The Role of Heparin-binding Proteins in Normal Pancreas and Acute Pancreatitis

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Abstract

Acute pancreatitis (AP) is a leading cause for hospitalisation and has significant quality of life implications for the patient and cost implications for the National Health Service. Although most episodes of AP are mild and self-limiting, the severe form of the disease is associated with a high mortality. In the absence of definitive treatment, management is mainly supportive. There is an urgent need to develop more effective biomarkers and drugs to manage AP. Genome-wide studies have demonstrated that proteins that bind to heparin (HBPs) form highly interconnected networks which are functionally important in health and disease. It was hypothesized that this is true in the pancreas and in AP.

Testing this hypothesis, using mRNA as a proxy for protein, it was shown that HBPs constitute an important extracellular sub-proteome within the normal pancreas and in major pancreatic diseases that is likely to provide a rich repository of potential biomarkers and drug targets. Building upon this work, a proteomic analysis of HBPs in normal pancreas (NP) and in caerulein-induced mouse AP was undertaken. This has more than doubled the number of HBPs to 883, with 460 new HBPs identified. These may represent the most interconnected set of extracellular proteins and therefore with the greatest regulatory potential. Non canonical HBPs such as NDUFS4, NDUFS6, NDUFS7, NDUFS8, NDUFA9, NDUFA10, NDUFA9 and NDUFA10 were identified and found to be underexpressed in AP as compared to NP. These may have potential moonlighting roles, not previously known. By virtue of being extracellular and binding to

heparin, HBPs are accessible and are potential biomarkers and drug targets in AP. In addition to identifying existing biomarkers in AP such as pancreatic amylase, a number of HBPs with biomarkers potential such as HRG, CD14 and FN1 were identified and need further investigation. HBPs such as SERPINC1, VEGFA and PIP5K1C need further evaluation in drug development. These along with modified heparins, heparin mimetics and matrix therapy in AP provide exciting areas for future research.

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Author's declaration

I declare that the work in this dissertation was carried out in accordance with the regulations of the University of Liverpool. The work described is original and has not been submitted for any other degree. All aspects of the experimental design and planning for the study were conducted by me in conjunction with my supervisors, Professor R Sutton and Professor D Fernig. The experimental work in this dissertation has been undertaken by me, with specific contributions that I have indicated below and in the text.

Dr. Wei Huang and Dr. Tao Jin assisted me in the induction of experimental acute pancreatitis, specifically with caerulein injections. Mr. Changye Sun and Mr. Yong Li assisted me in the pilot heparin affinity chromatography experiments. Dr. Philip Brownridge aided with the design and performed the mass spectrometry experiments. Professor R Beynon provided expert guidance in the design of the mass spectrometry experiments and analyses. Dr. Daniel Rigden provided specialist bioinformatics advice on the proteomic data analyses.

Any views in this thesis are those of the author and in no way represent those of the University of Liverpool. This thesis has not been presented to any other university for examination in the United Kingdom or overseas.

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List of Abbreviations

AP	Acute pancreatitis
ATP	Adenosine triphosphate
AT-III	Antithrombin-III
BP	Biological process
CC	Cellular component
CP	Chronic pancreatitis
DAMPS	Damage associated pattern molecules
ECM	Extracellular matrix
ER	Endoplasmic reticulum
FDR	False discovery rate
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GAG	Glycosaminoglycan
GCLP	Good Clinical and Laboratory Practice
GCP	Good Clinical Practice
GlcA	Glucuronic acid
GlcNAc	N-acetyl-D-glucosamine
GO	Gene ontology
HBP	Heparin-binding protein
HMGB1	High-mobility group box 1
HPLC	High-performance liquid chromatography

HS	Heparan sulfate
HSPG	Heparan sulfate proteoglycan
IdoA	Iduronic Acid
IPA	Ingenuity Pathways Analysis
MF	Molecular function
MMP-2	Matrix metalloproteinase-2
MODS	Multiple organ dysfunction syndrome
mRNA	messenger RNA
NA	N-acetylated
NAS	N-sulfated S-domain
NDUF	NADH dehydrogenase (ubiquinone) Fe
NDUFA10	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10
NDUFA9	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 9
NDUFS4	NADH dehydrogenase (ubiquinone) Fe-S protein 4
NDUFS6	NADH dehydrogenase (ubiquinone) Fe-S protein 6
NDUFS7	NADH dehydrogenase (ubiquinone) Fe-S protein 7
NDUFS8	NADH dehydrogenase (ubiquinone) Fe-S protein 8
NF-kB	Nuclear factor kappa-B
NP	Normal pancreas
PDAC	Pancreatic ductal adenocarcinoma

PPI	Protein-protein interaction
RCT	Randomised clinical trial
RGTA	Regenerative agent
ROS	Reactive oxygen species
SAP	Severe acute pancreatitis
SCOP	Structural classification of proteins
STARD	STAndards for Reporting of Diagnostic accuracy
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment receptor
TCA	Trichloroacetic acid
TGFβ1	Transforming growth factor - beta 1
TEF	Transcriptional enhancer factor

Chapter 1 – INTRODUCTION

1.1 The pancreas

The pancreas is a retroperitoneal organ located in the upper abdomen. It develops from endodermal cells in the embryonic foregut and has important exocrine and endocrine functions (Zaret and Grompe, 2008). The exocrine pancreas consists of acinar and ductal cells, which are important for nutrient digestion (Hegyi and Petersen, 2013). The endocrine pancreas is made up of five types of islets cells and regulates glucose homeostasis (Shih et al., 2013).

1.2 Major pancreatic diseases

The major pancreatic diseases are acute pancreatitis, chronic pancreatitis and pancreatic cancer. While the *in silico* work described in Chapter 2 investigates the major pancreatic diseases, the main and further focus of this thesis is acute pancreatitis.

1.2.1 Acute pancreatitis

Acute pancreatitis (AP) is acute inflammation of the pancreas and is mainly caused by gallstones and alcohol (Pandol et al., 2007). The diagnosis of acute pancreatitis requires the fulfillment of '2 out of 3' of the following criteria: clinical (upper abdominal pain), laboratory (serum amylase or lipase **>**3 times the upper limit of normal) and/or imaging (computed tomography, magnetic

resonance imaging, ultrasonography) criteria (2013). Its incidence is approximately 30 per 100,000 per year in the United Kingdom (Roberts et al., 2013). Although most episodes are mild and self-limiting, the severe form of the disease is associated with mortality of the order of 5% (Schneider et al., 2010, Banks and Freeman, 2006). It is the most frequent gastrointestinal cause of hospitalisation in the United States and has major socio-economic impacts on a health system (Peery et al., 2012). In the absence of specific treatments for AP, therapy is mainly supportive (Tenner et al., 2013).

1.2.2 Chronic pancreatitis

Chronic pancreatitis (CP) is a progressive inflammatory disease associated with exocrine and endocrine insufficiency, abdominal pain, significant quality of life and nutritional implications. Environmental factors together with numerous genetic mutations and polymorphisms predispose to the disease cause CP (Gupte and Forsmark, 2014). The pain in CP is multifactorial with inflammatory and neuropathic components and its management poses significant challenges. Exocrine and endocrine insufficiency occurs as a result of destruction of pancreatic tissue. CP is a known risk factor for pancreatic cancer (Lowenfels et al., 1993) and its diagnosis often poses a diagnostic dilemma being difficult to distinguish it from pancreatic ductal adenocarcinoma (PDAC) clinically and radiologically (Johnson and Outwater, 1999).

1.2.3 Pancreatic adenocarcinoma

Pancreatic ductal adenocarcinoma is the fifth most common cancer worldwide

(Parkin, 2008). Most patients are diagnosed with advanced disease, which rules out potentially curative surgery (Vincent et al., 2011). Even after surgery, the prognosis remains poor with a 5-year survival of around 10% (Cress et al., 2006). There is a need to develop more reliable biomarkers and drugs, with a view to improving outcome in PDAC. Novel therapeutic strategies targeting the pancreatic cancer microenvironment as well as PDAC cells are evolving (Costello et al., 2012).

1.3 Pathogenic mechanisms underlying acute pancreatitis

Pathological processes underlying acute pancreatitis include intra-acinar activation of digestive enzymes, the induction of pro-inflammatory mediators, such as the transcription factor nuclear factor kappa-B (NF-kB), resulting in inflammatory cell infiltration in the pancreas, systemic inflammatory response and acinar cell death through apoptosis and necrosis (Gukovskaya and Pandol, 2004, Pandol et al., 2007, Saluja et al., 2007). Recent advances have provided new insights into the pathogenic mechanisms underlying acute pancreatitis (Criddle et al., 2007, Booth et al., 2011, Voronina et al., 2014, Gukovsky et al., 2011) and have primarily focussed on signalling pathways involved in these processes (Gukovsky et al., 2011, Sah et al., 2012).

1.3.1 Abnormal Calcium signalling

Disruption of normal Ca^{2+} signaling has been suggested as a trigger for acute pancreatitis over a decade ago. Major precipitants of acute pancreatitis such as bile salts and non-oxidative metabolites of ethanol generate toxic elevations of

Ca^{2+} that result in cellular necrosis. A sustained rise in cytosolic Ca^{2+} is pathologic and results in a sustained increase in mitochondrial Ca^{2+}. This in turn causes a decreased ATP production, which impedes the clearance of Ca^{2+} from the cell, resulting in trypsinogen activation, vacuolisation, and necrosis (Booth et al., 2011). Aberrant Ca^{2+} influx via store operated Ca^{2+} channels (SOCs) has been implicated in many diseases including AP (Lee et al., 2010). The entry of Ca^{2+} through SOCs involves the interaction of the stromal interaction molecule (STIM) and Orai at the ER-PM (endoplasmic reticulum-plasma membrane) junction and phosphoinositides contribute to the accumulation of STIM1 at this location (Walsh et al., 2010).

1.3.2 Mitochondrial dysfunction

ER–mitochondria membrane microdomains meet the Ca^{2+} demand for ATP generation and oxidative phosphorylation in the mitochondria, regulate apoptosis and mediate ATP and redox signaling from mitochondria to endoplasmic reticulum (Cardenas et al., 2010, Pinton et al., 2008). The ER–mitochondria interactions may be crucial in containing/propagating pathologic Ca^{2+}. Mitochondrial permeability transition pore (PTP) opening and loss of mitochondrial membrane potential are recognized events during pancreatitis, leading to release of mitochondrial contents into the cytosol, ATP depletion and oxidative stress (Mukherjee et al., 2008).

1.3.3 Endoplasmic reticulum stress response

Endoplasmic reticulum (ER) stress and oxidative stress are known to occur

early in pancreatitis (Sah and Saluja, 2011). Mitochondrial dysfunction results in oxidative stress, which in turn results in the generation of reactive oxygen species (ROS). This modulates ATP generation, which when inhibited results in acinar necrosis (Section 1.3.1, 1.3.2) (Booth et al., 2011). Neutrophil generated ROS and mitochondrial ROS result in the activation of inflammatory pathways in acute pancreatitis.

1.3.4 Impaired autophagy due to lysosomal dysfunction

Mitochondrial dysfunction and ER stress induce autophagy (Gukovsky et al., 2012, Lugea et al., 2011). Autophagy protects the acinar cell from the deleterious effect of released activated enzymes in acute pancreatitis (Grasso et al., 2011). Impaired lysosomal digestion of autophagic contents in turn results in impaired autophagy and so a loss of protection of the cell by this mechanism (Gukovsky et al., 2012).

1.3.5 Impaired cellular trafficking

As a result of a loss of acinar cellular and cytoskeletal reorganization, apical secretion is impaired in acute pancreatitis (Gorelick and Thrower, 2009). Consequently, basolateral exocytosis occurs, which exposes the basolateral soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) complex. This in turn results in zymogen granules attaching to the basolateral membrane causing subsequent acinar injury (Gaisano and Gorelick, 2009). VAMP8 (vesicle-associated membrane protein 8) has been shown to be the SNARE that mediates basolateral exocytosis in a mouse model of acute

pancreatitis (Cosen-Binker et al., 2008).

1.4 Experimental murine models of acute pancreatitis

Various animal models have been created over the years with a view to investigate the pathogenic mechanisms underlying acute pancreatitis. While many species have been used for these models, most investigators use rodent models, either mice or rats, as they are better standardised, less expensive to maintain and, in the case of mice, can be easily manipulated genetically for studies.

1.4.1 Secretagogue hyperstimulation model

This has been the most widely used murine model of acute pancreatitis (Lerch and Gorelick, 2013) and supramaximal cholinergic stimulation of the pancreas with caerulein, which is an ortholog of the intestinal hormone cholecystokinin, is physiologically relevant. The most widely used protocol for the induction of acute pancreatitis in mice today involves 7 intraperitoneal injections, 1 hour apart, of 50 μg/kg body weight of synthetic caerulein. The advantages of the secretagogue model of acute pancreatitis include its lack of requirement for surgery or complicated manipulations and its suitability for studying the whole spectrum of the disease from mild to severe acute pancreatitis (Lerch and Gorelick, 2013).

1.4.2 Duct obstruction and bile acid infusion model

This model seeks to mimic gall stone pancreatitis and is based on the hypothesis that if a gallstone at the papilla obstructs the common channel that connects the common bile duct to the pancreatic duct, bile could enter the pancreas and thus would induce pancreatitis. Common channel ligation models of acute pancreatitis are relevant to human disease (Kamisawa et al., 2009, Kamisawa et al., 2007). While duct ligation in opossum results in pancreatic necrosis (Lerch et al., 1993), rodent models are poorly suited for investigations, as they lack severity (Samuel et al., 1994) . Infusion of bile acids into the pancreatic duct results in acute pancreatitis of varying severity depending on the concentration of the infused bile acid (Perides et al., 2010).

1.4.3 Basic amino acid induced model

This non-invasive model induces severe acute pancreatitis in rats by the intraperitoneal injection of high concentrations of L-arginine (Mizunuma et al., 1984). The advantage of this model is its severity, while a major disadvantage is that its mechanistic relevance to human acute pancreatitis is uncertain (Lerch and Gorelick, 2013).

1.4.4 Diet induced model

In this model, mice are fed a choline-deficient diet enriched with ethionine, a derivative of methionine and develop severe necrotizing pancreatitis (Lombardi et al., 1975), with up to 100% dying within 5 days. This model shares many common features with the secretagogue hyperstimulation model, though with a

higher severity of the disease (Gilliland and Steer, 1980).

1.5 The need for more accurate prognostic biomarkers and for drug development in acute pancreatitis

Most patients with acute pancreatitis have a mild course, which settles within 3-5 days (Raraty et al., 2004). However, about 20 percent of patients have a severe course, which is associated with a high mortality (Whitcomb, 2006). These patients require specialist intervention and multi-organ support (Raraty et al., 2004) and also have major cost implications for a health system (Peery et al., 2012). The early identification of patients with the severe form of the disease is key to effective management and allocation of resources (Raraty et al., 2004, Gomatos et al., 2014). A number of clinical features and laboratory markers have been used over the years, alone or in combination with scoring systems, to determine prognosis. However, no single laboratory marker or score can accurately predict the outcome in acute pancreatitis (Mounzer et al., 2012).

1.5.1 Scoring Systems in acute pancreatitis

The Ranson score is the earliest multifactorial scoring system to be proposed in the management of acute pancreatitis and is based on eleven factors calculated at admission and at 48 hours (Ranson et al., 1974). This system is not accurate until 48 hours into the course of the disease and the sensitivity of the score decreases with incomplete data collection (Ranson et al., 1974). The modified Glasgow score includes eight factors, namely, serum albumin, blood urea nitrogen, calcium, white cell count, lactate dehydrogenase, age and glucose

Scoring System	Time	Parameters	Sensitivity (%)	Specificity (%)
Ranson Score	At admission and at 48 hours	At admission: Age (>55 years), WBC (>16,000/mL), glucose (>200 mg/dL), LDH (>350 IU/mL), AST (>250 IU/mL) At 48 hours: haematocrit decrease (>10%), BUN increase (>5 mg/dL), calcium (<8 mg/dL), PaO_2 <60 mm of Hg, base deficit >4 mEq/L, fluid sequestration (>6L)	46	80
Modified Glasgow Score	At admission and at 48 hours	Age (>55 years), WBC (>15,000/mL, glucose (>180 mg/dL), BUN (>45 mg/dL), PaO_2 (<60 mm Hg), calcium (<8 g/dL), albumin (<3.2 g/dL), LDH (>600 IU/L)	65	82
APACHE II	At admission and at 48 hors	At admission and at 48 hours: Temperature, MAP, heart rate, respiratory rate, PaO_2, arterial pH, HCO_3, sodium, potassium, creatinine, haematocrit, WBC, Glasgow Coma Score, age, chronic health points	97	44
BISAP	At admission and at 48 hours	At admission and at 48 hours: BUN (>25 mg/dL, impaired mental status (Glasgow Coma Score <15), SIRS (>/= 2), age (>60 years), pleural effusion	62	76

Table 1.1: *Comparing the clinical scoring systems in acute pancreatitis.* WBC White blood count, LDH Lactate dehydrogenase, AST Aspartate aminotransferase, BUN Blood urea nitrogen, MAP Mean arterial pressure, SIRS Systemic Inflammatory Response Syndrome
The Glasgow score has been shown to be the best predictor of organ failure at admission.

levels. The APACHE (Acute Physiology and Chronic Health Evaluation) II system, which was initially developed for patients admitted to critical care units, was similar in predictive accuracy as the Ranson and Glasgow scores (Larvin and McMahon, 1989). Also, the Glasgow score requires the collection of a large number of parameters that can be tedious (Mounzer et al., 2012). The 'Bedside Index of severity in acute pancreatitis' (BISAP) scoring system was developed using data collected within the first 24 hours of hospitalisation for acute pancreatitis and includes five parameters: blood urea nitrogen levels of greater or equal to 25 mg/dL, impaired Glasgow coma scale of <15, a systemic inflammatory response syndrome (SIRS) score of greater or equal to 2 (heart rate >90 beats/min, core temperature <36 or >38ºC, white cell count <4 or >12 10^9/L, respirations >20/min or PaCO2 <32 mmHg), age greater than 60 years and pleural effusion (Wu et al., 2008). The Glasgow score was found to be the best classifier for predicting persistent organ failure at admission in a study (Table 1.5.1) comparing the Ranson's, Glasgow, APACHE II and BISAP scoring systems (Mounzer et al., 2012). The Atlanta Symposium in 1992 attempted to establish a global consensus on a classification system for acute pancreatitis (Bradley, 1993). This was recently revised in keeping with advances in the understanding of the pathophysiology of acute pancreatitis, as well as the advances in diagnostics (Banks et al., 2013). This revised system uses the modified Marshall score for organ dysfunction (Marshall et al., 1995). A determinant-based classification of acute pancreatitis, based on local and systemic determinants of severity of the disease, has also been recently proposed (Dellinger et al., 2012). This classification system uses the sepsis-related organ failure assessment scoring system for organ dysfunction (Vincent

et al., 1996). A consensus on the same has yet to be reached (Talukdar and Rau, 2014). A study to comparing it with the Revised Atlanta Classification showed that the Determinant Classification predicted the length of hospital stay better, whereas the Revised Atlanta Classification predicted interventions in acute pancreatitis better (Acevedo-Piedra et al., 2014).

1.5.2 Molecular markers of acute pancreatitis

1.5.2.1 Biomarker definitions

A biomarker is defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological or pathogenic processes, or pharmacologic responses to therapeutic intervention" (Biomarkers_Definitions_Working_Group, 2001). Biomarkers may be classified as prognostic, predictive or pharmacodynamics and ideal biomarkers are highly sensitive and specific (Amur et al., 2008). Prognostic biomarkers indicate the likely course of a disease irrespective of treatment, whereas predictive markers suggest the population of responders to a particular treatment (Drucker and Krapfenbauer, 2013). Pharmacodynamic biomarkers indicate the therapeutic and adverse outcome of interactions between a drug and its targets. Clinical biomarkers may be stratification markers, efficacy markers, toxicity markers or surrogate endpoint markers (Lassere et al., 2007). Guidelines such as "STAndards for Reporting of Diagnostic accuracy (STARD)" have been introduced to help in the description of key elements of biomarker study design and execution (Bossuyt et al., 2003). The STARD initiative included key elements of biomarker study design, execution, patient recruitment, diagnostic

tests and statistical analyses to guide researchers.

1.5.2.2 Biomarkers in acute pancreatitis

C-reactive protein (CRP) is a non-specific marker, which is probably the most widely used predictor of severity in AP (Schutte and Malfertheiner, 2008). Serum amyloid A is an acute phase reactant that reaches significantly high levels in patients with pancreatic necrosis and is more accurate than CRP in predicting severity (Pezzilli et al., 2000). Procalcitonin is the biologically inactive form of calcitonin and is an acute phase reactant is useful in predicting severity as well as infected pancreatic necrosis and sepsis (Rau et al., 1997). Cytokines are inflammatory mediators and inducers of synthesis of acute phase reactants. Interleukin-6 significantly increases in severe acute pancreatitis, however, its levels decrease rapidly during the course of the disease and it is unreliable in predicting mortality (Ikei et al., 1998). Interleukin-8, on the other hand, may predict multi-organ failure and maybe be used as a marker of progression (Mayer et al., 2000, Dugernier et al., 2003). Trypsinogen is cleaved by trypsin itself or by duodenal enterokinase into its active form trypsin and trypsinogen activation peptide (TAP). The kidneys excrete TAP in urine. Urinary TAP has been shown to be superior to CRP at 24 hours after admission in predicting the severity of acute pancreatitis (Johnson et al., 2004). However, as its levels decrease rapidly it cannot be used to monitor disease progression. Polymorphonuclear (PMN) elastase is released by PMN granulocytes and is a good predictor of severity of acute pancreatitis, peaking 24 hours after disease onset (Dominguez-Munoz et al., 1991). Studies show that a combination of

markers, such as interleukin-10 and serum calcium (Mentula et al., 2005), PMN elastase and CRP (Viedma et al., 1994), CRP and urinary TAP (Lempinen et al., 2001), hold promise.

1.5.3 Drug development in acute pancreatitis

Various therapeutic agents have been investigated to treat acute pancreatitis. Inhibition of pancreatic secretion used to be thought to be protective in acute pancreatitis. However, while several randomized controlled trials with anti-pancreatic secretion therapy using somatostatin have failed to show any significant clinical benefit in acute pancreatitis (Choi et al., 1989, D'Amico et al., 1990, Luengo et al., 1994, Gjorup et al., 1992), a meta-analysis of 7 studies showed an overall mortality advantage benefit with somatostatin in the severe form of the disease (Andriulli et al., 1998). Similarly, the clinical benefit of the somatostatin analogue, octreotide, is yet to be demonstrated convincingly (Uhl et al., 1999, Heinrich et al., 2006). Both somatostatin and octreotide need further investigation with respect to the dosage, timing of administration, the target population and their cost-effectiveness (Li et al., 2011). There is no concrete evidence to support the use of antiprotease therapy in treating acute pancreatitis (Kitagawa and Hayakawa, 2007). While the use of the protease inhibitor gabexate mesilate has shown no clinical benefit in acute pancreatitis (Valderrama et al., 1992, Buchler et al., 1993), continuous regional arterial infusion with Nafomostat in combination with antibiotics warrants further investigation (Imaizumi et al., 2004, Piascik et al., 2010). Lexipafant, a platelet activating factor receptor antagonist and anti-inflammatory drug, failed to show

a reduction in multiple organ dysfunction syndrome (MODS) and mortality in patients with severe acute pancreatitis (SAP) compared to placebo treatment in a double-blinded randomised clinical trial (RCT) (Johnson et al., 2001). The prophylactic use of antibiotics has not been shown to significantly reduce mortality, or the requirement for surgical intervention (Hackert and Werner, 2011), while antioxidant therapy has not shown to have a protective effect, but on the contrary seemed to augment the severity of acute pancreatitis (Siriwardena et al., 2007). In a large multicentre RCT antibiotic prophylaxis in combination with probiotic strains did not reduce the risk of infectious complications severe acute pancreatitis and was associated with an increased risk of mortality (Besselink et al., 2008). Despite an increased understanding of the pathophysiology of acute pancreatitis in the last two decades, a specific therapy for AP is lacking and treatment remains largely supportive (Tenner et al., 2013).

1.6 Systems biology and systems medicine

'Reductionism', in principle, is useful in investigating diseases, particularly in conditions where one or a few components are responsible for the overall outcome. It is useful and effective when an isolatable problem exists and where a quick and effective solution is available e.g. diseases such as acute appendicitis and urinary tract infections. However, this approach is less effective in investigating systems in which interactions between components dominate the systems themselves, thereby influencing behaviour and outcomes. This is the case in complex organs such as the pancreas and complex diseases such as acute

pancreatitis (Ahn et al., 2006). This has had an impact on drug design and the number of successful novel, single-target drugs developed over the past decades (Butcher, 2005). Previously, the reductionist approach to drug development has resulted in many successful single-target drugs. Single target drugs, however, are less able to combat the complex pathologies of inflammatory diseases and cancer, which are regulated by multiple and often partly redundant molecular inputs (Leung et al., 2012). Hence, single target drugs are sometimes used in combination which each other.

The last decade has seen enormous advances in the 'omics' technologies and the explosion of public databases for molecular interaction data (Kwoh and Ng, 2007). These data pose challenges of representation and analysis. This period has also seen the emergence of the concept of 'Systems Biology'. Systems biology adopts a holistic approach integrating experimental and computational research to investigate biological systems and this is particularly useful in understanding complex diseases such as acute pancreatitis, where a number of biological processes and pathways are involved. Spatial and temporal variability provide useful information regarding a system and are particularly important in a systems biology approach (Ahn et al., 2006). The analyses of the genetic profiles of various complex diseases such as lymphoma (Rosenwald et al., 2002), breast cancer (Zhang et al., 2003) and lung cancer (Au et al., 2004) have shown us that seemingly single phenotypes can have multiple etiologic and pathologic processes underlying them, which in turn can impact prognosis and response to therapeutic strategies (McCarthy et al., 2013, Gustafsson et al., 2014, Hagg et al., 2009). The 'MammaPrint' is a commercially available diagnostic biomarker product to stratify breast cancer based on gene

expression profiling (Kittaneh et al., 2013). A disease module-based approach to drug discovery in rheumatoid arthritis (Okada et al., 2014) and a combinatorial approach integrating mathematical models and high-throughput data have been used to generate disease-specific models for the identification of drug targets in tuberculosis (Rienksma et al., 2014). 'Systems medicine' uses spatial and temporal information with a view to achieving a holistic approach to an individual. In future, optimal treatment of diseases is likely to be based on 'personalised medicine' (Ahn et al., 2006) as evidenced by the optimisation of warfarin-based anticoagulant therapy using genotyping (Pirmohamed et al., 2013). Adopting a 'Systems' approach is likely to increase the possibility of developing more effective drugs with an improved safety profile, as well as developing multi-target drugs (Schrattenholz et al., 2010).

1.7 The extracellular matrix

The extracellular matrix (ECM) is a three-dimensional component of the microenvironment containing cells and bioactive molecules and lies between the plasma membranes of cells. The ECM is a dynamic space and plays an important role in homeostasis. It acts as a reservoir for bioactive molecules such as growth factors, cytokines and so on. Its biophysical and biochemical properties vary in health and disease (Hynes, 2009). There is an abnormal accumulation of ECM in inflammation and cancer that increases the stiffness of the tissue and leads to increased tissue hypoxia (Kong and Mooney, 2007). The altered ECM may also affect the transmission of bioactive molecules in the

microenvironment and may thereby influence cell signalling (Duchesne et al., 2012).

Extracellular proteins, including those of the ECM, are suggested to have played a central role in vertebrate evolution (Huxley-Jones et al., 2009). A genome-wide analysis by Vogel and Chothia has shown that these proteins expanded significantly with a concomitant expansion in their contribution to function as the biological complexity of organisms increased (Vogel and Chothia, 2006). Subsequently, Ori *et al.* demonstrated that within the extracellular proteins those that bind to heparin, form an important integrated functional network (Ori et al., 2011). Thus, the ECM could constitute an important repository for biomarker discovery and drug development. In AP, the regenerative response to the damaged pancreas is determined by a balance between the synthesis and degradation of ECM. Various constituents of the ECM play important roles in maintaining this balance. Transforming growth factor beta 1 (TGFβ1) promotes angiogenesis, stimulating the production of fibronectin and collagen (Border and Noble, 1994, Nakamura et al., 2007). It also reduces the synthesis of proteases and increases the production of protease inhibitors (Sporn and Roberts, 1992) and can induce fibrosis. Matrix metalloproteinase-2 (MMP-2) has been shown to promote healing following an attack of AP (Kihara et al., 2001).

1.8 Heparan sulfate and heparin

1.8.1 Structure

Heparan sulfate (HS) is a linear sulfated polysaccharide expressed by most

animal cells (Xu and Esko, 2014). Structurally, HS contains a characteristic disaccharide-repeating unit (Fig. 1.1) and belongs to the family of glycosaminoglycans (GAG). The basic building block consists of β1–4-linked D-glucuronic acid (GlcA) and α1–4-linked N-acetyl-D-glucosamine (GlcNAc). HS chains have a molecular weight between 20 to 100 kDa and typically consist of 50 to 250 disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine.

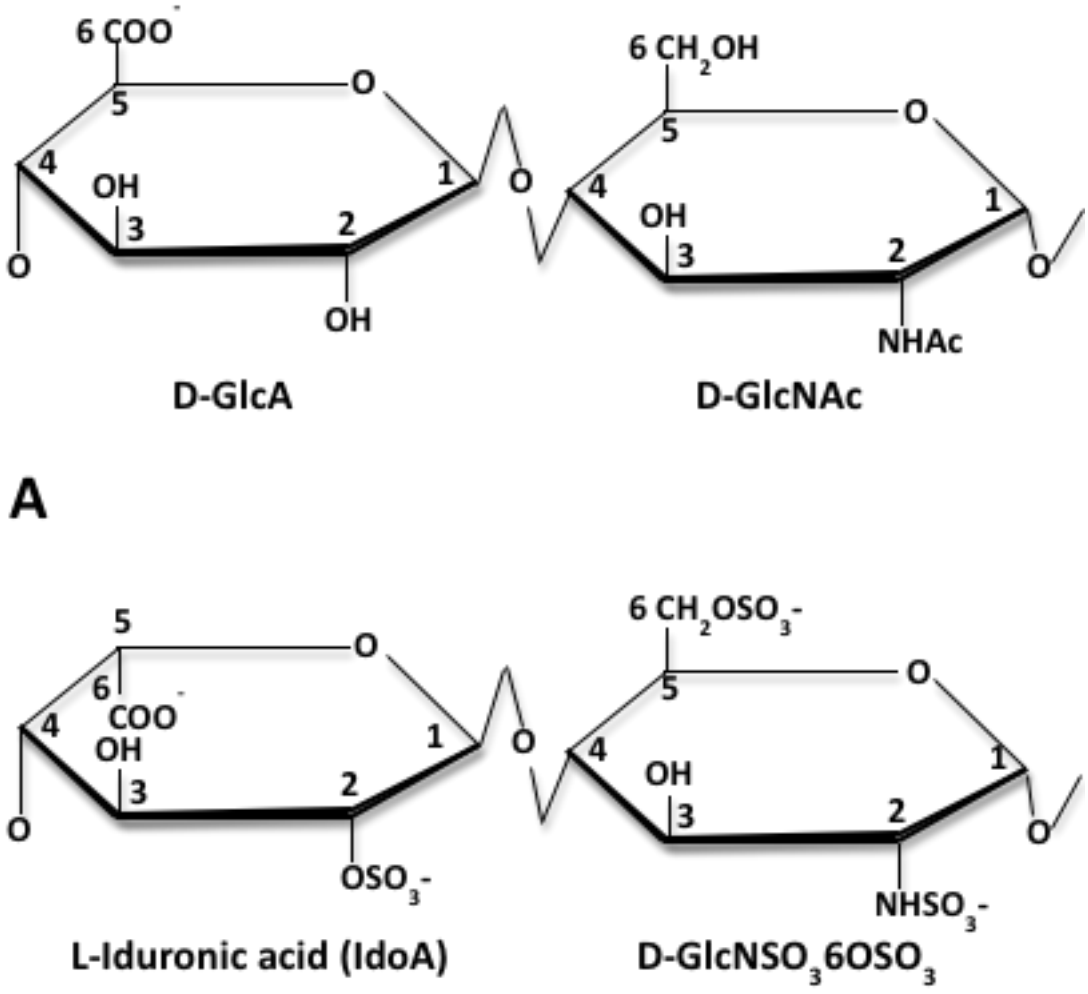

Figure 1.1 *The disaccharide units of heparan sulfate and heparin* (A) the GlycA β 1-4 GlcNAc unit, which is the initial product of polymerisation. (B) N-deacetylation/N-sulfation of the glucosamine marks the disaccharide unit for further modification, including epimerisation of GlcA to IdoA and O-sulfation at C2 of the IdoA, C6 of GlcNS. The trisulfated disaccharide makes up ~75% of heparin, whereas it is less frequent in the S-domains of heparan sulfate, which are less sulfated.

The formation of HS chains is initiated by the formation of a tetrasaccharide linkage region, synthesized by stepwise addition of xylose, followed by two galactose units and a glucuronic acid residue to a serine residue in the core protein. The first N-acetyl-glucosamine residue is then transferred to the linkage region, followed by extensive addition of glucuronic acid and N-acetyl-glucosamine units in alternating sequence. The nascent GAG chain is modified by sulfotransferases and an epimerase. First, some of the N-acetylglucosamine residues are N-deacetylated and N-sulphated by a dual-activity N-deacetylase/ N-sulphotransferase enzyme, using 3′-phosphoadenosine-5′-phosphosulphate as sulphate donor. The partially N-sulfated polysaccharide is then acted upon by glucuronyl C5-epimerase. This converts glucuronic into iduronic acid residues. The modification process is completed through 6-O- and 3-O-sulfation of glucosamine units. At each step only a fraction of substrates are modified which is responsible for considerable sequence diversity in HS chains.

These modification reactions all depend on the presence of N-sulfated glucosamine; that is they occur on the N-sulfated disaccharide unit or on the adjacent unit. There are four N-deacetylase/ N-sulfotransferase enzymes encoded in the human genome, but they all have in common the property of modifying blocks of N-sulfated glucosamine, with N-deacetylase/ N-sulfotransferase modifying very long blocks and the others leaving substantial tracts of unmodified disaccharides. The consequence is that heparan sulfate has a domain structure. From the tetrasaccharide linker there is an unmodified domain, termed NA (N-acetylated). This is followed by a transition domain, where between one in two and one in three disaccharides is N-sulfated (termed NAS domain) and an S-domain (sulfated), where each glucosamine is N-sulfated.

The S-domain is flanked on the non-reducing end by another NAS domain, which is followed by an NA domain and so on. The S domains are the most heavily sulfated.

After secretion to the cell exterior, the HS chains may be edited through SULF-catalysed 6-O-desulphation. HS chains are structurally dynamic and change with time and cellular physiology (Lindahl and Lindahl, 1997, Feyzi et al., 1998, Jayson et al., 1998). Characteristic differences in the structure of HS have been demonstrated with certain diseases, age and transition to malignancy (Lindahl and Lindahl, 1997, Lindahl et al., 1995). The factors that control the biosynthetic machinery in the Golgi and so determine the size and composition of HS chains in different cells and at different times are yet to be unraveled.

Heparin is used as an experimental proxy for cellular heparan sulfate, because it resembles a very large S-domain, but is somewhat more sulfated even than these, due to its synthesis being uniquely driven by N-deacetylase/ N-sulphotransferase-1. Heparin is the largest drug in production worldwide (Xu and Esko, 2014).

1.8.2 Heparan sulfate proteoglycans

Heparan sulfate proteoglycans (HSPGs) consist of a core protein with one or more covalently linked HS chains. There are 17 HSPGs that have been identified, which reside either in the plasma membrane or in the extracellular matrix. It is not yet clear whether the core protein influences the structure of HS (Xu and Esko, 2014).

1.8.3 Heparin-binding proteins

Proteins that bind to heparin at physiologically relevant ionic strength and pH, are called heparin-binding proteins (HBPs). Approximately 435 HBPs are known (Ori et al., 2011). HBPs belong to a wide range of functional categories ranging from cytokines and chemokines to enzymes and matrix proteins. They can bind to more than one sequence in HS and may also bind to other glycosaminoglycans (GAGs) such as chondroitin sulfate, dermatan sulfate and hyaluronic acid (Xu et al., 2012).

1.8.4 Interaction between HS and HBPs

1.8.4.1 Molecular basis of binding

At the molecular level, ionic bonding is an important feature of the interaction of HBPs with HS and other sulfated glycosaminoglycans. Thus, the negatively charged sulfate and carboxyl groups of HS interact with positively charged lysine and arginine residues in the heparin-binding proteins (HBPs). Polar residues and the amide of the peptide backbone also interact with these charged groups on the sugar, as well as participating in hydrogen bonds with the various oxygen and hydrogen atoms of HS (Ori et al., 2008). Although the kinetics of HBP binding to HS are dominated by the ionic interactions, energetically these are not necessarily dominant, evidenced by the lack of correlation between the concentration of electrolytes required to abrogate binding and binding affinity (Ori et al., 2008, Xu et al., 2012). One reason is that HBP binding to HS often causes substantial conformational change in the protein, as seem, for example,

by differential scanning fluorimetry and circular dichroism (Uniewicz et al., 2010, Xu et al., 2012, Uniewicz et al., 2014, Xu et al., 2013) and classically seem in the interaction of Antithrombin-III (AT-III) with anti coagulant heparin (Evans et al., 1992, Huntington, 2003).

The specificity and selectivity of the interactions between HBPs and HS is the matter of some debate. The high specificity of AT-III for a pentasaccharide sequence with a 3-O sulfate (the basis of heparin's anticoagulant activity) has led to the argument that many HBPs will possess a similar "high affinity" site in HS, but the sequence complexity of HS means that these have yet to be discovered. At the other extreme is the view that the interaction of HBPs is simply due to charge and that there is little selectivity or specificity, as found with thrombin, and so the interaction of AT-III is an exception. A more pragmatic view is that all HBPs can bind a range of structures, even AT-III, with varying affinity and it is possible to generate on, for example, an unrelated plant polysaccharide, functional, high affinity binding structures for HBPs (Rudd et al., 2010). Analysis of a group of evolutionarily related HBPs, the fibroblast growth factors (FGFs), has suggested that there is indeed specificity in the structures recognised by FGFs in HS, and though this is not absolute (so 1;1), it has arisen through natural selection and so likely to be of functional significance (Xu et al., 2012).

1.8.4.2 Functions of interaction with HS

The binding of HBPs to HS has many functions, which include protein stabilisation, scaffolding two proteins so they may interact more effectively,

including formation of ligand-receptor complexes, and localisation and transport.

The conformational change often observed upon HS binding increases protein stability (the basis of the above differential scanning fluorimetry assay) (Uniewicz et al., 2010, Xu et al., 2012). This improves the life span of some HBPs by preventing their degradation by proteases in the extracellular environment and is likely also important in the highly reactive environments seen for example, in inflammation.

The scaffold provided by HS can also act as a catalyst of encounters to promote more effective interactions of HBPs with one another. The combination of conformational change and catalysis of encounters is illustrated by the consequences of the binding of AT-III to heparin. This induces a structural rearrangement in AT-III that results in the expulsion of a loop containing a protease-reactive site; since the AT-III targets also bind heparin, the degrees of freedom of the collision between AT-III and its targets are reduced. Together, these two effects dramatically increases the inhibitor activity of AT-III against coagulation factors thrombin by 9000-fold and Factor X by 17000-fold, respectively (Whisstock et al., 2000). Different crystal structures of AT-III complexed with different saccharides and in a ternary complex with its substrates have been demonstrated to elucidate this mechanism (Imberty et al., 2007, Whisstock et al., 2000). HS may also be considered an allosteric modulator of HBPs, as evidenced by the crystallization studies of HS-dependent conformational change of AT-III (Langdown et al., 2009).

A further example of a scaffolding function is provided by the HS-dependent ligands (growth factors, morphogens, chemokines and cytokines). To generate

signals through their cognate membrane receptor, these have to form a ternary complex that also includes HS. The FGFs provide the paradigm for what is termed the "co-receptor" function of HS. Thus, the stimulation of biological processes such as cell proliferation requires the formation of a ternary complex of FGF, FGF receptor (FGFR) and HS (Yayon et al., 1991, Rapraeger et al., 1991). In this complex, HS binds both the FGF ligand and the FGF receptor (Schlessinger et al., 2000). In the absence of HS, though the FGF ligand still binds its cognate receptor, signalling kinetics are very different and in this instance, cell proliferation is not stimulated (Zhu et al., 2010)

HS will act as a tether, and so immobilise HBPs and concentrating them at a specific location in the extracellular space, which is likely important for regulating and directing the activity of HBPs that are enzymes. Such binding is also critical for the formation of gradients of HBPs that are signalling molecules, so cytokines, chemokines, growth factors and morphogens. For example, recent work has demonstrated that the transport of FGF-2 in pericellular matrix is controlled by its binding to HS (Duchesne et al., 2012). Indeed, disruption of the interactions of such HBPs with HS has been found to have a profound effect on cell communication in development, homeostasis and disease, in part due to the disruption of the transport of these effectors from source to target cell.

1.8.5 Roles of HS, HSPGs and HBPs in health and disease

HS and HSPGs play critical roles in embryogenesis and the development of various organs (Maeda et al., 2011, Miner, 2011, Thompson et al., 2010). The absence of HS is not compatible with life (Lin et al., 2000, Habuchi et al., 2007).

The targeted deletion of genes involved in the biosynthesis of HS results in embryonic and perinatal lethality in mice. The homozygous deletion of Ext1 or Ext2 genes, which encode HS polymerase enzymes, causes failure of gastrulation, while the heterozygous condition (Ext2) is associated with the development of exostoses and multiple cartilage deformities (Wang et al., 2005, Fuster et al., 2007). Loss of glucuronyl C5-epimerase is lethal for neonates and its targeted disruption caused synthesis of HS chains devoid of IdoA with a resultant loss of kidneys, poorly inflated, immature lungs and skeletal abnormalities (Li et al., 2003). The unique structure of HS affords it the ability to bind to variety of proteins thereby influencing their activity (Ori et al., 2008). HSPGs create preferential diffusive paths for their ligands by altering the shape of morphogen gradient residues thereby influencing diffusivity through the ECM (Lander, 2007), as evidenced directly in fibroblast pericellular matrix (Duchesne et al., 2012). The elucidation of the mechanism of activation of AT-III was the first example of how heparin influences protein function by inducing conformational change (Langdown et al., 2009). Many HBPs are important signalling molecules in the microenvironment and regulate fundamental biological processes such as cell adhesion, differentiation, proliferation and migration, organogenesis, lipid metabolism, inflammation and cancer (Ori et al., 2011). On a genome-wide basis, HBPs have been shown collectively to play a pivotal role in driving biological complexity and influence fundamental processes underlying complex diseases (Ori et al., 2011). Thus, the identification of HBPs and the understanding of HS-HBP interactions would be useful in developing heparin-based and HBP targeted therapeutic interventions. This has already been initiated in the field of regenerative medicine, in the

treatment of non-healing ulcers (Barbier-Chassefiere et al., 2009).

1.8.6 Therapeutic potential of HS-HBP interactions

Heparin is the largest drug by weight in production today and has been in clinical use for decades (Xu and Esko, 2014). There is great potential for drug development based on HS-HBP interactions in cancer and inflammation (Coombe and Kett, 2005). The phosphosulfomannan PI-88, which is based on a heparin-like structure, was designed with a view to targeting a number of HBPs in cancer and was tested as such in clinical trials in cancer patients (Yu et al., 2002). More recently, its heparanase inhibitory activity, which will be anti-metastatic and anti-angiogenic, has provided the basis for Phase III trials in post-resection hepatocellular carcinoma (Kudchadkar et al., 2008, Liu et al., 2009). Some drugs have been developed to specifically target single HBPs. A drug that has been developed using this approach is Fondaparinux, which is the first synthetic heparin (Bauer, 2003). This drug does not bind to platelet factor 4 and does not cross react with antibodies generated as a result of heparin-induced thrombocytopenia, thereby eliminating many of the side effects of heparin therapy (115). SST0001 is a modified heparin derivative that has shown anti-angiogenic activity in the preclinical setting and has recently entered Phase I clinical trials in multiple myeloma (Ramani et al., 2013). PG545 is a synthetic HS mimetic has been shown to have antitumour and antimetastatic properties in a breast carcinoma model (Hammond et al., 2012).

1.9 Proteomics as a tool to investigate the extracellular matrix in acute pancreatitis

Global strategies such as proteomics, along with genomics and transcriptomics present a novel methodological approach to investigating disease and have been used in AP (Anderson and Anderson, 1998, Pociot and Karlsen, 2002, Buchholz et al., 2001). However, mRNA levels often do not correlate to those of the corresponding proteins (Schwanhausser et al., 2011). Extensive protein analyses are, therefore, necessary to gain comprehensive information of cellular transactions and correlate them with disease (Lohr and Faissner, 2004). Protein analyses carry the advantage of representing the actual metabolic state of the cell, however, they are challenging (Lohr and Faissner, 2004). While serum proteomic analysis of ECM proteins in AP has been undertaken (Lohr et al., 1999), tissue proteomics remains largely underexplored due to the technical challenges associated with it (Wilson, 2010).

1.10 Hypothesis and outline of thesis

It is hypothesized that the high level of connectivity of HBPs demonstrated in previous genome-wide studies would also be true in the normal pancreas and in some of its disease states. If this hypothesis is true, by virtue of their binding to heparin, HBPs would provide an easily accessible sub-proteome, which could be mined for biomarkers and drug targets in pancreatic diseases. The first objective of this thesis is to undertake a comprehensive meta-analysis of mRNA datasets in the major pancreatic diseases. Chapter 2 describes this *in silico* study (Nunes et al., 2013). While this work provides strong support for the

hypothesis, it assumes mRNA to be representative of translation and that the existing list of HBPs (Ori et al., 2011) to be reasonably representative of those expressed in the pancreas. Both these assumptions could undermine the validity of the conclusions drawn from the meta-analysis of mRNA.

Consequently, the second objective of this thesis is to implement an affinity proteomic strategy that would tackle in a systematic way the identification of new HBPs in the normal pancreas (NP) and in AP. Biases often derive from experimental compromises necessary to ensure the high throughput of the procedures (Mackay et al., 2007). Further biases can arise from the disruption of cellular anatomical structures, typically in pull-down or affinity experiments that are performed on cell extracts. Also in this case, the information on the original sub-cellular localisation of interacting partners is lost, increasing, therefore, the probability of observing spurious interactions not likely to occur in vivo. Bearing this in mind, in Chapter 3, an affinity proteomic strategy for the identification of HBPs in murine normal pancreas and experimental acute pancreatitis was implemented. The strategy was focused on the cell surface and extracellular proteome derived from mouse pancreas extracts, the rationale being that targeting the compartment where HSPGs are expressed increases the probability of identifying more relevant binding partners, likely to co-localise with HS *in vivo*. Despite the need for validation of the newly described interactions, the identification of new heparin/HS binding partners can help to unravel new molecular circuits involving HSPGs. This chapter also describes the construction and network-level analysis of the heparin interactome, i.e. the protein-protein interactions of the HBPs. The heparin interactome was built

using the HBPs from the heparin affinity proteomics experimental data. This dataset was then used to investigate the network properties, and functional and structural features that characterise the heparin interactome. Network level approaches have been widely used in the last decade for the analysis of protein-protein interactions (PPIs) (Jeong et al., 2001), transcription factor binding specifities (Grove et al., 2009), metabolic pathways (Ideker et al., 2001), and signalling pathways (Linding et al., 2007), revolutionising the representation and interpretation of biological processes. The network-level analysis provides important general functional insights on HBPs that could be extremely valuable in the design of future experiments, and also exemplifies a new way of investigating protein-GAG interactions that has the potential for widespread application in the field of glycobiology.

Chapter 4 discusses the findings of this thesis and compares the *in silico* and experimental work. It focuses on some of the key pathways relevant to pancreatic homeostasis in health and in acute pancreatitis and explores the potential role of candidate HBPs as biomarkers and drug targets.

The main body of this thesis has been presented in the form of 2 manuscripts. The *in silico* work has been published (Nunes et al., 2013) and has been included in Chapter 2. The proteomics work is a manuscript in preparation and has been included in Chapter 3. The papers included are in the journal-specific submitted format with modifications for the purpose of convenience. The font style and size are identical to rest of the thesis text. Sections, tables and figures have been numbered and included in the table of contents. As the supplemental data for the manuscript included in Chapter 3 are large, these have been copied to the attached CD. The proteomics data has been uploaded into PRIDE database

(http://www.ebi.ac.uk/pride/archive/)(Vizcaino et al., 2013).

Chapter 2 – *In silico* analyses

2.1 Introduction

Extracellular proteins have played a central role in vertebrate evolution (Huxley-Jones et al., 2009). A genome-wide study has shown that these proteins showed a significant expansion with a concomitant expansion in function as the complexity of organisms increased (Vogel and Chothia, 2006). It was subsequently shown that among the extracellular proteins, HBPs form important functional modules within the extracellular space (Ori et al., 2011). This *in silico* analysis was undertaken to test the first hypothesis (Chapter 1; 1.9) that HBPs would form important functional modules in the extracellular space when investigating the normal pancreas (NP) and major pancreatic diseases namely acute pancreatitis, chronic pancreatitis and pancreatic ductal adenocarcinoma. If this is true, HBPs, by virtue of their binding to heparin, would provide an easily accessible repository within the extracellular space for biomarker discovery and drug development. In this study, we used mRNA expression as a proxy for protein and identified putative HBPs in NP, AP, CP and PDAC. Network analysis of the putative HBP interacting networks showed that HBPs form important, well-connected modules within the extracellular space in the normal pancreas, AP, CP and PDAC. The analysis also identified HBP candidates as potential biomarkers and drug targets in these diseases (Nunes et al., 2013). These candidates were further investigated using a literature-mining tool, Pubmatrix (Becker et al., 2003), to assign functional relevance to HBPs in the major pancreatic diseases. While this work provides strong support for the

hypothesis, it assumes mRNA to be representative of translation and that the existing list of HBPs to be reasonably representative of those expressed in the pancreas. Building on the results from the *in silico* analysis, it was necessary to implement an affinity proteomic strategy that would tackle in a systematic way the identification of new HBPs in the normal pancreas (NP) and in the major pancreatic diseases. In the first instance, it was decided to perform the proteomics experiments in NP and an experimental model of one of the major pancreatic diseases. As AP affords the benefit of several time-tested experimental models (Chapter 1; 1.4), it was decided to undertake the affinity proteomics experiments in NP and experimental murine AP (Chapter 3).

2.2 Various terms explained

2.2.1 Node

A node is a gene, mRNA or protein. In this study it refers to an HBP.

2.2.2 Edge

An edge is an interaction between 2 nodes.

2.2.3 Degree

The most elementary characteristic of a node is its degree or connectivity. This is denoted by 'k' and informs about the number of links a node has to other nodes, e.g., in an undirected network (Fig. 2.1A), node A has degree k = 4. In

directed networks in which each edge has a selected direction (Fig 2.1B) there is an incoming degree, k_{in}, which represents the number of edges that point to a node. Similarly, an outgoing degree k_{out}, denotes the number of edges that start from it. Node A has $k_{in} = 4$ and $k_{out} = 1$ (Fig 2.1B). An undirected network with N nodes and L links or edges is characterized by an average degree <k> = 2L/N (where <> denotes the average).

2.2.4 Interactome

An interactome is an interacting network.

2.2.5 Plugin

A plugin is a tool used to analyse a network.

2.2.6 Number of connected components

In undirected networks, two nodes are connected if there is a path of edges between them. Within a network, all nodes that are pairwise connected form a connected component. The number of connected components indicates the connectivity of a network – a lower number of connected components suggests a stronger connectivity (Fig. 2.2).

2.2.7 Clustering coefficient

If 'n' is the number of nodes, the clustering coefficient is a ratio N / M, where 'N' is the number of edges between the neighbours of n, and 'M' is the maximum

number of edges that could possibly exist between the neighbors of *n*. The clustering coefficient of a node is always a number between 0 and 1.

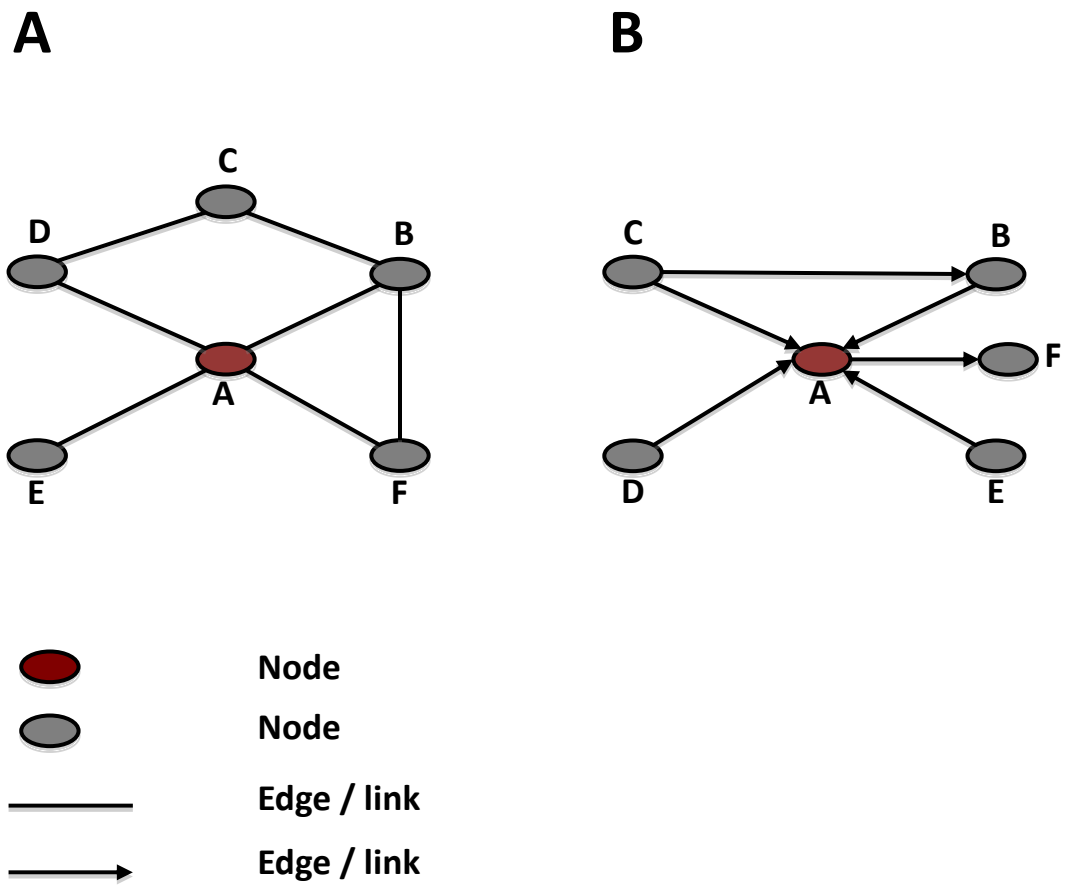

Figure 2.1: *Network connectivity* A) in an undirected network, node A has degree k = 4. B) in a directed network Node A has an incoming degree, k_{in} = 4 and an outgoing degree, k_{out} = 1.

The network clustering coefficient is the average of the clustering coefficients for all nodes in the network (Barabasi and Oltvai, 2004, Watts and Strogatz, 1998). Nodes with less than two neighbors are assumed to have a clustering coefficient of 0.

In undirected networks, the clustering coefficient C_n of a node n is defined by the equation $C_n = 2e_n/(k_n(k_n\text{-}1))$, where k_n is the number of neighbors of n and e_n is the number of connected pairs between all.

In directed networks, the equation is $C_n = e_n/(k_n(k_n\text{-}1))$ neighbors of n.

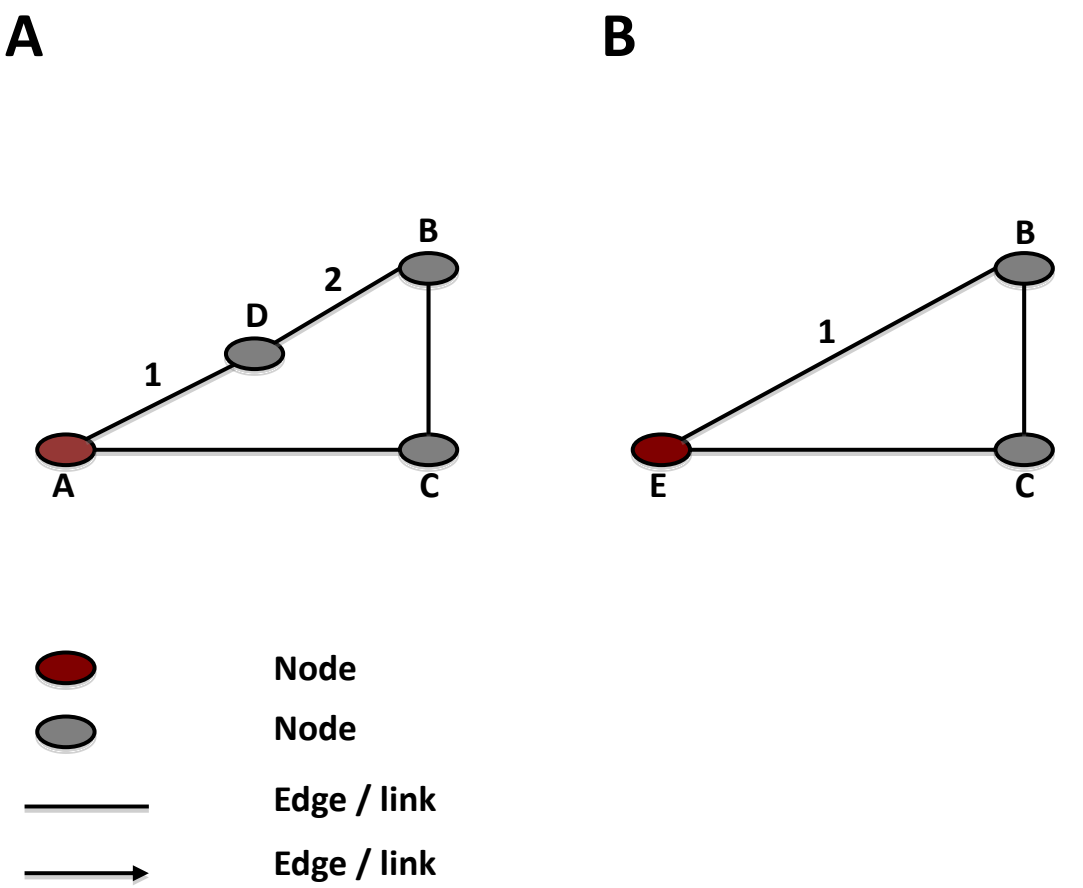

Figure 2.2: *Number of connected components* A) Node A, which is connected to Node B via 2 edges, is less well connected as compared to B) Node E, which is connected to Node B via a single edge

2.2.8 Gene ontology

Gene ontology (GO) is a major bioinformatics initiative to standardize gene and gene product attributes across species and covers three major domains namely biological process (BP), cellular component (CC) and molecular function (MF).

2.2.9 Canonical pathway

Canonical pathways are well characterized metabolic and cell signalling pathways that have been curated from original publications. These do not change according to data input.

2.3 Bioinformatics pipeline

A number of bioinformatics resources were used for network construction and data analysis (Fig. 2.3)

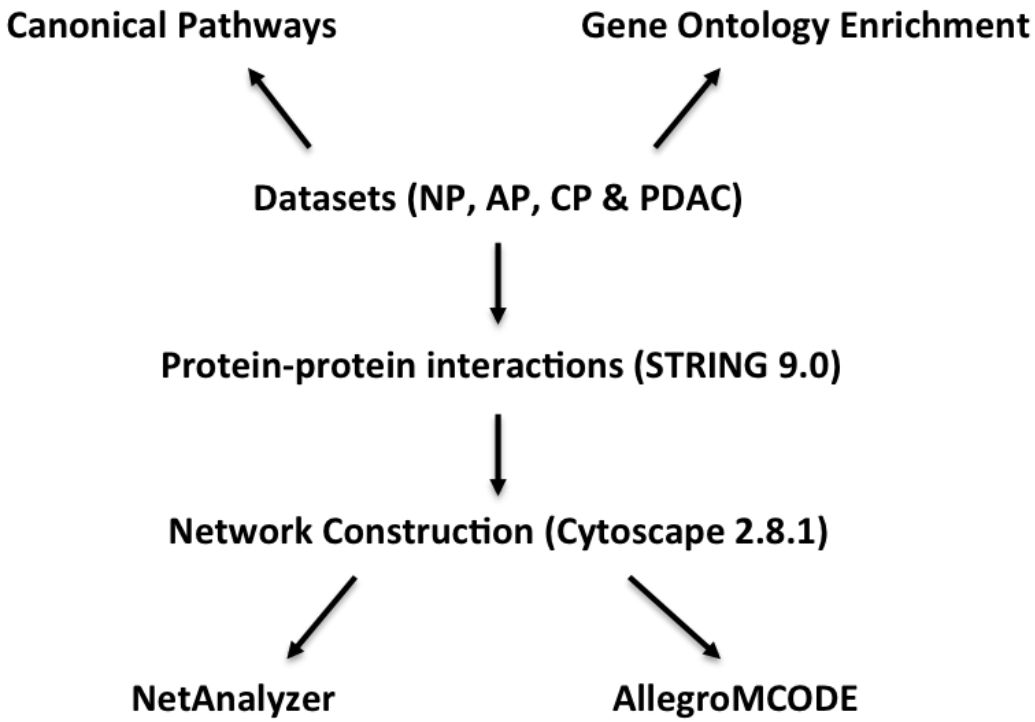

Figure 2.3: *Bioinformatics pipeline used to analyse the role of HBPs in the NP, AP, CP and PDAC*. Networks of HBPs were constructed using interactions from the STRING database. Canonical pathways analyses (Ingenuity Pathways Analysis), Gene ontology enrichment and top clusters using AllegroMCODE (Cytoscape 2.8.1) were some of the bioinformatics tools used to identify potential biomarkers and drug targets.

2.4 Manuscript: The heparin-binding protein interactome in pancreatic diseases

Nunes QM, Mournetas V, Lane B, Sutton R, Fernig DG, Vasieva O.

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Quentin M. Nunes performed the analyses, wrote the paper

Virginie Mournetas aided with the analyses, edited the paper

Brian Lane aided with the analyses, edited the paper

Robert Sutton conceived the study, edited the paper

David G. Fernig conceived the study, co-wrote the paper

Olga Vasieva aided with the analyses, edited the paper

For copyright reasons, this is the final submitted version of the manuscript and not the published version.

The heparin-binding protein interactome in pancreatic diseases.

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Short title: The heparin interactome in the pancreas.

Key words: Bioinformatics; heparan sulfate; heparin; heparin-binding protein

2.4.1 Abstract

Background: The cellular microenvironment plays an important role in the regulation of homeostasis and is a source of potential biomarkers and drug targets. In a genome-wide analysis the extracellular proteins that bind to heparin (HBPs) have been shown to form highly modular and interconnected extracellular protein regulatory networks. Using a systems biology approach, we have investigated the role of HBP networks in the normal pancreas and pancreatic digestive diseases.

Methods: Lists of mRNAs encoding for HBPs associated with the normal pancreas (NP), acute pancreatitis (AP), chronic pancreatitis (CP) and pancreatic ductal adenocarcinoma (PDAC) were obtained using public databases and publications. Networks of the putative protein interactomes derived from mRNA expression data of HBPs were built and analysed using cluster analysis, gene ontology term enrichment and canonical pathways analysis.

Results: The extracellular heparin-binding putative protein interactomes in the pancreas were better connected than their non heparin-binding counterparts, having higher clustering coefficients in the normal pancreas (0.273), acute pancreatitis (0.457), chronic pancreatitis (0.329) and pancreatic ductal adenocarcinoma (0.269). 'Hepatic Fibrosis / Hepatic Stellate Cell Activation' appears to be a significant canonical pathway in pancreatic homeostasis in health and disease with a large number of important HBPs.

Conclusions: Our analyses clearly demonstrate that HBPs form disease-specific and highly connected networks that can be explored for potential biomarkers and as collective drug targets via the modification of heparin binding properties.

2.4.2 Introduction:

The microenvironment of tissue matrix, cells and bioactive molecules plays an important role in the regulation of biological homeostasis. The extracellular matrix (ECM) is a dynamic three-dimensional medium and its biophysical and biochemical properties vary in health and disease (Hynes, 2009). There is an abnormal accumulation of ECM in inflammation and cancer that increases the stiffness of the tissue and leads to increased tissue hypoxia (Kong and Mooney, 2007). The altered ECM may also affect the transmission of bioactive molecules in the microenvironment and may thereby influence cell signalling (Duchesne et al., 2012). Within the microenvironment of the pancreas, the pancreatic stellate cell (PSC) is a specialised cell, which plays an important role in maintaining the architecture of the pancreas and the composition of the ECM. The PSC secretes excessive amounts of ECM proteins in pancreatic diseases, which increase fibrosis within the tissue and contribute to ECM stiffness (Apte et al., 2012).

Extracellular proteins, including those of the ECM, are suggested to have played a central role in vertebrate evolution (Huxley-Jones et al., 2009). Moreover, a genome-wide analysis by Vogel and Chothia has shown that these proteins expanded significantly with a concomitant expansion in their contribution to function as the biological complexity of organisms increased (Vogel and Chothia, 2006). Subsequently, Ori *et al.* demonstrated that within the extracellular proteins those that bind to heparin, form an important integrated functional network (Ori et al., 2011). Heparin is used as an experimental proxy for the related cellular heparan sulfate (HS) for practical reasons of convenience and

cost and heparin affinity chromatography affords a tool to analyse HBPs. HS is a linear polysaccharide in the ECM containing a characteristic disaccharide-repeating unit and belongs to the family of glycosaminoglycans. HS chains attach to core proteins to form HS proteoglycans and are major components of the pericellular matrix/glycocalyx and of the ECM (Ori et al., 2008). Many HBPs are important signalling molecules in the microenvironment and regulate fundamental biological processes such as cell adhesion, differentiation, proliferation and migration (Ori et al., 2011).

The pancreas has important exocrine and endocrine functions that develop from endodermal cells in the embryonic foregut (Zaret and Grompe, 2008). AP, CP and PDAC are major diseases of the pancreas that pose diagnostic and therapeutic challenges. AP is acute inflammation of the pancreas and is mainly caused by gallstones and alcohol (Pandol et al., 2007). Although most episodes are mild and self-limiting, the severe form of the disease is associated with a high mortality (Schneider et al., 2010). In the absence of specific treatments for AP, therapy is mainly supportive. CP is a progressive inflammatory disease of the pancreas and a known risk factor for pancreatic cancer (Lowenfels et al., 1993). PDAC and CP present with similar signs and symptoms and are associated with an intense fibrosis. These pose a diagnostic dilemma, as they are difficult to distinguish clinically and radiologically (Johnson and Outwater, 1999). PDAC is a major cause of cancer death and is one of the most difficult cancers to treat (Thomas et al., 2010). Surgical resection continues to form the mainstay of treatment in about 15-20% of patients, the rest presenting with unresectable disease (Alexakis et al., 2004). There is a need

to develop reliable biomarkers for early detection and more effective drugs in the management of PDAC

On a genome-wide basis, HBPs have been shown collectively to play a pivotal role in driving biological complexity and influence fundamental processes underlying complex diseases (Ori et al., 2011). We hypothesized that HBPs would form well-connected modules, which play important regulatory roles in the extracellular space in the normal pancreas and in some of its disease states. If this hypothesis were true, by virtue of their binding to heparin, HBPs would provide an easily accessible sub-proteome, which could be mined for biomarkers and drug targets in pancreatic diseases.

Using mRNA expression as a proxy for protein, we have identified the putative HBPs in normal pancreas (NP) and in three pancreatic diseases (AP, CP and PDAC). Analyses of the putative interactomes of the HBPs demonstrate that they indeed form an important integrated functional network. Many of the putative HBPs are recognised as regulating fundamental biological processes underlying pancreatic physiology and pathology. The analyses also identify potential biomarkers and drug targets in pancreatic diseases, such as fibroblast growth factor receptor -2 (FGFR2), which may be explored in pancreatic stellate cell (PSC) targeted therapy in PDAC.

2.4.3 Materials and methods:

2.4.3.1 Building putative protein interactomes for the pancreas

Lists of genes and gene products associated with NP, AP, CP and PDAC

Lists of mRNAs and proteins associated with NP, CP and PDAC were obtained using the Pancreatic Expression Database. This database is a comprehensive open-access mining tool for large-scale genomic, transcriptomic and proteomic datasets (Chelala et al., 2007, Cutts et al., 2011). The lists were generated using the filters 'Normal Pancreas', 'Chronic Pancreatitis', and 'Pancreatic Ductal Adenocarcinoma'. A list of genes associated with AP was obtained from the NCBI 'Gene' database using the search term "Acute Pancreatitis".

2.4.3.2 Lists of HBPs associated with NP, AP, CP and PDAC

A list of 435 HBPs was obtained from supplementary information in Ori *et al*, 2011 (Ori et al., 2011). This list was built using a combination of literature curation, data retrieval from public databases and experimental data using an affinity proteomic approach. The *'compare'* tool of Ingenuity Pathways Analysis (IPA, Ingenuity Systems, www.ingenuity.com) was used to generate lists of HBPs associated with NP, AP, CP and PDAC, derived from the IPA Knowledge Base, a repository of biological interactions and functional annotations that are reviewed regularly for accuracy.

2.4.3.3 Interactions and construction of networks of HBPs

Lists of interactions between HBPs were obtained using the online database resource 'Search Tool for the Retrieval of Interacting Genes' (STRING). STRING 9.0 is a database of known and predicted functional interactions and served as a 'one-stop' comprehensive resource that could be easily used with Cytoscape (Szklarczyk et al., 2011). The interactions in STRING are provided with a probabilistic confidence score that is an estimate of how likely an interaction describes a functional linkage between two proteins. A higher score indicating a higher confidence is given when more than one type of information supports a given association. Only interactions with the highest confidence score (0.900 and above) were used to build networks using Cytoscape 2.8.1, which is an open source, Java based bioinformatics package for biological network visualization (Smoot et al., 2011). The resulting networks were termed 'putative protein interactomes', because the HBP lists were derived from mRNA expression data and the interactions between the listed entities were retrieved from STRING. As for nearly any expression dataset used for *in silico* network analysis, there was uncertainty with respect to the actual presence of the expressed proteins and the potential interactions in a cultured cell or in a tissue. However, correspondence between the major protein and mRNA expression pools and the resistance of general network properties to slight variations in the connectivity of each component support the legitimacy of this widely used approach (Cirillo, 2012). In the putative protein interactomes, HBPs or 'nodes' that are unique to a dataset are coloured red, while the rest of the HBPs are coloured grey. Black lines connecting the HBPs denote the interactions or 'edges'.

2.4.3.4 Network Analysis

2.4.3.4.1 Network parameters

Additional 'plugins' or tools to analyse networks are available in Cytoscape. The networks were treated as undirected and the following parameters were computed using the *'NetworkAnalyzer'* plugin (Assenov et al., 2008): diameter, average number of neighbours, number of connected pairs of nodes, node degree, average clustering coefficient, topological coefficient, and shortest path length. We used the 'clustering coefficient' and 'number of connected components' as measures of network connectivity. Clustering coefficient is a ratio of the number of edges between neighboring nodes and the maximum number of edges that could possibly exist between them. The clustering coefficient of a node is always a number between 0 and 1. The clustering coefficient of a network is the average of the clustering coefficients for all nodes in the network. In undirected networks, two nodes are connected if there is a path of edges between them. Within a network, all nodes that are connected pairwise form connected components. A high clustering coefficient and a low number of connected components are present in well-connected networks(Barzel and Biham, 2009). Clusters, or highly interconnected hubs of nodes within the networks, were identified with the *'AllegroMCODE'* plugin.

2.4.3.4.2 Comparing the connectivity of the heparin-binding putative protein interactomes with other pancreatic putative protein interactomes

Clustering coefficients of extracellular putative protein interactomes in the normal and diseased pancreas were compared in order to ascertain if the heparin-binding putative protein interactomes formed important modules within the extracellular space in the pancreas. The lists of all extracellular proteins were extracted by applying filters based on Gene Ontology (GO) cellular component terms to the lists. This was performed using the 'Database for annotation, visualization and integrated discovery' (DAVID) and the GO FAT annotation. DAVID 6.7 is a bioinformatics resource that extracts biological meaning from gene / protein lists (Huang da et al., 2009). GO FAT is a subset of the GO term set that is created by filtering out the broadest ontology terms, so as not to overshadow more specific ones. The terms used were: GO:0005576 (extracellular region), GO:0005615 (extracellular space), GO:003102 (extracellular matrix- ECM) and GO:0005604 (basement membrane). The non-HBP lists were generated by subtraction of HBP names from the datasets of all extracellular proteins. The extracellular non heparin-binding putative protein interactome (Ec_not hepint) and the extracellular heparin-binding putative protein interactome (Ec_hepint) for NP, AP, CP and PDAC, were built using the interaction data retrieved from the STRING database and their clustering coefficients were compared.

2.4.3.4.3 Comparison with random networks

The heparin-binding putative protein interactomes (Ec_hepint) in NP, AP, CP and PDAC were each compared with 10 of their corresponding degree-preserving randomised versions in order to determine whether the network

parameters arising from the interactions of the putative HBPs were random. This is a quality assurance exercise. The randomised networks (Ec_hepint_random) were generated, by shuffling the edges of the respective heparin-binding putative protein interactomes, using the *'Random Networks'* plugin with Cytoscape. The average clustering coefficient of Ec_hepint was compared with the average clustering coefficient of the corresponding random network in NP, AP, CP and PDAC. A network is deemed to be well connected if its average clustering coefficient is significantly higher than that of its corresponding random networks.

A Shapiro-Wilk test was performed to test if the clustering coefficients were normally distributed (data are considered to be normally distributed if $p>0.05$)(Henderson, 2006). The clustering coefficients of the various networks were then compared using the independent t-test; $p<0.05$ was considered to be significant. The statistical analyses were performed using SPSS version 20.

2.4.3.5 Identification of potential biomarkers and therapeutic targets

2.4.3.5.1 Canonical pathways analysis

Canonical pathways are well characterized metabolic and cell signalling pathways that have been curated from original publications and do not change according to data input. Canonical pathways analysis used the IPA library of canonical pathways to identify those that were most significant to each of the datasets of HBPs associated with NP, AP, CP and PDAC. The significance of the association between the datasets and the canonical pathway was measured by calculating the p-value using Fisher's exact test to determine the probability of

the association between the HBPs in the dataset and the canonical pathway. Canonical pathways analysis is a useful tool, when used on its own or in conjunction with other tools such as the Cytoscape plugin AllegroMCODE, to identify potential biomarkers and drug targets. It also helps identify biological pathways that play important roles in homeostasis and complex diseases.

2.4.3.5.2 Functional Analysis of HBPs

Functional analyses of the datasets were performed using tools for GO term enrichment. GO is a major bioinformatics initiative to standardize gene and gene product attributes across species and covers three major domains namely biological process (BP), cellular component (CC) and molecular function (MF). GO term enrichment for the lists of HBPs associated with NP, AP, CP and PDAC was performed using DAVID and the GO FAT annotation. GO term enrichment provides biological context to the HBP datasets and so helps to identify potential biomarkers and drug targets.

2.4.4 Results:

2.4.4.1 Building putative protein interactomes for the pancreas

The HBPs were identified using the Pancreatic Expression Database, the NCBI 'Gene' database and a list of HBPs from Ori *et al*, 2011 (Ori et al., 2011). The lists of HBPs associated with NP (n= 115), AP (n= 31), CP (n=112) and PDAC (n=141) are in Supplementary Tables 1-4. A number of HBPs were uniquely associated with the NP (n=1), AP (n=16), CP (n=12) and PDAC (n=23) datasets in our study (Table 2.3.1). The lists of HBPs were used to obtain interactions from STRING, which were then imported into Cytoscape to build heparin-binding putative protein interactomes. As a result of the stringent criteria adopted, for interaction selection, some HBPs from the datasets in our study are notably absent from the interacting networks. The key features of the putative protein interactomes are illustrated with the HBPs identified in the PDAC-associated expression data (Figure 2.3.1). HBPs or 'nodes' that are unique to the PDAC dataset in the study are coloured red, while the rest of the HBPs are coloured grey. Black lines connecting the HBPs denote the interactions or 'edges'. The regulatory importance of these interactomes was quantified by network analysis.

2.4.4.2 Network analysis

The topological parameters of the heparin-binding putative protein interactomes associated with NP, AP, CP and PDAC were obtained using the 'NetworkAnalyzer' plugin in Cytoscape. A high clustering coefficient compared with those of its corresponding random networks and a lower number of

connected components indicate a strong interconnectivity of a network (Dong and Horvath, 2007, Barzel and Biham, 2009). HBPs form highly interconnected networks in the normal pancreas with four connected components and a high clustering coefficient of 0.273. The heparin-binding putative protein interactome (Ec_hepint) of the normal pancreas was better interconnected than its corresponding random network (Ec_hepint_random) and importantly, of the extracellular proteins minus the HBPs (Ec_not_hepint) (Figure. 2.3.2). This indicates that the HBPs indeed form a densely interconnected network module in the extracellular space in the normal pancreas.

The heparin-binding putative protein interactomes in AP, CP and PDAC were also found to be highly interconnected with one, three and five connected components. They also have high clustering coefficients of 0.457, 0.329 and 0.269 (Figure 2.3.2). Comparing the topological parameters of the HBP networks with the other protein networks in the pancreas, Ec_hepint in AP, CP and PDAC were also better interconnected than their corresponding random networks (Ec_hepint_random) and the extracellular non heparin-binding putative protein interactomes (Figure 2.3.2). Thus, as for NP, the heparin-binding putative protein interactomes of AP, CP and PDAC also formed highly interconnected modules of extracellular proteins.

To determine if the network parameters between HBPs arose randomly, the'*Random Networks*' plugin was used to perform shuffles on the heparin-binding putative protein interactomes to generate degree-preserving random networks of HBPs (Ec_hepint_random) (Materials and Methods). The clustering

coefficients of the various networks were normally distributed (Shapiro-Wilk test, Supplementary Table 5). In NP, AP, CP and PDAC the respective Ec_hepint had a higher clustering coefficient than the corresponding Ec_hepint_random (Figure 2.3.2). Therefore, these analyses demonstrated that the interactions and network parameters for the NP, AP, CP and PDAC heparin-binding putative protein interactomes did not arise randomly. The dense networks of HBPs in normal and in pancreatic disease exhibit properties similar to highly connected networks, sometimes termed 'small world' networks, and indeed represent real and probably important functional modules (Watts and Strogatz, 1998).

2.4.4.3 Identification of potential biomarkers and therapeutic targets

Canonical pathways analysis, cluster analysis and GO enrichment were used to identify potential biomarkers and therapeutic targets.

2.4.4.3.1 Canonical Pathways

Canonical pathways analysis provides insights into well-defined biological regulatory processes and signalling cascades underlying normal organ physiology and diseases. HBPs are enriched in a number of important biological pathways (Figure 2.3.3). 'Hepatic Fibrosis / Hepatic Stellate Cell Activation' is the top canonical pathway linked to HBPs associated with NP (p=7.98E-16, 16/147 molecules), AP (p=3.32E-18, 12/147 molecules) and PDAC (p=2.57E-18, 19/147 molecules). Six HBPs, which are unique to the AP dataset, namely CCl2, IFNG, Il6, TNF, TGFβ1 and VEGFA and 2 HBPs that are unique to the PDAC dataset in our study, FGFR2 and VEGFB, are linked to this canonical pathway.

'Coagulation System' (p=3.09E-17, 12/38 molecules) and 'Intrinsic Prothrombin Activation Pathway' (p=3.03E-14, 10/34 molecules) are other significant pathways associated with the PDAC dataset. The top canonical pathway associated with CP dataset is the 'Coagulation System' pathway (p=1.74E-18, 12/38 molecules). Other important canonical pathways associated with the CP dataset are 'Intrinsic Prothrombin Activation Pathway' (p=3.12E-17, 11/34 molecules) and 'Hepatic Fibrosis / Hepatic Stellate Cell Activation' (p=2.52E-13, 14/147 molecules). Canonical pathways analysis indicates that stellate cells play important roles in the pancreas and may be explored further to develop cell specific therapies.

2.4.4.3.2 Cluster analysis

Clusters are groups of highly interconnected nodes. Clusters are scored depending on the number of constituent nodes and the edges (interactions) between them. Top clusters associated with the HBP networks (Table 2.3.2) were generated using the AllegroMCODE plugin in Cytoscape. These top clusters were analysed further to explore their potential as biomarkers and drug targets, using IPA. Each of the top clusters in NP, AP, CP and PDAC contained HBPs that had similar biological functions notably inflammatory response, cell-cell signalling and cellular movement, indicating that they were biologically relevant (Barabasi and Oltvai, 2004). The top cluster in the AP heparin-binding putative protein interactome consists of 6 HBPs unique to the AP HBP dataset, namely CCl2, IFNG, Il6, TNF, TGFβ1 and VEGFA (Figure 2.3.4). This cluster also enriches to the 'Hepatic Fibrosis / Hepatic Stellate Cell Activation' canonical pathway,

which as shown above is also the top pathway associated with the AP HBP dataset. Thus, the canonical pathways and cluster analyses identify key molecules that may lead to biomarkers and drug targets.

2.4.4.3.3 GO term enrichment

GO term enrichment provides a means to identify key processes that can be attributed to a gene or protein set. The GO terms 'Response to wounding' (p=3.52E-23), 'Wound healing' (p=5.92E-16) and 'Regulation of body fluids' (p=8.88E-16) are the top terms of the 'Biological Process sub-ontology' enriched by HBPs associated with the normal pancreas. 'Response to wounding' (p=1.32E-17), 'Defence response' (p=1.34E-13) and 'Leucocyte migration' (p=1.73E-13) are the top terms enriched by HBPs associated with AP. 'Response to wounding' (p=8.31E-24), 'Extracellular matrix organisation' (p=1.90E-15) and 'Wound healing' (p=5.14E-15) are the top 'Biological Process' terms enriched by HBPs associated with CP. 'Response to wounding' (p=6.15E-25), 'Wound healing' (p=2.52E-15) and 'Cell adhesion' (p=3.60E-15) are the top 'Biological Process' terms enriched by HBPs associated with PDAC.

2.4.5 Discussion

The *in silico* analyses of putative interactomes of HBPs demonstrate that these form highly connected networks in the normal pancreas and in three major pancreatic digestive diseases that may define important extracellular protein regulatory modules. This is strongly supported by the high clustering coefficients associated with the heparin-binding putative protein interactomes

of the normal pancreas, AP, CP and PDAC, compared to their corresponding random networks. Moreover, the heparin-binding putative protein interactomes have higher clustering coefficients, as compared to the extracellular non heparin-binding putative protein interactomes in NP, CP and PDAC (Figure 2.3.2).

Previously, the reductionist approach to drug development has resulted in many successful single-target drugs over the past decades, although systematic analysis of interactomes has not featured prominently in drug design. Single target drugs, however, are less able to combat the complex pathologies of inflammatory diseases and cancer, which are regulated by multiple and often partly redundant molecular inputs (Leung et al., 2012). This difficulty is particularly evident in pancreatic diseases, where there are very few specific therapies. In contrast, a holistic 'Systems Biology' approach based on the heparin interactome may provide small groups of biomarkers and drug targets in pancreatic digestive diseases and is complementary to the reductionist approach to drug development. Even though the meta-analytical approach adopted here assumes mRNA to be representative of translation and our knowledge of the nature and functions of specific proteins is incomplete and variably informative, there is a plethora of original findings that it behoves us to inter-relate.

These conclusions are also supported by GO term enrichment and canonical pathways analysis, since these identified cell-signalling cascades and molecular functions that are coherent with NP, AP, CP and PDAC. For example, the top GO term of the sub-ontology Biological Process enriched to HBPs associated with AP, CP and PDAC was 'response to wounding'. Other Biological

Process sub-ontology terms significantly enriched to the AP dataset included defence response, leukocyte migration, inflammatory response and leukocyte migration, which one would associate with an acute inflammatory disease such as AP. Leukocytes and neutrophils in particular are known to play an important role in AP (Abdulla et al., 2011, Nakamura et al., 2010). The desmoplastic reaction associated with CP and PDAC is reflected by the enrichment of GO term 'extracellular matrix organization' to the HBP datasets associated with these diseases (Johnson and Outwater, 1999). GO terms such as cell adhesion, blood vessel development, vasculature development and regulation of cell migration enriched to the PDAC dataset are clearly key processes in cancer.

Canonical pathways represent well-characterized metabolic and cell-signalling cascades that have been curated from original literature and do not change according to data input. They are important in informing our understanding of cell function and predicting cell behaviour (Melas et al., 2011). The canonical pathways that are enriched by the HBP datasets in pancreatic digestive diseases provide useful insights into important pathways influencing these diseases. 'Hepatic Fibrosis / Hepatic Stellate Cell Activation' is the top canonical pathway linked to the AP and PDAC datasets and is a significant pathway in CP. Stellate cells play an important role in inflammatory diseases and cancer of the liver as in the pancreas (Xu et al., 2010b, Friedman, 2008, Masamune et al., 2009). The presence of a well-defined fibrosis and activation pathway associated with the hepatic stellate cell rather than its pancreatic counterpart is probably due to the fact that the former was discovered earlier and has been studied more extensively (Geerts, 2001). As information relating to PSCs evolves, pathways relating to PSC activity may become better defined.

Hepatic and pancreatic stellate cells exhibit great similarities, are activated by common cytokines and growth factors, and may share a common origin (Buchholz et al., 2005, Omary et al., 2007). Experiments relating to PSCs indicate that they play an important role in the normal and diseased pancreas (Vonlaufen et al., 2007, Algul et al., 2007). These cells maintain normal pancreatic architecture, as well as contribute to the increased stiffness of the ECM in CP and PDAC by inducing fibrosis (Apte et al., 2012). The increased stiffness of the ECM in turn may affect the transmission of signals between cells in the pancreatic microenvironment. Thus, HBPs unique to the AP and PDAC datasets and also enriched to the 'Hepatic Fibrosis / Hepatic Stellate Cell Activation' pathway may be explored as potential PSC specific biomarkers and drug targets. FGFR2 is a target, which may enable the development of PSC-directed drug therapy in PDAC. In pancreatic cancer, FGFR2 is over-expressed in both cancer cells and the adjacent pancreatic parenchyma (Ishiwata et al., 1998). Stromal FGF10 (fibroblast growth factor 10) – FGFR2 signalling induces migration and invasion in pancreatic cancer cells, and is associated with a poor prognosis (Nomura et al., 2008). FGFR2 is uniquely associated with the PDAC dataset in our study and this is in the context of a common expression in NP, CP and PDAC of two ligands, FGF-3 and FGF-5 (Table 2.3.1 and Supplementary Tables 1, 3 and 4). This ligand pair is able to activate both the FGFR2b and FGFR2c splice variants (Ornitz et al., 1996). Thus, these data suggest that FGFR2 inhibitors may be of use in the treatment of PDAC, and these are readily available because FGFR2 is already been being explored as a drug target in other solid organ tumours (Byron and Pollock, 2009).

The top cluster in the AP heparin-binding putative protein interactome is also linked with the 'Hepatic Fibrosis / Hepatic Stellate Cell Activation' canonical pathway. Within this cluster, 6 HBPs namely CCl2, IFNG, IL-6, TNF, TGFβ1 and VEGFA are in the AP HBP dataset. Interleukin-6 (IL-6), which is a cytokine produced by macrophages and a mediator in the synthesis of acute-phase proteins, forms the hub of this top cluster. IL-6 has been suggested as a therapeutic target in acute pancreatitis and is a well-established drug target in other diseases such as rheumatoid arthritis(Nishimoto and Kishimoto, 2006). PSCs are stimulated by TGFβ1 and PDGFβ (Apte et al., 2000). The various HBPs in the top cluster in AP might be thus used to develop PSC-specific or PSC-related therapy in AP.

The property of heparin binding, which defines the regulatory importance of HBPs through their physiological interactions with HS, can be elegantly exploited in a direct proteomic approach, since heparin affinity chromatography is a simple and effective means to extract HBPs from a tissue or body fluid (Xiong et al., 2008, Ori et al., 2011). Therefore, given the clear importance of HBPs, a direct analysis of HBPs by proteomics of healthy and diseased pancreas is likely to yield substantial insights.

2.4.6 Conclusions

HBPs are shown to constitute a highly regulatory extracellular sub-proteome in the normal and diseased pancreas and thus are a likely source of targets for therapy and biomarkers. The present analyses have identified HBPs already established as having a role in pancreatic disease, e.g., FGFR2, VEGFA.

Importantly, these analyses identify clusters of HBPs working together to execute a common function, e.g., Hepatic Stellate Cell activation in AP and PDAC. The present work demonstrates the power of systems analysis on meta-expression data where admitted uncertainty in protein presence and interactivity do not limit the ability to produce useful predictions and recommendations.

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Condition	Unique HBPs	Common HBPs
NP	AMBP	APOE, IL8, IL10, MMP9, PRSS1, PRSS3, SOD1
AP	APOA5, CCL2, CXCL10, IFNγ, IL6, LIPC, LPL, MBL2, MPO, PDGFβ, PON1, PROC, SELP, TGFβ1, TNF, VEGFA	
CP	AIBG, AKR1B1, COL14A1, HP, HRG, HSD17B12, PLG, SERPINC1, SOD3, TPSAB1, TPSB2, TXN	
PDAC	APP, ATP1B3, ATP5A1, C3, C6, CCL24, CD47, CTSG, CXCL2, CYCS, FGFR2, FSTL1, HBEGF, NAV2, PCSK6, SEMA5A, SERPINA6, SERPINE2, SLC2A2, SLC3A2, STEAP4, SYNGR1, VEGFB	

Table 2.4.1: Unique and common HBPs associated with NP, CP and PDAC datasets. These were obtained using IPA's comparison tool.

	NP	**AP**	**CP**	**PDAC**
Nodes	10	8	4	16
Edges	33	24	6	52
Score	3.3	3	1.5	3.25
Node IDs	ITGB1, COL1A1, VTN, ITGA5, FN1, ITGB3, COL1A2, ITGAV, ITGA1, TNC	MMP9, IL8, IL10, **TNF**, **IFNγ**, **VEGFA**, **CCL2, IL6, TGFβ1**	ITGB1, COL1A1, COL1A2, ITGA1	ITGB1, COL1A1, VTN, SERPINE1, ITGA5, FN1, ITGB3, COL1A2, ITGAV, ITGA1, TNC, CLU, SERPINA1, SERPING1, A2M, **APP**

Table 2.4.2: Top clusters in networks of HBPs associated with NP, AP, CP and PDAC. These were identified using 'AllegroMCODE' with Cytoscape 2.8.1. HBPs unique to each dataset are highlighted in bold. The HBPs within the clusters have similar biological functions.

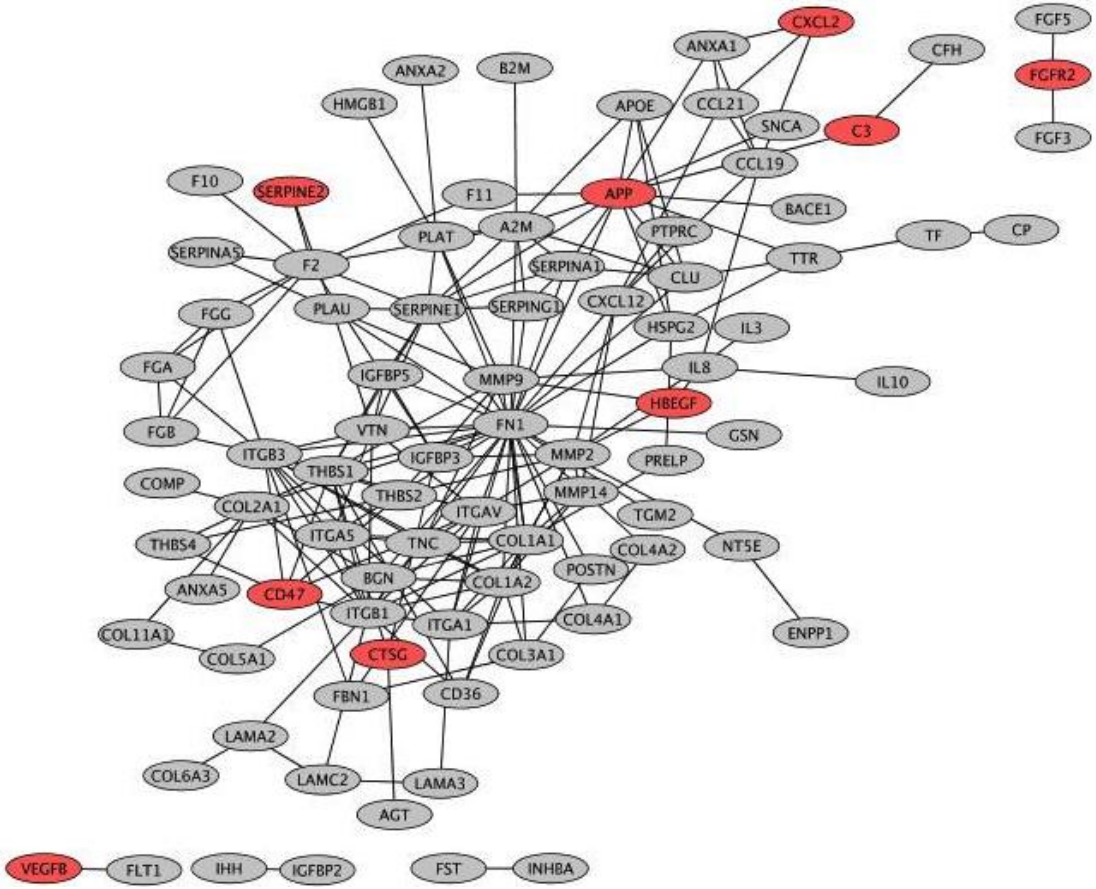

Figure 2.4.1: The heparin-binding putative protein interactome in PDAC constructed using Cytoscape 2.8.1. 'Nodes' coloured red are HBPs unique to the PDAC dataset in the study. Black lines connecting the HBPs denote 'edges' or interactions. As a result of the stringent criteria for selecting interactions, some HBPs from the datasets in our study are notably absent from the interacting networks.

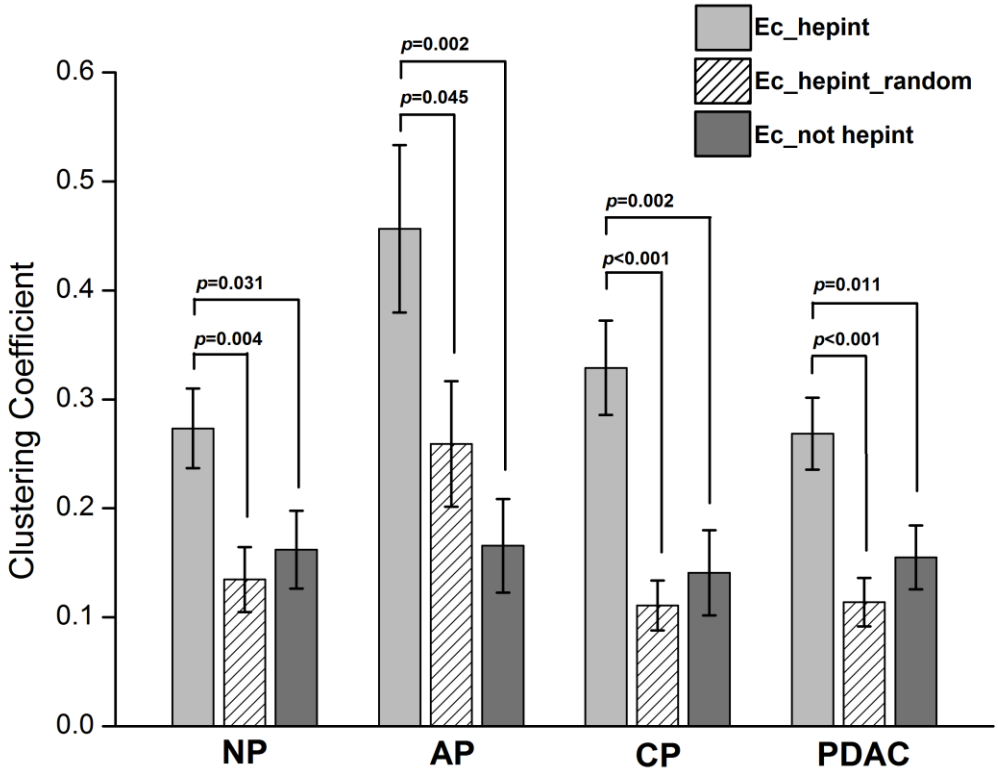

Figure 2.4.2: Comparison of the clustering coefficients. Clustering coefficients were calculated for the pancreatic extracellular heparin binding putative protein interactome (Ec_hepint), the corresponding random network (Ec_hepint_random) and the pancreatic extracellular non heparin-binding putative protein interactome (Ec_not hepint) in NP, AP, CP and PDAC. The Ec_hepint describes a densely interconnected network module in the extracellular space in the pancreas with a significantly higher clustering coefficient ($p<0.05$, independent t-test, SPSS) as compared to those of the random networks and Ec_not hepint in NP, AP, CP and PDAC.

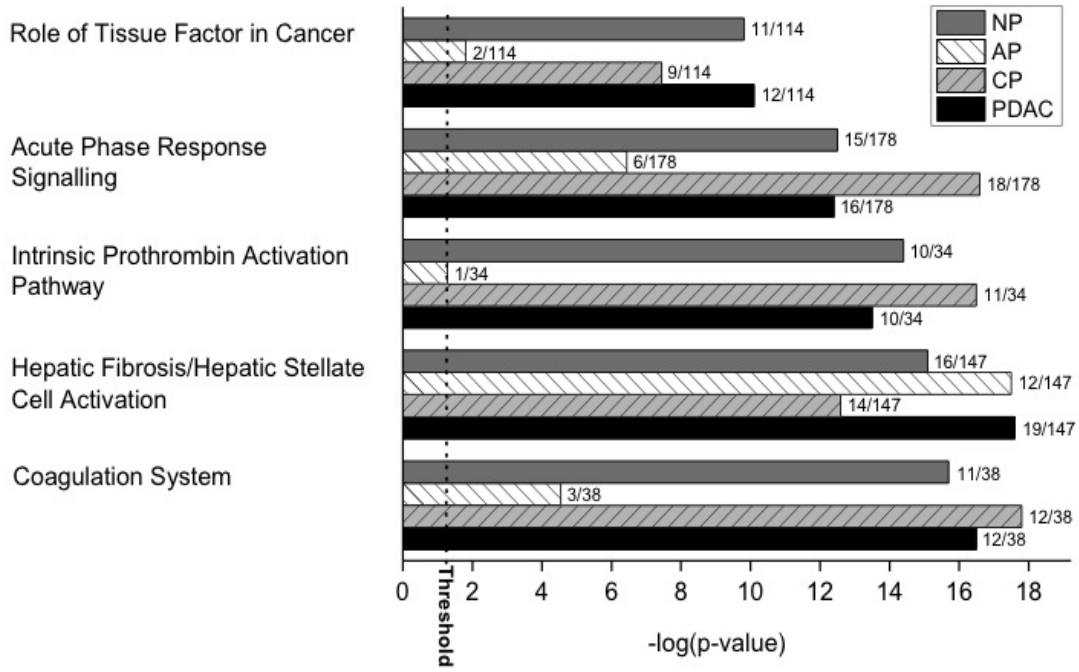

Figure 2.4.3: Comparison of canonical pathways involving HBPs associated with NP, AP, CP and PDAC using IPA. The 'Hepatic Fibrosis / Hepatic Stellate Cell Activation' pathway is an important pathway enriched to the HBP datasets in the normal and diseased pancreas. The ratio of the number of HBPs from a particular dataset to the total number of molecules in a canonical pathway is indicated beside the corresponding bar.

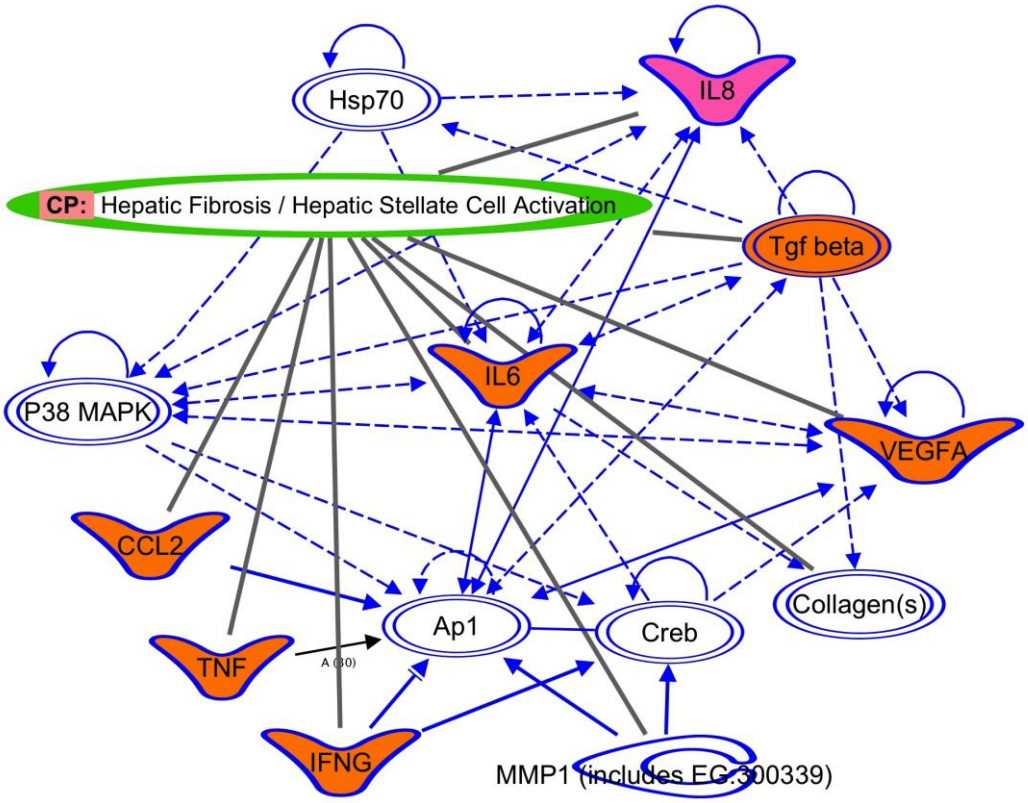

Figure 2.4.4: Top cluster in the AP heparin binding putative protein interactome. The nodes coloured orange are uniquely associated with the AP HBP dataset. IL-8 is not unique to the AP HBP dataset. IL-6, which is a cytokine produced by macrophages and a mediator in the synthesis of acute-phase proteins, forms the hub of this cluster. The top cluster in AP enriched to the 'Hepatic Fibrosis / Hepatic Stellate Cell Activation' canonical pathway (CP).

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Supplementary Table 1: List of HBPs associated with NP

Symbol	Entrez Gene Name
ABP1	amiloride binding protein 1 (amine oxidase (copper-containing))
AGT	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)
AMBP	alpha-1-microglobulin/bikunin precursor
ANXA1	annexin A1
ANXA2	annexin A2
ANXA5	annexin A5
APOE	apolipoprotein E
APOH	apolipoprotein H (beta-2-glycoprotein I)
AQP1	aquaporin 1 (Colton blood group)
ARG1	arginase, liver
ATP2B1	ATPase, Ca++ transporting, plasma membrane 1
B2M	beta-2-microglobulin
BACE1	beta-site APP-cleaving enzyme 1
BGN	biglycan
C4BPA	complement component 4 binding protein, alpha
CCL19	chemokine (C-C motif) ligand 19
CCL21	chemokine (C-C motif) ligand 21
CD36	CD36 molecule (thrombospondin receptor)
CEL	carboxyl ester lipase (bile salt-stimulated lipase)
CFH	complement factor H
CLU	clusterin
COL11A1	collagen, type XI, alpha 1
COL12A1	collagen, type XII, alpha 1
COL1A1	collagen, type I, alpha 1
COL1A2	collagen, type I, alpha 2
COL2A1	collagen, type II, alpha 1
COL3A1	collagen, type III, alpha 1
COL4A1	collagen, type IV, alpha 1
COL4A2	collagen, type IV, alpha 2
COL5A1	collagen, type V, alpha 1
COL6A3	collagen, type VI, alpha 3
COMP	cartilage oligomeric matrix protein
CP	ceruloplasmin (ferroxidase)
CTSB	cathepsin B
CXCL12	chemokine (C-X-C motif) ligand 12
DCC	deleted in colorectal carcinoma
ECE1	endothelin converting enzyme 1
EFNA1	ephrin-A1
ENO1	enolase 1, (alpha)
ENPP1	ectonucleotide pyrophosphatase/phosphodiesterase 1
F2	coagulation factor II (thrombin)
F10	coagulation factor X
F11	coagulation factor XI
FBN1	fibrillin 1
FGA	fibrinogen alpha chain
FGB	fibrinogen beta chain
FGF3	fibroblast growth factor 3
FGF5	fibroblast growth factor 5
FGG	fibrinogen gamma chain
FLT1	fms-related tyrosine kinase 1
FN1	fibronectin 1
FST	follistatin
GJB1	gap junction protein, beta 1, 32kDa

GPNMB	glycoprotein (transmembrane) nmb
GSN	gelsolin
HDGF	hepatoma-derived growth factor
HSPG2	heparan sulfate proteoglycan 2
IGFBP2	insulin-like growth factor binding protein 2, 36kDa
IGFBP3	insulin-like growth factor binding protein 3
IGFBP5	insulin-like growth factor binding protein 5
IHH	Indian hedgehog
IL3	interleukin 3 (colony-stimulating factor, multiple)
IL8	interleukin 8
IL10	interleukin 10
INHBA	inhibin, beta A
ITGA1	integrin, alpha 1
ITGA5	integrin, alpha 5 (fibronectin receptor, alpha polypeptide)
ITGAV	integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)
ITGB1	integrin, beta 1
ITGB3	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)
KAL1	Kallmann syndrome 1 sequence
LAMA2	laminin, alpha 2
LAMA3	laminin, alpha 3
LAMC2	laminin, gamma 2
LTBP1	latent transforming growth factor beta binding protein 1
LTF	lactotransferrin
MDK	midkine (neurite growth-promoting factor 2)
MET	met proto-oncogene (hepatocyte growth factor receptor)
MMP2	matrix metallopeptidase 2
MMP7	matrix metallopeptidase 7 (matrilysin, uterine)
MMP9	matrix metallopeptidase 9
MMP14	matrix metallopeptidase 14 (membrane-inserted)
MYL9	myosin, light chain 9, regulatory
NT5E	5'-nucleotidase, ecto (CD73)
OCLN	occludin
P4HB	prolyl 4-hydroxylase, beta polypeptide
PEBP1	phosphatidylethanolamine binding protein 1
PLAT	plasminogen activator, tissue
PLAU	plasminogen activator, urokinase
POSTN	periostin, osteoblast specific factor
PRDX4	peroxiredoxin 4
PRELP	proline/arginine-rich end leucine-rich repeat protein
PRSS1	protease, serine, 1 (trypsin 1)
PRSS3	protease, serine, 1 (trypsin 1)
PTPRC	protein tyrosine phosphatase, receptor type, C
RPL22	ribosomal protein L22
SERPINA1	serpin peptidase inhibitor, clade A, member 1
SERPINA3	serpin peptidase inhibitor, clade A, member 3
SERPINA5	serpin peptidase inhibitor, clade A, member 5
SERPINE1	serpin peptidase inhibitor, clade E, member 1
SERPING1	serpin peptidase inhibitor, clade G, member 1
SLC39A4	solute carrier family 39, member 4
SLC4A4	solute carrier family 4, sodium bicarbonate cotransporter, member 4
SLPI	secretory leukocyte peptidase inhibitor
SNCA	synuclein, alpha (non A4 component of amyloid precursor)
SOD1	superoxide dismutase 1, soluble
TGM2	transglutaminase 2
THBS1	thrombospondin 1
THBS2	thrombospondin 2
THBS4	thrombospondin 4
TNC	tenascin C

TNFAIP6	tumor necrosis factor, alpha-induced protein 6
TNXB	tenascin XB
TTR	transthyretin
VTN	vitronectin

Supplementary Table 2: List of HBPs associated with AP

Symbol	Entrez Gene Name
A2M	alpha-2-macroglobulin
APOA5	apolipoprotein A-V
APOE	apolipoprotein E
CCL2	chemokine (C-C motif) ligand 2
CTSB	cathepsin B
CX3CL1	chemokine (C-X3-C motif) ligand 1
CXCL10	chemokine (C-X-C motif) ligand 10
HMGB1	high-mobility group box 1
IFNG	interferon, gamma
IL6	interleukin 6 (interferon, beta 2)
IL8	interleukin 8
IL10	interleukin 10
LIPC	lipase, hepatic
LPL	lipoprotein lipase
MBL2	mannose-binding lectin (protein C) 2, soluble
MET	met proto-oncogene (hepatocyte growth factor receptor)
MIF	macrophage migration inhibitory factor (glycosylation-inhibiting factor)
MMP9	matrix metallopeptidase 9
MPO	myeloperoxidase
PDGFB	platelet-derived growth factor beta polypeptide
PON1	paraoxonase 1
PROC	protein C (inactivator of coagulation factors Va and VIIIa)
PRSS1	protease, serine, 1 (trypsin 1)
PRSS3	protease, serine, 1 (trypsin 1)
SELP	selectin P (granule membrane protein 140kDa, antigen CD62)
SERPINA1	serpin peptidase inhibitor, clade A, member 1
SOD1	superoxide dismutase 1, soluble
TF	transferrin
TGFβ1	transforming growth factor, beta 1
TNF	tumor necrosis factor
VEGFA	vascular endothelial growth factor A

Supplementary Table 3: List of HBPs associated with CP

Symbol	Entrez Gene Name
A1BG	alpha-1-B glycoprotein
A2M	alpha-2-macroglobulin
ABP1	amiloride binding protein 1 (amine oxidase (copper-containing))
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1
AGT	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)
AKR1B1	aldo-keto reductase family 1, member B1 (aldose reductase)
ANXA1	annexin A1
ANXA2	annexin A2
ANXA5	annexin A5
APOE	apolipoprotein E
APOH	apolipoprotein H (beta-2-glycoprotein I)
AQP1	aquaporin 1 (Colton blood group)
ARG1	arginase, liver
ATP2B1	ATPase, Ca++ transporting, plasma membrane 1
B2M	beta-2-microglobulin
BGN	biglycan
C9	complement component 9
C4BPA	complement component 4 binding protein, alpha
CCL19	chemokine (C-C motif) ligand 19
CCL21	chemokine (C-C motif) ligand 21
CD36	CD36 molecule (thrombospondin receptor)
CEL	carboxyl ester lipase (bile salt-stimulated lipase)
CFH	complement factor H
CLU	clusterin
COL11A1	collagen, type XI, alpha 1
COL14A1	collagen, type XIV, alpha 1
COL1A1	collagen, type I, alpha 1
COL1A2	collagen, type I, alpha 2
COL2A1	collagen, type II, alpha 1
COL3A1	collagen, type III, alpha 1
COL4A1	collagen, type IV, alpha 1
COL4A2	collagen, type IV, alpha 2
COL5A1	collagen, type V, alpha 1
COL6A3	collagen, type VI, alpha 3
COMP	cartilage oligomeric matrix protein
CP	ceruloplasmin (ferroxidase)
CXCL12	chemokine (C-X-C motif) ligand 12
ECE1	endothelin converting enzyme 1
ENO1	enolase 1, (alpha)
ENPP1	ectonucleotide pyrophosphatase/phosphodiesterase 1
F2	coagulation factor II (thrombin)
F10	coagulation factor X
F11	coagulation factor XI
FBN1	fibrillin 1
FGA	fibrinogen alpha chain
FGB	fibrinogen beta chain
FGF3	fibroblast growth factor 3
FGF5	fibroblast growth factor 5
FGG	fibrinogen gamma chain
FN1	fibronectin 1
FST	follistatin
GJB1	gap junction protein, beta 1, 32kDa
GPNMB	glycoprotein (transmembrane) nmb
GSN	gelsolin
HP	haptoglobin

HRG	histidine-rich glycoprotein
HSD17B12	hydroxysteroid (17-beta) dehydrogenase 12
HSPG2	heparan sulfate proteoglycan 2
IGFBP2	insulin-like growth factor binding protein 2, 36kDa
IGFBP3	insulin-like growth factor binding protein 3
IGFBP5	insulin-like growth factor binding protein 5
IGFBP6	insulin-like growth factor binding protein 6
IHH	Indian hedgehog
IL3	interleukin 3 (colony-stimulating factor, multiple)
IL8	interleukin 8
IL10	interleukin 10
INHBA	inhibin, beta A
ITGA1	integrin, alpha 1
ITGA5	integrin, alpha 5 (fibronectin receptor, alpha polypeptide)
ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide)
LAMA2	laminin, alpha 2
LAMA3	laminin, alpha 3
LAMC2	laminin, gamma 2
LTBP1	latent transforming growth factor beta binding protein 1
LTF	lactotransferrin
MDK	midkine (neurite growth-promoting factor 2)
MIF	macrophage migration inhibitory factor (glycosylation-inhibiting factor)
MMP9	matrix metallopeptidase 9
MYL9	myosin, light chain 9, regulatory
NT5E	5'-nucleotidase, ecto (CD73)
OCLN	occludin
P4HB	prolyl 4-hydroxylase, beta polypeptide
PEBP1	phosphatidylethanolamine binding protein 1
PLAT	plasminogen activator, tissue
PLG	plasminogen
POSTN	periostin, osteoblast specific factor
PRDX4	peroxiredoxin 4
PRELP	proline/arginine-rich end leucine-rich repeat protein
PRSS1	protease, serine, 1 (trypsin 1)
PRSS3	protease, serine, 1 (trypsin 1)
PTPRC	protein tyrosine phosphatase, receptor type, C
RPL22	ribosomal protein L22
SERPINA3	serpin peptidase inhibitor, clade A, member 3
SERPINA5	serpin peptidase inhibitor, clade A, member 5
SERPINC1	serpin peptidase inhibitor, clade C (antithrombin), member 1
SERPINE1	serpin peptidase inhibitor, clade E, member 1
SFRP1	secreted frizzled-related protein 1
SLC39A4	solute carrier family 39 (zinc transporter), member 4
SLPI	secretory leukocyte peptidase inhibitor
SNCA	synuclein, alpha (non A4 component of amyloid precursor)
SOD1	superoxide dismutase 1, soluble
SOD3	superoxide dismutase 3, extracellular
TF	transferrin
THBS1	thrombospondin 1
THBS2	thrombospondin 2
THBS4	thrombospondin 4
TNC	tenascin C
TNXB	tenascin XB
TPSAB1/TPSB2	tryptase alpha/beta 1
TTR	transthyretin
TXN	thioredoxin
VTN	Vitronectin

Supplementary Table 4: List of HBPs associated with PDAC

Symbol	Entrez Gene Name
A2M	alpha-2-macroglobulin
ABP1	amiloride binding protein 1 (amine oxidase (copper-containing))
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1
AGT	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)
ANXA1	annexin A1
ANXA2	annexin A2
ANXA5	annexin A5
APOE	apolipoprotein E
APP	amyloid beta (A4) precursor protein
AQP1	aquaporin 1 (Colton blood group)
ARG1	arginase, liver
ATP1B3	ATPase, Na+/K+ transporting, beta 3 polypeptide
ATP2B1	ATPase, Ca++ transporting, plasma membrane 1
ATP5A1	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1
B2M	beta-2-microglobulin
BACE1	beta-site APP-cleaving enzyme 1
BGN	biglycan
C3	complement component 3
C6	complement component 6
C9	complement component 9
CCL19	chemokine (C-C motif) ligand 19
CCL21	chemokine (C-C motif) ligand 21
CCL24	chemokine (C-C motif) ligand 24
CD36	CD36 molecule (thrombospondin receptor)
CD47	CD47 molecule
CEL	carboxyl ester lipase (bile salt-stimulated lipase)
CFH	complement factor H
CLU	clusterin
COL11A1	collagen, type XI, alpha 1
COL12A1	collagen, type XII, alpha 1
COL1A1	collagen, type I, alpha 1
COL1A2	collagen, type I, alpha 2
COL2A1	collagen, type II, alpha 1
COL3A1	collagen, type III, alpha 1
COL4A1	collagen, type IV, alpha 1
COL4A2	collagen, type IV, alpha 2
COL5A1	collagen, type V, alpha 1
COL6A3	collagen, type VI, alpha 3
COMP	cartilage oligomeric matrix protein
CP	ceruloplasmin (ferroxidase)
CTSG	cathepsin G
CX3CL1	chemokine (C-X3-C motif) ligand 1
CXCL2	chemokine (C-X-C motif) ligand 2
CXCL12	chemokine (C-X-C motif) ligand 12
CYCS	cytochrome c, somatic
DCC	deleted in colorectal carcinoma
ECE1	endothelin converting enzyme 1
EFNA1	ephrin-A1
ENO1	enolase 1, (alpha)
ENPP1	ectonucleotide pyrophosphatase/phosphodiesterase 1
F2	coagulation factor II (thrombin)
F10	coagulation factor X
F11	coagulation factor XI
FBN1	fibrillin 1

FGA	fibrinogen alpha chain
FGB	fibrinogen beta chain
FGF3	fibroblast growth factor 3
FGF5	fibroblast growth factor 5
FGFR2	fibroblast growth factor receptor 2
FGG	fibrinogen gamma chain
FLT1	fms-related tyrosine kinase 1
FN1	fibronectin 1
FST	follistatin
FSTL1	follistatin-like 1
GJB1	gap junction protein, beta 1, 32kDa
GPNMB	glycoprotein (transmembrane) nmb
GSN	gelsolin
HBEGF	heparin-binding EGF-like growth factor
HDGF	hepatoma-derived growth factor
HMGB1	high-mobility group box 1
HSPG2	heparan sulfate proteoglycan 2
IGFBP2	insulin-like growth factor binding protein 2, 36kDa
IGFBP3	insulin-like growth factor binding protein 3
IGFBP5	insulin-like growth factor binding protein 5
IGFBP6	insulin-like growth factor binding protein 6
IHH	Indian hedgehog
IL3	interleukin 3 (colony-stimulating factor, multiple)
IL8	interleukin 8
IL10	interleukin 10
INHBA	inhibin, beta A
ITGA1	integrin, alpha 1
ITGA5	integrin, alpha 5 (fibronectin receptor, alpha polypeptide)
ITGAV	integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)
ITGB1	integrin, beta 1
ITGB3	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)
KAL1	Kallmann syndrome 1 sequence
LAMA2	laminin, alpha 2
LAMA3	laminin, alpha 3
LAMC2	laminin, gamma 2
LTBP1	latent transforming growth factor beta binding protein 1
MDK	midkine (neurite growth-promoting factor 2)
MET	met proto-oncogene (hepatocyte growth factor receptor)
MMP2	matrix metallopeptidase 2
MMP7	matrix metallopeptidase 7 (matrilysin, uterine)
MMP9	matrix metallopeptidase 9
MMP14	matrix metallopeptidase 14 (membrane-inserted)
MYL9	myosin, light chain 9, regulatory
NAV2	neuron navigator 2
NT5E	5'-nucleotidase, ecto (CD73)
OCLN	occludin
P4HB	prolyl 4-hydroxylase, beta polypeptide
PCSK6	proprotein convertase subtilisin/kexin type 6
PEBP1	phosphatidylethanolamine binding protein 1
PLAT	plasminogen activator, tissue
PLAU	plasminogen activator, urokinase
POSTN	periostin, osteoblast specific factor
PRDX4	peroxiredoxin 4
PRELP	proline/arginine-rich end leucine-rich repeat protein
PRSS1	protease, serine, 1 (trypsin 1)
PRSS3	protease, serine, 1 (trypsin 1)
PTPRC	protein tyrosine phosphatase, receptor type, C
RPL22	ribosomal protein L22

SEMA5A	semaphorin 5A
SERPINA1	serpin peptidase inhibitor, clade A, member 1
SERPINA3	serpin peptidase inhibitor, clade A, member 3
SERPINA5	serpin peptidase inhibitor, clade A, member 5
SERPINA6	serpin peptidase inhibitor, clade A, member 6
SERPINE1	serpin peptidase inhibitor, clade E, member 1
SERPINE2	serpin peptidase inhibitor, clade E, member 2
SERPING1	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1
SFRP1	secreted frizzled-related protein 1
SLC2A2	solute carrier family 2 (facilitated glucose transporter), member 2
SLC39A4	solute carrier family 39 (zinc transporter), member 4
SLC3A2	solute carrier family 3, member 2
SLC4A4	solute carrier family 4, sodium bicarbonate cotransporter, member 4
SLPI	secretory leukocyte peptidase inhibitor
SNCA	synuclein, alpha (non A4 component of amyloid precursor)
SOD1	superoxide dismutase 1, soluble
STEAP4	STEAP family member 4
SYNGR1	synaptogyrin 1
TF	transferrin
TGM2	transglutaminase 2
THBS1	thrombospondin 1
THBS2	thrombospondin 2
THBS4	thrombospondin 4
TNC	tenascin C
TNFAIP6	tumor necrosis factor, alpha-induced protein 6
TNXB	tenascin XB
TTR	transthyretin
VEGFB	vascular endothelial growth factor B
VTN	vitronectin

2.5 Drug targets in pancreatic diseases

Pubmatrix, which is a literature-mining tool (Becker et al., 2003), was used to assign functional relevance to HBPs in the major pancreatic diseases. Lists of HBPs, identified for further investigation, along with MeSH (Medical Subject Headings) such as "drugs" and "drugs in acute pancreatitis" were searched in Pubmatrix (Table 2.5.1). The search revealed that a number of the HBPs have been investigated in drug development in AP, some of which have been listed (Table 2.5.2). Similar searches were carried out in CP and PDAC, using Pubmatrix. FGFR2, which was identified as a potential PSC-specific drug target in PDAC in the *in silico* study described Section 2.3 of this chapter, is being investigated in the treatment of other cancers (Byron and Pollock, 2009).

HBPs	Drugs	Drugs in acute pancreatitis
APOA5	14	0
CCL2	517	0
CXCL10	122	0
IFNγ	254	0
IL6	288	1
IL10	1040	16
LIPC	10	0
LPL	231	2
MBL	84	0
MPO	689	10
PDGFβ	2	0
PON1	92	0
PROC	9396	1
SELP	25	0
TGFβ1	119	1
TNF	9601	37
VEGFA	537	0

Table 2.5.1: *HBPs as potential drug targets in acute pancreatitis.* The literature-mining tool "Pubmatrix" was searched using HBPs shortlisted as potential drug targets and MeSH terms "drugs" and "drugs in acute pancreatitis".

A number of drugs against FGFR2 (Table 2.5.3) are in various stages of investigation. The top canonical pathways (Fig. 2.5) were also used to identify potential pathway-specific drug targets.

HBPs	Drugs in acute pancreatitis
IL10	FTY720, Emblica offiinalis
LPL	Alipogene tiparvovec
MPO	Ethyl pyruvate, Ligustrazine
TGFβ1	Emodin and Sandostatin
TNF	ND-07, Flavocoxid

Table 2.5.2: *Drugs against HBPs in acute pancreatitis.* A number of drugs against HBPs have been investigated in acute pancreatitis.

Drugs against FGFR2	Cancers
TKI-258	Gastrointestinal Stromal tumour
AZD2171	Renal cell carcinoma, breast cancer
BIFB-1120	Non-small cell lung cancer
Brivanib	Metastatic solid tumours

Table 2.5.3: *Drugs against FGFR2 in the treatment of cancer*. A number of drugs against FGFR2 are being investigated in clinical trials in various cancers. FGFR2 has been identified as potential drug target against pancreatic stellate cells in the treatment of pancreatic ductal adenocarcinoma.

2.6 Discussion

This work addressed the hypothesis that the high level of connectivity of HBPs discovered on a genome-wide basis would also be true at the level of an organ (Chapter 1; 1.9). It showed that HBPs constitute an important extracellular sub-proteome within the pancreas and is likely to provide a rich repository of potential biomarkers and drug targets. However, it is based on two assumptions. The first is that the current list of HBPs (Ori et al., 2011) is reasonably comprehensive. The latter study used a combination

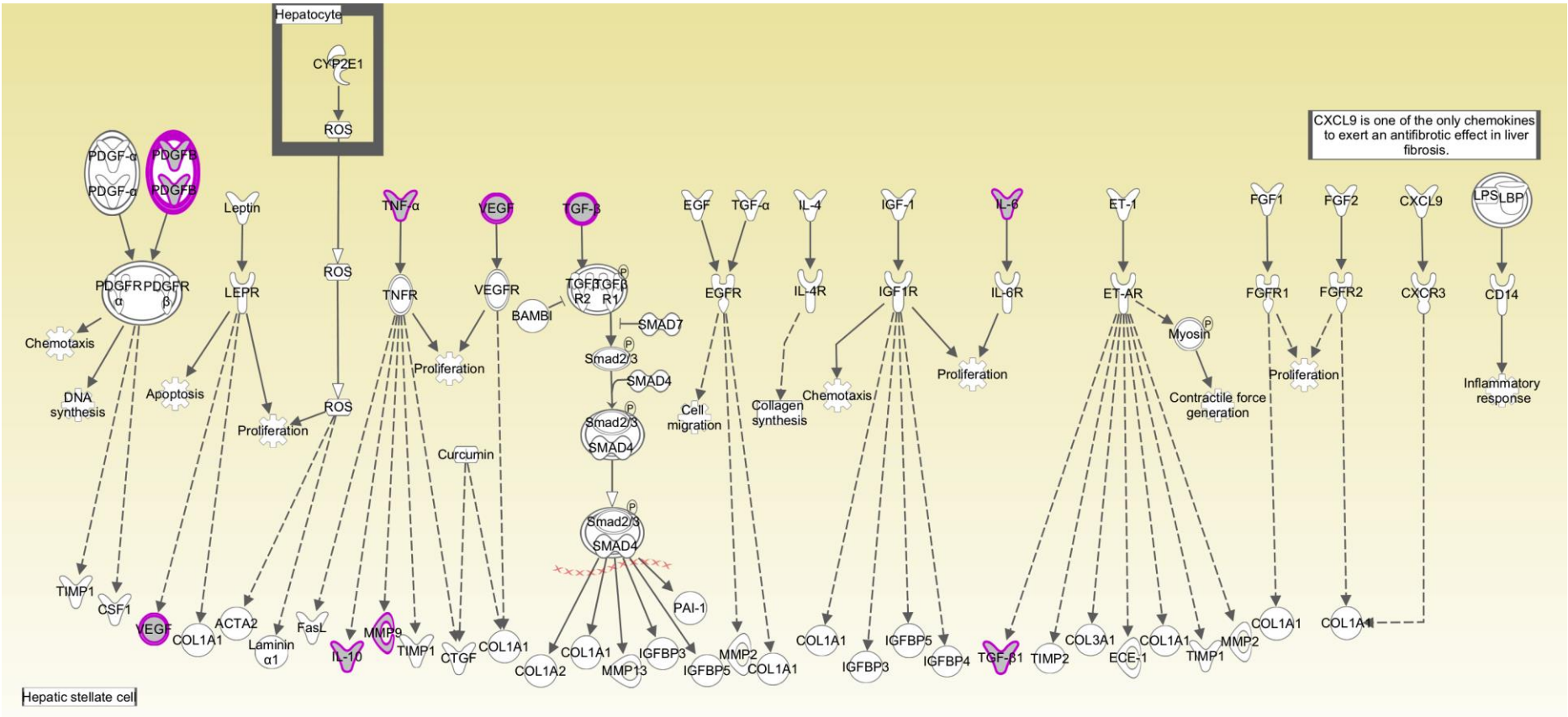

Figure 2.5: *Top canonical pathway in AP*. 'Hepatic Fibrosis / Hepatic Stellate Cell Activation' canonical pathway (CP) was the top canonical pathway enriched to the AP HBP dataset, using Ingenuity Pathways Analysis. The nodes outlined in magenta are HBPs from the AP dataset. The top cluster in the AP HBP dataset enriches to this pathway.

of literature curation and affinity mass spectrometry of HBPs derived from rat liver. There is no indication of the depth of the affinity proteomics, that is how sensitive the measurements were and so how comprehensive they might be. Moreover, one might expect differences between liver and pancreas, since they have different endocrine and exocrine functions. In addition, there has, as yet, been no follow up investigations in rat liver (the tissue used as a source of HBPs) or other tissues to determine how comprehensive the list actually is. The second assumption is that mRNA is reasonably representative of protein. It is established that the correlation is not that strong. For example, translation efficiency can vary considerably between mRNAs (Schwanhausser et al., 2011), and the methods used to measure mRNA levels can reduce the correlation further (Mournetas et al., 2014). The need to perform a meta analysis across different experimental measurements of mRNA, discussed in Section 2.3, may reduce further such correlation,

Thus, while the analysis of HBPs in pancreatic diseases supports the hypothesis that HBPs may provide the key to understanding how cell communication is altered in disease, the evidence is indirect. Heparin binding, which defines the regulatory importance of HBPs, may be exploited by a direct proteomic approach using heparin-affinity chromatography on tissue or a body fluid. This approach, first used in (Ori et al., 2011), will be used in Chapter 3 to identify HBPs in the normal mouse pancreas and in a mouse model of AP. This approach will test directly the hypothesis that HBPs are central to one pancreatic disease. It will also determine the extent to which the above assumptions hold. The systems biology approach

outlined in Section 2.2 and applied in Section 2.3 will provide the means to analyse the data.

Chapter 3 – Heparin binding proteins in NP and AP

3.1 Introduction

Individual studies have demonstrated a variety of mechanisms whereby binding to HS regulates the function of extracellular proteins. However, only recently has it been demonstrated on a global scale that, of the extracellular proteins, HBPs form a highly interconnected network that is functionally linked to physiological and pathological process in more complex multicellular organisms (Ori et al., 2011). Importantly from the perspective of pancreatic diseases, HBPs, because they bind to heparin and are extracellular, represent easily accessible new targets for the development of biomarkers and drugs. We have shown that HBPs are indeed a key subclass of extracellular proteins, whose pattern of expression differs in the healthy and diseased pancreas (Chapter 3 and (Nunes et al., 2013)). However, this work has important weaknesses. mRNA expression was by necessity used as a proxy for that of protein, but this is known to not always be the case (Schwanhausser et al., 2013, Li et al., 2014). Therefore, a proteomic analysis of HBPs in normal mouse pancreas and in the caerulein-induced mouse model of acute pancreatitis was undertaken, with the aim of identifying HBPs. This would, therefore, allow the hypothesis elaborated in Section 1.9, that HBPs form highly connected regulatory modules in the cell microenvironment and are an important source of potential new biomarkers and drug targets in pancreatic diseases to be tested directly.

The proteomics analysis will be submitted as a manuscript to the Journal of Biological Chemistry (3.5), which describes the bulk of the data. The mass spectrometry proteomics data has been uploaded into PRIDE database (http://www.ebi.ac.uk/pride/archive/)(Vizcaino et al., 2013), via its partner repository, the ProteomeXchange Consortium (Vizcaino et al., 2014), with the dataset identifier PXD001950. Additional information relating to methods are provided in Sections 3.2-3.4) and further discussion in Section 3.6).

3.2 Methodology

Studies were conducted in compliance with UK Home Office regulations, and with the Institutional ethical review processes of the University of Liverpool. The isolation of plasma membrane was performed as in Ori *et al.,* 2011 (Ori et al., 2011) The workflow to isolate and detect the plasma membrane fraction is depicted below (Fig. 3.1). Pancreases were obtained from 6-8 week old male adult CD1 mice (weight range, 24-30 g) with normal pancreas (NP) or experimental acute pancreatitis (AP). The pancreatic tissue was homogenized and then subjected to sequential steps of centrifugation to obtain the various subcellular fractions. The supernatant obtained after removal of the nuclear, mitochondrial and cytosolic fractions was subjected to ultracentrifugation to obtain a microsomal pellet, which contained ER membranes and plasma membranes. This was then subjected to differential ultracentrifugation on a sucrose gradient. The fractions with the highest caveolin-1 signal intensity on Western Blot, using a polyclonal

antibody against caveolin-1, were pooled together to obtain the plasma membrane fraction. This was then subjected to heparin chromatography to obtain the heparin-bound fraction, which was prepared for mass spectrometry (MS). Detailed description of isolation of the plasma membrane fraction, the sample preparation for MS and the MS analysis are outlined in the manuscript attached later in this chapter (3.5).

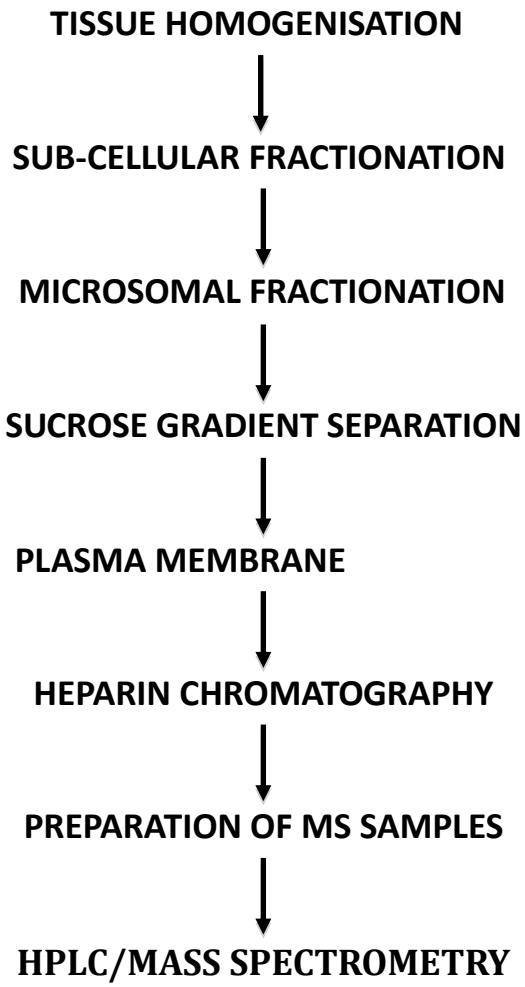

Figure 3.1: *A schematic representation of the various steps involved in extracting HBPs from mouse pancreases*

3.2.1 Method modifications

Some of the modifications to the methods described by Ori *et al*., 2011 (reference) are described in the sections that follow.

3.2.1.1 Measurement of protein concentration

The BCA (bicinhoninic acid) assay (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific, U.K.) was used instead of the Bradford assay to measure protein concentration at all steps, as it was compatible with most detergents including Triton-X-100.

3.2.1.2 Heparin affinity chromatography

Isolation of HBPs using heparin affinity chromatography and their separation into 3 fractions depending on their affinity was attempted using a reverse step gradient on a heparin column, as described by Ori *et al*., 2011 (reference). In order to preserve precious samples of pancreas, lung tissue from mice with caerulein induce acute pancreatitis was used. The high affinity fraction (H) was obtained by adjusting the resuspended pellets to 0.6 M NaCl and 1 % (v/v) Triton X-100 and centrifuged for 5 min at 4,000 x g to remove any insoluble material. The supernatant was applied to a 1 ml Hi-Trap heparin column (GE Healthcare Life Sciences) equilibrated with buffer WH (0.6 M NaCl, 13.7 mM Na_2HPO_4, 6.3 mM NaH_2PO_4, 0.1 % (v/v) Triton X-100, pH 7.2). After loading, the column was extensively washed with buffer WH until the absorbance at 280 nm reached the baseline. Bound proteins (fraction H) were then eluted with a one column volume of buffer E (2 M NaCl, 13.7 mM Na2HPO4, 6.3 mM NaH2PO4, 0.1 % (v/v) Triton X-100, pH 7.2). The unbound fraction from the 0.6 M NaCl load and wash was then

diluted to 0.4 M NaCl using phosphate buffer (13.7 mM Na_2HPO_4, 6.3 mM NaH_2PO_4, pH 7.2) and reapplied to the heparin column equilibrated in buffer WM (0.4 M NaCl, 13.7 mM Na_2HPO_4, 6.3 mM NaH_2PO_4, 0.1 % (v/v) Triton X-100, pH 7.2). After extensive washing with buffer WM, the medium affinity fraction (M) was eluted with 2 M NaCl, as described above.

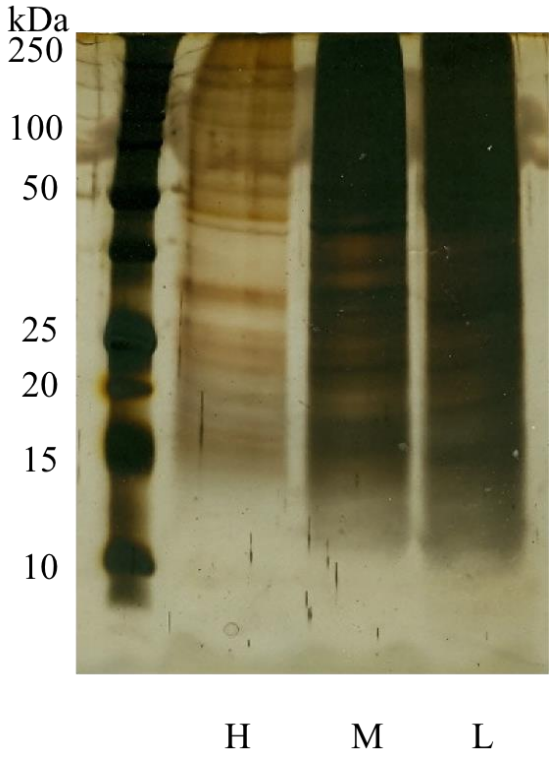

Figure 3.2.1: *Silver stained SDS-PAGE gels of the high affinity (H), medium affinity (M) and low affinity (L) heparin-bound plasma membrane fractions*. Lung tissue from mice with caerulein-induce acute pancreatitis was used in the above experiments.

The unbound fraction following elution of M was diluted to 0.15 M NaCl in the same phosphate buffer, reapplied to the heparin column equilibrated in buffer WL (0.15 M NaCl, 13.7 mM Na_2HPO_4, 6.3 mM NaH_2PO_4, 0.1 % (v/v) Triton X-100, pH 7.2) and eluted (fraction L) after extensive washing, as above. The SDS-PAGE gels stained with silver nitrate (Fig. 3.2.1) showed that the low affinity fraction using buffer WL included bands present in the other

fractions. Though the identity of these polypeptides was not established, this is consistent with what was observed previously, that is the three fractions contain very substantially overlapping proteins (Ori et al 2011). Thus, little information is gained by producing fractions eluted with

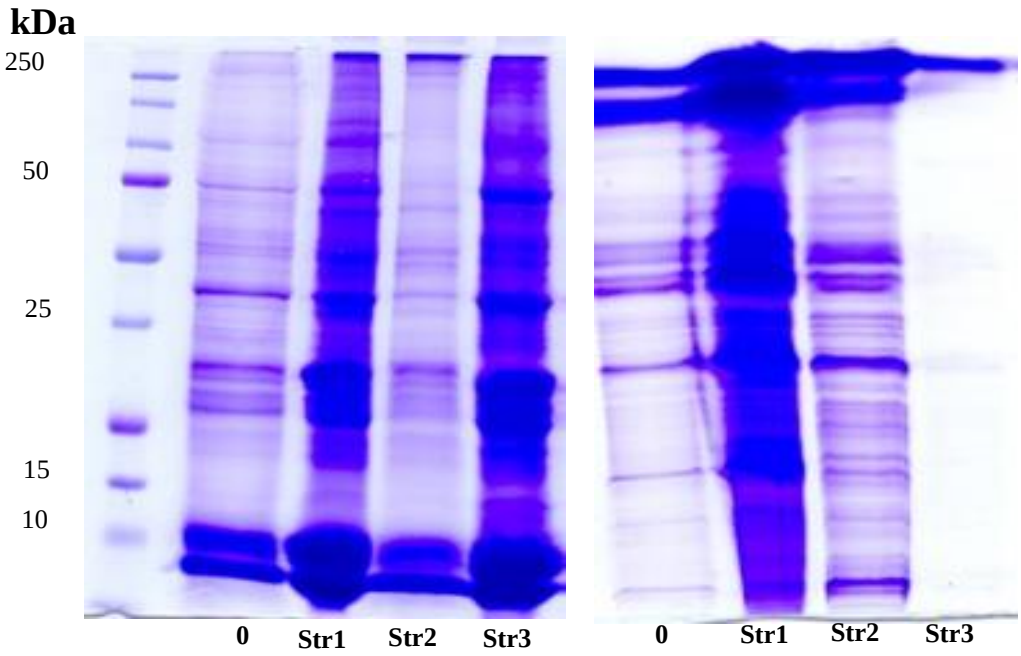

Figure 3.2.2: *Coomassie stained SDS-PAGE gels of various fractions (Str1, Str2, Str3) of cytosolic proteins from pancreatic tissue post-adsorption using StrataClean Resin™ with (A) and without 0.1% (v/v) Triton X-100 (B).* The experiment showed that Triton X-100 was affecting the adsorption of protein onto the StrataClean Resin.

different concentrations of NaCl, while such a procedure will reduce the amount of protein in each fraction. Hence a single buffer (WL) was used to isolate the heparin bound fraction from the pancreas, so as to increase the amount of protein for analysis.

3.2.1.3 Processing of the heparin-bound plasma membrane fraction

Triton-X-100 is a very effective detergent for solubilising membranes, but it is polydisperse and not compatible with mass spectrometry. StrataClean Resin (Stratagene, Hycor Biomedical Ltd., Edinburgh, U.K.) was investigated as a means to process the heparin-bound plasma membrane fraction and remove Triton-X-100. The maximum binding capacity of 10 µL of StrataClean resin is reported to be 100 µg of protein by the manufacturer. Ten µL of premixed StrataClean resin was added to 1 ml of protein solution. This was then vortexed for 1 minute. The protein-bound StrataClean resin (Str1) was collected by centrifugation at 2000 rpm for 1 minute. The supernatant (S1) was removed and stored. To the pelleted protein-resin mixture, 10 µL of 2X SDS-PAGE

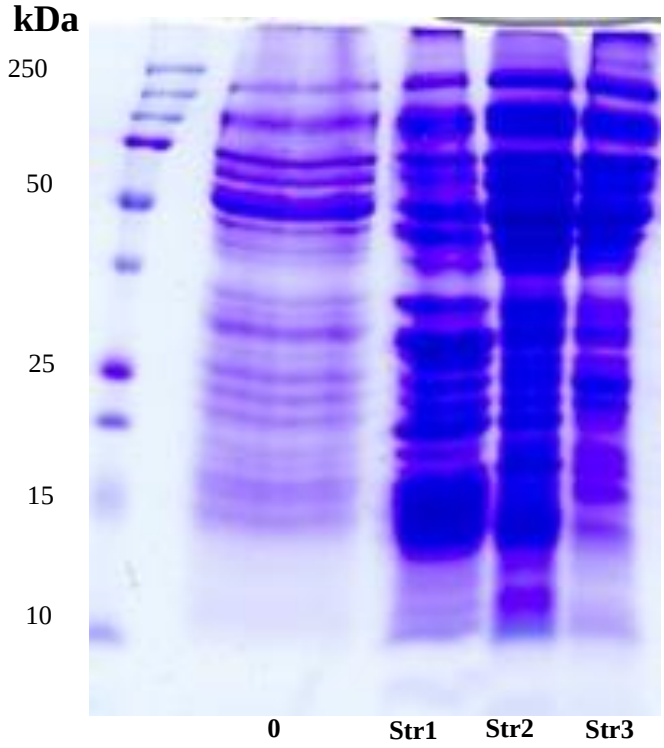

Figure 3.2.3: *Coomassie stained SDS-PAGE gels of various fractions (Str1, Str2, Str3) of whole cell lysate from pancreatic tissue post-adsorption using StrataClean Resin™ with 0.1% (v/v) Triton X-100.* The experiment showed that Triton X-100 was affecting the adsorption of protein onto the StrataClean Resin.

protein-loading buffer was added and this was applied onto an SDS-PAGE gel. The supernatant (S1) was processed as above to obtain Str2. Similarly the supernatant from processing Str2 was processed to obtain Str3. Adsorption using samples of protein with and without 0.1% (v/v) Triton X-100 was investigated (Fig. 3.2.2). Whole cell lysate (Fig.3.2.3) and heparin-bound plasma membrane fractions (Fig.3.2.4) were also used to investigate the use of StrataClean.

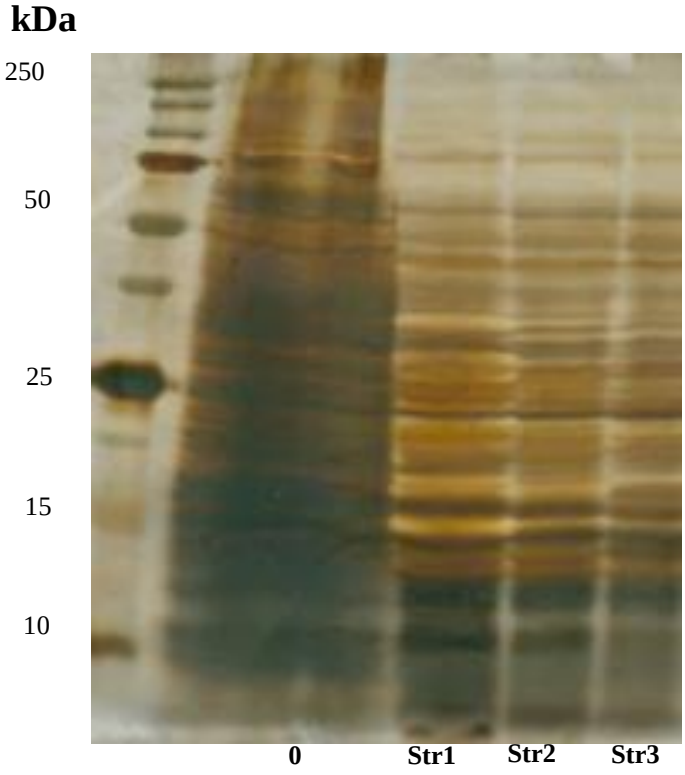

Figure 3.2.4: *Silver nitrate stained SDS-PAGE gels of various fractions (Str1, Str2, Str3) of heparin-bound plasma membrane fraction from pancreatic tissue post-adsorption using StrataClean Resin™ with 0.1% (/v) Triton X-100.* The experiment showed that Triton X-100 was affecting the adsorption of protein onto the StrataClean Resin.

Since Triton-X-100 was found to interfere with protein binding to Strataclean resin and so causing loss of sample, TCA precipitation was

investigated as an alternative. This was found to be satisfactory for processing the samples post-heparin affinity chromatography (Fig. 3.2.5). To avoid wasting experimental samples the correlation of pellet size post-TCA precipitation was done using BSA (Bovine Serum Albumin, Sigma Aldrich UK). Varying concentrations of BSA were precipitated using TCA as described earlier. The pellets were then washed 5 times with 5% (w/v) TCA and freeze-dried overnight. They were then washed with diethyl ether to remove the excess TCA and centrifuged at 5000 rpm. The supernatant was removed and the diethyl ether wash was repeated 2 more times. The final pellets were then left to dry in the fume hood.

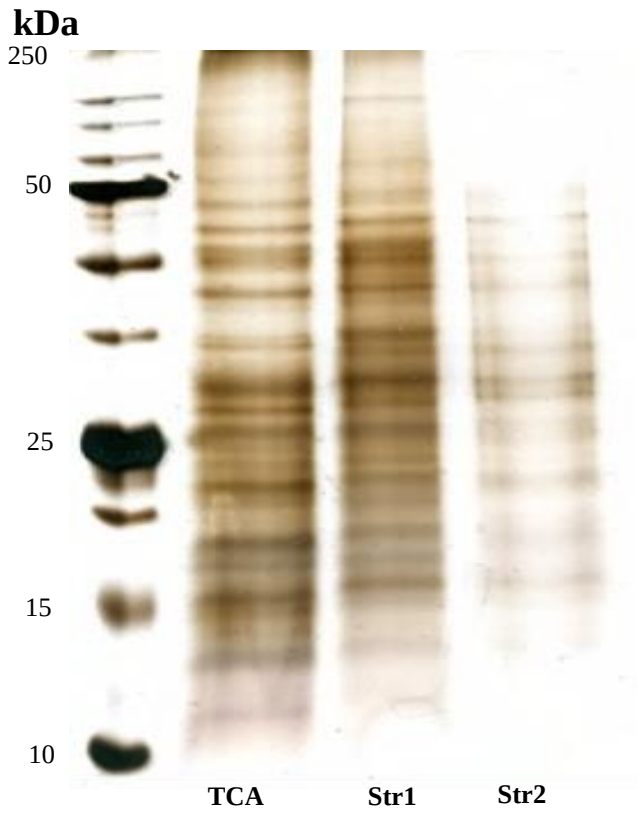

Figure 3.2.5: *Silver nitrate stained SDS-PAGE gels of the heparin-bound plasma membrane fraction from pancreas in 0.1% (v/v) Triton X-100 post-TCA precipitation and post-adsorption using StrataClean Resin*^{TM}. The experiment showed that TCA precipitation was more effective in processing the samples as compared to StrataClean Resin (Str1 and Str2).

The pellets were then stained with Coomasie Blue (Fig. 3.2.6) to obtain a visual correlation between pellet size and protein concentration.

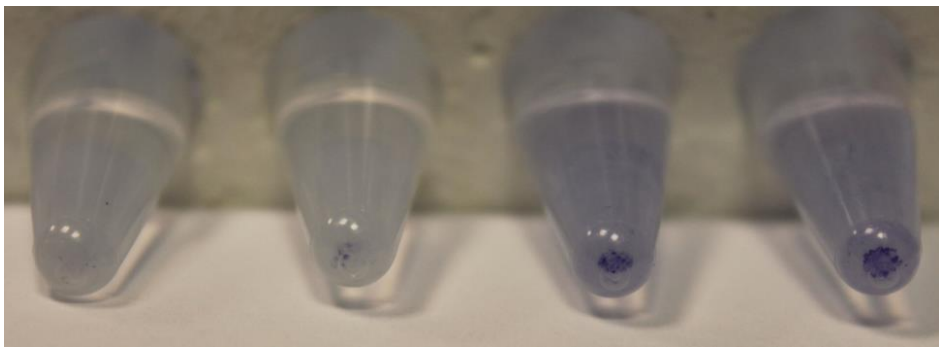

Figure 3.2.6: *Coomasie Blue stained pellets of BSA post-TCA precipitation.* This experiment provides an approximate correlation between protein concentration and size of the pellet.

3.3 Bioinformatics pipeline

Extracellular proteins were identified using a combination of bioinformatics tools, in order to achieve the widest and most accurate coverage. SignalP 4.1, which predicts the presence of a secretory signal peptide, was used to identify extracellular proteins (Petersen et al., 2011) with Phobius, which is a combined transmembrane topology and signal peptide prediction tool, to obtain a wider coverage for extracellular protein identification (Kall et al., 2007).

A third tool, namely, SecretomeP 2.0, which produces *ab initio* predictions of protein secretion not based on a secretory signal peptide (Bendtsen et al., 2004) and a fourth tool based on ontology, Ingenuity Pathways Analysis (IPA), was used to identify extracellular and plasma membrane proteins. The HBPs that were not identified using SignalP, Phobius, or SecretomeP, but which were identified using IPA were further investigated using a manual approach. Each candidate HBP was examined using UniProtKB for

the presence of an extracellular signature. A search in Pubmed, using the terms “extracellular” or “secreted” was performed to investigate those proteins that still had an ambiguous subcellular location to identify at least one publication demonstrating an unequivocal plasma membrane and or extracellular localisation. Finally, the outputs of the various approaches were merged to obtain the final list of HBPs in NP and AP.

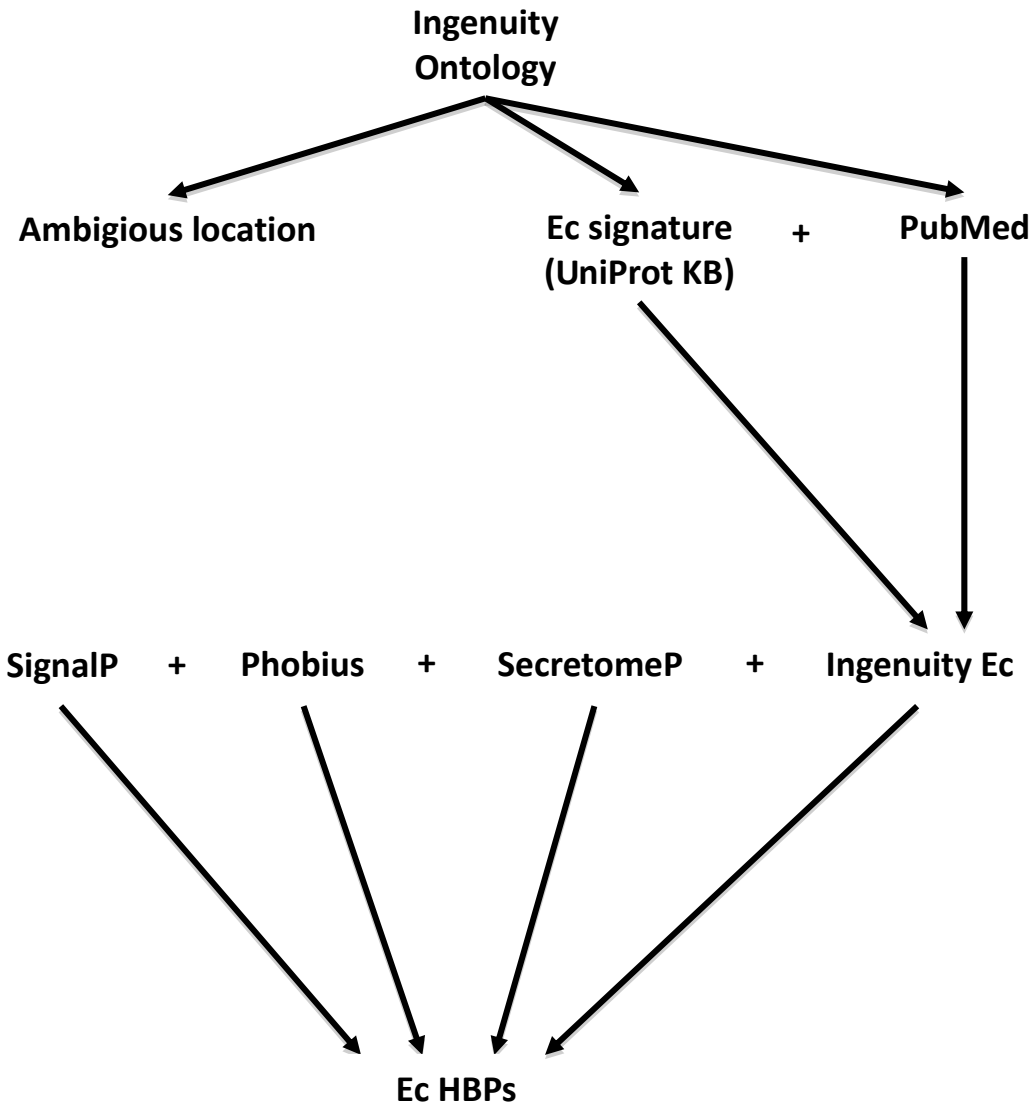

Figure 3.3: *Bioinformatics pipeline used to identify HBPs (Ec =extracellular).* A combination of various bioinformatics tools and manual curation was used to identify HBPs.

3.4 Identification of potential biomarkers and drug targets in AP

Label-free quantification was performed following the "Top3" methodology (Silva et al., 2006) by spiking the sample prior to analysis with an internal standard of 50 fmol yeast alcohol dehydrogenase digest (Uniprot P00330, Waters). Proteins were annotated as differentially expressed if they achieved a FDR corrected q value of 1%. The Bonferroni correction was used to increase the stringency and further reduce the number of false positives for the purpose of biomarker identification (Ting et al., 2009). Canonical pathways and cluster analysis were used in conjunction with differential expression as tools to identify potential functional biomarkers and drug targets.

3.5 Manuscript

The heparin-binding proteome in normal pancreas and murine experimental acute pancreatitis.

Nunes QM, Brownridge PJ, Sun C, Li Y, Huang W, Rigden DJ, Beynon RJ, Sutton R, Fernig DG.

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Quentin M. Nunes performed the analyses, wrote the paper

Philip J. Brownidge performed the mass spectrometry, aided with the analyses, edited the paper

Changye Sun aided with the analyses, edited the paper

Yong Li aided with the analyses, edited the paper

Wei Huang aided with the analyses, edited the paper

Daniel J. Rigden, aided with the analyses, edited the paper

Robert J Beynon aided with the design of the mass spectrometry experiments and analyses, edited the paper

Robert Sutton conceived the study, edited the paper

David G. Fernig conceived the study, co-wrote the paper

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The heparin-binding proteome in nomal pancreas and murine experimental acute pancreatitis.

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Running title: Heparin-binding proteins in acute pancreatitis.

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3.5.1 Capsule

Background: Extracellular heparin-binding proteins (HBPs) are key regulators of cell communication.

Results: HBPs in normal mouse pancreas (NP) and in a mouse model of acute pancreatitis (AP), including 460 new ones, were identified.

Conclusion: The HBPs form highly interconnected protein-protein interaction networks describing cell communication pathways in NP and in AP.

Significance: HBPs are a source of potential biomarkers and of drug targets in AP and are accessible by virtue of their extracellular location and heparin binding property.

3.5.2 Abstract

Acute pancreatitis (AP) is an acute inflammation of the pancreas, mainly caused by gallstones and alcohol, and driven by changes in communication between cells. Heparin-binding proteins (HBPs) of the plasma membrane and extracellular matrix play a central role in cell communication. Therefore, we used heparin affinity proteomics to identify the extracellular HBPs in mouse normal pancreas (NP) and in a caerulein mouse model of AP. Many new HBPs (460) were discovered more than doubling their total number to 883. A number of the new HBPs are proteins with well-characterised intracellular functions, e.g. NDUFS4, NDUFS6, but which also have a documented extracellular presence with potential 'moonlighting' roles. The HBPs

form highly interconnected protein-protein interaction networks in both NP and AP, as well as globally. Thus, HBPs may represent the most interconnected set of extracellular proteins and so those with the greatest regulatory potential. HBPs in NP are associated with biological functions such as molecular transport, cellular movement and tissue architecture that underlie pancreatic homeostasis. However, in AP HBPs are additionally associated with processes such as acute phase response signalling, complement system and mitochondrial dysfunction. By virtue of their extracellular location and heparin binding property, HBPs are easily accessible and are potential biomarkers and drug targets in AP.

3.5.3 Introduction

The pancreas develops from endodermal cells in the foregut and has important exocrine and endocrine functions (Zaret and Grompe, 2008). Acute pancreatitis (AP) is acute inflammation of the pancreas and is mainly caused by gallstones and alcohol (Pandol et al., 2007). It is the leading cause for hospitalization in the United States and has significant quality of life implications for the patient and cost implications health systems (Wu and Banks, 2013). Although most episodes of AP are mild and self-limiting, the severe form of the disease, accompanied by a systemic inflammatory response syndrome and multi-organ failure, is associated with a high mortality. At a cellular level disruption in calcium homeostasis and signaling, and mitochondrial dysfunction have been implicated in the

pathogenesis of AP (Gerasimenko et al., 2014, Mukherjee et al., 2008). The clinical characteristics of AP suggest that an important molecular component is change in cell communication within the pancreas and, in severe AP, systemically between the pancreas and other organs. Cell communication occurs through the medium of the extracellular matrix, with cell receptors responsible for generating the cellular response.

The importance of extracellular proteins in mediating communication between cells in multicellular organisms is underscored by the demonstration that the increase in complexity of multicellular organisms is accompanied by an expansion and increase in complexity of extracellular proteins and their complexity (Vogel and Chothia, 2006). A key non-protein component of the extracellular space is the glycosaminoglycan heparan sulfate (HS), because it binds and regulates the activity of a large number of extracellular proteins involved in cell communication (Xu and Esko, 2014, Ori et al., 2008). HS is formed of linear repeats of a characteristic disaccharide of 1,4 linked uronic acid (α-L-iduronate, IdoA, or β-D-glucuronate, GlcA) and α-D-glucosamine (GlcN), with variable O-sulfation of C2 on the uronic acid, of C3 and C6 on the glucosamine; the glucosamine being either N-acetylated or N-sulfated (Xu and Esko, 2014, Ori et al., 2008). These modifications are hierarchical (Xu and Esko, 2014), resulting in chains with a distinct domain structure (Murphy et al., 2004); HBPs bind to the sulfated domains and their flanking transition domains. HS chains are attached to proteins to form proteoglycans (HSPGs), with the core proteins serving in part to direct chains to particular extracellular locations (Uniewicz et al., 2012, Xu and Esko, 2014) Heparin is often used as a proxy

for the sulfated domains of HS, though it is more homogenous and sulfated than these (Ori et al., 2008, Xu and Esko, 2014).

Many individual studies have demonstrated a variety of mechanisms whereby binding to HS regulates the function of extracellular proteins, but only recently has this been analysed on a global scale (Ori et al., 2011). This work demonstrated that of the extracellular proteins, the HBPs form a highly interconnected functional network through protein-protein interactions that are functionally linked to physiological and pathological process in more complex multicellular organisms. This and other work identify HBPs as important signalling molecules in the cellular microenvironment that influence fundamental biological processes in development, homeostasis and disease (Ori et al., 2011, Malavaki et al., 2011, Xu and Esko, 2014).

We previously used mRNA expression data as a proxy for protein, to test the hypothesis that HBPs, which are demonstrably functionally important at a genome-wide level (Ori et al., 2011), are equally important in the context of a single organ, the pancreas, and its associated digestive diseases (Nunes et al., 2013). This work showed that HBPs are indeed a key subclass of extracellular proteins, whose pattern of expression differs in the healthy and diseased pancreas. Importantly from the perspective of pancreatic diseases, HBPs, because they bind to heparin and are extracellular, represent easily accessible new targets for the development of biomarkers and drugs. However, this work has important weaknesses. mRNA expression was by necessity used as a proxy for that of protein, but this is known to not always be the case (Schwanhausser et al., 2013, Li et al.,

2014). Moreover, the use of mRNA assumes that the existing set of 435 HBPs (Ori et al., 2011) is reasonably representative of those expressed in pancreas and that this organ does not express substantial, novel HBPs.

We have, therefore, undertaken a proteomic analysis of HBPs in normal mouse pancreas and in the caerulein-induced mouse model of acute pancreatitis. A large number of HBPs were identified, more than doubling their number to 883. These HBPs are highly interconnected in NP, AP and globally. They may represent the most interconnected set of extracellular proteins, and, therefore, those with the greatest regulatory potential. Non-canonical extracellular HBPs such as NDUFS4, NDUFS6, NDUFS7, NDUFS8, NDUFA9 and NDUFA10 were found to be under-expressed in AP compared to NP, using label-free quantification. These may have potential moonlighting roles not previously known. HBPs are functionally important in NP and AP and being accessible, by virtue of their extracellular location and heparin binding property, are potential biomarkers and drug targets in AP.

3.5.4 Experimental procedures

3.5.4.1 Pancreatic murine models

CD1 mice were used in all experiments as they are genetically heterogeneous and more likely to represent the human population (Festing, 2010). Pancreases were obtained from 6-8 week old male adult CD1 mice (weight range, 24-30 g) with normal pancreas (NP) or experimental acute pancreatitis (AP). In order to induce experimental acute

pancreatitis, CD1 mice were fasted for 12 hours before each experiment, following which they were administered seven hourly intraperitoneal injections of caerulein (50 μg /kg; Sigma Chemical Co., St. Louis, MO) dissolved in 0.9% (w/v) saline (Braun Medical Ltd., Aylesbury, England). Pancreatitis was confirmed in the experimental mice 24 hours after the first intraperitoneal injection. All mice (NP and AP) were euthanized by cervical dislocation. Studies were conducted in compliance with UK Home Office regulations, and with the Institutional ethical review processes of the University of Liverpool.

A blood sample was collected for serum amylase determination. Serum amylase was tested in the Clinical Biochemistry Department in Royal Liverpool University Hospital using a kinetic method. A sliver of pancreatic tissue from each of the pancreases was fixed in formalin for H&E staining and histological examination. The pancreases were removed, weighed, pooled together and stored in buffer H (10 mM HEPES pH 7.5, 5 mM $MgCl_2$, 25 mM KCl, 0.25 M sucrose supplemented with Complete™ protease inhibitors cocktail, Roche Products Ltd, Welwyn Garden City, UK) at 4°C. Complete™ protease inhibitor was used in all experiments as it has been shown to be particularly effective in experiments involving pancreatic tissue (Wandschneider et al., 2001). Sixteen CD1 mice were used for each HBP isolation experiment. Each experiment was performed thrice for NP and AP.

3.5.4.2 Isolation of a plasma membrane enriched fraction

The isolation of the plasma membrane from murine pancreases was performed as in Ori *et al*., 2011, with a few minor modifications (Ori et al., 2011). All the steps were performed on ice or at 4°C. Briefly, mouse pancreases were minced and homogenised with buffer H using a 30 mL Potter-Elvehjem homogeniser (30-40 strokes). Subcellular fractionation was performed using sequential steps of centrifugation. The homogenate was centrifuged for 20 min at 1,000 g in a Sorvall centrifuge (SS-34 rotor) (DuPont UK, Stevenage, UK). The pellet was resuspended with buffer H, homogenised and centrifuged at the same speed. The two supernatants were then combined (S1) and centrifuged for 20 min at 25,000 g. This supernatant was transferred to a fresh tube and the centrifugation repeated. The final supernatant (S2) was centrifuged for 45 min at 135,000 g in a Sorvall Ultra Pro 80 ultracentrifuge (T.865.1 rotor) to produce a microsomal pellet, which was washed with 8 ml of buffer H (W) by resuspension and centrifuged as above.

The final microsomal pellet was resuspended by homogenisation in a Dounce homogeniser (5 mL) with 4 mL of 1.55 M sucrose in buffer H and placed on a 2 mL 2 M sucrose cushion in a swing-bucket ultracentrifuge tube. It was then overlaid with 2.5 mL 1.33 M, 2 mL 1.2 M, 2 mL 1.1 M, 1 mL 0.77 M and 1 mL 0.25 M sucrose all in buffer H. The sucrose gradient was centrifuged for 16 h at 116,000 g in a Sorvall Ultra Pro 80 ultracentrifuge (AH-629 rotor). Purdenz™ (GENTAUR Belgium BVBA) was pumped into the bottom of the tube using a 20 mL syringe to collect 1 mL fractions (F1-F12) from the top of the sucrose gradient. The first eleven

fractions (F1-F11) were diluted eight times with buffer H and centrifuged for 45 min at 135,000 g. The last fraction was discarded. The pellets thus obtained (P2-P11) were resuspended with 400 µL 2 % (v/v) Triton X-100 (Sigma Aldrich) in phosphate-buffered saline (PBS; 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl and 2.7 mM KCL, pH 7.4) using a Dounce homogeniser (1 mL). Protein concentration was measured by the bicinchoninic acid (BCA) protein assay (Pierce, Thermo Fisher Scientific, Northumberland, UK) in 1:20 dilutions of the resuspended pellets (final Triton X-100 concentration: 0.1 % (v/v)). Equal amounts of the resuspended pellets P1-P11 were analysed by SDS-PAGE and by western blot, using a polyclonal antibody to caveolin1 (sc-890, Santa Cruz Biotechnology Inc., Insight Biotechnology, Wembley, UK). After gel electrophoresis, samples were transferred to HybondTM nitrocellulose membrane (GE Healthcare Life Sciences) using a wet system (Mini Trans-Blot Cell, Bio-Rad Laboratories Ltd) for 1 h at 100 V. Membranes were blocked with TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.5) supplemented with 10 % (w/v) skimmed milk powder for 1 h at room temperature. The membranes were then incubated in TBST-1% (w/v) milk (TBS supplemented with 0.1 % (v/v) Tween 20 (TBST) and 1 % (w/v) skimmed milk powder) with anti-caveolin1 (1:200) overnight at 4 °C. After three 5 min washes with TBST, the membranes were incubated in TBST-5 % milk (TBST supplemented with 5 % (w/v) skimmed milk powder) with anti-rabbit-HRP secondary antibody (A0545, Sigma Aldrich Ltd) (1:5,000) for 1 h at room temperature. After at least three 5 min washes with TBST, the membranes were developed using the SuperSignal West Pico

chemiluminescent substrate (Pierce, Thermo Fisher Scientific, Northumberland, UK).

3.5.4.3 Heparin affinity chromatography

Membrane pellets P2-P5 were selected according to their sedimentation profile and caveolin-1 signal intensity. The resuspended pellets were pooled, adjusted to 0.15 M NaCl and 1 % (v/v) Triton X-100 and were applied to a 1 ml Hi-Trap heparin column (GE Healthcare Life Sciences) equilibrated with a modified phosphate-buffered saline (PBS), buffer WL (0.15 M NaCl, 13.7 mM Na_2HPO_4, 6.3 mM NaH_2PO_4, 0.1 % (v/v) Triton X-100, pH 7.2). After loading, the column was extensively washed with buffer WL until the absorbance at 280 nm reached the baseline. Bound proteins were then eluted with a 1-column volume of buffer E (2 M NaCl, 13.7 mM Na_2HPO_4, 6.3 mM Na_2HPO_4, 0.1 % (v/v) Triton X-100, pH 7.2). Protein concentration was measured by the BCA protein assay.

3.5.4.4 Sample preparation for mass spectrometry

Seventy percent (w/v) trichloroacetic acid (TCA) was added to an equal volume of the heparin-bound fraction to obtain a final concentration of 35% (w/v) TCA. This was placed at -20°C for 1 hour, followed by centrifugation at 14,000 rpm for 10 minutes. After removing the supernatant carefully with a glass pipette, the pellet was washed 5 times with 5% (w/v) TCA. The pellet was freeze-dried overnight, washed subsequently with 0.5 mL of diethyl ether to remove the excess TCA and centrifuged at 5000 rpm. The supernatant was removed and the diethyl ether wash was repeated 2 more times. The final pellet was left to dry in

the fume hood. The ether-washed, TCA-precipitated pellets were resolublised in either 200 µL (normal pancreas (NP)) or 600 µL (acute pancreas (AP)) of 50 mM ammonium bicarbonate, 0.05% (v/v) Rapigest (Waters, Manchester, UK) and shaken at 550 rpm for 10 min at 80°C. The sample was then reduced (addition of 10 µL (NP) or 30 µL (AP) of 60 mM DTT and incubation at 60 °C for 10 minutes) and alkylated (addition of 10 µL (NP) or 30 µL (AP) of 180 mM iodoacetamide and incubation at room temperature for 30 minutes in the dark). Trypsin (Sigma, Poole, UK, proteomics grade) was reconstituted in 50 mM acetic acid to a concentration of 0.2 µg/µl and 10 µL (NP) or 30 µl (AP) added to the sample followed by overnight incubation at 37°C. The digestion was terminated and RapiGest™ removed by acidification (3 µL (NP) or 9 µL (AP) of TFA and incubation at 37°C for 45 min) and centrifugation (15,000 x g for 15 min). To check for complete digestion each sample was analysed pre- and post-acidification by SDS-PAGE.

3.5.4.5 Mass spectrometry data acquisition and analysis

For LC-MS/MS analysis each digest was diluted to 250 ng/µL with 97/3/0.1 % (v/v) water/acetonitrile/formic acid and mixed 2:1 with a protein digest standard (50 fmol/µL yeast alcohol dehydrogenase, Mass PREP™ Digestion Standard, Waters). A 3 µL injection of this mixture, corresponding to 500 ng of sample and 50 fmol of standard was analysed using an Ultimate 3000 RSLC™ nano system (Thermo Scientific, Hemel Hempstead, UK) coupled to a QExactive™ mass spectrometer (Thermo Scientific). The sample was loaded onto the trapping column (Thermo Scientific, PepMap100, C18, 300

μm X 5 mm), using partial loop injection, for 7 minutes at a flow rate of 4 μL/min with 0.1% (v/v) TFA. The sample was resolved on the analytical column (Easy-Spray C18 75 μm x 500 mm 2 μm column) using a gradient of 97% A (0.1% formic acid) 3% B (99.9% acetonitrile, 0.1% formic acid) to 60% A 40% B (all v/v) over 90 minutes at a flow rate of 300 nL/min. The data-dependent program used for data acquisition consisted of a 70,000 resolution full-scan MS scan (automatic gain control (AGC) set to 1e6 ions with a maximum fill time of 250 ms) the 10 most abundant peaks were selected for MS/MS using a 17,000 resolution scan (AGC set to 5e4 ions with a maximum fill time of 250 ms) with an ion selection window of 3 *m/z* and a normalised collision energy of 30. To avoid repeated selection of peptides for MS/MS, the program used a 30 second dynamic exclusion window.

The data were processed with Progenesis QI (version 2 Nonlinear Dynamics, Newcastle upon Tyne, UK). Samples were aligned according to retention time using a combination of manual and automatic alignment. Default peak picking parameters were applied and features with charges from 1^+ to 4^+ featuring three or more isotope peaks were retained. Database searching was performed using Mascot (Matrix Science, London, UK). A Mascot Generic File, created by Progenesis QI, was searched against the reviewed entries of the reference proteome set of *M. musculus* from Uniprot (19/02/2014, 43238 sequences) with the sequence of yeast alcohol dehydrogenase (UniProt: P00330) added. A fixed carbamidomethyl modification for cysteine and variable oxidation modification for methionine were specified. A precursor mass tolerance of 10 ppm and a

fragment ion mass tolerance of 0.01 Da were applied. The results were then filtered to obtain a peptide false discovery rate of 1% and a requirement of two peptides per protein was applied. Label-free quantification was performed following the "Top3" methodology (Silva et al., 2006) by spiking the sample prior to analysis with an internal standard of 50 fmol yeast alcohol dehydrogenase digest (Uniprot P00330, Waters). Proteins were annotated as differentially expressed if they achieved a false discovery rate (FDR) corrected q value of 1%. Additionally, an adjusted threshold p value of less than 0.001 following the Bonferroni correction was used to identify the HBPs as potential biomarkers in the first instance (Ting et al., 2009).

3.5.4.6 Identification of the extracellular HBPs in NP and AP

Extracellular proteins were identified using a combination of bioinformatics tools. SignalP 4.1, which predicts the presence of a secretory signal peptide, was used to identify extracellular proteins (Petersen et al., 2011) with Phobius, which is a combined transmembrane topology and signal peptide prediction tool, to obtain a wider coverage for extracellular protein identification (Kall et al., 2007). A third tool was SecretomeP 2.0, which produces *ab initio* predictions of protein secretions not based on a secretory signal peptide (Bendtsen et al., 2004). A fourth tool based on ontology, Ingenuity Pathways Analysis (IPA), was used to identify extracellular and plasma membrane proteins. The HBPs that were not identified using SignalP, Phobius, or SecretomeP but which were identified using IPA were further investigated using a manual approach. Each candidate HBP was examined using UniProtKB for the presence of an

extracellular signature. A search in Pubmed, using the terms “extracellular” or “secreted” was performed to investigate those proteins that still had an ambiguous subcellular location to identify at least one publication demonstrating an unequivocal plasma membrane and or extracellular localisation. Finally, the outputs of the various approaches were merged to obtain the final list of HBPs in NP and AP.

3.5.4.7 Interactions and construction of networks of HBPs

Interactions between HBPs were obtained from the online database resource ‘Search Tool for the Retrieval of Interacting Genes’ (STRING). STRING 9.1 is a database of known and predicted functional interactions and served as a ‘one-stop’ comprehensive resource that could be easily used with Cytoscape (Franceschini et al., 2013). The interactions in STRING are provided with a probabilistic confidence score that is an estimate of how likely an interaction describes a functional linkage between two proteins. A higher score indicating a higher confidence is given when more than one type of information supports a given association. Only interactions with a high confidence score (0.70 and above) were used to build networks using Cytoscape 2.8.1, which is an open source, Java based bioinformatics package for biological network visualization (Smoot et al., 2011). The resulting networks were termed ‘protein interactomes’. In the protein interactomes, HBPs or ‘nodes’ are coloured blue, while the black lines connecting the HBPs denote the interactions or ‘edges’.

3.5.4.8 Network Analysis

3.5.4.8.1 Network parameters

Additional 'plugins' or tools in Cytoscape were used for analysis. The networks were treated as undirected and the following parameters were computed using the *'NetworkAnalyzer'* plugin (Assenov et al., 2008): diameter, average number of neighbours, number of connected pairs of nodes, node degree, average clustering coefficient, topological coefficient, and shortest path length. We used the 'clustering coefficient' and 'number of connected components' as measures of network connectivity. A high clustering coefficient and a low number of connected components are present in well-connected networks (Barzel and Biham, 2009).

3.5.4.8.2 Identification of potential biomarkers and therapeutic targets

Canonical pathways and cluster analysis were used in conjunction with differential expression as tools to identify potential functional biomarkers and drug targets.

Canonical pathways and bio-functions analyses

Canonical pathways are well-characterized signalling pathways that have been curated from original data. Bio-functions are molecular and cellular functions that play important roles in homeostasis in health and disease. The significance of the association between the datasets and the canonical pathway/bio-function was measured by calculating the p-value using Fisher's exact test to determine the probability of the association between the HBPs in the dataset and the canonical pathway/bio-function. Canonical

pathways and bio-function analyses are useful tools for the identification of potential biomarkers and drug targets. They also help identify biological pathways that play important roles in homeostasis and complex diseases.

Cluster Analysis

Clusters, which are highly interconnected hubs of nodes (HBPs) within the networks, were identified using the *'AllegroMCODE'* plugin in Cytoscape. Clusters are scored depending on the number of constituent nodes and the edges (interactions) between them.

3.5.4.8.3 Comparing the connectivity of the heparin-binding protein interactome with other extracellular protein interactomes

An updated list of HBPs was obtained combining the list of HBPs from Ori *et al.* 2011 (Ori et al., 2011) and the HBPs from the experiments described here. The complete human proteome was obtained from the protein knowledgebase in UniProtKB, using the following search "Homo sapiens (Human) [9606]" AND keyword: "Complete proteome [KW-0181]". The extracellular proteome was extracted using the following Gene Ontology (GO) terms: GO:0005576 (extracellular region), GO:0005615 (extracellular space), GO:003102 (extracellular matrix- ECM) and GO:0005604 (basement membrane). Clustering coefficients of extracellular protein interactomes were compared in order to ascertain if the heparin-binding protein interactomes formed important modules within the extracellular space. The extracellular non-HBP protein list was generated by subtraction of the HBP list from the whole extracellular protein list. The extracellular protein

interactome (Ec), the extracellular non heparin-binding protein interactome (Ec_not hepint) and the extracellular heparin-binding protein interactome (Ec_hepint) were built in Cytoscape using the respective lists obtained, as described above, and the interaction data retrieved from the STRING database. The heparin-binding protein interactomes (Ec_hepint) were also compared with their corresponding degree-preserving randomised versions in order to determine whether the network parameters arising from the interactions of the HBPs were random. The randomised networks (Ec_hepint_random) were generated, by shuffling the edges of the respective heparin-binding putative protein interactomes, using the *'Random Networks'* plugin in Cytoscape Cytoscape. A network is deemed to be well connected if its average clustering coefficient is significantly higher than that of its corresponding random networks.

3.5.5 Results

Identification of HBPs from plasma membrane enriched fractions from normal and a murine model of acute pancreatitis - Each HBP isolation experiment was performed three times using sixteen pancreases from control (NP) and AP mice. Acute pancreatitis was induced in animals by injection of caerulein. Histological analysis of samples of these pancreases demonstrated the classic features of normal pancreas with preserved acinar pattern (Fig 3.5.1A) and those associated with AP namely marked oedema, vacuolisation, neutrophil infiltration in the ductal margins and parenchyma of the pancreas, with focal acinar cell necrosis (Figs 3.5.1B).

Serum amylase was observed to increase ~8- to 10-fold in AP compared to NP (Figs 3.5.1A, B). These data are consistent with successful induction of AP (Carvalho et al., 2014).

Differential centrifugation (De Duve, 1971, Ray, 1970) of pancreas homogenates produced a series of cellular sub-fractions, including the final microsomal pellet (lane Mc, Figs 3.5.2A.A, 35.2B.A), which was floated on a sucrose gradient (0.77-1.55 M) to separate its individual constituents (Figs 3.5.2A.B, 3.5.2B.B). The gradient was harvested into 12 fractions, with the final fraction being discarded. Eleven fractions were assessed by Western blot for the plasma membrane marker (caveolin-1) (Figs 3.5.2A.B, 3.5.2B.B). The caveolin-1 content was inversely correlated with the equilibrium density of the sucrose fractions, consistent with plasma membranes, which possess lower density than other microsomal membranes. Fractions 2 to 4, which had the strongest caveolin-1 immunoreactivity, were pooled and selected as the plasma membrane enriched fraction (PM). This was solubilised in Triton-X-100 and subjected to heparin affinity chromatography. After extensive washing the heparin column with PBS, proteins that remained bound were deemed to have a sufficiently strong interaction with the polysaccharide to be considered as HBPs. These were eluted with 2 M NaCl and precipitated with TCA to remove Triton-X-100, followed by digestion with with trypsin. After ascertaining the optimal loading concentration, the sample order was randomised across biological (three each NP and AP) and technical repeats prior to LC-MS. The LC-MS runs were then individually searched using MASCOT protein search engine (www.matrixscience.com) (Perkins et al.,

1999). There was little variation between the technical replicates for the samples, which can be attributed to the high quality of the sample preparation and MS analysis (Fig 3.5.3). Each technical replicate produced between 1500-1900 protein hits at a peptide false discovery rate (FDR) of 1%. To obtain a fuller coverage, the data were run through Progenesis label-free software. The merged file yielded over 1900 hits at a peptide false discovery rate of 1%. Using a 2-peptide stringency, these were reduced to 1602 proteins in NP and 1866 proteins in AP (Supplementary Tables 1 and 2).

The 1602 proteins in NP and 1866 proteins in AP were then filtered by the bioinformatics pipeline described in "Experimental Procedures" to identify those possessing extracellular (partially or wholly) amino acid sequence. A total of 396 proteins in NP (Supplementary Table 3) and 419 proteins in AP (Supplementary Table 4) were identified as heparin binding and extracellular. Combining these two sets of proteins yielded 559 HBPs, of which 460 HBPs had not been identified previously as heparin binding. With such a substantial number of new HBPs, it was important to analyse their functions and relationships to gain insight into the significance of the pancreas and AP associated HBPs. This was achieved by an analysis of their protein-protein interactions.

Label-free quantification

Using the “Top3” methodology, proteins were annotated as differentially expressed if they achieved a FDR corrected q value of 1% (Supplementary Tables 5 and 6). Introduction of a p value cut off of 0.001, following the

Bonferroni correction, resulted in the identification of 103 HBPs that were overexpressed and 116 HBPs that were under expressed in AP as compared to NP. Known biomarkers of AP, such as carboxypeptidase (CPB1 and CPB2) and pancreatic amylase (AMY2A) (Gomatos et al., 2014) were found to be overexpressed in the AP group. TAP-binding protein (TABP or Tapasin) that binds to TAP (trypsinogen activation peptide) was also found to be overexpressed. The top 20 HBPs with the highest fold change in each group (Tables 3.5.1 & 3.5.2) may provide potential biomarkers for AP.

Network construction and analysis of HBPs in the pancreas - The lists of HBPs in NP and AP were used to obtain protein-protein interactions from STRING. Only interactions with a high confidence score (0.70 and above) were used. These were imported into Cytoscape to build heparin-binding protein interactomes in NP and AP. The nodes, corresponding to each HBP, are coloured grey and are connected to each other by black lines, which are also termed edges and represent the known interactions of the HBPs in NP (Fig 3.5.4). 'Nodes' or HBPs are coloured depending on their fold change value, based on label-free quantification, in the AP interactome (Fig 3.5.5). The HBPs that are under expressed in AP relative to NP appear green and those that are over expressed appear red. Grey lines connecting the HBPs denote 'edges' or interactions. The topological parameters of the HBP interactomes were obtained using 'NetworkAnalyser', which is a plugin in Cytoscape. The HBP interactomes of NP and AP have high clustering coefficients (NP = 0.375 and AP = 0.390). The clustering coefficients of the HBP interactomes were also significantly higher than those of their

corresponding random networks (Ec_hepint_random) in NP ($p=0.001$) and AP ($p<0.001$). These suggest that the HBP interactomes form highly interconnected modules in the extracellular space of the pancreas (Barzel and Biham, 2009, Dong and Horvath, 2007). This would mean that the HBP interactomes are likely to be central to the homeostasis of the normal pancreas and the HBPs identified in AP may have key roles in mediating the altered cell communication that is associated with AP. To identify which HBPs are most likely to be disease biomarkers or targets for the development of therapy, specific tools in Cytoscape were used, such as canonical pathways, bio-functions and cluster analysis. These allow the HBPs in NP and AP to be associated with biological pathways and functions that play important roles in health and disease.

Analysis of Canonical Pathways, Bio-functions of HBPs in NP and AP - The context of a particular HBP, that is the other HBPs, will be important, particularly since these proteins are clearly highly interconnected. Therefore, a first analysis was performed of the HBPs in NP and of AP. A subsequent analysis was done on the HBPs unique to either NP or AP.

HBPs in NP and AP clearly have functions important in cell communication (Fig 3.5.6) and they enrich to a number of canonical pathways (Tables 3.5.3 and 3.5.4, Supplementary Tables 7 and 8) underlying homeostasis and complex diseases both at a systemic and an organ / disease level (NP and AP). For example, the top canonical pathway associated with the NP dataset, signalling by 'Rho family GTPases' plays an important role in cell adhesion, as do a number of the other highly ranked pathways, 'RhoGDI

Signaling', 'Ephrin B Signaling', 'Tec Kinase Signaling', 'Actin Cytoskeleton Signaling' and 'Ephrin Receptor Signaling'. Cell adhesion is essential to the maintenance of tissue architecture and so a type of cell communication appropriate for homeostasis (Maitre and Heisenberg, 2013). In contrast, the top pathways associated with the HBPs in AP are associated with inflammatory responses. Thus, the top four pathways in AP are 'Intrinsic Prothrombin Activation Pathway', 'Coagulation System', 'Acute Phase Response Signalling' and 'LXR/RXR Activation'. Most of the HBPs enriching to these pathways were found to be overexpressed in AP relative to NP (Supplementary Table 5). The presence of pathways linked to cell adhesion further down the list ('Actin Cytoskeleton Signaling', 'RhoGDI Signaling', 'Signaling by Rho Family GTPases', 'Ephrin Receptor Signaling', 'Ephrin B Signaling' and 'Integrin Signaling'), would then reflect the predominance of cell motility and a change in tissue architecture, driven by the inflammatory pathways.

Analysis of the bio-functions associated with the HBPs in NP and AP support the conclusions reached from the analysis of canonical pathways. Thus, the highest ranked bio-functions of HBPs in NP is transport of molecules and more specific related bio-functions are also highly ranked, e.g., amino acid metabolism, transport of ions, and these relate to the numerous anabolic functions of the pancreas (Supplementary Table 7). The various bio-functions relating to "Cellular Movement", "Cell Morphology" and "Cellular Assembly and Organization' will similarly reflect the architecture of homeostasis of the pancreas (Supplementary

Table 9). In AP, there is a marked change in bio-functions associated with the HBPs, with a loss of all those associated with anabolism from the most highly ranked positions. Instead, bio-functions linked to inflammation, cell death and disruption of tissue architecture feature (Supplementary Table 10).

The analysis of the HBPs that were unique to NP or to AP (Supplementary Tables 11 & 12) shows that these subsets of HBPs are also associated with canonical pathways and biofunctions characteristic of homeostasis and inflammation. Thus, top canonical pathways enriching to the HBPs unique to the NP dataset (Supplementary table 13) also relate to anabolism, but specifically to mitochondrial function, whereas the unique HBPs of AP are again associated with inflammation (Supplementary Table 14 and Fig. 3.5.7). In terms of bio-functions, the HBPs unique to the NP dataset similarly enrich to anabolic bio-functions (Supplementary Table 15) and those of AP to ones associated with inflammation (Supplementary Table 16).

Cluster analysis

The top clusters in AP include HBPs that are mainly over expressed compared to NP using label-free quantification (Supplementary Table 17). These clusters enrich to a number of canonical pathways that are linked to the AP dataset.

The global heparin interactome - A total of 559 extracellular HBPs were identified in the present work, of which 460 proteins have not been previously identified previously as being heparin binding (Ori et al., 2011). This takes the number of extracellular HBPs from 435 previously known to 883 (Supplementary Table 18), which is a significant increase. It was, therefore, important to determine whether the enlarged HBP interactome retained functional relevance in the extracellular space. This was accomplished by a comparison of clustering coefficients of various extracellular protein-protein interaction networks. The clustering coefficient of the extracellular HBP interactome (Ec_hepint) was significantly higher than that of the whole extracellular interactome (Ec) and the extracellular non-HBP interactome, as well that of the corresponding randomised version of Ec-hepint (Ec-hepint_random) (Fig. 3.5.8). This indicates that even with 460 additional members the HBPs remain highly interconnected and form an important regulatory module in the extracellular space. The global heparin interactome enriches to important canonical pathways (Supplementary Table 19); some have a clear pancreas bias, due to the fact that the new HBPs have been discovered in this organ. For example, 'Hepatic Fibrosis / Hepatic Stellate Cell Activation' (-log (p-value) = 3.78E01), which is the top pathway associated with the global HBP interactome is of particular relevance in pancreatic homeostasis, since the pancreatic stellate cell is known to play an important role in this tissue (Vonlaufen et al., 2007).

3.5.6 Discussion

Extracellular HBPs have been shown previously to be functionally associated to physiological and pathological processes. The aim of the present work was to determine if this was true for a single organ, the pancreas and one of its diseases, AP. The focus was on extracellular HBPs, because these could readily be distinguished from intracellular contaminants. However, it is important to note that there is clear evidence for intracellular HS (Courvalin et al., 1982, Bornens, 1973) However, there is no means at present to distinguish in the output from a proteomic experiment intracellular proteins that interact with intracellular HS from those that interact solely with another intracellular polyanion, such as phosphorylated lipids and nucleic acids. For this reason intracellular proteins are excluded from the analysis, though there are substantial numbers present in the analysis.

Differential centrifugation followed by density gradient centrifugation was used to isolate a plasma membrane enriched subcellular fraction, which was subsequently used as a source of extracellular HBPs (Ray, 1970). In addition to membrane proteins, which are often underrepresented in proteomic analyses, this fraction would also encompass intra- and extracellular associated membrane proteins, as well as proteins associated with the pericellular matrix. The plasma membrane enriched fraction was solubilized using a non-ionic detergent and subjected to heparin-affinity chromatography. In contrast to previous work (Ori et al., 2011), here a single elution at 2M NaCl was used to recover heparin-binding proteins. The rationale was that the concentration of NaCl required for elution from

heparin does not necessarily correlate with affinity for HS (Xu et al., 2012).

A number of tools were used to identify proteins with significant sequences in the extracellular space, including SignalP, Phobius, SecretomeP, IPA and manual curation for the presence of an extracellular signature using Uniprot-KB and PubMed. In a previous analysis of rat liver a bioinformatics pipeline based on ontology (gene ontology and ingenuity ontology, IPA) was combined with manual curation to identify 62 HBPs, of which 12 were previously known to bind to heparin (Ori et al., 2011). Middaugh et al., employing an antibody array on total cell lysate before and after depletion of HBPs by heparin agarose beads, identified 29 proteins whose signal was significantly reduced after incubation of the cell lysate with heparin beads, though only three were extracellular (Jones et al., 2004). Our present approach identified 460 new extracellular HBPs and has more than doubled the number of proteins that may be HBPs (Ori et al., 2011). This increase is likely due to the new source of tissue and an associated disease state, the purification of a plasma membrane enriched sub-proteome, the depth of the mass spectrometry analysis and the use of parallel approaches to identify proteins that possess significant extracellular sequence.

An important question is how representative are the HBPs identified here of the proteins in NP and AP whose function depends on, or is modified by, interaction with HS. The mass spectrometry is limited by the depth of the analysis is limited by only accepting identifications based on at least two peptides and a maximum false discovery rate of 1%. This in itself leads to false positives and false negatives. The Bonferroni correction was used to increase the stringency and further reduce the number of false positives for

the purpose of biomarker identification, (Ting et al., 2009).

There are additional factors that are likely to contribute to the false positives and false negatives. Some higher abundance HBPs may escape detection, because they are part of large macromoleuclar assemblies that are not solubilised by Triton-X-100, associated with, for example, matrix fibrils, membrane microdomains or cytoskeleton. Moreover, not all HS-binding proteins will necessarily bind the heparin column and may, instead remain bound to HSPGs during affinity chromatography. Indeed, the transition and S-domains of HS have far more diverse structures than heparin (Ori et al., 2008, Xu and Esko, 2014). Thus, the present analysis will have a bias towards HBPs that bind structures present in the trisulfated disaccharide repeat of heparin, which makes up 75% of the polysaccharide and contains 2-O sulfated iduronate, N-, 6-O sulfated glucosamine. A source of false positives would be proteins that are bound to an HBP with a sufficiently slow dissociation rate constant that they would be carried though the affinity chromatography, an interaction, which would be consistent with biological function.

A recurrent question is how specific or selective is a protein-HS interaction (Xu and Esko, 2014, Ori et al., 2008, Xu et al., 2012). This will be important in terms of understanding the mechanism whereby HS regulates the activity of a protein. HS has been shown directly to control the movement of a protein, fibroblast growth factor-2 in the pericellular matrix (Duchesne et al., 2012). Thus, even an unselective charge-dependent interaction of a protein with HS will restrict the movement of the protein in the extracellular space and so affect its function in terms of location and local

concentration. Therefore, proteins that bind heparin in PBS and that remain so during the extensive washing of the affinity column have a relatively slow dissociation rate constant and would be expected to have at least their movement and extracellular location regulated by HS. Taking into account the caveats described above regarding false positives, the HBPs identified here are thus likely have at least this aspect of their function regulated in this way by HS in the extracellular space of NP and AP. Any changes in the structure of HS that might accompany AP may, therefore, alter the movement/location of the HBP and so its contribution to cell physiology.

Apart from the limits imposed on the mass spectrometry analysis, the other sources of false positives and negatives are not currently quantifiable, until such time as the interaction of each HBP with the polysaccharide is measured directly.

Given the above caveats, the heparin interactome is now substantially larger that previously described (Ori et al., 2011). Importantly, the protein-protein interaction network of the HBPs retains the key properties of the earlier, smaller interactome and hence its functional relevance to the regulation of cell communication by extracellular proteins (Ori et al., 2011). Thus, the HBP interactome is highly interconnected and HBPs form numerous regulatory modules in the extracellular space. Importantly, this is true for the subset of HBPs expressed by a single organ, the pancreas, one of its associated diseases, AP, and for the global interactome. Thus, it may be that for most, if not all organs, HBPs represent the most interconnected set of extracellular proteins, and, therefore, those with the greatest

regulatory potential.

Intriguingly, the present work identified some non-canonical extracellular HBPs such as NDUFS4, NDUFS6, NDUFS7, NDUFS8, NDUFA9, NDUFA10 in NP, and NDUFA9 and NDUFA10 in NP and AP. These are mitochondrial proteins that are nuclear-encoded and were identified by SignalP, Phobius or SecretomeP as having a secretory signal. It seems unlikely that they are simply contaminants with polyanion binding properties that allow interaction with heparin. Direct evidence of extracellular localisation has been obtained for multiple subunits of the ATP synthase complex, including the OSCP (Oligomycin sensitivity-conferring protein) and D subunits, which have been observed at the plasma membrane (Moser et al., 1999, Vantourout et al., 2008, Yonally and Capaldi, 2006). Such proteins with a clearly established classic subcellular localisation, which are subsequently found, associated with other organelles or extracellularly, have been termed to be 'moonlighting'. We, therefore, included the NDUF proteins as extracellular HBPs. Though their plasma membrane/extracellular localisation remains to be confirmed by orthogonal measurements, the observation that these are all present in NP, but are under-expressed in AP is consistent with the contribution of mitochondrial dysfunction to AP (Cardenas et al., 2010, Pinton et al., 2008, Koopman et al., 2010).

A number of biomarkers that have been previously investigated in AP, such as carboxypeptidase B and pancreatic amylase,were identified in the AP dataset (Table 1 and Supplementary Table 5). LBP, an acute phase reactant, with a rise and fall in serum that is similar to CRP (Rau et al., 2003) and which is protective in AP, was identified to be over expressed in the AP

dataset. HRG (Table 1) has been shown to recruit IgG to facilitate clearance of necrotic cells by pahagocytes (Poon et al., 2010). Thus, the HBPs exhibiting the greatest fold change (Table 1) alone or in combination with existing biomarkers may provide an early stratification of AP, identifying the severe form of the disease earlier and improving outcomes. The top canonical pathways and clusters, to which the HBPs in the AP dataset enrich, provide a repository for functional biomarker and drug discovery in AP. Neutrophils have been shown to play an important role in AP, activating the complement system and promoting lung injury (Awla et al., 2012, Shrivastava and Bhatia, 2010). They release platelet activating factor, which has been implicated as a key mediator in the progression of AP and is associated with increased complication rates and mortality (Xia et al., 2007). HBPs such as PIP5K1C that participate in these canonical pathways regulate neutrophil adhesion by facilitating RhoA GTPase and integrin activation through chemoattractants (Xu et al., 2010a). Small molecule inhibitors of PIP5K1C have been investigated in the treatment of other diseases (Wright et al., 2014) and it may, useful to explore these in the treatment of AP. SERPINC1, has anti-inflammatory properties and has been shown to improve acute pancreatitis in the rat model (Hagiwara et al., 2009). Thus, its presence among the HBPs whose expression is increased to the greatest extent may reflect a normal physiological reaction to the insult of AP.

“Hepatic Fibrosis / Hepatic Stellate Cell Activation”, which is the top canonical pathway associated with the global HBP interactome and one of the top pathways enriching to the AP dataset is particularly relevant in

pancreatic homeostasis. Stellate cells play an important role in inflammatory diseases and cancer of the liver, as in the pancreas (Vonlaufen et al., 2007, Algul et al., 2007, Masamune et al., 2009). The enrichment of a well-defined fibrosis and activation pathway associated with the hepatic stellate cell, rather than its pancreatic counterpart is probably due to the fact that the former was discovered earlier and has been studied more extensively (Geerts, 2001). Hepatic and pancreatic stellate cells exhibit similarities of morphology and function and are activated by common cytokines and growth factors, and may share a common origin (Buchholz et al., 2005). FN1 and CD14, which are unique to the AP dataset (so highly increased relative to NP) and also enriched to the 'Hepatic Fibrosis / Hepatic Stellate Cell Activation' pathway may thus have potential as a PSC specific biomarker and drug target in the progression of AP to chronic pancreatitis. Indeed, Rhein, which is a natural anthraquinone targeting the SHH/GLI1 signalling pathway in pancreatic fibrosis, is presently being investigated as an anti- fibrotic drug in the pancreas (Tsang et al., 2013). HBPs such as vascular endothelial growth factor A (VEGFA) and fibroblast growth factor2 (FGF2) enrich to this pathway. Thus, although they are not amongst the most highly expressed in AP because they enrich to this pathway and because there are already drugs targeting their receptors undergoing clinical trials, particularly for the treatment of various cancers, they may have application in the treatment of AP.

'CXCR4 Signalling' regulates cell differentiation, cell chemotaxis cell survival and apoptosis, and has important roles in the embryonic development of the pancreas (Katsumoto and Kume, 2013). Its importance

as a key canonical pathway in AP is supported by the identification of its protective role in AP, which may be mediated by facilitating the migration of bone marrow derived stem cells towards the pancreas (Gong et al., 2014). Early 'Complement system' activation occurs in pancreatic necrosis and suggests this pathway may enable the development of treatment of leukocyte-associated injury in AP (Hartwig et al., 2006). In this respect, heparin-based compounds (Coombe and Kett, 2005) might be particularly interesting as potential therapeutics for AP. They may be tuneable to modulate a gamut of HBPs important in the cell communication underlying the progression of AP, which could include complement system activation, growth factors and cytokines, and, because heparin can modulate the activity of some transporters, metabolism (Chen et al., 2014).

The HBPs define a group of proteins that have a clear functional importance in cell communication in homeostasis and in at least one disease, AP. HBPs are experimentally accessible, because they bind to heparin and are extracellular, and in at least some instances they will be present in serum. Therefore, the HBPs of pancreas and AP are likely to yield much-needed biomarkers and targets for therapy, a conclusion reinforced by the observation that some of the HBPs identified are involved known mechanisms of AP and some are current targets for therapy in AP and other diseases.

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HBP	Max fold change
ERP27	412
CPB2	90
Ngp	79
HRG	77
Try4	58
SERPINC1	44
ITIH2	43
PLG	43
COL6A3	41
SERPIND1	37
CTRC	36
FN1	35
AHSG	30
Pzp	30
SERPINA1	28
COL1A2	26
PRG2	23
SERPINA3	21
F2	20
Ear3	20

Table 3.5.1. Top 20 HBPs upregulated in AP. The upregulated HBPs were filtered depending on the maximum fold change values. An adjusted threshold p value of less than 0.001 following the Bonferroni correction was used to identify the top HBPs to be validated as potential biomarkers.

HBP	Max fold change
KLB	-46
SOSTDC1	-20
CRIP2	-18
SLC4A7	-13
COMTD1	-13
MRGPRF	-12
RNASE1	-12
GPR39	-11
SLC35A2	-11
SLC4A2	-11
PDHA1	-10
KCNQ1	-10.
ARCN1	-10
COMMD10	-10
TMEM115	-9
INSR	-9
TFRC	-8
CLPS	-8
ADCY6	-7
CLTB	-7

Table 3.5.2. Top 20 HBPs downregulated in AP. The downregulated HBPs were filtered depending on the maximum fold change values. An adjusted threshold p value of less than 0.001 following the Bonferroni correction was used to identify the top HBPs to be validated as potential biomarkers.

Canonical Pathways	-log (p-value)
Signaling by Rho Family GTPases	8.45
G Beta Gamma Signaling	8.44
RhoGDI Signaling	8.16
CXCR4 Signaling	7.28
Caveolar-mediated Endocytosis Signaling	6.94
IL-1 Signaling	6.77
Thrombin Signaling	6.76
Clathrin-mediated Endocytosis Signaling	6.32
Ephrin B Signaling	5.91
Tec Kinase Signaling	5.71
Role of NFAT in Regulation of the Immune Response	5.23
CREB Signaling in Neurons	5.17
Cardiac Hypertrophy Signaling	5.12
Role of Tissue Factor in Cancer	5.11
Relaxin Signaling	4.76
Actin Cytoskeleton Signaling	4.75
Gαi Signaling	4.73
Triacylglycerol Degradation	4.71
Ephrin Receptor Signaling	4.55
Sertoli Cell-Sertoli Cell Junction Signaling	4.30

Table 3.5.3. Top 20 canonical pathways in normal pancreas using Ingenuity Pathways Analysis. The significance of the association between the datasets and the canonical pathway was measured by calculating the p-value using Fisher's exact test to determine the probability of the association between the HBPs in the dataset and the canonical pathway.

Canonical Pathways	-log (p-value)
Intrinsic Prothrombin Activation Pathway	14.3
Coagulation System	13.2
Acute Phase Response Signaling	11.4
LXR/RXR Activation	11.0
Clathrin-mediated Endocytosis Signaling	10.6
FXR/RXR Activation	10.4
Extrinsic Prothrombin Activation Pathway	9.08
G Beta Gamma Signaling	9.02
Complement System	7.82
Actin Cytoskeleton Signaling	7.70
RhoGDI Signaling	7.70
Role of Tissue Factor in Cancer	7.30
Signaling by Rho Family GTPases	7.23
Ephrin Receptor Signaling	7.01
CXCR4 Signaling	6.88
Caveolar-mediated Endocytosis Signaling	6.64
Ephrin B Signaling	6.58
Virus Entry via Endocytic Pathways	6.40
Thrombin Signaling	6.34
Integrin Signaling	6.19

Table 3.5.4. Top 20 canonical pathways in experimental acute pancreatitis using Ingenuity Pathways Analysis. The significance of the association between the datasets and the canonical pathway was measured by calculating the p-value using Fisher's exact test to determine the probability of the association between the HBPs in the dataset and the canonical pathway.

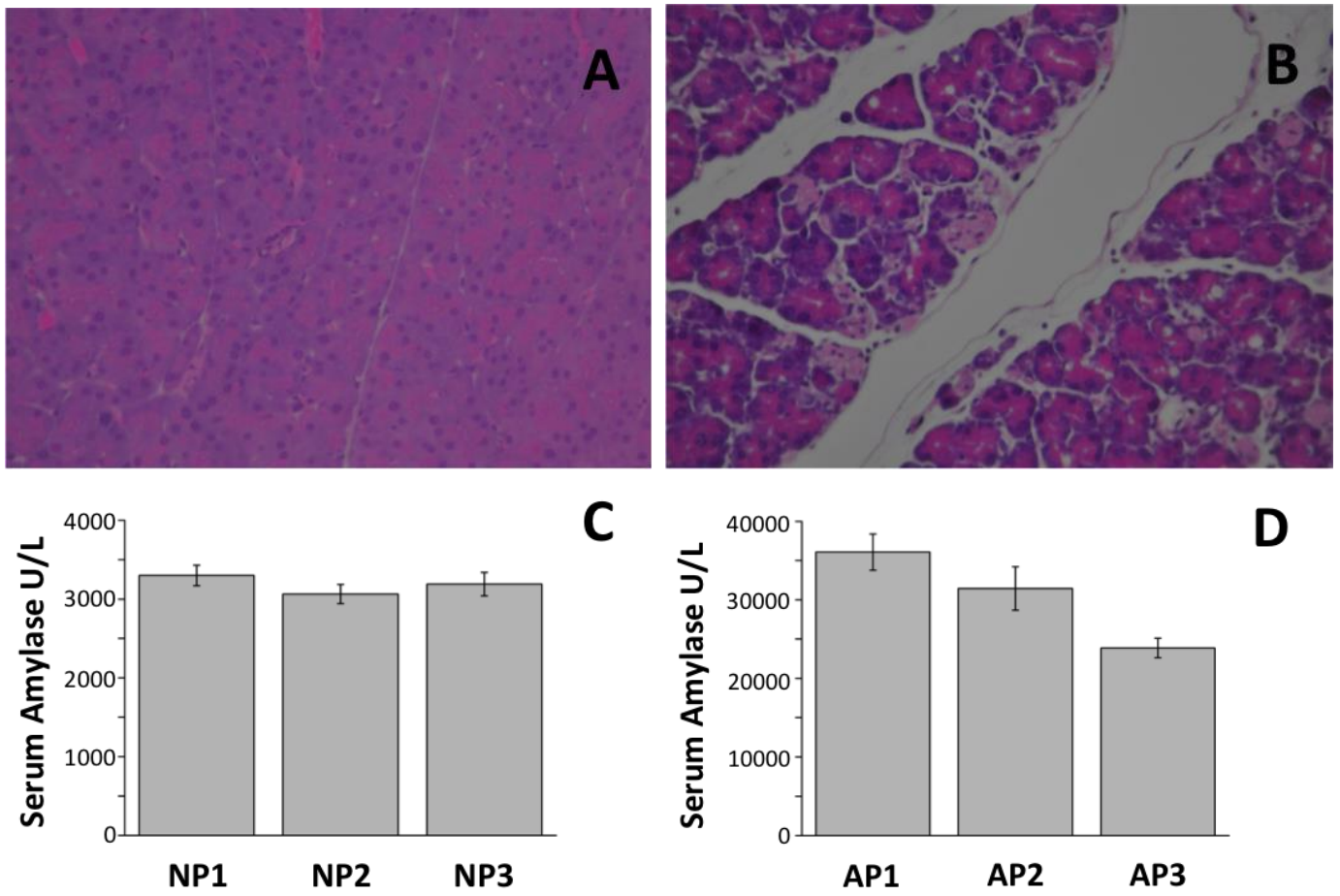

Figure 3.5.1: Normal pancreas (NP) and caerulein-induced acute pancreatitis (AP). Representative images of H&E stained histology slides of A) NP and B) AP. Mean serum amylase levels in (C) NP and (D) AP in each experiment consisting of 16 individuals.

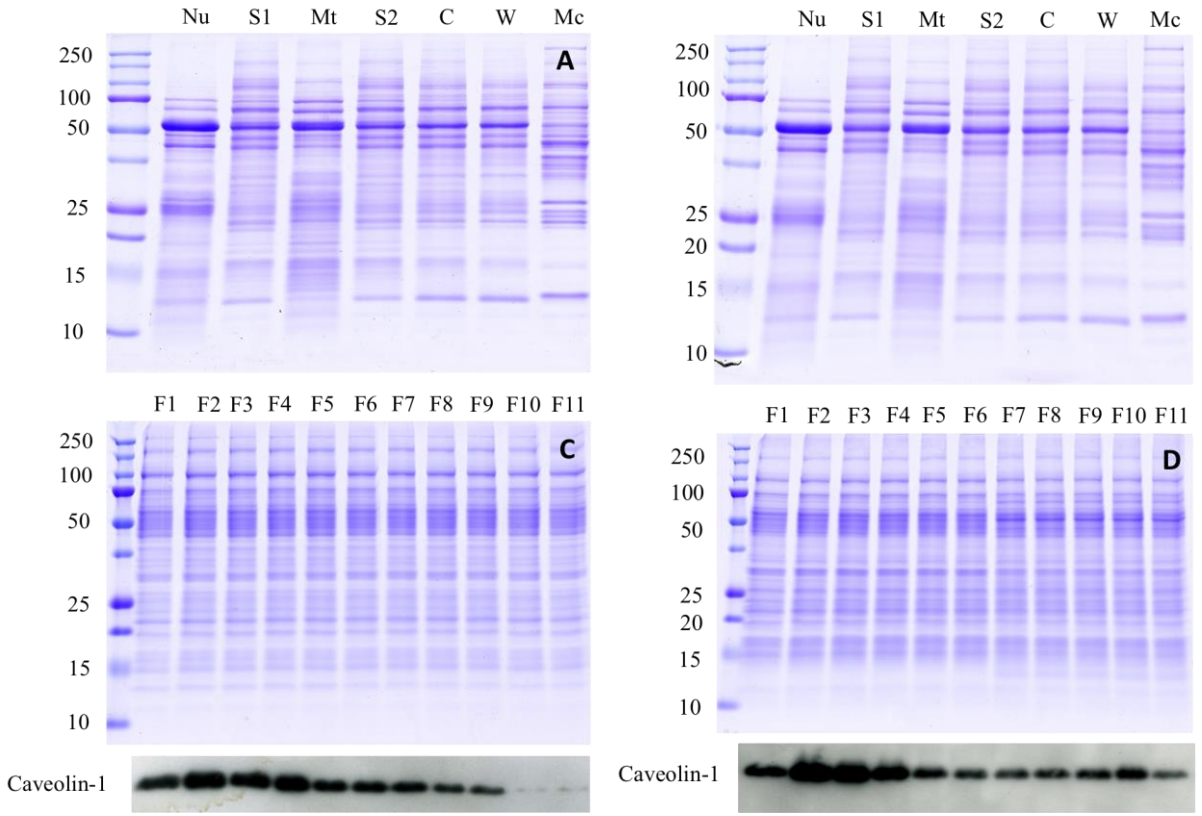

Figure 3.5.2: Preparation of a plasma membrane enriched fraction. Coomassie-stained SDS-PAGE gel of (A) NP and (B) AP samples obtained during homogenisation and fractionation by sequential steps of centrifugation. Nu = nuclear pellet; S1 = post-nuclear supernatant; Mt = mitochondrial pellet; S2 = post-mitochondrial supernatant; C = cytosol (post-microsomal supernatant); W = wash of the microsomal pellet; Mc = microsomal pellet. Coomassie-stained SDS-PAGE and western blot analysis of 10 fractions (F1-F11) from the microsomal pellet after flotation on a sucrose gradient (0.25 - 2 M) in (C) NP and (D) AP. Fractions are ordered depending on their equilibrium density from light (left) to heavy (right). The enrichment of plasma membrane was assessed by western blot using an antibody against caveolin-1, which is a specific plasma membrane marker.

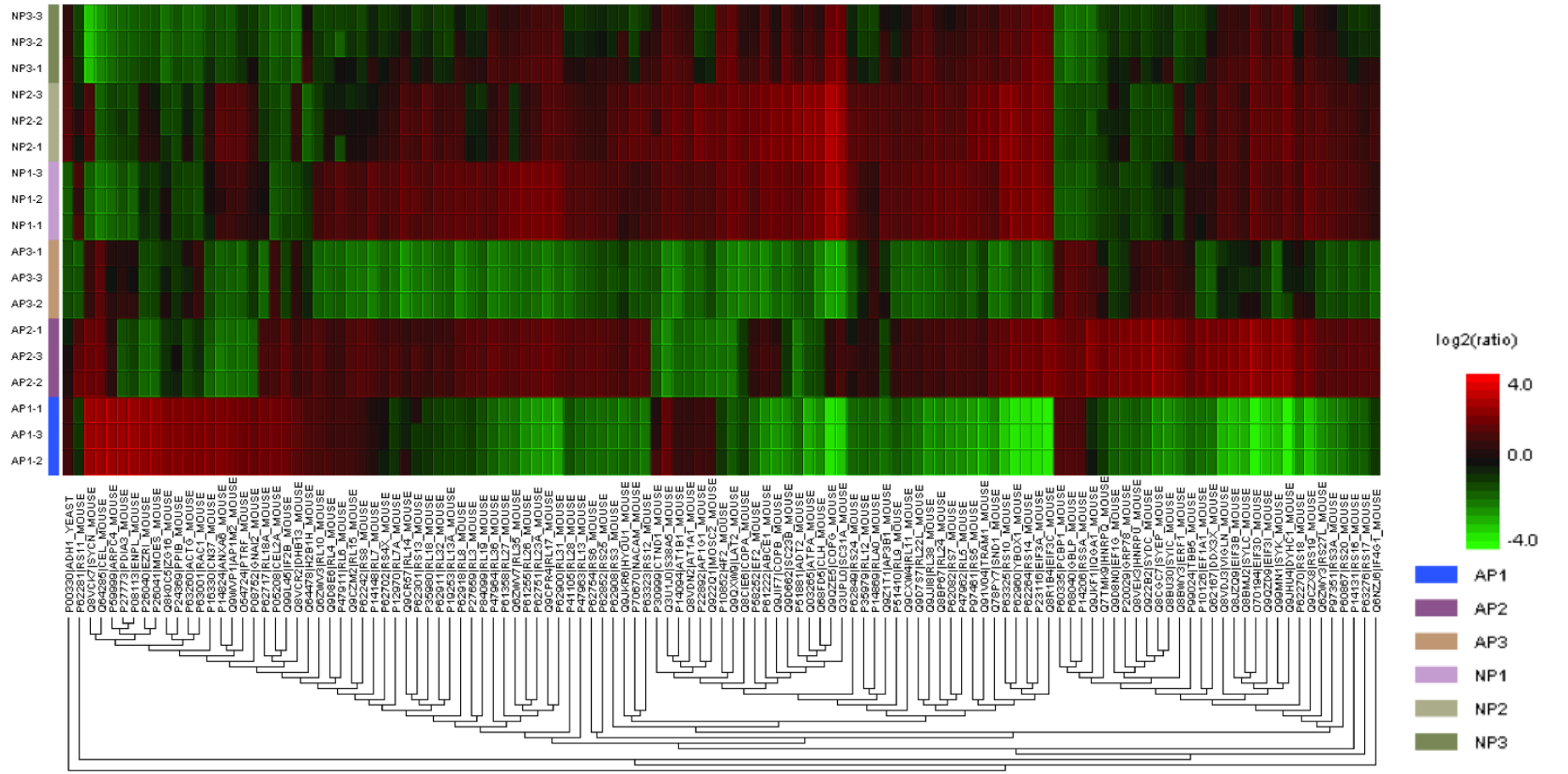

Figure 3.5.3: Heat map depicting the variation across the biological and technical replicates. The rows represent the various biological replicates in normal pancreas (NP) and acute pancreatitis (AP), while the columns represent proteins. Red represents over expression and green represents under expression.

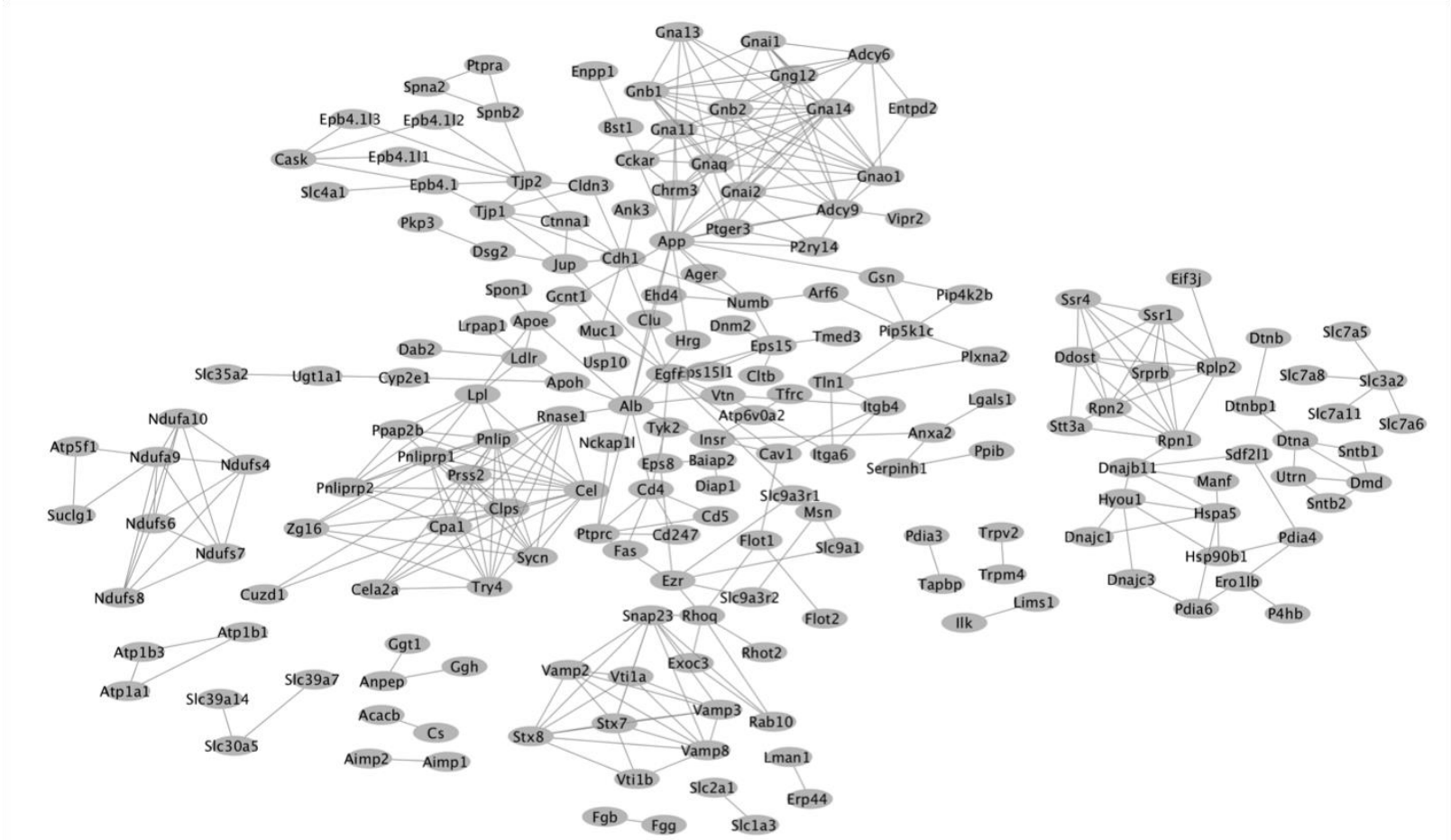

Figure 3.5.4: The heparin-binding putative protein interactome in normal pancreas (NP) constructed using Cytoscape 2.8.1. 'Nodes' or HBPs are coloured grey. Grey lines connecting the HBPs denote 'edges' or interactions.

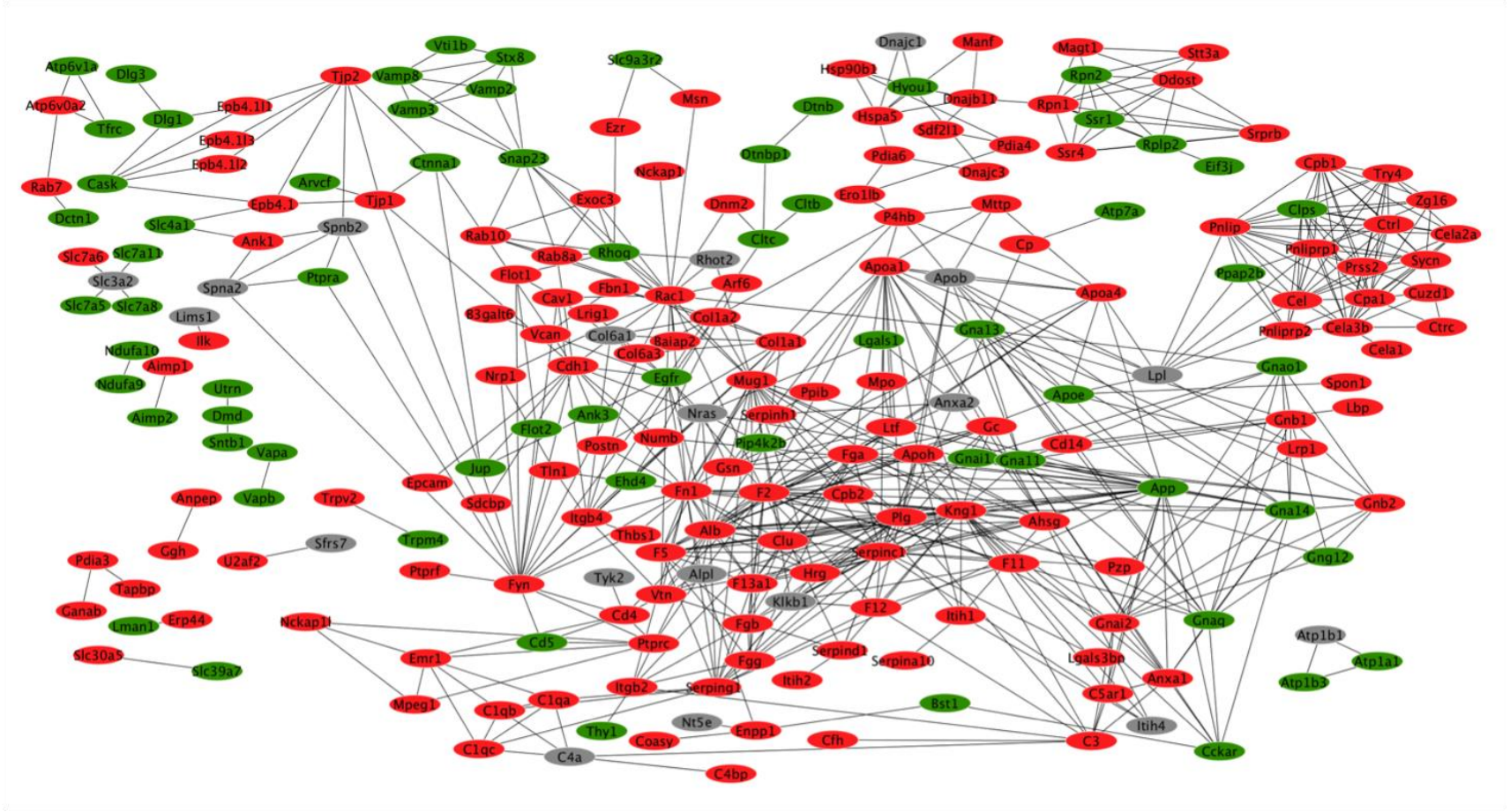

Figure 3.5.5: The heparin-binding putative protein interactome in acute pancreatitis (AP) constructed using Cytoscape 2.8.1. 'Nodes' or HBPs are depending on their fold change value, based on label-free quantification. The HBPs that are under expressed in AP appear in green and those that are over expressed appear in red. Grey lines connecting the HBPs denote 'edges' or interactions.

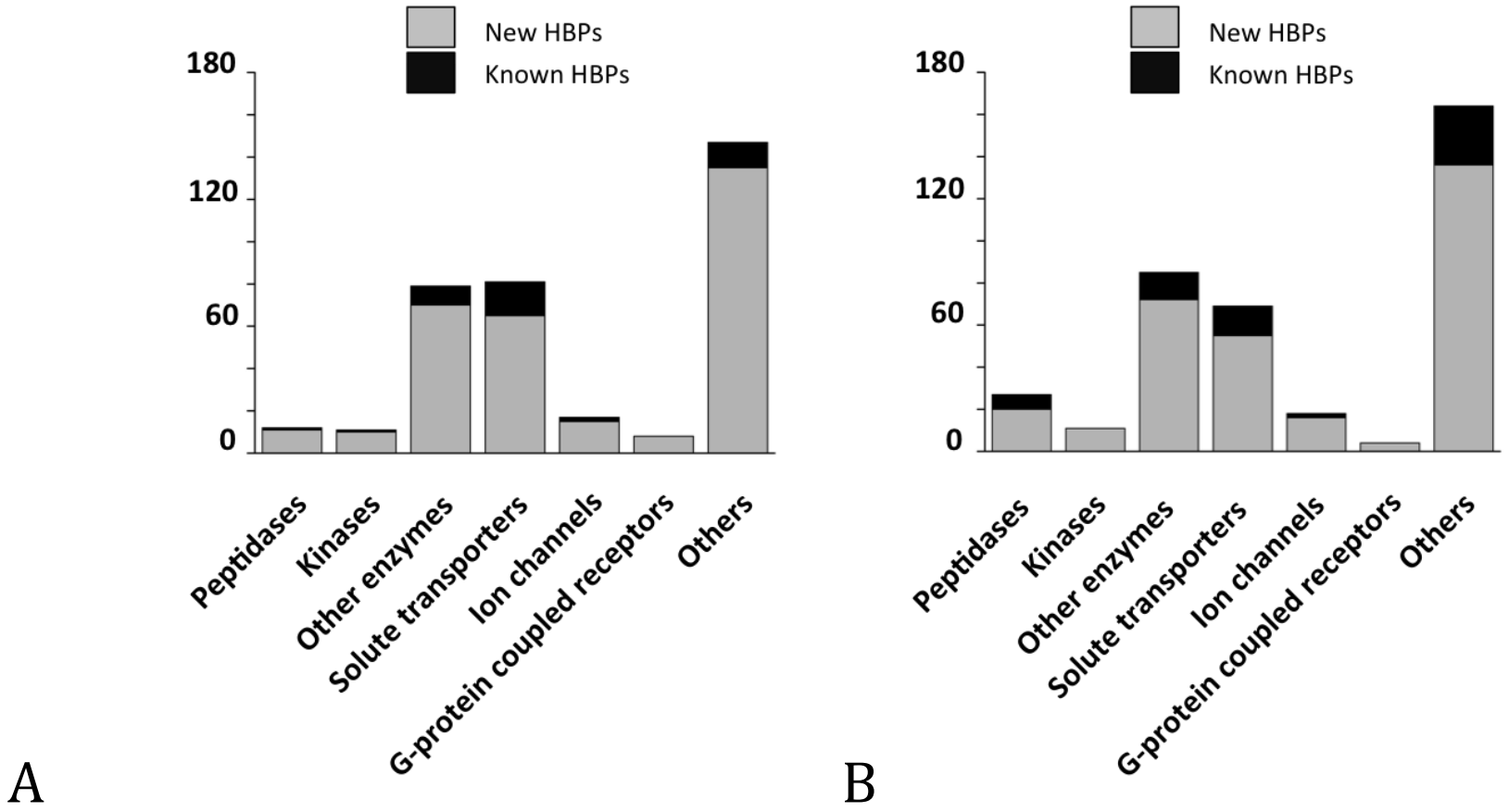

Figure 3.5.6: Functional groups of HBPs in (A) Normal pancreas (NP) and (B) Acute pancreatitis (AP). HBPs constitute important functional groups in NP and AP. A number of HBPs not previously known to bind to heparin have been identified in this study.

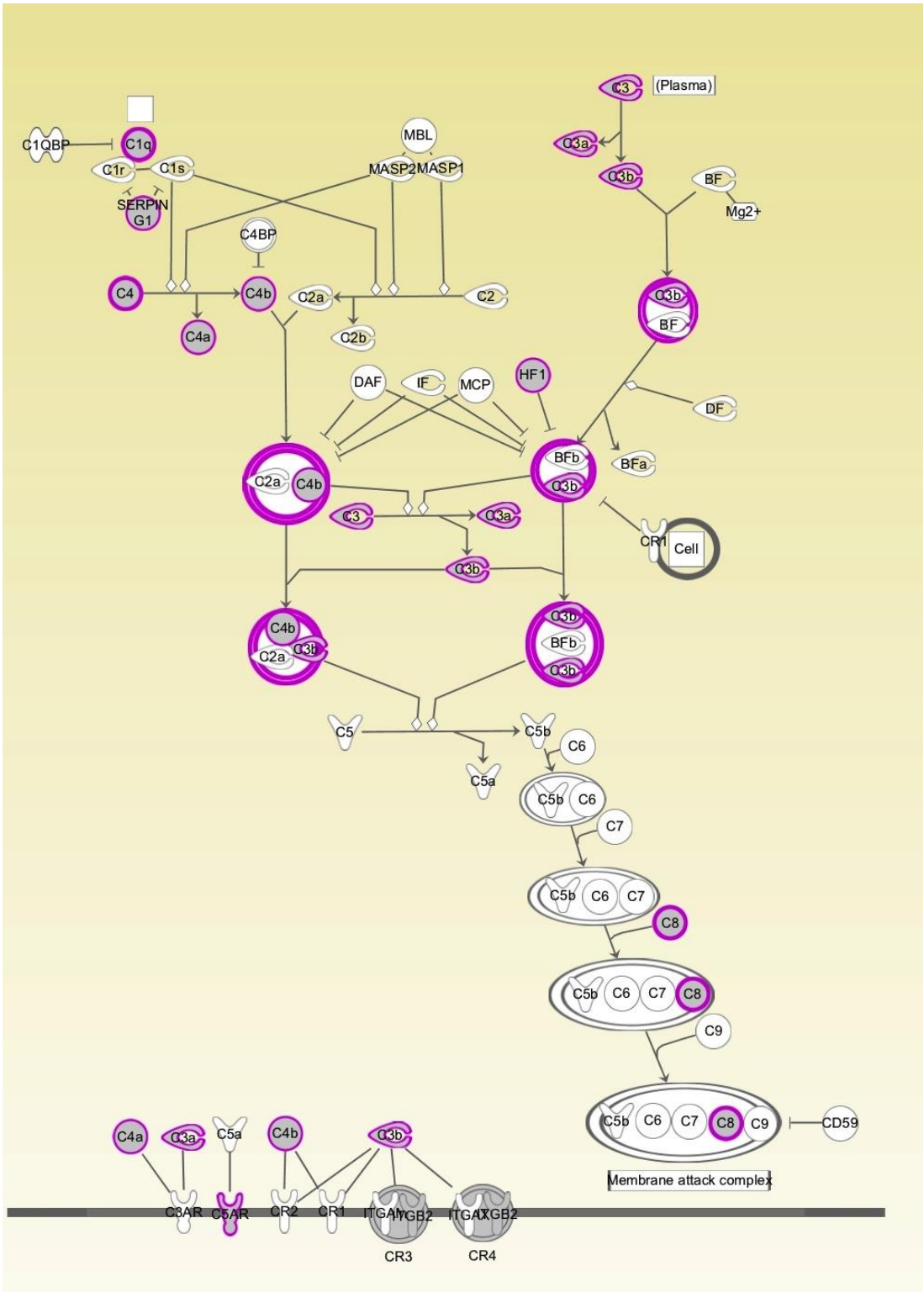

Figure 3.5.7: Canonical pathway analysis in AP. 'Acute phase Reaction' is one of the top canonical pathways enriched to the AP HBP dataset, using Ingenuity Pathways Analysis. The nodes outlined in magenta are HBPs from the AP dataset.

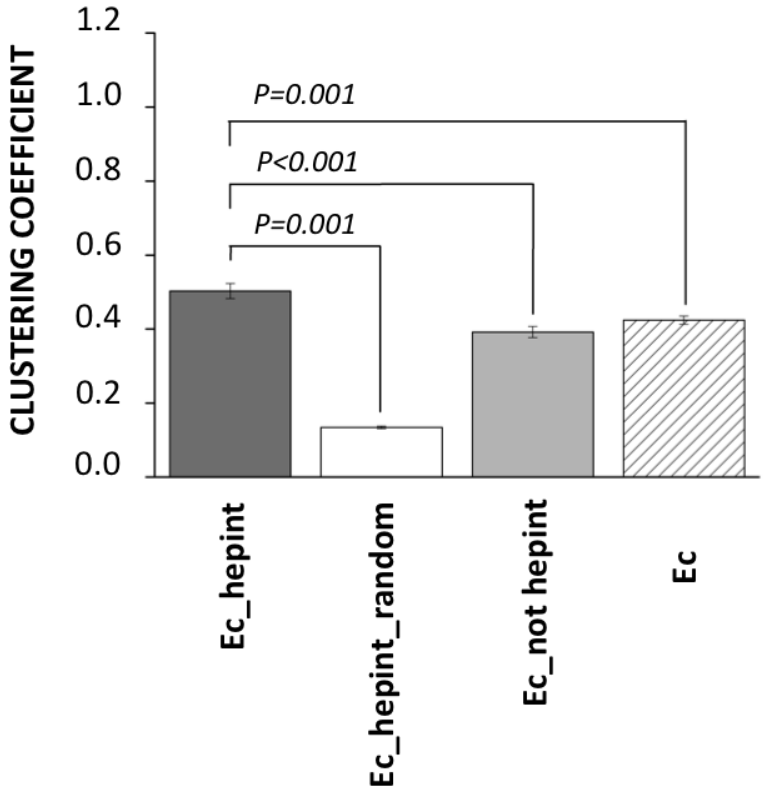

Figure 3.5.8: The relevance of the global heparin-binding protein interactome in the extracellular space. Comparison of the clustering coefficients of the global extracellular heparin binding protein interactome (Ec_hepint), corresponding random networks (Ec_hepint_random), the extracellular non heparin-binding protein interactome (Ec_not hepint) and the whole extracellular protein interactome (Ec). The Ec_hepint is a densely interconnected module in the extracellular space with a significantly higher clustering coefficient as compared to that of the Ec_hepint_random, Ec_not hepint and Ec.

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3.6 Comparison between transcriptomics and proteomics studies

A comparison of the whole mRNA and proteomic datasets from the 2 studies showed that there were only 193 candidates common in NP and 17 common in AP respectively (Fig. 3.6A). When comparing the HBP datasets from the proteomics and mRNA studies in NP and AP, there were 17 common in NP and 3 in AP respectively (Fig. 3.6B). This demonstrated that mRNA was not representative of protein in most cases as has been shown previously (Schwanhausser et al., 2011).

	Proteomics	mRNA	Common
NP	1602	1258	193
AP	1866	103	17

Table 3.6. *Comparison between whole mRNA and proteomics datasets in NP and AP*. The table outlines the common candidates in NP and AP from the 2 studies.

	Proteomics	mRNA	Common
NP	396	115	17
AP	419	31	3

Table 3.6B. *Comparison between HBP mRNA and proteomics datasets in NP and AP*. The table outlines the common candidates in NP and AP from the 2 studies.

3.7 Discussion

The aim of the present work was to determine if extracellular HBPs were functionally associated with physiological and pathological processes in the pancreas and in acute pancreatitis. As the focus of this work was on extracellular HBPs, intracellular proteins were excluded from the analysis. However, further to the bioinformatics pipeline that was used, a number of intracellular proteins that bind to HS, but which have a documented extracellular presence were included. These proteins may have possible 'moonlighting' roles. In contrast to recent work (Ori et al., 2011), a single elution

at 2 M NaCl was used to recover HBPs, the rationale being that the concentration of NaCl required for elution does not necessarily correlate with affinity for HS (Xu et al., 2012). The present work has more than doubled the number of HBPs (Ori et al., 2011). This may be due to the new source of tissue used with its associated disease state, the purification of a plasma membrane enriched sub-proteome and the depth of mass spectrometry analysis used. Even though the resultant heparin-binding proteome is much larger, the protein-protein interacting network retains the properties of the earlier, smaller interactome. It is highly interconnected, with HBPs forming important regulatory modules in the extracellular space. A number of biomarkers that have been previously investigated in AP, such as carboxypeptidase B and pancreatic amylase, were identified in AP dataset. LBP, an acute phase reactant, with a rise and fall in serum that is similar to CRP (Rau et al., 2003) and which is protective in AP, was identified to be over expressed in the AP dataset. FN1 and CD14, which are highly increased in AP relative to NP and also enriched to the 'Hepatic Fibrosis / Hepatic Stellate Cell Activation' pathway may thus have potential as a pancreatic stellate cell-specific biomarker and drug target in the progression of AP to chronic pancreatitis. HRG has been shown to recruit IgG to facilitate clearance of necrotic cells by phagocytes (Poon et al., 2010). Neutrophils have been shown to play an important role in AP, activating the complement system and promoting lung injury (Awla et al., 2012, Shrivastava and Bhatia, 2010). HBPs such as PIP5K1C that participate in these canonical pathways regulate neutrophil adhesion by facilitating RhoA GTPase and integrin activation through chemoattractants (Xu et al., 2010a). Small molecule inhibitors of PIP5K1C have been investigated in the treatment of other diseases

(Wright et al., 2014) and may be explored in the treatment of AP. SERPINC1, has anti-inflammatory properties and has been shown to improve acute pancreatitis in the rat model (Hagiwara et al., 2009). Thus, its presence among the HBPs whose expression is increased to the greatest extent may reflect a normal physiological reaction to the insult of AP.

The HBPs define a group of proteins that have a clear functional importance in cell communication in homeostasis and in AP. By virtue of binding to heparin and being extracellular, the HBPs of pancreas and AP are likely to yield much-needed biomarkers and targets for therapy.

Chapter 4 – Discussion and future perspectives

4.1 Cell communication and HBPs

A genome-wide analysis by Vogel and Chothia investigated the correlation between the expansion of superfamilies with the expansion in biological complexity in 38 unicellular and multicellular organisms (Vogel and Chothia, 2006) . They used a database of 1219 hidden Markov models, based on the structural classification of proteins (SCOP) classification of domains, called Superfamily, to map the occurrence of the different superfamilies (Gough et al., 2001). For each genome, they annotated one-domain proteins and the individual domains of multi-domain proteins to their respective superfamily. They calculated the abundance of each superfamily, as the number of proteins that contain at least one domain belonging to that particular superfamily. Normalised abundance profiles were then used to calculate a Pearson Correlation coefficient 'R', which described the correlation between superfamily abundance and the estimated number of cell types per genome. In order to establish a link between domain functions and organismal complexity, functional categories were assigned to each superfamily. They observed that 194 superfamilies have a strong positive correlation ($R \geq 0.8$) with organism complexity. However among these, 2 functional categories were responsible for a disproportionately high contribution, which amounted to nearly half of the positively correlated superfamilies. The 2 functional categories are superfamilies associated with extracellular processes (20 %) and regulation (29%) (Vogel and Chothia, 2006). Similarly, Ori et al. showed that the

correlation is even stronger for superfamilies that are associated with the heparin interactome and that HBPs form important regulatory modules in the extracellular space (Ori et al., 2011). Subsequently, it has been shown that HBPs form important regulatory modules in the pancreas and in major pancreatic diseases (Nunes et al., 2013). This was supported by the meta analysis of mRNA expression data, though such an approach rests on several assumptions (Section 2.4). For this reason, HBPs were analysed directly by affinity proteomics. The structure of this section is wrong. In general, it is better to put key findings first and then discuss their validity and reliability, limitations and implications.

4.2 Advance in HBP identification

Although nearly 30% of naturally occurring proteins are predicted to be embedded in biological membranes, membrane proteins have been traditionally understudied due to difficulties in solubilisation and separation (Tan et al., 2008). The work carried out as part of this thesis has more than doubled the number of proteins identified as binding to heparin. This is a significant advance in HBP proteomics and may be due to a number of factors. One is the new source of tissue and an associated disease state. Another is the streamlining of the HBPs purification. Previously (Ori et al., 2011), these were split into three elution groups: low (0.15 M to 0.4 M NaCl), medium (0.4 M NaCl to 0.6 M NaCl) and high (0.6 M NaCl to 2 M NaCl). However, it was noted that many proteins were found in two or more of these fractions and so this would dilute the sample. This would increase the possibility of loss during subsequent sample processing. In addition, the samples necessarily contained Triton-X-100 to

solubilise membrane proteins and the detergent had to be removed before mass spectrometry, as it is polydisperse and generates a lot of artefactual signal in the spectrum. Originally this was achieved with a C18 Zip-tip, which is likely to result in considerable losses (Stewart et al., 2001). This step was replaced by trichloroacetic acid (TCA) precipitation, which was validated by analysis of Coomassie stained gels in terms of efficacy. While not perfect this is likely to be an improvement (Polson et al., 2003, Jiang et al., 2004). Whereas the mass spectrometer used by Ori *et al.* (Ori et al., 2011) was an Orbitrap Velos, the instrument used for the present work was a QExactive which is housed in the University's Protein Function Group and is carefully maintained and calibrated. This would increase the depth of the analysis. Finally, a more sophisticated bioinformatics pipeline was used in the present work (Section 3.1), which would increase the number of HBPs.

However, there are caveats associated with this dataset. One is that in some instances, a protein that has been identified as an HBP may in fact be bound to another HBP rather than directly to heparin. If such an interaction had a sufficiently slow dissociation rate, the protein would be carried through the purification. Such an interaction would be consistent with biological functionality, because of its long lifetime and would consequently produce a false positive. Direct heparin binding will need to be demonstrated in the newly discovered HBPs, but given the size of the dataset, this information will only be produced slowly by current biophysical methods, e.g., which require a one protein at a time analysis (West et al., 2005). Sample preparation assumes that Triton-X-100 will efficiently solubilise all HBPs. However, those HBPs in large

macromolecular assemblies, associated with matrix, membrane domains or cytoskeleton may not be solubilised, and so would not figure in the analysis. It is also assume that all HBPs will bind the heparin column. This seems reasonable, but there are no data. For example, if a HBP binds a structure found in HS, but not in heparin, then it could not be identified. There is a precedent for this, proteins that bind to sequences containing a free amino group such a cyclophilin-B (Vanpouille et al., 2007). Heparin affinity columns are usually made by reacting these free amines with an activated column matrix, and so such proteins will not be retained on the heparin affinity column. In addition, the plasma membrane/matrix fraction contains both HBPs and the resident HS proteoglycans, so during the affinity chromatography there is likely to be competition between the heparin on the column and the HS of the proteoglycans. Some HBPs may exchange repeatedly between the immobilised heparin and the HS in the mobile phase and only those that bind preferentially to heparin will be retained. Thus, the affinity chromatography selects for proteins that engage efficiently with structures present in the classic trisulfated disaccharide (IdoA, 2S, GlcNS, 6S) repeat of heparin (75% of is disaccharides).

These technical issues clearly need addressing, alongside a further question: does binding to heparin have any physiological significance without further evidence? This is the focus of Section 4.5.

4.3 HBPs and the pancreas

The roles of HBPs in pancreas homeostasis and AP are discussed in Sections 3.2 and 3.3. The pancreas has important endocrine and exocrine functions and

plays an important role in in glucose homeostasis (Shih et al., 2013). HBPs play important roles in biological pathways and functions such as ion transport, cellular movement, adhesion, and mitochondrial function etc. underlying pancreatic homeostasis. Acute pancreatitis is a leading cause of hospital admissions and the severe form of the disease is associated with a high mortality (Wu and Banks, 2013, Schneider et al., 2010). In the absence of definitive treatment, the management remains mainly supportive. Also, no single laboratory marker or score can accurately predict outcome in AP (Mounzer et al., 2012). A number of HBPs have been identified, such as HRP, CD14 and FN1, that may be explored alone or in combination with existing biomarkers and scoring systems. These need to be validated using high quality samples with the associated clinical data from patients with acute pancreatitis. By virtue of binding to heparin, the HBP sub-proteome is easily accessible in tissue and body fluids, which is particularly important in biomarker and drug development. Interestingly, the top canonical pathway that the new global HBP interactome enriches to is the "Hepatic Fibrosis / Hepatic Stellate Cell Activation". Hepatic and pancreatic stellate cells exhibit similarities of morphology and function and are activated by common cytokines and growth factors, and may share a common origin (Buchholz et al., 2005). Experiments relating to pancreatic stellate cells indicate that they play an important role in AP (Vonlaufen et al., 2007). HBPs such as VEGFA and FGF2 that enrich to this canonical pathway may be used in developing pancreatic stellate cell-specific therapies. Other HBPs that may be explored in drug development in AP include SERPINC1 (Hagiwara et al., 2009), which has potent anti-inflammatory

properties and PIP5K1C that has been investigated in other diseases (Wright et al., 2014).

4.4 Moonlighting roles of HBPs

The heparin affinity proteomics approach identified a number of non-canonical extracellular HBPs such as NDUFS4, NDUFS6, NDUFS7, NDUFS8, NDUFA9, NDUFA10 in NP, and NDUFA9 and NDUFA10 in NP and AP. These are nuclear-encoded mitochondrial proteins. Sub-cellular fractionation using differential centrifugation removes mitochondria efficiently and it seems unlikely that these HBPs are simply contaminants with polyanion binding properties that allow them to interact with heparin. In support of this argument is the fact that nuclear encoded mitochondrial nucleic acid binding proteins were not identified. Glycosylated isoforms of a number of nuclear encoded mitochondrial proteins have been identified at the plasma membrane (Burnham-Marusich and Berninsone, 2012, Moser et al., 1999, Vantourout et al., 2008, Yonally and Capaldi, 2006). Nuclear proteins such as histones and HMGB1 (high-mobility group box 1), as well as cytosolic proteins, which are found extracellularly, under conditions of cell stress, are termed DAMPS (damage associated pattern molecules) (Agalave and Svensson, 2014, Krysko et al., 2012). HBPs with a clearly established classic subcellular localization and which are subsequently found associated with other organelles or extracellularly, have been termed to be 'moonlighting'. 'Moonlighting proteins' are being increasingly identified and studied with a view to unraveling their roles in biological processes as well as in drug development. The enzyme adenosine deaminase has been shown to act as

a catalyst for intracellular and extracellular purine metabolism, as well as a costimulator for the proliferation of T-cells (Cortes et al., 2015). G protein coupled-receptors (GPCRs) heteroreceptor protomers act as moonlighting proteins and are being investigated as targets in neurotherapeutics (Fuxe et al., 2014). Computational methods are also being used to characterize moonlighting proteins (Khan and Kihara, 2014, Khan et al., 2012, Gomez et al., 2003, Gomez et al., 2011). The NDUF proteins have therefore been included as extracellular HBPs, though their extracellular localisation needs documentation by orthogonal means.

4.5 The non-extracellular HBPs

In both NP and AP, approximately 75% of proteins identified by mass spectrometry were deemed not to be extracellular. In due course, evidence may accrue to suggest that some of these may turn out to be moonlighting proteins. However, this is likely to only explain the presence of some of these proteins in the heparin-bound fraction. Polyanion-binding proteins from cell lysate have been analysed previously (Jones et al., 2004). In this work no subcellular fractionation was performed and only 3 proteins were extracellular. The large numbers of intracellular polyanion binding proteins derive this property from interactions with nucleic acids and phospholipids, including phosphoinositols, and anionic surfaces on proteins e.g. transcriptional enhancer factor TEF-1. In the present work, a plasma membrane fraction was applied to the heparin affinity column; intracellular proteins binding to the membrane would be expected to be contaminants.

However, classing these proteins as simple contaminants may miss important functional associations and is not necessarily the best explanation for the presence of other intracellular proteins. For example, a considerable number of cytoskeletal and intracellular signalling-associated proteins were identified Some of these may be present due to physical association with plasma membrane receptor systems, which are themselves engaged with extracellular heparin-binding proteins. That there are differences in these proteins between NP and AP may be reporting of fundamental differences in cell adhesion and cell signalling, though this would require a fully quantitative measurement of proteins to establish the biological significance of such differences. Other proteins may simply relocate during tissue homogenisation, from one polyanion to another. However, this would require that they are relatively weakly bound, able to exchange and bind more strongly to either plasma membrane phospholipids or extracellular HS.

There is also an intriguing possibility that some of the of intracellular proteins identified in the present work may in fact be HBPs. This is because of the growing evidence for the translocation of HS from the extracellular space to the nucleo-cytoplasmic compartment. Early papers used heparin in procedures to stain nuclear proteins without having to hydrolyse DNA or to purifiy nuclear components (Labelle and Briere, 1971, Courvalin et al., 1982, Bornens, 1973). Possibly building on the idea that nuclear proteins bind heparin, pioneering work indicated that HS was present in the nucleus of cultured hepatocytes and involved in the regulation of their growth (Ishihara et al., 1986, Fedarko and Conrad, 1986, Fedarko et al., 1989). This remained a curiosity, until proteoglycan core proteins were characterised and new tools were developed.

There has since been a steady growth of papers relating to the presence of intracellular HS and HSPGs in a variety of contexts, such as translocation mechanisms from the extracellular space to the nucleus, sperm nuclei decondensation, esophageal keratinocyte differentiation and cancer (Richardson et al., 2001, Cheng et al., 2014, Romanato et al., 2008, Kobayashi et al., 2006, Purushothaman et al., 2011, Cheng et al., 2001). Allied to this work, is the growing body of evidence for the translocation of extracellular HBPs to the nucleus from the extracellular space, for example FGFs and their tyrosine kinase receptors, FGFRs (Coleman et al., 2014a), which may be particularly relevant to pancreatic stellate cell function and pancreatic cancer (Coleman et al., 2014b). Thus, at least some of the intracellular proteins identified here may be genuine HBPs, in that binding to intracellular HS affects their function and so cell signalling. However, it is not possible at present to distinguish adventitious binding to heparin of intracellular proteins during the affinity chromatography step from physiologically relevant binding.

4.6 Future perspectives

The present work has identified a number of areas for further investigation.

4.6.1 Biomarker development

4.6.1.1 Development of high through put measurement of HBPs

Immunoassays are the gold standard to measure protein/peptide targets due to their high sensitivity, high throughput and cost-effectiveness (Lequin, 2005). However, the development of these for new targets is expensive and would be

an unattractive proposition for candidate evaluation in which a high attrition rate may be anticipated. Multiple reaction monitoring (MRM) is an emerging MS high throughput approach and uses specific peptides as surrogates of proteins (Huttenhain et al., 2009). The ability of multiplexing with MS enables the rapid and cost-effective evaluation of a large list of candidate biomarkers and may facilitate the development of personalized medicine strategies in treating disease (Percy et al., 2014).

4.6.1.2 Serum biomarker development

Identification of putative candidates in clinical samples is an important first step in biomarker development. Serum or plasma is routinely analyzed in the clinical laboratory for biomarkers. However, these are the most challenging samples to analyze by proteomic techniques, as serum has a wide dynamic range of protein concentrations (Anderson and Anderson, 2002). This wide dynamic range allows for identification of high and medium abundance proteins, while low abundance proteins are often overlooked. Modification of sample preparation with enrichment techniques such as affinity chromatography to isolate the heparin-bound fraction may help reduce the sample complexity and obviate this problem in the case of HBPs.

4.6.1.3 Validation of potential biomarkers

Validation has to be done to make sure the putative biomarkers are specifically associated with AP. Before these biomarkers can be developed into useful clinical assays, they have to be validated and their sensitivity and specificity

needs to be established. Samples should be obtained not only from AP patients and healthy donors, but also from patients who have similar diseases and a broad range of individuals within the population. This requires a very large number of appropriately collected and documented clinical samples. The NIHR Liverpool Pancreas Biomedical Unit AP biobank stores high quality samples from patients with AP and healthy volunteers. It is ethically approved and functions within HTA, GCP (Good Clinical Practice) and GCLP (Good Clinical Laboratory Guidelines) and so would provide the source of material for such further studies.

The data presented in this thesis indicated that the proteins in Table 3.5.1 are the most likely candidates for the identification of biomarkers. This is based on their relative expression in AP *versus* NP and on the knowledge that these are secreted proteins, rather than membrane- or matrix-associated.

4.6.2 Drug development

As noted in Section 3.5.6, the protoemics analysis has identified a number of HBPs that are more highly expressed in AP for which there are existing drugs, either approved or undergoing clinical trials for other diseases. Thus, repurposing trials in AP may lead to improved patient outcome.

There is the possibility of a very different approach, which draws inspiration from regenerative medicine strategies of some Glycotech companies. These have targeted HBPs using chemically modified heparins and heparin mimetic polymers. For example, Regenerative agent (RGTA) is a carboxymethylated

dextran that is statistically sulfated, which is used for ocular and dermal wound healing (the latter includes lower limb ulcers of diabetics) to treat lower limb ulcers of diabetics with some success. These polymers do not have a mechanism mode of action, and this may underlie their efficacy: they target multiple HBPs and so may be able to reduce, for example, the activity of DAMPs while promoting the activity of HBPs that drive tissue regeneration. The definition of the statistical substitution that is clinically effective has hitherto been empirical; their application in a trial in AP similarly has to determine which range of sulfation is effective. However, the further development of the systems analysis of HBPs in this thesis alongside a structural analysis of the heparin binding motifs using high throughput methods such as differential scanning fluorimetry (Uniewicz et al., 2010, Xu et al., 2012) may provide a means to define prior to embarking on a trail the likely sulfation substitution of these polymers that will be effective.

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Appendix I

List of supplementary files (please refer to attached CD)

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