The Role of Genetic Variation in Predisposition to Alcohol-related Chronic Pancreatitis

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

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Abstract

Background

Chronic pancreatitis (CP) is a disease of fibrosis of the pancreas for which alcohol is the main causative agent. However, only a small proportion of alcoholics develop chronic pancreatitis. Genetic polymorphism may affect pancreatitis risk.

Aim

To determine the factors required to classify a chronic pancreatic population and identify genetic variations that may explain why only some alcoholics develop chronic pancreatitis.

Methods

The most appropriate method of diagnosing CP was assessed using a systematic review. Genetics of different populations of alcohol-related chronic pancreatitics (ACP) were explored using four different techniques: genome-wide association study (GWAS); custom arrays; PCR of variable nucleotide tandem repeats (VNTR) and next generation sequencing (NGS) of selected genes.

Results

EUS and sMR were identified as giving the overall best sensitivity and specificity for diagnosing CP.

GWAS revealed two associations with CP (identified and replicated) at *PRSS1-PRSS2*_rs10273639 (OR 0.73, 95% CI 0.68-0.79) and X-linked *CLDN2*_rs12688220 (OR 1.39, 1.28-1.49) and the association was more pronounced in the ACP group (OR 0.56, 0.48-0.64)and OR 2.11, 1.84-2.42).

The previously identified VNTR in *CEL* was shown to have a lower frequency of the normal repeat in ACP than alcoholic liver disease (ALD; OR 0.61, 0.41-0.93). Homozygosity of the normal variant was more common in ALD than ACP (OR 0.53, 0.3-0.96) or Healthy Controls (OR 0.55, 0.3-1.00)).

The NGS discovery phase lead on to validation of the 21 most significant SNPs with Sequenom array. This showed significance difference between ACP and ALD in allele frequency of the synonymous SNP, *PRSS1*_rs6666, (OR 1.99, 1.46-2.72)

Conclusion

A range of potential exonic and intronic sites have been identified that have association with a predisposition to developing chronic pancreatitis. These findings show that further work is justified to fully assess the interaction of the different polymorphisms and their phenotypic significance in development of the disease.

Declaration

I declare that this thesis and the research upon which is based is the result of my own work. Whereever I have incorporated the work of others it has been clearly stated. This work has not previously been submitted in any substance for any degree, not is it concurrenly being submitted in candidiature for this or at any other university. Part of this work has been published in:

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Common genetic variants in the *CLDN2* and *PRSS1-PRSS2* loci alter risk for alcohol-related and sporadic pancreatitis

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Figures

Tables

Abbreviations

ABO – ABO Blood Group
AC – Alcoholic Control
ACACA - Acetyl-CoA Carboxylase A
ACACB - Acetyl-CoA Carboxylase B
ACP – Alcohol-related Chronic Pancreatitis
ACSS2 - Acyl-CoA Synthetase Short-chain Family Member 2
ACtrl – Alcoholic Control Patients
AD – Alcohol Dependence
ADGC - Alzheimer's Disease Genetics Consortium
ADH - Alcohol Dehydrogenase
AHC – Alcoholic Healthy Controls
AIP – Autoimmune Pancreatitis
ALD – Alcoholic Liver Disease
ALDH - Aldehyde Dehydrogenase
ALT - Alanine Aminotransferase
AP – Acute Pancreatitis
APA – American Pancreatic Association
APOA – Apolipoprotein A
APOB – Apolipoprotein B
APOC – Apolipoprotein C
APOE – Apolipoprotein E
ASH - Alcoholic Steatohepatitis
AST - Aspartate Aminotransferase
ATry - Anionic Trypsinogen
AUS – Abdominal Ultrasound
AXR – Abdominal X-Ray
bp – Base Pair
BSA – Bovine Serum Albumin
CAB39L - Calcium Binding Protein 39-like
CCK - Cholecystokinin
CEL - Carboxyl-ester Lipase
CEPH - Utah Residents with Northern and Western European Ancestry
CES – Carboxylesterase
CDC42BPG - CDC42 Binding Protein Kinase Gamma (DMPK-like)
CF - Cystic Fibrosis
CFTR - Cystic Fibrosis Transmembrane Conductance Regulator
Chr - Chromosome
CI – Confidence Intervals
CLPS - Colipase, Pancreatic
CM – Chylomicrons
CNIO - Spanish National Cancer Research Centre
CNV – Copy Number Variant
CP – Chronic Pancreatitis

CPT1A - Carnitine Palmitoyltransferase 1A (liver)
CT – Computer Tomography
CTRB1 - Chymotrypsinogen B1
CTry - Cationic Trypsinogen
CYP2E1 - Cytochrome P450, Family 2, Subfamily E, Polypeptide 1
D – Deviation
D' – Normalised Deviation (range 0, 1)
DNA – Deoxyribonucleic Acid
DOR – Diagnostic Odds Ratio
dNTPs - Deoxyribonucleotides Triphosphatase
EDTA - Ethylenediaminetetraacetic Acid
ERCP - Endoscopic Retrograde Cholangiopancreatography
ESS - Effective Sample Size
EUS – Endoscopic Ultrasound Scan
FABP - Fatty-acid-binding Proteins
FADS - Fatty Acid Desaturase
FAEE – Fatty Acid Ethyl Esters
FCP – Familial Chronic Pancreatitis
FN - False Negative
FP - False Positive
GALNT2 - UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 2
GCKR - Glucokinase (regulator)
GCLP - Good Clinical Laboratory Practice
GCP - Good Clinical Practice
GGT - Gamma-glutamyltranspeptidase
GPDH - Glycerol-3-phosphate Dehydrogenase
GWAS – Genome-wide Association Study
HC – Healthy Controls
HnC – Honeycombing
HNF1A - HNF1Homeobox A
HSROC - Bayesian Hierarchical Summary Receiver Operating Characteristic
HWE – Hardy-Weinberg Equilibrium
ICP – Idiopathic Chronic Pancreatitis
IDL - Intermediate-density Lipoprotein
IDST - Intraductal Secretin Stimulation Test
IL – Interleukin
iPM - i.Patient Manager
IPMN - Intraductal Papillary Mucinous Neoplasm
L-CPT1 - Carnitine Palmitoyltransferase 1A
LCTU Liverpool Cancer Trials Unit
LD – Linkage Disequilibrium
LDH - Hepatic Lactate Dehydrogenase
LDL - Low-density Lipoproteins
LDLR - Low-density Lipoprotein Receptor
LEDDU - Liverpool Early Drug Development Unit

LIMS – Laboratory Information Systems
LIPA – Lipase A
LIPE – Lipase E
LPL – Lipoprotein Lipase
M – Megabases (million base pairs)
MAF – Minor Allele Frequency
MCMC - Monte Carlo Markov Chain
MCP – Minimal Change Pancreatitis
MCV - Mean Corpuscular Volume
MEOS - Microsomal Ethanol Oxidizing System
MGLL - Monoacylglycerol Lipase
MODY - Maturity-onset Diabetes of the Young
MRCP - Magnetic Resonance Cholangiopancreatography
MREC - Multicentre Research Ethics Committee
MRI – Magnetic Resonance Imaging
mRNA – Messenger Ribonucleic Acid
MTry - Mesotrypsinogen
NA – Not Applicable
NAD^+ - Nicotinamide Adenine Dinucleotide (oxidised)
NADH - Nicotinamide Adenine Dinucleotide (reduced)
$NADP^+$ - Nicotinamide Adenine Dinucleotide Phosphate (oxidised)
NADPH - Nicotinamide Adenine Dinucleotide Phosphate (reduced)
NAFLD – Non-alcohol Fatty Liver Disease
NaOH – Sodium Hydroxide
NHNST – Neonatal Hypertrypsinaemia with Normal Sweat Chloride Tests
NHS - National Health Service
NIHR – National Institute of Health Research
NGRC - NeuroGenetics Research Consortium
NGS – Next Generation Sequencing
NL – in reference to the length of *CEL* VNTR in both alleles
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NQO - NAD(P)H Dehydrogenase, Quinone
NR5A2 - Nuclear Receptor Subfamily 5, Group A, Member 2
NS – Not Significant
OR – Odds Ratio
PABA - Para-aminobenzoic Acid
PEI - PABA Excretion Index
PBRU – NIHR Liverpool Pancreas Biomedical Research Unit
PBS – Phosphate Buffered Saline
PCR – Polymerases Chain Reaction
PDAC – Pancreatic Ductal Adenocarcinoma Carcinoma
PI – Pancreatic Insufficiency
PIS – Patient Information Sheet
PNLIP - Pancreatic Lipase
PNLIPRP - Pancreatic Lipase-related Protein

PPE - Personal Protective Equipment
PRSS - Protease, Serine, (Trypsin)
PSU - Pennsylvania State University
PTPRR - Protein Tyrosine Phosphatase, Receptor Type, R
Pub MAF – Published MAF (taken from NCBI dbSNP database Nov 2014)
r^2 – Correlation of Linkage Disequilibrium
RAP - Recurrent Acute Pancreatitis
RLBUHT – Royal Liverpool and Broadgreen University Hospital Trust
RNA - Ribonucleic Acid
ROS – Reactive Oxygen Species
SCARB1 - Scavenger Receptor Class B, Member 1
sAUS – Secretin Abdominal Ultrasound
Se – Standard Error
SEM – Standard Error of the Mean
sMRCP – Secretin Magnetic Resonance Cholangiopancreatography
sMRI – Secretin Magnetic Resonance Imaging
SL – Short/Long; in reference to the length of *CEL* VNTR in both alleles
SN – Short/Normal; in reference to the length of *CEL* VNTR in both alleles
SNP – Single Nucleotide Polymorphisms
SOP – Standard Operating Procedure
SPINK1 - Serine Protease Inhibitor Kazal-type 1
SPT - Secretin-Pancreozymin Test
SS – Short/Short; in reference to the length of *CEL* VNTR in both alleles
TAG – Triacylglycerols
TBE - Tris/Borate/EDTA
TCP – Tropical Calcific Pancreatitis
TN - True Negative
TP - True Positive
Tris-HCL - Tris(hydroxymethyl)aminomethane Hydrochloride
UCD - University Clinical Department
US - Ultrasound
USS – Ultrasound Scan
UTR – Untranslated Region
VLDL - Very-low-density Lipoproteins
VNTR – Variable Nucleotide Tandem Repeat

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1 Chapter 1: Introduction

1.1 *Chronic Pancreatitis*

Chronic pancreatitis (CP) is an inflammatory condition that causes irreversible fibrosis of the pancreas, leading to both structural and functional sequelae.[1] CP is detrimental to an individual's quality of life in many ways: severe abdominal pain, exocrine and endocrine failure and increased risk of developing pancreatic cancer.

In countries where alcohol excess is common, about 70% of chronic pancreatitis is caused by alcoholism, however less than 3% of heavy alcohol users develop clinical CP.[2,3] Chronic pancreatitis may suffer with additional alcohol-related co-morbidities and have complex social problems hindering medical and surgical management plans.

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1.1.1 Epidemiology

Chronic pancreatitis has a prevalence of about 30 per 100,000 and annual incidence of 3-10 per 100,000 per year; the incidence varies from 5-11 per 100,000 in men to 2-3 per 100,000 in women.[4-7] In a population of those with a history of alcohol-excess the incidence is increased to 92 per 100,000 in men and 82 per 100,000 in women.[7] The average life expectancy is 72 years, which is lower than that of idiopathic chronic pancreatitis (ICP; 80 years).[8] The over-all mortality directly due to chronic pancreatitis has been calculated as 36% over 20 years.[9]

Acute and chronic pancreatitis are discreet disease processes but there is some overlap, with acute developing into chronic, or acute-on-chronic flare ups of the disease.[1] It is thought that in many cases ACP is a progression from an initial attack or multiple attacks of acute pancreatitis (AP), which may occur 1-19 years before manifestation of chronic pancreatitis.[10] CP can develop as a result of acute attacks of pancreatitis; around a quarter of those who have AP go on to develop CP.[11]

Acute pancreatitis is diagnosed by the presence of two out of three of the following: a clinical history of abdominal pain in keeping with acute pancreatitis; increased amylase greater than three times the upper limit of normal; radiological changes in keeping with pancreatic inflammation.[12] Acute pancreatitis should be followed by full recovery of pancreatic function.[13] Many individuals go on to experience recurrent episodes of acute alcoholic pancreatitis, which is classified as recurrent acute pancreatitis. When symptoms fail to resolve between attacks then a diagnosis of chronic pancreatitis can be considered. Unfortunately no such internationally agreed diagnostic criteria for the diagnosis of chronic pancreatitis has been established.

Chronic pancreatitis can present in a variety of ways; it can often be associated with acute or chronic abdominal pain, but some individuals may be pain free. Pain is the presenting feature in 77% of those with ACP.[8] Pancreatic pain is often localized to the upper abdomen and may radiate to the back. This pain is often refractory to analgesia. In severe cases the pain can lead to absence from work, hospitalizations and addiction to opioids.[10,14,15]

Painless chronic pancreatitis tends to present with steatorrhoea or be detected incidentally when a patient has a CT scan for another purpose and is sometimes difficult to distinguish from pancreatic cancer.[16]

As yet there is no clear international consensus as to either the diagnosis or management of pancreatitis. Individual groups from Italy, China, Germany, Spain, Belgium and Japan have produced guidance documents.[17-23] To date there are no management guidelines produced for application in a British population and the first American guidelines on the diagnosis of pancreatitis were only published at the end of 2014, and therefore have not yet been widely adopted into clinical practice.[24]

1.1.2 Pathogenesis of Chronic Pancreatitis

Despite the fact that CP can be caused by multiple different aetiologies (1.1.3) the consequence is fibrosis of the pancreas gland.

Pancreatic stellate cells are the mediators of fibrosis in the pancreas, which leads to the formation of an extracellular matrix in interstitial spaces.[25] This process leads to progressive loss of the lobular morphology and structure of the pancreas, deforming the large ducts and leading to changes in the islets.

This pancreatic fibrosis is initiated by injury to the pancreas, the mechanism of which depends on the aetiology. The exact pathogenesis of chronic pancreatitis is unknown, but several theories have emerged as to its mechanism, reflecting the different processes that may apply due to the variety of different aetiological factors implicated in the disease.[26] The pathological development of CP is still unclear, but several common theories are discussed below including tryspin activation, by-products of alcohol metabolism, and oxidative stress.

1.1.2.1 Enzyme activation

Trypsinogen is the inactive form of trypsin and cationic trypsinogen is the most plentiful isoform in human pancreatic juice, but there are several isoforms (Figure 1). When trypsin becomes active (normally by entropeptidases in the gut) it plays a central role in pancreatic exocrine physiology as it initiates the activation of other pancreatic digestive enzymes. In normal pancreatic acinar cells, only small amounts of trypsin are activated whilst still in the cell, and there is continued inactivation ongoing to prevent the digestion enzymes activation cascade and pancreatic auto digestion. When there is any disruption to this pathway there is risk of pancreatic damage.

Pancreatic secretory trypsin inhibitor (*SPINK1*) inhibits up to 20% of the trypsin activity. If *SPINK1* fails to inhibit the trypsin activity, trypsin and other zymogens will be hydrolyzed. Hence, a mutation in the *SPINK1* gene reduces this innate protection system and may lead to pancreatic auto digestion (acute pancreatitis) if the level of trypsin activity is increased above that of the protective threshold of *SPINK1*.

Trypsin activation, such as in hereditary pancreatitis (HP), is believed by some to be insufficient to lead to chronic pancreatitis and in fact it is the sustained activation of inflammatory pathways by persistent pathogenic stimuli in the acinar cell that may be responsible for the development chronic pancreatitis.[26] However other studies have shown that trypsin activation alone is insufficient to induce CP.[27]

Exon 2[28]

Codon	15	20	25	30	35	40	45	50	55	60	65
CTry	AAPFD	DDDKI	VGGYN	CEENS	VPYQV	SLNSG	YHFCG	GSLIN	EQWVV	SAGHC	YKS
ATry	AAPFD	DDDKI	VGGYI	CEENS	VPYQV	SLNSG	YHFCG	GSLIS	EQWVV	SAGHC	YKS
MTry	AVPFD	DDDKI	VGGYT	CEENS	LPYQV	SLNSG	YHFCG	GSLIS	EQWVV	SAAHC	YKT
TRY6	AVPFD	DDDKI	VGGYT	CEENS	VPYQV	SLNSG	YHFCG	GSLIS	EQWVV	SAGHC	YKP

Exon 3

Codon		70	75	80	85	90	95	100	105	110	115	120	125	130	135	140	145	150
CTry	RI	QVRLG	EHNIE	VLEGN	EQFIN	AAKII	RHPQY	DRKTL	NNDIM	LIKLS	SRAVI	NARVS	TISLP	TAPPA	TGTKC	LISGW	GNTAS	SGA
ATry	RI	QVRLG	EHNIE	VLEGN	EQFIN	AAKII	RHPKY	DSRTL	NNDIL	LIKLS	SPAVI	NSRVS	AISLP	TAPPA	AGTES	LISGW	GNTLS	SGA
MTry	RI	QVRLG	EHNIK	VLEGN	EQFIN	AAKII	RHPKY	DRKTL	NNDIM	LIKLS	SPAVI	NARVS	TISLP	TAPPA	AGTKC	LISGW	GNTLS	FGA
TRY6	RI	QVRLG	EHNIE	VLEGN	EQFIN	AAKII	RHPKY	DRITL	NNDIM	LIKLS	TPAVI	NAHVS	TISLP	TAPPA	AGTKC	LISGW	GNTLS	SGA

Exon 4

Codon		155	160	165	170	175	180	185	190	195
CTry	DY	PDELQ	CLDAP	VLSQA	KCEAS	YPGKI	TSNMF	CVGFL	EGGKD	SCQ
ATry	DY	PDELQ	CLDAP	VLSQA	ECEAS	YPGKI	TNNMF	CVGFL	EGGKD	SCQ
MTry	DY	PDELK	CLDAP	VLTQA	ECKAS	YPGKI	TSNMF	CVGFL	EGGKD	SCQ
TRY6	DY	PDELQ	CLDAP	VLTQA	KCKAS	YPLKI	TSKMF	CVGFL	EGGKD	SCQ

Figure 1 Different isoforms of trypsinogen 1

Variation in amino acid sequence of exons 2-4 between between the isoenzymes of trypsinogen; cationic trypsinogen (CTry), anionic trypsinogen (ATry), meso trypsinogen (MTry) and Trypsinogen C (TRY6). Differences for cationic trypsinogen are underlined.

1.1.2.2 Fatty Acid Ethyl Esters and Calcium Overload

It has been suggested that higher serum calcium levels may contribute to pancreatitis, as hypercalcaemia may predispose the pancreatic acinar cell to abnormal, sustained calcium levels, which lead to premature pancreatic protease activation and consequently pancreatitis.[29]

Fatty acid ethyl-esters (FAEE), which are products of non-oxidative ethanol metabolism, increases calcium concentration through inositol trisphosphate receptors by causing calcium-ATPase pump failure due impaired mitochondrial ATP production. Fatty acid ethyl esters have been shown to induce pancreatic injury *in vivo* and *in vitro*.[30] Lowering cellular fatty acid substrate concentrations may reduce cell injury in pancreatitis.[29]

It has been shown that different FAEEs and the enzyme responsible for their synthesis (FAEE synthase) are present predominantly in the organs most often damaged by ethanol abuse, notably the pancreas and liver.[31] Specific work done in animal models has shown that there is a direct link between the non-oxidative metabolism of alcohol to produce FAEE, and the damage of early pancreatic cell damage to cause pancreatitis (Figure 2).[29,32]

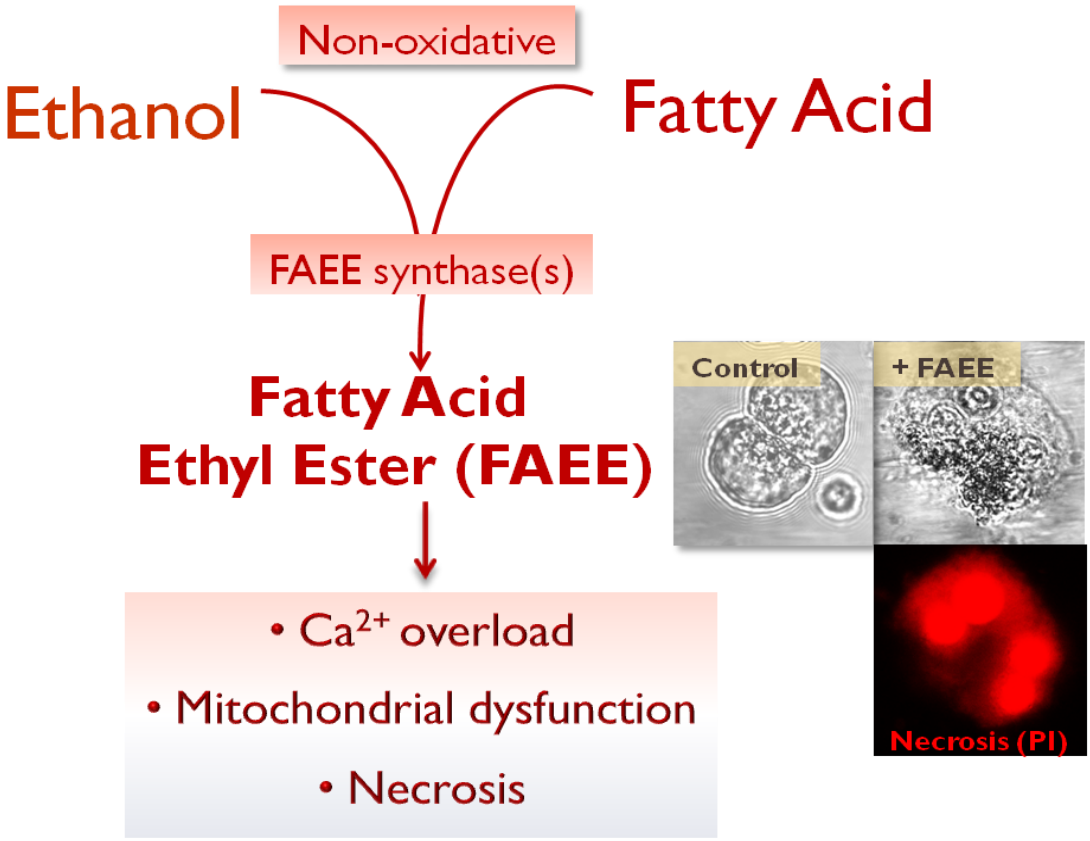


Figure 2 Schematic diagram of the effects of fatty acid ethyl esters on the pancreas

(Photos courtesy of Dr D Criddle)

Demostarates how the non oxidative metabolism of ethonal and fatty acids can lead to pancreatic damage.

1.1.2.3 Oxidative Stress

The oxidative stress hypothesis is that by-products of hepatic oxidase activity damage the pancreas through chronic reflux of bile into the pancreatic duct.[33] This theory has been better established in the development of AP.[34]

1.1.2.4 Toxic-metabolic

The toxic-metabolic theory is that alcohol is directly toxic to the acinar cell through a change in intracellular metabolism. This metabolic effect results in pancreatic lipid accumulation, fatty degeneration, cellular necrosis, and eventual widespread fibrosis. This association may be linked to the damage caused by calcium overload, which can lead to an abundance of FAEE, which in turn leads to pancreatitis.[35]

1.1.2.5 Stone and Duct Obstruction

It has been postulated that alcohol increases the lithogenicity of pancreatic juice, leading to stone formation within the pancreatic ducts. The stones continued contact with the surrounding ductal epithelium may lead to ulceration and scarring. Eventually, this can cause atrophy to the pancreas. Fibrosis occurs due to the chronic obstruction of flow from the acinar cells causing back pressure.[36]

1.1.2.6 Necrosis-fibrosis

The necrosis-fibrosis theory assumes that all chronic pancreatitis is caused by multiple attacks of acute pancreatitis leading to eventually chronic pancreatitis. The inflammation and necrosis associated with acute pancreatitis leads to scarring and fibrosis over time. There is also extrinsic compression of the pancreatic ducts due to oedema leading to obstruction, with a consequence of stasis, leading to stone formation and atrophy of the pancreas.[37]

1.1.3 Aetiology

The frequency of different aetiologies varies by geographical location, but in the UK, alcohol is the most common aetiology of chronic pancreatitis.[38]

1.1.3.1 Alcohol-related Chronic Pancreatitis

The most common cause of CP in the Western world is alcohol excess (70-90%).[8,9,39] It is thought that ACP may only be clinically present in around 3% of those who drink to excess.[3] Chronic pancreatitis is less prevalent than alcohol-related cirrhosis amongst those who drink to excess, and both pancreatitis and cirrhosis are not commonly found in the same individuals, suggesting that there may be other factors, which lead to susceptibility of either disease in some individuals.[40]

Commonly accepted value of excess alcohol range from 20g/day to 80g/day.[8,41,42] When the threshold was specifically sought by examining a large population of veterans and their relationship to alcohol consumption and the development of ACP, a threshold of 40g/day was given as the lower limit for very heavy drinking.[43] No studies have given a specific time period over which alcohol needs to be consumed to develop ACP, but some patients appear to exhibit symptoms over the first few years, whereas in the majority there will be a decade or more before ACP is diagnosed.[10]

The age of onset of ACP varies but on average is around 40 years of age.[10] Once diagnosed with ACP, the 5-year survival rate has been estimated at 65%, 10-year survival 43% and the 20-year survival at 20%.[44]

1.1.3.2 Idiopathic Chronic Pancreatitis

About 10% of cases are diagnosed as idiopathic.[5] Calcifications, exocrine insufficiency and diabetes develop more slowly in early onset idiopathic chronic pancreatitis than in late onset and alcoholic pancreatitis.[8]

Identified genes that contribute to idiopathic pancreatitis are *SPINK1* (serine protease inhibitor, Kazal type 1), and *CFTR* (cystic fibrosis transmembrane conductance regulator).[45,46] These are only

associated factors and lead to no clear pattern of inheritance, therefore they are classified as idiopathic rather than hereditary pancreatitis.

1.1.3.3 Hereditary Pancreatitis

About 1% of chronic pancreatitis is classified as hereditary pancreatitis (HP).[5] Hereditary chronic pancreatitis is inherited in an autosomal dominant fashion with penetrance of approximately 70%.[47] In the majority of HP families there is a known mutation within the cationic trypsinogen gene (*PRSS1*). The most common recognised disease causing mutations are p.R122H, p.N29I and arguably p.A16V that lead to increased stability of the trypsin product causing autodigestion.[48-50] There are some families displaying the typical patterns of inheritance for which no disease causing mutation has yet been identified.[51] The onset of symptoms is typically at 10-14 years of age although diagnosis may be delayed in individuals who are part of an as yet unidentified HP family.[19,20]

1.1.3.4 Hypertriglyceridaemia

Hypertriglyceridaemia is a rare cause of chronic pancreatitis that tends to involve the whole gland, but more commonly causing acute than chronic pancreatitis.[52,53] Familial hypertriglyceridaemia (type I, IV or V; Table 1) or secondary triglyceridaemia with levels of triglyceride more than 1000 mg/dL may predispose individuals to recurrent acute pancreatitis.[3]

Table 1 Classification of different types of familial hypertriglyceridaemia

Modified from Tsuang 2009[53]

		Blood lipids		Risk of
Classification	**Lipoprotein**	**Triacylglycerol**	**Combined hyperlipidaemia**	**developing CP**
I	CM	↑↑↑	↑	Yes
IIa	LDL	-	↑ ↑	No
IIb	LDL, VLDL	↑↑	↑↑	No
III	IDL	↑↑	↑↑	No
IV	VLDL	↑↑	-	Yes
V	VLDL, CM	↑↑↑	↑	Yes

CM – Chylomicrons; LDL – Low Density Lipoprotein; VLDL – Very Low Density Lipoprotein;
IDL -Intermediate-density Lipoprotein

1.1.3.5 Less Common Aetiology

Obstructive pancreatitis is a distinct entity according to the Marseilles classification, although this no longer seems to be recognised as such.[1] However, obstruction can be an aetiological factor, for example in the case of CP caused by an obstructing pancreatic head tumour.

Tropical pancreatitis occurs in geographical areas where low protein diet and cassava intake are common.[54] The aetiology of tropical calcific pancreatitis is related to genetic mutations in the *SPINK1* gene, but it is still unknown if environmental factors have an influence.[55]

Pancreatitis can be caused by hypercalcaemia, either in its own right or secondary to hyperparathyroidism.[56] Gallstones, the most common cause of acute pancreatitis, have not clearly been shown to cause chronic pancreatitis, although there is some evidence to support the development of fibrosis following gallstone pancreatitis.[57-59]

1.1.4 Risk Factors for Chronic Pancreatitis

Risk factors associated with the development of chronic pancreatitis are commonly classified using the M-ANNHIEM classification (Table 2).[42]

Table 2 M-ANNHEIM classification of risk factors of chronic pancreatitis

A	Alcohol consumption Excessive consumption (>80 g/day) Increased consumption (20–80 g/day) Low-Moderate consumption (<20 g/day)
N	Nicotine consumption (In cigarette smokers: description of nicotine consumption by pack-years)
N	Nutritional factors Nutrition (e.g., high caloric proportion of fat and protein) Hyperlipidemia
H	Hereditary factors Hereditary pancreatitis (defined according to Whitcomb)[48] Familial pancreatitis (defined according to Whitcomb)[48] Early-onset idiopathic pancreatitis Late-onset idiopathic pancreatitis Tropical pancreatitis
E	Efferent duct factors Pancreas divisum Annular pancreas and other congenital abnormalities of the pancreas Pancreatic duct obstruction (e.g., tumors) Posttraumatic pancreatic duct scars Sphincter of Odd dysfunction
I	Immunological Factors Autoimmune pancreatitis Sjögren syndrome-associated chronic pancreatitis Inflammatory bowel disease-associated chronic pancreatitis Chronic pancreatitis with autoimmune diseases (e.g., primary sclerosing cholangitis, primary biliary cirrhosis)
M	Miscellaneous and rare metabolic factors Hypercalcaemia and hyperparathyroidism Chronic renal failure Drugs Toxins

1.1.5 Diagnosis of Chronic Pancreatitis

There have been several attempts to reach a consensus on the classification of CP. The Marseilles criteria of 1963, 1984[1] and 1988[60] provided a clear category defining acute pancreatitis and removed the terms acute relapsing and chronic relapsing pancreatitis. The Cambridge classification set in 1984[61] classified the severity of disease but is predominately related to ERCP imaging results, and mainly idenfies large duct diease., The use of the Cambridge classification in ERCP is now becoming obsolete as a diagnostic test due to the high risk of ERCP induced pancreatitis.[62] There is currently no clear consensus into the best way to diagnose or classify chronic pancreatitis. The 2014 APA guidelines have provided new approaches to this, but they have not yet been validated in clinical practice.[24]

Imaging is commonly used to confirm the diagnosis and may also assess of the morphology of disease to diagnose complications of pancreatitis such as pseudocysts or pancreatic cancer. The diagnosis of advanced CP may be more straightforward if there are calcifications, which may be picked up with a plain abdominal X-ray, although these are not completely exclusive to CP.[63,64] Calcifications typically develop years after the onset of symptoms in alcoholics (median 8.7 years) and even longer in early onset idiopathic pancreatitis (24.9 years).[8] The main diagnostic challenge is detecting early stage disease.

1.1.5.1 Endoscopic Retrograde Cholangiopancreatography

Traditionally the most commonly used diagnostic test was Endoscopic Retrograde Cholangiopancreatography (ERCP), which could clearly demonstrate the morphology of the duct system. ERCP has been traditionally used as the gold-standard against which other modalities have been assessed.[65] However, it has more recently been recommended that it is only used for therapeutic procedures due to the high risk of post-ERCP pancreatitis.[62] This is not necessarily the case as in individuals with hereditary pancreatitis, who have such extensive damage to the pancreas, further pancreatitis is unlikely to be induced.[66] However, in this instance it is unlikely that the ERCP will be carried out for diagnosis, but rather for screening of this population for the development of cancer.[67]

1.1.5.2 Magnetic Resonance Imaging and Magnetic Resonance Cholangiopancreatography

Magnetic Resonance Cholangiopancreatography (MRCP) is increasingly being used, as it is a safe, non-invasive modality, which does not involve radiation, and does not cause acute pancreatitis. Magnetic Resonance Imaging (MRI) can also show extra-pancreatic anatomy. The delineation of the pancreatic ducts can be maximized by the administration of secretin. MRCP can be used to measure main pancreatic duct size.[68] These measurements tend to be less than that in an ERCP probably because contrast injection distends the duct in ERCP and this is not the case in MRCP.[69] Side branches smaller than 1mm are not well demonstrated at MRCP and therefore the sensitivity for side branch abnormalities is low in MRCP.[69,70] After secretin injection, duodenal fluid volume can be calculated and used as a marker of secretary function, but the value of routine use of secretin in MRI is unclear.[71] MRI may be superior to CT in detection of fibrosis, but more work needs to be done to assess this further.[72]

1.1.5.3 Computer Tomography

In the early stages of disease, the Computer Tomography (CT) may be normal or show features related to acute pancreatitis. CT cannot demonstrate the subtle changes in the side branches that can be appreciated using ERCP.[73] CT can be useful in the differential diagnosis of carcinoma. Chronic pancreatitis may be picked up incidentally when a CT is performed for other reasons. Its specific use for the diagnosis of CP has not been well evaluated but it has been recommended as the first line imaging method by the APA 2014 guidelines.[24]

1.1.5.4 Endoscopic Ultrasound

Endoscopic Ultrasound (EUS) has an increasing role in the diagnosis and management of chronic pancreatitis. EUS in some studies has been shown to be as effective as ERCP in diagnosing CP.[74] EUS can also demonstrate abnormalities in many individuals without definite ERCP features.[75] EUS allows high resolution imaging of both the parenchyma and pancreatic ducts. Experienced clinicians can detect early stage CP with good inter-observer agreement (92%).[75]

Features include focal hypoechoic areas, irregularity of the main pancreatic duct, dilated side branches and echogenic foci.[75] The parenchymal features of early chronic pancreatitis are lobulated parenchymal pattern and alternating echo-rich and echo-poor areas. Parenchymal changes reflect chronic pancreatitis and are independent of age and Body Mass Index (BMI).[76] The sensitivity for diagnosis of CP in individuals with typical histories and minimal changes on ERCP are quoted as being around 86-88%.[75,77]

Recent guidance has been given in the diagnosis of CP using EUS, which is referred to as the Rosemont classification.[78] This has been formed by consensus opinion, and has yet been validated for use in diagnostic accuracy.

In some centres elastography has been used as an adjunct to enhance the interpretation of EUS. Studies into the efficacy of this are limited at present, but results are promising and this warrants further investigation.[79]

1.1.5.5 Pancreatic Exocrine Function Tests

Impaired pancreatic enzyme secretion is a common feature in advanced disease but is not pathognomonic for chronic pancreatitis. Due to the large functional reserve of the exocrine pancreas, function tests may not become abnormal until a large proportion of the parenchyma is destroyed.[80] Steatorrhoea only becomes clinically evident after about 90% of the pancreas has been damaged.[80] Pancreatic tumours often cause pancreatic insufficiency and this is an important differential diagnosis.[81] Those with mild morphological evidence of chronic pancreatitis on imaging are expected to have normal pancreatic function tests.[81] The purpose of testing is to aid diagnosis but abnormalities are insufficient in themselves to diagnose chronic pancreatitis.[23]

Formalised direct pancreatic function tests are seldom available outside a few academic centres and have problems of standardization, are time consuming and expensive.

Indirect tests such as faecal elastase measurement or the pancreolauryl test (PLT) are the most acceptable form of testing for the majority of people. Elastase is stable in transit through the gut and can be measured in stool using ELISA. This test is superior to faecal chymotrypsin but is not able to diagnose chronic pancreatitis at an early stage.[81-85]

1.1.5.6 Biopsy

The gold standard for diagnosis of chronic pancreatitis is histopathology, which is rarely available unless surgery has been undertaken.[65] A few studies have looked at the diagnostic benefit of pancreatic biopsy.[86,87] However, the biopsy procedure can cause pancreatitis, haemorrhage or fistula and there is commonly high false negative results due to potential biopsying of spared areas of pancreas.[86]

1.1.5.7 Challenges to Diagnosis of Chronic Pancreatitis

1.1.5.7.1 Pancreatic Cancer

Pancreatic cancers and chronic pancreatitis can be potential differentials of pancreatic pathology and both can co-exist, making their differentiation challenging.[88] Histological diagnosis, which would normally be the gold standard to confirm or refute neoplasia, can be difficult as disease may be patchy and absence of neoplastic, or fibrosed cells in a biopsy sample, does not necessarily rule out the diagnosis.[87] Therefore in some cases a definitive diagnosis can only be confirmed with resection, with a high risk of morbidity and mortality to the patient.

1.1.5.7.2 Minimal Change Pancreatitis and Early Chronic Pancreatitis

A small number of individuals with a clinical diagnosis of CP have equivocal or minor abnormalities of the pancreas evident on imaging. This minimal change pancreatitis is more commonly identified in middle-aged women and alcohol excess is uncommon.[89] Pancreatitics may have normal or abnormal pancreatic function tests.[90] Due to the difficulty in obtaining objective evidence despite clinical symptoms, these cases may often end up being undiagnosed.

1.1.5.7.3 Groove Pancreatitis

Groove pancreatitis is a form of segmental chronic pancreatitis that occurs between the dorsocranial part of the head and the duodenum. The diagnosis is typically made in men aged 40-50 years with a history of alcohol excess with a relatively short history of pain (often less than one year).[91] The main significance of groove pancreatitis is that the clinical findings can conflict with pancreatic cancer causing diagnostic dilemma preoperatively.[91]

1.1.6 Consequences of the Development of Chronic Pancreatitis

Those with CP may exhibit some or all of the associated problems below. There is no classical pattern as to which problems develop, which adds to the difficultly of diagnosing the disease. Traditionally, before current investigations were available, the triad of pain, exocrine and endocrine insufficiency would be diagnostic of chronic pancreatitis, but now with more sensitive tests, some individuals may even be picked up incidentally without any suggestive clinical features.

1.1.6.1 Chronic Pain

The most significant consequence for the majority of individuals with CP is the chronic pain that can be associated with fibrosis of the pancreas.[92] Once pain develops, it can be very difficult to treat and patients will often become opiate dependant. Interventions which can be attempted when opiates are no longer effective in pain management include: EUS-guided coeliac plexus block, thoracoscopic splanchnicectomy or decompression procedures which can either be endoscopic or surgical but the outcomes are often poor, as once pain is established, even with removal of the tissue the nerve innovation continues.[93]

1.1.6.2 Endocrine Insufficiency

One of the two main functions of the pancreas is the endocrine release of insulin, and therefore chronic damage to the pancreas parenchyma often leads to the development of diabetes.[94] Diabetes purely induced by reduction of parenchyma is referred to as a distinct entity, Type 3c diabetes mellitus.[95] This is thought to account for around 10% of all cases of diabetes, and the most common cause of Type 3c diabetes mellitus is CP.[95]

1.1.6.3 Exocrine Insufficiency

The second role of the pancreas is the production and excretion of digestive juices. Therefore damage and loss of cell function leads to exocrine insufficiency in a proportion of pancreatitics. This leads predominately to a reduction in fat absorption, and therefore by default, insufficiency of the

fat-soluble vitamins, A, D, E and K. Pancreatitics can end up with malnutrition related to their CP, but this may overlap with aetiology such as alcohol that compounds the lack of absorption with low dietary intake.[96] Exocrine insufficiency can often be effectively treated with enzyme replacement therapy.[97]

1.1.6.3.1 Osteoporosis

Osteoporosis is becoming increasingly recognised as a complication of chronic pancreatitis.[98] Due to the poor absorption of the fat-soluble vitamins as a result of the exocrine insufficiency, there is poor uptake of Vitamin D, leading to osteoporosis and increased fracture rate.[99] There are no current guidelines into the management of osteoporosis risk in those with chronic pancreatitis.[100]

1.1.6.4 Pancreatic Cancer

The link between pancreatic cancer and chronic pancreatitis is complex. One is often found to co-exist with the other, and both can potentially be the causative agent for the other: CP can cause neoplasia due to dysplastic changes in the fibrosed tissues over time; tumours can cause obstruction of outflow from the pancreas leading to chronic pancreatitis changed in the distal pancreas.[101] There is an increased incidence of PDAC with CP patients.[101] Although at present there are no suitable methods for screening of pancreas cancer, lifelong surveillance maybe advisable in these individuals should such a test be found.[102-104]

1.1.7 Summary

Chronic pancreatitis has a prevalence of about 30 per 100000 with the incidence rising tenfold in a population of those with a history of heavy drinking. Chronic pancreatitis is caused by fibrosis of the pancreas leading to progressive loss of both structure and function of the gland. There are multiple causes identified, of which alcoholism is the most common. In addition, an individual's risk can be increased by smoking, poor diet and genetic factors.

Alcohol-related chronic pancreatitis may develop due to a build-up of FAEE within the pancreas, a breakdown of non-oxidative metabolism of alcohol and fat. Although there is the suggestion that different drinking patterns and different levels of fat consumption may bear some part in this process, it is remains unclear if there is any genetic component.

No international consensus has been developed as to how to diagnose chronic pancreatitis. This is partly contributed to by the lack of clear definition on what the disease is, and no current diagnostic gold standard that is suitable to compare current and future studies against. Therefore, there would be benefit in systematically reviewing the available literature to assess and identify the best methods for the diagnosis of chronic pancreatitis.

1.2 *Alcohol Metabolism*

As discussed, there are many reasons why individuals develop chronic pancreatitis, however, to quote Apte, the most obvious response to the question, why do individuals develop chronic pancreatitis is, "it's the alcohol, stupid".[105]

1.2.1 Ethanol Metabolism

Ethanol is a two-carbon alcohol and due to its small size and alcoholic hydroxyl group is soluble in both aqueous and lipid solutions. These factors allow ethanol to pass freely through the cells of the intestinal wall and travel via the portal circulation to the liver, where the majority of alcohol metabolism occurs.[106]

Alcohol can be metabolised through several different pathways (Figure 3).

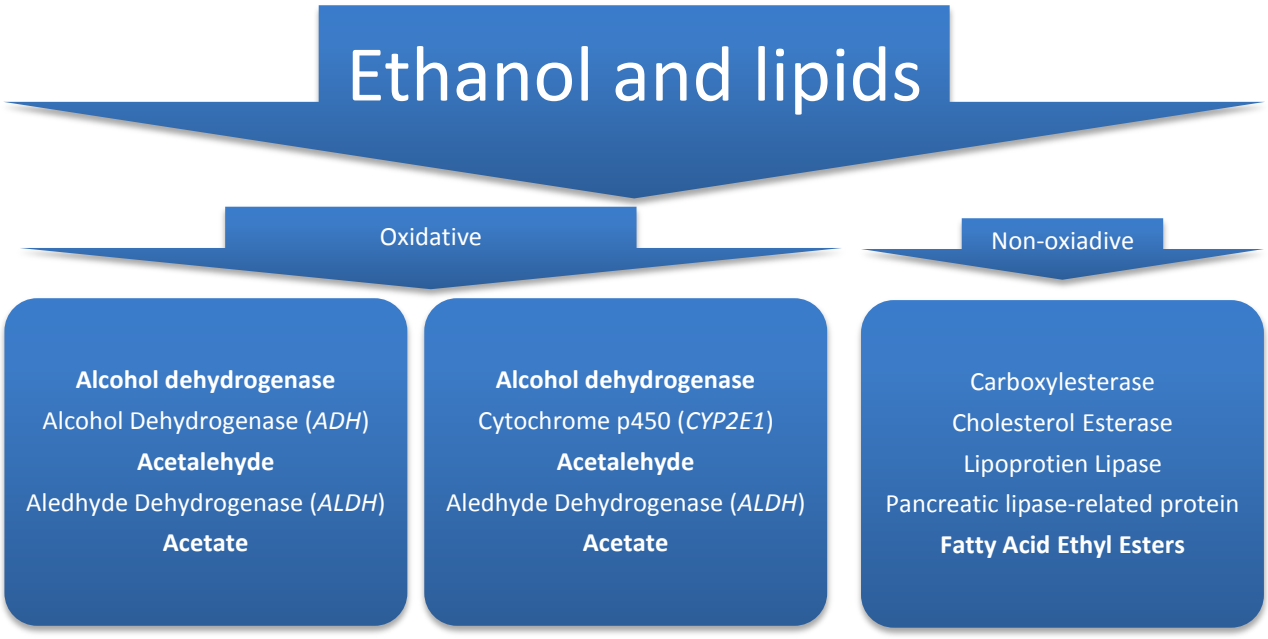


Figure 3 Oxidative and non-oxidative metabolism pathways of ethanol and lipids

Oxidation of ethanol can also occur in peroxisomes with catalase activity but this oxidation pathway requires hydrogen peroxide (H_2O_2) and therefore plays no significant role under normal physiological conditions.

1.2.1.1 Alcohol Dehydrogenase Pathway

The main pathway responsible for the majority of ethanol metabolism is initiated by alcohol dehydrogenase (*ADH*; Table 6). ADH is commonly found in hepatocytes and requires NAD^+ to oxidize ethanol to acetaldehyde, which upon entering the mitochondria is oxidized by aldehyde dehydrogenases (*ALDH*) to produce acetate.

There are two primary *ALDH* enzymes involved in this process; *ALDH1A1* and *ALDH2*.[107] *ALDH1A1* is a cytosolic enzyme while *ALDH2* resides in the mitochondria. The majority of acetaldehyde oxidation utilises *ALDH2* in the mitochondria, however, a small amount of oxidation occurs in the cytosol via *ALDH1*.

1.2.1.2 Microsomal Ethanol Oxidation System

The alternate ethanol metabolism pathway is the microsomal ethanol oxidizing system (MEOS). This pathway uses *CYP2E1* and requires NADPH. This pathway is more commonly used in individuals with chronic alcohol consumption.[108]

1.2.1.3 Non-oxidative Pathway

The non-oxidative pathway is catalysed by fatty acid ethyl ester (FAEE) synthase (carboxylesterase), which results in the formation of FAEE and takes place primarily in the pancreas.[109] Due to this association with fatty acids, the pathways of alcohol and lipid metabolism are interlinked.

Alcohol can be metabolised through several different but predominately oxidative pathways. However it has been demonstrated that inhibition of oxidative pathways can lead to an increase in both hepatic and pancreatic FAEE levels, which are products of non-oxidative metabolism (Figure 3).[110] This demonstrates that the oxidative and non-oxidative pathways are potential inversely linked.[32] The preference towards non-oxidative metabolism appears to be stronger in the pancreas than in other organs.[111]

1.2.2 Metabolism of Triglycerides

Alcohol and triglyceride metabolism are closely linked with increased alcohol intake having a J-shaped relationship with triglyceride levels.[112]

Apolipoprotein (*APOB*), which is related to VLDL synthesis, has been shown to reduce expression when alcohol is ingested.[113] Acute alcohol intake has been shown to decrease the activity of lipoprotein lipase, therefore reducing the lipolysis of VLDL and chylomicrons, causing an increase in their presence with increasing alcohol consumption.[114]

Chronic alcohol consumption has been demonstrated to increase free fatty acid release, mediated by activation of hormone sensitive lipase (*LIPE*) and a decrease in insulin sensitivity.[113] This mechanism may contribute to the development of alcoholic steatosis of the liver.

1.2.3 Effects of Ethanol Metabolism

The metabolic effects of inebriation mostly derive from the action of ADH and ALDH and the NADH/NAD^+ ratio imbalance they cause. The NADH produced during alcohol metabolism must be reduced back to NAD^+, therefore the continued ability to metabolize ethanol is dependent upon the capacity of hepatocytes to reduce NADH.[115] The reduction of NADH is moderated by the Krebs cycle in the mitochondria, which in turn is affected by the NADH produced by the ADH and ALDH reactions. There is an increase of hepatic lactate production due to the effect of NADH on the hepatic lactate dehydrogenase (LDH) reaction leading to a decrease in the liver's capacity to deliver glucose.[115]

The altered NADH/NAD^+ ratio also leads to a decrease in fatty acid oxidation, as this process requires NAD^+. An increase in fatty acid synthesis and triglyceride production by the liver are associated with reduced fatty acid oxidation.[116] In the mitochondria, the metabolism of acetaldehyde to acetate leads to increased levels of acetyl-CoA, which is diverted to fatty acid synthesis.[116] The reduction in cytosolic NAD^+ leads to reduced activity of glycerol-3-phosphate dehydrogenase (GPDH) resulting in increased levels of glycerol 3-phosphate, which aids in the synthesis of the triglycerides. This leads to fatty acid deposition in the liver, causing fatty liver syndrome and hyperlipidaemia.[106]

Acetaldehyde is produced as a part of alcohol metabolism. The acetaldehyde combines with proteins, nucleic acids and other compounds resulting in impaired activity of the affected compounds. Acetaldehyde compounds are known to promote cancer development.[106]

Ethanol metabolism can lead to an oxygen deficit in the liver; the metabolism of ethanol requires oxygen, therefore there are higher demands on oxygen from the blood. Hepatocytes residing beside arteries supplying oxygenated blood to the liver will tend to take up more than their normal share of oxygen and therefore the perivenous hepatocyte may have depleted levels of oxygen; therefore this hypoxic area is normally the first to show evidence of damage from chronic alcohol consumption.[117]

The metabolism of ethanol via the *CYP2E1* pathway results in increased reactive oxygen species (ROS) production, including superoxide, hydrogen peroxide, and hydroxyl radicals.[118] ROS production is associated with cancer development, atherosclerosis, diabetes, inflammation, aging, and other harmful processes.[119] Under normal conditions, a balance between ROS production and antioxidant removal exists in cells but this balance can be disturbed. Alcohol consumption increases ROS production which leads to oxidative stress, an established pathway in the development of chronic pancreatitis.[120]

Under conditions of acute ethanol consumption, the majority of ethanol is degraded by the hepatic oxidative pathways, predominantly the alcohol dehydrogenase mediated pathway. However, under conditions of chronic ethanol consumption, hepatic MEOS activity and non-oxidative pathways are induced and quantitatively make a greater contribution to ethanol catabolism.

1.2.3.1 Organ Specific Effects of Alcohol

It remains unclear why organs other than the liver develop alcohol-induced damage, especially the heart, pancreas, and brain, which lack or show minimal oxidative metabolism of ethanol and therefore are free of substantial acetaldehyde production.[31]

Alcohol encourages the collection of fat in the liver predominately through replacement of ethanol with fatty acids as the main hepatic fuel; the degree of fat deposition depends on the dietary supply of lipids.[121]

A statistically significant increase of class III alcohol dehydrogenase (*ADH3*) isoenzymes has been demonstrated in serum from individuals with both acute and chronic pancreatitis. In addition the activity of the class I ADH isoenzyme was significantly higher in serum from ACP patients.[122]

1.2.4 Ethanol Metabolism and Alcoholism

In addition to the predisposition of individuals to develop chronic pancreatitis in relation to their alcohol consumption, it must also be considered that some individuals have a greater predisposition for substance abuse.

There are several different commonly described polymorphisms of both *ADH* and *ALDH* genes (Appendix 10.1). Several of these variations have been linked with alcohol dependence (AD).[123]

ADH1A, *ADH1B*, *ADH1C* and *ALDH2* all play a central role in alcohol metabolism.[124] It is thought that genetic variability in these genes is associated with an individual's susceptibility to developing alcoholism and this also has potential to mediate alcohol-related tissue damage. Common polymorphisms exist in both *ADH1B* and *ADH1C* genes, which are associated with varying levels of enzymatic activity (Table 6).[125]

The *ADH1B* alleles occur at different frequencies in different populations. For example, the *ADH1B*1* form is found predominantly in Caucasian and Black populations, whereas *ADH1B*2* is more common in Chinese and Japanese populations and is seen in around 25% of people with a Jewish background.[126-128]

Although many *ALDH* genes have been identified, only *ALDH2* and to a lesser extent *ALDH1* have been shown to metabolise acetaldehyde.[129]

Because polymorphisms of *ADH* and *ALDH2* play an important role in determining peak blood acetaldehyde levels, they also influence vulnerability to alcohol dependence; polymorphisms that elevate acetaldehyde levels reduce the amount of alcohol drunk. As a consequence, alcohol-induced tissue damage is reduced in populations that harbour these polymorphisms due to the reduced preference for drinking.[130]

1.2.5 Summary

Ethanol metabolism has many different pathways, which commonly produce disease-causing products such as acetaldehyde, reactive oxygen species, and fatty acid ethyl esters. Oxidative metabolism is chosen preferentially but under some circumstances, especially within the pancreas, the non-oxidative pathway may be used.

Genetic variations in genes related to alcohol metabolism have been shown to influence those who become dependent on alcohol. Alcohol excess can lead to various disease processes effecting the liver, pancreas, heart and brain, but not all diseases seem to be directly dose-related, and some individuals may be more predisposed to one disease process rather than another.

1.3 *Alcoholic Liver Disease*

This thesis examines why some individuals who abuse alcohol get pancreatitis while others do not. Alcohol can cause damage to other organs in the body other than the pancreas, the most prominent of which is the liver. Alcoholic liver disease (ALD) arises in around 30% of heavy drinkers and liver cirrhosis in 10%.[131,132]

1.3.1 Definition of Alcoholic Liver Disease

ALD is a combination of different disease processes with the liver, as a result of alcohol excess, which include fatty liver, hepatitis and cirrhosis.

1.3.1.1 Alcoholic Fatty Liver Disease

Heavy alcohol consumption, even over only a few days, starts the build-up of fats within the liver (steatosis), which is the first step of the development of ALD. Steatosis does not cause any systemic symptoms and is reversible, but if excess alcohol consumption continues the extent of the liver disease will progress.

1.3.1.2 Alcoholic Hepatitis

Alcoholic hepatitis is normally the second stage of the development of ALD. Excess alcohol intake over a more prolonged period of time leads to inflammation of the liver. The liver damage associated with mild alcoholic hepatitis is usually reversible if alcohol consumption is stopped.

1.3.1.3 Cirrhosis

Cirrhosis is the final stage of ALD, when the disease is well established and the liver becomes significantly scarred. Once decompensated cirrhosis is established then life expectancy at 5 years can be as low as 15%.[133]

1.3.2 Pathophysiology of Alcoholic Liver Disease

In the presence of alcohol excess, liver disease is due to production of toxic metabolite acetaldehyde in the liver. Acetaldehyde is toxic to the mitochondria, which promotes leakage.[134]

In addition, the generation of NADH, which promotes steatosis of the liver by stimulating the synthesis of fatty acids and opposing their oxidation. Excess dietary lipids and their replacement with triglycerides also promote steatosis.

The *CYP2E1* metabolism pathway releases free radicals, which cause oxidative stress and in turn can lead to altered enzyme activity. Oxidative stress and associated cellular injury promote inflammation in the liver.

Intracellular concentration of free fatty acids may be in sufficient levels to damage membranes, leading to necrosis and inflammation, which can progress to fibrosis and ultimately cirrhosis.[134]

1.3.3 Natural History of Alcoholic Liver Disease

Studies of individuals with a strong history of alcohol excess suggest that 80% develop steatosis, which is the earliest and most common histopathological manifestation of ALD.[131] Steatosis occurs in most people consuming alcohol in excess of 80g/day, but normally resolves within 2–4 weeks of abstinence; only 20–40% individuals with steatosis go on to develop alcoholic steatohepatitis (ASH) and of them only around 40% will go on to develop cirrhosis.[135,136] In some cases the hepatitis stage may be bypassed but the overall figure for the development of alcoholic cirrhosis is approximately 10% of heavy drinkers.

There is an increased susceptibility of women as compared with men to develop alcoholic liver disease, however overall the disease process in more common in men, as they are more likely to consume alcohol to excess.[137]

1.3.4 Diagnosis of Alcoholic Liver Disease

It can be very difficult to predict the clinical stage in ALD before the development of decompensated cirrhosis.[131] In addition, some ALD patients may present with decompensated chronic liver disease without cirrhosis on biopsy. Therefore staging of ALD is most accurately performed though liver biopsy, which is not always available or suitable, especially for those with deranged clotting function.

Typical laboratory findings in ALD include transaminase levels with aspartate aminotransferase (AST) greater than alanine aminotransferase (ALT) as well as increased mean corpuscular volume (MCV), and gamma-glutamyltranspeptidase (GGT). In unclear cases, the diagnosis can be supported by imaging and liver biopsy. The histological features of ALD can ultimately define the diagnosis according to the typical presence and distribution of hepatic steatosis, inflammation, and Mallory-Denk bodies (inclusions found in the cytoplasm of liver cells).[138]

1.3.5 Chronic Pancreatitis and Alcoholic Liver Disease

There has been shown to be an inverse relationship between the risk of developing pancreatitis symptoms and the risk of developing liver symptoms, suggesting that the mechanism of the development of these disease processes is different.[139] Even when histologic samples are examined the correlation of presence of the two disease processes is low.[140]

Chronic alcoholic liver disease and chronic pancreatitis have a well-described association due to their common aetiology.[40] Although most individuals with alcoholic pancreatitis do not have cirrhosis, 72% of those with chronic pancreatitis have abnormal liver biopsies (12.5% have cirrhosis, and the remaining patients have steatosis, cholestasis, hepatitis or fibrosis).[40] About 15% of individuals with known cirrhosis have evidence of chronic pancreatitis.[40]

Several studies have looked at lifestyle factors to assess if alcohol intake or type predisposed patients to the develop ACP or ALD (Table 3).[141] These studies show no conclusive evidence that specific drinking habits affect a patient's predisposition to develop either ALD or ACP.

It has been shown that ACP, rather than ALD, may be more common in alcoholics who smoke.[142] Studies looking at the consumption of a high-protein and high-fat diet as a factor in the development of chronic pancreatitis in the alcoholic patient have not shown any direct association.[137]

Table 3 Review of published effects of drinking habits on the development of Alcohol-related Chronic Pancreatitis and Alcoholic Liver Disease

Paper	Year	Population	Number of participants (ACP vs. ALD)	Age started drinking (ACP vs. ALD)	Type of alcohol consumed (ACP vs. ALD)	Daily intake (ACP vs. ALD)
Wilson[141]	1985	Australia	20 vs. 33	No comment	Beer (70% vs. 48%; NS) Spirits (25% vs. 55%; NS) Wine (5% vs. 27%; p = 0.07)	No comment
Mezey[137]	1988	USA	42 vs. 18	No comment	Beer (7% vs. 33%; p = 0.01) Spirits (50% vs. 39%; NS) Wine (14% vs. 6%; NS)	180 vs. 218g/day; NS
Levy[143]	1995	France	56 vs. 50	19 vs. 19.6; NS	Beer (77% vs. 84%; NS) Spirits (75% vs. 76%; NS) Wine (80% vs. 98%; p < 0.005)	2.1 vs. 2.5g/day/kg; NS
Nakamura[144]	2004	Japan	59 vs. 17	No comment	Spirits (71% vs. 53%; p = 0.05)	>150g/day 58% vs. 71%; NS
Veena[145]	2012	India	119 vs. 188	24.3 vs. 22.8 years; NS	No comment	129 vs. 131g/day; NS
Spicak[146]	2012	Czech	66 vs. 80	Started before age 15 (29% vs. 88%; p = 0.03)	Beer (77% vs. 56%; p = 0.02)	58 vs. 64g/day; NS

1.3.6 Summary

Alcoholic liver disease is present in around 30% of all patients with a strong history of alcohol dependence. Clinically, there is little overlap in patients who develop ACP and ALD, suggesting that it is not simply the amount of alcohol consumed leading to a disease process but in fact there may be genetic aspects that leads to development of one disease process over another.

Life style and environmental factors have been compared between the two groups to try and account for the different disease processes, but no clear trend has been identified to explain this.

1.4 *Genetics*

1.4.1 Characterising Genetic Differences

Variations can be in several different forms such as Single Nucleotide Polymorphisms (SNPs), Insertions or Deletions (Indels), Variable Nucleotide Tandem Repeat (VNTR) and Copy Number Variants (CNV) as detailed below in Table 4.

Table 4 Summary of commonly found polymorphisms

Polymorphism	Description
Single Nucleotide Polymorphisms (SNPs)	SNPs are when a single genomic position has more than one potential base. In non protein-coding regions may still affect transcription on gene coding, but often their effect cannot be clearly quantified. These could be due to codon bias, enhanced binding or be at a splice site.[147]
Insertion and Deletions (Indel)	**In**sertion or the **del**etion of bases in DNA. If the indel is not a multiple of three then this will cause a frame shift in the amino acids produced, therefore, natural selection makes their presence uncommon in coding regions. Indels can be difficult to pick up accurately in some forms of genetic sequencing depending on the mechanism of base reads.
Variable Nucleotide Tandem Repeat (VNTR	The nulecotide repeats cluster together and oriented in the same direction. Individual repeats can be removed from (or added to) the VNTR via recombination or replication errors, leading to alleles with different numbers of repeats, commonly trinucleotide repeats.[148] Due to the repetitive nature of the sequence, many sequencing techniques that use short amplicons to eventually build the genomic sequence can fail to sequence VNTRs; the fragmentation of the DNA prevents accurate reconstruction of the repeating sequence.
Copy Number Variants (CNV)	CNVs are a duplication of a gene, or a section of a gene, meaning that the copied area may be more active. It is often difficult to identify CNVs as most sequencing techniques will only read a sequence and not quantify the proportionate amount of it.

1.4.2 Genetics of Chronic Pancreatitis

In the majority of cases the genetics of chronic pancreatitis is poorly understood. Only small numbers of patients are either classified as having hereditary or familial CP.[149] The development of CP is complex and multifactorial with potential gene-gene interactions in addition to gene-environmental factors. Genetic influence can be divided into those with hereditary pancreatitis (a clear pattern of autosomal dominant inheritance), familial pancreatitis (those with a family predisposition with no clear autosomal link) and those with potential associated genetic factors.

Genetic risk factors that link alcohol to susceptibility to CP have not been clearly defined.[150]

1.4.2.1 Hereditary Pancreatitis

Those patients classified with HP exhibit an autosomal dominant pattern of inheritance that accounts for around 1% of all known cases of chronic pancreatitis.[151] Around 80% of hereditary pancreatitis cases can be attributed to known variants in the serine protease 1 (*PRSS1*) gene, which is the only identified disease-causing gene, with the remaining 20% due to an as yet unknown cause.[152] *PRSS1* codes for cationic trypsinogen and disease causing variants within this gene have a generally accepted penetrance of around 70%.[47] Within the hereditary pancreatitis subpopulation, the cumulative risk of pancreatitis cancer rises from a 6-7-fold increase in sporadic CP patients to around 69 fold.[101]

The first identified and most commonly found variant within *PRSS1* is p.R122H, accounting for around 50-65% of known HP cases.[67,153,154] The variant p.R122H results in increased trypsin stability, increased zymogen stability and increased autoactivation leading to increased intrapancreatic trypsin activity which can lead to pancreatitis.[48,155]

An additional polymorphism affecting the same codon but with a different affect is p.R122C.[156] This adds an extra cysteine leading to partial misfolding of the recombinant protein causing reduced activity, autolysis, and autoactivation similar to p.R122H.[157]

The p.N29I variant accounts for 20% of HP cases.[47] It has no effect on trypsin or trypsinogen stability but increased autoactivation.[158] The less common p.N29T variation exhibits a phenotype similar to that of p.R122H and therefore as increased autoactivation was the common factor observed within the previously documented variations, the conclusion was put forward that autoactivation is the common pathogenic mechanism of hereditary pancreatitis.[155,159]

p.A16V has been identified as a less frequent variant of *PRSS1* in patients with pancreatitis.[50,160] p.A16V affects the first amino acid of mature trypsinogen, residing at the start of the signal peptide, although not forming a part of it, and therefore it is considered to influence secretion. However, the low penetrance of p.A16V (44%) may suggest this is a fairly conservative substitution, and has been identified in idiopathic CP cases with no family history.[47]

Copy number variants have also been demonstrated within *PRSS1*.[156] A list of commonly identified variations is found in Table 5. An up-to-date list of known *PRSS1* variants is collated at http://www.pancreasgenetics.org/.

Table 5 Time line of identification of disease causing mutations reported in *PRSS1*

(modified from Chen[161])

Missense mutation		
1996	p.R122H[48]	Increases trypsin stability and enhances trypsinogen autoactivation[162]
1997	p.N29I[49]	Enhances trypsinogen autoactivation and increases trypsin stability[155]
1999	p.A16V[50]	Possibly increases the rate of chymotrypsinogen C-mediated trypsinogen activation[163]
1999	p.K23R[164]	Increases trypsinogen autoactivation[165]
2000	p.D22G[166]	Increases trypsinogen autoactivation[165]
2001	p.R116C[167,168]	Results in protein misfolding that possibly leads to endoplasmic reticulum stress[169]
2001	p.R122C[170]	Increases trypsin stability and enhances trypsinogen autoactivation[157]
2002	p.N29T[171]	Enhances trypsinogen autoactivation and increases trypsin stability[155]
2003	p.D19A[165]	Increases trypsinogen autoactivation[165]
Copy number mutation		
2006	605-kb triplication[172]	Gene dosage effect
2008	605-kb duplication[156]	Gene dosage effect
2010	320-kb complex CNM[173]	Gene dosage effect
Double gain-of-function mutation		
2008	*PRSS2/PRSS1* hybrid[174]	Gene dosage effect plus the effect of *PRSS1 p.*N29I
Microduplication		
2011	p.K23 I24insIDK[175]	Increases trypsinogen autoactivation[175]
PRSS1-PRSS2 loci		
2012	*PRSS1-PRSS2* SNP[176]	Disease susceptibility by altering expression of the trypsinogen gene

1.4.2.2 Familial Chronic Pancreatitis

Familial chronic pancreatitis (FCP) are cases in which there is not a clear autosomal dominant pattern of inheritance, but there is a familial trend. The two genes that have been commonly associated with this form of pancreatitis are *SPINK1* and *CFTR*.

1.4.2.2.1 Serine protease inhibitor, Kazal-type 1

SPINK1 encodes PSTI (pancreatic secretory trypsin inhibitor; one of the defensive mechanisms against premature trypsin activition within the acinar cells) and *SPINK1* mutations are thought to be a disease modifying factor rather than being a disease-causing factor in chronic pancreatitis.[177,178]

SPINK1 was first associated with chronic pancreatitis with the indication of p.N34S variant in 18.75% of idiopathic CP patients, but not present in any controls.[45] However, p.N34S variant has not been demonstrated to result in a functional defect. By expression studies, p.L14P and p.L14R mutations demonstrated a markedly reduced *SPINK1* expression and resulted in loss of function.[179]

A few other polymorphisms have been noted in *SPINK* (including p.D50E, p.P55S and p.C58C), but their clinical significance remains unclear.[180]

There is some evidence that *SPINK1* may work in synergy with other variants. It has been recently reported that the coinheritance of *CFTR* p.R75Q and *SPINK1* p.N34S variants is significantly higher in patients with idiopathic chronic pancreatitis than in controls (8.75% vs. 0.38%).[181] In addition there is some evidence that p.N34S variant may be more potent in a specific haplotype with other variations.[182]

A calcium-sensing receptor (*CASR*) gene variant (p.L173P) has been associated with a family with the p.N34S *SPINK1* variant, who also have a history of familial hypocalciuric hypercalcaemia.[158] An association has also been demonstrated with the presence of the p.R990G *CASR* variant, with the common *SPINK1* variant, especially in the presence of heavy alcohol consumption.[183]

p.P163R, p.I427S, p.D433H, p.V477A, all lying within *CASR,* have also been described as being associated with CP in the presence of p.N34S.[184]

1.4.2.2.2 Cystic fibrosis transmembrane conductance regulator

Cystic fibrosis transmembrane conductance regulator (*CFTR*) is most commonly associated with cystic fibrosis (CF), a disease caused by high chloride output from secretory cells, predominately in the lungs and the pancreas, and therefore commonly causes pancreatic dysfunction. Symptoms of pancreatitis can be present in the absence of any other associated CF features.[185]

CFTR disease causing variants are common in the normal population (1 in 25 patients) but normally these variants need to be inherited from both parents to cause CF itself. However, some of the heterozygous variants can lead to chronic pancreatitis.[186-188]

Initial studies into the effect of *CFTR* variations in CP showed 134 patients with CP for 22 mutations of the *CFTR* gene. None of the patients had a mutation on both copies of the *CFTR* gene.[46] Eighteen patients (13.4%) had a *CFTR* mutation on a single chromosome, as compared with a frequency of 5.3% among 600 controls ($p < 0.001$). A total of 10.4% (14/134) of the patients had the 5T-allele (intron 8), which is twice the expected frequency ($p = 0.008$). The most commonly recognised *CFTR* variants are p.R75Q, p.I148T, 5T-allele and p.E528E.[189]

A specific *CFTR* haplotype has been shown to be associated to CP compared to healthy controls ($p=0.045$).[186] The haplotype contains three common synonymous SNPS, *CFTR*_rs213950 (p.V470M), *CFTR*_rs1042077 (p.T854T) and *CFTR*_rs1800136 (p.Q1463Q), none of which are known to be associated with CP.

1.4.2.2.3 Other Genes Associated with Familial Chronic Pancreatitis

1.4.2.2.3.1 PRSS1

Although some mutations in *PRSS1* are very clearly associated with hereditary pancreatitis (Chapter 1.4.2.1) the penetrance of some other variants is so low that they are better described as being associated with FCP. This could be extended to include the p.A16V variant described in the HP section.[47]

Other non-synonymous polymorphisms, such as p.K170E and p.E79K have been shown to have an association with CP, but their true pathogenic nature is unclear.[190]

1.4.2.2.3.2 PRSS2

PRSS2, which encodes anionic trypsinogen, lies close to the genetic position of *PRSS1*.[191] The variant p.G191R in *PRSS2* has been shown to be protective against the development of chronic pancreatitis.[192] The polymorphism was present in 3.4% controls but in only 1.3% of CP patients (OR 0.37); these findings were confirmed in Japanese populations.[159] p.G191R is thought to moderate intrapancreatic trypsin activity and thereby protects against chronic pancreatitis.[191]

A *PRSS1/PRSS2* hybrid, containing exon 1 and 2 from *PRSS2* and exons 3 to 5 from *PRSS1,* has been demonstrated in a French family.[174] This was thought to be disease causing by acting simultaneously as a 'quantitative' copy number mutation and a 'qualitative' missense mutation.

There has been reported CNV associated with both duplication and triplication of a 605kb segment on chromosome 7q35 associated with chronic pancreatitis. This has led to both increased copy number of *PRSS1* as well as *PRSS2*.[193]

1.4.2.2.3.3 PRSS3

Protease, serine, 3 (*PRSS3*) encodes a trypsinogen (mesotrypsin), a member of trypsin family. This enzyme is expressed in the brain and pancreas and is resistant to common trypsin inhibitors. Four transcript variants encoding different isoforms have been described for this gene.

Although *PRSS3* is thought to be a likely candidate gene for development for CP, several studies have failed to develop a clear causative relationship.[194,195] There has been a possible association with p.E32del, a frequent polymorphic variant, but has not been demonstrated to a statistically significant level.[195] No CNVs have not been demonstrated in *PRSS3*.[196]

1.4.2.2.3.4 Chymotrypsin C

Chymotrypsin C (*CTRC*) codes for a protein that promotes the degradation of trypsin and has been shown to be associated with idiopathic and alcoholic chronic pancreatitis.[197] The *CTRC* variants are known to exhibit diminished secretion and/or activity.[189]

One study identified two common variants and 19 rare variants in patients with idiopathic chronic pancreatitis.[198] These rare variants, when combined, were shown to be significantly higher in ICP than controls (12% vs. 1.1%; OR, 11.8; $p < 0.001$)).

p.R254W and p.K247_R254del were significantly overrepresented in patients with alcoholic, idiopathic or hereditary chronic pancreatitis (2.1% vs. 0.6% and 1.2% vs. 0.1% respectively).[197] Functional analysis of these and other associated *CTRC* variants showed impaired chymotrypsin C activity and/or reduced secretion.

1.4.3 The Genetics of Alcohol Metabolism

There are several key genes associated with both the oxidative and non-oxidative pathways of alcohol dependence.[199] Several of these genes have common polymorphisms and are linked to protection against the development of alcohol dependence, predominately through causing adverse reactions due to high acetaldehyde levels caused by the enzyme dysfunction.

1.4.3.1 Alcohol Metabolism and Alcohol Dehydrogenase

There are several different genes, and gene classes of alcohol dehydrogenase (Table 6). One group has claimed specific expression of *ALDH1A1*, *ALDH2*, *ADH1*, and *ADH5* in pancreas, although no other group has substantiated this.[200]

Table 6 Enzymes within the Alcohol Dehydrogenase (*ADH*) family

(Modified from Hurley 2012[125])

Gene Name and Common Haplotypes[$]	Gene Class	Protein Name	K_M(mM) for Ethanol	Primary Tissue
ADH1	I	α	4.0	Liver
*ADH2*1*	I	β_1	0.05	Liver, lung
*ADH2*2*		β_2	0.9	
*ADH2*3*		β_3	40	
*ADH3*1*	I	γ_1	1.0	Liver, stomach
*ADH3*2*	I	γ_2	0.6	
ADH4	II	π	30	Liver, cornea
ADH5	III	χ	>1000	Widely expressed
ADH6	V	*ADH6*	Unknown	Stomach
ADH7	IV	μ or σ	30	Liver, stomach

[$]Description of haplotypes is given in Appendix 10.1.

1.4.3.1.1 Alcohol Dehydrogenase 1A

Two common genetic variants have been identified in *ADH1A1*, *ADH1A1*2* (17bp deletion in the promoter region present in many different populations) and *ADH1A1*3* (3bp insertion in the promoter region only described in those of African descent); both polymorphisms have been associated with alcohol dependence.[201]

1.4.3.1.2 Alcohol Dehydrogenase 1B

The three most studied alleles of *ADH1B* are referred to as *ADH1B*1* (the reference allele, encoding β1 with arginine at both positions 48 and 370 in the amino acid chain), *ADH1B*2* (encoding β2 by p.R48H (*ADH1B*_rs1229984)), and *ADH1B*3* (encoding β3 p.R370C (*ADH1B*_rs2066702); Table 39).

*ADH1B*2* has been shown to be negatively associated with alcohol dependence, due to the problems of alcohol metabolism present in this population group.[202] The frequency of the *ADH1B*2* carriers has not been shown to be different in ACP than other alcohol dependent populations.[203]

1.4.3.1.3 Alcohol Dehydrogenase 1C

The two most studied polymorphism *ADH1C* are *ADH1C*1* and *ADH1C*2* (Appendix 10.1, Table 40). These two alleles differ at two sites, resulting in two amino acid changes: the enzyme encoded by *ADH1C*2* (γ2) has amino acid variation of p.R272Q (*ADH1C*_rs1693482) and p.I350V (*ADH1C*_rs698). The functional change in relation to p.I350V may be related to linkage disequilibrium with p.R48H within *ADH1B*.[204]

In a study of Polish populations, those homozygous for *ADH1C*2* were thought to be protected from chronic pancreatitis compared to other populations exposed to alcohol excess.[205] Other studies report no association of *ADH1C*1* with alcohol dependance (AD) in European samples.[206]

1.4.3.2 Alcohol metabolism and Aldehyde Dehydrogenase

An intron identified in *ALDH1A1*_rs8187974 may be associated with alcohol consumption behavior, but needs to be replicated to prove these data.[207] No other associations have been well described.

This is more common in individuals with a polymorphism in *ALDH2* (Appendix 10.1) that leads to low acetaldehyde oxidizing capacity, which is functionally significant even in the heterozygous form.[208] This polymorphism is prevalent in those of Chinese, Japanese, and Korean descent, leading to high leaves of intoxication in these populations after relatively little intake, meaning this variant is strongly protective against alcohol dependence. The polymorphism is not normally demonstrated in European or African populations.[209]

Other than the well-described polymorphism of *ALDH2*, there have not been any other disease associated SNPs described within the coding gene. *ALDH2*2* is common in oriental populations, being present in around 50% Chinese and Japanese populations.[127,128] *ALDH2*2* heterozygotes and particularly homozygotes show increased acetaldehyde levels after alcohol consumption and therefore experience significant negative physiological responses to alcohol.[210]

1.4.3.3 Alcohol metabolism and Cytochrome P450

There are several different polymorphisms of *CYP2E1* (Appendix 10.1, Table 41) which vary between populations.[211]

The *CYP2E1*1D* polymorphism is found more commonly in non-Caucasian populations, and has been shown to increase the risk of alcohol dependence.[212] This trend was also present in Caucasian populations, but did not reach significance.

There is a well described relationship with both alcohol dependence and ALD with *CYP2E1*5B* (also known as *CYP2E1*c2*), and to a lesser extent with *CYP2E1*5A*.[213]

1.4.3.4 Genes of Alcohol Metabolism Associated with Chronic Pancreatitis

Although there is a clear association between the consumption of alcohol and chronic pancreatitis, the sensitivity of the pancreas to injury from alcohol is dependent on other factors (environmental, dietary or genetic).[214] Specifically within the subgroup of patients with ACP, certain genetic polymorphisms have been identified within the genes commonly associated with alcohol metabolism that appear to predispose patients to the development of alcohol dependence and chronic pancreatitis.

The frequency of the *ADH1B*2* carriers has not been shown to be different in ACP than other alcohol dependent populations.[203]

Those homozygous for *ADH1C*2*, in a Polish population, have been shown in one study to be protected from chronic pancreatitis compared to controls exposed to alcohol excess and did not develop chronic pancreatitis.[205]

The common *ALDH2*2* has been shown to be more common in ACP (71%) than in alcoholics without signs of the disease (48%), but was statistically lower than in the normal population (76%) which was replicated in a similar study.[203,215]

Heterozygotes *CYP2E1*5A*/*CYP2E1*5B* were present only in patients consuming excessive amounts of ethanol, in 7% of patients with alcoholic cirrhosis and in 4.5% of those with ACP.[216] This has not been demonstrated in other studies but they may be insufficiently powered to identify these rare polymorphisms.[217,218] Carboxyl-ester lipase (*CEL*) has been examined previously in two different studies.[41,219] The original work, carried out in Japan, showed an association between both the absence of ACP and being homozygous for the normal 16-repeat alleles and an increase incidence of ACP in individuals with a repeat longer than 16-repeats.[219] However, when attempts were made to replicate this in a second Germany population, the findings were not substantiated.[41]

In addition to its established association with hypertriglyceridemia, the *LPL* polymorphism, p.S447X has been specifically linked with patients with a history of pancreatic calcification and steatorrhoea.[220]

APOC2 deficiency is an uncommon, autosomal dominant condition that is normally identified in childhood and can lead to pancreatitis.[221] There are individual reports of loss of function polymorphism such as p.V40T in *APOC2* or p.T133R in *APOA5* can lead to hypertriglyceridemia, which therefore can lead to pancreatitis.[222]

1.4.3.5 ABO Blood Group System

The ABO blood type is well recognized to be associated with some disease processes such as gastric cancer. In the case of pancreatic diseases, several studies have identified an association between those who developed pancreatic cancer and a SNP in linkage disequilibrium with the O blood group, giving a raised OR of 1.2 (95% confidence interval 1.12-1.28).[223,224]

Although there is some association with pancreatic cancer, other studies have failed to show an association with chronic pancreatitis.[153,225,226]

1.4.4 Genetics of Alcohol Liver Disease

Other than the established association of *ADH1B*1*, *ADH1C*2* and *ALDH2*1* with the risk of developing AD, there is no evidence that any of the commonly recognised enzymes involved in the metabolism of ethanol have any effect on the development of ALD.[227]

*CYP2E1*5B* (intronic SNPs *CYP2E1*_rs3813867 and *CYP2E1*_rs2031920) has been shown to be an associated factor with the development of ALD in both an Asian, and a Caucasian population.[213]

Interleukin 1B has been shown to have some association with the development of ALD in one study.[228] The SNP *IL1B*_rs16944 is present significantly more often in patients with alcoholic cirrhosis than in those with non-cirrhotic ALD ($p = 0.026$), heavy drinkers without ALD ($p = 0.001$), and HC ($p = 0.032$). The frequencies of *IL1B*_rs16944 ($p = 0.030$) and of *IL1B*_rs1143634 ($p = 0.027$) were both significantly higher in heavy drinkers without ALD than in patients with ALD. The haplotype, *IL1B*_rs16944/*IL1B*_rs1143634 was associated with the development of alcoholic cirrhosis ($p < 0.05$).

Several studies have shown a polymorphism within *PNPLA3* is associated with ALD, *PNPLA3*_rs738409 (p.I148M).[227] The presence of the minor SNP has been shown in four studies to significantly increase the OR of developing ALD.[229]

1.4.5 Methods for Assessing Genetic Variation

When assessing different modalities of genetic sequencing for their suitability for a specific project, several factors need to be taken into account: the type of disease processes (germline, somatic); the type of variation to be assessed; area to be assessed; time scale of the project; and budget of the project.

There are many different techniques that are currently available for genetic sequencing but each has different implications in terms of throughput, area to be sequenced, time, and cost. This is a field that is constantly evolving, but below are a description of the more traditional methods used over the last few decades, moving into Next Generation Sequencing (NGS) options which became available following the first whole genome sequencing in 2002.

1.4.5.1 Sanger Sequencing

The classical chain-termination method requires a single-stranded DNA template, a DNA primer, DNA polymerase and both normal and modified di-deoxynucleotidetriphosphates (ddNTPs), the latter of which terminate DNA strand elongation.[230] The different ddNTPs can be selectively fluorescently labelled. The various products are then measured and the sequence derived by ordering the lengths.

This system works well when small-scale projects require long continuous sequences, of over 500bp reads, or when the sequence to be read is largely unknown as there is no reference sequence to compare sequenced fragments to; otherwise it has been mostly superseded by other techniques.

1.4.5.2 Polymerase Chain Reaction (PCR) based approaches

There are a variety of techniques based on the selective ability to produce a product by PCR (e.g. allele specific PCR) or differential sizes of products from different alleles (e.g. Small Tandem Repeat analysis), which can be used to distinguish known alleles.

1.4.5.3 Single Nucleotide Polymorphism (SNP) arrays

SNP arrays potentially contain hundreds of thousands of probes that are designed to specifically hybridize with the complimentary section of DNA. Fluorescently labelled target sequences then bind to the probe. The total strength of the signal depends upon the amount of target sample binding to the probes allowing some quantitation, as well as the identity of the target sample sequence by binding to a probe with a known position on the chip.

SNP arrays are a common way of assessing SNPs over a large genetic area. Panels can be off-the-shelf or custom made to look at specific SNPs or genetic areas. They are commonly used for large-scale discovery experiments such as Gene-Wide Association Studies (GWAS) where hundreds of thousands of SNPs can be read on a single chip.

1.4.5.4 Next Generation Sequencing (NGS)

Following the first human genome being sequenced in 2000, there has been a range of different commercial sequencing machines that have become available to re-sequence the large section of the genome. These are based on massively parallel sequencing: the sequencing of many single fragments in individual reactions. These include products produced by Roche, Illumina, Applied Biosystems and Life Technologies that are described further in Table 7.

Depending on the accuracy required and whether germline or somatic mutations are to be identified, the depth of coverage must be taken into account with NGS. In the case of germline mutation, coverage of x20-x100 would be expected and this may be over x1000 in the case of somatic mutations.

1.4.5.4.1 Next Generation Sequencing Methods

Roche 454 uses pyrosequencing techniques to sequence DNA. It is a fast, high throughput machine, but is proportionally much more expensive to run than other similar products.

Both the HiSeq and MiSeq from Illumina use polymerase-based reactions to sequence. HiSeq processing time is over a week per run. It is good for sequencing a large amount of DNA from a limited number of samples, but is poor at multiplexing many samples. The MiSeq is the smaller quicker, benchtop version of the same technique.

Applied Biosystems SOLID has high throughput but long sequencing times and has the advantage of a low error rate.

The Ion Torrent™ uses a semi-conductor chip that detects hydrogen ions that are released during polymerization of DNA. Hydrogen ions are released when the appropriate next sequential base is incorporated, leading to a peak in amplitude; when two or more matching bases are incorporated sequentially, then the amplitude in increase proportionately.

The system has the advantages that it is relatively inexpensive to buy the machine and is quick to run. Its accuracy at detecting SNPs is much better than those of Indels or homopolymers (a sequence of identical bases).

Table 7 Comparison of the different properties of different Next Generation Sequence platforms

Data taken from manufactures websites and in correct as of 5th September 2014

Manufacturer	Platform	NGS chemistry	Read length	Run time	Gb per run	Accuracy	Cost per Mb	Pros	Cons
Roche	454	Pyrosequencing	700 bp	1day	0.45	0.999	$10 USD	Longer reads improve mapping in repetitive regions; fast runs	High reagent cost; high error rates in homo-polymers repeats
Illumina	HiSeq	Polymerase-based sequence-by-synthesis	100 bp	3–10 days	600 Gb	0.999	$0.07 USD	Currently the most widely used platform in the field	Low multiplexing capability of samples
Illumina	MiSeq	Polymerase-based sequence-by-synthesis	300 bp	27 hrs	8.5	0.999	$0.50 USD		
Applied Biosystems	SOLID	Ligation-based sequencing	50 bp	7–14 days	600 Gb	0.9994	$0.13 USD	Two-base encoding provides inherent error correction	Long run times
Life Technologies	Ion Torrent™	Ion semiconductor sequencing	200-400 bp	2 hrs	100-200 Mb	0.99	$1.00 USD	Low cost, speed	Difficulty with reading homopolymers

1.4.5.5 Sequence Capture

Whichever NGS method might be adopted, there needs to be some method of selection of the DNA to be sequenced, as whole genome sequencing is costly, time consuming and makes data analysis very challenging. In the case of small sections (up to 1000bp) traditional PCR may be appropriate, however, in most cases this will be too limited to cover the required area, and therefore sequence capture techniques should be used. These are commercially available, and can be used to isolate the necessary genes.

Two of the main modalities to achieve this are Haloplex and SureSelect. Both methods use similar processes. Haloplex works by first denaturing or shearing the DNA, then hybridizing with specific probes and barcodes for multiplexing. These specific regions are then captured using magnetic beads and a PCR amplification is undertaken to prepare the samples for downstream NGS.

1.4.6 Summary

A common type of variation in the human genome, accounting for phenotypical differences, are SNPs. At the current time there is a wide array of different techniques to obtain specific subsets of genetic data; whole genome sequencing is unfeasible, due to time, cost and difficulty with data analysis. Therefore a more focused approach is required. This can either be in the form of sampling of different SNPs throughout the genome (GWAS), targeting SNPs in specific areas such as in custom arrays or targeted sequencing of specific genetic areas (focused NGS). In addition other areas such as VNTR cannot be sequenced by these common methods and in these cases specific PCR techniques can be used with either Sanger sequencing or frequent analysis performed to assess the product.

When concentrating on specific areas of investigation, this can been done by analysis of a wide range of SNPs regardless of disease type (GWAS), a focus on SNPs thought to be associated with that disease processes, or a similar mechanism through custom arrays or selection of areas of interest based on a hypothesis of the mechanism of disease. The use of a variety of different but complimentary methods will give the most inclusive approach to identifying the role of genetic variations in predisposition to alcohol-related chronic pancreatitis.

2 Chapter 2: Aim and Objectives

2.1 *Aim*

- To determine the factors required to classify a chronic pancreatic population and identify genetic variations that may explain why only some alcoholics develop chronic pancreatitis.

2.2 *Objectives*

- Systematically review methods of diagnosis of chronic pancreatitis to assess the most effected method of diagnosing the disease and defining a disease population.
- To identify polymorphisms in selected genes associated with lipid and ethanol metabolism.
- To identify polymorphisms in selected genes associated with trypsinogen activation.

3 Chapter 3: Systematic Review of the Diagnosis of Chronic Pancreatitis

While clinical features can be indicative, consensus is lacking as to which diagnostic tests reliably confirm the presence or absence of CP.[12] Many tests have been used either individually or in combination, but no single gold standard has emerged. The most widely used reference tests have been the identification of ductal changes viewed by ERCP, now considered too high risk to perform as a diagnostic procedure, and histology that is usually only available in those with severe disease undergoing surgery.[62,231-233]

3.1 *Materials and Methods*

3.1.1 Data Sources and Search Strategy

The systematic review was carried out in reference to the Cochrane Handbook for Diagnostic Test Accuracy.[234] The author (MJ) and James Nicholson (JACN) independently performed searches of databases for publications from 1980 to 2012: MEDLINE (PubMed), Web of Science, and Cochrane Central Register of Controlled Trials & Database of Systematic Reviews (CENTRAL). The following terms were used to search the databases (where * is a wildcard): “chronic pancreatitis”[Title] AND ((“imaging” OR “etiolog*” OR “aetiolog*” OR “clinical” OR “history” OR “abdominal x-ray” OR “tube” OR “tubeless” OR “CCK” OR “CT” OR “topography” OR “tomography” OR “ultrasound*” OR “US” OR “USS” OR “endoscop*” OR “EUS” OR “ERCP” OR “magnetic” OR “MR” OR “MRI” OR “MR” OR “function” OR “PFT” OR “faecal elast*” OR “fecal elast*” OR “secretin” OR “Ca199” OR “Ca-199” OR “Ca19.9” OR “biomarker*” OR “guideline*”) AND (“diagnose*” OR “diagnostic*” OR “diagnosi*”)). Only full text articles in English were included. Abstracts were reviewed and potentially relevant papers were obtained and evaluated in detail by two independent authors; where there was failure of consensus, a third author adjudicated (Thomas Hanna, TH). Once the papers were selected a detailed review of referenced papers was carried out to obtain any studies that may have been missed in the original search.

3.1.2 Study Selection

Studies assessing the specificity and sensitivity of diagnostic tests in a population of patients with suspected chronic pancreatitis (classed as cohort), or in a group of known pancreatitis patients compared with either healthy controls or individuals with other gastrointestinal diseases (classed as case control) were eligible for inclusion.[235]

Exclusion criteria included: abstracts, reviews, letters and case reports; studies only including animals; studies in which the aim was not the diagnosis of chronic pancreatitis; studies aiming to differentiate mass forming CP from cancer; studies that did not attempt to measure diagnostic value of a test; studies not stating how the diagnosis of CP was arrived at and studies for which data could not be successfully extracted either from the presented data or following correspondence with the authors.

3.1.3 Data Extraction

MJ and TH independently extracted data using a predesigned form, and any discrepancies in data extraction were resolved by consensus (JACN). In studies where a cohort of patients with suspected CP were compared to a group of normal controls, data on normal controls was excluded and the study was classified as a cohort study.

3.1.4 Data Synthesis and Analysis

Data provided from different diagnostic tests were considered separately. When it was not possible to extract data from the paper, the source data was requested from the authors for use in the systematic review. In cases where data were presented giving both the pre-determined cut-off level for diagnosis and the optimum level for that study, only data using the pre-determined cut-off levels were used.

3.1.5 Quality Assessment

The quality of included studies was assessed using factors that would influence the outcome of diagnostic studies (Figure 5). Size of study was not included as this was taken into account by weighting of the studies in the model.

3.1.6 Statistical Analysis

Data are extracted from each study in terms of true positive (TP), false negative (FN), false positive (FP) and true negative (TN) frequencies by MJ and TH. Each study is summarised in the form of sensitivities and specificities with associated 95% confidence intervals. Meta-analyses techniques were carried out by Richard Jackson using a Bayesian Hierarchical Summary Receiver Operating Characteristic (HSROC) technique.[236] This approach calculates the position and shapes of the receiver operator curve for each diagnostic test and allows for variability both within and between studies. Diagnostic test is included as a covariate in the model as opposed to using different models for each test. This ensures that model summaries are accounted for within study variability as many studies report on more than one test. For the analysis of the full dataset, a two-step approach is carried out to account for study quality within the analysis. Here, studies with a quality score of one or two are considered as low quality studies and those with a score of three or greater as high quality studies. Low score studies are initially analysed with vague uninformative priors. The posterior results of the first stage are used to inform the analysis of the high quality studies through the prior distributions. In this second step, the results from the low quality study are penalised by increasing the posterior variances by a factor of two. This approach is equivalent to a power priors approach and ensures that higher quality studies have a larger effect upon the analysis.[237] For subgroup analysis, a simpler single step analysis is carried out due to the lack of data available. For the subgroup analyses of endoscopic data, cut-off values were included as covariates in the analysis.

Model summaries are presented in terms of summary sensitivity and specificity estimates with associated 95% credibility intervals for each summary statistic individually. The shape of the

credibility interval is determined by the observed correlation between model parameters and the size set to contain 95% of the observed posterior estimates. The diagnostic odds ratio, given by (sensitivity x specificity) / ((1-sensitivity) * (1-specificity)) is also presented as a summary measure.

Publication bias due to sample size is investigated by plotting the log diagnostic odds ratio against the effective sample size (ESS).[238] Analyses are carried out using the statistical packages WinBUGS and results compiled using R (version 3.01).[239,240] Parameter estimates are obtained via a Monte Carlo Markov Chain (MCMC) procedure (10000 draws with a thin of 20 following burn in and convergence).

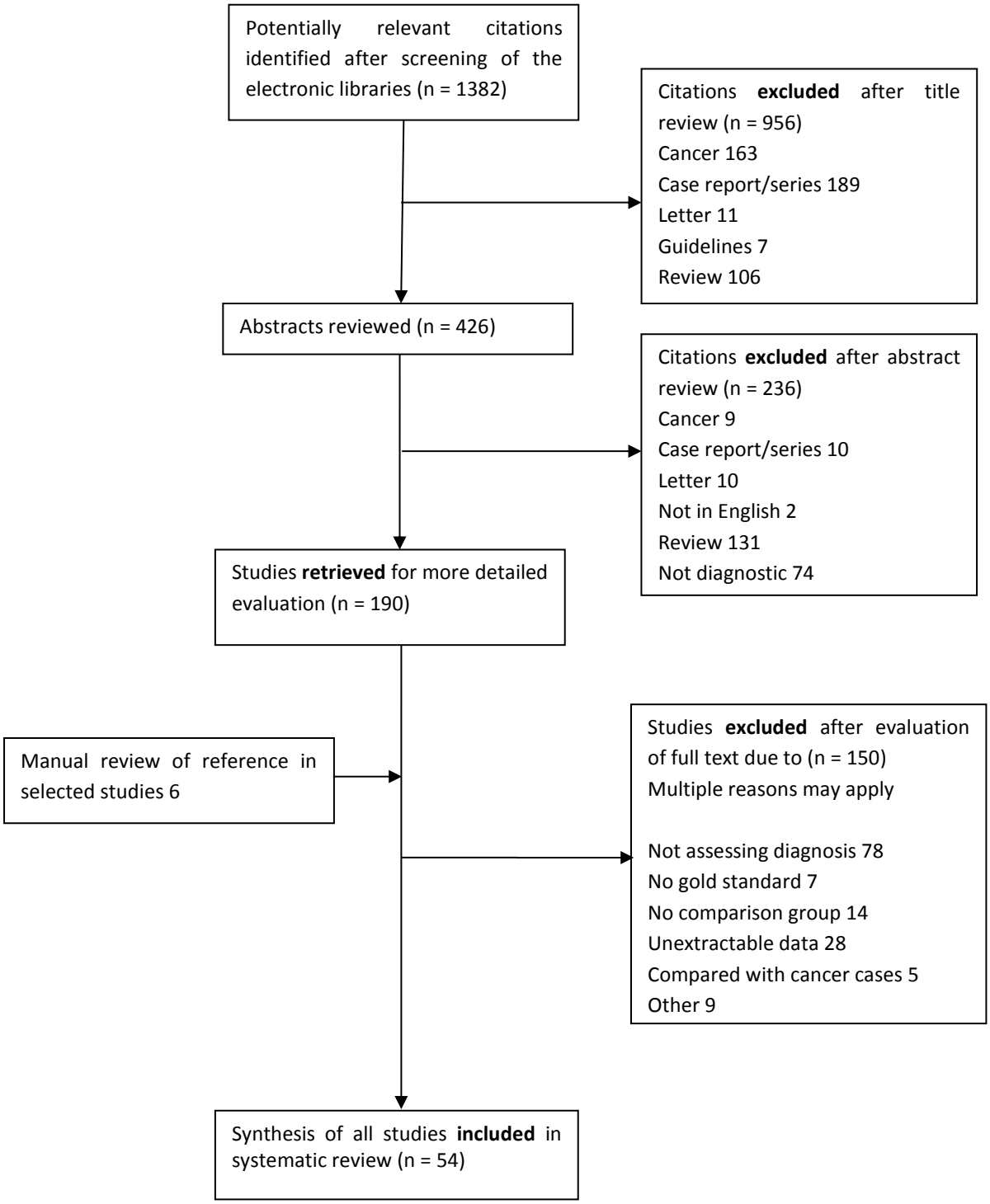


Figure 4 Flow diagram of studies considered in the systematic review

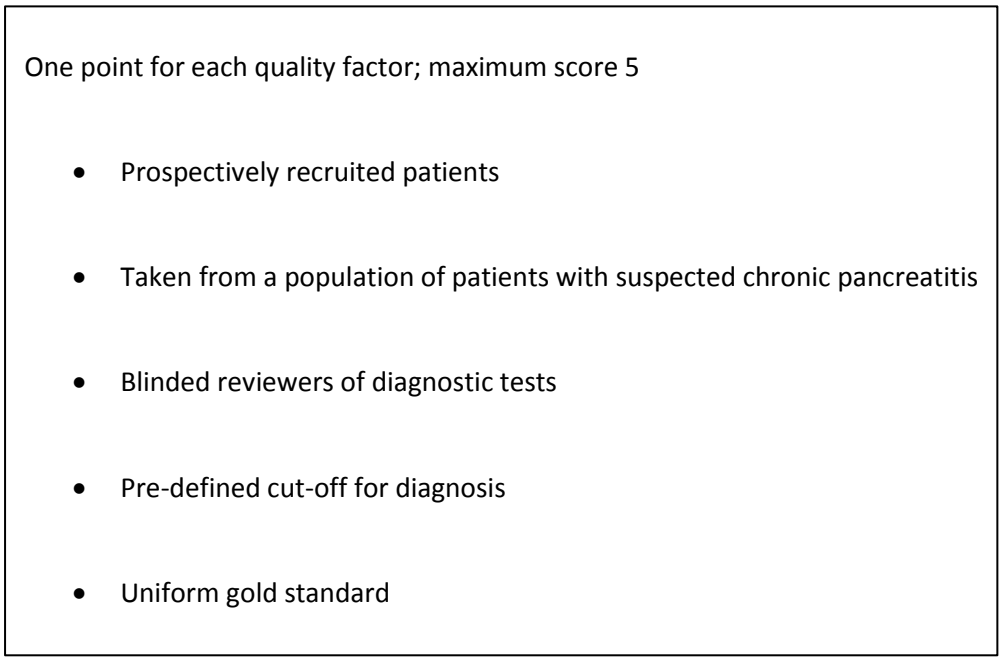


Figure 5 Method used for assigning quality of evidence for systematic review

3.2 *Results*

3.2.1 Population

Using the search criteria 1373 studies were identified, of which 190 were retrieved for detailed review; 47 of these met the inclusion/exclusion criteria (Figure 4). A further six studies were identified from papers cited within the references of the selected papers and one further study was added after the authors provided data that was previously unextractable.[241] In total, 54 studies were identified which assessed the use of a diagnostic tests against defined criteria (Table 8). For the diagnosis of CP in a population of individuals with and without CP this gave a total of 5287 individuals tested, of which 2298 (43%) had chronic pancreatitis. Of these studies 35 were cohort-type and 19 were case-control. In the cohort studies the pooled prevalence of chronic pancreatitis was 45% (95% CI, 40-50%); individual studies ranged from a prevalence of CP of 12% to 94%.

3.2.2 Gold Standards

Histology was used as the gold standard in nine of the published studies (two as the sole gold standard and seven in combination with other factors); and in all studies describing histology, tissue was obtained from resected pancreatic specimens. In those studies where the classification of histology was specifically stated, the Ammann fibrosis classification was used.[37]

ERCP was used as the gold standard in 40 (74%) of the studies and was either applied to all patients (19) or in a proportion of patients (21). Criteria for ERCP diagnosis of CP included the Marseille criteria, and criteria described by Kasugai; following the development of the Cambridge classification in 1984, the majority of studies use this classification (25).[61,242,243]

A mixed diagnostic standard was used to identify CP patients in 25 (46%) studies. Calcification was used as one of multiple factors in 8 (32%) of these studies.

3.2.3 Index Tests

Data are presented by modality of test in Table 9, with sensitivities and specificities for each individual tested provided. Investigations were broadly classified into pancreatic function tests (PFT; direct, serum, or faecal) or imaging (conventional or endoscopic). This saw ten, eight, two, four and two different diagnostic modalities being analysed respectively, with the addition of one study using FNA with EUS, which was the only study using pathology as the evaluated diagnostic method.

Table 10 shows those tests with three or more values, which could be pooled to provide combined sensitivities, specificities and diagnostic odds ratios (DOR; Figure 6).

3.2.4 Variation Over Time

Figures 7 and 8 show the variation of the examination of different diagnostic tests and gold standards over the decades. This demonstrates the decline of ERCP being used as a gold standard for the diagnosis of CP, with an increase in the use of EUS and MR as a suitable diagnostic modality.

3.2.5 Comparison of Specific Test

Several studies compared the utility of faecal elastase and faecal chymotrypsin in the diagnosis of CP. Both sensitivity and specificity were higher in pooled elastase results (Table 10 and Figure 6).

Five studies examined the use of MR, and most also assessed the effect of secretin in improving its diagnostic performance. Although secretin improved the specificity, it reduced the sensitivity of the test (Table 10 and Figure 6).

Endoscopic ultrasound was the most assessed diagnostic modality, although the diagnostic criteria varied between studies (Table 11). When the number of criteria required for a diagnosis was considered the sensitivity decreased, but the specificity increased with each extra criterion required (Figure 9 and Table 12).

3.2.6 Publication bias

Publication bias was assessed by both study size and quality score of each publication. There was no publication bias demonstrated in smaller studies (Figure 10, p=0.15), but poor quality studies showed higher diagnostic odds ratios (Figure 11, p=0.019).

Table 8 Details of included studies in systematic review

Study	Country	P/ R	Co/ CC	No. of pts[&]	No. CP pts (%)	Investigations tested	Gold standard	Consecutive patients	Blinded	Diagnostic Cut-off	Q
Ruddell 1981[244]	UK	P	CC	297	27 (9)	Serum Immunoreactive Trypsin	Lundh test[245]	No	Unclear	Set in paper	2
Stern 1981[246]	Australia	P	CC	83	50 (60)	Serum Pancreatic Polypeptide	PFT/Calc/ERCP/Op	No	Unclear	Pre-set	2
Valentini 1981[247]	Italy	P	Co	59	7 (12)	Duodenal Bicarbonate	ERCP	No	No	Set in paper	3
Noda 1983[248]	Japan	P	CC	262	79 (49)	Secretin-Pancreozymin test, Duodenal Dimethadione	Calc/Histology	No	Unclear	Set in paper	1
Enselv 1984[249]	Denmark	P	Co	80	25 (31)	Serum Immunoreactive Trypsin, Pancreatic Polypeptide, Isoamylase	PI/Calc/Previous AP	Yes	Unclear	Pre-set	3
Tait 1984[250]	Australia	P	CC	76	38 (50)	Serum Amylase, Pancreatic Isoamylase	ERCP/Calc/Histology	No	Unclear	Pre-set	2
Gaia 1985[251]	Italy	P	CC	98	40 (41)	Duodenal Lactoferrin	ERCP/Calc	Unclear	Unclear	Set in paper	1
Hamilton 1986[252]	UK	P	Co	18	6 (33)	Duodenal Bicarbonate, Trypsin	ERCP	Unclear	Yes	Pre-set	5
Heij 1987[253]	Netherlands	R	Co	69 (58)	36 (52)	Secretin CCK test	ERCP	Unclear	No	Optimised	1
Moller-Peterson 1988[254]	Denmark	P	Co	105	36 (34)	Serum Trypsin Immunoreactivity, Lipase, Pancreatic Isoamylase	ERCP/CT/AUS	Yes	Yes	Optimised	3
Bolondi 1989[255]	Italy	P	CC	33	15 (45)	Secretin AUS	History + PI	Yes	No	Not defined	1
Riedel 1991[256]	South Africa	P	CC	76	16 (21)	Faecal Chymotrypsin	ERCP/PFT	No	Unclear	Pre-set	2
Dominguez-Munoz 1993[257]	Germany	P	Co	296	167 (56)	Serum Amylase, Lipase, Immunoreactive Trypsin	ERCP/CT/ PFT	Yes	Unclear	Optimised	2
Dominguez-Munoz[258]	Germany	P	CC	144	90 (63)	Serum Pancreolauryl test	ERCP/CT/Clinical	No	Yes	Pre-set	3
Natterman 1993[77]	Germany	P	CC	94	51 (54)	EUS	ERCP	Unclear	Unclear	Not defined	2
Wiersema 1993[75]	USA	P	Co	69 (67)	19 (28)	EUS, Duodenal Bicarbonate	ERCP	Unclear	Yes	Pre-set	5
Bozkurt 1994[259]	Germany	P	Co	48	38 (79)	Duodenal Lipase, Amylase, Bicarbonate CT, AUS	ERCP	No	Yes	Pre-set	4
Buscail 1995[260]	France	P	CC	62	44 (71)	AUS, CT, EUS, ERCP	Histology/Calc/PI	Yes	Yes	Unclear	2
Dominguez-Munoz 1995[81]	Germany	P	CC	69	20 (29)	Serum Pancreolauryl	ERCP/CT/Clinical	No	Unclear	Pre-set	2
Lankisch 1996[261]	Germany	R	Co	202	110 (54)	Secretin-Pancreozymin test	ERCP	Unclear	Unclear	Not defined	2
Glasbrenner 1996[262]	Germany	P	CC	188	63 (34)	Serum Pancreolauryl test, Faecal Elastase, Chymotrypsin	ERCP	No	Unclear	Pre-set	3
Amann 1996[263]	USA	P	CC	36	14 (39)	Faecal Elastase	ERCP/Calc/Op/PI	No	Unclear	Pre-set	2
Kitagawa 1997[264]	Japan	P	Co	424(324	197 (61)	Secretin test	AUS/CT/ERP	No	Unclear	Set in paper	2
Katischinski 1997[265]	Germany	P	CC	33	11 (33)	Faecal Elastase, Chymotrypsin	AUS/CT/ERCP	No	Unclear	Pre-set	2
Lock 1997[266]	Germany	R	Co	60	40 (67)	Serum Pancreolauryl test	ERCP	Unclear	Unclear	Optimised	2
Sahai 1998[76]	USA	P	Co	126	96 (76)	EUS	ERCP	Yes	Yes	Optimised	5
Dominguez-Munoz 1998[267]	Germany	P	Co	271	127 (47)	Serum Pancreolauryl test	AUS/CT/ERCP	Yes	Yes	Pre-set	4
Catalano 1998[268]	USA	P	Co	80	38 (48)	EUS	ERCP/PFT	Yes	Yes	Pre-set	4

Gullo 1999[82]	Italy	P	CC	140	44 (31)	Faecal Elastase, Chymotrypsin	History/ERCP/AUS	No	Unclear	Optimised	2
Hastier 1999[269]	France	P	CC	104	14 (13)	EUS	ERCP	Yes	Unclear	Not defined	2
Pezzilli 2000[270]	Italy	P	CC	81	50 (62)	Serum Amylase, Trypsinogen, Lipase, Pancreatic Isoamylase, Elastase-1	ERCP/Calc/Histology/ Clinical	Unclear	Unclear	Pre-set	2
Hollerbach 2001[86]	Germany	P	Co	37	31 (84)	EUS with FNA	ERCP	No	Yes	Optimised	5
Hardt 2002[271]	Germany	P	Co	251(213	201 (94)	Faecal Elastase	ERCP	Yes	Yes	Pre-set	5
Kahl 2002[74]	Germany	P	Co	130	92 (71)	EUS	ERCP	No	Unclear	Pre-set	3
Keim 2003[272]	Germany	P	Co	212	45 (21)	Faecal Elastase	ERCP	Yes	Yes	Pre-set	5
Conwell 2003[273]	USA	P	Co	18	6 (33)	Duodenal bicarbonate	ERCP	Yes	Yes	Pre-set	4
Chowdhury 2005[274]	USA	R	Co	74	28 (38)	EUS	Duodenal Bicarbonate	Unclear	Yes	Optimised	2
Draganov 2005[275]	USA	P	Co	23 (19)	9 (47)	Standard secretin stimulation test, Intraductal secretin stimulation test	ERCP	No	Unclear	Optimised	3
Conwell 2007[276]	USA	R	Co	56	23 (41)	EUS	Duodenal Bicarbonate	Unclear	Yes	Pre-set	4
Conwell 2007[276]	USA	R	Co	36	17 (47)	Duodenal Bicarbonate	ERCP	No	No	Pre-set	4
Miyakawa 2007[277]	Japan	R	Co	106	41 (39)	EUS	ERCP/MR/CT/PFT	Unclear	Unclear	Not defined	2
Pungpapong 2007[278]	USA	P	Co	79	38 (48)	Duodenal Interleukin-8, EUS	ERCP/CT/MR/Histology	No	Yes	Pre-set	5
Pungpapong 2007[279]	USA	P	Co	99	40 (40)	MR, EUS	ERCP/Histology/Clinical	Yes	No	Pre-set	3
Varadarajulu 2007[280]	USA	P	Co	42	21 (50)	EUS	Histology	Yes	Yes	Optimised	4
Bilgin 2008[281]	Germany	R	Co	81	25 (31)	MR	Faecal Elastase	Yes	No	Not defined	2
Parsi 2008[282]	USA	P	Co	35	24 (69)	Duodenal Lipase	ERCP/MR/Histology/PI	Yes	Yes	Pre-set	4
Schlaudaff 2008[283]	Germany	P	Co	62	9 (15)	sMR	AUS/CT/ERCP	Yes	Yes	Not defined	3
Akisik 2009[284]	USA	R	Co	89	52 (58)	MR, sMR	ERCP/CT/Clinical	No	Yes	Optimised	2
Akisik 2009[285]	USA	P	CC	28	12 (43)	MR	ERCP	Unclear	Yes	Optimised	3
Stevens 2009[286]	USA	P	CC	84 (50)	14 (28)	Duodenal Lipase, EUS	CT	Yes	Yes	Optimised	3
Stevens 2009[287]	USA	P	Co	100	41 (41)	EUS (Radial/Linear)	Duodenal Bicarbonate	Yes	Yes	Optimised	4
Balci 2010[288]	USA	R	Co	36	12 (33)	MR, sMR	Duodenal Bicarbonate	No	No	Not defined	2
Albashir 2010[289]	USA	R	Co	25	21 (84)	EUS, Duodenal Bicarbonate	Histology	No	Yes	Pre-set	4
Law 2012[241]	USA	P	Co	69	16 (23)	Duodenal Lipase, Amylase, Bicarbonate	EUS	No	Unclear	Not defined	3

& - Values in brackets are the actual number of patients included in the results.

P – Prospective; R – Retrospective

CC – Case control; Co - cohort

PEI - (%6hr urinary PABA in 1st day)/(%6hr urinary PABA in 2nd day); PABA - Para-aminobenzoic acid

Calc – Calcifications

Q – Quality score as calculated from factors in Figure 5.

Op – underwent operation for chronic pancreatitis; AP – Acute pancreatitis

ERCP – Endoscopic retrograde cholangiopancreatography; AUS – Abdominal Ultrasound; MR – Magnetic resonance; sMR – Secretin enhanced Magnetic resonance; CT – Computer Tomography; EUS – Endoscopic Ultrasound; PFT – Pancreatic function test; PI – Pancreatic insufficiency

Table 9 Individual tests separately extracted from each study, grouped by modality with sensitivity and specificity with 95% confidence intervals

Test	Diagnostic test	Study	Gold standard	Criteria	Cut-off diagnosis	TP	FN	FP	TN	Sensitivity (CI 95%)	Specificity (CI 95%)
Direct Pancreatic function Test	Amylase	Bozkurt 1994[259]	ERCP	Cambridge≥1[61]	>5000 IU/h	27	11	0	10	0.71 [0.54, 0.84]	1.00 [0.66, 1.00]
		Law 2012[241]	EUS	Wiersema[75] ≥5	>5000 IU/L	8	8	10	43	0.50 [0.26, 0.74]	0.81 [0.68, 0.90]
	Lactoferrin (LF)	Gaia 1985[251]	ERCP/Radiology	Kasugai[243]	-	29	11	5	53	0.73 [0.56, 0.85]	0.91 [0.80, 0.97]
	LF/Chymotrypsin				-	34	6	5	53	0.86 [0.71, 0.94]	0.91 [0.80, 0.97]
	LF/Lipase				-	38	2	3	55	0.95 [0.82, 0.99]	0.95 [0.85, 0.99]
	Dimethadione	Noda 1983[248]	Histology/Calc	Japan 1971[290]	<32.8 mg/h	77	2	12	71	0.97 [0.90, 1.00]	0.86 [0.76, 0.92]
	Interleukin-8	Pungpapong 2007[279]	ERCP/CT/MR/Histology	Cambridge[61]	≥20 pg/ml	18	20	3	38	0.47 [0.31, 0.64]	0.93 [0.79, 0.98]
	Trypsin	Hamilton 1986[252]£	ERCP	Cambridge[61]	<50 mg/h	2	4	0	12	0.33 [0.06, 0.76]	1.00 [0.70, 1.00]
	Bicarbonate	Valentini 1981[247]	ERCP	Any criteria	-	2	5	21	30	0.29 [0.05, 0.70]	0.59 [0.44, 0.72]
		Hamilton 1986[252]£	ERCP	Cambridge[61]	<20mmol/h	2	4	0	12	0.33 [0.06, 0.76]	1.00 [0.70, 1.00]
		Wiersema 1993[75]	ERCP	Cambridge[61]	<105meq/dl	3	1	6	6	0.75 [0.22, 0.99]	0.50 [0.22, 0.78]
		Bozkurt 1994[259]	ERCP	Cambridge[61]	<30mmol/h	31	7	0	10	0.82 [0.65, 0.92]	1.00 [0.66, 1.00]
		Conwell 2003[273]	ERCP	Cambridge[61]	<80 mEq/L	5	1	5	7	0.83 [0.36, 0.99]	0.58 [0.29, 0.84]
		Draganov 2005[275]	ERCP	Cambridge[61]	SST <80 mEq/L	5	4	4	6	0.56 [0.23, 0.85]	0.60 [0.27, 0.86]
					IDST <105 mEq/L	10	0	4	5	1.00 [0.66, 1.00]	0.56 [0.23, 0.85]
		Conwell 2007[276]	ERCP	Cambridge[61]	<80 mEq/L	16	1	4	15	0.94 [0.69, 1.00]	0.79 [0.54, 0.93]
		Stevens 2009[286]	CT	*	<80 mEq/L	13	1	11	25	0.93 [0.64, 1.00]	0.69 [0.52, 0.83]
		Albashir 2010[289]	Histology	Ammann[37]	<80 mM	12	2	1	2	0.86 [0.56, 0.97]	0.67 [0.13, 0.98]
		Law 2012[241]	EUS	Wiersema[75] ≥5	<80 mM[286]	9	7	11	42	0.56 [0.31, 0.79]	0.79 [0.66, 0.89]
	Lipase	Bozkurt 1994[259]	ERCP	Cambridge[61]	<100000 IU/h	38	0	0	10	1.00 [0.89, 1.00]	1.00 [0.66, 1.00]
		Parsi 2008[282]	ERCP/MR/PI/Histology	Cambridge[61]	<800 IU/mL	23	1	7	4	0.96 [0.77, 1.00]	0.36 [0.12, 0.68]
		Stevens 2009[286]	CT	*	<300000 IU/L	11	3	13	23	0.79 [0.49, 0.94]	0.64 [0.46, 0.79]
		Law 2012[241]	EUS	Wiersema[75] ≥5	<300000 IU/L[286]	16	0	48	5	1.00 [0.76, 1.00]	0.09 [0.03, 0.21]
	Secretin CCK	Heij 1987[253]	ERCP	Kasugai[243]	Z score	30	6	2	20	0.83 [0.67, 0.93]	0.91 [0.69, 0.98]
	Secretin test~	Kitagawa 1997[264]	AUS/CT/ERP	Japan 1995[291]	See paper[264]	118	17	6	121	0.87 [0.80, 0.92]	0.95 [0.90, 0.98]
	SPT[292]	Noda 1983[248]	Histology/Calc	Japan 1971[290]	-	70	9	28	55	0.89 [0.79, 0.94]	0.66 [0.55, 0.76]
		Lankisch 1996[261]	ERCP	Cambridge[61]	HCO_3<70mEq/l[292]	87	23	7	85	0.79 [0.70, 0.86]	0.92 [0.84, 0.97]
Serum	Amylase	Tait 1984[250]	ERCP/Calc/Histology	Kasugai[243]	>198 IU/L	7	31	0	38	0.18 [0.08, 0.35]	1.00 [0.89, 1.00]
		Dominguez-Munoz 1993[257]	ERCP/CT/Fluorescence	Cambridge[61]	>240 IU/L	37	130	8	121	0.22 [0.16, 0.29]	0.94 [0.88, 0.97]
		Pezzilli 2000[270]	ERCP/Calc/Histology	-	>392 IU/L	13	37	4	26	0.26 [0.15, 0.41]	0.87 [0.68, 0.96]
	Elastase-1	Pezzilli 2000[270]	ERCP/Calc/Histology	-	>343 ng/dl	25	25	6	24	0.50 [0.36, 0.64]	0.80 [0.61, 0.92]
	Trypsinogen	Pezzilli 2000[270]	ERCP/Calc/Histology	-	<15 ng/ml	14	36	0	30	0.28 [0.17, 0.43]	1.00 [0.86, 1.00]
	Trypsin Immunoreactivity	Ruddell 1981[244]	Lundh test[245]	9.5µEqH$^+$/ml/min	<117 µg/l	7	20	11	259	0.26 [0.12, 0.47]	0.96 [0.93, 0.98]
		Enselv 1984[249]	PI/Calc/Previous AP	Marseille[293]	<140 pg/ml	10	15	2	53	0.40 [0.22, 0.61]	0.96 [0.86, 0.99]
		Moller-Peterson 1988[254]	CT/AUS/ERCP	Marseille[293]	<110 mcg/l	16	20	2	67	0.44 [0.28, 0.62]	0.97 [0.89, 0.99]
		Dominguez-Munoz 1993[257]	ERCP/CT/PFT	Cambridge[61]	<80 ng/ml	34	133	2	127	0.20 [0.15, 0.27]	0.98 [0.94, 1.00]

	Lipase	Moller-Peterson 1988[254]	CT/AUS/ERCP	Marseille[293]	<7.0 mcg/l	11	25	1	68	0.31 [0.17, 0.48]	0.99 [0.91, 1.00]
		Dominguez-Munoz 1993[257]	ERCP/CT/PFT	Cambridge[61]	<8 ng/ml	54	113	10	119	0.32 [0.25, 0.40]	0.92 [0.86, 0.96]
		Pezzilli 2000[270]	ERCP/Calc/Histology	-	<8IU/L	7	43	1	29	0.14 [0.06, 0.27]	0.97 [0.81, 1.00]
	Pancreatic polypeptide	Stern 1981[246]	PFT/ERCP/Calc/Op	-	Peak/basal ratio<5	45	5	3	30	0.90 [0.77, 0.96]	0.91 [0.75, 0.98]
		Enselv 1984[249]	PI/Calc/Previous AP	Marseille[293]	<50 pg/ml	11	14	3	52	0.44 [0.24, 0.65]	0.95 [0.84, 0.99]
	Isoamylase	Enselv 1984[249]	PI/Calc/Previous AP	Marseille[293]	<43 U/L	12	13	1	54	0.48 [0.28, 0.68]	0.98 [0.89, 1.00]
		Tait 1984[250]	ERCP/Calc/Histology	Kasugai[243]	<36 IU/L	19	19	2	36	0.50 [0.34, 0.66]	0.95 [0.81, 0.99]
		Moller-Peterson 1988[254]	CT/AUS/ERCP	Marseille[293]	<36 U/l	14	22	4	65	0.39 [0.24, 0.56]	0.94 [0.85, 0.98]
		Dominguez-Munoz 1993[257]	ERCP/CT/PFT	Cambridge[61]	<40 U/L	45	122	5	124	0.27 [0.20, 0.34]	0.96 [0.91, 0.99]
		Pezzilli 2000[270]	ERCP/Calc/Histology	-	<27 IU/L	6	44	0	30	0.12 [0.05, 0.25]	1.00 [0.86, 1.00]
	Pancreolauryl (Fluorescein)	Glasbrenner 1996[262]	ERCP	Not stated	≤4.5 mcg/ml	36	15	17	49	0.71 [0.56, 0.82]	0.74 [0.62, 0.84]
		Dominguez-Munoz 1993[258]	ERCP/CT/Clinical	Cambridge[61]	≤4.5 mcg/ml	75	15	5	49	0.83 [0.74, 0.90]	0.91 [0.79, 0.97]
		Dominguez-Munoz 1995[81]	ERCP/CT/Clinical	Cambridge[61]	≤4.5 mcg/ml	14	6	10	26	0.70 [0.46, 0.87]	0.72 [0.55, 0.85]
		Lock 1997[266]	ERCP	Cambridge[61]	≤4.5 mcg/ml	27	13	10	10	0.68 [0.51, 0.81]	0.50 [0.28, 0.72]
		Dominguez-Munoz 1998[267]	AUS/CT/ERCP	[294, 295]	≤4.0 mg/L	101	26	22	122	0.80 [0.71, 0.86]	0.85 [0.78, 0.90]
Faecal	Elastase	Dominguez-Munoz 1995[81]	ERCP/CT/Clinical	Cambridge[61]	<200 mcg/g	14	6	6	41	0.70 [0.46, 0.87]	0.87 [0.74, 0.95]
		Amann 1996[263]	History/ERCP/PFT/Calc/Op	-	<200 mcg/g	10	4	4	18	0.71 [0.42, 0.90]	0.82 [0.59, 0.94]
		Glasbrenner 1996[262]	ERCP	-	<200 mcg/g	50	13	28	97	0.79 [0.67, 0.88]	0.78 [0.69, 0.84]
		Katischinski 1997[265]	AUS/CT/ERCP	-	<200 mcg/g	7	4	1	21	0.64 [0.32, 0.88]	0.95 [0.75, 1.00]
		Gullo 1999[82]	History/ERCP/AUS	Cambridge[61]	<190 mcg/g	34	10	4	94	0.77 [0.62, 0.88]	0.96 [0.89, 0.99]
		Hardt 2002[271]	ERCP	Cambridge[61]	<200 mcg/g	91	110	3	9	0.45 [0.38, 0.52]	0.75 [0.43, 0.93]
		Keim 2003[272]	ERCP	Cambridge[61]	scheBo 200mcg/g	31	14	38	129	0.69 [0.53, 0.81]	0.77 [0.70, 0.83]
					BIOSERV200mcg/g	35	10	40	127	0.78 [0.63, 0.88]	0.76 [0.69, 0.82]
	Chymotrypsin	Riedel 1991[256]	ERCP/PFT	-	>5 U/g	15	1	32	28	0.94 [0.68, 1.00]	0.47 [0.34, 0.60]
		Dominguez-Munoz 1995[81]	ERCP/CT/Clinical	Cambridge[61]	>3 U/g	8	12	3	33	0.40 [0.20, 0.64]	0.92 [0.76, 0.98]
		Glasbrenner 1996[262]	ERCP	-	>3 U/g	30	33	22	103	0.48 [0.35, 0.60]	0.82 [0.74, 0.88]
		Katischinski 1997[265]	AUS/CT/ERCP	-	>3 U/g	3	8	1	21	0.27 [0.07, 0.61]	0.95 [0.75, 1.00]
		Gullo 1999[82]	History/ERCP/AUS	Cambridge[61]	>6 U/g	25	19	15	82	0.57 [0.41, 0.71]	0.85 [0.75, 0.91]
		Keim 2003[272]	ERCP	Cambridge[61]	>13.2 U/g	26	19	79	88	0.58 [0.42, 0.72]	0.53 [0.45, 0.60]
Imaging	AUS	Bozkurt 1994[259]	ERCP	Cambridge[61]	Any changes	33	5	5	5	0.87 [0.71, 0.95]	0.50 [0.20, 0.80]
		Buscail1995[260]	Calc/Histology/PI	-	Any changes	25	5	19	13	0.83 [0.65, 0.94]	0.41 [0.24, 0.59]
	sAUS	Bolondi 1989[255]£	History + PI	Gullo[296]	>50%MPD change	8	7	2	16	0.53 [0.27, 0.78]	0.89 [0.64, 0.98]
	CT	Bozkurt 1994[259]	ERCP	Cambridge[61]	Any changes	33	5	4	6	0.87 [0.71, 0.95]	0.60 [0.27, 0.86]
		Buscail 1995[260]	Calc/Histology/PI	-	Any changes	33	1	11	17	0.97 [0.83, 1.00]	0.61 [0.41, 0.78]
	MR	Pungpapong 2007[279]	ERCP/Histology/Clinical	Cambridge[61]	Paper specific	37	3	4	55	0.93 [0.79, 0.98]	0.93 [0.83, 0.98]
		Schlaudraff 2008[283]	AUS/CT/ERCP	-	[&]	6	3	4	49	0.67 [0.31, 0.91]	0.92 [0.81, 0.98]
		Bilgin 2008[281]	Faecal elastase	<200mcg/g	Cambridge[61]	21	4	31	46	0.84 [0.63, 0.95]	0.60 [0.48, 0.71]
		Akisik 2009[284]	ERCP/CT/Clinical	Cambridge[61]	ADC179x$10^{-5}$$mm^2$/s	37	13	13	24	0.74 [0.60, 0.85]	0.65 [0.47, 0.79]
		Balci 2010[288]	Bicarbonate	<80mEq/L	Cambridge[61]	7	5	9	15	0.58 [0.29, 0.84]	0.63 [0.41, 0.80]

sMR	Schlaudraff 2008[283]	AUS/CT/ERCP	-	[&]	6.5	2.5	2	51	0.72 [0.35, 0.94]	0.96 [0.86, 1.00]
	Akisik 2009[284]	ERCP/CT/Clinical	Cambridge[61]	ADC 210x10^{-5}mm^2/s	33	19	7	29	0.63 [0.49, 0.76]	0.81 [0.63, 0.91]
	Akisik 2009[285]	ERCP	Cambridge[61]	ADC 220x10^{-5}mm^2/s	13	0	4	11	1.00 [0.72, 1.00]	0.73 [0.45, 0.91]
	Balci 2010[288]	Bicarbonate	<80mEq/L	Duodenal filling	12	0	0	24	1.00 [0.70, 1.00]	1.00 [0.83, 1.00]
ERCP	Buscail 1995[260]	Histology/Calc/PI	-	Any changes[295]	19	7	0	18	0.73 [0.52, 0.88]	1.00 [0.78, 1.00]
EUS	Natterman 1993[77]	ERCP	Cambridge[61]	≥1[77]	50	1	27	36	0.98 [0.88, 1.00]	0.57 [0.44, 0.69]
	Wiersema 1993[75]	ERCP	Cambridge[61]	≥3[75]	19	0	10	38	1.00 [0.79, 1.00]	0.79 [0.65, 0.89]
	Buscail 1995[260]	Calc/Histology/PI	-	≥1[260]	39	5	0	18	0.89 [0.75, 0.96]	1.00 [0.78, 1.00]
	Sahai 1998[76]	ERCP	Cambridge[61]	Radial ≥4[76]	60	36	9	21	0.63 [0.52, 0.72]	0.70 [0.50, 0.85]
				Radial ≥6	36	60	1	29	0.38 [0.28, 0.48]	0.97 [0.81, 1.00]
	Catalano 1998[268]	ERCP/ST/PPJ	Cambridge[61]	≥3[268]	36	0	27	17	1.00 [0.88, 1.00]	0.39 [0.25, 0.54]
	Hastier 1999[269]	ERCP	Kasugai[243]	≥1[297]	13	1	17	40	0.93 [0.64, 1.00]	0.70 [0.56, 0.81]
	Hollerbach 2001[86]	ERCP	Cambridge[61]	≥1[86]	30	1	2	4	0.97 [0.81, 1.00]	0.67 [0.24, 0.94]
	Kahl 2002[74]£	ERCP	Cambridge[61]	≥1[75]	92	0	36	6	1.00 [0.95, 1.00]	0.14 [0.06, 0.29]
	Chowdhury 2005[274]	IDST	<80 mEq/L	≥4[274]	16	12	16	30	0.57 [0.37, 0.75]	0.65 [0.50, 0.78]
	Pungpapong 2007[279]	ERCP/Histology/Clinical	Cambridge[61]	≥4[279]	26	14	6	53	0.65 [0.48, 0.79]	0.90 [0.79, 0.96]
	Conwell 2007[298]	Bicarbonate	<80 mEq/L	≥6[298]	12	34	0	10	0.26 [0.15, 0.41]	1.00 [0.66, 1.00]
	Miyakawa 2007[277]	ERCP/MR/CT/PFT	Japan 1995[291]	≥1[277]	33	8	19	46	0.80 [0.65, 0.91]	0.71 [0.58, 0.81]
	Varadarajulu 2007[280]	Histology	Ammann[37]	Radial ≥4[76]	19	2	3	18	0.90 [0.68, 0.98]	0.86 [0.63, 0.96]
				Radial ≥6	9	12	0	21	0.43 [0.23, 0.66]	1.00 [0.81, 1.00]
	Stevens 2009[286]	CT	*	Radial≥4[76]	14	0	5	31	1.00 [0.73, 1.00]	0.86 [0.70, 0.95]
	Stevens 2009[287]	Bicarbonate	<80mmol/L	Radial ≥4[76]	28	13	3	56	0.68 [0.52, 0.81]	0.95 [0.85, 0.99]
				Radial ≥6	17	24	0	51	0.41 [0.27, 0.58]	1.00 [0.91, 1.00]
				Linear ≥4	18	23	3	56	0.44 [0.29, 0.60]	0.95 [0.85, 0.99]
				Linear ≥6	13	28	1	58	0.32 [0.19, 0.48]	0.98 [0.90, 1.00]
	Albashir 2010[289]	Histology	Ammann[37]	≥4[76]	16	3	0	4	0.84 [0.60, 0.96]	1.00 [0.40, 1.00]
EUS FNA	Hollerbach 2001[86]	ERCP	Cambridge[61]	Feteke[87]	24	0	1	2	1.00 [0.83, 1.00]	0.67 [0.13, 0.98]

~ The total volume, total amylase output, and maximal bicarbonate concentration were measured in each sample during a 60-min period after injection of secretin.

* Calcification/PD dilation/atrophy

[$] Main pancreatic duct dilation in the absence of structural obstruction, dilated side branches, intraductal stones, ductal irregularity, reduced T1-signal intensity, atrophy

& pancreatic duct stenosis or dilatation, dilatation of the ductal side branches, presence of pseudocysts, and extrapancreatic abscess formation.

1- slight, output of only one or more enzymes impaired; 2 - moderate, bicarbonate concentration and enzyme output reduced, stool fat excretion still normal; and 3 - severe, abnormal SPT result plus steatorrhoea

£ - MCP – Minimal change pancreatitis

IDST - Intraductal secretin stimulation test; SPT - Secretin-pancreozymin test

CCK – Cholecystokinin; PEI - PABA excretion index; PABA - Para-aminobenzoic acid

PI – Pancreatic insufficiency

ERCP – Endoscopic retrograde cholangiopancreatography; AUS – Abdominal Ultrasound; sAUS – Secretin Abdominal Ultrasound; MR – Magnetic resonance; sMR – Secretin Magnetic resonance; CT – Computer Tomography; EUS – Endoscopic Ultrasound; PFT – Pancreatic function test; PI – Pancreatic insufficiency

ADC - Apparent Diffusion Coefficient; FNA – Fine needle aspiration; PI – Pancreatic insufficiency; ST – standard secretin test; PPJ – pure pancreatic juice test

Table 10 Pooled sensitivity and specificity of tests to diagnose chronic pancreatitis where at least three studies were available

Study type	Pooled sensitivity (95% CI)	Pooled specificity (95% CI)
Duodenal bicarbonate	0.78 (0.61, 0.90)	0.72 (0.59, 0.87)
Lipase	0.96 (0.76, 1.00)	0.57 (0.23, 0.90)
Serum Amylase	0.40 (0.16, 0.80)	0.93 (0.74, 0.99)
Serum Trypsin Immunoreactivity	0.33 (0.20, 0.57)	0.97 (0.88, 0.99)
Serum Lipase	0.28 (0.13, 0.63)	0.95 (0.80, 0.99)
Serum Isoamylase	0.35 (0.22, 0.62)	0.96 (0.92, 0.98)
Serum Pancreolauryl	0.75 (0.60, 0.86)	0.78 (0.58, 0.89)
Faecal Elastase	0.69 (0.55, 0.81)	0.85 (0.75, 0.91)
Faecal Chymotrypsin	0.54 (0.20, 0.85)	0.78 (0.51, 0.95)
MR	0.79 (0.47, 0.92)	0.77 (0.51, 0.94)
sMR	0.87 (0.47, 0.99)	0.88 (0.58, 0.98)
EUS	0.87 (0.72, 0.95)	0.82 (0.68, 0.91)

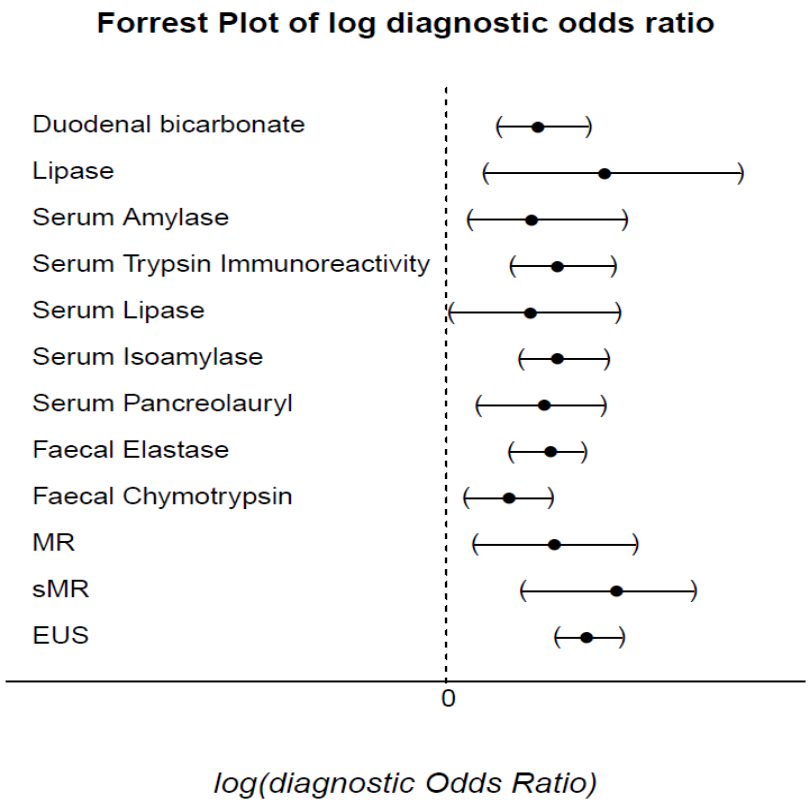


Figure 6 Forest plot showing pooled diagnostic odds rations for individual tests where at least three studies were available

Table 11 Evaluation of the variation of EUS criteria employed between different studies

		Parenchymal					Ductal							Other
Number of Criteria		**Hypo-echoic foci**	**Hyper-echoic foci**	**Cysts**	**Lobularity**	**Micro-calcifications**	**Hyperechoic strands**	**Hyperechoic duct margins**	**Irregular calibre MPD**	**Dilation of MPD**	**Dilatation of side branches**	**Calculi**	**Narrow Duct**	
Nattermann 1993[77]	≥1	Yes	Yes	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes	No	No
Wiersema 1993[75]	≥3	Yes	>3 mm	>3mm	Yes	No	No	Yes	Irregular contour	Yes	Yes	Yes	Yes	No
Buscail 1995[260]	≥1	Hetero-geneous	>3 mm	Yes	No	No	No	No	No	No	No	Yes	No	No
Sahai 1998[76]	-	No	1-2mm	>2mm	2-5mm lobules	No	Yes	Yes	Yes	>3mm head, >2mm body, >1mm tail	Viable side braches	Yes	No	No
Catalano 1998[268]	≥3	Hetero-geneous	Yes	> 5 mm	No	No	Septa	Yes	Yes	>3mm	Side-branch ectasia	Yes	No	No
Hastier 1999[269]	≥1	No	No	Yes	HC	Yes	No	Yes	Duct wall irregular	>3mm increased diameter	Yes	Stones or protein plugs	No	~
Hollerbach 2001[86]	≥1	No	Yes	Pseudocysts	Yes	Yes	No	No	?	Yes	No	Yes	Yes	No
Chowdhury 2005[274]	-	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Viable side braches	No	No	No
Conwell 2007[298]	≥6	No	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	No	No
Miyakawa 2007[277]	≥1	No	Yes	No	Yes	No	Yes	No	Yes	No	No	No	No	No
Pungpapong 2007[279]	≥4	No	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	No	No
Rosemont Criteria 2009[78]	§	No	MajorA (shadow); Minor (w/o shadow)	Minor	MajorB (HnC); Minor (w/o HnC)	No	Minor	Minor	Minor	Minor	Minor (≥1mm)	Major A	No	No

§Rosemont criteria - Consistent -1 MajorA + ≥3 Minor; 1 MajorA + MajorB; or 2 MajorA. Suggestive 1 MajorA + <3 Minor; 1 MajorB ≥3 Minor; ≥5 minor. Indeterminate 3-4 Minor; MajorB +<3 Minor

HnC – Honeycombing

~ Other complications - Microcyst formation; Vascular thrombosis; Biliary stenosis

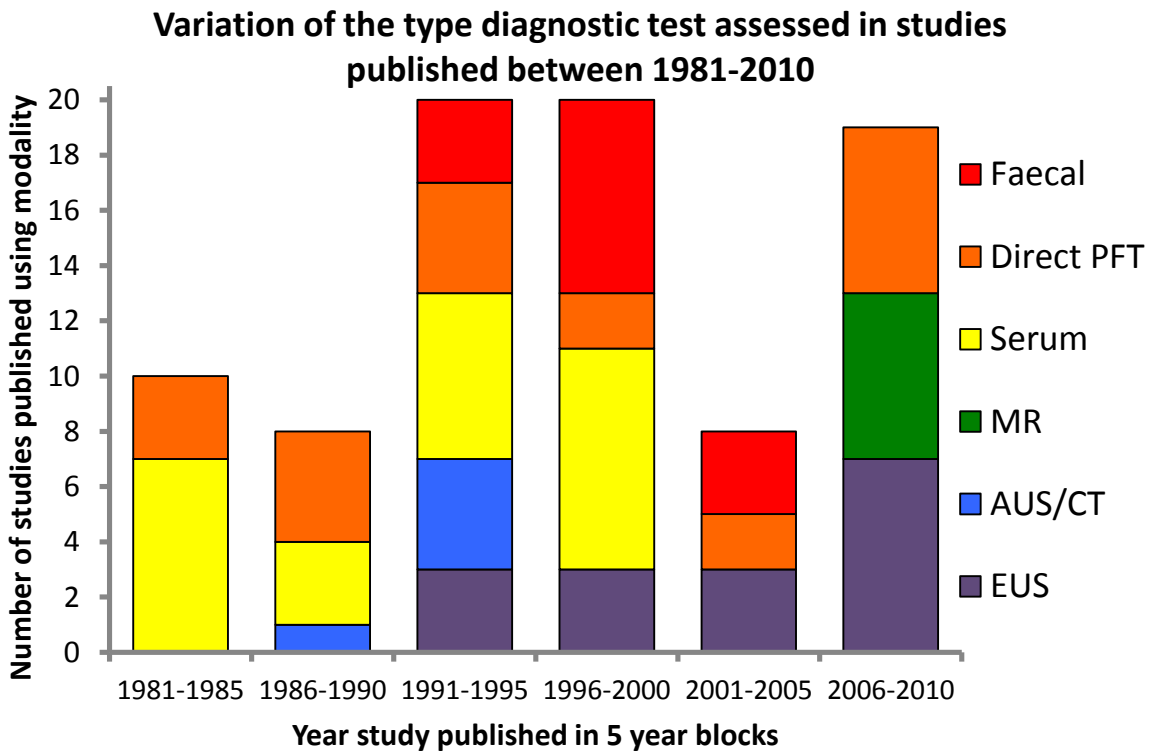


Figure 7 Variation of test assessed for diagnosing chronic pancreatitis over time

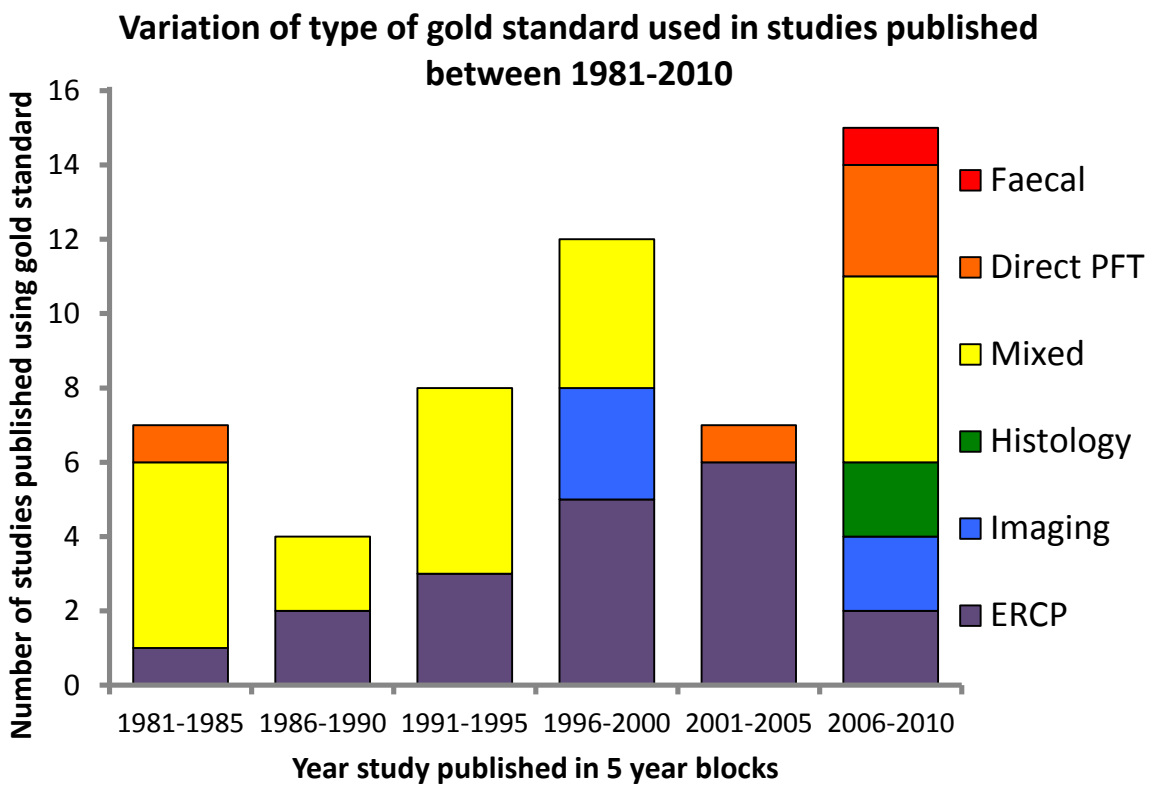


Figure 8 Variation of gold standard used for diagnosing chronic pancreatitis over time

Imaging includes a combination of AUS, CT and ERCP

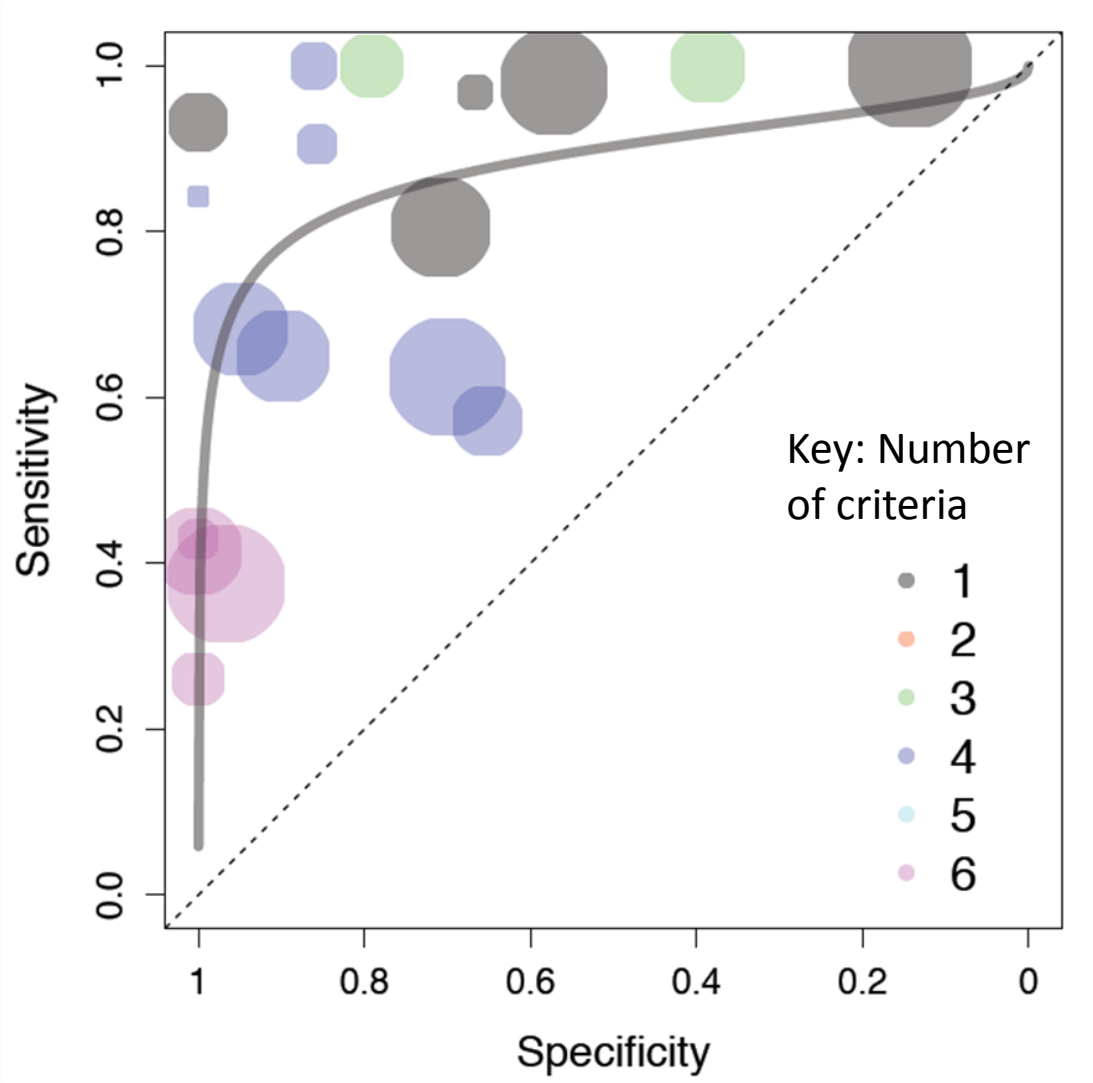


Figure 9 Pooled studies for EUS including sensitivity and specificity by cut-off point

Circle diameter represents sample size and colour represented the minimum number of EUS criteria needed to diagnosis chronic pancreatitis. ROC curve developed from pooled sensitivities and specificities of the different cut-off levels.

Table 12 Variation of sensitivity and specificity using different numbers of EUS diagnostic criteria

Cut-off level	Sensitivity	Specificity	Diagnostic Odds Ratio
1	0.98 (0.86, 1)	0.59 (0.22, 0.91)	60.08 (8.88, 634.6)
2	0.94 (0.75, 0.99)	0.8 (0.49, 0.97)	61.55 (15.84, 388.12)
3	0.85 (0.56, 0.97)	0.92 (0.69, 0.99)	67.15 (19.16, 565.91)
4	0.68 (0.33, 0.93)	0.97 (0.8, 1)	79.8 (13.73, 2074.33)
5	0.46 (0.13, 0.86)	0.99 (0.84, 1)	98.76 (6.15, 19796.12)
6	0.25 (0.04, 0.77)	1 (0.86, 1)	127.35 (2.15, 515905.38)

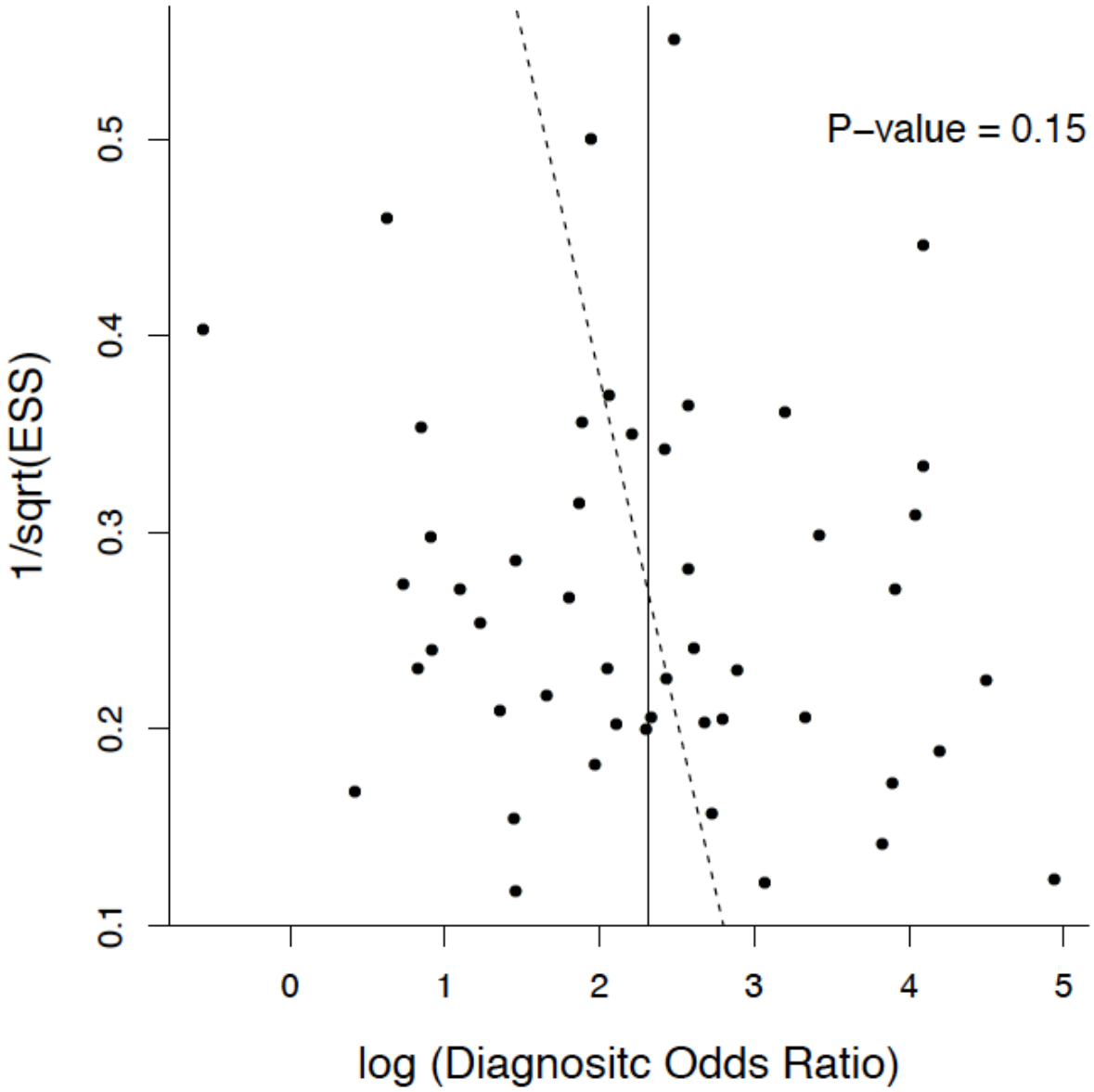


Figure 10 Funnel plot demonstrating publication bias on the basis of study sample size

ESS: Effective sample size. Technique taken from Deeks et al.[238]

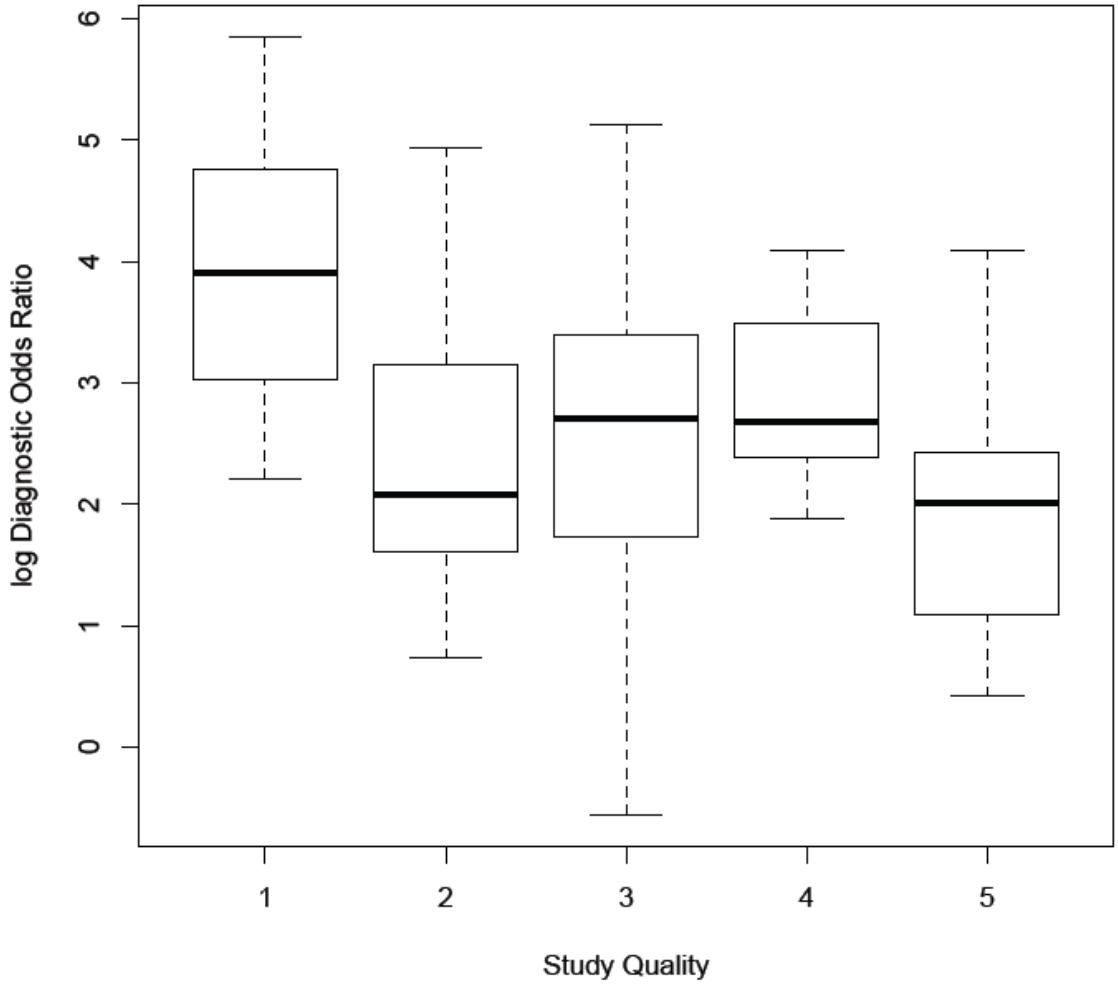


Figure 11 Box plot demonstrating study bias between low quality studies and high quality studies

There was a statistically significant difference between the diagnostic odds ratio between the lowest quality studies (score 1) and the highest (score 5; p=0.019).

3.3 *Discussion*

This is the first systematic review of tests used to diagnose CP and provides a comprehensive analysis of all tests evaluated over four decades. Despite the lack of standardisation and the possibility to undertake meta-analyses on only 12 of 27 tests, we found clear evidence to support the use of EUS and MRI, the latter preferably with secretin. These two tests were found to have the highest pooled sensitivities and specificities, each in excess of 0.80, making them the most accurate tests evaluated to establish a diagnosis of CP. Either or both would therefore be appropriate as first line tests when there is a strong suspicion of CP.

None of the nine function tests performed as well as EUS or secretin MRI, although some were found to have either pooled sensitivities or pooled specificities that were comparable, such as the pooled sensitivity of duodenal lipase or pooled specificity of faecal elastase. This is likely in part because deficiency is apparent only after very marked parenchymal loss;[299] also, loss of function may be secondary to other diseases.[300] Where faecal tests are deemed appropriate, the data supports the use of faecal elastase over faecal chymotrypsin. Poor sensitivity and specificity of pancreatic function tests mean, however, that it is likely to be more appropriate to confirm structural changes either in the form of imaging or histology to make a diagnosis. Simple function testing may be useful as a screening tool, provided the relatively low pooled sensitivity (e.g. of faecal elastase) is recognised, thus when clinical suspicion remains despite normal function testing, EUS or secretin MRI would be preferable. Furthermore, the addition of endoscopic pancreatic function testing, digital image analysis and/or elastography to EUS may increase sensitivity and/or specificity, but we have not been able to assess these or other combinations.

Tools commonly used for quality assessment of diagnostic tests include STARD, which does not evaluate study design, and QUADAS2, which does not give an objective value to compare the quality of one study against another.[301,302] In our review the quality of studies was assessed using a newly defined composite measure that included prospective design, individuals with the suspected

disease, blinding, pre-defined diagnostic criteria and uniform application of the chosen gold standard (Figure 5). This quantifiable score allowed for appropriate weighting of the studies in the pooled analysis, to reduce bias in the calculation of accuracy. Consistent with this, the quality score demonstrated higher diagnostic ORs in lower quality studies (Figure 11).

Pre-defined diagnostic criteria are necessary to evaluate any test that has been developed for general application, but in several studies diagnostic thresholds were selected after testing to optimise diagnostic efficiency (13); in a further 12 it was not stated what the diagnostic cut-off was or how it was obtained.[234] *Post hoc* selection of diagnostic cut-off levels will tend to inflate diagnostic sensitivity and specificity, unlikely to be reproduced by subsequent validation.[303] Well-established diagnostic criteria are available for a minority of tests e.g. faecal elastase at 200 μg/g, duodenal bicarbonate at 80 mEq/L and ERCP using the Cambridge criteria.[61,81,304] Some studies used the same scoring systems for EUS, but in the main these varied (Table 11). Increasing the number of EUS criteria increased specificity while decreasing sensitivity (Table 12); four diagnostic criteria gave the highest accuracy. No study has yet evaluated the Rosemont criteria, developed in the search for consensus in scoring EUS; randomised studies using predefined criteria are needed, preferably with long-term follow up. [78]

We excluded studies evaluating the diagnosis of CP in the context of suspected pancreatic cancer. In such studies the suspicion of neoplasm is the diagnostic priority and affects the nature of diagnostic tests chosen, such as CT and positron emission tomography.[305] The number of studies included in this review that examined the efficiency of CT in the primary diagnosis of CP was small. None of the studies used contemporary CT technology, which can more readily identify calcification, duct dilation and atrophy indicative of CP.[306,307] Therefore, assessment of the utility of CT in the diagnosis of CP requires further evaluation.

Three studies specifically addressed the detection of early CP, but not surprisingly either sensitivity or specificity was low; complicating factors include fibrosis and atrophy that are more frequent in

elderly, asymptomatic individuals.[74,252,255] Accurate identification of minimal change CP could significantly improve patient management by avoiding extensive diagnostic delay, which can otherwise result in pursuit of alternative diagnostic pathways and inappropriate treatment. Both EUS and secretin MRI are capable of identifying modest changes, e.g. when functional assessment and CT imaging is normal. Notably, EUS has been shown to be more sensitive than ERCP in the identification of early changes.[74] Nevertheless treatment decisions require substantial confidence in results, essential in the planning of surgical treatment of CP. When available, histology remains the final arbiter in the identification of CP. While wide bore needle sampling can provide sufficient pancreatic tissue to identify CP at an early stage, sampling may be misleading, not representative of the whole pancreas and itself carries additional risk.[37,233] Further studies are needed to determine the clinical utility of these and more recently developed technologies in suspected minimal change CP, both singly and in combination, preferably in randomised studies that also address outcomes. This will minimise bias inherent in comparisons made between those with a normal or highly abnormal pancreas, as in the 19 diagnostic case-control studies evaluated in retrospective studies.[308] Accordingly here the quality score was weighted towards prospective cohort studies.

We made the basis for the assessment of diagnostic tests comparison with a gold standard. Ductal changes on ERCP were the most commonly used gold standard, but recommendations that it should only be used as a therapeutic intervention mean that diagnostic ERCP is no longer appropriate.[62] Histology was used as the gold standard in a minority of studies, most frequently from resected specimens. This approach is likely to have introduced bias, as those individuals who undergo resection tend to be those with the most severe disease or those in whom there is a strong suspicion of cancer. In addition, scoring of histology can be subjective and is aligned to that found in alcoholic CP.[37] Composite gold standards were used in 25 (46%) of the 54 studies examined, but lacked uniformity. In practice CP requires a combination of all diagnostic approaches, including clinical features, functional assessment, imaging and histology. As with most diseases some criteria may not need to be positive for the diagnosis to be made, e.g. CP can be painless or there may be no evident

loss of function, but the priority is to identify CP in the presence of either so that appropriate advice and treatment can be given promptly. Further work is required to develop consensus, incorporating all informative components in the diagnosis of CP.

Studies that analyse the diagnosis of CP are wide ranging in both modality and study design. The most analysed test of EUS shows good overall sensitivity and specificity, and should be recommended for diagnosis of CP, either in addition to, or in place of secretin MRI which shows promising results but requires further evaluation.

4 Chapter 4: Genome-wide Association Study

Although alcohol has long been known to be of the primary causative agents of chronic pancreatitis, it has also been recognised that genetic contributions are relevant since the identification of *PRSS1*, *CFTR* and *SPINK1* variants as associated factors with pancreatitis risk. This idea was explored further with a genome wide approach to identify further genes that may have an association with the development of chronic pancreatitis.

As part of this thesis samples from well-characterised patients with chronic pancreatitis were selected, DNA extracted and the DNA sent along with anonymised clinical data by myself to a collaborative project in Pittsburgh combining samples from USA, Germany and Spain. This work has been published by Whitcomb et al, and contains the materials and methods and results for the study.[176]

4.1 *Discussion*

The GWAS carried out in collaboration with the Pittsburgh group, was the first GWAS to be carried out in chronic pancreatitis. Collaboration between many groups was required to obtain the large number of samples needed to run a GWAS. Over 300 samples were provided from the biobanks held in the Liverpool Pancreas Biomedical Research Unit.

Through discovery and validation stages, two highly significant and reproducable SNPs were identified one in the region of PRSS1 and the other in the not previously associated Claudin-2 gene; PRSS1-PRSS2_rs10273639 and CLDN_rs12688220. These produced an odds ratio (OR) of 0.734 ($p = 2.0 \times 10^{-14}$) and 1.385 ($p = 2.3 \times 10^{-27}$) respectively.

Overall, the when assessing patients with established alcohol-related pancreatitis the estimated odds ratio (OR) were greater for both *PRSS1-PRSS2*_rs10273639 and *CLDN*_rs12688220 than those produced for cases with pancreatitis of other etiology. This suggests that the effects of both loci may be increased in the presence of alcohol consumption.

The GWAS study used a combination of different patient populations including those with recurrent acute pancreatitis (RAP). This is due to the theory that there is a progression of disease from acute, through to recurrent acute and then on to chronic pancreatitis, but not all individuals will necessarily go through all stages, or some patients may no as of yet developed chronic pancreatitis, although with time this will occur.[49] However, this has not been unequivocally proven, and therefore the pathophysiology of the RAP group may not exactly mirror that of CP. Nonetheless, in this study there is the same distribution of *PRSS1-PRSS2*_rs10273639 between the RAP and the CP cohorts suggesting that the polymorphism has a similar effect on the two populations. In contrast the *CLDN2* polymorphism was more strongly associated with CP than RAP, suggesting that it may act to accelerate the transition from RAP to CP.

The major allele at the *PRSS1-PRSS2* locus has been shown to be associated with reduced *PRSS1* expression. This effect is independent of the previously reported rare gain-of-function *PRSS1* variants that increase susceptibility to both RAP and chronic pancreatitis.[48] As this allele occurs commonly, there would need to be a combination of other factors present in association with this polymorphism for chronic pancreatitis to develop.

Claudin-2 acts as a tight junction protein, forming cation-selective ion and water channels between endothelial cells. It is normally expressed at low levels in the tight junction between pancreatic ducts and in pancreatic islets.[309-311] Although it has been infrequently examined in humans, in a porcine model Claudin-2 has been identified in higher levels in acinar cells under stress.[312] As calcium levels have previously been shown to be associated with the development of acute pancreatitis, poor regulation of this in association with the CLDN2 haplotype may explain the association with chronic pancreatitis.

5 Chapter 5: Next Generation Sequencing

As the break down products of fat and alcohol metabolism have been shown to have a clear association with the development of chronic pancreatitis, it was hypothesised that variations in genes involved in these pathways may impact on the risk of developing chronic pacnreatitis.[110] Therefore full deep sequencing was undertaken of the exons of the selected genes to identify potential loci of interest.

5.1 *Materials and Methods*

5.1.1 Patients and samples

To assess for genetic polymorphisms clinical samples were required from both disease and control populations. Table 13 details the different ways samples were obtained.

5.1.1.1 Existing Resectional Sample Biobank

There is an existing biobank of samples from pancreatic patients at RLBUHT; these samples have predominately been collected from patients undergoing operative procedures on the pancreas, with samples of blood (stored as serum, plasma and cell pellets) and urine, being taken alongside operative specimens including pancreatic juice, trucut biopsies of the pancreas and histological specimens of diseased and normal pancreas, bile duct and duodenum. This work has been on-going since 1997, meaning within the larger bank there is a collection of over 100 samples from patients with histologically confirmed CP that was available before the start of this specific project.

In most cases these previously recruited individuals had the most severe phenotype of the disease, i.e. requiring surgical intervention; the only exceptions to this being those patients with mass forming pancreatitis who may have undergone resection for suspicion of cancer, rather than in relation to symptoms of CP. The CP samples were also derived from a range of different aetiologies, but patients' more commonly had the idiopathic form of this disease.

5.1.1.2 Chronic Pancreatitis Biobank

Therefore, at the start of this body of work, ethical approval was sought for a chronic pancreatitis biobank that would allow the attainment of samples of blood from patients with suspected or confirmed CP (10/WN003/46).

To identify the patients with CP, clinic lists for the four weekly pancreato-biliary clinics were screened prior to clinic by cross-referencing with a list of patients who had previously been identified as having CP through clinic letter review, EUS results or through the pancreas resection database.

Patients were approached in accordance with the ethical approval, given the appropriate patient information sheet (PIS) and fully consented. After the consent form was completed, the blood was collected as per standard operating procedures (SOP) of the GCLP facility (GCLPTSS055/2 and GCLPTSS040/1).

Samples were processed into serum, plasma and cell pellets and stored in the PBRU -80^{o}C freezers until they were required for further use in the project.

5.1.1.3 Control Biobank

In conjunction with this, a mirrored group of patients required recruitment to be used as a control group, and a separate ethical approval for blood samples for this group was obtained (11/NW/0347).

Control patients with a history of alcohol excess were identified patients within the “Lifestyles” clinic (for alcohol dependence) or through the hepatology weekly clinic. Once collected they were processed using the same methods as the CP samples, and stored for further use.

Additional healthy controls (HC) were collect through local donors at the University of Liverpool who volunteered for involvement in the research project (08/H1017/19).

5.1.1.4 Liver Transplant Tissue Samples

Additional samples were obtained for the NIHR Birmingham Liver Biomedical Research Unit. These were samples of liver tissue collected from patients undergoing liver transplants due to alcohol related cirrhosis in line with NREC approval (09/H1010/75). These samples were kept at -80^{o}C for long-term storage.

5.1.1.5 Discovery Phase

5.1.1.5.1 Chronic Pancreatitis

Participants included in the *CEL* study had to meet the following criteria

- symptoms typical of chronic pancreatitis
- alcohol excess of greater than 35u/wk (equivalent of >40g/day)[43] for at least 5 years
- radiological evidence of chronic pancreatitis (CT/MRI/EUS)
- cell pellet available for DNA extraction
- patient consented to take part in research projects

5.1.1.5.2 Alcoholic Control

For patients to be included in the discovery phase on the NGS section they had to meet the following criteria

- no personal or family history of chronic pancreatitis
- alcohol excess of greater than 35u/wk (equivalent of >40g/day)[43] for at least 5 years
- radiological evidence of a morphologically normal pancreatitis (CT/MRI/EUS)
- cell pellet available for DNA extraction
- patient consented to take part in research projects

5.1.1.6 Validation

5.1.1.6.1 Chronic Pancreatitis

Patients included in the validation phase on the NGS section had met the following criteria

- symptoms typical of chronic pancreatitis
- alcohol excess of greater than 35u/wk (equivalent of >40g/day)[43] for at least 5 years
- cell pellet available for DNA extraction
- patient consented to take part in research projects

5.1.1.6.2 Alcoholic Control

Patients included in the validation phase on the NGS section had met the following criteria

- no personal or family history of chronic pancreatitis
- alcohol excess of greater than 35u/wk (equivalent of >40g/day)[43] for at least 5 years
- cell pellet available for DNA extraction
- patient consented to take part in research projects

Table 13 Diagnostic criteria for classification into the different groups used in the genetic studies

	Clinical history of CP	Clinical history of ALD	Confirmation of disease	Confirmation of alcohol excess
Alcohol-related Chronic Pancreatitis (ACP)	Good clinical history suggesting CP	No clinical history that would be suspicious of ALD	Histology, CT scan, or EUS confirming a diagnosis of CP	A history of alcohol excess greater than 35units/week for over 5 years
Alcoholic Control (ACtrl)	No clinical history that would be suspicious of CP		CT scan of abdomen showing a morphologically normal pancreas	
Alcoholic Liver Disease (ALD)		Good clinical history suggesting ALD		
Alcoholic Health Controls (AHC)		No clinical history that would be suspicious of ALD		
Health Controls (HC)				No history suggestive of alcohol excess

5.1.2 DNA Preparation

5.1.2.1 Cell Pellets

DNA was extracted from the previously stored red cell pellets, using MagnaPure System as per SOP of the GCLP facility (GCLPEQU020/3).

The red cell pellet stored in 2ml Eppendorf tube was diluted with 0.6ml of PBS. 0.4ml of the resuspended cell pellet was used per sample for DNA extraction using the 100-400 DNA extraction protocol, to elute 0.1ml of DNA. All extracted DNA was stored at -20^{o}C until further use. All details relating to each elution was recorded on Matrix LIMS (Autoscribe, Reading) and on a MagnaPure machine (Roche Diagnostics, UK).

5.1.2.2 Liver Tissue

A Cryostat (Thermo Scientific, UK) was used to keep the liver tissue frozen while slicing thin sections of liver tissue before DNA was extracted. QIAamp DNA Micro Kit (Qiagen, Baltimore USA) was used to extract DNA from the liver tissue going through the processes of lysis, binding, washing and elution as detailed in the May 2010 handbook.

5.1.3 DNA Quality Control

DNA extracted by both methods was quality controlled and quantified using three methods as detailed below. If standards were not met in any of the three methods, then the sample was not used in any further experiments and a new sample was eluted from the stock of cell pellets.

Once DNA samples had satisfied all three quality control tests, the 225ng of DNA was diluted in 45µl of nuclease-free water to make a concentration of 5ng/µl ready for use in the sequence capture.

5.1.3.1 Gel Electrophoresis

2µl of the stock DNA solution was added to 2µl of nuclease-free water, and 1µl of loading buffer. This was loaded on to a 100ml 1% agarose gel containing Gelred™ (Biotium, California) with a 1kb ladder. The gel was run at 75v for 90 minutes before photographing under UV light. The DNA run on the gel was deemed acceptable if there was a smear of product greater than 2.5 KB with no evidence of a smear of degraded DNA below this.

5.1.3.2 NanoDrop Spectrophotometry

Before any readings were taken, the NanoDrop Spectrophotometer (Thermo Scientific, Delaware USA) was cleaned with nuclease-free water, and a blank reading was carried out using 1µl of a "blank" created by running a sample of nuclease-free water through the MagnaPure protocol. Then 1µl of each sample ware run through the machine and the DNA concentration and the 260/280 ratios were recorded. Sample with a 260/280 ratio outside 1.8 to 2.0 were excluded from further processing as per SOP of the GCLP facility (GCLPEQU029/1).

5.1.3.3 Qubit® dsDNA HS Assay

Concentration on the DNA was quantified using Qubit® dsDNA HS Assay (Life Technologies, Paisley). Samples were processed in batches of 8 or less. Before quantification of any samples the machine was calibrated using two standards provided in the kits following the manufacturer's instructions.

5.1.4 Selection of Genes of Interest

An extensive review of the literature in relation to genes known to be involved in the lipid and alcohol metabolism pathways was undertaken (http://geneontology.org//). This gave the list shown in Appendix 10.2 Table 42. Many of these genes were considered as too peripheral to the main metabolism pathways describe in Chapter 1.1, while others were eliminated from further consideration because there was no literature reference to significant impacts on the overall pathway in deletion studies. After eliminating genes of less interest, a final list of potential candidate genes was formed. These were inputted into a gene ontology program (Ingenuity pathway analysis, IPA 6, Qiagen Redwood city, USA), to establish the pathways between the potential genes, and to identify if there were other key genes within the pathway that had not yet been identified.

Given previous literature in relation to alcohol-related chronic pancreatitis, the main interest was the genetics of lipid and alcohol metabolism, and therefore the majority of genes included related directly to these processes. However, as *PRSS1*, *SPINK1* and *CFTR* have an established associated with chronic pancreatitis, it was felt to be beneficial to include them in the sequencing to allow any interaction with variants in these genes, and the genes of alcohol metabolism to be assessed.

5.1.5 Sequence Capture (Haloplex)

The specific design for the sequence capture was created using the Haloplex design tool (Life Technologies, Paisley, UK), selecting the exonic regions from the candidate genes; the resulting design coverage is shown in Appendix 10.2, Table 43.

For each batch of sequence capture performed, 15 clinical samples were run with one control sample provided with the kit. During the process, individual barcodes were added to each amplicon to allow multiplexing of up to 16 different samples on one NGS reaction. Samples were processed in accordance with Protocol Version D.1, November 2012. 21 cycles were used in the PCR step.

5.1.5.1 Bioanalyzer

The 2100 Bioanalyzer instrument (Agilent, Santa Clara, California) was used with Agilent High Sensitivity DNA Kit to quantify the product produced by each Haloplex reaction to allow multiplexing of the samples with equal distribution. Samples with insufficient product (defined as less than 300pg/l, and variation from expected trace; see example in Figure 12) were deemed unsuitable for NGS, and therefore additional DNA from the same patient was run through the Haloplex, before being quantified by the Bioanalyzer again to confirm its suitability. Samples were processed in accordance with Protocol Agilent High Sensitivity DNA Kit Guide, Version 5/09.

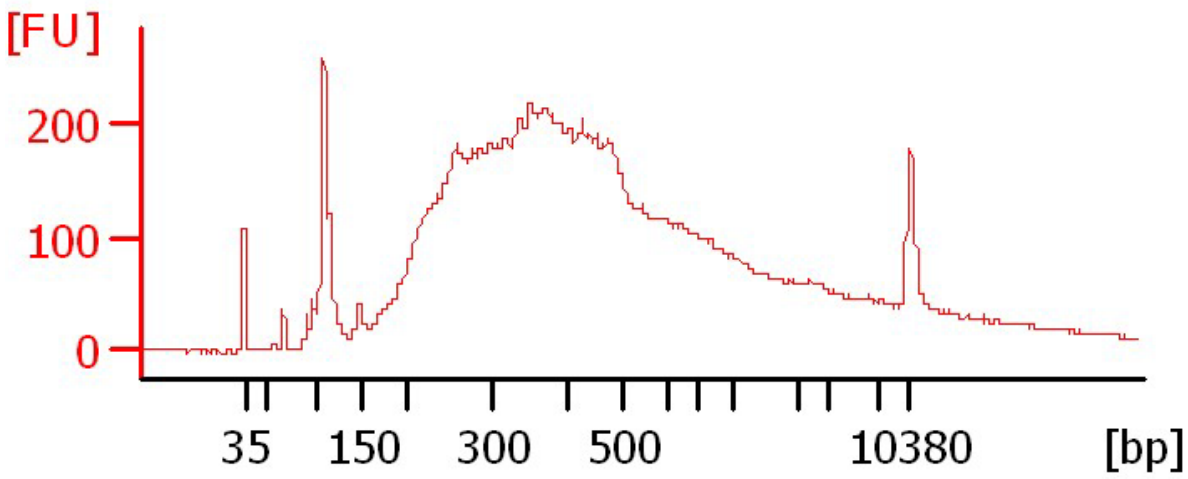


Figure 12 Typical output of the Haloplex capture product read with the Bioanalyzer

Peaks at 35bp and 10380bp are the ladder markers. The peak at 125bp indicated the PCR primers than have failed to bind to DNA. If this equated to greater than 10% of the overall product then the sample would need to be washed (this was not required for any of our samples). The majority of product from the Haloplex, including barcodes was between 200bp to 550bp as demonstrated on the graph.

5.1.6 Ion Torrent™

5.1.6.1 Ion OneTouch™

Following the quality and quantification measurements of each of the Haloplex products with the Bioanalyzer, the samples were multiplexed (15 or 16 samples with different Haloplex barcodes) with equivalent quantities of amplicons to give 20µl of product with 0.01328nM of multiplexed amplicons.

The sample preparation of the sequencing on the Ion Torrent™ was carried out using the Ion OneTouch™ (Life Technologies, Paisley, UK) system with the Protocol Ion OneTouch™ 200 Template Kit v2 DL (Cat Number 4480285, Publication Number MAN0006957, Revision 5). During this process, there is clonal amplification of the individual amplicons, and then each of the individual amplicons is combined with a bead within a detergent bubble to create an Isolate Positive Ion Sphere™.

5.1.6.2 Ion Torrent™

The prepared samples from the Ion OneTouch™ were then loaded onto a 318 chip following the protocol Simplified Ion PGM™ Chip Loading with the Ion PGM™ Weighted Chip Bucket, Publication Number MAN0007517 Revision 1.0.

The Ion Torrent PGM machine was then set up following the Ion PGM™ Sequencing 200 Kit v2 Ion 316™ Chip v2 Publication Number MAN0007273 Revision 3.0. Two chips were run in sequential pairs over a 24hour period to maximize the efficiency of the machine

5.1.7 Data Output from Ion Torrent™

The data produced from each chip run was stored on a specific server, and data was initially out putted from the Torrent Suite™. Basic data was provided on the number of read, length of reads and quality of reads (Figure 13). The fingerprint of the chip loading was provided to show the efficiency of live beads loaded onto the chip (Figure 13). Overall run results for each chip are presented in Appendix 10.3; by chip in Table 45 and by sample in Table 46.

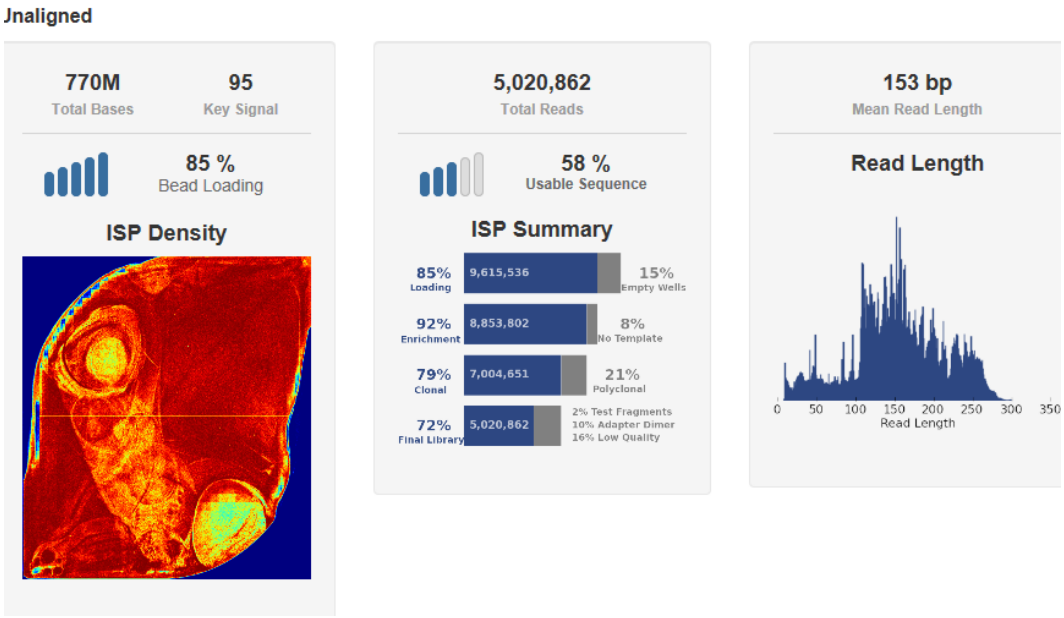


Figure 13 Sample of summary statistics from Torrent Suite™

The figure shows the total number of bases read, the percentage of beads loaded onto the chip which contained a single amplicons sequence, the total number of individual usable reads, the mean read length and a histogram of the variation in read lengths. This is for 16 multiplexed samples run on an individual chip. The image shows the evenness of loading with red indicating high levels of leading, green/yellow poorly levels of loading and blue is out with the loading area of the chip.

5.1.8 Data Analysis from Ion Torrent™

The reads for each sample were downloaded from the Ion Torrent™ PGM in Fastq format. With the assistance of Roy Chaudhuri (Centre for Genenomic Research, University of Liverpool) the reads were mapped to the human reference genome version hg19 using Bowtie2 version 2.1.0, using the option "-very-sensitive-local". Reads which mapped ambiguously, defined as those with a mapping quality score below 10, were excluded from further analysis.[313] Reads containing indels were realigned using IndelRealigner from GATK version 2.0, and variants were called using the GATK UnifiedGenotyper.[314]

5.1.8.1 Calling individual SNPs

All genetic variation from the base line reads were produced per run in individual .csv files. All potential SNP positions were identified, and where the number of reads at that position for each run was <6, then this was called as not read. Where no variant was called at greater than 6 reads, this was considered as no variant called. For any sample that called a variant, but less than 25%, this was also classed as not read. All variant read at between 25-75% of the reads were classed as heterozygous, and variant reads of >75% were classed as homozygous.

5.1.8.2 SNPs of Interest

The *SNPs of Interest*, selected for further validation of their significance, where selected by performing a χ^2 analysis of both the heterozygous and homozygous frequency of each SNP between the ACP and ACtrl groups. A p value of less than 0.05 was considered significant.

This long-list of potential SNPs were then individually examined to confirm that the polymorphism had been read in both directions, and did not follow a homopolymer, approximate insertions or deletions which may lead to a misread of the data. Only SNPs that had been found to have a significant difference between the groups, and appeared to be genuine calls were included in the final list of *SNPs of Interest*, which were then validated, in a second population.

These SNPs were assessed for their previous inclusion in reports of disease association in the literature.

5.1.9 Linkage Disequilibrium

Deviation (D) is the difference between the observed and expected frequency of each haplotype where 0 indicates complete linkage equilibrium.

Equation 1 Standard measure of Linkage Disequilibrium

$$D = (x_{11})(x_{22}) - (x_{12})(x_{21})$$

Standardisation of D (which can range from -0.25 to 0.25) can be performed based on the theoretical maximum and minimum relative to D (Figure 14).

Equation 2 Normalisation of deviation

$$D' = \frac{|D|}{D_{max}}$$

Figure 14 Relationship between allele frequencies and deviation from expected values

	A_1	A_2	Total
B_1	$x_{11} = p_1q_1 + D$	$x_{21} = p_2q_1 - D$	q_1
B_2	$x_{12} = p_1q_2 - D$	$x_{22} = p_2q_2 + D$	q_2
Total	p_1	p_2	

Correlation (r^2) between a pair of loci is calculated using the following formula

Equation 3 Correlation between loci

$$r^2 = \frac{D^2}{p_1p_2q_1q_2}$$

As data was only available to pairs of alleles, and due to the short reads produced by the Ion Torrent™ the probability of each haplotype occurring had to be imputed from the source data.

5.1.9.1.1 Tagging SNPs

To identify if the *SNPs of Interest* had been previously reported, tagging SNP were identified using data taken for 1000 genomes website (http://browser.1000genomes.org/index.html) included SNPs with r^2>0.8 and D' >0.9, and the tagging SNPs were then investigated in the literature.

5.1.10 Concordance of Results between Modalities

The Liverpool subsection of the data produced by the GWAS was compared to identify comparable SNPs analysed and overlap of patient samples. Results from the two methods were directly compared to calculate the correlation between the two techniques, and specifically between different SNPs.

5.1.11 Sequenom Validation

The MassARRAY® System (Agena, San Diego, California) using MALDI-TOF based technology was utilized to run the selected SNPs in Stage 1 for the Stage 2 analysis. This work was carried out with the assistance of Daniel Carr from the Wolfson Centre for Personalised Medicine, University of Liverpool.

Where the Sequenom was unable to assess the SNPs, then TaqMan PCR was designed to assess the SNPs. This data was compared to the original Ion Torrent™ results to calculate a correlation between the two techniques.

5.1.12 Haplotype Analysis

To assess for disease haplotypes in the most strongly associated SNPs, the other SNPs read on the same chromosome were compared to the reference SNP, and those SNPs that correlated with the reference SNP in either the ACP or the ACtrl population (as tested by χ^2 with a p value of less than 0.05), were then included in the haplotype design. Each of these loci were combined in location order to form an allelotype (e.g. 0/0 0/1 0/1 1/1 0/0); OR were calculated with a 95% confidence interval.

To assess for a haplotype association, considering all the possible alternative haplotypes from each allelotype, the counts were based on the total number of haplotypes possible per allelotype and these haplotypes were tabulate as the most likely imputed haplotype for each group. A χ^2 test was run to give a measure of the significance of disease association OR. Where there were no patients in one group (making computation of the OR impossible), 0.5 is added to all groups.[315]

Once the significance of each OR was identified, the least significant factors were removed in a stepwise fashion to see if this affected the model, until the most significant model was identified.

5.2 *Results*

5.2.1 Patients

A total of 203 patients (102 for ACP and 101 ACtrl) successfully went through Haloplex sequence capture successfully and were sequenced on the Ion Torrent™. The demographics of the patients sequenced are given in Table 14.

Table 14 Patient demographics of Alcohol-related Chronic Pancreatitis and Alcohol Control groups

		Alcohol-related Chronic Pancreatitis (102)	**Alcohol Controls (101)**
Age	Median (range)	44 years (26-77)	50 years (25-89)
Sex	Male	87	61
	Female	15	40
Smoking	Never	9	30
	Former	69	44
	Current	17	26
	Missing	7	1
Alcohol	Former	64	50
	Current	32	37
	Missing	6	14
Diabetes	Not known to be diabetic	58	81
	Diabetic	36	20
	Missing	8	0
Diagnosis of CP confirmed or refuted	CT	91	101
	EUS	18	0
	Histology	42	0
History of ALD	No	102	9
	Yes	0	92
Source of DNA	Blood	102	88
	Liver biopsy	0	13

5.2.2 Next Generation Sequencing Analysis

Within the sequenced area 2776 different SNPs were identified within at least one of the samples, of which only 1484 (53.5%) were known to be SNPs, as demonstrated by possession of an rs or a CM identifier.

SNPs were identified in all of the sequenced genes. The variation of each SNP between the ACP and the ACtrl group is demonstrated in Figure 15. Those SNPs that either demonstrated significant difference between the groups for either presence of the SNP either as a heterozygote or a homozygote SNP were identified for further validation (Table 23), and will be referred to as the *SNPs of Interest*. Significance was defined as $p < 0.05$ when analysed with χ^2.

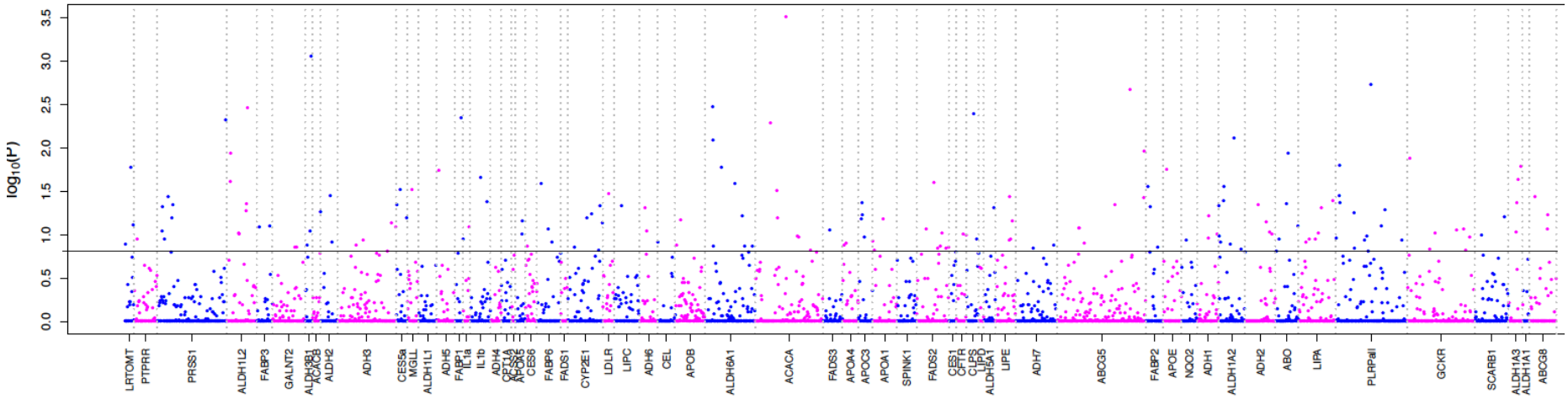


Figure 15 Manhattan plot of Next Generation Sequencing data presented by gene

Width of gene on base access represents the number of SNPs identified within that gene, and not the overall size of that gene.

5.2.3 Known Chronic Pancreatitis Associated Variant Analysis

5.2.3.1 PRSS1

No patients exhibited variants in the most common recognised *PRSS1* mutations of p.R122H or p.N29I. Two individuals appeared to show p.A16V variant from the Ion Torrent™ alignment (Table 15). Analysis of these patients is documented below.

p.D162D and p.N246N are common polymorphisms in linkage disequilibrium and were thought to have no pathogenic consequences. p.K170E has only been described in four separate reports, and it is unclear if there is any pathologic consequence.[190]

There were significantly more patients exhibiting the p.D162D polymorphism in the ACP group than in the ACtrl group (OR 1.80, 1.2-2.7; p = 0.004). This is discussed further in Chapter 5.3.2.8.1.

Table 15 Genotypes of commonly known *PRSS1* SNPs

			Alcohol-related Chronic Pancreatitis			Alcoholic Controls		
		Pub MAF	Major	Hetero	Minor	Major	Hetero	Minor
*PRSS1*_rs202003805[50]	p.A16V	-	102	0	0	97	2	0
*PRSS1*_rs6666[164]	p.D162D	0.3966	26	41	29	40	42	15
*PRSS1*_rs201550522[190]	p.K170E	0.0016	87	9	1	87	8	0
*PRSS1*_rs6667[164]	p.N246N	0.3938	8	47	45	17	42	37

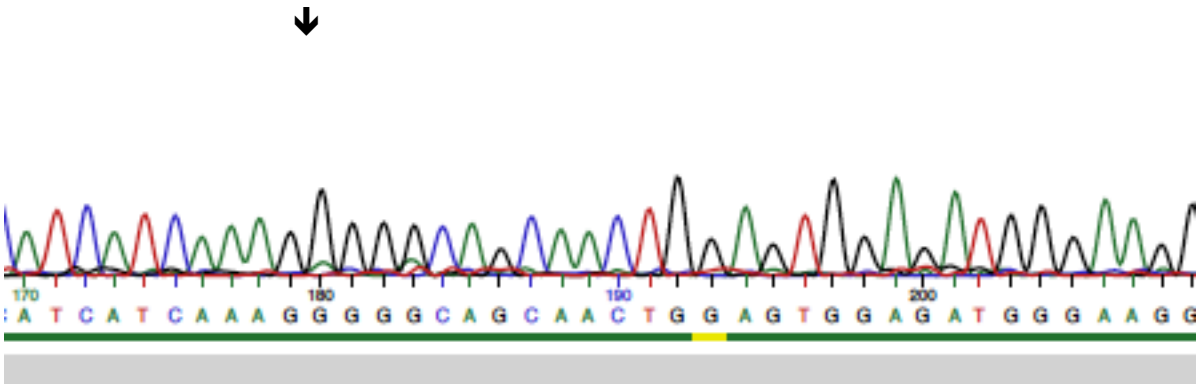
MAF – Published minor allele frequency; Major – Homozygous for major allele; Hetero – Major and minor allele; Minor – Homozygous for minor allele frequency.

5.2.3.1.1 p.A16V

To confirm the p.A16V variants identified in the two control patients, Sanger sequencing of the genetic area was carried out using the primers and methods described by Threadgold *et al*.[177] The sequences from both the patients' samples are as show in Figure 16, with no clear G to A base transition at point 183. On further analysis of the surrounding sequence, the isoform of cationic trypsin, mesotrypsin, has Valine rather than Alanine at codon position 16 (Figure 1), and it is therefore likely that both the sequence capture and alignment tool lead to mesotrypsin being misread as cationic trypsin at this point.

Figure 16 Sanger sequencing trace showing the area of potential p.A16V polymorphism

The potential polymorphism lies at point 180, where GA was read in this sample with the Ion Torrent.



5.2.3.2 SPINK1

Table 16 Genotypes of commonly recognised *SPINK1* polymorphisms

			Alcohol-related Chronic Pancreatitis			Alcoholic Controls		
		Pub MAF	Major	Hetero	Minor	Major	Hetero	Minor
*SPINK1*_rs17107315[45]	p.N34S	0.006	67	6	0	58	1	0
*SPINK1*_rs111966833[180]	p.P55S	0.003	89	2	0	78	2	0

Pub MAF – Published minor allele frequency; Major – Homozygous for major allele; Hetero – Major and minor allele; Minor – Homozygous for minor allele frequency

The commonly known SNPs in relation to *SPINK1* are presented in Table 16.

Due to the small numbers there was no difference in the prevalence of p.N34S between the ACP group and the ACtrl group, although there was a trend towards more of the polymorphisms in the ACP group (Fisher's exact p = 0.13). However, the frequencies demonstrated are representative of those that would be expected to be found in an ACP population.[178]

There was no difference in the presence of p.P55S, and none of the other variants were identified in any of the samples tested.

5.2.3.3 Cystic Fibrosis Transmembrane Conductance Regulator

Table 17 Genotypes of commonly reported *CFTR* SNPs

Pathogenic polymorphisms are identified in bold

			Alcohol-related Chronic Pancreatitis			Alcoholic Controls		
		MAF	Major	Hetero	Minor	Major	Hetero	Minor
*CFTR*_rs1800076[189]	p.R75Q	0.0064	87	11	0	83	5	1
***CFTR*_rs78655421**[316]	**p.R117H**	0.0005	98	4	0	97	2	0
*CFTR*_rs35516286[189]	p.I148T	0.0014	102	0	0	96	1	0
*CFTR*_rs143486492[317]	p.R297Q	0.002	99	1	0	89	0	0
***CFTR*_rs121908753**[316]	**p.R352Q**	rare	64	5	1	53	5	1
*CFTR*_rs213950[318]	p.V470M	0.4179	32	52	15	35	43	12
*CFTR*_rs1801178[189]	p.I507V	rare	69	1	0	62	0	0
*CFTR*_rs1800095[189]	p.E528D	0.0106	63	5	0	58	0	0
*CFTR*_rs1800098[319]	p.G576A	0.0024	91	3	0	82	1	0
*CFTR*_rs1800100[320]	p.R668C	0.0041	99	3	0	96	1	0
***CFTR*_rs1800103**[321]	**p.I807M**	0.001	88	1	0	85	0	0
*CFTR*_rs1042077[322]	p.T854T	0.4792	39	51	10	39	39	12
*CFTR*_rs1800109[323]	p.T966T	0.0062	100	1	0	96	1	0
*CFTR*_rs1800118	p.T1095T	0.0018	100	0	0	94	2	0
*CFTR*_rs1800121[324]	p.Q1186Q	0.002	91	1	0	80	0	0
*CFTR*_rs34911792[189]	p.S1235R	0.0037	90	0	0	80	1	0
*CFTR*_rs1800130[325]	p.P1290P	0.0647	89	9	0	92	4	0
*CFTR*_rs1800135	p.Y1424Y	0.0042	98	3	1	95	1	0
*CFTR*_rs1800136[326]	p.Q1463Q	0.1861	73	25	0	70	23	1

	Alcohol-related Chronic Pancreatitis		Alcoholic Controls	
Cumulative *CFTR* variants	Any variation	No variation	Any variation	No variation
Total Pathogenic	13	89	8	93
Total	82	20	70	31

The result for the common *CFTR* SNPs are presented in Table 17; underlining indicates SNP thought to have a pathogenic consequence. Although there were high number of *CFTR* variants identified in the ACP group compared to ACtrl, none of the variants were significantly associated with ACP and when either the presence of the disease causing variants or all variants were taken into account, there was no statistical difference between the groups (p = 0.369 and 0.097 respectively).

The previously described *CFTR* haplotype was examined in this population (*CFTR*_rs213950, *CFTR*_rs1042077 and *CFTR*_rs1800136).[186] The haplotypes were imputed using the method described in Chapter 6.1.12. The results are presented in Table 18. Unlike previous reports, there were no differences identified in any of the most common haplotypes between those with alcohol-related chronic pancreatitis and those with a history of alcohol excess. We see more A-G-G and less A-G-A than in previous reports, which could be potentially due to different populations used, or different control type.

Table 18 *CFTR* risk haplotype assessed by Next Generation Sequencing data

	Allele frequency			
***CFTR* Haplotype**	**ACP**	**ACtrl**	**OR (95%CI)**	**p value**
G-T-G	0.46	0.48	0.944 (0.485-1.835)	NS
G-T-A	0.03	0.05	0.734 (0.133-4.069)	NS
A-T-G	0.13	0.09	1.518 (0.517-4.451)	NS
A-G-G	0.15	0.18	0.807 (0.331-1.967)	NS
A-G-A	0.05	0.05	1.200 (0.258-5.571)	NS
Other	0.17	0.16	1.049 (0.426-2.580)	NS

5.2.4 Known Variants in Genes of Alcohol Metabolism

Below is a review of the previously reported polymorphisms in the genes of alcohol metabolism.

5.2.4.1 Alcohol Dehydrogenase

Previously described SNPs *ADH1B*_rs2066702, *ADH1B*_rs2018417 and *ADH1B*_rs1041969 were covered but no variants were identified in the population samples; the sequencing did not cover *ADH1B_rs1789882*. Results are presented in Table 19.

Table 19 Distribution of recognised polymorphisms in the exons of *ADH1B, ADH1C and ADH4*

			Alcohol-related Chronic Pancreatitis			Alcoholic Controls		
		Pub MAF	Major	Hetero	Minor	Major	Hetero	Minor
*ADH1B*_rs1229983[207]	p.K20K	0.0289	75	6	1	66	2	1
*ADH1B*_rs1229984[202]	p.R48H	0.2117	92	4	4	92	2	2
*ADH1B*_rs6413413[327]	p.T60S	0.0078	98	3	0	91	5	0
*ADH1C*_rs698[202]	p.I350V	0.2143	25	36	17	22	38	15
*ADH1C*_rs1693482[202]	p.R272Q	0.2143	67	21	10	81	8	7
*ADH1C*_rs1693425[328]	p.V158V	0.2145	60	13	10	50	15	10
*ADH1C*_rs2241894[328]	p.T151T	0.4716	76	17	3	65	18	2
*ADH1C*_rs1789915[328]	p.C104C	0.1502	60	14	8	48	16	6
*ADH4*_rs1126670[329]	p.P255P	0.1528	77	12	11	70	18	9
*ADH4*_rs1126671[329]	p.I309V	0.1530	18	24	9	23	24	8
*ADH4*_rs1126672[329]	p.L351L	0.1172	52	32	13	54	23	13

MAF – Minor allele frequency; Major – Homozygous for major allele; Hetero – Major and minor allele; Minor – Homozygous for minor allele frequency).

All previously reported exonic SNPs in Alcohol Dehydrogenase 1C were covered, and their distribution between the groups is demonstrated in Table 19.

The only one of these SNPs which shows a significant difference between the groups is *ADH1C*_rs1693482 (OR 2.04, 1.17-3.59; p = 0.017), which has been previously associated with alcohol dependence.

Those SNPs which have been previously been assessed in relation to pancreatitis and had variants called in our population are presented in Table 19. In addition, the sequencing covered SNP *ADH4*_rs139053416 and *ADH1B*_rs143067434, but no polymorphisms were identified within our study population.

The previously reported SNP, *ADH6*_rs3857224, lies within the intronic region, and therefore was not covered by the Next Generation Sequencing.

The previously reported area of *ADH7* associated with alcohol dependence, *ADH7*_rs2654849, was not read as it lies outside of the exonic region.[330] The polymorphism *ADH7*_rs971074 was within the sequence data but no polymorphisms were identified.

5.2.4.2 Aldehyde Dehydrogenase

The commonly recognized variant *ALDH2*_rs671, known as *ALDH2*2* was covered by the analysis, but no variations were identified, which is in keeping with its preponderance in Asian populations.

5.2.4.3 Cytochrome P450

There have been several different haplotypes described in the literature; a summary is given in Appendix 10.1.

Of the different polymorphisms previously described within *CYP2E1, CYP2E1*2* was covered but no polymorphism was identified in any of the samples, and *CYP2E1*5B* was not read as the polymorphisms lie within the intronic region. Results of *CYP2E1*3* and *CYP2E1*4* are given in Table 20.

Table 20 Distribution of recognised polymorphisms in the exons of *CYP2E1*

			Alcohol-related Chronic Pancreatitis			Alcoholic Controls		
		Pub MAF	Major	Hetero	Minor	Major	Hetero	Minor
*CYP2E1*_rs55897648[209]	p.I389V	0.0004	95	0	0	92	1	0
*CYP2E1*_rs6413419[211]	p.V179I	0.0778	92	5	0	92	5	0
*CYP2E1*_rs915909[211]	p.I321I	0.027	101	1	0	100	0	0
*CYP2E1*_rs2515641[211]	p.P421P	0.2991	73	26	0	72	22	0

5.2.4.4 Genes of Lipid Metabolism

5.2.4.4.1 Lipoprotein Lipase

The previous described SNPs in relation to *LPL* are presented in Table 21. There were no differences between groups.

Table 21 Previously identifed polymorphisms in the exons of *LPL, APOA5 and APOE*

			Alcohol-related Chronic Pancreatitis			Alcoholic Controls		
		Pub MAF	Major	Hetero	Minor	Major	Hetero	Minor
*LPL*_rs328[220]	p.S474X	0.0925	85	14	1	75	18	1
*LPL*_rs1801177[114]	p.D36N	0.0176	98	4	0	94	2	0
*LPL*_rs268[114]	p.N318S	0.0052	98	3	0	95	2	0
*APOA5*_rs3135506[114]	p.S19W	0.0557	86	5	0	78	7	0
*APOE*_rs429358[114]	p.C130R	0.1506	69	13	2	61	20	3
*APOE*_rs7412[114]	p.R176C	0.0751	73	9	3	74	11	3

Only one previously significant SNP was reported in *APOA5*, and this had no difference between groups. The previously described SNPs in *APOE*, with no significant difference between groups, and no deviation from the published MAF.

5.2.4.5 ABO Blood System

The SNP *ABO*_rs505922 commonly used to establish the ABO group of an individual was not sequenced as it lies in an intronic region. The SNPs that were sequenced are presented in Table 22; there was no difference between groups.

Table 22 Genotypes of previously identified SNPs in *ABO*

			Alcohol-related Chronic Pancreatitis			Alcoholic Controls		
		Pub MAF	Major	Hetero	Minor	Major	Hetero	Minor
*ABO*_rs8176746[225]	p.L266M	0.1528	80	12	3	73	10	0
*ABO*_rs8176747[226]	p.G268A	0.0106	81	12	2	73	9	0

5.2.5 Next Generation Sequencing *SNPs of Interest*

The key *SNPs of Interest*, which were shown to have statistically significant results between the groups, are presented in Table 23. SNP *MGLL_r*s116367069, did show significance with initial analysis, without correction factors, but has lost significance when reanalysed with χ^2 using Yate's correction factor. A comparison is made of the OR by allele frequency for each of these significant SNPs in Table 24.

Table 23 Next Generation Sequencing significant results from *SNPs of Interest*

					Alcohol-related Chronic Pancreatitis			Alcoholic Controls			P values	
SNP	**Chr,position**		**Amino**	**Pub MAF**	**Major**	**Hetero**	**Minor**	**Major**	**Hetero**	**Minor**	**Hetero**	**Minor**
*ACACB*_rs17848835	12,109692053	C/T	p.T2027I	0.0757	88	10	0	68	23	6	**0.0006**	**0.0140**
*ADH1C*_rs1693482	4,100263965	C/T	p.R272Q	0.2143	67	21	10	81	8	7	**0.0111**	0.6131
*ADH5*_rs116010022	4,99997409	C/A	Intron	0.0052	100	1	0	91	7	0	**0.0332**	-
*ADH6*_rs4147545	4,100128753	T/C	Intron	0.4307	39	39	13	47	37	3	0.1768	**0.0326**
*APOB*_novel	2,21228282	A/G	p.L3820L	-	42	34	4	16	46	1	**0.0012**	0.3841
*APOB*_rs72653066	2,21246382	C/G	Intron	0.0042	89	7	0	90	0	0	**0.0142**	-
*APOC3*_rs4520	11,116701535	T/C	p.G34G	0.4030	4	56	40	9	67	21	0.1598	**0.0058**
*CES5A*_rs7500040	16,55897519	G/A	Intron	0.2742	50	14	8	46	13	1	0.4335	**0.0395**
*CES5A*_rs200911306	16,55983724	C/T	p.V45I	0.0002	27	15	3	22	3	0	**0.0159**	0.5479
*FABP1*_rs2241883	2,88424066	T/C	p.T94A	0.2232	41	30	15	53	26	5	**0.0466**	**0.0303**
*FABP3*_rs11578034	1,31842153	C/T	Intron	0.0172	97	1	0	86	8	0	**0.0168**	-
*FABP6*_rs10056214	5,159665485	G/C	Intron	0.1188	33	18	49	48	17	29	**0.0133**	**0.0127**
*IL1B*_rs1143633	2,113590467	C/G	Intron	0.3109	34	46	9	49	28	3	**0.0034**	0.1385
*LDLR*_rs1003723	19,11224181	C/T	Intron	0.2768	33	51	13	22	48	27	0.1107	**0.0203**
*LIPA*_rs1051338	10,91007360	T/G	p.T16P	0.2861	49	16	3	51	4	1	**0.0111**	0.6260
*LIPC*_rs690	15,58834741	G/T	p.V155V	0.4780	20	38	40	26	44	22	0.2373	**0.0139**
*LIPC*_rs3751542	15,58856033	T/C	Intron	0.3992	57	35	8	55	35	1	0.6610	**0.0182**
*LPL*_novel	8,19809449	C/T	p.N120S	-	35	35	3	18	62	2	**0.001**	0.58
*MGLL*_rs116367069	3,127411206	A/G	Intron	0.0146	88	7	2	89	2	0	0.0592	0.4978
*PRSS1*_rs6666	7,142460313	T/C	p.D162D	0.3966	26	41	29	40	42	15	**0.0484**	**0.0167**
*PTPRR*_rs10506608	12,71155380	A/G	p.F166F	0.0475	79	16	2	86	7	0	**0.0313**	**0.4976**

Column 3 – reference allele/allele change; Pub MAF – Published Minor allele frequency; Major – Homozygous for major allele; Hetero – Major and minor allele; Minor – Homozygous for minor allele frequency.

Table 24 Next Generation Sequencing *SNPs of Interest* odds ratio in ACP group compared to ACtrl

SNP	Amino	OR	Lower CI	Upper CI	p value
*ACACB*_rs17848835	p.T2027I	4.094	1.965	8.530	**0.002**
*ADH1C*_rs1693482	p.R272Q	0.489	0.279	0.858	**0.013**
*ADH5*_rs116010022	Intron	7.444	0.907	61.080	0.051
*ADH6*_rs4147545	Intron	0.591	0.373	0.935	**0.025**
*APOB*_novel	p.L3820L	1.729	1.045	2.860	**0.033**
*APOB*_rs72653066	Intron	0.073	0.004	1.303	0.067
*APOC3*_rs4520	p.G34G	0.603	0.400	0.910	**0.016**
*CES5A*_rs7500040	Intron	0.543	0.277	1.065	0.076
*CES5A*_rs200911306	p.V45I	0.210	0.059	0.743	**0.016**
*FABP1*_rs2241883	p.T94A	0.509	0.314	0.826	**0.006**
*FABP3*_rs11578034	Intron	8.667	1.073	69.981	**0.043**
*FABP6*_rs10056214	Intron	0.481	0.321	0.721	**<0.001**
*IL1B*_rs1143633	Intron	0.481	0.295	0.782	**0.003**
*LDLR*_rs1003723	Intron	1.685	1.126	2.520	**0.011**
*LIPA*_rs1051338	p.T16P	0.293	0.115	0.751	**0.011**
*LIPC*_rs690	p.V155V	0.606	0.403	0.910	**0.016**
*LIPC*_rs3751542	Intron	0.746	0.461	1.206	0.232
*LPL*_novel	p.N140S	1.725	1.070	2.780	**0.025**
*MGLL*_rs116367069	Intron	0.185	0.040	0.846	**0.030**
*PRSS1*_rs6666	p.D162D	0.554	0.369	0.832	**0.004**
*PTPRR*_rs10506608	p.F166F	0.340	0.140	0.825	**0.017**

5.2.6 Biological Effects of *SNPs of Interest*

The potential effect of the *SNPs of Interest* was evaluated using commonly used web based tools (http://sift.jcvi.org and http://genetics.bwh.harvard.edu/pph2/). The data from these tools is presented in Table 25.

Table 25 Analysis of predicted effects of the *SNPs of Interest*

Predicted effects were assessed using SIFT and Polyphen web based tools.

Gene	Chr,position	Amino	SIFT Orthologues	SIFT Homologues	Polyphen
*ACACB*_rs17848835	12,109692053	p.T2027I	0.22 TOLERATED	0.01 DAMAGING	BENIGN
*ADH1C*_rs1693482	4,100263965	p.R272Q	0.05 DAMAGING	0.11	BENIGN
*ADH5*_rs116010022	4,99997409	Intron	-	-	-
*ADH6*_rs4147545	4,100128753	Intron	-	-	-
*APOB*_novel	2,21228282	p.L3820L	TOLERATED		
*APOB*_rs72653066	2,21246382	Intron	-	-	-
*APOC3*_rs4520	11,116701535	p.G34G	TOLERATED		1
*CES5A*_rs7500040	16,55897519	Intron	-	-	-
*CES5A*_rs200911306	16,55983724	p.V45I	TOLERATED		BENIGN
*FABP1*_rs2241883	2,88424066	p.T94A	0.52 TOLERATED	0.35 TOLERATED	0.68
*FABP3*_rs11578034	1,31842153	Intron	-	-	-
*FABP6*_rs10056214	5,159665485	Intron	-	-	-
*IL1B*_rs1143633	2,113590467	Intron	-	-	-
*LDLR*_rs1003723	19,11224181	Intron	-	-	-
*LIPA*_rs1051338	10,91007360	p.T16P	0.23 TOLERATED	0.10 TOLERATED	0.21
*LIPC*_rs690	15,58834741	p.V155V	TOLERATED		0.58
*LIPC*_rs3751542	15,58856033	Intron	-	-	-
*LPL*_novel	8,19809449	p.N140S			BENIGN
*MGLL*_rs116367069	3,127411206	Intron	-	-	-
*PRSS1*_rs6666	7,142460313	p.D162D	TOLERATED		1
*PTPRR*_rs10506608	12,71155380	p.F166F	TOLERATED		1

5.2.7 Linkage Disequilibrium

Linkage disequilibrium could not be calculated between the majority of the *SNPs of Interest* as they did not lie on the same chromosome. Among those SNPs that were lying on the same gene, then linkage analysis was undertaken as described in the methods.

There was no linkage disequilibrium demonstrated between any of the *SNPs of Interest* (Table 26).

5.2.8 Tagging SNPs

A list of tagging SNPs associated with the 21 *SNPs of Interest* is available in Appendix 10.3. Fifteen tagging SNPs were also mapped by the NGS sequencing covering five of the *SNPs of Interest* (Table 27). The correlation between the pairs of SNPs was assessed from the available NGS data. The absolute correlation ranged from 0.225 to 0.977 between SNPs.

Table 26 Linkage disequilibrium analysis for *SNPs of Interest*

				Alcohol-related Chronic Pancreatitis		Alcoholic Controls	
			Distance (bp)				
Chr	**1st SNP**	**2nd SNP**		**r^2**	**D'**	**r^2**	**D'**
2	*APOB*_novel	*APOB*_rs72653066	18100	0.8691	0.7624	0.1101	0.0767
	*APOB*_novel	*FABP1*_rs2241883	67195784	0.0979	0.1993	0.0106	0.0235
	*APOB*_novel	*IL1B*_rs1143633	92362185	0.0556	0.0409	0.0008	0.0029
	*APOB*_rs72653066	*FABP1*_rs22418831	67177684	0.2743	0.7194	0.0214	0.3913
	*APOB*_rs72653066	*IL1B*_rs1143633	92344085	0.0505	0.0174	0.0594	0.0001
	*FABP1*_rs2241883	*IL1B*_rs1143633	25166401	0.0754	0.1612	0.0036	0.0198
4	*ADH5*_rs116010022	*ADH6*_rs4147545	135212	0.0253	0.1487	-	0.0019
	*ADH5*_rs116010022	*ADH1C*_rs1693482	266556	0.0366	0.3188	-	0.0238
	*ADH6*_rs4147545	*ADH1C*_rs1693482	131344	0.0045	0.2167	-	0.0034
12	*PTPRR*_rs10506608	*ACACB*_rs17848835	38536673	0.1889	0.1900	0.0313	0.0262
15	*LIPC*_rs690	*LIPC*_rs3751542	21292	0.0240	0.6895	0.0004	0.0135

Table 27 Correlation of tagging SNPs within the Next Generation Sequencing data

The *SNPs of Interest* were assessed for any tagging SNPs that had been identified and for which sequencing data was available from the Next Generation Sequencing results.

				Actual correlation	
Reference SNP	**Tagging SNPs**	**r^2**	**D'**	**ACP**	**ACtrl**
*PTPRR*_rs10506608	*PTPRR*_rs11178399	1	1	0.977	0.976
*LDLR*_rs1003723	*LDLR*_rs12710260	1	1	0.912	0.716
	*LDLR*_rs688	0.889	0.976	0.364	0.225
	*LDLR*_rs5925	0.889	0.976	0.821	0.747
*IL1B*_rs1143633	*IL1B*_rs1143643	0.971	1	0.857	0.759
*ADH1C*_rs1693482	*ADH1C*_rs1612735	1	1	0.670	0.426
	*ADH1C*_rs698	1	1	0.658	0.474
	*ADH1C*_rs1693471	1	1	0.692	0.636
	*ADH1C*_rs1789912	1	1	0.779	0.897
	*ADH1C*_rs1693425	1	1	0.716	0.720
	*ADH1C*_rs1693426	1	1	0.682	0.608
	*ADH1C*_rs1693431	1	1	0.690	0.500
*PRSS1*_rs6666	*PRSS1*_rs6667	1	1	0.680	0.606
	*PRSS1*_rs4726576	1	1	0.618	0.561

5.2.9 Sensitivity Sub-analysis of Next Generation Sequencing Samples

5.2.9.1 Quality control

In cases where the initial NGS run was believed to be of poor quality (less than 90% converge at 6 bases), then the samples were either re-run from the same Haloplex extraction, or if the quality of the Haloplex samples was called into question, the DNA was re-run from the start of the Haloplex phase and then re-run on the Ion Torrent™. In those cases where the second run had good coverage, this run was used exclusively for analysis; in those run where there was also concern to the coverage of the second run of sequencing results, the first and second runs results were concatenated, and if the overall reads at the position of the previously identified SNPs was greater than 90% at x6 depth, the concatenated data was used, otherwise that patient was discarded (occurred in one ACtrl case). When these "pure" datasets were compared with the "concatenated" data sets, there were significant differences in the number of SNP variant calls made (Table 28).

Those samples that had poor coverage of more than 50% of the SNPs read in the dataset (less than 6 reads per SNP) were removed from the analysis. This included 10 samples (two ACP, eight ACtrl). There were no statistically difference in the results between the original and sub-analysis (Table 47).

5.2.9.2 Alcoholic Liver Disease compared to Disease-free Alcoholic Controls

Of the 101 original ACtrl samples run on NGS, only 91 had a clear diagnosis of ALD. There was no statistical difference determined between the groups, but the numbers were very small in the disease free alcoholic control group (AHC).

5.2.9.3 Control Blood Samples compared to Control Liver Samples

Twelve of the control DNA samples were extracted from liver tissue using Qiagen kits, as opposed to MagNA Pure extracted samples from peripheral blood. Differences in the number of SNPs called and overall coverage was analysed and no difference was demonstrated between either sample groups.

5.2.9.4 Disease Status According to History of Alcohol Consumption

Sub-analysis was attempted to identify and compare heavy drinkers to lighter drinkers within the study groups (based on frequency of alcohol consumption). However, over 50% of data were missing for both ACP and ACtrl groups due to different sample collection methods and difficulty in obtaining meaningful histories from patients. We had sufficient data to confirm that there was high alcohol consumption, but did not have information on the exact quantities of consumption, especially from the Birmingham liver samples. Therefore this sub-analysis could not be undertaken.

Table 28 Comparison on the frequency of call types between individual samples, or those where samples were concatenated from two runs

	Individual Samples		Concatenated Samples		
	Mean calls	**SEM**	**Mean calls**	**SEM**	**p value**
Not called	1033.60	56.31	1270.44	117.01	0.149
No variant	2878.39	50.72	2642.13	101.16	0.109
Heterozygous for variant	208.80	5.12	153.30	20.65	**0.001**
Homozygous for variant	105.76	2.24	160.13	30.68	**<0.001**

A sub-analysis of the whole dataset was performed excluding those patients who did not have a good enough quality score in their own right. The sensitivity analysis led to 6 of the SNPs no longer being of significance. For most there was a minor change in the numbers, and changes in significance were likely due to the reduced number of reads. However, in the case of *IL1B*_rs1143633, there was a significant shift in both OR and p value, suggesting this original difference may have been purely an artefact. The data are available in Appendix 10.2.

5.2.10 Internal Validation - Next Generation Sequencing compared with Genome-wide Association Study

Forty-six of the ACP samples were analysed by both the GWAS method and NGS. A total of 219 SNPs were covered by both modalities, giving over 10,000 comparable SNPs. Due to drop out of reads from some areas of the NGS results, there were 9300 points that were comparable, of which 8916 were concordant. This gave an overall accuracy of the dataset of 96%. 126 of the 219 SNPs analysis were 100% concordant. Of the remaining 97 SNPs with some element of discordance, there was a range of 44% to 98%.

5.2.11 Next Generation Sequencing Validation with Sequenom (Stage 1)

Of the initial batch of 203 samples, 170 were rerun to confirm the results using the Sequenom array. Details of the demographics of the samples used are presented in Table 31. An example of the genotype cluster is given in Figure 17. The Sequenom results are presented in Table 32.

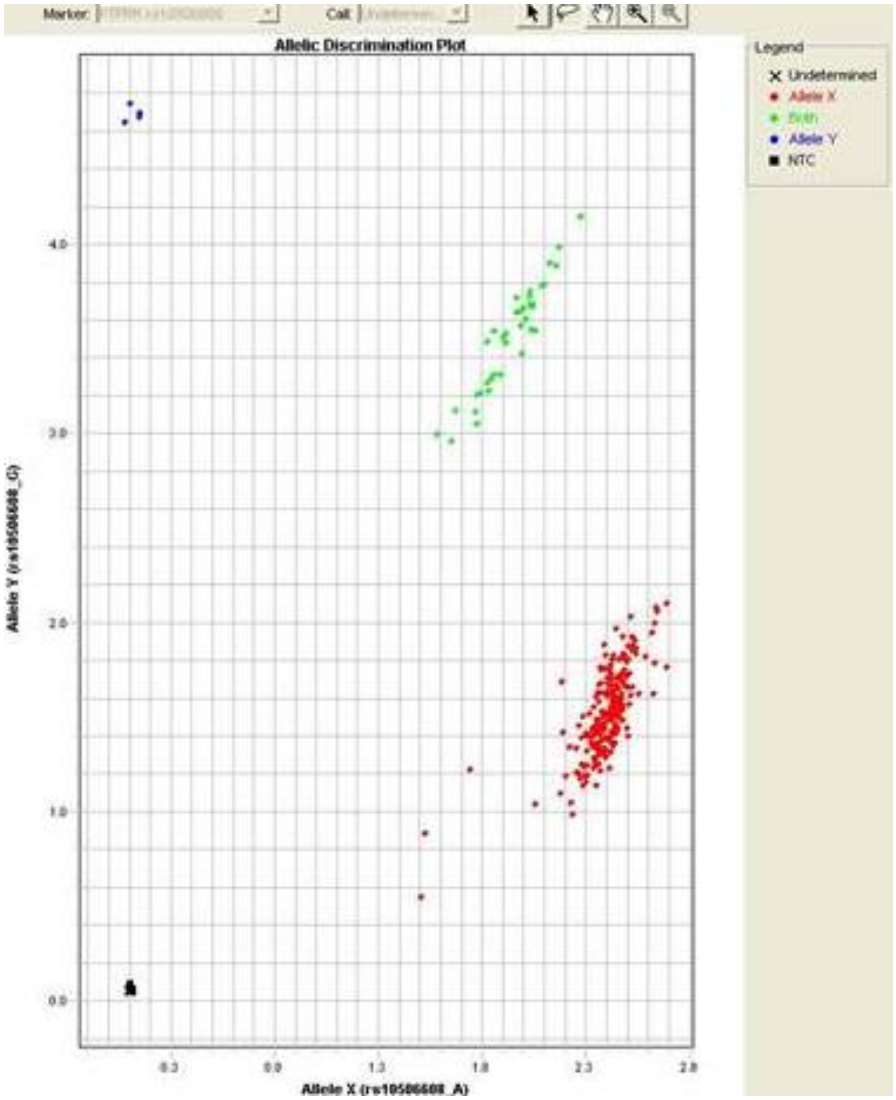


Figure 17 Example SNP genotype clusters on Sequenom for *PTPRR*_rs10506608

Red and blue indicate called homozygotes, green indicates heterozygotes, and black is non-called (missing) genotypes. This graph shows the distinct separation between the different groups.

5.2.12 Next Generation Sequencing Validation with Sequenom (Stage 2)

The validation set were then run through the same Sequenom chip, using a second set of patient samples as described in Table 31. Results are presented in Table 32 and concordance of samples is presented in Table 30.

In addition to the ACP groups and ALD groups, 49 HC who had a history of alcohol excess with no evidence of ACP or ALD, were also sequenced with the Sequenom. The results are presented in Table 33, in comparison with the combined ACP and ALD groups.

An assessment was made of the correlation of SNPs read between the two sequencing modalities, comparing exact matches, those that had 50% discordance (on matching allele) and 100% (complete) discordance (neither allele matching); Table 29.

Table 29 Correlation between Next Generation Sequencing and Sequenom data

SNP	Exact Match	50% discordant	100% discordant	% Correlation	% Discordant
*ACACB*_rs17848835	147	18	19	89.09	22.62
*ADH1C*_rs1693482	76	87	0	46.63	0
*ADH5*_rs116010022	165	3	0	98.21	0
*ADH6*_rs4147545	120	27	2	81.63	1.24
*APOB*_rs72653066	157	0	12	100	12.77
*APOC3*_rs4520	118	48	10	71.08	11.11
*CES5A*_rs7500040	91	16	8	85.05	8.6
*FABP1*_rs2241883	124	15	0	89.21	0
*FABP3*_rs11578034	153	3	5	98.08	4.2
*FABP6*_rs10056214	81	81	63	50	41.72
*IL1B*_rs1143633	95	49	6	65.97	6.82
*LDLR*_rs1003723	129	35	3	78.66	3
*LIPA*_rs1051338	65	34	11	65.66	11
*LIPC*_rs3751542	122	38	1	76.25	0.89
*LIPC*_rs690	130	28	2	82.28	2
*MGLL*_rs116367069	154	4	0	97.47	0
*PRSS1*_rs6666	89	73	2	54.94	2.15
*PTPRR*_rs10506608	154	7	0	95.65	0

Table 30 Comparison of additional validation step between using Sanger sequencing in comparison to Next Generation Sequencing and Sequenom results

		Sanger results			
	Number that are discordant	**Agrees with NGS**	**Agrees with Sequenom**	**Agrees with neither**	**Misread**
*ACACB*_rs17848835	18	6 (33%)	12 (67%)	0	0
*ADH1C*_rs1693482	88	6 (7%)	6 (7%)	16 (18%)	60 (68%)
*ADH5*_rs116010022	3	1 (33%)	2 (67%)	0	0
*FABP1*_rs2241883	15	2 (13%)	13 (87%)	0	0
*FABP3*_rs11578034	3	0 (0%)	3 (100%)	0	0
*IL1B*_rs1143633	49	4 (8%)	44 (90%)	1 (2%)	0
*LIPA*_rs1051338	34	3 (9%)	31 (91%)	0	0
*LIPC*_rs690	28	4 (14%)	24 (86%)	0	0
*LIPC*_rs3751542	37	4 (11%)	33 (89%)	0	0
*MGLL*_rs116367069	4	1 (25%)	3 (75%)	0	0
*PRSS1*_rs6666	71	4 (6%)	53 (75%)	1 (1%)	13 (18%)
*PTPRR*_rs10506608	7	2 (29%)	5 (71%)	0	0

Table 31 Characterisation of the demographics of the samples used in the Sequenom validation

		Stage 1		Stage 2		Combined	
		ACP (114)	**ACtrl (87)**	**ACP (80)**	**ACtrl (85)**	**ACP (194)**	**ACtrl (172)**
Age	Median (range)	53 years (26-90)	52 years (26-77)	43 years (22-73)	57 years (34-94)	49 years (22-90)	55 years (26-94)
Sex	Male	87	55	68	54	155	109
	Female	27	32	12	30	39	62
Smoking	Never	9	27	6	23	15	50
	Former	28	19	10	21	38	40
	Current	76	36	57	39	133	75
	Missing	1	5	7	1	8	6
Alcohol	Former Current	70	44	52	46	132	90
	Missing	40	27	22	37	62	64
		4	16	6	1	10	26
Diabetes	No	74	80	44	69	118	149
	Diabetic	39	7	28	15	67	22
	Missing	1	0	8	0	9	0
Diagnosis of CP	CT	102	80	71	79	173	159
confirmed or refuted	EUS	19	0	16	0	35	0
	Histology	52	0	32	0	84	0
History of ALD	No	114	4	80	8	194	12
	Yes	0	83	0	77	0	160
Source of DNA	Blood	114	40	80	85	194	125
	Liver biopsy	0	47	0	0	0	47

Table 32 Sequenom results from the Stage 1, Stage 2 and both stages combined comparing Alcohol-related Chronic Pancreatitis and Alcoholic Controls

	Stage 1				Stage 2				Stage 1 & 2 Combined			
	Presence of variant		Homozygous for variant		Presence of variant		Homozygous for variant		Presence of variant		Homozygous for variant	
	OR	p value	OR	p value	OR	p value	OR	p value	OR	p value	OR	p value
ACACB rs17848835	0.412	**0.039**	0.134	0.082	1.541	0.291	3.960	0.210	0.930	0.499	0.492	0.437
*ADH1C*_rs1693482	0.604	0.214	0.833	0.865	0.655	0.844	0.320	-	0.667	**0.035**	0.715	0.344
*ADH5*_rs116010022	0.215	0.194	1.125	-	0.640	0.448	-	-	0.362	0.140	-	-
*ADH6*_rs4147545	1.435	0.386	3.428	0.093	0.740	0.470	1.535	0.228	0.967	0.970	1.989	**0.033**
APOB rs72653066	4.684	**0.040**	-	-	2.527	0.594	-	-	3.106	0.066	-	-
*APOC3*_rs4520	0.994	0.945	1.743	0.405	1.213	0.434	0.632	0.464	1.058	0.660	0.994	0.930
*CES5A*_rs7500040	0.419	0.080	0.522	0.078	0.198	0.174	0.783	0.321	0.369	**0.044**	0.660	**0.045**
*FABP1*_rs2241883	0.620	0.159	0.745	0.454	1.407	0.292	0.977	0.790	0.882	0.779	0.854	0.477
*FABP3*_rs11578034	0.845	0.123	-	-	1.939	0.210	-	-	0.637	0.657	-	-
*FABP6*_rs10056214	2.104	0.217	-	-	1.381	0.407	-	-	1.788	0.106	-	-
*IL1B*_rs1143633	0.718	0.648	0.659	0.119	1.035	0.774	0.912	0.907	0.873	0.654	0.802	0.269
*LDLR*_rs1003723	0.879	0.609	0.635	0.282	1.048	0.781	0.690	0.221	0.961	0.872	0.658	0.106
*LIPA*_rs1051338	1.793	0.058	1.141	0.810	0.676	0.089	0.413	0.067	1.033	0.775	0.649	0.275
*LIPC*_rs3751542	0.576	**0.043**	0.933	0.761	0.676	0.313	1.087	0.822	0.616	**0.027**	1.071	0.914
*LIPC*_rs690	0.789	0.153	1.284	0.535	0.893	0.591	0.957	0.984	0.859	0.546	1.107	0.689
*MGLL*_rs116367069	1.775	0.347	2.278	0.241	1.589	0.300	-	-	2.112	0.164	0.970	0.307
*PRSS1*_rs6666	3.529	**0.004**	1.421	0.131	5.930	**<0.001**	2.534	**0.011**	4.432	**<0.001**	1.840	**0.003**
*PTPRR*_rs10506608	3.122	**0.025**	1.114	0.250	0.348	**0.013**	0.15	**0.046**	0.924	0.930	0.320	0.323

Table 33 Combined results of Stage 1 and Stage 2 of Sequenom validation comparing allele frequencies between Alcohol-related Chronic Pancreatitis, Alcoholic Liver Disease and Alcoholic Healthy Controls

			Allele frequency			ACP compared to ALD		ACP compared to AHC		ALD compared to AHC	
	Alleles		ACP	ALD	AHC	OR	p value	OR	p value	OR	p value
ACACB rs17848835	C	T	0.11	0.13	0.102	0.93	0.623	0.816	0.539	0.878	0.792
*ADH1C*_rs1693482	C	T	0.251	0.321	0.143	0.734	0.057	1.410	0.141	1.921	**0.007**
*ADH5*_rs116010022	C	A	0.008	0.021	0.01	0.362	0.126	0.944	0.961	2.605	0.359
*ADH6*_rs4147545	A	G	0.331	0.298	0.327	0.967	0.407	0.906	0.627	0.937	0.932
APOB rs72653066	G	C	0.025	0.008	0	3.106	0.081	-	0.087	-	0.327
*APOC3*_rs4520	C	T	0.262	0.25	0.224	1.058	0.833	1.409	0.264	1.331	0.330
*CES5A*_rs7500040	G	A	0.251	0.178	0.163	0.652	**0.021**	0.582	**0.038**	0.893	0.579
*FABP1*_rs2241883	T	C	0.29	0.267	0.316	0.882	0.46	1.135	0.600	1.286	0.295
*FABP3*_rs11578034	C	T	0.006	0.008	0	0.637	0.622	-	0.425	-	0.319
*FABP6*_rs10056214	C	G	0.083	0.054	0.056	1.788	0.084	1.314	0.564	0.735	0.532
*IL1B*_rs1143633	G	A	0.395	0.359	0.357	0.873	0.347	0.573	0.398	0.656	0.843
*LDLR*_rs1003723	C	T	0.406	0.444	0.52	0.961	0.297	0.637	0.053	0.663	0.230
*LIPA*_rs1051338	A	C	0.312	0.322	0.357	1.033	0.653	1.063	0.672	1.029	0.912
*LIPC*_rs3751542	A	G	0.285	0.34	0.245	0.616	0.098	1.211	0.531	1.965	0.082
*LIPC*_rs690	G	T	0.428	0.426	0.490	0.859	1	1.398	0.389	1.628	0.389
*MGLL*_rs116367069	A	G	0.058	0.035	0.051	2.187	**0.045**	0.761	0.636	0.360	**0.039**
*PRSS1*_rs6666	T	C	0.702	0.539	0.488	1.991	**<0.001**	3.063	**<0.001**	1.538	**0.020**
*PTPRR*_rs10506608	A	G	0.072	0.079	0.102	0.924	0.596	0.771	0.626	0.834	0.905

5.2.13 Next Generation Sequencing Haplotype Analysis

The most consistent polymorphism throughout the NGS analysis was *PRSS1*_rs6666. This SNP has been previously described, but is believed to be benign in nature and is common within both populations, therefore, it was felt it could in fact be a marker for a haplotype, which holds the disease causing polymorphism. Therefore, all other SNPs on Chromosome 7, where *PRSS1*_rs6666 lies, were examined to look at their association with *PRSS1*_rs6666 using χ^2 in both the disease and control populations. Those SNPs which showed significance at the <0.05 level were included in the haplotype analysis. The OR was calculated for the common haplotypes in both the disease group and the control group. If there was no effect on the model exhibited by the presence of a SNP, it was dropped from the haplotype and the new, smaller haplotype were analysed. This was done sequentially until the most significant haplotype was formed. The results of this analysis are shown in Table 34. No haplotype was produced that gave better OR than simply using the polymorphism *PRSS1*_rs6666 as a factor.

Table 34 Comparison of different haplotypes and the association with Alcohol-related Chronic Pancreatitis in relation to Alcoholic Controls

CFTR		*PRSS1*					
rs1042077	rs1800136	rs6666	rs201550522				
p.T854T	p.Q1463Q	p.D162D	p.K170E	OR	Upper CI	Lower CI	p value
T	G	T	A	0.516	0.305	0.873	**0.01**
T	G	T	G	1.942	0.178	21.159	NS
T	G	C	A	1.947	1.147	3.306	**0.013**
T	G	C	G	2.051	0.073	57.828	NS
T	A	T	A	1.177	0.256	5.411	NS
T	A	T	G	-			NS
T	A	C	A	1.273	0.316	5.127	NS
T	A	C	G	-			NS
G	G	T	A	0.728	0.399	1.329	NS
G	G	T	G	0.281	0.016	4.852	NS
G	G	C	A	1.294	0.624	2.684	NS
G	G	C	G	0.075	0.000	24.121	NS
G	A	T	A	1.110	0.290	4.251	NS
G	A	T	G	-			NS
G	A	C	A	1.299	0.380	4.441	NS
G	A	C	G	-			NS
T	G	T		0.542	0.323	0.911	**0.020**
T	G	C		1.969	1.163	3.333	**0.013**
T	A	T		1.373	0.313	6.028	NS
T	A	C		1.307	0.327	5.226	NS
G	G	T		0.688	0.381	1.243	NS
G	G	C		1.163	0.571	2.368	NS
G	A	T		1.139	0.300	4.332	NS
G	A	C		1.325	0.389	4.510	NS
T		T		0.581	0.351	0.961	**0.035**
T		C		1.963	1.179	3.269	**0.009**
G		T		0.733	0.420	1.280	NS
G		C		1.217	0.645	2.297	NS
		T		0.554	0.369	0.832	**0.004**
		C		1.804	1.202	2.707	**0.004**

5.3 *Discussion*

5.3.1 Previous Identified Variants

5.3.1.1 Protease, Serine, 1 (Trypsin 1)

The most well-known disease causing variants within *PRSS1* are autosomal dominant in expression, and therefore would not be expected to be represented within this population of patients with no familial history of pancreatic disease. Initially results of the NGS work suggested some patients were exhibiting the p.A16V polymorphism, but on further investigation this was shown to be a misread. This is not unexpected within the trypsin coding genes as there are many genetic similarities between them (Figure 1) which may lead to either cleavage of the wrong amplicons initially or misalignment of amplicons within the Ion Torrent™ leading to apparent polymorphisms. In the case of p.A16V, this variant mirrors the natural variation within *PRSS3*, leading to misreading of this region in a few of the samples.

The previously reported, but believed to not be disease related, polymorphisms p.D162D and p.N264N will be addressed in Chapter 6.3.2.8.1.

5.3.1.2 Serine Protease Inhibitor Kazal Type I

p.N34S *SPINK1* was first identified as an associated factor in 2000 at which time it was thought to be a clearly causative factor with 23% of tested CP patients having the variant, and none of the controls.[45] However, subsequent studies have shown the variant could be present in healthy controls, but the frequency was still higher in patients with CP (most commonly those who developed idiopathic CP from childhood). Therefore it is unsurprising that both ACP and ACtrl groups showed the presence of p.N34S, and although the prevalence was higher in the ACP group, this did not reach significance in the small sample size used.

5.3.1.3 Cystic Fibrosis Transmembrane Conductance Regulator

There has been a previously reported risk haplotype within *CFTR* associated with chronic pancreatitis, which includes two of the significant *CFTR* SNPS identified. The haplotypes were imputed using three SNPs in the haplotype (*CFTR*_rs213950, *CFTR*_rs1042077 and *CFTR*_rs1800136).[186] The previously described *CFTR* haplotype was reconfirmed by the SNP array data (Table 18).[186] Both these populations used a mix of ACP and ICP patients compared to the normal population. When we examined the NGS results of an ACP population compared to an ACtrl group for these polymorphisms, no difference was shown (Table 34). It may be that this association is only exhibited within the ICP group, but this theory will need to be tested in further in an ICP population.

In all cases the haplotype analysis has been carried out using software based on probabilistic models to create the likely haplotypes, therefore it is likely that some haplotypes will be over, or under called in frequency.[331] The best way to create haplotypes is using points that can be sequencing on the same strand with long distance sequencing, but this was not possible within our study populations and therefore imputation was the only method of analysis available.

The Whitcomb group recently reported that the coinheritance of *CFTR* p.R75Q and *SPINK1* p.N34S variants is significantly higher in patients with idiopathic chronic pancreatitis than in controls (8.75% vs. 0.38%).[181] In our population, none of the participants were found to have both the p.R75Q and p.N34S variation in either subgroup.

5.3.2 Significant SNPs identified in the NGS analysis

5.3.2.1 Alcohol Dehydrogenase

The polymorphism, *ADH1C*_rs1693482 (p.R272Q), has been frequently described in the literature in relation to alcohol dependence, and has also been associated with other alcohol risk diseases such as gastric cancer.[202,332]

It has been shown that the duodenal and jejunal activity of *ADH1C* is reduced in the presence of the *ADH1C*_rs1693482.[6]

Many different populations have shown to have a strong association with this polymorphism and alcoholism.[123,127] Analysis of a Korean GWAS has showed an association of *ADH1C*_rs1693482 to alcohol dependence but it was felt that this association might be driven through linkage disequilibrium with *ADH1B*_rs1229984 (p.R48H), rather than be a pathogenic polymorphism in its own right.[123] Similar results have been reported in a British and Irish population although the level of linkage disequilibrium was not as strong.[202]

Some other studies have been less sure of the AD risk of *ADH1C*_rs1693482 in Europeans.[206,333,334] However it is felt that there is some independent effect of both *ADH1B* and *ADH1C* on the drinking habits of European populations.[334,335]

One paper has suggested that there is also an association of this polymorphism with the development of ALD in combination with *ADH1C*_rs698 (p.I350V) and *ADH1B*_rs1229984 (p.R48H).[336] p.R272Q and p.I350V were shown to be in almost complete linkage disequilibrium (Appendix 10.4, Table 48). The 272Q/350V allele increased the risk of alcohol dependence in the homozygous form (OR 1.78, 1.11-2.85; p = 0.016). When *ADH1B* 48H was combined with the wild type *ADH1C* R272/I350 allele, it was protective against ALD (OR 0.37, 0.16-0.85; p = 0.019).

The significance of *ADH1C*_rs1693482 was maintained following the validation stage, but only between the ALD group and the AHC (OR 4.44, 1.71-11.52); ACP and AHC approached but did not

reach significance. Both these finding are supported by the previous findings in relation to alcohol dependence which is likely to be the genuine significance from this polymorphism.

A meta-analysis of 6796 cases and 6938 controls including samples of Asian, European, African, and Native American origins showed strong evidence of association between *ADH1C*_rs698 (p.I350V) and alcohol dependence (OR 1.51, 1.31-1.73; $p = 1 \times 10^{-8}$)[337] with Asian population demonstrating a stronger association (OR 2.14, 1.89-2.43; $p = 4 \times 10^{-33}$). The findings support that *ADH1C*_rs698 may increase the risk of AD as well as alcohol-related cirrhosis in pooled populations, with the most consistent effects in Asians.[337] There was no difference demonstrated between ACP and ACtrl in our population (Table 33).

The common polymorphism *ADH1B*2* has been well described to be heavily associated with alcohol dependence.[123] As all patients used in this analysis had a history of alcohol dependence, there was no association shown between the groups (8/100 vs. 4/96).

It has been shown that *ADH1C*_rs698 (p.I350V) substitution is in linkage disequilibrium with the *ADH1B*_rs1229984 (p.R48H) substitution, and that the *ADH1B*_rs1229984 polymorphism is responsible for differences in ethanol metabolism and alcoholism among Taiwanese populations, with the *ADH1C*_rs698 variant showing association only because of linkage disequilibrium.[204]

*ADH5*_rs116010022 is a previously identified intronic SNP, which has not been reported as being involved in any disease processes before. This SNP has been shown to be in complete linkage disequilibrium with 6 other intronic SNPs (Appendix 10.4, Table 48), none of which have been identified in the literature.

5.3.2.2 Lipase and Lipoprotein

The SNP *LDLR*_rs1003723 lies in the intronic region. It has been reported in two different studies by the same group to have an association with an increased risk of bile duct cancer and also has been show to raise total cholesterol ($p = 0.002$) and LDL ($p = 0.01$).[338,339]

There are several tagging SNPs associated with *LDLR*_rs1003723, two of which lie within coding regions of *LDLR*, but both are synonymous variations.

The closely associated tagging SNP, *LDLR*_rs688, is non-synonymous and codes for p.N591N. In previous work, despite its non-synonymous nature, it has been shown to exhibit functional changes to *LDLR*, leading to increased plasma total and LDL cholesterol.[340] This is thought to be due to an association with increased exon 12 alternative splicing.[341,342]

The associated SNP *LDLR*_rs5925 leads to the synonymous variant, p.V653V. A meta-analysis of studies assessing the role of *LDLR* in cerebral infarction showed that *LDLR*_rs5925 had been shown in three studies to be associated with an increased risk.[342]

*LDLR*_rs2738446 and *LDLR*_rs2738450 are both closely linked intronic SNPs, which have been examined in relation to modification of stroke presentation. In one study, a significant overall association between small vessel occlusion and the CC genotype was observed when compared to non-small vessel occlusion (OR 2.0, 1.08-3.70; $p = 0.025$).[343]

*LIPA*_rs1051338 lies within exon 2 and causes an amino acid change (p.T16P). It has previously been reported in a study related to obesity that, after adjustments for the effect of age, sex, diabetes, and medication, the C allele of SNP *LIPA*_rs1051338 was associated with lower blood pressure (systolic p = 0.004; diastolic p = 0.006).[344] It has also been shown to cause a significant reduction in 24S-hydroxycholesterol/cholesterol in one study.[345]

A previous GWAS examining coronary artery disease showed a tagging SNP, *LIPA*_rs1412444 was strongly associated with expression of the *LIPA* transcript itself but was not associated with hyperlipidemia.[346] This was supported by a separate GWAS.[347]

Another tagging SNP, *LIPA*_rs2246942, within the *LIPA* gene was associated with an increased risk of coronary heart disease (cases vs. HC: OR 1.63 (1.02-2.60), p = 0.04).[348]

*LIPC*_rs690 is a common synonymous SNP (p.V155V), which is only reported in one previous publication in relation to cleft lip, where it was found to have a weak association with the development of both cleft palate and dental abnormalities. However, when multiple testing is taking into account, the significance is lost.[349] There have been no recognised tagging SNPs in association with this variation.

MGLL_rs116367069 is a SNP that codes for an intronic region of monoglyceride lipase. This SNP has not been noted in the literature, nor does it have any associated tagging SNPs.

Following the validation process, *MGLL* was shown to be significantly different between ACP and ALD groups (OR 2.19, 0.01-4.64) and ALD and AHC groups (OR 0.38, 0.15-0.98). There was no difference between the ACP and AHC suggesting that the association may be with ALD rather than with ACP.

5.3.2.3 Trypsin

5.3.2.3.1 PRSS1_*rs6666*

This SNP codes for a common synonymous variant in *PRSS1*_rs666 (p.D162D).[164] Previous reports on this SNP are listed in Table 35. The normally reported allele frequencies for different populations are given in Table 36. It is reported to be in complete linkage disequilibrium with *PRSS1*_rs6667 (p.N246N).[168]

The majority of reports have suggested that there is no difference between with ACP or ICP populations.[350,351] However, there have been two studies (one published and one in abstract) suggesting there may be a relationship. One small study showed that the frequency of the C allele was higher in ACP group compared to HC (OR 8.44, 2.39-29.79).[352] The study presented in abstract also suggested that the C allele was in higher frequency in the ACP and ICP patients compared to healthy controls.[353]

These two studies support our findings produced by the NGS and the validation with Sequenom of *PRSS1*_rs6666.[352,353] Of note, these studies are both carried out within European populations. Table36 shows that there is a great variation in the frequency of this SNP within different populations, and this may account for the absence of a difference being identified in some other studies.

As this is a synonymous SNP, there is no clear reason why it could be causative, therefore it was postulated that it may be a marker for a disease haplotype. Using the technique described in the methods, several potential haplotypes were devised using other SNP identified within chromosome 7, however, the attrition of other potential SNPs did not increase the OR or the probity of the effect of *PRSS1*_rs6666. As only the exons of *PRSS1* and *CFTR* were specifically sequenced as part of NGS, we cannot rule out other significant variants within non-coding regions, which could be important in the disease haplotype. This could be further investigated using long-range sequencing of the *PRSS1* area.

Table 35 Previous published reports of *PRSS1*_rs6666

Paper	Year	Country	Nomenclature used by paper	Population group	Frequency of polymorphism
Gorry[49]	1997	USA	Asp162	HP families	Not stated
Teich[354]	1998	Germany	nt 133807 (C/T)	HP vs. relatives of HP	7/7 (100%) vs. 3/14 (21%)
Ferec[164]	1999	France	D162D	HP families	No comment
Nishimori[355]	1999	Japan	[162]Asp	HP families	No comment
*Gomez Lira[356]	2001	Italy	133807 C/T	NHNST	13/50 (26%) NHNST
O'Reilly[352]	2001	UK	C133807T	ACP vs. HC	9/29 (31%) vs. 19/24 (79%); p < 0.001
Tautermann[168]	2001	Germany	D162D	ICP	34/109 (31%)
Chandak[357]	2002	India	D162D	TCP vs. HC	58/68 (85%) vs. 90/100 (90%); p = 0.5
*Patuzzo[357]	2002	Italy	133807 C/T	NHNST	21/50 (24%)
Guarner[353]	2003	Spain	D162D	5.3.2.3.2 44 ACP vs. 22 ICP vs. 34 HC	23/44 (52%) vs. 12/22 (55%) vs. 28/34 (82%)
Bernardino[351]	2003	Brazil	N264N	(64 ACP, 16 ICP, 2 HP) vs. 200 HC	47/164 (29%) vs. 85/300 (28%) p = 1
Chandak[358]	2004	India	162Asp	(37 HP, 120 ICP, 41 ACP) vs. 290 HC	88% vs. 90%
Tzetis[350]	2007	Greece	p.D162D	ICP/RAP vs. HC	9/25 (36%) vs. 10/25 (40%); p = 1
Liu[359]	2009	China	133807 C/T	(22 ACP, 16 ICP, 30 TG, 35 HP, 150 GS)	27/253 (11%)
Johnstone	2014	UK	p.D162D	ACP vs. ALD vs. AHC	105/352 (30%) vs. 155/342 (45%) vs. 54/102 (53%)

NHNST – Neonatal Hypertrypsinaemia with normal sweat chloride tests.

*Gomez Lira 2001 and Patuzzo 2002 may be using the same population.

Table 36 MAF of *PRSS1*_rs6666 in different populations

Region	C	T
Africa	0.41	0.59
American	0.53	0.47
Asian	**0.22**	**0.78**
Han Chinese, Beijing	0.27	0.73
Southern Han Chinese	0.24	0.76
Europe	**0.58**	**0.42**
British	0.53	0.47
Spain	0.58	0.42
Italy	0.58	0.42
South Asia	**0.28**	**0.72**
India	0.3	0.7
Sri Lanka	0.2	0.8
All	0.4	0.6

Data taken from 1000 genomes database 4th Nov 2014.

The T allele polymorphism is reported to have a lower frequency in Europeans (0.4), whereas it is more frequent in subjects of Asian origin (0.75) or subjects from those from India (0.9).[360]

To assess what the functional significance of rs666 may be, we examined codon usage, which showed no bias to either codon, there was no splice site at the region of the variant and in a search for micro RNA that bound to *PRSS1*, only has-miR-185 was shown to be associated. There was no association with the sequence in exon 4. When assessing for enhancer binding, one potential transcription factor that would bind preferentially to the T variant was identified. This gave an 18% increase in function, but this is unlikely to be the main promoter of this gene.

5.3.2.3.2.1 *PRSS1*_rs6667

The most common tagging SNP is p.N246N, which has been reported alongside *PRSS1*_rs6666 in most papers, the only exception being Bernardino *et al*.[351] In this paper there was no difference in frequency of *PRSS1*_rs6667 in either CP or control groups in a Brazilian population.

In this study there was no significance found in *PRSS1*_rs6667. Where *PRSS1*_rs66667 was compared to *PRSS1*_rs6666 there was only between a 60-68% correlation in reads, accounting for

the difference in significance. The difference in reads may be related to the difficultly in sequencing the trypsin regions, due to the multiple isoforms, which can lead to misalignment and misreading of these readings as demonstrated in Figure 1.

*PRSS1*_rs6667 was examined in a similar way to assess for codon bias, splice sites, micro RNA and binding enhancers, but nothing of significance was found.

Other tagging SNPs are reported in Appendix 10.4, Table 48. None lie within exonic regions or have been previously reported on in the literature.

5.3.3 Quality of Next Generation Sequencing Results

Sub-analysis was performed removing the ten samples that had insufficient coverage to produce 50% on the SNPs analysed, and this had no effect on outcome. This cut off may have been fairly conservative, 28 samples lay beneath 70% covered and 69 samples had less than 90% coverage, but due to the small sample size used, such a large reduction in number would be likely to remove any true significance from the results.

Quality control of the Next Generation Sequencing results showed that results were less reliable from samples where the results from two chips had been concatenated and including these results may have given false positive readings. These samples were excluded from sub-analysis, and showed better consistency of results (Table 47). As a consequence, the sub-analysis has reduced down the number of samples that can be analysed, and therefore some samples may lose significance simply due to the reduced numbers.

5.3.3.1 Calling of Variants

The appropriate calling of variants by the Ion Torrent™ first required correct alignment of the amplicons to the reference structure, and also requires the appropriate criteria to identify whether there is truly a variant at this point, or simply a misread in the DNA. As with all tests there is a balance between sensitivity and the specificity that must be achieved. We selected a custom designed approach to both the alignment and the SNP calls to try and optimise the sensitivity and specificity of the test. The identification of false reads is less common in the case of germline mutations, and a fairly high threshold (>25% calls) is required to ensure this. It is more probable that some true variants may be missed due to exclusion of individuals with any reads under 25%. There may also be miscalling between homozygous and heterozygous calls, which lie at a cut-off of above and below 75% of all calls, respectively. This system may lead to bias towards the calling of SNPs, rather than the normal variant.[361]

5.3.3.2 Nature of Variants

The most commonly recognised reading errors, specific to the Ion Torrent™ system are homopolymers.[362] Due to the nature of the reading system the number of bases in homopolymers is calculated by the amplitude of the pH change, which has more variability with the more consecutive reads at this point. As a result of this, when the potential *SNPs of Interest* were examined, many were discarded as they were related to a long homopolymer, and all variant reads appeared to be in relation to this. As a result any variants either directly related to the homopolymers or which immediately juxtapose a homopolymer, are likely to be assumed to be this common read error.

5.3.3.3 Phenotypic Significance of Variants

To assess the potential phenotypic sequelae of some of the variants expresses, different online tools (SIFT and Polyphen) were used to assess the potential likelihood of an effect by looking at homologues and orthologues of the sequence.[363] These tools are only useful in the case of non-synonymous SNPs, and only give a predication related to other species, or similar polymorphism identified within the same species, which may not be related to the genuine significance of the SNP in question.

5.3.3.4 Tagging SNPs

The use of Tagging SNPs to identify the true area of genetic interest is a common approach, and relies on close LD between the SNPs selected.[364] In the NGS work, there were some unexpected inconsistencies between the calls of pairs of tagging SNPs, making the validity of this difficult to assess.

5.3.3.5 Next Generation Sequencing Validation

The NGS data was independently validated in part by cross over with the GWAS data and Sequenom sequencing and with Sanger sequencing in the case of p.A16V. There were some SNPs with poor concordance between the different modalities. The individual SNP analysis performed as part of the

GWAS and Sequenom sequencing, exhibit similar sequencing techniques, and this may explain consistent errors between different modalities. The NGS uses different technology, but relies on good use of alignment and calling tools to produce an accurate result.

Due to the discrepancies in some SNPs between the NGS and Sequenom validation data, further Sanger sequencing was undertaken as a second stage of validation, to determine which of the modalities are more consistent, or whether any differences are just natural variation in the reads between different modalities. This showed that for the most part that the Sequenom provided more reliable reads than the analysis of the NGS data. This may have been in part due to the analysis pipeline used to interpret the ion torrent data that may have been due to difficulties with alignment or with call thresholds in relation to the interpretation of the data.

These steps of validation have allowed us to hone the analysis to identify a few key SNPs that have been replicated in two populations with more than one modality of analysis. Further work can be done to reassess the original NGS output to identify if there are other key SNPs that may be been overlooked with the original analysis. In addition the key SNPs, which have been shown to be significant in this work need to be further, assess in similar and different populations to confirm the validity of results. Once a clear association is established, further research will need to be conducted to confirm what the physiological process is that leads to disease predisposition.

5.3.4 Statistical Analysis

Due to the large amount of data create by the Next Generation Sequencing, there is potential risk of identifying false positive results due to multiple testing. In larger studies such as GWAS, there is commonly alteration to the p value using Bonferroni correction.[365] However it was not felt appropriate in this study due to the small sample size in the discovery set, and the specific selection of the genetic areas for study.

6 Chapter 6: Variable Nuculeotide Tandam Repeat in Carboxyl-ester Lipase

FAEEs are a product of the non-oxidative metabolism of ethanol and fatty acids; carboxyl-ester lipase (CEL) is a key enzyme involved in this metabolic pathway.[29,366,367]

There is a 33bp Variable Number Tandem Repeat (VNTR) in exon 11 (the final exon) of CEL.[368] This VNTR sits in a functional domain of the gene and is most commonly represented by a 16–repeat variant, but between 3 and 23 repeats have been reported, with segment duplication also present in up to 5% of individuals.[369]

Due to the lack of consistency in the literature, we sought to further investigate potential associations between this polymorphism and ACP using a combined strategy with both a matched control cohort of alcoholic liver disease patients and a group of healthy controls from a British population.

6.1 *Materials and Methods*

6.1.1 Patients

6.1.1.1 Chronic Pancreatitis

Participants included in the *CEL* study had to meet the following criteria

- symptoms typical of chronic pancreatitis
- alcohol excess of greater than 35u/wk (equivalent of >40g/day)[43] for at least 5 years
- radiological evidence of chronic pancreatitis (CT/MRI/EUS)
- cell pellet available for DNA extraction
- patient consented to take part in research projects

6.1.1.2 Alcoholic Liver Disease

Participants included in the *CEL* study had to meet the following criteria

- no personal or family history of chronic pancreatitis
- reviewed by the hepatology clinic with a diagnosis of alcoholic liver disease or having undergone a liver transplant for alcoholic cirrhosis
- alcohol excess of greater than 35u/wk (equivalent of >40g/day)[43] for at least 5 years
- radiological evidence of a morphologically normal pancreatitis (CT/MRI/EUS)
- cell pellet available for DNA extraction
- patient consented to take part in research projects

6.1.1.3 Healthy Controls

Participants included in the *CEL* study had to meet the following criteria

- no personal or family history of chronic pancreatitis
- no personal or family history of alcoholic liver disease
- no history of alcohol excess
- DNA available
- patient consented to take part in research projects

These criteria parallel the type of patient samples used for the NGS project, but the actual groups used had some overlap, were not the same.

6.1.2 Association of Carboxyl-Ester Lipase Variable Nucleotide Tandem Repeat

6.1.2.1 Polymerase Chain Reaction

PCR was performed using minor modifications on methodology previously described by Torsvik *et al.*[369] A PCR reaction volume of 10µl was used, containing 1 x GC buffer I, 0.34mM dNTPs, 0.2U LaTaq Polymerase (TaKaRa, Japan RR02AG), 1.0M betaine (Sigma-Aldrich, USA), 0.2µM CEL_VNTR_F primer (5'-ACCGACCAGGAGGCCACCC-3'), 0.2µM CEL-VNTR_R2 primer (5'-CCTGGGGTCCCACTCTTGT-3'; labelled with HEX) and 20ng template DNA. The PCR cycle was 94^{o}C for 1min 30s; then 94^{o}C for 30s, 61^{o}C for 30s and 72^{o}C for 1min 10s for 40 cycles, followed by 72^{o}C for 5min, and cooling to 4^{o}C.

6.1.2.2 Fragment Analysis

The PCR product (2µl) was mixed with 10 µl of Hi-Di™ Formamide and 0.5 µl GeneScan™ 1000 ROX Size Standard (both Life Technologies, Paisley) heated at 95^{o}C for 2 min and snap chilled on ice prior to loading on a 3130 Genetic Analyser (Life Technologies, Paisley). Electrophoretic separation utilised a 36cm capillary array and POP7 polymer. The GeneMapper software v2.0 (Life Technologies, Paisley) was used to analyse the detected peaks.

6.1.2.3 Statistical Analysis

Haplotypes were subdivided into short (S), normal (N) or long (L) VNTR alleles; the most common number of repeats (16) was classified as normal, those with less than 16 repeats as short, and those with more than 16, as long.[219] Data were presented using medians (interquartile range). Differences between cohorts were examined using χ^2 or Fisher's exact test for categorical data and Mann-Whitney-U for continuous data (SPSS 20.0, Chicago, Illinois).

To test whether there was selection for or against homozygotes and whether populations were representative, Hardy Weinberg equilibrium was used ($p^2 + 2pq + q^2 = 1$). This was tested using the χ^2 test of expected numbers of homozygote and heterozygote based on allele frequency, taking the 16-repeat VNTR as the major allelotype (p) and any variation from this as the minor allelotype (q).

6.2 *Results*

In total 284 samples were analysed, 102 ACP, 91 ALD, and 91 HC: median age ACP 42 (37-49) years, ALD 57 years (50-63; $p < 0.001$). Male distribution between the groups was 86 ACP (84%), 60 ALD (66%) and 36 HC (40%; Table 37).

Results were assessed using both gel electrophoresis (Figure 19) and capillary electrophoresis (Figure 20).

Data on individual allele frequencies by group are presented in Table 38. The overall frequency of the normal, 16-repeat allele was statistically different between ACP (56%) and ALD (67%; $p = 0.020$). There was no significant difference between ACP and the HC (58%) groups ($p = 0.729$) and the difference between ALD and the HC groups tended towards, but did not reach statistical significance ($p = 0.062$). The long allele was more common in patients with ACP (6%) than ALD (2%; $p = 0.036$), but there was no significant difference in either group from HC (4%; $p > 0.1$).

Genotypes of patients by cohort group are presented in Table 39. Again there was a significant difference between the number of individuals homozygous for the normal 16-repeat (NN) between ACP (33%) and ALD (47%; $p = 0.034$). This difference was upheld between the ALD and HC (33%) groups ($p = 0.049$) but not between ACP and HC ($p = 0.928$). There was also a greater representation of NL genotype in the ACP patients (9%) compared to both HC (2%) and ALD patients (0%; ACP vs. ALD, $p = 0.004$). Duplicates of the *CEL* VNTR were identified in 8 (3%) patients with no variation between groups.

The level of selection of the different groups for or against the normal allele was tested using the Hardy-Weinberg equation. For all groups, there was no selection for or against homozygotes.

There was no significant difference between the distribution of alleles between males and females (S, 36% vs. 37%; N, 60% vs. 62%; L, 5% vs. 3%; $p>0.1$).

Table 37 Demographics of the Alcohol-related Chronic Pancreatitis and Alcohol Control groups

		Alcohol-related Chronic Pancreatitis (101)	Alcohol Controls (91)
Age (years)	Median (range)	43 (20-72)	57 (34-80)
Sex	Male	85	60
	Female	16	31
Smoking	Never	10	31
	Former	18	21
	Current	69	38
	Missing	4	1
Alcohol	Former	71	58
	Current	30	33
Diabetes	Yes	39	21
	No	62	70
CP confirmed/refuted			
	CT	97	91
	Histology	55	0

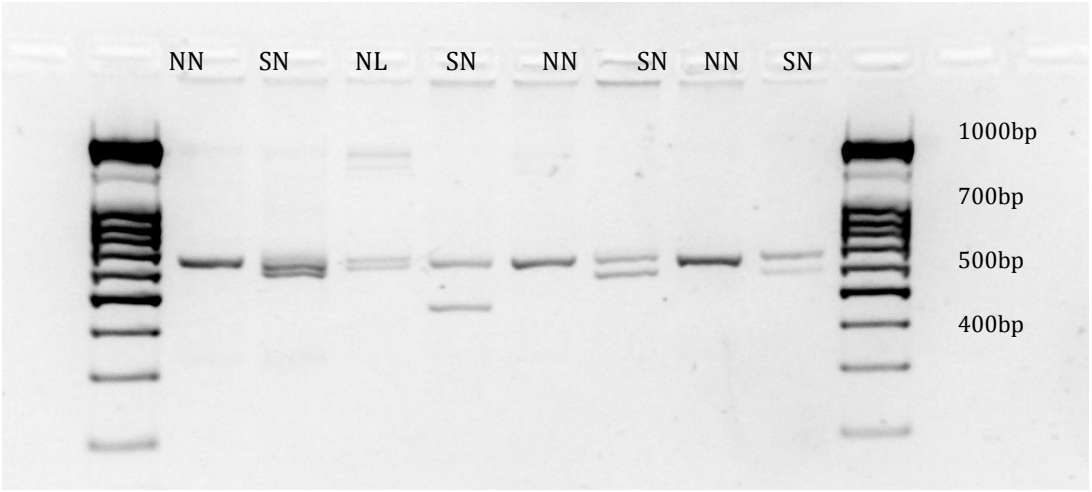


Figure 18 Electrophoresis gel showing *CEL* PCR product for different combinations of Variable Tandem Repeats

Different samples are represented: NN, homozygous for the normal 16 repeat at 617bp; SN, heterozygous for normal (16 repeats) and the shorter 10, 14 or 15 repeat VNTR; and NL, heterozygous for the normal 16-repeat and the longer 17 repeat.

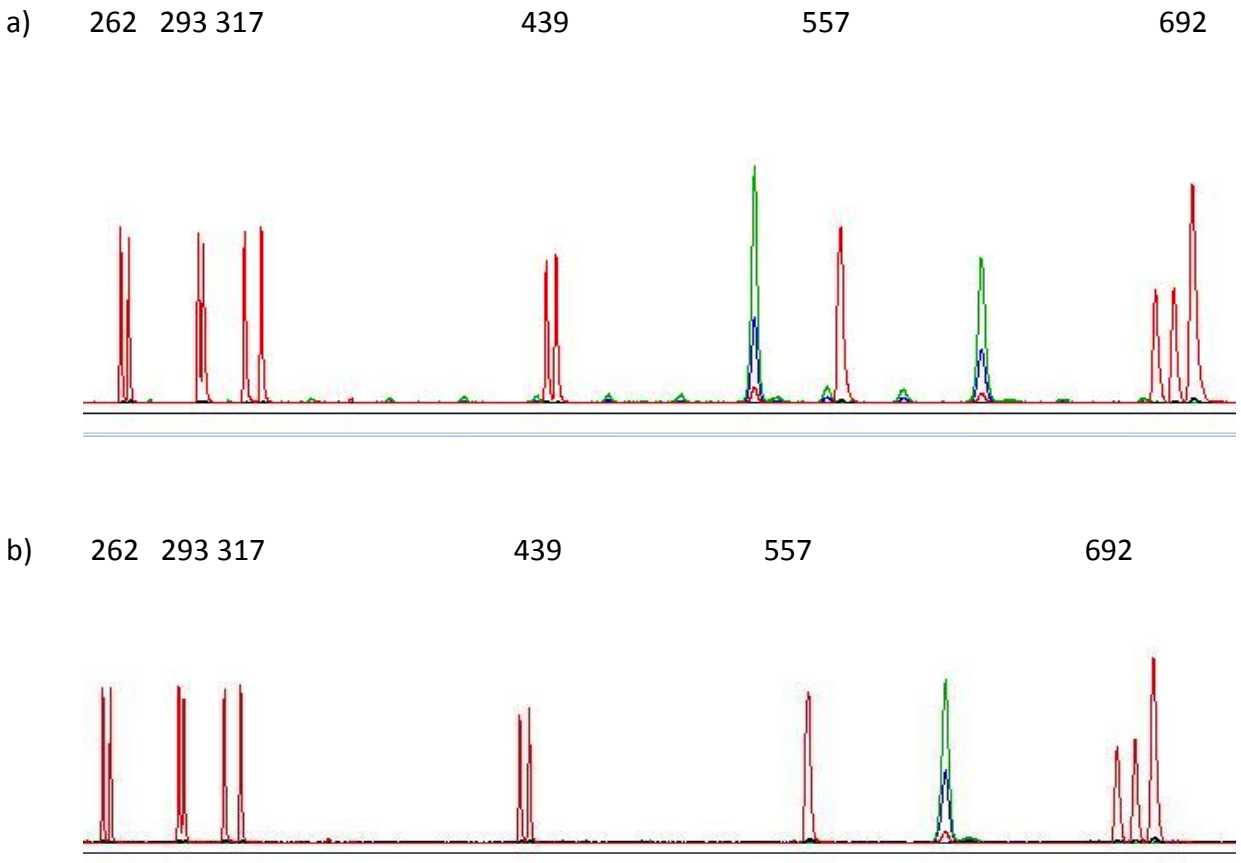


Figure 19 Capillary electrophoresis output for a) short/normal and b) homozygous normal VNTR

Red -ROX1000 ladder; Blue/green - marked PCR product showing a double peak at 518bp (13 repeats) and 617bp (16 repeats) in trace a, and only a single peak at 617bp for trace b.

Table 38 Total allele frequencies of the *CEL* VNTR by cohort

Number of VNTR repeats	ACP (%)	ALD (%)	HC (%)	ACP vs. ALD p value
7	2 (1.0)	0 (0)	0 (0)	0.501
10	0 (0)	1 (0.5)	0 (0)	0.472
11	1 (0.5)	0 (0)	0 (0)	1.000
12	8 (3.9)	2 (1.1)	1 (0.5)	0.110
13	12 (5.8)	8 (4.3)	14 (7.6)	0.508
14	29 (14.0)	19 (10.3)	29 (15.8)	0.260
15	28 (13.5)	28 (15.1)	27 (14.7)	0.650
16	115 (55.6)	124 (67.0)	106 (57.6)	**0.020**
17	10 (4.8)	3 (1.6)	7 (3.8)	0.094
18	2 (1.0)	0 (0)	0 (0)	0.501
Total	207	185	184	

p value was calculated using χ^2 or Fisher's exact test as appropriate.

$p < 0.05$ was considered significant and is highlighted in bold.

Table 39 Frequency of genotypes by groups of VNTR repeats

Genotypes	ACP (%)	ALD (%)	HC (%)	ACP vs. ALD p value
SS	18 (17.6)	8 (8.8)	11 (12.1)	0.072
SN	37 (36.3)	35 (38.5)	41 (45.0)	0.754
SSN	2 (2.0)	2 (2.2)	2 (2.2)	0.908
SL	2 (2.0)	2 (2.2)	4 (4.4)	0.908
NN	33 (32.6)	43 (47.3)	30 (33.0)	**0.034**
SNL	1 (1.0)	1 (1.1)	0 (0)	0.935
NL	9 (8.8)	0 (0)	3 (3.3)	**0.004**
LL	0 (0)	0 (0)	0 (0)	1.000
Total	102	91	91	

S – short (15 repeats or fewer); N – normal (16 repeats); L – long (17 or 18 repeats)

p value was calculated using χ^2 or Fisher's exact test as appropriate.

$p < 0.05$ was considered significant and is highlighted in bold.

6.3 *Discussion*

This work corroborates the original findings of a Japanese study, which reported a reduced number of homozygous normal (16-repeat, NN) genotype in ACP populations and an increased frequency of NL genotype as compared to a non-pancreatic disease alcoholic population.[219] This study did not establish an association of VNTR length with ACP compared to healthy controls, consistent with the subsequent European study.[41] Therefore, although the results of the two original studies appear to be conflicting, it has been shown the difference in results could be explained by the difference in the control groups selected rather than any difference in ethnicity or other confounding factor.

In this study, ALD patients were exclusively used rather than individuals defined as simply alcoholic without pancreatitis. This was due to the availability of individuals; those individuals who attended hospital with alcohol dependence normally did so as they had some pathological process, which in most cases was either liver disease or pancreatic disease. The majority of individuals presenting to hospital with problems related to alcohol have either liver or pancreatic disease. The alcoholic controls used by Miyasaka *et al* were only expressly stated as being free from pancreatic disease; liver disease can be assumed but was not mentioned.[219] Therefore, a difference in distribution between VNTR length in ALD and other groups could have been the true effect seen in the original Japanese paper, but the association was missed due to lack of classification of the ALD population.

Ragvin *et al* did not use any individuals with a history of alcohol excess, other than those with confirmed ACP.[41] The lack of any association with length of VNTR is therefore consistent with the hypothesis that the true difference in VNTR length is associated with ALD.

The present study includes an ALD group with VNTR length in Hardy Weinberg equilibrium, but with NN group over-represented compared to other groups (ACP or HC).

There is ambiguity in relation to the association of ACP and ALD. It has been reported that clinical ACP and ALD occur concurrently in around 16-20% of ACP/ALD cases, but the prevalence may be

higher at a histological level.[140,370,371] However it is generally agreed that there is some unknown factor(s) that predispose individuals to one or other disease process.[139,144] We appreciate there is a risk that members of either ALD group or the ACP group could subsequently develop the opposite disease process. The median age of patient within the ALD group was significantly higher than those with ACP, reducing the possibility of including individuals at high risk of ACP in the ALD group. The difference in age and gender distribution is typical of other studies.[137,141]

Further work needs to be done on how *CEL* variants may protect those with problems of alcohol abuse from ALD.

7 Chapter 7: Overall Discussion

7.1 *Patients*

7.1.1 Definition of Alcohol Excess

There is no clear consensus as to how much alcohol is required to classify a patient as having alcohol induced chronic pancreatitis. Due to the difficulty in obtaining and recording a clear alcohol history and the difficult groups of patients included in the study there was no clear way of comparing the volumes or types of alcohol consumed in our patient group. This problem is further augmented by the fact that different countries classify their alcohol consumption in different units, making extrapolation of results more challenging. Alcohol units, which are the usual method of quantification in the UK, equate to 10g of alcohol.[372] However most studies arising from the USA utilise "drinks" which contain 8g grams, and therefore this can make superficial interpretation of studies confusing.[43]

Previous studies have not shown that the differential outcome (ACP or ALD) is determined by the relative volume or type of consumption (Table 3). Therefore, although the lack of adequate classification remains a concern it is unlikely to impact on the final conclusions of this thesis.

7.1.2 Defining the Presence or Absence of Pancreatitis

The majority of genome wide studies such as the GWAS project, which require large populations, normally from multiple different institutes, inevitably have to rely on the appropriate diagnosis of CP by the local investigators.[176] This poses a problem because there is a lack of international consensus on the diagnosis of CP.[17,18,24] Therefore, in all such studies there is a risk of some individuals being included in either disease or control groups inappropriately.

The systematic review of the diagnosis of chronic pancreatitis showed that the best diagnostic modalities were EUS or MR to confirm or refute the diagnosis of pancreatitis; in the NGS study we

used CT scan to predominately diagnose the presence or absence of chronic pancreatitis. Where other modalities were available they were also used in the diagnosis of pancreatitis.

Although CT has been poorly examined as a specific modality for the diagnosis of CP, in clinical practice it is commonly use to both diagnosis CP and to rule out other pathology. The APA has now recommended CT as the first line diagnostic tool.[24]

This study was not carried out on the basis of a clinical trial and so no systematic use of imaging was applied to the patients without pancreatitis. There is therefore reliance on the clinical interpretation of the clinicians who managed and reported on these patients. This is not ideal but is in line with most other studies of this type.[24,373]

7.1.3 Control Groups

Several different control groups were used for different parts of the analysis. For the GWAS a different control cohort was utilised.[374,375] This meant inclusion of both an unselected cohort group and groups specifically selected to balance against a different disease process.[375] The prevalence of CP is low so it is unlikely that many (if any) CP patients would have been inadvertently included within the control groups. If any had been included this would reduce the power to identify risk genotypes and would not have led to false associations.

In the NGS section of the study, two different control populations were obtained: participants with a strong history of alcohol excess and no history of any alcohol related disease; and participants with a strong history of alcohol excess and a history of ALD. In most cases it was possible to review recent CT scans to eliminate the possibility of CP although clearly otherwise healthy controls would not routinely have imaging of the pancreas. Individuals with ALD were an exception to this as they were being scanned as part of their management. Therefore this well characterised group was selected as the main comparator for the ACP group.

Comparison of genetic differences in patients with ACP and ALD could identify polymorphisms related to ACP or ALD (or potentially to both in opposite directions). To determine if features were related to alcohol rather than disease process a second analysis was done using the alcohol excess healthy controls (AHC), to indicate how the frequency mediated from the population base line. The AHC group was chosen rather than a traditional HC group as several polymorphisms have been described in relation to alcohol dependence, and these may have falsely been shown to be related to either ACP or ALD, when in fact it is related to the increased consumption of alcohol, inherent in these patient groups.[123]

7.1.4 Heterogeneity between Groups

Although it would be preferable to have been able to use direct standardisation for all factors other than the presence or absence of ACP, this was not possible as there are natural differences between groups, such as higher rates of diabetes within ACP patients.[95] There was a higher ratio of men in the ACP group than the ALD group. Men are more likely to be alcoholics than women, but there has been a purported increased susceptibility to ALD in women,[137,376] but ACP is more common in men[6].

It was difficult to assess differences between drinking patter in in our groups due to missing data. Various studies have shown difference between alcohol drinking patterns in AP and ALD, but these findings have not been replicated across different studies (Table 3).

The pattern of smoking was different between the two groups with more ACP patients smoking. Smoking is a known risk factor for ACP, but this may also be true for ALD.[43,377]

7.1.5 Bias in Sample Source

All different sample groups will have inherent sources of bias, which may affect the final results produced. One such example is the chronic pancreatitis samples obtained for the NGS work. The original source available were all from operative samples, and these would have been derived from two distinct groups of individuals: either end-stage chronic pancreatitis making it an extreme

phenotype; or individuals who may have been resected as their disease was presumed to be cancer, but only found to be CP on histology. The first group would not give a representative picture of all chronic pancreatitics, and may skew findings towards a sub group, which may either augment, or hide the true genetic results. The second group are not a typical CP group and again would likely hide any true finding. To attempt to mitigate for this, additional criteria were added including the need for a clear clinical history of pancreatitis, and samples were collected from less severe cases of chronic pancreatitis, where they were not yet requiring surgical intervention.

7.2 *Synonymous SNPs*

Synonymous SNPs are normally classified as polymorphisms, which are considered to be neutral in nature; however, this may not always be the case. Through analysis of the mRNA produced from variant alleles, there is evidence that many human disease genes contain exonic variants that affect pre-mRNA splicing therefore inactivating genes by inducing the splicing machinery to skip mutant exons.[147] Alternative splicing is a regulated process during gene expression that can result in a single gene coding for multiple proteins.[341] It has been shown at least 74% of human multi-exon genes are alternatively spliced, and this may give an explanation for the effects of some of the polymorphisms identified.[378]

8 Chapter 8: Conclusion

This thesis sought to establish the role of genetic variation in the predisposition of alcohol-related chronic pancreatitis. Initially, the patient group of patients with clinically proven diagnosis of CP had to be defined. Assessment of the literature has suggested that either EUS or MR scanning may be the most sensitive way of identifying patient with a good clinical history, however in current practice CT scanning is the most commonly used modality, diagnosis is specific to CP and does allow appropriate characterisation of the group.

In terms of the genetic disposition, this was explored in several ways, including GWAS assessing an array of predefined loci, and exploring previously identified role of fatty acid ethyl esters by looking at *CEL* VNTR and Next Generation Sequencing of targeted genes.

GWAS revealed two associations with CP (identified and replicated) at *PRSS1-PRSS2*_rs10273639 (OR 0.73, 95% CI 0.68-0.79) and X-linked *CLDN2*_rs12688220 (OR 1.39, 1.28-1.49) and the association was more pronounced in the ACP group (OR 0.56, 0.48-0.64 and OR 2.11, 1.84-2.42). CLDN polymorphism has been shown to produce a difference in phenotypes; its role in chronic pancreatitis may be related to calcium transport, and this needs to be explored further.

The previously identified VNTR in *CEL* was shown to have a lower frequency of the normal repeat in ACP than ALD (OR 0.61, 0.41-0.93). Homozygosity of the normal variant was more common in ALD than ACP (OR 0.53, 0.3-0.96) or Healthy Controls (OR 0.55, 0.3-1.00)). The variations in the results identified in this work are most likely to be related to differences in the control groups used. This may be most useful in explaining difference between development of one pathology or the other.

The NGS discovery phase led on to validation of the 21 most significant SNPs with Sequenom array. This showed a significance difference between ACP and ALD in allele frequency of the synonymous SNP, *PRSS1*_rs6666, (OR 1.99, 1.46-2.72) and between presence or absence of a polymorphism in *ADH1C*_rs1693482 (OR 1.49, 1.11-2.01).

p.D162D lies within PRSS1, which is a gene of known significance in chronic pancreatitis, but the direct significance of the polymorphisms itself in unknown. This needs to be explored further in larger groups to identify if the association is held out in all populations, and if this SNP holds the true relationship, or is a marker for a disease associated variant.

The polymorphisms identified within this thesis are linked with their roles in metabolism of alcohol in the development of pancreatitis, which is a cascade of calcium influx, leading to activation of trypsinogen. Where we have identified significant differences the mechanism is not always clear, and this may represent linkage of genotype or subtitle difference that may impact only in specific tissues.

This work has identified a range of potential exonic and intronic sites associated with a predisposition of developing chronic pancreatitis on a background of alcohol excess. Further work needs to be done on how these factors interact, and assess their presence in further populations.

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10 Chapter 10: Appendices

10.1 *Common Alleles of Genes of Alcohol Metabolism*

10.1.1 Alcohol Dehydrogenase 1B

Table 40 Table demonstrating the amino acid difference between the different *ADH1B* alleles

		Amino acid position 48 ***ADH1B*_rs1229984**	Amino acid position 370 ***ADH1B*_rs2066702**
*ADH1B*1*	**β1**	R	R
*ADH1B*2*	**β2**	H	R
*ADH1B*3*	**β3**	R	C

10.1.2 Alcohol Dehydrogenase 1C

Table 41 Table demonstrating the amino acid difference between the different *ADH1C* alleles

		Amino acid position 272 ***ADH1C*_rs1693482**	Amino acid position 350 ***ADH1C*_rs698**
*ADH1C*1*	**γ**1	R	I
*ADH1C*2*	**γ**2	Q	V

10.1.3 Aldehyde Dehydrogenase 2

*ALDH2*2* varies from the normal allele with a polymorphism *ALDH2*_rs671 (p.E504K)

10.1.4 Cytochrome P450

Table 42 Table demonstrating the amino acid difference between the different *CYP2E1* alleles

	Repeats intronic	**rs2070676** intronic	**rs72559710** p.R76H	**rs55897648** p.I389V	**rs6413419** p.V1791	**rs3813867** intronic	**rs6413432** intronic	**rs2031920** intronic	**rs2070673** intronic	**rs2070672** intronic
*CYP2E1*1A*										
*CYP2E1*1B*		x								
*CYP2E1*1C*	Six									
*CYP2E1*1D*	Eight									
*CYP2E1*2*			x							
*CYP2E1*3*				x						
*CYP2E1*4*					x					
*CYP2E1*5A*						x	x	x		
*CYP2E1*5B*						x		x		
*CYP2E1*6*							x			
*CYP2E1*7A*									x	
*CYP2E1*7B*								x		
*CYP2E1*7C*									x	x

10.2 Genes of Interest

10.2.1 Genes of Alcohol Metabolism

Table 43 Long list of genes known to be related to alcohol metabolism

Gene symbol	Gene name
ABCA1	ATP-binding cassette sub-family A member 1
ABCG1	ATP-binding cassette sub-family G member 1
ACAA2	3-ketoacyl-CoA thiolase, mitochondrial
ACACA	Acetyl-CoA carboxylase alpha
ACACB	Acetyl-CoA carboxylase beta
ACADL	Long-chain specific acyl-CoA dehydrogenase, mitochondrial
ACADVL	Very long-chain specific acyl-CoA dehydrogenase, mitochondrial
ACER1	Alkaline ceramidase 1
ACER2	Alkaline ceramidase 2
ACER3	Alkaline ceramidase 3
ACHE	Acetyl cholinesterase
ACSL3	Long-chain-fatty-acid--CoA ligase 3
ACSS1	Acetyl-coenzyme A synthetase 2-like, mitochondrial
ACSS2	Acetyl-coenzyme A synthetase, cytoplasmic
ADH1A	Alcohol dehydrogenase 1A
ADH1B	Alcohol dehydrogenase 1B
ADH1C	Alcohol dehydrogenase 1C
ADH4	Alcohol dehydrogenase 4
ADH5	Alcohol dehydrogenase class-3
ADH6	Alcohol dehydrogenase 6
ADH7	Alcohol dehydrogenase class 4 mu/sigma chain
AGPAT6	Glycerol-3-phosphate acyltransferase 4
AKR1B10	Aldo-keto reductase family 1 member B10
AKR1C3	Aldo-keto reductase family 1 member C3
AKR1D1	3-oxo-5-beta-steroid 4-dehydrogenase
ALDH18A1	Aldehyde dehydrogenase 18 family, member A1
ALDH1A1	Aldehyde dehydrogenase 1 family, member A1
ALDH1A2	Aldehyde dehydrogenase 1 family, member A2
ALDH1A3	Aldehyde dehydrogenase family 1 member A3
ALDH1B1	Aldehyde dehydrogenase X, mitochondrial
ALDH1L1	Aldehyde dehydrogenase 1 family, member L1
ALDH1L2	Aldehyde dehydrogenase 1 family, member L2
ALDH2	Aldehyde dehydrogenase 2, mitochondrial
ALDH3A2	Fatty aldehyde dehydrogenase
ALDH3B1	Aldehyde dehydrogenase family 3 member B1
ALDH3B2	Aldehyde dehydrogenase family 3 member B2
ALDH5A1	Aldehyde dehydrogenase 5 family, member A1

ALDH6A1	Aldehyde dehydrogenase 6 family, member A1
ALDH7A1	Alpha-aminoadipic semialdehyde dehydrogenase
ANGPTL3	Angiopoietin-related protein 3
APLP2	Amyloid-like protein 2
APOA1	Apolipoprotein A-I
APOA2	Apolipoprotein A-II
APOA4	Apolipoprotein A-IV
APOA5	Apolipoprotein A-V
APOB	Apolipoprotein B
APOC1	Truncated apolipoprotein C-I
APOC3	Apolipoprotein C-III
APOE	Apolipoprotein E
APOF	Apolipoprotein F
APOL1	Apolipoprotein L1
APOL2	Apolipoprotein L2
APP	Amyloid beta A4 protein
ASM	Acid sphingomyelinase
AWAT2	Acyl-CoA wax alcohol acyltransferase 2
BCHE	Butyrylcholinesterase, isoform CRA_a
BCMO1	Beta, beta-carotene 15,15'-monooxygenase
BMP2	Bone morphogenetic protein 2
BMP5	Bone morphogenetic protein 5
BMP6	Bone morphogenetic protein 6
CACNA1H	Voltage-dependent T-type calcium channel subunit alpha-1H
CALM1	Calmodulin
CAT	Catalase
CDS1	Phosphatide cytidylyltransferase 1
CEBPA	CCAAT/enhancer-binding protein alpha
CEL	Bile salt-activated lipase
CEPT1	Choline/ethanolaminephosphotransferase 1
CES1	Carboxylesterase1
CES2	Carboxylesterase 2
CES3	Carboxylesterase 3
CES5a	Carboxylesterase 5A
CES6	Carboxylesterase 6
CETP	Cholesteryl ester transfer protein
CFTR	Cystic fibrosis transmembrane conductance regulator
CH25H	Cholesterol 25-hydroxylase
CHAT	Choline O-acetyltransferase
CHDH	Choline dehydrogenase, mitochondrial
CHKA	Choline kinase alpha
CHKB	Choline/ethanolamine kinase
CHPT1	Cholinephosphotransferase 1
CLN3	Battenin
CLN6	Ceroid-lipofuscinosis neuronal protein 6

CLPS	Colipase, pancreatic
CNBP	Cellular nucleic acid-binding protein
COQ2	4-hydroxybenzoate polyprenyltransferase, mitochondrial
COQ3	Hexaprenyldihydroxybenzoate methyltransferase, mitochondrial
CUBN	Cubilin
CYB5R1	NADH-cytochrome b5 reductase 1
CYB5R2	NADH-cytochrome b5 reductase 2
CYB5R3	NADH-cytochrome b5 reductase 3
CYP11A1	Cholesterol side-chain cleavage enzyme, mitochondrial
CYP11B1	Cytochrome P450 11B1, mitochondrial
CYP11B2	Cytochrome P450 11B2, mitochondrial
CYP17A1	Steroid 17-alpha-hydroxylase/17,20 lyase
CYP19A1	Aromatase
CYP1A1	Cytochrome P450 1A1
CYP1B1	Cytochrome P450 1B1
CYP21A2	cytochrome P450, family 21, subfamily A, polypeptide 2
CYP24A1	cytochrome P450, family 24, subfamily A, polypeptide 1
CYP27A1	cytochrome P450, family 27, subfamily A, polypeptide 1
CYP2E1	cytochrome P450, family 2, subfamily E, polypeptide 1
CYP2R1	Vitamin D 25-hydroxylase
CYP39A1	24-hydroxycholesterol 7-alpha-hydroxylase
CYP3A4	Cytochrome P450 3A4
CYP46A1	Cholesterol 24-hydroxylase
CYP51A1	Lanosterol 14-alpha demethylase
CYP7A1	Cholesterol 7-alpha-monooxygenase
CYP7B1	25-hydroxycholesterol 7-alpha-hydroxylase
CYP8B1	7-alpha-hydroxycholest-4-en-3-one 12-alpha-hydroxylase
DBI	Diazepam binding inhibitor (GABA receptor modulator, acyl-CoA binding protein)
DEGS2	Sphingolipid delta(4)-desaturase/C4-hydroxylase DES2
DGAT1	Diacylglycerol O-acyltransferase 1
DGAT2	Diacylglycerol O-acyltransferase 2
DHCR24	Delta(24)-sterol reductase
DHCR7	7-dehydrocholesterol reductase
DHRS3	Short-chain dehydrogenase/reductase 3
DHRS4	Dehydrogenase/reductase SDR family member 4
DHRS9	Dehydrogenase/reductase SDR family member 9
DKK3	Dickkopf-related protein 3
DMGDH	Dimethylglycine dehydrogenase, mitochondrial
DPAGT1	UDP-N-acetylglucosamine--dolichyl-phosphate N-acetylglucosaminephosphotransferase
DPM1	Dolichol-phosphate mannosyltransferase subunit 1
DPM2	Dolichol phosphate-mannose biosynthesis regulatory protein
EBP	3-beta-hydroxysteroid-Delta(8),Delta(7)-isomerase
EBPL	Emopamil-binding protein-like
ENPP2	Ectonucleotide pyrophosphatase/phosphodiesterase family member 2

ENPP6	Ectonucleotide pyrophosphatase/phosphodiesterase family member 6
ENPP7	Ectonucleotide pyrophosphatase/phosphodiesterase family member 7
EPHX2	Bifunctional epoxide hydrolase 2
EPM2A	Laforin
FABP1	Fatty acid-binding protein 1
FABP2	Fatty acid-binding protein 2
FABP3	Fatty acid-binding protein 3
FABP5	Fatty acid-binding protein 5
FABP6	Fatty acid-binding protein 6
FADS1	Fatty acid desaturase 1
FADS2	Fatty acid desaturase 2
FADS3	Fatty acid desaturase 3
FDFT1	Farnesyl-diphosphate farnesyltransferase 1
FDPS	Farnesyl pyrophosphate synthase
FDX1	Adrenodoxin, mitochondrial
FDXR	Ferredoxin reductase
FECH	Ferrochelatase, mitochondrial
FGF1	Fibroblast growth factor 1
FGF2	Fibroblast growth factor 2
FGF23	Fibroblast growth factor 23
FGL1	Fibrinogen-like protein 1
G6PD	Glucose-6-phosphate 1-dehydrogenase
GALK1	Galactokinase
GALNT2	Polypeptide N-acetylgalactosaminyltransferase 2
GBA	Glucosylceramidase
GC	Vitamin D-binding protein
GCKR	Glucokinase regulator
GDE1	Glycerophosphodiester phosphodiesterase 1
GDPD1	Glycerophosphodiester phosphodiesterase domain-containing protein 1
GDPD2	Glycerophosphoinositol inositolphosphodiesterase GDPD2
GDPD3	Glycerophosphodiester phosphodiesterase domain-containing protein 3
GDPD4	Glycerophosphodiester phosphodiesterase domain-containing protein 4
GDPD5	Glycerophosphodiester phosphodiesterase domain-containing protein 5
GFI1	Zinc finger protein Gfi-1
GGPS1	Geranylgeranyl pyrophosphate synthase
GK	Glycerol kinase
GK2	Glycerol kinase 2
GK5	Putative glycerol kinase 5
GOT1	Aspartate aminotransferase, cytoplasmic
GPD2	Glycerol-3-phosphate dehydrogenase, mitochondrial
GPER1	G-protein coupled estrogen receptor 1
GPLD1	Phosphatidylinositol-glycan-specific phospholipase D
HAO1	Hydroxyacid oxidase 1
HDLBP	high density lipoprotein binding protein
HMGCR	3-hydroxy-3-methylglutaryl-coenzyme A reductase

HMGCS1	Hydroxymethylglutaryl-CoA synthase, cytoplasmic
HMGCS2	Hydroxymethylglutaryl-CoA synthase, mitochondrial
HRH1	Histamine H1 receptor
HSD17B1	Estradiol 17-beta-dehydrogenase 1
HSD17B3	Testosterone 17-beta-dehydrogenase 3
HSD17B7	3-keto-steroid reductase
IDI1	Isopentenyl-diphosphate Delta-isomerase 1
IDI2	Isopentenyl-diphosphate delta-isomerase 2
IFNG	Interferon gamma
IGF1	Insulin-like growth factor I
IL1a	Interleukin-1 alpha
IL1B	Interleukin-1 beta
IL4	Interleukin-4
IMPA2	Inositol monophosphatase 2
IMPAD1	Inositol monophosphatase 3
INPP1	Inositol polyphosphate 1-phosphatase
INPP5D	Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 1
INPP5E	72 kDa inositol polyphosphate 5-phosphatase
INPP5J	Phosphatidylinositol 4,5-bisphosphate 5-phosphatase A
INPP5K	Inositol polyphosphate 5-phosphatase K
INPPL1	Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 2
INSIG1	Insulin-induced gene 1 protein
INSIG2	Insulin-induced gene 2 protein
IP6K1	Inositol hexakisphosphate kinase 1
IP6K2	Inositol hexakisphosphate kinase 2
IP6K3	Inositol hexakisphosphate kinase 3
IPMK	Inositol polyphosphate multikinase
IPPK	Inositol-pentakisphosphate 2-kinase
ISYNA1	Inositol-3-phosphate synthase 1
ITPK1	Inositol-tetrakisphosphate 1-kinase
ITPKA	Inositol-trisphosphate 3-kinase A
ITPKB	Inositol-trisphosphate 3-kinase B
ITPKC	Inositol-trisphosphate 3-kinase C
CPT1A	Carnitine palmitoyltransferase 1A (liver)
LBR	Lamin-B receptor
LCAT	Phosphatidylcholine-sterol acyltransferase
LDLR	Low-density lipoprotein receptor
LDLRAP1	Low density lipoprotein receptor adapter protein 1
LEP	Leptin
LEPR	Leptin receptor
LGMN	Legumain
LHCGR	Lutropin-choriogonadotropic hormone receptor
LIPA	Lipase A, lysosomal acid, cholesterol esterase
LIPC	Hepatic triacylglycerol lipase
LIPE	Hormone-sensitive lipase

LMF1	Lipase maturation factor 1
LPCAT1	Lysophosphatidylcholine acyltransferase 1
LPCAT2	Lysophosphatidylcholine acyltransferase 2
LPCAT3	Lysophospholipid acyltransferase 3
LPCAT4	Lysophospholipid acyltransferase 4
LPIN1	Phosphatidate phosphatase LPIN1
LPIN2	Phosphatidate phosphatase LPIN2
LPIN3	Phosphatidate phosphatase LPIN3
LPL	Lipoprotein lipase
LRAT	Lecithin retinol acyltransferase
LRP1	Prolow-density lipoprotein receptor-related protein 1
LRP2	Low-density lipoprotein receptor-related protein 2
LRP5	Low-density lipoprotein receptor-related protein 5
LSS	Lanosterol synthase
MAS1	Proto-oncogene Mas
MBOAT2	Lysophospholipid acyltransferase 2
MBTPS1	Membrane-bound transcription factor site-1 protease
MBTPS2	Membrane-bound transcription factor site-2 protease
MECP2	Methyl-CpG-binding protein 2
MGLL	Monoglyceride lipase
MINPP1	Multiple inositol polyphosphate phosphatase 1
MIOX	Myo-Inositol oxygenase
MOGAT1	2-acylglycerol O-acyltransferase 1
MOGAT2	2-acylglycerol O-acyltransferase 2
MOGAT3	2-acylglycerol O-acyltransferase 3
MSMO1	Methylsterol monooxygenase 1
MT3	Metallothionein-3
MTMR2	Myotubularin-related protein 2
MTMR7	Myotubularin-related protein 7
MVD	Diphosphomevalonate decarboxylase
MVK	Mevalonate kinase
NFKB1	Nuclear factor NF-kappa-B p105 subunit
NPC1	Niemann-Pick C1 protein
NPC1L1	Niemann-Pick C1-like protein 1
NPC2	Epididymal secretory protein E1
NQO1	NAD(P)H dehydrogenase, quinone 1
NQO2	NAD(P)H dehydrogenase, quinone 2
NR0B2	Nuclear receptor subfamily 0 group B member 2
NR1H4	Bile acid receptor
NSDHL	Sterol-4-alpha-carboxylate 3-dehydrogenase, decarboxylating
NUDT10	Diphosphoinositol polyphosphate phosphohydrolase 3-alpha
NUDT11	Diphosphoinositol polyphosphate phosphohydrolase 3-beta
NUDT3	Diphosphoinositol polyphosphate phosphohydrolase 1
NUDT4	Diphosphoinositol polyphosphate phosphohydrolase 2
OCRL	Inositol polyphosphate 5-phosphatase OCRL-1

OSBPL1A	Oxysterol-binding protein-related protein 1
OSBPL5	Oxysterol-binding protein-related protein 5
P2RY1	P2Y purinoceptor 1
PAFAH1B1	Platelet-activating factor acetylhydrolase IB subunit alpha
PARK7	Protein DJ-1
PCK1	Phosphoenolpyruvate carboxykinase, cytosolic [GTP]
PCSK9	Proprotein convertase subtilisin/kexin type 9
PCTP	Phosphatidylcholine transfer protein
PCYT1A	Choline-phosphate cytidylyltransferase A
PCYT1B	Choline-phosphate cytidylyltransferase B
PECR	Peroxisomal trans-2-enoyl-CoA reductase
PEMT	Phosphatidylethanolamine N-methyltransferase
PEX2	Peroxisome biogenesis factor 2
PHOSPHO1	Phosphoethanolamine/phosphocholine phosphatase
PLA2G10	Group 10 secretory phospholipase A2
PLA2G12A	Group XIIA secretory phospholipase A2
PLA2G15	Group XV phospholipase A2
PLA2G16	Phospholipase A2, group XVI
PLA2G1B	phospholipase A2, group IB
PLA2G2A	Phospholipase A2, membrane associated
PLA2G2D	Group IID secretory phospholipase A2
PLA2G2E	Group IIE secretory phospholipase A2
PLA2G2F	Group IIF secretory phospholipase A2
PLA2G3	Group 3 secretory phospholipase A2
PLA2G4A	Cytosolic phospholipase A2
PLA2G4B	Cytosolic phospholipase A2 beta
PLA2G4C	Cytosolic phospholipase A2 gamma
PLA2G4D	Cytosolic phospholipase A2 delta
PLA2G4E	Cytosolic phospholipase A2 epsilon
PLA2G4F	Cytosolic phospholipase A2 zeta
PLA2G5	Calcium-dependent phospholipase A2
PLA2G6	85/88 kDa calcium-independent phospholipase A2
PLB1	Phospholipase B1, membrane-associated
PLBD1	Phospholipase B-like 1
PLCB1	phospholipase C, beta 1
PLCB2	phospholipase C, beta 2
PLCB3	phospholipase C, beta 3
PLCB4	phospholipase C, beta 4
PLCD1	phospholipase C, delta 1
PLCD3	phospholipase C, delta 3
PLCE1	phospholipase C, epsilon 1
PLCG1	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-1
PLCG2	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-2
PLCH1	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase eta-1
PLCH2	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase eta-2

PLCZ1	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase zeta-1
PLD4	Phospholipase D4
PLEK	Pleckstrin
PMVK	Phosphomevalonate kinase
PNLIP	Pancreatic lipase
PNLIPRP1	Pancreatic lipase-related protein 1
PNLIPRP2	Pancreatic lipase-related protein 2
PNPLA6	Neuropathy target esterase
PNPLA8	Calcium-independent phospholipase A2-gamma
PON1	Serum paraoxonase/arylesterase 1
POR	NADPH--cytochrome P450 reductase
POU1F1	Pituitary-specific positive transcription factor 1
PPARD	Peroxisome proliferator-activated receptor delta
PPIP5K1	Inositol hexakisphosphate and diphosphoinositol-pentakisphosphate kinase 1
PPIP5K2	Inositol hexakisphosphate and diphosphoinositol-pentakisphosphate kinase 2
PRKAA1	5'-AMP-activated protein kinase catalytic subunit alpha-1
PRKAA2	5'-AMP-activated protein kinase catalytic subunit alpha-2
PRKAG2	5'-AMP-activated protein kinase subunit gamma-2
PRKCD	Protein kinase C delta type
PTAFR	Platelet-activating factor receptor
PTH1R	Parathyroid hormone/parathyroid hormone-related peptide receptor
PTK2B	Protein-tyrosine kinase 2-beta
PTPMT1	Phosphatidylglycerophosphatase and protein-tyrosine phosphatase 1
PTPRR	Protein tyrosine phosphatase, receptor type, R
RBP4	Retinol-binding protein 4
RDH10	Retinol dehydrogenase 10
RDH11	Retinol dehydrogenase 11
RDH12	Retinol dehydrogenase 12
RDH5	Retinol dehydrogenase 5
RDH8	Retinol dehydrogenase 8
REST	RE1-silencing transcription factor
RETSAT	All-trans-retinol 13,14-reductase
RPE65	Retinoid isomerohydrolase
RXRA	Retinoic acid receptor RXR-alpha
SAMD8	Sphingomyelin synthase-related protein 1
SC5D	Lathosterol oxidase
SCAP	Sterol regulatory element-binding protein cleavage-activating protein
SCARB1	Scavenger receptor class B member 1
SCARF1	Scavenger receptor class F member 1
SCP2	Non-specific lipid-transfer protein
SDR16C5	Epidermal retinol dehydrogenase 2
SEC14L2	SEC14-like protein 2
SGMS1	Phosphatidylcholine:ceramide cholinephosphotransferase 1
SGMS2	Phosphatidylcholine:ceramide cholinephosphotransferase 2
SGPP1	Sphingosine-1-phosphate phosphatase 1

SGPP2	Sphingosine-1-phosphate phosphatase 2
SLC27A1	Long-chain fatty acid transport protein 1
SLC34A1	Sodium-dependent phosphate transport protein 2A
SLC5A3	Sodium/myo-inositol cotransporter
SMPD1	Sphingomyelin phosphodiesterase 1
SMPD2	Sphingomyelin phosphodiesterase 2
SMPD3	Sphingomyelin phosphodiesterase 3
SMPD4	Sphingomyelin phosphodiesterase 4
SMPDL3A	Acid sphingomyelinase-like phosphodiesterase 3a
SMPDL3B	Acid sphingomyelinase-like phosphodiesterase 3b
SNAI1	Zinc finger protein SNAI1
SNAI2	Zinc finger protein SNAI2
SNCA	Alpha-synuclein
SNX17	Sorting nexin-17
SOAT1	Sterol O-acyltransferase 1
SOAT2	Sterol O-acyltransferase 2
SOD1	Superoxide dismutase [Cu-Zn]
SORD	Sorbitol dehydrogenase
SORL1	Sortilin-related receptor
SPHK1	Sphingosine kinase 1
SPHK2	Sphingosine kinase 2
SPTLC1	Serine palmitoyltransferase 1
SPTLC2	Serine palmitoyltransferase 2
SQLE	Squalene monooxygenase
SRD5A3	Polyprenol reductase
SREBF1	Sterol regulatory element-binding protein 1
SREBF2	Sterol regulatory element-binding protein 2
STAR	Steroidogenic acute regulatory protein, mitochondrial
STARD3	StAR-related lipid transfer protein 3
SYNJ1	Synaptojanin-1
TM7SF2	Transmembrane 7 superfamily member 2
TNF	Tumor necrosis factor
TNFSF4	Tumor necrosis factor ligand superfamily member 4
TRERF1	Transcriptional-regulating factor 1
TTR	Transthyretin
VDR	Vitamin D3 receptor
VLDLR	Very low-density lipoprotein receptor
WNT4	Protein Wnt-4
IMPA1	inositol(myo)-1(or 4)-monophosphatase 1

10.2.2 Background on *Genes of Interest*

Information presented on the chosen *Genes of Interest*. Data obtained from the NCBI website (http://www.ncbi.nlm.nih.gov/gene and http://www.ncbi.nlm.nih.gov/omim).

10.2.2.1 *ABO* - ABO blood group

Also known as *GTB; NAGAT; A3GALNT; A3GALT1*

Gene ID: 28 **Location:** 9q34.2

Summary Encodes for proteins related to the ABO blood group system. The 'O' blood group is caused by a deletion of guanine at position 258 resulting in a frameshift, leading to production of different protein.

10.2.2.2 *ACACA* - Acetyl-CoA carboxylase alpha

Also known as *ACC; ACAC; ACC1; ACCA; ACACAD*

Gene ID: 31 **Location:** 17q21

Summary Encodes an enzyme which catalyses the carboxylation of acetyl-CoA to malonyl-CoA which is the rate limiting step in the synthesis of fatty acids. ACC-alpha is highly enriched in lipogenic tissues.

10.2.2.3 *ACACB* - Acetyl-CoA carboxylase beta

Also known as *ACC2; ACCB; HACC275*

Gene ID: 32 **Location:** 12q24.11

Summary Like *ACACA*, encodes an enzyme which catalyses the carboxylation of acetyl-CoA to malonyl-CoA which is the rate limiting step in the synthesis of fatty acids. *ACACB* controls fatty acid oxidation by preventing the rate-limiting step in fatty acid uptake and mitochondrial oxidation. *ACACB* may in fact be involved in the regulation of fatty acid oxidation, rather than fatty acid synthesis. Increased concentrations of catecholamines promote the ultilisation of lipid stores from adipose tissue during fast, which is the rate-limiting enzyme in fatty acid synthesis.

10.2.2.4 *ACSS2* - Acyl-CoA synthetase short-chain family member 2

Also known as *ACS; ACSA; ACAS2; ACECS; dJ1161H23.1*

Gene ID: 55902 **Location:** 20q11.22

Summary Encodes for a cytosolic enzyme that helps catalyses activation of acetate, which is use for lipid synthesis and generation of energy. The protein acts as a monomer and produces acetyl-CoA from acetate in a reaction that requires ATP.

10.2.2.5 *ADH1A* - Alcohol dehydrogenase 1A (class I), alpha polypeptide

Also known as *ADH1*

Gene ID: 124 **Location:** 4q23

Summary Encodes for a protein member of the alcohol dehydrogenase family, which catalyses the oxidation of alcohols to aldehydes. This gene is active in the liver in early fetal life but only weakly active in adult liver. This gene is clustered with six other alcohol dehydrogenase genes, on chromosome 4. Mutations in this gene are associated with substance dependence. The class I *ADH* genes account for the majority of the ethanol-oxidizing capacity of the liver.

10.2.2.6 *ADH1B* - Alcohol dehydrogenase 1B (class I), beta polypeptide

Also known as *ADH2; HEL-S-117*

Gene ID: 125 **Location:** 4q23

Summary Encodes for a protein is a member of the alcohol dehydrogenase family, which includes a wide variety of substrates, including ethanol, retinol, other aliphatic alcohols, and lipid peroxidation products. It is highly active in ethanol oxidation and plays a major role in ethanol catabolism.

10.2.2.7 *ADH1C* - Alcohol dehydrogenase 1C (class I), gamma polypeptide

Also known as *ADH3*

Gene ID: 126 **Location:** 4q23

Summary Encodes for class I alcohol dehydrogenase, which is a member of the alcohol dehydrogenase family. Similar properties to *ADH1B*.

10.2.2.8 *ADH4* - Alcohol dehydrogenase 4 (class II), pi polypeptide

Also known as *ADH-2; HEL-S-4*

Gene ID: 127 **Location:** 4q22

Summary Encodes class II alcohol dehydrogenase 4 (pi subunit), a member of the alcohol dehydrogenase family. Has high oxidation activity with aliphatic and aromatic alcohols but is less sensitive with pyrazole. There is cluster of alcohol dehydrogenase genes chromosome 4 in relation to this gene. It is a distinct from other human liver alcohol dehydrogenases and this therefore termed it Pi-alcohol dehydrogenase. This enzyme accounts for up to 40% of the total ethanol oxidation when intoxicating levels of alcohol have been consumed.

10.2.2.9 *ADH5* - Alcohol dehydrogenase 5 (class III), chi polypeptide

Also known as *FDH; ADHX; ADH-3; FALDH; GSNOR; GSH-FDH*

Gene ID: 128 **Location:** 4q23

Summary Encodes a protein for a member of the alcohol dehydrogenase family. It oxidizes ethanol very poorly but oxidases long-chain primary alcohols well. The human genome contains several non-transcribed pseudogenes related to this gene.

10.2.2.10 *ADH6* - Alcohol dehydrogenase 6 (class V)

Also known as *ADH-5*

Gene ID: 130 **Location:** 4q23

Summary This gene encodes class V alcohol dehydrogenase, which is a member of the alcohol dehydrogenase family. This gene is expressed in the stomach as well as in the liver, and it contains a glucocorticoid response element upstream of its 5' UTR, which is a steroid hormone receptor-binding site.

10.2.2.11 *ADH7* - Alcohol dehydrogenase 7 (class IV), mu or sigma polypeptide

Also known as *ADH4*

Gene ID: 131 **Location:** 4q23-q24

Summary This gene encodes class IV alcohol dehydrogenase 7 mu or sigma subunit, which is a member of the alcohol dehydrogenase family. The enzyme encoded by this gene is inefficient in ethanol oxidation, but is the most active as a retinol dehydrogenase; thus it may participate in the synthesis of retinoic acid, a hormone important for cellular differentiation. The expression of this gene is much more abundant in stomach than liver, thus differing from the other known gene family members.

10.2.2.12 *ALDH1A2* - Aldehyde dehydrogenase 1 family, member A2

Also known as *RALDH2; RALDH2-T; RALDH(II)*

Gene ID: 8854 **Location:** 15q21.3

Summary This protein belongs to the aldehyde dehydrogenase family of proteins. The product of this gene is an enzyme that catalyzes the synthesis of retinoic acid from retinaldehyde. Retinoic acid, the active derivative of vitamin A (retinol), is a hormonal signaling molecule that functions in developing and adult tissues.

10.2.2.13 *ALDH1A3* - Aldehyde dehydrogenase 1 family, member A3

Also known as *ALDH6; MCOP8; RALDH3; ALDH1A6*

Gene ID: 220 **Location:** 15q26.3

Summary Encodes for an enzyme, part of aldehyde dehydrogenase isozymes, playing a major role in the detoxification of aldehydes generated by alcohol metabolism and lipid peroxidation.

10.2.2.14 *ALDH1L1* - Aldehyde dehydrogenase 1 family, member L1

Also known as *FDH; FTHFD; 10-fTHF; 10-FTHFDH*

Gene ID: 10840 **Location:** 3q21.3

Summary The loss of expression or function of this enzyme is associated with reduced apoptosis, increased cell motility, and as a consequence, progression of cancer.

10.2.2.15 *ALDH1L2* - Aldehyde dehydrogenase 1 family, member L2

Also known as *mtFDH*

Gene ID: 160428 **Location:** 12q23.3

Summary It has an essential role in the distribution of carbon groups between cytosolic and mitochondrial cell compartments.

10.2.2.16 *ALDH2* - Aldehyde dehydrogenase 2 family (mitochondrial)

Also known as *ALDM; ALDHI; ALDH-E2*

Gene ID: 217 **Location:** 12q24.2

Summary Aldehyde dehydrogenase is the second enzyme of the main oxidative pathway for metabolism of alcohol. The higher frequency of acute alcohol intoxication with the Oriental population compared to Caucasians may be related to the mitochondrial isozyme that is absence of catalytic activity; these individuals may also have a greater susceptibility to many types of cancer.

10.2.2.17 *ALDH3B1* - Aldehyde dehydrogenase 3 family, member B1

Also known as *ALDH4; ALDH7*

Gene ID: 221 **Location:** 11q13

Summary This gene is expressed in kidney and lung, however, the functional significance of this gene is unknown. It shares a close evolutionary relationship with *ALDH7* and *ALDH8*.

10.2.2.18 *ALDH5A1* - Aldehyde dehydrogenase 5 family, member A1

Also known as *SSDH; SSADH*

Gene ID: 7915 **Location:** 6p22

Summary A deficiency of this enzyme is a rare inborn error in the metabolism of the neurotransmitter 4-aminobutyric acid. In response to the defect, physiologic fluids from individuals accumulate GHB, a compound with numerous neuromodulatory properties.

10.2.2.19 *ALDH6A1* - Aldehyde dehydrogenase 6 family, member A1

Also known as *MMSDH; MMSADHA*

Gene ID: 4329 **Location:** 14q24.3

Summary Encodes a protein that plays a role in the valine and pyrimidine catabolic pathways. This protein catalyzes the irreversible oxidative decarboxylation of malonate and methylmalonate semialdehydes to acetyl- and propionyl-CoA.

10.2.2.20 *APOA1* - Apolipoprotein A-I

Gene ID: 335 **Location:** 11q23-q24

Summary Encodes for the major protein component of high-density lipoprotein. IT promotes the movement of cholesterol to the liver from tissues for excretion, and is responsible for the formation of most plasma cholesteryl esters. This gene is closely linked with two other apolipoprotein genes on chromosome 11. Furthermore to removing cholesterol from cells, HDL also delivers cholesterol to cells through cholesteryl esters that are selectively transferred from HDL particles into the cell.

10.2.2.21 *APOA4* - Apolipoprotein A-IV

Gene ID: 337 **Location:** 11q23

Summary This protein is primarily made in the intestine and is associated with chylomicron particles. Its exact function is unclear.

10.2.2.22 *APOA5* - Apolipoprotein A-V

Also known as *RAP3; APOAV*

Gene ID: 116519 **Location:** 11q23

Summary Encodes a protein that plays a key role in regulating the plasma triglyceride levels. It is a component of high-density lipoprotein. Mutations in this gene have been associated with hypertriglyceridemia and hyperlipoproteinaemia.

10.2.2.23 *APOB* - Apolipoprotein B

Also known as *FLDB; LDLCQ4*

Gene ID: 338 **Location:** 2p24-p23

Summary Encodes for the main apolipoprotein of chylomicrons and low-density lipoproteins, both intestinal and the hepatic forms. Mutations in this gene cause hypobetalipoproteinemia, normotriglyceridaemic hypobetalipoproteinemia, and hypercholesterolemia which are all diseases affecting plasma cholesterol levels.

10.2.2.24 *APOC3* - Apolipoprotein C-III

Also known as *HALP2; APOCIII*

Gene ID: 345 **Location:** 11q23.3

Summary Encodes for a very low-density lipoprotein protein. It inhibits both lipoprotein lipase and hepatic lipase, delaying catabolism of triglyceride-rich particles. The *APOA1, APOC3* and *APOA4* genes are closely linked. Increased *APOC3* levels leads to hypertriglyceridemia.

10.2.2.25 *APOE* - Apolipoprotein E

Also known as *AD2; LPG; LDLCQ5*

Gene ID: 348 **Location:** 19q13.2

Summary Apolipoprotein E, a main apoprotein of the chylomicron, binds to a specific receptor on liver cells and peripheral cells lowing chylomicron remnants and very low-density lipoprotein remnants to be rapidly removed from the circulation. *APOE* is essential for the normal catabolism of

triglyceride-rich lipoprotein constituents. Defects in *APOE* result in familial dysbetalipoproteinaemia, or hyperlipoproteinaemia, which cause increased plasma cholesterol and triglycerides due to impaired clearance of chylomicron and VLDL remnants.

10.2.2.26 *CEL* - Carboxyl ester lipase

Also known as *BAL; FAP; BSDL; BSSL; CELL; FAPP; LIPA; CEase; MODY8*

Gene ID: 1056 **Location:** 9q34.3

Summary Encodes a glycoprotein secreted by the pancreas into the digestive tract, hydrolysing cholesterol and lipid-soluble vitamin ester hydrolysis, aiding absorption and promoting large chylomicron production in the intestine. It is also thought to modulate the progression of atherosclerosis. This gene contains a VNTR in the coding region that may influence the function of the encoded protein.

10.2.2.27 *CES1* - Carboxylesterase 1

Also known as *CEH; REH; TGH; ACAT; CE-1; CES2; HMSE; SES1; HMSE1; PCE-1; hCE-1*

Gene ID: 1066 **Location:** 16q22.2

Summary Encodes a member of the carboxylesterase large family, which is responsible for the hydrolysis or transesterification of various xenobiotics, and endogenous substrates with ester or amide bonds. It has a role in fatty acyl and cholesterol ester metabolism. This enzyme is one of the main liver enzymes and is key for liver drug clearance.

10.2.2.28 *CES4A* - Carboxylesterase 4A

Also known as *CES6; CES8*

Gene ID: 283848 **Location:** 16q22.1

Summary This enzyme is involved in fatty acyl and cholesterol ester metabolism, and may play a role in the detoxification of drugs and xenobiotics tissues of the body and in the cerebrospinal fluid.

10.2.2.29 *CES5A* - Carboxylesterase 5A

Also known as *CES5; CES7; CAUXIN; CES4C1*

Gene ID: 221223 **Location:** 16q12.2

Summary This enzyme Involved in fatty acyl and cholesterol ester metabolism, regulating the production of a pheromone precursor and may contribute to lipid and cholesterol transfer.

10.2.2.30 *CFTR* - Cystic fibrosis transmembrane conductance regulator

Also known as *CF; MRP7; ABC35; ABCC7; CFTR/MRP; TNR-CFTR*

Gene ID: 1080 **Location:** 7q31.2

Summary Encodes for a protein, which functions as a chloride channel and helps control its transport pathway. It is a member of the ATP-binding cassette (*ABC*) transporter superfamily. *ABC* proteins transport various molecules across extra- and intra-cellular membranes. Mutations in this gene are associated with the autosomal recessive disorders cystic fibrosis and congenital bilateral aplasia of the vas deferens.

10.2.2.31 *CLPS* - Colipase, pancreatic

Gene ID: 1208 **Location:** 6p21.31

Summary Encodes for a protein, which is a cofactor required by pancreatic lipase to efficiently hydrolyse dietary lipids. Colipase is thought to help anchor lipase to the surface of lipid micelles, negating any destabilising influence of intestinal bile salts. This gene is only been identified as being expressed in pancreatic acinar cells.

10.2.2.32 *CPT1A* - Carnitine palmitoyltransferase 1A (liver)

Also known as *CPT1; CPT1-L; L-CPT1*

Gene ID: 1374 **Location:** 11q13.2

Summary *CPT1A* is the key enzyme in the carnitine-dependent transport across the mitochondrial inner membrane and its deficiency results in a decreased rate of fatty acid beta-oxidation. Alternatively spliced transcript variants encoding different isoforms have been found for this gene.

Major control over fatty acid oxidation process is exerted at the level of *CPT1* by virtue of the unique inhabitability of this enzyme by malonyl-CoA. This fuel 'cross talk' was first recognized in the context of hepatic cytogenesis and its regulation and thereafter emerged as a central component of metabolism in a variety of tissues.

10.2.2.33 *CTRB1* - Chymotrypsinogen B1

Gene ID: 1504 **Location:** 6q23.1

Also known as *CTRB*

Summary This gene encodes for a protein, which is part of a family of serine proteases. It is secreted into the gastrointestinal tract as an inactive precursor, and is then activated by proteolytic cleavage with trypsin.

10.2.2.34 *CYP2E1* - Cytochrome P450, family 2, subfamily E, polypeptide 1

Gene ID: 1571 **Location:** 10q26.3

Also known as *CPE1; CYP2E; P450-J; P450C2E*

Summary This gene encodes a member of the cytochrome P450 superfamily of enzymes, which are monooxygenases that catalyze many reactions involved in synthesis of cholesterol and steroids and in drug metabolism. This protein localizes to the endoplasmic reticulum and is induced by ethanol, the diabetic state, and starvation. The enzyme metabolizes both endogenous substrates, such as ethanol, acetone, and acetal, as well as exogenous substrates including benzene, carbon tetrachloride, ethylene glycol, and nitrosamines, which are premutagens, found in cigarette smoke. Due to its many substrates, this enzyme may be involved in such varied processes as gluconeogenesis, hepatic cirrhosis, diabetes, and cancer.[217,218,379,380]

10.2.2.35 *FABP1* - Fatty acid binding protein 1, liver

Also known as *FABPL; L-FABP*

Gene ID: 2168 **Location:** 2p11

Summary This gene encodes the fatty acid binding protein found in liver. Fatty acid binding proteins

are a family of small, highly conserved, cytoplasmic proteins that bind long-chain fatty acids and other hydrophobic ligands. This protein and *FABP6* are also able to bind bile acids. It is thought that *FABPs* roles include fatty acid uptake, transport, and metabolism and is required for cholesterol synthesis.

10.2.2.36 *FABP2* - Fatty acid binding protein 2, intestinal

Also known as *FABPI; I-FABP*

Gene ID: 2169 **Location:** 4q28-q31

Summary *FABPs* are divided into at least three distinct types, namely the hepatic-, intestinal- and cardiac-type. They form 14-15 kDa proteins and are thought to participate in the uptake, intracellular metabolism and/or transport of long-chain fatty acids. They may also be responsible in the modulation of cell growth and proliferation. *FABP2* contains four exons and is an abundant cytosolic protein in small intestine epithelial cells. It may participate in the uptake, intracellular metabolism and/or transport of long chain fatty acids. This gene has a polymorphism at codon 54 that identified an alanine-encoding allele and a threonine-encoding allele (*FABP2*_rs1799883). Thr-54 protein is associated with increased fat oxidation and insulin resistance.

10.2.2.37 *FABP3* - Fatty acid binding protein 3, muscle and heart

Also known as *MDGI; FABP11; H-FABP; M-FABP; O-FABP*

Gene ID: 2170 **Location:** 1p33-p32

Summary The intracellular fatty acid-binding proteins (FABPs) belong to a multi-gene family and are divided into at least three distinct types, namely the hepatic-, intestinal- and cardiac-type. They form 14-15 kDa proteins and are thought to participate in the uptake, intracellular metabolism and/or transport of long-chain fatty acids. They may also be responsible in the modulation of cell growth and proliferation. *FABP3* contains four exons and its function is to arrest growth of mammary epithelial cells. This gene is a candidate tumor suppressor gene for human breast cancer.

10.2.2.38 *FABP6* - Fatty acid binding protein 6, ileal

Also known as *ILBP; I-15P; I-BAP; ILBP3; ILLBP; I-BABP; I-BALB*

Gene ID: 2172 **Location:** 5q33.3-q34

Summary This gene encodes the ileal fatty acid binding protein. Fatty acid binding proteins are a family of small, highly conserved, cytoplasmic proteins that bind long-chain fatty acids and other hydrophobic ligands. *FABP6* and *FABP1* (the liver fatty acid binding protein) are also able to bind bile acids. It is thought that FABPs roles include fatty acid uptake, transport, and metabolism.

10.2.2.39 *FADS1* - Fatty acid desaturase 1

Also known as *D5D; TU12; FADS6; FADSD5; LLCDL1*

Gene ID: 3992 **Location:** 11q12.2-q13.1

Summary The protein encoded by this gene is a member of the fatty acid desaturase (*FADS*) gene family. Desaturase enzymes regulate unsaturation of fatty acids through the introduction of double bonds between defined carbons of the fatty acyl chain. *FADS* family members are considered fusion products composed of an N-terminal cytochrome b5-like domain and a C-terminal multiple membrane-spanning desaturase, both of which are characterized by conserved histidine motifs. This gene is clustered with family members *FADS1* and *FADS2* at 11q12-q13.1; this cluster is thought to have arisen evolutionarily from gene duplication based on its similar exon/intron organization. The *FADS1* substrate/product pair ratio is associated with LDL cholesterol, HDL cholesterol and triglycerides.

10.2.2.40 *FADS2* - Fatty acid desaturase 2

Also known as *D6D; DES6; TU13; FADSD6; LLCDL2; SLL0262*

Gene ID: 9415 **Location:** 11q12.2

Summary Desaturase enzymes regulate unsaturation of fatty acids through the introduction of double bonds between defined carbons of the fatty acyl chain. Similar profile to *FADS1*.

10.2.2.41 *FADS3* - Fatty acid desaturase 3

Also known as *CYB5RP; LLCDL3*

Gene ID: 3995 **Location:** 11q12-q13.1

Summary Desaturase enzymes regulate unsaturation of fatty acids through the introduction of double bonds between defined carbons of the fatty acyl chain. Similar profile to *FADS1*.

10.2.2.42 *GALNT2* - UDP-N-acetyl-alpha-D-galactosamine: N-acetylgalactosaminyltransferase 2

Also known as *GalNAc-T2*

Gene ID: 2590 **Location:** 1q41-q42

Summary This gene encodes polypeptide N-acetylgalactosaminyltransferase 2, a member of the GalNAc-transferases family. This family transfers an N-acetyl galactosamine to the hydroxyl group of a serine or threonine residue in the first step of O-linked oligosaccharide biosynthesis. Individual GalNAc-transferases have distinct activities and initiation of O-glycosylation in a cell is regulated by a repertoire of GalNAc-transferases.

10.2.2.43 *GCKR* - Glucokinase (regulator)

Also known as *GKRP; FGQTL5*

Gene ID: 2646 **Location:** 2p23

Summary Encodes for a regulatory protein that inhibits glucokinase by forming an inactive complex with the enzyme in both the liver and pancreatic islet cells. This gene is considered a susceptibility gene candidate for a form of maturity-onset diabetes of the young (MODY).

10.2.2.44 *IL1A* - Interleukin 1, alpha

Also known as *IL1; IL-1A; IL1F1; IL1-ALPHA*

Gene ID: 3552 **Location:** 2q14

Summary Encodes for a protein by this gene is a member of the interleukin 1 cytokine family. This cytokine is involved in various immune responses and inflammatory processes. It is released in response to cell injury-causing apoptosis.

10.2.2.45 *IL1B* - Interleukin 1, beta

Also known as *IL-1; IL1F2; IL1-BETA*

Gene ID: 3553 **Location:** 2q14

Summary Encodes for a protein by this gene is a member of the interleukin 1 cytokine family. This cytokine is produced by activated macrophages, which is proteolytically processed to its active form by caspase 1. This cytokine is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. The induction of cyclooxygenase-2 (by this cytokine in the central nervous system is found to contribute to inflammatory pain hypersensitivity. This gene and eight other interleukin 1 family genes form a cytokine gene cluster on chromosome 2. The *IL1* system contains genetic polymorphisms that are associated with fat mass in young men.[381]

10.2.2.46 *LDLR* - Low density lipoprotein receptor

Also known as *FH; FHC; LDLCQ2*

Gene ID: 3949 **Location:** 19p13.2

Summary The low-density lipoprotein receptor gene family consists of cell surface proteins involved in receptor-mediated endocytosis of specific ligands. *LDL* is normally bound at the cell membrane and taken into the cell ending up in lysosomes where the protein is degraded and the cholesterol is made available for repression of microsomal enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-limiting step in cholesterol synthesis. At the same time, a reciprocal stimulation of cholesterol ester synthesis takes place. Mutations in this gene cause the autosomal dominant disorder, familial hypercholesterolemia. Alternate splicing results in multiple transcript variants.

10.2.2.47 *LIPA* - Lipase A, lysosomal acid, cholesterol esterase

Also known as *LAL; CESD*

Gene ID: 3988 **Location:** 10q23.2-q23.3

Summary This gene encodes lipase A, the lysosomal acid lipase (cholesterol ester hydrolase). This

enzyme functions in the lysosome to catalyze the hydrolysis of cholesteryl esters and triglycerides. Mutations in this gene can result in Wolman disease and cholesteryl ester storage disease. Alternatively spliced transcript variants encoding the same protein have been found for this gene.

10.2.2.48 *LIPC*- Lipase, hepatic

Also known as *HL; HTGL; LIPH; HDLCQ12*

Gene ID: 3990 **Location:** 15q21-q23

Summary *LIPC* encodes hepatic triglyceride lipase, which is expressed in liver. *LIPC* has the dual functions of triglyceride hydrolase and ligand/bridging factor for receptor-mediated lipoprotein uptake. SNPs in the *LIPC* gene (*LIPC*_rs369262181, *LIPC*_rs12594375, *LIPC*_rs8023503, *LIPC*_rs4775047, and *LIPC*_rs11634134) are linked with HDL cholesterol levels.

10.2.2.49 *LPL* - Lipoprotein lipase

Also known as *LIPD; HDLCQ11*

Gene ID: 4023 **Location:** 8p22

Summary Lipoprotein lipase is expressed in heart, muscle, and adipose tissue. *LPL* functions as a homodimer, and has the dual functions of triglyceride hydrolase and ligand/bridging factor for receptor-mediated lipoprotein uptake. Severe mutations that cause *LPL* deficiency result in type I hyperlipoproteinaemia, while less extreme mutations in *LPL* are linked to many disorders of lipoprotein metabolism.

10.2.2.50 *LIPE* - Lipase, hormone-sensitive

Also known as *HSL; LHS*

Gene ID: 3991 **Location:** 19q13.2

Summary The protein encoded by this gene has a long and a short form, generated by use of alternative translational start codons. The long form converts cholesteryl esters to free cholesterol for steroid hormone production. The short form is expressed in adipose tissue, among others, where it hydrolyzes stored triglycerides to free fatty acids. Hormone-sensitive lipase has a vital role in the

mobilization of free fatty acids from adipose tissue by controlling the rate of lipolysis of the stored triglycerides. *LIPE* regulates energy homeostasis by catalyzing the rate-limiting step in adipose tissue lipolysis. Like glycogen phosphorylate, the corresponding enzyme in carbohydrate metabolism, LIPE is under acute neuronal and hormonal control. In both cases activation by catecholamines occurs through the cAMP-mediated phosphorylation of a single serine residue. The dephosphorylation of *LIPE* by insulin is responsible for the antilipolytic effect of this hormone, one of its most important actions.

10.2.2.51 *MGLL* - Monoglyceride lipase

Also known as *MGL; HUK5; MAGL; HU-K5*

Gene ID: 11343 **Location:** 3q21.3

Summary This gene encodes a serine hydrolase of the AB hydrolase superfamily that catalyzes the conversion of monoacylglycerides to free fatty acids and glycerol. The encoded protein plays a critical role in several physiological processes including pain and nociperception through hydrolysis of the endocannabinoid 2-arachidonoylglycerol. Expression of this gene may play a role in cancer tumour genesis and metastasis. *MGLL* functions together with *LIPE*; to hydrolyze intracellular triglyceride stores in adipocytes and other cells to fatty acids and glycerol. *MGLL* may also complement *LPL* in completing hydrolysis of Monoglyceride resulting from degradation of lipoprotein triglycerides.

10.2.2.52 *NQO1* - NAD(P)H dehydrogenase, quinone 1

Also known as *DTD; QR1; DHQU; DIA4; NMOR1; NMORI*

Gene ID: 1728 **Location:** 16q22.1

Summary This gene is a member of the NAD dehydrogenase (quinone) family and encodes a cytoplasmic 2-electron reductase. This FAD-binding protein forms homodimers and reduces quinones to hydroquinones. This protein's enzymatic activity prevents the one electron reduction of quinones that results in the production of radical species. Mutations in this gene have been

associated with tardive dyskinesia, an increased risk of haematotoxicity after exposure to benzene, and susceptibility to various forms of cancer. Altered expression of this protein has been seen in many tumors and is also associated with Alzheimer's disease. Alternate transcriptional splice variants, encoding different isoforms, have been characterized.

10.2.2.53 *NQO2* - NAD(P)H dehydrogenase, quinone 2

Also known as *QR2; DHQV; DIA6; NMOR2*

Gene ID: 4835 **Location:** 6p25.2

Summary *NQO2* is a flavoprotein that catalyzes the 2-electron reduction of various quinones, redox dyes, and the vitamin K menadione. *NQO2* predominantly uses dihydronicotinamide riboside as the electron donor. Mutations in this gene have been associated with neurodegenerative diseases and several cancers.

10.2.2.54 *PNLIP* - Pancreatic lipase

Also known as *PL; PTL; PNLIPD*

Gene ID: 5406 **Location:** 10q26.1

Summary This gene is a member of the lipase gene family. It encodes a carboxyl esterase that hydrolyzes insoluble, emulsified triglycerides, and is essential for the efficient digestion of dietary fats. This gene is expressed specifically in the pancreas.

10.2.2.55 *PNLIPRP1*- Pancreatic lipase-related protein 1

Also known as *PLRP1*

Gene ID: 5407 **Location:** 10q25.3

Summary Pancreatic lipase fulfills a key function in dietary fat absorption by hydrolyzing triglycerides into diglycerides and subsequently into monoglycerides and free fatty acids.

10.2.2.56 *PNLIPRP2* - Pancreatic lipase-related protein 2

Also known as *PLRP2*

Gene ID: 5408 **Location:** 10q25.3

Summary *PNLIPRP2* is essential for fat digestions. The expression level of *PNLIPRP2* in pancreas was approximately 6-fold lower than the expression levels of *PNLIPRP1*.

10.2.2.57 *PRSS1* - Protease, serine, 1 (trypsin 1)

Also known as *TRP1; TRY1; TRY4; TRYP1*

Gene ID: 5644 **Location:** 7q34

Summary This gene encodes a trypsinogen, which is a member of the trypsin family of serine proteases. This enzyme is secreted by the pancreas and cleaved to its active form in the small intestine. It is active on peptide linkages involving the carboxyl group of lysine or arginine. Mutations in this gene are associated with hereditary pancreatitis due to the trypsin self-destruct mechanism of trypsin designed to prevent pancreatic autodigestion; active trypsin is inhibited normally by a limited supply of trypsin inhibitor (e.g. *SPINK1*).[48]

10.2.2.58 *PTPRR* - Protein tyrosine phosphatase, receptor type, R

Also known as *PTPRQ; EC-PTP; PCPTP1; PTP-SL; PTPBR7*

Gene ID: 5801 **Location:** 12q15

Summary Encodes for a protein that is a member of the protein tyrosine phosphatase (*PTP*) family. PTPs are signalling molecules, regulating a range of cellular processes including cell growth, differentiation, mitotic cycle, and oncogenic transformation.

10.2.2.59 *SCARB1* - Scavenger receptor class B, member 1

Gene ID: 949 **Location:** 12q24.31

Also known as *CLA1; SRB1; CLA-1; SR-BI; CD36L1; HDLQTL6*

Summary Encodes a protein that is a plasma membrane receptor for HDL cholesterol. The encoded protein mediates cholesterol transfer to and from HDL. A polymorphism (p.P297S) has been associated with familial HDL hypercholesterolaemia.

10.2.2.60 *SPINK1* - Serine peptidase inhibitor, Kazal type

Gene ID: 6690 **Location:** 5q32

Also known as *TCP; PCTT; PSTI; TATI; Spink3*

Summary Encodes a protein that inhibits trypsin, an enzyme that is secreted by pancreatic acinar cells into pancreatic juice. It prevents the premature trypsin-catalyzed activation of zymogens within the pancreas. Mutations in this gene are associated with pancreatitis.

10.2.3 Coverage of *Genes of Interest*

Table 44 Selected genes for Next Generation Sequencing with Haloplex Coverage

Table displaying number of exons/regions covered by the Haloplex capture, the number of base pairs in the combined sequence, and overage coverage and the number of regions with either 90% total coverage or less than 90% total coverage.

Gene	Regions	Size	Coverage	≥90%	<90%
ABO	7	1064	100%	7	0
ACACA	58	7205	100%	58	0
ACACB	53	7377	100%	53	0
ACSS2	22	2145	100%	22	0
ADH1A	9	1128	100%	9	0
ADH1B	10	1128	99.41%	10	0
ADH1C	9	1128	99.54%	9	0
ADH4	11	1143	100%	11	0
ADH5	11	1125	100%	11	0
ADH6	9	1132	100%	9	0
ADH7	10	1239	100%	10	0
ALDH1	13	1506	100%	13	0
ALDH18A1	17	2388	100%	17	0
ALDH1A2	17	1557	100%	17	0
ALDH1A3	14	1539	100%	14	0
ALDH1L1	24	2709	100%	24	0
ALDH1L2	24	2772	100%	24	0
ALDH2	14	1554	100%	14	0
ALDH3B1	9	1404	100%	9	0
ALDH5A1	11	1647	100%	11	0
ALDH6A1	12	1608	100%	10	0
APOA1	3	804	100%	3	0
APOA4	3	1191	99.52%	3	0
APOA5	3	1101	100%	3	0
APOB	30	13692	100%	3	0
APOC3	3	300	100%	3	0
APOE	4	954	100%	4	0
CEL	11	2271	80.21%	8	3
CES1	15	1707	83.09%	11	4
CES2	12	1872	100%	12	0
CES3	14	1716	100%	14	0
CES5a	17	1888	100%	17	0

CES6	15	1404	100%	15	0
CFTR	27	4443	100%	27	0
CLPS	4	339	100%	4	0
CYP2E1	9	1482	100%	9	0
FABP1	4	384	100%	4	0
FABP2	4	399	100%	4	0
FABP3	4	402	100%	4	0
FABP6	7	534	100%	7	0
FADS1	15	1506	100%	15	0
FADS2	17	1335	100%	17	0
FADS3	12	1338	100%	10	0
GALNT2	18	1716	100%	18	0
GCKR	21	1878	100%	21	0
IL1a	6	816	100%	6	0
IL1b	6	810	100%	6	0
L-CPT1	20	2358	100%	20	0
LDLR	19	2583	100%	19	0
LIPA	10	1200	93.98%	9	1
LIPC	10	1500	100%	10	0
LIPE	10	3231	99.91%	10	0
LPL	11	1428	100%	11	0
MGLL	11	942	100%	11	0
NQO1	7	825	100%	7	0
NQO2	8	696	98.07%	7	1
PNLIP	12	1398	100%	12	0
PNLIPRP1	13	1404	100%	13	0
PNLIPRP2	14	1412	100%	14	0
PRSS1	6	744	100%	6	0
PTPRR	17	1974	100%	17	0
SCARB1	15	1650	98.44%	14	1
SPINK1	4	240	100%	4	0

10.3 *Ion Torrent™ Coverage*

10.3.1 By Chip

Table 45 Assessment of SNP coverage of samples by chip run on the Ion Torrent™

Chip 8 and Chip 9 both failed and those samples were run on alternative chips.

Chip Number	Chip Loading %	Total Reads (M)	Mean Read Length (bp)	Q20 bases (M)	Output (M)	Minimum % read	Maximum % read	Average % reads	Number of successful samples
1	69	4.09	136	472	557	69.8	99.0	89.2	15/15
2	78	4.24	129	507	551	81.1	99.0	92.1	15/15
3	71	5.02	153	652	768	50.2	100.0	83.5	6/15
4	86	5.2	141	623	735	59.3	99.2	90.5	13/15
5	82	4.12	148	518	610	61.8	98.3	90.5	8/16
6	80	4.24	155	562	658	84.5	99.8	95.5	15/16
7	76	6.21	148	779	920	44.6	100.0	93.0	16/16
10	73	3.07	141	372	435	64.7	97.7	89.8	12/16
11	83	3.4	97	269	331	60.6	98.8	80.2	8/16
12	76	3.13	120	325	378	47.6	99.6	84.8	11/16
13	86	6.44	139	781	898	66.4	100.0	93.5	12/16
14	84	3.88	114	374	446	57.7	99.8	90.2	14/16
15	76	5.30	142	671	754	25.3	100.0	78.2	15/16
16	84	4.35	132	509	577	6.9	98.5	65.8	15/16
17	61	3.23	88	234	286	42.5	98.0	83.2	5/16

10.3.2 By Sample

Table 46 Assessment of coverage of the SNPs within the *Genes of Interest*

Those with less than six reads coverage were classed as not covered.

CP patients include CP numbers, L numbers, M numbers, R numbers and T numbers
Control patients include Ctrl numbers, CEM numbers and S numbers

Patient ID	Reads	Mean read (bp)	Mapped reads	No. of SNPs read	% of SNPs read	Chips used
CP007	19911886	117	192076	2768	75.6	Single
CP012	43848817	152	342043	3540	96.6	Single
CP013	26484080	154	202046	3630	99.1	Single
CP014	191589	139	170691	3427	93.6	Single
CP017	13254499	135	113741	2556	69.8	Single
CP018	7452426	130	69539	2746	75	Combined
CP022	9159246	125	88639	2644	72.2	Single
CP027	34680040	135	278106	3546	96.8	Single
CP028	13493242	142	109875	3346	91.3	Combined
CP040	31152624	150	243265	3632	99.2	Single
CP042	19262379	146	151977	3360	91.7	Single
CP043	26476979	154	203744	3532	96.4	Single
CP044	245083936	152	1909228	3573	97.5	Combined
CP051	18805688	140	155140	3571	97.5	Combined
CP052	17681752	150	139128	3213	87.7	Single
CP056	10839376	95	141307	2645	72.2	Combined
CP061	51394225	143	413499	3443	94	Single
CP064	31368678	137	271408	3254	88.8	Single
CP077	76388158	148	600871	3599	98.3	Single
CP078	45846323	136	391590	3602	98.3	Single
CP079	5984144	102	71327	2834	77.4	Combined
CP080	36563513	146	291702	3641	99.4	Single
CP081	27310654	158	205072	2872	78.4	Combined
CP083	72860853	150	567479	3534	96.5	Single
CP085	36602136	121	372470	3620	98.8	Single
CP086	8512676	161	62742	3485	95.1	Combined
CP090	21464466	151	166953	3470	94.7	Single
CP092	25565426	129	213748	2811	76.7	Single
CP094	52213300	150	410378	2227	60.8	Combined
CP097	38264283	158	288810	3430	93.6	Combined
CP101	33237521	161	241343	3097	84.5	Single
CP108	27660642	127	251984	3451	94.2	Single
CP122	49752488	152	385131	3662	100	Single

CP135	21777358	140	181040	3566	97.4	Combined
CP136	29262359	163	209141	3549	96.9	Single
CP140	39197526	160	285029	3606	98.4	Single
CP141	61873692	153	473188	3661	99.9	Single
CP147	33890556	160	248496	3647	99.6	Single
CP151	18909506	143	155684	3532	96.4	Single
CP152	11990284	151	95300	3312	90.4	Single
CP153	20330747	153	157513	3509	95.8	Single
CP154	18060625	152	140759	3514	95.9	Single
CP156	18133891	151	140346	3407	93	Single
CP159	8047163	134	68133	2173	59.3	Single
CP164	44054545	145	355359	3564	97.3	Single
CP177	24415643	143	199977	3543	96.7	Single
CP183	34013071	137	292622	3303	90.2	Single
CP193	14139595	153	110824	2900	79.2	Combined
CP199	30851134	133	269847	3243	88.5	Single
CP203	37787751	140	318543	3132	85.5	Single
CP207	34243856	132	302539	2969	81.1	Single
CP212	38421792	141	319121	3628	99	Single
CP214	768973	159	759278	3064	83.6	Combined
CP215	91798416	141	751807	3661	99.9	Combined
CP222	28560266	145	230386	3427	93.6	Single
CP223	55732646	140	457295	3662	100	Single
CP224	27919215	158	207533	1839	50.2	Single
CP227	27230459	140	231042	3566	97.4	Single
CP229	36121595	161	260703	3542	96.7	Single
CP234	20174156	151	156435	3605	98.4	Single
CP241	29636154	112	319443	3637	99.3	Single
CP245	29763883	155	225516	3641	99.4	Single
CP254	75859428	154	584976	3654	99.8	Single
CP257	34249599	162	245336	3595	98.1	Single
CP264	36269771	160	266252	3459	94.4	Single
CP269	8610050	125	82715	3149	86	Single
CP278	53699131	153	409486	3662	100	Single
CP287	39208649	153	301129	3205	87.5	Single
CP292	48668322	148	386452	3662	100	Single
CP299	22038260	134	179671	3256	88.9	Single
CP310	25104659	136	200904	3098	84.6	Single
CP315	44833595	136	378456	3611	98.6	Single
CP316	22105237	137	175039	3527	96.3	Single
CP322	26385564	136	209737	3607	98.5	Single
CP328	31113267	137	245041	3552	97	Single
CP329	48627256	133	397094	3537	96.6	Single

CP330	23609261	160	172686	3292	89.9	Single
CP333	139218191	123	1087074	2488	67.9	Single
CP335	14693055	123	128673	2794	76.3	Single
CP338	65005793	133	528681	3650	99.6	Single
CP343	64244375	134	519733	3268	89.2	Single
CP353	44315567	155	341561	2934	80.1	Single
CP367	52706886	150	409771	2688	73.4	Single
CP368	33298455	160	242728	3596	98.2	Single
CP397	43175487	136	366622	3025	82.6	Single
CP414	46230626	134	392047	3086	84.2	Single
CP415	62548991	138	525329	2431	66.4	Single
CP438	35889375	110	388145	3465	94.6	Single
CP453	1628769	134	14183	1020	27.8	Single
CP456	3548172	123	34075	3601	98.3	Single
CP457	2561874	118	25676	1556	42.5	Single
L1083	23734542	120	227315	3208	87.6	Single
L1230	9996173	140	83656	2306	63	Single
M1477	42822362	133	368685	3586	97.9	Single
M1533	15703008	145	131231	2219	60.6	Single
M1704	43133700	160	312236	3647	99.6	Single
M1839	20826945	141	165126	2307	63	Single
M1847	17081166	125	164655	3503	95.6	Single
M1861	17356658	158	128783	3320	90.6	Single
R2404	13052769	142	115834	2542	69.4	Single
R2431	19692423	144	159069	3343	91.3	Single
T451	37991108	131	289726	3591	98	Single
CEM19	3047695	147	23647	3352	91.5	Single
CEM49	11118780	97	134130	3089	84.3	Single
CEM60	9579093	99	118159	3280	89.5	Single
Ctrl027	10749873	129	100492	2335	63.7	Combined
Ctrl028	17011350	131	153914	3473	94.8	Combined
Ctrl029	30941898	139	256451	3442	94	Single
Ctrl030	9621955	132	87562	2547	69.5	Combined
Ctrl031	32974861	140	273870	3648	99.6	Single
Ctrl032	9581697	144	77340	2371	64.7	Single
Ctrl033	33676044	162	248253	3631	99.1	Single
Ctrl034	32693119	160	242209	3386	92.4	Single
Ctrl035	22961811	109	250037	3616	98.7	Single
Ctrl036	38709871	156	294205	2262	61.8	Single
Ctrl037	14345845	141	119089	3239	88.4	Single
Ctrl038	39523388	145	317848	3602	98.3	Single
Ctrl039	192410495	146	1270079	3569	97.4	Single
Ctrl040	38629209	151	304987	3439	93.9	Single

Ctrl041	2911528	127	28230	1987	54.2	Combined
Ctrl042	71232608	122	723170	3584	97.8	Single
Ctrl043	3833212	114	39830	2353	64.2	Single
Ctrl044	6348693	128	59745	2437	66.5	Combined
Ctrl045	75049692	153	577852	3660	99.9	Single
Ctrl046	11090860	116	111193	2794	76.3	Single
Ctrl048	18062276	129	151321	3096	84.5	Single
Ctrl049	16452443	90	195669	3084	84.2	Single
Ctrl050	25853741	122	228449	3575	97.6	Single
Ctrl052	66555893	87	851901	1596	43.6	Single
Ctrl054	22463420	144	183326	3579	97.7	Single
Ctrl055	32143885	120	306904	3551	96.9	Single
Ctrl056	2028618	162	14772	1633	44.6	Single
Ctrl057	15070029	87	212578	2172	59.3	Single
Ctrl058	19599204	121	175550	3256	88.9	Single
Ctrl059	59961311	159	446107	3643	99.5	Single
Ctrl060	53499194	147	411028	3608	98.5	Single
Ctrl061	18842916	147	150232	3273	89.4	Single
Ctrl062	43401843	161	319627	3654	99.8	Single
Ctrl065	45459346	148	360991	3662	100	Single
Ctrl066	76672912	154	579649	3648	99.6	Single
Ctrl067	34472167	132	297945	3432	93.7	Single
Ctrl068	29310907	141	238442	3538	96.6	Single
Ctrl070	36600822	122	358918	3654	99.8	Single
Ctrl071	39371273	146	317250	3550	96.9	Single
Ctrl073	95054783	131	857178	3653	99.7	Single
Ctrl074	17081903	118	174871	3392	92.6	Single
Ctrl075	23465798	137	198480	3340	91.2	Single
Ctrl076	7860989	136	67564	1743	47.6	Single
Ctrl077	29502445	154	223415	3601	98.3	Single
Ctrl078	63225638	143	518176	3285	89.7	Single
Ctrl079	24583770	133	213354	3296	90	Single
Ctrl080	34563885	147	276103	3480	95	Single
Ctrl081	31850865	145	258233	3572	97.5	Single
Ctrl082	19745228	143	160690	3169	86.5	Single
Ctrl083	24042183	152	186364	3537	96.6	Single
Ctrl085	10213130	144	81891	2988	81.6	Single
Ctrl086	25866066	152	201105	3305	90.2	Single
Ctrl087	42828878	148	339741	3562	97.2	Single
Ctrl089	31625921	141	257510	2992	81.7	Single
Ctrl090	71534815	136	591031	2343	64	Single
Ctrl091	29862027	139	248123	3187	87	Single
Ctrl092	22872559	146	178653	3346	91.3	Single

Ctrl093	14820102	97	184711	3408	93	Single
Ctrl095	18968843	144	153432	3489	95.2	Single
Ctrl096	25928521	142	211407	3577	97.7	Single
Ctrl097	21551164	147	171567	3393	92.6	Single
Ctrl099	27037512	147	206219	3443	94	Single
Ctrl100	6750860	145	54784	2803	76.5	Single
Ctrl103	16370113	139	137794	2913	79.5	Single
Ctrl104	46518318	141	385476	3647	99.6	Single
Ctrl105	37674270	145	300981	3640	99.4	Single
Ctrl108	47948723	130	423241	3401	92.8	Combined
Ctrl111	14447003	142	120070	2606	71.1	Single
Ctrl113	67121077	148	509786	784	21.4	Single
Ctrl116	58989418	138	492367	3622	98.9	Single
Ctrl117	69177009	145	538919	927	25.3	Single
Ctrl118	3719003	128	33744	963	26.3	Single
Ctrl119	45297531	149	344345	3239	88.4	Single
Ctrl121	8280025	144	67782	3167	86.5	Single
Ctrl122	95976251	142	757842	3663	100	Single
Ctrl123	30899611	137	251190	2197	60	Single
Ctrl124	34615355	120	330951	3489	95.2	Single
Ctrl128	73082706	145	568212	3643	99.5	Single
Ctrl130	28760925	136	248725	3503	95.6	Single
Ctrl131	99933399	143	789911	3546	96.8	Single
Ctrl136	89660544	142	714274	2030	55.4	Single
Ctrl138	4354294	125	40773	1697	46.3	Single
Ctrl141	5219456	114	53145	3562	97.2	Single
Ctrl151	4238081	122	40795	2575	70.3	Single
Ctrl152	1711571	133	14904	252	6.9	Single
S038305	14591406	140	119867	3512	95.9	Single
S038310	42607252	135	351857	2982	81.4	Single
S038312	13486193	121	127499	3516	96	Single
S038324	50993582	135	432986	3633	99.2	Single
S038327	35838234	133	308785	3569	97.4	Single
S038330	44598485	134	374320	2421	66.1	Single
S038334	3057051	133	26502	2115	57.7	Single
S038336	92781017	138	752359	3641	99.4	Single
S038341	58970491	135	504539	3635	99.2	Single
S038342	50997197	140	418151	3655	99.8	Single
S038344	2046388	141	16298	3585	97.9	Single
S038345	2786863	136	23526	2592	70.8	Combined
S038360	16487839	133	141023	3609	98.5	Combined

10.3.3 Comparison of SNP Calls Removing Results from Concatenated Samples

Table 47 Sensitivity analysis excluding results that had been concatenated from two runs

The sensitivity analysis contains 85 patients in the alcohol-related chronic pancreatitis group and 89 patients in the alcoholic control group.

		Original analysis				Sensitivity analysis			
SNP	**Amino**	**OR**	**Lower CI**	**Upper CI**	**p value**	**OR**	**Lower CI**	**Upper CI**	**p value**
*ACACB*_rs17848835	p.T2027I	4.094	1.965	8.530	**0.002**	3.507	1.665	7.385	**0.001**
*ADH1C*_rs1693482	p.R272Q	0.489	0.279	0.858	**0.013**	0.483	0.267	0.875	**0.016**
*ADH5*_rs116010022	Intron	7.444	0.907	61.080	0.051	6.035	0.719	50.658	0.098
*ADH6*_rs4147545	Intron	0.591	0.373	0.935	**0.025**	0.646	0.397	1.050	0.078
*APOB*_novel	p.L3820L	1.729	1.045	2.860	**0.033**	1.914	1.108	3.307	**0.020**
*APOB*_rs72653066	Intron	0.073	0.004	1.303	0.067	0.069	0.004	1.230	0.062
*APOC3*_rs4520	p.G34G	0.603	0.400	0.910	**0.016**	0.625	0.405	0.964	**0.018**
*CES5A*_rs7500040	Intron	0.543	0.277	1.065	0.076	0.670	0.332	1.352	0.264
*CES5A*_rs200911306	p.V45I	0.210	0.059	0.743	**0.016**	0.337	0.090	1.269	0.108
*FABP1*_rs2241883	p.T94A	0.509	0.314	0.826	**0.006**	0.442	0.262	0.746	**0.002**
*FABP3*_rs11578034	Intron	8.667	1.073	69.981	**0.043**	8.244	1.020	66.649	**0.048**
*FABP6*_rs10056214	Intron	0.481	0.321	0.721	**<0.001**	0.428	0.278	0.659	**<0.001**
*IL1B*_rs1143633	Intron	0.481	0.295	0.782	**0.003**	0.916	0.530	1.583	0.753
*LDLR*_rs1003723	Intron	1.685	1.126	2.520	**0.011**	1.608	1.051	2.461	**0.029**
*LIPA*_rs1051338	p.T16P	0.293	0.115	0.751	**0.011**	0.277	0.108	0.716	**0.008**
*LIPC*_rs690	p.V155V	0.606	0.403	0.910	**0.016**	0.636	0.414	0.978	**0.039**
*LIPC*_rs3751542	Intron	0.746	0.461	1.206	0.232	0.841	0.505	1.401	0.506
*MGLL*_rs116367069	Intron	0.185	0.040	0.846	**0.025**	0.221	0.047	1.038	0.056
*PRSS1*_rs6666	p.D162D	0.554	0.369	0.832	**0.030**	0.559	0.364	0.858	**0.008**
*PTPRR*_rs10506608	p.F54F	0.340	0.140	0.825	**0.004**	0.358	0.145	0.881	**0.025**

10.4 *Tagging SNPs*

Table 48 Tagging SNPs for the *SNPs of Interest*

Data on the *SNPs of Interest* identify recognised tagging SNPs with r^2>0.8 and D' >0.9. Data taken for 1000 genomes website (http://browser.1000genomes.org/index.html)

Reference SNP	Tagging SNPs	Amino acid	r^2	D'
*ACACB*_rs17848835	NA	non coding		
*ADH1C*_rs1693482	rs1789892	non coding	1	1
	rs1154433	non coding	1	1
	rs1229978	non coding	1	1
	rs1662031	non coding	1	1
	rs1612735	non coding	1	1
	rs1789898	non coding	1	1
	rs1442479	non coding	1	1
	rs1442480	non coding	1	1
	rs1789900	non coding	1	1
	rs1442481	non coding	1	1
	rs1693456	non coding	1	1
	rs1662060	non coding	1	1
	rs1789902	non coding	1	1
	rs1693476	non coding	1	1
	rs1662059	non coding	1	1
	rs698	p.I350V	1	1
	CM033593	non coding	1	1
	rs3114048	non coding	1	1
	rs1693471	non coding	1	1
	rs1662058	non coding	1	1
	rs1693477	non coding	1	1
	rs1789906	non coding	1	1
	rs904093	non coding	1	1
	rs904094	non coding	1	1
	rs904095	non coding	1	1
	rs1662057	non coding	1	1
	rs1789908	non coding	1	1
	rs904096	non coding	1	1
	rs1789910	non coding	1	1
	rs1693480	non coding	1	1
	rs1789911	non coding	1	1

rs1693481	non coding	1	1
rs1789912	non coding	1	1
CM860053	non coding	1	1
rs1631460	non coding	1	1
rs41496344	non coding	1	1
rs1693424	non coding	1	1
rs1625439	non coding	1	1
rs62307297	non coding	1	1
rs62307298	non coding	1	1
rs1789914	non coding	1	1
rs1693425	p.V158V	1	1
rs1693426	non coding	1	1
rs1662053	non coding	1	1
rs1662052	non coding	1	1
rs1789916	non coding	1	1
rs1693427	non coding	1	1
rs1693428	non coding	1	1
rs1789917	non coding	1	1
rs1662051	non coding	1	1
rs1693430	non coding	1	1
rs1789919	non coding	1	1
rs1693431	non coding	1	1
rs1629270	non coding	1	1
rs1662049	non coding	1	1
rs1789924	non coding	1	1
rs1629838	non coding	1	1
rs2584453	non coding	1	1
rs1789925	non coding	1	1
rs2453980	non coding	1	1
rs980972	non coding	1	1
rs1693470	non coding	1	1
rs1662039	non coding	1	1
rs2851300	non coding	1	1
rs1234584	non coding	1	1
rs1662021	non coding	1	1
rs1662020	non coding	1	1
rs1235415	non coding	1	1
rs1229858	non coding	1	1
rs1229856	non coding	1	1
rs1229855	non coding	1	1
rs1229854	non coding	1	1

	rs1229851	non coding	1	1
	rs1154435	non coding	1	1
	rs1154438	non coding	1	1
	rs1154439	non coding	1	1
	rs1154440	non coding	1	1
	rs1229973	non coding	1	1
	rs1583974	non coding	1	1
	rs1154442	non coding	1	1
	rs1154444	non coding	1	1
	rs1154445	non coding	1	1
	rs1154447	non coding	1	1
	rs2851299	non coding	0.978	1
	rs1789913	non coding	0.978	1
	rs1662048	non coding	0.978	1
	rs1693469	non coding	0.978	1
	rs1238015	non coding	0.978	1
	rs1229857	non coding	0.978	1
	rs1693483	non coding	0.956	1
	rs283415	non coding	0.935	1
	rs283408	non coding	0.935	1
	rs11499826	non coding	0.893	1
	rs4093924	non coding	0.872	1
*ADH5*_rs116010022	rs144904000	non coding	1	1
	rs142452522	non coding	1	1
	rs148416104	non coding	1	1
	rs149709952	non coding	1	1
	rs146788033	non coding	1	1
	rs146829324	non coding	1	1
*ADH6*_rs4147545	rs4333190	non coding	0.95	1
	rs2903166	non coding	0.95	1
	rs3857224	non coding	0.95	1
	rs10030920	non coding	0.95	1
*APOB*_rs72653066	rs116359745	non coding	1	1
	rs113190464	non coding	1	1
*APOC3*_rs4520	NA	non coding		
*CES5A*_rs7500040	rs3859105	non coding	0.957	1
	rs3859106	non coding	0.957	1
	rs3933468	non coding	0.957	1
	rs8049600	non coding	0.957	1
	rs8048974	non coding	0.957	1
	rs6499794	non coding	0.957	1

	rs12445603	non coding	0.957	1
	rs6499792	non coding	0.957	1
	rs6416762	non coding	0.916	1
	rs6499793	non coding	0.871	0.954
	rs11647975	non coding	0.811	1
*FABP1*_rs2241883	rs1545223	non coding	1	1
	CM042701	non coding	1	1
*FABP3*_rs11578034	rs34406633	non coding	1	1
	rs34278290	non coding	1	1
	rs11589125	non coding	1	1
	rs12744206	non coding	1	1
	rs12749261	non coding	1	1
	rs2275437	p.A109A	1	1
	rs35961897	non coding	1	1
	rs4949405	non coding	1	1
	rs12756878	non coding	0.829	1
*FABP6*_rs10056214	rs10071871	non coding	1	1
	rs10071756	non coding	0.847	1
	rs10056227	non coding	0.847	1
	rs10079413	non coding	0.847	1
	rs10058512	non coding	0.847	1
	rs4921267	non coding	0.847	1
	rs4921268	non coding	0.847	1
	rs4921122	non coding	0.847	1
	rs2042259	non coding	0.847	1
*IL1B*_rs1143633	rs3917368	non coding	0.971	1
	rs3181067	non coding	0.971	1
	rs1143643	non coding	0.971	1
*LDLR*_rs1003723	rs12983082	non coding	1	1
	rs12710260	non coding	1	1
	CS022877	non coding	1	1
	CS040544	non coding	1	1
	rs1962352	non coding	0.889	0.976
	rs688	p.N591N	0.889	0.976
	CM984053	non coding	0.889	0.976
	rs28786710	non coding	0.889	0.976
	rs2738448	non coding	0.889	0.976
	rs2738449	non coding	0.889	0.976
	rs2738450	non coding	0.889	0.976
	rs2738452	non coding	0.889	0.976
	rs2915967	non coding	0.889	0.976

	rs9789302	non coding	0.889	0.976
	rs9789303	non coding	0.889	0.976
	rs34554139	non coding	0.889	0.976
	rs5925	p.V653V	0.889	0.976
	CM080431	non coding	0.889	0.976
	rs17248819	non coding	0.872	1
	rs111867267	non coding	0.868	0.975
	rs9789328	non coding	0.845	0.93
	rs10422256	non coding	0.816	1
	rs67475684	non coding	0.808	0.974
	rs2738453	non coding	0.808	0.974
*LIPA*_rs1051338	rs2246828	non coding	1	1
	rs2250645	non coding	1	1
	rs1332327	non coding	1	1
	rs2243547	non coding	0.977	1
	rs1412444	non coding	0.954	1
	rs2246833	non coding	0.932	1
	rs1332328	non coding	0.932	1
	rs1412445	non coding	0.932	1
	rs2246941	non coding	0.911	1
	rs2246942	non coding	0.911	1
	rs1332329	non coding	0.911	1
	rs2250644	non coding	0.908	0.975
*LIPC*_rs690	NA	non coding		
*LIPC*_rs3751542	rs3829460	non coding	1	1
	rs7175421	non coding	1	1
	rs4562992	non coding	1	1
	rs190316834	non coding	0.843	1
	rs28619338	non coding	0.831	1
	rs2242064	non coding	0.831	1
*MGLL*_rs116367069	NA	non coding		
*PRSS1*_rs6666	rs6667	p.N246N	1	1
	rs10258394	non coding	1	1
	rs4726576	non coding	1	1
	rs10273639	non coding	1	1
	rs9969188	non coding	1	1
	rs2855972	non coding	0.978	1
	rs13246726	non coding	0.956	0.978
	rs35031873	non coding	0.956	0.978
	rs13230029	non coding	0.956	0.978
	rs13230134	non coding	0.956	0.978

	rs28706856	non coding	0.956	0.978
	rs1969595	non coding	0.956	0.978
	rs13225332	non coding	0.956	0.978
	rs11765409	non coding	0.956	0.978
	rs12534573	non coding	0.956	0.978
	rs12534595	non coding	0.956	0.978
	rs4726580	non coding	0.956	0.978
	rs11769872	non coding	0.956	0.978
	rs11770572	non coding	0.956	0.978
	rs10952531	non coding	0.956	0.978
	rs4726581	non coding	0.956	0.978
	rs4726582	non coding	0.956	0.978
	rs4726583	non coding	0.956	0.978
	rs71529533	non coding	0.956	0.978
	rs4726586	non coding	0.956	0.978
	rs34500324	non coding	0.956	0.978
	rs4726587	non coding	0.956	0.978
	rs4726588	non coding	0.956	0.978
	rs13229600	non coding	0.956	0.978
	rs13229701	non coding	0.956	0.978
	rs13228878	non coding	0.956	0.978
	rs2367484	non coding	0.956	0.978
	rs2886990	non coding	0.956	0.978
	rs10952532	non coding	0.956	0.978
	rs1811090	non coding	0.895	1
	rs1985888	non coding	0.895	1
	rs2011216	non coding	0.895	1
	rs4726578	non coding	0.895	1
	rs4726575	non coding	0.853	0.976
	rs375656559	non coding	0.853	0.976
	rs71529532	non coding	0.853	0.976
	rs10246334	non coding	0.853	0.976
	rs2367343	non coding	0.853	0.976
	rs34708921	non coding	0.853	0.976
	rs10081384	non coding	0.853	0.976
	rs2187629	non coding	0.853	0.976
	rs10447734	non coding	0.853	0.976
	rs3757377	non coding	0.819	1
	rs3757378	non coding	0.819	1
*PTPRR*_rs10506608	rs11178395	non coding	1	1
	rs10506607	non coding	1	1

rs73144109	non coding	1	1
rs11178397	non coding	1	1
rs11178399	non coding	1	1
rs138264731	non coding	1	1
rs145149665	non coding	1	1
rs149570049	non coding	1	1
rs7313937	non coding	1	1
rs7132092	non coding	1	1
rs7299834	non coding	1	1
rs12369285	non coding	1	1
rs73144199	non coding	1	1
rs11178394	non coding	1	1
rs59474063	non coding	0.941	1
rs138137514	non coding	0.878	1
rs11178393	non coding	0.841	1