Investigating Notch and Related Pathways

for New Biomarkers in Pancreatic Cancer

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By

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This thesis is dedicated to my parents

Professor Abul Fazal Muhammad Masood (1935 – 2014), known as the
"Father of Paediatric Surgery in Bangladesh"
And my mother
Dr Meherun Nessa Masood

"Whatever good I have done in my life so far is to make these two
wonderful human beings proud of me"

Glory be to Almighty God. Without His blessings, it would not have been
possible for me to complete this thesis

ABSTRACT

Investigating Notch and related pathways for new biomarkers in pancreatic cancer

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Pancreatic ductal adenocarcinoma (PDAC) is the 5th commonest cause of cancer related death in the UK with a 5-year survival rate of only 4%. This is due to vague symptoms, late presentation and lack of availability of decent biomarkers, making most PDAC unsuitable for curative resections at the time of diagnosis. Therefore, there is an urgent need for new biomarkers.

Previous work in our lab has shown that Notch 1, Notch 3 and Notch 4 signalling pathway are upregulated in PDAC compared to normal pancreas. The aim of this this study was to examine the Notch signalling pathway and related miRNAs as potential biomarkers for PDAC.

In this thesis, we describe the method development of a process of detecting Notch Nβ-fragments in the plasma of human at concentrations of 121.67 fmol/µL. Notch Nβ-fragments are released between the two cleavage sites S3 and S4 as part of γ-secretase mediated cleavages following ligand activation. We could detect Notch 3 Nβ-fragments in plasma of PDAC and healthy volunteers but were unable to find any statistically significant differences between them. We were unable to detect Notch 1 Nβ-fragments.

We investigated Notch 2 expression in human tissue samples using immunohistochemistry and found Notch 2 to be upregulated in resectable PDAC and further upregulated in non-resectable locally advanced cancer, but down regulated in metastatic PDAC tissue in comparison to non-resectable locally advanced tissue. This suggests the role of Notch 2 proteins as diagnostic and prognostic biomarkers.

Using RT-PCR we identified miR-200c, miR–155a and miR–375 which could help differentiate between the plasma of healthy volunteers, PDAC and chronic pancreatitis (CP). Adding age to a binary logistic regression model we could differentiate between PDAC and CP achieving ROC curve values of above 0.95. This suggests the role of these miRNAs as diagnostic biomarkers.

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ABBREVIATIONS

ABC	Avidin-Biotin complex
ADAM/TACE	A desintegrin and metallopeptidase/tumour necrosis factor α converting enzyme
AGO	Argonaute
APP	Amyloid precursor protein
CADASIL	cerebral autosomal dominant arteriopathy
cDNA	Complementary DNA
CHCA	α- cyano-hydroxy cinnamic acid matrix
CLL	Chronic lymphocytic leukaemia
CP	Chronic pancreatitis
CSL	CBF1, Suppressor of Hairless, Lag1
DMP	Dimethyl Pimelimidate Dihydrochloride
DNA	Deoxy ribonucleic acid
DSL	Delta/Serrate/LAG -2
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ERCP	Endoscopic retrograde cholangiopancreatography
ESI	Electrospray ionisation
ESI-MS	Electrospray ionisation mass spectrometry
EUS	Endoscopic ultrasound
FDP	Freeze dried pellets
FNA	Fine needle aspiration
GSI	Gamma secretase inhibitor
HIAR	Heat-induced antigen retrieval

HV	Healthy volunteer
HRP	Horseradish peroxidase
IA-MS	Immunoprecipitation using antibody bound dynabeads followed by MS analysis
IHC	Immunohistochemistry
IMS	Industrial Methylated spirit
LSAB	Labelled Streptavidin Biotin
mRNA	messenger RNA
miRNA	micro RNA
MS	Mass spectrometry
MALDI	Matrix-assisted laser desorption ionisation
MALDI-TOF	Matrix-assisted laser desorption ionisation time of flight
NEXT	Notch extracellular truncation
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NICD	Notch intracellular domain
NRARP	Notch regulated Ankyrin repeat protein
PanIN	Pancreatic intraepithelial neoplasia
PDAC	Pancreatic ductal adenocarcinoma
PCR	Polymerase chain reaction
RAM	RBP-jk associate molecule
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RT-PCR	Real time polymerase chain reaction
SKIP	Ski-interacting protein
SPE	Solid Phase Extraction
TAD	Transcriptional activator domain/transactivation domain

T-ALL	T cell acute lymphoblastic leukaemia/lymphoma
TBS	Tris Buffered Saline
TFA	Trifluroacetic acid
TGF–β	Transforming growth factor beta
VSMC	Vascular smooth muscle cells

CHAPTER 1.

INTRODUCTION

1.1 Pancreatic Cancer

Pancreatic cancer (PANC) is the 5th most common cause of cancer related deaths in the UK (Cancer Research UK, 2016). This is despite it being the 10th most common cancer and making up only 3% of all cancer cases in the UK. 4716 men (50%) and 4692 women (50%) were diagnosed with the disease in 2013. (Cancer Research UK, 2016) Despite advances in chemotherapy and radiotherapy, the only definitive curative option is surgical resection. However due to factors such as late presentation, aggressive local spread and early metastasis, only 10% of tumours are amenable for surgical resection at the time of diagnosis. (Stocken *et al*, 2005) 79% of patients with known staging of pancreatic cancer at diagnosis, present with advanced disease (stage 3 & 4) unsuitable for curative resection. PANC is also a cancer associated with aging, and on average each year almost half of the patients (47%) diagnosed with this cancer in the UK are above 75 years old (Cancer Research UK, 2016).

Complete resection of the cancer involves a Whipples procedure (also known as a pancreaticoduodenectomy), which also carries an operative mortality rate of approximately 1 to 16% (National Cancer Institute, 2016). Thus, pancreatic adenocarcinoma has one of the worst prognoses of all gastrointestinal tumours, with a 5-year survival rate of between 0.4- 4%. The European Study Group for Pancreatic Cancer (ESPAC) 1 trial showed that median survival for patients with curative surgery and adjuvant chemotherapy was 20.1 months and the 5-year survival rate of these patients are 29% (Ghaneh *et al*, 2008; Stocken *et al*, 2005).

The majority (95%) of pancreatic cancers develop as Pancreatic Ductal Adenocarcinoma (PDAC), from the ductal cells of the exocrine pancreas. Other variants

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of the adenocarcinoma include adenosquamous carcinoma, colloid carcinoma, hepatoid carcinoma, medullary carcinoma, signet ring cell carcinoma and undifferentiated carcinoma. (Weledji *et al*, 2016; Ralph and Fukushima, 2007). Tumours of endocrine origin or neuroendocrine tumours as they are commonly known, such as insuloma and glucagonoma, account for a maximum of 5 % of all pancreatic tumours and are usually associated with much more favourable outcomes that the ductal carcinoma of the exocrine origin (National Cancer Institute, 2015). This thesis focuses on pancreatic ductal adenocarcinoma.

Despite advances in radiological imaging techniques, diagnosis remains difficult. In particular, there is the need to differentiate between pancreatic carcinoma and chronic pancreatitis when presented with a pancreatic mass. In the absence of accessible metastatic disease, obtaining tissue for histological diagnosis is often challenging, despite the use of modalities such as endoscopic ultrasound and diagnostic laparotomy. (Kloppel and Adsay, 2009) Patients often proceed to laparotomy and pancreatic resection and the associated morbidity without formal histology. There is therefore a need to develop biomarkers for early detection/differentiation of chronic pancreatitis and pancreatic ductal adenocarcinoma and to aid treatment of pancreatic ductal adenocarcinoma at an early and potentially curable stage.

An understanding of the pathways involved in pancreatic adenocarcinoma progression will allow us to identify new biomarkers as well as identify potential novel therapeutic targets.

1.2 Cell Signalling in Carcinogenesis

We are learning more about the process of carcinogenesis day by day. Carcinogenesis or tumourigenesis, is the process of cancer development from a normal cell. The life history of cancer can be divided into stages. It was initially thought it could simply be divided into initiation (single cell mutation), promotion (mutant cell proliferation) stages. Progression can be described as the additional changes which occur after cancer has formed. (King, 2000; Weinberg, 1989). However, despite the simplicity of the above statement, tumourigenesis has long been thought to be a complex multistep process. Weinburg (1989) described tumourigenesis as a succession of five to six independent rate-limiting steps, each step representing a physiological barrier that must be breached for the cell to progress further towards the end-point of malignancy. Such multiple barriers are necessary to ensure that the successful completion to a tumourigenic event is a rare process (Weinberg, 1989). Fearon *et al* described the first step in tumourigenesis as initial mutational activation of oncogenes coupled with mutational in-activation of tumour suppressor genes. Secondly at least 4 to 5 gene mutations are required for the formation of a malignant tumour. Third, the cumulative effects of the genetic alterations are responsible for determining the tumour's biological properties. They also said that in some cases, tumour suppressor genes tend to exert an effect at the cellular level even in the presence of cancer (Fearon and Vogelstein, 1990).

It was originally suggested by Hanahan and Weinburg in 2000, that cancer progression is the culmination of six essential alterations in cell physiology which collectively dictate malignant growth (Hanahan and Weinberg, 2000). They were:

- Self-sufficiency in growth signals

- Insensitivity to growth-inhibitory (antigrowth) signals

- Evasion of programmed cell death (apoptosis)

- Limitless replicative potential

- Sustained angiogenesis

- Tissue invasion and metastasis

Since then, Hanahan and Weinberg has released the next generation "Hallmarks of Cancer" (Hanahan and Weinberg, 2011) in which they have added two enabling characteristics and two more emerging hallmarks as essential for tumour progression. The enabling characteristics are genome instability and mutation; and tumour-promoting inflammation. The two more hallmarks are:

- Reprogramming energy metabolism

- Evading immune destruction

It is now well established that cells are continuously under the influence of intracellular signalling pathways. It is these pathways which normally targets their effector proteins to carry out the normal activities of the cell. In cancer, certain pathways target proteins which ultimately decide whether a cell proliferates, becomes senescent or dies. In healthy cells these signals are finely regulated to maintain equilibrium. However, if the regulation fails it can lead to abnormal signalling (O'Connor and Adams, 2010). Any signalling changes which lead to uncontrolled proliferation may predispose to cancerous changes by further incorporation of mutations compared to normally proliferating cells. A cascade of changes in several signalling pathways is necessary for a cancer to develop. It is important to understand the sequence of events in intracellular pathways which result in these changes. Understanding the malfunction in different

pathways may enable the identification of targets for novel treatment as well as new biomarkers which could identify the disease whilst still in a potentially curable stage (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011).

Several pathways including KRAS, CDKN2A, TGF-β, TP53, HEDGEHOG, NOTCH, WNT, EGFR FAMILY, NF-κB and MYC have been identified as having roles in pancreatic adenocarcinoma (Muller *et al*, 2007; McCleary-Wheeler, McWilliams and Fernandez-Zapico, 2012). This thesis will examine the Notch Signalling Pathway.

1.3 The Notch Signalling Pathway

The Notch signalling pathway was first discovered in 1917 when strains of *Drosophila* were seen with notches in their wing blades. This was attributed to a gene insufficiency which was later identified as a transmembrane receptor, named Notch. Since the discovery of the involvement of a Notch 1 translocation in human T cell acute lymphoblastic leukaemia, there has been considerable interest in the role of Notch signalling in carcinogenesis (Jun, Stevens and Kaplan-Lefko, 2008).

1.3.1. Notch receptors

The Notch genes encodes members of a family of receptors that mediate short range signalling events. In mammals, there are four Notch genes designated Notch 1, 2, 3 and 4 (Borggrefe and Oswald, 2009). A typical Notch gene encodes a single transmembrane receptor. The extracellular region of the receptor is made of an array of up to 36 epidermal growth factors (EGF)-like repeats, which is involved in ligand interaction. (Borggrefe and Oswald, 2009) There are three juxtamembrane repeats known as Lin-

12-Notch (LN) repeats which modulate interactions between the extracellular and the membrane-tethered intracellular domains (Bolos, Grego-Bessa and De La Pompa, 2007; Bianchi, Dotti and Federico, 2006; Fiuza and Arias, 2007). One of the most important features of Notch is that it acts as a transcription factor as well as a transmembrane receptor. At the cell surface, Notch is present as a heterodimer consisting of extracellular EGF repeats and juxtamembranous LN repeats linked non-covalently by a heterodimerization region to the rest of the molecule (Fiuza and Arias, 2007).
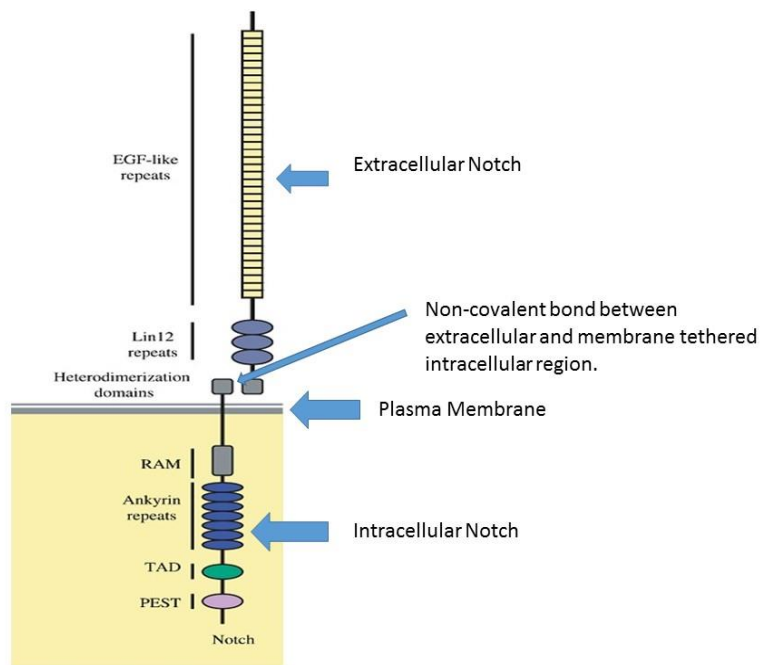
Figure 1.1 Schematic diagram displaying the structure of Notch.
The extracellular Notch is composed of up to 36 EGF-like repeats with 3 juxtamembrane Lin-12 repeats, which is in close proximity with the heterodimerization domain. The heterodimerization domain binds non-covalently, the extracellular Notch with the membrane tethered intracellular Notch. The intracellular Notch has regions called RAM, Ankyrin repeats, a transactivation domain (TAD) and a PEST domain. Adapted from Fiuza and Arias, 2007.

Notch proteins are synthesized as a 360 kDa precursor protein. The C- terminal heterodimerization domain of the extracellular Notch forms a stable complex with the extracellular region of the juxtamembranous Notch. It is this site, the S1 site, which is

the cleavage site of this structure. During maturation, the unprocessed Notch is cleaved at the S1 site by furin-like proteases in the trans-Golgi, yielding a 180 kDa extracellular domain and a 120 kDa fragment consisting of the membrane tethered intracellular domain with a short extracellular sequence (Logeat *et al*, 1998; Baron, 2003).

The intracellular region of Notch has four distinct regions. They include the RAM (RBPjk Associate Molecule) domain, the seven ankyrin repeats, a transcriptional activator domain/transactivation domain (TAD) and the PEST (proline, glutamine, serine, threonine-rich) sequence. The ankyrin repeats are flanked by two nuclear localisation sequences, prior to and following the Ankyrin repeats (Baron, 2003).

1.3.2 Notch Receptor Activation

Notch receptors are normally inactive in the absence of ligands. As previously mentioned, Notch is a single transmembrane protein and the extracellular region is present on the cell surface. The ligands for Notch are also transmembrane proteins thereby making it essential for cell-cell contact to trigger the signalling event (Fiuza and Arias, 2007).

The extracellular portion of the cleaved Notch (S1 site) during the secretory phase undergoes extensive glycosylation. This glycosylation is critical for proper folding of the receptor and its subsequent interactions with the ligands, which are also glycoproteins (D'Souza, Miyamoto and Weinmaster, 2008).

It is well established that Notch receptor activation is mediated by a sequence of proteolytic events (Kopan and Ilagan, 2009). The ligands mediate the interaction with Notch EGF like repeats 11 and 12. This interaction leads to a conformational change within the extracellular Notch that exposes an extracellular metalloprotease site (S2 site). This S2 site is susceptible to cleavage by transmembrane proteases of the ADAM/TACE (a desintegrin and metallopeptidase/tumour necrosis factor α converting enzyme) family. (Fiuza and Arias, 2007) Cleavage at the S2 site results in shedding of the Notch extracellular domain and creates a membrane tethered intermediate called Notch extracellular truncation (NEXT). NEXT is a substrate for γ Secretase, which is a member of a family of intramembrane cleaving proteases. Gamma Secretase activity of a membrane protein complex containing presenilin as the catalytic component then cleaves the transmembrane part of NEXT at two intramembranous sites (S3 & S4). This releases the Notch Intracellular domain which translocates into the nucleus and subsequently regulates Notch gene expression (Roy, Pear and Aster, 2007; Kopan and Ilagan, 2009).

In mammals, presenilin have also been found in large protein complexes with gamma-secretase activity, which cleaves the amyloid precursor protein (APP) in its transmembrane domain. This suggests that the mechanism leading to cleavage of APP and Notch might be related. Abnormal processing of APP has been implicated in Alzheimer's disease (Haass and De Strooper, 1999).
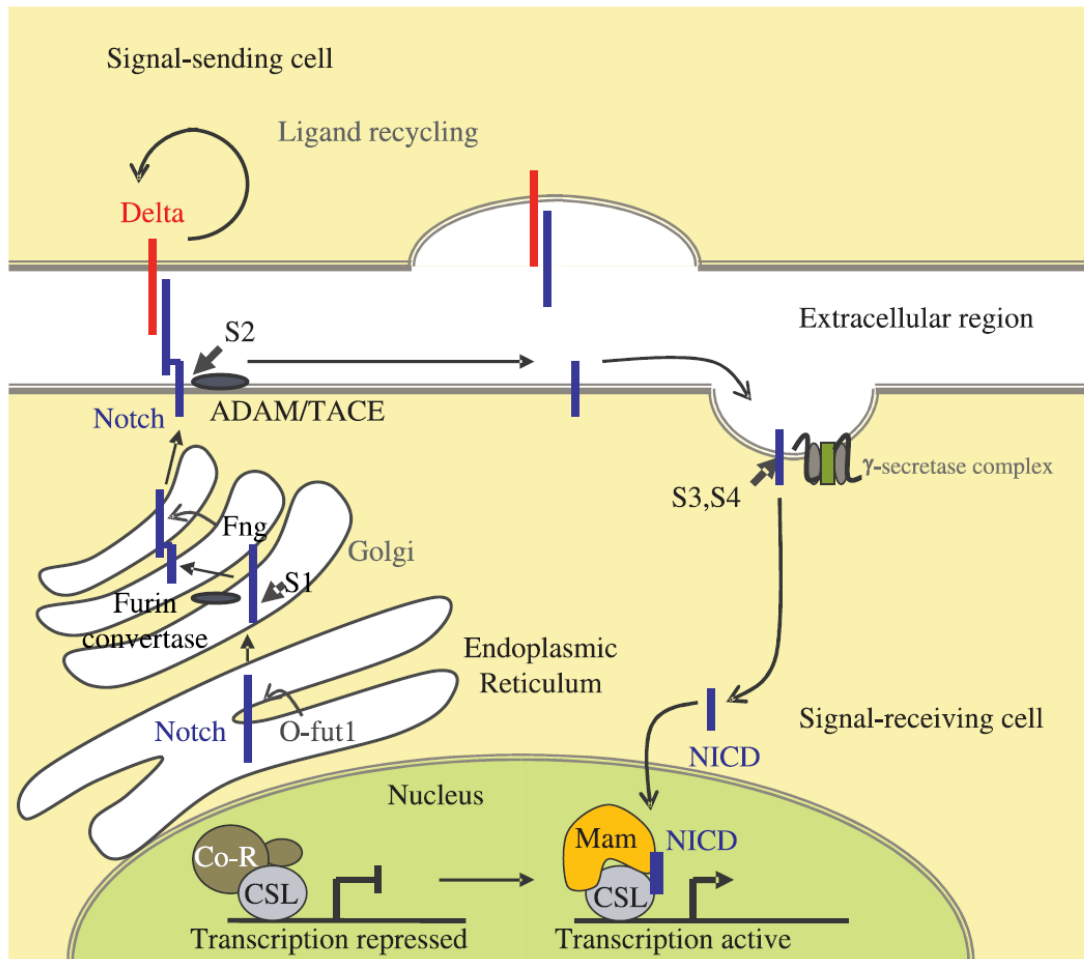
Figure 1.2 Notch signalling Pathway.

S1 cleavage happens at the trans-Golgi apparatus. Ligand binding activates Notch and elicits several steps of cleavage. The first step is the cleavage at the extracellular S2 by proteases ADAM10 or by TACE. Two further intramembranous cleavages, S3 and S4 by γ secretase activity, results in the release of Notch Intracellular Domain (NICD). NICD translocates into the nucleus and recruits transcription factors activating target gene expression. Figure taken from Fiuza and Arias, 2007.

1.3.3 Notch Ligands

As mentioned in the previous section, Notch ligands are also transmembrane glycoproteins and so cell-cell contact is necessary for activation of Notch (Fiuza and Arias, 2007; D'Souza *et al*, 2008). There are two types of Notch ligands in mammals, Delta and Jagged/Serrate, and they both belong to the DSL family of proteins (Fiuza

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and Arias, 2007). The largest types of Notch ligands are characterized by three structural blocks: a N-terminal DSL (Delta/Serrate/LAG-2) motif, specialized tandem EGF repeats called the DOS (Delta and OSM-11-like proteins) domain, and EGF like repeats (Kopan and Ilagan, 2009). DSL is an extracellular cysteine rich region and is present in both Delta and Jagged ligands. DSL interacts with EGF-like repeats 11 and 12 of the Notch receptor protein and subsequently triggers cleavage at the S2 site which results in activation of the Notch signalling pathway (D'Souza *et al*, 2008; Fiuza and Arias, 2007).

DSL ligands are classified according to the presence or absence of a cysteine-rich domain and the presence or absence of a DOS domain. (Kopan and Ilagan, 2009). There are three Delta like proteins (Delta 1,3 and 4) and two Serrate proteins (Jagged 1 and 2). The main structural differences between the Delta and Jagged families are as follow:

- The Jagged ligands contain a greater number of EGF repeats in the extracellular region. They also have insertions within the EGF repeats.
- The Jagged/Serrate ligands also contain a cysteine-rich region, closer to the membrane, that is completely absent from the Delta ligands (Fiuza and Arias, 2007).
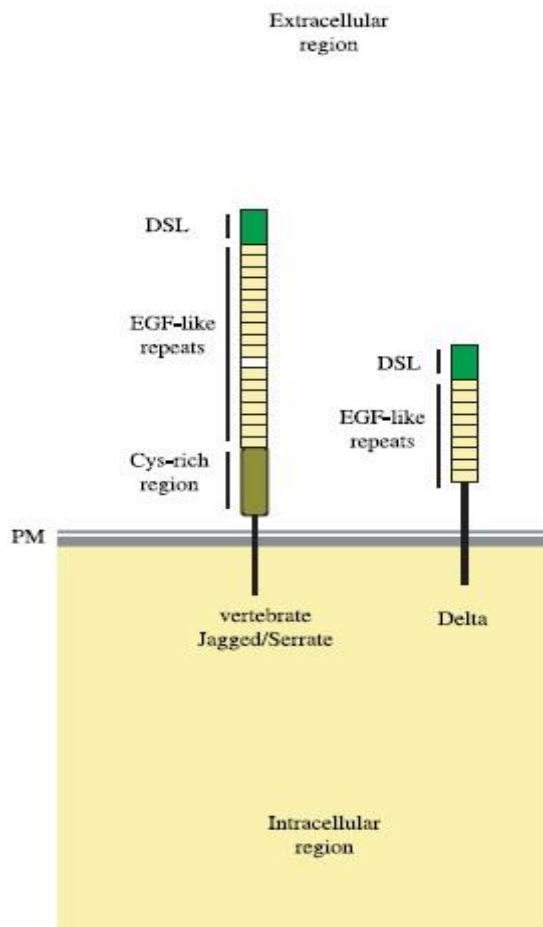
Figure 1.3 Structure of the Notch ligands.

Delta and Jagged/Serrate ligands are composed of an N terminal DSL region responsible for interacting with EGF like repeats 11 and 12 of the Notch receptor. Jagged/Serrate ligands have longer EGF like repeats compared to the Delta ligand and also contains an extracellular cysteine-rich region. Figure taken from Fiuza and Arias, 2007.

1.3.4 DSL ligands as inhibitors of Notch Signalling

DSL ligands can also interact with Notch proteins in the same cell. However, in these circumstances, instead of inducing Notch signalling, they have an inhibitory effect. The molecular basis of this interaction and its effects are not well understood, but it is thought to be important in a subset of Notch dependent developmental events (Klein, Brennan and Martinez Arias, 1997; Fiuza and Arias, 2007). This inhibitory action has

been shown to promote retinal neurogenesis and neurite outgrowth, as well as inhibiting keratinocyte differentiation within epidermal stem cells (Lowell and Watt, 2001; Dorsky *et al*, 1997).

1.3.5 Target Gene Transcription

The interaction of Notch with its ligands and subsequent proteolysis of the intact, membrane-bound Notch receptor results in the release of a soluble fragment consisting of the entire intracellular domain, termed the Notch intracellular domain (NICD). NICD enters the nucleus and participates directly in the transcriptional regulation of target genes (Fiuza and Arias, 2007).

This direct translocation of an active Notch signalling fragment to the nucleus is a unique characteristic about Notch as it does not rely upon multiprotein phosphorylation cascades, second messengers and other signal-relaying mechanisms (Fortini, 2009). Since Notch does not use second messengers, the level of signalling activity is solely dependent on the nuclear concentration of NICD and this is very tightly controlled (Fortini, 2009). The nuclear responses to the NICD translocation are sensitively modulated by several transcriptional mechanisms. The primary effectors of the NICD in the nucleus are transcription factors of the conserved mammalian CBF1/Drosophila Su(H)/C, elegans LAG-1 (CSL) family. CSL is also known as CBF-1 and RBP-Jκ. Suppressor of Hairless (Su(H)) is a DNA-binding protein component of the Notch signalling pathway that is thought to be required for target gene activation. (Fortini, 2009; Oswald *et al*, 2002; Fiuza and Arias, 2007).

In the absence of NICD, CSL proteins bind to specific sequences of the regulator elements of several Notch target genes and act as transcriptional repressors, recruiting histone deacetylase and other corepressors (NCoR/SMRT, MINT/SHARP/SPEN, CIR, Hairless, CtBP, and Groucho/TLE) to form a transcription repressor complex (Fortini, 2009; Bianchi *et al*, 2006; Fiuza and Arias, 2007). Upon ligand induced activation, NICD enters the nucleus and binds to CSL, removes the histone deacetylase/corepressor complex and, together with coactivator Mastermind, forms a transcriptionally active ternary complex (Borggrefe and Oswald, 2009; Fiuza and Arias, 2007; Fortini, 2009). This active complex recruits general transcription factors, promoting increased expression of Notch target genes (Fryer, White and Jones, 2004).

Several Notch target genes have been identified, but the most recognised targets in mammals belong to a family of basic helix-loop-helix (HLH) transcription factors. They are the HES (Hairy-Enhancer of Split) and the Hes-related factor families (HRT, also known as HESR, HERP or CHF; HRP; HEY). These target genes function as transcriptional repressors (Bolos *et al*, 2007; Bianchi *et al*, 2006; Borggrefe and Oswald, 2009). Two other Notch target genes, NRARP (Notch regulated ankyrin repeat protein) and Deltex-1 were shown to be potent negative regulators of Notch signalling. Although NRARP's transcription is activated by Notch signalling, it forms an inhibitor complex with NICD-CSL, thereby functioning as a negative feedback regulator (Yamamoto *et al*, 2001; Yun and Bevan, 2003; Krebs *et al*, 2001).

1.3.6 Degradation of Notch

Once the NICD is generated by irreversible proteolysis of the Notch receptor, the potent NICD signalling fragment can no longer be controlled by ligand binding or cell-surface

events. It is therefore essential that the NICD turnover is tightly controlled to prevent sustained signalling for an inappropriately long time or at very high levels (Kopan and Ilagan, 2009; Fiuza and Arias, 2007). Sustained levels of NICD can be detrimental in mammals with Weng *et al* showed that stabilising the NICD can cause T cell acute lymphoblastic leukaemia (Weng *et al*, 2004).

Thus, in addition to the different regulatory mechanisms on the DSL ligands which primarily control NICD production, optimal levels of NICD are regulated in cells by ensuring that NICD has a very short half-life, lasting in most cases a fraction of the cell cycle (Kopan and Ilagan, 2009). Therefore Notch mediated transcriptional activation is stopped by the degradation of NICD. Protein degradation is a very effective method of signalling regulation and one that is clearly used to keep the levels of NICD just above the functional threshold. (Fiuza and Arias, 2007).

The active CSL-NICD-Mastermind ternary complex, in the nucleus, associates with a protein called Ski-interacting protein (SKIP). SKIP is unique in that it can associate with both the CSL co-repressors and with the CSL- NICD- Mastermind ternary complex (Zhou *et al*, 2000; Kovall, 2007). SKIP and Mastermind recruit kinases that specifically phosphorylate NICD in the TAD and PEST domains (O'Neil *et al*, 2007; Fryer *et al*, 2004; Fiuza and Arias, 2007). Ubiquitination of the phosphorylated sites by Fbw7/Sel 10 ubiquitin ligase leads to NICD degradation. This stops the signalling process until the arrival of new NICD in the nucleus. Addition of ubiquitin by E3 ligases to DSL ligands, is termed ubiquitination, and this modification regulates ligand signalling activity and cell-surface expression (Fryer *et al*, 2004).
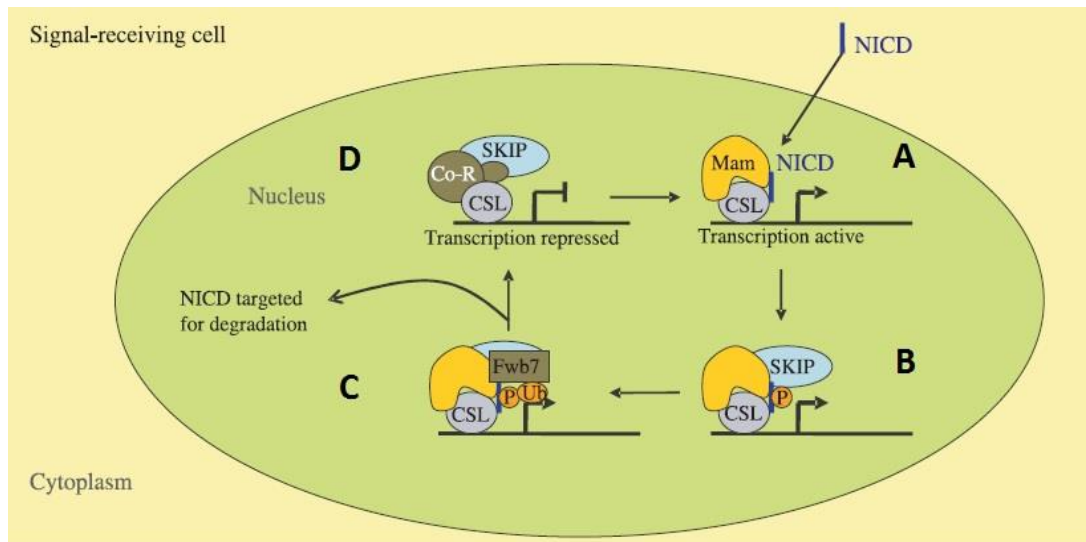
Figure 1.4 Notch signalling is tightly regulated through an efficient process of NICD degradation.
A) Upon ligand induced activation, NICD enters the nucleus and binds to CSL, removes the histone deacetylase/corepressor complex and, together with coactivator Mastermind, forms a transcriptionally active ternary complex. B) The active CSL-NICD-Mastermind ternary complex, in the nucleus, associates with a protein called Ski-interacting protein (SKIP). C) SKIP and Mastermind recruit kinases that specifically phosphorylate NICD in the TAD and PEST domains. Ubiquitination of the phosphorylated sites by Fbw/Sel 10 ubiquitin leads to NICD degradation. D) In the absence of NICD, CSL proteins bind to the specific sequences of the regulator elements of Notch target genes and act as transcriptional repressors, recruiting histone deacetylase and other corepressors to form a transcription repressor complex. Adapted from Fiuza and Arias, 2007.

1.3.7 CSL-independent Notch signalling

There is increasing evidence of a CSL-independent Notch signalling pathway. Shawber *et al.* looked at the differentiation of mouse myoblasts into myotubes and found expression of truncated forms of Notch lacking the ability to interact with CSL but still inhibiting myoblast differentiation (Shawber *et al*, 1996). More work on *Drosophila,* showed that mutant forms of Notch still exhibit gain of function phenotypes during neurogenesis that are independent of Su(H), but dependent on shaggy, which plays a central role in Wnt signalling. This study suggested a functional connection between Notch and Wnt signalling, which other studies have confirmed (Lawrence *et al*, 2000;

Hayward *et al*, 2005; Brennan *et al*, 1999; Brennan *et al*, 1997). Nakhai *et al* found that RBP-Jκ (another name for CSL) has a Notch independent role in pancreatic organogenesis. They demonstrated an essential role for Rbpj, not Notch 1 and Notch 2 in pancreatic organogenesis (Nakhai *et al*, 2008).

Wnt signalling is mediated by β-catenin and it is thought that Notch modulates Wnt signalling by setting up a threshold for the function of β-catenin. For example, Notch has been able to suppress the development of osteoblasts in favour of chrondoblasts by the suppression of β-catenin activity (Deregowski *et al*, 2006; Hayward *et al*, 2005).

1.3.8 Notch in normal tissue development

Notch signalling is used in many developmental events. It has been shown to regulate a broad range of events in both embryonic and post-natal development. These include proliferation, apoptosis, border formation, and cell fate decisions (Kloppel and Adsay, 2009). There are three basic functions of Notch which enable it to exhibit all the phenotypic effects described later in this section (Fiuza and Arias, 2007).

The functions of Notch are:

1. **Lateral inhibition:** This is a notion that during cell development, only some cells adopt their potential out of a group of cells with a common development potential. Cells which adopt the potential suppress the same fate in the others. This is known as lateral inhibition (Gibert and Simpson, 2003). For example, during development, groups of ectodermal cells with a neural and epidermal potential are inhibited by Notch and thereby do not achieve their neural fate

(Chiba, 2006). Similarly, in mammals, lateral inhibition plays an important part in hair and cell development in the inner ear (Kiernan *et al*, 2005; Radosevic, Fargas and Alsina, 2014).

2. **Asymmetric cell fate assignation**: Notch is mainly involved in binary cell fate decisions. One way of involvement is by lateral inhibition and the other is by asymmetric cell division which relies on cell polarisation, with Notch signalling being localised to one part of the cell (Fiuza and Arias, 2007). Asymmetric distribution of Notch signalling activity determines whether the daughter cells will be a signal sending or a signal- receiving cell. Once this is determined, the cells differentiate according to binary cell fate decisions mediated by Notch (Hutterer and Knoblich, 2005; Le Borgne and Schweisguth, 2003).
An important example of Notch's involvement in binary cell fate decision is its role in the maintenance of stem cell population. Notch mediates many decisions on whether a cell remains undifferentiated or differentiates in both embryonic and post-embryonic cell stem systems (Chiba, 2006).

3. **Boundary formation:** Notch is involved in establishing boundaries between different cell types during development. An example is the formation of boundaries between the prospective somites during somitogenesis (Giudicelli and Lewis, 2004). Although the full mechanisms are not fully understood, this is thought to be achieved by a complex process of multiple Notch regulatory mechanisms and also involving the Wnt pathway.

The role of Notch in the development of the central nervous system (CNS) in vertebrates is very well documented (Bolos *et al*, 2007). Notch maintains neuronal progenitors (neuronal stem cells) in a progenitor state and inhibits differentiation. Several studies have shown that Notch influences multiple choice points in the neural progenitor lineage. It has been shown that Hes1 (Notch target gene) mutation produces severe defects in neural development, including lack of cranial neural tube closure and anencephaly (Bolos *et al*, 2007). In contrast to neuronal differentiation, Notch has a more instructive role in gliogenesis directly promoting the differentiation of many glial subtypes. Notch signalling favours the generation of Müller glia cells at the expense of neurons (Bolos *et al*, 2007). There are suggestions that Notch signalling has a role in neuronal function in the adult brain (Bolos *et al*, 2007; Saura *et al*, 2004; Ables *et al*, 2011). High levels of Notch signalling are present in the adult brain and in particular Notch is also thought to maintain neuronal stem cells in quiescence (Urbán and Guillemot, 2014).

As mentioned previously the role of Notch in boundary formation is extremely important in the patterning process leading to somite boundary formation and the establishment of the anterior and posterior compartments of somites. Following Notch 1 mutation, irregular somites have been generated with abnormal segmental boundaries (Bolos *et al*, 2007).

Notch is an essential regulator of the cardiovascular system and alterations in Notch signalling lead to abnormal vascular development at multiple stages and to varying degrees (Bolos *et al*, 2007; T. Wang, Baron and Trump, 2008; Zhang *et al*, 2014). Mutation in Notch 3 is associated with CADASIL (cerebral autosomal dominant

arteriopathy with subcortical infarction and leukoencephalopathy), characterised by strokes and vascular dementias (T. Wang *et al*, 2008). An insufficiency of Jagged 1 ligand causes Alagille syndrome, which is characterised by vascular anomalies amongst other features (Bolos *et al*, 2007). Notch 1 and 4 have been found to be predominant in the endothelium and Notch 1 and 3 are present in smooth muscle cells. Notch also plays an important role in arterial/venous specification and patterning during development and a mutation results in a loss of arterial identity and arteriovascular malformations (Bolos *et al*, 2007; Zhang *et al*, 2014).

Notch is also involved in the development of some classical endocrine systems. For example, Notch target genes Hes 1 and Hes 5 have been shown to control the progenitor cell pool in pituitary gland development and their absence leads to severe hypoplasia exhibited in mice lacking the genes (Kita *et al*, 2007). Notch has a similar effect on the development of the pancreas as Hes 1 null mice pancreas precursor cells have shown premature differentiation leading to severe hypoplasia (Avila and Kissil, 2013; Jensen *et al*, 2000). Notch also has an additional role in the cell fate decision between progenitor/exocrine and endocrine pancreas (Avila and Kissil, 2013).

Similarly, Notch signalling plays a role in regulating stem cell differentiation towards a secretory or absorptive cell fate, through lateral inhibition, in the largest endocrine gland, the gut (C. S. Lee and Kaestner, 2004). It has also been suggested that Notch downregulates osteoclastogenesis and commits mesenchymal cells to the osteoblastic cell lineage (Yamada *et al*, 2003).

Since the Notch pathway can lead to so many different and sometimes opposing outcomes, it is thought that Notch function is context dependent, depending on dosage and cell lineages (Bolos *et al*, 2007). Tight control of Notch is essential for the development of most tissues and once development is complete, Notch is normally down regulated in mature tissues.

1.3.9 The role of Notch signalling in malignancies

Tumour formation involves re-activation of down regulated pathways which were used during embryonic development (Bolos *et al*, 2007) . In the same context, aberrant Notch signalling, can lead to tumour formation (Bolos *et al*, 2007). Notch is interesting as it can act as both an oncogene or as a tumour suppressor. It has been suggested that Notch signalling plays a key role in haematological and solid malignancies. The outcome of Notch action depends on signal strength, timing, cell type and context. The consequence of abnormal Notch functioning depends on its normal function in the given tissue (Radtke and Raj, 2003; Maillard and Pear, 2003). If the normal function of Notch is as a gatekeeper or as a regulator of stem cells or as a regulator of precursor cell fate, then abnormal functioning leads it to act as an oncogene; whereas it acts as a tumour suppressor in tissues where it normally initiates terminal differentiation events (Radtke and Raj, 2003; Bolos *et al*, 2007).

The Notch signalling pathway has oncogenic as well as tumour suppressive effects in cancer, depending on cellular context. It has been identified as an oncogene in multiple cancers, including leukaemia, colorectal, lung, cervical, breast and oral squamous carcinoma (Avila and Kissil, 2013). Notch 1 function as a tumour suppressor in mice non-melanoma skin cancer and may also function as a tumour suppressor in human

non-melanoma skin cancer. Notch has also been implicated as a tumour suppressor in prostate cancer, hepatocelullar carcinoma and small cell lung cancer (Avila and Kissil, 2013).

Notch alone might not be a very efficient oncogene and must collaborate with other oncoproteins to become a very efficient oncogene. In *in-vitro* experiments, in various cell types, expressing NICD with certain oncoproteins, have induced transformation to tumour cells (Bolos *et al*, 2007). Evidence suggests that in tumours where Notch acts as an oncogene, Notch signalling inhibition can be used as a viable strategy for treatment of certain solid and haemopoietic tumours (Nickoloff, Osborne and Miele, 2003; Aster, 2005). There is also evidence to suggest that in addition to its role as an oncogene Notch also plays a big role in tumour angiogenesis (Zeng *et al*, 2005).

1.3.9.1 Haematological malignancies

Notch plays a key role in haematopoiesis. In fact, Notch 1 receptor was first detected in human haematopoietic cells through its involvement in a chromosomal translocation in leukaemic T cells (Ellisen *et al*, 1991). It was soon found to be also expressed by multiple cells within the haematopoietic hierarchy, including bone marrow haematopoietic progenitors (Milner *et al*, 1994). Notch 1 signalling is essential for normal T cell progenitor development and specification in the thymus, as well as for splenic marginal zone B cell development (Maillard, He and Pear, 2003). It also plays a positive role in megakaryocyte development (Mercher *et al*, 2008). In addition to mediating haemopoietic cell fate determination in the embryo and in the adult, Notch signalling is also a critical factor in the maintenance of a pool of self-renewing haematopoietic stem cells (HSC) (Milner and Bigas, 1999). Any dysregulation of the

Notch signalling pathway could therefore lead to the development of haematological malignancies. The most obvious haematological cancer associated with Notch dysregulation is human acute T cell acute lymphoblastic leukaemia/lymphoma (T-ALL). This constitutes approximately 15-20% of ALL in both children and adults (Bolos *et al*, 2007).

As mentioned earlier, Notch 1 was first discovered due to its involvement in chromosome translocation (t) in human T-ALL. Chromosome translocation is seen in less than 1% of all T-ALL. However, aberrant Notch gain-of-function activity has been seen in the majority of human T-ALL. This places the Notch pathway at the centre of T-ALL pathogenesis (Weng *et al*, 2004).

The theory that Notch functions as an efficient oncogene only when it is in association with other dysregulated proteins is supported by the detection of mutations in multiple molecular subtypes of T-ALL (Aster, 2005). c-MYC has also been identified as an important direct target of Notch in T-ALL and in normal T-cell development. c-MYC inhibitors have been shown to interfere with progrowth effects of activated Notch 1, whereas forced c-MYC expression has been shown to rescue Notch 1 dependent T-ALL cell lines from Notch withdrawal (Weng *et al*, 2006). These findings led to a phase I/II clinical trial using a Notch pathway inhibitor to treat patients with refractory T-ALL (Bolos *et al*, 2007). Despite a 48% reduction in mediastinal mass in one patient with mutated Notch 1 out of 7 T-ALL patients after 28 days of treatment with Gamma secretase inhibitors (GSI), there was subsequent disease progression in this patient and none of the patients received an objective durable response. Furthermore, there was a

lot of gastro-intestinal toxicity associated with the impact of Notch inhibition on intestinal linings (Tosello and Ferrando, 2013).

1.3.9.2 Solid Tumours

There is evidence that mammary stem cells might be the target of transformation during mammary carcinogenesis. Notch signalling pathway has been implicated in the self-renewal of normal mammary stem cells and there is a suggestion that the Notch pathway plays a role in breast cancer (Bolos *et al*, 2007). Elevated expression of Notch signalling pathway components have been reported in invasive breast cancer. Elevated expression of Notch 1 and Jagged 1 has been linked to poor prognosis in breast cancer patients (Acar *et al*, 2016). Work in mice has shown that activated Notch 4 leads to arrested mammary gland development and eventually poorly differentiated adenocarcinoma (Bolos *et al*, 2007). The role of Notch signalling pathway in breast cancer is further supported by the study of NUMB expression, a cell fate determinant. NUMB mediated negative regulation of Notch signalling has been found to be lost in 50% of human mammary carcinomas. This loss is due to increased Notch activation which leads to specific NUMB ubiquitination and proteasomal degradation (Acar *et al*, 2016; Pece *et al*, 2004).

It has already been mentioned that Notch is essential for gut development and homeostasis. However, the Wnt pathway has also been implicated in intestinal crypt progenitor cell maintenance and its mutational activation is considered a play a major role in the proliferative potential of intestinal adenomas and adenocarcinomas, due to the loss of the intestinal suppressor gene *Apc* (Sancho, Batlle and Clevers, 2004).

Studies on the role of Notch signalling in intestine development and cancer have indicated that Notch might function downstream of Wnt and both pathways synergize as gatekeepers in the intestinal epithelium. Van Es *et al* have shown high levels of *Hes-1* expression in intestinal adenomas in mice, indicating Notch activation. Notch inhibition by γ-secretase inhibitors, in these same cells, induces goblet cell differentiation and reduces adenoma proliferation (van Es *et al*, 2005). Cross talk between active Notch and WNT is important for tumourigenesis and cell proliferation in colorectal cancer (Vinson *et al*, 2016). These results suggest that Notch signalling and Wnt inhibitors could be combined in an approach for colorectal neoplasia treatment.

Notch 1 acts as a tumour suppressor gene in mouse keratinocytes, promoting exit from the cell cycle and entry into differentiation, whereas *ras* and Wnt signalling both work upstream of Notch and suppress the activity of Notch (Bolos *et al*, 2007; Rangarajan *et al*, 2001). Notch signalling does not inhibit melanoma, as it is does in keratinocyte derived carcinomas. Experiments have shown that Notch activation is insufficient to transform melanocytes into melanoma, but does have the ability to enable primary melanoma cells to gain metastatic capability (Balint *et al*, 2005; Thelu, Rossio and Favier, 2002).

Abnormal Notch expression has been reported in several other solid tumours. Cervical (Zagouras *et al*, 1995), lung and brain malignancies (Cuevas *et al*, 2005; Sriuranpong *et al*, 2001) are other examples of Notch 1 involvement. It has recently been found that the Notch signalling has different effects on different stages of cervical cancer. In the early stages of the disease Notch signalling is upregulated, whilst in the late stages Notch

signalling is downregulated (Sun *et al*, 2016). Notch 2 over-expression has been
reported in colorectal and cervical cancers, and meningioma. Notch 3 is involved in
melanoma and Notch 4 in breast cancer. Other malignancies which express high levels
of Notch ligands are prostatic, cervical and brain cancers (Cuevas *et al*, 2005; Santagata
et al, 2004; Gray *et al*, 1999).

1.3.10 Notch in Pancreatic Cancer

Notch signalling plays an important role in development of the pancreas by maintaining
pancreatic epithelial cells in a progenitor state and thereby delaying their differentiation
until the appropriate time (Mysliwiec and Boucher, 2009). It has been shown in mice
that reduced Notch signalling in the developing pancreas results in premature
differentiation and hypoplasia (Nakhai *et al*, 2008).

Although Notch 1 and Notch 2 are found to be expressed in the embryonic pancreas,
there is not much evidence of them being detected in the adult exocrine pancreatic cells.
Hes1 expression has been detected in the adult pancreas in only centroacinar cells and a
few ductal cells (Jensen *et al*, 2000; Fujikura *et al*, 2007). Increased expression of
Notch 1, Notch 2, Jagged 2 and Hes 1 were detected in the mouse exocrine tissue of
caerulein-induced regenerating pancreas (Jensen *et al*, 2000) and caerulein-induced
acute pancreatitis (Gomez *et al*, 2004). Pancreatic-specific Notch 1 deficiency also
results in impaired regeneration after caerulein-induced pancreatitis in mice (Siveke *et
al*, 2008). All these evidence supports the theory that although the Notch signalling
pathway remains relatively inactive in normal adult pancreas, it participates in the
regeneration process following pancreatic injury.

26

Pancreatic intraepithelial Neoplasia (PanIN) represents a pre-invasive form of pancreatic ductal adenocarcinoma (PDAC)(Winter, Maitra and Yeo, 2006). There is now an association of genetic alteration between the histological progression of low-grade PanIN (PanIn-1) to intermediate grade PanIN (PanIn-2), to high grade PanIN (PanIn-3), and finally to PDAC (Bardeesy and DePinho, 2002; Schneider *et al*, 2005; Hezel *et al*, 2006). Compared to very minimal/no expression levels of Notch and their ligands in normal adult pancreas, there is a moderate-to-high level expression of Notch receptors, ligands and their target genes (Hes 1, Hes 4, Hey 1, Hey L) in metaplastic ductal epithelium (Miyamoto *et al*, 2003).

Aberrant Notch 1 and/or Hes1 expression have been displayed in mutant mouse models of PanIn-1 to PanIn-3 and PDAC. This supports the cumulative observations that sustained re-activation of the Notch signalling pathway contributes to initiation, progression and maintenance of pancreatic cancer in both humans and mice (Pasca di Magliano *et al*, 2006; Miyamoto *et al*, 2003). A model, supported by several studies, suggests that acinar cells are progressively replaced by duct-like epithelia (acinar-to-ductal metaplasia), which can ultimately progress into PanIN and PDAC (Hezel *et al*, 2006; Bardeesy and DePinho, 2002; Schneider *et al*, 2005).

Treatment of primary acinar cells with TGFα causes acinar-to-ductal cell metaplasia, a conversion associated with Notch 1 cleavage and Hey 1 expression. However, prevention of Notch activation by γ-secretase inhibitors in these cell lines prevents acinar to ductal metaplasia (Sawey, Johnson and Crawford, 2007). It has also been shown that the Notch pathway can be activated in premalignant pancreatic epithelium and malignant pancreatic cancer by transgenic overexpression of TGFα driven by an

acinar promoter (Sawey *et al*, 2007; Miyamoto *et al*, 2003). Since increased TGFα

signalling is seen as an initiating event (Bardeesy and DePinho, 2002; Schneider *et al*,

2005), these results indicate that sustained Notch re-activation, leading to PDAC, is an

early event.
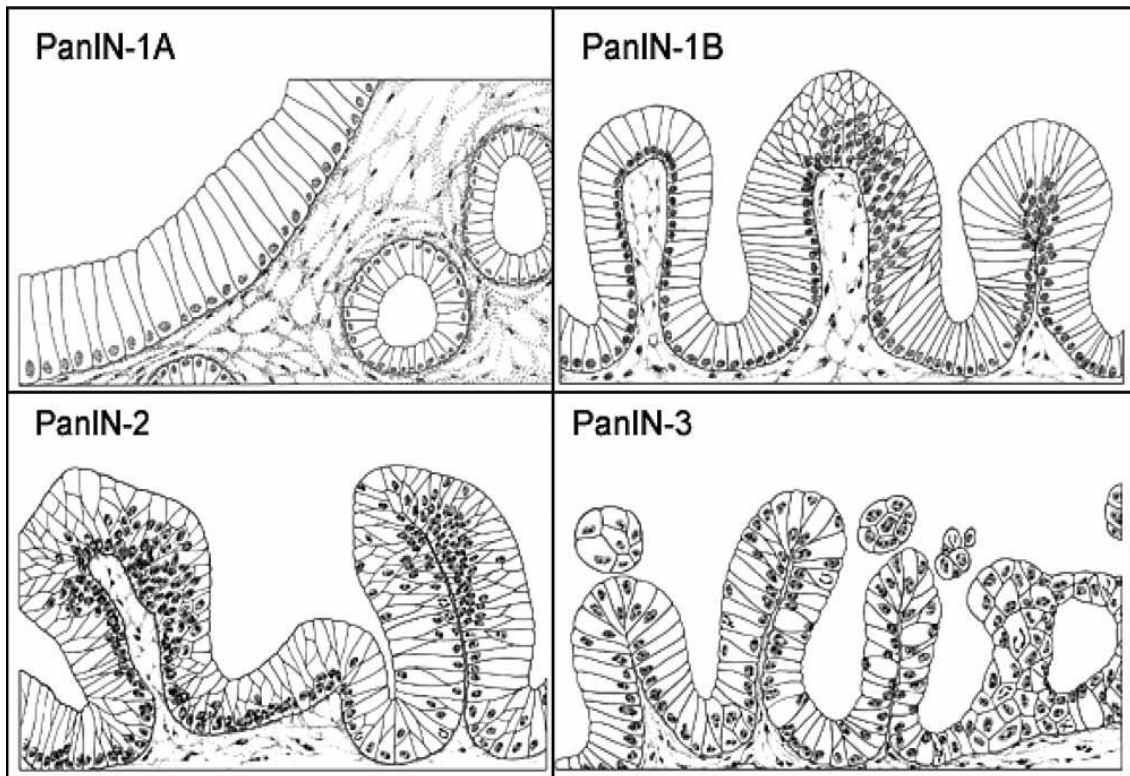
Figure 1.5 Pancreatic intraepithelial neoplasia. Pancreatic intraepithelial neoplasia: PanIN-1A
(flat), PanIN-1B (papillary), PanIN-2 (papillary with nuclear changes), and PanIN-3 (severely atypical with mitoses,
budding, and luminal necrosis). Figure taken from Winter, Maitra and Yeo, 2006.

Although overexpression of NICD was sufficient to induce acinar-to-ductal metaplasia

in primary acinar cell cultures, it was not sufficient to initiate metaplastic lesions or

PanIN after 4 months. This led to the suggestion that NICD co-operates with other

signalling pathways to initiate pancreatic carcinogenesis (De La *et al*, 2008). Hes 1

expression has also been well documented in the Kras signalling model. It was possible

to form metaplastic and PanIN lesions in either pancreatic progenitors or mature acini,

by expressing NICD with an oncogenic Kras, at a time point where no such lesions were observed in either NICD expressing or Kras expressing mice. This supports the theory that both activated Kras signalling and aberrant Notch activity are needed to initiate pancreatic carcinogenesis (De La *et al*, 2008).

Wang *et al* have shown that the acquisition of epithelial-mesenchymal transition (EMT) phenotype of gemcitabine-resistant pancreatic cancer cells is linked with the activation of the Notch signalling pathway (Z. Wang *et al*, 2009). Furthermore, they have demonstrated partial reversal of EMT by inhibiting Notch activation. Their results suggest that Notch signalling is associated with a chemoresistance phenotype of pancreatic cancer cells, and so inactivation of Notch signalling by novel strategies could be a potential targeted therapeutic approach for overcoming chemoresistance in pancreatic cancer cells (Z. Wang *et al*, 2009) .

Interestingly, Plentz *et al* showed that γ-secretase activity is required for the progression of pre-malignant to malignant pancreatic cells *in vivo*. Their experiment, using mice exhibiting isolated PanIN lesions without detectable PDAC, involved treating animals with γ-secretase inhibitors for 11 -13 weeks. Subsequent autopsies revealed that 12 out of 34 mice without γ-secretase inhibitors developed PDAC, compared with none of the 25 mice treated with γ-secretase inhibitors. The findings were very important as it not only confirmed the requirement of Notch signalling for PDAC progression, but also offered a potential therapeutic target in this treatment-refractory malignancy (Plentz *et al*, 2009).

The role of gamma-secretase as a potential therapeutic target for Notch signalling pathway induced cancers was also further enforced by studies which demonstrated that gamma-secretase inhibitors reduced the cell viability of T-ALL cell lines by inducing cell cycle arrest and apoptosis (Lewis *et al*, 2007; Weng *et al*, 2004).

Gamma-secretase has been actively investigated in the past as a potential target which could be exploited to prevent Alzheimer's disease. Amyloid precursor protein is cleaved sequentially by β secretase and gamma-secretase, and therefore releases the amyloid β-peptide. Amyloid β-peptide is the precursor of the amyloid plaques found in the brain of patients with Alzheimer's disease (Shih and Wang, 2007). There are toxicities associated with gamma-secretase inhibitors and these usually result from the inhibition of Notch signalling. The toxicities primarily affect the intestine, thymus and spleen (Jun *et al*, 2008). However, a phase 2 safety clinical trial demonstrated that γ-secretase inhibitors can be safely tolerated for 14 weeks, and therefore prolonged use of gamma-secretase inhibitor is conceivable (Fleisher *et al*, 2008). Since Notch signalling does not play a major role in the normal adult pancreas, only cancerous cells should be affected by gamma-secretase inhibition. Studies have shown that inhibition of Notch signalling does not have deleterious effects on normal pancreatic exocrine and endocrine functions (Fujikura *et al*, 2006; Siveke *et al*, 2008).

1.3.11 Notch Signalling as a potential Biomarker for Pancreatic Cancer

There is a need for the discovery of specific blood biomarkers in the non-invasive detection of pancreatic cancer as only 10% of tumours are amenable for surgical

resection at the time of diagnosis. The ineligibility of the other tumours are due to factors such as late presentation, aggressive local spread and early metastasis (Stocken *et al*, 2005). The most commonly used marker for pancreatic cancer is CA 19-9. This has a median sensitivity of 79%, a median specificity of 82%, a median positive predictive value of (PPV) 72 and a median negative predictive value of 81 (Goonetilleke and Siriwardena, 2007). CA 19-9 is a high-molecular-weight glycoprotein and is expressed by several epithelial cancers, as well as in normal pancreatic and biliary ductal epithelial cells, and it is also detectable in salivary mucus and meconium (Marrelli *et al*, 2009). CA 19-9 is also elevated in patients with other malignancies such as colorectal, liver, breast, and lung cancers, as well as non-malignant diseases such as obstructive jaundice, pancreatitis, cirrhosis, and lung disorders. Furthermore, CA 19-9 lacks sensitivity for early or small diameter pancreatic cancer. Poorly differentiated pancreatic cancers also appear to produce less CA 19-9 compared to moderately or well-differentiated pancreatic cancer (Duffy *et al*, 2009; Wu *et al*, 2013). Given all these limitations, CA 19-9 serum levels alone cannot distinguish between benign, precursor lesions, and malignant pancreatic and biliary tract conditions. The American Society of Clinical Oncology claimed the specificity and sensitivity of Ca 19-9 alone is inadequate for a reliable diagnosis of pancreatic cancer (Wu *et al*, 2013).

Biomarkers can be defined as molecules that acts as indicators of the physiological state and are the hallmarks of change in tissues or bodily fluids during a disease process (Yang *et al*, 2008). Cancer cells can secrete biomarkers into the bloodstream which are absent or altered concentrations in healthy individuals (Yang *et al*, 2008). Measurement of biomarkers in blood is a relatively non-invasive technique. Considerable and

dramatic improvement to genomic and proteomic technologies have contributed to the molecular landscape of PANC being much greater understood (Bailey *et al*, 2016; Geng *et al*, 2011).

The canonical pathway for Notch signalling is mediated by regulated intramembrane proteolysis (RIP). Ligand binding generates sequential cleavages at S2, S3 and S4 sites of the transmembrane receptor resulting in the release of NICD which translocate into the nucleus and modifies target genes. Whereas the S2 cleavage occurs in the extracellular juxtamembranous region, the resulting transmembrane fragment undergoes intramembranous proteolysis at S3 and S4 sites. The S3 cleavage happens between the cytosol and the membrane and results in the release of NICD into the cytosol, whereas the S4 cleavage occurs in the transmembrane domain resulting in the release of a putative Notch 1 fragment (Nβ) into the extracellular fluid (Okochi *et al*, 2002). By methods of autoradiography and immunoprecipitation (using an antibody raised against a peptide which corresponds to the N terminus of the Notch extracellular truncation), Okochi *et al* were able to detect two Notch 1 peptides of ~3 and 6 kDa in methionine-labelled HEK293 cells expressing Notch 1. They were also able to show that the level of Notch 1 peptide increased with increased cleavage at S2 sites and decreased with the addition of γ-secretase inhibitors. They were also able to show that the secretion of Notch 1 peptides is greatly enhanced in cells expressing both Jagged-1 and Notch 1, but it is hardly detectable in cells expressing either one of them. These results indicated that the Notch 1 peptide secretion is a product of Notch receptor proteolysis and is a result of Notch activation by its respective ligand. The production of the Notch 1 peptide is also associated with the cleavage efficiency at S2 and S3/S4 sites (Okochi *et al*, 2006).

Using an antibody to immunoprecipitate the Notch 1 peptide, Okochi et al analysed the molecular mass by matrix-assisted laser desorption ionisation time of flight (MALDI-TOF) mass spectrometry (MS) and were able to pick up 2 major peaks with molecular masses of 2306 and 2694 Da. The peptide sequences were determined based on the sequence surrounding the epitope for the antibody and the Notch 1 peptides were found to be composed of peptides S2 to S4. The shorter Nβ length was named Nβ21 and the longer Nβ named Nβ25 (the numbers indicated their peptide length). (Okochi *et al*, 2006)

Artificial synthesis of these peptides and their subsequent examination using immunoblotting on Tris-Tricine SDS-PAGE revealed that Nβ21 peptide corresponds to a 3 kDa band and the Nβ25 peptide corresponds to a 6 kDa band. The results also showed that there are extremely low levels of intracellular Nβ; Nβ21 is more stable in SDS-PAGE; and the two species of Nβ fragments do not form a heterodimer. Based on these results Okochi *et al* concluded that the secreted Nβ species corresponds to the sequence of peptides between S2 and S4 cleavage, and Nβ21 is predominantly present in cell culture. The N terminal of the Nβ is the same, whereas the C terminal is dependent on the site of S4 cleavage. In Nβ25, the S4 cleavage takes place four amino acids terminal to the site for Nβ21 cleavage (Okochi *et al*, 2006).

Ligand-induced Notch signalling is required for the metaplastic conversion of acinar-to-ductal cells and ultimately pancreatic ductal adenocarcinoma and Notch signalling inhibition could be used as a potential therapeutic target to prevent tumour progression. The knowledge that Nβ fragments are secreted extracellularly during Notch activation

by their ligands suggests the possibility of exploring the idea of using Notch Nβ

fragments as biomarkers in malignancy of the pancreas.
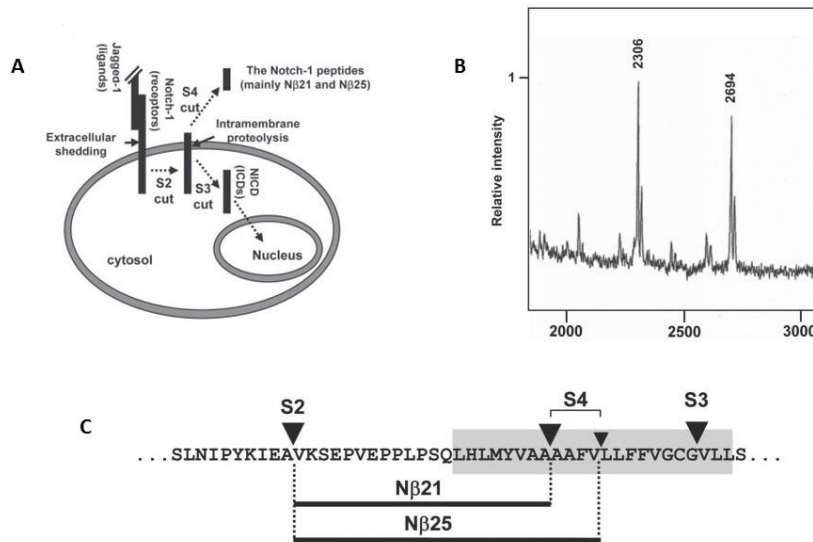
Figure 1.6 Extracellular release of the Notch 1 peptides and its detection.
A. Schematic representation of the Notch 1 peptide release during activation. Cleavage
at S4 results in the extracellular secretion of Nβ peptide; whereas cleavage at S3 results
in NICD. B. Detection of the two Notch extracellular Nβ peptides on Mass
Spectrometry. C. schematic representation of two different Nβ peptides and the S2, S3,
S4 in Notch 1. The *grey box* indicates the putative transmembrane domain of murine
Notch 1. Figure adapted from Okochi *et al* 2006.

1.4. MicroRNAs

MicroRNAs (miRNAs) are a class of RNAs which are recently discovered and are

small (~22 nt), noncoding RNAs with gene-regulating functions. They are recognised

as epigenetic regulators of gene expression (J. Wang, Chen and Sen, 2016) Unlike most

biomarkers that are currently available, miRNAs appear to be cell type and disease

specific. They have been implicated in the regulation of a multitude of cellular

processes and also in the expression of cancer-related genes (Mardin and Mees, 2009; J. Wang *et al*, 2016).
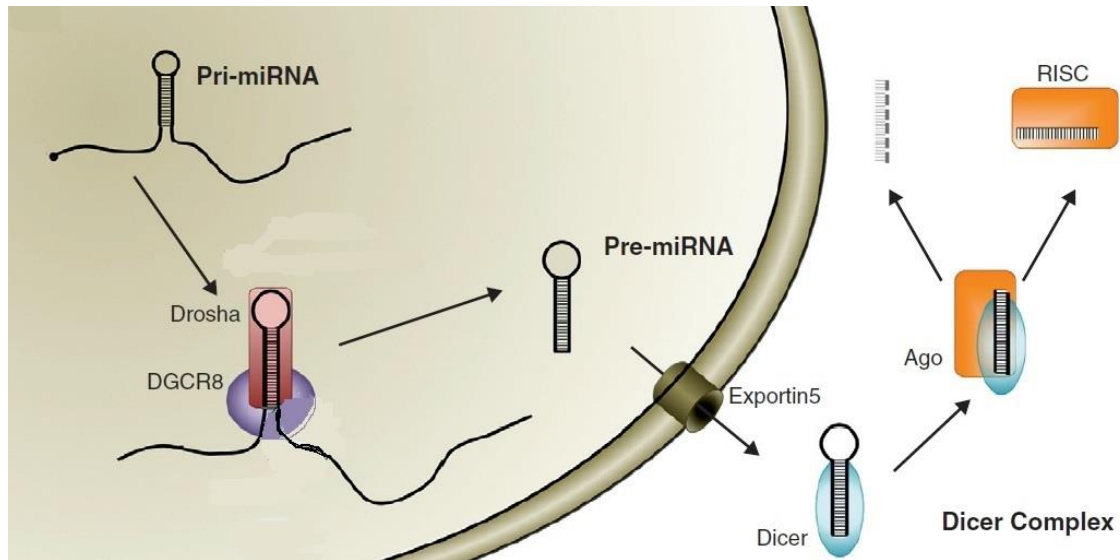
Figure 1.7 Biogenesis of miRNA.

miRNA begins as long primary miRNA (pri-miRNA) and is cleaved by Drosha and cofactor DiGeorge Critical Region 8 protein complex to generate precursor miRNA (pre-miRNA). Following the export of pre-miRNA from the nucleus by exportin 5 (EXP5), Dicer processes the pre-miRNA into double stranded mature miRNA. Mature miRNA will be loaded into Argonaute proteins (Ago), which separates mature miRNA into two single stranded miRNAs. The functional strand becomes the mature miRNA and binds onto RNA-induced silencing complex (RISC) and acts as its guide. Figure adapted from Blahna *et al*, 2012.

miRNAs are initially transcribed as long primary miRNAs (pri-miRNA), which are then cleaved into precursor miRNA (pre-miRNA) in the nucleus by a nuclear microprocessor complex composed of RNase III enzyme (Drosha) and cofactor DiGeorge Critical Region 8 protein. The pre-miRNA is then exported into the cytoplasm by a nuclear transport receptor, called Exportin 5, and then further cleaved (by Dicer, another RNase III) into an imperfect miRNA double stranded RNA (dsRNA) duplex that contains both the mature miRNA and its complimentary strand (miRNA*). The dsRNA complex does not persist in the cell for long, and under the mediation of argonaute 2 dissociates to a single functional guide strand (which becomes the mature

miRNA) and a passenger strand which disappears. The argonaute (Ago) are a group of four proteins which arbitrate the effector function of the miRNAs either through inhibiting translation of the target mRNA or by directly degrading the mRNA transcript. The miRNA then loads onto the RNA-induced silencing complex (RISC) and acts as its guide. The RISC binds to messenger RNA by the imperfect base pairing of the miRNA to the 3' untranslated region of the messenger RNA. The miRNA acts as a guide thereby choosing which messenger RNA to bind to, whereas the RISC causes posttranscriptional gene silencing by either cleaving the target messenger RNA or by inhibiting the translational process. This essentially makes the miRNAs negative regulators of gene expression, which can specifically and efficiently silence target genes (Du and Zamore, 2005; Y. Li *et al*, 2010; Blahna and Hata, 2012; J. Wang *et al*, 2016).

1.4.1 MicroRNAs in cancer

In the last few years, there has been a lot of work documenting the biological significance of miRNAs in tumour progression. miRNAs have been implicated in a wide array of biological cell functions including cell proliferation, differentiation, apoptosis, and stress resistance and also been shown to be key players in human cancers (Z. Wang *et al*, 2010). miRNAs are involved in the process of cell proliferation and apoptosis, the two key intimately linked process that are critical in the development and progression of human malignancies. Aberrant expression of miRNAs has been reported when comparing various types of cancer with normal tissues (Z. Wang *et al*, 2010). It is thought that some miRNAs act as oncogenic and others act as tumour suppressors. Oncogenic miRNAs are upregulated in cancers whereas tumour suppressive miRNAs tend to be downregulated in malignancies (Z. Wang *et al*, 2010). Calin *et al* were the first to show evidence of miRNA involvement in human cancer. They showed that miR-

15 and miR-16 were absent or downregulated in about 70% of chronic lymphocytic leukaemia (CLL) cases (Calin *et al*, 2002). These miRNAs were later confirmed as tumour suppressors (J. Y. Park *et al*, 2011). Several studies have identified some miRNAs which are upregulated in specific cancers and other miRNAs which are downregulated in other cancers (J. Y. Park *et al*, 2011). The studies have also shown good correlation between miRNA expression signatures and specific clinical cancer characteristics, which suggests that miRNA expression signatures could be used to differentiate between normal and cancerous tissue (J. Y. Park *et al*, 2011).

1.4.2. MicroRNAs as potential biomarkers of pancreatic cancer

miRNAs from solid tumours are detectable in peripheral blood samples and are also found to be highly stable at room temperature (J. Y. Park *et al*, 2011). Many tissues have been shown to have specific miRNA expression patterns (Mitchell *et al*, 2008; Fan *et al*, 2008). Since Pancreatic Ductal Adenocarcinoma (PDAC) is a disease resulting from both genetic and epigenetic alterations (Mardin and Mees, 2009), miRNAs could be used a potential biomarker and therapeutic agent for this disease. miRNAs-216 and 217 have been identified to be specific for normal pancreas tissue, with only the duodenum exhibiting a 15-25-fold lower expression on their tissue. These miRNAs were shown to be downregulated more than 200-fold in PDAC samples, making them potential biomarker candidates (Szafranska *et al*, 2007). miRNA-196a and miRNA-196b were identified as miRNAs which are expressed in PDAC, but completely absent in normal pancreatic and chronic pancreatitis tissue (Mardin and Mees, 2009; Szafranska *et al*, 2007).

Using miRNA microarray expression profiling, Bloomston *et al* were able to identify 21 miRNAs with increased expression and 4 with decreased expression which differentiated PDAC from benign pancreatic tissue in 90% of the samples. They were also able to identify 15 overexpressed and 8 under-expressed miRNAs which differentiated PDAC from chronic pancreatitis with 93% accuracy. Combining the results, 11 miRNAs were found to differentiate cancer specimens from both chronic pancreatitis and benign tissue (Bloomston *et al*, 2007). miRNA-196a-2 was found to be higher in patients who had a lower median survival (14.3 months) compared to patients with low expression levels of miRNA-196a-2 (median survival 26.5 months) (Bloomston *et al*, 2007). The studies described so far have relied on expression profiles from surgically excised tissue. This is not available pre-operatively and therefore not useful in making therapy decisions.

Pancreatic juice can be collected during Endoscopic retrograde cholangiopancreatography (ERCP) or by Endoscopic Ultrasound (EUS) guided Fine needle aspiration (FNA) and examined for the presence of specific miRNAs. EUS FNA is becoming a common modality used for preoperative diagnosis and staging of pancreatic cancer (Jing *et al*, 2009). Szafranska *et al* were able to isolate miRNA from FNAs of PDAC patients and controls. miRNA-196a was found to be specific for PDAC, whilst miRNA-217 was found to be expressed in healthy normal pancreas (Szafranska *et al*, 2007). miRNA-21 and miRNA-155 have been identified as the miRNAs demonstrating the highest fold changes in intraductal papillary mucinous neoplasms (IPMNs), which are non-invasive precursor lesions of pancreatic cancer (Habbe *et al*, 2009). EUS-FNA is still an invasive procedure and development of plasma or serum-based biomarkers still remains the desired outcome.

Wang *et al* selected a panel of only four miRNAs, miRNA-21, miRNA-210, miRNA-155, miRNA-196a, and were able to show that these miRNAs were overexpressed in blood plasma/serum and varying expression profiles of these samples can be used to distinguish PDAC from normal healthy individuals with a sensitivity of 64% and a specificity of 89%. It is important to remember that although these results are encouraging, the small sample size consisting of extremes of adenocarcinomas and healthy controls allow it to have limited clinical implications towards developing into pancreatic cancer screening and an early detection modality currently (J. Wang *et al*, 2009). More work needs to be done with adequate sample size and varying grades and stages of the disease before the miRNAs can be developed as biomarkers for early detection of pancreatic cancer.

1.4.3 MicroRNAs and Notch

It is well established that loss of p53 tumour suppressor protein is a causative event in the pathogenesis of at least 50% of all human malignancies (Ji *et al*, 2009; Fridman and Lowe, 2003). Chang *et al.* demonstrated that miRNA-34a is a direct transcriptional target of p53. They were also able to demonstrate that induction of miRNA-34a promotes apoptosis through p53 dependent and independent mechanisms. MiRNA-34a responsive genes are also highly enriched for those genes that regulate cell cycle progression, cellular proliferation, apoptosis, DNA repair and angiogenesis (Chang *et al*, 2007).

Since miRNA-34 has been implicated as a potential tumour suppressor, it has been examined in some detail. Bommer *et al* reported that the anti-apoptotic Bcl-2 protein is regulated directly by miRNA-34 (Bommer *et al*, 2007). He *et al* indicated that

expression of miRNA-34 induces cell cycle arrest in both primary and tumour-derived cell lines (He *et al*, 2007). There are reports which link miRNA-34 to tumour initiating cells, or cancer stem cells, in cancer initiation and progression. miRNA-34 is therefore thought to target Notch, c-Met and Bcl-2 genes. These are genes which are involved in the self-renewal and survival of cancer stem cells (L. L. Song and Miele, 2005; Bommer *et al*, 2007; He *et al*, 2007).

Chang *et al* also observed a reduced expression of miRNA-34a of at least 2-fold in 15 pancreatic cell lines, when compared to normal pancreatic ductal epithelial cell lines (Chang *et al*, 2007). A recent study by Ji *et al* examined the effects of functional restoration of miRNA-34 on human p53-mutant pancreatic cancer cells. MiaPaCA2 and BxPC3 cells were found to have very low expression levels of miRNA-34a, b, c but high levels of their target genes Bcl-2 and Notch 1. They were able to show that miRNA-34 restoration in human pancreatic cancer cells inhibited the expression of Bcl-2, Notch 1 and 2. This significantly inhibited clonogenic cell growth and metastasis, induced apoptosis and cell cycle arrest at G1 and G2/M. miRNA-34 restoration also sensitised the cells to radiation and chemotherapy, and at least in part restored p53 tumour suppressive function in p53-deficient cancer cells. Most importantly, Ji *et al* were able to show miRNA-34 restoration inhibits cancer stem cells accompanied by a significant inhibition of tumour growth in both *in vitro* and *in vivo* (Ji *et al*, 2009). All these findings suggest that miRNA-34 may provide a novel therapeutic approach for p53 deficient pancreatic cancer.

1.5 Hypothesis, Aim and Objectives

Our overarching hypothesis is that there is sufficient evidence to suggest that Notch plays a key role in pancreatic carcinogenesis and has the potential to be used as a diagnostic and prognostic biomarker. There is also sufficient evidence to suggest that miRNA can used as potential biomarkers in pancreatic ductal adenocarcinoma.

1.5.1 Aim

The aim of this study is to further investigate the role of the Notch 1 & 2 peptides and miRNA and their potential as biomarkers in PDAC.

1.5.2 Objectives

1. To explore the potential of the Notch receptors as a biomarker in pancreatic adenocarcinoma using solid phase extraction and mass spectrometry.

2. To investigate the expression of the Notch receptors in pancreatic adenocarcinoma *in vivo.*

3. To explore the potential of miRNAs as a biomarker to differentiate between healthy, early pancreatic cancer, late pancreatic cancer and chronic pancreatitis.

CHAPTER 2.

MATERIALS AND METHOD

2.1 Clinical Materials

A substantial part of this project involved work on human blood plasma obtained from patients receiving treatment at University Hospitals of Leicester NHS Trust. Research was also carried out on human pancreatic tissue, collected from the Department of Pathology archives at the Leicester General Hospital. Ethics committee approval for the use of this tissue in the study was obtained (LREC number 7176). A copy of the ethics approval letter is contained in Appendix 1.

2.1.1 Patient recruitment for collection of blood

Along with healthy volunteers, patients included in the study were those treated for pancreatic ductal adenocarcinoma (PDAC) or chronic pancreatitis, between 2010 and 2012 at the University Hospitals of Leicester NHS Trust. This timeframe was chosen in order to allow for prospective plasma collection using the same collection and preparation method for the different groups. PDAC patient lists were generated from Leicester Hepatobiliary Multidisciplinary Team records. Chronic pancreatitis patients were recruited from general Hepatobiliary clinics. Healthy volunteers were recruited by asking relatives accompanying PDAC patients to clinics. This was done to include age-matched volunteers for better comparison. Case notes were reviewed by a single observer (Clinical Investigator) to confirm suitability for inclusion. Inclusion and exclusion criteria for all patients and volunteers for the study are summarised below.

Inclusion criteria:

- Patients must be over 18 years of age

- Patients must be of sound mind to give written informed consent

- Patients must have a diagnosis of the following conditions

    ➢ Primary pancreatic ductal adenocarcinoma

    ➢ Chronic pancreatitis

- All patients with pancreatic malignancy must have disease amenable to

    surgical exploration +/- resection/palliation or be suitable for treatment

    with gemcitabine chemotherapy.

- All healthy volunteers must be over 18 years of age.

Exclusion criteria:

- Patients unfit for general anaesthesia.

- Patients with Alzheimer's disease.

- For healthy volunteers, those with previous malignancy were excluded.

2.1.2 Patient Recruitment for immunohistochemistry

Patients included in this study had either resectable pancreatic cancer or advanced

pancreatic cancer which was deemed non-resectable. They were treated in Leicester

General Hospital, University Hospitals of Leicester NHS trust. The resectable group

were treated between October 2000 and May 2007. The non-resectable group were

treated between January 2003 and January 2007. Patients were identified from the

Leicester Hepatopancreatobiliary Multidisciplinary team database. The case notes were reviewed by a single observer (previous PhD candidate Dr Chris Mann) to confirm suitability for inclusion.

Inclusion criteria:

- Resectable group must have a surgical resection of pancreatic adenocarcinoma.

- Non-resectable group is defined as patients with advanced cancer which have been deemed unsuitable for curative resection.

- There must be tissue available for immunohistochemistry.

- Accurate follow up information must be available.

Exclusion criteria:

- Malignancy other than primary pancreatic ductal adenocarcinoma.

- Previous history of malignancy.

- Pre-operative chemoradiotherapy.

- Chemoradiotherapy before biopsy for tissue diagnosis in non-resectable patients.

- Peri-operative mortality.

From the medical case notes and computerised hospital records, a database was created containing the patients' demographics and clinicopathological factors. These included ages and genders, blood results including CA 19.9, treatment administered,

histopathological factors and survival data (updated as of 1st of January 2011). The database was created by a previous PhD Candidate (Chris Mann) to investigate the relationship of Notch 1,3 and 4 in pancreatic adenocarcinoma.

2.1.3 Tissue selection and assessment

Once suitable patients were identified from the case notes, original slides which were used for histological diagnosis were retrieved from the Leicester General Hospital pathology archive. The slides were then reviewed by an experienced Consultant Histopathologist who specialised in Gastrointestinal pathology and blocks containing PDAC and healthy tissue were identified. The selected blocks were then cut into sections for immunohistochemical assessment.

2.2 Laboratory Materials

2.2.1 Materials for immunohistochemistry (chapter 4)

Details of materials used for immunohistochemistry are listed in table 2.1.

Table 2.1 Materials for immunohistochemistry

Material	Company obtained from
Superfrost Plus™ slides	Menzel-Glazer via Fisher Scientific
Glass cover slips	Menzel-Glazer via Fisher Scientific
Haematoxylin-plus	Vector Laboratories
DPX mountant	Sigma
Envision+ detection kit	Dakocytomation

2.2.2 Antibodies

Details of primary antibodies used for immunohistochemistry and mass spectrometry work are shown in **Table 2.2**.

Table 2.2 Primary antibodies

Antibody against	Species	Clone	Isotype	Supplier
Notch 2 intracellular domain (N2ICD)	Rabbit	Polyclonal	IgG	Abcam
Notch 1 Nβ21 fragment	Rabbit	Monoclonal	IgG	Davids Biotechnology

2.2.3 Materials for plasma preparation, solid phase extraction and mass spectrometry (chapter 3)

Details of materials used for plasma preparation, solid phase extraction and mass spectrometry are listed in table 2.3.

Table 2.3 Materials used for plasma preparation, solid phase extraction and mass spectrometry

Material	
Lithium Heparin tubes with Green BD Hemogard™ Closure for blood collection	BD Vacutainer®
Oasis® HLB 1cc (30 mg) Extraction Cartridges	Waters. Part no: WAT094225
Amicon Ultra Centrifugal Filters 0.5 mL – 10K membrane	Millipore

Microcon Centrifugal Filter Devices; Regenerated Cellulose 10,000 MWCO	Millipore
Dynabeads® Protein G.	Invitrogen
Waters Q-ToF Ultima Global in MALDI mode.	Waters

2.2.4 Materials for microRNA extraction and PCR (chapter 5)

Details of materials used for microRNA extraction and PCR are listed in table 2.4.

Table 2.4 Materials used for microRNA extraction and PCR

Material	Company
EDTA KEA monovette collection tubes used for blood collection	Sarstedt
miRneasy Mini Kit	Qiagen
miScript II RT Kit	Qiagen
miScript SYBR® Green PCR Kit	Qiagen
Primers for use with miScript SYBR ® Green PCR Kit	Biomers.net Germany
Optical 96-Well Reaction Plate	Applied Biosystems

The primers for use with miScript SYBR ® Green PCR Kit were made into a concentration of 5 pmol/µL by dissolving in Tris-HCl pH 7.5. The miRNA Primers used were miR-10b, miR–21, miR-34a, miR–143, miR-148a, miR–155a, miR–196a, miR-200c, miR–206, miR–375 and miR–503 (Table 2.5).

Table 2.5 miRNA Primers for use with MiScript SYBR® Green PCR Kit

Primer Name	Sequence (5' -3')
miR-10b	ATCCTGTAGAACCGAATTTGTG
miR-21	GCTAGCTTATCAGACTGATGTTGA
miR-34a	GGCAGTGTCTTAGCTGGTTG
miR-143	TGAGATGAAGCACTGTAGCTC
miR-148a	TCTCAGTGCACTACAGAACTTTGT
miR-155	TAATGCTAATCGTGATAGGGGTA
miR-196a	GCTAGGTAGTTTCATGTTGTTGG
miR-200c	TAATACTGCCGGGTAATGATG
miR-206	AGGAATGTAAGGAAGTGTGTGG
miR-375	TTGTTCGTTCGGCTCG
miR-503	TAGCAGCGGGAACAGTTCT

2.3 Solutions and Buffers

2.3.1 Solutions and buffers for immunohistochemistry

TBS (10x)

0.5 M Tris base (60.5 g); 1.5M NaCl (87.6 g) in 1 litre of distilled water and adjusted to pH 7.6. The stock solution was kept at room temperature. Prior to use, the solution was diluted to x1 with distilled water.

Tris-EDTA antigen retrieval buffer *10 mM Tris base, 1 mM EDTA.*

This buffer was prepared fresh when required.

Tris base 1.818 g

EDTA 0.555 g

Tween-20 1 mL

The Tris base and EDTA were dissolved in distilled water. Tween was added and the volume was made up to 1.5 L using distilled water

Tris Buffered Saline (TBS) (10x) wash buffer

Tris base 60.5 g Sodium chloride 87.6 g

The Tris base and NaCl were dissolved in distilled water and the pH adjusted to 7.6 using concentrated HCl. The volume was made up to 1 L using distilled water. The solution (500 mM Tris, 1.5 M NaCl) was stored at room temperature and diluted 1:10 prior to use.

Tris-HCl buffer for dilution of primary antibody

0.785 g Tris hydrochloride (MW 157.6) added to 100ml distilled water and was stored at room temperature (50 mM, pH 7.2-7.6). Immediately prior to use the appropriate volume was aliquoted and 1% BSA added.

Envision$^+$ detection system

1. Peroxidase blocking solution

0.03% hydrogen peroxide containing sodium azide.

2. Secondary antibody

Peroxidase-labelled polymer conjugated to goat anti-rabbit immunoglobulin, in Tris-HCl buffer containing stabilising protein and an anti-microbial agent.

3. Detection

One drop of 3,3-diaminobenzidine (DAB) added to 1ml substrate buffer, containing hydrogen peroxide and a preservative immediately before use.

2.3.2 Buffers for Solid Phase Extraction

4% Phosphoric acid. Stored at room temperature

2 mL of concentrated phosphoric acid was added to 48 mL HPLC grade water. This

was stored at room temperature.

40% Acetonitrile, 0.1% trifluoroacetic acid (TFA)

40 mL acetonitrile was added to 60 mL HPLC grade water. 100 µL TFA was then

added to the solution. This solution was stored at room temperature.

50 mM Ammonium bicarbonate (NH_4HCO_3) pH 7.4

0.3953 g of Ammonium bicarbonate was dissolved in 100 mL HPLC grade water. pH

was adjusted to 7.4 using concentrated HCl, prepared fresh.

50 mM Tris pH 7.4

3.03 g Trizma™ was dissolved in 500 mL HPLC grade water and stirred until

dissolved. pH was adjusted to 7.4 using concentrated HCl. Buffer stored at room

temperature.

Citrate Phosphate Buffer pH 5.0

4.7 g of monohydrous citric acid and 9.2 g dibasic sodium phosphate were dissolved in

HPLC grade water and volume adjusted to 1 L after pH was adjusted to 5.0 using

concentrated HCL. Fresh buffer was made for each sample batch.

Citrate Phosphate Buffer + 0.01% Tween 20

2 µL of Tween 20 was added to 20 mL of the Citrate Phosphate Buffer.

Dimethyl Pimelimidate Dihydrochloride (DMP) in Triethanolamine

5.4 mg DMP (stored in -20°C) was dissolved in 1 mL triethanolamine pH 8.2 (stored in 4°C) immediately prior to use.

Phosphate buffered saline (PBS) with 0.01% Tween 20

To 40 mL of PBS was added 4µL Tween 20 and mixed.

0.1 M Citric acid pH 1.5

2.10 g of citric acid was dissolved in HPLC grade water and pH adjusted to 1.5 with concentrated HCl. Final volume was made to 100 mL. Stored at room temperature. pH was always checked before use.

α-Cyano-hydroxycinnamic acid matrix (CHCA)

2 mg of α-cyanno-hydroxycinnamic acid dissolved in 1 mL methanol:acetonitrile = 1:1 This solution was prepared freshly every week and stored in -20°C.

0.1% Trifluoroacetic acid (TFA)

40 µL TFA was added to 40 mL of HPLC grade water. This was stored at room temperature.

2.4 Methods – Immunohistochemistry

2.4.1 The EnVision[+] immunohistochemistry technique

The technique employed for this study was the EnVision[+] system, manufactured by

DAKO. Sabattini *et al* found this technique to compare favourably with other detection

methods such as LSAB and ABC (Sabattini *et al*, 1998) . The EnVision+ System, HRP

(horseradish peroxidase) is a two-step IHC staining technique and is based on an HRP

labelled polymer which is conjugated with secondary antibodies. The labelled polymer

does not contain avidin or biotin. As a result, nonspecific staining resulting from

endogenous avidin-biotin activity is eliminated or significantly reduced.
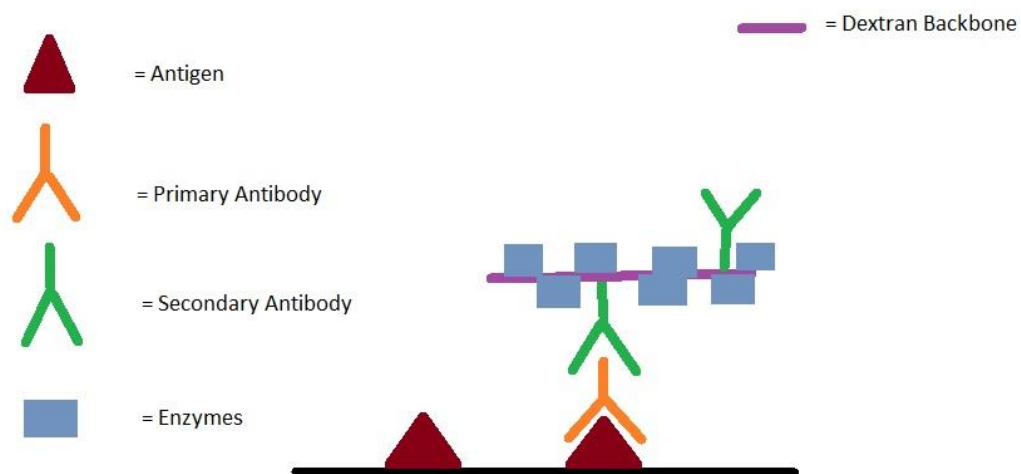
Figure 2.1 Schematic diagram depicting the Envision immunohistochemistry system.
The primary antibody binds to the antigen. The secondary antibody binds to the primary antibody.
The secondary antibody is attached to a dextran backbone. The dextran backbone has about 20
secondary antibodies and 100 enzymes, consisting of horseradish peroxidase (HRP) attached to it.
HRP reacts with a chromogen (DAB) to produce a brown colour.

The secondary antibody is conjugated with a dextran backbone (polymer) which is bound to a large number of enzymes containing HRP molecules. Each dextran backbone holds up to 20 secondary antibodies and 100 enzymes. This leads to marked signal amplification. The advantages of this system include increased sensitivity, minimal non-specific background staining and a reduction in the total number of assay steps as compared to LSAB and ABC methods (Sabattini *et al*, 1998). (**Figure 2.1**) The HRP forms a visible end product is by forming a complex with its substrate, hydrogen peroxide. This complex reacts with a chromogen to produce a coloured molecule and water. The chromogen in the EnVision+ System is 3,3 diaminobenzidine (DAB) which produces a brown colour. It also has the advantage of being insoluble in alcohol and so it can be dehydrated and mounted in conventional mounting media.

2.4.2 Preparation of tissue slides

The embedded tissues were processed by Karen Kulbicky (Department of Cancer Studies and Molecular Medicine). The formalin-fixed, paraffin-embedded dehydrated tissue blocks were cut into 5 µm sections onto Superfrost plus ^{TM} microscope slides using a Shandon Citadel 2000 processor. The first section from each block was stained with haematoxylin and eosin in order to identify different areas of histology. The rest of the sections from the block were cut onto numbered slides so that serial sections from the block could be examined.

A Shandon Varistain 24-4 staining machine (Shandon Inc, Pittsburgh, Pennsylvania, USA) was used to perform the haematoxylin and eosin staining. Slides were initially put through two changes of xylene for 3 min, followed by two changes of 100% industrial methylated spirit (IMS), one change of 70% IMS, and one of distilled water,

for a minute each. The slides were then stained with haematoxylin for 1.5 min followed by being rinsed in water for a minute. They were then dipped in 1% acid water for 15 s followed by being washed 3 times in water. Eosin staining was carried out for 3 min, followed by a 2 min wash in water. Slides were then put through 70% IMS, 4 changes of 100% IMS and two changes of xylene.

2.4.3 Envision$^+$ Immunohistochemistry protocol

Optimisation of immunohistochemistry for anti-Notch 2 antibody is described in Section 2.4.4. The general method is described below.

Paraffin sections were deparaffinised in two changes of xylene for 5 min each. The sections were then rehydrated following two washes in 99% industrial methylated spirits (IMS) and one in 95% IMS, all for 5 min each. The slides were washed in running tap water for 5 min followed by distilled water for 5 min.

Antigen retrieval. One of the retrieval protocols recommended for the Envision kit is microwave oven heating. This is a commonly used and easy method of heat-induced antigen retrieval (HIAR). Tris-EDTA buffer was used. The buffer was preheated to boiling and the slides then heated for 11 min at 900W. This was followed by heating at 360W for a further 21 min. Slides were then allowed to cool for 30 min at room temperature in the Tris-EDTA buffer.

Peroxidase block. Following antigen retrieval, the slides are washed in tap water again for 5 min and then in TBS for 5 min. Excess liquid was wiped from the slide around the sections and one-two drops of the supplied peroxidase blocking solution placed over the

tissue (enough to cover the section). The slides were then incubated for 10 min in a humidified chamber at room temperature. They were then rinsed with TBS before being washed in a TBS bath for 5 min. The peroxidase block is done in order to prevent generalised non-specific staining from small amounts of endogenous peroxidase which are present within normal tissues.

Primary antibody. The primary antibody was diluted in 0.05M Tris-HCl buffer, to which 1% BSA was added before use. 150–200 µL of the diluted primary antibody was applied to each section. The slides were then incubated in a humidified chamber. Optimised antibody concentrations, incubation times and conditions are mentioned in section 2.4.4. In order to exclude non-specific staining, a negative control was included in each run. This consisted of a section incubated with a non-specific immunoglobulin, of the same class as anti-Notch 2 used, which was diluted to the same concentration as the diluted antibody in Tris-HCl buffer. A section of normal liver (positive control tissue) was included in each run to ensure consistent staining between experiments. Following incubation with the primary antibody, the slides were rinsed with TBS and then washed twice in TBS for 5 min baths.

Peroxidase labelled polymer. Excess liquid was again wiped from around the sections and adequate drops of the supplied peroxidase labelled polymer was added to each section and incubated for 40 min in a humidified chamber at room temperature. Following incubation, sections were rinsed with TBS and then washed twice in TBS baths for 5 min each.

Substrate-chromogen. One drop of DAB+ chromagen was added to 1 mL of DAB+ substrate buffer and the solution was vortexed to mix. Excess liquid was wiped from around the sections and two drops of the mixed DAB+ solution was added to each section. Sections were incubated in a humidified chamber at room temperature for 10 min. They were then rinsed with distilled water, followed by two distilled water baths for 5 min each.

Counterstaining. The sections were then rinsed in tap water, followed by counterstaining with vector haematoxylin QS for 5 s, and rinsed again in tap water. The slides were then put back through 95% IMS for 3 min, followed by 99% IMS for 3 min x 2 and two changes of xylene (3 min each). The slides were then wet mounted with DPX mountant using glass coverslips.

2.4.4 Optimisation of immunohistochemistry

Tissue fixation by standard methods, such as the use of formalin, could result in masking of antigenic sites due to crosslinking of proteins. This is especially the case for the monoclonal antibodies, as there is a less of a chance of masking all epitopes recognised by polyclonal antibodies. During the 1990s, it was shown that formalin-induced cross linkages of proteins could be broken down by heat treatment with subsequent successful antibody usage in formalin-fixed specimens (Shi, Key and Kalra, 1991). After review of the literature it was established that heat-mediated antigen retrieval technique by microwaving would be used.

Optimisation of staining was achieved using suitable positive control tissue. Normal healthy liver was identified as a good positive control for Notch 2 (Gao *et al* 2007).

Experiments were conducted using serial dilutions of primary antibody to find the best IgG concentration for Notch 2. A series of experiments were also done to check whether the microwaving time for HIAR or primary antibody incubation conditions (at room temperature for 1 h vs. 4°C for 18 h) was resulting in excessive background staining. In this manner, an optimised protocol for Notch 2 was produced (shown in **Table 2.6**).

Table 2.6 Optimal conditions for Notch 2 antibody

Antibody	Concentration	Incubation duration	Positive control tissue
Notch 2	1/500	1 h at RT	Normal Liver tissue.

2.4.5 Scoring of immunohistochemical staining

An Axioskop 2 Plus microscope was used for slide interpretation and image capture (Carl Zeiss Ltd, Hertfordshire, UK). The scoring system was previously devised in the department with the assistance of a consultant histopathologist and was already used to investigate the expression of Notch 1, 3 and 4 in PDAC. Slides were initially assessed under 100x magnification and areas of staining were identified, which were then examined at a higher power (400x) to identify stain localisation (cytoplasmic or nuclear). Cytoplasmic staining was measured by a semi-quantitative assessment of the proportion of positive tumour cells/ductal epithelial cells, ranging from 0% to 100%, with scores in 10% increments according to the percentage of the tumour cells/ductal epithelial cells stained. Nuclear staining was graded positive if > 10% of tumour/ductal epithelial cell nuclei stained positive. If less than 10% of tumour/ductal epithelial cell nuclei stained positive, then the nuclear staining was graded negative. Staining intensity was avoided since the degree of staining intensity has been shown to vary by tumour

type, as well as being partially influenced by the choice of fixatives and inversely correlated with storage time of the unstained tissue sections. The inter-observer agreement of staining intensity assessed by multiple observers was also noted to be only moderate to poor (Zlobec *et al.*, 2007). Slides were graded independently by two observers (post graduate student Dr Masood and a consultant histopathologist Dr Kevin West) blinded to the clinical data. In very few cases (<5%) where there were discrepancies in the score given by each observer, the slides were re-reviewed by both observers simultaneously and a final score agreed

2.5 Methods – Patient Plasma Preparation

Blood from patients was taken in both heparin coated tubes and EDTA tubes. The tubes were immediately placed in ice. Within 2 h the bloods were fractionated into plasma, white blood cell layer and red blood cell layer by centrifuging the sample for 10 min at 2000 g at 4°C. The resulting plasma was aliquoted into labelled cryotubes and stored at -80°C.

2.6 Methods – MicroRNA

2.6.1 Extraction of miRNA from plasma (miRNeasy Kit)

The plasma samples were thawed on ice with gentle inversion periodically to mix the sample. 4 mL (10 times the volume of plasma) of QIAzol lysis reagent was added to 400 µL of plasma and vortexed vigorously. This homogenous solution was left in a tube on the bench top at room temperature for 5 min. The solution was then spiked with 5 µL of synthetic C *elegans* (5 fmol/µL) as control miRNA and vortexed to mix the

spiked synthetic with the homogenous solution. The homogenised plasma was combined with 800 µL of chloroform (0.2 mL of chloroform per 1mL of QIAzol lysis re-agent used) followed by at least 30 s of vigorous mixing by using a vortex at maximum speed. The solution was then incubated at room temperature for 3 min following which it was centrifuged at 12,000g for 15 min at 4°C. This separated the solution into 3 phases: an upper, colourless, aqueous phase containing RNA and miRNAs; a white interphase containing DNA; and a lower red phenol-chloroform phase. The RNA containing aqueous phase was then transferred into a fresh tube and the volume of aqueous phase measured and 1.5 times the volume of 100% ethanol was added and vortexed. At least 700 µL portions of the sample was then applied into an RNeasy Mini spin column in a 2 mL collection tube. The spin column was then centrifuged at ≥ 8000g for 30 s at room temperature. Flow-through from the column was discarded. This process was repeated as many times as necessary until all the aqueous phase-ethanol-mix sample was exhausted.

The following step is the DNase treatment and was done to remove any possible traces of DNA from the preparation. 350 µL of Buffer RWT was pipetted into the RNeasy Mini spin column and centrifuged for 15 s at ≥ 8000g and the subsequent follow through was discarded. 10 µL of DNase 1 stock solution was added to 70 µL of Buffer RDD. This is mixed by gently inverting the tube. 80 µL of the DNase 1 incubation mix was then pipetted directly onto the RNeasy Mini spin column membrane and placed on the benchtop at room temperature (20–30°C) for 15 min. 350 µL of Buffer RWT was then pipetted into the RNeasy Mini spin column and centrifuged for 15 s at ≥ 8000g. The follow through was discarded.

After the DNase treatment, 500 µL of Buffer RPE was pipetted onto the RNeasy Mini spin column. The lid was closed and centrifuged for 15 s at ≥ 8000g to dry the RNeasy Mini spin column membrane and ensure that no ethanol was carried over during RNA elution. Another 500 µL of Buffer RPE was added to the RNeasy Mini spin column and the process repeated with centrifugation now lasting for 2 min. The RNeasy Mini spin column was carefully removed from the collection tube with special consideration taken to ensure that no contact was made with the follow-through. The RNeasy Mini spin collection was transferred to a new 1.5 mL collection tube and 50 µL of RNase-free water was pipetted directly onto the RNeasy Mini spin column membrane. The lid was closed and the tube left standing in room temperature for 10 min, following which the tube was centrifuged at ≥ 8000g for 1 minute at room temperature. This process was repeated with 50 µL of RNase-free water. The total 100 µL eluent, containing the total RNA and miRNA was collected and transferred to an eppendorf before being stored at -20°C.

2.6.2 miScript Reverse Transcription and SYBR® Green R -T qPCR

The RNA obtained from human plasma was reversed transcribed using the miScript II RT Kit. The template RNA was thawed on ice. The 10x miScript Nucleics Mix and 5x miScript HiSpec Buffer was thawed at room temperature. The reverse-transcription master mix was prepared on ice with the different volume of the components of which are outlined in Table 2.7. The template RNA was added to the tube containing reverse-transcription master mix. This was mixed gently, followed by brief centrifugation and then placed on ice. The solution was incubated for 60 min at 37°C, after which it was

incubated for 5 min at 95°C, to inactivate the miScript Reverse Transcriptase Mix, and

placed on ice. The 20 µL mix (containing cDNA) was diluted to 100 µL by adding 80

µL of RNase-free water and mixed by pipetting. The cDNA was either used for real-

time PCR immediately or stored in -20°C until used for real-time PCR.

Table 2.7 Reverse-transcription reaction components

Component	Volume
5x miScript HiSpec Buffer	4 µL
10x miScript Nucleics Mix	2 µL
miScript Reverse Transcriptase Mix	2 µL
Template miRNA	12 µL
Total	20 µL

The miScript SYBR® Green PCR Kit was used to perform real-time qPCR on the

diluted cDNA. 2x QuantiTect SYBR Green PCR Mater Mix, 10x miScript Universal

Primer, 10x miScript Primer Assay and the template cDNA were thawed on ice. A

miScript mastermix was made with the different volume of the components as outlined

in Table 2.8. Three microlitres of the diluted cDNA template was added into the

individual wells of the 96-well PCR plate. The mastermix was thoroughly mixed and 13

µL of the mastermix was aliquoted into each well of the PCR plate. An optical adhesive

cover was used to seal the PCR plates. The plate was gently mixed followed by

centrifugation at 1000g for 1 minute. The samples were then analysed using a 7300

Real Time PCR system (Applied Biosystems). The cycling conditions are shown in

Table 2.9.

Table 2.8 Reaction setup for real-time PCR

Component	Volume for 1 reaction
2x QuantiTect SYBR Green PCR Master Mix	8 µL
10x miScript Universal Primer	2.5 µL
10x miScript Primer Assay	2.5 µL
Template cDNA	3 µL
Total	16 µL

Table 2.9 Cycling conditions for real-time PCR

Step	Time	Temperature
PCR Initial activation step	15 min	95°C
	3 -step cycling	
Denaturation	15 s	94°C
Annealing	30 s	55°C
Extension	34 s	70°C
Cycle number	44 cycles	
	Melting curve Analysis	
Denaturation	15 s	95°°C
Annealing	30 s	60°C
Extension	15 s	95°C
Cooling		4°C

2.7 Methods – Mass Spectrometry

2.7.1 Steps used for sample preparation.

2.7.1.1 Preparation, solid phase extraction and freeze drying

The samples were mixed in equal volume of 4% phosphoric acid and incubated in ice

for an hour. This was done to break the tertiary bonds of the proteins and therefore

make them more amenable to SPE as well as to make more binding sites available for

immunoprecipitation by antibody-bound magnetic dynabeads. SPE was carried out

using hydrophilic-lipophilic-balanced reversed phase 1 cc Oasis columns obtained from

Waters. They were primed before use and were placed on a vacuum manifold

maintaining a suction pressure of ~ 5 mmHg. The SPE cartridges were activated with 1

mL of HPLC grade methanol and then 1 mL of HPLC grade water was used to wash off

the activation solvent. The samples were then loaded onto the primed SPE columns.

Following SPE, the samples were placed in a centrifugal evaporator for 1 h to remove

any volatile liquids, which were acquired during SPE during elution, and also to

concentrate the eluates. After centrifugal evaporation, the samples were freeze dried to

remove all the solvent and to further concentrate the analytes. Freeze Dried Pellets

(FDP) which contained the sample were maintained at -80°C prior to the next step.

2.7.1.2 Dissolving the FDP and size-exclusion filtration

The freeze-dried pellets were then resuspended in 50 µL of 0.1% trifluoroacetic acid

(TFA) and passed through centrifugal filter devices with a molecular weight cut off at

10,000 Da.

2.7.1.3 Immunoprecipitation using magnetic dynabeads

Following size-exclusion filtration, the sample was incubated in 4°C with antibody

coupled magnetic dynabeads. The dynabeads were then washed and the peptides eluted.

The main steps during immunoprecipitation were washing of the dynabeads with

appropriate buffers; capture of IgG to the dynabeads; crosslinking of the antibody to the

dynabeads; resuspension of the FDP in ammonium bicarbonate followed by size-

exclusion filtration; capture of the Notch 1 peptide to the antibody-bound-dynabeads; and elution of the Notch 1 peptide. The procedure was adapted from the product leaflet provided by Invitrogen.

2.7.1.3.1 Dynabeads washing steps

Prior to using the dynabeads, the solution in which it was suspended was vortexed to ensure that the dynabeads went back into suspension evenly. Dynabeads (100 µL) were taken and placed in a magnetic rack (magnarack provided by Invitrogen) for two min. The magnarack resulted in the beads coming out of suspension and sticking to a side of the eppendorf allowing the removal of the solvent without disturbing the beads. The beads were washed twice with 500 µL of citrate phosphate buffer pH 5.0 containing 0.01% Tween-20, using the magnetic rack after each wash to remove the buffer. This method of using the magnetic rack to isolate the dynabeads was used at the end of every step, whether it was to wash, or to remove the buffer.

Invitrogen recommended the use of PBS or citrate phosphate buffers with a pH range of 5-7, but their results indicated that the dynabeads captured more IgG at pH 5.0 compared to pH 7.4. Based on these findings citrate phosphate buffer, pH 5.0 with 0.01% Tween-20 was used as the wash buffer.

2.7.1.3.2 IgG capture procedure

The amount of Ig captured by the Dynabeads is dependent on the concentration of Ig and the Dynabeads protein G in the starting sample. It has been recommended by the manufacturer that the concentration of Dynabeads protein G in the antibody solution

should be similar to the concentration of the Dynabeads protein G in the starting vial. Since 100 µL of suspended Dynabeads was used from the vial, the dynabeads stuck to the side of the eppendorfs in the magnarack following the two washes were resuspended in a total volume of 100 µL. This volume was composed of 50 µL of buffer and 50 µL of antibody solution.

Invitrogen provided a graph which shows the relationship between the amount of IgG captured and concentration (IgG/mL) in the sample. This shows a rapid increase in IgG captured until 25 µg IgG/mL is reached. After this, the increase in IgG capture is very slow and begins to plateau at 100 µg IgG/mL. Twenty-five µg IgG equates to 19 µL of Notch 1 antibody and 100 µg IgG equates to 76 µL of antibody. Since there was a limited supply of polyclonal antibody to Notch 1 peptide (5 mLs), 50 µLs of antibody was used for each sample.

Fifty microlitres of antibody was diluted with 50 µL of citrate phosphate buffer and the dynabeads were suspended in this solution and mixed gently using a rotating table for 2 h at room temperature to ensure maximum amount of Ig-binding, as recommended by the manufacturer. Following capture of the antibody, the Dynabeads were washed three times with the citrate phosphate buffer, pH 5.0 with 0.01% Tween-20.

2.7.1.3.3 Cross-linking of Ig to Protein G

The Dynabead protein G- Ig complex was washed twice in 1 mL of 0.2 M triethanolamine, pH 8.2 using the magnetic rack. The beads were then resuspended in 1 mL freshly made 20 mM dimethyl pimelimidate hydrochloride (DMP) in 0.2 M triethanolamine, pH 8.2. They were left to incubate in a gentle mixer for 30 min at room

temperature. They were again placed on the magnetic rack for 2 min and the supernatant discarded. The dynabeads were then resuspended in 1 mL of 50 mM Tris, pH 7.5, and incubated for 15 min by gentle mixing to stop the cross-linking reaction. Following this, the supernatant was again discarded and the beads were washed three times using 100 µL PBS containing 0.01% Tween-20 and finally the supernatant was discarded leaving behind the cross-linked dynabeads.

2.7.1.3.4 Immunoprecipitation of Notch 1 peptide

The freeze-dried pellets (which were stored in -80°C until required) were dissolved in 100 µL of 50 mM ammonium bicarbonate buffer, pH 7.4. The solution was then passed through a centrifugal filtration device with a cut off of 10,000Da. The resulting filtrate which should contain the Notch peptide was incubated with the Notch 1 antibody crosslinked-dynabeads at 4°C and gently mixed overnight for a minimum of 12 h.

2.7.1.3.5 Target Protein Elution Procedure

The suspension was placed on the magnetic rack for 2 min and the supernatant discarded. The beads were washed with 1 mL of 50 mM ammonium bicarbonate (pH 7.4) three times. After each wash the supernatants were collected and retained as wash 1, 2 and 3 respectively. It was recommended by Invitrogen that 30 µL of 0.1 M citric acid, pH 2-3 should be used to elute the target peptide from the cross-linked Ig G-protein G complex. Following mixing for 2 min, the samples were placed on the magnetic rack for 2 min and the supernatant collected as eluant 1. This step was repeated again for eluant 2. The eluants were then mixed with 0.1% TFA and further mixed with matrix for spotting onto the target plate for MALDI-MS analysis.

2.7.2. Preparing the sample for MALDI analysis

The final enriched sample was mixed with 1:1 with matrix {αCHCA dissolved in an acetonitrile:methanol solution (1:1, V/V) in a ratio of 2 mg/mL}. The resulting solution was then loaded onto MALDI target plates by using 1 µL for each spot and air dried. The plate was analysed using MALDI-MS. All experiments were done using a Waters Q-ToF Ultima Global in MALDI mode (Walters, Milford, US). Each spot was subjected to 100 laser ablations giving a high resolution MALDI mass spectrum containing ∼ 1.2 million events tagged with a sensitivity of m/z 0.01 or better covering a range of m/z 800-3000. Thus, combining the spectra from 4 wells provided ∼ 5 million events which are sufficient to produce high resolution spectra with a bin size of $\Delta\, m/z \approx$ 1. The resolution of the spectrometer is a function of m/z. The Full Width Half Maximum (FWHM) rises from 2 to 7 over the m/z range 800-3000. The scatter of the point about the regression line is largely introduced by the counting statistics on individual peaks. The intrinsic resolution of the machine is better than this but the resolution of peptide peaks is limited by the isotopic spread and confusion (see below). The gain and calibration of the MS were regularly checked and accuracy achieved repeatable to ≤ m/z 0.5 over the entire spectral range.

Part 1 - Solid Phase Extraction

50 µL of Plasma + 50 µL of 4% Phosphoric Acid

1 hour room temperature

Solid Phase Extraction

Oasis HLB 1cc Cartridges
Washed – 1 ml HPLC H_2O
Eluted – 40% Acetonitrile .1% TFA

Centrifugal Evaporator

1 hour

-80°C for half an Hour until Frozen

Freeze Dried Overnight

Freeze Dried Pellets. Stored in
-80°C until required

Part 2 - Immunoprecipitation

100 µL of Dynabeads extracted from Soultion

Washed with Citrate Phosphate Buffer

50µL Antibody + 50 µL Citrate Phosphate Buffer + Dynabeads

Mixed at room temperature for 2 hours

Crosslinking of Ig's to Dynabeads Protein G

Freeze Dried Pellets dissolved in Ammonium Bicarbonate

Centrifugal Filer 10,000 MWCO (Microcon/Amicon)

Dissolved Peptide & Crosslinked Dynabeads incubated overnight at -4°C

Washed with Ammonium Bicarbonate

Peptide Eluted using 0.1M Citric Acid pH 1.5

Samples analysed using Mass Spectometry

Figure 2.2 Schematic diagram of the steps involved in sample purification and enrichment.
This is a diagram showing the steps involved in sample purification and enrichment. It has been divided into two parts for simplification. Part 1 shows the steps involved in purification of the sample. Part 2 shows the steps involved in enrichment of the peptides and its subsequent preparation for MALDI-MS analysis. Before the start of immunoprecipitation, the sample undergoes size-exclusion filtration.

CHAPTER 3.

INVESTIGATING NOTCH 1 AND NOTCH 3 AS POTENTIAL BIOMARKERS FOR PANCREATIC DUCTAL ADENOCARCINOMA USING MASS SPECTOMETRY

3.1 Introduction

This chapter describes the use of Notch signalling pathway products as potential biomarkers in pancreatic ductal adenocarcinoma. Notch is mediated by intramembrane proteolysis as described in chapter 1. Ligand binding generates sequential cleavages at S2, S3 and S4 sites of the transmembrane receptor resulting in the release of Notch Intracellular Domain (NICD) which translocates into the nucleus and modifies expression of target genes. Whereas the S2 cleavage occurs in the extracellular juxtamembranous region, the resulting transmembrane fragment undergoes intramembranous proteolysis at S3 and S4 sites. The S3 cleavage happens between the cytosol and the membrane and results in the release of NICD into the cytosol (Roy *et al*, 2007; Kopan and Ilagan, 2009). Okochi *et al* showed in murine cell line that the S4 cleavage in the transmembrane domain resulted in the release of a putative Notch 1 fragment (Nβ) into the extracellular fluid (Figure 3.1). They were further able to detect two Nβ fragments, Nβ21 and Nβ25 by a process of immunoprecipitation and analysis of the molecular masses of the immunoprecipitated peptides by matrix-assisted laser desorption ionisation time of flight (MALDI-TOF) mass spectrometry. Nβ21 had a molecular mass of 2306 and Nβ25 has a molecular mass of 2694 Daltons and both these Notch 1 peptide sequences were found to be composed of peptides from the S2 to S4 cleavage sites (Okochi *et al*, 2006) (Figure 3.2).

It was also shown that the secretion of Notch 1 peptides was greatly enhanced in cells expressing both Jagged-1 and Notch 1, but was hardly detectable in cells expressing either one of them indicating that the Nβ peptide secretion is a product of Notch receptor proteolysis as a result of activation by its respective ligands (Okochi *et al*, 2006). It is also known that ligand-induced Notch signalling is required for the

metaplastic conversion of acinar-to-ductal cells and ultimately pancreatic ductal

adenocarcinoma. Notch signalling inhibition is considered a potential therapeutic target

to prevent tumour progression (Sawey *et al*, 2007; Miyamoto *et al*, 2003). The

knowledge that Nβ fragments are secreted extracellularly during Notch activation by

their ligands suggests possible exploration of the idea of using Nβ fragments of Notch

as biomarkers in pancreatic malignancy.
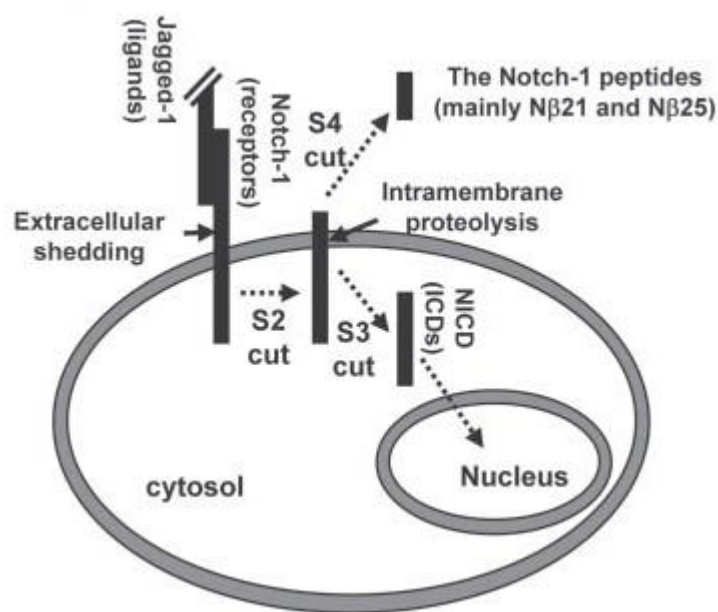
Figure 3.1 Schematic diagram of Notch 1 signalling.

Notch receptors, when activated by their ligands, initiates a sequential cleavage of the
transmembrane receptor. S2 cleavage occurs at the extracellular juxtamembranous region. The S3
cleavage occurs between the cytosol and the membrane. The S4 cleavage occurs in the
transmembrane domain resulting in the release of the Nβ peptide fragment. Figure taken from
Okochi et al 2006.

The aim of this chapter is to determine whether Notch 1 Nβ fragment could be detected

in human plasma and assess its potential as a biomarker for pancreatic ductal

adenocarcinoma. If successful, the intention is to carry out similar analysis for other

Notch receptors.
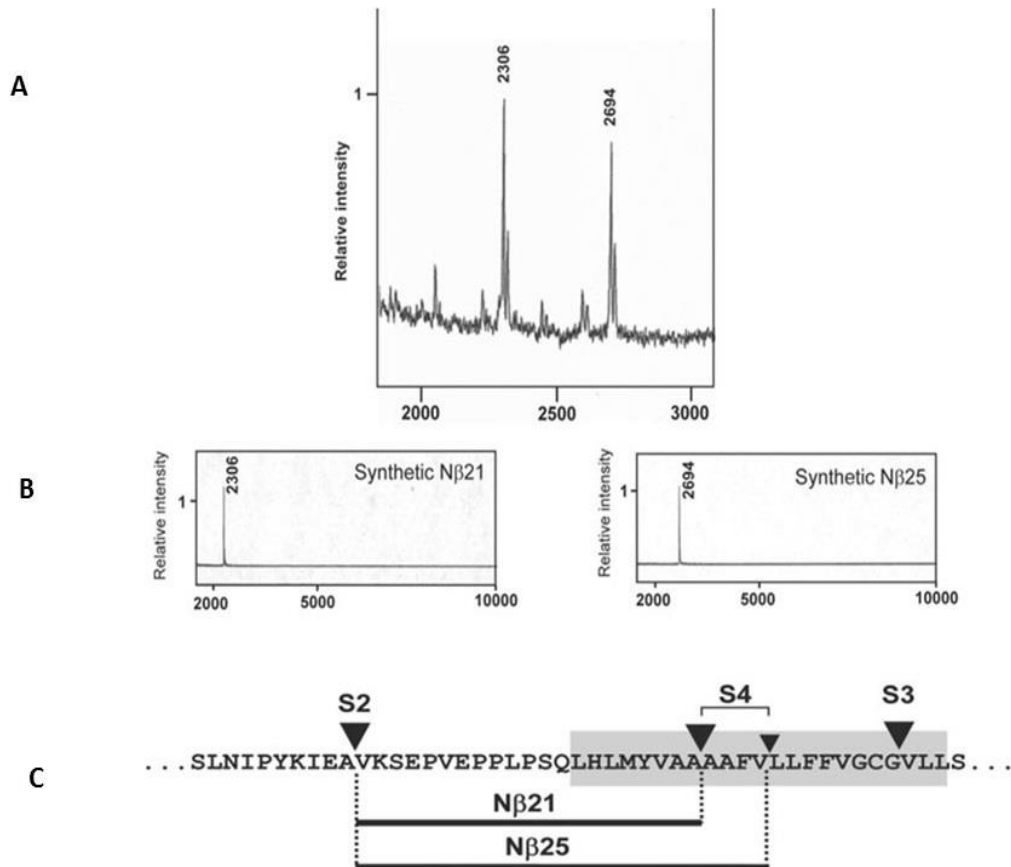
Figure 3.2 Detection and sequencing of Notch 1 Nβ peptide.
A. Okochi et al were able to detect 2 major ions of *m/z* 2306 and 2694 in Notch 1-expressing murine cells using their methods of immunoprecipitation. *B.* MALDI-TOFMS spectra of the synthetic N21 and N25 peptides. C. schematic representation of two different N species and the S2, S3, S4 cleaved sites in Notch 1. The grey box indicates the putative transmembrane domain of murine Notch 1. Figure taken from Okochi *et al* 2006.

3.2 Background information on Mass Spectrometry

The principle of mass spectrometry relies on the formation of gas phase ions (either positively or negatively charged) and their separation depending on the mass to charge ratio *(m/z)*. A typical mass spectrometer consists of three parts: an ionisation source which ionises the sample, a mass analyser where the charged ions are separated depending on their mass and charge, and an ion detector which detects the current and amplifies it (El-Aneed, Cohen and Banoub, 2009). With the advent of soft ionisation

techniques, first described in 1974 (Karas and Hillenkamp, 1988), which did not destroy thermally unstable, polar biological compounds, MS could be used for proteomics studies. Soft ionisation means that minimal energy is used to ionise the analytes. Matrix Assisted Laser Desorption Ionisation (MALDI) and ElectroSpray Ionisation (ESI) represent the advancements required to allow researchers to use MS easily in the study of biological substances, such as glycoconjugates, DNA and proteins (Karas and Hillenkamp, 1988; Yamashita and Fenn, 1984; Whitehouse *et al*, 1985; El-Aneed *et al*, 2009).

3.2.1 Electrospray Ionisation-Mass Spectrometry (ESI-MS)

This technique was optimised in the 1980s. It involved the samples being dissolved in a polar solvent which is then fed into an ionisation source via a metal capillary, which is electrically charged. The charged droplets are then bathed by a warm neutral gas, such as nitrogen, resulting in the droplets becoming smaller and the charge on their surface exerting a greater repulsive force against each other. Eventually when the repulsive force exceeds the surface tension which holds the droplet together, ions desorb into the gas phase. As this technique allows the desorbtion of ions into the gaseous phases without destruction of molecules, it made it easier to study large proteins (Kebarle, 2000).

3.2.2 Matrix–Assisted Laser Desorption Ionisation (MALDI)

MALDI, a laser-based soft ionisation technique, is one of the most successful methods for MS analysis of large biological molecules and a technique used for protein sequencing and proteomic research. Samples are dissolved in a suitable solvent and

then mixed with a matrix. This solution is then spotted on a MALDI plate and allowed to dry, forming a crystalline compound with the matrix. This complex is then placed in the ionisation source where a laser beam is directed at the crystals. The energy from the laser is absorbed by the matrix resulting in desorption and ionisation of the analytes in the sample into the gas phase (El-Aneed *et al*, 2009).

The choice of matrix depends on the characteristics of the analytes as well as the charge induced on the analytes. Acidic matrices are favoured where there is a proton donor and base matrices are preferred for negative ionisation mode. The matrix, α-cyano-4-hydroxy-cinnamic acid (CHCA), is normally used for analysing peptides with molecular weights below 10 kDa (Baldwin, 2005).

3.2.3 Comparison of MALDI and ESI

In MALDI the sample is introduced into the ion source in the solid state; whereas in ESI, the sample is introduced as a liquid. Although both these techniques can be used for qualitative assessment for analytes in very low concentrations, ESI, especially when interfaced with liquid chromatography, can produce efficient quantitative measurements since it utilises dissolved samples. MALDI readings are dependent on the position where the laser beam hits the sample, and are qualitative rather than quantitative (El-Aneed *et al*, 2009). MALDI is more robust than ESI in the presence of contaminants such as salts and detergents. In ESI, the contaminants can actually compete with the ions of the analyte for ionisation and therefore influence results. In MALDI it is less of a problem since analytes can escape impurities in the sample similar to the way they escape the matrix when they absorb the laser energy (Breaux *et al*, 2000).

Another important difference between MALDI and ESI is the number of charges acquired during each ionisation process. ESI can produce multiple charged analytes for proteins and peptides and therefore multiple *m/z* values for the same analytes. This makes identifying the analytes quite difficult in the spectrum of a complex sample. MALDI, however, only tends to produce single charged analytes and therefore identifying them is much easier (Karas, Gluckmann and Schafer, 2000).

Since our study was to determine the presence of Notch Nβ fragment in human plasma (qualitative) and model of study was based on the work done by Okochi *et al*, MALDI-MS was used.

3.3 Purification of Samples – The Principle

The objective was to detect a Notch 1 Nβ fragment in human plasma. Direct analysis of patient plasma samples by MS would lead to too much interference making detection very difficult. This is due to ionisation suppression from interfering proteins which are present in the sample in large quantities relative to the protein of interest. Examples include albumin present at 50 mg/mL and globulin at 35 mg/mL. Enrichment is required to remove interfering substances in order to be able to detect the peptide of interest which is likely to be present at femtomolar levels.

Okochi *et al* used agarose gel electrophoresis to immunoprecipitate Nβ fragments from murine cell line media. The immunoprecipitates were washed three times with wash buffer to remove further impurities and finally eluted before analysing using MALDI-TOF MS (Okochi *et al*, 2006). Whiteaker *et al* (2007) were able to demonstrate ion signal enhancement of $>10^3$ which is sufficient for quantifying biomarkers in plasma at

the ng/mL range, by using an antipeptide antibody bound magnetic bead based platform for peptide enrichment. We used three levels of purification during namely solid phase extraction (SPE), size-exclusion filtration and immunoprecipitation using antibody bound magnetic dynabeads.

3.3.1 Solid Phase Extraction -The principle

Solid Phase Extraction (SPE) is a chromatographic technique which is commonly used for sample purification prior to analysis. Samples are applied to columns or cartridge devices containing a bed of chromatographic packing material (solid phase). If the analytes which are to be extracted have a greater affinity for the solid phase compared to the sample matrix they are dissolved in, they bind to the solid phase whilst the rest of the sample matrix passes through the column or cartridge to waste. The analytes which are retained on the solid phase can then be eluted by a solvent for which the analytes have a greater affinity to than the solid phase (Berrueta, Gallo and Vicente, 1995).

SPE can be used to remove specific impurities from samples which, when present in large quantities, can suppress the signal of the analyte of interest during mass spectrometry analysis. This is especially important when analysing plasma extracts, as phospholipids and several large proteins present in the plasma can cause severe ionisation suppression. An optimised SPE protocol would provide a cleaner extract and minimize any ionisation suppression. It can also be used to increase the concentration of the analyte in the original sample to improve detection limits and also to separate a sample into separate fractions of analytes, by using eluting agents of differing strengths to obtain several fractions (Berrueta *et al*, 1995).

SPE involves several steps: pre-treatment of the sample, followed by priming and conditioning of the cartridge, loading the sample and finally the elution of the cartridges. Priming and conditioning involves activation of the solid phase in the cartridge by passing an appropriate solvent through under positive pressure which activates the surface of the solid phase, followed by passing a liquid solvent which is similar to the sample matrix to remove the activation solvent. The samples are then loaded onto the cartridges, followed by a washing step to remove any unwanted substances and finally eluting the analytes required. The washing step of the SPE is applicable during sample preparation in order to remove any unwanted interfering compounds. This however does not affect the analytes since they are bound to the solid phase and have a greater affinity for the solid phase (Berrueta *et al*, 1995).

There are several principal separation modes of chromatography used in SPE. Reversed Phase Chromatography, the most commonly used SPE mode, was the method chosen in this study. It involves the use of a non-polar solid phase and a polar, or moderately polar, sample matrix to attract a non-polar analyte of interest. This mode can be used to separate peptides, proteins and oligonucleotides according to their hydrophobicity. It is the main technique for the purification of synthetic peptides and is used for desalting of peptide and oligonucleotide samples (Thurman and Mills, 1998).

3.3.2 Size-exclusion filtration – The Principle

As our molecule of interest is in the region of 2,000 to 3,000 Daltons, a size-exclusion filtration step will remove larger unwanted molecules and effectively provide a cleaner sample for analysis. Centrifugal filter devices with a molecular weight cut off at 10,000 Daltons were used.

3.3.3 Immunoprecipitation using Magnetic Dynabeads – The principle

Following SPE purification the samples will still contain large amounts of additional peptides and proteins which will interfere with the mass spectrometry and lead to suppression of the signal for the Notch 1 peptide. Therefore, further sample preparation steps are still necessary to attain the best possible results. Immunoprecipitation can be used to isolate the target antigen by binding it to specific antibodies. The use of magnetic beads in the isolation and purification of peptides and proteins is a commonly used purification method. Different types of magnetic beads with different surfaces are commercially available (Whiteaker *et al*, 2007).

A Dynabeads capture system with a high antibody coupling affinity as well as good crosslinking ability, which would bind to background serum proteins was required to generate clean samples for mass spectrometric analysis. A panel of seven Dynal magnetic beads (including Protein G, Myone streptavidin, M280 streptavidin, M270 streptavidin, tosylactivated, epoxy, and carboxyl beads) were previously assessed and Protein G magnetic Dynal beads were found to provide optimal results based on their low background binding, lack of chemical contamination, high coupling efficiency and proper orientation of cross-linked antibody. (Whiteaker *et al*, 2007) Based on these findings it was decided to use Dynabeads Protein G in the current study.

3.4 Methods

3.4.1 Synthetic Notch Nβ peptide

3.4.1.1 Sequencing the Notch Nβ peptide

Okochi *et al* determined the amino acid sequences of murine Notch 1 Nβ21 and Nβ 25 as VKSEPVEPPLPSQLHLMYVAA and VKSEPVEPPLPSQLHLMYVAAAAFV, respectively. Using this peptide sequence to compare with human Notch 1 and Notch 3 sequences, available from UniProtKB/Swiss-Prot database, the following amino acid sequences were found (Table 1). The predicted Nβ 21 peptide sequence for human was considered to start from the site of the S2 cleavage and continue 21 amino acids distally towards the site of the S3 cleavage.

Table 3.1 Amino acid sequence of 1) Notch 1 Nβ21 in mouse and human 2) Notch 3 Nβ21 in human

Notch 1 mouse	VKS	EPV	EPP	LPS	QLH	LMY	VAA
Notch1 human	VQS	ETV	EPP	PPA	QLH	FMY	VAA
Notch 3 human	VRG	EPL	EPP	EPS	VPL	LPL	LVA

3.4.1.2 Determination of the mass of synthetic Notch 1 Nβ peptide fragment by MALDI

A synthetic peptide for human Notch 1 Nβ was synthesised and a rabbit polyclonal antibody was raised against it by Davids Biotechnology. The synthetic Human Notch 1 peptide was dissolved in 0.1% TFA and this solution was mixed 1:1 with matrix {α-cyano-4-hydroxy-cinnamic acid dissolved in an acetonitrile:methanol solution (1:1,

V/V) in a ratio of 2 mg/mL}. The resulting solution was then loaded onto MALDI target plates by using 1μL for each spot and air dried. The plate was then analysed using MALDI-MS. Each spot received 100 laser ablations to acquire a chromatogram which was extracted to produce a spectrum of mass/charge ratio for the detection range from *m/z* 800-3000.

The Human Notch 1 peptide gave a signal of *m/z* 2311. This was the expected value as the correct molecular weight of the synthetic peptide is 2310.14 Da and a positive ion is added since the MS was operated in positive ionisation mode (Figure 3.3A). There was a delay obtaining the synthetic Notch 1 human peptide and so to minimise delays for the initial method development work involving sample purification, a Notch 1 peptide fragment of murine species, which was already available, was used and this gave a reading of *m/z* of 2303 (Figure 3.3B). Due to the similarities in sequencing and ultimately weight, it was thought that this peptide would be a suitable alternative to use for the initial method development work involving SPE using pancreatic cancer cell medium. For the latter stages of SPE method development and the Dynabeads extraction stage, a human form of Notch 1 peptide fragment and its corresponding antibody (made by David's Biotechnology) were used.

Prior to sample analysis the MALDI-MS was always calibrated by using either the Notch1 synthetic peptide or using a commercially available synthetic peptide, human Glu Fibrinopeptide B (Glu FiB) with a molecular weight of 1570.57 Da. In most of the MALDI-MS spectra a peak corresponding to the expected *m/z* of the Notch peptide + 16 Da was seen. This peak corresponds to methionine oxidation {M+16 (oxygen)} (Larsen and Roepstorff, 2000) of the Notch 1 peptide and so *m/z* values of 2322

(mouse) and 2327 (human) were observed. (Figure 3.3). For the purposes of our study, *m/z* 2322 was also recognised as Notch 1 Nβ fragment during the MALDI-MS spectrum analysis.
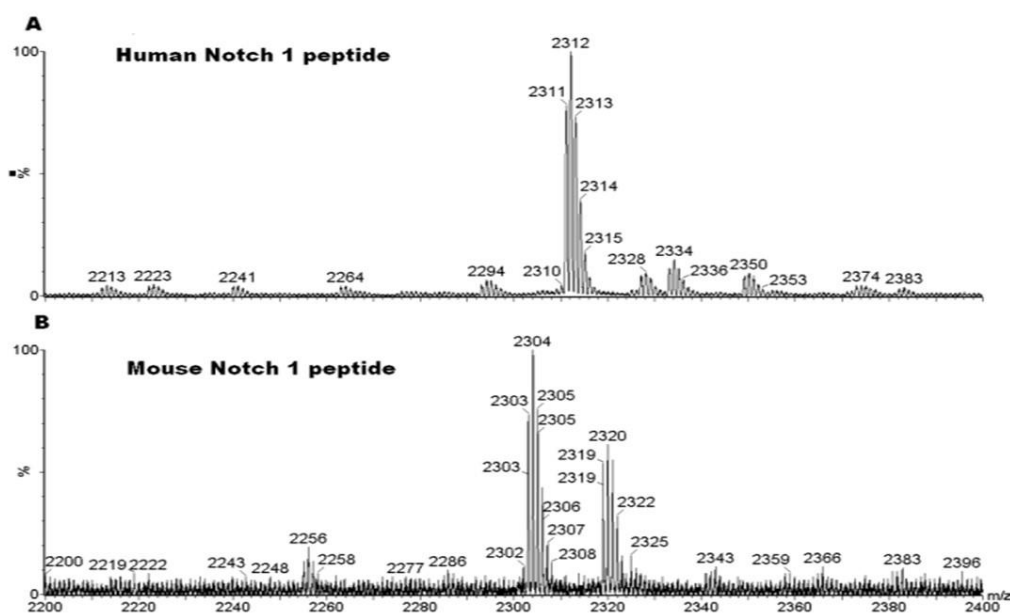
Figure 3.3 MALDI-MS signals for Notch 1 Human and Mouse peptide.

A. Human Notch 1 peptide gave a signal at 2311. B. Mouse Notch 1 peptide gave a signal at 2303. Please note a signal at 2319 which represents a M+16 oxidation of the methionine in the peptide sequence.

3.4.2 Sample preparation

The steps used for sample preparation are described in Chapter 2, section 2.7.

3.5 Method Development

The method was developed using synthetic Notch 1 peptides. The areas requiring development were SPE, size-exclusion filtration, and immunoprecipitation using Dynabeads followed by mass spectrometric analysis.

3.5.1 Solid Phase Extraction

The initial work for the SPE method development was carried out using pancreatic ductal adenocarcinoma cell culture medium. The medium was prepared by centrifuging at 2,000g for 5 min and collecting the supernatant to remove the cells. The following experiments were carried out to optimise the SPE.

3.5.1.1 Detection of Notch 1 and Notch 3 peptide in ASPC cell medium

Cell medium (50 µL) was mixed with an equal volume of 4% phosphoric acid and then left incubated on ice for 1 h. The samples were then loaded onto the primed SPE columns. Washing is designed to remove any non-bound salts and interfering substances. Previous work in the lab involving SPE on Notch peptides used 10% acetonitrile/0.1% TFA as the washing agent and 80% acetonitrile/0.1% TFA as the eluting agent (Mann, 2012). Using these agents, SPE was performed.

Following the steps required for sample purification, samples were analysed using MALDI-MS as already described. No signals were observed for Notch 1 peptides (2311) nor Notch 3, which were expected to be *m/z* 2311 and 2223 in PDAC cancer cell medium (Figure 3.4). The experiment was repeated with 500 µL of medium and although it gave a similar result, there was a lot more background noise. This shows that increasing sample size introduces a lot more impurities which might result in ionisation suppression in MALDI-MS analysis (Figure 3.5).
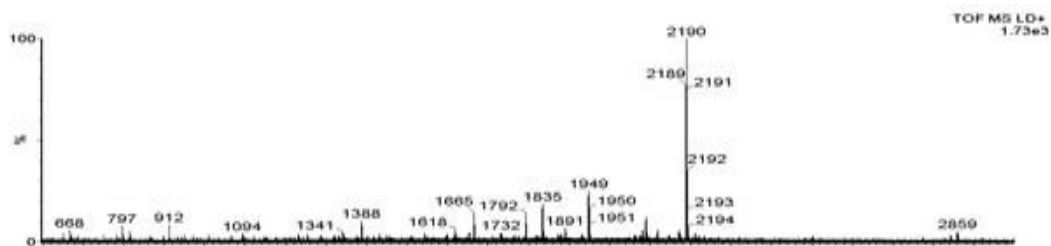
Figure 3.4 MALDI-MS spectrum reading of 50 µL of ASPC cell culture medium.

No signal for Notch 1 peptide (2311) nor Notch 3 peptide (2223) can be seen. The signals which can be seen are unknown peptides which are not significant in the current study. The TOF MS LD+ reading on the top right side represents the signal strength of the most abundant peptide which in this spectrum is 2190.
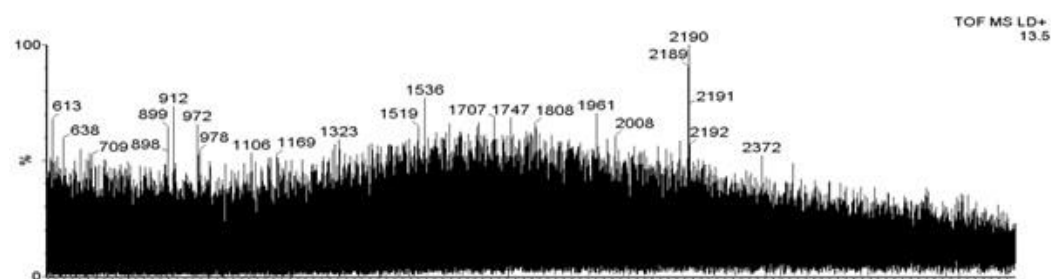
Figure 3.5 MALDI-MS spectrum of 500 µL ASPC cell culture medium.

Neither Notch 1 nor Notch 3 peptide signals can be detected. Please note a lot more background noise, indicating impurities. The signal strength of the 2190 ion is 13.5 compared to 1730 in figure 4, where 50 µL of medium was used. This proves that 500 µl medium introduces more impurities and therefore suppresses ionisation.

3.5.1.2 Development of the SPE washing step

Washing the columns is designed to remove any salts and interfering substances by using an aqueous substance similar to the sample matrix. This method was modified in the current study by washing with HPLC grade water instead of 10% acetonitrile/0.1% TFA as previously used. To validate whether this change in washing protocol altered the final results, experiments were carried out comparing the two washes. For both samples, no signals could be observed for Notch 1 (m/z 2311) or Notch 3 (m/z 2223)

following MALDI-MS analysis. However, it was noticeable that washing with HPLC

grade water resulted in much less background noise (Fig 3.6).
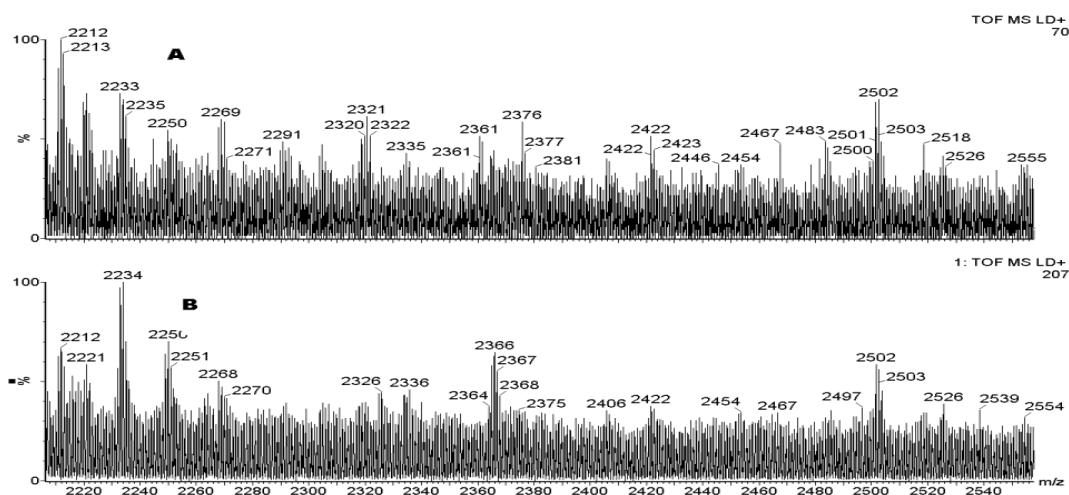
Figure 3.6 MALDI-MS spectra of 50 µL ASPC cell culture medium after different washes.
A. This sample was washed by 10% acetonitrile/0.1% TFA. B. This sample was washed with
ultra-pure water. Notice the higher signal reading in B compared to A indicating more ionisation
occurs when washed with water.

3.5.1.3 Detection of Notch1 peptide in spiked culture medium

Synthetic mouse Notch 1 peptide (50 pmol), dissolved in a solution of 0.1%TFA, was

added to 50 µL of culture medium and subjected to SPE as described previously. The

MALDI-MS was able to detect Notch 1 peptide for this sample. This experiment

validated the changes made to the SPE procedure and showed that in principle Notch 1

peptide can be detected in a solution such as cell culture medium/plasma using the

methods described so far, provided it is present in high enough concentrations (Figure

3.7).
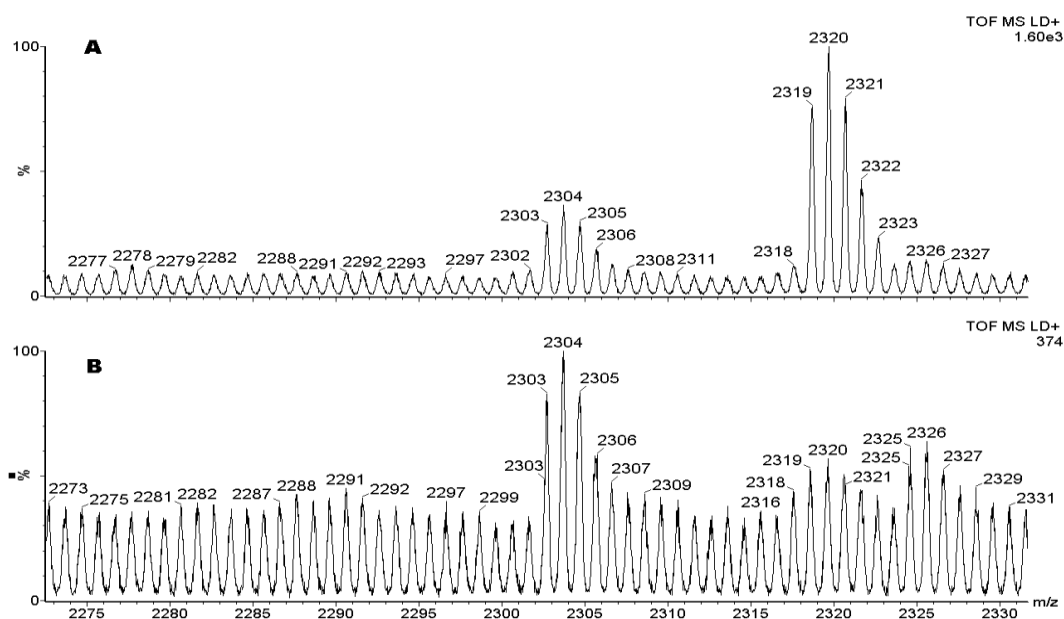
Figure 3.7 MALDI-MS spectrum of ASPC cell culture medium spiked with mouse Notch 1 peptide.

A. 50 µL of medium spiked with 50 pmol of peptide. Note that the Notch 1 signal can be detected at 2303 and 2319 (M+ 16). B. 500 µL of cell medium was spiked with 50 pmol of Notch 1 peptide. Please note that the signal intensity in B is considerably less than that of A, indicating that with increased sample volume there is less ionisation.

3.5.1.4 Determination of the volume of medium required for SPE

It was hypothesised that the amount of Notch 1 peptide present in culture medium and patient plasma samples would be at femtomolar levels and that it should be possible to detect it in 50 µL or 500 µL of sample using MALDI-MS. Notch 1 peptide (50 pmol) was added to 50 µL and 500µL of medium and mixed with an equal volume of 4% phosphoric acid. MALDI analysis detected Notch 1 peptide with much higher signal intensity in 50 µL medium (Figure 3.7). Although 500 µL of cell culture medium will contain more Notch 1 peptide than 50 µL, it also contained a lot more impurities. This resulted in ionisation suppression, leading to much poorer detection of peptide. Following the results of this experiment the volume of patient plasma/ medium to be analysed was fixed at 50 µL.

3.5.1.5 Determination of the optimal solvent for SPE

The ultimate aim of sample preparation is to provide an enriched sample with the least amount of impurities. A less hydrophobic eluting agent than 80% acetonitrile/0.1% TFA might result in less contaminating compounds. The optimal concentration of the eluting agent was determined in order to release the Notch 1 peptide from the SPE columns with the fewest impurities.

Medium (50 µL) was spiked with 50 pmol of Notch1 peptide and SPE was carried out on the sample. The eluting solvent initially used was 10% acetonitrile/0.1% TFA and this was increased in 10% increments to 80% acetonitrile. Samples were collected and processed separately. Notch 1 peptide was detected in samples with the eluting agents at 30% acetonitrile/0.1% TFA and 40% acetonitrile/0.1% TFA. This suggests that all detectable Notch peptides are eluted by 40% acetonitrile/0.1% TFA and 80% acetonitrile/0.1% TFA is not necessary (Figure 3.8).

When a further experiment was done to compare 30% acetonitrile/0.1%TFA and 40% acetonitrile/0.1% TFA as eluting solvents, the 40% acetonitrile/0.1% TFA gave much higher signal intensity. These experiments suggested that although some of the Notch 1 peptide was eluted using 30% acetonitrile/0.1% TFA, most the peptide was eluted using 40% acetonitrile/0.1% TFA. Since peptides are present at femtomolar levels and some are bound to be lost during the purification process, 40% acetonitrile/0.1%TFA was deemed as the optimal concentration of the eluting solvent (Figure 3.9).
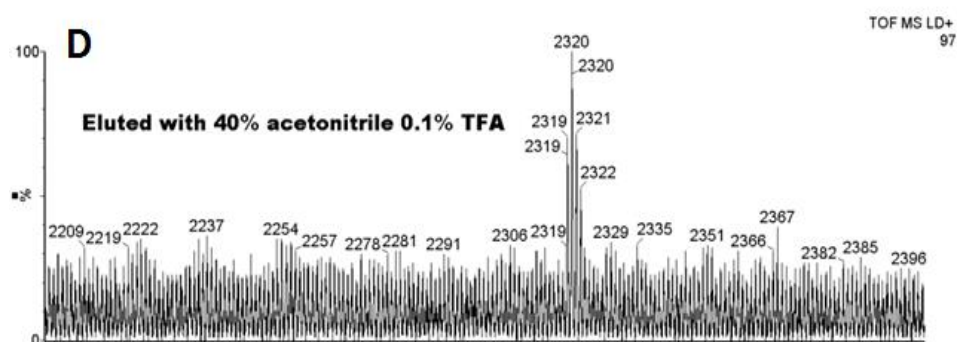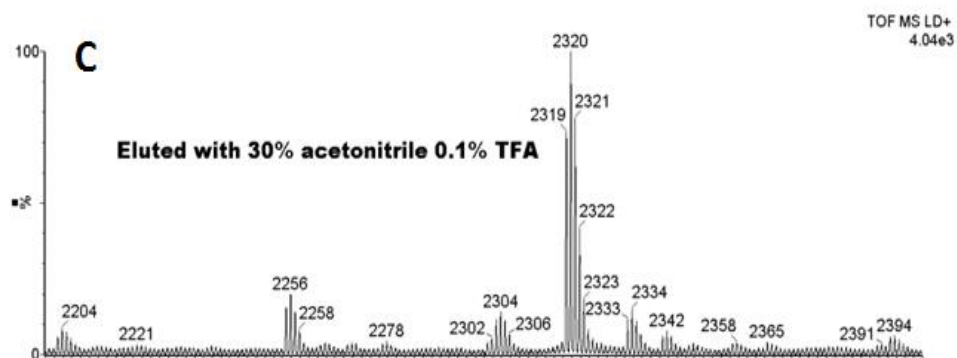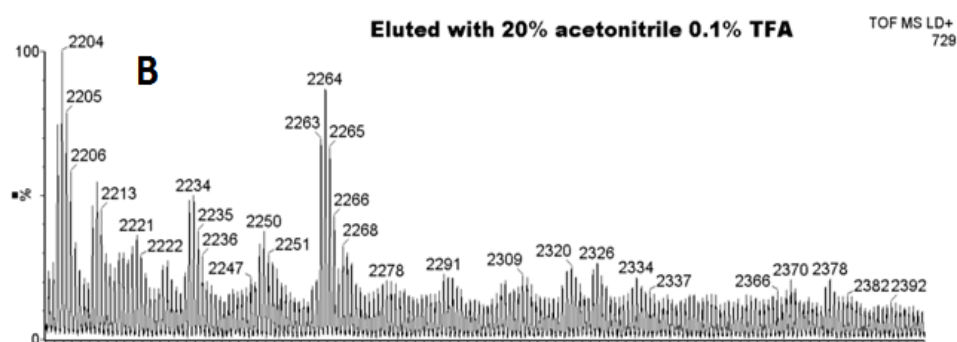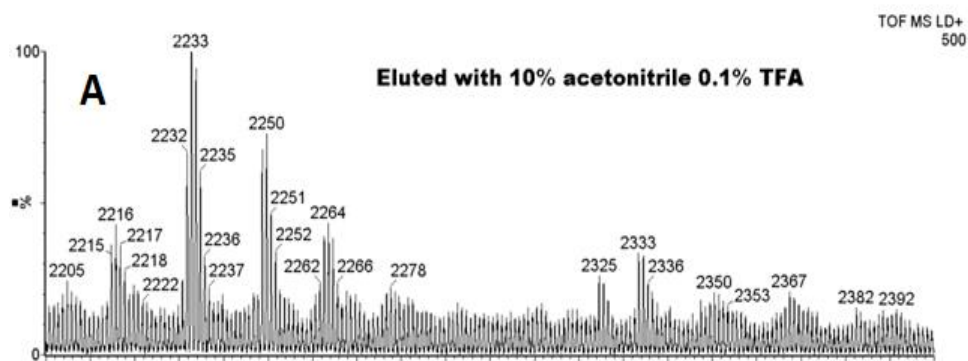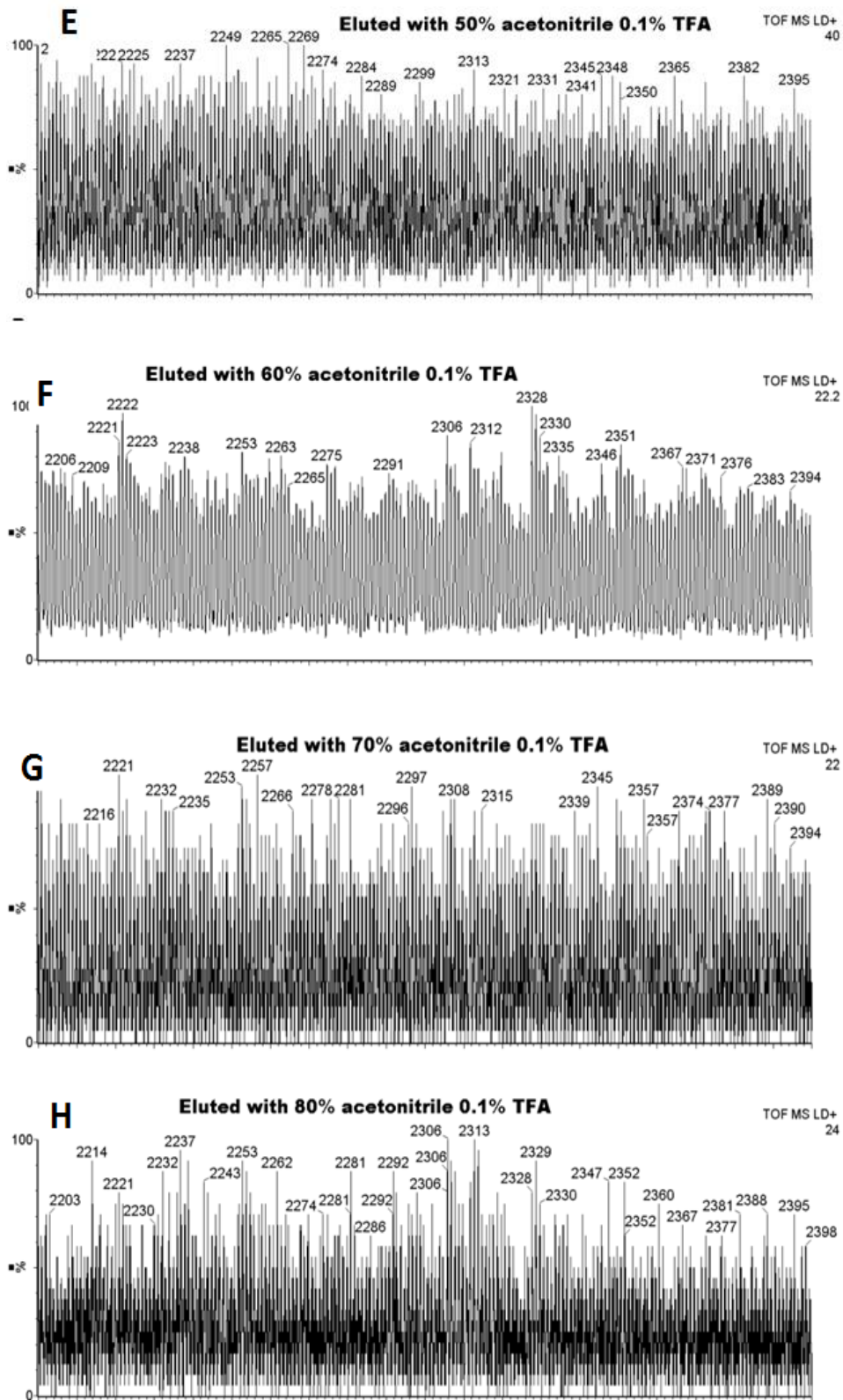
Figure 3.8 MALDI-MS spectra of 50 µL ASPC culture medium spiked with 50 pmol of mouse Notch 1 peptide.

A – H are increasing concentrations of acetonitrile/0.1% TFA used to elute the peptides during SPE. The Notch 1 mouse peptide (2306 & 2320) can only be detected in C and D (30% and 40% acetonitrile/0.1% TFA) meaning that all Notch peptides are eluted at 40% acetonitrile/0.1% TFA.
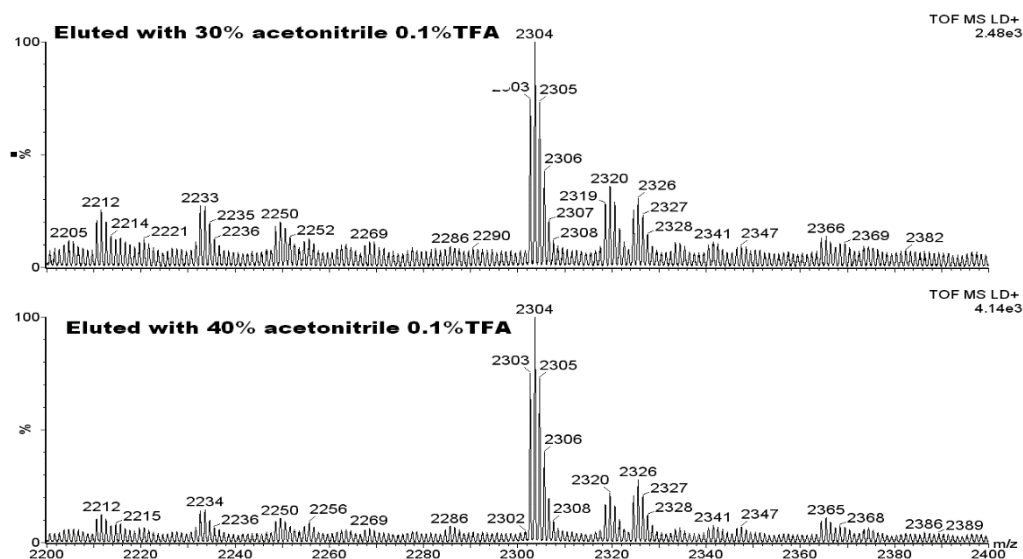
Figure 3.9 MALDI-MS spectrum of 50 µL of ASPC cell culture medium spiked with 50 pmol of Mouse Notch 1 peptide.

The sample which was eluted with 40% acetonitrile 0.1% TFA during SPE gives the higher signal intensity for Notch 1 peptide. This indicates that 40% acetonitrile/0.1% TFA is the optimal eluting agent during SPE for this peptide.

3.5.1.6 Recovery of the Notch 1 Peptide following SPE

An experiment was performed to check whether any Notch 1 peptide samples were lost during SPE, centrifugal evaporation, freeze drying, size-exclusion filtration. Cell medium (50 µL) was spiked with 50 pmol of Notch1 peptide and underwent SPE and the subsequent preparation process and eventual MALDI-MS analysis. A 50 µL of cell medium also went through the same process and just prior to mixing with matrix, this sample was spiked with 50 pmol of Notch 1 peptide. A control experiment was also done with 50 µL of cell medium to ensure that the MALDI-MS was not giving any false positive results. The MALDI-MS results between the first and the second set of samples were very similar. This showed that there was almost 100% recovery of the Notch 1 peptide during SPE and all the other purification processes (Figure 3.10).
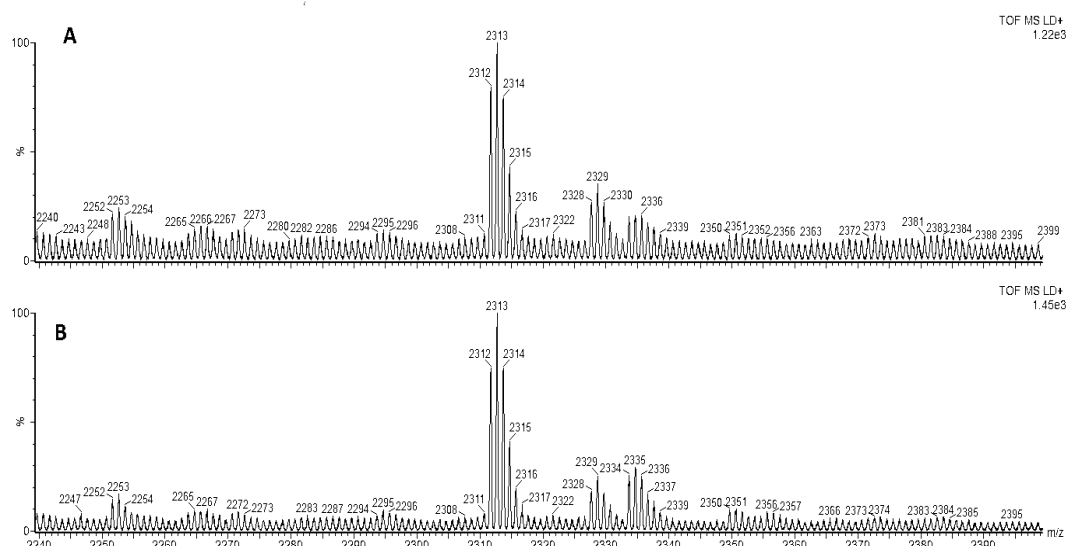
Figure 3.10 MALDI-MS spectra of ASPC cell culture medium spiked with 50p mol of human Notch 1 peptide before and after SPE.

A) Sample spiked before SPE. B) Sample spiked after SPE, centrifugal evaporation and freeze drying, just prior to mixing with matrix before loading onto target plate for MALDI-MS analysis. Note that the signal intensity for both spectrums are roughly similar. This indicates that hardly any Notch 1 peptide is lost during the process of SPE, centrifugal evaporation and freeze-drying.

3.5.2 Validation of antibody

A dot blot analysis was carried out to validate the anti-Notch 1 peptide antibody obtained from Davids Biotechnology. Several dilutions of peptide were spotted onto a nitrocellulose membrane and this was incubated in anti-Notch 1 antibody diluted 1:10,000 in 5% milk at 4°C overnight. Positive results were obtained with 20 pmol, 1 pmol, 500 fmol and 250 fmol of peptide indicating that the antibody has an affinity for Notch 1 peptides.

3.5.3 Immunoprecipitation using magnetic Dynabeads.

Method development of the immunoprecipitation procedure using dynabeads was done using healthy human plasma. The plasma was either spiked with synthetic Notch1

peptide or not depending on the experiment. Each sample then underwent SPE, centrifugal evaporation, freeze-drying, and the freeze-dried pellet would be stored in - 80°C, until required for immunoprecipitation with the antibody-coupled dynabeads. As mentioned in section 3.4.2.6, the FDP were resuspended in 50 mM ammonium bicarbonate (pH 7.4) in order to provide an optimal environment for the peptide to bind to the antibody bound magnetic dynabeads.

3.5.3.1 Validation of immunoprecipitation of Notch 1 peptide using Dynabeads.

To validate the method of immunoprecipitation using dynabeads, 100 pmol of Notch 1 peptide, diluted in 100 µL of ammonium bicarbonate, was immunoprecipitated as described in section 2.7. A signal for the Notch 1 peptide was detected following MALDI-MS analysis of this sample. This experiment was repeated using 25 pmol of Notch 1 peptide with positive results (Figure 3.11).

3.5.3.2 Determination of the elution conditions of the Notch 1 peptide from the IgG-protein G complex.

The final sample preparation step was elution of the bound peptide. It was initially thought that eluting the peptide from the antigen IgG-protein G complex using 0.1% TFA might reduce the interference and suppression of the peptide ionisation to give an improved response. An experiment was set up where two samples each containing 25 pmol of Notch 1 peptide were analysed using the method described above. One of the samples was eluted using 0.1% TFA, pH 2.0 and the other using 0.1 M citric acid, pH 2.0. Notch 1 peptide signal was detected in both the samples. However, the better
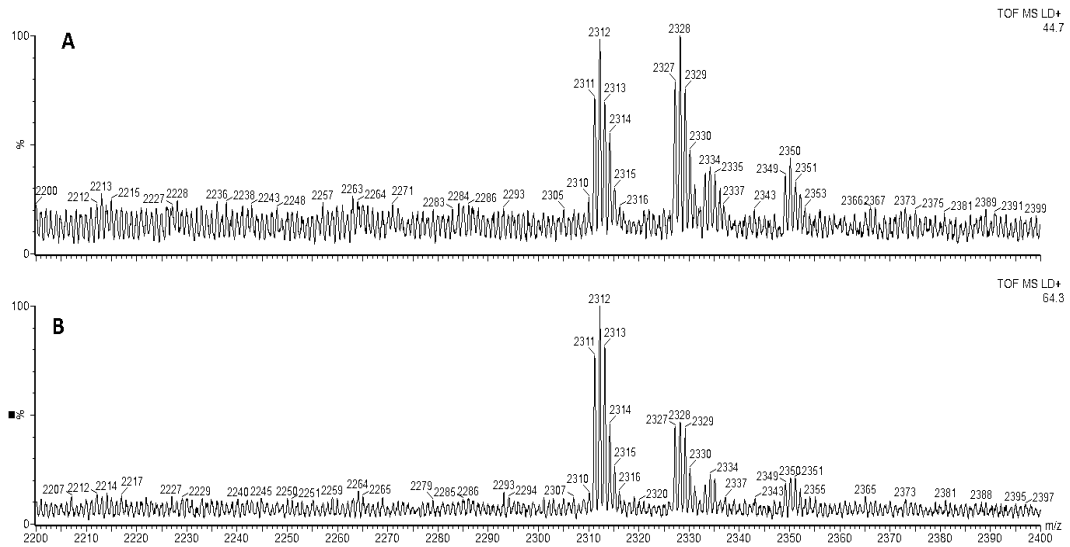
Figure 3.11 MALDI-MS spectra of human Notch 1 peptide dissolved in ammonium bicarbonate and immunoprecipitated using dynabeads.

A) 100 pmol of Notch 1 peptide. Peaks of 2311 and 2327 (M+16) can be seen. This proves that the method of immunoprecipitation works. B) The method also works with 25 pmol of Notch 1 peptide. The peak ratios of 2311:2327 differ between the two samples due to the differing levels of methionine oxidation.
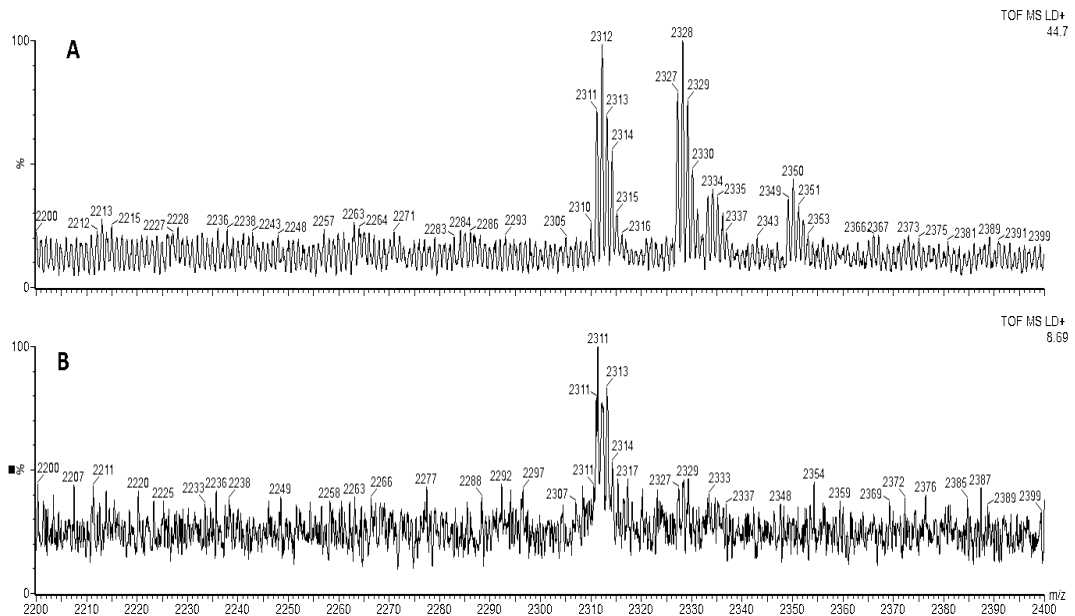
Figure 3.12 MALDI-MS spectrum of 25 pmol of human Notch 1 peptide eluted with differing acids during immunoprecipitation using magnetic dynabeads.

A) Sample eluted using 0.1 M citric acid pH 2 B) Sample eluted using 0.1% TFA pH 2. Elution with citric acid gives a higher signal intensity.
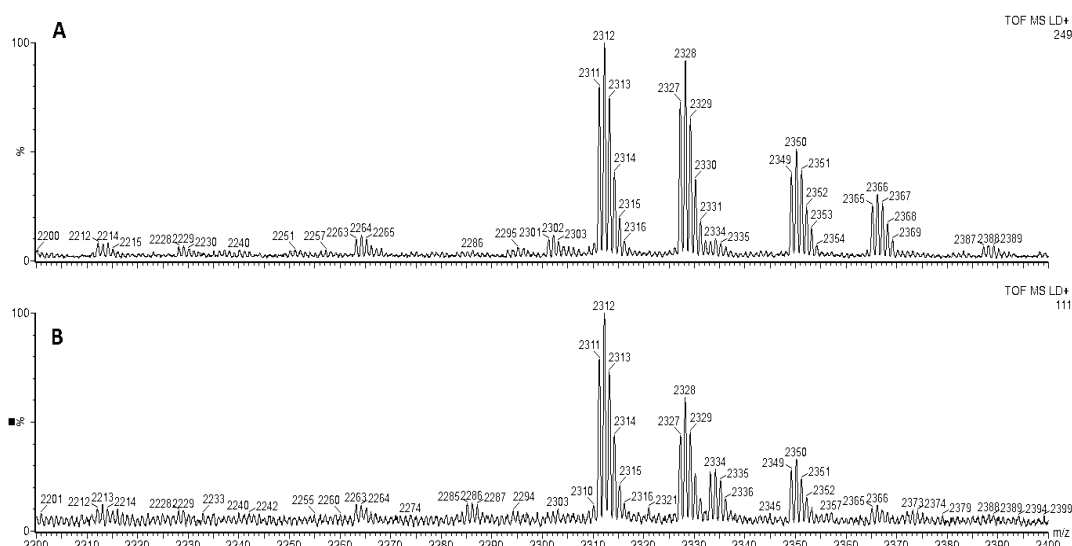
Figure 3.13 MALDI-MS spectra of 100 pmol of human Notch 1 peptide eluted with differing pH of 0.1 M citric acid during immunoprecipitation using magnetic dynabeads.

A) Sample eluted using pH 1.5 B) Sample eluted using pH 2. Stronger signal intensity was obtained using 0.1 M citric acid pH 1.5.

response was obtained using citric acid and so it was chosen for elution in subsequent experiments. (Figure 3.12)

3.5.3.3 Determination of the optimal pH of the eluting agent

Although Notch 1 peptide signals were detected for samples where 0.1 M citric acid pH 2.0 was used as the eluting agent, the response was weak. It was therefore decided to investigate whether further peptide could be eluted. An experiment using two samples of 1 pmol/µL of peptide dissolved in 100 µL of ammonium bicarbonate was carried out. One sample was eluted using 0.1 M citric acid pH 2.0 and the other sample was eluted using 0.1 M citric acid pH 1.5. It was decided not to try pH less than 1.5, since denaturation of the peptide will happen in the presence of a very strong acid. The sample eluted with 0.1 M citric acid, pH 1.5 gave stronger Notch 1 peptide signals

when compared with that using 0.1M citric acid, pH 2.0. This showed that it was possible to increase the number of peptides eluted by reducing the pH. (Figure 3.13)

3.5.3.4 Validation of the method by spiking Notch 1 peptide in healthy human plasma samples

A 50 µL sample of healthy human plasma was pre-treated with an equal volume of 4% phosphoric acid and processed as described. A second sample spiked with 100 pmol of Notch 1 peptide was processed in parallel. Following MALDI-MS analysis, no signal for the Notch 1 peptide was detected in the unspiked sample. This result was expected since the Notch 1 peptide signal was not expected to be observed in healthy individuals. However, signals for Notch 1 peptide were seen in the spiked samples, indicating that the method works (Figure 3.14).
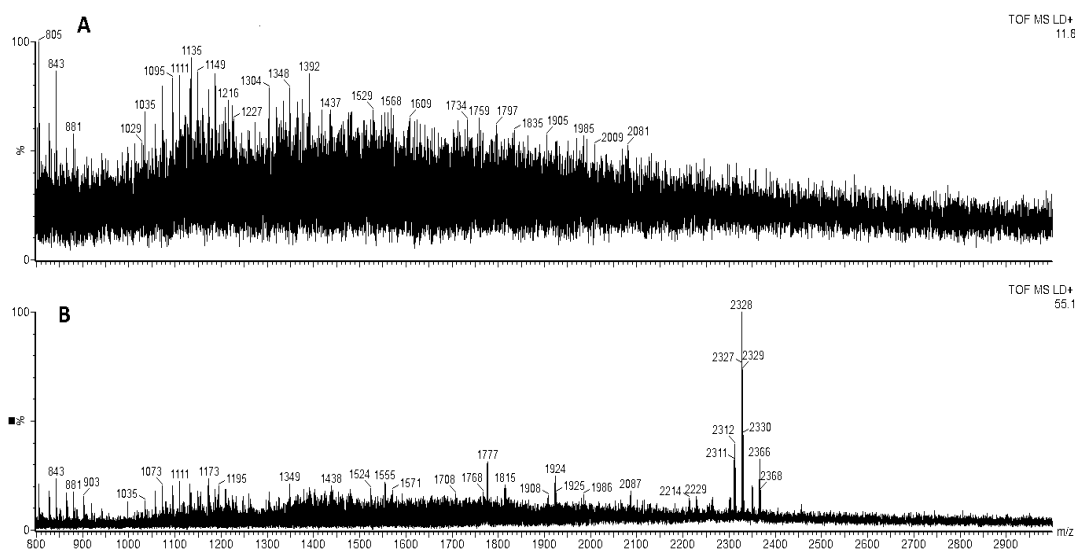
Figure 3.14 MALDI-MS spectra of healthy human plasma and plasma spiked with Notch 1 peptide.

A) Healthy human plasma shows no Notch 1 peptide signals. B) Healthy human plasma spiked with 100 pmol of Notch 1 peptide. Signals can be seen at m/z 2311 and 2327 (M+16) indicating that the method works n=3.

3.5.3.5 Determination of the number of elutions required for optimum detection of Notch 1 by MALDI-MS analysis

As described in section 2.7.1.3.5, eluents were collected from each sample. When 50 µL of healthy plasma was spiked with 100 pmol of Notch 1 peptide, eluant 1 gave the strongest signals, followed by eluant 2 and in eluant 3 the signal was very weak. Thus, most of the Notch 1 peptide is eluted in the first two samples (Figure 3.15). Following this, for the remainder of the method development, the samples were eluted twice.

The first eluant had the strongest signal for Notch 1, whereas the second eluant gave a considerably less signal. This showed that most of the Notch 1 peptide was eluted in the first eluant (Figure 3.15). Since this study was designed for detection of Notch 1 peptides rather than quantification, it was decided to elute patient samples only once before MALDI-MS analysis.

3.5.3.6 Determination of the optimal incubation time for antigen capture

There are several factors which can determine the binding of target antigen to the Dynabeads. These include the concentration of the target antigen, the concentration of Dynabeads ProteinG-Ig complex, the affinity of the immobilised Ig for the target protein/antigen and the incubation time and temperature. It was previously shown that there was no improvement in the yield of the Notch 1 peptide when analysing 500 µL of medium compared to 50 µL of medium. Similarly, this should be applicable to the plasma samples as well. The information about the optimal amount of Dynabeads to be used and the appropriate amount of corresponding antibody was provided by Invitrogen.
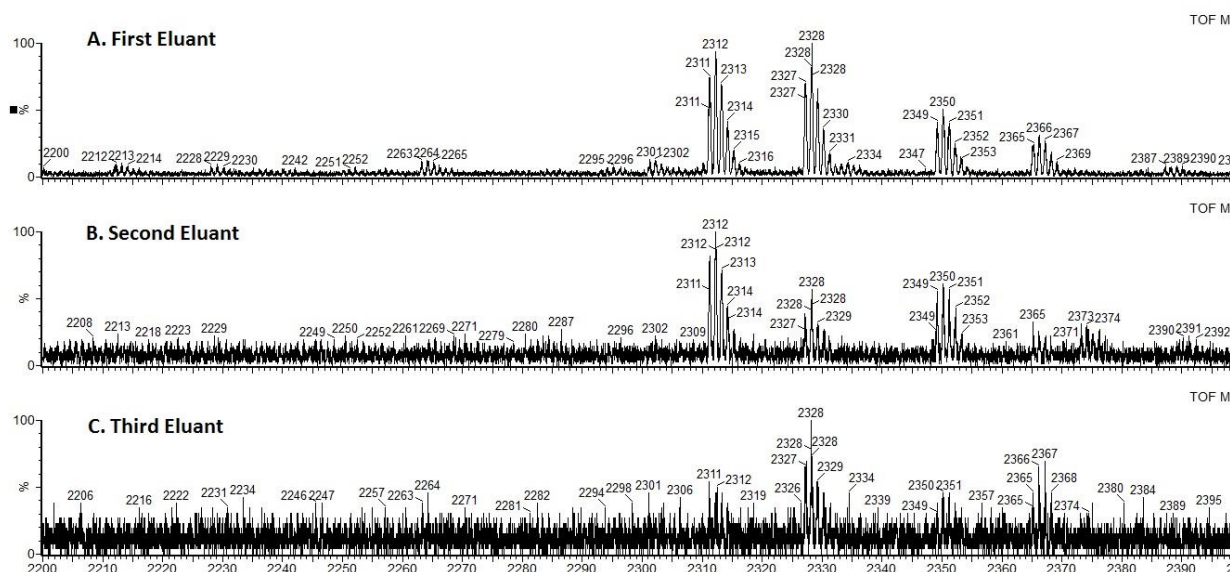
Figure 3.15 MALDI-MS spectrum of healthy human plasma spiked with 100 pmol Notch 1 peptide.
Eluant 1 shows the highest signal intensity for Notch 1 peptides. This indicates that most of the Notch 1 peptide is recovered during the first elution with >95% found in the first and second eluants.

According to the Invitrogen product sheet, an incubation time of 1 h would be sufficient for binding for most peptides. In this experiment, it was attempted to reduce the incubation time to speed up the analysis. Two healthy human plasma samples spiked with 25 pmol of Notch 1 peptide were processed using the methods already described. For one sample, the incubation time of the Notch 1 peptide with the Ig G–protein G complex was 2 h at 4°C and for the other sample the incubation time was 12 h at 4°C. Samples which were incubated overnight gave better Notch 1 peptide signals and therefore it was decided to continue incubating antigens with Ig G–protein G complexes for a minimum of 12 h at 4°C (Figure 3.16).
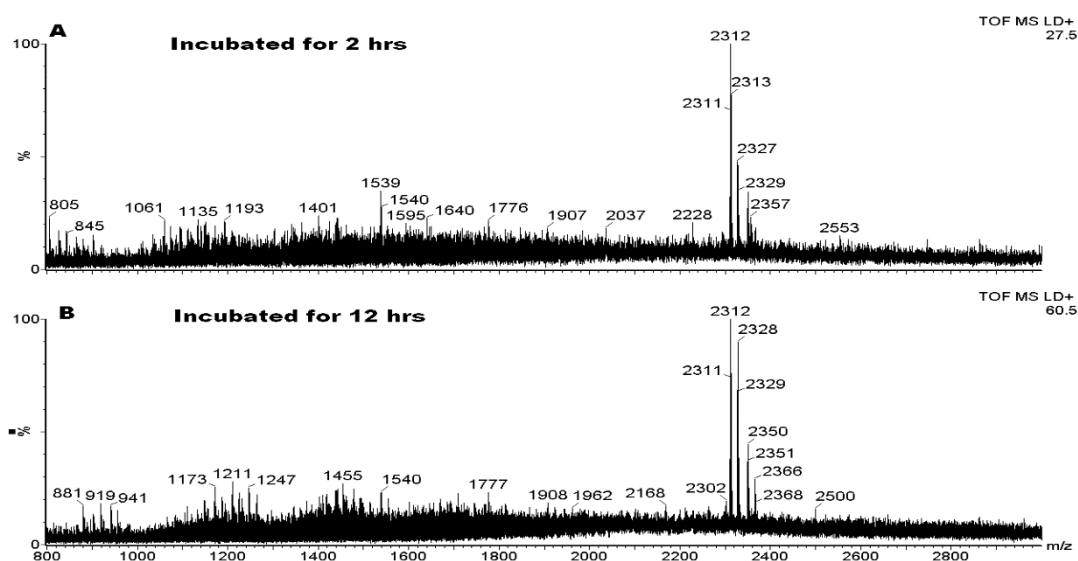
Figure 3.16 MALDI-MS spectra of healthy human samples spiked with 25 pmol of Notch 1 peptide, incubated with antibody for 2 h and 12 h.

A) Sample incubated for 2 h at 4°C. B) Sample incubated for 12 h at 4°C. The sample which has been incubated for 12 h gives a higher signal intensity.

3.5.3.7 Determination of the optimal amount of antibody for Notch 1 response on the MALDI-MS

An experiment was carried out to check whether Notch 1 could be detected on the MALDI-MS using less antibody in order to maximise the number of analysis which could be carried out with the limited amount of antibody available.

As mentioned in section 2.7.1.3.2, between 25 µg Ig G/mL (25 µg Ig G =19 µL of antibody) and 100 µg Ig G/mL (100 µg Ig G =76 µL of antibody) there was not a great increase in the amount of Ig G captured. The starting volume for this part of the method development was 50 µL of antibody. An experiment was carried out using 50 µL (65 µg Ig G), 25 µL (33 µg Ig G) and 20 µL (26 µg Ig G) of antibody with 50 µL of healthy plasma spiked with 10 pmol of synthetic Notch 1 peptide respectively. Only the sample using 50 µL of antibody gave a Notch 1 signal at three times the background signal. For

MALDI-MS analysis, a signal was only considered above background if it was at least 3 times the baseline noise. From the result of this experiment, it was determined that the optimal amount of antibody to be used for this method is 75 µg which equates to a volume of 50 µL (Figure 3.17).
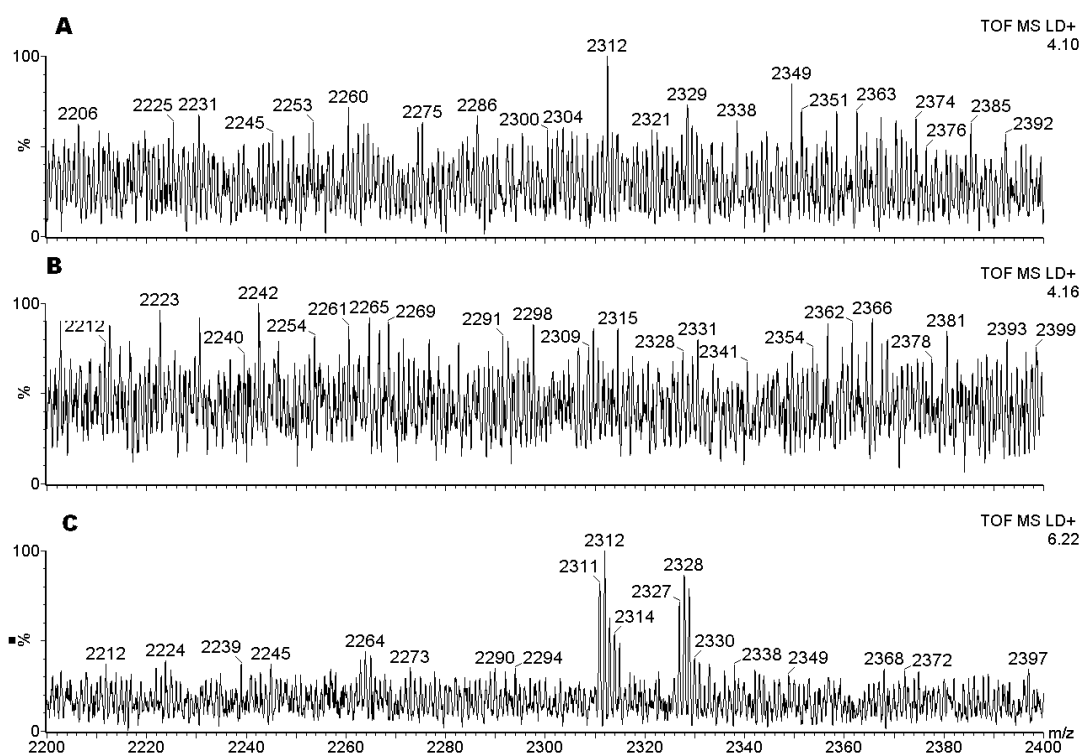
Figure 3.17 MALDI-MS spectra of healthy human plasma spiked with 10 pmol of Notch 1 peptide.
Sample incubated with A) 20 µL of antibody. B) 25 µL of antibody. C) 50 µL of antibody. Only the sample incubated with 50 µL gives a reading which is three times the background noise.

3.5.3.8 Determination of peptide loss during washing steps prior to elution

Prior to elution, the peptide bound dynabeads are washed three times in ammonium bicarbonate (section 2.7.1.3.5). The washes were analysed in MALDI-MS to check if any peptides were eluted during the washes. None of the washes gave any reading for

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Notch 1 peptide, even when diluted 5 times with trifluoroacetic acid. The washes gave

signals for spiked control ACTH, proving that the Notch peptide was genuinely not

present in the washes and its ionisation was not being suppressed. This step proved that

Notch peptides were not lost during the washes prior to elution by citric acid. (Figure

3.18)
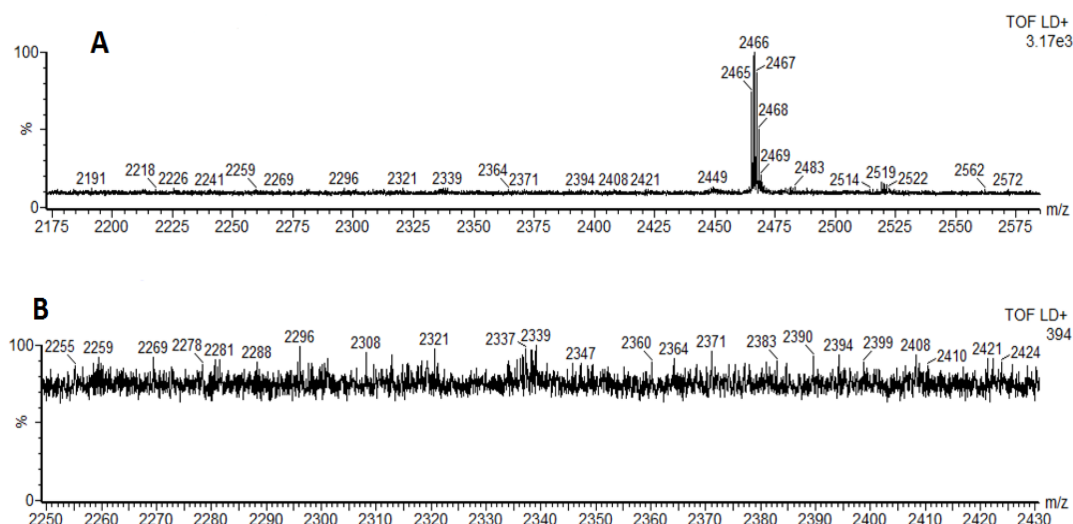
**Figure 3.18 MALDI-MS spectra of the first wash with ammonium bicarbonate prior to
elution with 0.1 M citric acid pH 1.5.**

A) Shows that no signals for Notch 1 peptide can be detected, but signals for ACTH (*m/z* 2466)
can be detected proving that there is no ionisation suppression in the sample. B) A magnified
image of A which shows that there is no Notch 1 peptide detected, suggesting that Notch 1
peptide is not lost during the washing procedure.

3.5.3.9 Determination of the limit of detection of Notch 1 in plasma samples

To determine the limit of detection, 50 µL of plasma was spiked with 5 pmol, 10 pmol

or 30 pmol of synthetic Notch 1 peptide and immunoprecipitation was carried out using

50 µL of antibody with an incubation period of at least 12 h at 4°C. The sample spiked

with 10 pmol provided a Notch 1 signal which was above (3 times) background and

therefore was considered the limit of detection. This was equivalent to a concentration

of 121.67 fmol/µL of plasma. Therefore, assuming no loss of the Notch 1 peptide during the preparation steps, it would have to be present in the plasma at a concentration equal to or greater than 121.67 fmol/µL for detection by our method and subsequent MALDI-MS analysis (Figure 3.19).
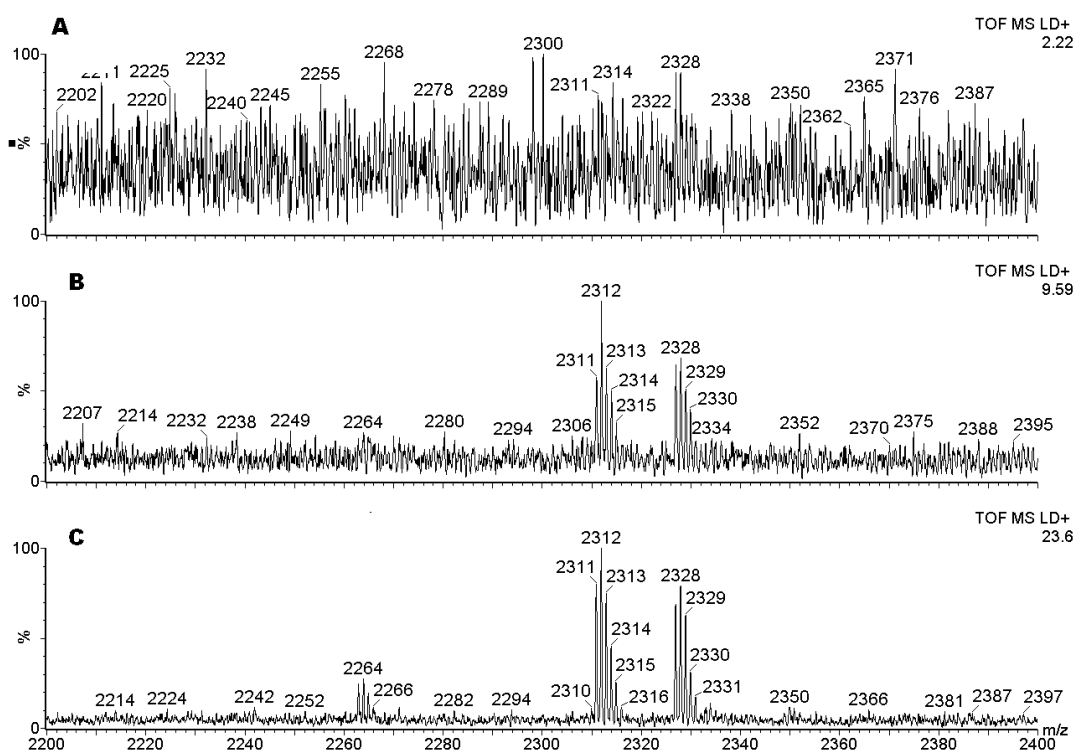
Figure 3.19 MALDI-MS spectra of healthy human plasma spiked with Notch 1 peptide.
Sample spiked with A) 5 pmol B) 10 pmole C) 30 pmol. In the samples with 10 pmol and 30 pmol signal at 2311 or 2327 (M+16) were detected.

3.5.4 Development of the size exclusion filtration method

The size exclusion filtration was initially carried out using Microcon Centrifugal Filter Devices (Regenerated Cellulose 10,000 MWCO provided by Millipore). They were pre-rinsed twice using 500 µL ultrapure water to remove trace amounts of glycerine which might have interfered with analysis. The filter devices were centrifuged at 14,000 g at 4°C, for different times to investigate whether all the water had been removed but

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without the filter drying out. It was found that 20 min was the optimal centrifugation time. Freeze dried samples, dissolved in either ammonium bicarbonate or 0.1% TFA, were loaded onto the pre-rinsed Microcon devices and centrifuged at 14,000 g at 4°C. The optimal time for centrifugation was 45 min for the samples to be filtered through at 4°C without any damage to the membrane.

Microcon filters were discontinued by Millipore during the study. Therefore, method development for future patient sample analysis was undertaken using Amicon filters. When the Amicon filters were used initially, no Notch 1 peptide was detected in healthy plasma which was spiked with 30 pmol of synthetic Notch 1 peptide. In a simultaneous experiment performed using Microcon filters Notch 1 peptide was detected (Figure 3.20).

It was possible that the Notch 1 signal was not being detected by MALDI-MS due to suppression of ionisation by salt in the eluants where the Amicon filters were used. Some of the Amicon-filtrated eluants were therefore diluted and re-analysed but with no improvement. This suggested that ionisation suppression of the Notch 1 peptide was not occurring. Some of the original Amicon-filtrated eluants were then spiked with synthetic Notch 1 peptide (500fmol of Notch 1 on each spot) and in these samples a signal was detected suggesting that the loss of Notch 1 peptide signal was not due to ionisation suppression and was due to the Notch 1 peptide being retained by the Amicon filters (Figure 3.20).

To confirm whether this was the case or whether it was a faulty batch of Amicon filters, four samples of freeze dried pellets of healthy plasma were each dissolved in ammonium bicarbonate. Two of them were spiked with Notch 1 peptide prior to
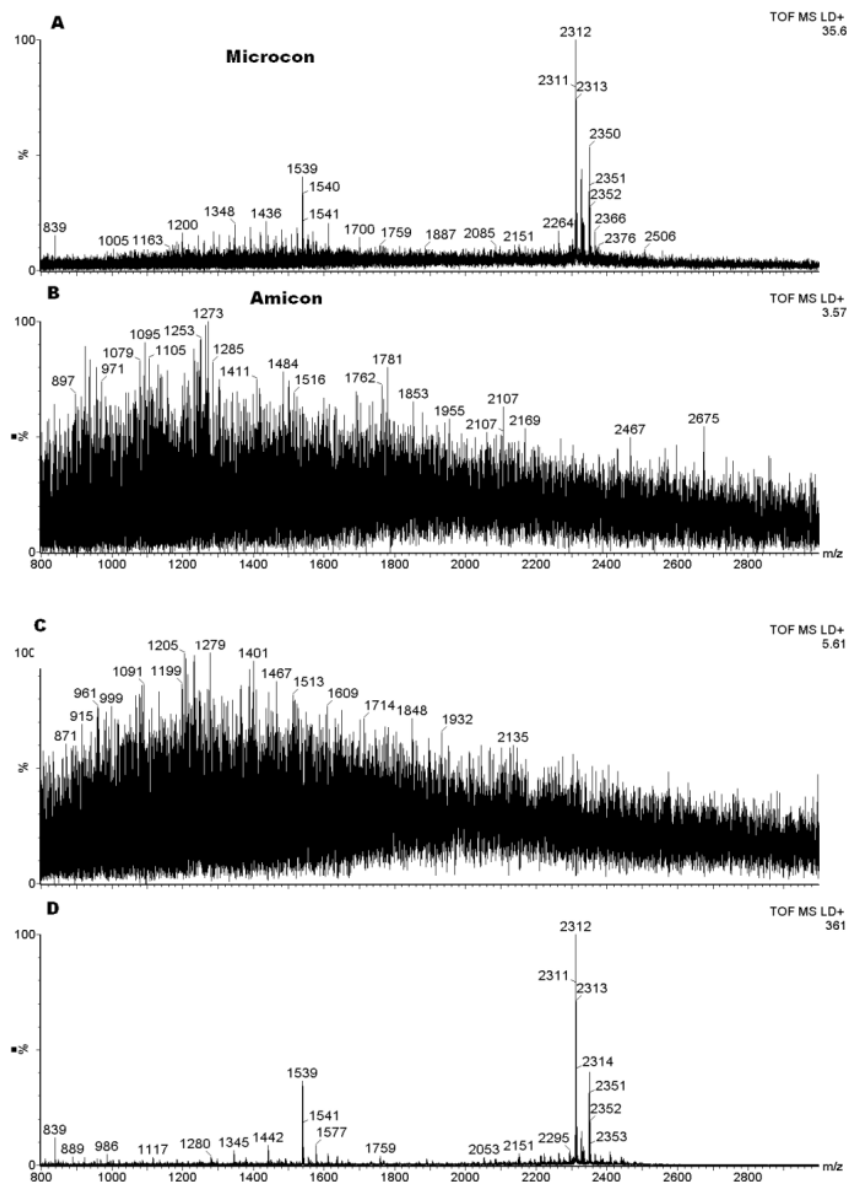
Figure 3.20 MALDI-MS spectra of 50 μL of healthy human plasma spiked with 50 pmol of Notch 1 peptide.

A) Sample filtered using microcon filter, where Notch 1 signal can be seen. B) Sample filtered using amicon filter with no Notch 1 signal. C) Eluant from sample B further diluted with TFA to reduce possible ionisation suppression. Still no Notch 1 signal can be seen. D) Eluant from sample B spiked with Notch 1 peptide so there would be approximately 500 fmol of Notch 1 peptide on target MALDI plates. Notch 1 signal can be seen. This proves that the loss of Notch 1 signal is due to the peptide being lost during filtration and is not due to ionisation suppression.

filtration to give about 500 fmol on the MALDI-MS target plate and two were spiked post filtration. One sample of each set was centrifuged using an old batch of Amicon filters and the other two were centrifuged using a new batch of Amicon filters. For the samples which were spiked prior to filtration, no signal was detected with either batch of Amicon filters. For both samples which were spiked following filtration, the peptide was detected. This experiment suggested that the failure to detect the Notch 1 peptide was because the peptide was being retained by the Amicon filters during filtration and not because of a faulty batch (Figure 3.21).

It was assumed that the Notch 1 peptide was binding to the plastic structure of the Amicon device. Passivation (pre-treating) of the device before use could be used to block any binding sites and potentially make the device suitable for use with Notch 1 peptides. It was mentioned in the literature provided by Millipore that 1% Milk or 5% Tween-20 could be used as a passivation/pre-treatment agent. An experiment was performed where the Amicon filters were pre-treated with either 1% milk or 5% Tween-20 and left overnight. Samples of healthy plasma spiked with synthetic Notch 1 peptide were analysed using the pre-treated Amicon filters and Notch 1 peptide signal was detected only in the samples where the Amicon filters were pre-treated with 5% Tween- 20 (Figure 3.22).

A solution of 5% Tween-20 produced significant amount of foam which could interfere with sample analysis. Therefore, an attempt was made to lower the concentration of Tween-20 comparing 5%, 1%, 0.5%, 0.25% and 0.05%. Since one percent Tween-20 gave good signals for Notch 1 peptide it was decided to use this as the passivation agent for Amicon filters (Figure 3.23).
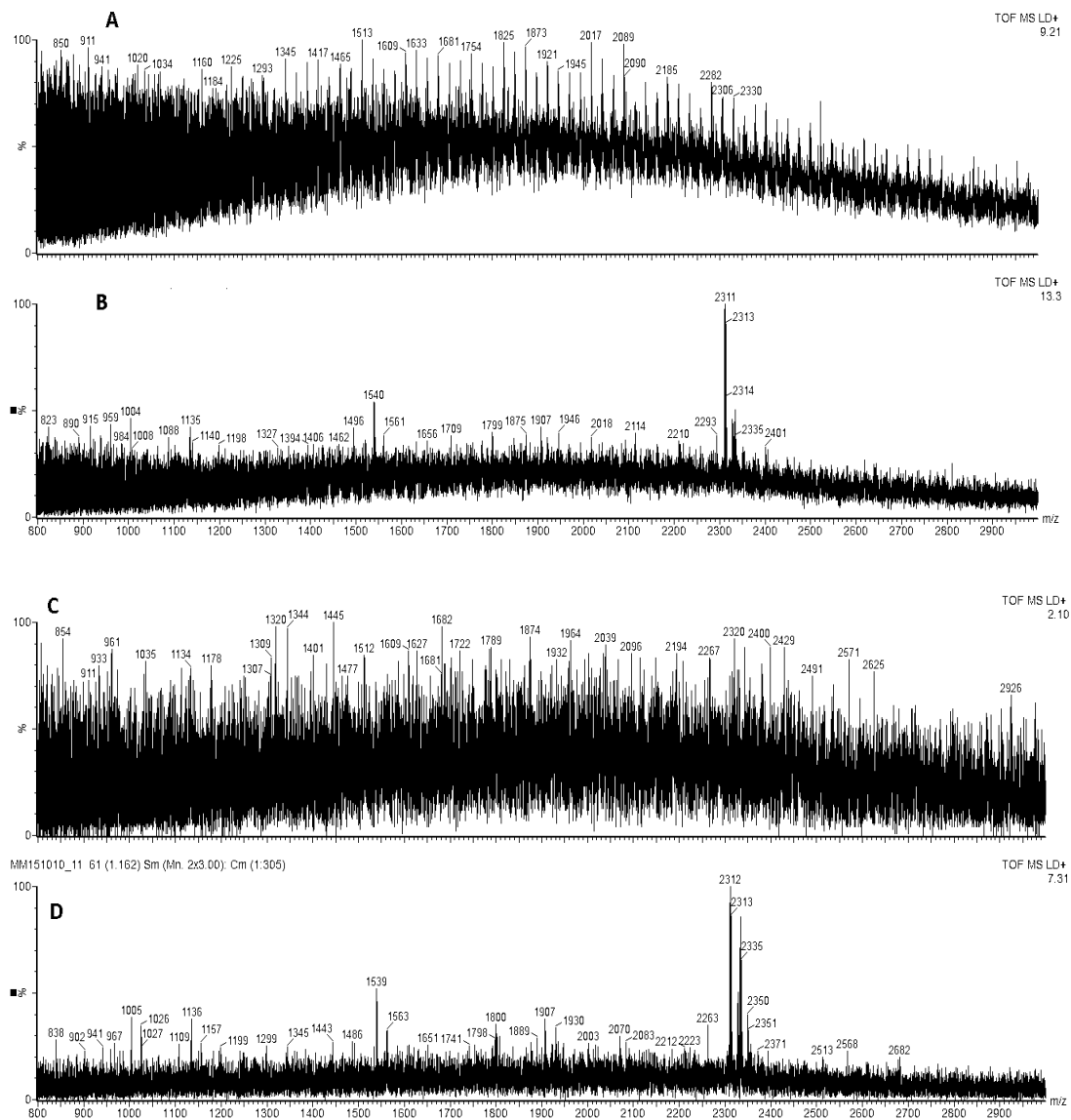
Figure 3.21 MALDI-MS spectrum showing development of the size exclusion filtration method.
Healthy plasma spiked with Notch 1 peptide A) before filtration using old Amicon filters. No Notch 1
signal can be detected. B) after filtration using old Amicon filters. Notch 1 signal can be detected in
this sample. C) before filtration using new batch of Amicon filters. No Notch 1 signal can be
detected. D) after filtration using new batch of Amicon filters. Notch 1 signals can be detected. This
suggests that the loss of signals was due to the filters retaining the peptide.
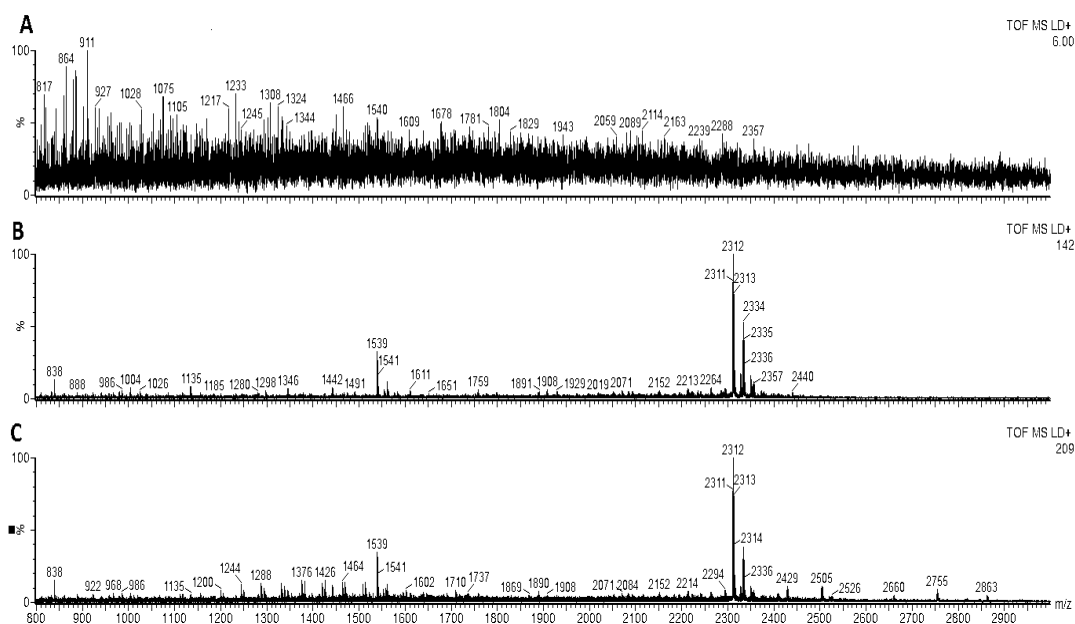
Figure 3.22 MALDI-MS spectra following passivation of the Amicon filters.
Healthy plasma spiked with Notch 1 peptides were used as samples. A) Amicon filters treated with
1% milk. No signals for Notch 1 peptide can be detected. B) The eluant from A was spiked with
Notch 1 peptide. Notch 1 signals can be seen, indicating that no salt induced ionisation suppression
was taking place in A and Notch 1 was genuinely absent from the eluant C) Amicon filters treated
with 5% Tween-20. Notch 1 signals can be seen in this sample, indicating that Tween-20 can be
used as a passivation solution for Amicon filters.

Passivation thus involved adding 500 µL of 1% Tween-20 into the sample reservoir
which was left overnight at room temperature. The following day the devices were
rinsed thoroughly with ultrapure water. A further 500 µL of ultrapure water was then
added to the reservoir and the filter centrifuged for 20 min at 14,000 g at 4°C. Any
remaining water was discarded by inverting the reservoir and the washing step was
repeated with a further 500 µL of ultrapure water. To remove any remaining water, the
reservoir was then inverted again in the filtrate vial, and then centrifuged once at 1,000
g for 3 min making the Amicon filters before use.
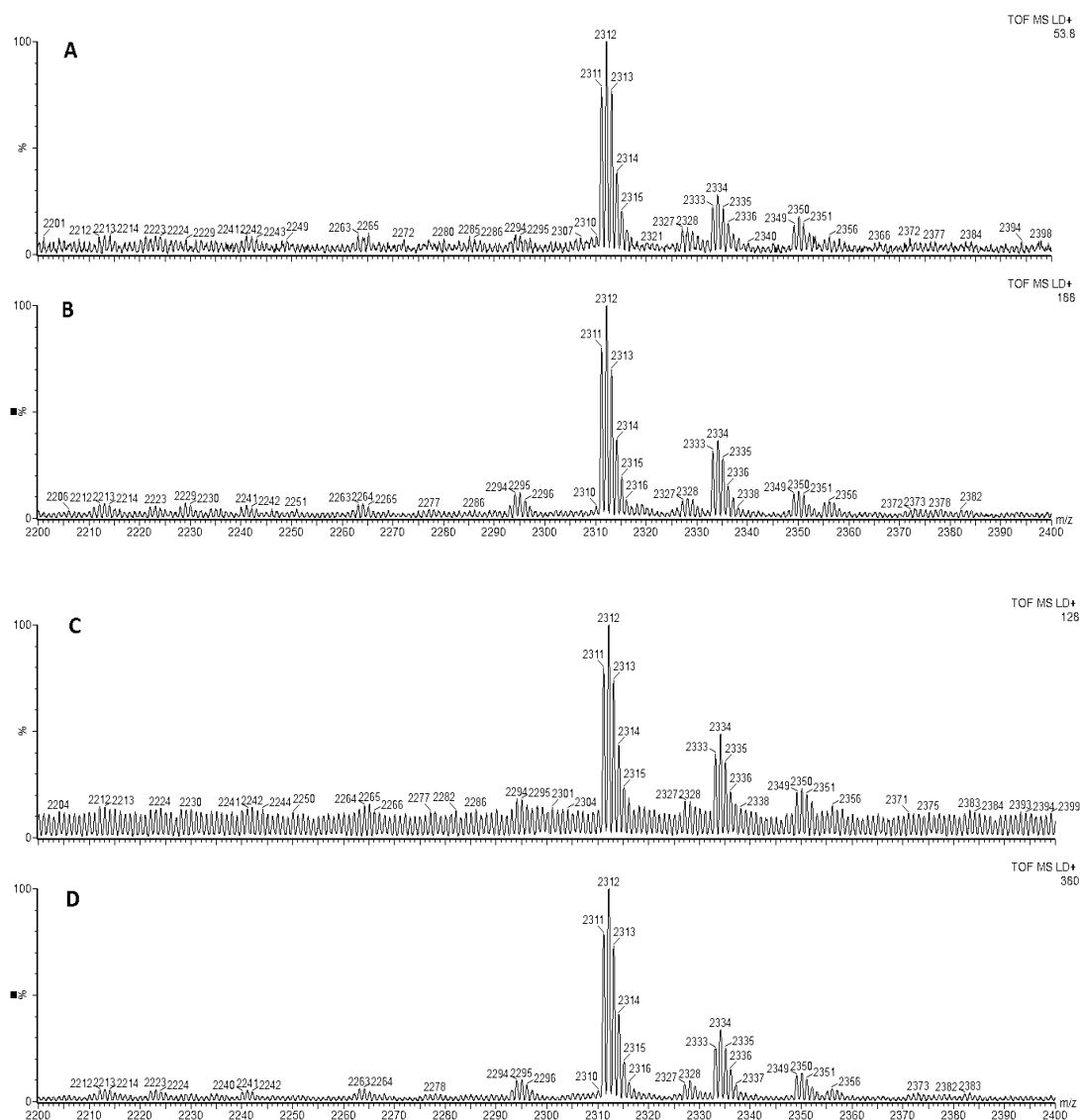
Figure 3.23 MALDI-MS spectra following Tween-20 passivation of Amicon filters.

Healthy plasma was spiked with Notch 1 peptide. Amicon filter was treated overnight with A) 0.05% Tween-20. B) 0.25% Tween-20 overnight. C) 0.5% Tween-20 D) 1% Tween-20. One percent Tween-20 gave the best signal intensity for Notch 1 peptide.

3.6 Results

3.6.1 Analysis of Notch 1 peptide in patient samples

Prior to analysing patient samples, the MALDI-MS was calibrated using a standard peptide Glu-fibrinopeptide B (GFP) (MW 1570.57). A Notch 1 peptide standard was also analysed.

Plasma samples from 20 non-resectable pancreatic ductal adenocarcinoma patients and 10 healthy volunteers were analysed. Notch 1 signals for either $N\beta_{21}$ and $N\beta_{25}$ could not be detected in any of the samples. The samples were also reanalysed using another MALDI-MS instrument which produced the same result. (MW: Notch 1 $N\beta_{21}$ = 2310.14 Da; Notch 1 $N\beta_{25}$= 2700 Da). There were also no peaks detected at +16 for both $N\beta_{21}$ and $N\beta_{25}$ (Figure 3.24).

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3.6.2 Analysis of Notch 3 peptide in patient samples

Using the amino acid sequence available for human Notch 3 and comparing it to that of Notch 1, it was possible to predict the sequence of a putative $N\beta$ fragment. This is the site of the S2 cleavage {N terminal of the Notch Extracellular Truncation (NEXT)} at amino acid number 1629. Assuming that Notch 3 also has $N\beta_{21}$ fragment, a peptide sequence of 21 amino acids was synthesised for Notch 3 $N\beta_{21}$ fragment. The sequence for Notch 3 $N\beta_{21}$ is VRG EPL EPP EPS VPL LPL LVA {GAVLLLVILVLG}. The area in grey shading is part of the transmembrane domain just prior to where the N terminus for the NICD starts and is not part of Notch 3 $N\beta_{21}$. Davids Biotechnology

were commissioned to synthesise a synthetic Notch 3 Nβ21 peptide and to raise a polyclonal antibody against the peptide.
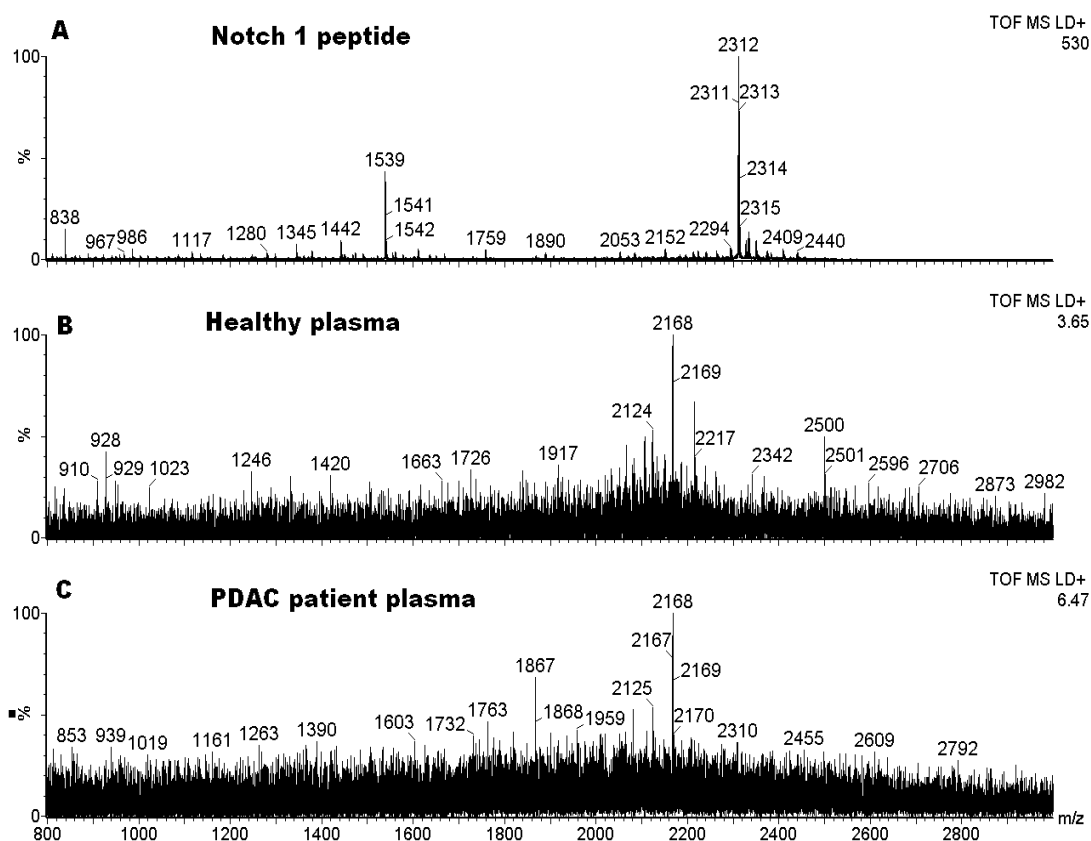
Figure 3.24 MALDI-MS spectra analysis of PDAC patient and healthy volunteer plasma.
A) Notch 1 standard peptide giving a signal of *m/z* of 2311. B) A healthy plasma showing no Notch 1 peptide signals. The signal at *m/z* of 2168 is not relevant in the current study and we do not know what it is. C) A patient plasma showing no Notch 1 peptide signals.

Analysis of Notch 3 Nβ21 synthetic peptide by MALDI-MS resulted in a major ion at *m/z* 2223. This was the correct signal as the calculated MW of the peptide is 2222.65 Da and the MALDI-MS analysis was performed in positive ionisation mode (Figure 3.25).

Using the method described for the Notch 1 peptide, 5 patient samples and 5 healthy volunteers were initially analysed for the Notch 3 Nβ21 fragment. The Notch 3 peptide

was present in all samples. An internal standard adrenocorticotrophic hormone (ACTH) {m/z 2466} was incorporated into the matrix (25 fmol per spot). For every sample, 4 spectra were obtained from 4 different spots on the MALDI target plate and the signal intensity for Notch 3 peptide averaged and normalised to ACTH intensity (Figure 26). There was no significant difference in Notch 3 signal intensity between the two groups (p= 0.142, p< 0.05 Mann-Whitney U test).

To investigate whether there were any differences in Notch 3 peptide levels in plasma from healthy volunteers and non-resectable PDAC patients, a further 20 patients and 16 healthy volunteers' plasma were analysed. The experiment was carried out by a BSc student in the department, using the author's method and patient samples. The MALDI-MS analysis and subsequent statistical calculations were carried out by the author.

There was no significant difference in Notch 3 signal intensity between healthy volunteers and non-resectable PDAC patients. (p= 0.65, p<0.05 Mann-Whitney U test). Healthy volunteers had a slightly higher median signal intensity (0.37186) compared to PDAC patients (0.301470).

The distribution of age across the two groups was significantly different (p=0.04, Mann-Whitney U test) and the median of the patient group (68.8 years) was significantly higher compared to the healthy volunteers (56 years). {p=0.07, Median test}. To compare evenly across the two groups, samples from healthy volunteers aged 55 and above were compared with patient samples. There were no significant differences in Notch 3 signal intensity between the two groups. (p= 0.420, p<0.05 Fishers exact test).
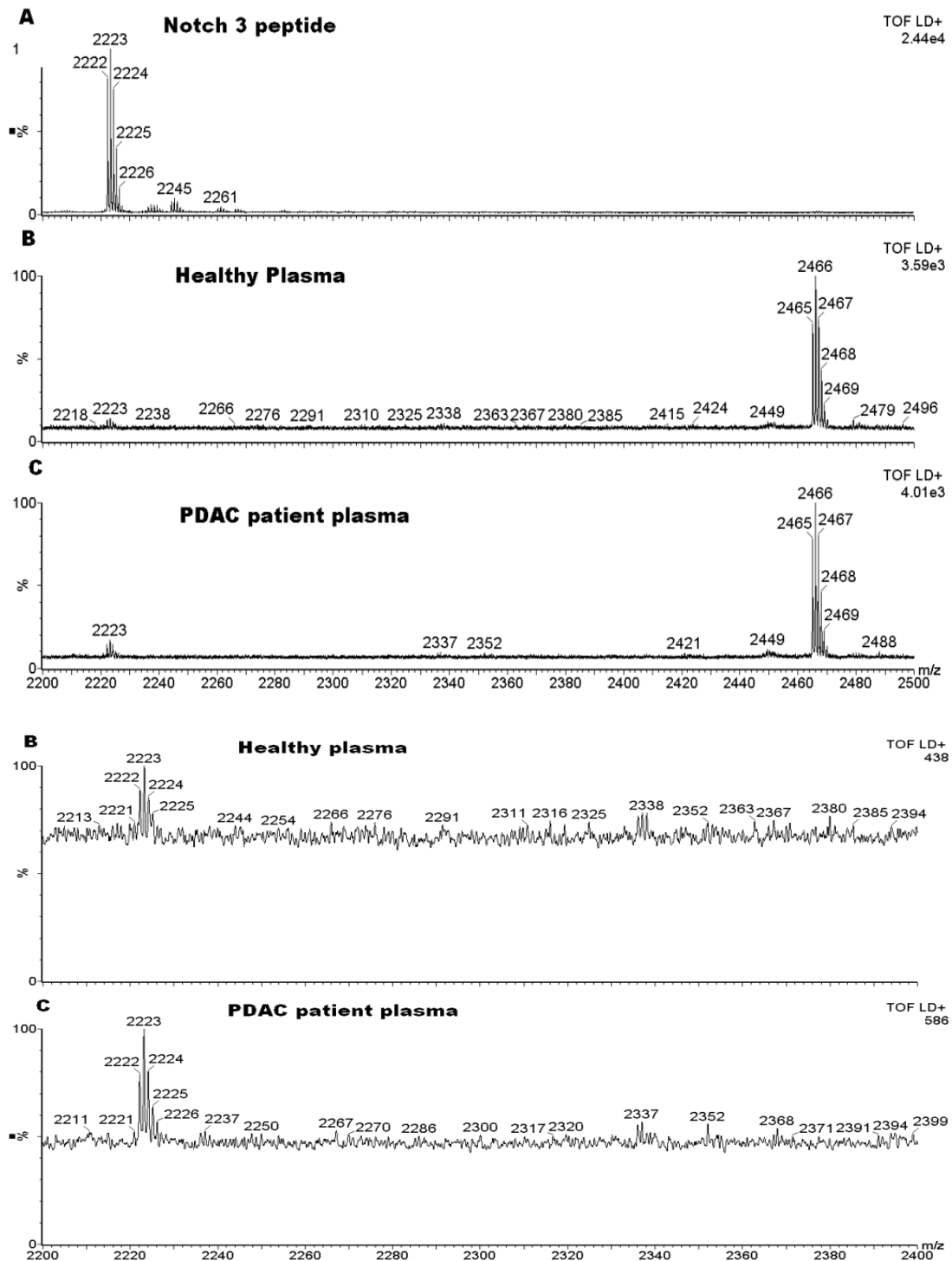
Figure 3.25 MALDI-MS spectrum of Notch 3 peptide in healthy plasma and PDAC patient's plasma.

A) Notch 3 standard peptide giving a m/s signal of 2223. B) Notch 3 can be detected in healthy plasma. ACTH (*m/z* 2466) can also be seen as the internal control. The fourth image also identified B, is a magnified version of the peak in interest. C) Notch 3 can also be detected in PDAC patient's plasma with roughly the same signal intensity. The fifth image also identified C, is a magnified version of the peak in interest.

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3.7 Discussion

In this study, an optimal method for detecting Notch peptides in human plasma using MALDI-MS analysis was developed. It was determined that Notch 1 peptide needs to be present at a concentration of at least 121.67 fmol/µL in the human plasma for this method to be able to detect the peptide. Although Notch 1 Nβ fragment could not be detected in any of the patient and healthy volunteer samples, Notch 3 Nβ fragment could be detected in both groups. This provides evidence that this is a valid method of detecting circulating Notch Nβ fragments, and is the first report of Notch 3 peptides detected in human plasma.

It could be argued that enzyme-linked immunosorbent assay (ELISA), which is the gold standard for detecting potential protein biomarkers, should have been used for our study instead of MS. Although ELISA has extraordinary sensitivity and specificity for quantifying the target analyte, its development is extremely costly and time consuming and has a high failure rate. This leads to reduced validations of potential diagnostic biomarkers and therefore less than optimal patient outcomes. Purification of samples and immunoprecipitation using antibody bound dynabeads followed by MS analysis (IA-MS) has several advantages over ELISA. IA-MS requires only one antibody to be developed, whereas ELISA requires two different antibodies for the sandwich assay, associated with higher costs as well as a longer developing time. It is also easier to generate antibodies for IA-MS because there are no restrictions to just target the nonoverlapping epitopes as well as the intact protein. IA-MS can also monitor several targets within one sample, whereas the standard ELISA can only monitor one target per sample. This leads to more throughput from one sample as well as lower sample consumption. All these advantages have led us to use IA-MS (Whiteaker *et al*, 2007).

Although previous studies have suggested that Notch 1 is a potential oncogene in PDAC, Mazur *et al* (2010) have proved with their work on murine models that Notch 2 is required for the progression of pancreatic intraepithelial neoplasia and the development of pancreatic ductal adenocarcinoma. Moreover, Hanon *et al* (2010) were also able to prove in murine models of *K-ras* induced tumourigenesis, that depletion of Notch 1 actually resulted in increased tumour incidence and progression of PDAC. This suggests that Notch 1 acts more as a tumour suppressor gene rather than an oncogene in PDAC. These findings could possible explain why Notch 1 circulating levels were not high enough in patients with non-resectable PDAC, in order to be detected by our method.

Notch 3 peptides were detected in both healthy volunteers and non-resectable PDAC with no significant difference in levels between them. This result validates the method of detecting Notch Nβ fragments using IA-MS, but limits its potential as a biomarker.

Previous work had established that Notch 3 is associated with an aggressive PDAC phenotype (Doucas *et al*, 2008). Notch 3 is also shown to be important in other solid tumours such as ovarian and breast carcinomas (Xiao *et al*, 2011; Jung *et al*, 2010). It not only plays a vital role in the development of vascular smooth muscle cells (VSMCs) during embryogenesis but is also expressed in adult VSMCs. Notch 3 is considered to be a downstream mediator of growth factors in vascular injury as well as playing a part in regulating VSMC growth and apoptosis. Such is the importance of Notch 3 in VSMCs homeostasis, mutations in Notch 3 have resulted in a genetic stroke syndrome CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) due to a loss of VSMCs (T. Wang *et al*, 2008). This role in the

vasculature would explain the presence of Notch 3 Nβ fragments in healthy volunteers. Due to the limitations of time and consumption of the entire Notch 3 peptide antibody, a further method was not developed using ESI-MS, which could provide more accurate quantification of the Notch 3 Nβ fragments between healthy human plasma and non-resectable PDAC patients.

Therefore, in conclusion we have determined that Notch 1 Nβ fragments cannot be detected in human plasma and therefore cannot be used as a potential biomarker for PDAC. Notch 3 Nβ fragments can be detected in plasma of patients with PDAC but more work is required using ESI Mass Spectrometry for accurate quantification.

CHAPTER 4.

NOTCH 2 EXPRESSION IN

PANCREATIC

ADENOCARCINOMA *IN VIVO*

4.1 Introduction

Notch plays a critical role in the early development of pancreas organogenesis by maintaining pancreatic epithelial cells in a progenitor state until it is appropriate for the cells to differentiate (Z. Wang *et al*, 2011). Little is known about the role of Notch signalling in the adult pancreas and most studies suggest that the expression and activation of Notch receptors are downregulated in the adult pancreas under normal physiological conditions (Z. Wang *et al*, 2011; Avila and Kissil, 2013). However, there is mounting evidence that the Notch signalling pathway contributes to pancreatic cancer development and progression (Miyamoto *et al*, 2003; Avila and Kissil, 2013). Notch is a ligand receptor pathway and is involved in cell proliferation, apoptosis, migration, invasion, metastases, and angiogenesis in a variety of human cancers including pancreatic ductal adenocarcinoma (PDAC) (Ma *et al*, 2013).

Whilst Notch had already been demonstrated to be upregulated in PDAC with specific upregulation of Notch 1, Notch 2, Jagged 1, Jagged 2 and Notch downstream target genes Hes 1 and Hey 1 (Miyamoto *et al*, 2003; Büchler *et al*, 2005), previous work in our laboratory was the first to examine the expression of Notch pathway constituents in PDAC, using immunohistochemistry (IHC), with reference to disease progression and correlate Notch expression with clinicopathological characteristics and prognosis (Mann, 2012). Mann investigated Notch 1, Notch 3, Notch 4, Hes 1 and Hey 1. This chapter is a continuation of the same work and investigates the role of Notch 2 in PDAC in different stages of the disease and correlates Notch 2 expression with clinicopathological characteristics and disease prognosis.

4.2 Patient Demographics

The database used in this study is the same used by a previous PhD candidate Dr Chris Mann for his study on immunohistochemistry for Notch 1, 3, 4, Hes 1 and Hey 1. Local ethics approval (REC 7176) was obtained for the study. Dr Mann had identified the patients for his study from the hepatobiliary MDT database at the University Hospitals of Leicester NHS Trust. A combination of archival tissue and freshly collected specimens from patients with pancreatic adenocarcinoma undergoing surgery at biopsy were used by him. Patient data was obtained from case notes, pathology and laboratory computer systems. Survival status was determined by checking hospital patient information systems and general practice records (Mann, 2012).

The patients were separated into two groups. The resectable group (42) consisted of patients who underwent curative surgery for pancreatic ductal adenocarcinoma between October 200 and May 2007. Pancreatic tissue was available from all these patients. In addition, non-cancerous healthy pancreatic tissue (control) were obtained from 35 of these patient samples. 16 patients had locally advanced lymph nodes out of which 9 were available for us to stain for immunohistochemistry.

The unresectable group (50) consisted of two types of patients. Twenty-six patients were deemed unresectable at the time of the operation due to locally advanced disease and so pancreatic tissue sample were obtained for these patients. 24 patients had metastatic disease and out of these, 14 samples were from liver metastases, 8 from peritoneal metastases and 2 from distant lymph node metastases.

The full clinicopathological data of the patients who underwent potentially curative resections are illustrated in Table 4.1. Patients were followed up in the hepatopancreatobiliary and oncology clinics. Patient survival was last updated on 1st January 2011, at which point seven patients were alive, all of them disease-free. Median survival of patients who underwent potentially curative resections was 30.5 months with a 1 year, 3 year and 5 year survival of 87.5%, 32.5% and 21.3%. Median value of disease free survival was 25 months giving 1, 3, and 5 year disease free survival rates of 64.1%, 25.6% and 16.6%. Following recurrence, median survival was 5.2 months (Mann, 2012) .

In the unresectable group the median survival was 5.9 months (range 1.0 – 18.2 months) giving a 6 and 12 months survival of 48.9% and 17.0% (p <0.0001 vs the overall survival in the resectable group). At the time of last data collection, all the patients in this group had died (Mann, 2012).

Table 4.1 Clinicopathological data of patients undergoing potentially curative resection for pancreatic ductal adenocarcinoma.
Taken from Mann 2012.

	Number	%	Median	Range
Gender				
Male	24	57.1	-	-
Female	18	42.9	-	-
Age (years)	-	-	64	30-80
Serology at presentation				
CA 19-9 (U/ml)	-	-	320	3-10,000
Alkaline Phosphatase (IU/L)	-	-	258	39-6881
Alanine transaminase (IU/L)	-	-	91	18-474
Bilirubin (μmol/L)	-	-	146	5-410
WCC (x10^9/L)	-	-	7.5	3.8-20.6
Lymphocytes (x10^9/L)	-	-	1.5	0.7-3.0
Neutrophils (x10^9/L)	-	-	5.0	2.0-16.2
NLR	-	-	3.7	1.1-11.9
Albumin (g/L)	-	-	36	29-47
Creatinine (μmol/L)	-	-	72	49-139
Operative intervention				
Pancreaticoduodenectomy	36	85.7	-	-
Distal pancreatectomy	4	9.5	-	-
Total pancreatectomy	2	4.8	-	-
Tumour characteristics				
Tumour diameter (mm)	-	-	29	10-50
Differentiations				
Well	4	9.5	-	-
Moderate	19	45.2	-	-
Poor	18	42.9	-	-
No data	1	2.4		
Nodal status				
Positive	22	52.4	-	-
Negative	20	47.6	-	-
Microvessel invasion				
Yes	14	33.3	-	-
No	25	59.5	-	-
No data	3	7.1		
Perineural infiltration				
Yes	28	66.7	-	-
No	12	28.6	-	-
No data	2	4.8		
Resection margin				
Positive	15	35.7	-	-
Negative	27	64.3	-	-

WCC, White cell count; NLR, Neutrophil/lymphocyte ratio.

4.3 Results

4.3.1 Notch 2 expression

There was quite a degree of cytoplasmic staining in the pancreatic ductal cells across the 3 groups with all of them ranging between 0–100%. In background pancreas, involved lymph nodes and in advanced disease, a median of 100% of ductal carcinoma cells stained positive for cytoplasmic Notch 2, ranging from 0-100%. Cytoplasmic Notch 2 expression was downregulated somewhat in the resectable cancer cells with a median of 85% of pancreatic ductal adenocarcinoma (PDAC) cells staining positive for cytoplasmic Notch 2. Although the median of percentage of cells staining positive for Notch 2 is 100% for most of the groups, there is a statistically significant difference in the distribution of Notch 2 cytoplasmic staining between groups (p = 0.014, Kruskal-Wallis test). There is a statistically significant difference in the distribution of cytoplasmic Notch 2 staining between healthy tissue and resectable PDAC (p = 0.001 Related-Samples Wilcoxon Signed Rank Test); between resectable cancer tissue and non-resectable cancer tissue (p = 0.013, Independent samples Mann-Whitney U test); and between resectable and locally advanced cancer tissue (p = 0.039).

Although Notch 2 was positively expressed in the nuclei of 3 (7.9%) healthy tissue samples, it was statistically significantly lower when compared to all the other groups: versus resectable cancer group (42.9%, p = 3.86E-04, Pearson Chi-square); versus involved lymph nodes (77.8%, p = 4E-06,); versus non-resectable group (62.2%, p =

Table 4.2 Expression of Notch 2 in normal pancreas, early and advanced pancreatic adenocarcinoma.

 * p< 0.05 compared to background pancreas; ψ p <0.05 compared to resectable pancreatic carcinoma tissue; ʒ compared to locally advanced disease.

		Notch 2 Expression		
		Nuclear n (%)	**Cytoplasmic median** % (range)	**Cytoplasmic mean %** (standard deviation)
Normal	Background pancreas (n=38)	3 (7.9%)	100% (0-100)	87.9% (28.9)
Resectable disease	Pancreatic tissue (n=42)	18 (42.9%)*	85% (0-100) *	61.3% (41.8)
	Local lymph nodes (n=9)	7 (77.8%)*	100% (0-100)	71.1% (43.4)
Advanced disease	Overall (n=45)	28 (62.2%)*	100% (0-100) ψ	82.44 (30.9)
	Locally advanced (n=22)	18 (81.8%)* ψ	100% (0-100) ψ	85.0% (26.0)
	Metastatic (n=23)	10 (43.5%)*ʒ	100% (0-100)	80.0% (35.4)

0.071); versus locally advanced (81.8%, p = 7.24E-09); versus metastatic disease (43.5%, p = 0.001). There is also significant upregulation of positive Notch 2 nuclear staining in locally advanced PDAC tissue when compared to resectable PDAC tissue (p = 0.003) and when compared to metastatic PDAC tissue (p = 0.008). There is no significant difference in the frequency of Notch 2 nuclear staining between resectable PDAC tissue and metastatic PDAC tissue (p =0.961).

Cytoplasmic Notch 2 expression was significantly positively correlated with nuclear Notch 2 expression in PDAC cells (p = 1.23E-04, Table 4.3). There were no significant

relationships between Notch 2 cytoplasmic expression and Notch 1, Notch 3, Notch 4, Hes 1 and Hey 1 cytoplasmic and nuclear expressions. Similarly, there were no significant relationships between Notch 2 nuclear expression and Notch 1, Notch 3, Notch 4, Hes 1 and Hey 1 cytoplasmic and nuclear expressions (Table 4.3). The data for Notch 1, Notch 3, Notch 4, Hes 1 and Hey 1 nuclear and cytoplasmic expression was obtained from Dr Chris Mann

Nuclear Notch 2 expression was not found to be significantly associated with any of the clinicopathological factors investigated (Table 4.4). Cytoplasmic Notch 2 expression was found to be significantly associated with higher CA 19.9 levels ($p = 0.030$), and positive resection margins ($p = 0.012$); however, reduced cytoplasmic Notch expression (median = 15%) was associated with positive microvessel invasion ($p = 0.041$).
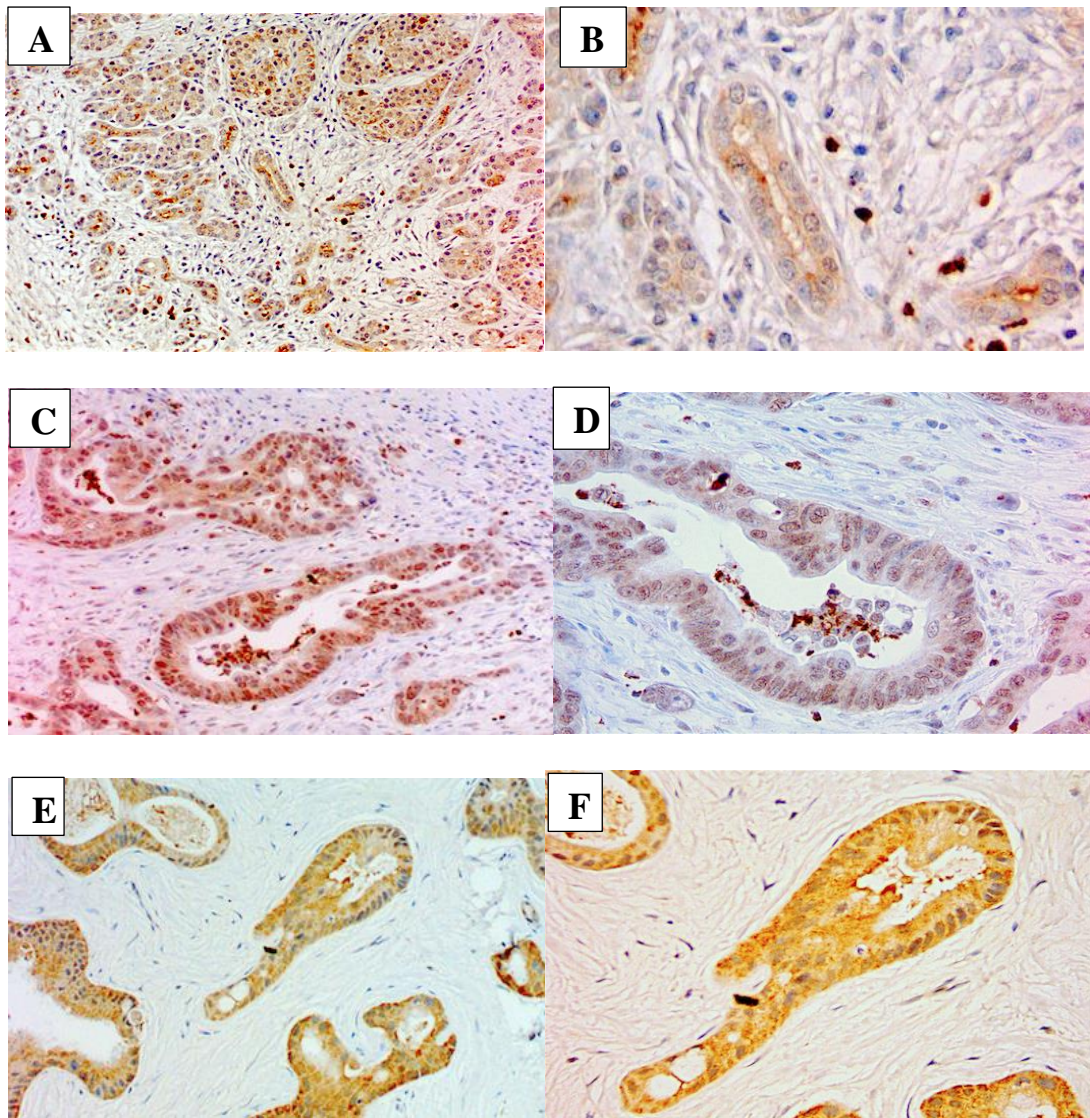
Figure 4.1 Immunohistochemical expression of Notch 2 in normal pancreas and pancreatic adenocarcinoma.

A) normal pancreas using a x 20 magnification. B) normal pancreas with a x 40 magnification. There is Notch 2 cytoplasmic staining but minimal nuclear staining. C) resectable PDAC using a x 20 magnification and a D) x 40 magnification. There is Notch 2 cytoplasmic and nuclear staining present. E) Non-resectable advanced PDAC using a x 20 magnification and a F) x 40 magnification. There is Notch 2 cytoplasmic staining and further upregulation of Notch 2 nuclear staining.

Table 4.3 Correlations between Notch 2 protein expression and other Notch proteins and pathway constituents.

Nucl = nuclear; cyto = cytoplasmic; * = correlation is significant at the 0.01 level (2 - tailed), Spearman's rank correlation coefficient.

	Notch 2 nucl		Notch 2 cyto	
	Coeff.	Sig.	Coeff.	Sig.
Notch 2 nucl	1.000	.	0.565*	0.000
Notch 2 cyto	0.565*	0.000	1.000	.
Notch 1 nucl	0.031	0.844	0.238	0.128
Notch 1 cyto	-0.059	0.710	0.082	0.608
Notch 3 nucl	0.138	0.385	0.210	0.182
Notch 3 cyto	0.121	0.445	-0.013	0.937
Notch 4 nucl	0.193	0.222	0.089	0.575
Notch 4 cyto	-0.248	0.114	-0.232	0.139
HES 1 nucl	0.075	0.644	0.148	0.361
HES 1 cyto	-0.073	0.657	-0.107	0.509
HEY 1 nucl	0.031	0.844	-0.040	0.803
HEY 1 cyto	0.168	0.288	0.052	0.745

Table 4.4 Associations of nuclear and cytoplasmic Notch 2 expression in resectable PDAC tissue with clinicopathological variables.

 * = Chi squared or Fisher's exact test as appropriate; ** = Mann–Whitney U test. Alk Phos = alkaline phosphatase; ALT = alanine transaminase; NLR = neutrophil/lymphocyte ratio.

Factor	Category	Nuclear Notch 2 expression			Cytoplasmic Notch 2 expression	
		Present	Absent	P*	Median (range)	P**
Gender	Male	11	13	0.767	77.5% (0-100%)	0.804
	Female	7	10		90% (0-100%)	
Age	≥64 years	8	13	0.533	80% (0-100%)	0.520
	<64 years	10	11		90% (0-100%)	
CA19.9	≥320 (U/ml)	10	8	0.150	100% (10-100%)	**0.030**
	<320 (U/ml)	8	16		40% (0-100%)	
Alk phos	≥258 (IU/L)	8	12	0.721	85% (0-100%)	0.990
	<258 (IU/L)	10	12		70% (0-100%)	
ALT	≥91 (IU/L)	9	11	0.789	95% (0-100%)	0.407
	<91 (IU/L)	9	13		50% (0-100%)	
Bilirubin	≥146 (μmol/L)	7	13	0.327	77.5% (0-100%)	0.318
	<146 (μmol/L)	11	11		95% (0-100%)	
WCC	≥7.5 (x 10^9/L)	6	13	0.179	50% (0-100%)	0.210
	<7.5 (x 10^9/L)	12	11		90% (0-100%)	
Lymphocytes	≥1.5 (x 10^9/L)	11	9	0.162	50% (0-100%)	0.989
	<1.5 (x 10^9/L)	7	14		90% (0-100%)	
Neutrophils	≥5.0 (x 10^9/L)	8	12	0.721	50% (0-100%)	0.216
	5.0 (x 10^9/L)	10	12		95% (0-100%)	
NLR	≥5.0	5	6	0.972	80% (0-100%)	0.599
	<5.0	13	16		90% (0-100%)	
Albumin	≥36 (g/L)	11	11	0.327	85% (0-100%)	0.486
	<36 (g/L)	7	3		70% (0-100%)	
Creatinine	≥72 (μmol/L)	11	9	0.129	95% (0-100%)	0.242
	<72 (μmol/L)	7	15		50% (0-100%)	
Tumour diameter	≥29mm	9	10	0.591	90% (0-100%)	0.579
	<29mm	9	14		75% (0-100%)	

Tumour differentiation	Poor	9	9	0.565	82.5% (0-100%)	0.702
	Well/Mod	9	13		85% (0-100%)	
Nodal status	Positive	9	13	0.678	50% (0-100%)	0.446
	Negative	9	10		90% (0-100%)	
Microvessel invasion	Present	4	10	0.197	15% (0-100%)	**0.041**
	Absent	12	12		90% (0-100%)	
Perineural invasion	Present	12	16	0.677	50% (0-100%)	0.183
	Absent	6	6		100% (0-100%)	
Resection margin	Positive	7	8	0.786	100% (10-100%)	**0.012**
	Negative	11	15		50% (0-100%)	

4.3.2 Survival Analyses

Survival analyses were performed to associate the expression of Notch 2 with overall and disease- free survival following resection with curative intent of pancreatic adenocarcinoma. In the univariate Cox regression model, neither Notch 2 nuclear expression nor cytoplasmic expression made any significant contribution to the overall survival or disease-free survival (Table 4.5). To investigate if Notch 2 expression had any effect on overall or disease-free survival following clear resections margins, another univariate Cox regression model was done. Again, Notch 2 nuclear expression nor cytoplasmic expression made any significant contribution to the overall survival or disease-free survival (Table 4.6).

There was no significant difference in overall survival between expression of nuclear Notch 2 and no expression of nuclear Notch 2 (p =0.642 Log Rank test). For patients undergoing curative resections, the median survival was 30.1 months, 95% CI 22.8 – 37.4 months for patients with positive Notch 2 nuclear expression, whereas the median survival was 30.5 months, 95% CI 25.2 – 37.8 months for patients without nuclear

Notch 2 expression. 5-year survival for patients with positive nuclear Notch 2 expression was 27.8% and for patients without nuclear Notch 2 expression was 21.7%. (Figure 4.2). There was also no significant difference in disease free survival between expression of nuclear Notch 2 and no expression of nuclear Notch 2 (p =0.867 Log Rank test) with a median of 25.0 months (95% CI 12.6 -37.4 months) and 21.8 months (95% CI 13.2 – 30.4 months) respectively (Figure 4.3).

Similarly, there was no significant difference in overall survival between upregulated cytoplasmic expression of Notch 2 ($\geq$ 50%) and low cytoplasmic expression of Notch 2 ($\leq$ 50%) (p =0.813 Log Rank test). For patients undergoing curative resections, the median survival was 27.5 months (95% CI 24.1 – 30.9 months) for patients with upregulated cytoplasmic expression of Notch 2 ($\geq$ 50%) whereas the median survival was 31.3 months (95% CI 29.3 – 33.3 months) for patients with lower cytoplasmic Notch 2 expression. 5-year survival for patients with upregulated cytoplasmic expression of Notch 2 was 26.9% and for patients with low cytoplasmic Notch 2 expression was 18.8%. (Figure 4.2). There was also no significant difference in disease free survival between upregulated expression of cytoplasmic Notch 2 and low expression of cytoplasmic Notch 2 (p =0.763 Log Rank test) with a median of 21.8 months (95% CI 6.9 – 36.7 months) and 28.7 months (95% CI 19.1 – 38.3 months) respectively (Figure 4.3).

Univariate Cox regression model analysis on overall survival was done using clinicopathological factors as variables. CA 19.9 $\geq$ 320 (p = 0.028), Alanine Transaminase $\geq$ 91 (p = 0.049), lymph node involvement (p = 0.002), microvessel invasion (p = 0.023) and positive resection margins (p = 0.015) gave statistically

significant results. WCC $\geq$ 7.5 (p = 0.080) and neutrophils $\geq$ 5 (p = 0.090) were the other variables who gave p values of less than 0.1. (Table 4.7) Therefore, a forward logistic multivariate Cox regression model analysis was done on overall survival using the above variables. Alanine Transaminase $\geq$ 91 (p = 0.018, HR 2.437), lymph node involvement (p = 0.003, HR 3.526), microvessel invasion (p=0.026, HR 2.407) and involved resection margin (p = 0.009, HR 2.852) all made significant contributions to the model, with lymph node involvement giving the highest hazard ratio for overall survival.

A similar univariate cox regression model analysis for disease free survival found CA 19.9 $\geq$ 320 (p = 0.028), Lymph node involvement (p = 0.001), microvessel invasion (p=0.034), and neural invasion (p =0.075) gave statistically significant results. Alanine Transaminase $\geq$ 91 (p = 0.071) was the other variable which gave p values of less than 0.1. (Table 4.7) A forward logistic multivariate Cox regression model analysis was done on overall survival using the above variables. Alanine Transaminase $\geq$ 91 (p = 0.037, HR 2.269) and lymph node involvement (p = 0.001, HR 4.184) made statistically significant contributions to the model with lymph node involvement giving the higher hazard ratio for disease-free survival.

Table 4.5 Univariate Cox Regression survival analyses for Notch 2 pathway biomarkers for all patients undergoing potentially-curative resection for pancreatic adenocarcinoma (n=42)

	Overall Survival		Disease-free Survival	
	Hazard Ratio (95% CI)	P	Hazard Ratio (95% CI)	P
Notch 2 nuclear expression: +/-	0.850 (0.428-1.688)	0.643	0.941 (0.463-1.913)	0.867
Notch 2 cytoplasmic expression: ≥50%/<50% staining	0.922 (0.470-1.809)	0.813	0.896 (0.437-1.837)	0.763

Table 4.6 Univariate Cox Regression survival analyses for Notch 2 pathway biomarkers for patients who had a clear R0 resection margin following surgery for pancreatic adenocarcinoma (n=27)

	Overall Survival		Disease-free Survival	
	Hazard Ratio (95% CI)	P	Hazard Ratio (95% CI)	P
Notch 2 nuclear expression: +/-	0.897 (0.366-2.200)	0.813	1.005 (0.395-2.559)	0.992
Notch 2 cytoplasmic expression: ≥50%/<50% staining	0.623 (0.260-1.490)	0.287	0.618 (0.247-1.549)	0.305
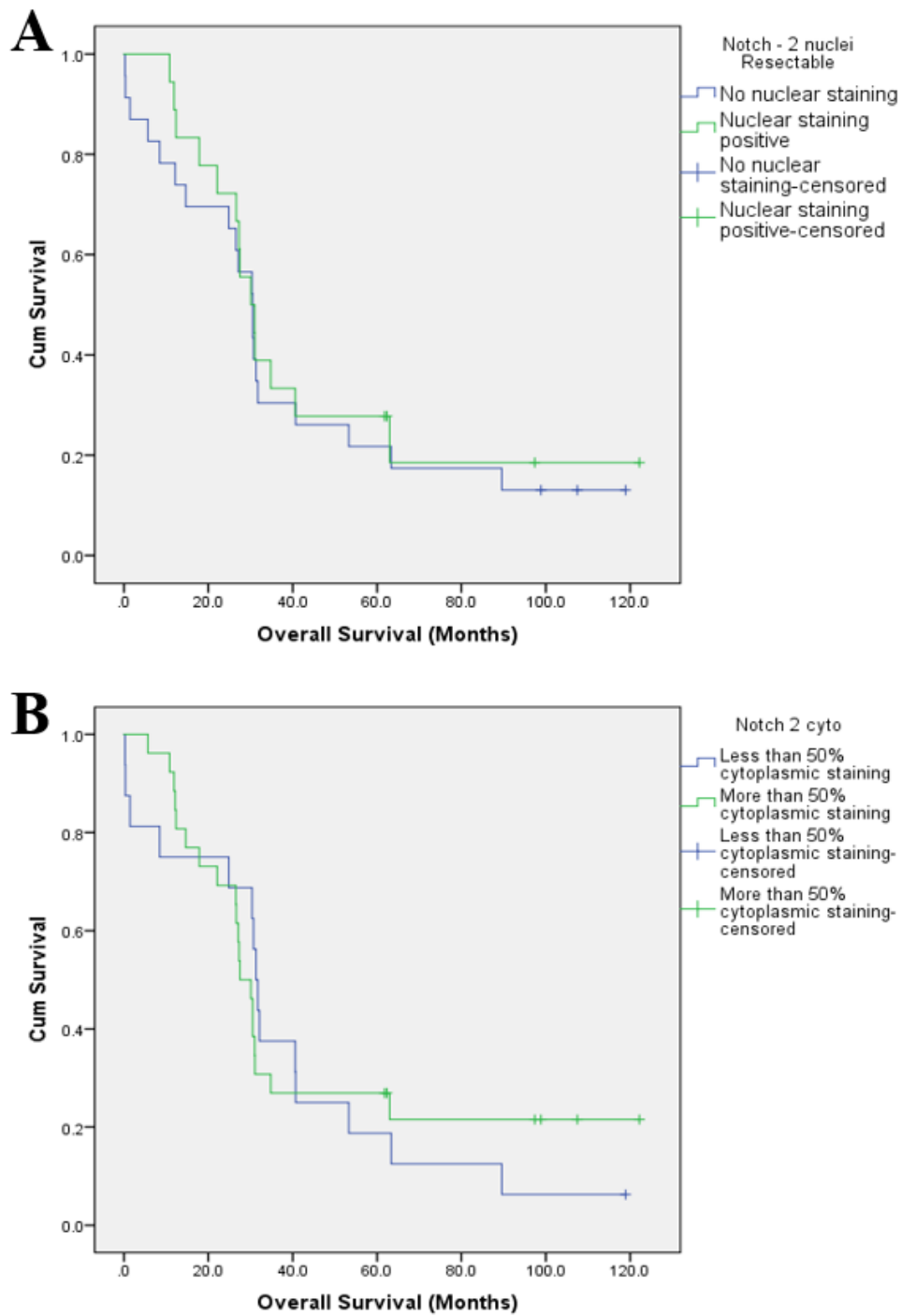
Figure 4.2 Kaplan Meier curve demonstrating the impact of Notch 2 expression on overall survival in patients who had potentially curative resection (n = 42)

A = Nuclear Notch 2 expression. No significant difference (p =0.642). B = Cytoplasmic Notch expression. No significant difference (p= 0.813). Log-rank test)
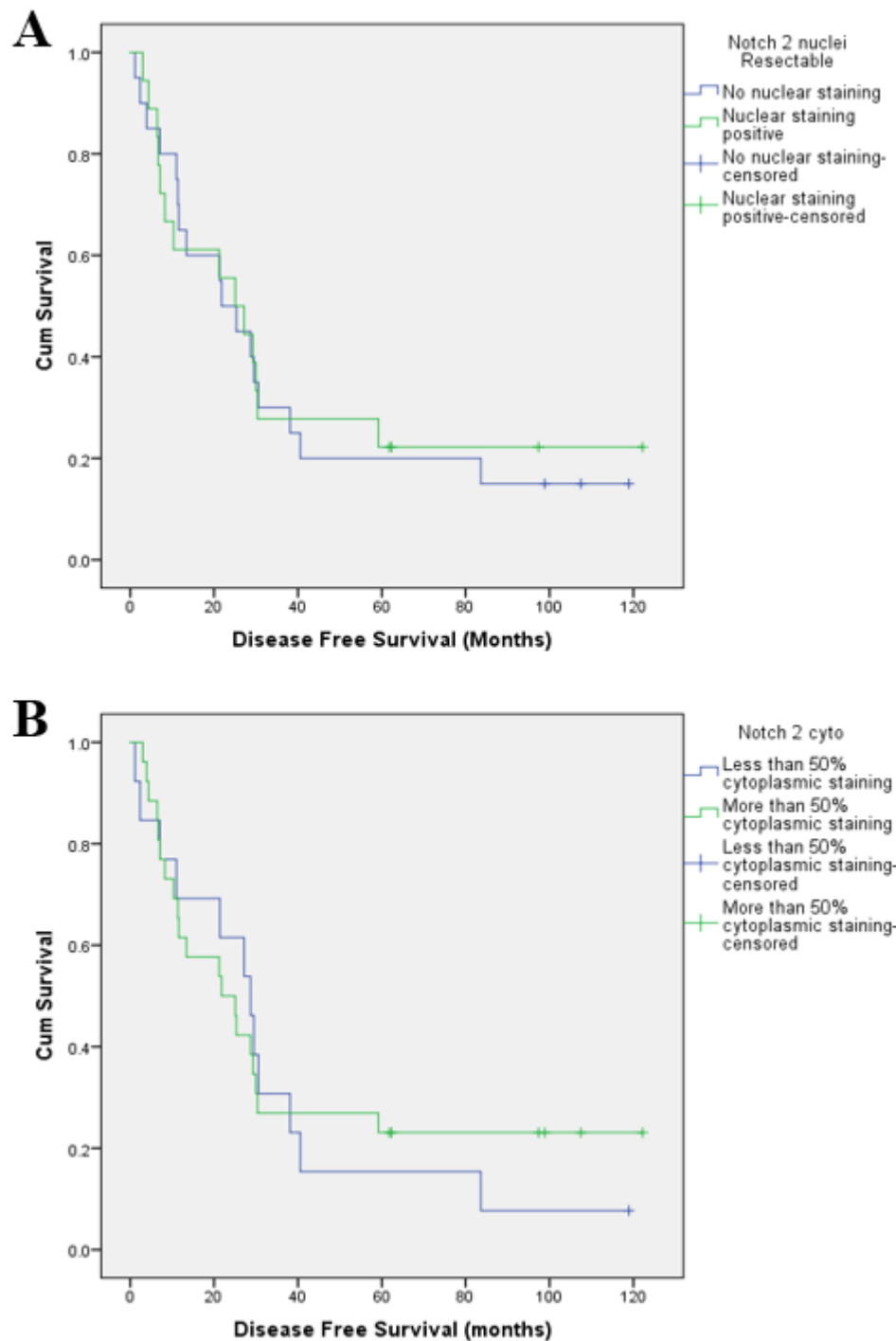
Figure 4.3 Kaplan Meier curve demonstrating the impact of Notch 2 expression on disease-free survival in patients who had potentially curative resection (n = 42)

A = Nuclear Notch 2 expression. No significant difference (p =0.867). B = Cytoplasmic Notch expression. No significant difference (p= 0.763). Log-rank test)

Table 4.7 Univariate Cox regression survival using clinicopathologic factors for patients undergoing potentially-curative resection for pancreatic adenocarcinoma (n = 42). Variables with P values < 0.1 are highlighted in bold.

Factor	Variables	Overall Survival		Disease Free Survival	
		Hazard ratio (95% CI)	P value	Hazard ratio (95% CI)	P value
Gender	Male/ Female	0.611 (0.313 – 1.192)	0.149	1.243 (0.617 – 2.506)	0.545
Age	≥64 /<64 years	1.158 (0.592 – 2.264)	0.668	1.056 (0.524 – 2.127)	0.880
CA19.9	≥320/<320 (U/ml)	**2.142 (1.084 – 4.231)**	**0.028**	**2.245 (1.091 – 4.618)**	**0.028**
Alkaline phosphatase	≥258/<258 (IU/L)	0.955 (0.488 – 1.866)	0.892	1.018 (0.506 – 2.047)	0.960
Alanine Transaminase	≥91/<91 (IU/L)	**1.975 (1.004 – 3.882)**	**0.049**	**1.920 (0.592 – 2.264)**	**0.071**
Bilirubin	≥146/<146 (μmol/L)	1.121 (0.577 – 2.180)	0.736	1.109 (0.552 – 2.230)	0.771
White Cell count	≥7.5/<7.5 (x 10^9/L)	**0.547 (0.279 – 1.074)**	**0.080**	0.695 (0.344 – 1.405)	0.311
Lymphocytes	≥1.5/<1.5 (x 10^9/L)	0.865 (0.441 – 1.698)	0.674	1.113 (0.547 – 2.268)	0.767
Neutrophils	≥5.0/<5.0 (x 10^9/L)	**0.558 (0.284 – 1.095)**	**0.090**	0.693 (0.344 – 1.394)	0.303
Neutrophil Lymphocyte ratio	≥5.0/<5.0	0.790 (0.375 – 1.663)	0.535	0.891 (0.418 – 1.900)	0.766
Albumin	≥36/<36 (g/L)	1.045 (0.534 – 2.045)	0.899	0.944 (0.470 – 1.896)	0.872
Creatinine	≥72/<72 (μmol/L)	0.696 (0.354 – 1.370)	0.294	0.643 (0.316 – 1.308)	0.233
Tumour diameter	≥29/<29 mm	0.959 (0.490 – 1.876)	0.902	0.835 (0.415 – 1.680)	0.612
Tumour differentiation	Poor/ Well/Mod	0.833 (0.415 – 1.673)	0.608	0.864 (0.417 – 1.791)	0.694
Nodal status	Positive/ Negative	**3.312 (1.566 – 7.004)**	**0.002**	**4.047 (1.786 – 9.172)**	**0.001**
Microvessel invasion	Present/ Absent	**2.304 (1.124 – 4.719)**	**0.023**	**2.275 (1.065 – 4.860)**	**0.034**
Perineural invasion	Present/ Absent	1.790 (0.824 – 3.890)	0.142	**2.108 (0.929 – 4.786)**	**0.075**
Resection margin	Positive/ Negative	**2.373 (1.186 – 4.747)**	**0.015**	1.645 (0.803 – 3.369)	0.174

4.4 DISCUSSION

This chapter reports the immunohistochemical assessment of Notch 2 receptors in PDAC. It is the first study to examine Notch 2 expression throughout disease progression from early, through advanced local disease to metastatic disease. This study is also the first to explore the association of Notch 2 expression with clinicopathological factors. Finally, this study also looks at the correlation of Notch 2 with other Notch receptors and its target genes Hes 1 and Hey 1, and the effect of Notch 2 expression on overall and disease-free survival. There were only two widely recognized studies exploring the expression of Notch 2 in human PDAC tissue using immunohistochemistry (Miyamoto *et al*, 2003; Büchler *et al*, 2005).

Miyamoto *et al* found Notch 2 cRNA to be upregulated in PDAC tissue, with a 2-3-fold increase compared to normal pancreas. Using real-time quantitative RT-PCR analysis, they demonstrated a 3.4-fold overexpression of Notch 2 in infiltrating pancreatic ductal adenocarcinomas compared to normal pancreas. Using 34 resected human pancreas tissue samples which contained both PDAC and PanIN lesions, they demonstrated little-to-no expression in normal pancreatic ductal epithelium but moderate to high levels of detectable Notch 2 in 47% of the cancer samples (Miyamoto *et al*, 2003). Bucher *et al* found Notch 2 to be consistently expressed in 6 different human pancreatic cancer cell lines, at different levels, with the highest levels of Notch 2 expression in the undifferentiated cell lines. They examined the expression of the Notch gene family by real-time quantitative PCR using 31 pancreatic cancer patients and 22 healthy organ donors. They detected all 4 members of the Notch gene family in normal pancreatic tissue, however higher levels of Notch 2 mRNA were detected in both normal and pancreatic cancer specimens. Furthermore, the Notch 2 gene was not significantly

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upregulated in pancreatic cancer samples in comparison to normal pancreatic tissue. Using IHC, Bucher *et al* reported strong Notch 2 expression present in ductal pancreatic cancer cells and in vascular smooth muscle cells of the tumour blood vessels, but disappointingly there was no comparison with normal pancreatic tissue (Büchler *et al*, 2005).

The results of this study found cytoplasmic Notch 2 was expressed highly in the cytoplasm of the ductal epithelial cells of both normal pancreas tissue and PDAC, including lymph node metastases, locally advanced disease and distant metastasis. This increased expression is in keeping with the findings of Bucher *et al* (2005) which detected Notch 2 mRNA in both normal and pancreatic cancer specimen. However, there is a statistically significant difference in the cytoplasmic expression of Notch 2 between healthy tissue and resectable PDAC tissue with reduced expression in the cancer tissues. This result is slightly unexpected since we were expecting Notch 2 to be upregulated in cancer. One possible explanation for this could be due to the relatively high proportion of positive cytoplasmic staining and our choice of semi-quantitative method of scoring. Our median value for all the tissue types, except for resectable cancer, was 100% and therefore any reduction in the median value could have resulted in a difference. The alternate explanation could be that Notch 2 signalling is complex and there is still a lot of it that we do not understand. We found minimal nuclear expression in healthy pancreas tissue in keeping with the findings of Miyomoto *et al* (2003)*.* We also found nuclear expression of Notch 2 to be significantly upregulated in all cancer tissues (including resectable tissues, locally advanced non-resectable tissues, involved lymph nodes and distant metastases). This is in keeping with the finding of Miyomoto *et al* (2003) and also confirms the immunohistochemical presence of Notch

2 proteins in cancer specimens as described by Bucher *et al* (2005). We found that the nuclear expression of Notch 2 was also significantly upregulated in locally advanced non-resectable disease when compared to resectable disease. This is similar to the findings of Mann (2012) in which he found the expression of Notch 1, 3,4 and its downstream targets Hes 1 and Hey 1 upregulated in locally advanced non-resectable cancers. However, we also found that there is significant downregulation in Notch 2 nuclear expression in distant metastasis of PDAC when compared to locally advanced non-resectable tissue and resectable (early) PDAC tissue. This is different from Mann 2012, where he found that the expression of the other receptors in the Notch family was upregulated in metastatic PDAC, in comparison to resectable (early) PDAC tissue. This finding is potentially interesting and could suggest that Notch 2 does not behave like the rest of the Notch receptors but in a rather complex manner depending on the stage of the disease. This could also suggest that Notch 2 might have a differing role to the rest of its family in relation to pancreatic cancer. Our findings that the nuclear expression of Notch 2 in distant metastasis is significantly lower compared to locally advanced non-resectable tissue and resectable (early) PDAC tissue seems to suggest a theory that Notch 2 is required for progression of PDAC up to a certain point, after which its role is somewhat reduced. This theory is supported by a study examining the role of Notch signalling in radiologically evident pancreatic tumours in mice, which demonstrated that GSI treatment failed to extend lifespan thus suggesting that Notch signalling may not be involved in the maintenance of advanced pancreatic tumours (Cook *et al*, 2012).

We have found that Notch 2 nuclear and cytoplasmic expression are significantly positively correlated as is expected, but found no other significant associations with the other Notch family and the downstream targets Hes 1 and Hey 2. This suggests that

Notch 2 works independently of the Notch family and its downstream targets are not
Hes 1 or Hey 1. Among the 4 members of the Notch family protein, only Notch 1 and
Notch 2 are thought to have significant roles in pancreatic embryonic development and
pancreatic carcinogenesis (Hu *et al*, 2013). Notch 1 and Notch 2 have also been shown
to have different effects on pancreatic organogenesis as well as carcinogenesis (Mazur
et al, 2010). So, the lack of association with the other Notch family is not surprising. It
is also unsurprising that there is no association with Hes 1 and Hey 1. Hes 1 is
commonly used as an identification marker of the activation of the Notch pathway, but
Hes 1 expression does not necessarily correlate with Notch activation and does not
differentiate between Notch receptors (Avila and Kissil, 2013). There has been several
studies where the inhibition of Notch signalling in pancreatic cancer has not led to
decreased expression of Hes 1(Hanlon *et al*, 2010; Mazur *et al*, 2010). Similarly, Hes 1
expression has also failed to recreate the effects of Notch activation (Miyamoto *et al*,
2003; Plentz *et al*, 2009). There has also been reports of Hes 1 activation in a Notch-
independent manner (Mazur *et al*, 2010; Hashimoto *et al*, 2006). A very recent study by
Liu *et al* in human pancreatic cancer cells lines have found that only a small subset of
Notch 1 and Notch 2 binding sites overlap with the binding sites of transcription factor
CSL (CBF1, Suppressor of Hairless, Lag1), whilst about half of the CSL binding sites
overlap with that of Notch 1 or Notch 2, indicating most Notch signalling activities are
actually CSL independent (H. Liu *et al*, 2017). Furthermore, they have also identified
TPM3, NFκ-BIA, NFκ-B, GSK -3β and GNAQ as Notch 2 target genes in pancreatic
cell lines (H. Liu *et al*, 2017).

We also found an association of higher level of Notch 2 cytoplasmic staining with
higher levels of CA 19.9 and positive resection margins. This is again in-keeping with

the findings that Notch 2 activation is upregulated and required for the progression of PDAC (Mazur *et al*, 2010). We found no statistical difference in overall survival and disease-free survival between patients with positive Notch 2 expression and patients with negative Notch 2 expression in patients who had a potentially curative resection for PDAC. This is somewhat surprising as Mazur *et al* were able to show that Notch 2 knockdown in *Kras* mice led to significantly longer median survival (Mazur *et al*, 2010).

There has been contrasting reports on the role of Notch 1 and Notch 2 in pancreatic carcinogenesis. Miyamoto *et al reported* that the increased expression of Notch receptors and its ligands promoted the activation of Notch in early PanIN lesions. In mice cells, they were able to show that activation of Notch 1 and Notch 2 resulted in metaplastic conversion of acinar -cell predominant epithelium to a ductal cell-predominant epithelium and that transforming growth factor alpha (TGFα)- induced γ-secretase- dependent Notch activation is required for initiation of the metaplasia/neoplasia sequence (Miyamoto *et al*, 2003). Mazur *et al* found that, in *Kras*^{G12D} mice, Notch 2 is the predominant Notch receptor in ductal, centroacinar and PanIN cells and by far the most prominently expressed Notch receptor during PanIN development and in PDAC, whilst Notch 1 is predominantly in the acinar cells (Mazur *et al*, 2010). They were also able to show that Notch 2 knockdown in *Kras* mice led to significantly longer median survival, whereas Notch 1 knockdown led to no significant change. This led to the suggestion that Notch 2 is involved in the progression of PanIN and is associated with decreased survival, whereas Notch 1 has no oncogenic role in *Kras* mice. Interestingly Mazur *et al* did not observe loss of Hes 1 expression in either Notch 1 or Notch 2 knocked down pancreas, suggesting that Hes 1 may be regulated by

other signalling pathways. Mazur *et al* also found evidence to support the theory that Notch 2 modulates Myc signalling in Kras mice and Notch–dependent Myc signalling is a key regulator of the carcinogenic process in the pancreas (Mazur *et al*, 2010). Mullendore *et al* showed that loss of Notch 2 in a mouse model of pancreatic cancer resulted in inhibition of PanIN progression, highlighting the important role of Notch 2 in PanIN progression (Mullendore *et al*, 2009).

By working in human foetal tissue, Hu *et al* (2013) found that Notch 1/Hes 1 signalling pathway is activated during early pancreatic embryogenesis and reaches the highest level at birth. Once the pancreas is fully developed, the Notch 1/Hes 1 pathway is inactivated. They also show that the expression of both Notch 1 and Hes 1 are present in 50% of PDACs, but not in PanINs, indicating that Notch 1 activation is only apparent in late stage pancreatic carcinogenesis (Hu *et al*, 2013). Notch 2 has also been implicated in the chemoresistance of PDAC. Gugnor *et al* (2011) found that chemotherapy-induced MK (a heparin- binding growth factor) secretion, triggered activation of extracellular Notch 2 that resulted in up-regulation downstream targets, Hes 1 and NF-κB, which in turn, promoted the epithelial-mesenchymal transition (EMT) that accompanies increased resistance to chemotherapy and migration potential (Güngör *et al*, 2011; Gungor *et al*, 2014). Notch signalling pathway is believed to play an important role in pancreatic cancer stem cells (CSC). Several studies have shown high expression of Notch 1 and Notch 2 in CSC and it is thought that that there is a link between CSC and EMT cells and gemcitabine-resistant cells, both of which are linked with Notch 2 activation (Z. Wang *et al*, 2011).

As mentioned earlier, Liu *et al* (2017) identified Notch 2 target genes in the cancer pathway, from a pancreatic cancer cell line. Two of the target genes are NFκ-BIA and NFκ-B. NFκ-BIA is an inhibitor of NFκ-B and both these genes are highly expressed in the pancreatic cell line. Whereas NFκ-B plays a key role in pancreatic cancer cell proliferation, cell survival and invasion, NFκ-BIA seeks to inhibit it resulting in chemosensitization and apoptosis. Therefore Notch 2 has the potential to act as an oncogene and tumour suppressor in the same cancer cell (H. Liu *et al*, 2017).

Notch 2 therefore has multiple complex roles in pancreatic carcinogenesis and only some of the functions are now known. More work needs to be done on Notch 2 signalling pathway to fully understand its role in pancreatic carcinogenesis and therefore to evaluate it potential as a therapeutic option and as a possible biomarker for prognosis and detection.

CHAPTER 5.

INVESTIGATING miRNA EXPRESSION IN PLASMA OF PATIENTS WITH PANCREATIC DUCTAL ADENOCARCINOMA AND CHRONIC PANCREATITIS AS POTENTIAL BIOMARKERS

5.1 Introduction

miRNAs (Micro RNAs) are a class of small (~22 nucleotides) noncoding RNAs which act as negative regulators of gene expression by either cleaving the target messenger RNA or by inhibiting the translational process (Mardin and Mees, 2009). Due to their ability to alter gene expression they play a significant physiological role in the maintenance of cellular homeostasis and development. As a result, their dysregulation also has an adverse effect on biological processes, including proliferation, differentiation and apoptosis (Hwang and Mendell, 2006; Dykxhoorn, 2010).

There have been wide reports of aberrant expression of miRNAs in human cancers. They have been found to be both overexpressed and under expressed in neoplastic cells compared with their normal healthy counterparts (J. Wang *et al*, 2009). miRNAs have been profiled in many different malignancies including breast, lung, colorectal cancer and pancreatic cancer (P. Xu *et al*, 2016; Iorio *et al*, 2005; Johnson *et al*, 2005; C. Xu *et al*, 2015; Bloomston *et al*, 2007). miRNAs have been found to be very stable in tissue, plasma, stool and other fluids. They can also be quantified in very small sample sizes making it an excellent choice for biomarkers (Hernandez and Lucas, 2016). The first circulating miRNAs in serum to be used as biomarkers were in patients with diffuse large B-cell lymphoma (Lawrie *et al*, 2007).

Using miRNA microarray expression profiling, Bloomston *et al* identified 30 miRNAs that were upregulated in pancreatic cancers and 3 miRNAs that were down-regulated when compared to normal pancreatic tissue. 15 miRNAs were overexpressed, and 8 miRNAs were under expressed in pancreatic cancer tissue when compared with chronic pancreatitis tissue. 22 miRNAs were overexpressed in chronic pancreatitis tissue and 2

miRNAs were under-expressed when compared with normal pancreatic tissue (Bloomston *et al*, 2007).

Szafranska *et al*, through the process of miRNA expression profiling of tissue samples and pancreatic cancer cell lines, found a total of 94 miRNAs were differentially expressed at significant level between any two of the 3 tissue types (normal pancreas, pancreatic ductal adenocarcinoma and chronic pancreatitis). Out of this, they selected the top 26 miRNAs which they thought represented the best novel potential candidates for biomarkers and therapeutic target selection for PDAC (Szafranska *et al*, 2007). Szafranska *et al* also found miR–196a and miR–196b to be upregulated in PDAC and also expressed in cancer cell lines, but completely absent in normal pancreatic and chronic pancreatitis tissue. This led them to suggest miR–196 overexpression is specific to PDAC and its potential as an excellent biomarker and therapeutic potential (Szafranska *et al*, 2007). Interestingly, Bloomston *et al* found miR-196a-2 did not help differentiate between pancreatic cancers and non-cancers but found it to be overexpressed in pancreatic cancer patients with a lower median survival compared to pancreatic cancer patients with a slightly higher median survival (Bloomston *et al*, 2007).

Wang *et al* selected a panel of only four miRNAs, miRNA-21, miRNA-210, miRNA-155, miRNA-196a, and were able to show that these miRNAs were overexpressed in blood plasma/serum and varying expression profiles of these samples can be used to distinguish PDA from normal healthy individuals with a sensitivity of 64% and a specificity of 89% (J. Wang *et al*, 2009).

It was therefore thought that expression levels of certain miRNAs can be used to differentiate between PDAC patients, chronic pancreatitis patients and healthy volunteers. The aim of this chapter is to examine the plasma for 11 miRNAs of interest in PDAC patients, chronic pancreatitis patents and healthy volunteers in order to examine their potential as a biomarker.

5.2 Results

5.2.1 Micro RNAs investigated

The miRNAs which were investigated were miR–10b, miR–21, miR–34a, miR–143, miR–148, miR–155, miR–196a[2], miR–200c, miR–206, miR–375 and miR–503. Our study was aimed as a proof of principle pilot study to evaluate the utility of a few selected miRNAs in developing a blood-plasma biomarker assay for pancreatic cancer and chronic pancreatitis. MicroRNA–21, miR–143, miR-155a and miR–196a were already known to be upregulated in PDAC tissue, whereas miR–10b and miR–143 were known to be upregulated in both PDAC and chronic pancreatitis tissues (Szafranska *et al*, 2007; Habbe *et al*, 2009; Bloomston *et al*, 2007). MicroRNA–148a and miR-375 have been shown to be downregulated in PDAC compared to healthy tissues (Bloomston *et al*, 2007; Szafranska *et al*, 2007). MicroRNA– 34a have been shown to inhibit human pancreatic cancer tumour initiating cells and Chang *et al* have shown the expression of miR–34a to be reduced 2-fold in 15 pancreatic cancer cells lines when compared to normal pancreas (Ji *et al*, 2009; Chang *et al*, 2007). Song *et al* found miR– 206 to be a pro-apoptotic activator of cell death by inhibiting Notch 3 signalling and tumour formation (G. Song, Zhang and Wang, 2009). MicroRNA 200c was already linked with increased aggressiveness and chemoresistance in female reproductive

malignancies and Park et al reported that miR–200c regulates epithelial to mesenchymal transition (EMT) in breast and ovarian cancer cells (S. Park *et al*, 2008). Yu et al found that increased levels of miR–200c was associated with a significantly higher survival rates in PDAC and there was a strong positive correlation with E Cadherin expression and this led them to suggest this miRNA as a prognostic marker (Yu *et al*, 2010). Micro RNA–503 is shown to be required for the development of islet cells in the pancreas and was also shown to be upregulated in endometrial cancer. (Lynn *et al*, 2007; Wentz-Hunter and Potashkin, 2011). Simultaneous work in our laboratory on 4 pancreatic cell lines by a PhD candidate (Lara Lewis) found miR–503 clearly downregulated in all 4 pancreatic cells line when compared to normal pancreatic tissue. We also found that miR– 206 to be slightly upregulated in 3 cell lines; miR–200c to be downregulated in 3 of the 4 cancer cell lines; and miR–34a to be clearly upregulated in 3 cell lines whilst being slightly downregulated in one cell line. These results along with the evidence from the literature prompted us to finally decide on the 11 miRNAs of interest. A summary of the expression profiling is found in Table 5.1. The methodology of the miRNA extraction and real-time qPCR are described in section 2.6.

5.2.2 Micro RNA statistical analysis

Following quantitative real-time qPCR, the cycle threshold (Ct) values were recorded for the tested miRNAs and the internal spike control miR-cel-238. The Ct value is the quantitative endpoint for a PCR cycle. The numerical value of the Ct is inversely related to the amount of cDNA created by reverse transcription and therefore is inversely related to the amount of miRNA in the sample (Schmittgen and Livak, 2008).

Table 5.1 Summary of expression profiles in literature of the 11 miRNAs which were chosen for our study to differentiate between PDAC patients, chronic pancreatitis and healthy volunteers.

miRNA	Reason
miR-10b	upregulated in PDAC and chronic pancreatitis tissue
miR-21s	upregulated in PDAC tissue
miR-34a	downregulated in PDAC tissue
miR-143	Upregulated in PDAC and chronic pancreatitis tissue
miR-148a	Downregulated in PDAC
miR-155a	Upregulated in PDAC tissue
miR-196a	Upregulated in PDAC tissue
miR-200c	Increased levels associated with significantly higher survival rates in PDAC
miR-206	Pro-apoptotic activator of cell death. Slightly upregulated in ¾ PDAC cell lines in our lab.
miR-375	Downregulated in PDAC tissue
miR-503	Required for development of islet cells in the pancreas. Found to be downregulated in 4/4 PDAC cell lines in our lab.

Relative quantification for the tested miRNAs were calculated by subtracting the Ct values of the internal spiked control from the Ct values of the miRNA is question. For each sample, the Δ Ct = Ct (miRNA) – Ct (cel-miR-238 of that sample). The Δ Ct for

every sample and each subgroup of patients were studied and spurious readings which were confirmed as definite outliers were further explored using the duplicate raw Ct values. If the samples were giving consistent extreme outlier Ct values, they were removed from the statistical analysis. Two healthy volunteers and 2 PDAC patient samples were consistently giving outlier readings on most of the miRNAs and their raw readings were observed. It was found that these four samples were processed at the same time and it was thought that there was either something wrong with the samples or the master mix and therefore all the readings from those samples were discarded. A normality test, Kolmogorov-Smirnov test was done for all Δ Ct values. The Δ Ct data for all the miRNAs except miR-206 and miR–155a and miR–10b, followed a normal distribution and so a parametric test ANOVA, followed by post-hoc test with Bonferroni corrections were used for their analysis. Non-parametric tests Kruskal Wallis followed by Mann Whitney U test were performed for calculations involving miR-206, miR–155a and miR-10b.

5.2.1 Patient Demographics

There was a total of 56 patients included in this analysis, 18 pancreatic ductal adenocarcinoma patients (PDAC), 20 chronic pancreatitis patients (CP) and 18 healthy volunteers (HV). (Table 5.2) The process of selecting patients has been described in section 2.1.1.

There was a significant statistical difference between the mean age of healthy volunteers and pancreatic cancer patients ($p = 0.028$) and between patients with pancreatic cancer and chronic pancreatitis ($p = 7.9E -5$). There was no significant statistical difference in mean age between healthy volunteers and chronic pancreatitis

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patients (p = 0.218). There was no statistical significance between the frequency of

male and female patients amongst the three patient groups (p = 0.061, chi square test).

Table 5.2 Age and Gender of patients included in this study.

	Male	**Female**	**Mean Age (years); (Range)**
Healthy Volunteers	5 (28%)	13 (72%)	58.5 (40.3 – 82.7)
Pancreatic Cancer	10 (56%)	8 (44%)	69.6 (39.9 – 84.4)
Chronic Pancreatitis	13 (65%)	7 (35%)	51.1 (19.3 – 73.8)

5.2.3 Micro RNA Results

An ANOVA test with post hoc analysis of the miRNAs with normally distributed data

was done. A non-parametric (Kruskal Wallis test) was done for the miRNAs with non-

normally distributable data. The full list of miRNAs and their results are listed in

Table 5.3. It can be seen from the table that there are statistically significant differences

in means/medians between groups in miR–155a, miR–196a, miR–200c, miR–375 and

miR-503. Although both miR–10b and miR–34a had a p value of 0.0503 and 0.0504

respectively, there was a difference in medians of Δ Ct values between HV and PDAC

groups in miR–10b (p = 0.0331); and a difference in means of Δ Ct values between HV

and PDAC in miR–34a (p = 0.049).

The miRNAs where there is a significant statistical difference in mean/median values of

Δ Ct between HV and PDAC are miR–10b (p = 0.0331), miR–34a (p = 0.049), miR–155a

(p = 0.049) and miR–200c (p = 0.002) (Figure 5.1). The miRNAs where there is a

significant statistical difference in the mean/median Δ Ct values between HV and CP

groups are miR–155a (p = 0.001) and miR–375 (p = 0.001) (Figure 5.2). The miRNAs where there is a significant statistical difference in the mean/median Δ Ct values between PDAC and CP groups are miR–155a (p = 0.009) and miR–196a (p = 0.028). miR–503 is almost significant with a p value of 0.055 (Figure 5.3). miR–155a is the only miRNA where there is a significant statistical difference between all patient groups highlighting its potential as a biomarker (Figure 5.2).

Table 5.3 The mean/median of the Δ Ct values of the miRNAs and p value of the ANOVA/Kruskal Wallis test for Δ Ct values within groups.

Statistically significant results are highlighted in yellow. * denotes non-normally distributed data and where non-parametric tests were done.

miRNA	Patient type	Mean/median *Δ Ct	Standard Deviation/Interquartile Range for Median values*	(p value) between groups
miR–10b	Healthy Volunteer	12.0603	1.3899	
	Pancreatic Cancer	10.8550*	1.24*	0.053
	Chronic Pancreatitis	11.5708	0.7310	
miR–21s	Healthy Volunteer	4.7883	0.8218	
	Pancreatic Cancer	4.0583	1.0709	0.081
	Chronic Pancreatitis	4.4100	1.0085	
miR–34a	Healthy Volunteer	12.3867	0.8899	
	Pancreatic Cancer	11.5888	0.6346	0.054
	Chronic Pancreatitis	11.9480	1.1940	
miR-143	Healthy Volunteer	12.3150	1.3312	
	Pancreatic Cancer	11.9070	0.9673	0.641
	Chronic Pancreatitis	12.0381	1.2842	
	Healthy Volunteer	9.7419	1.3206	

miR–148a	Pancreatic Cancer	9.2881	1.60549	0.307
	Chronic Pancreatitis	9.0292	1.2853	
miR–155a	Healthy Volunteer	15.6919	2.3407	
	Pancreatic Cancer	14.2597	1.4398	0.001
	Chronic Pancreatitis	13.3225*	1.65*	
miR–196a	Healthy Volunteer	11.2938	0.8432	
	Pancreatic Cancer	11.8253	1.2234	0.033
	Chronic Pancreatitis	12.7024	0.6919	
miR–200c	Healthy Volunteer	11.1958	1.0107	
	Pancreatic Cancer	9.5969	1.7761	0.002
	Chronic Pancreatitis	10.4485	0.9645	
miR-206	Healthy Volunteer	12.4750*	4.20*	
	Pancreatic Cancer	11.8975*	5.07*	0.213
	Chronic Pancreatitis	12.3925*	4.58*	
miR-375	Healthy Volunteer	8.3542	0.7922	
	Pancreatic Cancer	7.3931	0.7141	0.001
	Chronic Pancreatitis	7.7195	0.6360	
miR-503	Healthy Volunteer	12.0856	0.9578	
	Pancreatic Cancer	11.4056	0.8914	0.035
	Chronic Pancreatitis	12.1471	0.9301	
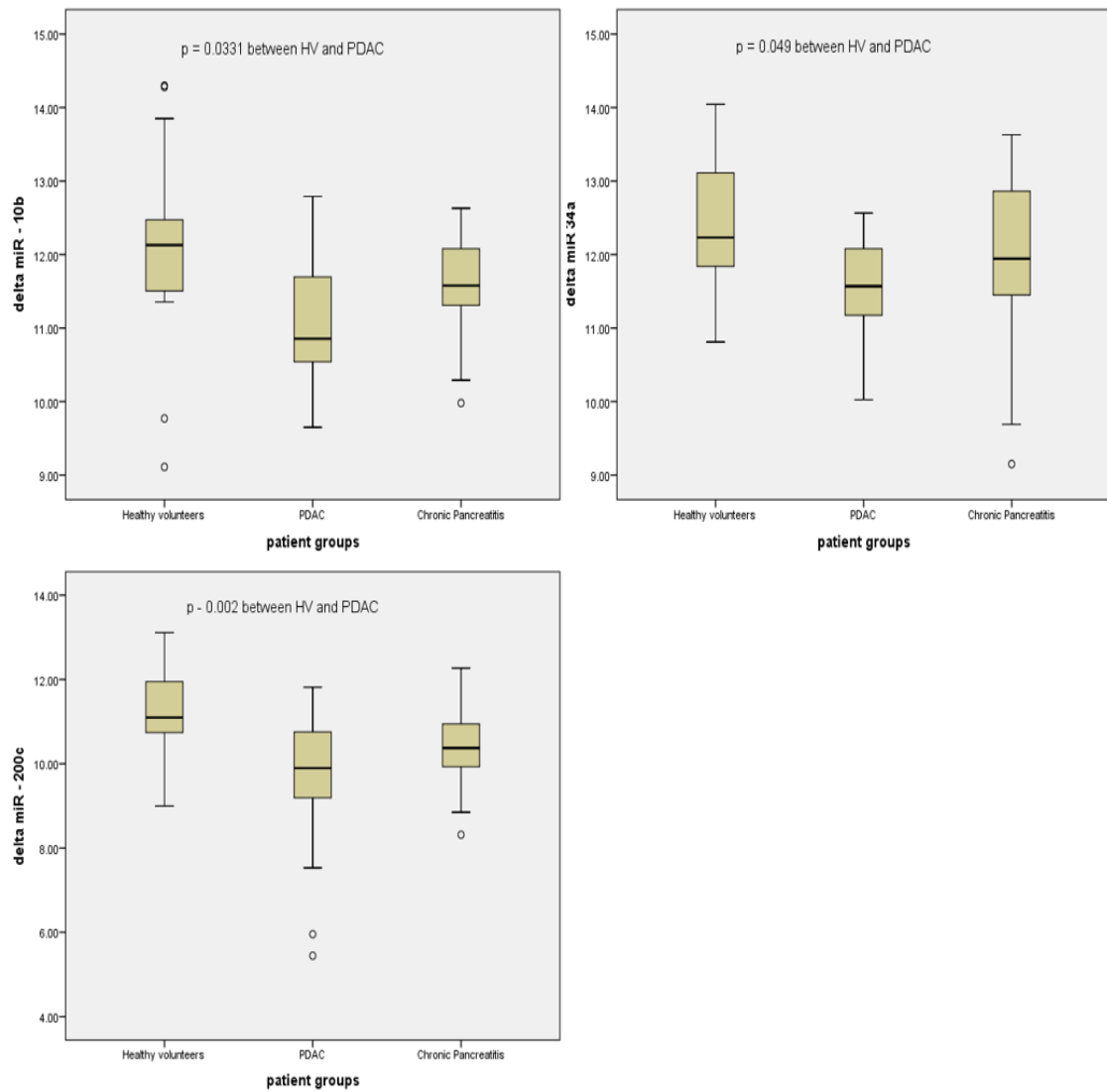
Figure 5.1 Box plot of Δ Ct of miR-10b, miR–34a, and miR–200c.

In these miRNAs, only the differences in mean/median between healthy volunteers (HV) and pancreatic cancer (PDAC) are statistically significant. In all of these miRNAs, the value of Δ Ct is higher for HV, meaning that the actual number of micro RNAs is actually lower in HV compared to PDAC.
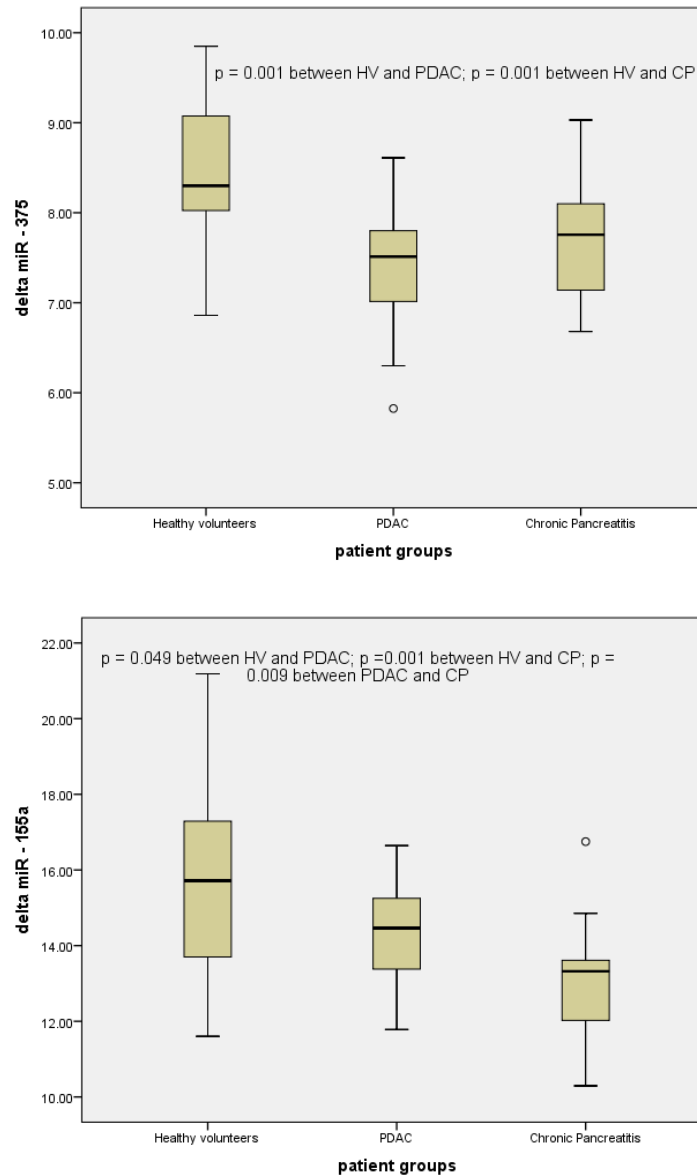
Figure 5.2 Box plot of Δ Ct of miR-375, miR–155a.

In miR-375 there is a statistically significant difference in Δ Ct values between HV and PDAC and between HV and CP (chronic pancreatitis). However, miR–155a is the only miRNA where there is a statistically significant difference in Δ Ct values between all three groups, indicating that this is potentially a suitable miRNA to differentiate chronic pancreatitis from PDAC and HV.
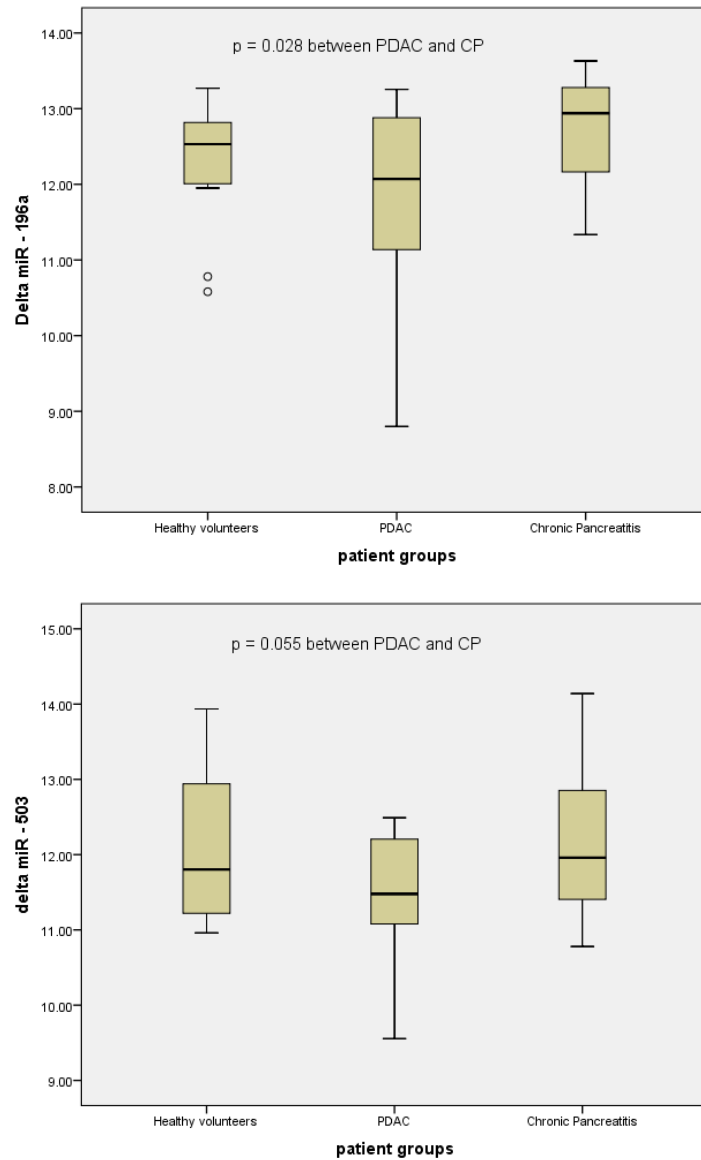
Figure 5.3 Box plot of Δ Ct of miR-196a, miR–503.

There is a statistically significant difference between PDAC and CP groups in miR-196a. miR–503 levels are almost significant. In both of these miRNAs, the Δ Ct value is lower for PDAC group indicating that the actual number of miRNA is higher in the PDAC group.

To further, explore the potential of the miRNAs as biomarkers to differentiate between PDAC and non-cancer, we entered all the miRNAs where there was a significant difference in the Δ Ct values between PDAC and healthy volunteers into a binary forward conditional logistic regression model. The miRNAs which were included were miR–10b, miR–34a, miR–155a, miR–196a, miR–200c, miR–375 and miR-503. The full model containing all the predictors was statistically significant, χ^2 (1, N = 42) = 9.284, p = 0.002, indicating that model could distinguish between pancreatic cancers and non-cancers. The model explained 27.2 % of the variance in disease status, and correctly classified 73.8% of the cases. This model has a sensitivity of 60%, a specificity of 92.6%, a positive predictive value of 81.8% and a negative predictive value of 80.6%. Only miR–200c made a unique statistically significant contribution to the model with an odd ratio of 0.451 (p = 0.016). This model therefore implies that for every 1 unit rise in Δ Ct value of miR–200c, the chances of having pancreatic cancer decreases by a factor of 0.451. A Receiver Operating Characteristics curve using the predicted probabilities form this model gave an area under the curve of 0.722 with a p value of 0.008 (Figure 5.4).

Since there is also a statistical difference in age between PDAC and healthy volunteers another binary logistic regression model was run with miR–200c and age (in years) as variables. This model was also statistically significant, χ^2 (2, N = 56) = 22.284, p = 1.4E -5, indicating that model could distinguish between pancreatic cancers and non-cancers. This model explains 45.9 % of the variance in disease status between PDAC and non-cancer. This model also correctly 75% of the cases with a sensitivity and specificity of 50% and 86.8% respectively. This model gives a positive predictive value of 64.2% and a negative predictive value of 78.6%.
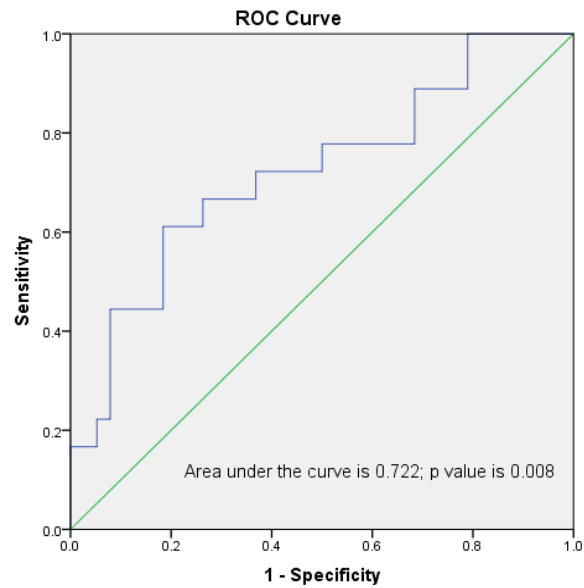
Figure 5.4 A ROC curve using the probabilities of the model where miR-200c was the only statistically significant variable and the binary outcome was PDAC vs non-cancer.

Please note that the area under the curve is 0.722 with a significant p value. This implies a fair test.

Figure 5.5 A ROC curve using the predicted probabilities of the model where age (in years) and miR-200c were the covariables and the binary outcome was PDAC vs non-cancer.

Please note that the area under the curve is 0.854 with a significant p value. This implies a good test.

Both age and miR–200c made statistically significant contributions to the model with odds ratio of 1.101 (p = 0.004) and 0.540 (p= 0.044). This model therefore implies that for every 1 year rise in age, the chances of having PDAC increases by a factor of 1.101 provided the miRNA values of miR–200c remains constant. However, if age is constant, for each one unit rise in Δ Ct of miR–200c odds of having PDAC decreases by a factor of 0.540. A Receiver Operating Characteristics curve using the predicted probabilities from this model gave an area under the curve of 0.854 with a p value of 2.2E -5 (Figure 5.5).

In order to identify a miRNA or a panel of miRNAs which would be effective in distinguishing HV from PDAC, a logistic regressions analysis was done including miR–10b, miR–34a, miR–155a, miR–200c and miR–375 as co-variables. This model was statistically significant, χ^2 (2, N = 32) = 16.727, p = 2.33E -4, indicating that model could distinguish between healthy volunteers and pancreatic ductal adenocarcinoma. The model explained 54.3 % of the variance in disease status, and correctly classified 71.9 % of the cases. This model has a sensitivity of 75%, a specificity of 68.8%, a positive predictive value of 70.6% and a negative predictive value of 73.3%. Both miR–200c and miR–375 made statistically significant contributions to the model (p = 0.027 and p = 0.046 respectively) with an odds ratio of 0.350 and 0.209 respectively. A ROC curve using the predicted probabilities from this model gave an area under the curve of 0.849 with a p value of 3.5E -4 (Figure 5.6).

Figure 5.6 A ROC curve using the predicted probabilities of the model where miR-200c and miR–375 made statistically significant contributions and the binary outcome was HV vs PDAC.

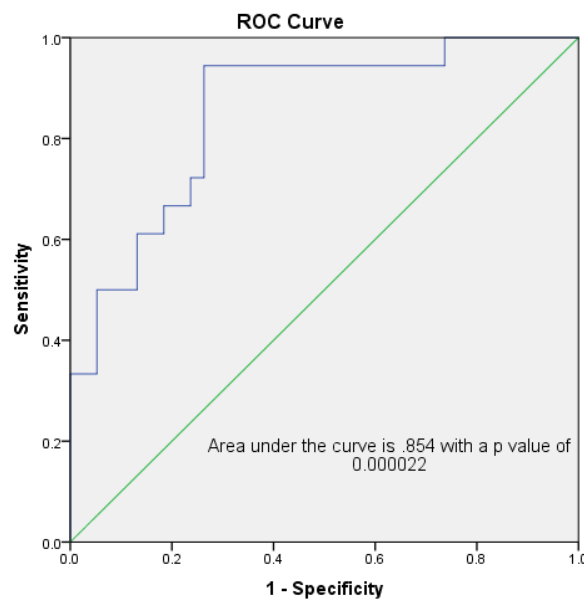Please note that the area under the curve is 0.849 with a significant p value. This implies a good test.

Once age (in years) is added into a new model containing the two significant variables from the previous model an even better fit of a model is achieved. This model is statistically significant, χ^2 (3, N = 36) = 24.383, p = 2.1E -5 and explains 65.6 % of the variance in disease status, and correctly classified 77.8 % of the cases. This model has a sensitivity of 83.3%, a specificity of 72.2%, a positive predictive value of 75% and a negative predictive value of 81.3%. However only miR – 375 made a statistically significant contribution to the model (p = 0.027) with an odds ratio of 0.131. Age (in years) almost makes a significant statistical contribution to the model with a p value of 0.054) and an odds ratio of 1.099. A ROC curve using the predicted probabilities form this model gave an area under the curve of 0.910 with a p value of 2.6E -5 (Figure 5.7).

Figure 5.7 A ROC curve using the predicted probabilities of the model where miR–375 made statistically significant contributions to the binary outcome of HV vs PDAC.

Please note that the area under the curve is 0.910 with a significant p value. This implies an excellent test.

A binary logistic regression analysis was done to identify the best miRNAs to differentiate between PDAC and CP. miR–155a, miR–196a and miR–503 were added as co-variables. The model was statistically significant, χ^2 (3, N = 33) = 18.138, p = 4.12E -4 and explains 56.5 % of the variance in disease status, and correctly classified 81.8 % of the cases. This model has a sensitivity and positive predictive value of 80%, a specificity and a negative predictive value of 83%. miR–503 and miR–155a made statistically significant contributions to the model (p = 0.030 and 0.021 respectively) with odds ratio of 0.208 and 3.176 respectively. A ROC curve using the predicted probabilities form this model gave an area under the curve of 0.881 with a p value of 1.96E -4 (Figure 5.8).
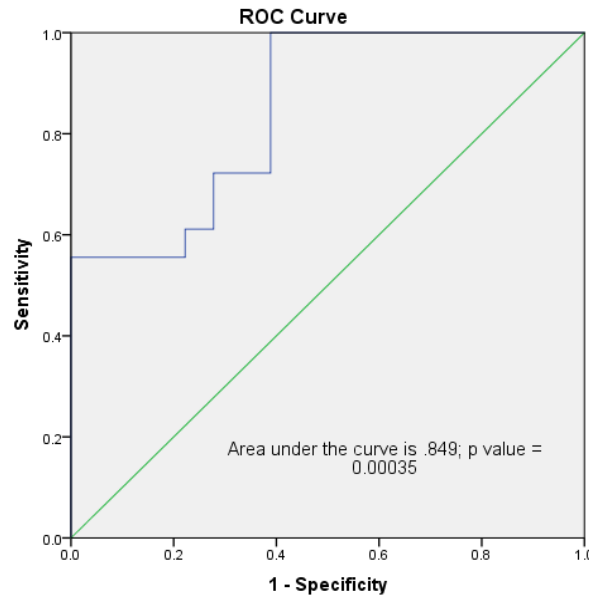
Figure 5.8 A ROC curve using the predicted probabilities of the model where miR-503 and miR–155a made statistically significant contributions and the binary outcome was PDAC vs chronic pancreatitis.

Please note that the area under the curve is 0.881 with a significant p value. This implies a good test.

Once age (in years) is added into a new model containing the two significant variables from the previous model an even better fit of a model is achieved. This model is statistically significant, χ^2 (2, N = 36) = 28.949, p = 5.1741E -7 and explains 73.7 % of the variance in disease status, and correctly classified 86.1 % of the cases. This model has a sensitivity of 88.2%, a specificity of 84.2%, a positive predictive value of 83.3% and a negative predictive value of 88.9%. However only age (in years) and miR–155a make statistically significant contributions to the model (p = 0.003 and p = 0.008 respectively). There was an odd ratio of 3.537 for miR–155a and an odds ratio of 1.262 for age in years. A ROC curve using the predicted probabilities form this model gave an area under the curve of 0.953 with a p value of 3E -6 (Figure 5.9).
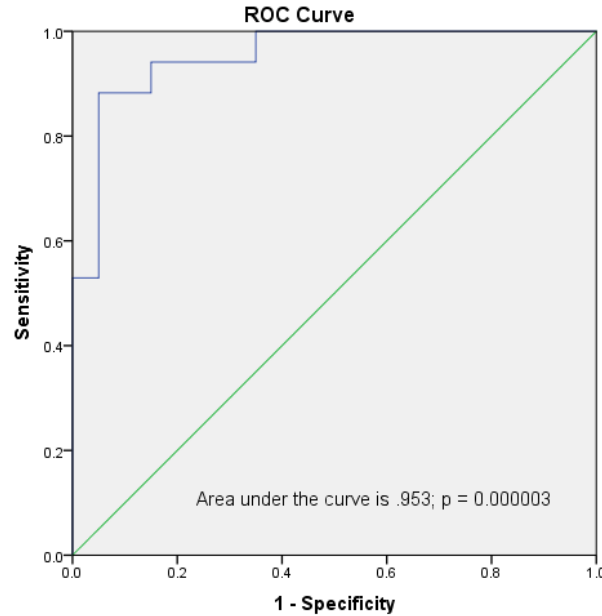
Figure 5.9 A ROC curve using the predicted probabilities of the model where age (in years) and miR – 155a made statistically significant contributions to the binary outcome of PDAC vs chronic pancreatitis.

Please note that the area under the curve is 0.953 with a significant p value. This implies an excellent test.

A final logistic regression analysis was done to find the best miRNA to differentiate between healthy volunteers and chronic pancreatitis patients and so miR-155a and miR–375 were included as co-variables. The model is statistically significant with a χ^2 (2, N = 38) = 20.091, p = 4.3E -5 and explains 54.8 % of the variance in disease status. This model has a sensitivity of 85%, a specificity of 77.8%, a positive predictive value of 81% and a negative predictive value of 82.4%. Only miR–155a makes a statistical significant contribution to the model (p = 0.007) with an odds ratio of 0.468. miR–375 almost makes a statistically significant contribution with a p value of 0.053 and an odds ratio of 0.321. A ROC curve using the predicted values of this model gives an area under the curve of 0.875 (p = 7.9E -5) (Figure 5.10). Since there was no statistical difference in age between healthy volunteers and patients with chronic pancreatitis, age

was not added to a logistic regression model to differentiate between healthy volunteers and chronic pancreatitis patients.
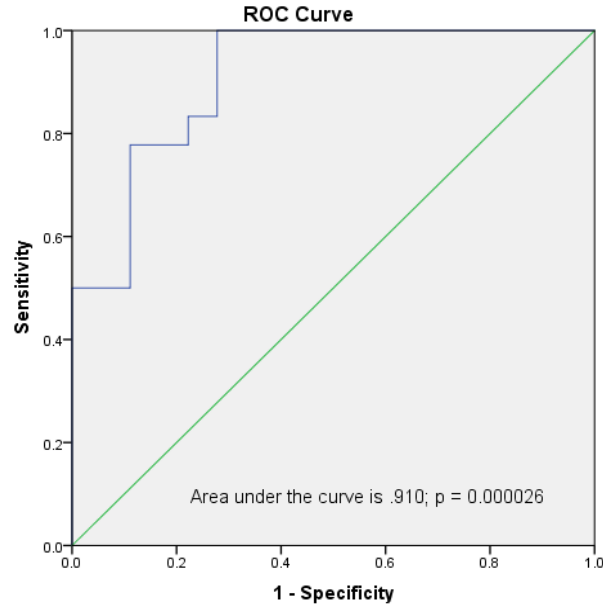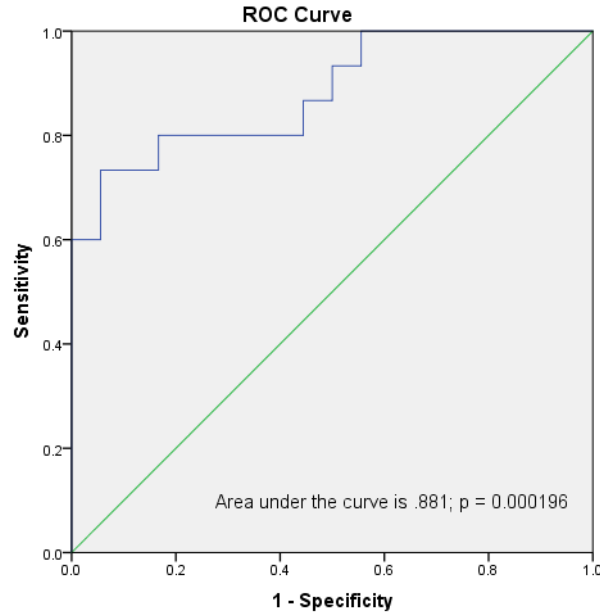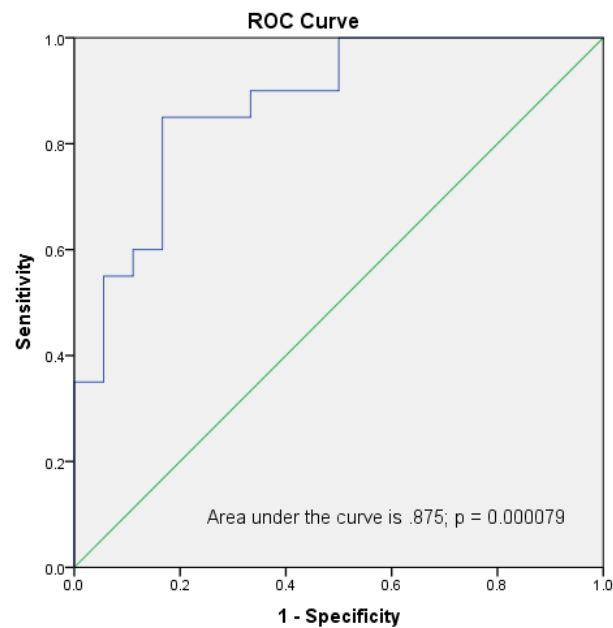

Figure 5.10 A ROC curve using the predicted probabilities of the model where miR–155a made statistically significant contributions to the binary outcome of healthy volunteers' vs chronic pancreatitis.

Please note that the area under the curve is 0.875 with a significant p value. This implies a good test.

5.3 Discussion

The purpose of this study was to examine the levels of certain miRNAs of interest in the plasma of patients with PDAC, chronic pancreatitis and healthy volunteers and to assess whether any of the chosen miRNAs had any potential to act as biomarkers. The miRNAs were chosen due to their previously established role in pancreatic carcinogenesis or carcinogenesis in general and also because some of the miRNAs were being researched in our laboratory in pancreatic cancer cell lines and showed upregulation compared to normal pancreas.

Our study was based on plasma samples which was collected in non-heparinised EDTA bottles and so did not require any additional treatment with heparin (Tiberio *et al*, 2015; Moldovan *et al*, 2014). The samples used were frozen rather than fresh as it has been shown to bear no significance (Tiberio *et al*, 2015). During the process of plasma preparation, visual analysis was made to ensure that the samples were not haemolysed in-vitro which would have otherwise impaired the quantification of plasma miRNAs.

The miRNeasy Mini kit (Qiagen) was used to extract the RNA from plasma as several studies have shown that this kit normally yields the greatest recovery of circulating RNA with regards to quantity and quality (Tiberio *et al*, 2015). There are several platforms available for micRNA profiling and the popular ones include Northern blotting, miRNA microarray technology, miRNA quantitative reverse transcription PCR (qRT-PCR) assays, next generation sequencing platforms and multiplex miRNA profiling (Hunt *et al*, 2015). Quantitative reverse transcription PCR (qRT-PCR) assays, is the amplification- based method used to detect and quantify the miRNAs of interest. This is because qRT-PCR is a well-established, highly sensitive method and is considered as the "gold standard" for miRNA detection and also because of our relatively low sample numbers and our choice of miRNA, this platform is the most cost effective (Tiberio *et al*, 2015).

There will always be technical variability amongst samples, due to issues such as variation in the starting material, amount of RNA extracted, and efficiency during the qRT-PCR process (Tiberio *et al*, 2015). There are several accepted methods in the literature on how to account for the variability and effectively normalise the data. Housekeeping transcripts have been suggested and miR–16 is probably the most

common housekeeping transcript which has been reported in the literature. However, it is also one of the miRNAs most affected by haemolysis and therefore should not be considered as a reference for data normalisation. There have been proposals of other miRNAs as candidates for housekeeping transcripts but without any global consensus (Tiberio *et al*, 2015). We followed the normalisation strategy suggested by Kroh *et al*. It involved the use of spiked-in synthetic *C. elegans* as control miRNAs during the first phase of the extraction which involved the denaturation of plasma (Kroh *et al*, 2010). This was done to normalise the biological variability that could otherwise could affect the extraction efficiency. It was not possible to extract miRNA from 60 plasma samples at the same time. It was spread out over several days, but equal number of random samples between the 3 groups (healthy volunteers, PDAC and chronic pancreatitis) were processed at the same time to reduce technical variability. Similarly, it was not possible to perform qRT-PCR on 60 samples for 11 miRNAs of interest at the same time on a 96 well plate. The qRT – PCR was carried out over a few days but again to reduce technical variability, equal number of random samples between the 3 groups were processed at the same time. We believe we have been successful in effectively minimising our technical variability as much as possible. This is reflected in our data. The Ct values of our synthetic spike-in miRNA miR-cel-238 follows a normal distribution. The Ct values of a miRNA of interest, miR-143a, also has a significant correlation with miR–cel–238 and also follows a normal distribution pattern. Furthermore, there was also no statistically significant difference between the mean Δ Ct of 143a between the 3 groups with an equal homogeneity of variances (p = 0.54). If needed, our data can also be normalised to miR–143a and this miRNA can act as an endogenous internal control. To normalise the data, the Δ Ct was calculated for every

miRNA. This was the Ct value for the miRNA of interest in that sample minus the Ct value of miR-cel–238 for that sample.

The Ct values are actually log-linear plot of the PCR signal versus the cycle number and has therefore already undergone a log transformation. It is therefore not necessary to use the $2^{-\Delta Ct}$ data and the Δ Ct would suffice (Schmittgen and Livak, 2008). We have presented our data as Δ Ct as we are interested in the relative gene expression and this reduces the variability of the data and normalises it (Livak and Schmittgen, 2001). Using Δ Ct also allows us to do much stronger statistical test such as T- test and ANOVA which yields $\Delta\Delta$ Ct (Yuan *et al*, 2006). Since we are using Δ Ct to present our data, it is important to remember than a decrease in Δ Ct suggests higher levels of the miRNA and an increase in Δ Ct suggest lower levels of the miRNA.

During statistical analysis, it was noticed that there was a lot of variability in data involving the healthy volunteer group. We now know that individual variability and external factors can all contribute to affect miRNA levels in the circulation. Although the exclusion criteria for healthy volunteers included anyone less than 18 years of age and also anyone who previously has been diagnosed with cancer, pancreatitis and Alzheimer disease (appendix 1), we do not know whether the individual had hypertension, diabetes or hypercholesterolemia or any other illnesses. We also did not consider the lifestyle of the healthy volunteers such as smoking status and food habits. These could have been contributing factors towards the variability of the healthy volunteers' samples and this is a weakness of this study.

At the time of the study (autumn 2011 to spring 2012), there were a few high-profile papers on the differential expression of miRNAs in tissue samples highlighting miRNAs which might be useful to differentiate between healthy controls, PDAC and chronic pancreatitis. (Szafranska *et al*, 2007; E. J. Lee *et al*, 2007; Bloomston *et al*, 2007) However, there were only two published papers which have looked at circulating miRNAs in blood for their usefulness as biomarkers in pancreatic cancer. Wang *et al* selected a panel of four miRNAs, miR–21, miR–210, miR–155 and miR–196a, which were reported to be over expressed in PDAC and reported a combined sensitivity of 64% and specificity of 89% with an area under the curve (for a ROC analysis) of 0.82%. This was detectable in plasma (J. Wang *et al*, 2009). Bauer *et al* adopted a slightly different approach and studied the variations of the miRNA levels in blood cells. They were able to distinguish between healthy and diseased pancreas from their blood analysis, but were unable to separate pancreatic inflammation from cancer. This was therefore not clinically useful (Bauer *et al*, 2012).

We have found that miR-200c is a significant variable in a binary logistics model of several micro RNAs when trying to differentiate between PDAC and non-cancer. This model gave us 60% sensitivity and 92.6% specificity with an area under the curve (ROC curve analysis) of 0.722, which indicates a fair test. Our result compared very well with Wang et al where they achieved sensitivity of 64% and a specificity of 89% and an area under the curve of 0.82 using a combination of miR–21, miR–210, miR–155 and miR–196a (J. Wang *et al*, 2009). Furthermore, when combining age with the miR-200c, we have managed to increase the area under the curve to 0.854, which indicates a good test. It is encouraging the odd ratio of miR-200c is 0.511 in this model which supported the theory that miR–200c was associated with a significantly higher

survival rates in PDAC and a strong positive correlation with E Cadherin expression

(Yu *et al*, 2010). MicroRNA–200c has also been shown to directly inhibit Jagged 1,

which is a ligand of Notch, resulting in stopping the proliferation of human metastatic

prostatic cancer cells(Vallejo, Caparros and Dominguez, 2011). Micro RNA–200c is

interesting since it has not been identified as a biomarker in any of the studies using

plasma, serum or whole blood (Diab *et al*, 2016). Li et al were able to distinguish

between PDAC and healthy control in serum using miR–200a and miR–200c as

markers achieving a sensitivity of 84.4% and a specificity of 87.5% for miR–200a and a

sensitivity of 71.1% and a specificity of 96.9% for miR -200b (A. Li *et al*, 2010).

We have also found that miR–375 is the significant variable when trying to distinguish

PDAC from healthy volunteers giving an odds ratio of 0.131. This suggests that miR–

375 is over-expressed in PDAC plasma compared to healthy volunteers. This is

interesting but not surprising. It has reported in the two major studies on miRNA

expression in pancreatic tissue that miR-375 is one of the few miRNA which is

downregulated in PDAC compared to healthy volunteers and chronic pancreatitis tissue

(Bloomston *et al*, 2007; Szafranska *et al*, 2007). However, Carlsen *et al* found increased

expression of miR–375 in plasma of PDAC patients as opposed to control. Carlsen *et al*

found that miR-375 did not improve detection rates nor predict prognosis in PDAC

patients when compared to CA 19-9 (Carlsen *et al*, 2013).

We were also able to differentiate between healthy volunteers and chronic pancreatitis

using a regression analysis model which identified miR–155a as the significant variable

with an odds ratio of 0.321 suggesting relatively higher levels of miR-155a in chronic

pancreatitis when compared to healthy volunteers. This is evident from our box plot in

figure 5.2 which shows that the highest levels of miRNA–155a is in the chronic pancreatitis group, followed by PDAC group and with the lowest levels in the healthy volunteers. This model has a sensitivity of 85%, a specificity of 77.8%, with an area under the curve (ROC curve analysis) of 0.875, which indicates a good test.

When comparing PDAC to chronic pancreatitis, we initially identified two significant micro RNAs which contributed significantly to a multiple binary logistic regression models and explained 56.5% of the variation in the outcome. This model gave a sensitivity of 80% and a specificity of 83% with an area under the curve (ROC curve analysis) of 0.881, which implies a good test. MicroRNA–155a gave an odds ratio of 3.176 which suggests that this miRNA is under-expressed in PDAC compared to chronic pancreatitis. This is somewhat different from the findings in other studies where miR–155a plasma levels were found to be elevated in pancreatic cancer compared to benign diseases (Cote *et al*, 2014; J. Wang *et al*, 2009). The odds ratio for miR–503 in this model is 0.208 which suggests that higher levels of miR–503 in PDAC compared to chronic pancreatitis. Both miR–503 and miR–155 have been known to have pro-differentiation roles in monocytes and miR–503 also has a role in the development of the endocrine function of the pancreas (Forrest *et al*, 2009; Lynn *et al*, 2007). Whilst little is known about miR–503's role in pancreatic cancer, miR–503 has been found to be involved in several cancers (H. Liu *et al*, 2015). It has shown to be up-regulated in parathyroid carcinoma, adrenocorticoid carcinoma and promotes tumour progression and acts as a prognostic biomarker for oesophageal cancer. It also acts as a tumour suppressor in gastric cancer, endometrial cancer and non-small cell lung cancer (H. Liu *et al*, 2015).

Our most impressive results came when we added age to the logistic regression analysis when comparing PDAC to chronic pancreatitis. This model explained 73.7% variation in the outcome with a sensitivity of 88.2% and a specificity of 84.2% and an area under the curve (ROC curve analysis) of an impressive 0.953, which is deemed an excellent test. The micro RNA which significantly contributed to this model is miR-155a. Since our study, there has been several pilot studies reported in the literature identifying micro RNAs as possible biomarkers (Hernandez and Lucas, 2016; Diab *et al*, 2016). However, the only study which has managed to achieve results similar to us when differentiating PDAC and chronic pancreatitis is Liu et al, who combined CA 19-9 with miR–16 and miR–196a, thereby achieving a sensitivity of 88.4% and a specificity of 96.3%. (J. Liu *et al*, 2012).

Our study suggests that miR–200c. miR-375 and especially miR–155a have significant roles to play as potential biomarkers for PDAC. Several studies have identified several circulating micro RNA as potential biomarkers, however there are limitation such as standardisation of extraction, reproducibility of results and small sample size (Hernandez and Lucas, 2016) . More understanding is required of the biological process of these miRNAs in PDAC. Further understanding is also required of the various ways miRNAs travel in blood and the external and internal factors which could affect their circulating levels before widespread application as biomarkers. However, despite these limitations miRNA expression has also been successfully analysed in pancreatic juices, saliva and stools which are very encouraging (Hernandez and Lucas, 2016).

CHAPTER 6.

DISCUSSION

Pancreatic cancer is one of the more lethal tumours presented at oncology clinics. Late presentation is a key factor in this lethal manifestation. The late presentation is mainly due to vague symptoms during the early stages and lack of suitable biomarkers. It is one of the most aggressive tumour and not very sensitive to current chemotherapeutic treatment agent gemcitabine (Cancer Research UK, 2016). Current biomarker CA 19.9 is neither very specific nor very sensitive and is also elevated in benign diseases (Wu *et al*, 2013). This creates a problem when the clinician is faced with a pancreatic mass and has to differentiate between PDAC or chronic pancreatitis without having to resort to an invasive open biopsy. The molecular mechanisms that underpin the development of pancreatic tumours are still far from understood. In this thesis, we have attempted to explore a couple of mechanisms namely Notch (and more precisely NOTCH 1 and 2) and miRNAs that could provide key clinical information in regard to the disease and progress of the disease, in the hope that these would help us develop novel biomarkers.

We have firstly developed a technique of detecting Notch Nβ-fragments in the plasma of human at concentrations of 121.67 fmol/µL. Notch Nβ-fragments are part of the Notch juxtamembranous receptor which is released between the two cleavage sites S3 and S4 as part of γ-secretase mediated cleavages following ligand activation. The Notch pathway has been implicated in several solid and haematological malignancies in both an oncogenic and a tumour suppressive capacity, as well as in pancreatic adenocarcinoma (H. Liu *et al*, 2017; Cook *et al*, 2012). Previous work in our lab by a PhD student has showed that that Notch 1, 3 and 4 receptors and its down-stream targets Hes 1 and Hey 1 were upregulated in pancreatic ductal carcinoma and further upregulated in non-resectable locally advanced pancreatic cancer and metastatic disease (Mann, 2012). Furthermore, there has also been several studies implicating Notch 1 in

pancreatic carcinogenesis (Hu *et al*, 2013). This led us to examine the hypothesis that Notch 1 Nβ-fragments might be detectable in the plasma of PDAC patients, especially after Okochi *et al* first identified these Notch Nβ-fragments in murine cell lines (Okochi *et al*, 2006). Although we were not able to detect Notch 1 in the plasma of patients with PDAC and healthy plasmas, we were able to prove that our method of immunoprecipitation followed by detection of signals on mass spectrometry works. Our results could also suggest that Notch 1 does not play a significant role in the progression of PDAC as initially thought. There have been some papers which have suggested that it is Notch 2, not Notch 1, which is required for the progression of PanIN and is associated with a worse prognosis (Mazur *et al*, 2010). In our study, we were able to detect Notch 3 Nβ-fragments in the plasma of PDAC patients but were also able to detect it in the plasma of healthy volunteers. This provides a proof of principle that our method works, but also raises questions about the role of Notch 3 in pancreatic carcinogenesis. We did not find a statistically significant difference in the signal levels of Notch 3 Nβ-fragments between the groups, suggesting that Notch 3 activation is something which is encountered on a daily basis in normal healthy people. Our findings were supported by reports of Notch 3 having a role in maintaining vascular smooth muscle cells and response to vascular injury (T. Wang *et al*, 2008). This probably explains why Notch 3 is highly upregulated and therefore is found in abundance in healthy people. Our study was a qualitative study and was designed in a way to check for the presence of Notch Nβ-fragments and therefore our methods are not the best for accurate quantification of Notch Nβ-fragments between groups. An alternative would be to examine the levels of Notch 3 between patient groups using liquid chromatography mass spectrometry selected reaction monitoring (LC-MS-SRM) assay for the specific, sensitive and selective determination of Notch fragments. Another

alternative would be to concentrate future efforts on Notch 1 as there are not any reports of Notch 1 being involved in any wide spread system in a developed adult, as opposed to Notch 3 which is involved in maintaining the vascular endothelial cells and is therefore present in abundance in both healthy people and those with cancer. This way, if Notch 1 Nβ- peptide fragment is detected, it is most likely due to the disease. One way of doing that would be to develop an enzyme-linked immunosorbent assay (ELISA) for Notch 1 Nβ peptide fragment. Although ELISA is the gold standard for detecting potential protein biomarkers and has extraordinary sensitivity and specificity for quantifying the target analyte, it comes with its own specific drawbacks. Its development is extremely costly and time consuming with a high failure rate. This therefore leads to reduced validations of potential diagnostic biomarkers (Whiteaker *et al*, 2007).

Our next section of work concentrated on the immunohistochemical expression of Notch 2 receptors in healthy tissue, resectable pancreatic cancer, non-resectable advanced disease and metastatic disease. We found Notch 2 expression to be upregulated in resectable PDAC and further upregulated in non-resectable locally advanced cancer, but down regulated in metastatic PDAC tissue in comparison to non-resectable locally advanced tissue. This data is interesting as this is the only Notch receptor which has behaved as such. Previous findings in our group has found that the expression of Notch receptors in the rest of the family has only gone up with increasing advancing disease (Mann, 2012). We have also found that Notch 2 has no effects on long term survival or disease-free survival. Some parts of our data are supported by a study, where Notch downregulation in mice with radiologically proven cancers has not resulted in increased survival suggesting that although Notch might play a role in initial

carcinogenesis, it has a reduced role in the maintenance of advanced PDAC disease (Cook *et al*, 2012). It was long thought that Notch 2 was involved in the process of Epithelial to Mesenchymal transition (EMT) which makes PDAC cells resistant to Gemcitabine and therefore a more aggressive tumour. More recently, it has been suggested that Notch 2 also generates cancer stem cells which is thought to contribute to chemoresistance and metastasis (Z. Wang *et al*, 2011). Despite several papers on Notch 2 expression and its effects in cell lines, human tissue and mice tissue being available, our study is probably the first to explore the expression of Notch 2 in differing stages of the disease and correlate it with clinicopathological factors and compare the effect of Notch 2 in patient survival following curative resection for PDAC.

There are a few limitations with our work on immunohistochemistry. Firstly, the number of resectable patients used in this study is small. However, because of the aggressive nature of the disease it is difficult to get large patient numbers in a single centre and the number of patients in our study are comparable if not larger than most of the other studies reported. To get more number, would necessitate a multi-centre study and more correlations would probably be observed. There are also the known concerns about antigen degradation in formalin-fixed, paraffin-embedded tissue (Xie *et al*, 2011; Vis *et al*, 2000). This is unavoidable in this type of research, but steps taken to reduce this included only using specimens collected after 2000. Lastly one of the problems with investigating Notch 2 is lack of availability of a good quality commercially available Notch 2 antibody. We have found it a struggle to find an antibody which worked well with both immunohistochemistry and western blotting and the same problems have been reported by other researchers (Hu *et al*, 2013). This probably

explains the lack of good quality papers which has investigated the immunohistochemical expression of Notch 2 in human tissue. Going forward, we suggest developing a validated antibody against Notch 2 and examining its expression in cell lines, including metastatic ones and exploring cell viability following specific knockdowns on Notch 2 in these cells lines to explore the significance of Notch 2 in advanced and metastatic PDAC.

Our final section of work concentrated on quantifying some miRNAs in the plasma of PDAC and chronic pancreatitis (CP) and comparing it to healthy volunteers to find a miRNA or a panel of miRNA which might act as potential biomarkers. There has been a lot of work on miRNAs recently and a large body of literature has emerged documenting the significance of miRNAs in tumour progression. miRNAs are thought to play a role in cancer is that because they play a critical role in the biological processes of cell proliferation, differentiation, apoptosis and stress resistance (Z. Wang *et al*, 2010). Some miRNAs are thought to have an oncogenic role and so are elevated in cancers whilst others are thought to have a tumour suppressive effect. It has been found that several miRNAs play critical roles in Notch signalling pathway. Although the exact nature of their roles is unclear, the effect is more obvious. miR–34 is meant to regulate Notch 1 and Notch 2 in pancreatic cancer and therefore acts as a tumour suppressor. It was also found that there is loss of miR-34 in PDAC cancer stem cells, but high levels of Notch 1 and 2 suggesting that miR-34 is involved in cancer stem cells self-renewal. Similarly, the miR–200 family has also been shown to regulate the Notch signalling pathway in pancreatic cancer and in PDAC stem cells. miR-21 is a well-known oncogene and is also found to upregulated with over-expression of Notch 1.(Ma *et al*, 2013) There are some other miRNAs which cross talk with Notch signalling

pathway but they have not been shown to play a role in pancreatic cancer. Keeping the relation between Notch and miRNAs in mind, we tested a panel of miRNAs and explored their potential as biomarkers. We have managed to identify miR-200c, miR–155a and miR–375 which could help differentiate between healthy volunteers, PDAC and chronic pancreatitis. Furthermore, when patient's age was added to the equation, we had some very impressive results with ROC curves of above 0.95.

It is also encouraging that we found miR-200c as a potential biomarker, as this is a part of the miR-200 family which has a tumour suppressive effect on EMT via the Notch signalling pathway (Zaravinos, 2015). Curcumin induces apoptosis in pancreatic cancer through inactivation of the Notch signalling pathway, but more recently has also been shown to upregulate miR–200 and downregulate miR-21 leading to increased gemcitabine sensitivity (Z. Wang *et al*, 2011). This points at potential therapeutic option for these tumour suppressive miRNAs.

The limitation of our miRNA work is mainly due to the sample numbers. This was a proof of existence study done with some remaining samples to look at the relationship of some miRNAs and PDAC and chronic pancreatitis. Reverse transcriptase RT-PCR is expensive. We were therefore limited to just 60 samples and only eleven miRNAs of choice. However, the results of our study are very encouraging and justifies further work on larger sample numbers and with some more miRNAs.

In summary, this thesis demonstrates that Notch 2 signalling plays a very complex role in pancreatic carcinogenesis but does not correlate or behave like the rest of the Notch family. Future work needs to concentrate on the role of Notch 2 signalling in pancreatic

carcinogenesis before using the qualitative method developed in this thesis to try and detect Notch 2 Nβ-fragments in plasma as a potential biomarker. This thesis has also identified 3 miRNAs which could play a role as biomarkers in differentiating PDAC, CP and health volunteers.

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APPENDIX

Ethics approval for this project

University Hospitals of Leicester **NHS**

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NHS Trust

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24 February 2004

Ms Helena Doucas
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Dear Ms Doucas

RE: **UHL Ref. 9170**   *[Please quote this number in all correspondence]*
Cell signalling in gastrointestinal tumours.   Ethics Ref. 7176

We have now been notified by the Ethics Committee that this project has been given a favourable opinion by the Ethics Committee (please see the attached letter from the Ethics Committee).

Since all other aspects of your UHL R+D notification are complete, I now have pleasure in confirming full approval of the project on behalf of University Hospitals of Leicester NHS Trust, Leicester General Hospital Site.

This approval means that you are fully authorised to proceed with the project, using all the resources which you have declared in your notification form.

The project is also now covered by Trust Indemnity, except for those aspects already covered by external indemnity (e.g. ABPI in the case of most drug studies).

We will be requesting annual and final reports on the progress of this project, both on behalf of the Trust and on behalf of the Ethical Committee.

In the meantime, in order to keep our records up to date, could you please notify the Research Office if there are any significant changes to the start or end dates, protocol, funding or costs of the project.

I look forward to the opportunity of reading the published results of your study in due course.

Yours sincerely

Mr Michael Roberts
Service Manager for Research and Development