCR products with *Alu I* gave fragments of 57bp, 144bp and 201bp for alleles 1 and 2 respectively. These two alleles gave rise to three genotypes: 1,1; 1,2; 2,2. The $T \rightarrow C^{1903}$ SNP destroys an *Alu I* restriction site. Homozygotes for allele 1 (57bp, 144bp) occur when the SNP is not present on either chromosome. Homozygotes for allele 2 (201bp) occur when the SNP is present on both chromosomes. Heterozygotes (57bp, 144bp, 201bp) occur when the SNP is present on only one chromosome (Figure 6.6).

Figure 6.6: 3% agarose gel illustrating the genotypes detected at the *Alu I* locus.



Two alleles were detected: allele 1 (57bp,144bp), allele 2 (201bp).
Lanes 1,2,4 and 7 - 1,2
Lane 3 - 2,2
Lanes 5,6,8,9,10,11 and 12 - 1,1
Lane 13 - Molecular weight marker XIII (50bp ladder)

Table 6.5A: Frequency of IL-1B *Alu I* genotypes in controls, patients and patient subgroups

Genotype	Controls %	Patients %	Mild AP %	Severe AP %	OFS=0 %	OFS≥1 %	OFS≥2 %	OFS≥3 %	OFS≥4 %	A AP %	I AP %	G AP %
1 , 1	**40.8**	**41.8**	**40.2**	**45.9**	**44.3**	**37.0**	**39.4**	**36.8**	**41.7**	**38.1**	**51.4**	**38.5**
	(69)	(56)	(39)	(17)	(39)	(17)	(13)	(7)	(5)	(8)	(18)	(30)
1 , 2	**39.6**	**48.5**	**48.5**	**48.6**	**45.5**	**54.3**	**54.5**	**57.9**	**58.3**	**61.9**	**40.0**	**48.7**
	(67)	(65)	(47)	(18)	(40)	(25)	(18)	(11)	(7)	(13)	(14)	(38)
2 , 2	**19.5**	**9.7[a]**	**11.3**	**5.4**	**10.2**	**8.7**	**6.1**	**5.3**	**0.0**	**0.0**	**8.6**	**12.8**
	(33)	(13)	(11)	(2)	(9)	(4)	(2)	(1)	(0)	(0)	(3)	(10)
Total (n)	**169**	**134**	**97**	**37**	**88**	**46**	**33**	**19**	**12**	**21**	**35**	**78**

This table shows the frequency of the 3 genotypes of the *Alu I* polymorphism in the IL-1B gene in controls and patient subgroups. n represents the number of genotypes in each group. The number in brackets is the actual number of subjects with a particular genotype. Comparisons were made between the control and patient group, control and patient subgroups, mild and severe groups, OFS=0 and OFS≥1,2,3,4 groups and different patient aetiologies for each genotype using the $\chi 2$ test and 2 x 2 contingency tables.

[a] = patients vs controls (9.7% vs 19.5%, $\chi 2$=5.6, p=0.018 (1df), Pc=0.036)

Table 6.5B: Frequency of IL-1B *Alu I* alleles in controls, patients and patient subgroups

Allele	Controls %	Patients %	Mild AP %	Severe AP %	OFS=0 %	OFS≥1 %	OFS≥2 %	OFS≥3 %	OFS≥4 %	A AP %	I AP %	G AP %
1	**60.7**	**66.0**	**64.4**	**70.3**	**67.0**	**64.1**	**66.7**	**65.8**	**70.8**	**69.0**	**71.4**	**62.8**
	(205)	(177)	(125)	(52)	(118)	(59)	(44)	(25)	(17)	(29)	(50)	(98)
2	**39.3**	**34.0**	**35.6**	**29.7**	**33.0**	**35.9**	**33.3**	**34.2**	**29.2**	**31.0**	**28.6**	**37.2**
	(133)	(91)	(69)	(22)	(58)	(33)	(22)	(13)	(7)	(13)	(20)	(58)
Total (n)	**338**	**268**	**194**	**74**	**176**	**92**	**66**	**38**	**24**	**42**	**70**	**156**

This table shows the frequency of the 2 alleles of the *Alu I* polymorphism in the IL-1B gene in controls and patient subgroups. n represents the number of alleles in each group. The number in brackets is the actual number of subjects with a particular allele. Comparisons were made between the control and patient group, control and patient subgroups, mild and severe groups, OFS=0 and OFS≥1,2,3,4 groups and different patient aetiologies for each allele using the $\chi 2$ test and 2 x 2 contingency tables. No significant differences were found.

Summary of the results obtained at the *Alu I* locus

All of the significant data are discussed, however, only the data that are significant following correction for the number of comparisons involved are included in the tables.

Genotype frequency

Controls vs patients

Comparisons of the *Alu I* genotype frequencies between patients and controls showed the 2,2 genotype was significantly decreased in the patient group compared to controls (9.7% vs 19.5%, $\chi 2$ =5.6, p=0.018, Pc=0.036).

Controls vs patient subgroups

When the patient group was subdivided according to severity, organ failure scores and aetiology, and the subgroups compared with controls, the 2,2 genotype was significantly decreased in the severe group compared to controls (5.4% vs 19.5%, $\chi 2$ =4.29, p=0.038*). The 2,2 genotype was significantly decreased in the alcoholic group compared to controls (0.0% vs 19.5%, $\chi 2$=4.96, p=0.026*).

Mild group vs severe group/ OFS=0 group vs OFS≥1,2,3,4 groups/ different combinations of the aetiological groups

No significant differences in genotype frequency were found when mild group was compared with the severe group, the OFS=0 group compared with OFS≥1,2,3,4 group or between different combinations of the aetiological groups.

Allele frequency

Controls vs patients/controls vs patient subgroups/ mild group vs severe group/ OFS=0 group vs OFS≥1,2,3,4 groups/ different combinations of the aetiological groups

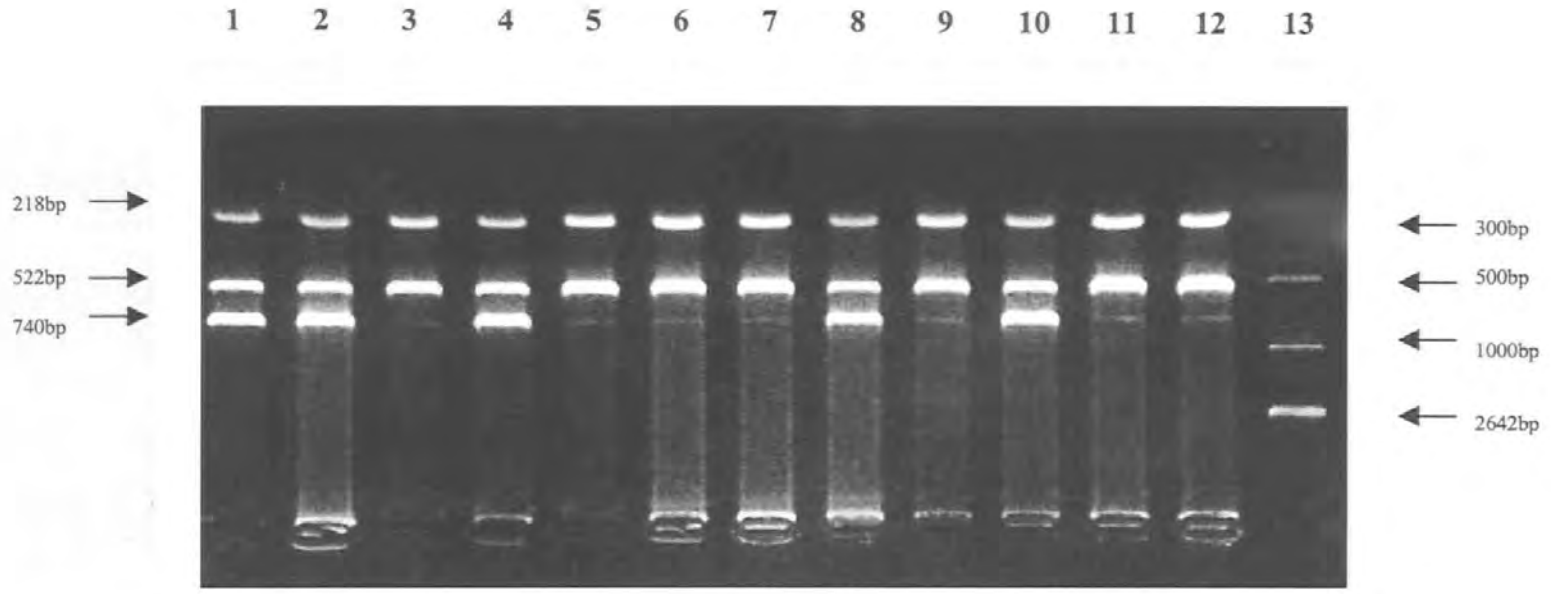
No significant differences were found between the *Alu I* allelic frequencies in controls and patients or between subdivisions of the patient group.

* p value became non-significant following correction for the number of comparisons made.

6.1.3.3 Taq I polymorphism

Figure 6.1 illustrates the location of the *Taq I* polymorphism in the IL-1 gene cluster. *Taq I* digestion of the PCR fragment amplified by amplimers *Taq I* and *II* detected fragments of 218bp, 522bp and 740bp for alleles 1 and 2 respectively. Three genotypes were observed: 1,1; 1,2; 2,2. The $C \rightarrow T^{5887}$ SNP creates a *Taq I* restriction site. Homozygotes for allele 1 (218bp, 522bp) occur when the SNP is present on both chromosomes. Homozygotes for allele 2 (740bp) occur when the SNP is not present on either chromosome. Heterozygotes (218bp, 522bp, 740bp) occur when the SNP is present on only one chromosome (Figure 6.7).

Figure 6.7: 3% agarose gel showing the genotypes detected at the *Taq I* locus.



Two alleles were detected: allele 1(218bp,522bp), allele 2 (740bp). (The 2,2 genotype is not shown).
Lanes 1,2,4,8 and 10 - 1,2
Lanes 3,5,6,7,9,11 and 12 - 1,1
Lane 13 - Molecular weight marker XIV (100bp ladder)

Table 6.6A: Frequency of IL-1B *Taq I* genotypes in controls, patients and patient subgroups

Genotype	Controls %	Patients %	Mild AP %	Severe AP %	OFS=0 %	OFS≥1 %	OFS≥2 %	OFS≥3 %	OFS≥4 %	A AP %	I AP %	G AP %
1 , 1	**67.6**	**63.2**	**66.7**	**55.6**	**70.6**	**52.2**	**48.5**	**50.0**	**41.7**	**72.2**	**56.3**	**64.1**
	(115)	(72)	(52)	(20)	(48)	(24)	(16)	(9)	(5)	(13)	(18)	(41)
1 , 2	**31.2**	**35.1**	**32.1**	**41.7**	**26.5**	**47.8[b]**	**51.5[ac]**	**50.0**	**58.3**	**27.8**	**43.8**	**32.8**
	(53)	(40)	(25)	(15)	(18)	(22)	(17)	(9)	(7)	(5)	(14)	(21)
2 , 2	**1.2**	**1.8**	**1.3**	**2.8**	**2.9**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**3.1**
	(2)	(2)	(1)	(1)	(2)	(0)	(0)	(0)	(0)	(0)	(0)	(2)
Total (n)	**170**	**114**	**78**	**36**	**68**	**46**	**33**	**18**	**12**	**18**	**32**	**64**

This table shows the frequency of the 3 genotypes of the *Taq I* polymorphism in the IL-1B gene in controls and patient subgroups. n represents the number of genotypes in each group. The number in brackets is the actual number of subjects with a particular genotype. Comparisons were made between the control and patient group, control and patient subgroups, mild and severe groups, OFS=0 and OFS≥1,2,3,4 groups and different patient aetiologies for each genotype using the $\chi 2$ test and 2 x 2 contingency tables.

[a] = OFS≥2 vs controls (51.5% vs 31.2%, $\chi 2$ =5.06, p=0.024 (1df), Pc=0.048)

[b] = OFS≥1 vs OFS=0 (47.8% vs 26.5%, $\chi 2$=5.49, p=0.019 (1df), Pc=0.038)

[c] = OFS≥2 vs OFS=0 (51.5% vs 26.5%, $\chi 2$=6.15, p=0.013 (1df), Pc=0.026)

Table 6.6B: Frequency of IL-1B *Taq I* alleles in controls, patients and patient subgroups

Allele	Controls %	Patients %	Mild AP %	Severe AP %	OFS=0 %	OFS≥1 %	OFS≥2 %	OFS≥3 %	OFS≥4 %	A AP %	I AP %	G AP %
1	**83.2**	**80.7**	**82.7**	**76.4**	**83.8**	**76.1**	**74.2**	**75.0**	**70.8**	**86.1**	**78.1**	**80.5**
	(283)	(184)	(129)	(55)	(114)	(70)	(49)	(27)	(17)	(31)	(50)	(103)
2	**16.8**	**19.3**	**17.3**	**23.6**	**16.2**	**23.9**	**25.8**	**25.0**	**29.2**	**13.9**	**21.9**	**19.5**
	(57)	(44)	(27)	(17)	(22)	(22)	(17)	(9)	(7)	(5)	(14)	(25)
Total (n)	**340**	**228**	**156**	**72**	**136**	**92**	**66**	**36**	**24**	**36**	**64**	**128**

This table shows the frequency of the 2 alleles of the *Taq I* polymorphism in the IL-1B gene in controls and patient subgroups. n represents the number of alleles in each group. The number in brackets is the actual number of subjects with a particular allele. Comparisons were made between the control and patient group, control and patient subgroups, mild and severe groups, OFS=0 and OFS≥1,2,3,4 groups and different patient aetiologies for each allele using the $\chi 2$ test and 2 x 2 contingency tables. No significant differences were found.

Summary of the results obtained at the *Taq I* locus

All of the significant data are discussed, however, only the data that are significant following correction for the number of comparisons involved are included in the tables.

Genotype frequency

Controls vs patients

No significant differences in genotype frequency were present between controls and patients.

Controls vs patient subgroups

When the patient group was subdivided according to severity, organ failure scores and aetiology and the subgroups compared with controls, the 1,2 genotype was significantly increased in the OFS≥1 group compared to controls (47.8% vs 31.2%, $\chi 2$=4.43, p=0.035*). The 1,1 genotype was significantly decreased in the OFS≥2 group compared to controls (48.5% vs 67.6%, $\chi 2$=4.43, p=0.035*), while the 1,2 genotype was significantly increased between the same groups (51.5% vs 31.2%, $\chi 2$=5.06, p=0.024, Pc=0.048).

Mild group vs severe group

No significant differences in genotype frequencies were found between the mild group and severe group.

OFS=0 group vs OFS≥1,2,3,4 groups

Analysis of the genotype data with respect to organ failure scores showed the 1,1 genotype was significantly decreased in the OFS≥1 group compared to the OFS=0 group (52.2% vs 70.6%, $\chi 2$=4.0, p=0.046*), whereas the 1,2 genotype was significantly increased between the same groups (47.8% vs 26.5%, $\chi 2$=5.49, p=0.019, Pc=0.038). The 1,1 genotype was significantly decreased in the OFS≥2 group compared to the OFS=0 group (48.5% vs 70.6%, $\chi 2$=4.68, p=0.031*) while the 1,2 genotype was significantly increased between the same groups (51.5% vs 26.5%, $\chi 2$=6.15, p=0.013, Pc=0.026). The 1,2 genotype was

significantly increased in the OFS≥4 group compared to OFS=0 group (58.3% vs 26.5%, $\chi 2=4.82$, p=0.028*).

Different combinations of the aetiological groups

No significant differences in genotype frequency were found between the different combinations of the aetiological groups.

Allele frequency

Controls vs patients/ controls vs patient subgroups/ mild group vs severe group/ OFS=0 group vs OFS≥1,2,3,4 groups/ different combinations of the aetiological groups

No significant differences were found between the *Taq I* allelic frequencies in controls and patients or between subdivisions of the patient group.

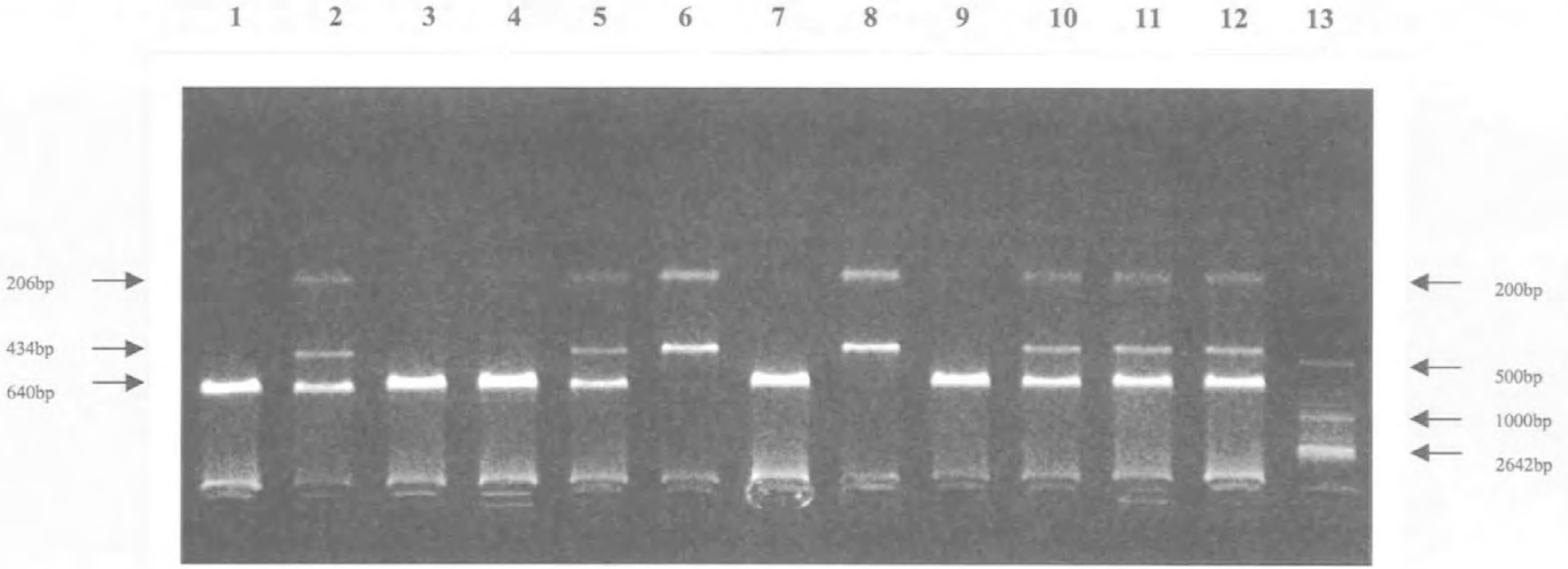
* p value became non-significant following correction for the number of comparisons made.

6.1.4 IL-1RN:

6.1.4.1 Msp I polymorphism

Figure 6.1 illustrates the location of the *Msp I* polymorphism in the IL-1 gene cluster. *Msp I* digestion of genomic DNA amplified with amplimers *Msp I* and *II* produced fragments of 640bp and 206bp, 434bp for alleles 1 and 2 respectively. Three genotypes were observed: 1,1; 1,2; 2,2. The $T \rightarrow C^{8006}$ SNP creates a *Msp I* restriction site. Homozygotes for allele 1 (640bp) occur when the SNP is not present on either chromosome. Homozygotes for allele 2 (206bp, 434bp) occur when the SNP is present on both chromosomes. Heterozygotes (206bp, 434bp, 640bp) occur when the SNP is present on only one chromosome (Figure 6.8).

Figure 6.8: 3% agarose gel displaying the genotypes detected at the *Msp I* locus



Two alleles were detected: allele 1 (640bp), allele 2 (434bp, 206bp).
Lanes 1,3,4,7 and 9 - 1,1
Lanes 2,5,10,11 and 12 - 1,2
Lanes 6 and 8 - 2,2
Lane 13 - Molecular weight marker XIV (100bp ladder)

Table 6.7A: Frequency of IL-1RN *Msp I* genotypes in controls, patients and patient subgroups

Genotype	Controls %	Patients %	Mild AP %	Severe AP %	OFS=0 %	OFS≥1 %	OFS≥2 %	OFS≥3 %	OFS≥4 %	A AP %	I AP %	G AP %
1 , 1	47.7	55.9	50.5	70.3[a]	53.9	59.6	61.8	68.4	66.7	52.4	66.7	51.9
	(71)	(76)	(50)	(26)	(48)	(28)	(21)	(13)	(8)	(11)	(24)	(41)
1 , 2	43.6	36	40.4	24.3	39.3	29.8	29.4	26.3	33.3	42.9	30.6	36.7
	(65)	(49)	(40)	(9)	(35)	(14)	(10)	(5)	(4)	(9)	(11)	(29)
2 , 2	8.7	8.1	9.1	5.4	6.7	10.6	8.8	5.3	0.0	4.8	2.8	11.4
	(13)	(11)	(9)	(2)	(6)	(5)	(3)	(1)	(0)	(1)	(1)	(9)
Total (n)	149	136	99	37	89	47	34	19	12	21	36	79

This table shows the frequency of the 3 genotypes of the *Msp I* polymorphism in the IL-1RN gene in controls and patient subgroups. n represents the number of genotypes in each group. The number in brackets is the actual number of subjects with a particular genotype. Comparisons were made between the control and patient group, control and patient subgroups, mild and severe groups, OFS=0 and OFS≥1,2,3,4 groups and different patient aetiologies for each genotype using the $\chi 2$ test and 2 x 2 contingency tables.

[a] = severe vs controls (70.3% vs 47.7%, $\chi 2=6.08$, p=0.014 (1df), Pc=0.028)

Table 6.7B: Frequency of IL-1RN *Msp I* alleles in controls, patients and patient subgroups

Allele	Controls %	Patients %	Mild AP %	Severe AP %	OFS=0 %	OFS≥1 %	OFS≥2 %	OFS≥3 %	OFS≥4 %	A AP %	I AP %	G AP %
1	69.5	73.9	70.7	82.4[a]	73.6	74.5	76.5	81.6	83.3	73.8	81.9[b]	70.3
	(207)	(201)	(140)	(61)	(131)	(70)	(52)	(31)	(20)	(31)	(59)	(111)
2	30.5	26.1	29.3	17.6[a]	26.4	25.5	23.5	18.4	16.7	26.2	18.1[b]	29.7
	(91)	(71)	(58)	(13)	(47)	(24)	(16)	(7)	(4)	(11)	(13)	(47)
Total (n)	298	272	198	74	178	94	68	38	24	42	72	158

This table shows the frequency of the 2 alleles of the *Msp I* polymorphism in the IL-1RN gene in controls and patient subgroups. n represents the number of alleles in each group. The number in brackets is the actual number of subjects with a particular allele. Comparisons were made between the control and patient group, control and patient subgroups, mild and severe groups, OFS=0 and OFS≥1,2,3,4 groups and different patient aetiologies for each allele using the $\chi 2$ test and 2 x 2 contingency tables.

[a] = severe vs controls (Allele 1: 82.4% vs 69.5%, $\chi 2$=4.95, p=0.026 (1df), Pc=0.026; Allele 2: 17.6% vs 30.5%, $\chi 2$=4.95, p=0.026 (1df), Pc=0.026)

[b]= idiopathic vs controls (Allele 1: 81.9% vs 69.5%, $\chi 2$=4.47, p=0.034 (1df), Pc=0.034; Allele 2: 18.1% vs 30.5%, $\chi 2$=4.47, p=0.034 (1df), Pc=0.034)

Summary of the results obtained at the *Msp I* locus

All of the significant data are discussed, however, only the data that are significant following correction for the number of comparisons involved are included in the tables.

Genotype frequency

Controls vs patients

No significant differences were found in the *Msp I* genotype frequencies between controls and patients.

Controls vs patient subgroups

When the patient group was subdivided according to severity, organ failure scores and aetiology, and comparisons made between the different subgroups and the controls, the 1,1 genotype was found to be significantly increased in the severe group compared to controls (70.3% vs 47.7%, $\chi 2=6.08$, p=0.014, Pc=0.028), while the 1,2 genotype was significantly decreased between the two groups (24.3 % vs 43.6%, $\chi 2=4.61$, p=0.032*). In addition, the 1,1 genotype was significantly increased in the idiopathic group compared to controls (66.7% vs 47.7%, $\chi 2 = 4.2$, p=0.041*).

Mild group vs severe group

Analysis of the genotypes with respect to severity showed the 1,1, genotype was significantly increased in the severe group compared to the mild group (70.3% vs 50.5%, $\chi 2=4.27$, p=0.039*).

OFS=0 group vs OFS≥1,2,3,4 groups/different combinations of the aetiological groups

There were no significant differences in *Msp I* genotype frequencies between the OFS=0 group and OFS≥1,2,3,4 groups or between the different combinations of the aetiological groups.

Allele frequency

Controls vs patients

No significant differences were found in the *Msp I* allelic frequencies between controls and patients.

Controls vs patient subgroups

When the patient group was subdivided according to severity, organ failure scores and aetiology and the subgroups compared with the controls, allele 1 was found to be significantly increased in the severe group compared to controls (82.4% vs 69.5% $\chi 2$=4.95, p=0.026, Pc=0.026), while allele 2 was significantly decreased between the same two groups (17.6% vs 30.5%, $\chi 2$=4.95, p=0.026, Pc=0.026). In addition, allele 1 was significantly increased in the idiopathic group compared to controls (81.9% vs 69.5%, $\chi 2$=4.47, p=0.034, Pc=0.034) while allele 2 was significantly decreased between the same groups (18.1% vs 30.5 %, $\chi 2$=4.47, p=0.034, Pc=0.034).

Mild group vs severe group

Analysis of the data with respect to severity showed allele 1 was significantly increased in the severe group compared to the mild group (82.4% vs 70.7%, $\chi 2$=3.84, p=0.05*) whereas allele 2 was significantly decreased between the 2 groups (17.6% vs 29.3%, $\chi 2$=3.84, p=0.05*).

OFS=0 group vs OFS≥1,2,3,4 groups, different combinations of the aetiological groups

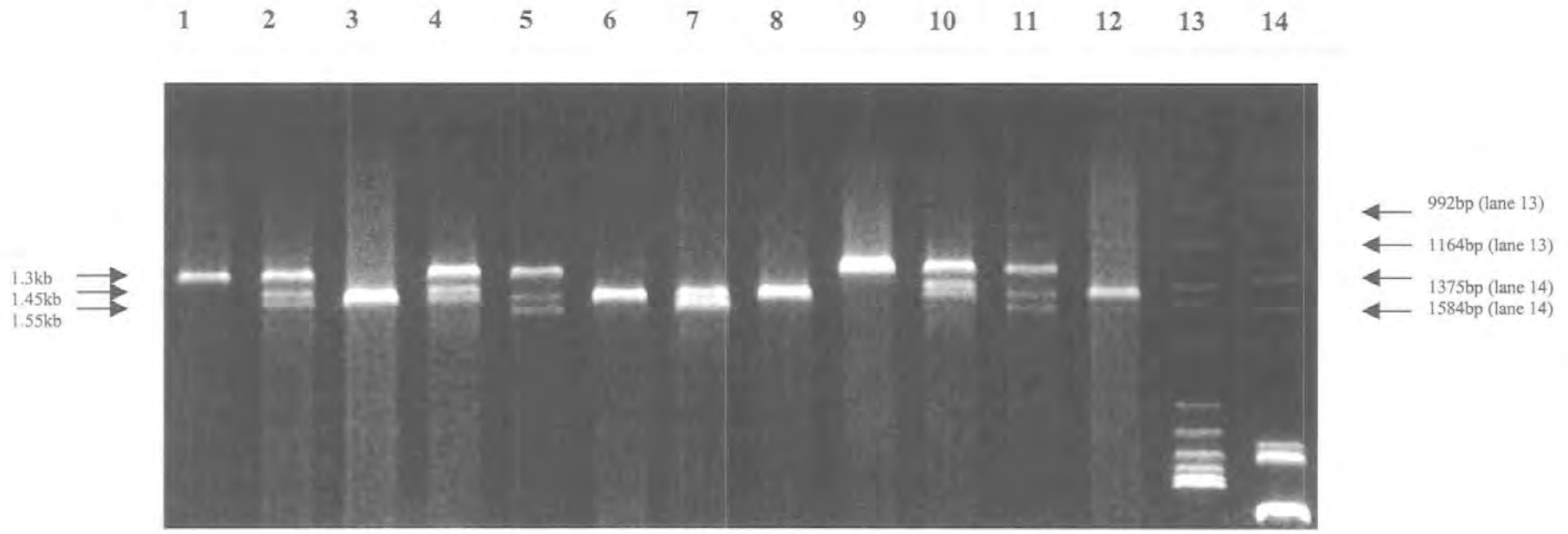
No significant differences in allele frequency were found when the mild group was compared with the severe group, OFS=0 group was compared with the OFS≥1,2,3,4 groups or between different combinations of the aetiological groups.

* p value became non-significant following correction for the number of comparisons made.

6.1.4.2 VLP polymorphism

Figure 6.1 illustrates the location of the VLP polymorphism in the IL-1 gene cluster. Three alleles (alleles 1-3) were detected in this study corresponding to 1.55kb, 1.45kb and 1.3kb fragments. Six genotypes were observed. Intermediate sized products of 1.4kb and 1.5kb were detected in 2,3 and 1,3 heterozygotes respectively. These artefacts were also present in the study by Steinkasserer et al, 1991 (Figure 6.9).

Figure 6.9: 2% agarose gel illustrating the genotypes observed at the VLP locus.



Three different alleles were detected: allele 1 (1.55kb), allele 2 (1.45kb), allele 3 (1.3kb).
Lanes 1 and 9 - 3,3
Lanes 2,4 and 10 - 2,3
Lanes 3,6,8 and 12 - 2,2
Lanes 5 and 11 - 1,3
Lane 7 - 1,2
Lane 13 - Molecular weight marker VII (0.37kbp-8.0kbp)
Lane 14 - Molecular weight marker III (0.12kbp-21.2kbp)
Intermediate sized artefacts of 1.4kb and 1.5kb were detected in 2,3 and 1,3 heterozygotes respectively. These were also present in the study by Steinkasserer et al, 1991.

Table 6.8A: Frequency of IL-1RN VLP genotypes in controls, patients and patient subgroups

Genotype	Controls %	Patients %	Mild AP %	Severe AP %	OFS=0 %	OFS≥1 %	OFS≥2 %	OFS≥3 %	OFS≥4 %	A AP %	I AP %	G AP %
1 , 1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
1 , 2	5.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	(8)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
1 , 3	2.7	1.5[a]	2	0.0	2.2	0.0	0.0	0.0	0.0	4.8	0.0	1.3
	(4)	(2)	(2)	(0)	(2)	(0)	(0)	(0)	(0)	(1)	(0)	(1)
2 , 2	42.5	55.9	50.5	70.3[b]	53.9	59.6	61.8	68.4	66.7	52.4	66.7[c]	51.9
	(62)	(76)	(50)	(26)	(48)	(28)	(21)	(13)	(8)	(11)	(24)	(41)
2 , 3	41.8	33.8	37.4	24.3	36	29.8	29.4	26.3	33.3	38.1	27.8	35.4
	(61)	(46)	(37)	(9)	(32)	(14)	(10)	(5)	(4)	(8)	(10)	(28)
3 , 3	7.5	8.8	10.1	5.4	7.9	10.6	8.8	5.3	0	4.8	5.6	11.4
	(11)	(12)	(10)	(2)	(7)	(5)	(3)	(1)	(0)	(1)	(2)	(9)
Total (n)	146	136	99	37	89	47	34	19	12	21	36	79

This table shows the frequency of the 6 genotypes of the VLP polymorphism in the IL-1RN gene in controls and patient subgroups. n represents the number of genotypes in each group. The number in brackets is the actual number of subjects with a particular genotype. Comparisons were made between the control and patient group, control and patient subgroups, mild and severe groups, OFS=0 and OFS≥1,2,3,4 groups and different patient aetiologies for each genotype using the $\chi 2$ test and 2 x 2 contingency tables.

[a] (sum total of 1,1;1,2;1,3)= patients vs controls (1.5% vs 8.2%, $\chi 2$=6.8, p=0.009 (1df), Pc=0.027)

[b] = severes vs controls (70.3% vs 42.5%, $\chi 2$=9.14, p=0.002 (1df), Pc=0.006)

[c] = idiopathics vs controls (66.7% vs 42.5%, $\chi 2$=6.79, p=0.009 (1df), Pc=0.027)

Table 6.8B: Frequency of IL-1RN VLP alleles in controls, patients and patient subgroups

Allele	Controls %	Patients %	Mild AP %	Severe AP %	OFS=0 %	OFS≥1 %	OFS≥2 %	OFS≥3 %	OFS≥4 %	A AP %	I AP %	G AP %
1	4.1	0.7[a]	1.0	0.0	1.1	0.0	0.0	0.0	0.0	2.4	0.0	0.6
	(12)	(2)	(2)	(0)	(2)	(0)	(0)	(0)	(0)	(1)	(0)	(1)
2	66.1	72.8	69.2	82.4[b]	71.9	74.5	76.5	81.6	83.3	71.4	80.6[c]	69.6
	(193)	(198)	(137)	(61)	(128)	(70)	(52)	(31)	(20)	(30)	(58)	(110)
3	29.8	26.5	29.8	17.6	27.0	25.5	23.5	18.4	16.7	26.2	19.4	29.7
	(87)	(72)	(59)	(13)	(48)	(24)	(16)	(7)	(4)	(11)	(14)	(47)
Total (n)	292	272	198	74	178	94	68	38	24	42	72	158

This table shows the frequency of the 3 alleles of the VLP polymorphism in the IL-1RN gene in controls and patient subgroups. n represents the number of alleles in each group. The number in brackets is the actual number of subjects with a particular allele. Comparisons were made between the control and patient group, control and patient subgroups, mild and severe groups, OFS=0 and OFS≥1,2,3,4 groups and different patient aetiologies for each allele using the $\chi 2$ test and 2 x 2 contingency tables.

[a] = patients vs controls (0.7% vs 4.1%, $\chi 2 = 6.62$, p=0.01(1df), Pc=0.02)

[b] = severe vs controls (82.4% vs 66.1%, $\chi 2 = 7.42$, p=0.006 (1df), Pc=0.012)

[c] = idiopathics vs controls (80.6% vs 66.1% $\chi 2=5.64$, p=0.018 (1df), Pc=0.036)

Summary of the results obtained at the VLP locus

All of the significant data are discussed, however, only the data that are significant following correction for the number of comparisons involved are included in the tables.

Genotype frequency

To make the genotype analysis easier, the genotypes were divided into 2,2; 2,3; 3,3 and "other" (1,1+1,2+1,3).

Controls vs patients

Comparisons of the VLP genotype frequencies between patients and controls showed that the 2,2 genotype was significantly increased in patients compared to controls (55.9% vs 42.5%, $\chi 2=5.07$, p=0.024*), whereas the 'other' genotype (1,1+1,2+1,3) was significantly decreased between the same groups (1.5% vs 8.2%, $\chi 2=6.8$, p=0.009, Pc=0.027).

Controls vs patient subgroups

When the patient group was subdivided according to severity, organ failure scores and aetiology and the subgroups compared with controls, the 'other' genotype (1,1+1,2+1,3) was significantly decreased in mild patients compared to controls (2.0% vs 8.2%, $\chi 2=4.21$, p=0.04*). The 2,2 genotype was significantly increased in the severe group compared to controls (70.3% vs 42.5%, $\chi 2=9.14$, p=0.002, Pc=0.006). The 2,2 genotype was significantly increased in the OFS≥1 group compared to controls (59.6% vs 42.5%, $\chi 2=4.18$, p=0.041*). The 'other' genotype (1,1+1,2+1,3) was significantly decreased in the OFS≥1 group compared to controls (0.0% vs 8.2%, $\chi 2=4.12$, p=0.042*). The 2,2 genotype was significantly increased in the OFS≥3 group compared to controls (68.4% vs 42.5 %, $\chi 2=4.57$, p=0.033*). The 2,2 genotype was significantly increased in the idiopathic group compared to controls (66.7% vs 42.5%, $\chi 2=6.79$, p=0.009, Pc=0.027). The 'other' genotype (1,1+1,2+1,3) was significantly decreased in the gallstone group compared to controls (1.3% vs 8.2%, $\chi 2=4.55$, p=0.033*).

Mild group vs severe group

Analysis of the data with respect to severity showed the 2,2 genotype was significantly increased in the severe group compared to the mild group (70.3% vs 50.5%, $\chi 2=4.27$, $p=0.039*$).

OFS=0 group vs OFS≥1,2,3,4 groups/different combinations of the aetiological groups

No significant differences in genotype frequency were observed when the OFS=0 group was compared with OFS≥1,2,3,4 groups or when the different combinations of the aetiological groups were compared.

Allele frequency

Controls vs patients

Comparisons of the VLP allelic frequencies between patients and controls showed allele 1 was significantly decreased in patients compared to controls (0.7% vs 4.1%, $\chi 2=6.62$, $p=0.01$, $Pc=0.02$).

Controls vs patient subgroups

When the patient group was subdivided according to severity, organ failure scores and aetiology and the subgroups compared with controls allele 1 was significantly decreased in the mild group compared to controls (1.0% vs 4.1%, $\chi 2=4.08$, $p=0.043*$). Allele 2 was significantly increased in the severe group compared to controls (82.4% vs 66.1%, $\chi 2 =7.42$, $p=0.006$, $Pc=0.012$) whereas allele 3 was significantly decreased between the same two groups (17.6% vs 29.8%, $\chi 2=4.44$, $p=0.035*$). Allele 1 was significantly decreased in the OFS≥1 group compared to controls (0.0% vs 4.1%, $\chi 2=3.99$, $p=0.046*$). Allele 2 was significantly increased in the idiopathic group compared to controls (80.6% vs 66.1%, $\chi 2=5.64$, $p=0.018$, $Pc=0.036$). Allele 1 was significantly decreased in the gallstone group compared to controls (0.6% vs 4.1%, $\chi 2=4.42$, $p=0.036*$).

Mild group vs severe group

Analysis of the data with respect to severity allele 2 was significantly increased in the severe group compared to the mild group (82.4% vs 69.2%, $\chi 2=4.77$, p=0.029*) whereas allele 3 was significantly decreased between the same groups (17.6% vs 29.8%, $\chi 2=4.14$, p=0.042*).

OFS=0 group vs OFS≥1,2,3,4 groups/different combinations of the aetiological groups

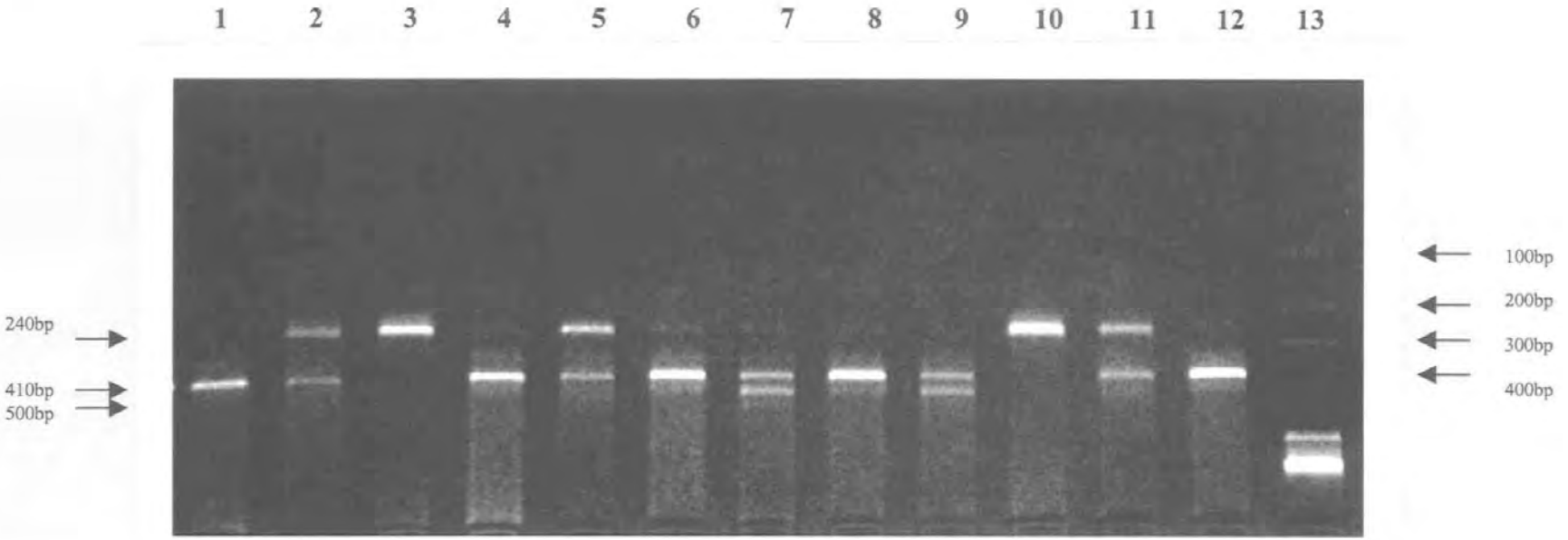
No significant differences in allele frequency were found between the OFS=0 group and OFS≥1,2,3,4 groups or between different combinations of the aetiological groups.

* p value became non-significant following correction for the number of comparisons made.

6.1.4.3. VNTR^{86} polymorphism

Figure 6.1 illustrates the location of the VNTR^{86} polymorphism in the IL-1 gene cluster. Three different alleles (alleles 1-3) were observed corresponding to 4 (410bp), 2 (240bp) and 5 (500bp) copies of the 86bp repeat sequence. Five genotypes were detected. Alleles 4 and 5 corresponding to 3 (325bp) and 6 (595bp) copies of the repeat sequence were not detected (Figure 6.10).

Figure 6.10: 2% agarose gel showing the genotypes detected at the IL-1RN VNTR^{86} locus.



Three different alleles (alleles 1-3) were observed corresponding to 4 (410bp), 2 (240bp) and 5 (500bp) copies of the 86bp repeat sequence.
Alleles 4 and 5 corresponding to 3 (325bp) and 6 (595bp) copies of the repeat sequence were not detected.
Lanes 1,4,6, 8 and 12 - 1,1
Lanes 2,5 and 11 - 1,2
Lanes 3 and 10 - 2,2
Lane 7 and 9 - 1,3
Lane 13 - Molecular weight marker XIV (100bp ladder)

Table 6.9A: Frequency of IL-1RN VNTR^{86} genotypes in controls, patients and patient subgroups

Genotype	Controls %	Patients %	Mild AP %	Severe AP %	OFS=0 %	OFS≥1 %	OFS≥2 %	OFS≥3 %	OFS≥4 %	A AP %	I AP %	G AP %
1 , 1	**42.0**	**54.3**	**47.5**	**69.4[a]**	**50.0**	**60.9[b]**	**63.6[c]**	**68.4**	**66.7**	**44.4**	**67.6[d]**	**50.0**
	(63)	(63)	(38)	(25)	(35)	(28)	(21)	(13)	(8)	(8)	(23)	(32)
1 , 2	**38.0**	**35.3**	**40.0**	**25.0**	**40.0**	**28.3**	**27.3**	**26.3**	**33.3**	**44.4**	**29.4**	**35.9**
	(57)	(41)	(32)	(9)	(28)	(13)	(9)	(5)	(4)	(8)	(10)	(23)
2 , 2	**12.0**	**10.3**	**12.5**	**5.6**	**10.0**	**10.9**	**9.1**	**5.3**	**0.0**	**11.1**	**2.9[e]**	**14.1**
	(18)	(12)	(10)	(2)	(7)	(5)	(3)	(1)	(0)	(2)	(1)	(9)
1 , 3	**4.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
	(6)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
2 , 3	**4.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
	(6)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
Total (n)	**150**	**116**	**80**	**36**	**70**	**46**	**33**	**19**	**12**	**18**	**34**	**64**

This table shows the frequency of the 5 genotypes of the VNTR^{86} polymorphism in the IL-1RN gene in controls and patient subgroups. n represents the number of genotypes in each group. The number in brackets is the actual number of subjects with a particular genotype. Comparisons were made between the control and patient group, control and patient subgroups, mild and severe groups, OFS=0 and OFS≥1,2,3,4 groups and different patient aetiologies for each genotype using the $\chi 2$ test and 2 x 2 contingency tables.

[a] = severe vs controls (69.4% vs 42.0%, $\chi 2$ =8.77, p=0.003 (1df), Pc=0.006)

[b] = OFS≥1 vs controls (60.9% vs 42.0%, $\chi 2$=5.04, p=0.025 (1df), Pc<0.05)

[c] = OFS≥2 vs controls (63.6% vs 42.0%, $\chi 2$=5.1, p=0.024 (1df), Pc=0.048)

[d] = idiopathics vs controls (67.6 % vs 42.0%, $\chi 2$ = 7.32, p=0.007 (1df), Pc=0.014)

[e] (sum total of 2,2;1,3;2,3) = idiopathics vs controls (2.9% vs 20.0%, $\chi 2$=5.76, p=0.016 (1df), Pc=0.032)

Table 6.9B: Frequency of IL-1RN VNTR^{86} alleles in controls, patients and patient subgroups

Allele	Controls %	Patients %	Mild AP %	Severe AP %	OFS=0 %	OFS≥1 %	OFS≥2 %	OFS≥3 %	OFS≥4 %	A AP %	I AP %	G AP %
1	63.0 (189)	72.0 (167)	67.5 (108)	81.9[cj] (59)	70.0 (98)	75.0 (69)	77.3 (51)	81.6[f] (31)	83.3 (20)	66.7 (24)	82.4[g] (56)	68.0 (87)
2	33.0 (99)	28.0 (65)	32.5 (52)	18.1[dj] (13)	30.0 (42)	25.0 (23)	22.7 (15)	18.4 (7)	16.7 (4)	33.3 (12)	17.6[h] (12)	32.0 (41)
3	4.0 (12)	0.0[a] (0)	0.0[b] (0)	0.0 (0)	0.0[e] (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0[i] (0)
Total (n)	300	232	160	72	140	92	66	38	24	36	68	128

This table shows the frequency of the 3 alleles of the VNTR^{86} polymorphism in the IL-1RN gene in controls and patient subgroups. n represents the number of alleles in each group. The number in brackets is the actual number of subjects with a particular allele. Comparisons were made between the control and patient group, control and patient subgroups, mild and severe groups, OFS=0 and OFS≥1,2,3,4 groups and different patient aetiologies for each allele using the $\chi 2$ test and 2 x 2 contingency tables.

[a] = patients vs controls (0.0% vs 4.0%, $\chi 2$=9.49, p=0.002 (1df), Pc=0.004)

[b] = mild vs controls (0.0% vs 4.0%, $\chi 2$=6.57, p=0.01 (1df), Pc=0.02)

[c] = severe vs controls (81.9% vs 63.0%, $\chi 2$=9.38, p=0.002 (1df), Pc=0.004)

[d] = severe vs controls (18.1% vs 33.0%, $\chi 2$=6.16, p=0.013 (1df), Pc=0.026)

[e] = OFS=0 vs controls (0.0% vs 4.0%, $\chi 2$=5.76, p=0.016 (1df), Pc=0.032)

[f] = OFS≥3 vs controls (81.6% vs 63.0%, $\chi 2$=5.12, p=0.024 (1df), Pc=0.048)

[g] = idiopathics vs controls (82.4% vs 63.0%, $\chi 2$ =9.33, p=0.002 (1df), Pc=0.004)

[h] = idiopathics vs controls (17.6% vs 33.0%, $\chi 2$=6.2, p=0.013 (1df), Pc=0.026)

[i] = gallstones vs controls (0.0% vs 4.0%, $\chi 2$=5.27, p=0.022 (1df), Pc=0.044)

[j] = severe vs mild (Allele 1: 81.9% vs 67.5%, $\chi 2$=5.14, p=0.023 (1df), Pc=0.046; Allele 2: 18.1% vs 32.5%, $\chi 2$=5.14, p=0.023 (1df), Pc=0.046)

Summary of the results obtained at the IL-1RN VNTR^{86} locus

All of the significant data are discussed, however, only the data that are significant following correction for the number of comparisons involved are included in the tables.

Genotype frequency

To make the genotype analysis easier, the genotypes were divided into 1,1; 1,2 and "other" (2,2+1,3+2,3).

Controls vs patients

Comparisons of the IL-1RN VNTR^{86} genotype frequencies between patients and controls showed the 1,1 genotype was significantly increased in patients compared to controls (54.3% vs 42.0%, $\chi 2=3.98$, $p=0.046*$) while the 'other' genotype (2,2+1,3+2,3) was significantly decreased between the groups (10.3% vs 20.0%, $\chi 2=4.59$, $p=0.032*$).

Controls vs patient subgroups

When the patient group was subdivided according to severity, organ failure scores and aetiology and the subgroups compared with controls, the 1,1 genotype was significantly increased in the severe group compared to controls (69.4% vs 42.0%, $\chi 2=8.77$, $p=0.003$, $Pc=0.006$) while the 'other' genotype (2,2+1,3+2,3) was significantly decreased between the two groups (5.6% vs 20.0%, $\chi 2=4.25$, $p=0.039*$). The 1,1 genotype was significantly increased in the OFS≥1 group compared to controls (60.9% vs 42.0%, $\chi 2=5.04$, $p=0.025$, $Pc<0.05$). The 1,1 genotype was significantly increased in the OFS≥2 group compared to controls (63.6% vs 42.0%, $\chi 2=5.1$, $p=0.024$, $Pc=0.048$). The 1,1 genotype was significantly increased in the idiopathic group compared to controls (67.6% vs 42.0%,$\chi 2=7.32$, $p=0.007$, $Pc=0.014$) whereas the 'other' group (2,2+1,3+2,3) was significantly decreased between the groups (2.9% vs 20.0%, $\chi 2=5.76$, $p=0.016$, $Pc=0.032$).

Mild group vs severe group

Analysis of the data with respect to severity showed the 1,1 genotype was significantly increased in the severe group compared to the mild group (69.4% vs 47.5%, $\chi 2$=4.82, p=0.028*).

OFS=0 group vs OFS≥1,2,3,4 groups

When the data was analysed with respect to organ failure scores, the 1,1 genotype was significantly increased in the OFS≥3 group compared to the OFS=0 group (68.4 % vs 50.0%, $\chi 2$=4.76, p=0.029*).

Different combinations of the aetiological groups

No significant differences in IL-1RN VNTR^{86} genotype frequency were found between the different combinations of the aetiological groups.

Allele frequency

Controls vs patients

Comparisons of the IL-1RN VNTR^{86} allelic frequencies between patients and controls showed allele 1 was significantly increased in the patient group compared to control group (72.0% vs 63.0%,$\chi 2$ =4.77, p=0.029*), whereas allele 3 was significantly decreased between the groups (0.0% vs 4.0%,$\chi 2$=9.49, p=0.002, Pc=0.004).

Controls vs patient subgroups

When the patient group was subdivided according to severity, organ failure scores and aetiology and compared with controls, allele 3 was significantly decreased in the mild group compared to controls (0.0% vs 4.0%,$\chi 2$=6.57, p=0.01, Pc=0.02). Allele 1 was significantly increased in the severe group compared to controls (81.9% vs 63.0%, $\chi 2$=9.38, p=0.002, Pc=0.004) whereas allele 2 was significantly decreased between the same two groups (18.1% vs 33.0%, $\chi 2$=6.16, p=0.013, Pc=0.026). Allele 3 was significantly decreased in OFS=0 group compared to controls (0.0% vs 4.0%, $\chi 2$=5.76,

p=0.016, Pc=0.032). Allele 1 was significantly increased in the OFS≥1 group compared to controls (75.0% vs 63.0%, $\chi 2$=4.51, p=0.034*). Allele 1 was significantly increased in the OFS≥2 group compared to controls (77.3% vs 63.0%, $\chi 2$=4.88, p=0.027*). Allele 1 was significantly increased in the OFS≥3 group compared to controls (81.6% vs 63.0%, $\chi 2$=5.12, p=0.024, Pc=0.048). Allele 1 was significantly increased in the OFS≥4 group compared to controls (83.3% vs 63.0%, $\chi 2$=4.01, p=0.045*). Allele 1 was significantly increased in the idiopathic group compared to controls (82.4% vs 63.0%, $\chi 2$=9.33, p=0.002, Pc=0.004). Allele 2 was significantly decreased in the idiopathic group compared to controls (17.6% vs 33.0%,$\chi 2$=6.2, p=0.013, Pc=0.026). Allele 3 was significantly decreased in the gallstone group compared to controls (0.0% vs 4.0%, $\chi 2$=5.27, p=0.022, Pc=0.044).

Mild vs severe

Analysis of the allele data with respect to severity showed allele 1 was significantly increased in the severe group compared to mild group (81.9% vs 67.5%, $\chi 2$=5.14, p=0.023, Pc=0.046) whereas allele 2 was significantly decreased between the same groups (18.1% vs 32.5%,$\chi 2$=5.14, p=0.023, Pc=0.046).

OFS=0 group vs OFS≥1,2,3,4 groups

No significant differences in allele frequency were seen when comparing OFS=0 group with OFS≥1,2,3,4 groups.

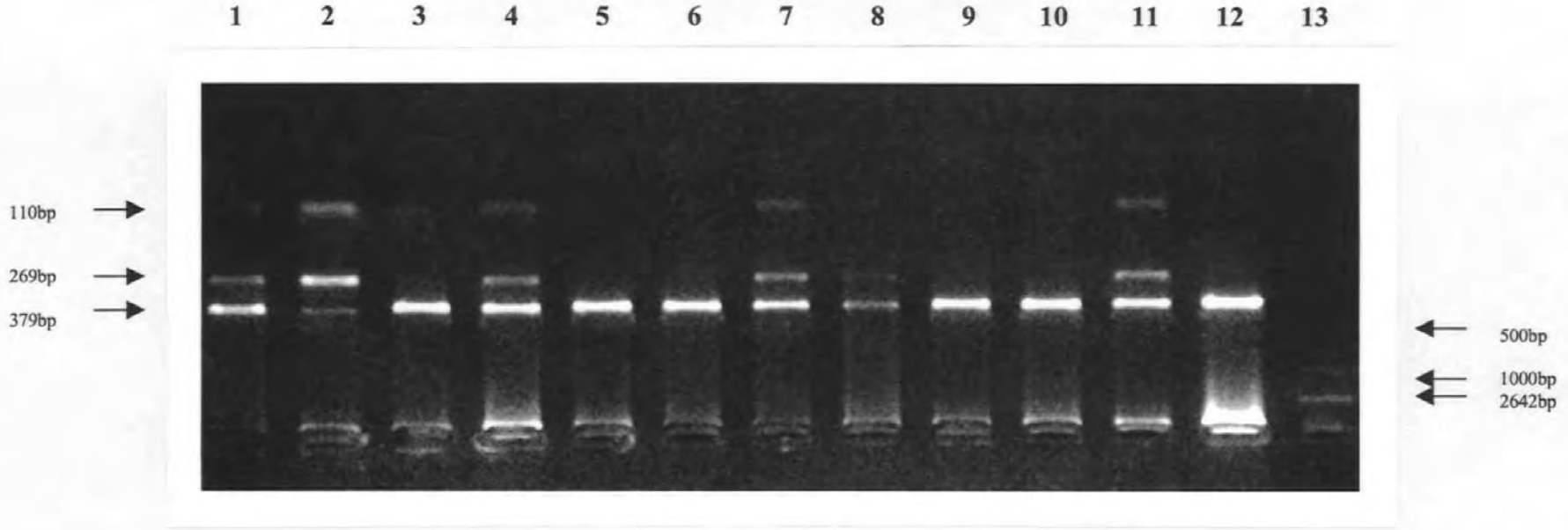
Different combinations of the aetiological groups

When the data was analysed with respect to aetiology, allele 1 was significantly increased in the idiopathic group compared to the gallstone group (82.4% vs 68.0%, $\chi 2$=4.66, p=0.031*) whereas allele 2 was significantly decreased between the same groups (17.6% vs 32.0%, $\chi 2$=4.66, p=0.031*). * p value became non-significant following correction for the number of comparisons made.

6.1.4.4 Ssp I polymorphism

Figure 6.1 illustrates the location of the *Ssp I* polymorphism in the IL-1 gene cluster. *Ssp I* digestion of the fragment amplified with amplimers *Ssp I* and *II* identified allele 1 of 379bp and allele 2 of 110bp, 269bp. Three genotypes were observed: 1,1;1,2;2,2. The $A \rightarrow T^{9589}$ SNP creates a *Ssp I* restriction site. Homozygotes for allele 1 (379bp) occur when the SNP is not present on either chromosome. Homozygotes for allele 2 (110bp, 269bp) occur when the SNP is present on both chromosomes. Heterozygotes (110bp, 269bp, 379bp) occur when the SNP is present on only one chromosome (Figure 6.11).

Figure 6.11: 3% agarose gel displaying the genotypes observed at the *Ssp I* locus.



Two alleles were detected: allele 1 (379bp), allele 2 (269bp,110bp).
Lanes 1,4,7,8 and 11 - 1,2
Lane 2 - 2,2
Lanes 3,5,6,9,10 and 12 - 1,1
Lane 13 - Molecular weight marker XIV (100bp ladder)

Table 6.10A: Frequency of IL-1RN *Ssp I* genotypes in controls, patients and patient subgroups

Genotype	Controls %	Patients %	Mild AP %	Severe AP %	OFS=0 %	OFS≥1 %	OFS≥2 %	OFS≥3 %	OFS≥4 %	A AP %	I AP %	G AP %
1 , 1	46.2	55.1	49.5	70.3[a]	52.8	59.6	61.8	68.4	66.7	52.4	63.9	51.9
	(67)	(75)	(49)	(26)	(47)	(28)	(21)	(13)	(80	(11)	(23)	(41)
1 , 2	46.2	37.5	42.4	24.3[b]	41.6	29.8	29.4	26.3	33.3	42.9	36.1	36.7
	(67)	(51)	(42)	(9)	(37)	(14)	(10)	(5)	(4)	(9)	(13)	(29)
2 , 2	7.6	7.4	8.1	5.4	5.6	10.6	8.8	5.3	0.0	4.8	0.0	11.4
	(11)	(10)	(8)	(2)	(5)	(5)	(3)	(1)	(0)	(1)	(0)	(9)
Total (n)	145	136	99	37	89	47	34	19	12	21	36	79

This table shows the frequency of the 3 genotypes of the *Ssp I* polymorphism in the IL-1RN gene in controls and patient subgroups. n represents the number of genotypes in each group. The number in brackets is the actual number of subjects with a particular genotype. Comparisons were made between the control and patient group, control and patient subgroups, mild and severe groups, OFS=0 and OFS≥1,2,3,4 groups and different patient aetiologies for each genotype using the $\chi 2$ test and 2 x 2 contingency tables.

[a] = severe vs controls (70.3% vs 46.2%, $\chi 2$=6.83, p=0.009 (1df), Pc=0.018)

[b] = severe vs controls (24.3% vs 46.2%, $\chi 2$ =5.8, p=0.016 (1df), Pc=0.032)

Table 6.10B: Frequency of IL-1RN *Ssp I* alleles in controls, patients and patient subgroups

Allele	Controls %	Patients %	Mild AP %	Severe AP %	OFS=0 %	OFS≥1 %	OFS≥2 %	OFS≥3 %	OFS≥4 %	A AP %	I AP %	G AP %
1	69.3	73.9	70.7	82.4[a]	73.6	74.5	76.5	81.6	83.3	73.8	81.9[b]	70.3
	(201)	(201)	(140)	(61)	(131)	(70)	(52)	(31)	(20)	(31)	(59)	(111)
2	30.7	26.1	29.3	17.6[a]	26.4	25.5	23.5	18.4	16.7	26.2	18.1[b]	29.7
	(89)	(71)	(58)	(13)	(47)	(24)	(16)	(7)	(4)	(11)	(13)	(47)
Total (n)	290	272	198	74	178	94	68	38	24	42	72	158

This table shows the frequency of the 2 alleles of the *Ssp I* polymorphism in the IL-1RN gene in controls and patient subgroups. n represents the number of alleles in each group. The number in brackets is the actual number of subjects with a particular allele. Comparisons were made between the controls and patient subgroups for each allele using the $\chi 2$ test and 2 x 2 contingency tables.

[a] = severe vs controls (Allele 1: 82.4% vs 69.3%, $\chi 2$ =5.03, p=0.025 (1df), Pc=0.025, Allele 2: 17.6 vs 30.7%, $\chi 2$ =5.03, p=0.025 (1df), Pc=0.025)

[b] = idiopathics vs controls (Allele 1 : 81.9% vs 69.3%, $\chi 2$ =4.55, p=0.033 (1df), Pc=0.033; Allele 2: 18.1% vs 30.7%, $\chi 2$ =4.55, p=0.033 (1df), Pc=0.033)

Summary of the results obtained at the *Ssp I* locus

All of the significant data are discussed, however, only the data that are significant following correction for the number of comparisons involved are included in the tables.

Genotype frequency

Controls vs patients

No significant differences in genotype frequency were seen between controls and patients.

Controls vs patient subgroups

When the patient group was subdivided according to severity, organ failure scores and aetiology and the subgroups compared with controls, the 1,1 genotype was significantly increased in the severe group compared to controls (70.3% vs 46.2%, $\chi 2=6.83$, p=0.009, Pc=0.018), whereas the 1,2 genotype was significantly decreased between the same groups (24.3% vs 46.2%, $\chi 2=5.8$, p=0.016, Pc=0.032). The 1,2 genotype was significantly decreased in the OFS≥1 group compared to controls (29.8% vs 46.2%, $\chi 2=3.92$, p=0.048*).

Mild group vs severe group

Analysis of the genotype data with respect to severity showed the 1,1 genotype was significantly increased in the severe group compared to the mild group (70.3 % vs 49.5%, $\chi 2=4.7$, p=0.03*).

OFS=0 group vs OFS≥1,2,3,4 groups

No significant differences in genotype frequency were found between the OFS=0 group and the OFS≥1,2,3,4 groups.

Different combinations of the aetiological groups

When the data was analysed with respect to aetiology, the 2,2 genotype was significantly decreased in the idiopathic group compared to the gallstone group (0.0% vs 11.4%, $\chi 2=4.45$, p=0.035*).

Allele frequency

Controls vs patients

No significant differences in allele frequency were found between controls and patients.

Controls vs patient subgroups

When the patient group was subdivided according to severity, organ failure scores and aetiology and the subgroups compared with controls, allele 1 was significantly increased in the severe group compared to controls (82.4% vs 69.3%, $\chi 2=5.03$, p=0.025, Pc=0.025) whereas allele 2 was significantly decreased in the severe group compared to controls (17.6% vs 30.7%, $\chi 2=5.03$, p=0.025, Pc=0.025). Allele 1 was significantly increased in the idiopathic group compared to controls (81.9% vs 69.3%, $\chi 2=4.55$, p=0.033, Pc=0.033) while allele 2 was significantly decreased between the same groups (18.1% vs 30.7%, $\chi 2=4.55$, p=0.033, Pc=0.033).

Mild group vs severe group

Analysis of the data with respect to severity showed allele 1 was significantly increased in the severe group compared to the mild group (82.4% vs 70.7%, $\chi 2=3.84$, p=0.05*) whereas allele 2 was significantly decreased between the 2 groups (17.6% vs 29.3%, $\chi 2=3.84$, p=0.05*).

OFS=0 group vs OFS≥1,2,3,4 groups/different combinations of the aetiological groups

No significant differences in allele frequency were observed between the mild and severe group, OFS=0 group and OFS≥1,2,3,4 groups or between different combinations of the aetiological groups.

* p value became non-significant following correction for the number of comparisons made.

6.1.5 Haplotype Analysis

In addition to analysing the genotypes of each polymorphism between controls, patients and patient subgroups, the genotypes of the different genes were combined to give haplotypes and analysed to determine whether the genes studied were linked and hence influence each others expression. Only 2 genes were compared at any one time since the combination of more than 2 genes would make the number of subjects too small following removal of the double heterozygotes that no significant data could be obtained. The haplotype analysis compared the total controls and total patient population only. All of the significant data are discussed, however, only the data that are significant following correction for the number of comparisons involved are included in the tables. With the exception of the (X)n haplotypes, the data in each of the following tables is displayed with the most significant result at the top and least significant at the bottom of the table.

Table 6.11A: Frequency of (AC)n-VNTR[46] haplotype in the control population

(AC)n-VNTR[46] Haplotype	observed (n)	frequency (%)	expected (n)	frequency (%)	χ^2
2-2	22	15.7	6.9	4.9	33.04[a]
2-1	7	5.0	24.9	17.8	12.87[b]
1-2	3	2.1	11.4	8.1	6.19
2-6	3	2.1	0.8	0.6	6.05
2-4	2	1.4	0.5	0.4	4.50
4-2	1	0.7	5.2	3.7	3.39
4-1	26	18.6	18.7	13.4	2.85
1-3	8	5.7	4.7	3.4	2.32
1-1	48	34.3	40.8	29.1	1.27
5-1	8	5.7	5.5	3.9	1.14
3-3	1	0.7	0.4	0.3	0.90
0-1	2	1.4	1.4	1.0	0.26
8-1	2	1.4	1.4	1.0	0.26
2-3	2	1.4	2.8	2.0	0.23
6-1	1	0.7	0.7	0.5	0.13
3-1	3	2.1	3.5	2.5	0.07
3-2	1	0.7	1.0	0.7	0.00
0-3	0	0.0	-	-	-
5-2	0	0.0	-	-	-
X-X	0	0.0	9.4	6.7	9.40[c]

This table shows the frequency and actual (observed) number of copies of the (AC)n-VNTR[46] haplotype detected within the control population. The X-X haplotype represents the sum of the haplotypes that were not detected. Haplotypes were assigned in those subjects who were homozygous at one or both loci. The expected haplotype frequencies were obtained from the individual allelic frequencies. (The haplotype frequency analysis does not take into account individuals who were heterozygous at more than one locus). Comparisons were made between the observed and expected (AC)n-VNTR[46] haplotypes and $\chi 2$ values were determined. The $\chi 2$ test was used to estimate linkage disequilibrium between the loci.

[a] 2-2 (15.7% vs 4.9%, $\chi 2=33.04$, $p<0.0000001$ (1df), $Pc<0.000002$)

[b] 2-1 (5.0% vs 17.8%, $\chi 2=12.87$, $p=0.0003$ (1df), $Pc=0.005$)

[c] X-X (0.0% vs 6.7% $\chi 2= 9.40$, $p=0.002$ (1df), $Pc=0.03$)

Table 6.11B Frequency of (AC)n-VNTR[46] haplotype in the patient population

(AC)n-VNTR[46] Haplotype	observed (n)	frequency (%)	expected (n)	frequency (%)	χ^2
2-2	17	13.7	5.9	4.8	20.88 [a]
2-1	9	7.3	23.8	19.2	9.20 [b]
1-2	1	0.8	8.4	6.8	6.52
2-6	3	2.4	0.9	0.7	4.90
4-1	21	16.9	14.2	11.5	3.26
0-3	1	0.8	0.2	0.2	3.20
4-2	1	0.8	3.5	2.8	1.79
1-3	12	9.7	8.8	7.1	1.16
1-1	39	31.5	33.5	27.0	0.90
3-1	4	3.2	2.6	2.1	0.75
6-1	3	2.4	1.9	1.5	0.64
2-3	8	6.5	6.2	5.0	0.52
5-1	4	3.2	3.2	2.6	0.20
5-2	1	0.8	0.8	0.6	0.05
0-1	0	0.0	-	-	-
2-4	0	0.0	-	-	-
3-2	0	0.0	-	-	-
3-3	0	0.0	-	-	-
8-1	0	0.0	-	-	-
X-X	0	0.0	10.1	8.1	10.10 [c]

This table shows the frequency and actual (observed) number of copies of the (AC)n-VNTR[46] haplotype detected within the patient population. The X-X haplotype represents the sum of the haplotypes that were not detected. Haplotypes were assigned in those subjects who were homozygous at one or both loci. The expected haplotype frequencies were obtained from the individual allelic frequencies. (The haplotype frequency analysis does not take into account individuals who were heterozygous at more than one locus). Comparisons were made between the observed and expected (AC)n-VNTR[46] haplotypes and $\chi 2$ values were determined. The $\chi 2$ test was used to estimate linkage disequilibrium between the loci.

[a] 2-2 (13.7% vs 4.8%, $\chi 2$=20.88, p=0.000005 (1df), Pc=0.00007)

[b] 2-1 (7.3% vs 19.2%, $\chi 2$=9.20, p=0.002 (1df), Pc=0.03)

[c] X-X (0.0% vs 8.1% $\chi 2$=10.10, p=0.001 (1df), Pc=0.01)

Summary of the results obtained for analysis of the (AC)n-VNTR^{46} haplotypes

All of the significant data are discussed, however, only the data that are significant following correction for the number of comparisons involved are included in the tables. Tables 6.11A and 6.11B show that the (AC)n-VNTR^{46} polymorphisms of the IL-1A gene are linked. In both control and patient groups, the most common haplotype was found to be 1-1 with an observed frequency of 34.3% in controls and 31.5% in patients.

Controls

Significantly more of the 2-2 (15.7% vs 4.9%, $\chi 2$=33.04, p<0.0000001, Pc<0.000002), 2-6 (2.1% vs 0.6%, $\chi 2$ =6.05, p=0.01*) and 2-4 (1.4% vs 0.4%, $\chi 2$ =4.50, p=0.03*) haplotypes and significantly less of the 2-1 (5.0% vs 17.8%, $\chi 2$=12.87, p=0.0003, Pc=0.005), X-X (0.0% vs 6.7%, $\chi 2$=9.40, p=0.002, Pc=0.03) and 1-2 (2.1% vs 8.1%, $\chi 2$ =6.19, p=0.01*) haplotypes were observed in controls than expected.

Patients

There were significantly more of the 2-2 (13.7% vs 4.8%, $\chi 2$ = 20.88, p=0.000005, Pc=0.00007) and 2-6 (2.4% vs 0.7%, $\chi 2$ = 4.90, p=0.03*) haplotypes than expected in the patient group and a significant reduction in the number of the X-X (0.0% vs 8.1%, $\chi 2$ =10.10, p=0.001, Pc=0.01), 2-1 (7.3% vs 19.2%, $\chi 2$ =9.20, p=0.002, Pc=0.03) and 1-2 (0.8% vs 6.8%, $\chi 2$ = 6.52, p=0.01*) haplotypes.

* p value became non-significant following correction for the number of comparisons made.

Table 6.12A: Frequency of *AvaI-AluI-TaqI* haplotype in the control population

AvaI-AluI-TaqI Haplotype	observed (n)	frequency (%)	expected (n)	frequency (%)	χ^2
2 - 2 - 1	40	33.9	11.4	9.7	71.75 [a]
2 - 1 - 1	0	0.0	22.2	18.8	22.20 [b]
1 - 2 - 1	0	0.0	22.2	18.8	22.20 [c]
1 - 1 - 2	19	16.1	8.3	7.0	13.79 [d]
1 - 1 - 1	59	50.0	43.3	36.7	5.69 [e]
2 - 1 - 2	0	0.0	4.3	3.6	4.30
1 - 2 - 2	0	0.0	4.3	3.6	4.30
2 - 2 - 2	0	0.0	2.2	1.9	2.20

This table shows the frequency and actual (observed) number of copies of the *AvaI-AluI-TaqI* haplotype detected within the control population. Haplotypes were assigned in those subjects who were homozygous at two or more loci. The expected haplotype frequencies were obtained from the individual allelic frequencies. (The haplotype frequency analysis does not take into account individuals who were heterozygous at more than one locus). Comparisons were made between the observed and expected *AvaI-AluI-TaqI* haplotypes and $\chi 2$ values were determined. The $\chi 2$ test was used to estimate linkage disequilibrium between the loci.

[a] 2-2-1 (33.9% vs 9.7%, $\chi 2$ =71.75, p<0.0000001 (1df), Pc<0.0000002)

[b] 2-1-1 (0.0% vs 18.8%, $\chi 2$=22.20, p=0.000002 (1df), Pc=0.000004)

[c] 1-2-1 (0.0% vs 18.8%, $\chi 2$=22.20, p=0.000002 (1df), Pc=0.000004)

[d] 1-1-2 (16.1% vs 7.0%, $\chi 2$=13.79, p=0.0002 (1df), Pc=0.0004)

[e] 1-1-1 (50.0% vs 36.7%, $\chi 2$=5.69, p=0.02 (1df), Pc=0.04)

Table 6.12B: Frequency of *AvaI-AluI-TaqI* haplotypes in the patient population

AvaI-AluI-TaqI Haplotype	observed (n)	frequency (%)	expected (n)	frequency (%)	χ^2
2 - 2 - 1	17	14.9	2.9	2.5	68.56 [a]
2 - 1 - 1	0	0.0	12.3	10.8	12.30 [b]
1 - 2 - 1	1	0.9	13.8	12.1	11.87 [c]
2 - 2 - 2	3	2.6	0.9	0.8	4.90
2 - 1 - 2	0	0.0	3.8	3.3	3.80
1 - 2 - 2	1	0.9	4.3	3.8	2.53
1 - 1 - 1	69	60.5	57.9	50.8	2.13
1 - 1 - 2	23	20.2	18.0	15.8	1.39

This table shows the frequency and actual (observed) number of copies of the *AvaI-AluI-TaqI* haplotype detected within the patient population. Haplotypes were assigned in those subjects who were homozygous at two or more loci. The expected haplotype frequencies were obtained from the individual allelic frequencies. (The haplotype frequency analysis does not take into account individuals who were heterozygous at more than one locus). Comparisons were made between the observed and expected *AvaI-AluI-TaqI* haplotypes and $\chi 2$ values were determined. The $\chi 2$ test was used to estimate linkage disequilibrium between the loci.

[a] 2-2-1 (14.9% vs 2.5%, $\chi 2=68.56$, $p<0.0000001$ (1df), $Pc<0.0000005$)

[b] 2-1-1 (0.0% vs 10.8%, $\chi 2=12.30$, $p=0.0005$ (1df), $Pc=0.003$)

[c] 1-2-1 (0.9% vs 12.1%, $\chi 2=11.87$, $p=0.0006$ (1df), $Pc=0.003$)

Summary of the results obtained for analysis of the *AvaI-AluI-TaqI* haplotypes

All of the significant data are discussed, however, only the data that are significant following correction for the number of comparisons involved are included in the tables. Tables 6.12A and 6.12B indicate that the *AvaI-AluI-TaqI* polymorphisms of the IL-1B gene are linked. In both control and patient groups, the most common haplotype was found to be 1-1-1 with an observed frequency of 50.0% in controls and 60.5% in patients.

Controls:

Significantly more of the 2-2-1 (33.9% vs 9.7%, $\chi 2$ =71.75, p<0.0000001, Pc<0.0000002), 1-1-2 (16.1% vs 7.0%, $\chi 2$ =13.79, p=0.0002, Pc=0.0004) and 1-1-1 (50.0% vs 36.7%, $\chi 2$ =5.69, p=0.02, Pc=0.04) haplotypes and significantly less of the 2-1-1 (0.0% vs 18.8%, $\chi 2$ =22.20, p=0.000002, Pc=0.000004), 1-2-1 (0.0% vs 18.8%, $\chi 2$ = 22.20, p=0.000002, Pc=0.000004), 1-2-2 (0.0% vs 3.6%, $\chi 2$ =4.30, p=0.04*) and 2-1-2 (0.0% vs 3.6%, $\chi 2$ =4.30, p=0.04*) haplotypes were observed in controls than expected.

Patients:

There was significantly more of the 2-2-1 (14.9% vs 2.5%, $\chi 2$ =68.56, p<0.0000001, Pc<0.0000005) and 2-2-2 (2.6% vs 0.8%, $\chi 2$ = 4.90 p=0.03*) haplotypes than expected in the patient group with a significant reduction in the number of 2-1-1 (0.0% vs 10.8%, $\chi 2$ =12.30, p=0.0005, Pc=0.003) and 1-2-1 (0.9% vs 12.1%, $\chi 2$ = 11.87, p=0.0006, Pc=0.003) haplotypes.

* p value became non-significant following correction for the number of comparisons made.

Table 6.13A Frequency of *MspI*-VLP-VNTR^{86}-*SspI* haplotype in the control population

MspI-VLP-VNTR^{86}-*SspI* Haplotype	observed (n)	frequency (%)	expected (n)	frequency (%)	χ^2
2 - 3 - 2 - 2	16	15.7	0.1	0.1	2528.10[a]
1 - 2 - 1 - 1	86	84.3	51.5	50.5	23.11[b]
1 - 2 - 2 - 1	0	0.0	9.6	9.4	9.60[c]
1 - 2 - 1 - 2	0	0.0	9.6	9.4	9.60[d]
2 - 3 - 2 - 1	0	0.0	0.3	0.3	0.30
X-X-X-X	0	0.0	30.9	30.3	30.90[e]

This table shows the frequency and actual (observed) number of copies of the *MspI*-VLP-VNTR^{86}-*SspI* haplotype detected within the control population. The X-X-X-X haplotype represents the sum of the haplotypes that were not detected. Haplotypes were assigned in those subjects who were homozygous at three or more loci. The expected haplotype frequencies were obtained from the individual allelic frequencies. (The haplotype frequency analysis does not take into account individuals who were heterozygous at more than one locus). Comparisons were made between the observed and expected *MspI*-VLP-VNTR^{86}-*SspI* haplotypes and $\chi 2$ values were determined. The $\chi 2$ test was used to estimate linkage disequilibrium between the loci.

[a] 2-3-2-2 (15.7% vs 0.1%, $\chi 2$=2528.10, p<0.0000001 (1df), Pc<0.0000001)

[b] 1-2-1-1 (84.3% vs 50.5%, $\chi 2$=23.11, p=0.000001 (1df), Pc=0.000001)

[c] 1-2-2-1 (0.0% vs 9.4%, $\chi 2$=9.60, p=0.002 (1df), Pc=0.002)

[d] 1-2-1-2 (0.0% vs 9.4%, $\chi 2$=9.60, p=0.002 (1df), Pc=0.002)

[e] X-X-X-X (0.0% vs 30.3%, $\chi 2$=30.90, p<0.0000001 (1df), Pc<0.0000001)

Table 6.13B Frequency of *MspI*-VLP-VNTR^{86}-*SspI* haplotype in the patient population

MspI-VLP-VNTR^{86}-*SspI* Haplotype	observed (n)	frequency (%)	expected (n)	frequency (%)	χ^2
2 - 3 - 2 - 2	21	14.0	0.1	0.1	4368.10 [a]
1 - 2 - 1 - 1	126	84.0	78.9	52.6	28.12 [b]
1 - 2 - 2 - 1	1	0.7	14.2	9.5	12.27 [c]
1 - 2 - 1 - 2	1	0.7	13.6	9.1	11.67 [d]
2 - 3 - 2 - 1	1	0.7	0.4	0.3	0.90
X-X-X-X	0	0.0	42.8	28.5	42.80 [e]

This table shows the frequency and actual (observed) number of copies of the *MspI*-VLP-VNTR^{86}-*SspI* haplotype detected within the patient population. The X-X-X-X haplotype represents the sum of the haplotypes that were not detected. Haplotypes were assigned in those subjects who were homozygous at three or more loci. The expected haplotype frequencies were obtained from the individual allelic frequencies. (The haplotype frequency analysis does not take into account individuals who were heterozygous at more than one locus). Comparisons were made between the observed and expected *MspI*-VLP-VNTR^{86}-*SspI* haplotypes and $\chi 2$ values were determined. The $\chi 2$ test was used to estimate linkage disequilibrium between the loci.

[a] 2-3-2-2 (14.0% vs 0.1%, $\chi 2$=4368.10, p<0.0000001 (1df), Pc<0.0000004)

[b] 1-2-1-1 (84.0% vs 52.6%, $\chi 2$=28.12, p<0.0000001 (1df), Pc<0.0000004)

[c] 1-2-2-1 (0.7% vs 9.5%, $\chi 2$=12.27, p=0.0005 (1df), Pc=0.002)

[d] 1-2-1-2 (0.7% vs 9.1%, $\chi 2$=11.67, p=0.0006 (1df), Pc=0.002)

[e] X-X-X-X (0.0% vs 28.5%, $\chi 2$=42.80, p<0.0000001 (1df), Pc<0.0000004)

Summary of the results obtained for analysis of the *MspI*-VLP-VNTR^{86}-*SspI* haplotypes

All of the significant data are discussed, however, only the data that are significant following correction for the number of comparisons involved are included in the tables. Tables 6.13A and 6.13B suggest that the *MspI*-VLP-VNTR^{86}-*SspI* polymorphisms of the IL-1RN gene are linked. The most common haplotype in both control and patient populations was found to be 1-2-1-1 that occurred with an observed frequency of 84.3% in the control group and 84.0% in the patient group.

Controls:

Significantly more of the 2-3-2-2 (15.7% vs 0.1%, $\chi 2$ =2528.11, p<0.0000001, Pc<0.0000001) and 1-2-1-1 (84.3% vs 50.5%, $\chi 2$ =23.11, p=0.000001, Pc=0.000001) haplotypes were observed in the control population than expected. In addition, there was a significant reduction in the X-X-X-X (0.0% vs 30.3%, $\chi 2$=30.90, p<0.0000001, Pc<0.0000001), 1-2-2-1 (0.0% vs 9.4%, $\chi 2$ =9.60, p=0.002, Pc=0.002) and 1-2-1-2 (0.0% vs 9.4%, $\chi 2$ =9.60, p=0.002, Pc=0.002) haplotypes.

Patients:

There were significantly more 2-3-2-2 (14.0% vs 0.1%, $\chi 2$ =4368.10, p<0.0000001, Pc<0.0000004) and 1-2-1-1 (84.0% vs 52.6%, $\chi 2$ =28.12, p<0.0000001, Pc<0.0000004) haplotypes than expected in the patient population. Significantly less X-X-X-X (0.0% vs 28.5%, $\chi 2$ =42.80, p<0.0000001, Pc<0.0000004), 1-2-2-1 (0.7% vs 9.5%, $\chi 2$ =12.27, p=0.0005, Pc=0.002) and 1-2-1-2 (0.7% vs 9.1%, $\chi 2$ =11.67, p=0.0006, Pc=0.002) haplotypes were also found in this group.

Table 6.14A Frequency of *PstI*-(AC)n-VNTR[46] haplotype in the control population

Haplotype *PstI*-(AC)n-VNTR[46]	observed (n)	frequency (%)	expected (n)	frequency (%)	χ^2
2 - 2 - 2	12	14.3	3.7	4.4	18.62 [a]
1 - 2 - 6	2	2.4	0.2	0.2	16.20 [b]
2 - 2 - 1	3	3.6	12.2	14.5	6.94
2 - 2 - 4	2	2.4	0.4	0.5	6.40
2 - 1 - 2	1	1.2	5.4	6.4	3.59
1 - 3 - 1	2	2.4	0.6	0.7	3.27
2 - 3 - 3	1	1.2	0.2	0.2	3.20
1 - 2 - 2	3	3.6	1.2	1.4	2.70
1 - 0 - 1	1	1.2	0.3	0.4	1.63
2 - 1 - 1	23	27.4	17.8	21.2	1.52
2 - 4 - 1	11	13.1	7.6	9.0	1.52
1 - 4 - 1	4	4.8	2.4	2.9	1.07
2 - 5 - 1	2	2.4	1.0	1.2	1.0
2 - 8 - 1	2	2.4	1.0	1.2	1.0
2 - 1 - 3	3	3.6	1.9	2.3	0.64
1 - 1 - 1	7	8.3	5.6	6.7	0.35
1 - 1 - 3	1	1.2	0.6	0.7	0.27
2 - 3 - 2	1	1.2	0.6	0.7	0.27
2 - 2 - 6	1	1.2	0.7	0.8	0.13
2 - 2 - 3	1	1.2	1.3	1.5	0.07
2 - 0 - 1	1	1.2	1.0	1.2	0.00
2 - 0 - 3	0	0.0	-	-	-
1 - 2 - 1	0	0.0	-	-	-
1 - 2 - 3	0	0.0	-	-	-
2 - 3 - 1	0	0.0	-	-	-
2 - 6 - 1	0	0.0	-	-	-
X-X-X	0	0.0	18.3	21.8	18.30 [c]

This table shows the frequency and actual (observed) number of copies of the *PstI*-(AC)n-VNTR[46] haplotype detected within the control population. The X-X-X haplotype represents the sum of the haplotypes that were not detected. Haplotypes were assigned in those subjects who were homozygous at two or more loci. The expected haplotype frequencies were obtained from the individual allelic frequencies. (The haplotype frequency analysis does not take into account individuals who were heterozygous at more than one locus). Comparisons were made between the observed and expected *PstI*-(AC)n-VNTR[46] haplotypes and $\chi 2$ values were determined. The $\chi 2$ test was used to estimate linkage disequilibrium between the loci.

[a] 2-2-2 (14.3% vs 4.4%, $\chi 2$ =18.62, p=0.00002 (1df), Pc=0.0004)

[b] 1-2-6 (2.4% vs 0.2%, $\chi 2$=16.20, p=0.00006 (1df), Pc=0.001)

[c] X-X-X (0.0% vs 21.8%, $\chi 2$=18.30, p=0.00002 (1df), Pc=0.0004)

Table 6.14B Frequency of *PstI*-(AC)n-VNTR[46] haplotype in the patient population

Haplotype *PstI*-(AC)n-VNTR[46]	observed (n)	frequency (%)	expected (n)	frequency (%)	χ^2
2 - 2 - 2	10	12.2	3.0	3.7	16.33 [a]
2 - 2 - 1	3	3.7	14.1	17.2	8.74 [b]
2 - 2 - 6	2	2.4	0.5	0.6	4.50
2 - 4 - 1	12	14.6	7.0	8.5	3.57
2 - 0 - 3	1	1.2	0.2	0.2	3.20
1 - 2 - 2	1	1.2	0.2	0.2	3.20
2 - 1 - 3	10	12.2	6.9	8.4	1.39
2 - 5 - 1	4	4.9	2.4	2.9	1.07
1 - 2 - 3	1	1.2	0.4	0.5	0.90
2 - 3 - 1	3	3.7	1.8	2.2	0.80
1 - 2 - 1	2	2.4	1.1	1.3	0.74
2 - 6 - 1	2	2.4	1.2	1.5	0.53
2 - 1 - 1	24	29.3	21.2	25.9	0.37
1 - 1 - 1	2	2.4	1.7	2.1	0.05
2 - 2 - 3	5	6.1	4.6	5.6	0.03
1 - 0 - 1	0	0.0	-	-	-
2 - 0 - 1	0	0.0	-	-	-
2 - 1 - 2	0	0.0	-	-	-
1 - 1 - 3	0	0.0	-	-	-
2 - 2 - 4	0	0.0	-	-	-
1 - 2 - 6	0	0.0	-	-	-
1 - 3 - 1	0	0.0	-	-	-
2 - 3 - 2	0	0.0	-	-	-
2 - 3 - 3	0	0.0	-	-	-
1 - 4 - 1	0	0.0	-	-	-
2 - 8 - 1	0	0.0	-	-	-
X-X-X	0	0.0	15.7	19.1	15.70 [c]

This table shows the frequency and actual (observed) number of copies of the *PstI*-(AC)n-VNTR[46] haplotype detected within the patient population. The X-X-X haplotype represents the sum of the haplotypes that were not detected. Haplotypes were assigned in those subjects who were homozygous at two or more loci. The expected haplotype frequencies were obtained from the individual allelic frequencies. (The haplotype frequency analysis does not take into account individuals who were heterozygous at more than one locus). Comparisons were made between the observed and expected *PstI*-(AC)n-VNTR[46] haplotypes and $\chi 2$ values were determined. The $\chi 2$ test was used to estimate linkage disequilibrium between the loci.

[a] 2-2-2 (12.2% vs 3.7%, $\chi 2$=16.33, p=0.00005 (1df), Pc=0.0007)

[b] 2-2-1 (3.7% vs 17.2%, $\chi 2$=8.74, p=0.003 (1df), Pc=0.04)

[c] X-X-X (0.0% vs 19.1%, $\chi 2$=15.70, p=0.00007 (1df), Pc=0.001)

Summary of the results obtained for analysis of the PstI-(AC)n-VNTR[46] haplotypes

All of the significant data are discussed, however, only the data that are significant following correction for the number of comparisons involved are included in the tables. Tables 6.14A and 6.14B suggest that the *Pst I* polymorphism of the IL-1R gene and (AC)n-VNTR[46] polymorphisms of the IL-1A gene are linked. The most common haplotype was 2-1-1 that occurred with an observed frequency of 27.4% in controls and 29.3% in patients.

Controls:

There were significantly more of the 2-2-2 (14.3% vs 4.4%, $\chi 2$ =18.62, p=0.00002, Pc=0.0004), 1-2-6 (2.4% vs 0.2%, $\chi 2$ =16.20, p=0.00006, Pc=0.001) and 2-2-4 (2.4% vs 0.5%, $\chi 2$=6.40 p=0.01*) haplotypes than expected in controls and a significant reduction in the number of the X-X-X (0.0% vs 21.8%, $\chi 2$=18.30, p=0.00002, Pc=0.0004) and 2-2-1 (3.6% vs 14.5%, $\chi 2$=6.94, p=0.008*) haplotypes.

Patients:

Significantly more of the 2-2-2 (12.2% vs 3.7%, $\chi 2$=16.33, p=0.00005, Pc=0.0007) and 2-2-6 (2.4% vs 0.6%, $\chi 2$ =4.50, p=0.03*) haplotypes were found than expected whereas the number of X-X-X (0.0% vs 19.1%, $\chi 2$=15.70, p=0.00007, Pc=0.001) and 2-2-1 (3.7% vs 17.2%, $\chi 2$=8.74, p=0.003, Pc=0.04) haplotypes were significantly reduced.

* p value became non-significant following correction for the number of comparisons made.

Table 6.15A: Frequency of *PstI-AvaI-AluI-TaqI* haplotype in the control population

PstI-AvaI-AluI-TaqI Haplotype	observed (n)	frequency (%)	expected (n)	frequency (%)	χ^2
2 - 2 - 2 - 1	26	27.7	9.1	9.7	31.39 [a]
1 - 2 - 2 - 1	10	10.6	3.1	3.3	15.36 [b]
2 - 1 - 2 - 1	0	0.0	14.6	15.5	14.60 [c]
2 - 2 - 1 - 1	0	0.0	14.6	15.5	14.60 [d]
2 - 1 - 1 - 2	9	9.6	3.1	3.3	11.23 [e]
2 - 1 - 1 - 1	35	37.2	23.5	25.0	5.63
1 - 1 - 2 - 1	0	0.0	5.0	5.3	5.00
1 - 2 - 1 - 1	0	0.0	5.0	5.3	5.00
2 - 1 - 2 - 2	0	0.0	1.9	2.0	1.90
2 - 2 - 1 - 2	0	0.0	1.9	2.0	1.90
1 - 1 - 1 - 1	12	12.8	8.1	8.6	1.88
2 - 2 - 2 - 2	0	0.0	1.2	1.3	1.20
1 - 1 - 1 - 2	2	2.1	1.1	1.2	0.74
1 - 1 - 2 - 2	0	0.0	0.7	0.7	0.70
1 - 2 - 1 - 2	0	0.0	0.7	0.7	0.70
1 - 2 - 2 - 2	0	0.0	0.4	0.4	0.40

This table shows the frequency and actual (observed) number of copies of the *PstI-AvaI-AluI-TaqI* haplotype detected within the control population. Haplotypes were assigned in those subjects who were homozygous at three or more loci. The expected haplotype frequencies were obtained from the individual allelic frequencies. (The haplotype frequency analysis does not take into account individuals who were heterozygous at more than one locus). Comparisons were made between the observed and expected *PstI-AvaI-AluI-TaqI* haplotypes and $\chi 2$ values were determined. The $\chi 2$ test was used to estimate linkage disequilibrium between the loci.

[a] 2-2-2-1 (27.7% vs 9.7%, $\chi 2=31.39$, $p<0.0000001$ (1df), $Pc<0.0000005$)

[b] 1-2-2-1 (10.6% vs 3.3%, $\chi 2=15.36$, $p=0.00009$ (1df), $Pc=0.0005$)

[c] 2-1-2-1 (0.0% vs 15.5%, $\chi 2=14.60$, $p=0.0001$ (1df), $Pc=0.0005$)

[d] 2-2-1-1 (0.0% vs 15.5%, $\chi 2=14.60$, $p=0.0001$ (1df), $Pc=0.0005$)

[e] 2-1-1-2 (9.6% vs 3.3%, $\chi 2=11.23$, $p=0.0008$ (1df), $Pc=0.004$)

Table 6.15B: Frequency of *PstI-AvaI-AluI-TaqI* haplotype in the patient population

PstI-AvaI-AluI-TaqI Haplotype	observed (n)	frequency (%)	expected (n)	frequency (%)	χ^2
2 - 2 - 2 - 1	12	12.5	1.9	2.0	53.69[a]
2 - 1 - 2 - 1	0	0.0	9.7	10.1	9.70[b]
1 - 2 - 2 - 1	2	2.1	0.3	0.3	9.63[c]
2 - 2 - 1 - 1	0	0.0	9.0	9.4	9.00[d]
2 - 2 - 2 - 2	2	2.1	0.5	0.5	4.50
2 - 2 - 1 - 2	0	0.0	2.4	2.5	2.40
1 - 1 - 2 - 1	0	0.0	1.5	1.6	1.50
2 - 1 - 1 - 2	16	16.7	11.8	12.3	1.49
1 - 2 - 1 - 1	0	0.0	1.4	1.5	1.40
1 - 1 - 1 - 1	10	10.4	7.0	7.3	1.29
2 - 1 - 1 - 1	52	54.2	45.1	47.0	1.06
2 - 1 - 2 - 2	1	1.0	2.5	2.6	0.90
1 - 1 - 2 - 2	0	0.0	0.4	0.4	0.40
1 - 2 - 1 - 2	0	0.0	0.4	0.4	0.40
1 - 1 - 1 - 2	1	1.0	1.8	1.9	0.36
1 - 2 - 2 - 2	0	0.0	0.1	0.1	0.10

This table shows the frequency and actual (observed) number of copies of the *PstI-AvaI-AluI-TaqI* haplotype detected within the patient population. Haplotypes were assigned in those subjects who were homozygous at three or more loci. The expected haplotype frequencies were obtained from the individual allelic frequencies. (The haplotype frequency analysis does not take into account individuals who were heterozygous at more than one locus). Comparisons were made between the observed and expected *PstI-AvaI-AluI-TaqI* haplotypes and $\chi 2$ values were determined. The $\chi 2$ test was used to estimate linkage disequilibrium between the loci.

[a] 2-2-2-1 (12.5% vs 2.0%, $\chi 2$=53.69, p<0.0000001 (1df), Pc<0.0000007)

[b] 2-1-2-1 (0.0% vs 10.1%, $\chi 2$=9.70, p=0.002 (1df), Pc=0.01)

[c] 1-2-2-1 (2.1% vs 0.3%, $\chi 2$=9.63, p=0.002 (1df), Pc=0.01)

[d] 2-2-1-1 (0.0% vs 9.4%, $\chi 2$=9.00, p=0.003 (1df), Pc=0.02)

Summary of the results obtained for analysis of the *PstI-AvaI-AluI-TaqI* haplotypes

All of the significant data are discussed, however, only the data that are significant following correction for the number of comparisons involved are included in the tables. Tables 6.15A and 6.15B indicate that the *Pst I* polymorphism of the IL-1R gene and *AvaI-AluI-TaqI* polymorphisms of the IL-1B gene are also linked. The most common haplotype was found to be 2-1-1-1 with an observed frequency of 37.2% in controls and 54.2% in patients.

Controls:

Significantly more of the 2-2-2-1 (27.7% vs 9.7%, $\chi 2$ =31.39, p<0.0000001, Pc<0.0000005), 1-2-2-1 (10.6% vs 3.3%, $\chi 2$ =15.36, p=0.00009, Pc=0.0005), 2-1-1-2, (9.6% vs 3.3%, $\chi 2$ =11.23, p=0.0008, Pc=0.004) and 2-1-1-1 (37.2% vs 25.0%, $\chi 2$ =5.63, p=0.02*) haplotypes were observed in controls than expected, whereas the number of 2-1-2-1, (0.0% vs 15.5%, $\chi 2$ =14.60, p=0.0001, Pc=0.0005), 2-2-1-1, (0.0% vs 15.5%, $\chi 2$ =14.60, p=0.0001, Pc=0.0005) 1-1-2-1 (0.0% vs 5.3%, $\chi 2$ =5.0, p=0.03*) and 1-2-1-1 (0.0% vs 5.3%, $\chi 2$ =5.0, p=0.03*) haplotypes were found to be significantly decreased.

Patients:

There were significantly more of the 2-2-2-1 (12.5% vs 2.0%, $\chi 2$=53.69, p<0.0000001, Pc=0.0000007), 1-2-2-1, (2.1% vs 0.3%, $\chi 2$ =9.63, p=0.002, Pc=0.01) and 2-2-2-2 (2.1% vs 0.5%, $\chi 2$ =4.50, p=0.03*) haplotypes than expected in the patient group, however, there were less of the 2-1-2-1, (0.0% vs 10.1%, $\chi 2$ =9.70, p=0.002, Pc=0.01) and 2-2-1-1, (0.0% vs 9.4%, $\chi 2$ =9.00, p=0.003, Pc=0.02) haplotypes than expected.

* p value became non-significant following correction for the number of comparisons made.

Table 6.16A Frequency of *PstI-MspI*-VLP-VNTR^{86}-*SspI* haplotype in the control population

PstI-MspI-VLP-VNTR^{86}-*SspI* Haplotype	observed (n)	frequency (%)	expected (n)	frequency (%)	χ^2
2 - 1 - 2 - 1 - 1	58	59.2	33.4	34.1	18.12 [a]
1 - 1 - 2 - 1 - 1	24	24.5	14.7	15.0	5.88
2 - 2 - 3 - 2 - 2	10	10.2	0.0	-	-
1 - 2 - 3 - 2 - 2	6	6.1	0.0	-	-
2 - 1 - 2 - 2 - 1	0	0.0	0.0	-	-
X-X-X-X-X	0	0.0	49.9	50.9	49.90 [b]

This table shows the frequency and actual (observed) number of copies of the *PstI-MspI*-VLP-VNTR^{86}-*SspI* haplotype detected within the control population. The X-X-X-X-X haplotype represents the sum of the haplotypes that were not detected. Haplotypes were assigned in those subjects who were homozygous at four or more loci. The expected haplotype frequencies were obtained from the individual allelic frequencies. (The haplotype frequency analysis does not take into account individuals who were heterozygous at more than one locus). Comparisons were made between the observed and expected *PstI-MspI*-VLP-VNTR^{86}-*SspI* haplotypes and $\chi 2$ values were determined. The $\chi 2$ test was used to estimate linkage disequilibrium between the loci.

[a] 2-1-2-1-1 (59.2% vs 34.1%, $\chi 2=18.12$, $p=0.00002$ (1df), $Pc=0.00006$)

[b] X-X-X-X-X (0.0% vs 50.9%, $\chi 2=49.90$, $p<0.0000001$ (1df), $Pc<0.0000003$)

Table 6.16B Frequency of *PstI-MspI*-VLP-VNTR^{86}-*SspI* haplotype in the patient population

PstI-MspI-VLP-VNTR^{86}-*SspI* Haplotype	observed (n)	frequency (%)	expected (n)	frequency (%)	χ^2
2 - 1 - 2 - 1 - 1	103	71.5	68.6	47.6	17.25 [a]
2 - 1 - 2 - 2 - 1	1	0.7	10.4	7.2	8.50 [b]
1 - 1 - 2 - 1 - 1	22	15.3	15.2	10.6	3.04
2 - 2 - 3 - 2 - 2	14	9.7	0	-	-
1 - 2 - 3 - 2 - 2	4	2.8	0	-	-
X-X-X-X-X	0	0.0	49.8	34.6	49.80 [c]

This table shows the frequency and actual (observed) number of copies of the *PstI-MspI*-VLP-VNTR^{86}-*SspI* haplotype detected within the patient population. The X-X-X-X-X haplotype represents the sum of the haplotypes that were not detected. Haplotypes were assigned in those subjects who were homozygous at four or more loci. The expected haplotype frequencies were obtained from the individual allelic frequencies. (The haplotype frequency analysis does not take into account individuals who were heterozygous at more than one locus). Comparisons were made between the observed and expected *PstI-MspI*-VLP-VNTR^{86}-*SspI* haplotypes and $\chi 2$ values were determined. The $\chi 2$ test was used to estimate linkage disequilibrium between the loci.

[a] 2-1-2-1-1 (71.5% vs 47.6%, $\chi 2$=17.25, p=0.00003 (1df), Pc=0.0001)

[b] 2-1-2-2-1 (0.7% vs 7.2%, $\chi 2$=8.50, p=0.004 (1df), Pc=0.02)

[c] X-X-X-X-X (0.0% vs 34.6%, $\chi 2$=49.80, p<0.0000001 (1df), Pc<0.0000004)

Summary of the results obtained for analysis of the *PstI-MspI*-VLP-VNTR^{86}-*SspI* haplotypes

All of the significant data are discussed, however, only the data that are significant following correction for the number of comparisons involved are included in the tables. Data in tables 6.16A and 6.16B suggest that linkage occurs between *PstI* polymorphism of the IL-1RI gene and the *MspI*-VLP-VNTR^{86}-*SspI* polymorphisms of the IL-1RN gene. The most common haplotype was found to be 2-1-2-1-1 with an observed frequency of 59.2% in controls and 71.5% in patients.

Controls

Significantly more of the 2-1-2-1-1 (59.2% vs 34.1%, $\chi 2$ =18.12 p=0.00002, Pc=0.00006) and 1-1-2-1-1 (24.5% vs 15.0%, $\chi 2$ =5.88, p=0.02*) haplotypes and significantly less of the X-X-X-X-X haplotype were found (0.0% vs 50.9%, $\chi 2$ =49.90 p<0.0000001, Pc<0.0000003) in controls than expected.

Patients

There were significantly more of the 2-1-2-1-1 (71.5% vs 47.6%, $\chi 2$ =17.25, p=0.00003, Pc=0.0001) than expected in the patient group. Significantly less of the X-X-X-X-X (0.0% vs 34.6%, $\chi 2$ =49.80, p<0.0000001, Pc<0.0000004) and 2-1-2-2-1 (0.7% vs 7.2%, $\chi 2$ =8.50, p=0.004, Pc=0.02) haplotypes were found than expected.

* p value became non-significant following correction for the number of comparisons made.

Table 6.17A: Frequency of (AC)n-VNTR[46]-*AvaI-AluI-TaqI* haplotype in the control population

Haplotype IL-1A-IL-1B	observed (n)	frequency (%)	expected (n)	frequency (%)	χ^2
2-2-2-2-1	6	13.0	0.2	0.4	168.20[a]
2-6-2-2-1	3	6.5	0.1	0.2	84.10[b]
1-1-1-1-2	5	10.9	0.6	1.3	32.27[c]
4-1-1-1-1	10	21.7	3.5	7.6	12.07[d]
5-1-1-1-1	5	10.9	1.5	3.3	8.17
3-1-1-1-1	3	6.5	0.9	2.0	4.90
1-1-2-2-1	2	4.3	0.7	1.5	2.41
2-1-1-1-1	1	2.2	3.8	8.3	2.06
4-1-1-1-2	1	2.2	0.5	1.1	0.50
4-1-2-2-1	1	2.2	0.7	1.5	0.13
1-1-1-1-1	4	8.7	3.8	8.3	0.01
1-2-1-1-1	1	2.2	1.1	2.4	0.01
2-2-1-1-1	2	4.3	1.1	2.4	0.74
1-3-2-2-1	1	2.2	0.0	-	0.00
2-4-2-2-1	1	2.2	0.0	-	0.00
1-1-1-2-2	0	0.0	-	-	-
1-1-2-2-2	0	0.0	-	-	-
1-3-1-1-1	0	0.0	-	-	-
2-3-1-1-1	0	0.0	-	-	0.00
2-3-2-2-1	0	0.0	-	-	0.00
6-1-1-1-1	0	0.0	-	-	0.00
X-X-X-X-X	0	0.0	27.5	59.8	27.50[e]

This table shows the frequency and actual (observed) number of copies of the (AC)n-VNTR[46]-*AvaI-AluI-TaqI* haplotype detected within the control population. The X-X-X-X-X haplotype represents the sum of the haplotypes that were not detected. Haplotypes were assigned in those subjects who were homozygous at four or more loci. The expected haplotype frequencies were obtained from the individual allelic frequencies. (The haplotype frequency analysis does not take into account individuals who were heterozygous at more than one locus). Comparisons were made between the observed and expected (AC)n-VNTR[46]-*AvaI-AluI-TaqI* haplotypes and $\chi 2$ values were determined. The $\chi 2$ test was used to estimate linkage disequilibrium between the loci.

[a] 2-2-2-2-1 (13.0% vs 0.4%, $\chi 2$=168.20, p<0.0000001 (1df), Pc<0.000001)

[b] 2-6-2-2-1 (6.5% vs 0.2%, $\chi 2$=84.10, p<0.00000001 (1df), Pc<0.000001)

[c] 1-1-1-1-2 (10.9% vs 1.3%, $\chi 2$=32.27, p<0.0000001 (1df), Pc<0.000001)

[d] 4-1-1-1-1 (21.7% vs 7.6%, $\chi 2$=12.07, p=0.0005 (1df), Pc=0.007)

[e] X-X-X-X-X (0.0% vs 59.8%, $\chi 2$=27.50, p<0.0000001 (1df), Pc<0.0000001)

Table 6.17B: Frequency of (AC)n-VNTR[46]-*AvaI-AluI-TaqI* polymorphism haplotype in the patient population

Haplotype IL-1A-IL-1B	observed (n)	frequency (%)	expected (n)	frequency (%)	χ^2
1-1-1-1-2	3	8.3	0.5	1.4	12.50[a]
1-1-1-2-2	1	2.8	0.1	0.3	8.10
4-1-1-1-1	9	25.0	3.9	10.8	6.67
3-1-1-1-1	3	8.3	1.3	3.6	2.22
5-1-1-1-1	2	5.6	0.9	2.5	1.34
2-2-1-1-1	2	5.6	1.0	2.8	1.00
2-1-1-1-1	3	8.3	5.2	14.4	0.93
6-1-1-1-1	1	2.8	0.4	1.1	0.90
1-3-1-1-1	1	2.8	0.4	1.1	0.90
2-3-1-1-1	1	2.8	0.6	1.7	0.27
1-1-1-1-1	4	11.1	3.9	10.8	0.00
2-2-2-2-1	3	8.3	0.0	-	0.00
2-6-2-2-1	2	5.6	0.0	-	0.00
2-3-2-2-1	1	2.8	0.0	-	0.00
1-1-2-2-1	0	0.0	-	-	-
1-1-2-2-2	0	0.0	-	-	-
1-3-2-2-1	0	0.0	-	-	-
2-4-2-2-1	0	0.0	-	-	-
4-1-1-1-2	0	0.0	-	-	-
4-1-2-2-1	0	0.0	-	-	-
X-X-X-X-X	0	0.0	17.8	49.4	17.80[b]

This table shows the frequency and actual (observed) number of copies of the (AC)n-VNTR[46]-*AvaI-AluI-TaqI* haplotype detected within the patient population. The X-X-X-X-X haplotype represents the sum of the haplotypes that were not detected. Haplotypes were assigned in those subjects who were homozygous at four or more loci. The expected haplotype frequencies were obtained from the individual allelic frequencies. (The haplotype frequency analysis does not take into account individuals who were heterozygous at more than one locus). Comparisons were made between the observed and expected (AC)n-VNTR[46]-*AvaI-AluI-TaqI* haplotypes and $\chi 2$ values were determined. The $\chi 2$ test was used to estimate linkage disequilibrium between the loci.

[a] 1-1-1-1-2 (8.3% vs 1.4%, $\chi 2=12.50$, p=0.0004 (1df), Pc=0.005)

[b] X-X-X-X-X (0.0% vs 49.4%, $\chi 2=17.80$, p=0.00002 (1df), Pc=0.0003)

Summary of the results obtained for analysis of the (AC)n-VNTR^{46}-*AvaI-AluI-TaqI* haplotypes

All of the significant data are discussed, however, only the data that are significant following correction for the number of comparisons involved are included in the tables. Tables 6.17A and 6.17B show that the (AC)n-VNTR^{46} polymorphisms of the IL-1A gene and *AvaI-AluI-TaqI* polymorphisms of the IL-1B gene are also linked. The most common haplotype was 4-1-1-1-1 that occurred with an observed frequency of 21.7% in controls and 25.0% in patients.

Controls:

There were significantly more of the 2-2-2-2-1 (13.0% vs 0.4%, $\chi 2$ =168.20, p<0.0000001, Pc<0.000001), 2-6-2-2-1 (6.5% vs 0.2%, $\chi 2$=84.10, p<0.0000001, Pc<0.000001), 1-1-1-1-2 (10.9% vs 1.3%, $\chi 2$=32.27, p<0.0000001, Pc<0.000001), 4-1-1-1-1 (21.7% vs 7.6%, $\chi 2$=12.07, p=0.0005, Pc=0.007), 5-1-1-1-1 (10.9% vs 3.3%, $\chi 2$=8.17, p=0.004*) and 3-1-1-1-1 (6.5% vs 2.0%, $\chi 2$=4.90, p=0.03*) haplotypes than expected in the control group. The X-X-X-X-X haplotype was significantly reduced in controls compared to expected (0.0% vs 59.8%, $\chi 2$=27.50, p<0.0000001, Pc<0.000001).

Patients:

Significantly more of the 1-1-1-1-2 (8.3% vs 1.4%, $\chi 2$=12.50, p=0.0004, Pc=0.005), 1-1-1-2-2 (2.8% vs 0.3%, $\chi 2$=8.10, p=0.004*) and 4-1-1-1-1 (25.0% vs 10.8%, $\chi 2$=6.67, p=0.01*) haplotypes and significantly less of the X-X-X-X-X haplotype (0.0% vs 49.4%, $\chi 2$=17.80, p=0.00002, Pc=0.0003) were observed in patients compared to expected.

* p value became non-significant following correction for the number of comparisons made.

Table 6.18A: Frequency of (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* haplotype in the control population

Haplotype IL-1A-IL-1RN	observed (n)	frequency (%)	expected (n)	frequency (%)	χ^2
2-2-1-2-1-1	6	12.5	0.5	1.0	60.50 [a]
4-1-1-2-1-1	13	27.1	6.2	12.9	7.46
1-1-1-2-1-1	15	31.3	7.8	16.3	6.65
2-1-1-2-1-1	1	2.1	2.9	6.0	1.24
8-1-1-2-1-1	1	2.1	0.4	0.8	0.90
5-1-1-2-1-1	3	6.3	2.1	4.4	0.39
1-2-1-2-1-1	1	2.1	1.3	2.7	0.07
1-1-2-3-2-2	3	6.3	0.0	-	-
4-1-2-3-2-2	2	4.2	0.0	-	-
5-1-2-3-2-2	2	4.2	0.0	-	-
3-1-2-3-2-2	1	2.1	0.0	-	-
0-3-1-2-1-1	0	0.0	-	-	-
1-3-1-2-1-1	0	0.0	-	-	-
2-1-2-3-2-2	0	0.0	-	-	-
2-2-2-3-2-2	0	0.0	-	-	-
2-3-1-2-1-1	0	0.0	-	-	-
2-6-2-3-2-2	0	0.0	-	-	-
3-1-1-2-1-1	0	0.0	-	-	-
4-2-1-2-1-1	0	0.0	-	-	-
5-2-1-2-1-2	0	0.0	-	-	-
6-1-1-2-1-1	0	0.0	-	-	-
X-X-X-X-X-X	0	0.0	26.8	55.8	26.80 [b]

This table shows the frequency and actual (observed) number of copies of the (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* haplotype detected within the control population. The X-X-X-X-X-X haplotype represents the sum of the haplotypes that were not detected. Haplotypes were assigned in those subjects who were homozygous at five or more loci. The expected haplotype frequencies were obtained from the individual allelic frequencies. (The haplotype frequency analysis does not take into account individuals who were heterozygous at more than one locus). Comparisons were made between the observed and expected (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* haplotypes and $\chi 2$ values were determined. The $\chi 2$ test was used to estimate linkage disequilibrium between the loci.

[a] 2-2-1-2-1-1 (12.5% vs 1.0%, $\chi 2=60.50$, $p<0.0000001$ (1df), $Pc<0.000001$)

[b] X-X-X-X-X-X (0.0% vs 55.8%, $\chi 2=26.80$, $p<0.0000001$ (1df), $Pc<0.000001$)

Table 6.18B Frequency of (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* haplotype in the patient population

Haplotype IL-1A-IL-1RN	observed (n)	frequency (%)	expected (n)	frequency (%)	χ^2
2-2-1-2-1-1	11	12.5	2.8	3.2	24.01 [a]
4-1-1-2-1-1	14	15.9	6.0	6.8	10.67 [b]
1-3-1-2-1-1	10	11.4	4.0	4.5	9.00 [c]
1-1-1-2-1-1	24	27.3	13.4	15.2	8.39
0-3-1-2-1-1	1	1.1	0.1	0.1	8.10
5-2-1-2-1-2	1	1.1	0.1	0.1	8.10
2-1-1-2-1-1	5	5.7	10.1	11.5	2.58
3-1-1-2-1-1	2	2.3	0.8	0.9	1.80
6-1-1-2-1-1	2	2.3	0.8	0.9	1.80
2-3-1-2-1-1	5	5.7	3.0	3.4	1.33
4-2-1-2-1-1	1	1.1	1.7	1.9	0.29
5-1-1-2-1-1	2	2.3	1.5	1.7	0.17
2-6-2-3-2-2	3	3.4	0.0	-	-
1-1-2-3-2-2	2	2.3	0.0	-	-
2-2-2-3-2-2	2	2.3	0.0	-	-
2-1-2-3-2-2	1	1.1	0.0	-	-
4-1-2-3-2-2	1	1.1	0.0	-	-
5-1-2-3-2-2	1	1.1	0.0	-	-
3-1-2-3-2-2	0	0.0	-	-	-
8-1-1-2-1-1	0	0.0	-	-	-
1-2-1-2-1-1	0	0.0	-	-	-
X-X-X-X-X-X	0	0.0	43.7	49.7	43.70 [d]

This table shows the frequency and actual (observed) number of copies of the (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* haplotype detected within the patient population. The X-X-X-X-X-X haplotype represents the sum of the haplotypes that were not detected. Haplotypes were assigned in those subjects who were homozygous at five or more loci. The expected haplotype frequencies were obtained from the individual allelic frequencies. (The haplotype frequency analysis does not take into account individuals who were heterozygous at more than one locus). Comparisons were made between the observed and expected (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* haplotypes and $\chi 2$ values were determined. The $\chi 2$ test was used to estimate linkage disequilibrium between the loci.

[a] 2-2-1-2-1-1 (12.5% vs 3.2%, $\chi 2$ =24.01, p=0.000001 (1df), Pc=0.00002)

[b] 4-1-1-2-1-1 (15.9% vs 6.8%, $\chi 2$=10.67, p=0.001 (1df), Pc=0.02)

[c] 1-3-1-2-1-1 (11.4% vs 4.5%, χ 2=9.00, p=0.003 (1df), Pc<0.05)

[d] X-X-X-X-X-X (0.0% vs 49.7%, $\chi 2$=43.70, p<0.0000001 (1df), Pc<0.000002)

Summary of the results obtained for analysis of the (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* haplotypes

All of the significant data are discussed, however, only the data that are significant following correction for the number of comparisons involved are included in the tables. The data in tables 6.18A and 6.18B indicate that the (AC)n-VNTR^{46} polymorphisms of the IL-1A gene and *MspI*-VLP-VNTR^{86}-*SspI* polymorphisms of the IL-1RN gene are linked. The most common haplotype was 1-1-1-2-1-1 that occurred with an observed frequency of 31.3% in controls and 27.3% in patients.

Controls:

There were significantly more of the 2-2-1-2-1-1 (12.5% vs 1.0%, $\chi 2$=60.50, p<0.0000001, Pc<0.000001), 4-1-1-2-1-1 (27.1% vs 12.9%, $\chi 2$=7.46, p=0.006*) and 1-1-1-2-1-1 (31.3% vs 16.3%, $\chi 2$=6.65, p=0.01*) haplotypes and significantly less of the X-X-X-X-X-X haplotype (0.0% vs 55.8%, $\chi 2$= 26.80, p<0.0000001, Pc<0.000001) than expected in controls.

Patients:

Significantly more of the 2-2-1-2-1-1 (12.5% vs 3.2%, $\chi 2$=24.01, p=0.000001, Pc=0.000002), 4-1-1-2-1-1 (15.9% vs 6.8%, $\chi 2$ =10.67, p=0.001, Pc=0.02), 1-3-1-2-1-1 (11.4% vs 4.5%, $\chi 2$=9.00, p=0.003, Pc<0.05) 1-1-1-2-1-1 (27.3% vs 15.2%, $\chi 2$=8.39, p=0.004*), 0-3-1-2-1-1 (1.1% vs 0.1%, $\chi 2$=8.10, p=0.004*) and 5-2-1-2-1-2 (1.1% vs 0.1%, $\chi 2$ =8.10, p=0.004*) haplotypes were found than expected while the X-X-X-X-X-X haplotype was significantly decreased (0.0% vs 49.7%, $\chi 2$=43.70, p<0.0000001, Pc<0.000002) in patients compared to expected.

* p value became non-significant following correction for the number of comparisons made.

Table 6.19A Frequency of *AvaI-AluI-TaqI-MspI*-VLP-VNTR[86]-*SspI* haplotype in the control population

Haplotype IL-1B-IL-1RN	observed (n)	frequency (%)	expected (n)	frequency (%)	χ^2
2-2-1-1-2-1-1	16	24.2	2.6	3.9	69.06 [a]
1-1-2-1-2-1-1	11	16.7	3.0	4.5	21.33 [b]
1-1-1-1-2-1-1	29	43.9	13.6	20.6	17.44 [c]
1-2-1-1-2-1-1	0	0.0	5.9	8.9	5.90
1-1-1-1-2-1-2	0	0.0	2.4	3.6	2.40
1-2-2-1-2-1-1	0	0.0	1.3	2.0	1.30
2-2-2-1-2-1-1	0	0.0	0.6	0.9	0.60
1-1-1-2-3-2-2	5	7.6	0.0	0.0	-
2-2-1-2-3-2-2	4	6.1	0.0	0.0	-
1-1-2-2-3-2-2	1	1.5	0.0	0.0	-
2-2-2-2-3-2-2	0	0.0	0.0	0.0	-
X-X-X-X-X-X-X	0	0.0	36.6	55.5	36.60 [d]

This table shows the frequency and actual (observed) number of copies of the *AvaI-AluI-TaqI-MspI*-VLP-VNTR[86]-*SspI* haplotype detected within the control population. The X-X-X-X-X-X-X haplotype represents the sum of the haplotypes that were not detected. Haplotypes were assigned in those subjects who were homozygous at six or more loci. The expected haplotype frequencies were obtained from the individual allelic frequencies. (The haplotype frequency analysis does not take into account individuals who were heterozygous at more than one locus). Comparisons were made between the observed and expected *AvaI-AluI-TaqI-MspI*-VLP-VNTR[86]-*SspI* haplotypes and $\chi 2$ values were determined. The $\chi 2$ test was used to estimate linkage disequilibrium between the loci.

[a] 2-2-1-1-2-1-1 (24.2% vs 3.9%, $\chi 2=69.06$, $p<0.0000001$ (1df), $Pc<0.0000005$)

[b] 1-1-2-1-2-1-1 (16.7% vs 4.5%, $\chi 2=21.33$, $p=0.000004$ (1df), $Pc=0.00002$)

[c] 1-1-1-1-2-1-1 (43.9% vs 20.6%, $\chi 2=17.44$, $p=0.00003$ (1df), $Pc=0.0002$)

[d] X-X-X-X-X-X-X (0.0% vs 55.5%, $\chi 2=36.60$, $p<0.0000001$ (1df), $Pc<0.0000005$)

Table 6.19B Frequency of *AvaI-AluI-TaqI-MspI*-VLP-VNTR[86]-*SspI* haplotype in the patient population

Haplotype IL-1B-IL-1RN	observed (n)	frequency (%)	expected (n)	frequency (%)	χ^2
1-1-1-1-2-1-1	48	54.5	25.5	29.0	19.85 [a]
1-1-2-1-2-1-1	21	23.9	9.6	10.9	13.54 [b]
2-2-1-1-2-1-1	3	3.4	0.8	0.9	6.05
1-2-1-1-2-1-1	1	1.1	4.8	5.5	3.01
1-1-1-1-2-1-2	1	1.1	4.4	5.0	2.63
2-2-2-1-2-1-1	1	1.1	0.3	0.3	1.63
1-2-2-1-2-1-1	1	1.1	1.8	2.0	0.36
1-1-1-2-3-2-2	4	4.5	0.0	-	-
2-2-1-2-3-2-2	7	8.0	0.0	-	-
2-2-2-2-3-2-2	1	1.1	0.0	-	-
1-1-2-2-3-2-2	0	0.0	0.0	-	-
X-X-X-X-X-X-X	0	0.0	40.8	46.4	40.80 [c]

This table shows the frequency and actual (observed) number of copies of the *AvaI-AluI-TaqI-MspI*-VLP-VNTR[86]-*SspI* haplotype detected within the patient population. The X-X-X-X-X-X-X haplotype represents the sum of the haplotypes that were not detected. Haplotypes were assigned in those subjects who were homozygous at six or more loci. The expected haplotype frequencies were obtained from the individual allelic frequencies. (The haplotype frequency analysis does not take into account individuals who were heterozygous at more than one locus). Comparisons were made between the observed and expected *AvaI-AluI-TaqI-MspI*-VLP-VNTR[86]-*SspI* haplotypes and $\chi 2$ values were determined. The $\chi 2$ test was used to estimate linkage disequilibrium between the loci.

[a] 1-1-1-1-2-1-1 (54.5% vs 29.0%, $\chi 2$=19.85, p=0.000008 (1df), Pc=0.00007)

[b] 1-1-2-1-2-1-1 (23.9% vs 10.9%, $\chi 2$=13.54, p=0.0002 (1df), Pc=0.002)

[c] X-X-X-X-X-X-X (0.0% vs 46.4%, $\chi 2$=40.80, p<0.0000001 (1df), Pc<0.0000009)

Summary of the results obtained for analysis of the *AvaI-AluI-TaqI-MspI*-VLP-VNTR^{86}-*SspI* haplotypes

All of the significant data are discussed, however, only the data that are significant following correction for the number of comparisons involved are included in the tables. The information shown in tables 6.19A and 6.19B suggests that the *AvaI-AluI-TaqI* polymorphisms of the IL-1B gene and *MspI*-VLP-VNTR^{86}-*SspI* polymorphisms of the IL-1RN gene are linked. The most common haplotype was found to be 1-1-1-1-2-1-1 with an observed frequency of 43.9% in controls and 54.5% in patients.

Controls:

Significantly more of the 2-2-1-1-2-1-1 (24.2% vs 3.9%, $\chi 2$=69.06, p<0.0000001, Pc<0.0000005), 1-1-2-1-2-1-1 (16.7% vs 4.5%, $\chi 2$=21.33 p=0.000004, Pc=0.00002) and 1-1-1-1-2-1-1 (43.9% vs 20.6%, $\chi 2$=17.44 p=0.00003, Pc=0.0002) haplotypes were observed in the controls than expected, whereas the number of the X-X-X-X-X-X-X (0.0% vs 55.5%, $\chi 2$ =36.60, p<0.0000001, Pc<0.0000005) and 1-2-1-1-2-1-1 (0.0% vs 8.9%, $\chi 2$ =5.90, p=0.02*) haplotypes were found to be significantly decreased.

Patients:

There were significantly more of the 1-1-1-1-2-1-1 (54.5% vs 29.0%, $\chi 2$=19.85, p=0.000008, Pc=0.00007), 1-1-2-1-2-1-1, (23.9% vs 10.9%, $\chi 2$=13.54, p=0.0002, Pc=0.002) and 2-2-1-1-2-1-1 (3.4% vs 0.9%, $\chi 2$=6.05, p=0.01*) haplotypes than expected in the patient population, while the X-X-X-X-X-X-X haplotype (0.0% vs 46.4%, $\chi 2$ =40.80, p<0.0000001, Pc<0.0000009) was significantly reduced.

* p value became non-significant following correction for the number of comparisons made.

6.1.6 Combined Genotype Analysis

Because the numbers of subjects are so small in the patient subgroups when the double heterozygotes are removed, there are not enough subjects to obtain significance for the haplotype analysis. In view of this, the genotypes of the polymorphisms were combined to determine whether there was preferential association of the markers within controls, patients and patient subgroups to determine whether a particular group expressed preferential association of genotypes. The results obtained from this analysis are shown in Appendix 2.

6.2 Protein studies

Studies were also performed to determine whether there was an association between the polymorphisms and protein levels. The most significant results obtained for the genetic studies in the IL-1A, IL-1B and IL-1RN genes were used as a basis for patient selection for the protein studies. Patients were recalled according to their genotype and depending on the hypothesis. For example, in the case of the IL-1B study, the 2,2 genotype of the *Alu I* polymorphism was found to be associated with susceptibility to acute pancreatitis, therefore patients with the 2,2 and 1,1 genotype were recalled to represent the 2 extremes of the genotype. Before the protein studies were performed the supernatants obtained in the optimisation experiment (Appendix 1) were used to determine the secretory profile of each protein over a 120 hour period. The same 10 Caucasian adults were used as controls for each of the following protein studies. These were 4 males and 6 females with an age range of 24-48 years and a median age of 27 years.

C^0= unstimulated controls
P^0= unstimulated patients
$A1^0$= unstimulated subjects with allele 1
$A2^0$= unstimulated subjects with allele 2

C^5= stimulated controls
P^5= stimulated patients
$A1^5$= stimulated subjects with allele 1
$A2^5$= stimulated subjects with allele 2

6.2.1 Interleukin-1 alpha protein

6.2.1.1 Interleukin-1 alpha time course experiment

The IL-1α time course experiment showed that the peak IL-1α protein secretion occurred at 24 hours (Figure 6.12).

6.2.1.2 Interleukin-1 alpha protein experiment

The most significant results in the polymorphism studies of the IL-1A gene were obtained from the (AC)n repeat polymorphism. The 2,4 genotype of this polymorphism was significantly increased in the alcoholic patients compared to controls (33.3% vs 12.0%, $\chi 2$= 6.72, p=0.01, Pc<0.05) and significantly decreased in patients with gallstone disease compared to patients with alcoholic disease (7.5% vs 33.3%, $\chi 2$=9.9, p=0.002, Pc=0.01). "*Hypothesis: the 2,4 genotype of the (AC)n polymorphism may be associated with susceptibility to alcoholic acute pancreatitis.*"

Patients with gallstone and alcoholic disease with the 2,4 genotype were recalled and the amount of IL-1α protein secreted from their PBMCs stimulated with 0-5μg/ml LPS for 0-72 hours was compared with that secreted from PBMCs taken from healthy controls. Unfortunately, only 3 patients with gallstone disease and 1 with alcohol-induced acute pancreatitis agreed to donate a blood sample for this study. In addition, because there are many different alleles in the polymorphism giving rise to may different genotypes, only 1 of the 10 controls had a 2,4 genotype and therefore it was not possible to compare 2,4 patients with 2,4 genotype controls so the hypothesis could not be tested.

Instead the amount of IL-1α protein secreted was compared between controls and patients so the samples that had been prepared would not be wasted. The genotypes, protein levels (individual and median) stimulated with 0 and 5μg/ml LPS for 0-72 hours in controls and patients are shown in Tables 6.20A and 6.20B respectively.

Figure 6.12: IL-1 alpha protein secreted from PBMCs cultured at $1x10^6$ cells per ml and stimulated with 5ug/ml LPS for 0-120 hours

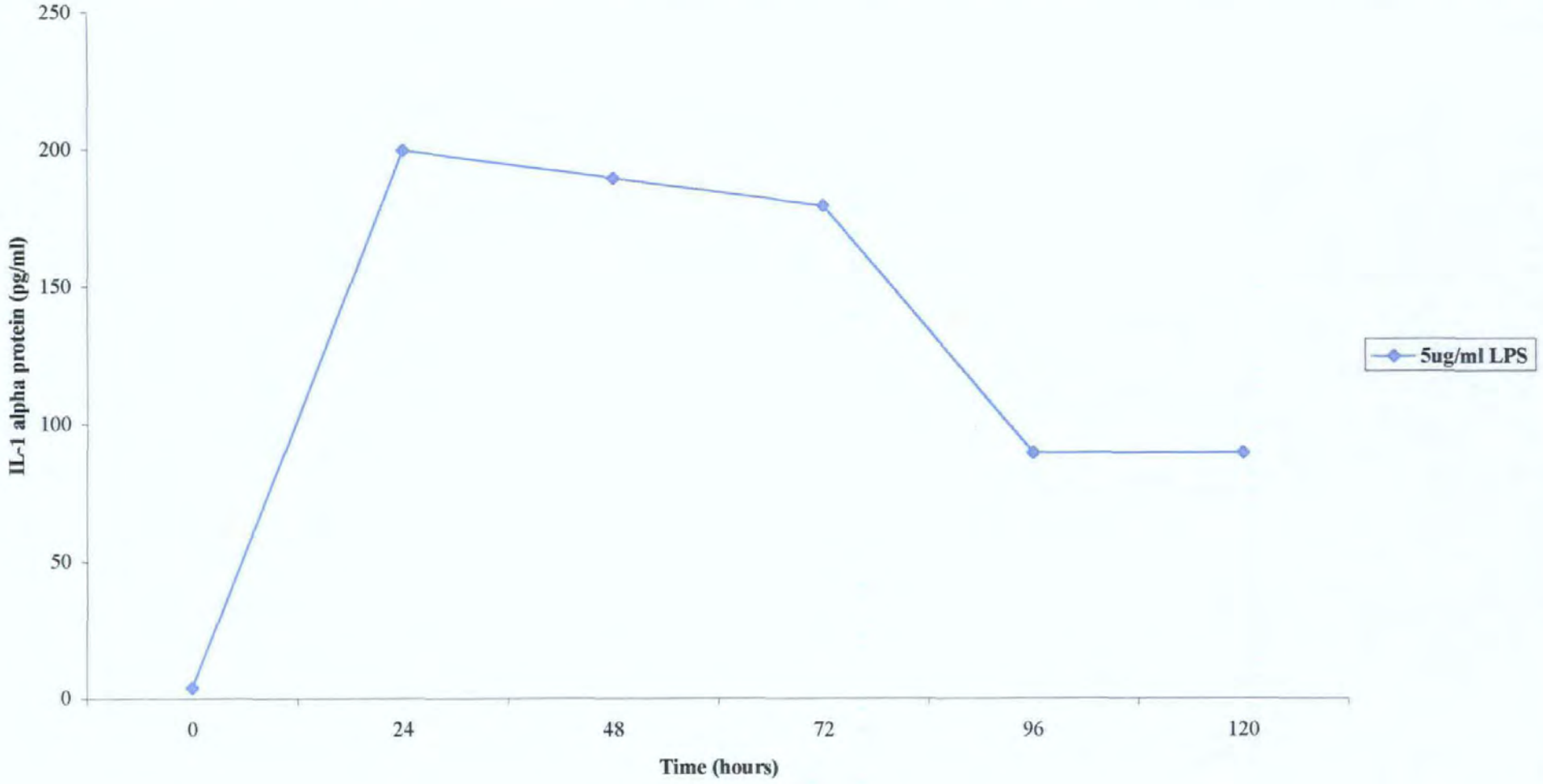


Table 6.20A: IL-1α protein (pg/ml) secreted by PBMCs cultured at $1x10^6$ cells per ml and stimulated with 0-5μg/ml LPS for 0-72 hours in controls (C^0 and C^5)

Time (hours)	G/T	0	24	48	72	0	24	48	72
LPS (μg/ml)		0	0	0	0	5	5	5	5
Control 1	2,3	0	0	0	0	7	440	400	150
Control 2	1,1	2	1	2	2	0	540	490	370
Control 3	4,4	3	2	2	1	3	350	300	260
Control 4	1,7	2	54	57	55	2	560	490	430
Control 5	1,4	3	4	8	13	0	490	410	250
Control 6	1,1	0	0	16	0	0	490	440	270
Control 7	0,2	0	0	8	0	2	600	540	530
Control 8	1,1	19	0	21	0	0	690	530	450
Control 9	1,3	1	3	5	7	0	1130	980	690
Control 10	2,4	1	1	3	0	0	370	350	160
n		10	10	10	10	10	10	10	10
Median		1.5	1.0	6.5	0.5	0.0	515	465	320
Range		0-19	0-54	0-57	0-55	0-7	350-1130	300-980	150-690
i.q.r.		0-3	0-3	2-16	0-7	0-2	440-600	400-530	250-450

Table 6.20B: IL-1α protein (pg/ml) secreted by PBMCs cultured at $1x10^6$ cells per ml and stimulated with 0-5μg/ml LPS for 0-72 hours in patients with acute pancreatitis (P^0 and P^5)

Time (hours)	G/T	0	24	48	72	0	24	48	72
LPS (μg/ml)		0	0	0	0	5	5	5	5
Patient 1	2,4	0	83	90	90	0	620	480	310
Patient 2	2,4	0	215	253	235	0	1020	640	460
Patient 3	2,4	0	512	574	619	0	1690	1250	1090
Patient 4	2,4	1	172	260	186	0	730	390	330
n		4	4	4	4	4	4	4	4
Median		0.0	193.5[a]	256.5[b]	210.5[c]	0.0	875[d]	560	395
Range		0-1	83-512	90-574	90-619	0-0	620-1690	390-1250	310-1090
i.q.r.		0-0.5	127.5-363.5	171.5-417.0	138-427	0-0	675-1355	435-945	320-775

G/T = genotype
i.q.r. = interquartile range

[a] =patients vs controls 0 LPS, 24 hours: 193.5 (127.5-363.5)pg/ml vs 1.0 (0.0-3.0)pg/ml, p=0.005
[b] =patients vs controls 0 LPS, 48 hours: 256.5 (171.5-417.0)pg/ml vs 6.5 (2.0-16.0)pg/ml, p=0.006
[c] =patients vs controls 0 LPS, 72 hours: 210.5 (138-427)pg/ml vs 0.5 (0-7)pg/ml, p=0.005
[d] =patients vs controls 5 LPS, 24 hours: 875 (675-1355)pg/ml vs 515 (440-600)pg/ml, p=0.028

The IL-1α protein levels for the individual controls and patients are shown in Figure 6.13. The protein secreted from all subjects peaked at 24 hours as in the time course study, however, the amount of protein produced varied in each case.

Figure 6.14 shows the median IL-1α protein levels in controls and patients separated according to whether the cells were unstimulated or stimulated with LPS (C^0 vs P^0 and C^5 vs P^5). The IL-1α protein levels were significantly increased in the unstimulated patients (P^0) compared to the unstimulated controls (C^0) at the following time points. Values are median (interquartile range): 24 hours: 193.5 (127.5-363.5) pg/ml vs 1.0 (0.0-3.0) pg/ml, p=0.005; 48 hours: 256.5 (171.5-417.0) pg/ml vs 6.5 (2.0-16.0) pg/ml, p=0.006; 72 hours: 210.5 (138-427) pg/ml vs 0.5 (0-7) pg/ml, p=0.005. The IL-1α protein level was also significantly increased in the stimulated patients (P^5) compared to the stimulated controls (C^5) at 24 hours. Values are median (interquartile range): 875 (675-1355) pg/ml vs 515(440-600) pg/ml, p=0.028. No other significant differences in protein levels were found.

C^0= unstimulated controls
P^0= unstimulated patients
C^5= stimulated controls
P^5= stimulated patients

Figure 6.13: IL-1 alpha protein secreted from PBMCs cultured at $1x10^6$ cells per ml and stimulated with 0-5ug/ml LPS for 0-72 hours in controls and patients with acute pancreatitis

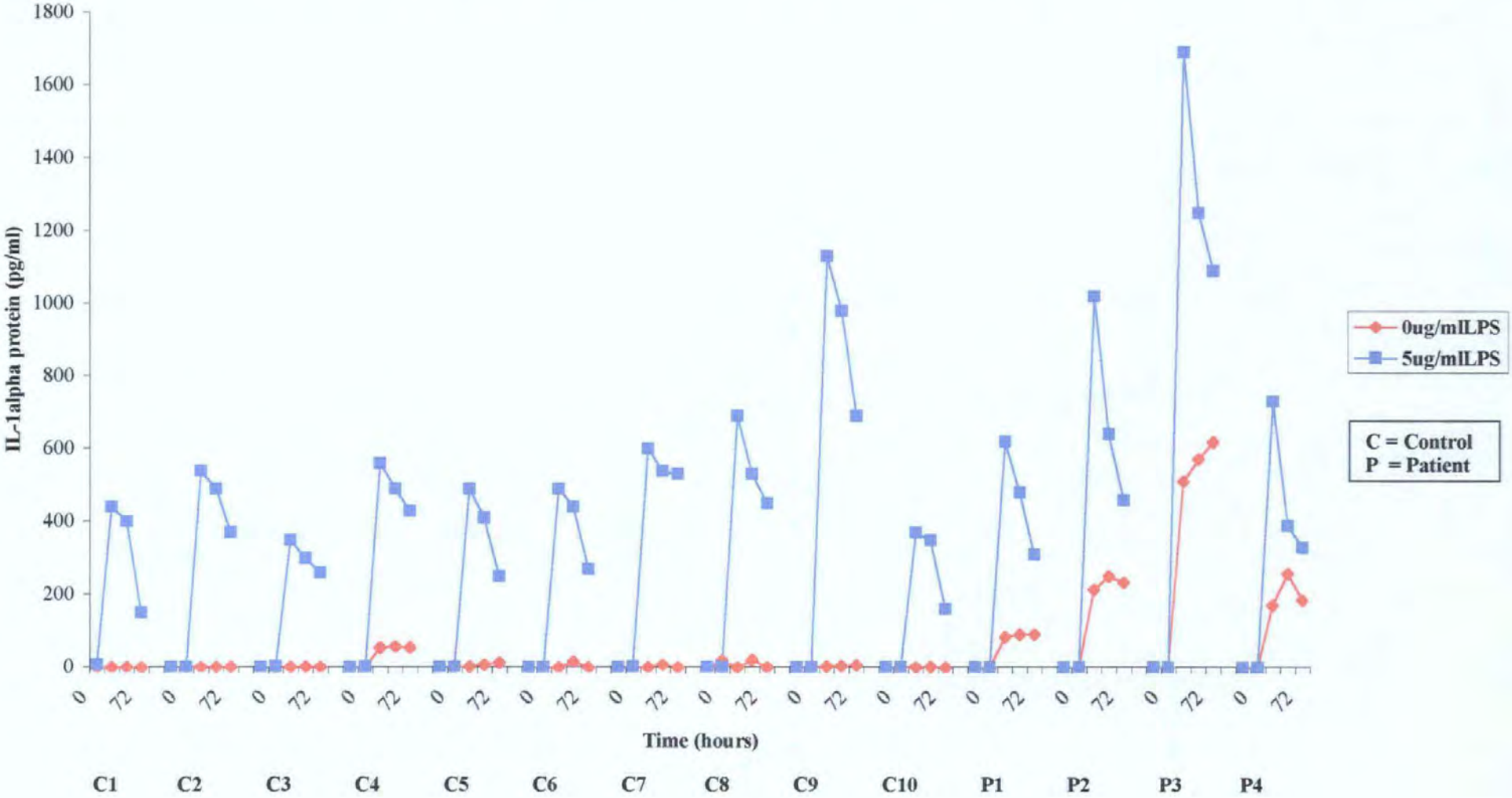
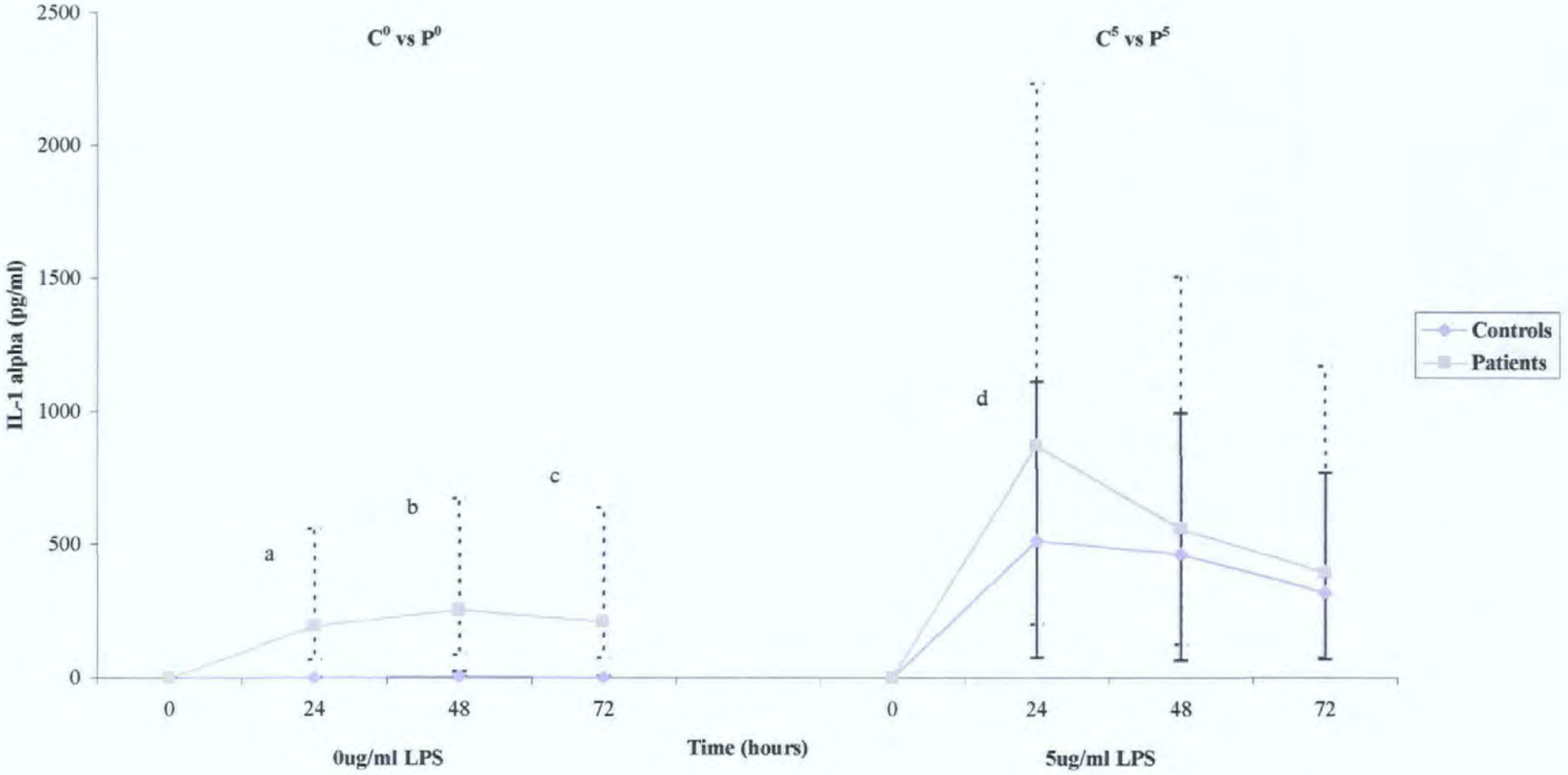


Figure 6.14: Median IL-1 alpha protein secreted from PBMCs cultured at $1x10^6$ cells per ml for 0-72 hours in controls and patients with acute pancreatitis separated according to whether the PBMCs were unstimulated or stimulated with 5ug/ml LPS



Values are median (interquartile range)
Solid vertical bars represent interquartile range for controls
Dashed vertical bars represent interquartile range for patients

[a] p=0.005 [c] p=0.005
[b] p=0.006 [d] p=0.028
(Mann-Whitney U test)

6.2.2 Interleukin-1 beta protein

6.2.2.1 Interleukin-1 beta time course experiment

The 120 hour time course experiment showed that the peak IL-1β protein secretion occurred at 24 hours (Figure 6.15).

6.2.2.2 Interleukin-1beta protein experiment

The most significant results in the polymorphism studies of the IL-1B gene were obtained from the *Alu I* polymorphism. The 2,2 genotype was found to be significantly decreased in patients compared to controls (9.7% vs 19.5%, $\chi 2=5.60$, p=0.018, Pc=0.036). "*Hypothesis: the 2,2 genotype of the IL-1B Alu I polymorphism may be associated with susceptibility to acute pancreatitis.*"

Patients with 1,1 and 2,2 genotypes were recalled for this study and the amount of IL-1β protein secreted from their PBMCs stimulated with 0-5μg/ml LPS for 0-72 hours was compared with IL-1β secreted from PBMCs taken from healthy controls. The genotypes, protein levels (individual and median) stimulated with 0 and 5μg/ml LPS for 0-72 hours in controls and patients are shown in Tables 6.21A and 6.21B respectively. The IL-1β protein levels for the individual controls and patients are shown in Figure 6.16. The protein secreted from all subjects peaked at 24 hours as in the time course study, however, the amount of protein produced varied in each case.

Figure 6.17 shows the median IL-1β protein levels in controls and patients separated according to whether the cells were unstimulated or stimulated with LPS (C^0 vs P^0 and C^5 vs P^5).

C^0= unstimulated controls
C^5= stimulated controls
P^0= unstimulated patients
P^5= stimulated patients

Figure 6.15: IL-1 beta protein secreted from PBMCs cultured at $1x10^6$ cells per ml and stimulated with 5ug/ml LPS for 0-120 hours

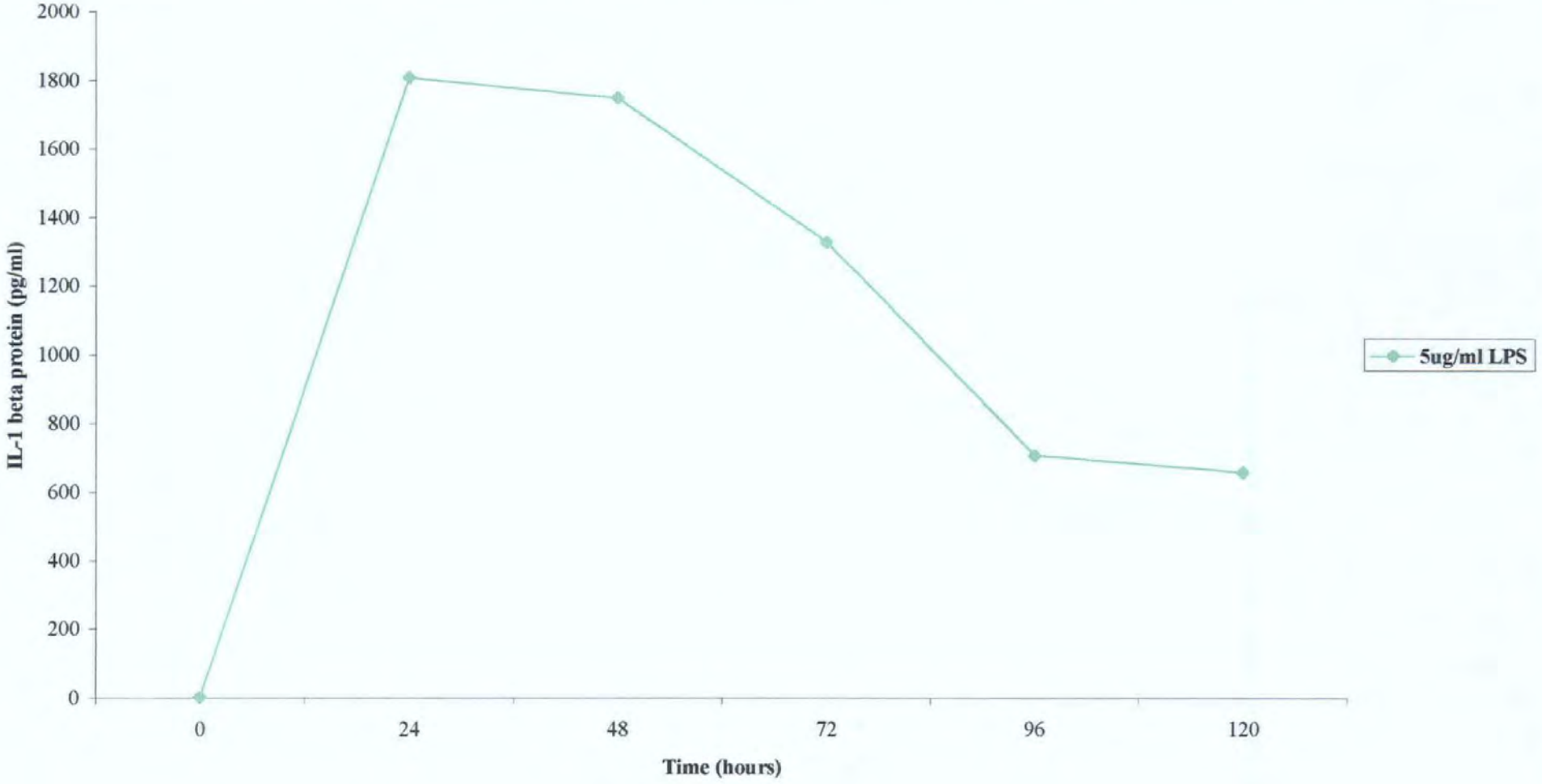


Table 6.21A: IL-1β protein (pg/ml) secreted by PBMCs cultured at $1x10^6$ cells per ml and stimulated with 0-5μg/ml LPS for 0-72 hours in controls (C^0 and C^5)

Time (hours) LPS (μg/ml)	G/T	0 0	24 0	48 0	72 0	0 5	24 5	48 5	72 5
Control 1	2,2	10	0	16	7	0	4200	3760	3580
Control 2	1,1	14	13	21	11	9	5150	4780	4410
Control 3	1,1	15	11	13	14	20	3430	3320	2990
Control 4	1,1	5	681	605	614	2	5780	5470	5250
Control 5	1,1	15	0	9	2	9	3440	3250	2650
Control 6	1,2	8	5	11	0	7	4300	3340	3070
Control 7	2,2	3	6	11	2	6	6180	5870	5610
Control 8	1,1	10	84	108	102	19	10190	9350	9040
Control 9	1,1	0	53	36	43	2	4260	3770	3700
Control 10	1,2	1	14	13	15	2	3070	2990	2780
n		10	10	10	10	10	10	10	10
Median		9	12	14.5	12.5	6.5	4280	3765	3640
Range		0-15	0-681	9-605	0-614	0-20	3070-10190	2990-9350	2650-9040
i.q.r.		3-14	5-53	11-36	2-43	2-9	3440-5780	3320-5470	2990-5250

Table 6.21B: IL-1β protein (pg/ml) secreted by PBMCs cultured at $1x10^6$ cells per ml and stimulated with 0-5μg/ml LPS for 0-72 hours in patients with acute pancreatitis (P^0 and P^5)

Time (hours) LPS (μg/ml)	G/T	0 0	24 0	48 0	72 0	0 5	24 5	48 5	72 5
Patient 1	2,2	2	507	508	442	2	3040	2740	2460
Patient 2	2,2	2	598	593	608	3	4780	4770	4240
Patient 3	2,2	0	728	647	483	1	4860	3560	2510
Patient 4	1,1	0	782	1080	771	0	4090	3920	3880
Patient 5	1,1	0	1032	1253	931	0	4680	4010	3630
Patient 6	1,1	0	433	570	422	0	2570	2030	1720
n		6	6	6	6	6	6	6	6
Median		0[a]	663[b]	620[c]	545.5[d]	0.5[e]	4385	3740	3070
Range		0-2	433-1032	508-1253	422-931	0-3	2570-4860	2030-4770	1720-4240
i.q.r.		0-2	507-782	570-1080	442-771	0-2	3040-4780	2740-4010	2460-3880

G/T= genotype
i.q.r. = interquartile range

[a] =patients vs controls 0 LPS, 0 hours: 0 (0-2)pg/ml vs 9 (3-14)pg/ml, p=0.010
[b] =patients vs controls 0 LPS, 24 hours: 663 (507-782)pg/ml vs 12 (5-53)pg/ml, p=0.004
[c] =patients vs controls 0 LPS, 48 hours: 620 (570-1080)pg/ml vs 14.5 (11-36)pg/ml, p=0.004
[d] =patients vs controls 0 LPS, 72 hours: 545.5 (442-771)pg/ml vs 12.5 (2-43)pg/ml, p=0.006
[e] =patients vs controls 5 LPS, 0 hours: 0.5 (0-2)pg/ml vs 6.5 (2-9)pg/ml, p=0.024

Figure 6.16: IL-1beta protein secreted from PBMCs cultured at $1x10^6$ cells per ml and stimulated with 0-5ug/ml LPS for 0-72 hours in controls and patients with acute pancreatitis

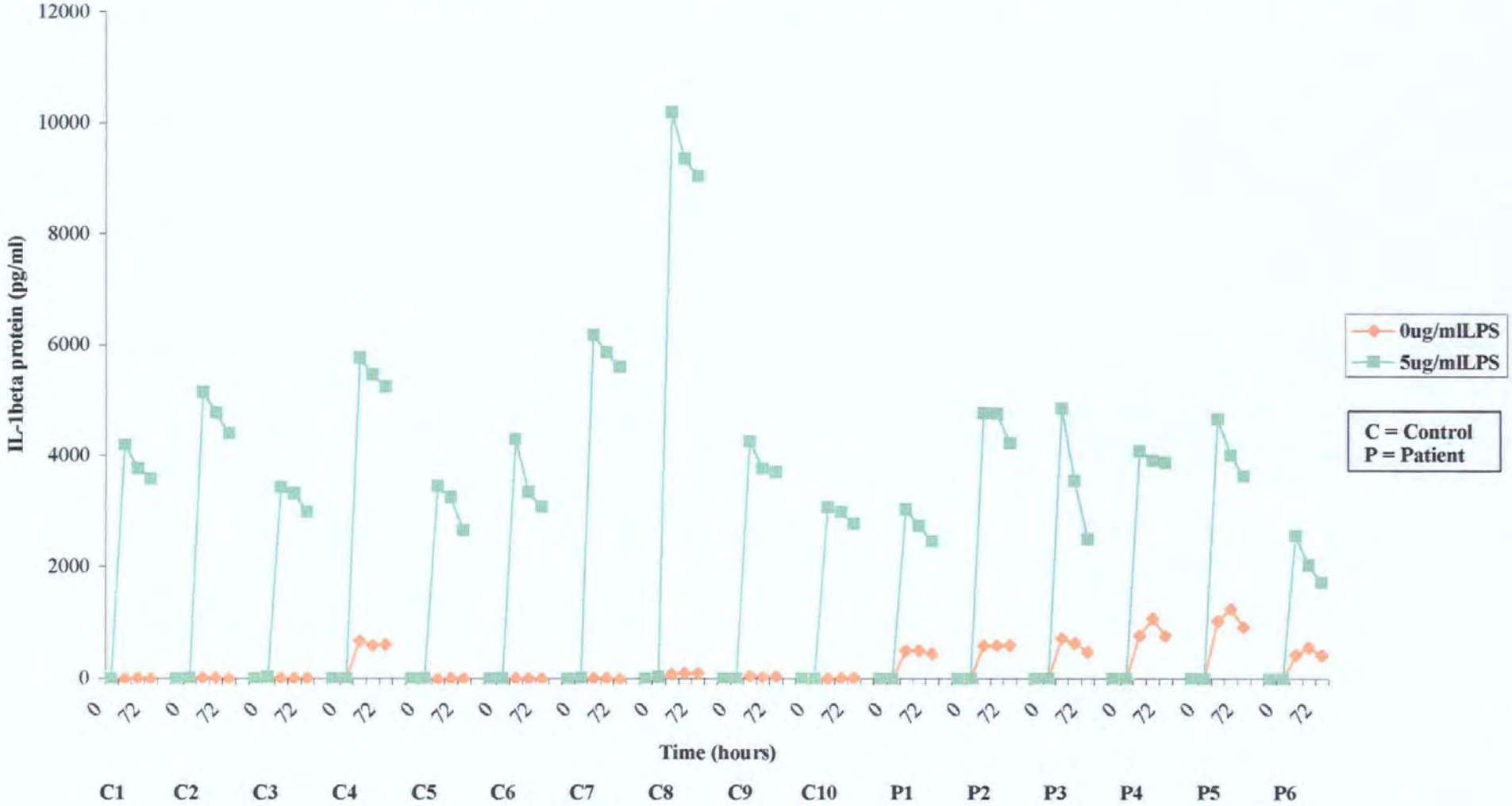
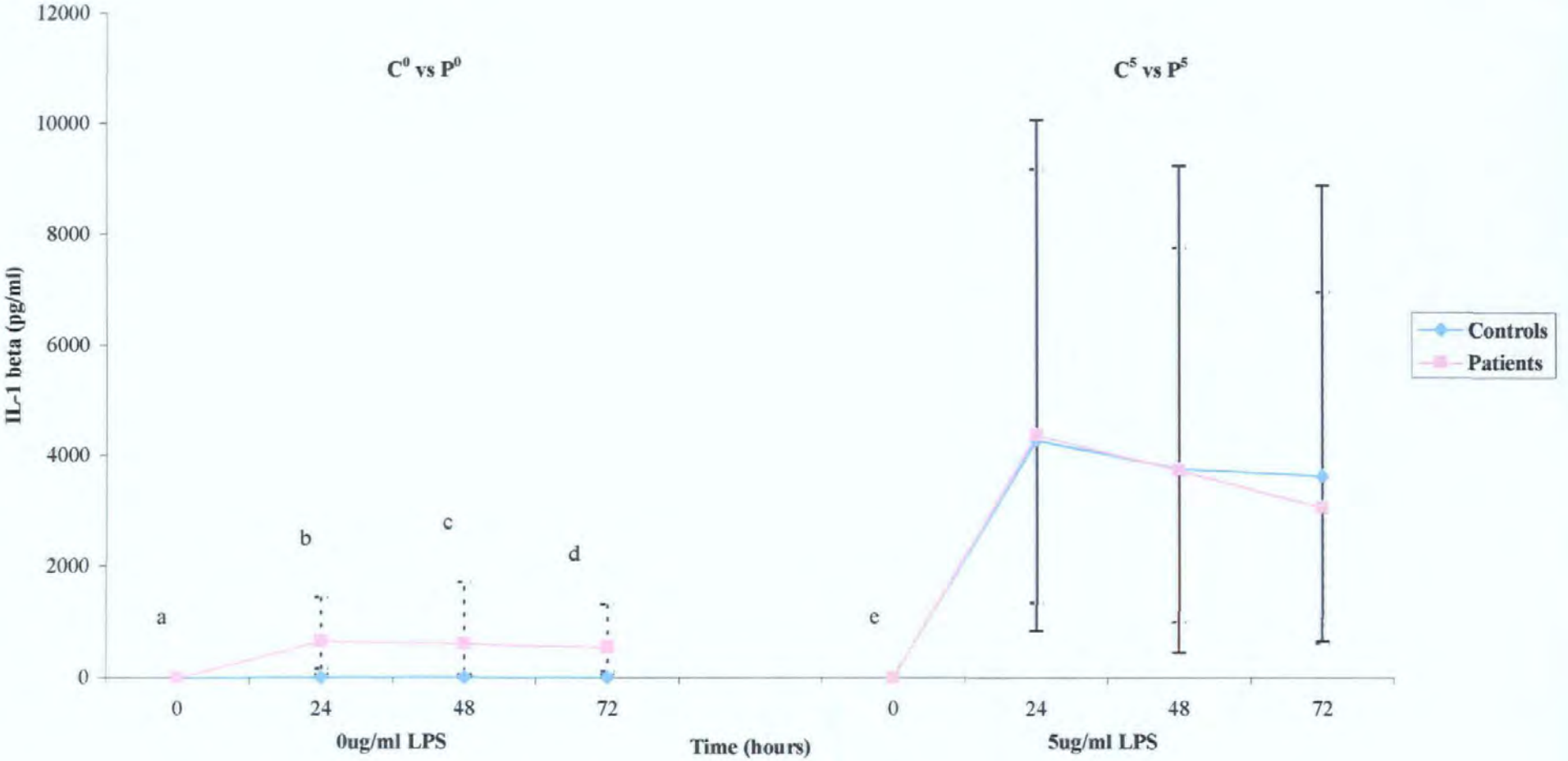


Figure 6.17: Median IL-1 beta protein secreted from PBMCs cultured at $1x10^6$ cells per ml for 0-72 hours in controls and patients with acute pancreatitis separated according to whether the PBMCs were unstimulated or stimulated with 5ug/ml LPS



Values are median (interquartile range)
Solid vertical bars represent interquartile range for controls
Dashed vertical bars represent interquartile range for patients

[a] p=0.010 [c] p=0.004 [e] p=0.024
[b] p=0.004 [d] p=0.006
(Mann-Whitney U test)

The IL-1β protein levels were significantly increased in the unstimulated patients (P^0) compared to the unstimulated controls (C^0) at the following time points. Values are median (interquartile range): 24 hours: 663 (507-782) pg/ml vs 12 (5-53) pg/ml, p=0.004; 48 hours: 620 (570-1080) pg/ml vs 14.5 (11-36) pg/ml, p=0.004; 72 hours: 545.5 (442-771) pg/ml vs 12.5 (2-43) pg/ml, p=0.006. The IL-1β protein level was significantly decreased in the unstimulated patients (P^0) compared to the unstimulated controls (C^0) at 0 hours: 0 (0-2) pg/ml vs 9 (3-14) pg/ml, p=0.010. The IL-1β protein level was also significantly decreased in the stimulated patients (P^5) compared to stimulated controls (C^5) at 0 hours: 0.5 (0-2) pg/ml vs 6.5 (2-9) pg/ml, p=0.024. No other significant differences in protein levels were found.

In order to determine whether particular alleles corresponded to protein levels, the subjects were separated according to whether their genotypes contained allele 1 (1,1 and 1,2) or allele 2 (2,2 and 1,2). The data are shown in Tables 6.21C and 6.21D. Figure 6.18 shows the median IL-1β protein levels in subjects with allele 1 and subjects with allele 2 separated according to whether the cells were unstimulated or stimulated with LPS ($A1^0$ vs $A2^0$ and $A1^5$ vs $A2^5$). No significant differences in IL-1β protein secretion were found between subjects with allele 1 or allele 2 at any time point for unstimulated or stimulated cells.

$A1^0$= unstimulated subjects with allele 1
$A1^5$= stimulated subjects with allele 1
$A2^0$= unstimulated subjects with allele 2
$A2^5$= stimulated subjects with allele 2

Table 6.21C: IL-1β protein (pg/ml) secreted by PBMCs cultured at $1x10^6$ cells per ml and stimulated with 0-5μg/ml LPS for 0-72 hours from subjects with allele 1 ($A1^0$ and $A1^5$)

Time (hours) LPS (μg/ml)	G/T	0 0	24 0	48 0	72 0	0 5	24 5	48 5	72 5
Subject 1	1,1	12	1033	2354	4210	93	14900	32860	26240
Subject 2	1,1	7	304	305	311	63	10140	16230	12320
Subject 3	1,1	97	2480	1562	813	118	20290	28790	27430
Subject 4	1,1	7	464	488	1263	125	10850	22820	19640
Subject 5	1,1	150	1321	3727	4610	173	9950	15340	12000
Subject 6	1,1	0	1735	2864	3322	217	7080	9820	8820
Subject 7	1,2	8	5	11	0	7	4300	3340	3070
Subject 8	1,2	1	14	13	15	2	3070	2990	2780
Subject 9	1,1	0	782	1080	771	0	4090	3920	3880
Subject 10	1,1	0	433	570	422	0	2570	2030	1720
Subject 11	1,1	0	1032	1253	931	0	4680	4010	3630
n		11	11	11	11	11	11	11	11
Median		7	782	1080	813	63	7080	9820	8820
Range		0-150	5-2480	11-3727	0-4610	0-217	2570-20290	2030-32860	1720-27430
i.q.r.		0-12	304-1321	305-2354	311-3322	0-125	4090-10850	3340-22820	3070-19640

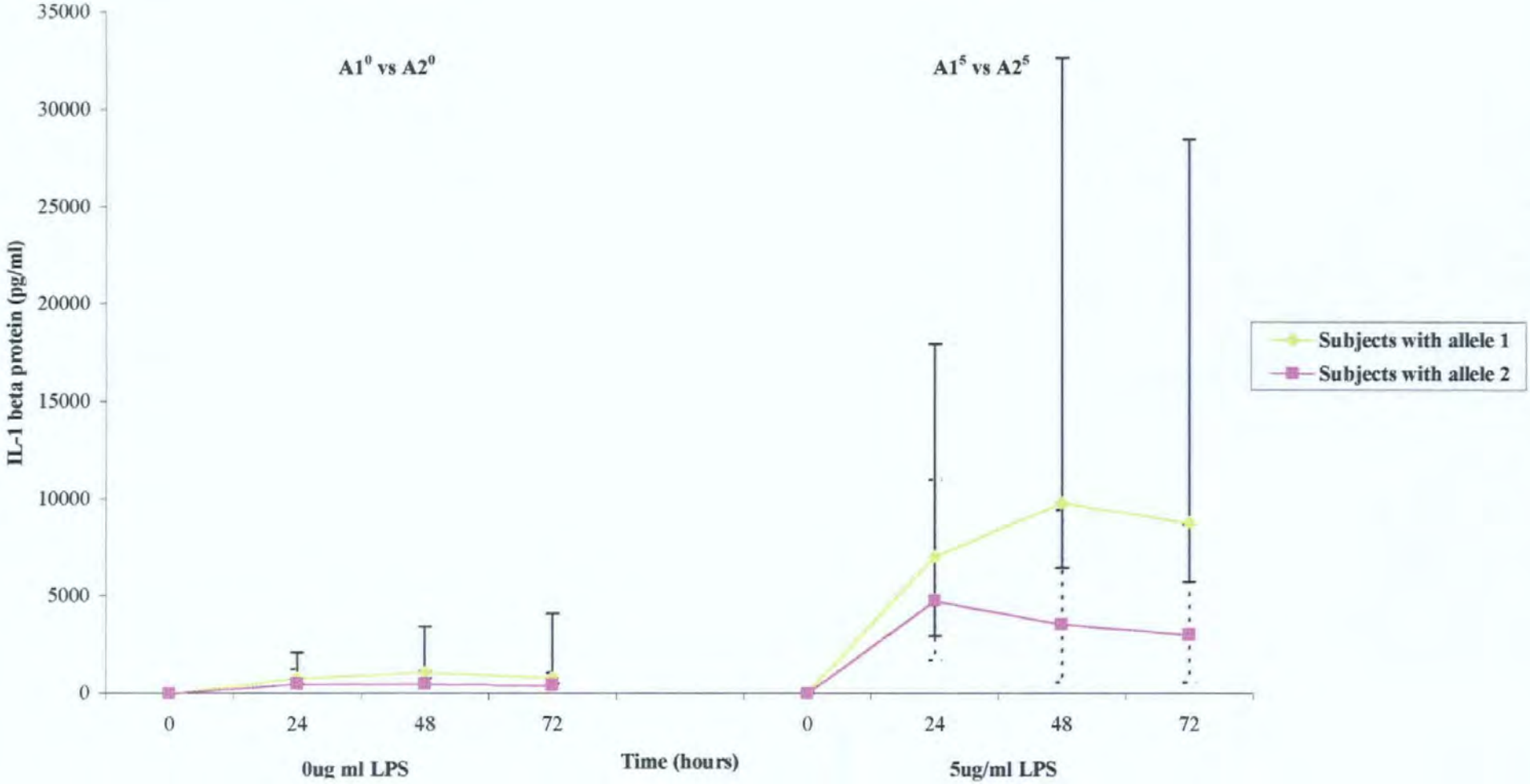
Table 6.21D: IL-1β protein (pg/ml) secreted by PBMCs cultured at $1x10^6$ cells per ml and stimulated with 0-5μg/ml LPS for 0-72 hours from subjects with allele 2 ($A2^0$ and $A2^5$)

Time (hours) LPS (μg/ml)	G/T	0 0	24 0	48 0	72 0	0 5	24 5	48 5	72 5
Subject 1	2,2	396	3108	3789	3836	354	12310	14190	13960
Subject 2	2,2	3	6	11	2	6	6180	5870	5610
Subject 3	1,2	8	5	11	0	7	4300	3340	3070
Subject 4	1,2	1	14	13	15	2	3070	2990	2780
Subject 5	2,2	2	598	593	608	3	4780	4770	4240
Subject 6	2,2	2	507	508	442	2	3040	2740	2460
Subject 7	2,2	0	728	647	483	1	4860	3560	2510
n		7	7	7	7	7	7	7	7
Median		2	507	508	442	3	4780	3560	3070
Range		0-396	5-3108	11-3789	0-3836	1-354	3040-12310	2740-14190	2460-13960
i.q.r.		1-8	6-728	11-647	2-608	2-7	3070-6180	2990-5870	2510-5610

G/T= genotype
i.q.r. = interquartile range

No significant differences in IL-1β protein secretion were found when unstimulated and stimulated cells from subjects with allele 1 were compared with those from subjects with allele 2 at any time point.

Figure 6.18: Median IL-1 beta protein secreted from PBMCs cultured at $1x10^6$ cells per ml in subjects with allele 1 and allele 2 separated according to whether the PBMCs were unstimulated or stimulated with 5ug/ml LPS



Values are median (interquartile range)
Solid vertical bars represent interquartile range for subjects with allele 1
Dashed vertical bars represent interquartle range for subjects with allele 2

6.2.3 Interleukin-1 receptor antagonist protein

6.2.3.1 Interleukin-1 receptor antagonist time course experiment

The 120 hour time course experiment showed that the peak IL-1ra protein secretion occurred at 48 hours (Figure 6.19)

6.2.3.2 Interleukin-1 receptor antagonist protein experiment

The most significant results in the polymorphism studies of the IL-1RN gene were obtained from the VNTR[86] polymorphism. Allele 1 was significantly increased in the severe group of patients compared to controls (81.9% vs 63.0%, $\chi 2=9.38$, p=0.002; Pc=0.004) and in the idiopathic patients compared to controls (82.4% vs 63.0%, $\chi 2=9.33$, p=0.002; Pc=0.004).

"*Hypothesis: allele 1 may be associated with severity of acute pancreatitis and also susceptibility to idiopathic acute pancreatitis.*"

Patients with idiopathic disease with the 1,1 and 1,2 genotype were recalled for this study and the amount of IL-1ra protein secreted from their PBMCs stimulated with 0-5μg/ml LPS for 0-72 hours was compared with IL-1ra secreted from PBMCs taken from healthy controls. The genotypes, protein levels (individual and median) stimulated with 0 and 5μg/ml LPS for 0-72 hours in controls and patients are shown in Tables 6.22A and 6.22B respectively. The protein levels for the individual controls and patients are shown in Figure 6.20. The protein secreted from all subjects peaked at 48 hours as in the time course study, however, the amount of protein produced varied in each case.

Figure 6.19: IL-1ra protein secreted from PBMCs cultured at $1x10^6$ cells per ml and stimulated with 5ug/ml LPS for 0-120 hours

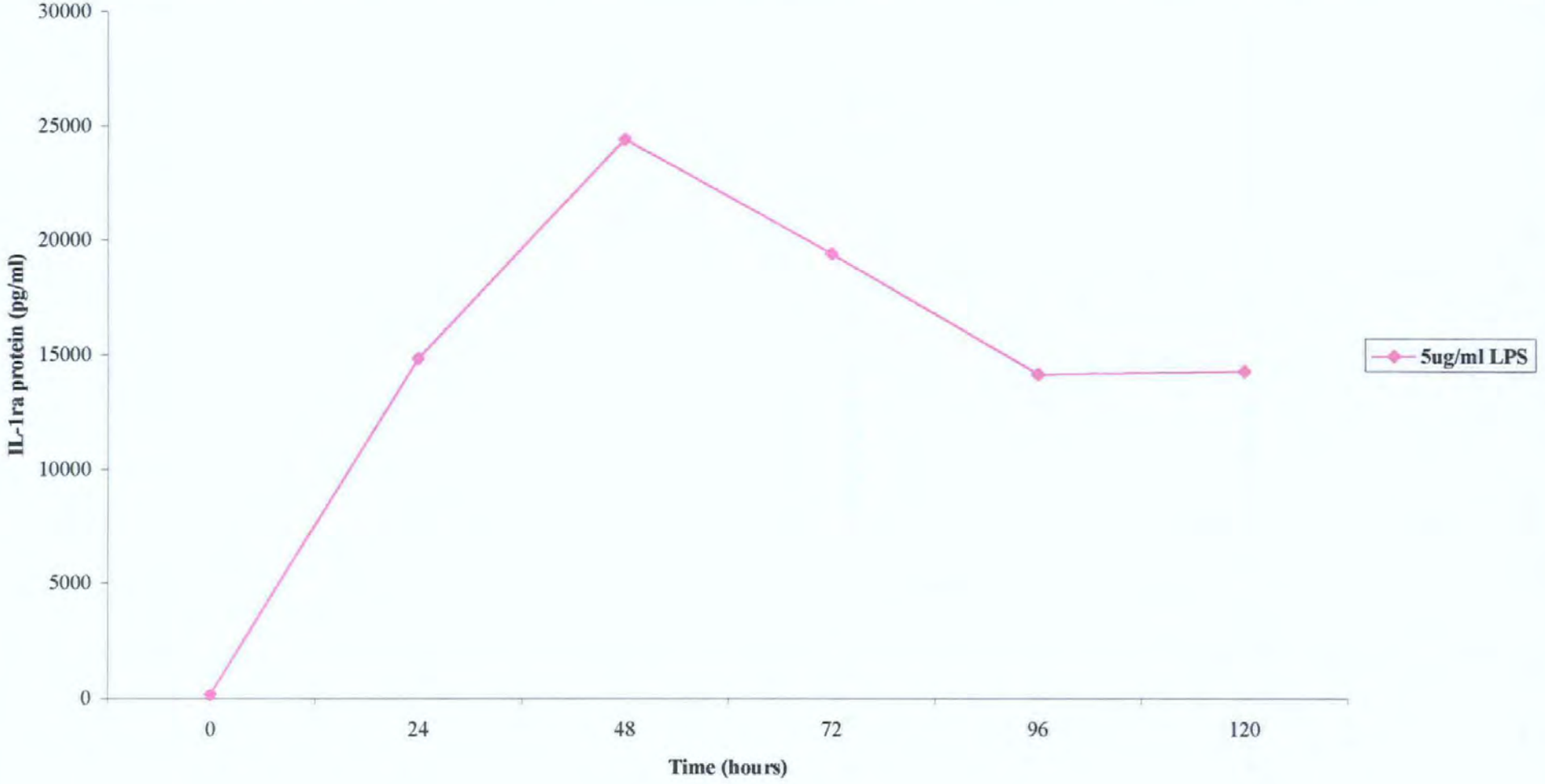


Table 6.22A: IL-1ra protein (pg/ml) secreted by PBMCs cultured at $1x10^6$ cells per ml and stimulated with 0-5μg/ml LPS for 0-72 hours in controls (C^0 and C^5)

Time (hours) LPS (μg/ml)	G/T	0 0	24 0	48 0	72 0	0 5	24 5	48 5	72 5
Control 1	1,2	396	3108	3789	3836	354	12310	14190	13960
Control 2	1,1	12	1033	2354	4210	93	14900	32860	26240
Control 3	1,2	7	304	305	311	63	10140	16230	12320
Control 4	1,2	97	2480	1562	813	118	20290	28790	27430
Control 5	1,1	7	464	488	1263	125	10850	22820	19640
Control 6	1,1	52	637	587	1035	86	12110	21520	16330
Control 7	1,1	149	759	1137	2430	112	13890	18640	17170
Control 8	1,2	150	1321	3727	4610	173	9950	15340	12000
Control 9	1,1	0	1735	2864	3322	217	7080	9820	8820
Control 10	1,1	108	164	159	345	44	7000	10440	9590
n		10	10	10	10	10	10	10	10
Median		74.5	896	1349.5	1846.5	115	11480	17435	15145
Range		0-396	164-3108	159-3789	311-4610	44-354	7000-20290	9820-32860	8820-27430
i.q.r.		7-149	464-1735	488-2864	813-3836	86-173	9950-13890	14190-22820	12000-19640

Table 6.22B: IL-1ra protein (pg/ml) secreted by PBMCs cultured at $1x10^6$ cells per ml and stimulated with 0-5μg/ml LPS for 0-72 hours in patients with acute pancreatitis (P^0 and P^5)

Time (hours) LPS (μg/ml)	G/T	0 0	24 0	48 0	72 0	0 5	24 5	48 5	72 5
Patient 1	1,1	0	241	288	344	0	2750	3140	2000
Patient 2	1,1	397	984	1373	1342	203	6610	11040	8870
Patient 3	1,1	17	5539	8092	6547	0	14480	24960	20520
Patient 4	1,1	237	1713	2780	2256	0	1140	3170	2520
Patient 5	1,1	21	209	268	293	16	20010	24340	19330
Patient 6	1,2	378	4802	6028	6079	385	19690	23860	21220
Patient 7	1,2	215	7766	10890	12010	0	20910	37820	33210
Patient 8	1,2	28	507	703	595	18	11730	15030	13130
n		8	8	8	8	8	8	8	8
Median		121.5	1348.5	2076.5	1799	8	13105	19445	16230
Range		0-397	209-7766	268-10890	293-12010	0-385	1140-20910	3140-37820	2000-33210
i.q.r.		19-307.5	374-5170.5	495.5-7060	469.5-6313	0-110.5	4680-19850	7105-24650	5695-20870

G/T= genotype
i.q.r.= interquartile range
There were no significant differences in IL-1ra protein secretion between controls and patients at any time point for unstimulated or stimulated cells.

Figure 6.20: IL-1ra protein secreted from PBMCs cultured at $1x10^6$ cells per ml and stimulated with 0-5ug/ml LPS for 0-72 hours in controls and patients with acute pancreatitis

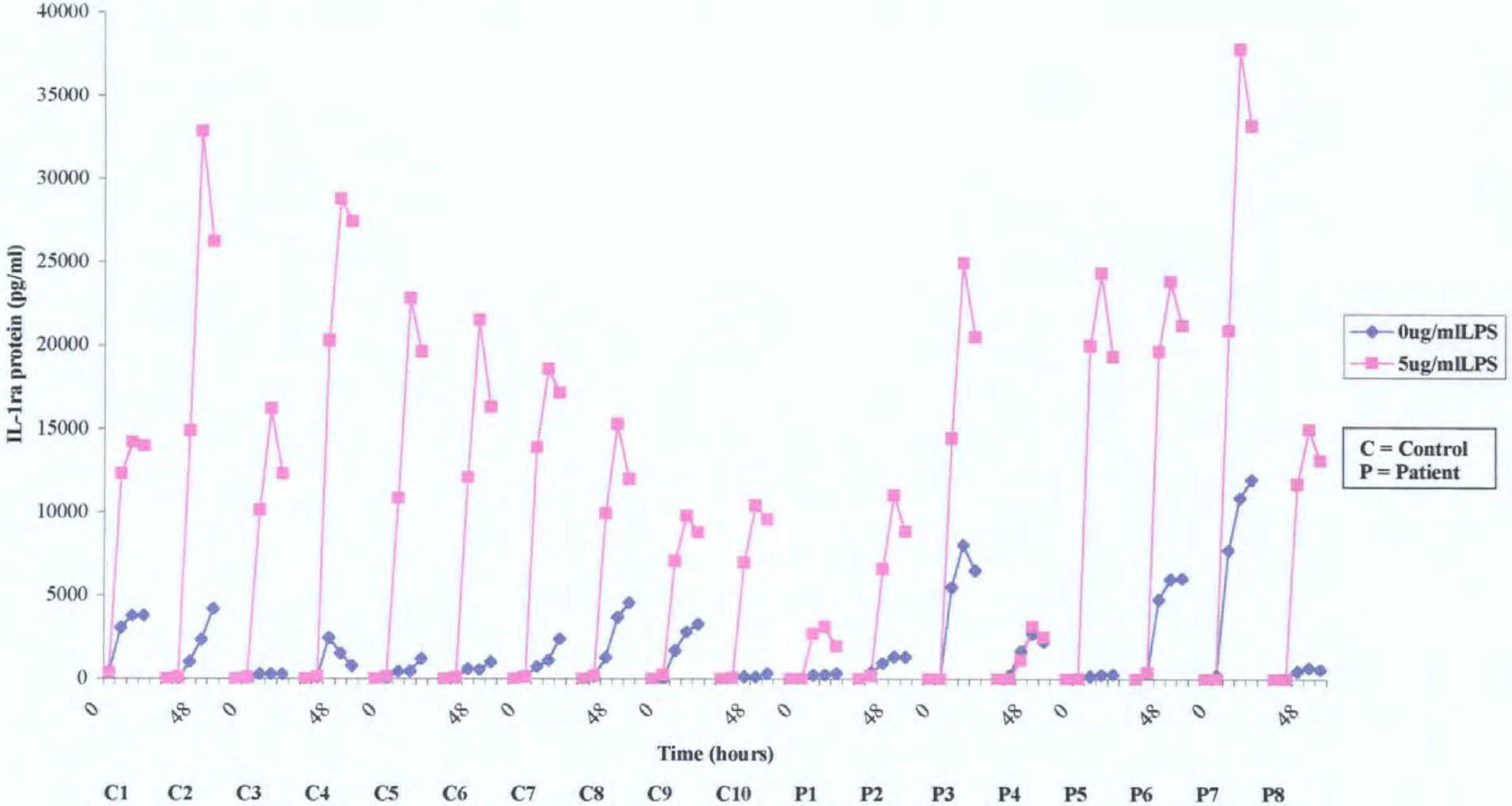
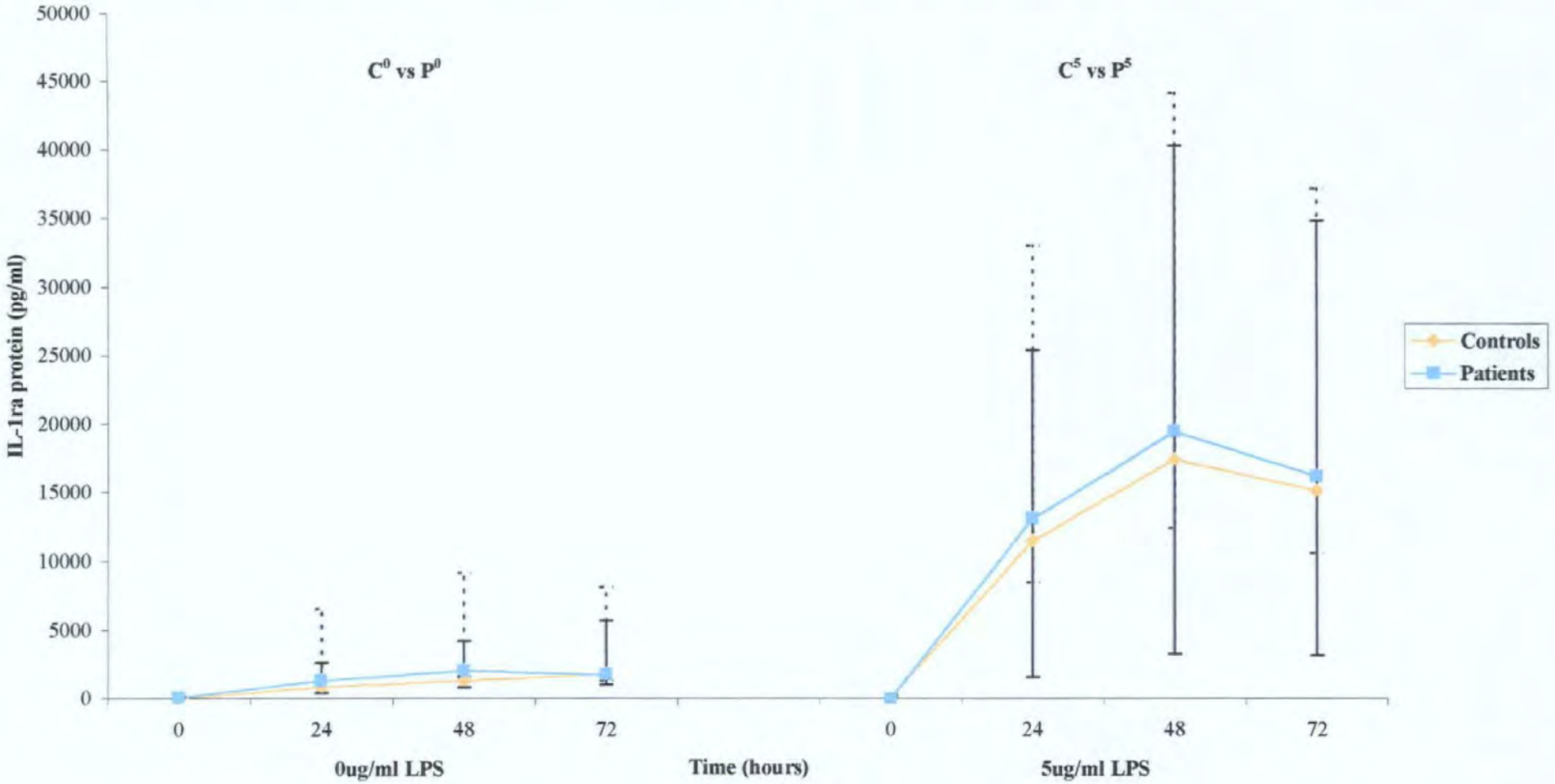


Figure 6.21 shows the median IL-1ra protein levels separated according to whether the cells were unstimulated or stimulated with LPS (C^0 vs P^0 and C^5 vs P^5). No significant differences in protein levels were found between unstimulated cells in controls (C^0) and patients (P^0) or between stimulated cells in controls (C^5) and patients (P^5).

In order to determine whether particular alleles corresponded to protein levels, the subjects were separated according to whether their genotypes contained allele 1 (1,1) and allele 2 (1,2). (The 2,2 genotype was rare and therefore subjects willing to take part in the study could not be found). The data are shown in Tables 6.22C and 6.22D. Figure 6.22 shows the median IL-1ra protein levels in subjects with allele 1 and subjects with allele 2 separated according to whether the cells were unstimulated or stimulated with LPS ($A1^0$ vs $A2^0$ and $A1^5$ vs $A2^5$). No significant differences in IL-1ra protein secretion were found between subject groups with allele 1 or allele 2 at any time point for unstimulated cells or stimulated cells.

C^0= unstimulated controls
C^5= stimulated controls
$A1^0$= unstimulated subjects with allele 1
$A2^0$= unstimulated subjects with allele 2
P^0= unstimulated patients
P^5= stimulated patients
$A1^5$= stimulated subjects with allele 1
$A2^5$= stimulated subjects with allele 2

Figure 6.21: Median IL-1ra protein secreted from PBMCs cultured at $1x10^6$ cells per ml for 0-72 hours in controls and patients separated according to whether the PBMCs were unstimulated or stimulated with 5ug/ml LPS



Values are median (interquartile range)
Solid vertical bars represent interquartile range for controls
Dashed vertical bars represent interquartile range for patients

Table 6.22C: IL-1ra protein (pg/ml) secreted by PBMCs cultured at $1x10^6$ cells per ml and stimulated with 0-5μg/ml LPS for 0-72 hours from subjects with allele 1 ($A1^0$ and $A1^5$)

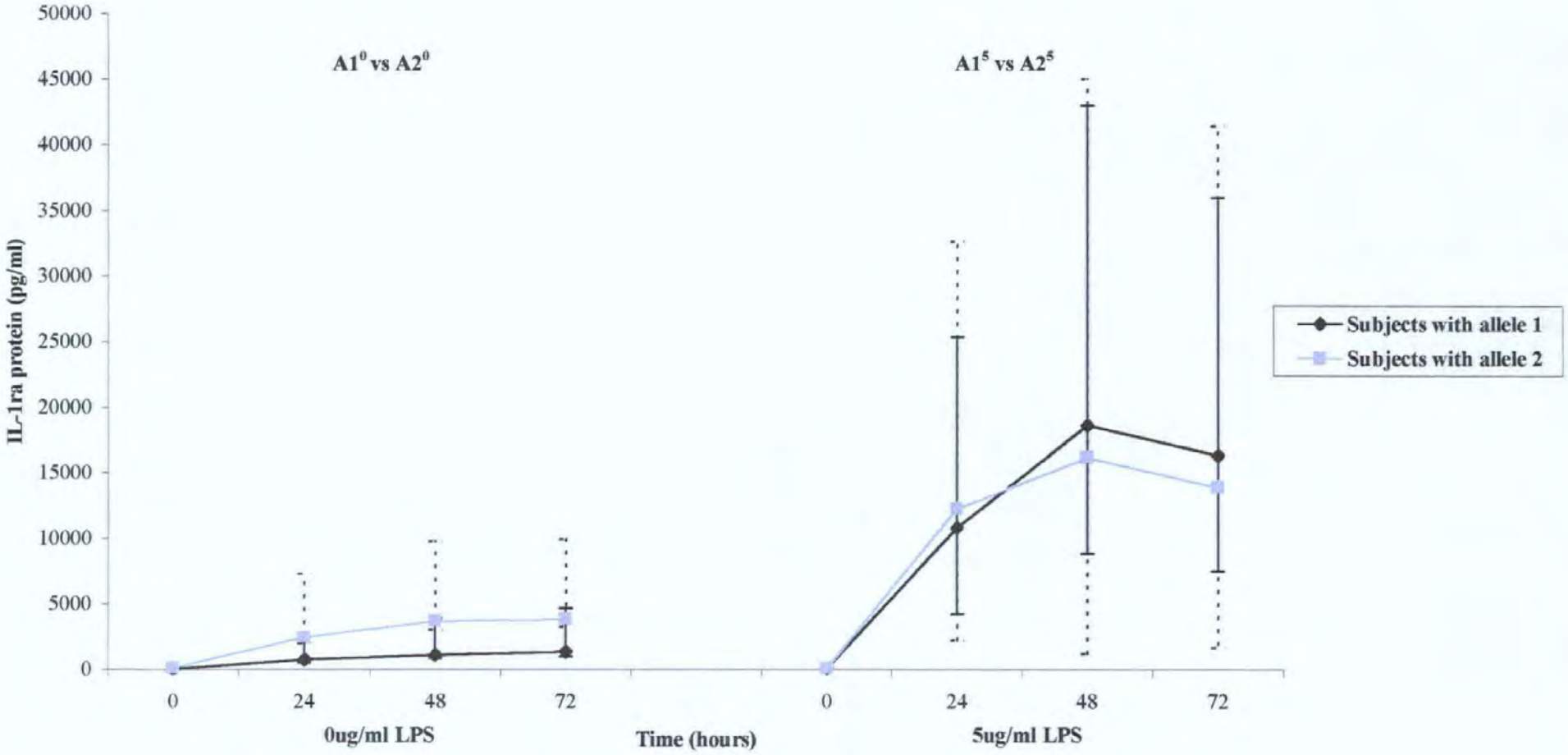
Time (hours) LPS(μg/ml)	G/T	0 0	24 0	48 0	72 0	0 5	24 5	48 5	72 5
Subject 1	1,1	12	1033	2354	4210	93	14900	32860	26240
Subject 2	1,1	7	464	488	1263	125	10850	22820	19640
Subject 3	1,1	52	637	587	1035	86	12110	21520	16330
Subject 4	1,1	149	759	1137	2430	112	13890	18640	17170
Subject 5	1,1	0	1735	2864	3322	217	7080	9820	8820
Subject 6	1,1	108	164	159	345	44	7000	10440	9590
Subject 7	1,1	0	241	288	344	0	2750	3140	2000
Subject 8	1,1	397	984	1373	1342	203	6610	11040	8870
Subject 9	1,1	17	5539	8092	6547	0	14480	24960	20520
Subject 10	1,1	237	1713	2780	2256	0	1140	3170	2520
Subject 11	1,1	21	209	268	293	16	20010	24340	19330
n		11	11	11	11	11	11	11	11
Median		21	759	1137	1342	86	10850	18640	16330
Range		0-397	164-5539	159-8092	293-6547	0-217	1140-20010	3140-32860	2000-26240
i.q.r.		7-149	241-1713	288-2780	345-3322	0-125	6610-14480	9820-24340	8820-19640

Table 6.22D: IL-1ra protein (pg/ml) secreted by PBMCs cultured at $1x10^6$ cells per ml and stimulated with 0-5μg/ml LPS for 0-72 hours from subjects with allele 2 ($A2^0$ and $A2^5$)

Time (hours) LPS (μg/ml)	G/T	0 0	24 0	48 0	72 0	0 5	24 5	48 5	72 5
Subject 1	1,2	396	3108	3789	3836	354	12310	14190	13960
Subject 2	1,2	7	304	305	311	63	10140	16230	12320
Subject 3	1,2	97	2480	1562	813	118	20290	28790	27430
Subject 4	1,2	150	1321	3727	4610	173	9950	15340	12000
Subject 5	1,2	378	4802	6028	6079	385	19690	23860	21220
Subject 6	1,2	215	7766	10890	12010	0	20910	37820	33210
Subject 7	1,2	28	507	703	595	18	11730	15030	13130
n		7	7	7	7	7	7	7	7
Median		150	2480	3727	3836	118	12310	16230	13960
Range		7-396	304-7766	305-10890	311-12010	0-385	9950-20910	14190-37820	12000-33210
i.q.r.		28-378	507-4802	703-6028	595-6079	18-354	10140-20290	15030-28790	12320-27430

G/T= genotype
i.q.r.= interquartile range
No significant differences in IL-1ra protein secretion were found when unstimulated and stimulated cells from subjects with allele 1 were compared with those from subjects with allele 2 at any time point.

Figure 6.22: Median IL-1ra protein secreted from PBMCs cultured at $1x10^6$ cells per ml for 0-72 hours in subjects with allele 1 and allele 2 separated according to whether the PBMCs were unstimulated or stimulated with 5ug/ml LPS



Values are median (interquartile range)
Solid vertical bars represent interquartile range for subjects with allele 1
Dashed vertical bars represent interquartile range for subjects with allele 2

Summary of the Interleukin-1 alpha, Interleukin-1 beta and Interleukin-1 receptor antagonist protein protein studies

Correlation of the (AC)n polymorphism with IL-1α protein levels.

IL-1α protein levels peaked at 24 hours in all subjects, but there was inter-individual variation in the amount secreted. Comparison of the median IL-1α protein levels between unstimulated patients (P^0) and unstimulated controls (C^0) showed that the levels were significantly increased in patients compared to controls. Significant differences in median IL-1α protein levels were found at 24 hours (193.5 (127.5-363.5) pg/ml vs 1.0 (0.0-3.0) pg/ml, p=0.005), 48 hours (256.5 (171.5-417.0) pg/ml vs 6.5 (2.0-16.0) pg/ml, p=0.006) and at 72 hours (210.5 (138-427) pg/ml vs 0.5 (0-7) pg/ml, p=0.005). Comparison of the median IL-1α protein levels between stimulated patients (P^5) and stimulated controls (C^5) showed that the levels were also increased in patients compared to controls, however, a significant difference was only found at 24 hours (875 (675-1355) pg/ml vs 515 (440-600) pg/ml, p=0.028). No genotype-protein correlation was performed for the (AC)n polymorphism of the IL-1A gene due to lack of subjects with the 2,4 genotype.

Correlation of the Alu I polymorphism with IL-1β protein levels.

IL-1β protein levels peaked at 24 hours in all subjects, but there was inter-individual variation in the amount secreted. Comparison of the median IL-1β protein levels between unstimulated patients (P^0) and unstimulated controls (C^0) showed that the levels were significantly increased in patients compared to controls. Significant differences in median IL-1β protein were found at 24 hours (663 (507-782) pg/ml vs 12 (5-53) pg/ml, p=0.004), 48 hours (620 (570-1080) pg/ml vs 14.5 (11-36) pg/ml, p=0.004) and at 72 hours (545.5 (442-771) pg/ml vs 12.5 (2-43) pg/ml, p=0.006). Comparison of the median IL-1β protein levels between unstimulated patients (P^0) and unstimulated controls (C^0) at time 0

hours showed that the levels were significantly decreased in patients compared to controls: 0(0-2) pg/ml vs 9(3-14) pg/ml, p=0.010).

Comparison of the median IL-1β protein levels between stimulated patients (P^5) and stimulated controls (C^5) showed that the levels were decreased in patients compared to controls, however, a significant difference was only found at 0 hours (0.5 (0-2) pg/ml vs 6.5 (2-9) pg/ml, p=0.024). No significant differences in the median IL-1β levels were found when the subjects were separated according to those with genotypes containing allele 1 or 2 ($A1^0$ vs $A2^0$ and $A1^5$ vs $A2^5$).

Correlation of the $VNTR^{86}$ polymorphism with IL-1ra protein levels

IL-1ra protein levels peaked at 48 hours in all subjects, but there was inter-individual variation in the amount secreted. No significant differences were found when comparing the median IL-1ra protein levels between unstimulated patients (P^0) and unstimulated controls (C^0) or between stimulated patients (P^5) and stimulated controls (C^5). No significant differences in the median IL-1ra levels were found when the subjects were separated according to those with genotypes containing allele 1 or 2 ($A1^0$ vs $A2^0$ and $A1^5$ vs $A2^5$).

CHAPTER 7: GENERAL DISCUSSION

This is the first genetic study to characterise the IL-1 gene cluster in patients with acute pancreatitis and therefore the results can only be compared with other diseases in which the polymorphisms have been studied. Figure 7.1 illustrates a map of the IL-1 gene cluster and IL-1 receptor genes and summarises the results obtained from the thesis. Although many results were found, only the most important are discussed. Strong associations were found with polymorphisms in the IL-1RN gene (*Msp I*, VLP, VNTR^{86}, *Ssp I*). Weak or no associations were found with polymorphisms in the IL-1RI (*Pst I*), IL-1A ((AC)n, VNTR^{46}) and IL-1B (*Ava I*, *Alu I*, *Taq I*) genes. The strong associations will be discussed first, followed by the weak associations.

Interestingly, a study of polymorphisms in the genes for other pro-inflammatory TNFα (TNFa, TNFb, TNFc, TNF-308) and anti-inflammatory cytokines IL-10 (IL-10.G, -1117, -854, -627) on the same group of patients with acute pancreatitis showed no association with disease susceptibility or severity (Sargen et al, 2000).

7.1 DNA Studies

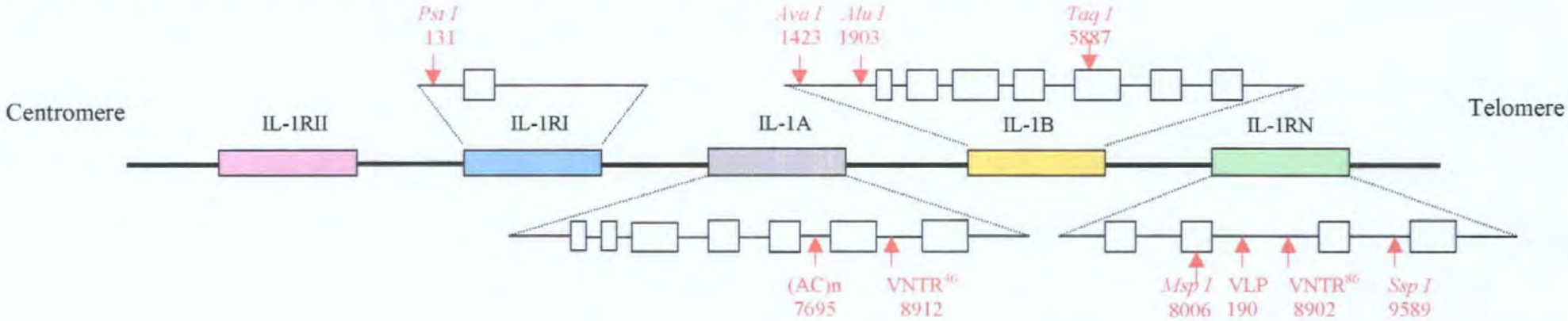
7.1.1 Genotype Analysis

7.1.1.1 IL-1RN gene

Msp I polymorphism

The association of the 1,1 genotype and allele 1 (640bp) with severe patients suggests that the *Msp I* polymorphism may be a severity marker for acute pancreatitis. An association was also found between allele 1 and idiopathic patients. This implies that the polymorphism may also be a susceptibility marker for idiopathic acute pancreatitis and suggests that genetic heterogeneity exists within the disease. This supports studies that show genetic heterogeneity exists within hereditary pancreatitis (Ferec et al, 1999).

Figure 7.1: Map of the IL-1 gene cluster and IL-1 receptor genes with a summary of the results



Subjects	Polymorphism									
	Pst I	**(AC)n**	**VNTR^{46}**	***Ava I***	***Alu I***	***Taq I***	***Msp I***	**VLP**	**VNTR^{86}**	***Ssp I***
Patients	++	-	-	-	++	-	-	+++ ++++	++++++	-
Mild	-	-	-	-	-	-	-	-	++++	-
Severe	-	-	-	-	-	-	+++ +++	+++++ +++++	+++++ ++++++ +++ +(vsmild) +(vsmild)	++++ +++ +++
OFS=0	-	-	-	-	-	-	-	-	+++	-
OFS≥1	+++	-	-	-	-	++(vsOFS=0)	-	-	+	-
OFS≥2	-	++ ++(vsOFS=0)	-	-	-	+ +++(vsOFS=0)	-	-	+	-
OFS≥3	-	-	-	-	-	-	-	-	+	-
OFS≥4	-	-	-	-	-	-	-	-	-	-
Alcoholics	-	+	-	-	-	-	-	-	-	-
Idiopathics	-	-	-	-	-	-	+++	+++ ++	+++++ +++ ++++++ +++	+++
Gallstones	+++	+++++(vs Alc)	-	-	-	-	-	-	++	-

++++++ = strong association + = weak association - = no association

All comparisons are with controls, unless stated differently

+ = association with genotype frequency

+ = association with allele frequency

To date, there have been no studies reporting an association of the *Msp I* polymorphism with disease or with functional protein levels. If allele 1 was found to correlate with low IL-1ra protein levels, 1,1 homozygotes would have reduced IL-1ra protein compared to individuals with 1,2 or 2,2 genotypes and would therefore be more at risk of developing acute pancreatitis or a more severe form of the disease.

Variable length polymorphism

The 2,2 genotype and allele 2 (1.45kb) were significantly increased in idiopathic patients compared to controls. These results suggest that the variable length polymorphism may be a susceptibility marker for acute pancreatitis and in particular idiopathic disease. The frequencies of the 2,2 genotype and allele 2 were also significantly increased in severe group compared to controls implying that the polymorphism may also be a marker of disease severity. As yet, there have been no studies reporting an association of the variable length polymorphism with disease or with functional protein levels. The results suggest that allele 2 may be associated with decreased IL-1ra production since this allele and the 2,2 genotype were significantly increased in patients with acute pancreatitis and in patients with severe disease.

VNTR^{86} polymorphism

The VNTR^{86} polymorphism is the most extensively studied polymorphism of the IL-1RN gene and of the IL-1 gene cluster. In this study, the 1,1 genotype and allele 1 (4 x 86bp repeats) were significantly increased in the severe group compared to controls implying that this polymorphism may act as a severity marker for acute pancreatitis. The 1,1 genotype and allele 1 were also significantly increased in the idiopathic group compared to controls. These observations suggest that the VNTR^{86} polymorphism may be a susceptibility marker for idiopathic acute pancreatitis.

To date, only one other group based in Edinburgh has studied the VNTR[86] polymorphism in patients with acute pancreatitis. In their study, no association was found between the genotype or allele frequencies suggesting that this polymorphism is neither a marker of susceptibility or severity for the disease (Powell et al, 2001). The possible reasons for the difference in results obtained between the two studies are discussed later.

Studies regarding an association between this polymorphism and functional IL-1ra protein levels are conflicting. One study suggests that allele 2 (2 x 86bp repeats) correlates with low IL-1ra protein level. The 86bp repeat sequence is comprised of 3 protein-binding sites: an α-interferon silencer A, a β-interferon silencer B and an acute phase response element. The activity of the enhancer sequences and hence the transcription of the IL-1RN gene may depend on the number of tandem copies present in the gene - the less 86bp repeats, the less protein-binding sites and the less IL-1ra protein is produced. Allele 2 homozygotes with 2 x 86bp repeats would therefore have reduced levels of IL-1ra protein compared to allele 1 homozygotes with 4 x 86bp repeats (Tarlow et al, 1993). Interestingly a similar VNTR polymorphism in the IL-1A gene which consists of 46bp repeating units has been found to have an inverse correlation with IL-1α production - the more repeats the less IL-1α is produced (Bailly et al, 1996). In contrast, a subsequent study has reported that allele 2 (2 x 86bp repeats) corresponds to high IL-1ra protein levels (Danis et al, 1995). If allele 2 corresponds to high IL-1ra secretion as suggested by Danis et al, then allele 1 may correspond to low IL-1ra secretion. This hypothesis has been supported by data from Mandrup-Poulsen et al, who found that the 1,1 genotype was associated with low plasma IL-1ra levels in type 1 diabetes (Mandrup-Poulsen et al, 1994).

The results obtained in this thesis support the studies by Danis and Mandrup-Poulsen and imply that allele 1 (4 x 86bp repeats) is associated with reduced IL-1ra protein levels. 1,1 homozygotes would have lower IL-1ra levels compared to individuals with other

genotypes and are therefore at greater risk of developing acute pancreatitis or a more severe form of the disease.

There have been many studies linking the VNTR^{86} polymorphism with disease and most have reported an association with allele 2. For example, increased frequency of this allele has been associated with susceptibility to ulcerative colitis (Mansfield et al, 1994) and multiple sclerosis (Crusius et al, 1995) and with severity of alopecia areata (Tarlow et al, 1994) and diabetic nephropathy (Blakemore et al, 1996). In contrast, increased frequency of allele 1 has been associated with susceptibility to hepatic fibrosis in alcoholic liver disease (Takamatsu et al, 1998), asthma in black South Africans (Pillay et al, 2000) and with severity and susceptibility to multiple sclerosis (Sciacca et al, 1999). For other disease associations with this polymorphism see Tables 4.2A-C.

Ssp I polymorphism

The association between the 1,1 genotype and allele 1 (379bp) in severe patients implies that the *Ssp I* polymorphism may be a severity marker for acute pancreatitis. Furthermore, an association was also found between allele 1 and the idiopathic patients. This suggests that the polymorphism may be a susceptibility marker for idiopathic acute pancreatitis and suggests genetic heterogeneity within the disease. To date, there have been no studies reporting an association of the *Ssp I* polymorphism with disease or with functional protein levels. As with the *Msp I* polymorphism, if allele 1 was found to correspond with low IL-1ra protein levels, then 1,1 homozygotes would have reduced IL-1ra protein compared to individuals with 1,2 or 2,2 genotypes and would therefore be more at risk of developing acute pancreatitis or a more severe form of the disease. It is interesting to note that all 4 of the polymorphisms studied in the IL-1RN gene confer susceptibility to idiopathic acute pancreatitis and with severity of the disease.

7.1.1.2 IL-1RI, IL-1A and IL-1B genes

Pst I Polymorphism

An association was found between the allele frequencies of the *Pst I* polymorphism in the IL-1RI gene and acute pancreatitis, but not the genotype frequencies. Allele 2 (97bp, 252bp) was significantly increased in patients compared to controls and in patients with gallstone disease compared to controls. This suggests that allele 2 may be a marker of disease susceptibility to acute pancreatitis and in particular patients with gallstone disease. The lack of an association with idiopathic or alcoholic disease again implies that there may be genetic heterogeneity within acute pancreatitis. Allele 2 was also significantly increased in the OFS≥1 group compared to controls, implying that the *Pst I* polymorphism may also be a marker of disease severity. However, since there was no association with the OFS≥2,3,or 4 groups that are progressively more severe forms of the disease, this association with disease severity is questionable. The lack of an association between the other organ failure score groups may have been due to the lower number of patients within these groups that meant the figures could not reach significance.

The *Pst I* polymorphism which was originally identified as a restriction fragment length polymorphism (RFLP) consisting of 2 fragments: a more common fragment (1.2kb) and a less common fragment (3.2kb) (Pociot et al, 1992) has been associated with susceptibility to IDDM in both Dutch (Pociot et al, 1994; Bergholdt et al, 1995) and British (Metcalfe et al, 1996) populations. The studies involving the Dutch population showed an association with genotype frequencies only, whereas the British study showed an association with both the *Pst I* genotype and allele frequencies. The 1,1 genotype and allele 1 (362bp) were significantly increased in patients compared to controls while the 2,2 genotype and allele 2 (256bp, 106bp) were significantly decreased between the same 2 groups. These trends in allele frequency are opposite to those found in this study for acute pancreatitis and may be due to the fact that IDDM is an autoimmune disease whereas acute

pancreatitis is an inflammatory disease. This suggests that the 2 alleles may act differently according to the disease pathogenesis.

Since the *Pst I* polymorphism is located in the promoter region of the gene, the T→C nucleotide substitution may affect the transcription. If allele 2 corresponded with increased IL-1RI expression it would cause an increase in the activity of IL-1 resulting in an augmented inflammatory response. Conversely, if allele 2 corresponded with decreased IL-1RI expression it would cause a decrease in IL-1 activity resulting in an attenuated inflammatory response. There is no evidence to date to suggest that this polymorphism has functional significance; however, it may be in linkage disequilibrium with another polymorphism in the IL-1RI gene that has not yet been identified. The haplotype analysis performed in this study suggests that the *Pst I* polymorphism is in linkage disequilibrium with other genes in the IL-1 gene complex. The functional effect of this polymorphism could be tested using reporter gene assays similar to those used in the study to determine the effect of the -174 (G→C) *NlaIII* polymorphism in the promoter region of the IL-6 gene (Fishman et al, 1998).

(AC)n repeat polymorphism

The 2,4 genotype of this polymorphism was significantly increased in the alcoholic group compared to controls and significantly decreased in the gallstone group compared to the alcoholic group. This suggests that the (AC)n repeat polymorphism may act as a marker for disease susceptibility to acute pancreatitis in particular alcoholic disease. It may also determine aetiologies most at risk whereby gallstone patients with 2,4 genotype are at less risk than alcoholic patients with 2,4 genotype. In addition, the 1,2 genotype was significantly increased in OFS≥2 group compared to controls and in the OFS≥2 group compared to the OFS=0 group. This suggests that the (AC)n polymorphism may act also act as a marker of severity for acute pancreatitis, however, since no significance was

obtained for OFS≥3 or OFS≥4 groups which are more severe forms of the disease, the link between severity is doubtful. Again, this may be due to the lower number of subjects within these groups.

Lack of an association was found between this (AC)n repeat polymorphism polymorphism and rheumatoid arthritis (Gomolka et al, 1995), juvenile chronic arthritis (Donn et al, 1999) and Tuberculosis (Bellamy et al, 1998). Polymorphisms located within introns of a gene can still affect protein levels (Beenken et al, 1991), however, as yet there have been no reports of an association between this polymorphism and functional IL-1α protein levels. Even if this dinucleotide repeat is not functional itself, the polymorphism may be in linkage disequilibrium with other polymorphisms in the gene. This is supported by the results from this thesis that imply the (AC)n repeat polymorphism is strongly linked with the $VNTR^{46}$ polymorphism in intron 6 and with polymorphisms in other genes of the IL-1 cluster.

$VNTR^{46}$ polymorphism

No significant differences in genotype or allele frequencies were found between controls, patients or patient subgroups for the $VNTR^{46}$ polymorphism suggesting that it is not a susceptibility or severity marker for acute pancreatitis. The involvement of this polymorphism in acute pancreatitis should not be totally disregarded, however, because as previously discussed it appears to be in linkage disequilibrium with the (AC)n repeat polymorphism that may be a susceptibility and/or severity marker for the disease.

Lack of an association was also found between this polymorphism and rheumatoid arthritis (Bailly et al, 1995). Each 46bp repeat contains 3 potential transcription factor-binding sites: SP1, viral enhancer element and glucocorticoid-responsive element (GRE) (Bailly et al, 1993). The presence of these transcription factor-binding sites implies that the polymorphism may be involved in IL-1α gene regulation. A recent study has shown that

there was an inverse relationship between the number of repeats and IL-1α production, suggesting that the polymorphism has a negative regulatory role on IL-1α gene expression (Bailly et al, 1996).

Ava I polymorphism

The lack of an association between this polymorphism and patients with acute pancreatitis suggests that *Ava I* polymorphism is not associated with susceptibility to or severity of the disease. However, it may be in linkage disequilibrium with another polymorphism that is associated with the disease. In fact the haplotype analysis performed in this study suggests that this polymorphism is strongly linked with the *Alu I* and *Taq I* polymorphisms of the IL-1B gene and with polymorphisms in other genes of the IL-1 gene cluster.

The *Ava I* polymorphism is located in the promoter region of the gene and may therefore affect transcription. This has been confirmed by a recent study which showed that allele 2 (717bp) was associated with high IL-1β secretion (Nemetz et al, 1999). The polymorphism has also been linked with a number of diseases. Increased carriage of allele 2 has been associated with susceptibility to alcoholic liver disease (Takamatsu et al, 2000) and gastric cancer (El-Omar et al, 2000) and with severity of ulcerative colitis, Crohn's disease (Nemetz et al, 1999) and rheumatoid arthritis (Di Giovine et al, 1992). For other associations see Tables 4.2A-C.

Alu I polymorphism

The association of the 2,2 genotype with patients implies that this polymorphism may be a susceptibility marker for acute pancreatitis. No significant differences in genotype or allele frequencies were found between the groups when they were separated according to

severity, organ failure scores or aetiology, suggesting that the *Alu I* polymorphism is not a susceptibility marker for a particular aetiology or a severity marker for acute pancreatitis.

A positive association has recently been found between this polymorphism and gastric cancer (El-Omar et al, 2000). The *Alu I* polymorphism is also located within the promoter region of the IL-1B gene and therefore may affect the expression of the gene, however, to date, no studies have demonstrated an association between this polymorphism and functional protein levels. Without these functional studies, it is only possible to speculate as to the effect of the *Alu I* polymorphism. If for example, allele 2 was found to be associated with low IL-1B protein levels, then 2,2 homozygotes would have low IL-1B levels and would be "protected". Since this low IL-1B genotype was decreased in patients compared to controls, the patients would be more susceptible to inflammation.

Taq I polymorphism

The *Taq I* polymorphism is the most extensively studied polymorphism of the IL-1B gene. The 1,2 genotype was significantly increased in the OFS≥2 group compared to controls, which implies that the polymorphism may be a susceptibility marker for acute pancreatitis. However, since no association was found between patients and controls or between different aetiological groups and controls, this link with susceptibility is unlikely. Furthermore, the same genotype was significantly increased in the OFS≥1 group compared to the OFS=0 group and was even more significantly increased in the OFS≥2 group compared to the OFS=0 group suggesting that the polymorphism may be a marker of severity. However, since there was no association with the OFS≥3 or OFS≥4 groups that are progressively more severe forms of the disease, this association with disease severity is questionable and probably represents a spurious result. As previously mentioned, the lack of an association between the other organ failure score groups may be due to the lower number of subjects within these groups.

Allele 2 (740bp) of the *Taq I* polymorphism of the IL-1B gene has been associated with high IL-1β protein production (Pociot et al, 1992). The *Taq I* polymorphism has been studied in a variety of diseases. Increased carriage of allele 2 has been associated with susceptibility to myasthenia gravis (Huang et al, 1998) and pancreatic cancer (Barber et al, 2000) and with the severity of periodontitis (Galbraith et al, 1999a) and multiple sclerosis (Kantarci et al, 2000). For other associations see Tables 4.2A-C.

Variability between different genetic studies

Many of the studies involving the IL-1 gene polymorphisms are not reproducible in different ethnic populations or within ethnic populations and often provide conflicting results. For example, the *Ava I* polymorphism was associated with rheumatoid arthritis in a British population (Di Giovine et al, 1992) but not in a French population (Cantagrel et al, 1999). The *Taq I* polymorphism polymorphism was associated with multiple sclerosis in an American population (Kantarci et al, 2000) but not in a Dutch population (Schrijver et al, 1999). The $VNTR^{86}$ polymorphism was associated with diabetic nephropathy in a British (Blakemore et al, 1996) but not in Danish population (Tarnow et al, 1997). Furthermore, Crusius et al found an association with multiple sclerosis in a Dutch population (Crusius et al, 1995) that could not be replicated by Schrijver et al in a similar cohort (Schrijver et al 1999).

A similar difference in result was obtained for the $VNTR^{86}$ and *Taq I* polymorphisms in acute pancreatitis (Powell et al, 2001) compared to this study. The reasons for no (or weak) associations may be related to a number of factors. The epidemiology of acute pancreatitis is known to vary between different geographical regions within the same country, therefore it might be expected that different subjects (controls and patients) taken from opposite ends of Britain might produce different results. This may represent a true difference in incidence or may be due to better diagnostic procedures in a

particular area of the country. Furthermore, population stratification may occur in areas such as Cornwall and Devon whereby because of the high percentage of Celtic population, the subjects do not represent a random selection. Also, the defining criteria that are to a certain extent subjective in nature may not be consistent with those used in this study. It is evident that the proportions of patients used in this thesis were different to those reported in other studies: 58% gallstones (80/137), 26% idiopathic (36/137) and 15% alcoholics (21/137) as compared to 40% gallstones, 30% idiopathic and 20% alcoholics reported by Lankisch et al (Lankisch et al, 1997). Another explanation is that there are different numbers of subjects in the cohorts. A small cohort of subjects might have a borderline non-significant value that would become significant in a larger cohort. Finally, there appears to be no universal method for correction of p values.

Whilst acknowledging that standards were not used on a day to day basis thus introducing the possibility of errors and differences in results between studies, in the case of the restriction digestion reactions, there were always samples which showed the 3 extremes of a restriction digestion reaction on a gel to show that the enzyme was working adequately (i.e. homozygotes for both alleles and heterozygotes). The optimum conditions for each enzyme were determined on test samples prior to the experiments on subjects and if on a particular day all subjects appeared to be heterozygotes, suggesting only partial digestion by the enzyme, the experiment was repeated. The allele frequencies of the polymorphisms studied in the thesis obeyed the Hardy-Weinberg equilibrium showing that the polymorphisms conformed to normal distribution within the population. Furthermore, the control allele frequencies for the studies involving the restriction digestion reactions were similar to those reported in other studies involving Caucasian controls. For example, in this *Ava I* study the allele frequencies were: A1=62.2%, A2=37.8% as compared with A1=59.0%, A2=41.0% (Di Giovine et al, 1992), whilst in this *Taq I* study the allele frequencies were: A1= 83.2%, A2=16.8% as compared with A1=79.8%, A2=20.2%

(Loughrey et al, 1998). The control allele frequencies of other polymorphisms such as the $VNTR^{86}$ and VLP were also similar to those reported in other studies. For example, in this $VNTR^{86}$ study the allele frequencies were: A1=63.0%, A2=33.0%, A3=4.0% as compared with A1=68.0%, A2=28.0%, A3+A4=4.0% (Danis et al, 1994) and in this VLP study the allele frequencies were: A1=4.1%, A2=66.1%, A3=29.8% as compared with A1=4.0%, A2=65.0%, A3=30.0% (Steinkasserer et al, 1991).

7.1.2 Haplotype Analysis

Recent haplotype studies in other diseases have shown that it is not simply a matter of correlating a particular polymorphism with certain protein level, since polymorphisms of the different genes within the IL-1 gene complex are often linked and coordinately regulate each others expression. Linkage disequilibrium is expected since the genes of the IL-1 cluster occupy a relatively short distance (430kb) on the long arm of chromosome 2. Cox et al, have recently shown that some of the IL-1 gene cluster polymorphisms are in significant linkage disequilibrium, indicating that specific groups of alleles are inherited together. The disequilibrium was strong for 3 markers in the IL-1A gene (222/223, gz5/gz6 and $NcoI^{889}$) but was weaker in the IL-1B gene ($Taq\ I^{+3953}$ and $Ava\ I^{-511}$) (Cox et al, 1998). A study by Guasch et al, also showed weak disequilibrium within the IL-1B gene between *Taq I* and *Alu I* (Guasch et al, 1996). Two studies have shown that the linkage between polymorphisms in the IL-1RN gene is very strong (Clay et al, 1996; Guasch et al, 1996).

Several different haplotype patterns have been identified and linked with disease. Recently, a haplotype consisting of $NcoI^{889}$ $(A2)^{+}$ $-TaqI^{+3953}$ $(A2)^{+}$ has been associated with susceptibility to severe adult periodontitis. It was named the "periodontitis-associated genotype (PAG) or "periodontitis susceptibility trait (PST)" (Kornman et al, 1997; Gore et al, 1998) and was found to be associated with increased IL-1 production (Engebretson et al, 1999). The same haplotype is associated with increased risk of Alzheimers' disease (Nicoll

et al, 2000). Another haplotype consisting of *Ava I*^{511} (A2)$^{+}$- IL-1RN $^{+2018}$ (A2)$^{+}$ is associated with susceptibility to cardiovascular diseases. The IL-1RN^{+2018} polymorphism is in strong linkage disequilibrium with VNTR^{86} (Kornman et al, 1999). The VNTR^{86}(A2)$^{+}$-*TaqI*$^{+3953}$(A2)$^{-}$ haplotype has been associated with ulcerative colitis (Bioque et al, 1995; Heresbach et al, 1997) and with the progression of multiple sclerosis (Schrijver et al, 1999). The VNTR^{86}(A2)$^{-}$ - *TaqI* $^{+3953}$ (A2)$^{+}$ haplotype has been associated with susceptibility to myasthenia gravis (Huang et al, 1998). The VNTR^{86}(A2)$^{-}$- *Taq I* $^{+3953}$ (A1) $^{+}$ haplotype (VNTR^{86}(A2)$^{-}$-*TaqI*$^{+3953}$(A2)$^{-}$ haplotype) is associated with Tuberculous pleurisy (Wilkinson et al, 1999) and found to be protective against Epstein-Barr Virus (Hurme and Helminen, 1998a). The *Ava I*^{511} (A2)$^{+}$- *TaqI* $^{+3953}$ (A1)$^{+}$ haplotype is associated with the development of alcoholic liver cirrhosis (Takamatsu et al, 2000). Finally, the *Ava I*^{511}(A1)$^{+}$-*NcoI*^{889}(A2)$^{+}$-VNTR^{86}(A1)$^{+}$ haplotype was significantly increased in patients with Schizophrenia compared to controls (Katila et al, 1999).The results obtained from the haplotype analysis in this study provide further evidence for linkage in the IL-1 gene cluster.

Since it is the polymorphisms of the IL-1RN gene that are strongly associated with acute pancreatitis, only the haplotypes containing these polymorphisms will be discussed. The other haplotypes involving different combinations of polymorphisms in the IL-1RI, IL-1A and IL-1B genes can be explained in a similar way with respect to those polymorphisms that are known to be functional, however, in each case the effect of the other polymorphisms on protein levels remains unknown. i.e. haplotypes containing the VNTR^{46} polymorphism produce IL-1α protein which is inversely proportional to the number of repeats in the allele (Bailly et al, 1996), whilst those containing *Ava I* allele 2 (Nemetz et al, 1999) or *Taq I* allele 2 (Pociot et al, 1992) are high IL-1β secretors.

The genotype studies showed an association of the IL-1RN gene with severe and idiopathic acute pancreatitis. It would have been useful to test the IL-1RN haplotypes in the

severe and idiopathic groups in order to determine whether there is a particular haplotype for susceptibility or severity, however, the study was limited by the number of subjects in each group. In view of this, the haplotypes were compared only in the total controls and total patient groups.

MspI-VLP-VNTR^{86}-SspI haplotypes

The 2-3-2-2 and 1-2-1-1 haplotypes were significantly increased in controls and patients, while the 1-2-2-1, 1-2-1-2 and X-X-X-X haplotypes were significantly decreased between the same 2 groups. It is expected that if one particular haplotype increases, then another will decrease to compensate and that if the polymorphisms are in linkage disequilibrium they will be linked in both controls and patients. Since the X-X-X-X haplotype represents the sum of all other haplotypes not detected in the study, it is not possible to comment on which of the undetected haplotypes is significantly decreased. One particular haplotype or all of the undetected haplotypes may be decreased and this applies to all of the haplotype analyses.

As previously discussed, there is controversy as to whether allele 2 of the VNTR^{86} polymorphism corresponds to high IL-1ra protein (Danis et al, 1995) or low IL-1ra protein (Tarlow et al, 1993). To date there are no functional studies for the *Msp I*, VLP or *Ssp I* polymorphisms. The 2-3-2-2 haplotype contains the high IL-1ra VNTR^{86} allele according to Danis et al (low IL-1ra VNTR^{86} allele according to Tarlow et al), the 1-2-1-1 haplotype contains the low IL-1ra VNTR^{86} allele according to Danis et al (high IL-1ra VNTR^{86} allele according to Tarlow et al), whereas the 1-2-2-1 and 1-2-1-2 haplotypes contain the high and low alleles respectively according to Danis et al. Since these haplotypes were increased in controls and patients, they are unlikely to be susceptibility haplotypes, but may be linked with other susceptibility haplotypes.

PstI-MspI-VLP-VNTR^{86}-SspI haplotypes

The 2-1-2-1-1 haplotype was significantly increased while the X-X-X-X-X haplotype was significantly decreased in both controls and patients (although more pronounced in the controls). This haplotype contains the low IL-1ra VNTR^{86} allele according to Danis et al (high IL-1ra VNTR^{86} allele according to Tarlow et al). Because these haplotypes were increased and decreased in both controls and patients they are unlikely to be susceptibility haplotypes but again may be in linkage with others. The 2-1-2-2-1 haplotype was also significantly decreased in patients but not controls suggesting susceptibility to disease. It contains the high IL-1ra VNTR^{86} allele according to Danis et al (low IL-1ra VNTR^{86} allele according to Tarlow et al). A decrease in this high IL-1ra allele in patients, may cause acute pancreatitis to occur. This explanation does not however take into account the influence of the *Pst I*, *Msp I*, VLP or *Ssp I* polymorphisms whose functional status remains unknown.

(AC)n-VNTR^{46}-MspI-VLP-VNTR^{86}-SspI haplotypes

The 2-2-1-2-1-1 haplotype was significantly increased in controls and patients while the X-X-X-X-X-X haplotype was significantly decreased between other groups. Since the same results were found in controls and patients, these particular haplotypes are unlikely to determine susceptibility but they may be linked with other haplotypes. Interestingly the 4-1-1-2-1-1 and 1-3-1-2-1-1 haplotypes were also significantly increased in the patient group but not in the control group suggesting that these may be susceptibility haplotypes. The 4-1-1-2-1-1 haplotype contains the VNTR^{46} high IL-1α allele (allele 1:9 repeats) (Bailly et al, 1996) and low IL-1ra VNTR^{86} allele (allele 1) according to Danis et al (high IL-1ra VNTR^{86} allele according to Tarlow et al). High IL-1α and low IL-1ra could therefore predispose to acute pancreatitis. The 1-3-1-2-1-1 haplotype contains another VNTR^{46} high IL-1α allele (allele 3: 8 repeats) with the low IL-1ra VNTR^{86} allele (allele 1)

according to Danis et al (high IL-1ra VNTR^{86} allele according to Tarlow et al). Again high IL-1α and low IL-1ra could lead to acute pancreatitis. This explanation does not however take into account the influence of the (AC)n, *Msp I*, VLP and *Ssp I* polymorphisms whose functional status remains unknown.

AvaI-Alu-I-TaqI-MspI-VLP-VNTR^{86}-SspI haplotypes.

The 1-1-2-1-2-1-1 and 1-1-1-1-2-1-1 haplotypes were significantly increased in both controls and patients whereas the X-X-X-X-X-X-X haplotype was significantly decreased between the 2 groups. Because the same haplotypes were increased or decreased in controls and patients, these are probably not susceptibility haplotypes but may be in linkage with other haplotypes. Interestingly, the 2-2-1-1-2-1-1 haplotype was also significantly increased in the control group but not the patient group, suggesting that this may be "protective". This haplotype contains the *Ava I* high IL-1β allele (Nemetz et al, 1999), *Taq I* low IL-1β allele (Pociot et al, 1992) and low IL-1ra VNTR^{86} allele according to Danis et al (high IL-1ra VNTR^{86} allele according to Tarlow et al). If co-dominant, the high and low IL-1β alleles may cancel to give a moderate amount of IL-1β and despite having the low IL-1ra allele may still be protective because the IL-1ra: IL-1 ratio may still be large enough to prevent acute pancreatitis. Again, this explanation does not take into account the influence of the *Alu I*, *Msp I*, VLP and *Ssp I* polymorphisms whose functional status remains unknown.

7.1.3 Combined Genotype Analysis

The combined genotype data showed that in general, different combinations of genotypes are more common in controls than in patients with acute pancreatitis e.g. 1,4-1,2 of the (AC)n-VNTR^{46} combined genotype is more common in controls whereas the 1,2-1,2 combination is more common in patients. In addition, the majority of patient subgroups

shared the same most common combined genotype, regardless of whether they were categorised according to severity, organ failure scores or aetiology. In cases where a particular group or groups varied from the majority, the most common genotype in the majority of groups was the second or third most common genotype in the varied group and was only less common by 1 or 2 patients e.g. for the (AC)n-VNTR[46] combined haplotype, the most common combined genotype in the majority of groups (total patients, mild, severe, OFS≥1, OFS≥2, OFS≥3, OFS≥4, idiopathic and gallstone groups) was 1,2-1,2 that occurred at frequencies of 16.9%, 15.2%, 25.0%, 29.8%, 33.3%, 31.6%, 33.3%, 24.2% and 17.2% respectively. The OFS=0 and alcoholic group varied from this trend whereby the most common combined genotype was 2,4-1,2 that occurred at frequencies of 11.2% and 22.2% respectively. The 1-2,1-2 combined genotype that was the most common combined genotype in the majority of patient groups was the second most common genotype in the OFS=0 group and third most common in the alcoholic group occurring at frequencies of 10.1% and 11.1% respectively. The presence of the 2,4-1,2 combined genotype in the OFS=0 group, but its absence in OFS≥1,2,3 and 4 groups suggests that this combination could act as a disease severity marker.

Although no statistical analysis could be performed, the data gives an indication of the most common combined genotype in a particular group of patients. If a larger cohort was used, more numbers would be present in the groups and statistical analysis could be applied to determine whether the differences in distribution of the most common combined genotypes were significant between controls and patients.

7.2 Protein Studies

It is well recognised that polymorphisms in the promoter and intron regions of cytokine genes such as IL-6 (Fishman et al, 1998), IL-10 (Eskdale et al, 1996; Turner et al, 1997), IL-13 (Van der Pouw Kraan et al, 1999), TNFα (Wilson et al, 1997; Huang et al,

1999) and IFNγ (Pravica et al, 1999) can have an effect on the expression of the gene. In view of this, the polymorphisms in genes such as IL-1RN studied in this thesis may also have a functional effect on the protein. The IL-1ra protein may be affected by polymorphisms in two main ways:

a) altered expression of protein levels

If there is an increase in IL-1ra expression, there would be a decrease in inflammation. Conversely, an increase in IL-1ra expression would cause an increase in inflammation.

b) altered structure of the protein

A change in the structure of the protein may affect the affinity of the IL-1ra for the IL-1 receptor. An increased affinity would decrease the inflammation, whereas a decreased affinity would increase the inflammation. Whether there is altered expression or structure of the protein depends on whether the polymorphism is in a coding, non-coding or promoter region of the gene.

When considering the *Msp I*-VLP-VNTR^{86}-*Ssp I* haplotype, the VLP, VNTR^{86} and *Ssp I* polymorphisms are located in introns 2 and 3 and therefore may affect the IL-1ra mRNA or protein levels, whereas the *Msp I* polymorphism is located in exon 2 and may affect the structure of the IL-1ra protein. At present, it is not known whether the T→C SNP which creates the *Msp I* restriction site alters an amino acid sequence, however, a method of testing this would be to sequence cDNAs. There may also be variability between subjects i.e. environmental factors such as alcohol consumption. Just because an individual has a particular IL-1ra phenotype, they may not necessarily develop acute pancreatitis, however, they may be more predisposed to the disease compared to an individual with an alternative IL-1ra phenotype.

To date, there are no studies of IL-1RN gene transgenic "knockout" mice in acute pancreatitis. It would be expected that these "knockout" mice would have increased severity since there would be no antagonist to block the action of IL-1. Conversely, if the

expression of IL-1ra was increased there would be reduced severity of the disease in much the same way as administering recombinant IL-1ra (Tanaka et al, 1995; Norman et al, 1995a).

Polymorphisms in the IL-1A and IL-1B genes may also affect the proteins they encode. It is not known whether the polymorphisms are located on the same chromosome or on different chromosomes. If they are located on the same chromosome, they may cause a cis effect resulting in the formation of normal protein, however, if they are located on separate chromosomes, they may cause a trans effect resulting in a mutated protein. IL-1 requires 2 receptor-binding sites to bind to the IL-1 receptor in order to initiate a signal. The binding of IL-1 to the receptor causes a structural change in the cytoplasmic domain of the receptor which allows the IL-1R-AcP to form a high affinity heterodimer complex with the initiation of signal transduction. A mutated protein may have only one receptor-binding site so that it binds to the receptor but does not result in a structural change of the receptor or formation of a high affinity complex and hence no signal transduction. In this way, the mutated protein would competitively inhibit the action of IL-1 in the same way as IL-1ra.

7.2.1 Secretion of Interleukin-1 alpha, Interleukin-1beta and Interleukin-1receptor antagonist

In order to determine whether the polymorphisms had a functional effect, protein studies were performed using LPS to induce the production of IL-1α, IL-1β and IL-1ra from PBMCs *in vitro*. IL-1α is the intracellular form of IL-1 that is only secreted by monocytes following cleavage by membrane-bound calpains or as a result of damaged cell membranes. Since the viability of the cells was confirmed using trypan blue at each time point, the IL-1α found in the supernatants must have been secreted following cleavage by calpain and not as a result of damaged cell membranes. IL-1β is the secretory form of IL-1 that is released from the cell following cleavage by interleukin-1 beta converting enzyme

(ICE) while the secretory form of IL-1ra possesses a leader sequence and is readily secreted from the cell without enzymatic cleavage.

7.2.1.1 Protein levels peak at particular time points

The 120 hour time-course experiments showed that *in vitro* secretion of IL-1α and IL-1β protein levels from PBMCs peaked at 24 hours, whereas the IL-1ra protein levels peaked at 48 hours. These findings were confirmed by all of the subjects in the main IL-1α, IL-1β and IL-1ra protein studies.

The time of peak protein secretion appears to vary greatly between studies. One study has reported that IL-1 protein peaks at 2 hours, whereas IL-1ra peaks at 4 hours *in vitro* (Vannier et al, 1992). In contrast, another 5 day time-course study showed that IL-1β levels from monocytes stimulated *in vitro* by LPS were high at day 1 of culture, gradually decreased to day 3 and rose again to day 5 (McKay et al, 1996). This is different to the IL-1β cytokine profile obtained from this study which showed that IL-1β protein levels continued to decrease and level off after 24 hours. A medline search was unable to find studies that demonstrated similar time-course experiments of 5 days or more for IL-1α and IL-1ra.

Although both IL-1 and IL-1ra protein levels peaked later in this study compared to that reported by Vannier et al, the expression kinetics were consistent. The IL-1 protein peaked in half the time of IL-1ra protein (24 hours (IL-1) compared to 48 hours (IL-1ra) and 2 hours (IL-1) compared to 4 hours (IL-1ra)). These findings are consistent with the IL-1 biology since IL-1 gene expression is known to occur before IL-1ra. The main functions of IL-1 during the inflammatory process are to cause: the increased expression of other cytokines that activate and attract leucocytes to the site of injury, upregulation of cell adhesion molecules involved in leucocyte migration and increased NO and prostaglandins production that cause increased vascular permeability and vasodilatation. The transcription

of IL-1ra is induced later by IL-1 itself as a compensatory mechanism to counteract the effects of IL-1 and limit the inflammatory process.

In further support of the results obtained for IL-1ra secretion in this thesis, it has been reported that within the first 24 hours following stimulation, monocytes use the secretory promoter to transcribe sIL-1ra. After 48 hours, the monocyte exhibits a macrophage phenotype and switches on the intracellular promoter so that it starts to transcribe icIL-1ra (Dinarello, 1996). In view of this, it would be expected that sIL-1ra would rise between 24-48 hours and then gradually decline after 48 hours as the icIL-1ra levels increase.

The differences in the IL-1α, IL-1β and IL-1ra cytokine profiles between studies may be due to a number of reasons. The various studies measured cytokine secretion from different cell populations: monocytes, PBMCs or neutrophils. PBMCs (mixed cell population comprised mainly of monocytes and lymphocytes) were used in this study since these cells are known to interact with one another and a mixed culture may therefore more accurately reflect actions *in vivo* compared to isolated monocytes. In addition, the studies use different ELISA kits. Some use "in house" ELISA kits which are more likely to introduce variation due to human error when preparing reagents whilst others use commercially prepared "ready to use" kits. Cell concentrations also vary between studies. High concentrations of cells cultured in well plates with a small well surface area may lead to "contact inhibition" when the cells become confluent and this may affect the results. A study comparing LPS, GM-CSF and TNFα-induced IL-1 and IL-1ra stimulation found that the cytokine profile also varied depending on the stimulant (Malyak et al, 1994). Other studies using uromodulin, *Streptococcus pneumoniae* and alpha CD3 to stimulate IL-1β secretion from PBMCs showed that the protein level peaked at 6-12, 24 and 72 hours respectively (Su and Yeh, 1999; Arva and Andersson, 1999; Ruschen et al, 1992). Moreover, certain stimulants preferentially stimulate the secretion of particular cytokines.

For example, LPS is more effective at inducing IL-1, IL-6 and TNFα production whereas phytohemaglutinin (PHA) is better at stimulating IL-2, IFNγ and GM-CSF production (De Groote et al, 1992). Different strains of LPS also produce different results. It has been shown that LPS from *Actinobacillus actinomycetemcomitans* was a more potent stimulant of IL-1β from PBMCs than LPS from *E. coli* 026:B6 (Schytte Blix et al, 1999). The studies also vary in the concentrations of LPS used. 5μg/ml of LPS from *E. coli* 0127:B8 was added to the cells in this study and this was a very high dose in comparison to others. Although this concentration was a potent stimulator of protein, other studies have shown that high concentrations of LPS may inhibit cytokine secretion. This is further supported by the results from the optimisation experiment (Figures A1.3-A1.6) that showed that LPS reduced IL-1ra protein secretion in a dose dependent manner. Although 5μg/ml was the lowest LPS concentration used in the optimisation experiment, with hindsight it may have been better to use an even lower LPS concentration (1ng/ml) that was more physiologically relevant. This raises the question as to whether the *in vitro* adequately reflects the events *in vivo*.

7.2.1.2 Inter-individual variability of protein levels

Although the protein from all the subjects consistently peaked at the same time in each study, 24 hours for IL-1α or IL-1β and 48 hours for IL-1ra, the amount of protein varied between individuals. This inter-individual variation has been demonstrated with other cytokine protein levels (IL-2, IL-4, IL-10 and IFNγ) (Kaminski et al, 1995; Cartwright et al, 1999).

Since cytokines interact in a network, other cytokines may influence the levels of IL-1 and IL-1ra. IL-4, IL-10 and IFNγ increase IL-1ra levels while simultaneously decreasing IL-1 levels. As with IL-1, the genes for these cytokines contain polymorphisms that may cause variation in the levels of these cytokines between individuals. If e.g. IL-10

levels are low in an individual this could cause lower IL-1ra levels than in an individual with higher IL-10 levels. Conversely low IL-10 levels would lead to increased IL-1 levels compared to an individual with high IL-10. A study investigating the effect of IL-10 on LPS-induced IL-1 and IL-1ra levels showed that IL-10 inhibited IL-1 production but stimulated IL-1ra production in monocytes (Jenkins et al, 1994). Furthermore, IL-1 has been shown to induce IL-1ra production, so low IL-1 levels may also lead to low IL-1ra levels in a particular individual (Marsh and Wewers et al 1994). The effect of IL-1ra on IL-1 levels has also been studied. Recombinant human IL-1ra administered to cultures was found to decrease LPS-induced IL-1mRNA and decrease IL-1 protein (Conti et al, 1992). An individual that produces high levels of IL-1ra *in vitro* may have lower IL-1 levels in the supernatant as compared to individuals with lower IL-1ra levels. Furthermore, IL-1 stimulates its own production and this stimulation may be more efficient in some individuals than in others, which means that these individuals may have more IL-1 protein present in the supernatant compared to individuals with a less efficient stimulation of IL-1.

7.2.1.3 Correlation of the (AC)n and Alu I polymorphism with protein levels.

The median IL-1α and IL-1β protein levels from unstimulated cells were significantly increased in patients compared to controls (P^0 vs C^0). This enhanced release of IL-1α and IL-1β by unstimulated cells from patients with acute pancreatitis suggests that these cells may be "primed" following an episode of the disease. Following stimulation by LPS, the median amount of IL-1α was significantly higher in patients than in controls (P^5 vs C^5) at 24 hours, while the median amount of IL-1β was significantly lower in patients than in controls (P^5 vs C^5) at 0 hours. No significant differences in the median IL-1β levels were found when the subjects were separated according to those with genotypes containing allele 1 or 2 ($A1^0$ vs $A2^0$ and $A1^5$ vs $A2^5$). This suggests that despite being located in the promoter region of the IL-1B gene, the *Alu I* polymorphism is not functional. Results from

this thesis and the study by Guasch et al, suggest that it may be in linkage disequilibrium with the *Taq I* polymorphism, which is functional (Guasch et al, 1996).

Although conclusions were drawn from the protein data, they were based on a relatively small number of subjects and hence a small number of alleles, which would affect the statistical analysis. The protein studies were originally designed to show whether the disease itself may influence the protein levels and therefore patients with a particular genotype were taken and compared with controls of the same genotype. Unfortunately, problems were encountered when recalling patients, so it was difficult to obtain blood from patients with the required genotype. Furthermore, the controls used in the genotype study were taken from a bank of cord bloods, which had been collected previously and these individuals were not available to obtain blood samples for protein studies. This meant that controls from the laboratory had to be used and their genotypes were not always the required genotype. This limited availability of subjects resulted in selected patients being compared with unselected controls, which are not directly comparable. Any significant differences noted could be simply due to the different genotypes and therefore the results obtained here are probably spurious. No genotype-protein correlation could be performed for the (AC)n polymorphism of the IL-1A gene due to lack of subjects with the 2,4 genotype. Since neither of these polymorphisms have previously been associated with functional protein levels it is not possible to compare with other studies.

7.2.1.4 Correlation of the VNTR^{86} polymorphism with protein levels

No significant differences were found in median IL-1ra protein levels between controls and patients for unstimulated (C^0 vs P^0) or stimulated (C^5 vs P^5) cells. This VNTR^{86} polymorphism has been previously associated with functional protein levels. Danis et al, found allele 2 was associated with high IL-1ra (Danis et al, 1995), whereas Tarlow et al, found it was associated with low IL-1ra (Tarlow et al, 1993). This thesis

could not reproduce the results of either study since on separating the subjects according to alleles, allele 1 appeared to produce a similar amount of IL-1ra as allele 2 ($A1^0$vs $A2^0$ and $A1^5$ vs $A2^5$). In contrast to Danis and Tarlow, this suggests that the polymorphism is not functional but may be in linkage with other polymorphisms that are functional. Again as with the IL-1α and IL-1β studies, this study involved the comparison of selected patients and unselected controls and a small number of alleles, which would affect the statistical analysis. In view of this, the results obtained here are probably spurious.

To my knowledge only 2 studies have investigated the LPS-induced cytokine secretion from cells taken from patients with acute pancreatitis. One study compared the cytokine secretion of TNFα, IL-6 and IL-8 (but not IL-1β) from PBMCs taken from 6 controls and 16 patients (De Beaux et al, 1996). The second study compared the cytokine secretion of IL-1β, TNFα, IL-6 and IL-8 from monocytes taken from 26 patients. This study compared protein levels between mild cases with severe cases, but did not compare against controls. No association was found between IL-1β levels and disease severity (McKay et al, 1996). Because the first study did not investigate IL-1α, IL-1β or IL-1ra protein levels and the second investigated only IL-1β protein levels with respect to severity, these studies are not directly comparable to those performed in this thesis.

The *in vitro* production of IL-1 and IL-1ra protein has been studied in other diseases. IL-1ra levels were decreased in unstimulated and LPS-stimulated neutrophils obtained from patients with active SLE compared to non-active SLE and controls (Hsieh et al, 1995). LPS-stimulated IL-1β production was increased in PBMCs cultured from patients with chronic fatigue syndrome (Chao et al, 1991), Parkinsons' Disease (Bessler et al, 1999) and multiple sclerosis compared with controls (Sarchielli et al, 1997). Interestingly, the LPS-induced stimulation of IL-1α and IL-1β from PBMCs and monocytes were decreased in 2 studies of sepsis implying that a regulatory mechanism downregulates the production of IL-1 during sepsis (Munoz et al, 1991; Kremer et al,

1996). Since acute pancreatitis is considered to be a model of sepsis (Wilson et al, 1998), this may also be the case in this disease.

7.2.2 Limitations of the protein studies

This project was originally designed as a genetic study in 1996. In view of this, blood was collected from all patients for DNA extraction and the serum was discarded. With hindsight the serum from this blood should have been removed, aliquoted and frozen for future use. This would have avoided the need to recall patients, a process that proved to be fraught with problems (see below). Disadvantages of this method are that the protein may start to degrade over time and the measurement of cytokine levels in the serum may not adequately reflect the cytokine producing potential of immune cells because of the short half-life and presence of inhibitors in the human sera. An alternative method to this is the *in vitro* stimulation of cells that was used in this study, however, even this raises the question of tissue variability because cultures of PBMCs stimulated with LPS may not adequately reflect the events that occur in the pancreas during acute pancreatitis.

Numerous problems were encountered when attempting to recall patients for blood donations. These included: death since the original admission 5 years ago and non-reply to the requesting letter. Some of the patients who did agree to donate blood proved to be not suitable since they lived too far from hospital to give a fresh blood sample that is required for cell culture experiments or they had recently received a vaccination and were therefore immune-compromised. Other patients who agreed to give blood samples were unable to attend outpatient appointments due to employment or holiday commitments. One elderly patient attended her appointment but due to her "poor" veins was unable to donate blood. Other patients, in particular the alcoholics, agreed to donate blood but failed to attend their outpatient appointment. In total 52 patients were recalled, however, only 18 donated blood (35%). Rewriting to the patients for a second time did not improve this recall rate. Patients

that did not donate blood following the first request were unlikely to donate following a second.

Studies that correlate genotypes with functional protein levels limit the number of subjects that can be used since only those individuals with the appropriate genotype can be studied. Gender and age may also have influenced the results obtained in the study since the controls used were mainly young and female. Elderly patients are known to produce more IL-1ra whilst females produce more IL-1 in the luteal phase of their menstrual cycle (Dinarello, 1996).

The protein experiments could be improved in a number of ways. More subjects would increase the number of alleles and improve the statistical analyses. They could be performed on controls only since the genotype should have the same functional effect regardless of whether controls or patients are used. In the case of the IL-1A study for example, where the 2,4 genotype was implicated, protein levels from controls with the 2,4 genotype could be compared with protein levels from controls without the 2,4 genotype.

Also triplicate ELISA samples should be used to improve accuracy, however, this technique is limited by expense. The use of one large ELISA microplate (as yet not commercially available) would have prevented the use a number of different plates and so limited inter-plate variability. This would also have meant that analysis of all samples could be performed on the same day so ensuring ambient conditions e.g. room temperature are constant. Furthermore, the author acknowledges that internal standards should also have been used on a day to day basis to show that the results obtained from each protein study were consistent with those obtained on other days.

Methods such as ELISA are sensitive to temperature changes since they are based on antigen-antibody binding. Comparison of "R square values", a measure of the daily accuracy as determined by the ELISA plate reader showed that hot days (>23°C) resulted in non-linear standard curves and inaccurate results. Only the results from ELISA plates with

linear standard curves with "R square values" close to 1 were used in the study. ELISA are expensive to repeat and are limited by the amount of supernatant available. Alternative methods of quantifying protein levels include radioimmunoassay (RIA), western blotting and ELIspot (for intracellular protein).

7.3 Further studies

Further studies to arise from this work include:

1) Correlation of IL-1 mRNA levels with protein levels

   Polymorphisms in the DNA can have an affect on mRNA and protein levels. It is not enough to measure just protein levels or just mRNA levels since protein levels do not necessarily correspond to mRNA levels. For example, a low protein level could indicate a low mRNA level, when actually mRNA levels may be high but protein is being metabolised rapidly by the cell. Similarly a high protein level does not necessarily mean that mRNA levels will be high, since mRNA is unstable and may degrade too rapidly to be measured effectively. A preliminary experiment was performed using RNA stat 60 to extract mRNA from PBMCs, however, not enough cells were present to detect an adequate amount of mRNA. At least $20x10^6$ cells ($10x10^6$ for unstimulated and $10x10^6$ for stimulated) requiring 20mls of blood were required to obtain a reasonable amount of mRNA. Since these patients were already donating 30mls for the protein study it was not ethical to collect a further 20mls from them for the mRNA study.

2) Investigation of other IL-1 gene cluster polymorphisms in acute pancreatitis since new polymorphisms are continually being reported or in the ICE, type II receptor or interleukin-1 accessory protein which are involved in IL-1 processing and signal transduction

3) Investigation of other pro-inflammatory cytokines and chemokines in acute pancreatitis

4) Investigation of the IL-1 polymorphisms in patients with chronic pancreatitis particularly the (AC)n repeat polymorphism which showed an association with alcoholic acute pancreatitis since most chronic pancreatitis are alcohol-induced.

5) Although not a genetic study, for completeness it would be interesting to compare IL-1α, IL-1β, IL-1ra protein levels and IL-1 receptor expression in specimens of pancreas obtained from patients with acute pancreatitis and healthy controls using techniques such as immunohistochemistry. This type of study is however limited by specimen availability. Pancreatic specimens are difficult to obtain from acute pancreatitis patients because the pancreas is rarely resected and control specimens would also be difficult to obtain unless donated by for example, road traffic accident victims.

7.4 Concluding Points

The main conclusions from this thesis are:

1) *Pst I* polymorphism of the IL-1RI gene may be associated with susceptibility to acute pancreatitis
2) (AC)n repeat polymorphism of the IL-1A gene may be associated with susceptibility to alcoholic acute pancreatitis (suggesting genetic heterogeneity in acute pancreatitis)
3) *Alu I* polymorphism of the IL-1B gene may be associated with susceptibility to acute pancreatitis
4) *Msp I*, VLP, VNTR[86] and *Ssp I* polymorphisms of the IL-1RN gene may be associated with idiopathic acute pancreatitis and with severity of the disease
5) Polymorphisms within genes of the IL-1 gene cluster appear to be in linkage disequilibrium
6) IL-1RI, IL-1A, IL-1B and IL-1RN genes appear to be in linkage disequilibrium
7) There appears to be preferential combinations of genotypes in controls and patients.
8) There appears to be inter-individual variation in IL-1α, IL-1β and IL-1ra protein levels

9) Unstimulated PBMCs from patients with acute pancreatitis (P^0) secrete significantly more IL-1α and IL-1β protein levels but the same amount of IL-1ra protein compared with unstimulated PBMCs from controls (C^0). There is no significant difference in IL-1α, IL-1β or IL-1ra secretion from PBMCs stimulated with LPS between controls (C^5) and patients with acute pancreatitis (P^5)

10) The (AC)n, *Alu I* and VNTR[86] polymorphisms do not appear to correspond to differences in functional protein levels

APPENDIX 1

Experiment to determine the optimum conditions for maximum protein secretion

Part I

An optimisation experiment was performed to determine the optimum cell culture conditions for peak protein secretion. IL-1ra was the first protein to be studied and this was used to determine optimum cell culture conditions for all 3 proteins.

20mls of peripheral venous blood were collected in a sodium heparin vacutainer (Becton Dickinson, Oxford, UK) from a healthy control. The PBMCs were then extracted using "Lymphoprep™" (Gibco GRL, Paisley, UK) by the method described in Section 5.9 to give 20 X 10^6 cells. The cell concentration was then adjusted to $1x10^6$ cells per ml. Next 1ml of the cell suspension was added to 20 wells on a 24 well flat-bottomed culture plate (Fischer Scientific, Loughborough, UK). Different amounts of LPS (0-20μg/ml LPS)(Sigma Chemicals, Poole, UK) were then added to each wells in rows 1-5 (Figure A1.1). The cells were stimulated for 1,6,12 and 24 hours at 37°C 5% CO_2 in a humidified container. Twenty-four hours was the maximum time chosen since previous studies have shown that IL-1ra protein expression peaks at 4 hours and IL-1 peaks at 2 hours *in vitro* (Vannier et al, 1992). The supernatant was removed after each time interval using a 1ml Gilson Pipetman and transferred to a 1.5ml Eppendorf. The supernatant was then centrifuged in a Heraeus Sepatech microcentrifuge (Heraeus, Germany) to pellet out the cells and the supernatant was then transferred to a clean Eppendorf and stored at -20°C ready for batch cytokine assay by ELISA. Exactly the same method was carried out on cells plated at $5x10^5$ and $1x10^5$ cells per ml. The various cell concentrations were used to determine whether contact inhibition would affect the level of protein secreted.

Figure A1.1: Layout of the 24 well cell-culture plate used in Part I of the optimisation experiment

0 1hour	5 1hour	10 1 hour	15 1 hour	20 1 hour	X
0 6 hours	5 6 hours	10 6 hours	15 6 hours	20 6 hours	X
0 12 hours	5 12 hours	10 12 hours	15 12 hours	20 12 hours	X
0 24 hours	5 24 hours	10 24 hours	15 24 hours	20 24 hours	X

0, 5, 10, 15 and 20μg/ml LPS

X = unused well

Figure A1.2: Layout of the 24 well cell-culture plate used in Part II of the optimisation experiment

0 0 hour	5 0 hour	0 24 hours	5 24 hours	0 48 hours	5 48 hours
0 72 hours	5 72 hours	0 96 hours	5 96 hours	0 120 hours	5 120 hours
X	X	X	X	X	X
X	X	X	X	X	X

0 and 5μg/ml LPS

X = unused well

Part II

Data obtained from the Part I of the optimisation experiment showed that the optimum cell culture conditions, i.e. those that produced the maximum protein yield were: $1x10^6$ cells per well stimulated with 5μg/ml. The optimum time for peak protein secretion could not be determined, however, because at 24 hours the curves were still increasing so maximum protein secretion must occur after 24 hours. In view of this a second part of the experiment was carried out which involved plating cells at $1x10^6$ and stimulating half with 5μg/ml LPS for 0, 24, 48, 72, 96 and 120 hours. The other half of the cells remained unstimulated (Figure A1.2). Cells were not cultured any later than 5 days because cell viability would become compromised after this time. The supernatant from each well was removed after each time point using a 1ml Gilson Pipetman and transferred into an Eppendorf where it was centrifuged in a Heraeus Sepatech microcentrifuge to pellet out the cells. The supernatant was then transferred to a clean Eppendorf and stored at -20°C for batch cytokine assay. After each time point, the cell viability was checked using 20μl trypan blue to determine whether the cell membranes were still intact. This showed that the protein was being secreted and not released due to cell membrane rupture dying cells. The cell/trypan blue mixture was added to a Neubauer Haemocytometer and observed under a microscope. The cells were considered to be still viable if at least 95% of cell membranes remained intact. The data are shown in Tables A1.1A to A1.1D and illustrated in Figures A1.3 to A1.6.

Table A1.1A: IL-1ra protein (pg/ml) secreted from $1x10^6$ PBMCs stimulated with 0-20μg/ml LPS for 1-24 hours

Time (Hours)	LPS (μg/ml) 0	5	10	15	20
1	660	600	920	120	600
6	1470	5300	3190	2080	2900
12	920	8500	7620	6250	7520
24	1170	20060	16240	13620	11510

Table A1.1B: IL-1ra protein (pg/ml) secreted from $5x10^5$ PBMCs stimulated with 0-20μg/ml LPS for 1-24 hours

Time (Hours)	LPS (μg/ml) 0	5	10	15	20
1	0	21	131	36	9
6	120	952	1243	1218	694
12	228	2191	2116	1874	1709
24	377	4743	3938	3440	3032

Table A1.1C: IL-1ra protein (pg/ml) secreted from $1x10^5$ PBMCs stimulated with 0-20μg/ml LPS for 1-24 hours

Time (Hours)	LPS (μg/ml) 0	5	10	15	20
1	6	70	111	47	20
6	83	708	263	183	118
12	242	1296	520	335	260
24	190	1714	890	861	454

Table A1.1D: IL-1ra protein (pg/ml) secreted from $1x10^6$ PBMCs stimulated with 0-5μg/ml LPS for 0-120 hours

Time (Hours)	LPS (μg/ml) 0	LPS (μg/ml) 5
0	290	189
24	356	14870
48	452	24440
72	437	19450
96	461	14200
120	381	14330

Figure A1.3: IL-1ra protein secreted from PBMCs cultured at $1x10^6$ cells per ml and stimulated with 0-20ug/ml LPS for 1-24 hours

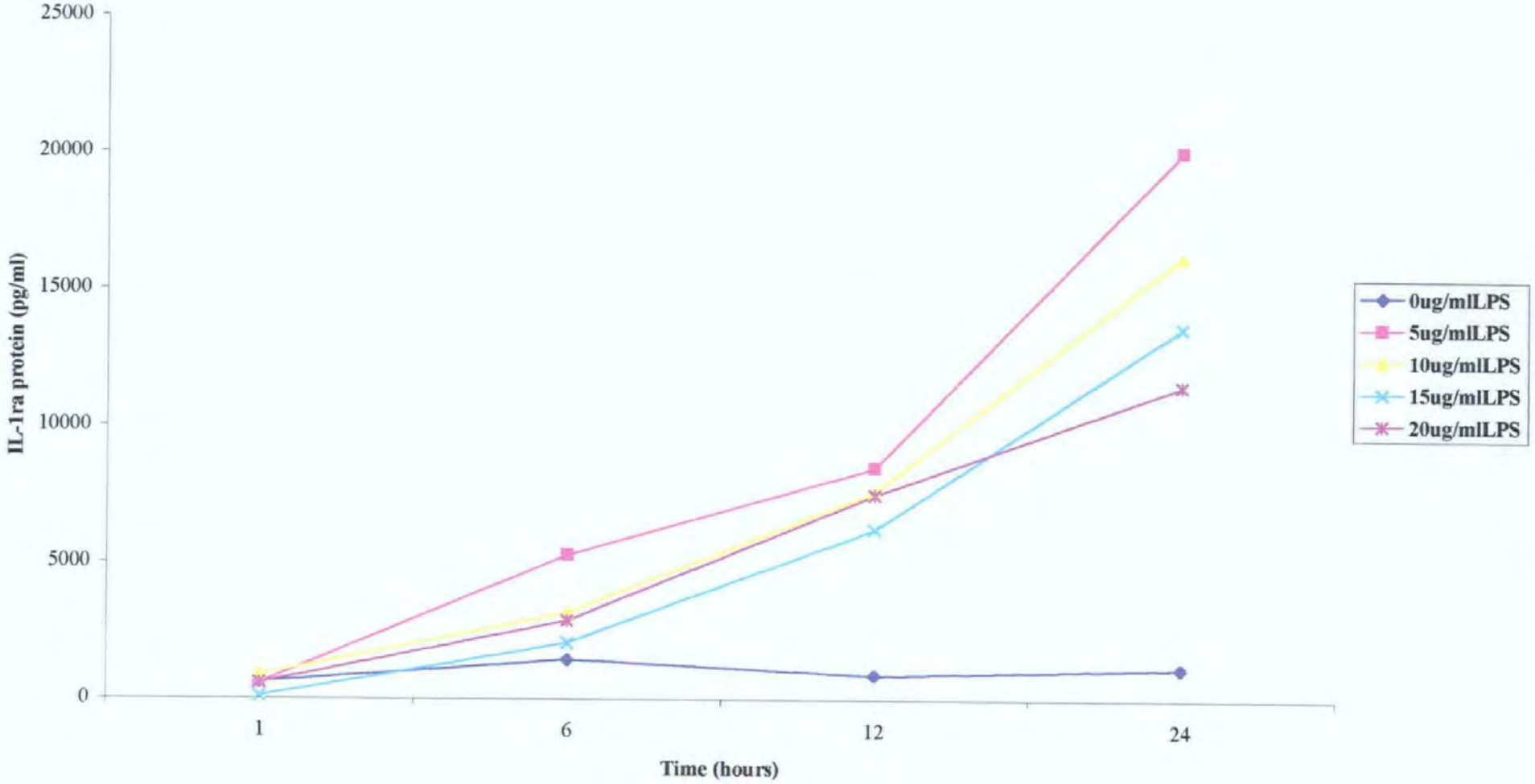


Figure A1.4: IL-1ra protein secreted from PBMCs cultured at $5x10^5$ cells per ml and stimulated with 0-20ug/ml LPS for 1-24 hours

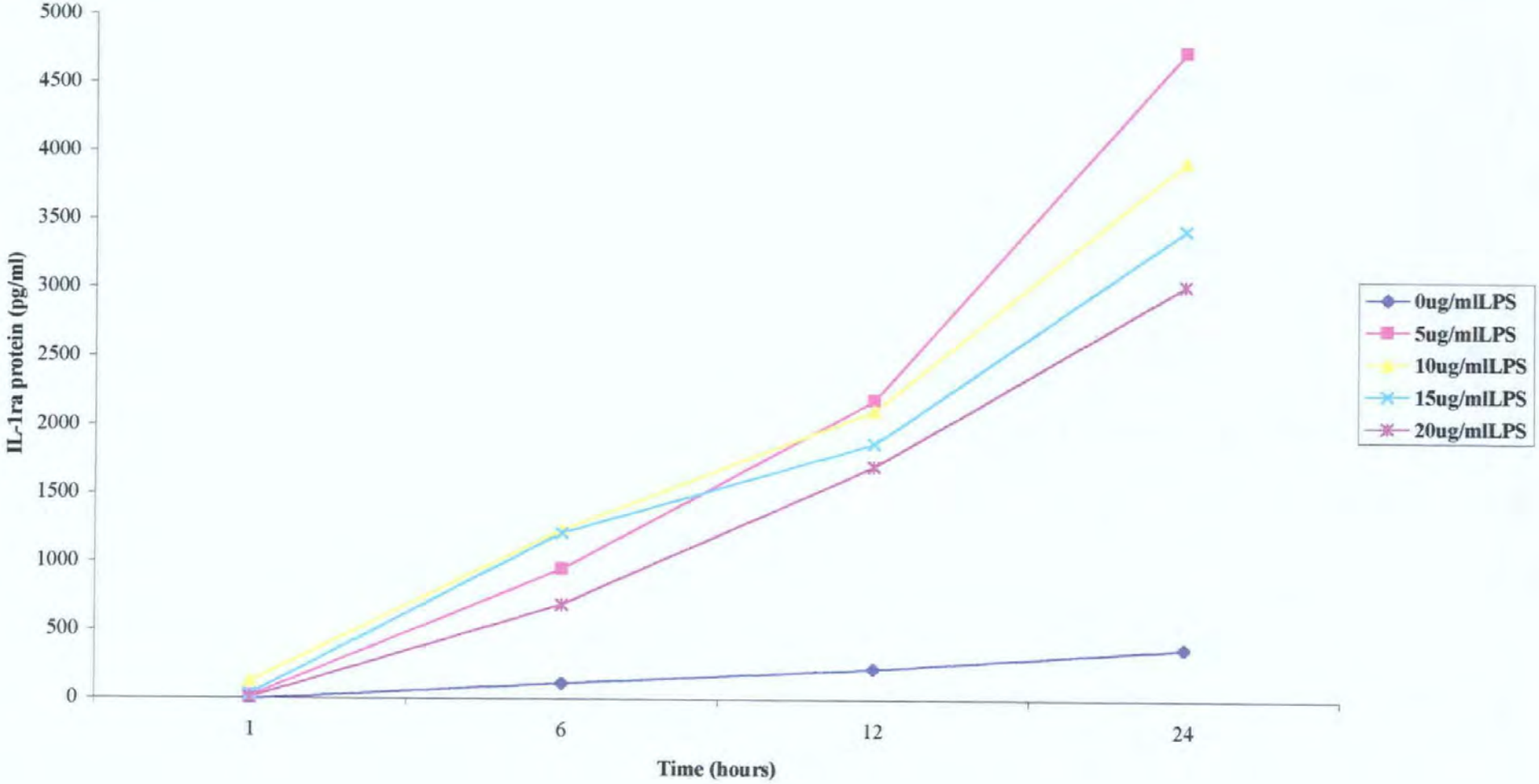


Figure A1.5: IL-1ra protein secreted from PBMCs cultured at $1x10^5$ cells per ml and stimulated with 0-20ug/ml LPS for 1-24 hours

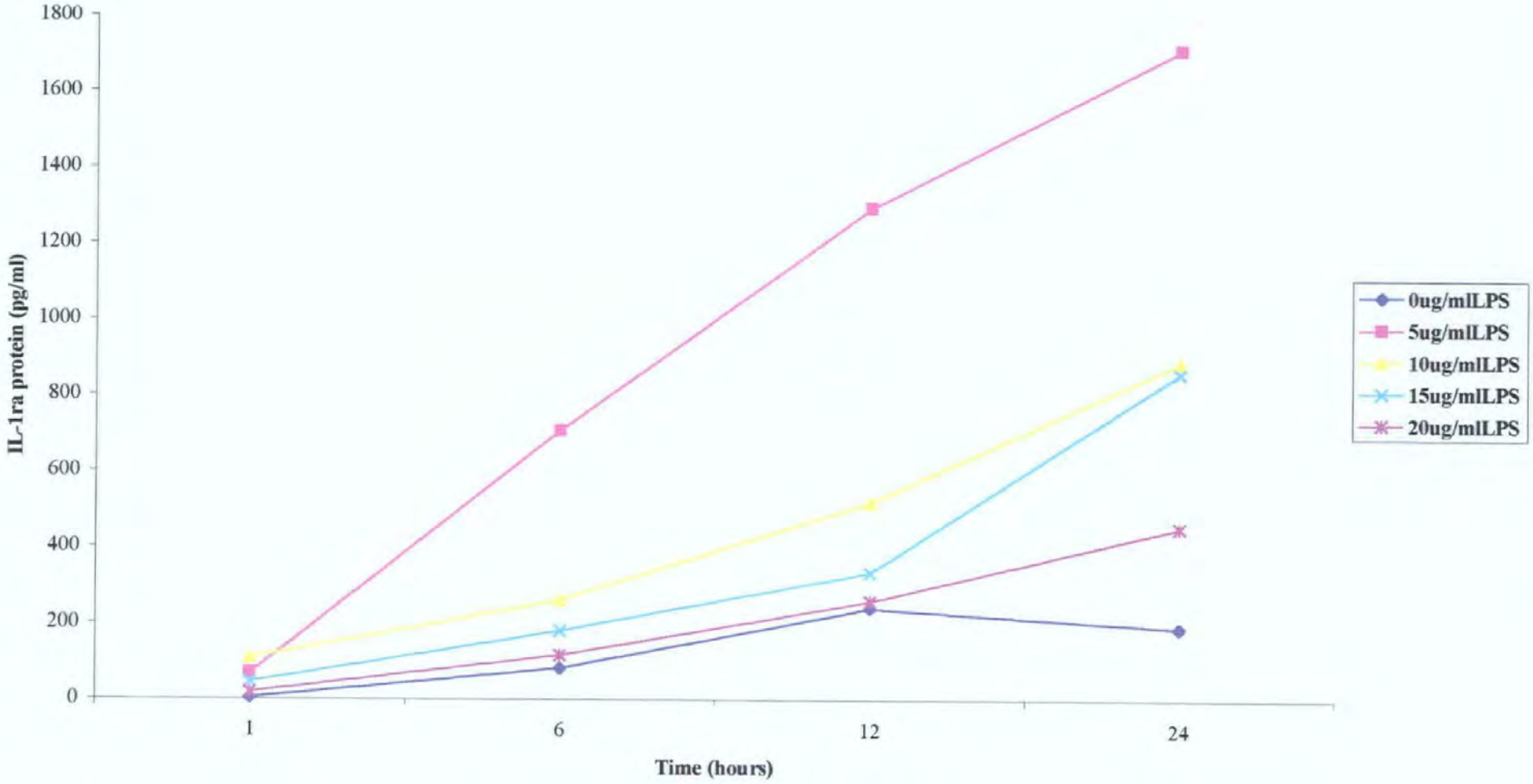
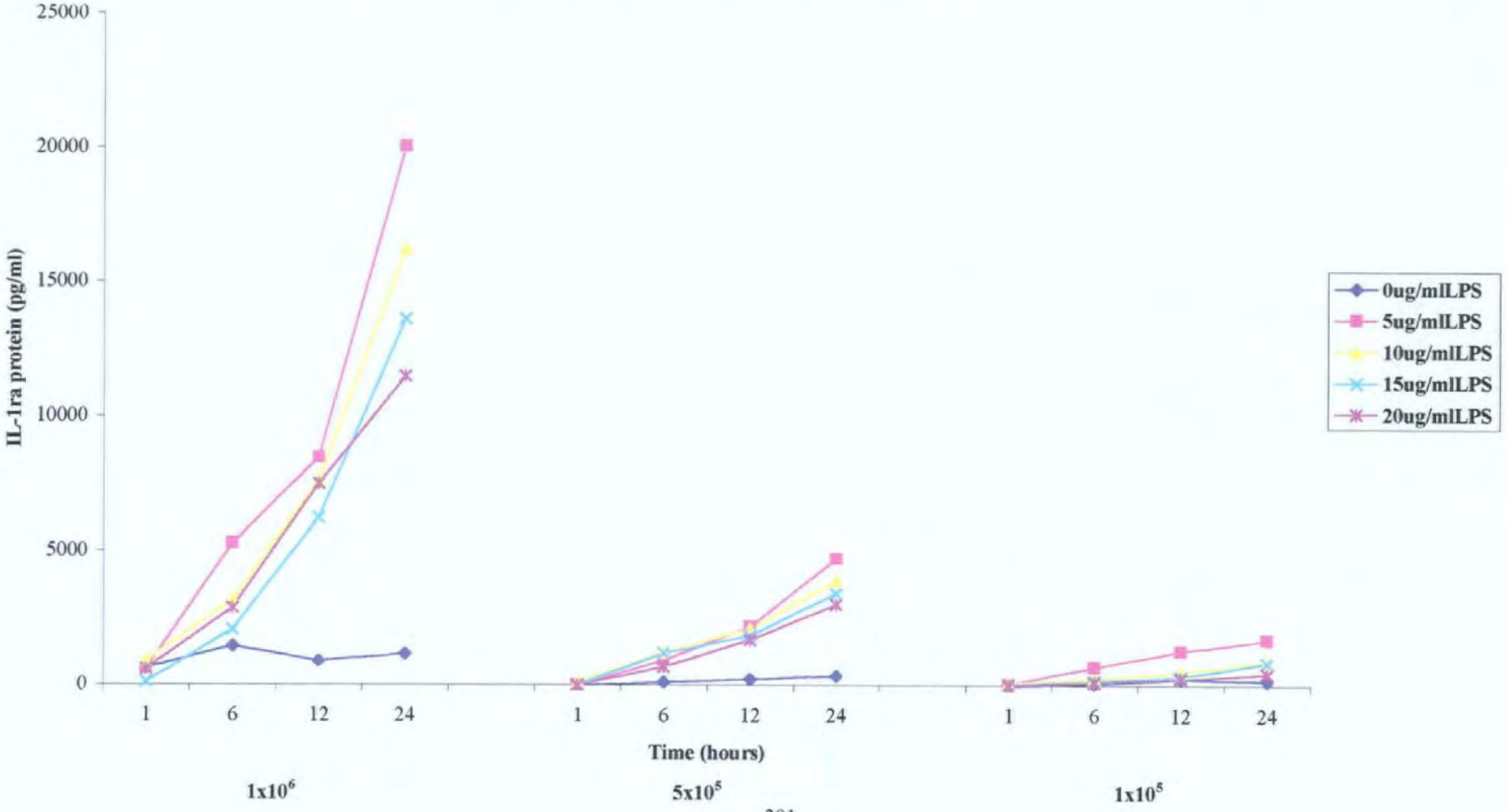


Figure A1.6: IL-1ra protein secreted from PBMCs cultured at $1x10^6$,$5x10^5$ and $1x10^5$ cells per ml and stimulated with 0-20ug/ml LPS for 1-24 hours



APPENDIX 2

Combined Genotype Analysis

The results of the combined genotype analyses are presented in the following format:

Table A2.XA: Frequency of the combined genotypes in the control population

Table A2.XB: Frequency of the combined genotypes in the patient population

Table A2.XC: Frequency of the combined genotypes in the mild group of patients

Table A2.XD: Frequency of the combined genotypes in the severe group of patients

Table A2.XE: Frequency of the combined genotypes in the OFS=0 group of patients

Table A2.XF: Frequency of the combined genotypes in the OFS≥1 group of patients

Table A2.XG: Frequency of the combined genotypes in the OFS≥2 group of patients

Table A2.XH: Frequency of the combined genotypes in the OFS≥3 group of patients

Table A2.XI: Frequency of the combined genotypes in the OFS≥4 group of patients

Table A2.XJ: Frequency of the combined genotypes in the alcoholic group of patients

Table A2.XK: Frequency of the combined genotypes in the idiopathic group of patients

Table A2.XL: Frequency of the combined genotypes in the gallstone group of patients

Table A2.1A: Frequency of (AC)n-VNTR[46] combined genotypes in the control population

(AC)n-VNTR[46]	Number (n)	Frequency (%)
1,4-1,1	16	12.7
1,2-1,2	12	9.5
2,4-1,2	10	7.9
2,2-2,2	8	6.3
1,1-1,1	8	6.3
Y - Y	72	57.1
Total	126	99.8

This table shows the (AC)n-VNTR[46] combined genotype in the control population. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,4-1,1 that occurred at a frequency of 12.7%.

Table A2.1B: Frequency of (AC)n-VNTR[46] combined genotypes in the patient population

(AC)n-VNTR[46]	Number (n)	Frequency (%)
1,2-1,2	23	16.9
2,4-1,2	12	8.8
1,4-1,1	10	7.4
1,2-2,3	10	7.4
1,1-1,3	8	5.9
1,1-1,1	8	5.9
Y - Y	65	47.8
Total	136	100.1

This table shows the (AC)n-VNTR[46] combined genotype in the patient population. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2 that occurred at a frequency of 16.9%.

Table A2.1C: Frequency of (AC)n-VNTR[46] combined genotypes in the mild group of patients

(AC)n-VNTR[46]	Number (n)	Frequency (%)
1,2-1,2	12	15.2
2,4-1,2	9	11.4
1,4-1,1	6	7.6
1,2-2,3	6	7.6
1,1-1,3	4	5.1
1,1-1,1	4	5.1
Y - Y	38	48.1
Total	79	100.1

This table shows the (AC)n-VNTR[46] combined genotype in the mild group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2 that occurred at a frequency of 15.2%.

Table A2.1D: Frequency of (AC)n-VNTR[46] combined genotypes in the severe group of patients

(AC)n-VNTR[46]	Number (n)	Frequency (%)
1,2-1,2	9	25.0
1,1-1,3	3	8.3
1,2-2,3	3	8.3
2,2-2,3	2	5.6
2,2-1,2	2	5.6
1,1-1,1	2	5.6
Y - Y	15	41.7
Total	36	100.1

This table shows the (AC)n-VNTR[46] combined genotype in the severe group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2 that occurred at a frequency of 25.0%.

Table A2.1E: Frequency of (AC)n-VNTR[46] combined genotypes in the OFS=0 group of patients

(AC)n-VNTR[46]	Number (n)	Frequency (%)
2,4-1,2	10	11.2
1,2-1,2	9	10.1
1,4-1,1	8	9.0
1,1-1,1	7	7.9
1,2-2,3	7	7.9
1,1-1,3	6	6.7
1,4-1,3	5	5.6
Y - Y	37	41.6
Total	89	100.0

This table shows the (AC)n-VNTR[46] combined genotype in the OFS=0 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 2,4-1,2 that occurred at a frequency of 11.2%.

Table A2.1F: Frequency of (AC)n-VNTR[46] combined genotypes in the OFS≥1 group of patients

(AC)n-VNTR[46]	Number (n)	Frequency (%)
1,2-1,2	14	29.8
1,2-2,3	3	6.4
1,5-1,3	3	6.4
Y - Y	27	57.4
Total	47	100.0

This table shows the (AC)n-VNTR[46] combined genotype in the OFS≥1 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2 that occurred at a frequency of 29.8%.

Table A2.1G: Frequency of (AC)n-VNTR[46] combined genotypes in the OFS≥2 group of patients

(AC)n-VNTR[46]	Number (n)	Frequency (%)
1,2-1,2	11	33.3
2,2-1,2	2	6.1
1,2-2,3	2	6.1
1,1-1,3	2	6.1
1,5-1,3	2	6.1
Y - Y	14	42.4
Total	33	100.1

This table shows the (AC)n-VNTR[46] combined genotype in the OFS≥2 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2 that occurred at a frequency of 33.3%.

Table A2.1H: Frequency of (AC)n-VNTR[46] combined genotypes in the OFS≥3 group of patients

(AC)n-VNTR[46]	Number (n)	Frequency (%)
1,2-1,2	6	31.6
1,2-2,3	2	10.5
1,1-1,3	2	10.5
Y - Y	9	47.4
Total	19	100.0

This table shows the (AC)n-VNTR[46] combined genotype in the OFS≥3 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2 that occurred at a frequency of 31.6%.

Table A2.1I: Frequency of (AC)n-VNTR[46] combined genotypes in the OFS≥4 group of patients

(AC)n-VNTR[46]	Number (n)	Frequency (%)
1,2-1,2	4	33.3
1,1-1,3	2	16.7
2,2-2,3	1	8.3
4,6-1,1	1	8.3
1,2-2,3	1	8.3
1,4-1,1	1	8.3
1,5-1,1	1	8.3
1,1-3,3	1	8.3
Total	12	99.8

This table shows the (AC)n-VNTR[46] combined genotype in the OFS≥4 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. The most common combined genotype was 1,2-1,2 that occurred at a frequency of 33.3%.

Table A2.1J: Frequency of (AC)n-VNTR[46] combined genotypes in the alcoholic group of patients

(AC)n-VNTR[46]	Number (n)	Frequency (%)
2,4-1,2	4	22.2
1,1-1,3	3	16.7
1,2-1,2	2	11.1
3,4-1,1	2	11.1
Y - Y	7	38.9
Total	18	100.0

This table shows the (AC)n-VNTR[46] combined genotype in the alcoholic group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 2,4-1,2 that occurred at a frequency of 22.2%.

Table A2.1K: Frequency of (AC)n-VNTR[46] combined genotypes in the idiopathic group of patients

(AC)n-VNTR[46]	Number (n)	Frequency (%)
1,2-1,2	8	24.2
2,2-2,3	3	9.1
2,4-1,2	3	9.1
2,2-1,2	2	6.1
2,7-1,2	2	6.1
1,2-2,3	2	6.1
1,4-1,1	2	6.1
Y - Y	11	33.3
Total	33	100.1

This table shows the (AC)n-VNTR[46] combined genotype in the idiopathic group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2 that occurred at a frequency of 24.2%.

Table A2.1L: Frequency of (AC)n-VNTR[46] combined genotypes in the gallstone group of patients

(AC)n-VNTR[46]	Number (n)	Frequency (%)
1,2-1,2	11	17.2
1,2-2,3	7	10.9
1,1-1,1	5	7.8
1,4-1,1	5	7.8
Y - Y	36	56.3
Total	64	100.0

This table shows the (AC)n-VNTR[46] combined genotype in the gallstone group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2 that occurred at a frequency of 17.2%.

Table A2.2A: Frequency of *AvaI-AluI-TaqI* combined genotypes in the control population

AvaI-AluI-TaqI	Number (n)	Frequency (%)
1,1-1,1-1,1	22	25.6
2,2-2,2-1,1	20	23.3
1,1-1,1-1,2	15	17.4
1,2-1,2-1,1	13	15.1
1,2-1,2-1,2	12	14.0
Y - Y - Y	4	4.7
Total	86	100.1

This table shows the *AvaI-AluI-TaqI* combined genotype in the control population. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,1-1,1-1,1 that occurred at a frequency of 25.6%.

Table A2.2B: Frequency of *AvaI-AluI-TaqI* combined genotypes in the patient population

AvaI-AluI-TaqI	Number (n)	Frequency (%)
1,2-1,2-1,1	39	34.5
1,1-1,1-1,1	24	21.2
1,1-1,1-1,2	20	17.7
1,2-1,2-1,2	17	15.0
2,2-2,2-1,1	7	6.2
Y - Y - Y	6	5.3
Total	113	99.9

This table shows the *AvaI-AluI-TaqI* combined genotype in the patient population. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2-1,1 that occurred at a frequency of 34.5%.

Table A2.2C: Frequency of *AvaI-AluI-TaqI* combined genotypes in the mild group of patients

AvaI-AluI-TaqI	Number (n)	Frequency (%)
1,2-1,2-1,1	27	34.6
1,1-1,1-1,1	18	23.1
1,1-1,1-1,2	12	15.4
1,2-1,2-1,2	11	14.1
2,2-2,2-1,1	6	7.7
Y - Y - Y	4	5.1
Total	78	100.0

This table shows the *AvaI-AluI-TaqI* combined genotype in the mild group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2-1,1 that occurred at a frequency of 34.6%.

Table A2.2D: Frequency of *AvaI-AluI-TaqI* combined genotypes in the severe group of patients

AvaI-AluI-TaqI	Number (n)	Frequency (%)
1,2-1,2-1,1	12	34.3
1,1-1,1-1,2	8	22.9
1,2-1,2-1,2	6	17.1
1,1-1,1-1,1	6	17.1
Y - Y - Y	3	8.6
Total	35	100.0

This table shows the *AvaI-AluI-TaqI* combined genotype in the severe group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2-1,1 that occurred at a frequency of 34.3%.

Table A2.2E: Frequency of *AvaI-AluI-TaqI* combined genotypes in the OFS=0 group of patients

AvaI-AluI-TaqI	Number (n)	Frequency (%)
1,2-1,2-1,1	22	32.4
1,1-1,1-1,1	20	29.4
1,2-1,2-1,2	9	13.2
1,1-1,1-1,2	8	11.8
2,2-2,2-1,1	5	7.4
Y - Y - Y	4	5.9
Total	68	100.1

This table shows the *AvaI-AluI-TaqI* combined genotype in the OFS=0 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2-1,1 that occurred at a frequency of 32.4%.

Table A2.2F: Frequency of *AvaI-AluI-TaqI* combined genotypes in the OFS≥1 group of patients

AvaI-AluI-TaqI	Number (n)	Frequency (%)
1,2-1,2-1,1	17	37.8
1,1-1,1-1,2	12	26.7
1,2-1,2-1,2	8	17.8
1,1-1,1-1,1	4	8.9
Y - Y - Y	4	8.9
Total	45	100.1

This table shows the *AvaI-AluI-TaqI* combined genotype in the OFS≥1 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2-1,1 that occurred at a frequency of 37.8%.

Table A2.2G: Frequency of *AvaI-AluI-TaqI* combined genotypes in the OFS≥2 group of patients

AvaI-AluI-TaqI	Number (n)	Frequency (%)
1,2-1,2-1,1	10	31.3
1,1-1,1-1,2	8	25.0
1,2-1,2-1,2	8	25.0
1,1-1,1-1,1	4	12.5
Y - Y - Y	2	6.3
Total	32	100.1

This table shows the *AvaI-AluI-TaqI* combined genotype in the OFS≥2 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2-1,1 that occurred at a frequency of 31.3%.

Table A2.2H: Frequency of *AvaI-AluI-TaqI* combined genotypes in the OFS≥3 group of patients

AvaI-AluI-TaqI	Number (n)	Frequency (%)
1,2-1,2-1,1	6	33.3
1,1-1,1-1,2	4	22.2
1,2-1,2-1,2	3	16.7
1,1-1,1-1,1	2	11.1
1,2-1,2-1,2	2	11.1
2,2-2,2-1,1	1	5.6
Total	18	100.0

This table shows the *AvaI-AluI-TaqI* combined genotype in the OFS≥3 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. The most common combined genotype was 1,2-1,2-1,1 that occurred at a frequency of 33.3%.

Table A2.2I: Frequency of *AvaI-AluI-TaqI* combined genotypes in the OFS≥4 group of patients

AvaI-AluI-TaqI	Number (n)	Frequency (%)
1,2-1,2-1,1	4	33.3
1,1-1,1-1,2	4	33.3
1,2-1,2-1,2	3	25.0
1,1-1,1-1,1	1	8.3
Total	12	99.9

This table shows the *AvaI-AluI-TaqI* combined genotype in the OFS≥4 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. The most common combined genotypes were 1,2-1,2-1,1 and 1,1-1,1-1,2 that each occurred at a frequency of 33.3%.

Table A2.2J: Frequency of *AvaI-AluI-TaqI* combined genotypes in the alcoholic group of patients

AvaI-AluI-TaqI	Number (n)	Frequency (%)
1,2-1,2-1,1	8	44.4
1,1-1,1-1,1	4	22.2
1,2-1,2-1,2	3	16.7
1,1-1,1-1,2	2	11.1
1,1-1,2-1,1	1	5.6
Total	18	100.0

This table shows the *AvaI-AluI-TaqI* combined genotype in the alcoholic group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. The most common combined genotype was 1,2-1,2-1,1 that occurred at a frequency of 44.4%.

Table A2.2K: Frequency of *AvaI-AluI-TaqI* combined genotypes in the idiopathic group of patients

AvaI-AluI-TaqI	Number (n)	Frequency (%)
1,2-1,2-1,1	10	31.3
1,1-1,1-1,2	9	28.1
1,1-1,1-1,1	6	18.8
1,2-1,2-1,2	4	12.5
2,2-2,2-1,1	2	6.3
Y - Y - Y	1	3.1
Total	32	100.1

This table shows the *AvaI-AluI-TaqI* combined genotype in the idiopathic group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2-1,1 that occurred at a frequency of 31.3%.

Table A2.2L: Frequency of *AvaI-AluI-TaqI* combined genotypes in the gallstone group of patients

AvaI-AluI-TaqI	Number (n)	Frequency (%)
1,2-1,2-1,1	21	33.3
1,1-1,1-1,1	14	22.2
1,2-1,2-1,2	10	15.9
1,1-1,1-1,2	9	14.3
2,2-2,2-1,1	5	7.9
Y - Y - Y	4	6.3
Total	63	99.9

This table shows the *AvaI-AluI-TaqI* combined genotype in the gallstone group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2-1,1 that occurred at a frequency of 33.3%.

Table A2.3A: Frequency of *MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the control population

MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
1,1-2,2-1,1-1,1	43	42.2
1,2-2,3-1,2-1,2	38	37.3
2,2-3,3-2,2-2,2	8	7.8
Y - Y - Y - Y	13	12.7
Total	102	100.0

This table shows the *MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the control population. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,1-2,2-1,1-1,1 that occurred at a frequency of 42.2%.

Table A2.3B: Frequency of *MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the patient population

MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
1,1-2,2-1,1-1,1	62	53.9
1,2-2,3-1,2-1,2	39	33.9
2,2-3,3-2,2-2,2	10	8.7
Y - Y - Y - Y	4	3.5
Total	115	100.0

This table shows the *MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the patient population. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,1-2,2-1,1-1,1 that occurred at a frequency of 53.9%.

Table A2.3C: Frequency of *MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the mild group of patients

MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
1,1-2,2-1,1-1,1	37	46.8
1,2-2,3-1,2-1,2	30	38.0
2,2-3,3-2,2-2,2	8	10.1
Y - Y - Y - Y	4	5.1
Total	79	100.0

This table shows the *MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the mild group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,1-2,2-1,1-1,1 that occurred at a frequency of 46.8%.

Table A2.3D: Frequency of *MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the severe group of patients

MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
1,1-2,2-1,1-1,1	25	69.4
1,2-2,3-1,2-1,2	9	25.0
2,2-3,3-2,2-2,2	2	5.6
Total	36	100.0

This table shows the *MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the severe group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. The most common combined genotype was 1,1-2,2-1,1-1,1 that occurred at a frequency of 69.4%.

Table A2.3E: Frequency of *MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the OFS=0 group of patients

MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
1,1-2,2-1,1-1,1	34	49.3
1,2-2,3-1,2-1,2	26	37.7
2,2-3,3-2,2-2,2	5	7.2
Y - Y - Y - Y	4	5.8
Total	69	100.0

This table shows the *MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the OFS=0 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,1-2,2-1,1-1,1 that occurred at a frequency of 49.3%.

Table A2.3F: Frequency of *MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the OFS≥1 group of patients

MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
1,1-2,2-1,1-1,1	28	60.9
1,2-2,3-1,2-1,2	13	28.3
2,2-3,3-2,2-2,2	5	10.9
Total	46	100.1

This table shows the *MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the OFS≥1 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. The most common combined genotype was 1,1-2,2-1,1-1,1 that occurred at a frequency of 60.9%.

Table A2.3G: Frequency of *MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the OFS≥2 group of patients

MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
1,1-2,2-1,1-1,1	21	63.6
1,2-2,3-1,2-1,2	9	27.3
2,2-3,3-2,2-2,2	3	9.1
Total	33	100.0

This table shows the *MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the OFS≥2 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,1-2,2-1,1-1,1 that occurred at a frequency of 63.6%.

Table A2.3H: Frequency of *MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the OFS≥3 group of patients

MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
1,1-2,2-1,1-1,1	13	68.4
1,2-2,3-1,2-1,2	5	26.3
2,2-3,3-2,2-2,2	1	5.3
Total	19	100.0

This table shows the *MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the OFS≥3 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. The most common combined genotype was 1,1-2,2-1,1-1,1 that occurred at a frequency of 68.4%.

Table A2.3I: Frequency of *MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the OFS≥4 group of patients

MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
1,1-2,2-1,1-1,1	8	66.7
1,2-2,3-1,2-1,2	4	33.3
Total	12	100.0

This table shows the *MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the OFS≥4 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. The most common combined genotype was 1,1-2,2-1,1-1,1 that occurred at a frequency of 66.7%.

Table A2.3J: Frequency of *MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the alcoholic group of patients

MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
1,1-2,2-1,1-1,1	8	44.4
1,2-2,3-1,2-1,2	8	44.4
2,2-3,3-2,2-2,2	1	5.6
1,2-1,3-2,2-1,2	1	5.6
Total	18	100.0

This table shows the *MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the alcoholic group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. The most common combined genotypes were 1,1-2,2-1,1-1,1 and 1,2-2,3-1,2-1,2 that each occurred at a frequency of 44.4%.

Table A2.3K: Frequency of *MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the idiopathic group of patients

MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
1,1-2,2-1,1-1,1	22	66.7
1,2-2,3-1,2-1,2	8	24.2
Y - Y - Y - Y	3	9.1
Total	33	100.0

This table shows the *MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the idiopathic group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,1-2,2-1,1-1,1 that occurred at a frequency of 66.7%.

Table A2.3L: Frequency of *MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the gallstone group of patients

MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
1,1-2,2-1,1-1,1	32	50.0
1,2-2,3-1,2-1,2	23	35.9
2,2-3,3-2,2-2,2	9	14.1
Total	64	100.0

This table shows the *MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the gallstone group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. The most common combined genotype was 1,1-2,2-1,1-1,1 that occurred at a frequency of 50.0%.

Table A2.4A: Frequency of *PstI*-(AC)n-VNTR[46] combined genotypes in the control population

PstI-(AC)n-VNTR[46]	Number (n)	Frequency (%)
1,2-1,4-1,1	8	6.7
2,2-1,2-1,2	8	6.7
2,2-1,4-1,1	8	6.7
1,2-2,4-2,2	6	5.0
Y - Y - Y	89	74.8
Total	119	99.9

This table shows the *PstI*-(AC)n-VNTR[46] combined genotype in the control population. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotypes were 1,2-1,4-1,1, 2,2-1,2-1,2 and 2,2-1,4-1,1 that each occurred at a frequency of 6.7%.

Table A2.4B: Frequency of *PstI*-(AC)n-VNTR[46] combined genotypes in the patient population

PstI-(AC)n-VNTR[46]	Number (n)	Frequency (%)
2,2-1,2-1,2	10	8.8
1,2-1,2-1,2	10	8.8
2,2-1,2-2,3	9	7.9
2,2-2,4-1,2	7	6.1
2,2-1,1-1,3	6	5.3
Y - Y - Y	72	63.2
Total	114	100.1

This table shows the *PstI*-(AC)n-VNTR[46] combined genotype in the patient population. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotypes were 2,2-1,2-1,2 and 1,2-1,2-1,2 that each occurred at a frequency of 8.8%.

Table A2.4C: Frequency of *PstI*-(AC)n-VNTR[46] combined genotypes in the mild group of patients

PstI-(AC)n-VNTR[46]	Number (n)	Frequency (%)
2,2-2,4-1,2	7	9.0
2,2-1,2-1,2	6	7.7
2,2-1,2-2,3	6	7.7
1,2-1,2-1,2	5	6.4
2,2-1,1-1,1	4	5.1
2,2-1,4-1,1	4	5.1
Y - Y - Y	46	59.0
Total	78	100.0

This table shows the *PstI*-(AC)n-VNTR[46] combined genotype in the mild group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 2,2-2,4-1,2 that occurred at a frequency of 9.0%.

Table A2.4D: Frequency of *PstI*-(AC)n-VNTR[46] combined genotypes in the severe group of patients

PstI-(AC)n-VNTR[46]	Number (n)	Frequency (%)
1,2-1,2-1,2	5	13.9
2,2-1,2-1,2	4	11.1
2,2-1,2-2,3	3	8.3
2,2-1,1-1,3	3	8.3
2,2-2,2-2,3	2	5.6
Y - Y - Y	19	52.8
Total	36	100.0

This table shows the *PstI*-(AC)n-VNTR[46] combined genotype in the severe group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2-1,2 that occurred at a frequency of 13.9%.

Table A2.4E: Frequency of *PstI*-(AC)n-VNTR[46] combined genotypes in the OFS=0 group of patients

PstI-(AC)n-VNTR[46]	Number (n)	Frequency (%)
2,2-2,4-1,2	8	9.1
2,2-1,2-2,3	7	8.0
2,2-1,1-1,1	6	6.8
2,2-1,2-1,2	5	5.7
1,2-1,4-1,3	5	5.7
Y - Y - Y	57	64.8
Total	88	100.1

This table shows the *PstI*-(AC)n-VNTR[46] combined genotype in the OFS=0 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 2,2-2,4-1,2 that occurred at a frequency of 9.1%.

Table A2.4F: Frequency of *PstI*-(AC)n-VNTR[46] combined genotypes in the OFS≥1 group of patients

PstI-(AC)n-VNTR[46]	Number (n)	Frequency (%)
1,2-1,2-1,2	8	17.4
2,2-1,2-1,2	6	13.0
2,2-1,2-2,3	3	6.5
Y - Y - Y	29	63.0
Total	46	99.9

This table shows the *PstI*-(AC)n-VNTR[46] combined genotype in the OFS≥1 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2-1,2 that occurred at a frequency of 17.4%.

Table A2.4G: Frequency of *PstI*-(AC)n-VNTR[46] combined genotypes in the OFS≥2 group of patients

PstI-(AC)n-VNTR[46]	Number (n)	Frequency (%)
1,2-1,2-1,2	6	18.2
2,2-1,2-1,2	5	15.2
2,2-1,5-1,3	2	6.1
2,2-1,2-2,3	2	6.1
2,2-1,1-1,3	2	6.1
Y - Y - Y	16	48.5
Total	33	100.2

This table shows the *PstI*-(AC)n-VNTR[46] combined genotype in the OFS≥2 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2-1,2 that occurred at a frequency of 18.2%.

Table A2.4H: Frequency of *PstI*-(AC)n-VNTR[46] combined genotypes in the OFS≥3 group of patients

PstI-(AC)n-VNTR[46]	Number (n)	Frequency (%)
1,2-1,2-1,2	5	26.3
2,2-1,2-2,3	2	10.5
2,2-1,1-1,3	2	10.5
2,2-2,2-2,3	1	5.3
2,2-4,6-1,1	1	5.3
2,2-1,5-1,3	1	5.3
2,2-1,1-1,1	1	5.3
2,2-2,2-6,6	1	5.3
1,2-1,4-1,1	1	5.3
2,2-1,5-1,1	1	5.3
2,2-1,1-3,3	1	5.3
1,2-2,4-1,1	1	5.3
2,2-1,2-1,2	1	5.3
Total	19	100.3

This table shows the *PstI*-(AC)n-VNTR[46] combined genotype in the OFS≥3 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. The most common combined genotype was 1,2-1,2-1,2 that occurred at a frequency of 26.3%.

Table A2.4I: Frequency of *PstI*-(AC)n-VNTR[46] combined genotypes in the OFS≥4 group of patients

PstI-(AC)n-VNTR[46]	Number (n)	Frequency (%)
1,2-1,2-1,2	3	25.0
2,2-1,1-1,3	2	16.7
2,2-2,2-2,3	1	8.3
2,2-4,6-1,1	1	8.3
2,2-1,2-2,3	1	8.3
1,2-1,4-1,1	1	8.3
2,2-1,5-1,1	1	8.3
2,2-1,1-3,3	1	8.3
2,2-1,2-1,2	1	8.3
Total	12	99.8

This table shows the *PstI*-(AC)n-VNTR[46] combined genotype in the OFS≥4 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. The most common combined genotype was 1,2-1,2-1,2 that occurred at a frequency of 25.0%.

Table A2.4J: Frequency of *PstI*-(AC)n-VNTR[46] combined genotypes in the alcoholic group of patients

PstI-(AC)n-VNTR[46]	Number (n)	Frequency (%)
2,2-2,4-1,2	3	16.7
1,2-1,1-1,3	2	11.1
1,2-1,2-1,2	2	11.1
2,2-3,4-1,1	2	11.1
1,2-2,4-2,2	1	5.6
2,2-1,1-1,3	1	5.6
2,2-2,4-1,1	1	5.6
1,2-1,4-1,3	1	5.6
2,2-1,5-1,1	1	5.6
2,2-1,2-3,3	1	5.6
1,2-2,4-1,2	1	5.6
2,2-4,5-1,1	1	5.6
1,1-1,2-1,1	1	5.6
Total	18	100.4

This table shows the *PstI*-(AC)n-VNTR[46] combined genotype in the alcoholic group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. The most common combined genotype was 2,2-2,4-1,2 that occurred at a frequency of 16.7%.

Table A2.4K: Frequency of *PstI*-(AC)n-VNTR[46] combined genotypes in the idiopathic group of patients

PstI-(AC)n-VNTR[46]	Number (n)	Frequency (%)
1,2-1,2-1,2	5	15.2
2,2-1,2-1,2	3	9.1
2,2-2,2-2,3	2	6.1
1,2-2,7-1,2	2	6.1
2,2-2,4-1,2	2	6.1
2,2-1,2-2,3	2	6.1
Y - Y - Y	17	51.5
Total	33	100.2

This table shows the *PstI*-(AC)n-VNTR[46] combined genotype in the idiopathic group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2-1,2 that occurred at a frequency of 15.2%.

Table A2.4L: Frequency of *PstI*-(AC)n-VNTR[46] combined genotypes in the gallstone group of patients

PstI-(AC)n-VNTR[46]	Number (n)	Frequency (%)
1,2-1,2-1,2	7	11.1
2,2-1,2-2,3	7	11.1
2,2-1,1-1,1	4	6.3
Y - Y - Y	45	71.4
Total	63	99.9

This table shows the *PstI*-(AC)n-VNTR[46] combined genotype in the gallstone group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotypes were 1,2-1,2-1,2 and 2,2-1,2-2,3 that each occurred at a frequency of 11.1%.

Table A2.5A: Frequency of *PstI-AvaI-AluI-TaqI* combined genotypes in the control population

PstI-AvaI-AluI-TaqI	Number (n)	Frequency (%)
2,2-2,2-2,2-1,1	11	14.1
2,2-1,1-1,1-1,1	11	14.1
1,2-1,2-1,2-1,2	7	9.0
1,2-1,2-1,2-1,1	7	9.0
2,2-1,1-1,1-1,2	7	9.0
1,2-1,1-1,1-1,1	6	7.7
1,2-1,1-1,1-1,2	5	6.4
2,2-1,2-1,2-1,1	4	5.1
1,2-2,2-2,2-1,1	4	5.1
Y - Y - Y - Y	16	20.5
Total	78	100.0

This table shows the *PstI-AvaI-AluI-TaqI* combined genotype in the control population. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotypes were 2,2-2,2-2,2-1,1 and 2,2-1,1-1,1-1,1 that each occurred at a frequency of 14.1%.

Table A2.5B: Frequency of *PstI-AvaI-AluI-TaqI* combined genotypes in the patient population

PstI-AvaI-AluI-TaqI	Number (n)	Frequency (%)
2,2-1,2-1,2-1,1	25	22.3
2,2-1,1-1,1-1,1	15	13.4
2,2-1,1-1,1-1,2	14	12.5
1,2-1,2-1,2-1,1	12	10.7
1,2-1,2-1,2-1,2	9	8.0
2,2-1,2-1,2-1,2	8	7.1
1,2-1,1-1,1-1,1	8	7.1
1,2-1,1-1,1-1,2	6	5.4
Y - Y - Y - Y	15	13.4
Total	112	99.9

This table shows the *PstI-AvaI-AluI-TaqI* combined genotype in the patient population. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 2,2-1,2-1,2-1,1 that occurred at a frequency of 22.3%.

Table A2.5C: Frequency of *PstI-AvaI-AluI-TaqI* combined genotypes in the mild group of patients

PstI-AvaI-AluI-TaqI	Number (n)	Frequency (%)
2,2-1,2-1,2-1,1	18	23.4
2,2-1,1-1,1-1,1	11	14.3
2,2-1,1-1,1-1,2	8	10.4
1,2-1,2-1,2-1,1	8	10.4
2,2-1,2-1,2-1,2	6	7.8
1,2-1,1-1,1-1,1	6	7.8
1,2-1,2-1,2-1,2	5	6.5
1,2-1,1-1,1-1,2	4	5.2
Y - Y - Y - Y	11	14.3
Total	77	100.1

This table shows the *PstI-AvaI-AluI-TaqI* combined genotype in the mild group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 2,2-1,2-1,2-1,1 that occurred at a frequency of 23.4%.

Table A2.5D: Frequency of *PstI-AvaI-AluI-TaqI* combined genotypes in the severe group of patients

PstI-AvaI-AluI-TaqI	Number (n)	Frequency (%)
2,2-1,2-1,2-1,1	8	22.9
2,2-1,1-1,1-1,2	5	14.3
1,2-1,2-1,2-1,2	4	11.4
2,2-1,1-1,1-1,1	4	11.4
1,2-1,1-1,1-1,2	3	8.6
1,2-1,2-1,2-1,1	3	8.6
1,2-1,1-1,1-1,1	2	5.7
2,2-1,2-1,2-1,2	2	5.7
Y - Y - Y - Y	4	11.4
Total	35	100.0

This table shows the *PstI-AvaI-AluI-TaqI* combined genotype in the severe group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 2,2-1,2-1,2-1,1 that occurred at a frequency of 22.9%.

Table A2.5E: Frequency of *PstI-AvaI-AluI-TaqI* combined genotypes in the OFS=0 group of patients

PstI-AvaI-AluI-TaqI	Number (n)	Frequency (%)
2,2-1,2-1,2-1,1	14	20.9
2,2-1,1-1,1-1,1	13	19.4
1,2-1,2-1,2-1,1	6	9.0
1,2-1,1-1,1-1,1	6	9.0
2,2-1,2-1,2-1,2	5	7.5
2,2-1,1-1,1-1,2	4	6.0
1,2-1,2-1,2-1,2	4	6.0
1,2-1,1-1,1-1,2	4	6.0
Y - Y - Y - Y	11	16.4
Total	67	100.2

This table shows the *PstI-AvaI-AluI-TaqI* combined genotype in the OFS=0 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 2,2-1,2-1,2-1,1 that occurred at a frequency of 20.9%.

Table A2.5F: Frequency of *PstI-AvaI-AluI-TaqI* combined genotypes in the OFS≥1 group of patients

PstI-AvaI-AluI-TaqI	Number (n)	Frequency (%)
2,2-1,2-1,2-1,1	11	24.4
2,2-1,1-1,1-1,2	10	22.2
1,2-1,2-1,2-1,1	6	13.3
2,2-1,2-1,2-1,2	3	6.7
1,2-1,2-1,2-1,2	3	6.7
Y - Y - Y - Y	12	26.7
Total	45	100.0

This table shows the *PstI-AvaI-AluI-TaqI* combined genotype in the OFS≥1 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 2,2-1,2-1,2-1,1 that occurred at a frequency of 24.4%.

Table A2.5G: Frequency of *PstI-AvaI-AluI-TaqI* combined genotypes in the OFS≥2 group of patients

PstI-AvaI-AluI-TaqI	Number (n)	Frequency (%)
2,2-1,2-1,2-1,1	7	21.9
2,2-1,1-1,1-1,2	7	21.9
1,2-1,2-1,2-1,2	5	15.6
2,2-1,2-1,2-1,2	3	9.4
1,2-1,2-1,2-1,1	3	9.4
1,2-1,1-1,1-1,1	2	6.3
2,2-1,1-1,1-1,1	2	6.3
Y - Y - Y - Y	3	9.4
Total	32	100.2

This table shows the *PstI-AvaI-AluI-TaqI* combined genotype in the OFS≥2 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotypes were 2,2-1,2-1,2-1,1 and 2,2-1,1-1,1-1,2 that both occurred at a frequency of 21.9%.

Table A2.5H: Frequency of *PstI-AvaI-AluI-TaqI* combined genotypes in the OFS≥3 group of patients

PstI-AvaI-AluI-TaqI	Number (n)	Frequency (%)
2,2-1,2-1,2-1,1	5	27.8
1,2-1,2-1,2-1,2	3	16.7
1,2-1,2-1,2-1,1	2	11.1
2,2-1,2-1,2-1,2	2	11.1
2,2-1,1-1,1-1,2	2	11.1
2,2-1,1-1,1-1,1	1	5.6
2,2-2,2-2,2-1,1	1	5.6
1,2-1,1-1,1-1,2	1	5.6
1,2-1,1-1,1-1,1	1	5.6
Total	18	100.2

This table shows the *PstI-AvaI-AluI-TaqI* combined genotype in the OFS≥3 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. The most common combined genotype was 2,2-1,2-1,2-1,1 that occurred at a frequency of 27.8%.

Table A2.5I: Frequency of *PstI-AvaI-AluI-TaqI* combined genotypes in the OFS≥4 group of patients

PstI-AvaI-AluI-TaqI	Number (n)	Frequency (%)
2,2-1,1-1,1-1,2	3	25.0
2,2-1,2-1,2-1,1	2	16.7
1,2-1,2-1,2-1,1	2	16.7
2,2-1,2-1,2-1,2	2	16.7
2,2-1,1-1,1-1,1	1	8.3
1,2-1,1-1,1-1,2	1	8.3
1,2-1,2-1,2-1,2	1	8.3
Total	12	100.0

This table shows the *PstI-AvaI-AluI-TaqI* combined genotype in the OFS≥4 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. The most common combined genotype was 2,2-1,1-1,1-1,2 that occurred at a frequency of 25.0%.

Table A2.5J: Frequency of *PstI-AvaI-AluI-TaqI* combined genotypes in the alcoholic group of patients

PstI-AvaI-AluI-TaqI	Number (n)	Frequency (%)
2,2-1,2-1,2-1,1	6	33.3
1,2-1,2-1,2-1,2	3	16.7
2,2-1,1-1,1-1,1	3	16.7
2,2-1,1-1,1-1,2	2	11.1
1,2-1,1-1,2-1,1	1	5.6
1,2-1,1-1,1-1,1	1	5.6
1,1-1,2-1,2-1,1	1	5.6
1,2-1,2-1,2-1,1	1	5.6
Total	18	100.2

This table shows the *PstI-AvaI-AluI-TaqI* combined genotype in the alcoholic group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. The most common combined genotype was 2,2-1,2-1,2-1,1 that occurred at a frequency of 33.3%.

Table A2.5K: Frequency of *PstI-AvaI-AluI-TaqI* combined genotypes in the idiopathic group of patients

PstI-AvaI-AluI-TaqI	Number (n)	Frequency (%)
2,2-1,1-1,1-1,2	5	15.6
2,2-1,2-1,2-1,1	5	15.6
1,2-1,1-1,1-1,2	4	12.5
1,2-1,2-1,2-1,1	4	12.5
1,2-1,2-1,2-1,2	3	9.4
2,2-1,1-1,1-1,1	3	9.4
1,2-1,1-1,1-1,1	3	9.4
1,2-2,2-2,2-1,1	2	6.3
Y - Y - Y - Y	3	9.4
Total	32	100.1

This table shows the *PstI-AvaI-AluI-TaqI* combined genotype in the idiopathic group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotypes were 2,2-1,1-1,1-1,2 and 2,2-1,2-1,2-1,1 that each occurred at a frequency of 15.6%.

Table A2.5L: Frequency of *PstI-AvaI-AluI-TaqI* combined genotypes in the gallstone group of patients

PstI-AvaI-AluI-TaqI	Number (n)	Frequency (%)
2,2-1,2-1,2-1,1	14	22.6
2,2-1,1-1,1-1,1	9	14.5
2,2-1,1-1,1-1,2	7	11.3
1,2-1,2-1,2-1,1	7	11.3
2,2-1,2-1,2-1,2	7	11.3
1,2-1,1-1,1-1,1	4	6.5
2,2-2,2-2,2-1,1	4	6.5
Y - Y - Y - Y	10	16.1
Total	62	100.1

This table shows the *PstI-AvaI-AluI-TaqI* combined genotype in the gallstone group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 2,2-1,2-1,2-1,1 that occurred at a frequency of 22.6%.

Table A2.6A: Frequency of *PstI-MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the control population

PstI-MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
2,2-1,1-2,2-1,1-1,1	20	21.3
1,2-1,1-2,2-1,1-1,1	18	19.1
2,2-1,2-2,3-1,2-1,2	17	18.1
1,2-1,2-2,3-1,2-1,2	14	14.9
Y - Y - Y - Y - Y	25	26.6
Total	94	100.0

This table shows the *PstI-MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the control population. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 2,2-1,1-2,2-1,1-1,1 that occurred at a frequency of 21.3%.

Table A2.6B: Frequency of *PstI-MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the patient population

PstI-MspI-VLP-VNTR^{86}-*SspI*	Number(n)	Frequency(%)
2,2-1,1-2,2-1,1-1,1	39	34.2
2,2-1,2-2,3-1,2-1,2	23	20.2
1,2-1,1-2,2-1,1-1,1	22	19.3
1,2-1,2-2,3-1,2-1,2	14	12.3
Y - Y - Y - Y - Y	16	14.0
Total	114	100.0

This table shows the *PstI-MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the patient population. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 2,2-1,1-2,2-1,1-1,1 that occurred at a frequency of 34.2%.

Table A2.6C: Frequency of *PstI-MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the mild group of patients

PstI-MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
2,2-1,1-2,2-1,1-1,1	23	29.5
2,2-1,2-2,3-1,2-1,2	19	24.4
1,2-1,1-2,2-1,1-1,1	13	16.7
1,2-1,2-2,3-1,2-1,2	10	12.8
2,2-2,2-3,3-2,2-2,2	4	5.1
Y - Y - Y - Y - Y	9	11.5
Total	78	100.0

This table shows the *PstI-MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the mild group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 2,2-1,1-2,2-1,1-1,1 that occurred at a frequency of 29.5%.

Table A2.6D: Frequency of *PstI-MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the severe group of patients

PstI-MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
2,2-1,1-2,2-1,1-1,1	17	47.2
1,2-1,1-2,2-1,1-1,1	8	22.2
2,2-1,2-2,3-1,2-1,2	4	11.1
1,2-1,2-2,3-1,2-1,2	4	11.1
Y - Y - Y - Y - Y	3	8.3
Total	36	99.9

This table shows the *PstI-MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the severe group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 2,2-1,1-2,2-1,1-1,1 that occurred at a frequency of 47.2%.

Table A2.6E: Frequency of *PstI-MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the OFS=0 group of patients

PstI-MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
2,2-1,1-2,2-1,1-1,1	22	32.4
2,2-1,2-2,3-1,2-1,2	15	22.1
1,2-1,1-2,2-1,1-1,1	11	16.2
1,2-1,2-2,3-1,2-1,2	9	13.2
Y - Y - Y - Y - Y	11	16.2
Total	68	100.1

This table shows the *PstI-MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the OFS=0 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 2,2-1,1-2,2-1,1-1,1 that occurred at a frequency of 32.4%.

Table A2.6F: Frequency of *PstI-MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the OFS≥1 group of patients

PstI-MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
2,2-1,1-2,2-1,1-1,1	19	41.3
1,2-1,1-2,2-1,1-1,1	9	19.6
2,2-1,2-2,3-1,2-1,2	8	17.4
1,2-1,2-2,3-1,2-1,2	5	10.9
2,2-2,2-3,3-2,2-2,2	3	6.5
Y - Y - Y - Y - Y	2	4.3
Total	46	100.0

This table shows the *PstI-MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the OFS≥1 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 2,2-1,1-2,2-1,1-1,1 that occurred at a frequency of 41.3%.

Table A2.6G: Frequency of *PstI-MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the OFS≥2 group of patients

PstI-MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
2,2-1,1-2,2-1,1-1,1	15	45.5
1,2-1,1-2,2-1,1-1,1	6	18.2
2,2-1,2-2,3-1,2-1,2	5	15.2
1,2-1,2-2,3-1,2-1,2	4	12.1
1,2-2,2-3,3-2,2-2,2	2	6.1
Y - Y - Y - Y - Y	1	3.0
Total	33	100.1

This table shows the *PstI-MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the OFS≥2 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 2,2-1,1-2,2-1,1-1,1 that occurred at a frequency of 45.5%.

Table A2.6H: Frequency of *PstI-MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the OFS≥3 group of patients

PstI-MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
2,2-1,1-2,2-1,1-1,1	9	47.4
1,2-1,1-2,2-1,1-1,1	4	21.1
1,2-1,2-2,3-1,2-1,2	3	15.8
2,2-1,2-2,3-1,2-1,2	2	10.5
2,2-2,2-3,3-2,2-2,2	1	5.3
Total	19	100.1

This table shows the *PstI-MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the OFS≥3 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. The most common combined genotype was 2,2-1,1-2,2-1,1-1,1 that occurred at a frequency of 47.4%.

Table A2.6I: Frequency of *PstI-MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the OFS≥4 group of patients

PstI-MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
2,2-1,1-2,2-1,1-1,1	7	58.3
1,2-1,2-2,3-1,2-1,2	3	25.0
1,2-1,1-2,2-1,1-1,1	1	8.3
2,2-1,2-2,3-1,2-1,2	1	8.3
Total	12	99.9

This table shows the *PstI-MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the OFS≥4 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. The most common combined genotype was 2,2-1,1-2,2-1,1-1,1 that occurred at a frequency of 58.3%.

Table A2.6J: Frequency of *PstI-MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the alcoholic group of patients

PstI-MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
2,2-1,2-2,3-1,2-1,2	5	27.8
1,2-1,1-2,2-1,1-1,1	4	22.2
2,2-1,1-2,2-1,1-1,1	4	22.2
1,2-1,2-2,3-1,2-1,2	2	11.1
2,2-1,2-1,3-2,2-1,2	1	5.6
2,2-2,2-3,3-2,2-2,2	1	5.6
1,1-1,2-2,3-1,2-1,2	1	5.6
Total	18	100.1

This table shows the *PstI-MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the alcoholic group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. The most common combined genotype was 2,2-1,2-2,3-1,2-1,2 that occurred at a frequency of 27.8%.

Table A2.6K: Frequency of *PstI-MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the idiopathic group of patients

PstI-MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
2,2-1,1-2,2-1,1-1,1	13	39.4
1,2-1,1-2,2-1,1-1,1	9	27.3
1,2-1,2-2,3-1,2-1,2	5	15.2
2,2-1,2-2,3-1,2-1,2	2	6.1
Y - Y - Y - Y - Y	4	12.1
Total	33	100.1

This table shows the *PstI-MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the idiopathic group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 2,2-1,1-2,2-1,1-1,1 that occurred at a frequency of 39.4%.

Table A2.6L: Frequency of *PstI-MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the gallstone group of patients

PstI-MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
2,2-1,1-2,2-1,1-1,1	24	38.1
2,2-1,2-2,3-1,2-1,2	16	25.4
1,2-1,2-2,3-1,2-1.2	7	11.1
1,2-1,1-2,2-1,1-1,1	7	11.1
1,2-2,2-3,3-2,2-2,2	4	6.3
2,2-2,2-3,3-2,2-2,2	4	6.3
Y - Y - Y - Y - Y	1	1.6
Total	63	99.9

This table shows the *PstI-MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the gallstone group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 2,2-1,1-2,2-1,1-1,1 that occurred at a frequency of 38.1%.

Table A2.7A: Frequency of (AC)n-VNTR^{46}-*AvaI-AluI-TaqI* combined genotypes in the control population

(AC)n-VNTR^{46}-*AvaI-AluI-TaqI*	Number (n)	Frequency (%)
1,4-1,1-1,1-1,1-1,2	5	6.3
2,4-1,2-1,2-1,2-1,1	4	5.0
1,2-1,2-2,2-2,2-1,1	4	5.0
Y - Y - Y - Y - Y	67	83.8
Total	80	100.1

This table shows the (AC)n-VNTR^{46}-*AvaI-AluI-TaqI* combined genotype in the control population. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,4-1,1-1,1-1,1-1,2 that occurred at a frequency of 6.3%.

Table A2.7B: Frequency of (AC)n-VNTR^{46}-*AvaI-AluI-TaqI* combined genotypes in the patient population

(AC)n-VNTR^{46}-*AvaI-AluI-TaqI*	Number (n)	Frequency (%)
1,2-1,2-1,2-1,2-1,2	8	7.1
1,2-1,2-1,2-1,2-1,1	6	5.3
1,2-1,2-1,1-1,1-1,2	6	5.3
1,4-1,1-1,1-1,1-1,2	6	5.3
Y - Y - Y - Y- Y	87	77.0
Total	113	100.0

This table shows the (AC)n-VNTR^{46}-*AvaI-AluI-TaqI* combined genotype in the patient population. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2-1,2-1,2-1,2 that occurred at a frequency of 7.1%.

Table A2.7C: Frequency of (AC)n-VNTR[46]-*AvaI-AluI-TaqI* combined genotypes in the mild group of patients

(AC)n-VNTR[46]-*AvaI-AluI-TaqI*	Number (n)	Frequency (%)
1,2-1,2-1,2-1,2-1,2	5	6.4
1,4-1,1-1,1-1,1-1,2	5	6.4
2,4-1,2-1,2-1,2-1,1	5	6.4
Y - Y - Y - Y- Y	63	80.8
Total	78	100.0

This table shows the (AC)n-VNTR[46]-*AvaI-AluI-TaqI* combined genotype in the mild group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotypes were 1,2-1,2-1,2-1,2-1,2, 1,4-1,1-1,1-1,1-1,2 and 2,4-1,2-1,2-1,2-1,1 that each occurred at a frequency of 6.4%.

Table A2.7D: Frequency of (AC)n-VNTR[46]-*AvaI-AluI-TaqI* combined genotypes in the severe group of patients

(AC)n-VNTR[46]-*AvaI-AluI-TaqI*	Number (n)	Frequency (%)
1,2-1,2-1,2-1,2-1,2	3	8.6
1,2-1,2-1,2-1,2-1,1	3	8.6
1,2-2,3-1,2-1,2-1,1	3	8.6
1,2-1,2-1,1-1,1-1,2	3	8.6
Y - Y - Y - Y - Y	23	65.7
Total	35	100.1

This table shows the (AC)n-VNTR[46]-*AvaI-AluI-TaqI* combined genotype in the severe group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotypes were 1,2-1,2-1,2-1,2-1,2, 1,2-1,2-1,2-1,2-1,1, 1,2-2,3-1,2-1,2-1,1 and 1,2-1,2-1,1-1,1-1,2 that all occurred at a frequency of 8.6%.

Table A2.7E: Frequency of (AC)n-VNTR[46]-*AvaI-AluI-TaqI* combined genotypes in the OFS=0 group of patients

(AC)n-VNTR[46]-*AvaI-AluI-TaqI*	Number (n)	Frequency (%)
1,2-1,2-1,2-1,2-1,2	4	5.9
1,4-1,1-1,1-1,1-1,2	4	5.9
2,4-1,2-1,2-1,2-1,1	4	5.9
Y - Y - Y - Y - Y	56	82.4
Total	68	100.1

This table shows the (AC)n-VNTR[46]-*AvaI-AluI-TaqI* combined genotype in the OFS=0 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotypes were 1,2-1,2-1,2-1,2-1,2, 1,4-1,1-1,1-1,1-1,2 and 2,4-1,2-1,2-1,2-1,1 that each occurred at a frequency of 5.9%.

Table A2.7F: Frequency of (AC)n-VNTR[46]-*AvaI-AluI-TaqI* combined genotypes in the OFS≥1 group of patients

(AC)n-VNTR[46]-*AvaI-AluI-TaqI*	Number (n)	Frequency (%)
1,2-1,2-1,1-1,1-1,2	5	11.1
1,2-1,2-1,2-1,2-1,2	4	8.9
1,2-1,2-1,2-1,2-1,1	4	8.9
1,2-2,3-1,2-1,2-1,1	3	6.7
1,5-1,3-1,2-1,2-1,1	3	6.7
Y - Y - Y - Y - Y	26	57.8
Total	45	100.1

This table shows the (AC)n-VNTR[46]-*AvaI-AluI-TaqI* combined genotype in the OFS≥1 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2-1,1-1,1-1,2 that occurred at a frequency of 11.1%.

Table A2.7G: Frequency of (AC)n-VNTR[46]-*AvaI-AluI-TaqI* combined genotypes in the OFS≥2 group of patients

(AC)n-VNTR[46]-*AvaI-AluI-TaqI*	Number (n)	Frequency (%)
1,2-1,2-1,2-1,2-1,2	4	12.5
1,2-1,2-1,1-1,1-1,2	3	9.4
1,2-1,2-1,2-1,2-1,1	3	9.4
1,5-1,3-1,2-1,2-1,1	2	6.3
1,2-2,3-1,2-1,2-1,1	2	6.3
Y - Y - Y - Y- Y	18	56.3
Total	32	100.2

This table shows the (AC)n-VNTR[46]-*AvaI-AluI-TaqI* combined genotype in the OFS≥2 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2-1,2-1,2-1,2-that occurred at a frequency of 12.5%.

Table A2.7H: Frequency of (AC)n-VNTR[46]-*AvaI-AluI-TaqI* combined genotypes in the OFS≥3 group of patients

(AC)n-VNTR[46]-*AvaI-AluI-TaqI*	Number (n)	Frequency (%)
1,2-1,2-1,2-1,2-1,2	3	16.7
1,2-1,2-1,2-1,2-1,1	2	11.1
1,2-2,3-1,2-1,2-1,1	2	11.1
2,2-2,3-1,2-1,2-1,1	1	5.6
4,6-1,1-1,1-1,1-1,1	1	5.6
1,5-1,3-1,2-1,2-1,1	1	5.6
2,2-6,6-2,2-2,2-1,1	1	5.6
1,4-1,1-1,1-1,1-1,2	1	5.6
1,5-1,1-1,1-1,1-1,2	1	5.6
1,1-3,3-1,2-1,2-1,2	1	5.6
2,4-1,1-1,1-1,1-1,1	1	5.6
1,2-1,2-1,1-1,1-1,2	1	5.6
1,1-1,3-1,2-1,2-1,2	1	5.6
1,2-1,3-1,1-1,1-1,2	1	5.6
Total	18	100.5

This table shows the (AC)n-VNTR[46]-*AvaI-AluI-TaqI* combined genotype in the OFS≥3 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. The most common combined genotype was 1,2-1,2-1,2-1,2-1,2 that occurred at a frequency of 16.7%.

Table A2.7I: Frequency of (AC)n-VNTR[46]-*AvaI-AluI-TaqI* combined genotypes in the OFS≥4 group of patients

(AC)n-VNTR[46]-*AvaI-AluI-TaqI*	Number (n)	Frequency (%)
1,2-1,2-1,2-1,2-1,1	2	16.7
2,2-2,3-1,2-1,2-1,1	1	8.3
4,6-1,1-1,1-1,1-1,1	1	8.3
1,2-2,3-1,2-1,2-1,1	1	8.3
1,4-1,1-1,1-1,1-1,2	1	8.3
1,5-1,1-1,1-1,1-1,2	1	8.3
1,1-3,3-1,2-1,2-1,2	1	8.3
1,2-1,2-1,1-1,1-1,2	1	8.3
1,1-1,3-1,2-1,2-1,2	1	8.3
1,1-1,3-1,1-1,1-1,2	1	8.3
1,2-1,2-1,2-1,2-1,2	1	8.3
Total	12	99.7

This table shows the (AC)n-VNTR[46]-*AvaI-AluI-TaqI* combined genotype in the OFS≥4 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. The most common combined genotype was 1,2-1,2-1,2-1,2-1,1 that occurred at a frequency of 16.7%.

Table A2.7J: Frequency of (AC)n-VNTR^{46}-*AvaI-AluI-TaqI* combined genotypes in the alcoholic group of patients

(AC)n-VNTR^{46}-*AvaI-AluI-TaqI*	Number (n)	Frequency (%)
2,4-1,2-1,2-1,2-1,1	3	16.7
1,2-1,2-1,2-1,2-1,2	2	11.1
3,4-1,1-1,1-1,1-1,1	2	11.1
2,4-2,2-1,1-1,2-1,1	1	5.6
1,1-1,3-1,2-1,2-1,2	1	5.6
1,1-1,3-1,2-1,2-1,1	1	5.6
1,1-1,3-1,1-1,1-1,2	1	5.6
2,4-1,1-1,2-1,2-1,1	1	5.6
1,4-1,3-1,2-1,2-1,1	1	5.6
1,5-1,1-1,1-1,1-1,2	1	5.6
1,2-3,3-1,2-1,2-1,1	1	5.6
2,4-1,2-1,1-1,1-1,1	1	5.6
4,5-1,1-1,1-1,1-1,1	1	5.6
1,2-1,1-1,2-1,2-1,1	1	5.6
Total	18	100.5

This table shows the (AC)n-VNTR^{46}-*AvaI-AluI-TaqI* combined genotype in the alcoholic group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. The most common combined genotype was 2,4-1,2-1,2-1,2-1,1 that occurred at a frequency of 16.7%.

Table A2.7K: Frequency of (AC)n-VNTR[46]-*AvaI-AluI-TaqI* combined genotypes in the idiopathic group of patients

(AC)n-VNTR[46]-*AvaI-AluI-TaqI*	Number (n)	Frequency (%)
1,2-1,2-1,2-1,2-1,2	3	9.4
1,2-1,2-1,1-1,1-1,2	3	9.4
1,2-1,2-1,2-1,2-1,1	2	6.3
1,4-1,1-1,1-1,1-1,2	2	6.3
Y - Y - Y - Y - Y	22	68.8
Total	32	100.2

This table shows the (AC)n-VNTR[46]-*AvaI-AluI-TaqI* combined genotype in the idiopathic group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotypes were 1,2-1,2-1,2-1,2-1,2 and 1,2-1,2-1,1-1,1-1,2 that each occurred at a frequency of 9.4%.

Table A2.7L: Frequency of (AC)n-VNTR[46]-*AvaI-AluI-TaqI* combined genotypes in the gallstone group of patients

(AC)n-VNTR[46]-*AvaI-AluI-TaqI*	Number (n)	Frequency (%)
1,2-1,2-1,2-1,2-1,1	4	6.3
1,4-1,1-1,1-1,1-1,2	4	6.3
1,2-2,3-1,2-1,2-1,1	4	6.3
Y - Y - Y - Y - Y	51	81.0
Total	63	99.9

This table shows the (AC)n-VNTR[46]-*AvaI-AluI-TaqI* combined genotype in the gallstone group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotypes were 1,2-1,2-1,2-1,2-1,1, 1,4-1,1-1,1-1,1-1,2 and 1,2-2,3-1,2-1,2-1,1 that each occurred at a frequency of 6.3%.

Table A2.8A: Frequency of (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the control population

(AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
1,4-1,1-1,1-2,2-1,1-1,1	8	8.5
2,4-1,2-1,1-2,2-1,1-1,1	5	5.3
Y - Y - Y - Y - Y - Y	81	86.2
Total	94	100.0

This table shows the (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the control population. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,4-1,1-1,1-2,2-1,1-1,1 that occurred at a frequency of 8.5%.

Table A2.8B: Frequency of (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the patient population

(AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
1,2-1,2-1,2-2,3-1,2-1,2	10	8.7
1,2-1,2-1,1-2,2-1,1-1,1	10	8.7
1,1-1,3-1,1-2,2-1,1-1,1	7	6.1
2,4-1,2-1,2-2,3-1,2-1,2	7	6.1
1,4-1,1-1,1-2,2-1,1-1,1	7	6.1
Y - Y - Y - Y - Y - Y	74	64.3
Total	115	100.0

This table shows the (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the patient population. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotypes were 1,2-1,2-1,2-2,3-1,2-1,2 and 1,2-1,2-1,1-2,2-1,1-1,1 that each occurred at a frequency of 8.7%.

Table A2.8C: Frequency of (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the mild group of patients

(AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
2,4-1,2-1,2-2,3-1,2-1,2	7	8.9
1,2-1,2-1,2-2,3-1,2-1,2	6	7.6
1,4-1,1-1,1-2,2-1,1-1,1	6	7.6
1,2-1,2-1,1-2,2-1,1-1,1	5	6.3
1,1-1,3-1,1-2,2-1,1-1,1	4	5.1
1,2-2,3-1,2-2,3-1,2-1,2	4	5.1
Y - Y - Y - Y - Y - Y	47	59.5
Total	79	100.1

This table shows the (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the mild group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 2,4-1,2-1,2-2,3-1,2-1,2 that occurred at a frequency of 8.9%.

Table A2.8D: Frequency of (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the severe group of patients

(AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
1,2-1,2-1,1-2,2-1,1-1,1	5	13.9
1,2-1,2-1,2-2,3-1,2-1,2	4	11.1
1,2-2,3-1,1-2,2-1,1-1,1	3	8.3
1,1-1,3-1,1-2,2-1,1-1,1	3	8.3
2,2-2,3-1,1-2,2-1,1-1,1	2	5.6
1,1-1,1-1,1-2,2-1,1-1,1	2	5.6
Y - Y - Y - Y - Y - Y	17	47.2
Total	36	100.0

This table shows the (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the severe group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2-1,1-2,2-1,1-1,1 that occurred at a frequency of 13.9%.

Table A2.8E: Frequency of (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the OFS=0 group of patients

(AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
2,4-1,2-1,2-2,3-1,2-1,2	6	8.7
1,2-1,2-1,2-2,3-1,2-1,2	5	7.2
1,1-1,3-1,1-2,2-1,1-1,1	5	7.2
1,4-1,1-1,1-2,2-1,1-1,1	5	7.2
1,2-2,3-1,2-2,3-1,2-1,2	4	5.8
Y - Y - Y - Y - Y - Y	44	63.8
Total	69	99.9

This table shows the (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the OFS=0 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 2,4-1,2-1,2-2,3-1,2-1,2 that occurred at a frequency of 8.7%.

Table A2.8F: Frequency of (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the OFS≥1 group of patients

(AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
1,2-1,2-1,1-2,2-1,1-1,1	9	19.6
1,2-1,2-1,2-2,3-1,2-1,2	5	10.9
Y - Y - Y - Y - Y- Y	32	69.6
Total	46	100.1

This table shows the (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the OFS≥1 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2-1,1-2,2-1,1-1,1 that occurred at a frequency of 19.6%.

Table A2.8G: Frequency of (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the OFS≥2 group of patients

(AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
1,2-1,2-1,1-2,2-1,1-1,1	7	21.2
1,2-1,2-1,2-2,3-1,2-1,2	4	12.1
2,2-1,2-1,1-2,2-1,1-1,1	2	6.1
1,5-1,3-1,2-2,3-1,2-1,2	2	6.1
1,2-2,3-1,1-2,2-1,1-1,1	2	6.1
1,1-1,3-1,1-2,2-1,1-1,1	2	6.1
Y - Y - Y - Y - Y - Y	14	42.4
Total	33	100.1

This table shows the (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the OFS≥2 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2-1,1-2,2-1,1-1,1 that occurred at a frequency of 21.2%.

Table A2.8H: Frequency of (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the OFS≥3 group of patients

(AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
1,2-1,2-1,2-2,3-1,2-1,2	3	15.8
1,2-1,2-1,1-2,2-1,1-1,1	3	15.8
1,2-2,3-1,1-2,2-1,1-1,1	2	10.5
1,1-1,3-1,1-2,2-1,1-1,1	2	10.5
2,2-2,3-1,1-2,2-1,1-1,1	1	5.3
4,6-1,1-1,1-2,2-1,1-1,1	1	5.3
1,5-1,3-1,2-2,3-1,2-1,2	1	5.3
1,1-1,1-1,1-2,2-1,1-1,1	1	5.3
2,2-6,6-2,2-3,3-2,2-2,2	1	5.3
1,4-1,1-1,1-2,2-1,1-1,1	1	5.3
1,5-1,1-1,2-2,3-1,2-1,2	1	5.3
1,1-3,3-1,1-2,2-1,1-1,1	1	5.3
2,4-1,1-1,1-2,2-1,1-1,1	1	5.3
Total	19	100.3

This table shows the (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the OFS≥3 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. The most common combined genotypes were 1,2-1,2-1,2-2,3-1,2-1,2 and 1,2-1,2-1,1-2,2-1,1-1,1 that both occurred at a frequency of 15.8%.

Table A2.8I: Frequency of (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the OFS≥4 group of patients

(AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI*	Number(n)	Frequency(%)
1,2-1,2-1,2-2,3-1,2-1,2	3	25.0
1,1-1,3-1,1-2,2-1,1-1,1	2	16.7
2,2-2,3-1,1-2,2-1,1-1,1	1	8.3
4,6-1,1-1,1-2,2-1,1-1,1	1	8.3
1,2-2,3-1,1-2,2-1,1-1,1	1	8.3
1,4-1,1-1,1-2,2-1,1-1,1	1	8.3
1,5-1,1-1,2-2,3-1,2-1,2	1	8.3
1,1-3,3-1,1-2,2-1,1-1,1	1	8.3
1,2-1,2-1,1-2,2-1,1-1,1	1	8.3
Total	12	99.8

This table shows the (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the OFS≥4 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. The most common combined genotype was 1,2-1,2-1,2-2,3-1,2-1,2 that occurred at a frequency of 25.0%.

Table A2.8J: Frequency of (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the alcoholic group of patients

(AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
2,4-1,2-1,2-2,3-1,2-1,2	4	22.2
1,1-1,3-1,1-2,2-1,1-1,1	3	16.7
2,4-2,2-1,1-2,2-1,1-1,1	1	5.6
1,2-1,2-1,1-2,2-1,1-1,1	1	5.6
2,4-1,1-1,1-2,2-1,1-1,1	1	5.6
1,4-1,3-1,1-2,2-1,1-1,1	1	5.6
1,5-1,1-1,2-2,3-1,2-1,2	1	5.6
1,2-3,3-1,2-1,3-2,2-1,2	1	5.6
4,5-1,1-2,2-3,3-2,2-2,2	1	5.6
1,2-1,1-1,2-2,3-1,2-1,2	1	5.6
3,4-1,1-1,1-2,2-1,1-1,1	1	5.6
3,4-1,1-1,2-2,3-1,2-1,2	1	5.6
1,2-1,2-1,2-2,3-1,2-1,2	1	5.6
Total	18	100.5

This table shows the (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the alcoholic group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. The most common combined genotype was 2,4-1,2-1,2-2,3-1,2-1,2 that occurred at a frequency of 22.2%.

Table A2.8K: Frequency of (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the idiopathic group of patients

(AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
1,2-1,2-1,1-2,2-1,1-1,1	5	15.2
1,2-1,2-1,2-2,3-1,2-1,2	3	9.1
2,2-2,3-1,1-2,2-1,1-1,1	3	9.1
1,2-2,3-1,1-2,2-1,1-1,1	2	6.1
1,4-1,1-1,1-2,2-1,1-1,1	2	6.1
Y - Y - Y - Y - Y - Y	18	54.5
Total	33	100.1

This table shows the (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the idiopathic group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2-1,1-2,2-1,1-1,1 that occurred at a frequency of 15.2%.

Table A2.8L: Frequency of (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the gallstone group of patients

(AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
1,2-1,2-1,2-2,3-1,2-1,2	6	9.4
1,4-1,1-1,1-2,2-1,1-1,1	5	7.8
1,2-1,2-1,1-2,2-1,1-1,1	4	6.3
1,2-2,3-1,2-2,3-1,2-1,2	4	6.3
Y - Y - Y - Y - Y - Y	45	70.3
Total	64	100.1

This table shows the (AC)n-VNTR^{46}-*MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the gallstone group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2-1,2-2,3-1,2-1,2 that occurred at a frequency of 9.4%.

Table A2.9A: Frequency of *AvaI-AluI-TaqI-MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the control population

AvaI-AluI-TaqI-MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
1,1-1,1-1,1-1,1-2,2-1,1-1,1	10	12.5
1,1-1,1-1,2-1,1-2,2-1,1-1,1	9	11.3
2,2-2,2-1,1-1,1-2,2-1,1-1,1	8	10.0
2,2-2,2-1,1-1,2-2,3-1,2-1,2	7	8.8
1,2-1,2-1,1-1,2-2,3-1,2-1,2	6	7.5
1,2-1,2-1,2-1,2-2,3-1,2-1,2	6	7.5
Y - Y - Y - Y - Y - Y - Y	34	42.5
Total	80	100.1

This table shows the *AvaI-AluI-TaqI-MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the control population. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,1-1,1-1,1-1,1-2,2-1,1-1,1 that occurred at a frequency of 12.5%.

Table A2.9B: Frequency of *AvaI-AluI-TaqI-MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the patient population

AvaI-AluI-TaqI-MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
1,2-1,2-1,1-1,2-2,3-1,2-1,2	19	16.8
1,1-1,1-1,2-1,1-2,2-1,1-1,1	18	15.9
1,1-1,1-1,1-1,1-2,2-1,1-1,1	14	12.4
1,2-1,2-1,1-1,1-2,2-1,1-1,1	13	11.5
1,2-1,2-1,2-1,1-2,2-1,1-1,1	10	8.8
1,2-1,2-1,2-1,2-2,3-1,2-1,2	7	6.2
1,1-1,1-1,1-1,2-2,3-1,2-1,2	7	6.2
Y - Y - Y - Y - Y - Y - Y	25	22.1
Total	113	99.9

This table shows the *AvaI-AluI-TaqI-MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the patient population. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2-1,1-1,2-2,3-1,2-1,2 that occurred at a frequency of 16.8%.

Table A2.9C: Frequency of *AvaI-AluI-TaqI-MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the mild group of patients

AvaI-AluI-TaqI-MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
1,2-1,2-1,1-1,2-2,3-1,2-1,2	13	16.7
1,1-1,1-1,2-1,1-2,2-1,1-1,1	11	14.1
1,1-1,1-1,1-1,1-2,2-1,1-1,1	8	10.3
1,2-1,2-1,1-1,1-2,2-1,1-1,1	8	10.3
1,1-1,1-1,1-1,2-2,3-1,2-1,2	7	9.0
1,2-1,2-1,2-1,1-2,2-1,1-1,1	6	7.7
1,2-1,2-1,2-1,2-2,3-1,2-1,2	5	6.4
Y - Y - Y - Y - Y - Y - Y	20	25.6
Total	78	100.1

This table shows the *AvaI-AluI-TaqI-MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the mild group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2-1,1-1,2-2,3-1,2-1,2 that occurred at a frequency of 16.7%.

Table A2.9D: Frequency of *AvaI-AluI-TaqI-MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the severe group of patients

AvaI-AluI-TaqI-MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
1,1-1,1-1,2-1,1-2,2-1,1-1,1	7	20.0
1,2-1,2-1,1-1,2-2,3-1,2-1,2	6	17.1
1,2-1,2-1,1-1,1-2,2-1,1-1,1	5	14.3
1,1-1,1-1,1-1,1-2,2-1,1-1,1	5	14.3
1,2-1,2-1,2-1,1-2,2-1,1-1,1	4	11.4
1,2-1,2-1,2-1,2-2,3-1,2-1,2	2	5.7
Y - Y - Y - Y - Y - Y - Y	6	17.1
Total	35	99.9

This table shows the *AvaI-AluI-TaqI-MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the severe group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,1-1,1-1,2-1,1-2,2-1,1-1,1 that occurred at a frequency of 20.0%.

Table A2.9E: Frequency of *AvaI-AluI-TaqI-MspI*-VLP-VNTR[86]-*SspI* combined genotypes in the OFS=0 group of patients

AvaI-AluI-TaqI-MspI-VLP-VNTR[86]-*SspI*	Number (n)	Frequency (%)
1,2-1,2-1,1-1,2-2,3-1,2-1,2	10	14.7
1,1-1,1-1,1-1,1-2,2-1,1-1,1	10	14.7
1,1-1,1-1,2-1,1-2,2-1,1-1,1	7	10.3
1,2-1,2-1,1-1,1-2,2-1,1-1,1	7	10.3
1,1-1,1-1,1-1,2-2,3-1,2-1,2	7	10.3
1,2-1,2-1,2-1,2-2,3-1,2-1,2	5	7.4
1,2-1,2-1,2-1,1-2,2-1,1-1,1	4	5.9
Y - Y - Y - Y - Y - Y - Y	18	26.5
Total	68	100.1

This table shows the *AvaI-AluI-TaqI-MspI*-VLP-VNTR[86]-*SspI* combined genotype in the OFS=0 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotypes were 1,2-1,2-1,1-1,2-2,3-1,2-1,2 and 1,1-1,1-1,1-1,1-2,2-1,1-1,1 that each occurred at a frequency of 14.7%.

Table A2.9F: Frequency of *AvaI-AluI-TaqI-MspI*-VLP-VNTR[86]-*SspI* combined genotypes in the OFS≥1 group of patients

AvaI-AluI-TaqI-MspI-VLP-VNTR[86]-*SspI*	Number (n)	Frequency (%)
1,1-1,1-1,2-1,1-2,2-1,1-1,1	11	24.4
1,2-1,2-1,1-1,2-2,3-1,2-1,2	9	20.0
1,2-1,2-1,2-1,1-2,2-1,1-1,1	6	13.3
1,2-1,2-1,1-1,1-2,2-1,1-1,1	6	13.3
1,1-1,1-1,1-1,1-2,2-1,1-1,1	4	8.9
Y - Y - Y - Y - Y - Y - Y	9	20.0
Total	45	99.9

This table shows the *AvaI-AluI-TaqI-MspI*-VLP-VNTR[86]-*SspI* combined genotype in the OFS≥1 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,1-1,1-1,2-1,1-2,2-1,1-1,1 that occurred at a frequency of 24.4%.

Table A2.9G: Frequency of *AvaI-AluI-TaqI-MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the OFS≥2 group of patients

AvaI-AluI-TaqI-MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
1,1-1,1-1,2-1,1-2,2-1,1-1,1	7	21.9
1,2-1,2-1,1-1,2-2,3-1,2-1,2	6	18.8
1,2-1,2-1,2-1,1-2,2-1,1-1,1	6	18.8
1,1-1,1-1,1-1,1-2,2-1,1-1,1	4	12.5
1,2-1,2-1,1-1,1-2,2-1,1-1,1	3	9.4
1,2-1,2-1,2-1,2-2,3-1,2-1,2	2	6.3
Y - Y - Y - Y - Y - Y - Y	4	12.5
Total	32	100.2

This table shows the *AvaI-AluI-TaqI-MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the OFS≥2 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,1-1,1-1,2-1,1-2,2-1,1-1,1 that occurred at a frequency of 21.9%.

Table A2.9H: Frequency of *AvaI-AluI-TaqI-MspI*-VLP-VNTR[86]-*SspI* combined genotypes in the OFS≥3 group of patients

AvaI-AluI-TaqI-MspI-VLP-VNTR[86]-*SspI*	Number (n)	Frequency (%)
1,2-1,2-1,2-1,1-2,2-1,1-1,1	4	22.2
1,2-1,2-1,1-1,1-2,2-1,1-1,1	3	16.7
1,2-1,2-1,1-1,2-2,3-1,2-1,2	3	16.7
1,1-1,1-1,2-1,1-2,2-1,1-1,1	3	16.7
1,1-1,1-1,1-1,1-2,2-1,1-1,1	2	11.1
2,2-2,2-1,1-2,2-3,3-2,2-2,2	1	5.6
1,1-1,1-1,2-1,2-2,3-1,2-1,2	1	5.6
1,2-1,2-1,2-1,2-2,3-1,2-1,2	1	5.6
Total	18	100.2

This table shows the *AvaI-AluI-TaqI-MspI*-VLP-VNTR[86]-*SspI* combined genotype in the OFS≥3 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. The most common combined genotype was 1,2-1,2-1,2-1,1-2,2-1,1-1,1 that occurred at a frequency of 22.2%.

Table A2.9I: Frequency of *AvaI-AluI-TaqI-MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the OFS≥4 group of patients

AvaI-AluI-TaqI-MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
1,1-1,1-1,2-1,1-2,2-1,1-1,1	3	25.0
1,2-1,2-1,1-1,1-2-2-1,1-1,1	2	16.7
1,2-1,2-1,1-1,2-2,3-1,2-1,2	2	16.7
1,2-1,2-1,2-1,1-2,2-1,1-1,1	2	16.7
1,1-1,1-1,1-1,1-2,2-1,1-1,1	1	8.3
1,1-1,1-1,2-1,2-2,3-1,2-1,2	1	8.3
1,2-1,2-1,2-1,2-2,3-1,2-1,2	1	8.3
Total	12	100.0

This table shows the *AvaI-AluI-TaqI-MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the OFS≥4 group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. The most common combined genotype was 1,1-1,1-1,2-1,1-2,2-1,1-1,1 that occurred at a frequency of 25.0%.

Table A2.9J: Frequency of *AvaI-AluI-TaqI-MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the alcoholic group of patients

AvaI-AluI-TaqI-MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
1,2-1,2-1,1-1,2-2,3-1,2-1,2	4	22.2
1,2-1,2-1,1-1,1-2,2-1,1-1,1	3	16.7
1,2-1,2-1,2-1,1-2,2-1,1-1,1	2	11.1
1,1-1,1-1,1-1,2-2,3-1,2-1,2	2	11.1
1,1-1,2-1,1-1,1-2,2-1,1-1,1	1	5.6
1,1-1,1-1,2-1,1-2,2-1,1-1,1	1	5.6
1,1-1,1-1,2-1,2-2,3-1,2-1,2	1	5.6
1,2-1,2-1,1-1,2-1,3-2,2-1,2	1	5.6
1,1-1,1-1,1-2,2-3,3-2,2-2,2	1	5.6
1,1-1,1-1,1-1,1-2,2-1,1-1,1	1	5.6
1,2-1,2-1,2-1,2-2,3-1,2-1,2	1	5.6
Total	18	100.3

This table shows the *AvaI-AluI-TaqI-MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the alcoholic group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. The most common combined genotype was 1,2-1,2-1,1-1,2-2,3-1,2-1,2 that occurred at a frequency of 22.2%.

Table A2.9K: Frequency of *AvaI-AluI-TaqI-MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the idiopathic group of patients

AvaI-AluI-TaqI-MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
1,1-1,1-1,2-1,1-2,2-1,1-1,1	8	25.0
1,2-1,2-1,1-1,1-2,2-1,1-1,1	5	15.6
1,1-1,1-1,1-1,1-2,2-1,1-1,1	5	15.6
1,2-1,2-1,1-1,2-2,3-1,2-1,2	3	9.4
1,2-1,2-1,2-1,2-2,3-1,2-1,2	2	6.3
1,2-1,2-1,2-1,1-2,2-1,1-1,1	2	6.3
Y - Y - Y - Y - Y - Y - Y	7	21.9
Total	32	100.1

This table shows the *AvaI-AluI-TaqI-MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the idiopathic group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,1-1,1-1,2-1,1-2,2-1,1-1,1 that occurred at a frequency of 25.0%.

Table A2.9L: Frequency of *AvaI-AluI-TaqI-MspI*-VLP-VNTR^{86}-*SspI* combined genotypes in the gallstone group of patients

AvaI-AluI-TaqI-MspI-VLP-VNTR^{86}-*SspI*	Number (n)	Frequency (%)
1,2-1,2-1,1-1,2-2,3-1,2-1,2	12	19.0
1,1-1,1-1,2-1,1-2,2-1,1-1,1	9	14.3
1,1-1,1-1,1-1,1-1,2-2,2-1,1-1,1	8	12.7
1,2-1,2-1,2-1,1-2,2-1,1-1,1	6	9.5
1,1-1,1-1,1-1,2-2,3-1,2-1,2	5	7.9
1,2-1,2-1,1-2,2-3,3-2,2-2,2	4	6.3
1,2-1,2-1,2-1,2-2,3-1,2-1,2	4	6.3
1,2-1,2-1,1-1,1-2,2-1,1-1,1	4	6.3
2,2-2,2-1,1-2,2-3,3-2,2-2,2	4	6.3
Y - Y- Y -Y -Y -Y -Y	7	11.1
Total	63	99.7

This table shows the *AvaI-AluI-TaqI-MspI*-VLP-VNTR^{86}-*SspI* combined genotype in the gallstone group of patients. n represents the number of subjects with each genotype combination. The most common combined genotype is shown at the top of the table and the least common is shown at the bottom. Y-Y-Y-Y-Y-Y-Y represents the sum total of the combined genotypes that occurred at a frequency of less than 5%. The most common combined genotype was 1,2-1,2-1,1-1,2-2,3-1,2-1,2 that occurred at a frequency of 19.0%.

Summary of the combined genotype results

(AC)n-VNTR[46] combined genotype

The most common combined genotype in controls was 1,4-1,1 that occurred with a frequency of 12.7%. The most common combined genotype in the total patients, mild, severe, OFS≥1, OFS≥2, OFS≥3, OFS≥4, idiopathic and gallstone groups was 1,2-1,2 that occurred at frequencies of 16.9%, 15.2%, 25.0%, 29.8%, 33.3%, 31.6%, 33.3%, 24.2% and 17.2% respectively. The most common combined genotype in the OFS=0 and alcoholic groups was 2,4-1,2 that occurred at frequencies of 11.2% and 22.2% respectively. Interestingly, the 2,4-1,2 combined genotype was the most common in the OFS=0 group, but was absent in OFS≥1,2,3,4 groups, while the 1,2-1,2 combined genotype was increased in these groups compared to the OFS=0 group.

Ava I-Alu I-TaqI combined genotype

The most common combined genotype in controls was 1,1-1,1-1,1 that occurred at a frequency of 25.6%. The most common combined genotype in the total patient, mild, severe, OFS=0, OFS≥1, OFS≥2, OFS≥3, OFS≥4, alcoholic, idiopathic and gallstone groups was 1,2-1,2-1,1 that occurred at frequencies of 34.5%, 34.6%, 34.3%, 32.4%, 37.8%, 31.3%, 33.3%, 33.3%, 44.4%, 31.3% and 33.3% respectively. The 1,1-1,1-1,2 combined genotype was equally most common in the OFS≥4 group occurring at a frequency of 33.3%.

MspI-VLP-VNTR[86]-SspI combined genotype

The most common combined genotype in controls was 1,1-2,2-1,1-1,1 occurring at a frequency of 42.2%. This was also the most common combined genotype in the total patient, mild, severe, OFS=0, OFS≥1, OFS≥2, OFS≥3, OFS≥4, alcoholic, idiopathic and gallstone groups occurring at frequencies of 53.9%, 46.8%, 69.4%, 49.3%, 60.9%, 63.6%, 68.4%, 66.7%, 44.4%, 66.7% and 50.0% respectively. The 1,2-2,3-1,2-1,2 combined

genotype was equally most common in the alcoholic group occurring at a frequency of 44.4%.

PstI-(AC)n-VNTR[46] combined genotype

The most common combined genotypes in the controls were 1,2-1,4-1,1, 2,2-1,2-1,2 and 2,2-1,4-1,1 that occurred at a frequency of 6.7%. The most common combined genotype in the total patient, severe, OFS≥1, OFS≥2, OFS≥3, OFS≥4, idiopathic and gallstone groups was 1,2-1,2-1,2 that occurred at frequencies of 8.8%, 13.9%, 17.4%, 18.2%, 26.3%, 25.0%, 15.2% and 11.1% respectively. The 2,2-1,2-1,2 combination was equally most common in the total patient group occurring at a frequency of 8.8%, while the 2,2-1,2-2,3 combined genotype was equally most common in the gallstone group occurring at a frequency of 11.1%. The most common combined genotype in the mild, OFS=0 and alcoholic groups was 2,2-2,4-1,2 that occurred at frequencies of 9.0%, 9.1% and 16.7% respectively. Interestingly, the 2,2-2,4-1,2 combined genotype was the most common in the OFS=0 group, but was absent in OFS≥1,2,3,4 groups.

PstI-AvaI-AluI-TaqI combined genotype

The most common combined genotypes in controls were 2,2-2,2-2,2-1,1 and 2,2-1,1-1,1-1,1 occurring at a frequency of 14.1%. The most common combined genotypes in the total patient, mild, severe, OFS=0, OFS≥1, OFS≥2,OFS≥3, alcoholic, idiopathic and gallstone groups was 2,2-1,2-1,2-1,1 that occurred at frequencies of 22.3%, 23.4%, 22.9%, 20.9%, 24.4%, 21.9%, 27.8%, 33.3%, 15.6% and 22.6% respectively. The 2,2-1,1-1,1-1,2 combination was equally most common in the OFS≥2 group occurring at a frequency of 21.9%, while the 2,2-1,1-1,1-1,2 combination was equally most common in the idiopathic group occurring at 15.6%. The most common combined genotype in the OFS≥4 group was 2,2-1,1-1,1-1,2 that occurred at a frequency of 25.0%.

PstI-MspI-VLP-VNTR^{86}-SspI combined genotype

The most common combined genotype in controls was 2,2-1,1-2,2-1,1-1,1 that occurred at a frequency of 21.3%. The most common combined genotype in the total patient, mild, severe, OFS=0, OFS≥1, OFS≥2, OFS≥3, OFS≥4, idiopathic and gallstone groups was 2,2-1,1-2,2-1,1-1,1 occurring at a frequencies of 34.2%, 29.5%, 47.2%, 32.4%, 41.3%, 45.5%, 47.4%, 58.3%, 39.4% and 38.1% respectively. The most common combined genotype in alcoholics was 2,2-1,2-2,3-1,2-1,2 that occurred at a frequency of 27.8%.

(AC)n-VNTR^{46}-AvaI-AluI-TaqI combined genotype

The most common combined genotype in controls was 1,4-1,1-1,1-1,1-1,2 that occurred at a frequency of 6.3%. The most common combined genotype in the total patient, mild, severe, OFS=0, OFS≥2, OFS≥3 and idiopathic groups was 1,2-1,2-1,2-1,2-1,2 that occurred at frequencies of 7.1%, 6.4%, 8.6%, 5.9%, 12.5%, 16.7% and 9.4% respectively. The 1,4-1,1-1,1-1,1-1,2 and 2,4-1,2-1,2-1,2-1,1 combinations were equally common in the mild group occurring at a frequency of 6.4%. The 1,2-1,2-1,2-1,2-1,1, 1,2-2,3-1,2-1,2-1,1 and 1,2-1,2-1,1-1,1-1,2 combinations were equally most common in the severe group occurring at a frequency of 8.6%. The 1,4-1,1-1,1-1,1-1,2 and 2,4-1,2-1,2-1,2-1,1 combinations were equally common in the OFS=0 group occurring at a frequency of 5.9%. The 1,2-1,2-1,1-1,1-1,2 combination was equally as common in the idiopathic group occurring at a frequency of 9.4%. The most common combined genotype in OFS≥1 group was 1,2-1,2-1,1-1,1-1,2 that occurred at a frequency of 11.1%. The most common combined genotype in the OFS≥4 group was 1,2-1,2-1,2-1,2-1,1 that occurred at a frequency of 16.7%. The most common combined genotype in the alcoholic group was 2,4-1,2-1,2-1,2-1,1 that occurred at a frequency of 16.7%. The most common combined genotypes in the gallstone group were 1,2-1,2-1,2-1,2-1,1, 1,4-1,1-1,1-1,1-1,2 and 1,2-2,3-1,2-1,2-1,1 that occurred at a frequency of 6.3%. Interestingly, the 2,4-1,2-1,2-1,2-1,1

combined genotype was the most common in the OFS=0 group, but was absent in OFS≥1,2,3,4 groups.

(AC)n-VNTR^{46}-MspI-VLP-VNTR^{86}-SspI combined genotype

The most common combined genotype in the controls was 1,4-1,1-1,1-2,2-1,1-1,1 that occurred at a frequency of 8.5%. The most common combined genotype in the total patient, OFS≥3, OFS≥4 and gallstone group was 1,2-1,2-1,2-2,3-1,2-1,2 that occurred at frequencies of 8.7%, 15.8%, 25.0% and 9.4% respectively. The 1,2-1,2-1,1-2,2-1,1-1,1 combination was equally common in the total patient group occurring at a frequency of 8.7%, while the 1,2-1,2-1,1-2,2-1,1-1,1 combination was equally most common in the OFS≥3 group occurring at a frequency of 15.8%. The most common combined genotype in the mild, OFS=0 and alcoholic groups was 2,4-1,2-1,2-2,3-1,2-1,2 that occurred at frequencies of 8.9%, 8.7% and 22.2% respectively. The most common combined genotype in the severe, OFS≥1, OFS≥2 and idiopathic groups was 1,2-1,2-1,1-2,2-1,1-1,1 that occurred at frequencies of 13.9%, 19.6%, 21.2% and 15.2% respectively. Interestingly, the 2,4-1,2-1,2-2,3-1,2-1,2 combined genotype was the most common in the OFS=0 group, but was absent in OFS≥1,2,3,4 groups.

AvaI-AluI-TaqI-MspI-VLP-VNTR^{86}-SspI combined genotype

The most common combined genotype in controls was 1,1-1,1-1,1-1,1-2,2-1,1-1,1 that occurred at a frequency of 12.5%. The most common combined genotype in the total patient, mild, OFS=0, alcoholic and gallstone groups was 1,2-1,2-1,1-1,2-2,3-1,2-1,2 that occurred at frequencies of 16.8%, 16.7%, 14.7%, 22.2% and 19.0% respectively. The 1,1-1,1-1,1-1,1-2,2-1,1-1,1 combination was equally common in the OFS=0 group occurring at a frequency of 14.7%. The most common combined genotype in the severe, OFS≥1, OFS≥2, OFS≥4 and idiopathic groups was 1,1-1,1-1,2-1,1-2,2-1,1-1,1 that occurred at frequencies of 20.0%, 24.4%, 21.9%, 25.0% and 25.0% respectively. The most common

combined genotype in the OFS≥3 group was 1,2-1,2-1,2-1,1-2,2-1,1-1,1 that occurred at a frequency of 22.2%.

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Investigation of the Interleukin 1 Gene Cluster and Its Association with Acute Pancreatitis

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Summary: The interleukin 1 (IL-1) gene cluster has been implicated in acute pancreatitis. Penta-allelic and bi-allelic polymorphisms exist in the IL-1RN and IL-1B genes, respectively. The aim of the study was to investigate these polymorphisms in acute pancreatitis. Genotype and allele frequencies were determined in patients (n = 116) and healthy controls (n = 170) using the polymerase chain reaction. PCR products from the IL-1B study were further digested with Taq I restriction endonuclease. Patients were categorised according to aetiology, severity, and organ-failure scores. Allele 1 of the IL-1RN polymorphism was significantly increased in patients compared with controls (72.0 vs. 63.0%; p = 0.029, Pc = 0.029), in severe cases compared with controls (81.9 vs. 63.0%; p = 0.002, Pc = 0.004), in idiopathics compared with controls (82.4 vs. 63.0%; p = 0.002, Pc = 0.006), and in severe cases compared with mild cases (81.9 vs. 67.5%; p = 0.023, Pc = 0.046). Allele 2 was significantly decreased in severe cases compared with controls (18.1 vs. 33.0%; p = 0.013, Pc = 0.026), in idiopathics compared with controls (17.6 vs. 33%; p = 0.013, Pc = 0.039), and in severe cases compared with mild cases (18.1 vs. 32.5%; p = 0.023, Pc = 0.046). No significant differences were found for the Taq I allele or genotype frequencies between controls and patients/subgroups of patients. IL-1RN appears to determine severity of acute pancreatitis and susceptibility to idiopathic acute pancreatitis. No association was found between IL-1B and the disease. **Key Words:** Interleukin-1 receptor antagonist (IL-1ra)—Interleukin 1β (IL-1β)—Polymorphism—Acute pancreatitis.

Acute pancreatitis is a relatively common disease with an annual incidence of 10–20 cases per 100,000 population in the Western world (1). The majority of cases are alcohol related or due to gallstones. Whatever the cause, the inflammatory process is triggered by ectopic intrapancreatic protease activation, and the disease then progresses in three phases: local inflammation of the pancreas, a systemic inflammatory response that can result in an organ or multiple organ failure, and finally the intervention of infection by translocation of bacteria from the gut (2). The local inflammatory response is amplified by changes in the microcirculation to increase permeability, which together with activation of leukocytes leads to migration of polymorphonuclear granulocytes into the interstitium of the pancreas. A number of cytokines are known to regulate this local inflammatory response, the most prominent being tumour necrosis factor alpha (TNFα), interleukin 1 (IL-1), and interleukin 10 (IL-10) (3,4). If there is an upregulated and inappropriate response to the initial injury, then the systemic inflammatory response syndrome (SIRS) will supervene. The systemic manifestations are responsible for the majority of pancreatitis-associated morbidity and mortality and are due to the actions of specific inflammatory cytokines. It has been suggested that the clinical course of an acute inflammatory illness such as acute pancreatitis may have a genetic basis because certain genetic cytokine polymorphisms may operate functional differences and hence the outcome of the inflammatory process (5).

IL-1 and IL-1-receptor antagonist (IL-1ra) are members of the IL-1 gene cluster, which has been mapped to a 430-kb stretch of DNA on the long arm of human chromosome 2 (2q13-q21) (6,7). IL-1 has two forms, IL-1α (encoded by IL-1A) and IL-1β (encoded by IL-1B), which share a wide variety of biologic activities that are both immunologic (e.g., regulating proliferation of T and B cells) and nonimmunologic (e.g., regulating collagen synthesis in fibroblasts) (3). IL-1ra (encoded by IL-1RN) also has two forms: a secretory form (sIL-1ra)

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produced by macrophages and an intracellular form (icIL-ra) produced by epithelial cells (8). IL-1ra competes with IL-1 for occupancy of the IL-1 cell-surface receptors, IL-1RtI and IL-1RtII, but fails to initiate an intracellular signal, thereby acting as a competitive inhibitor of the actions of IL-1 (9).

It has been suggested that an imbalance between IL-1 and IL-1ra protein levels predisposes an individual to disease (10,11). Evidence obtained from animal models of acute pancreatitis has shown that it is associated with elevated levels of IL-1, which correlate with the degree of pancreatic damage (12). In addition, blockade of the cytokine cascade at the level of the IL-1 receptor using IL-1ra, before or soon after induction of acute pancreatitis, inhibits the rise in the cytokines and is associated with decreased severity of pancreatitis and a reduction in pancreatic damage (13).

Imbalances in protein levels of IL-1 and IL-1ra have also been associated with endotoxemia/sepsis (14), Lyme arthritis (15), rheumatoid arthritis (16), acute myelogenous leukemia (17), Hodgkin's disease (18), and inflammatory bowel disease (11). Because the protein levels are determined by the genes that encode them, polymorphisms at the genetic level will result in a variation in the protein level, with an alteration in functional outcome.

The IL-1 genes are highly polymorphic. A bi-allelic polymorphism exists in the promoter region of the IL-1A gene and involves a C to T substitution. This polymorphism has been associated with the severity of juvenile rheumatoid arthritis and its ocular complications (19). Similarly a bi-allelic polymorphism is known to exist in exon 5 of the IL-1B gene, which involves a C to T substitution and which completes a Taq I restriction site. This polymorphism has been associated with type I diabetes (20). In addition, a polymorphism has been characterised (21) in intron 2 of the IL-1RN gene in which there are a variable number of tandem repeat units (VNTR) corresponding to between two and six copies of an 86-base pair sequence. Associations have been found between this polymorphism and a variety of diseases. Increased carriage of allele 2, in particular, has been linked with the severity of type I psoriasis (22); systemic lupus erythematosus (SLE) (23); lichen sclerosus (24), alopecia areata (25), and with nephropathy in diabetes mellitus (26). Increased carriage of the same allele also has been associated with the susceptibility to certain diseases including ulcerative colitis (27–30), Graves' disease (31), and relapsing/remitting multiple sclerosis (32).

The aim of this study was to investigate whether polymorphisms in the IL-1RN and IL-1B genes differentially affect the clinical course of acute pancreatitis.

MATERIALS AND METHODS

Subjects

After Ethical Committee approval, 10 mL of peripheral blood was collected from 170 unrelated healthy white individuals and from 116 acute pancreatitis patients admitted to Derriford Hospital in Plymouth (see Tables 1 and 2 for clinical characteristics). The patients were divided into groups according to aetiology (alcoholic/idiopathic/gallstone), severity (mild/severe), and organ-failure score groups (OFS $\geq$ 2, OFS $\geq$ 3, OFS $\geq$ 4) according to Kingsnorth et al. (33). High-molecular-weight DNA was prepared from all subjects using Nucleon DNA extraction kits (Scotlab, Coatbridge, U.K.). Two assays were carried out: (a) VNTR polymorphism of the IL-1RN gene, and (b) Taq I polymorphism of the IL-1B gene.

Assays

VNTR polymorphism of the IL-1RN gene

The variable number of tandem repeats region within intron 2 of the IL-1RN gene was amplified using the polymerase chain reaction (PCR) with the following primers: forward, 5'-CCC TCA GCA ACA CTC CTA TTG AC-3'; reverse, 5'-TCA TCT TCC TGG TCT GCA GGT AA-3' (MWG Biotechnology, Ebersberg, Germany).

TABLE 1. *Characteristics of controls and patients used in the IL-1RN gene VNTR polymorphism study*

	Normal controls (n = 150)[a]	AP patients (n = 116)[a]	Mild AP (n = 80)	Severe AP (n = 36)	OFS ≥2 (n = 33)	OFS ≥3 (n = 19)	OFS ≥4 (n = 12)	A AP (n = 18)	I AP (n = 34)	G AP (n = 64)
Age range (yr)	N/A	21–86	21–86	26–78	26–84	26–78	26–76	28–62	23–84	21–86
Av age (yr)	N/A	57	54	63	64	62	61	43	59	60
Sex (M/F)	77:73	53:63	37:43	16:20	16:17	11:8	7:5	17:1	17:17	19:45

AP, Acute pancreatitis, n, number of subjects in each group; M, male; F, female; mild and severe disease severity groups, according to the Atlanta convention classification; OFS, Organ-Failure Scores as defined by Kingsnorth et al., 1995 (33); A, alcoholic pancreatitis if daily consumption of ≥80 g alcohol per day for 6 months; G, gallstones if radiologic or ERCP evidence of cholelithiasis; I, idiopathic if no identifiable aetiology; N/A, age not applicable to controls as taken from a bank of cord bloods.

[a] Although the controls and patients used in the two studies were taken from the same populations, the numbers differ in each study because the two assays did not necessarily work for all subjects.

TABLE 2. *Characteristics of controls and patients used in the IL-1B gene Taq I polymorphism study*

	Normal controls (n = 170)[a]	AP patients (n = 114)[a]	Mild AP (n = 78)	Severe AP (n = 36)	OFS ≥2 (n = 33)	OFS ≥3 (n = 18)	OFS ≥4 (n = 12)	A AP (n = 18)	I AP (n = 32)	G AP (n = 64)
Age range (yr)	N/A	21–86	21–86	26–78	26–84	26–78	26–76	28–62	25–84	21–86
Av age (yr)	N/A	57	54	63	64	63	61	43	60	60
Sex (M/F)	79:91	52:62	36:42	16:20	16:17	11:7	7:5	17:1	17:15	18:46

AP, acute pancreatitis; n, number of subjects in each group; M, male; F, female; mild and severe disease severity groups, according to the Atlanta convention classification; OFS, Organ-Failure Scores as defined by Kingsnorth et al., 1995 (33); A, alcoholic pancreatitis if daily consumption of ≥80 g alcohol per day for 6 months; G, gallstones if radiologic or ERCP evidence of cholelithiasis; I, idiopathic if no identifiable aetiology; N/A, age not applicable to controls as taken from a bank of cord bloods.

[a] Although the controls and patients used in the two studies were taken from the same populations, the numbers differ in each study because the two assays did not necessarily work for all subjects.

PCR conditions consisted of 94°C for 4 minutes and then 30 cycles of 94°C for 30 seconds, 60°C for 2 minutes, and 72°C for 2 minutes. The PCR products were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and photographed to evaluate the presence or absence of the repeat polymorphism.

Taq I polymorphism of the IL-1B gene

The Taq I polymorphic site within exon 5 of the IL-1B gene was amplified using the PCR with the following primers: forward, 5′-GAC CTG AAG CTG GAA CCC ATG TC-3′; reverse, 5′-CGC TCC AGC ACT CTT GTT TCA GC-3′ (MWG Biotechnology).

PCR conditions consisted of 94°C for 4 minutes and then 30 cycles of 94°C for 30 seconds, 60°C for 2 minutes, and 72°C for 2 minutes. The PCR products were then digested with Taq I restriction endonuclease (Roche Diagnostic Ltd., Mannheim, Germany) at 65°C for 3 hours. The digestion products were analysed on a 1.5% agarose gel, stained with ethidium bromide, and photographed to determine whether the polymorphism was present.

Statistical analysis

The occurrence of each allele in the populations was expressed as a percentage of the total number of alleles present to give a frequency. The genotype frequency (i.e., the number of individuals carrying at least one copy of a specific allele as a proportion of the total number of individuals) also was calculated. The χ^2 test with contingency tables was performed to determine the p values, which were then corrected for the number of variables tested (Pc).

RESULTS

VNTR polymorphism of the IL-1RN gene

Only three of the five alleles reported by Tarlow et al. (21) were observed in this study: allele 1, allele 2, and allele 3, corresponding to four, two, and five copies, respectively, of the 86-bp sequence. Five genotypes were detected: 1,1; 1,2; 2,2; 1,3; and 2,3. The IL-1ra allelic and genotype frequencies are shown in Tables 3 and 4.

IL-1ra allelic frequencies

The frequency of allele 1 was significantly increased in total patients compared with total controls (72.0 vs. 63.0%; Pc = 0.029); however, the frequency of allele 3 was found to be significantly decreased between the two groups (0.0 vs. 4.0%; Pc = 0.002).

When analysing the patients according to severity,

TABLE 3. *IL-1ra allelic frequencies (%) in patients and controls*

Allele	Normal controls (n = 300)	AP patients (n = 232)	Mild AP (n = 160)	Severe AP (n = 72)	OFS ≥2 (n = 66)	OFS ≥3 (n = 38)	OFS ≥4 (n = 24)	Alcohol AP (n = 36)	Idiopath AP (n = 68)	G/stone AP (n = 128)
1	63.0	72.0[a]	67.5	81.9[b,d]	77.3	81.6	83.3	66.7	82.4[e]	68.0
	(189)	(167)	(108)	(59)	(51)	(31)	(20)	(24)	(56)	(87)
2	33.0	28.0	32.5	18.1[b]	22.7	18.4	16.7	33.3	17.6[f]	32.0
	(99)	(65)	(52)	(13)	(15)	(7)	(4)	(12)	(12)	(41)
3	4.0	0.0[c]	0.0[c]	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	(12)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)

n, number of alleles in each group

[a] p = 0.029, Pc = 0.029. [b] p = 0.023, Pc = 0.046; [c] p = 0.013, Pc = 0.026

[d] p = 0.002, Pc = 0.004 [e] p = 0.002, Pc = 0.006; [f] p = 0.013, Pc = 0.039

[g] p = 0.002, Pc = 0.002, [h] p = 0.01, Pc = 0.02

TABLE 4. *IL-1ra genotype frequencies (%) in patients and controls*

Genotype	Normal controls (n = 150)	AP patients (n = 116)	Mild AP (n = 80)	Severe AP (n = 36)	OFS ≥2 (n = 33)	OFS ≥3 (n = 19)	OFS ≥4 (n = 12)	Alcohol AP (n = 18)	Idiopath AP (n = 34)	G/stone AP (n = 64)
1,1	42.0	54.3[a]	47.5	69.4[c]	63.6	68.4	66.7	44.4	67.6[e]	50.0
	(63)	(63)	(38)	(25)	(21)	(13)	(8)	(8)	(23)	(32)
1,2	38.0	35.3	40.0	25.0	27.3	26.3	33.3	44.4	29.4	35.9
	(57)	(41)	(32)	(9)	(9)	(5)	(4)	(8)	(10)	(23)
2,2	12.0	10.3[b]	12.5	5.6	9.1	5.3	0.0	11.1	2.9[f]	14.1
	(18)	(12)	(10)	(2)	(3)	(1)	(0)	(2)	(1)	(9)
1,3	4.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	(6)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
2,3	4.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	(6)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)

n, number of genotypes in each group.
[a] p = 0.046, Pc = 0.046; [b] p = 0.007, Pc = 0.021; [c] p = 0.003, Pc = 0.006.
[d] (2,2 + 1,3 + 2,3) p = 0.016, Pc = 0.048; [e] (2,2 + 1,3 + 2,3) p = 0.032, Pc = 0.032.

there was a significantly increased frequency of allele 1 (81.9 vs. 63.0%; p = 0.002, Pc = 0.004) and corresponding decreased frequency of allele 2 (18.1 vs. 33.0%; p = 0.013, Pc = 0.026) in the severe group compared with controls. The same association was found between the allelic frequencies of alleles 1 and 2 when comparing the severe group with the mild group (81.9 vs. 67.5% and 18.1 vs. 32.5%; p = 0.023, Pc = 0.046). In addition, allele 3 was found to be increased in the control group compared with the mild group (4.0% vs. 0.0; p = 0.01, Pc = 0.02); however, there was no significant association with alleles 1 or 2.

There were no significant differences between the frequencies of the alleles in controls compared with organ-failure score groups or in the mild group compared with organ-failure scores groups.

When the patients were analysed according to aetiology, there was a significant increase in the frequency of allele 1 (82.4 vs. 63.0%; p = 0.002, Pc = 0.006), and corresponding decrease in the frequency of allele 2 (17.6 vs. 33.0%; p = 0.013, Pc = 0.039) in the idiopathic group of patients compared with controls; however, there was no association with any other aetiologic group and the alleles.

IL-1ra genotype frequencies

The frequency of the 1,1 genotype was found to be increased in the total patient group compared with the total control group (54.3 vs 42.0%; Pc = 0.046), whereas the 2,2; 1,3; and 2,3 genotypes were found to be decreased between the two groups (10.3 vs. 20.0%; Pc = 0.032).

When patients were categorised according to severity, there was a significantly increased frequency of the 1,1 genotype in the severe group compared with the controls (69.4 vs. 42.0%; p = 0.003, Pc = 0.006); however, no genotype association was found when comparing the mild group with the severe group, controls with organ-failure score groups, or the mild group with the organ-failure score groups.

When patients were categorised according to aetiology, there was an increased frequency in the 1,1 genotype (67.6 vs. 42.0%; p = 0.007, Pc = 0.021) and decreased frequency in the 2,2;1,3;2,3 genotypes (2.9 vs. 20.0%; p = 0.016, Pc = 0.048) in idiopathics compared with controls; however, there was no genotype association with any other aetiologic group.

Taq I polymorphism of the IL-1B gene

Two alleles (1 and 2) and three genotypes (1,1; 1,2; and 2,2) were observed in this study.

No significant differences were found between the Taq I allelic or genotype frequencies in acute pancreatitis patients and normal controls or between subdivisions of the patient group. The Taq I allelic and genotype frequencies are shown in Tables 5 and 6.

DISCUSSION

We describe here an association between the VNTR polymorphism in intron 2 of the IL-1RN gene and acute pancreatitis. Only three of the five alleles of the polymorphism were detected in this study. According to Tarlow et al. (21), the sum total of alleles 3, 4, and 5 accounts for only 4% of the total allele frequency in a given population; therefore, for us to detect the rarer genotypes, more subjects must be studied. Our data show significant differences in both allele and genotype frequencies between the groups. Allele 1 (4 × 86-bp repeat allele) was significantly increased in patients compared with controls, in severe cases compared with controls, in idiopathic patients compared with controls, and in severe cases compared with mild cases. In addition, allele 2 (2

TABLE 5. *Taq I allelic frequencies (%) in patients and controls*

Allele	Normal controls (n = 340)	AP patients (n = 228)	Mild AP (n = 156)	Severe AP (n = 72)	OFS ≥2 (n = 66)	OFS ≥3 (n = 36)	OFS ≥4 (n = 24)	Alcohol AP (n = 36)	Idiopath AP (n = 64)	G/stone AP (n = 128)
1	83.2	80.7	82.7	76.4	74.2	75.0	70.8	86.1	78.1	80.5
	(283)	(184)	(129)	(55)	(49)	(27)	(17)	(31)	(50)	(103)
2	16.8	19.3	17.3	23.6	25.8	25.0	29.2	13.9	21.9	19.5
	(57)	(44)	(27)	(17)	(17)	(9)	(7)	(5)	(14)	(25)

All p values were nonsignificant.
n, number of alleles in each group.

× 86-bp repeat allele) was found to be significantly decreased in severe cases compared with controls, in idiopathics compared with controls, and in severe cases compared with mild cases. The 1,1 genotype was found to be significantly increased in patients compared with controls, in severe cases compared with controls, and in idiopathics compared with controls, whereas the 2,2; 1,3; and 2,3 genotypes were significantly decreased in patients compared with controls and in idiopathics compared with controls. These results suggest that the IL-1RN gene could act as both a genetic marker for the severity of acute pancreatitis and a susceptibility marker for the idiopathic group.

It has been reported that each 86-bp repeat unit of the VNTR polymorphism comprises three protein-binding sites: an α-interferon silencer A, a β-interferon silencer B, and an acute-phase response element. These binding sites are believed to control IL-1ra transcription either by acting as promoter elements or by influencing the splicing efficiency of the IL-1ra transcripts. Individuals with different numbers of the repeat sequences have different numbers of the transcription binding sites, and this could have functional significance (i.e., the more repeats, the more protein binding sites and the more protein is produced). Allele 2 homozygotes who have 2 × 86-bp repeats might have reduced levels of IL-1ra mRNA and protein compared with allele 1 homozygotes who have 4×86bp repeats (21).

As previously mentioned, increased frequency of allele 2 of this polymorphism has been found to be a genetic marker of severity in alopecia areata, SLE, lichen sclerosus, and type 1 psoriasis, and a susceptibility marker for ulcerative colitis, relapsing/remitting multiple sclerosis, and Graves' disease. The majority of these inflammatory conditions are of epithelial origin. Because keratinocytes do not produce secretory IL-1ra (sIL-1ra), the results obtained in these studies are probably due to the intracellular form of IL-1ra (icIL-1ra). There has, however, been a lack of association between allele 2 of the polymorphism and susceptibility to nonepithelial conditions such as rheumatoid arthritis (25) and Crohn's disease (28), which are thought to involve sIL-1ra. Although both forms of the antagonist are produced by the same gene, they have different structures (icIL-1ra lacks a leader sequence), so their processing may be regulated differently. Acute pancreatitis may also fall into the same category as rheumatoid arthritis and Crohn's disease because macrophages, a source of sIL-1ra, are known to migrate into the pancreas early in the course of the disease.

Information regarding an association between genotype and functional protein levels is conflicting. Tarlow et al. (21) have suggested that allele 2 corresponds to low IL-1ra protein levels; however, Danis et al. (34) have found the reverse. If allele 2 does correspond to high IL-1ra secretion, as suggested by Danis et al., then allele 1 may correspond to low IL-1ra secretion. This hypothesis has been supported by data from Mandrup-Poulsen

TABLE 6. *Taq I genotype frequencies (%) in patients and controls*

Genotype	Normal controls (n = 170)	AP patients (n = 114)	Mild AP (n = 78)	Severe AP (n = 36)	OFS ≥2 (n = 33)	OFS ≥3 (n = 18)	OFS ≥4 (n = 12)	Alcohol AP (n = 18)	Idiopath AP (n = 32)	G/stone AP (n = 64)
1,1	67.6	63.2	66.7	55.6	48.5	50.0	41.7	72.2	56.3	64.1
	(115)	(72)	(52)	(20)	(16)	(9)	(5)	(13)	(18)	(41)
1,2	31.2	35.1	32.1	41.7	51.5	50.0	58.3	27.8	43.8	32.8
	(53)	(40)	(25)	(15)	(17)	(9)	(7)	(5)	(14)	(21)
2,2	1.2	1.8	1.3	2.8	0.0	0.0	0.0	0.0	0.0	3.1
	(2)	(2)	(1)	(1)	(0)	(0)	(0)	(0)	(0)	(2)

All p values were nonsignificant.
n, number of genotypes in each group.

et al. (35), who have found that the 1.1 genotype is associated with low plasma IL-1ra levels in type 1 diabetes. The results obtained in our study suggest that allele 1 is associated with increased severity in acute pancreatitis because of a reduced production of IL-1ra and inability to counteract the action of IL-1. Whether allele 1 corresponds to a high or low secretory phenotype will not be known until functional studies are carried out. These experiments will involve the in vitro stimulation of monocyte cell cultures obtained from acute pancreatitis patients and quantification of protein levels by techniques such as Western blotting.

The IL-1RN gene may have functional significance on its own, or it may be in linkage with other genes. Possible candidates are IL-1A, IL-1B, and IL-1RtI, which share the same gene complex on the long arm of chromosome 2 and which have previously been linked with disease. Analysis of the Taq I polymorphism in exon 5 of the IL-1B gene has found no significant differences between patients and controls; however, other polymorphic regions exist within this gene, and these must be studied before this gene can be eliminated. In conclusion, to our knowledge, this is the first report of an association between the IL-1 gene cluster and acute pancreatitis.

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Investigation of the IL-1 gene cluster and its association with acute pancreatitis

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Recent studies have implicated the IL-1 gene cluster in acute pancreatitis, an inflammatory condition of the pancreas. A penta-allelic polymorphism is known to exist in intron 2 of the IL-1ra gene in which there are a variable number of tandem repeat units each 86bp long. In addition, a biallelic polymorphism is present in exon 5 of the IL-1β gene which involves a C to T substitution. The aim of this study was to investigate these polymorphisms in acute pancreatitis. The genotype and allele frequencies were determined in patients (n=125) and healthy controls (n=150) using the polymerase chain reaction. Patients were categorised according to aetiology (alcohol, idiopathic, gallstone), severity (mild/severe) and organ failure scores (OFS≥>2, OFS≥3, OFS≥4). There was an increased frequency of allele 1 of the IL-1ra polymorphism in idiopathics compared to controls (82.4% vs 63%, $p<0.01$). No association was found with any other aetiological group. The frequency of allele 1 was also significantly increased in severe cases compared to mild cases (81.9% vs 68.2%, $p<0.05$), however, the increase did not correspond to the organ failure scores. IL-1ra may determine severity of acute pancreatitis and susceptibility to idiopathic acute pancreatitis. No association was found between IL-1β and the disease.

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Association between the IL-1 Gene Cluster and Acute Pancreatitis

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Introduction: The IL-1 (interleukin-1) gene cluster has been implicated in acute pancreatitis. Bi-allelic polymorphisms exist in the IL-1B and IL-1RtI genes which either generate or destroy restriction endonuclease sites (TagI, AvaI, AluI, PstI).
Aim: The aim of the study was to investigate these polymorphisms in acute pancreatitis.
Method: The genotype and allele frequencies were determined in patients (n = 114) and healthy controls (n = 170) using the polymerase chain reaction, followed by digestion with the appropriate restriction endonuclease. Patients were categorised according to etiology, severity and organ failure scores.
Results: IL-1B: There were no significant differences in the allele frequencies between controls and patients/subgroups of patients, however, the Alu I 2,2 genotype and Ava I 2,2 genotype was significantly decreased in patients vs controls (9.7% vs 19.5%, pc = 0.018 and 9.6% vs 18.0%, pc = 0.03 respectively). No other significant differences were found. IL-1RtI: Allele 1 was significantly decreased and allele 2 significantly increased in patients vs controls (21.3% vs 28.7%, pc = 0.037 and 78.7% vs 71.3%, pc = 0.037 respectively). No other significant differences were found.
Conclusion: Bi-allelic polymorphisms in the IL-1B and IL-1RtI genes appear to be associated with susceptibility to acute pancreatitis, but not with severity of the disease.

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TRYPSINOGEN GENE MUTATIONS IN ALCOHOLIC AND IDIOPATHIC CHRONIC PANCREATITIS.

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Background: Mutations of the trypsinogen gene have been identified in patients with hereditary pancreatitis. A mutation in exon 3 (R117H) produces an Arg (CGC) to His (CAC) substitution resulting in an inactivation-resistant trypsin. **Aim:** To investigate the relevance of this mutation in patients with alcoholic and idiopathic chronic pancreatitis (CP). **Methods:** DNA was extracted from blood samples of 27 patients with alcoholic CP, 13 with idiopathic CP, 20 alcoholics without pancreatitis and 20 normal controls. The R117H mutation was examined by restriction endonuclease (Afl III) digestion of polymerase chain reaction amplification products. **Results:** The R117H mutation was detected in 2 of the alcoholic CP patients. It was not detected in the idiopathic CP group or in the controls. **Conclusions:** This is the first reported finding of a trypsinogen gene mutation in alcoholic CP. The prevalence of this mutation should be determined and the need to screen this group of patients and their families assessed.

Association of the IL-1A Gene and Susceptibility to and Severity of Acute Pancreatitis

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Introduction. Recent studies have implicated the IL-1 gene cluster in acute pancreatitis. An (AC)n repeat and variable number of tandem repeat (VNTR) polymorphism exist within introns 5 and 6 of the IL-1A gene.

Aims. The aim of this study was to investigate these polymorphisms in acute pancreatitis.

Methods. Genotype and allele frequencies were determined in patients (n = 136) and healthy controls (n = 187) using the polymerase chain reaction. Patients were categorized according to etiology (alcoholic, idiopathic, gallstone) severity (mild/severe), and organ failure scores (OFS ≥ 2, OFS ≥ 3, OFS ≥ 4).

Results. (AC)n repeat study: allele 4 was significantly decreased in severe patients compared to mild patients (7.9% vs. 20.7%, Pc = 0.024) and in OFS > 2 patients compared to mild patients (7.1% vs. 20.7%, Pc = 0.03). The 2,4 genotype was significantly increased in alcoholics compared to controls (33.3% vs. 12.0%, Pc = 0.03) and decreased in gallstone compared to alcoholics (7.5% vs. 33.3%, Pc = 0.006). In addition, the 1,2 genotype was significantly increased in OFS ≥ 2 compared with controls (45.7% vs. 23.3%, Pc = 0.024). VNTR study: no significant differences in allele or genotype frequencies were found between controls and patients or subgroups of patients.

Conclusions. The (AC)n repeat polymorphism of the IL-1A gene is associated with susceptibility to and severity of acute pancreatitis.

Investigation of Trypsinogen Gene Mutations in Patients with Chronic Pancreatitis

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Mutations of the trypsinogen gene have been identified in patients with hereditary pancreatitis (HP). A mutation in exon 3 (R117H) produces an Arg (CGC) to His (CAC) substitution resulting in an inactivation resistant trypsinogen. Furthermore, neutral single C to T transitions in exons 4 and 5 at position 133807 and 134359, respectively, were also identified in the trypsinogen gene. The aim is to investigate the relevance of the R117H and the polymorphisms in exon 4 in patients with chronic pancreatitis (CP). DNA was extracted from blood samples of 35 patients with alcoholic CP, 13 with idiopathic CP, 20 alcoholic controls, and 38 normal controls (NC). The R117H mutation was examined by restriction endonuclease (*Afl*III) digestion of polymerase chain reaction (PCR) products and the positive samples were confirmed by direct sequencing. Direct sequencing of the PCR products was used to detect polymorphism in exon 4. The R117H mutation was detected in 1 of the alcoholic CP patients. The allelic frequency of the T of C(133807)T polymorphism was 0.52 (NC) and 0.29 (CP). The allelic frequencies between CP and NC was significant (χ^2 =6.28, $p < 0.02$). This is first report of a R117H mutation in alcoholic CP. Further studies are now required to elucidate these polymorphisms in chronic pancreatitis.

POLYMORPHISMS IN THE IL-1RN GENE ARE ASSOCIATED WITH SUSCEPTIBILITY TO IDIOPATHIC ACUTE PANCREATITIS AND WITH SEVERITY OF THE DISEASE.

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The IL-1 (interleukin-1) gene cluster has been implicated in acute pancreatitis. MspI, variable length (VLP) and SspI polymorphisms exist within exon 1, introns 2 and 3 respectively of the IL-1RN gene. The aim was to investigate these polymorphisms in acute pancreatitis. Genotype and allele frequencies were determined in patients (n=136) and controls (n=149) using DNA amplification and digestion with the appropriate enzyme. Patients were categorised according to severity, organ failure scores and aetiology. MspI and SspI loci: The 1,1 genotype was significantly increased in the severe group compared to controls (70.3% vs 47.7%, p=0.014 and 70.3% vs 46.2%, p=0.009 respectively). Allele 1 was significantly increased in the severe group compared to controls (82.4% vs 69.5%, p=0.026 and 82.4% vs 69.3% p=0.025 respectively) and in the idiopathic group compared to controls (81.9% vs 69.5%, p=0.034 and 81.9% vs 69.3%, p=0.033 respectively). VLP locus: The 2,2 genotype frequency was significantly increased in the severe group compared to controls (70.3% vs 42.5%, p=0.002) and in the idiopathic group compared to controls (66.7% vs 42.5%, p=0.009), and this was reflected in the allelic frequencies. Polymorphisms in the IL-1RN gene are associated with susceptibility to idiopathic acute pancreatitis and with severity of the disease.