Proteomics of the Effect of Gemcitabine with Intravenous Omega-3 Fish Oil Infusion in Patients with Unresectable Pancreatic Adenocarcinoma

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By

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Abstract:

Title: Proteomics of the Effect of Gemcitabine with Intravenous Omega-3 Fish Oil Infusion in Patients with Unresectable Pancreatic Adenocarcinoma

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Background: Pancreatic cancer carries the poorest prognosis of all solid organ tumours. Administration of intravenous omega-3 fatty acid (n-3FA) in advanced pancreatic adenocarcinoma patients receiving gemcitabine chemotherapy in our institute shows disease stabilisation and improved progression free survival. Uncovering the underlying biological mechanisms that are responsible for these clinical effects will be investigated using high definition plasma proteomics.

Methods: Plasma from patients with histologically confirmed un-resectable pancreatic adenocarcinoma, collected at baseline before treatment (Baseline group), after one-month treatment with intravenous gemcitabine and n-3FA (Treatment group) and intravenous gemcitabine only (Control group). Plasma was 99% immuno-depleted using Seppro IgY14 + Supermix columns, reduced, alkylated and tryptically digested. Two arms studies comparing Baseline vs Treatment groups and Treatment vs Control groups performed. Samples labelled with TMT-6plex, with a Quality Control sample comprising pooled samples. Combined TMT-labelled samples underwent high-pH reversed-phase fractionation. Fractions were injected into a QExactive-Orbitrap LC-MS/MS in triplicate and analysed on Proteome Discoverer 2.1 and Scaffold 4.7. Bioinformatic analysis was performed on Protein Centre for Gene Ontology Biological Process (GO-BP) enrichment analysis ($p < 0.05$, Bonferroni corrected), Cytoscape for visualisation and KEGG pathway analysis. Selective Reaction Monitoring (SRM) analysis for verification were performed on selected peptides.

Results: 3476 proteins identified. 125 proteins were significant markers of pancreatic cancer, including REG1A, LVVE and TFF. Anti-inflammatory markers (CRP, Haptoglobin and Serum Amyloid-A1) were reduced in the treatment group confirming the anti-inflammatory effects of n-3FA. GO-BP enrichment analysis showed angiogenesis downregulation, complement immune systems upregulation and epigenetic modifications on histones. KEGG pathway analysis identified direct action via the Pi3K-AKT pathway, with decreased HSP90 and increased inhibitory protein 14-3-3. Serum Amyloid-A1 significantly reduced ($p<0.01$) as a potential biomarker of efficacy for n-3FA.

Conclusion: Administration of n-3FA and gemcitabine has anti-inflammatory, anti-angiogenic and pro-apoptotic effect via direct mechanism on cancer signalling pathways in patients with advanced pancreatic adenocarcinoma.

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List of Abbreviations

n-3 Fatty Acid, ω-3 Fatty Acid – Omega-3 Fatty Acid

PDAC – Pancreatic ductal adenocarcinoma

APC – Advanced pancreatic cancer

EPA – Eicosapentaenoic acid

DHA – Docosahexaenoic acid

EFA – Essential fatty acids

MS – Mass Spectrometer

LC – Liquid Chromatography

ESI – Electro Spray Ionisation

m/z – Mass to charge ratio

UPLC – Ultra Performance Liquid Chromatography

HPLC – High Performance Liquid Chromatography

CID - Collision Induced Dissociation

DIA – Data Independent Acquisition

DDA – Data Dependent Acquisition

QE – QExactive-Orbitrap (Thermo Fisher Scientific, Waltham USA)

G2Si – SYNAPT-G2 Si HDMS (Waters, Milford USA)

TQ – XEVO MS Triple Quadrupole (Waters, Milford USA)

TMT – Tandem Mass Tag

SRM – Selective Reaction Monitoring

DNA – Deoxyribonucleic Acid

RNA – Ribonucleic acid

DTT – DL-Dithiothreitol

IAA – Iodoacetamide

Alcohol Dehydrogenase – ADH

hpRP-HPLC – High pH reverse-phase chromatography

PD – Protein Discoverer

FDR – False Discovery Rate

LRA – Lipid Removal Agent

PLGS – ProteinLynx Global SERVER

GO – Gene Ontology

GO-MF – Gene Ontology Molecular Function

GO-CC – Gene Ontology Cellular Component

GO-BP – Gene Ontology Biological Processes

TEAB – Triethyl Ammonium Bicarbonate

ECOG – Eastern Cooperative Oncology Group Performance Status

TNF – Tumour necrosis factor

TGF-β – Transforming growth factor Beta

VEGF – Vascular endothelial growth factor

CRP – C Reactive Protein

HSP – Heat Shock Protein

SAA – Serum Amyloid A

FA – Formic Acid

TFA - Trifluoroacetic Acid

1 Introduction

Pancreatic cancer is the fourth and fifth most common cancer in men and women respectively with an annual incidence of 8 and 13 per 100,000 [1,2]. In England, pancreatic cancer has the worst prognosis of all cancers, with a median survival of 12 weeks and a 5-year survival in the order of 2-3% [3]. The poor prognosis for pancreatic cancer is mainly due to late diagnosis of the disease, with the majority of patients presenting with locally advanced disease or distant metastases. Only 10% of patients with pancreatic cancers are suitable for surgical resection which remains the only curative option [4]. Therefore, identifying potential alternative treatment options and understanding the complex mechanism in pancreatic cancer to improve patient outcomes is a key goal in the management of this disease.

1.1 Pancreatic Cancer

Pancreatic cancer is responsible for an estimated 227 000 deaths per year worldwide [1,2]. Risk factors for pancreatic cancer include: smoking, a family history of chronic pancreatitis, advancing age, diabetes mellitus and male sex [5]. However, despite advances in cancer management and survival for other cancers, the outcome for pancreatic cancer has hardly changed since 1970. Histologically, 85% of pancreatic cancers are ductal adenocarcinoma (PDAC) with the rest comprising of rarer endocrine tumours [6].

Various imaging techniques exist for detection of pancreatic cancers. Ultrasound would be suitable as a non-invasive screening tool, however exhibits a low specificity and sensitivity in detecting smaller lesions. Computed Tomography, Magnetic Resonance Imaging and Endoscopic Retrograde Cholangiopancreatography are able to detect early stage disease, but are too invasive to be utilised as a screening tool. Ideally, serum-based cancer biomarkers would be a preferred screening strategy. Currently, the carbohydrate antigen (CA) 19.9 is used as a serum based biomarker for PDAC, with a sensitivity of 70% to 90% and specificity of a 70% to 98%, depending on tumour size. However, a Japanese cancer registry reported only 48.4% of patients with pancreatic

cancers of less than 2 cm showed increased levels [7]. Furthermore, 10% of patients are carriers of the Lewis-negative genotype and do not express CA 19.9 [8] . Ca 19.9 levels are also raised in other malignancies and benign conditions such as pancreatitis and biliary obstruction [9]. Blood based investigations are ideal as they are minimally invasive, easily accessible and do not present any radiation risks.

The progression of pancreatic cancer involves multiple steps and factors, some are avoidable, for example exposure to carcinogens (smoking, alcohol) and some are unavoidable, for example a familial genetic predisposition or mutation. Hanahan and Weinberg, proposed six hallmarks of cancers to describe a logical framework of cancer progression from normal cells to a neoplastic state [10,11]. These six hallmarks are: Sustaining proliferative signalling, Evading growth suppressors, Activating invasion and metastasis, Enabling replicative immortality, Inducing angiogenesis and Resisting cell death. PDAC exhibits features in keeping with these hallmarks through various pathways. K-*ras* mutation of codon 12 is commonly found in PDAC, which in turn leads to uncontrolled cell proliferation [12]. Mutation of the tumour suppressor gene *TP53* occurs relatively late in the neoplastic process and is found in approximately 70% of invasive PDAC, as the cells avoids growth suppression and undergoes uncontrolled proliferation and invasion [13]. A recent comprehensive integrated genomic analysis of PDAC summarised various genetic mutations aggregate into 10 common pathways: KRAS, TGF-β, WNT, NOTCH, ROBO/SLIT signalling, G1/S transition, SWI-SNF, Chromatin modification, DNA repair and RNA processing [14]. Understanding these pathways help in elucidating the path in which normal pancreatic cells progresses to cancerous adenocarcinomas, which in turn provides potential avenues to treat this debilitating disease.

1.2 Treatment of Pancreatic Adenocarcinoma

1.2.1 Clinical Overview of the Pancreas and Adenocarcinoma

The pancreas gland anatomically is a retro-peritoneal structure which is in close to relation to other important vascular, biliary and intestinal organs. The head of the pancreas is adjacent to the second part of the duodenum, the common bile duct, the

superior mesenteric artery (which supplies arterial oxygenated blood to the small intestine and proximal two thirds of the colon) and the portal vein (which is the confluence of the superior mesenteric vein and splenic vein, which drains the intestine of nutrient rich blood and supplies three quarters of blood to the liver). The two main functions of the pancreas denotes the types of cells within the pancreas: the endocrine function is primarily derived from the islet cells of the pancreas (which commonly consist of alpha and beta cells), whilst the exocrine function of the pancreas delivers pancreatic enzymes (for example amylase and trypsin) and the ductal cells secrete the bicarbonate rich fluid from the pancreas. These ductal cells are of glandular epithelial origin, which give rise to the pancreatic ductal adenocarcinoma [15].

Pancreatic adenocarcinoma typically presents insidiously, with patients complaining of vague symptoms of non-specific pain or weight loss. The only cure for pancreatic adenocarcinoma is surgical resection of the tumour, however only 10% of patients present with disease that can be surgically removed [5]. These are normally identified incidentally usually by CT scans arranged for different reasons. If identified early, the classic operation of a Whipple's Procedure (for head of pancreas tumours) offers a cure, however this is a major surgical undertaking involving removal of the pancreas, with the duodenum and bile ducts, whilst preserving the closely related superior mesenteric artery and portal vein (which is a high risk of bleeding) hence this operation is typically reserved for physiologically fit individuals [16]. Even with treatment, the overall 5 year survival following curative surgery for pancreatic adenocarcinoma remains bleak at 4% [5].

The remaining majority of patients present later in the disease with specific symptoms such as obstructive jaundice or difficulty in eating, during which usually the cancer is either locally advanced (usually affecting the head of the pancreas with involvement of the superior mesenteric artery or portal vein – which indicates irresectability due to the importance of these structures) or if it has metastasised to other organs, usually the liver or lungs. The remaining options for treatment at this stage is palliative chemotherapy to prolong survival [5].

1.2.2 Palliative Chemotherapy with Gemcitabine for Pancreatic Adenocarcinoma

The European Study Group for Pancreatic Cancer (ESPAC) is a multi-centre international European group led by J Neoptolemos which first met in 1994 and is credited with determining the current regime for chemotherapy in pancreatic cancer patients [17]. The details of the various ESPAC trials are beyond the scope of this thesis. However, the ESPAC-1 trial identified that single agent chemotherapy with gemcitabine was superior to combination chemoradiotherapy in pancreatic adenocarcinoma [18]. Further work since then has identified the use of combination chemotherapy, for example FOLFIRINOX (combination of oxiliplatin, irinotecan, leucovorin and fluorouracil) was found to be superior to gemcitabine alone. However, this was associated with significant side effects, complications and degradation in quality of life [19]. The addition of nab-paclitexal (a mitotic inhibitor, preventing breakdown of microtubules) with gemcitabine was associated with increased survival compared to gemcitabine monotherapy, however again was associated with significant side effects and higher costs which subsequently led to its withdrawal by NICE [20].

As such, currently the most commonly used palliative chemotherapy agents for patients with locally advanced non-resectable pancreatic adenocarcinoma is single agent chemotherapy with gemcitabine, due to its lower side effect profile compared to combination chemotherapy agents. Use of single agent gemcitabine for 6 months versus observation alone in resected pancreatic adenocarcinoma increased overall survival (22.8 versus 20.2 months) and disease free survival (13.4 vs 6.7 months) [21]. This effect though noted in resected pancreatic adenocarcinoma, forms the basis to which gemcitabine is also used in non-resectable pancreatic adenocarcinoma.

Gemcitabine is a pyrimidine (deoxycytidine) analogue with multiple modes of action inside the cell. Once it enters the cell cytoplasm, gemcitabine is phosphorylated to the active triphosphate form of gemcitabine diphosphate, by the enzyme deoxycytidine kinase [22]. The most important mechanism of action of gemcitabine is inhibition of DNA synthesis. It incorporates itself into DNA during synthesis, resulting in a masked chain

termination and prevents DNA polymerases from further chain elongation [23]. This prevents further DNA synthesis and apoptosis of the cells. This effect is illustrated in Figure 1.1.

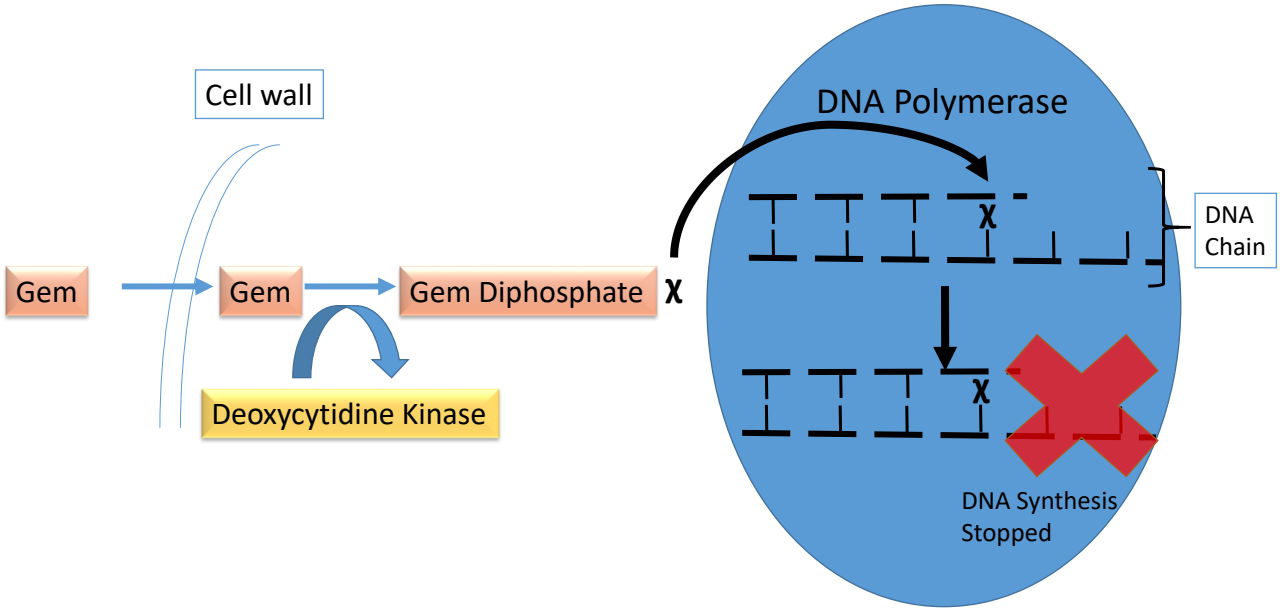


Figure 1.1: Diagram illustrating effect of gemcitabine (Gem), which enters the cell wall, is phosphorylated by deoxycytidine kinase to gemcitabine diphosphate which enters the cell nucleus and stops DNA synthesis and proliferation resulting in cell death.

1.3 Effect of Fish Oil Supplementation on the Pancreas

Fatty acids are compounds comprising hydrocarbon chains containing a carboxyl group at one end. Unsaturated fatty acids contain carbons connected by double bonds, with the higher the number of double bonds resulting in a greater degree of unsaturation. Omega-3 (n-3) fatty acids are a group of fatty acids with the first double bond three carbons from the methyl end, whilst n-6 fatty acids have the first double bond six carbons from the methyl end. The human body is unable to desaturate the n-3 or n-6 bond, hence both are termed essential fatty acids (EFA) and must be consumed from dietary sources [24]. The two main types of n-3 fatty acids are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Figure 1.2); both are found in cold water, fatty fish. The consumption of foods rich in these n-3 fatty acids is advocated by public

health organisations worldwide. Extensive research of the potential use of n-3 fatty acids in augmenting pancreas cancer therapy has focussed on optimising its clinical use and revealing the mechanisms by which n-3 fatty acids mediates its effect [25].

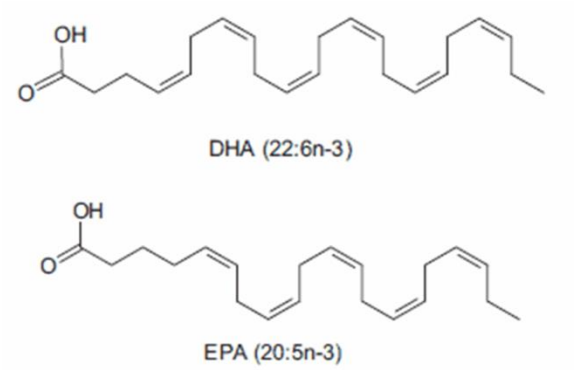


Figure 1.2: Structure of DHA and EPA. Note the first double bond three carbons from the methyl end.

1.3.1 Fish Oils in Pancreatic Cancers: Pre-clinical studies

The beneficial effect of n-3 fatty acids for the treatment of advanced pancreatic cancers has been previously reviewed [4]. In human pancreatic adenocarcinoma cell lines, EPA and DHA have been shown to inhibit growth in a dose dependent manner [26-28]. They also induce apoptosis in a dose dependent manner [27,29-32] and inhibit proliferation in gemcitabine-resistant cell lines irrespective of the level of gemcitabine resistance [33]. Postulated models for the therapeutic effect include induction of apoptosis in cancer cell lines via alteration at the cell-cycle phase [32,34], intracellular glutathione depletion [30] and inhibition of nuclear factor-kB (NF- kB) [24].

Angiogenesis is the growth of new blood vessels which must occur for cancers to grow. N-3 fatty acids suppress vascular endothelial growth factor-stimulated cell proliferation, migration and tube formation during angiogenesis [35-37]. They have also been shown to reduce levels of platelet derived growth factors from vascular endothelial cells and inhibit vascular smooth muscle proliferation [38,39]. n-3 fatty acids have also been shown to reduce the production of pro-inflammatory eicosanoids, such as prostaglandin E2 (PGE2) which promotes cell proliferation and tumour angiogenesis, via inhibition of the

COX pathway [40]. The COX pathway is also under partly regulated by NF - kB which as mentioned is inhibited by n-3 fatty acids.

Another factor of interest is the effect of n-3 fatty acids on peroxisome proliferator activated receptor (PPAR). These are ligand-activated transcription factors that are implicated in the regulation of lipid metabolism and homeostasis, but have also been found to be involved in cell proliferation, differentiation and inflammatory responses. PPARγ has been shown to be up-regulated by EPA and PPARδ by DHA: this has both anti-proliferative and pro-apoptotic effects shown in *in-vitro* and *in-vivo* studies [40]. These mechanisms are summarised in Figure 1.3.

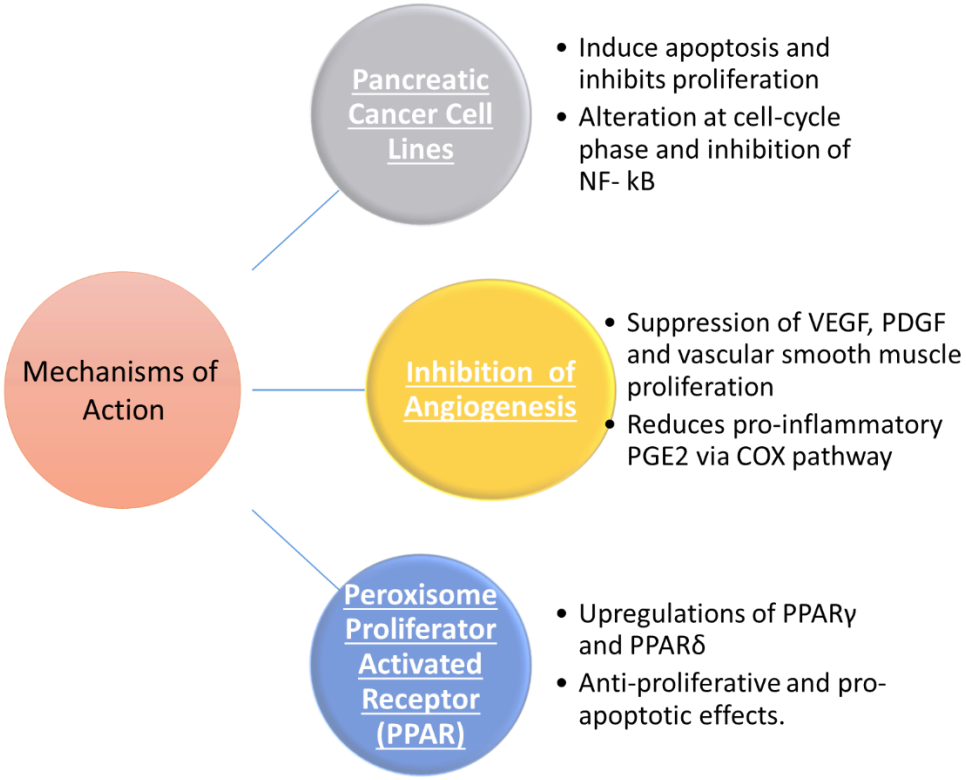


Figure 1.3: Summary of potential mechanism of action of fish oils in pancreatic adenocarcinoma. NF – kB - Nuclear Factor kappa beta, VEGF – Vascular endothelial growth factor, PDGF – Platelet derived growth factor, PGE2 – Prostaglandin E2, COX – Cyclo-oxygenase pathway.

1.3.2 Fish Oils in Pancreatic Cancers: Clinical Studies

A main detriment to the quality of life of patients with advanced pancreatic cancer is tumour-induced cachexia which is often refractory to treatment despite best medical management. Various clinical studies have shown that oral supplementation of fish oil in patients with advanced pancreatic cancers improves quality of life by arresting cachexia, improved appetite, increases lean body mass and induces significant weight gain within three weeks of oral fish oils supplementation [41-46]. A systematic evaluation of the literature by Ma *et al* showed the oral consumption of n-3 fatty acids improves clinical outcomes in terms of increased body weight, lean body mass, significant decrease in resting energy expenditure and an increased overall survival [47].

Metabolic changes induced by fish oils supplementation include a significant increase in fasting insulin concentration and a normalisation of energy expenditure in patients with advanced pancreatic cancer. The effect of fish oils in these patients was sufficient to normalise their metabolism rendering them equivalent to normal baseline healthy volunteers. The induced changes reversed their cachectic weight loss and induced weight gain [43]. Another postulated model suggests downward modulation of the hepatic acute phase protein response which is associated with accelerated weight loss and shortened survival in cancer patients. Advanced pancreatic cancer patients receiving 2 g of EPA daily for three weeks showed reduced albumin and fibrinogen synthetic rates which may have contributed to a net whole-body anabolism [48].

Fish oil supplementation has also been proven to modulate various mediators of catabolism. These include a significant reduction in interleukin-6 (a mediator of inflammation), a rise in serum insulin concentration, a fall in cortisol-to-insulin ratio and a fall in the proportion of patients excreting proteolysis inducing factor [49]. These effects are summarised in Figure 1.4.

These studies show that n-3 fatty acids have a direct effect at a cellular level following administration which leads to altering mechanistic pathways such that pancreatic cancer development is halted or reversed. These pathways involve the production of various

protein molecules which can be used as putative biomarkers including for diagnosis or efficacy of treatment. The specific protein changes can be analysed utilising advanced mass spectrometer based instruments to uncover the deep proteome following treatment with n-3 fatty acids.

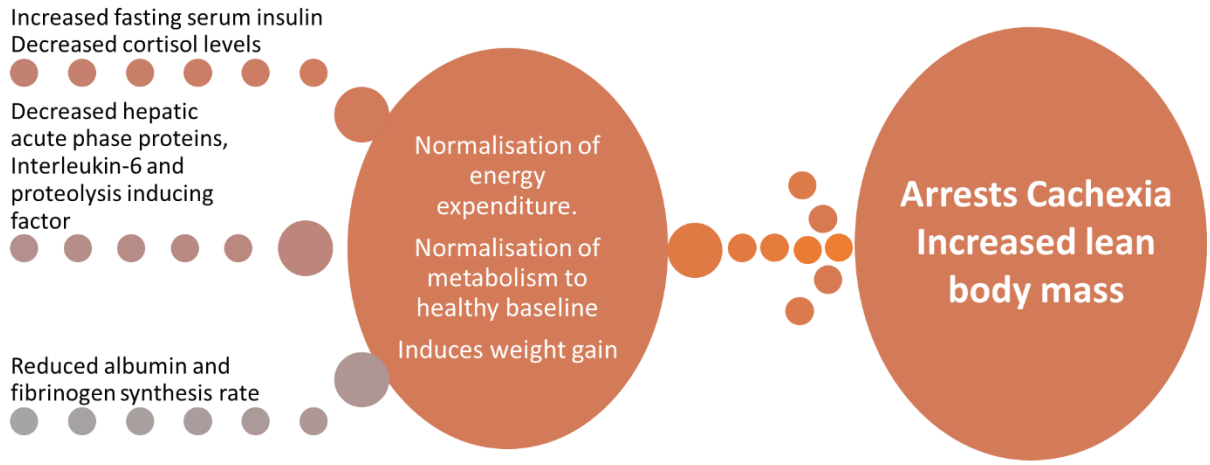


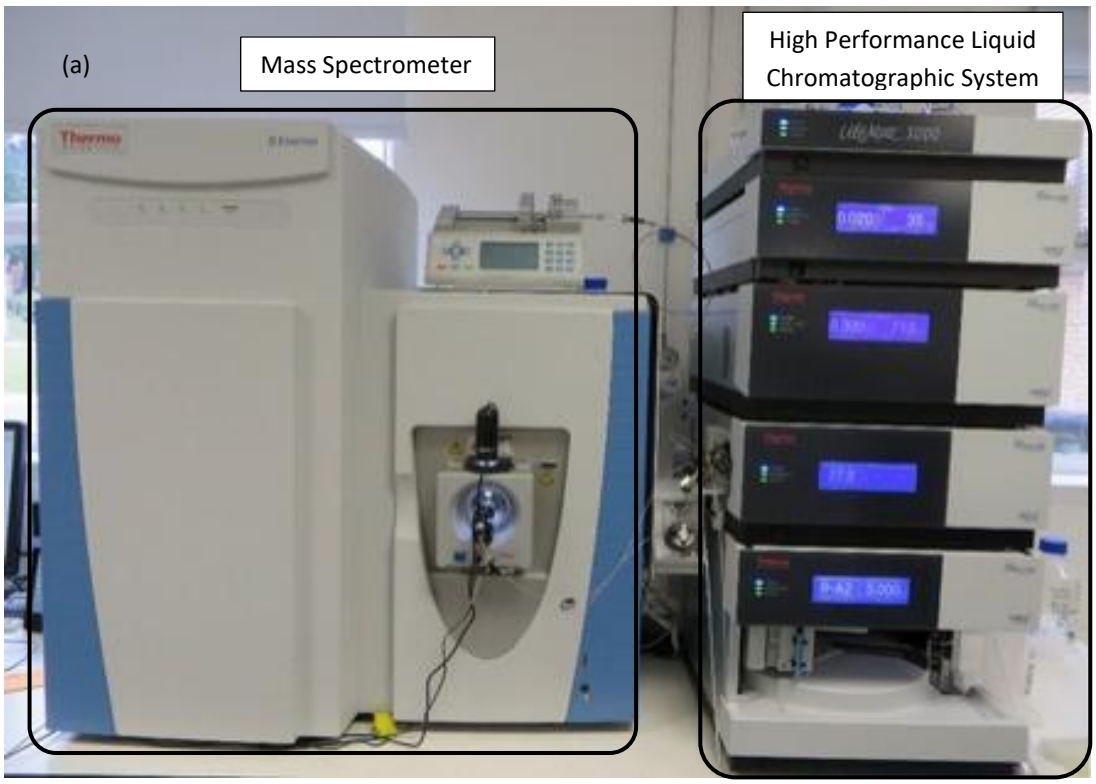
Figure 1.4: Summary of clinical action of fish oils in pancreatic cancer by modulating insulin, cortisol and inflammatory processes resulting in normalisation of energy expenditure and finally arresting tumour induced cachexia.

1.4 Principles of Proteomic Analysis

Proteomics is the large-scale determination of gene and cellular function directly at the protein level [50]. Mass-spectrometry based proteomic analysis allows high throughput analysis of various systems in human biology, including cell culture, plasma, tissues and urine [51]. In essence, the Mass Spectrometer (MS) measures the mass to charge (m/z) of a particular ionised molecule. The MS consists of three main parts: an ion source, a mass analyser that measures the mass to charge ratio (m/z) of the ionised analytes and a detector to measure the number of ions at each m/z value. Electro spray ionisation (ESI) is the most common ionisation source; it ionises analytes from a

solution converting them to gas-phase ions [52]. A MS is commonly paired with a Liquid Chromatographic system (LC-MS) which is the preferred modality in our laboratory for the analysis of complex samples.

The mass analyser is of central importance to MS based proteomic analysis: it measures the m/z of particular analytes. There are four main types of mass analysers: quadrupole (Q), ion trap, time of flight (TOF) and Fourier-transform ion cyclotron resonance (FTICR) mass analysers. They vary in their properties and have various advantages and disadvantages over each other. Systems can improve their sensitivity by pairing mass analysers. Such systems include: Quadrupole Ion trap (QIT), Linear Ion Trap (LTQ), triple- quadrupoles (Q), Q-TOF, LTQ-FTICR and LTQ-Orbitrap. In the Orbitrap, ions are trapped and orbit around a central spindle like electrode and oscillate harmonically along its axis with a frequency characteristic of their m/z values, inducing an image current in the outer electrodes that is Fourier transformed into the time domain producing mass spectra [53]. There are various reviews [50,53] documenting the pros and cons of each system which is beyond the scope of this report.



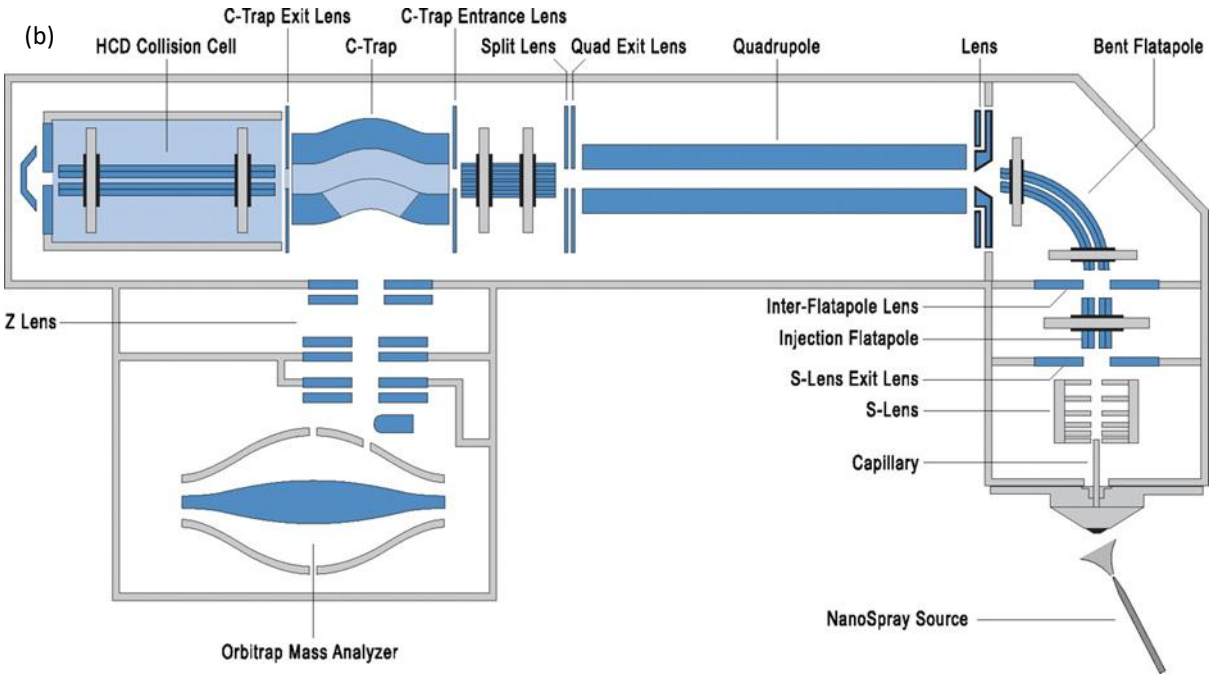


Figure 1.5 (a) An example of a LC-MS: QExactive-Orbitrap (ThermoFisher). (b) Schematic view of configuration of the QE.

Figure 1.5 illustrates the setup of an LC-MS: The QExactive-Orbitrap (QE) (ThermoFisher). The QE configuration means that a quadrupole is preceded by a "flatapole" and "S-lens" (see Figure 1.5(b)). The combination of these two means that clusters and droplets are removed prior to quadrupole injection. Ions can be isolated in the quadrupole and transmitted to a C-trap which is interfaced with a higher-energy collision dissociation (HCD) cell where fragmentation can occur. Ions are accumulated in the HCD cell and then transferred back into the C-trap, ejected into the Orbitrap analyser and analysed in a single Orbitrap detection cycle.

Each peak in a mass spectrum represents a unique m/z in the sample, and the height of the peak reflects the relative abundance in the sample. Information about the detected molecule can be gained from the m/z, the isotopic profile and the charge of the particular molecule. The MS instruments utilising ESI coupled with ion traps or quadrupoles generates fragment ion spectra (Collision Induced Dissociation, CID, spectra) of selected precursor ions [54]. In tandem MS, the original acquisition method is

via Data Dependent Acquisition (DDA). Here, peptides that are multiply charged are selected for CID fragmentation. In the first instance a short survey scan (eg 100ms) is done to establish which precursor ions are available for selection. Then 10 of the most abundant ions from the survey run are sequentially fragmented. The total cycle time for one DDA cycle i.e. 1 survey scan followed by 10 MS/MS scans equals 1s. Subsequent runs can choose to exclude previously chosen ions for MS/MS fragmentation to prevent repeated sampling of the same ion.

In Data Independent Acquisition no precursor ions are selected, instead all precursor ions that fall within a specified mass range are fragmented in a systematic and unbiased fashion. Depending on sample complexity, DIA leads to the co-fragmentation of many co-eluting peptides selected from the same precursor ion window resulting in highly multiplexed and complex fragment ion spectra [55]. The fragmentation pattern from the MS/MS spectrum is used in a database search, from which the precursor ion's m/z, charge state and mass accuracy of the measured precursor m/z is available. As all precursor ions are fragmented, DIA tends to increase the number of quantifiable proteins identified compared to DDA [56]. Specific examples of DIA methods include MSe [57] and SWATH-MS (Sequential Windowed Acquisition of All Theoretical Fragment Ion Mass Spectra). In SWATH-MS successive pre-defined ranges of precursor m/z values are isolated and subjected to co-fragmentation. Peptide-centric scoring of SWATH-MS data can be performed for example by using prior knowledge in form of a spectral library [55].

Each MS/MS represents the complete or partial sequence of a peptide. Thus a sequence database is downloaded for the organism in question, usually in FASTA format and an *in silico* digest is carried out. The computer simulated fragmentation is compared against the actual spectra and depending on a number of criteria the peptide is calculated to be associated with a protein in the database. These criteria include determining the enzyme used for protein cleaving and narrowing the precursor mass tolerance according to the mass analyser used. Comparing the experimental MS/MS spectrum against a candidate peptide, the database search algorithm evaluates how well

to match a spectrum to a peptide, allocating a search score. The software uses the scores to rank all the candidate peptides to a spectrum such that the correct peptide sits at the top of the list and at the end of the search the software determines the set of correctly identified peptides from the thousands of spectra. Multiple scoring algorithms exist, including SEQUEST, MASCOT and PLGS. This process can reveal a lot of false positives, thus decoy databases are generally used to establish false discovery rate. Typically the decoy database is either reversed or randomised. Protein identification relies on the robustness and comprehensiveness of the protein/peptide database to which it is cross-referenced. Currently there are 151,619 different proteins in the Human UniProt database (http://www.uniprot.org/), 20,199 of which are positively reviewed for in depth analysis.

Commercially available isobaric labels (for example Tandem Mass Tag Sixplex (TMT-6, Thermo Scientific) are useful to label peptides prior to MS/MS analysis. These tags attach to cleaved peptides and enables relative quantitation for comparison measured by the appropriate computing software. By pooling of samples, isobaric labels increase sample throughput and allows multiplexing experiments via setting a set label as an overall comparison quality control measure.

Selective Reaction Monitoring (SRM) allows identification of peptides down to the low attomoles per micro litre range [58]. Highly proteotypic peptides (peptides cleaved by trypsin) which are specific to a particular protein can be used for absolute or relative quantification in samples such as plasma. Peptides can be synthetically produced via commercial sources up to 99% purity. Additionally, these peptides can be modified by adding a heavy stable isotopic label of either $^{13}C_6{}^{15}N_2$ on the C-terminus of Lysine (K) or $^{13}C_6{}^{15}N_4$ on the C-terminus of Arginine (R). Using a triple quadrupole (QQQ) MS, the first and third quadrupoles act as filters to specifically select predefined m/z values corresponding to the peptide ion and a specific fragment ion of the peptide, while the second quadrupole acts as a collision cell [59]. Standards are used to formulate a calibration line. Using known concentrations of the unlabelled peptides and spiking the labelled peptides into the standards as well as the plasma sample allows for quantitation

of the specific protein. A review of plasma proteomic biomarker discovery identifies SRM analysis as verification of proteins of interest following the initial discovery phase of proteins in plasma [60].

Advances in the field of mass spectrometer based proteomics means this tool is an ideal avenue to identify changes in protein quantities in patients with pancreatic cancer.

1.4.1 Proteomics Studies Investigating the Effects of Fish Oils

Proteomics allows the global analysis of protein expression from a biological sample. It is thus an ideal technique by which to identify and evaluate a multitude of putative surrogate markers, and the effect of therapeutic regimens upon them. A literature review of fish oils (MeSH terms: fish oil, omega-3 fatty acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) and Proteomics revealed a number of different studies. A detailed summary of these studies is attached in Appendix 9.1 Table 1.

As described in Section 1.3, the potential mechanism of fish oil on pancreatic cancer involves inducing cell apoptosis, inhibition of angiogenesis and regulation of whole body metabolism via acute phase proteins and reduction of pro-inflammatory mediators. In the following section we describe putative biomarkers identified via proteomic methods in pancreatic cancer implicated in the above mechanisms.

1.4.1.1 Proteomic Biormarkers in Apoptosis and Proliferation

In a study comparing serum of patients with pancreatic cancer, gastric cancer and healthy individuals, Sun *et al* [61] independantly verfied up regulation of Cyclin I: Cyclins influence a wide range of cellular function including induction of apoptosis and differentiation. Guo *et al* [62] identified C14orf166 as a protein that was up-regulated in both tissue and serum sample of pancreatic cancer patients. It is postulated to be involved in centrosome architecture which leads to disruption of cellular architecture and cell death.

In malignant pancreatic cysts, Cuoghi *et al* [63] identified up-regulation of OLFM4. This protein is involved in pancreatic cancer cell cycle proliferation by promoting cancer-cell transition at the G2/M stage.

Annexin is another group of proteins associated with increased proliferation in pancreatic adenocarcinoma cell lines. Annexin II [64] and Annexin A5 [65] are two specific annexins identified independently by two separate groups.

1.4.1.2 Proteomic Biomarkers in Angiogenesis

As well as cell apoptosis, Cyclin I as identified by Sun *et al* [61] is associated with vascular endothelial growth factor which promotes angiogenesis. Reduction of platelet derived growth factor by fish oils also plays a role in angiogenesis. This may potentially postulate the mechanism by which downregulation of Platelet Factor 4, a protein identified by Fiedler *et al* [66] as strongly involved in patients with pancreatic cancer.

1.4.1.3 Proteomic Biomarkers in Metabolism Regulation (Acute Phase Protein Reaction)

In clinical studies as described above, it is postulated that fish oils induces downward metabolic changes by reducing the acute phase protein reactions. Proteomic studies identified serum amyloid A, which is an acute phase reactant, in the plasma of patients with pancreatic adenocarcinoma. Unsurprisingly, there was an increased in the levels of this protein compared to healthy individuals as well as pancreatitis patients [67].

An increase in the hepatic acute phase protein response results in worsening clinical symptoms in pancreatic cancer; this has been found to be reversed in patients receiving fish oils [48]. Thus, predictably, increased levels of the proteins Fibrinogen γ [68] and increased levels of Proly 4-hydroxylation of α-fibrinogen [69] is associated in pancreatic adenocarcinoma in proteomic studies.

Table 1.1 summarises the reviewed proteomic markers identified in pancreatic cancers.

1.4.2 Proteomic Biomarkers of Response to Fish Oil Supplementation in Pancreas and Other Related Tissues/Organs

To date, there have been only a handful of published studies looking into the effect of nutritional fish oils rich in n-3 fatty acids and proteomic studies. A PubMed/Medline (MESH) search of 'proteomic' AND 'omega 3' OR 'n-3 polyunsaturated fatty acids' OR 'EPA' OR 'DHA' was carried out and relevant articles were screened manually for inclusion in this review. Only one paper was related to pancreatic cancer and one other related to inflammatory bowel disease. The remainder concentrated on the proteomic biomarkers of the anti-inflammatory properties of fish oils in other disease states, which as described, is also relevant in carcinogenesis. Table 1.2 summarises the reviewed papers.

D'Allesandro *et al* [70] investigated the effect of supplementing the human PaCa-44 Pancreatic adenocarcinoma cell line with 200μM of DHA and investigating the onset of apoptosis on pancreatic cancer cells. They identified three main groups of protein-protein integrators, the first characterised by structural and chaperon-like proteins, the second a group of solute carriers targeting membrane fractions and thirdly, proteins involved in metabolism. It was postulated that apoptosis induced by DHA is related to DHA-induced over-activation of oxidative metabolism via effects on the Krebs cycle.

Cooney *et al* [71] investigated the effect of dietary EPA vs AA vs controls in IL10 deficient rats and performed proteomic analysis on colon. They found that dietary PUFAs ameliorate intestinal inflammation with down regulation of metabolism and digestion/absorption/excretion of nutrients and up regulation of cellular stress and immune response. Changes in protein expression suggest modulation of these effects via PPARα and Heat Shock Protein 90AB1 (HSP90AB1). Heat Shock Protein 90 has already been described in a separate proteomic study by Walsh *et al* [72]. In comparing two cloned MiaPaCa2 cell lines with different levels of invasion (high and low), they identified up-regulation of STIPI (Stress-induced phosphoprotein I) which mediates the association of the molecular chaperones HSP70 and HSP90 which are proteins implicated in pancreatic cancers.

Kalupahana *et al* [73] investigated the effect of EPA on the prevention and reversal of insulin resistance in high fat diet-induced diabetic mice. Proteomic analysis identified that mice fed with EPA showed increased levels of enzymes involved in carbohydrate metabolism, fatty acid metabolism and also in proteins involved in cellular metabolism, such as HSP 70 and HSP 1, which again is implicated in pancreatic cancers.

Similarly investigating cardiovascular risk factors, Mavrommatis *et al* [74] investigated the effect of dietary fish oils vs high fat in apoE knockout mice, and identified down-regulation of the hepatic proteome apoA1 and hepatic peroxiredoxin 3, which is involved in oxidative stress.

De Roos *et al* [75] investigated the effect of dietary fish oils versus oleic acid in healthy human volunteers, primarily looking into decreasing cardiovascular risk factors. It is postulated that fish oil supplementation caused a shift towards larger more cholesterol-rich HDL2 particles, which in turn activate anti-inflammatory and lipid modulating mechanisms. Of the ten proteins identified to be down regulated, α-1 antitrypsin precursor was also implicated in serum of PDAC patients. In an earlier study, de Roos *et al* [76] identified down-regulation of apoA1 hepatic proteome and also up-regulation of Annexin A5, which is implicated with pancreatic cancer.

1.4.3 Proteomics Studies Investigating the Effects of Gemcitabine in Pancreatic Cancer

The patient samples analysed in this study originates from a completed phase II clinical study investigating the effect of intravenous omega-3 fatty acids in patients with advanced pancreatic adenocarcinoma (locally advanced or metastatic – unsuitable for surgical resection) who received single agent gemcitabine chemotherapy [77]. The results showed that patients receiving additional intravenous omega-3 fatty acid had improvements in progression free survival and quality of life. A control arm of patients with advanced pancreatic adenocarcinoma, who only received single agent chemotherapy with gemcitabine without omega-3 fatty acid infusion was later recruited to provide a control arm for comparison in proteomic analysis. Further details on these

studies, patient cohorts and clinical results are described in Sections 2.2.1, 2.2.2 and 2.2.3.

As described in section 1.2.2, gemcitabine (Gemzar, Lilly: 2'-deoxy-2'-difluorodeoxycytidine) is the standard single agent chemotherapy used in advanced pancreatic adenocarcinoma, though currently the addition of other chemotherapeutic agents is becoming common place, including nab-paclitaxel as combination therapy to improve outcome[78]. The mechanism of action of gemcitabine is illustrated in Figure 1.1. Gemcitabine is a pyrimidine analogue, which disrupts DNA synthesis leading to cell death. The cohort includes patients with pancreatic adenocarcinoma receiving gemcitabine chemotherapy (Gemzar, Lilly: 2'-deoxy-2'-difluorodeoxycytidine), hence the effects of gemcitabine to the proteome in pancreatic cancer needs to be considered.

Resistance of pancreatic cancer cells to gemcitabine leads to poorer response and hence survival. The majority of current proteomic based investigation concentrates on human pancreatic cancer cell lines (e.g.: MiaPaCa-2, AsPC-1, Panc-1 and BxPC-3). Some of the cell lines have developed resistance to gemcitabine (e.g. PK45p and PK59) through prolonged exposure to gemcitabine. Many of these studies employed 2-Dimensional Gel Electrophoresis (2-DE) coupled to LC-MS/MS to determine differential proteomic expressions.

Various proteins and different mechanisms have been postulated as to how gemcitabine resistant cells develop, including up-regulation of Heat Shock Protein (HSP) 27 within the cell nucleus, which can lead to inhibition of cell apoptosis [79-81]. Proteins that are key to EMT such as alpha-enolase, vimentin, cAV1 and IQGAP-1 [82] have all been identified in pancreatic cancer studies to be differentially expressed. In particular, alpha-enolase led to increased phosphorylation of the P13K/AKT pathway leading to established EMT [83].

On a cellular level, gemcitabine enters pancreatic cells and phosphorylates via different enzymes prior to disrupting DNA synthesis. Disruption of DNA synthesis is the

primary mechanism of cell death. Ohmine *et al* [84,85] successfully applied combined proteomic and metabolomic methods to confirm suppression of dCK (which phosphorylates gemcitabine) leads to increased gemcitabine resistance and consequently higher levels of dCK in human pancreatic adenocarcinoma tissues leads to increased survival. They also identified transport proteins on the cell surface facilitating gemcitabine excretion (e.g. ENT1) and cytosolic proteins that inactivate gemcitabine (e.g. CDA). Both of these mechanisms lead to increased resistance.

Studies on protein post translation modifications (PTMs) have found that increasing Ubiquitin and Nedd8 specific pathways while a decreased Sumo1 specific pathway leads to increased gemcitabine resistance in pancreatic cancer cell lines [86]. Of note, there were only two studies which directly investigated human serum or plasma of patients with advanced pancreatic adenocarcinoma receiving gemcitabine monotherapy. Markers of tumour progression and cell-cell adhesion (Annexin II, α1-antitrypsin (AAT) and α1-antichymotrypsin (AACT)) have been found to be over-expressed in patients with poorer outcome and survival [87-89].

Appendix 9.2 Table 2 provides an in-depth summary of the current proteomic based investigations of gemcitabine on pancreatic cancer.

1.4.4 Summary of Proteomics in Fish Oils and Pancreatic Cancer

The advantage of high throughput mass-spectrometer based proteomic analysis is the large amounts of individual proteins identified and techniques such as SRM allows quantifying proteins compared to non-diseased states. This has resulted in a huge amount of potential protein biomarkers in the literature which is summarised above in terms of its actions on pancreatic cancer. The disadvantage with discovering large amounts of protein biomarkers is that they may not be all clinically relevant. Validation using further proteomic profiling is possible but expensive, and using commercially available ELISA kits are possible but time consuming and not necessarily available for all proteins.

However, perhaps identifying an individual biomarker is not the key but identifying a panel of markers within a known mechanistic pathway will act better as a diagnostic tool or prognostic marker of efficacy of treatment. This was successfully shown by Song Nie et al [90] who identified a panel of four glycoproteins (α1-antichymotrypsin (AACT), thrombospondin-1 (THBS1), haptoglobin (HPT) and Ca 19-9) using quantitative proteomics to distinguish between pancreatic adenocarcinoma from normal controls, diabetes, pancreatic cystadenoma and chronic pancreatitis. This can be performed using available web-based panel construction tools which employ multivariate analysis of disease biomarker candidates.[91]

Pancreatic adenocarcinoma remains a challenging disease with a poor prognosis and very little advancement in the last 40 years. Clinically, the use of intravenous fish oil has shown improvement in terms of progression free disease and quality of life [77]. Proteomics is a modern tool which although has been available for many years, is now becoming integrated into modern biomarker discovery strategies. Currently there is a lack of highly specific or sensitive protein biomarker to explain the response of pancreatic adenocarcinoma to fish oil. As such, this resulted in the following work in identifying the mechanistic action of intravenous omega-3 fatty acids in advanced pancreatic adenocarcinoma utilising advanced proteomic tools.

Table 1.1

Author [ref]	**Protein Biomarkers**	**Expression**	**Tissue analysed**	**Pancreatic cancer type**
Honda *et al* [67]	Serum amyloid A	↑	Plasma	PDAC vs healthy/ Pancreatitis
Bloomston *et al* [68]	Fibrinogen γ	↑	Serum	PDAC vs Healthy
Sun *et al* [61]	Cyclin I and Rab GDP dissociation inhibitor β (GDI2)	↑	Serum/ Tissue/ Bile Juice	Pancreatic ca vs Gastric ca vs Healthy
Guo *et al* [62]	C14orf166	↑	Serum	Pancreatic Ca vs healthy
Fiedler *et al* [66]	Platelet Factor 4	↓	Serum	Pancreatic Ca vs healthy
Ono *et al* [69]	Proly 4-hydroxylation of α-fibrinogen	↑	Serum	PDAC vs Healthy
Cuoghi *et al* [63]	OLFM4, MUC18	↑	Pancreatic Cyst Fluid	Malignant Cyst vs Healthy
Tian *et al* [64]	Nucleotide Diphosphatase Kinase (NDPK), Annexin II	↑	Tissue samples vs healthy adjacent tissue	PDAC vs Healthy

Cui *et al* [65]	Glucose regulated protein 78 (Grp78), macrophage migration inhibitory factor (MIF) and Annexin A5	↑	Pancreatic tissue vs healthy	PDAC vs Healthy

Table 1.1: Summary of reviewed publications applying proteomics to pancreatic cancer. Abbreviations: PaCa: Pancreatic cancers, PDAC: Pancreatic ductal adenocarcinoma, 2-DE: 2 dimensional electrophoresis, LC: Liquid Chromatography, MS: Mass Spectrometry, SDS-PAGE: 1-Dimensional electrophoresis, HPLC: High performance liquid chromatography, TOF: Time of flight, ESI: Electro Spray Ioniser, 2DICAL: Two-dimensional image converted analysis of liquid chromatography and mass spectrometry, MALDI: Matrix Assisted laser desorption/ionization, SELDI: Surface-enhanced laser desorption/ionization.

Table 1.2

Author [ref]	**Protein Biomarkers**	**Expression**	**Tissue analysed**	**Dietary Fish Oils**
D'Allesandro *et al* [70]	Keratin 19 (KRT19), Vimentin (Vim), calreticulin (CALR), HSP90AA1, GRP58, Dihydrolipoamide	↑	Pancreatic Cancer Cell line PACA44	DHA supplemented to cell medium (200 µM DHA bathed in cells for 24, 4 hrs)
Cooney *et al* [71]	PPARα, HSP90AB1	↑	Colon tissue of interleukin 10 deficient rats	Oral diets of control (lipid diet 5% cor Oleic Acid (OA) (1% corn oil with 3.7 Arachidonic Acid (AA) (1% corn oil w AA) and Eicopentaenoic acid (EPA) (oil with 3.7% EPA)
Kalupahana *et al* [73]	HSP 70, HSP1, Glutamate dehydrogenase 1	↑	Male C57Bl/6J mice – blood samples analysed	Oral diets of low fat (10% energy from high fat (45% energy from fat) vs EPA (36g/kg EPA)

De Roos *et al* [75]	Apo A1, Apo L1, zinc-α-2-glycoprotein (ZAG) Haptoglobin precursors, α-1 antitrypsin precursor, antithrombin III chain L, serum amyloid P (SAP), haemopexin	↓	Serum of healthy human volunteers	Oral supplementation of fish oil (daily 700mg EPA, 560mg of DHA and 260 other omega-3 fatty acids) vs oleic aci (placebo)
Mavrommatis *et al* [74]	ApoA1, hepatic peroxiredoxin 3	↓	Male Apoe Knockout mice	Oral diets high fat (diet supplemented w/w high-oleic acid sunflower seed oi DHA (diet supplemented with 2% w/ oil containing 40% DHA and 0% EPA oil (diet supplemented with 2% w/w f containing 22% EPA and 17% DHA)
De Roos *et al* [76]	Annexin A5	↑	APOE*3 Leiden Transgenic Mice	Oral diets of fish oils (diet supplement 3% w/w fish oil containing EPA and D linoleic acid (diet supplemented with 1 conjugated linoleic acid) vs elaidic aci supplemented with 3% w/w elaidic aci
	ApoA1	↓		

Table 1.2: Summary of published research of proteomic studies on fish oils. Abbreviations: 2-DE: 2 dimensional electrophoresis, LC: Liquid Chromatography, MALDI: Matrix Assisted laser desorption/ionization, MS: Mass Spectrometry, SDS-PAGE: 1-Dimensional electrophoresis, HPLC: High performance liquid chromatography, TOF: Time of flight, ESI: Electro Spray Ioniser.

1.5 Aims and Objectives

As described above, there are published works describing the mechanisms behind the beneficial effect of fish oils within pancreatic cancer (Section 1.3) and studies utilising proteomics to further elucidate these effects (Section 1.4.1). However, the variability of these works include experiments on cancer cell lines, rodent based and human studies, though often through the use of oral fish oil administration. Historically, oral administration of fish oil based studies has been proven difficult to validate, due to poor compliance of patients in taking the often fatty, milky emulsion of fish oil supplementation [44]. Published works have also described proteomics based analysis involving use of fish oils, though there are limited to cancer cell lines, rodent based and human based studies in small numbers and not specific to pancreatic cancer (Section 1.4.2). To the best of my knowledge, this work is novel, as it involves investigating the effect of a verified amount of omega-3 fatty acid, administered intravenously in advanced pancreatic adenocarcinoma by utilising advanced proteomic methods to discover the deep proteome changes in these patients. It is important to understand the mechanism behind which these patients exhibit their improvement in progression free survival and quality of life.

1.5.1 Hypothesis

- The null hypothesis for this study is that the administration of intravenous omega-3 fatty acid infusion will have no effect on the proteomics of plasma samples of patients with unresectable pancreatic adenocarcinoma receiving gemcitabine chemotherapy.

1.5.2 Aims

- To characterise phenotypic changes in the plasma of patients with unresectable pancreatic adenocarcinoma administered intravenous omega-3 fatty acid in combination with standard gemcitabine chemotherapy.
- Protein profiles from the group will be compared with patients receiving gemcitabine alone.
- The clinical arm of the study has confirmed that administration of intravenous omega-3 fatty acids in combination with standard gemcitabine chemotherapy

exhibits an improvement in progression free survival and quality of life. Deep proteome changes that reflect the mechanistic action of omega-3 fatty acid in improving the quality of life and progression free survival in pancreatic adenocarcinoma will be investigated.

1.5.3 Objectives

- Establish a workflow to investigate the deep proteome profile in human plasma. The workflow would begin with intense immuno-depletion of high and moderate abundant proteins in plasma, followed by methods to increase protein and peptide yield by using liquid chromatographic (LC) methods in fractionation.
- Develop the use of isobaric labels with TMT-6plex tagging to perform multiplex experiment and provide measurable protein quantitation for comparison of changes following treatment/intervention.
- Develop a suitable method to increase quantifiable protein yield using LC-MS/MS instruments by optimising the criteria and database search engines, and post processing software for protein identification on Protein Discoverer and Scaffold programmes.
- To test the above methodology on patient plasma to identify proteomic changes from baseline of Advanced Pancreatic Cancer (APC) to treatment with n-3 fatty acid and gemcitabine (Treatment).
- To test the above methodology on patient plasma to identify proteomic changes in APC following treatment with n-3 fatty acid and gemcitabine (Treatment) versus n-3 fatty acid naive, gemcitabine alone (Control).
- Protein changes elucidating the mechanistic action of n-3 fatty acid in improvement of progression free survival and quality of life using bioinformatics analysis.
- To verify the candidate proteins using LC-MS/MS based Selective Reaction Monitoring (SRM).

2 Materials and Methodology

2.1 Common Materials

- Optima Water (Thermo Fisher Scientific, Waltham USA), – LC/MS grade HPLC water.
- Acetonitrile, HPLC Grade (Sigma-Aldrich, Dorset) – HPLC grade Acetonitrile, CH_3CN.
- Formic Acid Optima LC/MS Grade (Fisher Scientific, Loughborough).
- Low Bind Eppendorf Tubes (Eppendorf, Hamburg) – Various size Eppendorf tubes.
- Amicon Ultra Centrifugal Filter Device (Merck Milipore, Darmstadt) – various size capacity 0.5 ml to 15 ml, 3000 Dalton filter size.
- DTT (DL-Dithiothreitol), (Sigma-Aldrich, Dorset) – Ultragrade
- IAA (Iodoacetamide), (Sigma-Aldrich, Dorset) – Ultragrade
- Rapigest^{TM} SF (Waters, Milford USA).
- 1M Triethylammonium bicarbonate (TEAB) for TMT (Thermo Fisher Scientific, Waltham USA).
- 50% Hydroxylamine for TMT (Thermo Fisher Scientific, Waltham USA).
- Sequencing Grade Modified Trypsin (Promega, Madison USA).
- Lys-C Protease MS Grade (Thermo Fisher Scientific, Waltham USA).
- Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham USA).
- Trifluoroacetic acid, 99+% for HPLC (Fisher Scientific, Loughborough).

2.2 Patient Samples

2.2.1 Patient Cohort

The clinical study was conducted from 2009 and concluded in 2013. The results of the study have been published [77] and the results are summarised in Section 2.2.2. As such, the samples that have been analysed in this body of work are from archived samples but stored appropriately in -80°C with full Research Ethics Committee (REC) and Human Research Authority (HRA) approval and compliance.

As part of a phase II single-arm trial [77] over a period of 48 months, patients presenting to a tertiary hepatobiliary multidisciplinary team meeting, which screened outpatient referrals from other hospitals within the region, with histologically confirmed locally advanced or metastatic pancreatic adenocarcinoma deemed unsuitable for surgical resection but suitable for gemcitabine chemotherapy were recruited.

2.2.1.1 Inclusion Criteria

Patients were included in the study if they met the histological diagnosis of unresectable pancreatic adenocarcinoma and:

- Aged >18 years
- Able to give informed written consent
- ECOG performance status of 0 or 1 (Eastern Cooperative Oncology Group).
- Life expectancy >12 weeks
- Adequate hepatic and renal function documented within 14 days prior to treatment
    - AST (Aspartate aminotransferase) and ALT(Alanine aminotransferase) ≤2.5x upper limit of normal (ULN), unless liver metastases present, in which case ≤5.0xULN
    - Total bilirubin ≤2.5xULN
    - Serum creatinine ≤1.5xULN or calculated creatinine clearance ≥60ml/min
    - Urinary protein <1+ by urine dipstick. If ≥1+, then 24-hour urine collection should be done and may only be enrolled if urine protein is <2g/24hours

- Adequate bone marrow function
    - Haemoglobin ≥9g/dL (can have transfusion or growth factors)
    - Platelets ≥100,000cells/mm^3
    - Neutrophil count ≥1500cells/mm^3
- No significant hyperlipidaemia
- Patients without severe blood coagulation disorders (anticoagulants allowed)
- Women of childbearing age must have a negative pregnancy test (urine or serum) at commencement of treatment
- Willingness to comply with scheduled visits, treatment, laboratory test, and other aspects of the trial

2.2.1.2 Exclusion Criteria

Patients were excluded from the trial if they had:

- Prior treatment with any systemic chemotherapy for metastatic disease
- Prior adjuvant radio- or chemotherapy within 4 weeks of starting the study
- Previous treatment with gemcitabine
- Hypersensitivity to fish-, egg-, or soy protein, or to any of the active substances or constituents in the lipid emulsion
- Any general contra-indications to infusion therapy – pulmonary oedema, hyperhydration, decompensated cardiac insufficiency
- Any unstable medical conditions – uncontrolled diabetes mellitus, acute myocardial infarction, stroke, embolic disease, metabolic acidosis, sepsis, pancreatitis

- Known HIV or AIDS
- Dementia or significantly altered mental status that would prohibit the understanding or rendering of informed consent and compliance with requirements of the protocol
- History of malignancy other than pancreatic cancer, with the exception of curative treatment for skin cancer (other than melanoma) or *in situ* breast or cervical carcinoma, or those treated with curative intent for any other cancer with no evidence of disease for 5 years
- Major surgical procedure or significant traumatic injury within 4 weeks of treatment
- Female patients must be surgically sterilised or postmenopausal or agree to use two adequate contraception measures during the period of therapy and continued for 6 months after the last dose of gemcitabine. Male patients must be surgically sterilised or agree to use adequate contraception for the same period.
- Patients deemed unsuitable for gemcitabine chemotherapy

2.2.1.3 Treatment Protocol Regime

Following informed consent and study enrolment they were treated in a chemotherapy suite of a university teaching hospital on an outpatient basis with weekly gemcitabine chemotherapy (Gemzar, Lilly: 1000 mg/m^3 over 30 minutes by peripheral intravenous infusion) immediately followed by an omega-3 fatty acid- rich lipid infusion, Lipidem (BBraun, Melsungen) of 50 g by peripheral intravenous infusion delivered at 25 g/hr)

The composition of Lipidem at a concentration of 200 mg/ml are:

50% Medium Chain Triglycerides (MCT) – 100 g per 1000 ml of Lipidem
40% Long Chain Triglycerides (LCT) – as Soya bean oil refined, 80 g per 1000 ml
10% Omega-3 Fatty Acids – 20 g per 1000 ml of Lipidem.
This is equivalent to each patient receiving omega-3 fatty acid in the form of Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA) at 2.2 to 4.3 g for each intravenous infusion.

This regimen was given weekly for 3 weeks (administered on Day 1, 8 and 15). This is followed by a rest week from all treatment (No treatment on Day 22): this constitutes one cycle of treatment (Cycle completes on Day 28). This regimen was continued until either death, withdrawal by investigator or participant or tumour progression on CT for a maximum of 6 cycles in total. Fifty patients were recruited. Patients were followed up until death in all cases. The trial was approved by the regional ethics committee, Medicines and Healthcare products Regulatory Agency (MHRA) and the institutional review board. The study was registered with clinicaltrials.gov: registration number NCT01019382.

Primary outcome measure was objective response rate on CT evaluated using Response Evaluation Criteria in Solid Tumours (RECIST) version 1.1 criteria by an experienced independent clinical trials radiologist. Tumour progression was defined by a >20% increase in target lesion dimensions or any new lesion occurrence since baseline CT. Evaluation by CT was carried out every 2 cycles of treatment (8 weeks). Secondary outcome measures included Overall Survival (OS), which was defined as the interval between first treatment and death, Progression free survival (PFS) which is defined as the interval between first treatment and progression of the disease and safety analysis of adverse events recorded by Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 criteria. Plasma samples were taken immediately prior to and after treatment each week, frozen and stored prior to analysis. These samples have already been collected and stored with their corresponding clinical outcomes.

2.2.2 Summary of Result from Clinical Study of Omega-3 Fatty Acid and Gemcitabine in Advanced Pancreatic Cancer

The results from the phase II clinical result have been published [77]. Fifty patients were recruited from which 35 who had completed at least 2 months of treatment with an interval CT scan were analysed. The baseline demographic included 30 (60%) male patients and a median age of 68 years old (Range 40 – 83 years old).

In summary the findings are:

- Disease stabilisation (Stable disease and partial response to n-3 fatty acid) was achieved in 85.7 % of patients.
- Median overall survival and progression free survival were 5.9 month and 4.8 months respectively – overall survival was comparable to single agent gemcitabine, whilst there was an improvement in the progression free survival due to improved disease stabilisation.
- Quality of life and pain scores were improved with administration of n-3 fatty acids – 47.2 % achieved an increase in global health of at least 10 points over baseline sustained for at least 4 weeks. More than half (52.8 %) had a 10 point or greater improvement in quality of life scores for at least 4 weeks.

Based on these results, the mechanism of action and associated changes in plasma protein following administration of n-3 fatty acids in patients with advanced pancreatic cancer was aimed to be discovered using high definition plasma proteomics.

2.2.3 Control Arm of n-3 fatty Acid Naïve Patients

The principal clinical study above [77] used historical data and baseline changes for comparison of the effect of n-3 fatty acids in advanced pancreatic cancer. This study superseded the work described herein. Hence a control arm of omega-3 fatty acid naive patients was subsequently recruited to collect plasma samples to enable a comparison of the plasma proteomic changes between n-3 fatty acid with gemcitabine and gemcitabine alone (without n-3 fatty acid). This was principally led by myself including the

application for ethical approval, production of patient information leaflets, identifying suitable patients from the tertiary hepatobiliary multi-disciplinary meeting, obtaining consent for involvement in the study, collection of plasma samples and processing of plasma samples prior to proteomic analysis.

The patients consisted of a similar cohort to the original study: histologically confirmed unresectable pancreatic adenocarcinoma who only receive gemcitabine, without intravenous omega-3 fatty acid, to act as a control group for comparison. These patients were matched to the intervention group and have tumour assessments as per normal clinical protocol to the above. Informed consent for the analysis of plasma samples was obtained prior to commencing into the study. Ethical approval was obtained from the regional ethics board (REC Reference number 12/EE/0425) and 9 patients were recruited.

2.2.4 Plasma

The typical human plasma concentration is ~70 mg/ml of protein. However, in patients with advanced pancreatic adenocarcinoma, this may be lower due to the cachectic effect the malignancy has on this cohort. In our study, ten millilitres (ml) of peripheral blood was obtained by percutaneous venous puncture of patients immediately before and after gemcitabine +/- omega-3 fatty acid infusion into heparinised monovette tubes. The samples were stored in ice to be processed within an hour. The tubes were centrifuged at 1000 x g for 15 minutes at 4°C. Aliquots of 250 µL were collected and stored in -80 ºC freezer until use.

In the treatment group (n-3 fatty acid with gemcitabine) the total number of treatments administered was 409 whilst in the control group (gemcitabine alone without n-3 fatty acid) the total number of treatment administered was 38. Hence as plasma samples were taken before and after treatment, there were 894 plasma samples available for analysis. In total 16 treatment patients (n-3 fatty acid and gemcitabine) and 5 control patients (gemcitabine alone) were analysed on, with treatment points up to 2 months –in total 40 patient samples underwent proteomic analysis.

2.3 Instrument Details

2.3.1 Liquid Chromatography for Immunodepletion

The High Performance Liquid Chromatography (HPLC) for immunodepletion utilised a Varian ProStar HPLC Solvent Delivery Module Model 230 (Varian, Walnut Creek USA) and a Varian ProStar UV-VIS Detector Model 310 (Varian, Walnut Creek USA). The UV detector lamp was set at 280 nm. Two different immunodepletion methods were explored: Multiple Affinity Removal Column Human 14 (MARS 14) (Agilent Technologies, Santa Clara USA) and Seppro IgY14 + Supermix Column (Sigma-Aldrich, Dorset). The various buffer solutions and gradient times are detailed further on in Section 2.5.1.

2.3.2 Liquid Chromatography for Fractionation

HPLC for fractionation also utilised the Varian ProStar HPLC Solvent Delivery Module Model 230 (Varian, Walnut Creek USA) and a Varian ProStar UV-VIS Detector Model 310 (Varian, Walnut Creek USA). A high pH Reverse-Phase chromatography for peptide fractionation protocol was established using XBridge BEH300 C18 5µm column (Waters, Milford USA), with mobile phases solutions of 200 mM Ammonium Formate, pH10 and 10% 200 mM Ammonium Formate pH10 + 90% HPLC Grade Acetonitrile. Details of the flow rate, gradient times and fractionation collections are detailed further. The UV detector lamp was set at 280 nm.

2.4 LC-MS/MS Instruments

Three LC-MS/MS instruments have been used for analysis of samples: A SYNAPT-G2 Si HDMS (Waters, Milford USA) (Hence forward described as G2Si), a QExactive-Orbitrap (Thermo Fisher Scientific, Waltham USA) (Hence forward described as QE) and a XEVO MS Triple Quadrupole (Waters, Milford USA) (Hence forward described as a TQ). The HPLC conditions were established methods within the group. Three LC-MS/MS instruments were used as these were available to conduct the experiments.

For the SYNAPT-G2 HDMS (High Definition Mass Spectrometry) instrument, the details are as follows.

2.4.1 Nano Ultra Performance Liquid Chromatography (NanoUPLC)

Sample analysis was performed using a Waters NanoAcquity UPLC system. The peptides were initially loaded onto a Waters 2G-V/M Symmetry C18 trap column (180 µm x 20 mm, 5 µm) to desalt and chromatographically focus the peptides prior to elution onto a Waters Acquity HSS T3 analytical UPLC column (75 µm x 250 mm, 1.8 µm). Single pump trapping was used with 99.9% solvent A and 0.1% solvent B at flow rate of 5 µL/min for 3 minutes. Solvent A was LC-MS grade water containing 0.1% formic acid and solvent B was acetonitrile containing 0.1% formic acid. For the analytical column the flow rate was set at 0.3 µL/min and the temperature maintained at 40 ºC. The 50 minute run time gradient elution was initiated as the peptides were eluted from the trap column. The following gradient was used: 0 minute- 3% B, 30 minutes- 40% B, 32 minutes- 85% B, 40 minutes- 85% B, 41 minutes- 3% B and 50 minutes- 3% B.

2.4.2 Nano Electrospray Ionisation Mass spectrometry

The NanoAcquity UPLC was coupled to a G2Si mass spectrometer. The instrument was operated in positive electrospray ionisation (ESI) mode. The capillary voltage was set at 2.4 kV and cone voltage at 30 V. PicoTip emitters (New Objective, US, 10 µm internal diameter) were used for the nanostage probe. A helium gas flow of 180 mL/min and ion mobility separator nitrogen gas flow of 90 mL/min with a pressure of 2.5 mbar were used. The IMS wave velocity was set at 650 m/s and the IMS wave height at 40 V. During the HDMS^E acquisition a low collision induced dissociation (CID) energy of 2V was applied across the transfer ion guide. For the high CID energy acquisition a ramp of 27 to 50 V was applied. Argon was used as the CID gas. Lockspray provided mass accuracy throughout the chromatographic run using [Glu1]-Fibrinopeptide (GFP) with 785.8427 *m/z*. The data was acquired using MassLynx 4.1.

The dried digested and fractionated samples are reconstituted into 97% 0.1% formic acid: 3% Acetonitrile, 10 µL and an equal amount (10 µL) of 100 fmol of yeast alcohol dehydrogenase 1 (ADH, Uniprot accession number P00330). Yeast ADH1 is used as an internal label for absolute quantitation. Thus the final sample has a known concentration of 50 fmol per µL injected sample into the LC-MS/MS.

2.4.3 Mass Spectrometry on the QExactive-Orbitrap (Thermo Fisher Scientific, Waltham USA)

Samples were digested as described. Dried pellets were re-constituted in 0.1% formic acid (FA)/ 50 fmol alcohol dehydrogenase (ADH) as an internal standard enabling relative quantitation of the proteins post-analysis. Tryptic peptides were separated on an Ultimate 3000 RSLC nano HPLC system (Dionex/Thermo Fisher Scientific, Bremen, Germany). Samples were loaded onto a Cartridge based trap column, using a 300 µm x 5 mm C18 PepMap (5 µm, 100A) and then separated using the Easy-Spray pepMap C18 column (75 um x 50 cm) with a gradient from 3-10% B in 10 minutes, 10-50% B in 37 minutes, 50-90% in 9 minutes and 90-3% in 26 minutes, where mobile phase A was 0.1% FA in water and mobile phase B, 80%/20% ACN/Water in 0.1% FA. Flow rate was 0.3 µL/min. The column was operated at a constant temperature of 40 °C.

The nanoHPLC system was coupled to a QE mass spectrometer (ThermoScientific, Bremen, Germany). The QE was operated in the data-dependent top10 mode; full MS scans were acquired at a resolution of 70,000 at m/z 200 to 2000, with an ACG (ion target value) target of 1e6, maximum fill time of 50 ms. MS2 scans were acquired at a resolution of 17,500, with an ACG target of 1e5, maximum fill time of 100 ms. The dynamic exclusion was set at 30.0 seconds, to prevent repeat sequencing of peptides.

2.4.4 Mass spectrometry on the XEVO MS Triple Quadrupole Instrument (Waters, Milford USA)

Nano Ultra Performance Liquid Chromatography (NanoUPLC)

Sample analysis was performed using a Waters NanoAcquity UPLC system. The peptides were initially loaded onto a Waters 2G-V/M Symmetry C18 trap column (180 µm x 20 mm, 5 µm) to desalt and chromatographically focus the peptides prior to elution onto a Waters Acquity HSS T3 analytical UPLC column (75 µm x 250 mm, 1.8 µm). Single pump trapping was used with 99.9% solvent A and 0.1% solvent B at flow rate of 5 µL/min for 3min. Solvent A was LC-MS grade water containing 0.1% formic acid and solvent B was acetonitrile containing 0.1% formic acid. For the analytical column the flow rate was set at 0.3 µL/min and the temperature maintained at 40 ºC. The 50 min run time gradient elution was initiated as the peptides were eluted from the trap column. The following gradient was used: 0 min- 3% B, 30 min- 40% B, 32 min- 85% B, 40 min- 85% B, 41 min- 3% B and 50 min- 3% B.

Nano Electrospray Ionisation Mass spectrometry

The NanoAcquity UPLC was coupled to a Waters Xevo TQ tandem quadrupole mass spectrometer. The instrument was operated in positive electrospray ionisation (ESI) mode. The temperature of the electrospray source was maintained at 70°C. The capillary voltage was set at 2.40 kV and cone voltage at 35 V. PicoTip emitters (10 µm internal diameter, New Objective, US) were used for the nanostage probe. Nitrogen was used for the purge gas set at 2 L/h, the cone gas set at 5 L/h and the nanoflow gas pressure was set at 0.24 bar. Samples were analysed using selected reaction monitoring (SRM) mode for the peptide transitions as detailed in Section 6.2.3 and Appendix Table 7 (Section 9.7). The collision gas was argon and the collision energy was set according to each optimised peptide, as set out in Section 6.2.3 and Appendix Table 7 (Section 9.7). The dwell time was set to 100 ms and the resolution was one m/z unit at peak base. The data was acquired using MassLynx 4.1.

2.5 High Definition Multiplexed Quantitative Protocol

A recent study by Keshishian *et al* [92] has revealed the promising use of a multiplexed, quantitative workflow to identify novel markers in early myocardial injury. They have managed to identify just over 5000 proteins from plasma sample. This methodology

will be the basis for the discovery phase in this cohort. The steps are summarised in Figure 2.1.

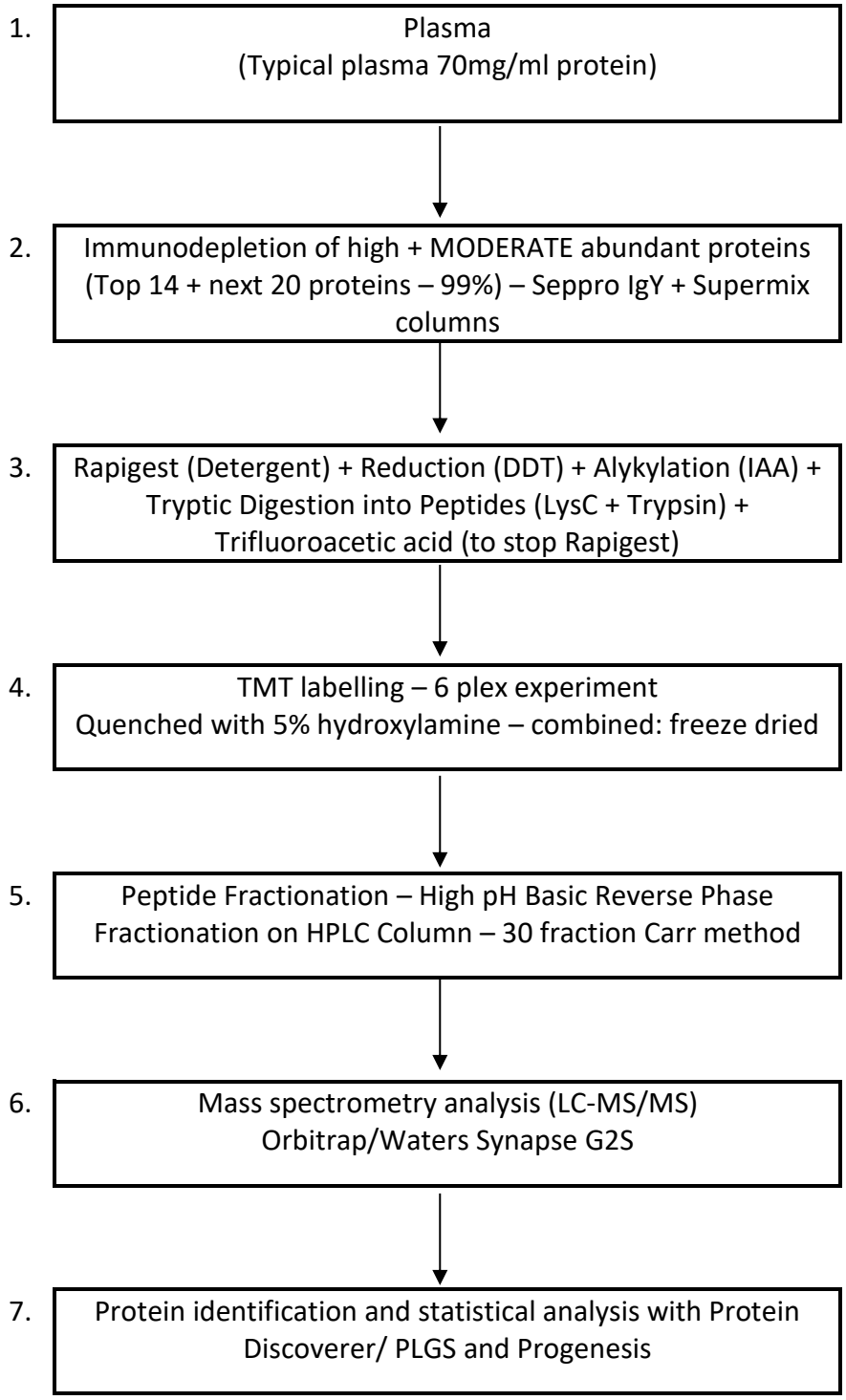


Figure 2.1: Summary of analytical steps for proteomic based discovery of plasma samples.

2.5.1 Immunodepletion

Human plasma has a high dynamic range and is incredibly complex with 95% constituted by highly abundant proteins, for example albumin, immunoglobulins and fibrinogen. The dynamic range spans up to 12 orders of magnitude [93].

Immunodepletion helps reduce the complexity and dynamic range of plasma, by removing highly abundant proteins. Two types of immunodepletion method were explored, first with the Multiple Affinity Removal Column Human 14 (MARS 14) (Agilent Technologies, Santa Clara USA). The MARS 14 column contains affinity ligands which bind to the top 14 human proteins (albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin, fibrinogen, alpha2-macroglobulin, alpha1-acid glycoprotein, IgM, apolipoprotein AI, apolipoprotein AII, complement C3 and transthyretin) and removes 94% of total protein. The column is connected to a High Performance Liquid Chromatography (HPLC) unit, with two buffers as supplied from the manufacturers and the timetable gradient as per manufacturer's instructions. The low abundant proteins are collected at the first peak on the chromatogram, and the high abundant proteins are eluted off in a second peak in the gradient. The advantage of this method is that the collection time is short (~ 6 minutes), total run time is relatively short (40 minutes per run) and the columns are cheaper (£400 per column for 100 uses) compared to a Seppro Supermix column. A typical immunodepletion chromatogram of a 40 µL plasma sample of human pancreatic adenocarcinoma patient is shown in Figure 2.2.

The second method explored involves the use of the Seppro IgY14 + Supermix Column (Sigma-Aldrich, Dorset). These columns employ avian antibody (IgY)-antigen interactions to remove selected proteins. The IgY14 column removes the top 14 highly abundant proteins similar to the MARS 14 column. The Supermix column resin contain avian antibodies which target the next 20 moderately abundant proteins. The columns are run in series, with injected plasma sample first running through the IgY14 column then the flow through runs into the Supermix column: this removes ~ 99% of total protein from the sample. Three buffer solutions as provided by the manufacturers are used, namely a Dilution Buffer (Tris-buffered saline TBS – 100 mM, Tris-HCl with 1.5

M NaCl, pH 7.4), a Stripping Buffer (1 M Glycine, pH 2.5) and a Neutralization Buffer (1 M Tris-HCl, pH 8.0) (all Sigma-Aldrich, Dorset). The manufacturers suggest utilising a larger LC5 sized IgY14 column coupled to the LC2 Supermix column. We have elected to use two similar sized (LC2) columns for both, as a compromise between cost and overall performance. Alterations were made to the gradient timetable to reflect these changes: 20 µL of plasma (diluted 5x with dilution buffer) was injected onto the HPLC with the columns in series with a run time of 30 minutes, where the low abundant proteins are collected from ~ minute 13 to minute 23. The columns are then uncoupled, with only the IgY 14 column connected for a wash, striping and neutralisation run, for 36 minutes. The Supermix column is then attached, for a wash, stripping and neutralisation run for 28 minutes. Figure 2.3 shows a typical combined chromatogram of an IgY 14 + Supermix immunodepletion run. The main advantage of this method is that 99% of total protein is removed, which further reduces the dynamic range of the plasma enabling identification of rich low abundant proteins. The disadvantages are the length of time per sample (94 minutes), smaller amount sample per run (20 µL vs 40 µL on MARS14) and cost (£6000 for both columns per 100 uses).

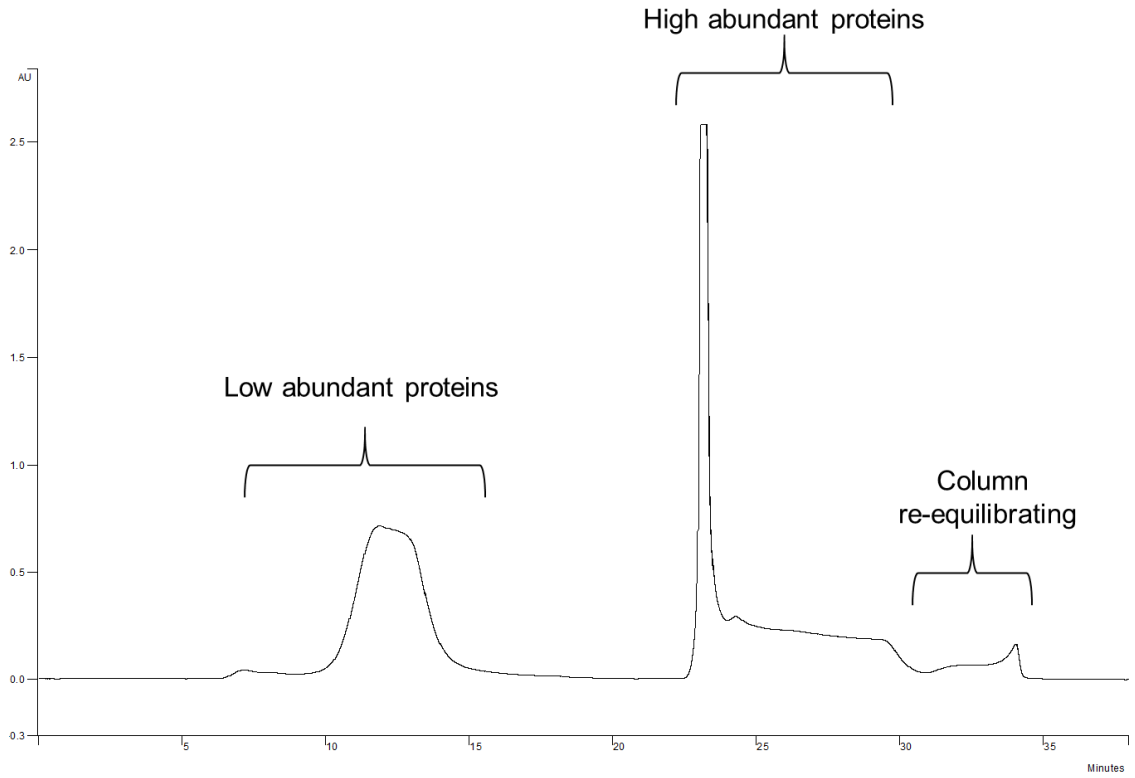


Figure 2.2: Typical MARS 14 chromatogram of human plasma with pancreatic adenocarcinoma. The total run time is 40 minutes. The first peak represents low abundant proteins of interest, which were collected. The second peak represents high abundant proteins and the final peak represents the column re-equilibrating.

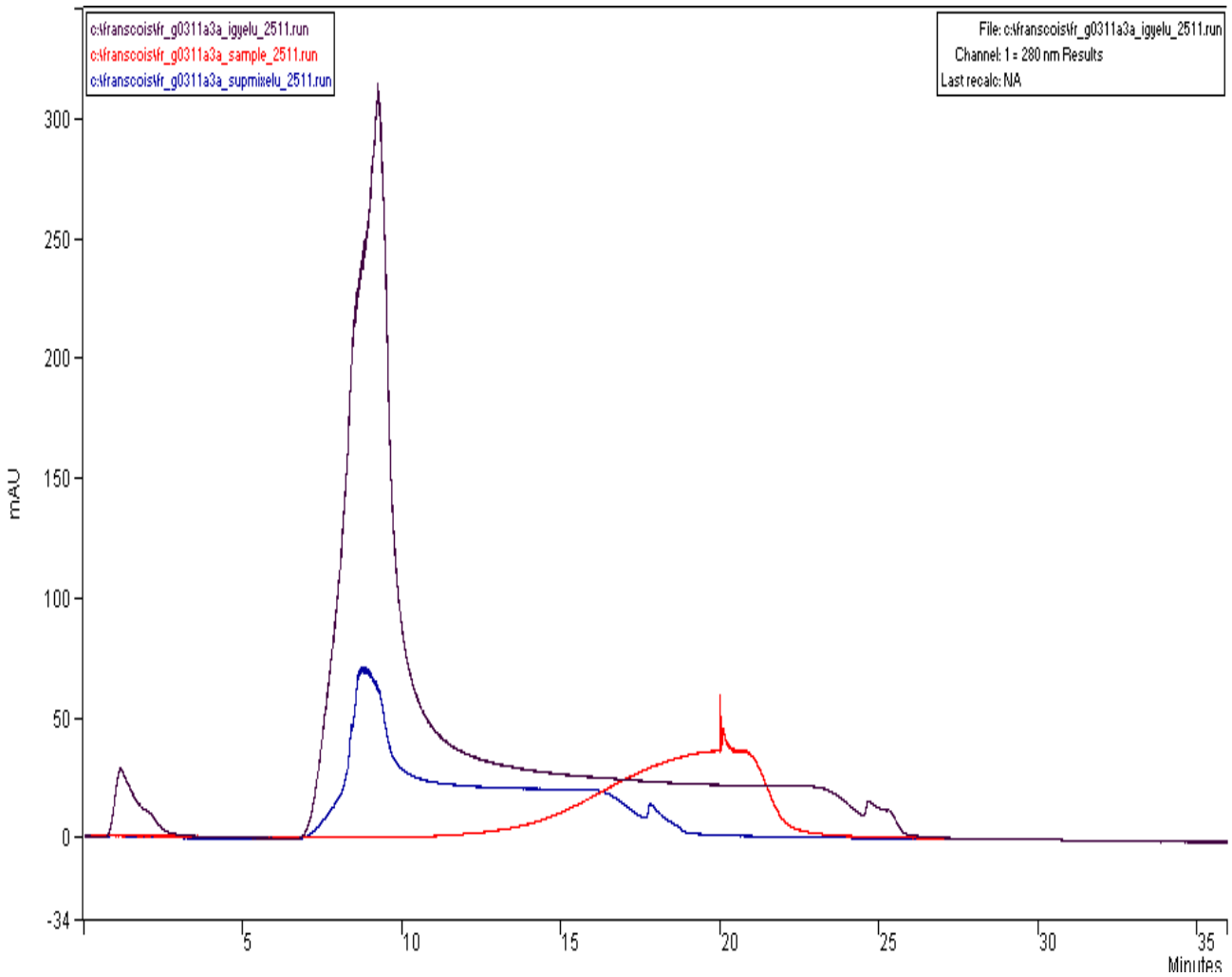


Figure 2.3: Typical chromatogram following injection of 20 µL plasma sample immunodepleted on IgY 14 + Supermix Columns: Red = Plasma Sample with low abundant protein collected from minute 13-23, Purple = IgY14 column with high abundant protein eluted and column re-equilibration, Blue = Supermix column elution and column re-equilibration. Total run time is 94 minutes.

2.5.2 Protein Concentration, Buffer Exchange and Protein Assays

Collected flow-through of low abundant proteins following immunodepletion is high volume (for MARS 14 typically ~ 1.5 ml, for IgY14 + Supermix column ~ 7.5 ml) and is high in salt concentration which makes it unsuitable for further analysis. Centrifugal filters are used to purify and concentrate the proteins. An Amicon Ultra 3K (either 0.5 ml or 15 ml volume limit) Filter (Merck Milipore, Darmstadt) is a filter unit with an Ultracell regenerated cellulose membrane restricting proteins up to 3000 molecular weight cut off. It concentrates and purifies the protein, typically reducing the end volume to about 200-300 µL. It can also be used to desalt and buffer exchange the protein solution. In these experiments, the solution is buffer exchanged to either 50 mM Ammonium Bicarbonate (ABC) or 50 mM Triethyl Ammonium Bicarbonate (TEAB).

Protein concentration in the sample is then determined using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham USA). The BCA protein assay utilises the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium, coupled with the colorimetric detection of the cuprous cation (Cu^{1+}) by bicinchoninic acid (BCA)[94]. The BCA/ Cu^{1+} complex exhibits a linear absorbance at 562 nm with increasing protein concentration. Using a known linear standard of protein (Bovine serum albumin, from concentrations 0 to 1 mg/ml), a reference line of known protein concentration to absorbance units, otherwise known as a calibration line, from which the concentration of the sample tested (concentrated and desalted plasma sample) can be deduced.

2.5.3 Proteins to Peptides: Rapigest, Reduction, Alkylation and Digestion

Once the concentration is known, together with the volume we are able to determine the total amount of protein per sample. The next step is breaking the complex protein structures into peptides for MS identification. Rapigest SF Surfactant (Waters Ltd) is a reagent used to enhance enzymatic digestion of proteins by ensuring that the protein structure is suitably unfolded for subsequent digestion. It is an acid labile detergent and thus helps solubilise proteins, making them more amenable to enzymatic cleavage without inhibiting enzyme activity. Unlike conventional detergents, it is MS compatible

as it can be removed by treatment through acid hydrolysis. Another advantage is it does not interfere with trypsin or Lys-C enzyme. Rapigest is added to our sample to produce a 0.1% final concentration. (e.g.: to a 100 µL sample, 10 µL of 1% Rapigest is added. This is incubated at 80°C for 45 minutes.

Dithiothreitol (DTT) is a reducing agent used to stabilise enzymes and proteins which possess free sulphydryl groups. It reduces disulphide bonds and maintains monothiols in a reduced state. The breakage of disulphide bonds in effect straightens the protein into a less complex form. A solution of 100 mM of DTT is made using a buffer solution of 50 mM ABC. DTT is added to the sample solution to give a final 15 mM final concentration. The sample is incubated at 60°C for 30 minutes.

Iodoacetamide (IAA) is an alkylating agent used for peptide mapping. It covalently binds to the thiol group of cysteine so the protein cannot form disulphide bonds. This prevents the peptide chains from reforming protein complexes. This minimizes the appearance of unknown masses from disulphide bond formation and side-chain modifications, improving detection of cysteine-containing peptides. Alkylation with IAA increased the mass of a peptide by 57.02 Da for each cysteine present. IAA is sensitive to light and is always freshly prepared. A 200 mM solution of IAA is dissolved in 50 mM ABC. The IAA solution is added to the sample to give a 20 mM final concentration of IAA. The sample is incubated in the dark at room temperature for 30 minutes.

Protein digestion occurs using two types of MS-grade endoproteinases: Sequencing Grade Modified Trypsin (Promega, Madison USA) and Lys-C Protease MS-Grade (Thermo Fisher Scientific, Waltham USA). Trypsin is a serine protease that specifically cleavages the protein on the carboxyl side of arginine and lysine residues. However, cleavage can be slowed or blocked in a sequence-dependant manner (e.g. with multiple adjacent cut sites). Peptide fragments with one or more missed cleavages are common, but still need to be considered during mass analysis. Lys-C specifically cleavages on the carboxyl side of lysine residues. The addition of Lys-C is to increase protein/peptide

coverage and reduce the number of missed cleavages. A 20 µL solution of HPLC grade water is added to a vial of 20 µg of Lys-C (to make a concentration of 1 µg/µL). Lys-C is added to the sample in a 1:25 volume: volume ratio (i.e.1 µg/µL of Lys-C per 25 µg/µL of protein) and incubated at 37°C for 2-4 hours. Following this, trypsin at 1 µg/µL (similarly adding 20 µL of 50 mM ABC to 20 µg of trypsin), which is then added on the sample at 1:25 volume: volume ratio. This is incubated at 37°C for 16-24 hours (overnight).

The enzymatic reactions and Rapigest SF is stopped by adding Trifluoroacetic Acid (TFA) (Fisher Scientific, Loughborough). TFA (initial concentration of 100%) is added to the solution to make up to 0.5% final concentration. This is incubated at 37°C for 45 minutes. The sample is then centrifuged at 13,000 RPM for 10 minutes to produce a pellet. The supernatant is transferred to a clean vial for the next stage of the analysis. Table 2.1 shows a sample workflow for reduction, alkylation and protein digestion. Once the peptide samples are ready, freeze dried and stored in -80°C until the next stage.

Sample volume	Total protein	Rapigest 1% to make 0.1%	DTT 100mM to make 15mM	IAA 200mM to make 20mM	Lys-C to add (1:25 v:v)	Trypsin to add (1:25 v:v)	TFA to make 0.5%
100 µL	100 µg	10 µL	16.5 µL	12.7 µL	4 µg	4 µg	0.7 µL

Table 2.1: Example workflow of concentration and volume for protein reduction, alkylation and tryptic digestion

2.5.4 TMT-6 Labelling

Once the proteins have been tryptically digested to peptides, isobaric tags are applied to each tryptic peptide sample. Isobaric tagging allows multiplexed relative quantitation

by MS, increased sample throughput and fewer missing quantitative channels among samples. Tandem Mass Tag Sixplex (TMT-6) (Thermo Fisher Scientific, Waltham USA) is the isobaric tag used in our experiments. Each mass-tagging reagent has the same nominal mass (i.e. isobaric) and a chemical structure composed of an amine-reactive NHS-ester group, a spacer arm and an MS/MS reporter (Figure 2.4). The Mass reporter in a TMT-6 reagent consists of masses 126, 127, 128, 129, 130 and 131 Daltons.

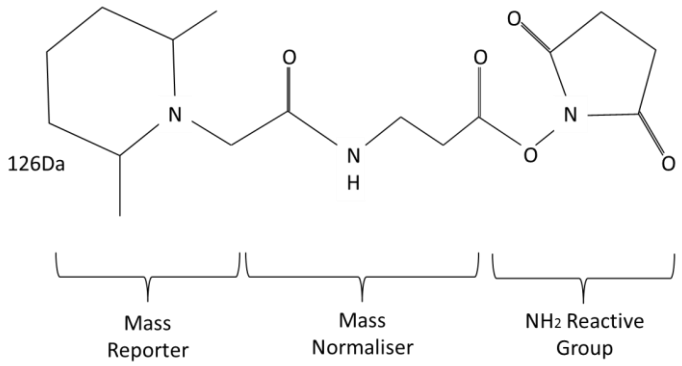


Figure 2.4: Structure of TMT isobaric tag label.

The amine reactive group binds to the peptides in each sample labelled, hence every peptide in that sample will be labelled with a unique reporter in the low mass region of the MS/MS spectrum (e.g. 126 to 131 Dalton in TMT-6 experiments) and this allows relative protein expression and quantitation. During multiple comparison experiments using more than one TMT-6 sets of labels, one of the channels (e.g.: TMT-126 Da) is set as a control channel for comparison and a Quality Control (e.g.: combined samples from all samples as a representative control) will also be prepared.

The steps in TMT labelling of the peptide samples are initial equilibration of the TMT label reagents to room temperature. Anhydrous acetonitrile at a volume of 41 µL is then added to each 0.8 mg TMT vial, allowing for the reagent to dissolve for 5 minutes with occasional vortexing. Between 25-100 µg of the dried digested peptides are reconstituted in 100 µL of 50 mM TEAB (Thermo Fisher Scientific, Waltham USA). The 41 µL of TMT label reagent is then carefully added to each 100 µL peptide sample.

(Note: labelling more than 100 µg of peptides requires more TMT) The sample is left to incubate at room temperature for 1 hour. Eight µL of 5% hydroxylamine (reconstituted by diluting 50% Hydroxylamine in 50 mM TEAB) is added to the sample and incubated for 15 minutes to quench the reaction. The samples are then combined together in equal amounts in a new microcentrifuge tube. This is then freeze dried and stored at -80°C, prior to the next step.

2.5.5 Fractionation

Fractionation offers another step in reducing the complexity and wide dynamic range of the plasma proteome in order to identify low abundant proteins of interest and potential biomarkers of disease. In essence, fractionation breaks up the sample into numerous parts (fractions) which increases the relative concentration of low abundant proteins. There are various methods of fractionation including Cation/Anion exchange, 1-D SDS-PAGE, peptide isoelectric focussing and peptide high pH (Basic) reverse-phase chromatography. 1-D SDS-PAGE is an example of fractionation method at the protein level, whilst the other three fractionates at the peptide level. The main drawback of fractionation is the increased sample number for MS analysis which has a dramatic effect on throughput.

Cao *et al* [95] performed a systematic comparison of fractionation methods comparing the performance of 1-D SDS-PAGE, peptide isoelectric focussing and peptide high pH reverse-phase chromatography (hpRP-HPLC). This comparison identified that hpRP-HPLC produced the highest peptide resolution and yielded the best depth of analysis with detection of the largest number of known low abundant proteins. As fractionation was executed at the peptide level another advantage of this method was that it made it more compatible with quantitative biomarker validation methods such as stable isotope dilution multiple reaction monitoring. As such, we have elected to use hpRP-HPLC as the method of choice for fractionation of our samples.

The current in-house method of hpRP-HPLC fractionation involved a protocol gradient run of 24 fractions over 80 minutes, which are then concatenated into 11 fractions.

Concatenation allows the combination of early, middle and late fractions together which pools the peptides equally: this improves analysis coverage while maintains high throughput [96]. This was compared to a method from Keshishian *et al* [92] who ran a gradient for 64.5 minutes, with a prolonged first and final fraction, and concatenation of 84 early, middle and late fractions at 36 seconds each, with a combined total of 30 fractions to be analysed by LC-MS/MS. A comparison of the coverage and number of proteins identified comparing these two methods was performed and presented in the results section.

In summary the steps are: Tryptic digested peptide is reconstituted into the starting condition (97% Mobile Phase A: 3% Mobile Phase B) and injected onto an XBridge BEH300 C18 5µm column (Waters, Milford USA). A smaller column (1.0 x 150 mm column, part number 186003617) was available for fractionation of total protein <150 µg, however after several attempts at developing this method, a reliable and reproducible yield of results was not possible. This methodology was abandoned for a larger column (2.1 x 250 mm, part number 186003621) which was used for fractionation of total protein up to 400 µg. The column is connected to a Varian ProStar HPLC Solvent Delivery Module Model 230 and a Varian ProStar UV-VIS Detector Model 310 (Varian, Walnut Creek USA). The Mobile Phases are: Solution A – 200 mM Ammonium Formate, pH 10, Solution B: 10% 200 mM Ammonium Formate pH 10 + 90% HPLC Grade Acetonitrile. Flow rate is set at 0.2 ml/minute. For the in-house fractionation protocol, the starting gradient is 97:3 A:B is static for 8 minutes, then reduces down to 60:40 A:B to minute 63, then 12:88 A:B to minute 72 then finally back to 97:3 A:B at 80 minutes. Fraction 1 runs for 25 minutes, followed by 22 x 2 minute fraction collected until minute 69, then the final fraction for 11 minutes to 80. Concatenation are: fractions 1+2+ 13, 3+4, 4+15, 5+16, 6+17, 7+18, 8+19, 9+20, 10+21, 11+22, 12+23+24: this makes a final fraction total of 11 fractions for LC-MS/MS. The modified Keshishian method utilises similar mobile phases A and B, with an initial static 97:3 A:B for 5 minutes, then gradient down to 85:15 A:B to minute 13, then down to 72:28 A:B to minute 46, then 66:34 A:B to minute 51.5, then finally 40:60 A:B to minute 64.5. The first fraction runs for 7.05 minutes, and the final fraction runs

from minute 57.45 to 64.50. The corresponding middle fractions, were collected at 36 second intervals, with the early, middle and late fraction concatenated to 28 fractions, which together with the first and final fraction make up the 30 total fractions for LC-MS/MS. The UV detector lamp was set at 280 nm.

Once the samples were collected and concatenated, the samples were freeze dried and stored in -80°C prior to the next step, which is LC-MS/MS. Details of the LC-MS/MS instruments used for analysis are detailed in Section 2.4 above.

2.6 Peptide – Protein Identification

The spectra obtained from LC-MS/MS are saved as .raw files. These raw spectra are then processed on bioinformatics programs to identify each spectral match to a peptide and thence identifying proteins from the known peptides. ProteinLynx Global SERVER (PLGS) and Progenesis QI are two programs used to process raw data obtained from G2Si instruments (Waters, Milford USA). Protein Discoverer 2.1 is the equivalent data processing software developed by Thermo Fisher Scientific, Waltham USA for use on raw files obtained on the QE instrument. Scaffold Q+ 4.7 (Proteome Software, Portland USA) is another data processing software used for further corroboration and visualisation of the results.

2.6.1 PLGS and Progenesis QI

On PLGS and Progenesis QI, the first step is uploading a FASTA file, which is a text based format for representing peptide sequences in which amino acids are represented using single letter codes. The peptide sequences are obtained from UniProtKB, restricting to ‘Reviewed’ and ‘Homo-Sapiens’. Currently the database has 20,199 reviewed human proteins. This forms the database from which the software determines the peptide spectral matches and proteins. The raw files are uploaded onto the programs. The peak processing parameters are: Automatic MS TOF resolution, lock mass charge 785.8426 Da/e, Lock mass window: 0.25Da, Low energy threshold: 150.0 counts, Elevated energy threshold: 15.0 counts, Intensity threshold: 750 counts. The

workflow parameters are: Search type: Electrospray MS^E, Peptide and Fragment tolerance: Automatic, Minimum fragment ion matches per Peptide: 2, Minimum fragment ion matches per Protein: 5, Minimum peptide matches per Protein: 2, Maximum protein mass: 100000, Primary digest reagent: Trypsin, Missed cleavages: up to 2, Fixed Modifier Reagent: Carbamidomethyl C, Variable Modifier Reagents: Oxidation M, Deamidation Q, Deamidation N, Phophoryl-STY, False Discovery Rate: 4%.

2.6.2 Protein Discoverer (PD) 2.1

MS spectra obtained from the QE are processed with Protein Discoverer v 2.1 (Thermo Fisher Scientific, Waltham USA) software package. The processing workflow and consensus workflow for determining and scoring the peptides/proteins were modified to determine the highest yielding protein counts. Single and multiple search engine 'nodes' were trialled, specifically SequestHT and MS Amanda search 'nodes' – these search engine nodes use computational algorithms to interpret the MS/MS spectra matching them to the best peptide: peptide spectral matching (PSMs)[97]. The use of multiple search nodes and the use of iterative search nodes increases the absolute number and confidence of proteins identified[98,99]. Generic search parameters included: Precursor mass tolerance of 10ppm, Fragment mass tolerance 0.02 Da, Trypsin and Lys-C enzyme digestion, up to 4 missed cleavages, minimum peptide length 6. For TMT-6 labels: Fixed modifications TMT6plex/ +229.163 (Any) at the peptide N-terminus and fixed modification TMT6plex/+229.163 Da (K). All nodes had fixed modification of Carbamidomethyl/+57.021at cysteine. Variable modifications were: Oxidation M, Deamidation N, Glu -> pyro-Glu, Acetyl N terminus, Gln ->pyro-Glu (N terminus) and CarbamidomethylDTT2 cysteine. Alteration of co-isolation of the TMT reporter ion was experimented on, between 10% to 100% to identify the optimum coisolation threshold. The false discovery rate (FDR) was set at 1% for high and 5% for low score calculations.

2.6.3 Scaffold Q+ 4.7

Once the raw files are entered into PD, the processing workflow generates the primary search results from the search engine (e.g. SequestHT) and extracts the raw quantification values (the extracted reporter peak intensities from the TMT-6 labels) to produce an MSF file output. This MSF file can then be entered onto Scaffold 4.7 (Proteome Software, Portland USA) as a second software for authenticating the proteins and visualising the results. Scaffold identifies and quantifies the peptides using a different computational algorithm and search engine (XTandem!) for further corroboration of putative peptides. The settings used were a minimum of 95% protein identification and 95% peptides identification with a minimum of two peptides per protein.

2.6.4 Skyline 3.7

Skyline 3.7 (MacCoss Lab, Washington USA) is a software used for quantifying SRM based MS results [100]. Once peptides for corroborating particular proteins of interest have been identified, the peptides are checked against databases (https://www.proteomicsdb.org and https://blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure its proteotypicity and uniqueness to the particular protein. SRMAtlas (http://www.srmatlas.org) lists the most optimum peptide ion and top three specific fragment ions. These act as the search parameters on the triple quadrupole TQ producing a chromatogram, which when viewed on Skyline 3.7 quantifies the peak area of the chromatogram to correspond with the amount of peptide identified. The retention windows for scanning can be further narrowed to increase the sensitivity. Known concentration of unlabelled standards and heavy labelled peptides are used to produce a calibration curve. On Skyline, the curve is set to linear through zero, with structural modification of carbamidomethylation of Cysteine and normalised to heavy peptides (^{13}C, ^{15}N for C-terminus of Lysine and Arginine).

2.7 Lipid Removal Agent

A second method for depleting, hence reducing the complexity of plasma is via Lipid Removal Agent (LRA), obtained from Supelco, Sigma Aldrich. It is a synthetic calcium silicate hydrate, which is commercially available and used in biopharmaceutical production for the removal of lipids by tightly binding to lipids and lipoproteins [101]. This compound is added onto the plasma following which lipids and associated tightly bound proteins are removed. The remaining proteins are subsequently denatured (DTT), reduced (IAA) and tryptically digested before LC-MS/MS analysis. When applied to our n-3 fatty acid enriched plasma samples, the aim is to remove direct measurements of these lipids and discover downstream proteomic changes following administration of the n-3 fatty acids.

2.8 Bioinformatics Analysis

Data set from identified proteins are typically in the mid hundreds to low thousands range from a typical proteomic experiment on human plasma. These are complicated by the large amount of data about peptides, false discovery rates (FDR) and relative quantitation using isobaric labels. Computational software is necessary to process such large complex data sets whilst maintaining a consistency between samples and the studies. Subsequent informatics tools can be used to interpret the underlying biological changes that occur.

2.8.1 Protein Centre 3.15

Protein Centre 3.15 (Thermo Fisher Scientific, Waltham USA) is software developed for interpreting complex data sets of proteins, integrating the results to produce biological data. The list of proteins (with any corresponding fold changes between control and treatment) is uploaded onto the software and analysed according to the Gene Ontology (GO) Consortium (http://www.geneontology.org/). GO describes the gene function (obtained from the protein list), analyses the relationships between them and classifies functions along three aspects: **molecular function (**GO-MF: molecular activities of gene products), **cellular component (**GO-CC: where gene products are

active within the cell**)** and **biological processes (**GO-BP: pathways and processes made up of the activities of multiple gene products**)**. Of these, GO-BP is most useful in providing direct processes or pathways which are up or down regulated from the identified protein set.

Enrichment analysis identifies the GO terms in a set of genes that are significantly over represented in a given set [102]. Protein Centre performs this on protein list and compares it to the whole reference set of known human proteins. It calculates the ratio of proteins from a particular ontology within the dataset versus the reference whole human set (for example in relation to the coagulation cascade: Five proteins identified from 100 in analysed dataset = 5% versus overall reference of 50 proteins from 10 000 = 0.5%). Protein Centre performs the statistical tests (5% False Detection Rate, p value <0.05, Bonferroni Corrected for set variation) to suggest possible mechanisms of regulation or functional pathways which are activated.

2.8.2 Cytoscape

Cytoscape [103] 3.5 (http://www.cytoscape.org/what_is_cytoscape.html) is an open source Java based software platform used for visualising protein interaction networks, biological pathways and integrating the networks using different Apps. On Cytoscape, each protein is visualised as nodes with interaction between them annotated. Fold changes (comparing control vs treatment) can also be visualised within each protein node.

2.8.3 KEGG Pathway

KEGG Pathway (Kanesiha Laboratories, Kyoto, http://www.genome.jp/kegg/pathway.html) is a collection of manually drawn pathways maps representing different molecular interaction, reaction and relation networks, which broadly covers different human diseases, metabolisms and cancer pathways. Protein Centre identifies significantly up or down regulated pathways by enrichment analysis. Reactome (http://reactome.org/) is an online analysis tool which aids further in

visualising up or down regulated proteins (based on fold changes) in multiple KEGG pathways.

3 Method Development

3.1 Introduction

The basic steps for proteomic analysis of plasma firstly entails depletion of highly abundant proteins to uncover low abundant proteins of interest. The next step is to cleave the proteins into peptides. Next, via fractionation of peptides, this further sub-compartmentalises the peptides into smaller groups, which aids identification of low abundant proteins of interest. Subsequently, the peptides are processed on the mass spectrometer and finally peptide-protein identification is performed on software packages such as Protein Discoverer 2.1 (Thermo Fisher Scientific, Waltham USA).

Keshishian *et al* [92] established a workflow (see Section 2.5 and Figure 2.1) for a high definition, multiplexed quantitative protocol which formed the basis for this work. To the best of our knowledge, this is the first documented work involving proteomic analysis specifically on human plasma with advanced pancreatic adenocarcinoma receiving n-3 fatty acid. As such, method development was required to establish the optimum conditions to increase peptide and protein identification yield. Specifically, development was required in the aspects of immunodepletion of high and moderate abundance proteins, high-pH-Reverse Phase fractionation and peptide-protein identification on Protein Discoverer 2.1 (Thermo Fisher Scientific, Waltham USA) using iterative search methods. Finally, method development was performed on Lipid Removal Agent (LRA, Supelco, Sigma Aldrich, Dorset).

3.2 Immunodepletion

Patients with advanced pancreatic adenocarcinoma receiving the treatment arm of intravenous gemcitabine and n-3 fatty acid are henceforth labelled as G## e.g.: G01 = patient on treatment arm number 1. The time point measured are at baseline (prior to any treatment, either gemcitabine or n-3 fatty acid infusion, labelled: G##1/1/A), after 1 month of treatment (G## 1/3/B), and 2-months of treatment (G## 2/3/B). The protocol for immunodepletion with the MARS 14 (Agilent Technologies, Santa Clara USA) column is well established in the laboratory, with the methods as described above. 8

patients (G01, G03, G05, G07, G11, G13, G15 and G19) at three time points, baseline, 1 month and 2 months following treatment had 40 µL of plasma, filtered then immunodepleted. BCA protein assays were performed to quantify the protein amount as shown in Table 3.1.

Patient	Protein conc µg/µL	Vol-ume µL	Total protein µg	Protein conc µg/µL	Vol-ume µL	Total protein µg	Protein conc µg/µL	Vol-ume µL	Total protein µg
	Baseline (1/1/A)			1 Month (1/3/B)			2 Month (2/3/B)		
G01	0.835	160	**133.7**	0.748	180	**134.6**	0.606	210	**127.2**
G03	0.488	180	**87.9**	0.685	185	**126.7**	0.633	190	**120.3**
G05	0.731	185	**135.2**	0.646	180	**116.3**	0.448	210	**94.1**
G07	0.651	175	**114.0**	0.769	180	**138.5**	0.437	210	**91.7**
G11	0.554	185	**102.6**	0.907	180	**163.3**	0.634	200	**126.9**
G13	0.696	165	**114.8**	0.743	180	**133.7**	0.546	205	**112.0**
G15	0.761	175	**133.1**	0.736	175	**128.7**	0.623	205	**127.7**

G19	0.946	180	**170.2**	1.321	180	**237.8**	0.915	205	**187.5**

Table 3.1: Sample volume and total protein following MARS immunodepletion for G01 - G19 patients at baseline, 1 month and 2 months of treatment.

3.2.1 Direct LC-MS/MS analysis of Plasma

Direct analysis of unfractionated plasma was analysed on the G2Si (Waters, Milford USA) instrument. The plasma of patient G01 with advanced pancreatic adenocarcinoma, who received the treatment arm of intravenous gemcitabine and n-3 fatty acid, was compared at baseline, 1 month and 2 months of treatment. The immunodepleted samples were reduced with DTT, alkylated with IAA and tryptically digested overnight as per methods (as described in Sections 2.5.1, 2.5.2 and 2.5.3) freeze dried and reconstituted in 0.1% Formic acid:ADH (50:50%) to 20 µL vials. An initial triplicate of 2 µL injection for 55 minutes yielded a very rich chromatogram (~1.69 e7) which led to detector saturation and subsequent loss of mass accuracy. When processed in PLGS (Waters, Milford USA) (see Section 2.6.1), there were ~90 proteins identified but no quantification. Subsequently, the samples were diluted 1:10 and injected into the G2SI (Waters, Milford USA) for a 110 minute gradient with improved chromatograms (Figure 3.1). The chromatograms were processed in PLGS (Waters, Milford USA), with the parameters as described above and the resulting protein yield was ~ 110 protein hits, with quantification over 3 dynamic ranges. The triplicates injections at the three time points (Baseline, 1 month and 2 months) was further processed in Progenesis QI (Waters, Milford USA): this yielded 135 quantifiable proteins.

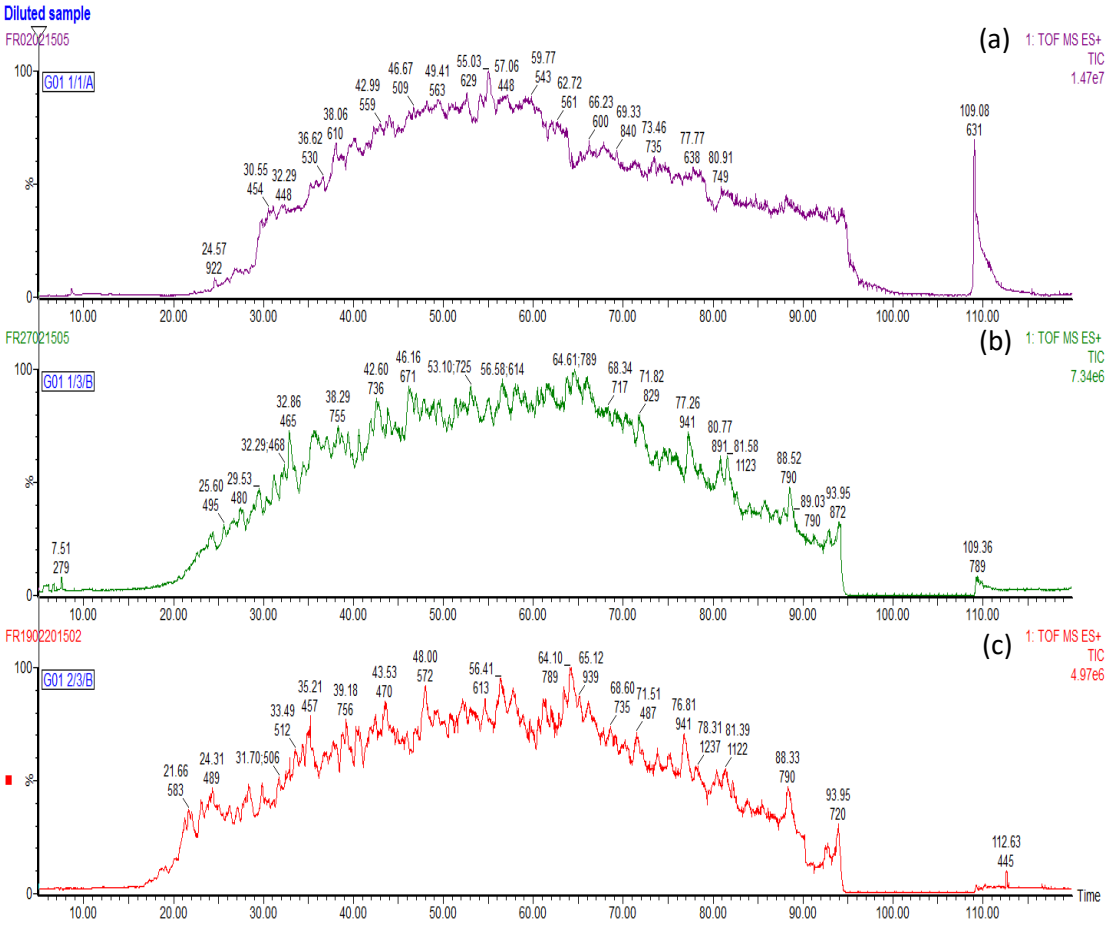


Figure 3.1: Chromatogram of diluted plasma G01 at baseline (a), 1 month (b) and 2 months (c), 110 minute gradient (See Section 2.4.2).

3.2.2 High pH-Reverse Phase HPLC Fractionation

An initial assessment of the current in house fractionation of an 80 minute run, over 24 fractions, concatenated to 11 fractions was examined. This method of injecting up to 400 µg of protein was tested on patient sample G03. Two time points were combined (1 month, 1/3/B + 2 month, 2/3/B) were combined to produce a 247 µg sample. The sample was reduced, alkylated, tryptically digested overnight and freeze dried as above. This was injected into an XBridge BEH300 C18 5 µm column (Waters, Milford USA), 2.1x250 mm (part number 186003621) connected to a VarianProstar HPLC (Varian, Walnut Creek USA) instrument using the protocol outlined above. An example fractionation chromatogram (which is typical for this method) is shown in Figure 3.4. The final 11 fractions were collected, freeze dried and processed on the G2Si instrument. (A chromatogram from a single fraction is shown in Figure 3.2). This was processed on PLGS (Waters, Milford USA): individually each fraction produced between 43 and 79 proteins. In combination, the total proteins yield was 140 proteins with a dynamic range of >3.5.

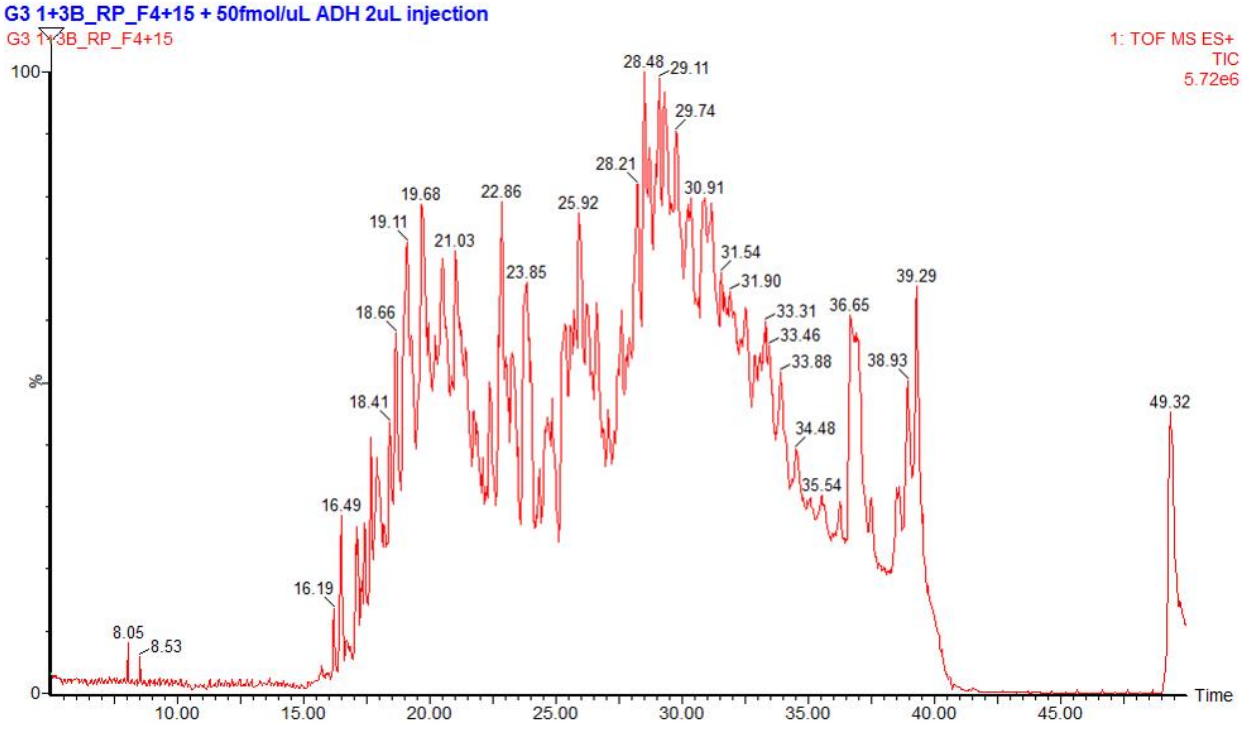


Figure 3.2: Typical chromatogram from fractionation of two combined fractions (Fractions 4 + 15). This fraction was injected onto a G2Si instrument with a 50 minute run and a satisfactory TIC value of 5.72e6 was obtained.

As the individual protein yield per sample after immunodepletion is less than 150 µg, hpRP-HPLC fractionation was tested for this amount of protein. Using the protocol for fractionation <150 µg using an XBridge BEH300 C18 5µm column (Waters, Milford USA) (1.0 x 150 mm column, part number 186003617), we prepared MARS 14 (Agilent Technologies, Santa Clara USA) immunodepleted patient sample G05 baseline 1 month and 2 month (each containing 135, 116 and 94 µg) of protein. The samples were reduced, alkylated and tryptically digested then freeze dried (as described in Sections 2.5.1, 2.5.2 and 2.5.3). This fractionation method however was unsuccessful despite repeated injections of different samples (including cell lysates), reconstituting the buffer solutions, changing the HPLC instrument and using an older column. The UV detection was unable to measure the peptides sufficiently and thus a judgement of its separation was unforthcoming: there was no reproducible fractionation of peptides on the chromatograms (Figure 3.3) and the collected fractions yielded minimal proteins

on LC-MS/MS. A decision to abandon fractionation at <150 µg was taken, and all further fractionation was performed up to 400 µg.

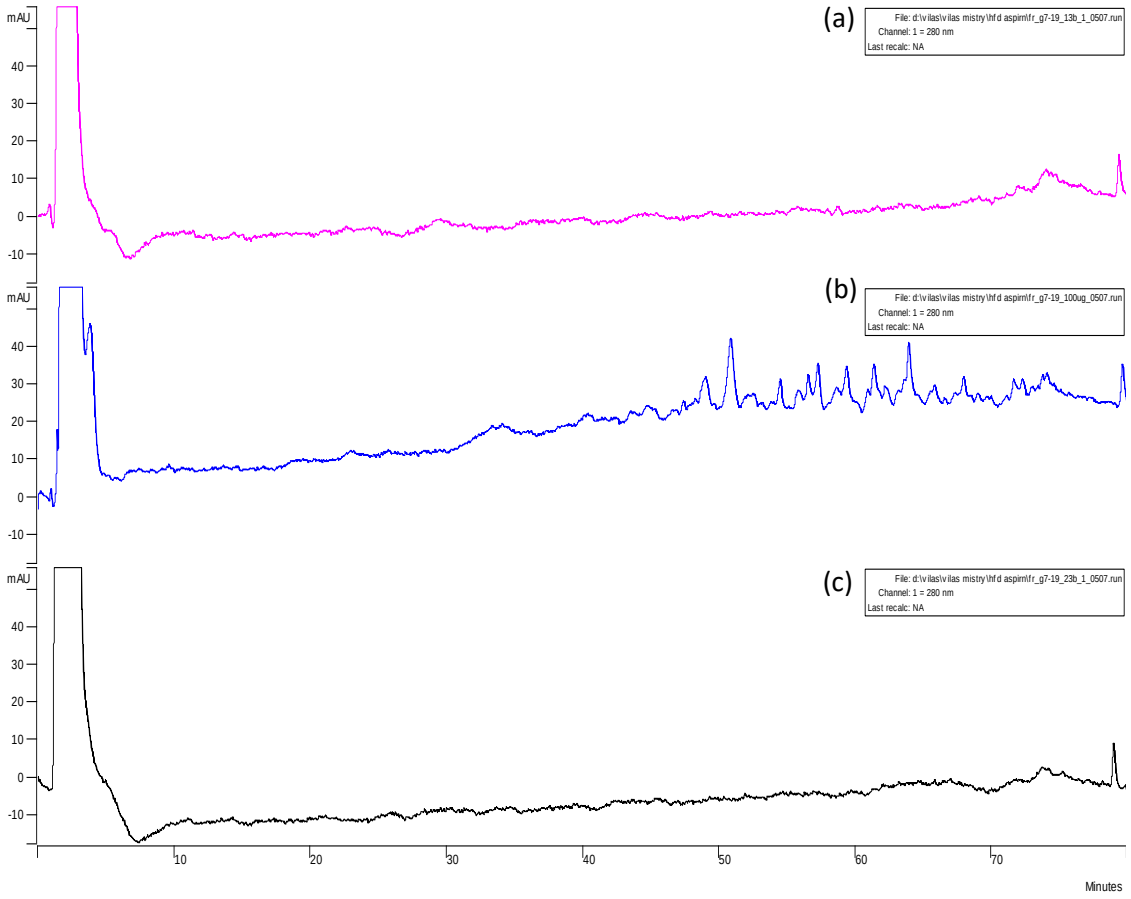


Figure 3.3: Chromatogram for hpRP-HPLC Fractionation <150 µg samples, no reproducible results and no peptides within the fractions. Injections of the immunodepleted G05 at baseline (a), 1 month of treatment (b) and 2 months of treatment (c) showed no significant peaks on chromatogram.

The second method of hpRP-HPLC fractionation modified from Keshishian *et al* [92] runs over 64.5 minutes with 86 fractions concatenated to 30 final fractions. We examined the efficacy of this method: a healthy volunteer human plasma (T3) was collected and prepared accordingly. Assuming a normal protein concentration of 70 µg/µL, two samples (T3a and T3b) 6 µL of neat plasma (un-immunodepleted ~ 420 µg protein per sample) was diluted, reduced, alkylated, tryptically digested, freeze dried and reconstituted for fractionation. The 11 fraction in house method vs the 30 fraction modified Keshishian method was performed (chromatogram shown in Figure 3.4), and

fractions were collected and prepared for LC-MS/MS analysis on the G2Si (Waters, Milford USA) instrument. Processing on Progenesis QI (Waters, Milford USA) revealed a 44% increased protein count on the 30 fraction method (215 vs 149 hits), with increased coverage of proteins (e.g. for albumin, 191 vs 165 unique peptides, confidence score 1450 vs 1291, in favour of 30 fraction method – Figure 3.5).

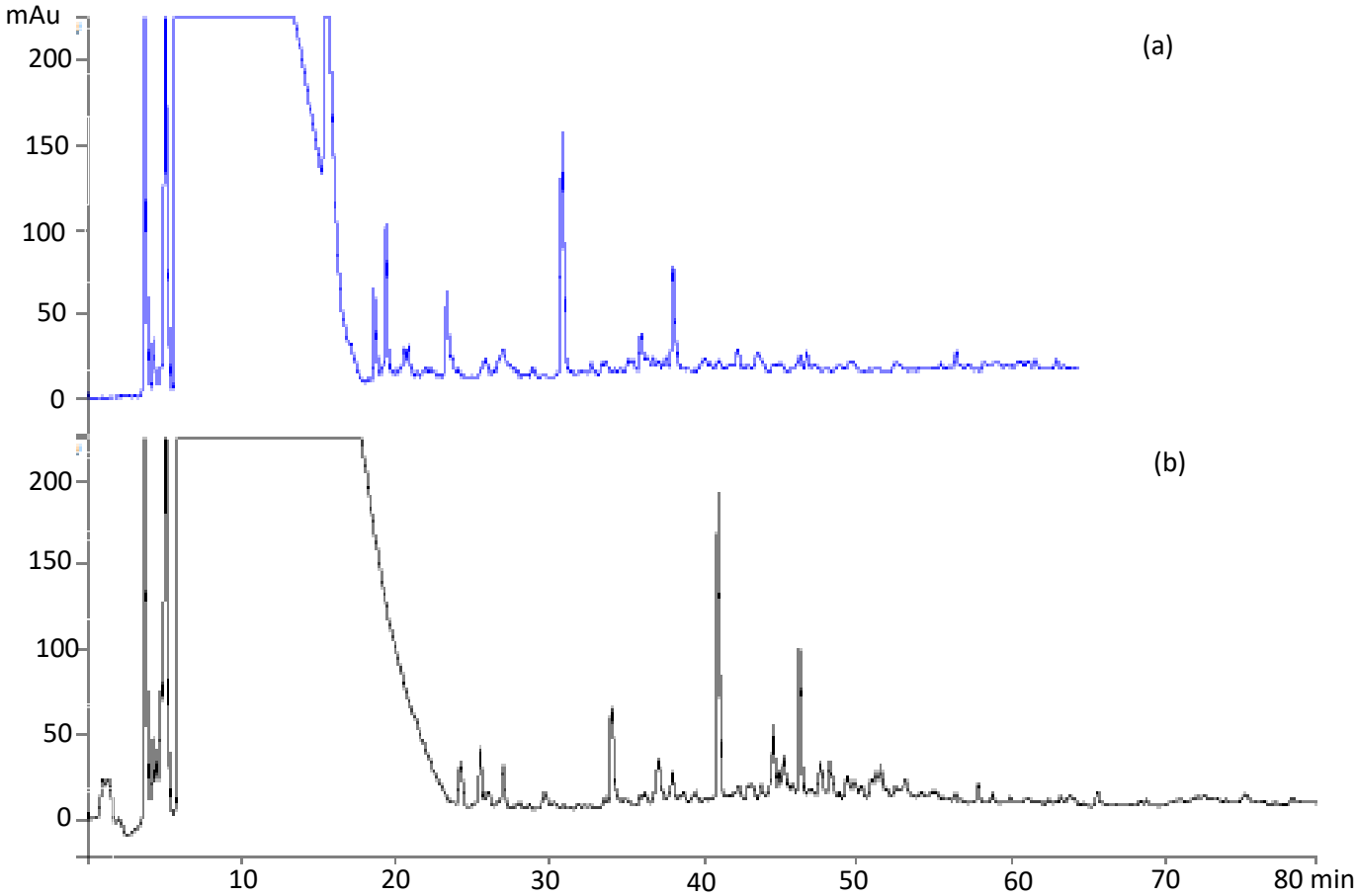
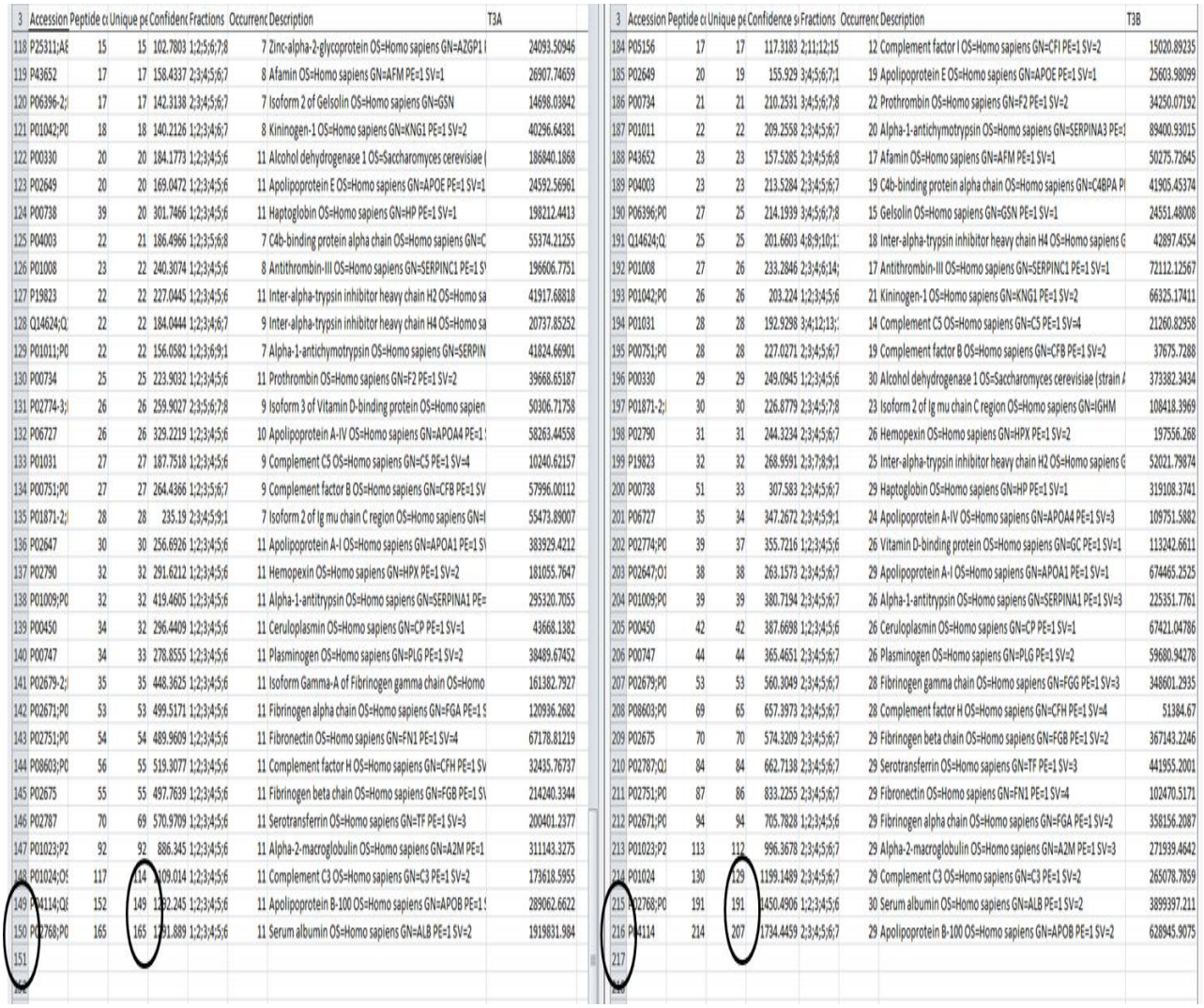


Figure 3.4: Chromatogram of patient T3a - 30 (a) vs T3b - 11 (b) fractionation method. Amount of protein injected ~ 420 µg each, 64.5 minute (a) vs 80 minutes in (b).



	Accession	Peptide cı	Unique pe	Confidenc	Fractions	Occurrenc	Description	T3A
118	P25311;AE	15	15	102.7803	1;2;5;6;7;8	7	Zinc-alpha-2-glycoprotein OS=Homo sapiens GN=AZGP1	24093.50946
119	P43652	17	17	158.4337	2;3;4;5;6;7	8	Afamin OS=Homo sapiens GN=AFM PE=1 SV=1	26907.74659
120	P06396-2;	17	17	142.3138	2;3;4;5;6;7	7	Isoform 2 of Gelsolin OS=Homo sapiens GN=GSN	14698.03842
121	P01042;P0	18	18	140.2126	1;2;3;4;6;7	8	Kininogen-1 OS=Homo sapiens GN=KNG1 PE=1 SV=2	40296.64381
122	P00330	20	20	184.1773	1;2;3;4;5;6	11	Alcohol dehydrogenase 1 OS=Saccharomyces cerevisiae (	186840.1868
123	P02649	20	20	169.0472	1;2;3;4;5;6	11	Apolipoprotein E OS=Homo sapiens GN=APOE PE=1 SV=1	24592.56961
124	P00738	39	20	301.7466	1;2;3;4;5;6	11	Haptoglobin OS=Homo sapiens GN=HP PE=1 SV=1	198212.4413
125	P04003	22	21	186.4966	1;2;3;5;6;8	7	C4b-binding protein alpha chain OS=Homo sapiens GN=C	55374.21255
126	P01008	23	22	240.3074	1;2;3;4;5;6	8	Antithrombin-III OS=Homo sapiens GN=SERPINC1 PE=1 S	196606.7751
127	P19823	22	22	227.0445	1;2;3;4;5;6	11	Inter-alpha-trypsin inhibitor heavy chain H2 OS=Homo sa	41917.68818
128	Q14624;Q	22	22	184.0444	1;2;3;4;6;7	9	Inter-alpha-trypsin inhibitor heavy chain H4 OS=Homo sa	20737.85252
129	P01011;P0	22	22	156.0582	1;2;3;6;9;1	7	Alpha-1-antichymotrypsin OS=Homo sapiens GN=SERPIN	41824.66901
130	P00734	25	25	223.9032	1;2;3;4;5;6	11	Prothrombin OS=Homo sapiens GN=F2 PE=1 SV=2	39668.65187
131	P02774-3;	26	26	259.9027	2;3;5;6;7;8	9	Isoform 3 of Vitamin D-binding protein OS=Homo sapien	50306.71758
132	P06727	26	26	329.2219	1;2;3;4;5;6	10	Apolipoprotein A-IV OS=Homo sapiens GN=APOA4 PE=1	58263.44558
133	P01031	27	27	187.7518	1;2;3;4;5;6	9	Complement C5 OS=Homo sapiens GN=C5 PE=1 SV=4	10240.62157
134	P00751;P0	27	27	264.4366	1;2;3;5;6;7	9	Complement factor B OS=Homo sapiens GN=CFB PE=1 SV	57996.00112
135	P01871-2;	28	28	235.19	2;3;4;5;9;1	7	Isoform 2 of Ig mu chain C region OS=Homo sapiens GN=I	55473.89007
136	P02647	30	30	256.6926	1;2;3;4;5;6	11	Apolipoprotein A-I OS=Homo sapiens GN=APOA1 PE=1 S	383929.4212
137	P02790	32	32	291.6212	1;2;3;4;5;6	11	Hemopexin OS=Homo sapiens GN=HPX PE=1 SV=2	181055.7647
138	P01009;P0	32	32	419.4605	1;2;3;4;5;6	11	Alpha-1-antitrypsin OS=Homo sapiens GN=SERPINA1 PE=	295320.7055
139	P00450	34	32	296.4409	1;2;3;4;5;6	11	Ceruloplasmin OS=Homo sapiens GN=CP PE=1 SV=1	43668.1382
140	P00747	34	33	278.8555	1;2;3;4;5;6	11	Plasminogen OS=Homo sapiens GN=PLG PE=1 SV=2	38489.67452
141	P02679-2;	35	35	448.3625	1;2;3;4;5;6	11	Isoform Gamma-A of Fibrinogen gamma chain OS=Homo	161382.7927
142	P02671;P0	53	53	499.5171	1;2;3;4;5;6	11	Fibrinogen alpha chain OS=Homo sapiens GN=FGA PE=1 S	120936.2682
143	P02751;P0	54	54	489.9609	1;2;3;4;5;6	11	Fibronectin OS=Homo sapiens GN=FN1 PE=1 SV=4	67178.81219
144	P08603;P0	56	55	519.3077	1;2;3;4;5;6	11	Complement factor H OS=Homo sapiens GN=CFH PE=1 SV	32435.76737
145	P02675	55	55	497.7639	1;2;3;4;5;6	11	Fibrinogen beta chain OS=Homo sapiens GN=FGB PE=1 S	214240.3344
146	P02787	70	69	570.9709	1;2;3;4;5;6	11	Serotransferrin OS=Homo sapiens GN=TF PE=1 SV=3	200401.2377
147	P01023;P2	92	92	886.345	1;2;3;4;5;6	11	Alpha-2-macroglobulin OS=Homo sapiens GN=A2M PE=1	311143.3275
148	P01024;O	117	114	709.014	1;2;3;4;5;6	11	Complement C3 OS=Homo sapiens GN=C3 PE=1 SV=2	173618.5955
149	P04114;Q	152	149	1282.245	1;2;3;4;5;6	11	Apolipoprotein B-100 OS=Homo sapiens GN=APOB PE=1	289062.6622
150	P02768;P0	165	165	1781.889	1;2;3;4;5;6	11	Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2	1919831.984
151								

	Accession	Peptide cı	Unique pe	Confidence sı	Fractions	Occurrenc	Description	T3B
184	P05156	17	17	117.3183	2;11;12;15	12	Complement factor I OS=Homo sapiens GN=CFI PE=1 SV=2	15020.89235
185	P02649	20	19	155.929	3;4;5;6;7;1	19	Apolipoprotein E OS=Homo sapiens GN=APOE PE=1 SV=1	25603.98099
186	P00734	21	21	210.2531	3;4;5;6;7;8	22	Prothrombin OS=Homo sapiens GN=F2 PE=1 SV=2	34250.07192
187	P01011	22	22	209.2558	2;3;4;5;6;7	20	Alpha-1-antichymotrypsin OS=Homo sapiens GN=SERPINA3 PE=1	89400.93015
188	P43652	23	23	157.5285	2;3;4;5;6;8	17	Afamin OS=Homo sapiens GN=AFM PE=1 SV=1	50275.72645
189	P04003	23	23	213.5284	2;3;4;5;6;7	19	C4b-binding protein alpha chain OS=Homo sapiens GN=C4BPA P	41905.45374
190	P06396;P0	27	25	214.1939	3;4;5;6;7;8	15	Gelsolin OS=Homo sapiens GN=GSN PE=1 SV=1	24551.48008
191	Q14624;Q	25	25	201.6603	4;8;9;10;1	18	Inter-alpha-trypsin inhibitor heavy chain H4 OS=Homo sapiens G	42897.4554
192	P01008	27	26	233.2846	2;3;4;6;14;	17	Antithrombin-III OS=Homo sapiens GN=SERPINC1 PE=1 SV=1	72112.12567
193	P01042;P0	26	26	203.224	1;2;3;4;5;6	21	Kininogen-1 OS=Homo sapiens GN=KNG1 PE=1 SV=2	66325.17411
194	P01031	28	28	192.9298	3;4;12;13;	14	Complement C5 OS=Homo sapiens GN=C5 PE=1 SV=4	21260.82958
195	P00751;P0	28	28	227.0271	2;3;4;5;6;7	19	Complement factor B OS=Homo sapiens GN=CFB PE=1 SV=2	37675.7288
196	P00330	29	29	249.0945	1;2;3;4;5;6	30	Alcohol dehydrogenase 1 OS=Saccharomyces cerevisiae (strain A	373382.3434
197	P01871-2;	30	30	226.8779	2;3;4;5;7;8	23	Isoform 2 of Ig mu chain C region OS=Homo sapiens GN=IGHM	108418.3969
198	P02790	31	31	244.3234	2;3;4;5;6;7	26	Hemopexin OS=Homo sapiens GN=HPX PE=1 SV=2	197556.268
199	P19823	32	32	268.9591	2;3;7;8;9;1	25	Inter-alpha-trypsin inhibitor heavy chain H2 OS=Homo sapiens G	52021.79874
200	P00738	51	33	307.583	2;3;4;5;6;7	29	Haptoglobin OS=Homo sapiens GN=HP PE=1 SV=1	319108.3741
201	P06727	35	34	347.2672	2;3;4;5;9;1	24	Apolipoprotein A-IV OS=Homo sapiens GN=APOA4 PE=1 SV=3	109751.5882
202	P02774;P0	39	37	355.7216	1;2;3;4;5;6	26	Vitamin D-binding protein OS=Homo sapiens GN=GC PE=1 SV=1	113242.6611
203	P02647;O1	38	38	263.1573	2;3;4;5;6;7	29	Apolipoprotein A-I OS=Homo sapiens GN=APOA1 PE=1 SV=1	674465.2525
204	P01009;P0	39	39	380.7194	2;3;4;5;6;7	26	Alpha-1-antitrypsin OS=Homo sapiens GN=SERPINA1 PE=1 SV=3	225351.7761
205	P00450	42	42	387.6698	1;2;3;4;5;6	26	Ceruloplasmin OS=Homo sapiens GN=CP PE=1 SV=1	67421.04786
206	P00747	44	44	365.4651	2;3;4;5;6;7	26	Plasminogen OS=Homo sapiens GN=PLG PE=1 SV=2	59680.94278
207	P02679;P0	53	53	560.3049	2;3;4;5;6;7	28	Fibrinogen gamma chain OS=Homo sapiens GN=FGG PE=1 SV=3	348601.2935
208	P08603;P0	69	65	657.3973	2;3;4;5;6;7	28	Complement factor H OS=Homo sapiens GN=CFH PE=1 SV=4	51384.67
209	P02675	70	70	574.3209	2;3;4;5;6;7	29	Fibrinogen beta chain OS=Homo sapiens GN=FGB PE=1 SV=2	367143.2246
210	P02787;Q1	84	84	662.7138	2;3;4;5;6;7	29	Serotransferrin OS=Homo sapiens GN=TF PE=1 SV=3	441955.2001
211	P02751;P0	87	86	833.2255	2;3;4;5;6;7	29	Fibronectin OS=Homo sapiens GN=FN1 PE=1 SV=4	102470.5171
212	P02671;P0	94	94	705.7828	1;2;3;4;5;6	29	Fibrinogen alpha chain OS=Homo sapiens GN=FGA PE=1 SV=2	358156.2087
213	P01023;P2	113	112	996.3678	2;3;4;5;6;7	29	Alpha-2-macroglobulin OS=Homo sapiens GN=A2M PE=1 SV=3	271939.4642
214	P01024	130	129	1199.1489	2;3;4;5;6;7	29	Complement C3 OS=Homo sapiens GN=C3 PE=1 SV=2	265078.7859
215	P02768;P0	191	191	1450.4906	1;2;3;4;5;6	30	Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2	3899397.211
216	P04114	214	207	1734.4459	2;3;4;5;6;7	29	Apolipoprotein B-100 OS=Homo sapiens GN=APOB PE=1 SV=2	628945.9075
217								

Figure 3.5: Comparison of plasma proteome analysis in un-immunodepleted plasma using 11 fractionation method (T3a) vs 30 fractionation method (T3b). Note in Human Serum albumin (P02768), there were higher number of unique peptides and a higher confidence score in the 30 fractionation method vs the 11 fractionation method.

3.2.3 MARS Immunodepletion + Fractionation

Immunodepleted samples from 5 patients (G07, G11, G13, G15 and G19) were combined at three separate time points (baseline, 1 month and 2 months of treatment) to assess serial changes in the plasma proteome. 80 µg of protein from each time point was combined for a total 400 µg at each time point (i.e.: G07-19 Baseline, G07-19 1 month, G07-19 2 month). The samples were reduced, alkylated, tryptically digested overnight and freeze dried, as described above. Reconstituted samples underwent hpRP-

HPLC fractionation with the in house, 11 fraction (400 µg) method with good separation (Figure 3.6).

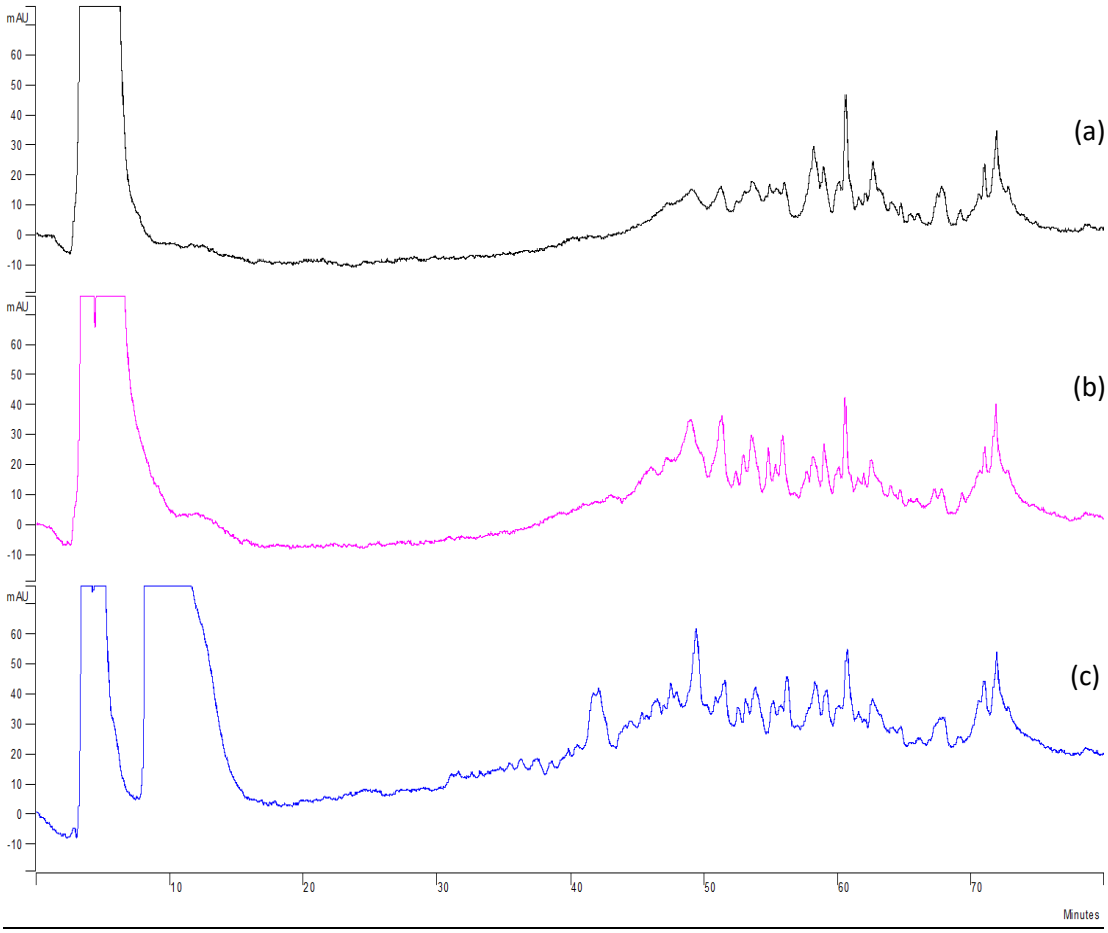


Figure 3.6: Successful fractionation of G07-19 Baseline (a) , 1 Month (b) and 2 Months (c) of treatment. Note the seperation of peptides with peaks occuring from minute 40 to 70 in all three injections, typical to the 11 fractionation methodology.

The collected fractions were freeze dried then reconstituted for triplicate injections onto the G2Si (Waters, Milford USA) instrument. The resulting fractionation spectra were combined and analysed on Progenesis QI (Waters, Milford USA): 543 protein groups were identified and quantified. 302 proteins had a p value <0.05 on ANOVA analysis based on changes from baseline, 1 month and 2 months of treatment with gemcitabine and n-3 fatty acids. Kim *et al* [104] compiled a list of 260 proteins related to pancreatic cancer with a p-value <0.05 from Oncomine, 18 studies from manual inspection filtered from the Plasma Proteome Database related to pancreatic cancer and four mutant forms

of KRAS, GNAS and AGER. Using this as a cross reference, 38 proteins of the 302 significant proteins were related to pancreatic cancer.

3.2.4 Immunodepletion with IgY14 + Supermix Columns

The method of immunodepletion using serial Seppro IgY14 + Supermix (Sigma-Aldrich, Dorset) columns to remove up to 99% of protein required development from the manufacturer's protocol, due to the size discrepancy of the suggested columns as described above. Patient G01 1/1/A was examined: A comparison of 40 µL plasma immune-depleted on the IgY-14 column alone produced a protein total of 201.4 µg vs 33.6 µg on the serial IgY14+Supermix column. Next, volume injections were compared for immunodepletion yield on the IgY14+Supermix column: 40 µL vs 20 µL plasma was depleted then prepared for LC-MS/MS injection into the G2Si instrument and processed on PLGS. The total number of proteins were broadly similar (82 vs 93) but the depletion of albumin was much lower in the 20 µL sample, 1.5% remaining albumin per total protein concentration vs 15% in the 40 µL sample. Following this, 20 µL of plasma was the ideal injection volume for optimum immuno-depletion of our sample using these columns.

3.2.5 Trial of Combined Immunodepletion, Fractionation and TMT-6plex

As the total protein per 20 µL depletion was low (~ 15-20 µg) and knowing that the amount required for fractionation is between 150 to 400 µg, the decision to immunodeplete 6 x 20 µL sample per patient was taken. Three patients (G03, G09 and G17) at two time points (Baseline vs 1-month) was performed (Table 3.2).

Patient		Total Protein (µg)	TMT-6plex Label
G03	Baseline (1/1/A)	131.1	126
	1-Month (1/3/B)	125.0	127
G09	Baseline (1/1/A)	99.0	128
	1-Month (1/3/B)	129.0	129
G17	Baseline (1/1/A)	54.4	130
	1-Month (1/3/B)	220.0	131

Table 3.2: Total protein concentration from IgY14 + Supermix column immunodepletion of 6x20 µL samples from 3 patients at 2 time points and the corresponding TMT-6plex label utilised for each sample.

A protein load of 60 µg from each (apart from all from G17 1/1/A) was prepared into individual Eppendorfs. Rapigest was added to the samples which were then reduced (DTT), alkylated (IAA) and tryptically digested, using 50 mM Ammonium Bicarbonate as the buffer solution. The freeze dried digested sample was then labelled with TMT-6plex as per Table 3.2. The samples were combined and freeze dried for hpRP-HPLC Fractionation (64.5 minute run, 30 concatenated fractions – chromatogram: Figure 3.7). Note that the first broad peak (Figure 3.7 – X axis minutes 5 to 20 represent the binding conditions of eluted peptides that interact weakly with the resin in the column.

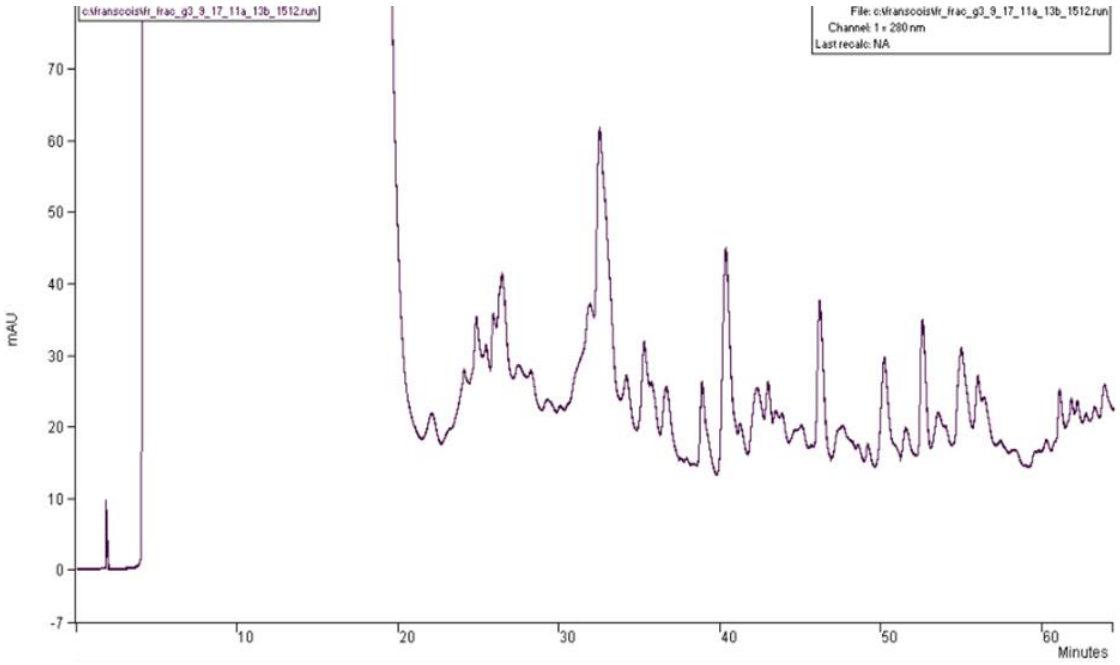


Figure 3.7: hpRP-HPLC Fractionation chromatogram of G030917, Baseline and 1 Month of treatment, all combined once dried digested immunodepleted peptides were labelled with TMT-6plex. Note the satisfactory separation of peptides with the corresponding peaks on the chromatogram.

All 30 concatenated fractions were freeze dried and reconstituted to 50:50 0.1% Formic Acid:ADH, 20 µL. These were injected into the QE (Thermo Fisher Scientific, Waltham USA) instrument for LC-MS/MS analysis. The raw files were processed on Protein Discoverer 2.1 (Thermo Fisher Scientific, Waltham USA).

3.3 Protein Discoverer 2.1

As described in the methods (See Section 2.6.2), iterative search engines with multiple search nodes increases the protein identification yield [98,99]. To optimise the processing and consensus workflow for our samples, a simple workflow (Figure 3.8(a)) with a single SequestHT search engine (with the parameters as described in methods) was applied onto a single Fraction 15 yielding 26 protein groups (Protein Groups constitute one master protein from which a peptide specifically identifies it). The search engine was subsequently expanded, with multiple search nodes (SequestHT and MSAmanda) each with individual fixed or variable modification (Figure 3.8(b)) yielding 120 protein groups. When the iterative search workflow was applied to all 30 fractions, without

quantitation channels on (no TMT fixed labels applied) the total protein groups identified was 731.

Coisolation is a problem in TMT labelling as the peptides from the samples are modified with isobaric labels. These labels fragment to create reporter tags that appear in the low-mass region of the fragment spectra: it is the intensity ratio of these tags that is used for relative quantification of peptides from different samples. However, coisolating peptides are fragments from other unrelated peptides which reduce the ratio intensity of the actual peptide of interest, rendering them indistinguishable. A series of experiments was performed to determine the ideal coisolation threshold on PD 2.1, starting from 10% with 10% increments up to 100%. A single fraction 15, from sample G030917 using a basic processing workflow and a consensus workflow with increasing coisolation threshold, with quantitative value corrections on. The total number of proteins identified was 26 protein groups, but the quantitation ratio increased at 60%, which is the default threshold for further analysis.

The multiple, iterative search engine processing workflow and quantitation consensus workflow with 60% quan corrected coisolation threshold was applied to all 30 Fractions for patient sample G03 + G09 + G17: 759 protein groups were identified. When cross referenced with the 260 known pancreatic cancer proteins, 98 were identified from the list compiled by Kim *et al*[104].

On further analysis however, it was discovered that label TMT-131 was positively identified in most of the sample, but the other labels (126, 127, 128, 129 and 130) were weakly identified. Further reading identified primary amine (such as Ammonium Bicarbonate) as a strong contra-indication to TMT labelling on peptides. Even though during the TMT labelling step, only 50 mM Triethyl Ammonium Bicarbonate (TEAB) was used as the buffer, it was discovered the use of 50 mM Ammonium Bicarbonate in the preceding steps (reduction, alkylation, tryptic digestion) contributed to the poor labelling of the peptides.

Table 3.3 summarises the samples analysed, experimental steps and final yield of proteins identified. Subsequent discovery work used combined immunodepletion (high and moderate abundant proteins) using Seppro IgY14 and Supermix Columns, followed by Rapigest, reduction with DTT, alkylation with IAA, protein digestion using Lys-C and Trypsin, TMT-6 labelling, 30 method fractionation, analysis on the QE (Thermo Fisher Scientific, Waltham USA) instrument and protein identification on Protein Discoverer 2.1 (Thermo Fisher Scientific, Waltham USA) using a multiple iterative search engine.

Cohort of patient and experimental analysis	LC-MS/MS Instrument and Processing Programme	Protein Yield
Patient G01, three time points (baseline, 1 month and 2 months), MARS immunodepletion, without fractionation	G2Si instrument + Progenesis QI	135
Patients G07, G11, G13, G15 and G19, three time points (baseline, 1 month and 2 months), MARS immunodepletion with 11 method fractionation.	G2Si instrument + Progenesis QI	543
Patients G03, G09 and G17, two time points (baseline and 1 month), IgY 14 + Supermix immunodepletion and 30 method fractionation	QE instrument + Protein Discoverer (Multiple Iterative search engine nodes without quantitation nodes)	731
Patients G03, G09 and G17, two time points (baseline and 1 month), IgY 14 +	QE instrument + Protein Discoverer (Multiple Iterative	759

Supermix immunodepletion and 30 method fractionation	search engine nodes with quantitation nodes)	

Table 3.3: Summary of different experimental models with increasing protein yield, when increasing intensity and depth of immunodepletion, increasing the number of fractions analysed and increasing the number of search engines utilised o PD 2.1.

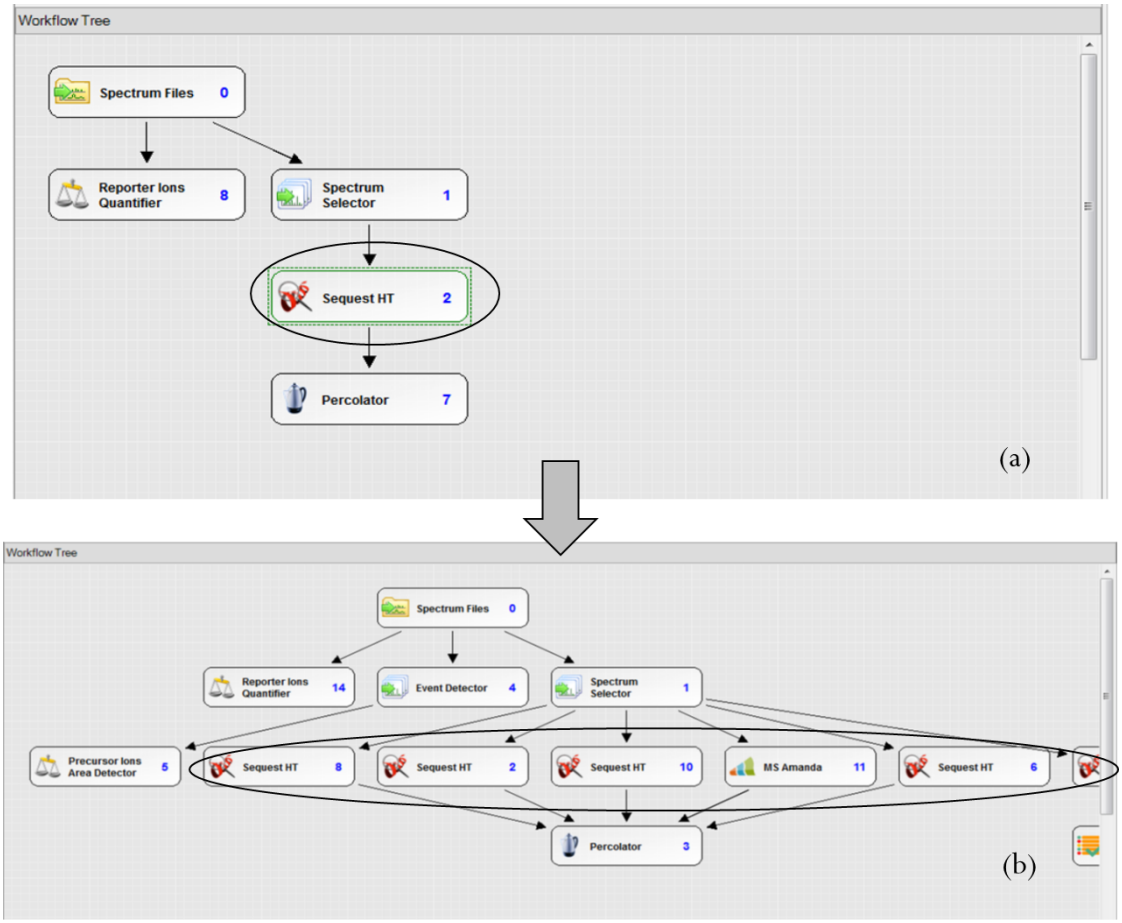
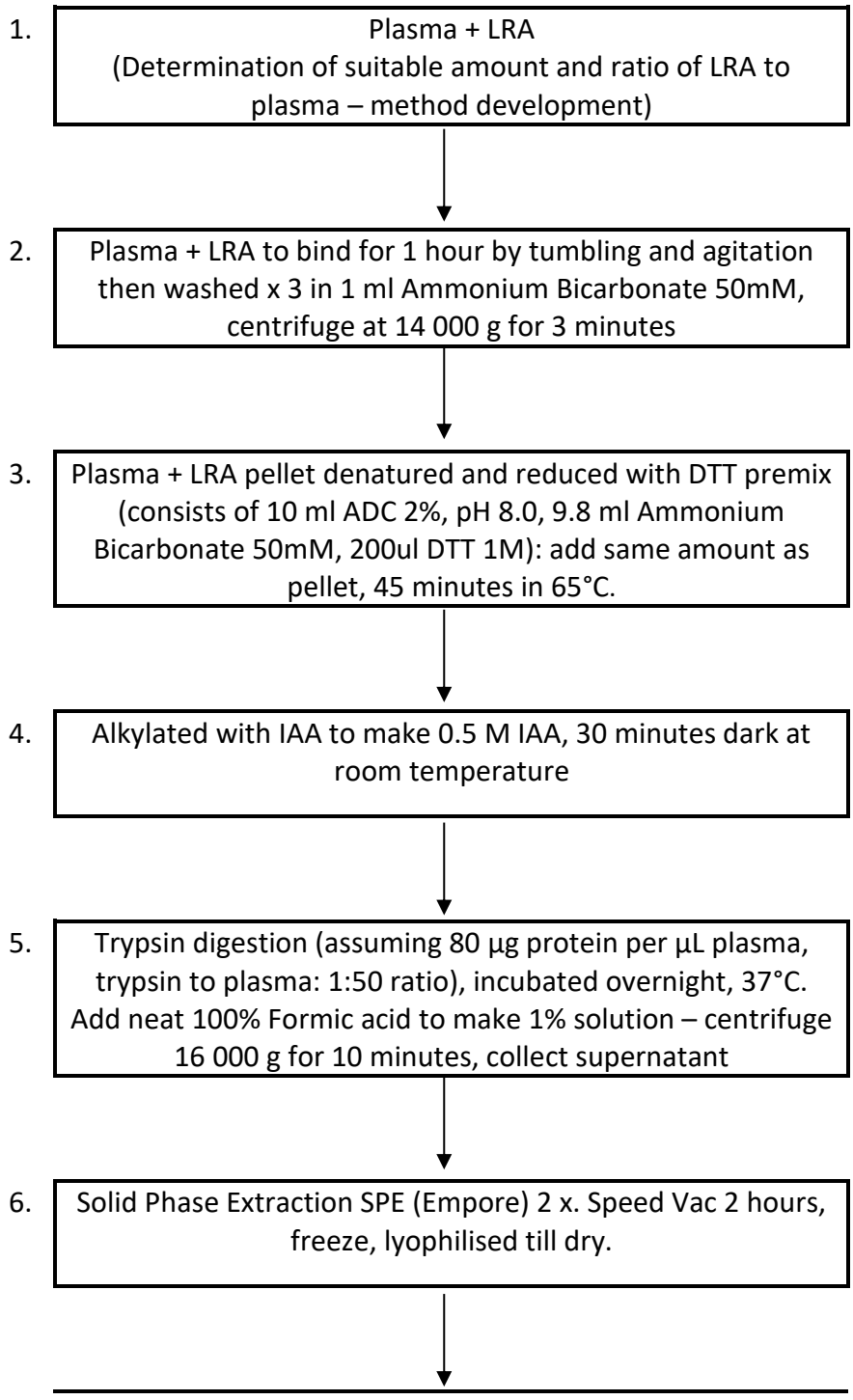


Figure 3.8: Processing workflow on Protein Discoverer 2.1, showing simple, single SequestHT search engine node (a) vs multiple, iterative search engine nodes (b).

3.4 Lipid Removal Analysis

The steps for LRA (Supelco, Sigma Aldrich, Dorset) is as follows in Figure 3.9.



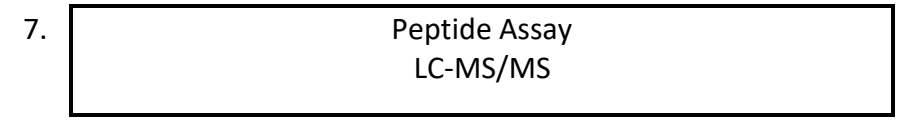


Figure 3.9: Workflow from plasma protein preparation using LRA protocol

LRA was utilised as a second method for plasma depletion. In determining the optimum ratio of LRA:plasma volume, an experiment with differing volumes (ranging from 6.25 μL to 50 μL plasma between a 1 part plasma to 2 or 4 parts LRA (1:2 vs 1:4). The corresponding protein hit numbers and protein concentration was obtained for comparison in Table 3.4.

Sample ratio plasma:LRA (A 1:4, B 1:2) & Plasma volume	Protein Hits				
	Replicate 1	Replicate 2	Replicate 3	Standard Deviation	Confidence Interval
A 50 µL	84	83	87	2.08	2.36
A 25 µL	92	94	86	4.16	4.71
A 12.5 µL	89	88	96	4.36	4.93
A 6.25 µL	95	90	95	2.89	3.27
B 50 µL	83	85	74	5.86	6.63
B 25 µL	78	79	79	0.58	0.65
B 12.5 µL	89	74	74	8.66	9.80
B 6.25 µL	63	72	60	6.24	7.07
	Protein amount (ng)				
A 50 µL	249.42	177.25	259.94	45.01	50.93
A 25 µL	246.46	224.76	230.82	11.19	12.67
A 12.5 µL	292.76	219.64	197.07	50.02	56.60
A 6.25 µL	223.85	153.84	189.05	35.01	39.61
B 50 µL	190.70	157.61	250.48	47.07	53.26
B 25 µL	159.29	137.72	122.97	18.27	20.68
B 12.5 µL	317.62	117.13	117.13	115.75	130.99
B 6.25 µL	69.68	98.11	58.63	20.37	23.05

Table 3.4: Protein hit number and amount (ng) for differing ratio (A 1:4 & B 1:2) and volume of plasma:LRA in triplicates. Based on these results, a 1:4 ratio of Plasma to LRA (A) and a higher volume of 25 to 50 µL is ideal.

Based on the above results, a ratio of plasma:LRA of 1:4 and a volume between 25-50 µL of plasma for the higher number of protein hits, protein amount and tighter confidence interval. A final volume of 40 µL of plasma (as the collected plasma were collected in 40 µL Eppendorfs) to 160 µL of LRA was decided upon.

3.5 Discussion

Intense immunodepletion of high and moderate abundant protein is applicable in plasma samples of patients with APC receiving n-3 fatty acids. Compared to immunodepletion of the top 14 highly abundant proteins using the MARS 14 (Waters, Milford USA) column, the number of proteins identified were broadly similar (93 vs 110 protein hits). However, the depletion of highly abundant proteins was more intense in the Seppro IgY14 + Supermix (Thermo Fisher Scientific, Waltham USA) with depletion of albumin down to 1.5% remaining in the sample. The depletion of these large, highly abundant proteins allows identification of the low and very low abundant proteins, which may elucidate the mechanism of action of n-3 fatty acid in patients with APC.

The biggest increase in protein identification was in immunodepleted samples coupled with hpRP-HPLC fractionation. Fractionation alone increased proteins identified 4 fold (543 from 135, see Table 3.3) on samples immunodepleted with MARS 14 (Waters, Milford USA) column. Using the 30 fraction method, the yield of proteins identified was increased further by 44%. A consequence of performing a higher number of fractions is the more frequent and labour intense collection of fractions from the HPLC, but mainly the higher number of samples which needed to be processed on the LC-MS/MS. As triplicate injections are typically performed, a single pooled experiment with the 30 fraction method will yield 90 injections into the LC-MS/MS, with a typical runtime of 82 minutes, incorporating blanks for cleaning, will take 164 hours ~ 7 days.

Once processed on the QE instrument, a multiple, iterative search workflow parameter on the processing software package PD 2.1 (Thermo Fisher Scientific, Waltham USA) increased the protein yield further. As described in Section 2.6.2, this workflow isolates each modification onto a single SequestHT or MS Amanda search ‘node’ compared to a single search ‘node’ with multiple modifications (as illustrated in Figure 3.8. The disadvantage of this workflow model is the computing processing required: a simple, single search node workflow will be completed in minutes, whereas a workflow

containing multiple, iterative search nodes will require hours or days depending on the size of the .raw file.

The Lipid Removal Agent, LRA (Supelco, Sigma Aldrich, Dorset) method was experimented on as an alternative method to immunodepletion. Subsequently the samples were analysed on the G2Si (Waters, Milford USA) with the proteins yields shown in Table 3.4. The individual yields were low (circa 90 proteins per sample) compared to the immunodepletion, fractionation and TMT labelling method. Even when multiple samples from different patients (baseline versus n-3 fatty acid versus control) were combined and analysed on the G2Si and Progenesis QI (Waters, Milford USA), the total protein hits numbered circa 200-300. As such, the chosen method for subsequent proteomic analysis in this cohort is based on the intense immunodepletion of high and moderate abundant proteins with Seppro IgY14 + Supermix Column (Sigma-Aldrich, Dorset), TMT-6 isobaric labelling, 30 method hpRP-HPLC fractionation, injection onto the QE instrument (Thermo Fisher Scientific, Waltham USA) and subsequent analysis using a multiple, iterative search parameter on PD 2.1 (Thermo Fisher Scientific, Waltham USA).

4 Results I: Comparison between Baseline versus Omega-3 Fatty Acid

4.1 Introduction

Upon administration of n-3 fatty acid intravenously, the active components of DHA and EPA enters the cell membrane immediately [105] to instigate its expected actions of reducing cellular inflammation, lowering pro-angiogenic factors and increasing pancreatic cancer cell death. These effects have been shown to occur in a dose dependant manner [26-28]. It is expected that various cancer specific pathways are altered on administration of n-3 fatty acid, with potential pathways including the nuclear factor-kB (NF- kB) pathway [24]. Other pancreatic adenocarcinoma related pathways of interest include the Pi3K-AKT, WNT and SMAD pathways, which will hopefully be elicited from this discovery phase of experiments. The cohort of patients investigated for this first discovery phase will compare treatment naïve (gemcitabine or n-3 fatty acid) patients at baseline compared to the same patients following a month of treatment with gemcitabine and n-3 fatty acid intravenously. As the effects of n-3 fatty acids are dose dependant, it is expected that the above effects are easily identified from the discovery phase.

Of interest is also the effect of administration of n-3 fatty acid on the plasma lipid profile, specifically the apolipoprotein lipids. It is expected that the apolipoproteins will reflect changes relating to increases in the high density lipoproteins, reduction of low and very-low density lipoproteins and lowering apolipoproteins related to acute phase reactions in keeping with its anti-inflammatory effects.

Utilising the method of intense immune-depletion, high-pH reverse phase fractionation and TMT-6 labelling described in Section 3.2.5, it is anticipated that this will provide high definition in depth changes to the proteome in plasma. As is often the case, biomarker proteins of interest are usually in the low femtomoles per micro litre region [106]. Identifying these proteins is challenging in itself, but with the use of TMT-6 it is

hoped that quantitative changes could be confidently reported on following treatment with n-3 fatty acid.

As the primary disease process is in advanced pancreatic adenocarcinoma, it is important that known markers of pancreatic adenocarcinoma identified confidently in order to corroborate this methodology. Another aspect for confirmation would be to confirm that inflammatory markers are downregulated in keeping with the known effect of n-3 fatty acid.

4.2 Materials and Methods

4.2.1 Patients

Three patients (G03, G09 and G17) were selected at random for the initial discovery phase of high definition plasma proteomic analysis, at baseline (prior to any treatment) and after one cycle (1 month) treatment with intravenous gemcitabine and n-3 fatty acid. The demographics are presented in Table 4.1.

Patient	Sex	Age	ECOG	Haemoglobin (g/l)	Neutrophil Count ($x10^9$/L)
G03	Male	54	0	116	6.5
G09	Male	69	0	126	5.4
G17	Male	62	0	128	7.2

	(Cont…)				
Patient	Serum Creati-nine (mmol/L)	Deranged Liver Function Tests	Previous / Other Cancers	Previous Chemo-therapy	Recent Major Surgery (within 4 weeks)
G03	83	No	No	No	No
G09	56	No	No	No	No
G17	55	No	No	No	No

Table 4.1: Demographic of patients analysed on commencement of trial, ECOG (Eastern Cooperative Oncology Group) Performance Status: 0 = Fully active, able to carry on all pre-disease performance without restriction.

4.2.2 Proteomic Plasma Analysis

The method as described by Keshishian *et al* [92] is summarised in Figure 2.1. In summary, it consists of intense immune-depletion (Seppro IgY 14 + Supermix columns), protein reduction, alkylation, two endoproteinases (Lys-C and Trypsin) digestion, TMT-6 plex labelling and 30 fraction high pH reverse-phase chromatography fractionation (Refer to Sections 2.5.1, 2.5.2, 2.5.3, 2.5.4 and 2.5.5). The fractions were subsequently injected onto the QExact-Orbitrap (QE, Thermo Fisher Scientific, Waltham USA) and processed on Protein Discoverer (PD, Thermo Fisher Scientific, Waltham USA) 2.1 and Scaffold 4.7 (Proteome Software, Portland USA) with bioinformatics analysis on Protein Centre 3.15 (Thermo Fisher Scientific, Waltham

USA) and Cytoscape [103] 3.5 (http://www.cytoscape.org/what_is_cytoscape.html)as described (Refer to Sections 2.4.3, 2.6.2, 2.6.3, 2.8.1 and 2.8.2).

4.3 Results

4.3.1 Protein Count

On PD 2.1 utilising the iterative methodology with a minimum one peptide count: A total of 2886 proteins were discovered using this methodology, from 8148 peptide groups, 507975 Peptide Spectral Matches and 990364 MS/MS spectrum (Section 9.3 Appendix Table 3). Mean number of peptide count 4.5 per protein, mean coverage 10.7 % [93.39 – 0.1%]. 90.23% had 0 missed cleavages, 9.01% 1 missed cleavage and 0.72% 2 missed cleavages. (Figure 4.1 a-e). 100% obtained high peptide confidence scores. Excluding isomers, 1403 with relative quantitation on TMT labels were identified. Fold changes were obtained comparing the two groups Baseline (TMT labels 126, 128 and 130) vs Treatment (TMT labels 127, 129 and 131). 828 proteins were up regulated (Fold change >1) and 575 proteins were down regulated (Fold change < 1) following one cycle of treatment with n-3 fatty acids and gemcitabine.

Within the proteomic field, it is generally accepted that fold changes of more than or less than 1.5 x (FC >1.5 or < 1.5) is the cut off to determine if there are any changes in the proteins identified following treatment. However, in this body of work, a generally positive or negative fold change (more or less than 1) was used in order to establish the overall mechanistic action of n-3 fatty acids in this cohort. Including more proteins in the bioinformatics analysis allows a more global approach in determining the mechanistic effect of how n-3 fatty acids improved progression free survival and quality of life.

Keshishian *et al* [92] identified >5000 proteins (with two peptides per proteins) in their plasma analysis using similar methods. Other groups have not since been able to reproduce this volume of protein discovery. In this experiment, with a minimum number of two peptides per proteins, 1488 proteins were identified. Our results may differ because of some modifications to the method: during plasma immunodepletion a

smaller sized LC2 IgY14 column was used (compared to the larger LC5 IgY14 column used by Keshishian *et al* [92]), due to restrictions in the more expensive cost of the larger column. With the larger column, a larger volume of plasma and hence higher amount of proteins were loaded for immunodepletion, which would lead to higher yield of proteins. However, compared to other plasma based proteomic analysis, a yield of 1500 proteins is accepted as a good amount and depth to cover the plasma proteome for identification of protein biomarker of interest [60]. Compared to other proteomic studies in pancreatic cancers, this experiment is again comparative with its yield and coverage, Kakisaka *et al* [107] identified 1200 proteins in pancreatic cancer plasma samples.

Figure 4.1 (a)

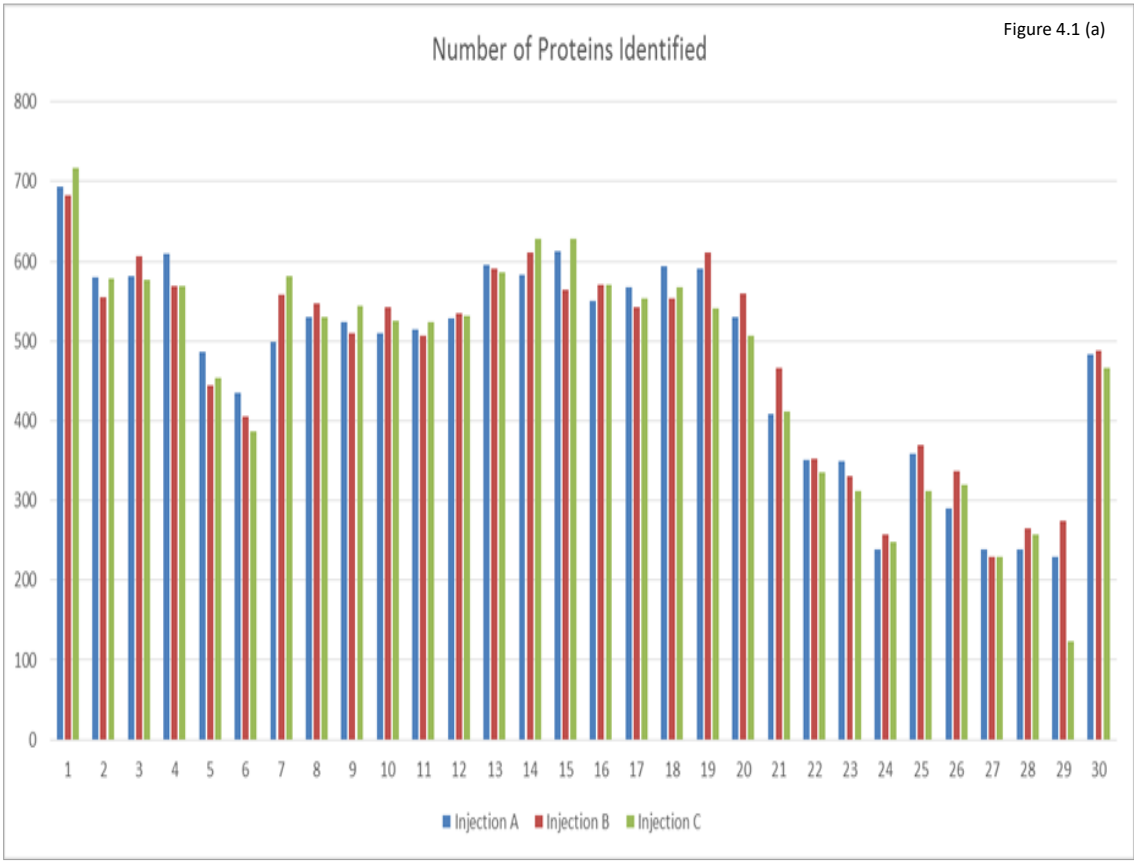
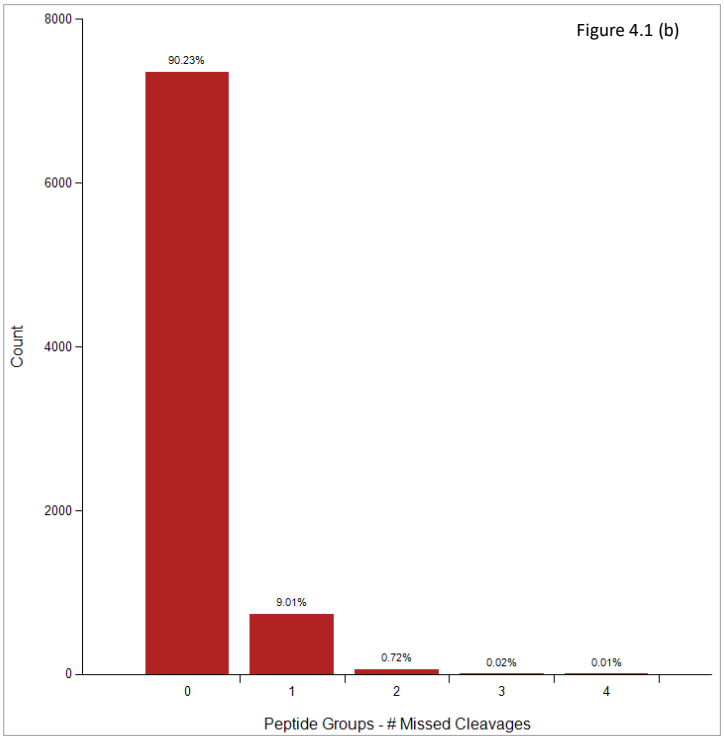
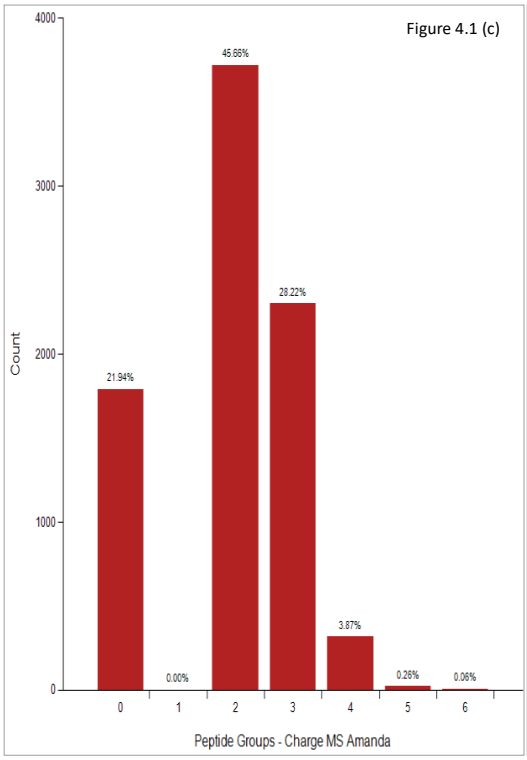
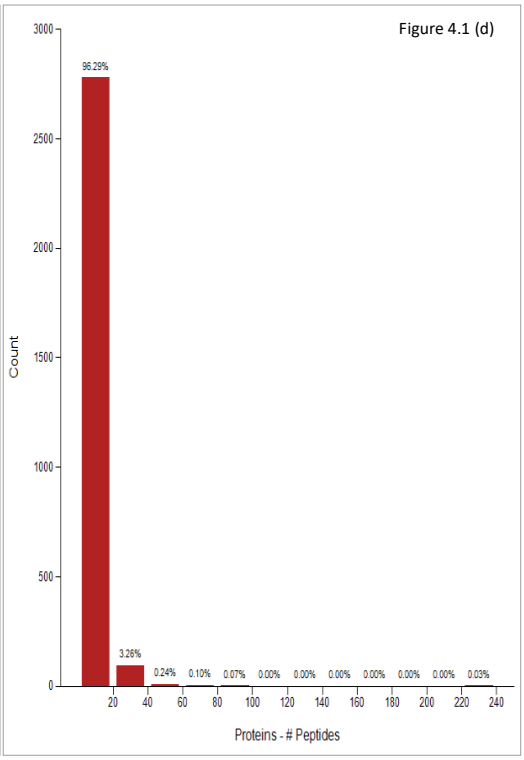


Figure 4.1 (b)







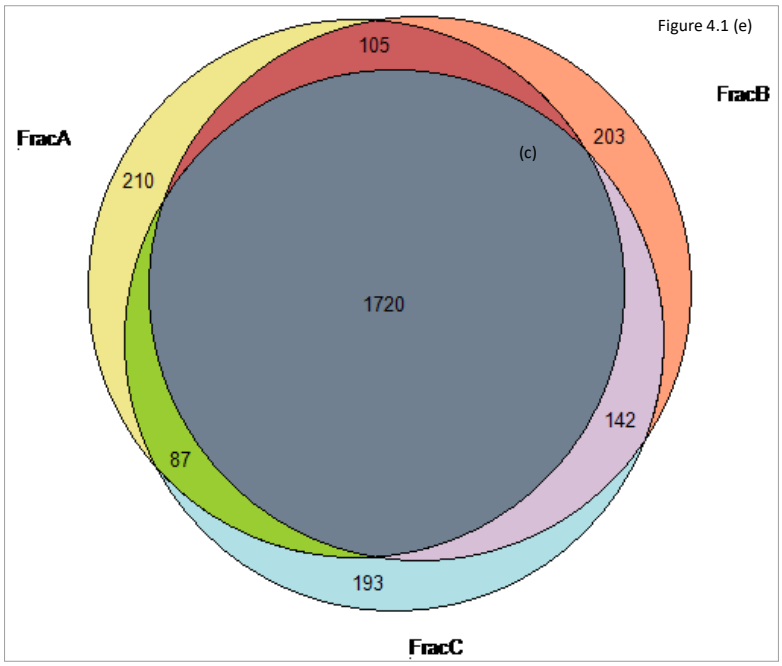


Figure 4.1: Data graphics from PD 2.1 obtained from patients G03, G09 and G17 comparing baseline versus 1 month of treatment with n-3 fatty acid and gemcitabine. (a) Number of protein hits per fraction per triplicate injections n = 30 fractions x 3 = 90 samples. (b) Number of missed cleavages:

90% no missed cleavages. (c) Charge of corresponding peptides. (d) Number of peptides per protein (mean = 4.5). (e) Venn diagram showing protein numbers from each triplicate injections.

4.3.2 Markers of Pancreatic Cancer

Kim *et al* [108] compiled a list of 260 known and verified pancreatic cancer protein biomarker, from a combination of searching on the public database Oncomine [109], manual inspection and filtering through the Plasma Proteome Database (PPD, http://www.plasmaproteomedatabase.org) [110]. This was cross referenced against the 2886 proteins and 121 proteins of known verified protein markers of pancreatic cancer. The mean fold change was positive at 1.28.

A landmark study by Radon *et al* [111] identified a panel of three protein biomarker panel for early detention of pancreatic adenocarcinoma in urine. The three proteins are: LYVE-1, REG1A and TFF1. From the 2886 proteins list, two proteins (LYVE-1 and REG1A) were positively identified and an isomer of TFF1 was also identified. This provides confidence that this methodology and its results are suited for the detection of pancreatic adenocarcinoma.

4.3.3 Bioinformatic Analysis

Gene Ontology Enrichment Analysis was performed on all 1403 proteins on Protein Centre (Thermo Fisher), analysed with an FDR at 5% against all reference sets and Bonferroni corrected. On Gene Ontology Slim Cellular Component (GO-CC), the highest proportion of protein action (% compared to all proteins) was on Membrane (53.8%), followed by Extracellular (41.9%) and Cytoplasm (33.7%). For Gene Ontology Slim Molecular Function (GO-MF), the main protein function affected were in: Protein Binding (77.9%), Catalytic Activity (43.6%) and metal ion binding (23.0%). Gene Ontology Biological Processes (GO-BP) revealed multiple up and down regulated processes which is described below and summarised in Figure 4.2, Figure 4.3, Figure 4.4, Figure 4.5 and Figure 4.6.

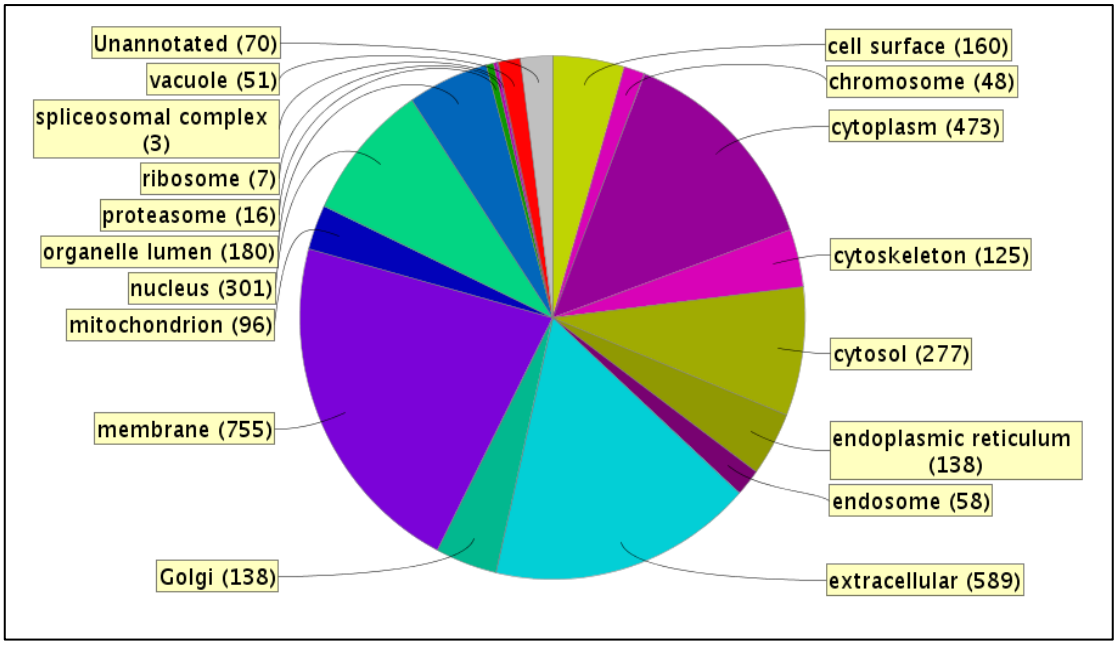


Figure 4.2: Pie Chart illustrating Gene Ontology Slim: Cellular Component for all proteins identified, with the number of proteins in (brackets). The highest proportion of proteins identified were from the cell membrane (755), followed by extracellular proteins (589) and the cytoplasm (473).

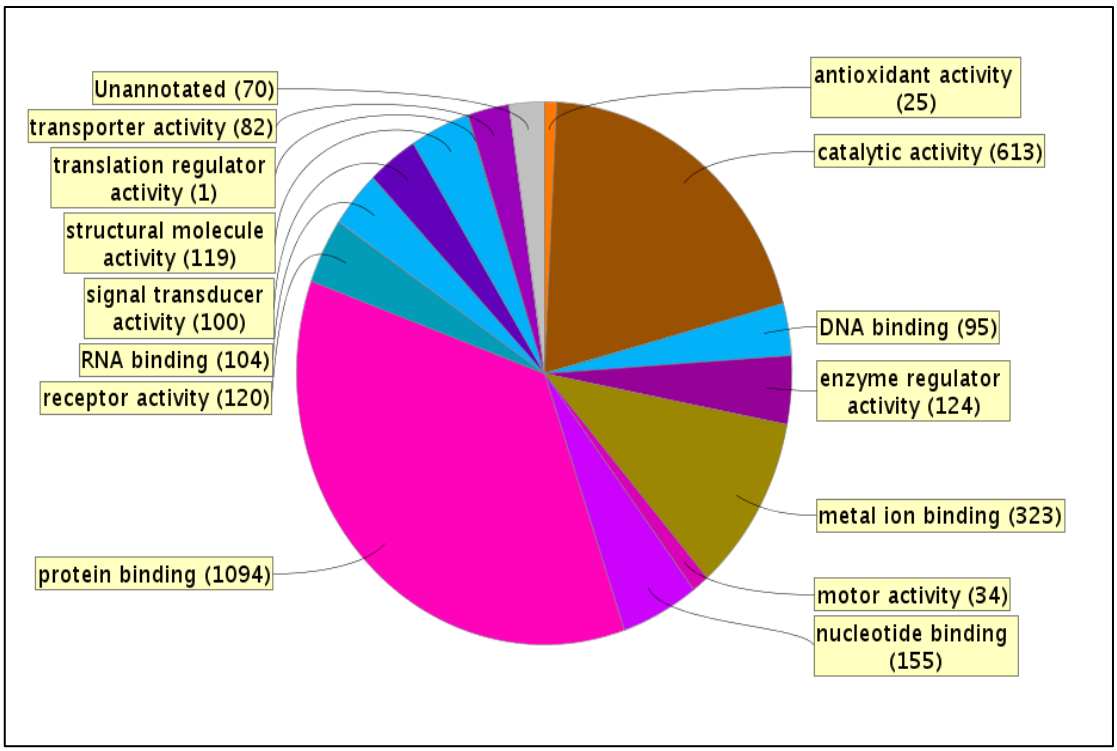


Figure 4.3: Pie Chart illustrating Gene Ontology Slim: Molecular Function for all proteins identified, with the number of proteins in (brackets). The highest proportion of proteins identified were involved in protein binding (1094), followed by catalytic activity (613) and metal ion binding (323).

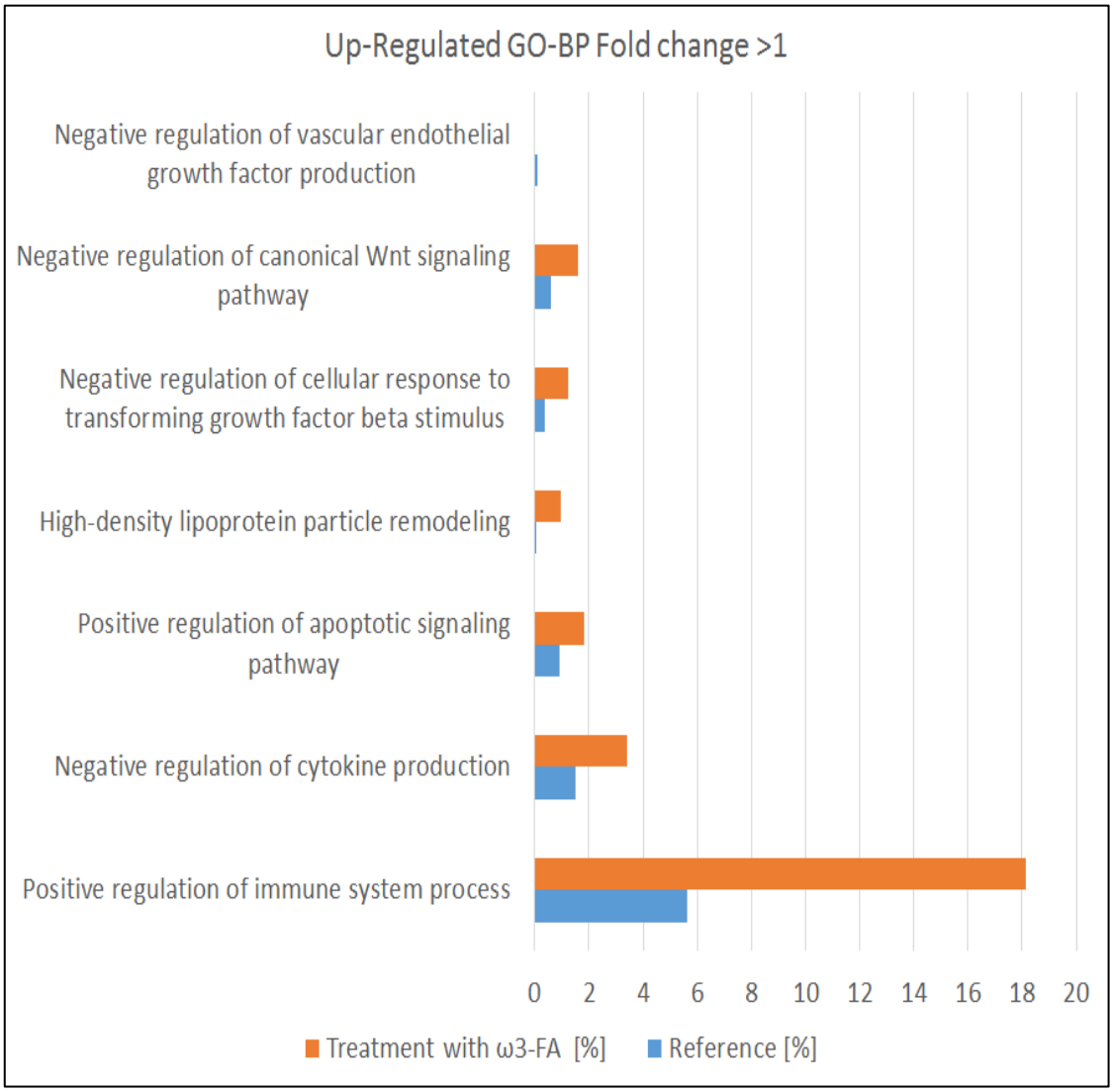


Figure 4.4: Over Represented Gene Ontology Biological Processes identified from protein with fold changes >1 following treatment with n-3 fatty acids and gemcitabine. All p values <0.05. There is an overwhelming positive regulation in the immune system process on treatment with n-3 fatty acids and gemcitabine.

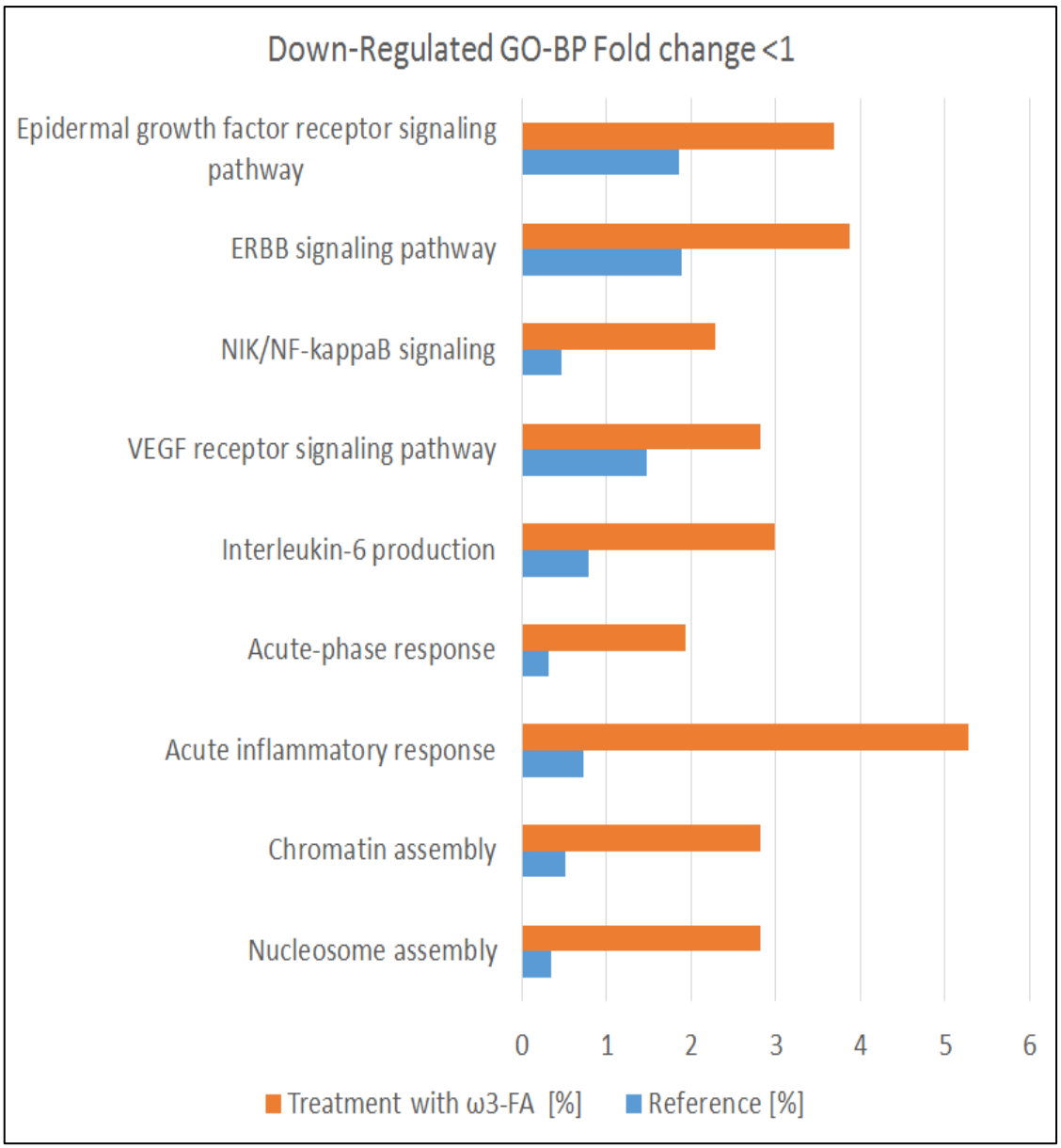


Figure 4.5: Over Represented Gene Ontology Biological Processes identified from protein with fold changes <1 following treatment with n-3 fatty acids and gemcitabine. All p values <0.05. As well as downregulation of various cancer pathways, there is a strong correlation of a reduction in the acute inflammatory processes, acute phase response and interleukin production (an inflammatory cytokine) on treatment with n-3 fatty acids and gemcitabine.

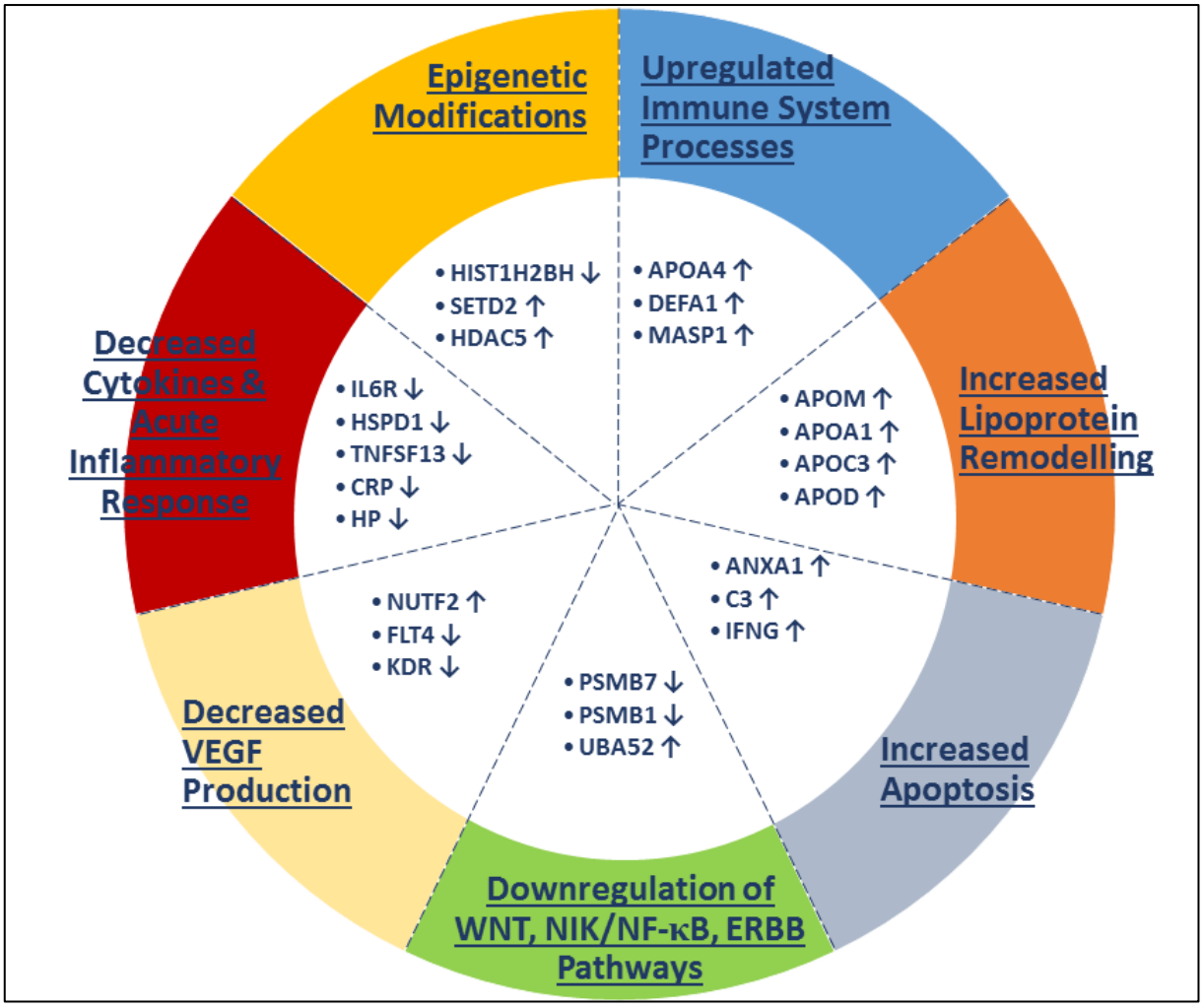


Figure 4.6: Pie chart illustrating different biological processes involved with specific proteins up or down regulated following n-3 fatty acid and gemcitabine treatment. Proteins based on fold changes identified correlates with different biological processes. Note the reduction in clinically used inflammatory markers (CRP and Haptoglobin) correlating with the known effect of n-3 fatty acids. Administration of n-3 fatty acid and gemcitabine show a reduction in various hallmarks in cancer, for example increased in cell death, reduction in vascular growth factors and downregulation of known pancreatic cancer markers.

4.3.4 Up Regulated Proteins: Fold Change > 1

Immune System Processes: One hundred and forty nine proteins identified (p=5.37E-34) corresponding to a positive regulation of immune system processes. The specific child terms up regulated include the positive regulation of innate immune response (22

proteins, p=2.09E-2) and innate immune response in mucosa (4 proteins: Apolipoprotein A-IV, Neutrophil defensin 1, Neutrophil defensin 3 and Lactotransferrin, p=8.01E-3).

Proteins involved in regulation of complement activation were also identified for all three pathways: Classical Pathway (75 proteins, p= 2.34E-80), Alternative pathway (6 proteins: VSIG4, Complement factor B, Complement C3, Complement factor D, Complement component C7 and Properdin, p= 2.85E-04) and Lectin Pathway (3 proteins: Mannan-binding lectin serin, Mannose-binding protein C, alpha-2-macroglobulin, p=2.64E-2).

Cytokine Production: Sixty proteins involved in cytokine production were identified with 28 involved in the negative regulation of cytokine production (p=7.45E-04). Specific cytokine pathways negatively regulated include Interleukin-2 (p=3.78E-02), Interleukin-8 (p=4.27E-02) and chemokine production (p=8.69E-03).

Fifteen proteins related to Tumour necrosis factor (TNF)-mediated signalling pathway were identified (p=2.87E-03), with 3 proteins (Glutathione S-transferase A1, Apolipoprotein A-I and Glutathione S-transferase P) negatively regulating TNF-signalling pathway (p=2.41E-02).

Other related pathways are the negative regulation of the JAK-STAT cascade, a cytokine-mediated immune response system (6 proteins: Reticulon-4 receptor-like 1, Biglycan, Decorin, Leptin receptor, Reticulon-4 receptor and Reticulon-4 receptor-like 2 p=8.20E-03) and negative regulation of JUN kinase activity (3 proteins: Glutathione S-transferase A1, Serpin B3 and Glutathione S-transferase P, p=4.33E-02).

Apoptotic Processes: Ninety five proteins were identified in regulation of apoptotic processes (p=6.69E-06). Specifically, 15 proteins involved in positive regulation of apoptotic signalling pathway (p=3.61E-02) were identified with 3 proteins (Complement C3, Complement C4-B and Complement C4-A) involved in the positive

regulation of apoptotic cell clearance (p=3.61E-02), 2 proteins (Annexin A1 and Interferon gamma) involved in neutrophil apoptosis (p=4.41E-02) and regulation of myeloid cell apoptotic process (4 proteins: Annexin A1, Beta-2-glycoprotein 1, Interferon regulatory factor 7 and Macrophage migration inhibitory factor, p=2.25E-02).

Lipoprotein Remodelling: A large proportion of apolipoproteins were identified and significantly upregulated including: Apolipoprotein M, Apolipoprotein A-I, Apolipoprotein A-II, Apolipoprotein C-I, Apolipoprotein C-II, Apolipoprotein C-III, Apolipoprotein D Apolipoprotein A-IV, Apolipoprotein(a) and Apolipoprotein F. The average upregulated fold change value is 1.67 compared to baseline. These proteins are involved in high-density lipoprotein particle remodelling (p=1.38E-06), negative regulation of very low density lipoprotein remodelling (p=3.96E-04), negative regulation of very-low-density lipoprotein particle clearance (p=5.26E-04), negative regulation of lipoprotein oxidation (p=1.25E-03), positive regulation of fatty acid biosynthetic process (p=1.67E-02) and positive regulation of fatty acid metabolic

process (p=2.03E-02). (Figure 4.7)

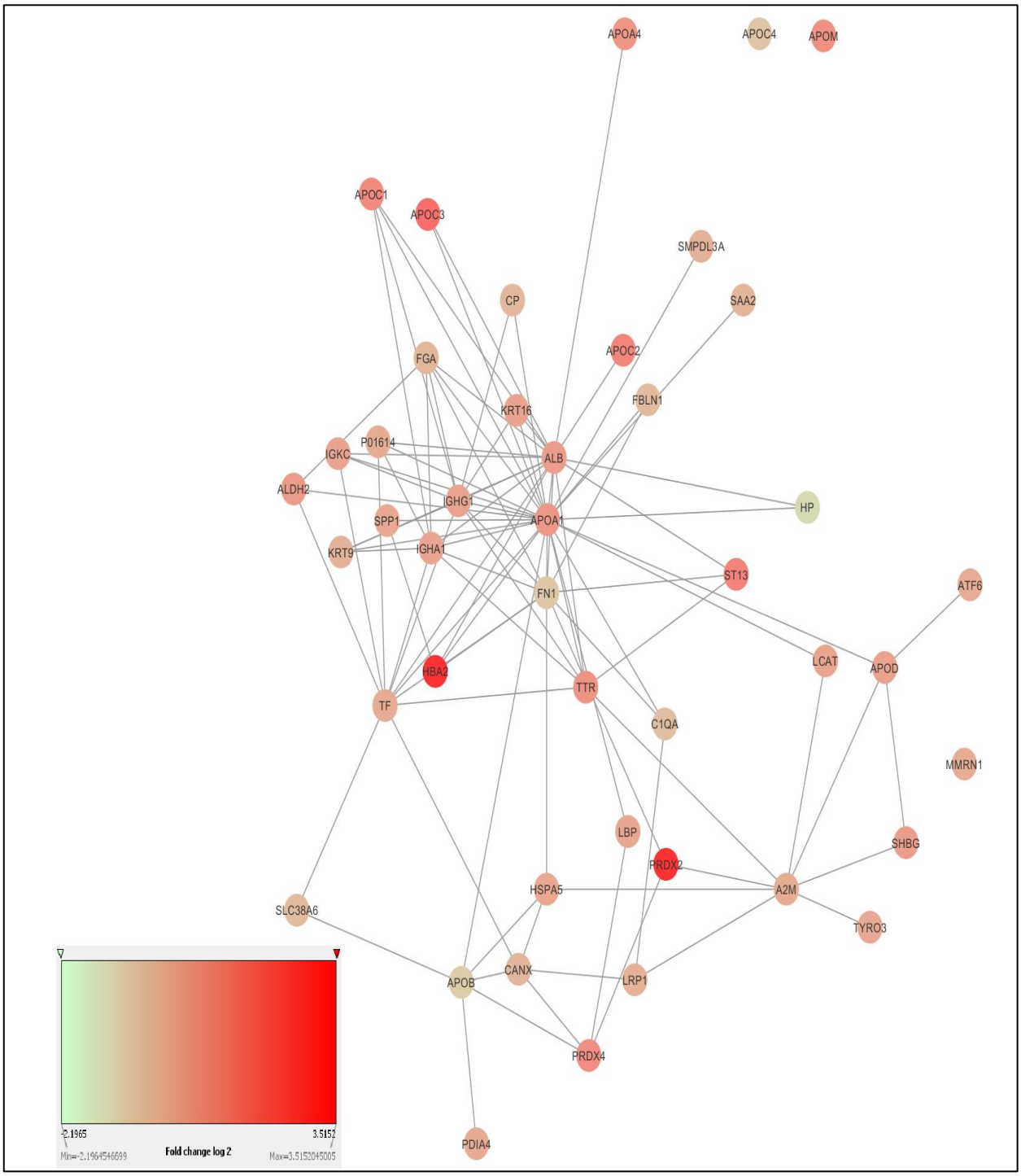


Figure 4.7: Cytoscape visualising apolipoproteins: Majority of the apolipoproteins identified have a strong positive fold change >1.5 with its associated proteins, suggesting overall positive effect following n-3 fatty acids infusion.

Growth Factor Stimulus: Twenty eight proteins were attributed to Vascular endothelial growth factor(VEGF) receptor signalling pathway (including cellular response and growth factor) – with a high fold change in protein Nuclear transport factor 2 (Fold change = 2.23): This protein is involved in negatively regulating VEGF production (p=3.04E-02). Complementing this is the finding of negative regulation of angiogenesis and negative regulation of vasculature development (8 proteins: Histone deacetylase 5, Beta-2-glycoprotein 1, Histidine-rich glycoprotein, Multimerin-2, Decorin, Thrombospondin-4, Thrombospondin-1 and Tyrosine-protein kinase receptor Tie-1, p=4.61E-02).

Transforming growth factor Beta (TGF-β) receptor signalling pathway and cellular stimulus were negatively regulated (10 proteins, p=8.20E-03).

Signalling Pathway: The ubiquitin proteins identified (Polyubiquitin-B, Polyubiquitin-C, Ubiquitin-like modifier-activating enzyme, Ubiquitin-40S ribosomal protein S27a , Ubiquitin-60S ribosomal protein L40, Ubiquitin-conjugating enzyme E2 L3, Probable E3 ubiquitin-protein ligase HERC1, Probable E3 ubiquitin-protein ligase HERC4 and E3 ubiquitin-protein ligase DZIP3) were highly upregulated (Mean Fold change = 1.69). Ubiquitination plays a role in multiple cell signalling pathways. Other signalling pathways affected included negative regulation of canonical WNT signalling pathway (13 proteins including ubiquitin and proteasome subunits, p=1.29E-02), positive regulation of ERBB signalling pathway (5 proteins 4 Ubiquitin proteins and Matrix metalloproteinase-9, p=4.37E-02) and regulation of Notch receptor processing (5 proteins, p=7.12E-03).

Glutathione Processes: Multiple Glutathione related proteins were identified, which were strongly up regulated (12 proteins, mean fold change = 2.14). These proteins were involved in the regulation of Glutathione derivative metabolic and biosynthetic process (p=5.06E-09).

4.3.5 Down Regulated Proteins: Fold Change < 1

Immune System Processes: GO-BP found corresponding negative regulation (hence up regulation) of the Innate immune response (23 proteins, p=5.38E-09), Complement activation, classical pathway (3 proteins, p=4.12E-03), humoral immune response mediated by circulating immunoglobulin (3 proteins, p=7.51E-03) and adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains, (5 proteins, p=2.45E-02). The common down regulated proteins found are C4b-binding protein beta chain, C4b-binding protein alpha chain and Complement receptor type 1.

Epigenetic Modification: A large proportion of Histone H2Bs and Histone H2As were found to be significantly down regulated (28 proteins, H2B fold change <1.67). These proteins were involved in nucleosome assembly and organisation (p=7.94E-09), chromatin assembly and silencing (p=1.10E-06), Protein DNA complex assembly (p=3.94E-06) and in positive regulation of gene expression, epigenetic (p= 4.02E-05). Of note Histone-Lysine N-methyltransferase and Histone deacetylase 5 were significantly up-regulated (Fold change > 1.5). Figure 4.8 illustrates the relationship between the various histone proteins identified from this cohort.

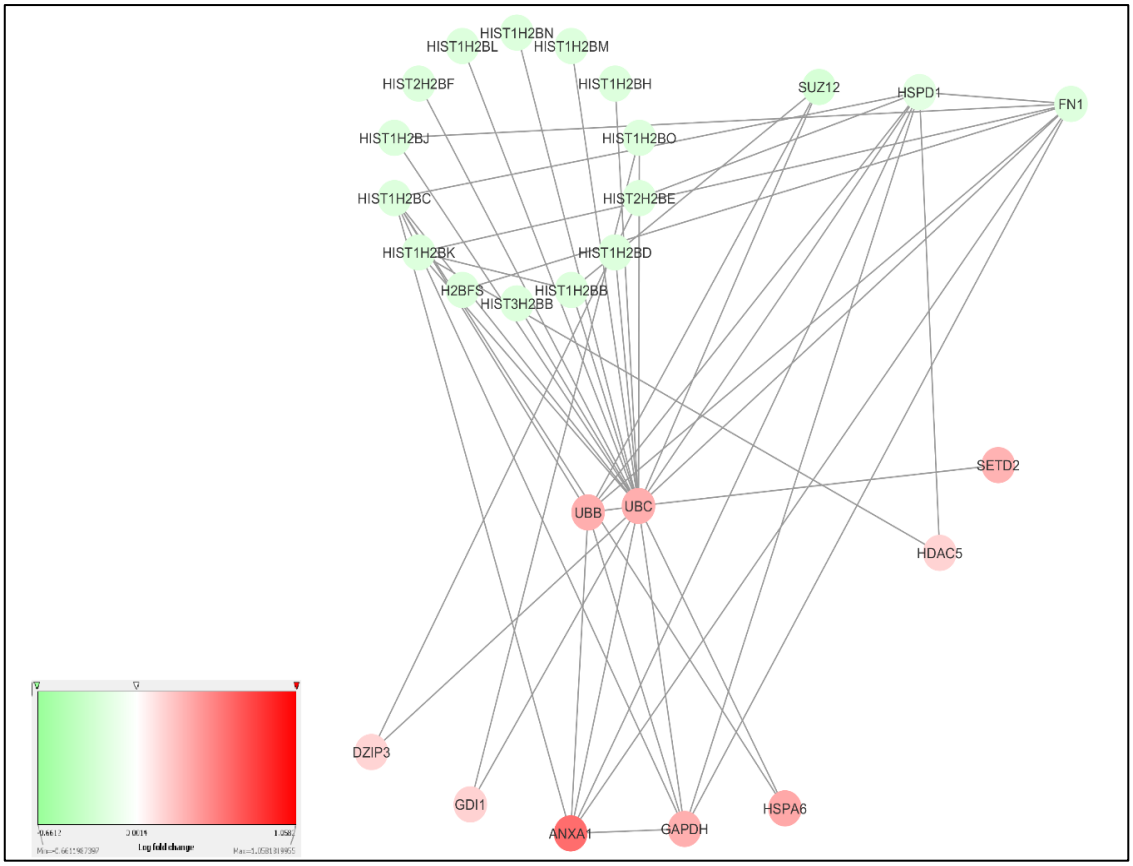


Figure 4.8: Cytoscape visualisation of epigenetic related modifications: Multiple Histone 2B genes identified are down-regulated, with corresponding positive fold change in deacetylation (HDAC5) and demethylation (SETD2) centred on ubiquitination proteins (UBB, UBC).

Acute Inflammatory Response: Thirty proteins corresponded to the down regulation of the acute inflammatory response (p=3.53E-15) with a specific emphasis on the Acute phase response (p=3.64E-05). Eleven proteins were identified with an average fold change < 1.64. Of particular interest, CRP reduced to a fold change of 3.85 whilst Haptoglobin (another acute phase marker) was down regulated by a factor of 2.33.

Cytokine Production: An overall trend of down regulation of cytokine production was observed (28 proteins, p=6.75E-04). Specific down regulated cytokines included production of interleukin-12 (16 proteins, p=2.11E-05), Interleukin-6 production and cellular response to Interleukin-6 (17 proteins, p=4.15E-05), Interleukin-8 production (9 proteins, p= 3.31E-04) and Cytokine production involved in immune response (7

proteins, p=1.16E-02). Common proteins identified were the HLA class I histocompatibility proteins, 60 kDa Heat shock protein and Interleukin-6 receptor subunit.

GO-BP relating to TNF down regulation included cellular response to TNF (20 proteins, p=4.87E-05) and TNF mediated signalling pathway (13 proteins, p=1.02E-03). Specific down regulated proteins include TNF ligand superfamily member 13, TNF receptor superfamily member 6 and Proteasome subunits.

Apoptotic Processes: Forty proteins involved in the increased regulation of apoptotic process were identified (p=3.90E-03): specifically affecting extrinsic apoptotic signalling pathway via death domain receptors (5 proteins, p=2.14E-02), Regulation of apoptotic signalling pathway (13 proteins, p=3.75E-02) and Regulation of epithelial cell apoptotic process (5 proteins, p=4.21E-02).

Growth Factor Stimulus: VEGF receptor signalling pathway was negatively regulated with 16 proteins identified (p=4.97E-02) with two specific proteins VEGF receptors 2 and 3 both down regulated. The general regulation of angiogenesis was also down regulated (17 proteins, p=5.80E-04). Another growth factor identified was the down regulation of fibroblast growth factor signalling pathway (2 proteins, p= 3.25E-02) and the cellular response to fibroblast growth factor stimulus (18 proteins, p=3.88E-02). Paradoxically, Platelet Derived Growth Factor (PDGF) receptor signalling pathway was up regulated.

Lipoprotein Remodelling: Three apolipoproteins (Apolipoprotein E, Apolipoprotein B-100 and Apolipoprotein C-IV) were found to be down regulated (Mean fold change < 1.37). These proteins were involved in the negative regulation of low density lipoprotein remodelling, regulation of plasma lipoprotein particle levels, low-density lipoprotein particle receptor catabolic process and lipoprotein modification/oxidation.

Signalling Pathway: WNT signalling pathway was found to be downregulated, specifically the canonical pathway (13 proteins, p=1.64E-04). Other pathways also down regulated are NIK/NF-kappa β signalling (13 proteins, p=4.97E-05), the ERBB signalling pathway (22 proteins, p=1.01E-02) and Epidermal growth factor receptor signalling pathway (21 proteins, p=1.53E-02). The proteasome subunit proteins were heavily linked to the downregulation of these pathways.

4.3.6 Correlation with Scaffold 4.7

The MSF file was reprocessed with Scaffold 4.7, under conditions of at least 95% protein probability, 95% peptide probability and minimum of two peptides per protein. A total of 1039 proteins were discovered. (Section 9.4 Appendix Table 4). The associated fold change between treatment (n-3 fatty acids & gemcitabine) vs baseline was obtained, then compared to the associated protein fold changes from PD 2.1 and normalised for comparison. There were 624 matching proteins, with a Pearson correlation score of 0.8019 with a P value <0.001 (Figure 4.9).

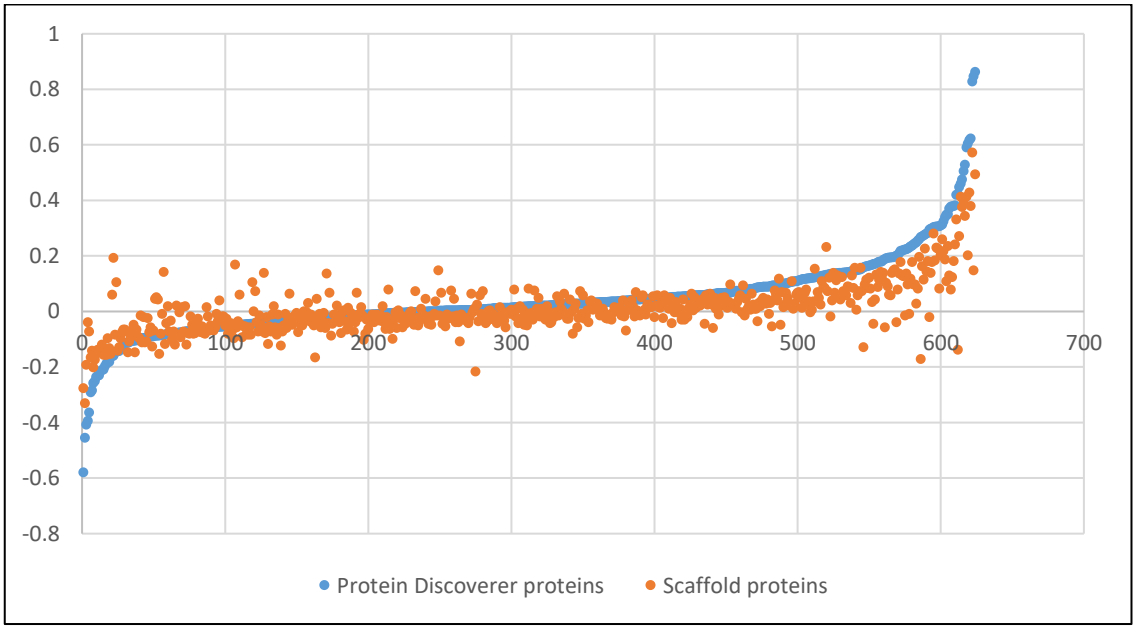


Figure 4.9: Correlation of Fold Changes between proteins discovered on PD 2.1 versus Scaffold 4.7, showing a significant (p<0.001) positive Pearson correlation (0.8019) for the 624 matching proteins.

4.4 Discussion

High definition proteomic analysis, utilising intensive immunodepletion, fractionation and isobaric TMT labelling, on human plasma of patients with APC demonstrates proteomic changes following treatment with n-3 fatty acids and gemcitabine. The identification of 121 known pancreatic cancer markers amongst our protein cohort as well as LYVE-1, REG1A and an isoform of TFF1 [111] corroborates the methodology for identifying pancreatic cancer in plasma. Whilst acknowledging the average fold change increased to 1.38 following treatment with n-3 fatty acids and gemcitabine, the markers are primarily for diagnostic purposes, not markers of efficacy or prognosis.

Intravenous infusion of n-3 fatty acids results in immediate uptake of EPA and DHA into cell membrane [105] – this correlates with GO-CC slim analysis where the majority of proteins are attributed to the cell membrane. Significant upregulation of various apolipoproteins confirms the biologic and pharmacokinetic effect following n-3 fatty acid and gemcitabine treatment. We have shown various biological processes affected, involving down regulation of low density and very-low density lipoproteins, increased high density lipoprotein remodelling, decreased lipoprotein oxidation and fatty acid metabolism. This corresponds to other evidence investigating effect of n-3 fatty acids in other settings including in human serum [75,112] and mice [73,74,113]. The anti-inflammatory effects of n-3 fatty acids are well established [114]. CRP, a widely used clinical marker of inflammation, reduced by 76% whilst GO-BP showed strongly significant down regulation of acute inflammatory and acute phase response following n-3 fatty acids and gemcitabine treatment. The combination of increased membrane activity, lipoprotein regulation and anti-inflammatory pathways further supports our methodology in detecting changes directly to the administration of n-3 fatty acids.

Following n-3 fatty acids and gemcitabine treatment, numerous proteins positively modulating both the humoral and innate immune response systems were discovered. Upregulation of the Complement system occurred via all three pathways: Alternative Pathway, Classical Pathway and Lectin Pathway. Upregulated Complement C3 and C4 proteins in our cohort were involved in apoptotic cell clearance. Furthermore, various

cytokines were down regulated including Interleukin-2, Interleukin-6, Interleukin-8, Interleukin-12, Chemokines and TNF. We have previously shown restoration of the Mannose-Binding Lectin pathway is beneficial in APC [115]. Raised Interleukin-6 is an associated poor prognostic marker in APC relating to cachexia [116] and administration of n-3 fatty acids reduces Interleukin-6 production [49]. TNF has been shown to induce tumour promotion in pancreatic cancer cell lines [117]. A direct effect of n-3 fatty acids in promotion of the immune response system, by way of the complement system is it has a net benefit in reducing harmful and tumorigenic cytokines.

Angiogenesis is a hallmark of cancer progression [10,11]. Following administration of n-3 fatty acids and gemcitabine, angiogenesis is downregulated, with a particular emphasis on reducing VEGF production (via protein Nuclear transport factor 2), reducing vasculature development and TGF-β receptor signalling pathway. This corresponds with other findings that n-3 fatty acids suppresses vascular endothelial growth factor-stimulated cell proliferation, migration and tube formation during angiogenesis [35-37].

Epigenetic changes secondary to nutritional intake is an emerging topic in cancer research with evidence of dietary bioactive compounds directly affecting DNA methylation and histone modifications [118]. This cohort has shown an obvious down regulation of histone bodies (H2B) and up regulation of histone deacetylase (HDAC 5 and HDAC 11) and methylation (SETD2) proteins. The overall effect results in down regulation of nucleosome and chromatin assembly and organisation. This may increase the stability of DNA reducing the chances of mutation. N-3 fatty acids may also directly affect methylation of DNA via the peroxisome proliferator activated receptor-α, though the overall effect to the DNA is unclear [119]. A recent genomic analyses of 456 PDAC identified 4 different molecular subtypes on pancreatic cancer based mutated genes found in 10 different molecular mechanisms [14]. SETD2, involved in histone modification, was identified in 24% of cases. Two other pathways of note are TGF-β signalling and WNT signalling defects (in 47% and 5% of cases). Both these signalling pathways were down regulated following n-3 fatty acids and gemcitabine treatment in

our cohort, which may indicate a positive outcome. However, future better powered studies would be needed to make a firm conclusion.

The main drawback from the above experiment is the inability to attribute the proteomic changes solely to the administration of n-3 fatty acids. As previously commented, the primary clinical study compared administration of n-3 fatty acid and gemcitabine with historical results, which affects the strength of the findings. Gemcitabine (Section 1.2.2, Figure 1.1) is a pyrimidine analogue which disrupts DNA synthesis and the effects of n-3 fatty acids on reducing inflammation, apoptosis and angiogenesis is well described. The proteomic changes identified above which corresponds to these known n-3 fatty acids effect strongly suggest this to be the mechanism of improving quality of life and progression free survival. Alternatively it may be a synergistic effect of n-3 fatty acids with gemcitabine that improves the outcomes in these patients. In either case, the reduction in inflammation will have a positive effect in terms of patient's quality of life and progression free survival [120]. Recent work in human pancreatic cancer xenografts also show that reducing inflammation improves cancer cachexia, which leads to improved quality of life [121].

Following on from this first discovery phase experiments on the effect of n-3 fatty acid and gemcitabine on treatment naïve patients with advanced pancreatic adenocarcinoma, the next chapter seeks to elucidate the effect of n-3 fatty acid independent of gemcitabine.

5 Results II: Comparison between Control versus Omega-3 Fatty Acid

5.1 Introduction

The proteomic changes in patients with APC from baseline (without any treatment) to one cycle of n-3 fatty acid infusion and gemcitabine can be summarised as: anti-inflammatory, positive modulation of the immune system leading to lower cytokine production, possible direct anti-angiogenic effect via VEGF and potential modulation of epigenetics via histones and SETD2. (See Section 4.3). This next chapter seeks to identify the effect of n-3 fatty acid infusion controlling for the effect of gemcitabine.

As outlined in Section 1.4.3, gemcitabine is a pyrimidine analogue which disrupts DNA synthesis leading to pancreatic cancer cell death. Various proteomic studies investigating the effect of gemcitabine in APC have been summarised in Section 1.4.3 and Appendix 9.2 Table 2. The majority of these studies concentrate on proteins which either enhance the effect of gemcitabine in killing pancreatic cancer cells or reduce the efficacy of gemcitabine by promoting chemo-resistance. A cohort of patients who only received intravenous gemcitabine (without n-3 fatty acid infusion) was recruited as a control group of patients. 9 patients were successfully recruited up to 6 months (cycles) of treatment.

5.2 Materials and Methods

5.2.1 Patients

A two arm experimental model comparing Control (APC with gemcitabine only, n-3 fatty acid naïve) versus Treatment (APC with n-3 fatty acid and gemcitabine treatment) at one cycle (1-month) of treatment. Five patients were recruited into each arm (5 vs 5). The demographics are presented in Table 5.1.

Patient	Sex	Age	ECOG	Haemoglobin (g/l)	Neutrophil Count ($x10^9$/L)
G05	Female	64	0	125	8.8
G13	Male	70	0	135	4.8
G15	Male	71	0	147	3.8
G19	Male	65	1	135	11.5
G21	Male	70	0	116	5.4
C02	Male	58	1	116	9.8
C03	Male	50	0	71	8.75
C04	Male	75	0	132	4.1
C06	Male	75	0	125	2.2
C08	Female	59	0	122	4.96

	(cont…)				
	Serum Creatinine (mmol/L)	Deranged Liver Function Tests	Previous / Other Cancers	Previous Chemo-therapy	Recent Major Surgery (within 4 weeks)
G05	57	No	No	No	No
G13	77	No	No	No	No
G15	69	No	No	No	No
G19	75	No	No	No	No
G21	67	No	No	No	No
C02	62	No	No	No	No
C03	57	No	No	No	No
C04	65	No	No	No	No

C06	84	No	No	No	No
C08	59	No	No	No	No

Table 5.1: Demographic of patients analysed in the treatment arm versus control arm. ECOG (Eastern Cooperative Oncology Group) Performance Status: 0 = Fully active, able to carry on all pre-disease performance without restriction. 1 = Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g. light house work.

Patients G05, G13, G15, G19 and G21 had received one cycle (1 month) of intravenous n-3 fatty acid and gemcitabine treatment, herein denoted as the Treatment group. Note that patients in the Treatment group were from the similar cohort from the primary clinical study as analysed in Section 4.2.1. Patients C02, C03, C04, C06 and C08 had received one cycle (1 month) of intravenous gemcitabine treatment only, herein denoted as the Control group.

5.2.2 Control Patients

As described in Section 2.2.3 a control arm of patients with advanced pancreatic adenocarcinoma who only received intravenous gemcitabine treatment were recruited after completion of the primary clinical study [77]. The primary clinical study was a single arm study comparing outcomes of patients receiving n-3 fatty acids and gemcitabine in APC to historical data from patients who only receive gemcitabine in APC. This meant there were no actual control patients in order to analyse their plasma to identify the proteomic plasma changes. A concurrently run two arm study would have provided direct clinical and plasma samples for comparative analysis, which would have yielded stronger results. In order to overcome these deficiencies, a separate control group was recruited later.

The demographic of the 9 control patients are summarised in Table 5.2.

Demographics		Control Patients N=9
Gender	Male Female	7 2
Age	Median age (range) >70 years <70 years	64 (50-75) 2 8
Ethnicity	White British Asian	8 1
Baseline Weight	Mean weight in Kilograms (range)	69 (54-86)
Total number of treatment time points (mean)		38 (3)
Number of patients completed 1 cycle (%)		5 (63%)
Number of patients completed 2 cycles (%)		2 (25%)
Number of patients completed 4 cycles (%)		1 (12.5%)
Number of patients completed 6 cycles (%)		1 (12.5%)

Table 5.2: Demographic of control patients recruited - Advanced pancreatic adenocarcinoma only receiving intravenous gemcitabine.

Though nine control patients were recruited, five were analysed in order to match up with the five Treatment group patients in the two arm TMT experiment. The five patients were also the patients who had completed one cycle (1 month) of treatment with intravenous gemcitabine.

5.2.3 Proteomic Plasma Analysis

The general proteomic analysis workflow according to Figure 2.1 was carried out. In brief, sufficient amount of heparinised plasma from each patient was intensely immunodepleted of both high and moderate abundant proteins using the IgY14 + Supermix Seppro columns (as detailed in Sections 2.5.1, 2.5.2 and 3.2.4) to obtain 60 µg of depleted protein from each patient sample. An additional 12 µg of proteins from each patient was then pooled together (12 µg x 10 patients = 120 µg) and split into two: each 60 µg pooled sample corresponded to a Quality Control (QC) group for a TMT multi-arm comparison experiment.

Each 60 µg depleted sample was subjected to Rapigest solubilisation, DTT reduction, IAA alkylation, endoproteinase digestion using Lys-C and Trypsin and finally TFA addition to halt the Rapigest (rendering it suitable for MS analysis) and endoproteinase reactions. (As detailed in Section 2.5.3). Samples were lyophilised prior to the next step.

Each patient sample and QC were labelled with TMT Sixplex reagents according to Table 5.3 , into each corresponding arm of the experiment. (See Section 2.5.4 for details of TMT-6 Labelling). All TMT-6 labels from each arm (i.e. Control (QC1 + C02, C03, C04, C06 and C08) and Treatment (QC2, G05, G13, G15, G19 and G21)) were then combined before undergoing lyophilisation into two sample groups: Control vs Treatment.

Experiment Group / TMT 6plex Label	Control Arm	Treatment Arm
126	QC1	QC2
127	C02	G05
128	C03	G13
129	C04	G15
130	C06	G19
131	C08	G21

Table 5.3: List of patients in each group with 60 µg of lyophilised immunodepleted tryptically digested peptides of each patient sample (Gxx and Cxx). Quality Control (QC – combination of all ten patients 12 µg each) was spiked with a TMT-6 label as described above, before each arm was pooled (eg. Control Arm TMT 126 - 131 combined).

Each group (Control and Treatment) proceeded to 30 fraction high pH reverse-phase chromatography fractionation. The fractions were subsequently injected onto the QExact-Orbitrap (QE) (Thermo Fisher Scientific, Waltham USA) and processed on Protein Discoverer (PD) 2.1 (Thermo Fisher Scientific, Waltham USA) and Scaffold 4.7 (Proteome Software, Portland USA) with bioinformatics analysis on Protein Centre 3.15 (Thermo Fisher Scientific, Waltham USA) and Cytoscape [103] 3.5

(http://www.cytoscape.org/what_is_cytoscape.html)as described (Refer to Sections 2.4.3, 2.6.2, 2.6.3, 2.8.1 and 2.8.2).

5.3 Results

5.3.1 Protein Count

On PD 2.1 utilising the iterative methodology with a minimum one peptide count: A total of 3476 proteins were discovered using this methodology, from 8862 peptide groups, 1228867 Peptide Spectral Matches and 1859016 MS/MS spectrum (Section 9.5 Appendix Table 5). Mean number of peptide count 4.0 per protein, mean coverage 9.7 % [96.22 – 0.1%]. 93.25% had 0 missed cleavages, 6.36% 1 missed cleavage and 0.68% 2 missed cleavages. Figure 5.1 (a-e). 100% obtained high peptide confidence scores. Excluding isomers, 1705 with relative quantitation on TMT labels were identified. Fold changes were obtained comparing the two arms Control vs Treatment with TMT channel 126 acting as the Quality Control for baseline normalisation.

As has been described in Section 4.3.1, either a positive or negative fold change (more or less than 1) were used in order to give a general overview of the mechanistic changes in the plasma proteome following treatment with n-3 fatty acids. When at least two peptide counts were required for protein identification, 1533 proteins were identified. Again, this correlates well with other plasma based proteomic experiments and in proteomic experiments related to pancreatic cancers [60,107].

125 proteins matched known pancreatic cancer markers as collated by Kim *et al* [108] , Oncomine [109], and Plasma Proteome Database (PPD, http://www.plasmaproteomedatabase.org) [110]. The same proteins 121 discovered from the discovery experiment (Refer Section 4.3.2) were also discovered in this list of proteins. The average fold change amounted to 1.25 comparing the Treatment arm vs Control arm. Similarly, two proteins (LYVE-1 and REG1A) and an isomer of TFF1 were identified, which comprised the three protein biomarker for early detection of pancreatic adenocarcinoma by Radon *et al* [111] . This provides confidence that this methodology and its results are suited for the detection of pancreatic adenocarcinoma.

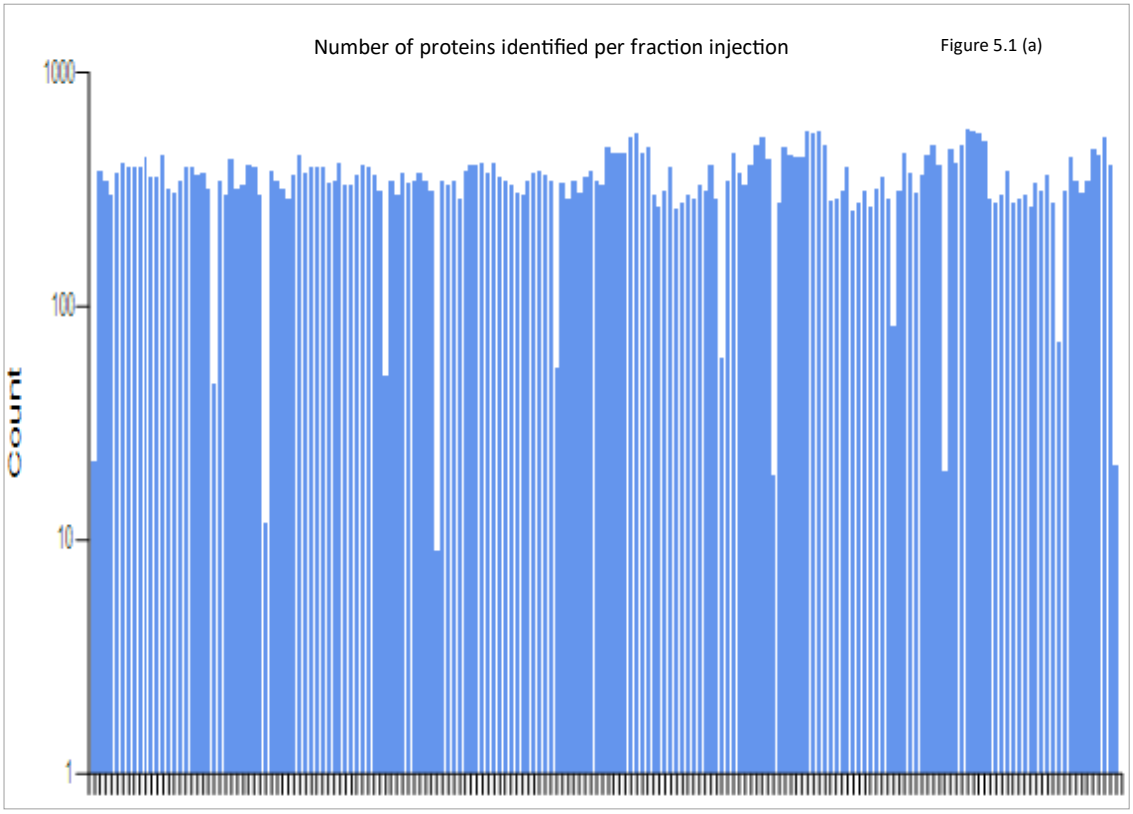


Figure 5.1 (a)

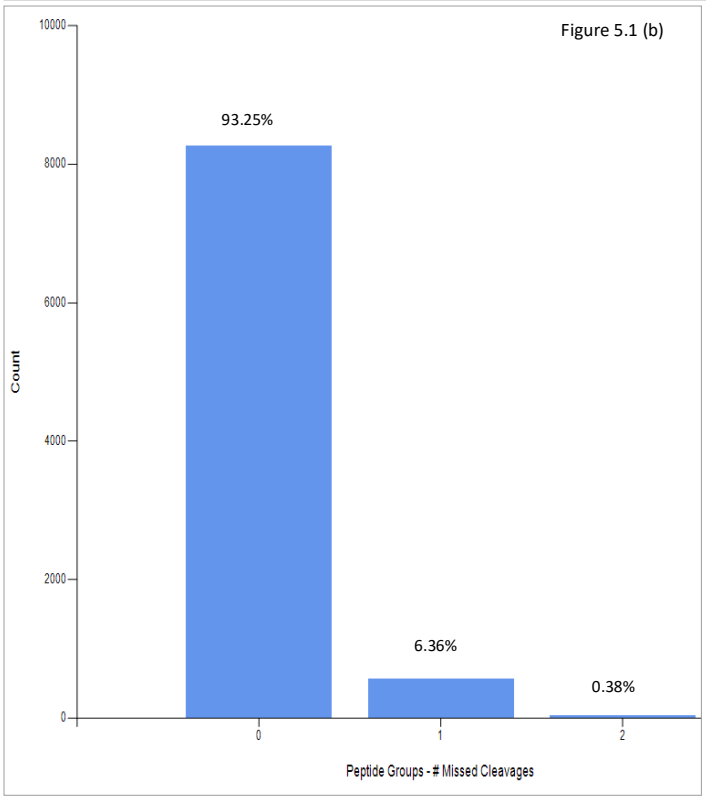


Figure 5.1 (b)

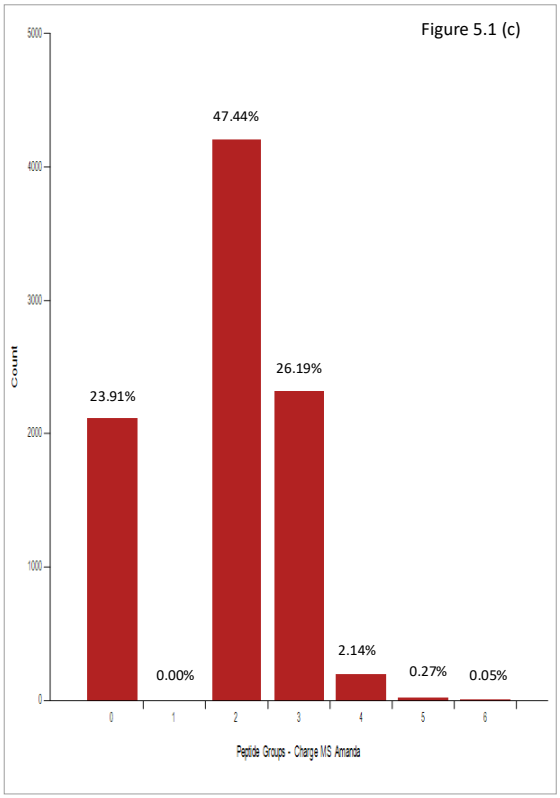


Figure 5.1 (c)
Count
5000
4000
3000
2000
1000
0
23.91%
0.00%
47.44%
26.19%
2.14%
0.27%
0.05%
0
1
2
3
4
5
6
Peptide Groups - Charge MS Amanda

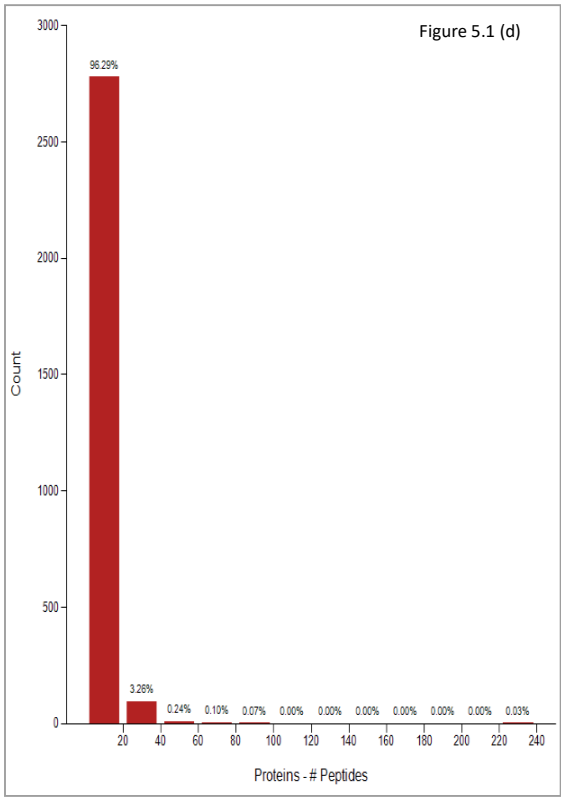


Figure 5.1 (d)
Count
3000
2500
2000
1500
1000
500
0
96.29%
3.26%
0.24%
0.10%
0.07%
0.00%
0.00%
0.00%
0.00%
0.00%
0.00%
0.03%
20
40
60
80
100
120
140
160
180
200
220
240
Proteins - # Peptides

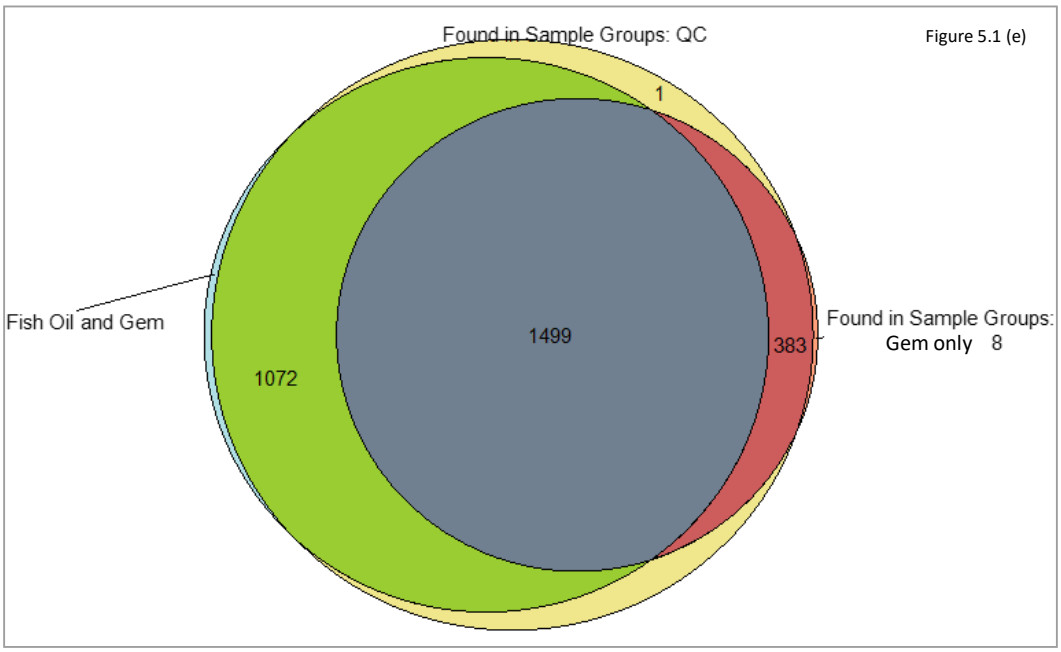

Found in Sample Groups: QC
Figure 5.1 (e)
1
Fish Oil and Gem
1499
383
Found in Sample Groups:
Gem only 8
1072

Figure 5.1: Illustrations from PD 2.1 (a) Number of protein hits per fraction per fraction injections. (b) Number of missed cleavages. (c) Charge of corresponding peptides. (d) Number of peptides per protein. (e) Venn diagram showing protein numbers from each triplicate injections.

5.3.2 Bioinformatic Analysis

Gene Ontology Enrichment Analysis was performed on all 1705 proteins on Protein Centre 3.15 (Thermo Fisher Scientific, Waltham USA), analysed with an FDR at 5% against all reference sets and Bonferroni corrected. On Gene Ontology Slim Cellular Component (GO-CC), the highest proportion of protein action (% compared to all proteins) was on intracellular proteins (62.7%), followed by organelle proteins (51.3%) and cytoplasm (49.0%). For Gene Ontology Slim Molecular Function (GO-MF), the main protein function affected were in: Protein Binding (57.5%), Catalytic Activity (34.1%) and hydrolase activity (19.3%). Gene Ontology Biological Processes (GO-BP) revealed multiple up and down regulated processes which is described below and summarised in Figure 5.2, Figure 5.3, Figure 5.4 and Figure 5.5.

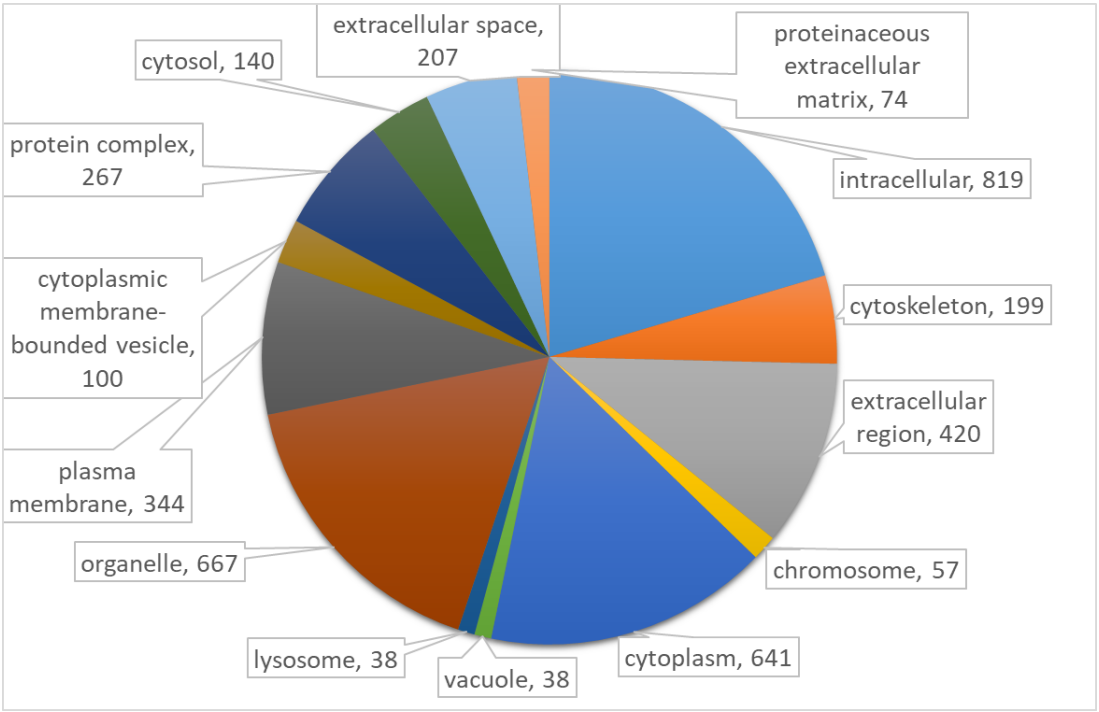


Figure 5.2: Pie Chart illustrating Gene Ontology Slim: Cellular Component pie chart for all proteins identified, with the number of proteins in (brackets). The highest proportion of proteins identified were from intracellular proteins (819), followed by organelle proteins (667) and the cytoplasm (641).

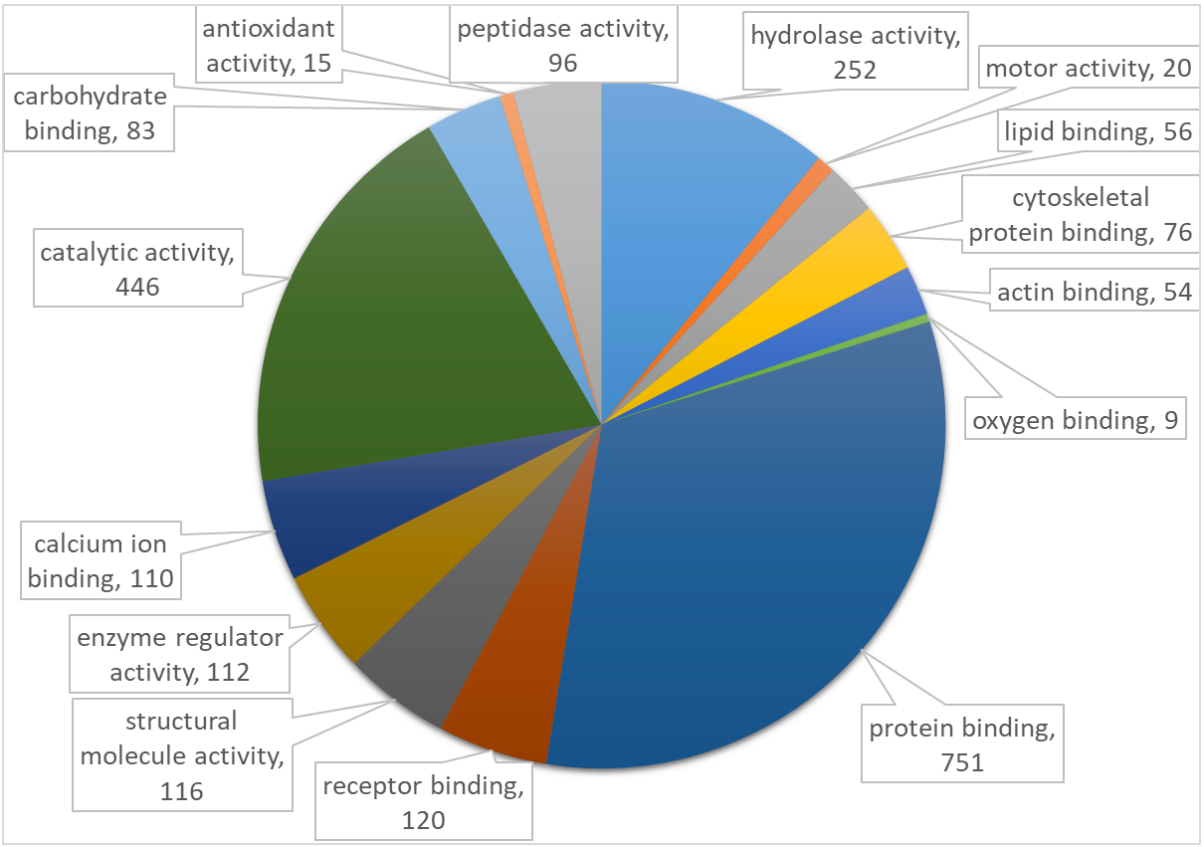


Figure 5.3: Pie Chart illustrating Gene Ontology Slim: Molecular Function pie chart for all proteins identified, with the number of proteins in (brackets). The highest proportion of proteins identified were involved in protein binding (751), followed by catalytic activity (446) and hydrolase activity (252).

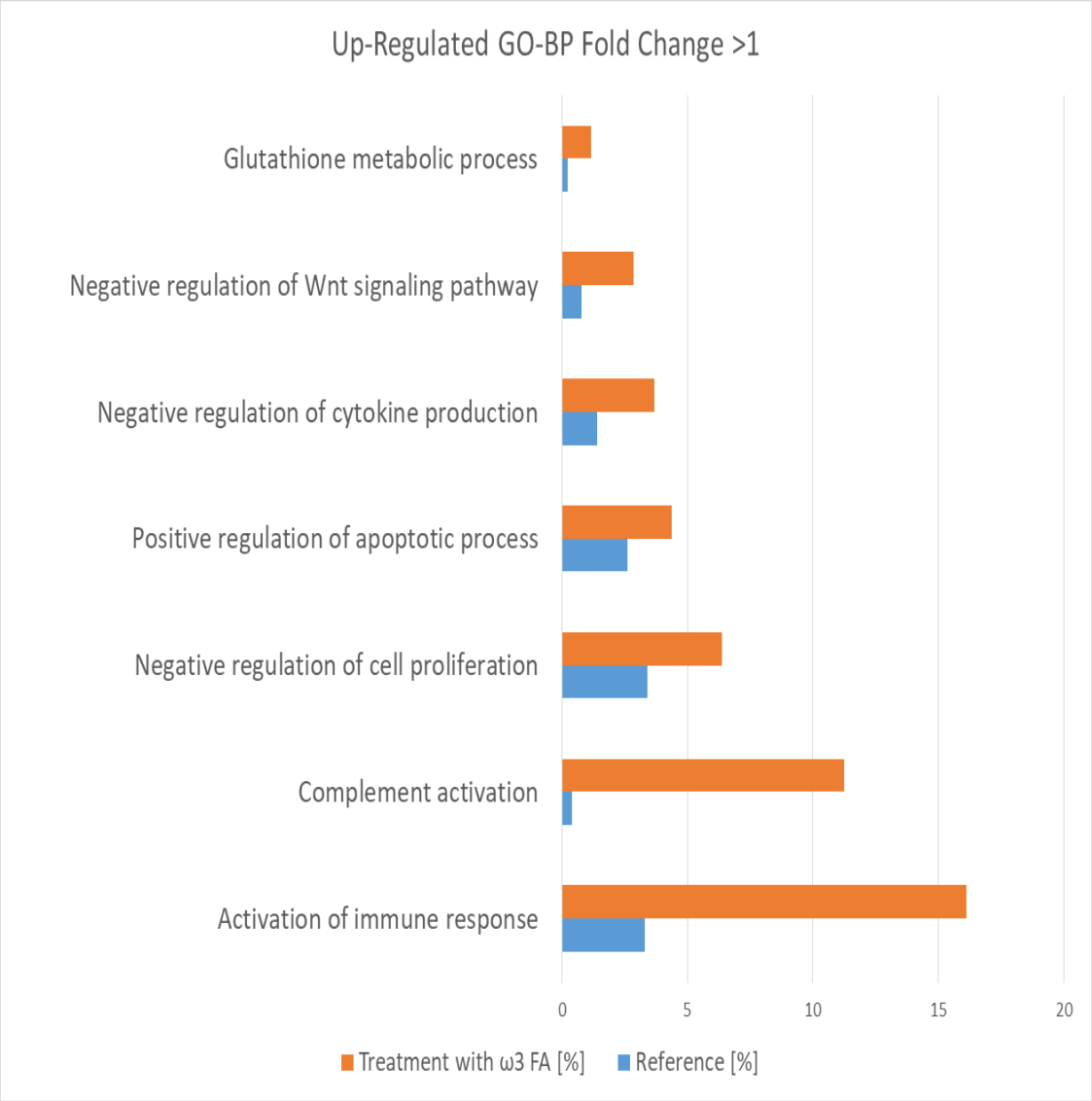


Figure 5.4: Over Represented Gene Ontology Biological Processes identified from protein with fold changes >1 following treatment with n-3 fatty acids and gemcitabine versus gemcitabine alone. All p values <0.05. There is a positive regulation in the immune system process and apoptotic pathway and negative effects on cytokines and cancer signalling pathway on treatment with n-3 fatty acids.

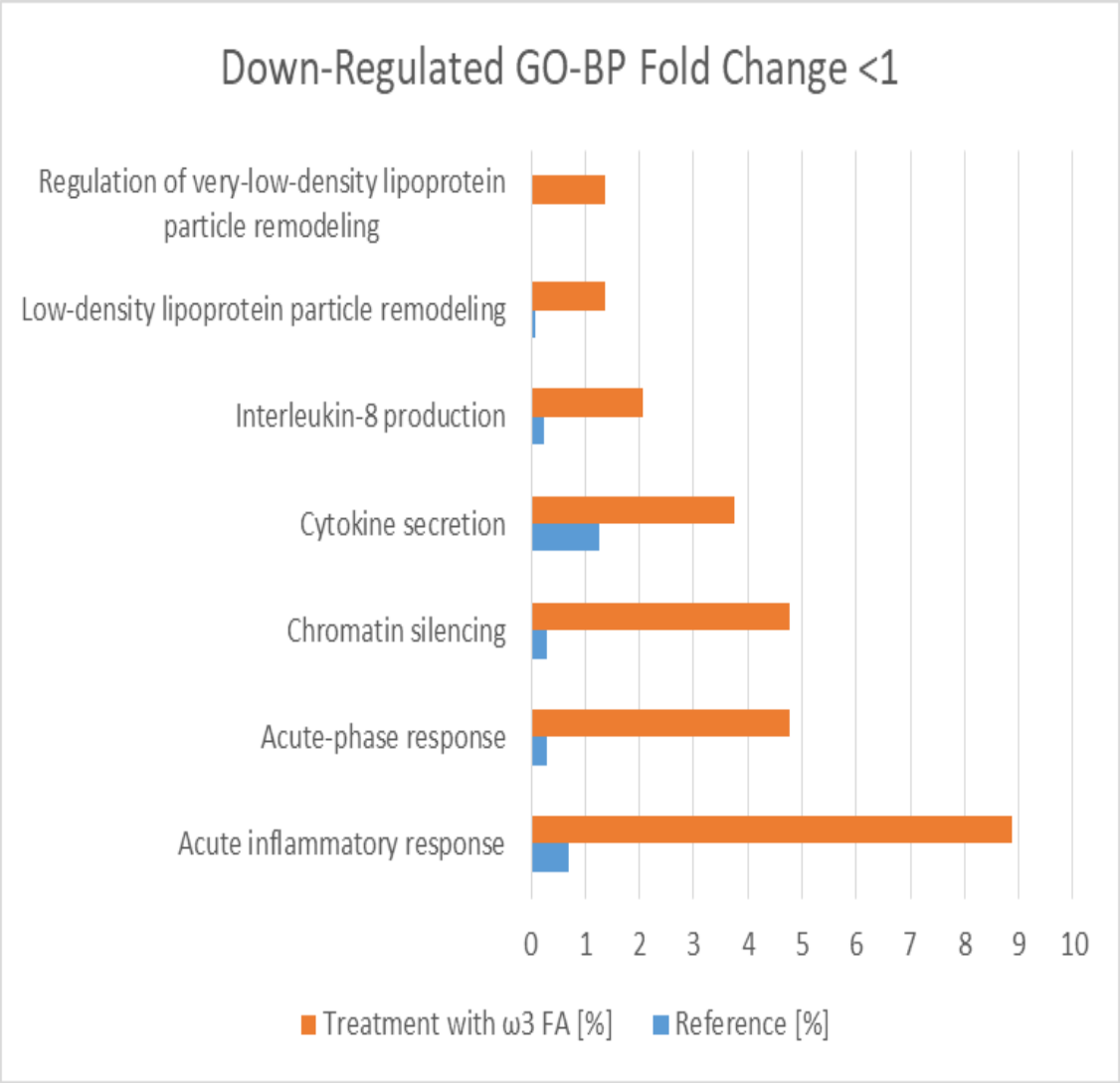


Figure 5.5: Over Represented Gene Ontology Biological Processes identified from protein with fold changes <1 following treatment with n-3 fatty acids and gemcitabine versus gemcitabine alone. All p values <0.05. Strong reduction in the acute inflammatory processes, acute phase response and cytokine including interleukin 8 production on treatment with n-3 fatty acids. Associated chromatin silencing pointing towards epigenetic changes and down regulation of low and very low density lipoprotein particles.

5.3.3 Up Regulated Proteins: Fold change >1

Activation of Immune Response: Ninety six proteins identified corresponding to activation of the immune response systems (p = 4.23E-35). Proteins with high fold changes included the immunoglobulins heavy constant epsilon, lambda and heavy

variables (all with FC> 1.5). These immunoglobulins aid in fighting infections by taking part in various parts of the immune response.

Complement Activation: Sixty seven proteins were up regulated in relation to complement system activation ($p = 3.46E-71$). Of these, all three pathways of the complement system (classical, lectin and alternative) were positively up regulated, with strong significance ($p<0.001$). Various immunoglobulins and complement factors were significantly up regulated with fold changes > 1.5, of these, Complement component 6 (P13671) obtained a high fold change of 3.5: this protein is part of the membrane attack complex (MAC) which forms pores in the plasma membrane of target cells. Figure 5.6 illustrates all the component factors identified which showed an overall positive fold change.

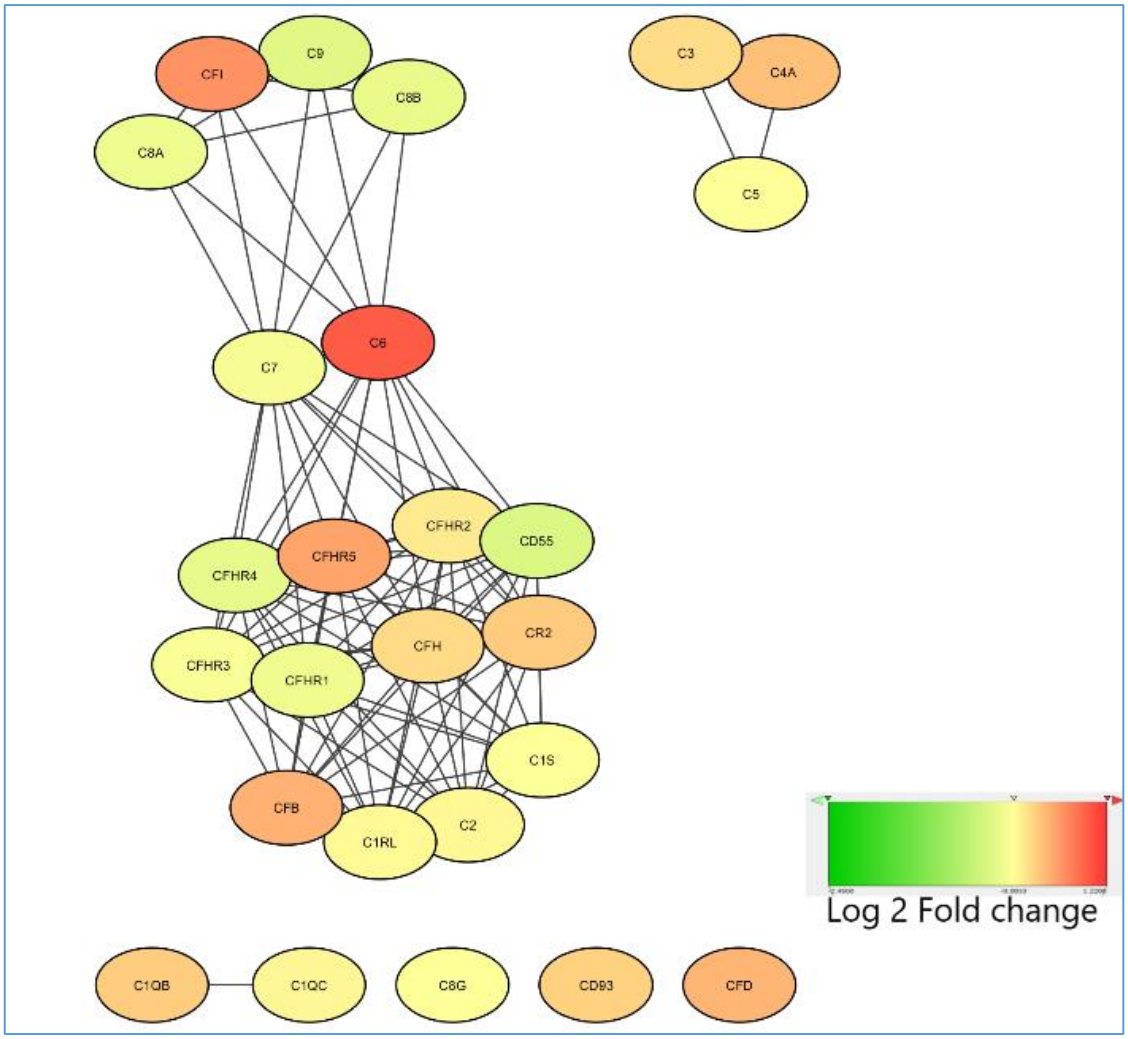


Figure 5.6: Complement protein nodes identified, mainly positively upregulated, highest fold change seen in complement factor C6 (FC 3.5)

Negative regulation of cell proliferation: Thirty eight proteins were involved in negative cell proliferation (p= 0.00187). Of these Tumour necrosis factor receptor superfamily member 8 (TNFRSF8) a member of the TNF family showed a positive fold change of 4.55. This protein is involved in regulation of cellular growth and regulates gene expression via the NF-Kappa β pathway. Glutathione S-transferase P was also strongly elevated (Fold change 9.19), similar to our previous findings. Another interesting up-regulated protein is Superoxide dismutase [Mn], mitochondrial, SOD2 (Fold change = 6.38). This protein is involved in the destruction of superoxide anion molecules.

Positive regulation of apoptosis: Twenty six proteins were involved in the positive regulation of apoptotic processes (p= 0.0415) which is directly related to the regulation of programme cell death and is one of the hallmarks of cancers. In relation to the negative regulation cell proliferation, Tumour Necrosis Factors was strongly up-regulated. Protein DJ-1 (Q99497) was strongly up regulated in relation to apoptosis (Fold change >10). This protein is involved in protein and nucleotide deglycase for nucleotide repair. The other interesting up-regulated proteins were the 14-3-3 proteins (sigma, epsilon and zeta/delta: fold change >1.5). These are constituent proteins involved in a large number of generalised and specialised pathways, including cancer specific pathways.

Negative regulation of cytokine production: Twenty two proteins were involved in the negative regulation of cytokine production (p = 0.000572). Of interest, Lactotransferrin is an iron binding transport protein which interferes with the lipopolysaccharide (LPS)-stimulated Toll Like Receptor (TLR) - 4 signalling. Tyrosine-protein kinase receptor UFO (P30530), similarly is upregulated which is associated with interfering with the TLR signalling.

5.3.4 Down Regulated Proteins: Fold Change <1

Acute Inflammatory Response and Acute Phase Response: There were strong reductions in both the acute inflammatory response (26 proteins, p= 9.14E-19) and the acute phase response (14 proteins, p= 2.55E-11) processes. Of interest, multiple immunoglobulins (lambda, kappa, heavy variables) were strongly downregulated with fold changes <1.5.

The acute phase proteins Serum Amyloid A-1, Serum Amyloid A-2 and Haptoglobin (HP) were all strongly down regulated FC <1.5. CRP was also down regulated. This is illustrated in Figure 5.7.

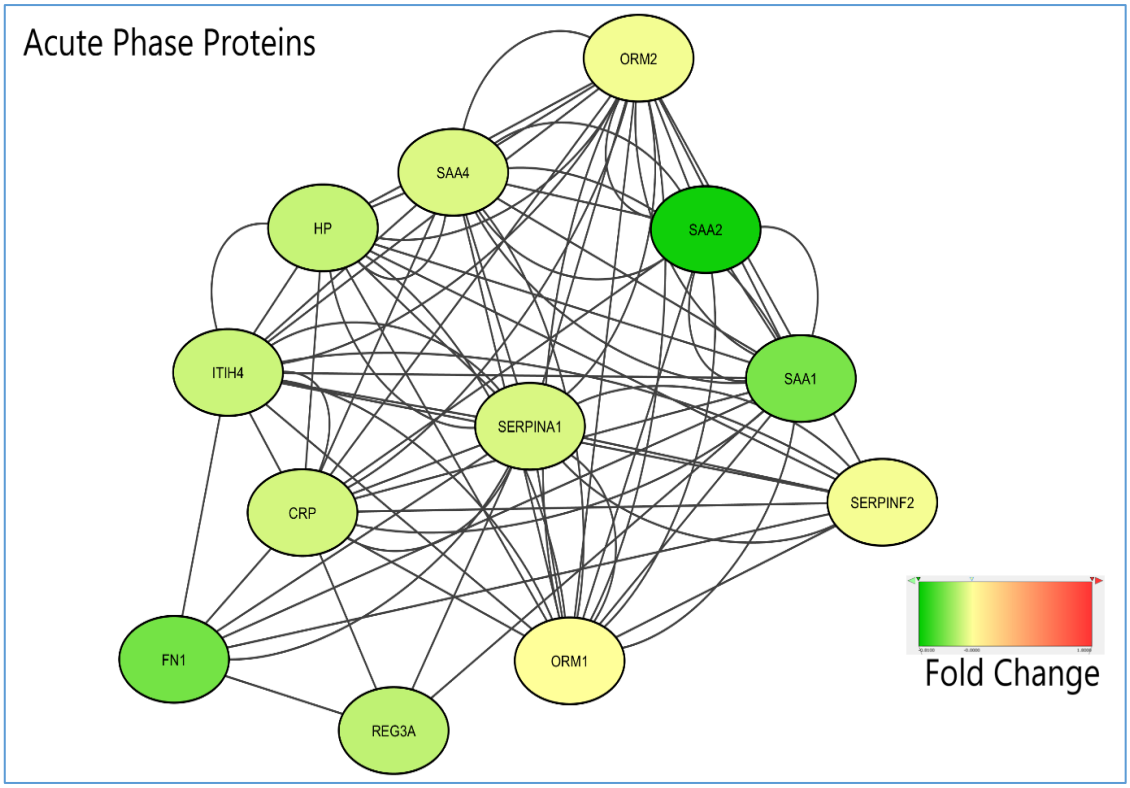


Figure 5.7: Down regulation of the acute phase proteins.

Chromatin Silencing: There was a strong down regulation of the histone H2 and H4 proteins, following administration of n-3 fatty acids. Fourteen proteins were involved (p = 1.97E-11). This again reiterates the epigenetic effect n-3 fatty acid has in plasma, correcting for gemcitabine. There was also up-regulation of the Histone-lysine N-methyltransferase 2C (Fold change 3.89) a methylation protein involved in the epigenetic translational activity. Figure 5.8 illustrates these effects.

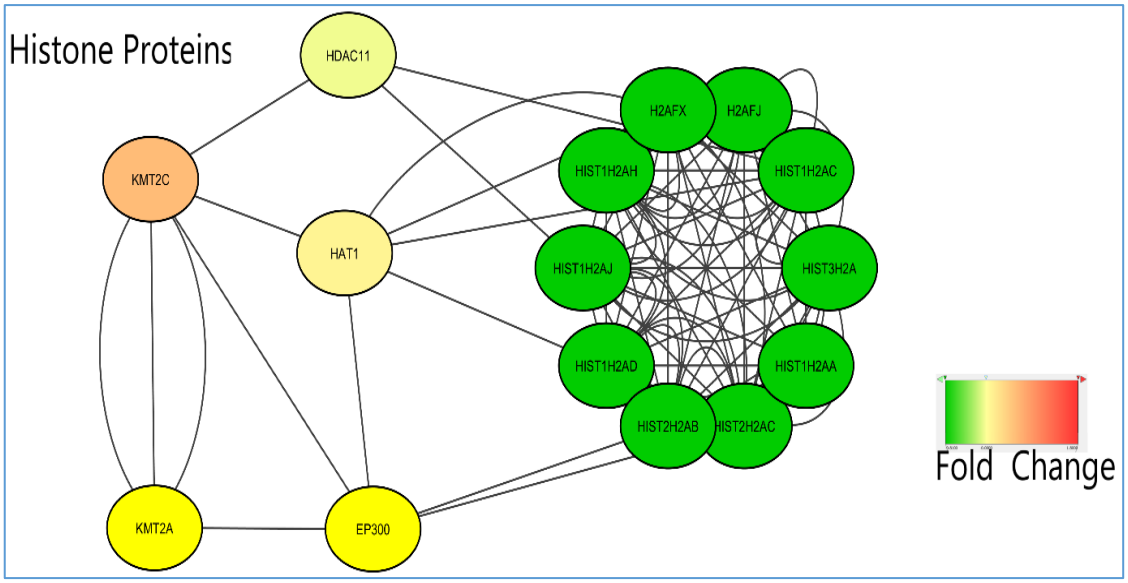


Figure 5.8: Down regulation of Histone H2 bodies and up regulation of the methylation protein Histone-lysine N-methyltransferase 2C (KMT2C).

Cytokine Secretion: Cytokine secretion was down regulated (11 proteins, p= 0.00973), particularly interleukin-8 production. The common proteins involved in this process included CRP and Soluble scavenger receptor cysteine-rich domain-containing protein SSC5D. Specific to interleukin-8, Lipopolysaccharide-binding protein (Fold change <2.09), a protein which facilitates CD14 to lipopolysaccharides, thereby reducing cytokine production, was down regulated.

5.3.5 Pathways

Cancer Pathway: Correlating our protein changes following treatment with n-3 fatty acids versus gemcitabine alone, several proteins involved in pancreatic cancer pathways have been identified. In the PI3k-AKT pathway, there is a combination of down regulation of HSP90 alpha and beta proteins (Fold change < 1.3) and up regulation of the 14-3-3 protein family (epsilon, gamma, sigma and zeta/delta, fold change > 1.5). Heat Shock Protein 90 family proteins are chaperone proteins which stabilise multiple processes including cell cycle control and transduction. In the Pi3K-AKT pathway it promotes the formation of AKT leading to further oncogenic downstream changes. Inhibitors of HSP 90 have been shown to lead to pancreatic cancer cell deaths [122] and

epigenetic changes leading to DNA methylation [123]. Our results also identified protein Phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchanger 1, which belongs in the same family as PI3K, which was down regulated (Fold change <1.43) (Figure 5.9). Another protein involved in a cancer pathway is Protein Wnt-6, which is a direct constituent of the WNT cancer pathway. There was down regulation of this protein (Fold change <1.41) and it has been shown that WNT inhibitors are tumorigenic to pancreatic cancer cell lines [124].

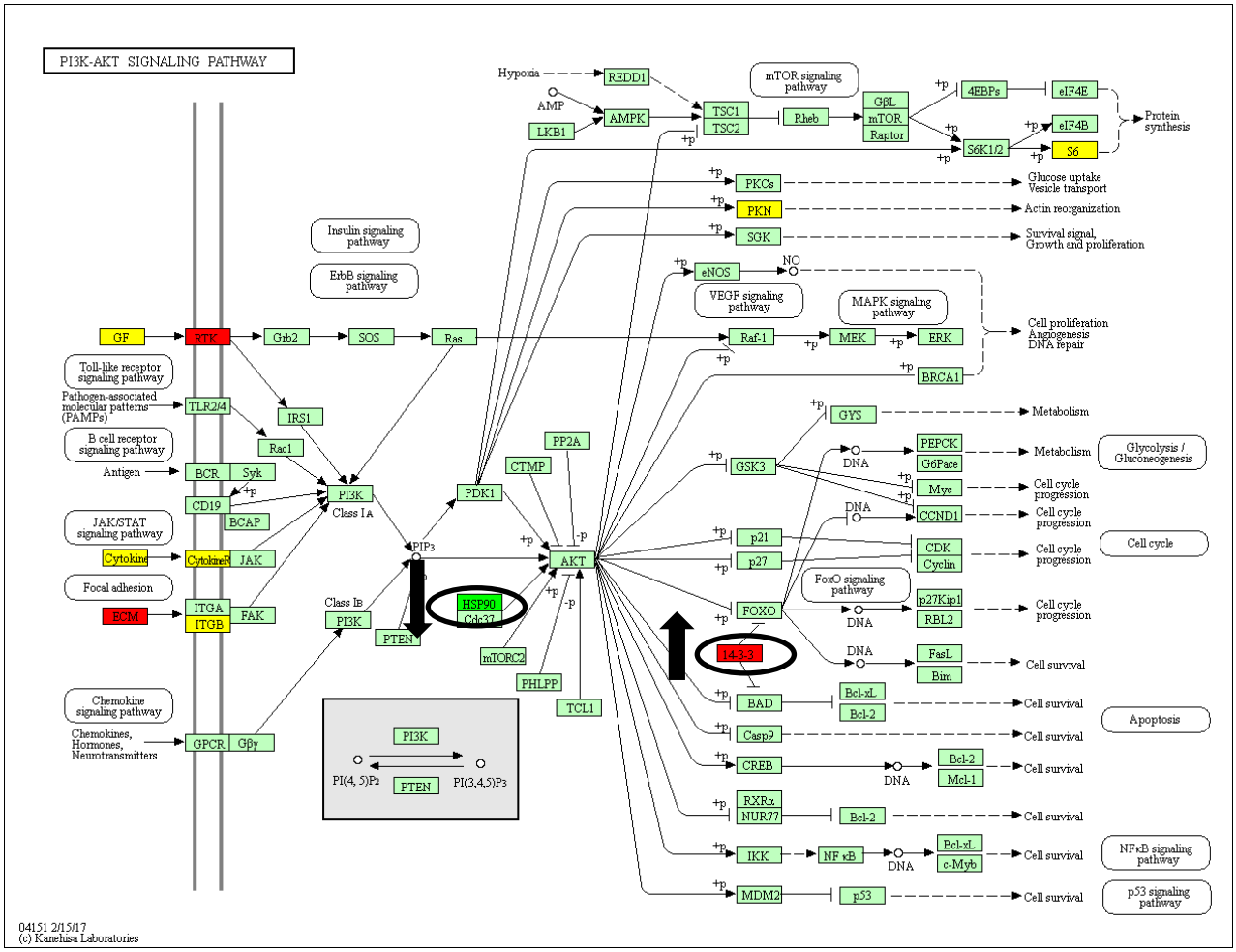


Figure 5.9: PI3k-AKT pathway showing downregulation of HSP90 and up regulation of 14-3-3 proteins.

Coagulation and Complement Cascade: Proteomic analysis was performed on plasma samples. As such, a large majority of proteins found would be constituents of plasma, particularly the coagulation and complement cascade. As has been mentioned, on Gene

Ontology enrichment analysis, the complement cascade is up regulated. There is also multiple up regulation of various coagulation factors 2, 5, 7 11, 12 and 13. This may be due to the pro-coagulopathic effect in plasma of patients with advanced pancreatic cancer. Figure 5.10 illustrates this effect.

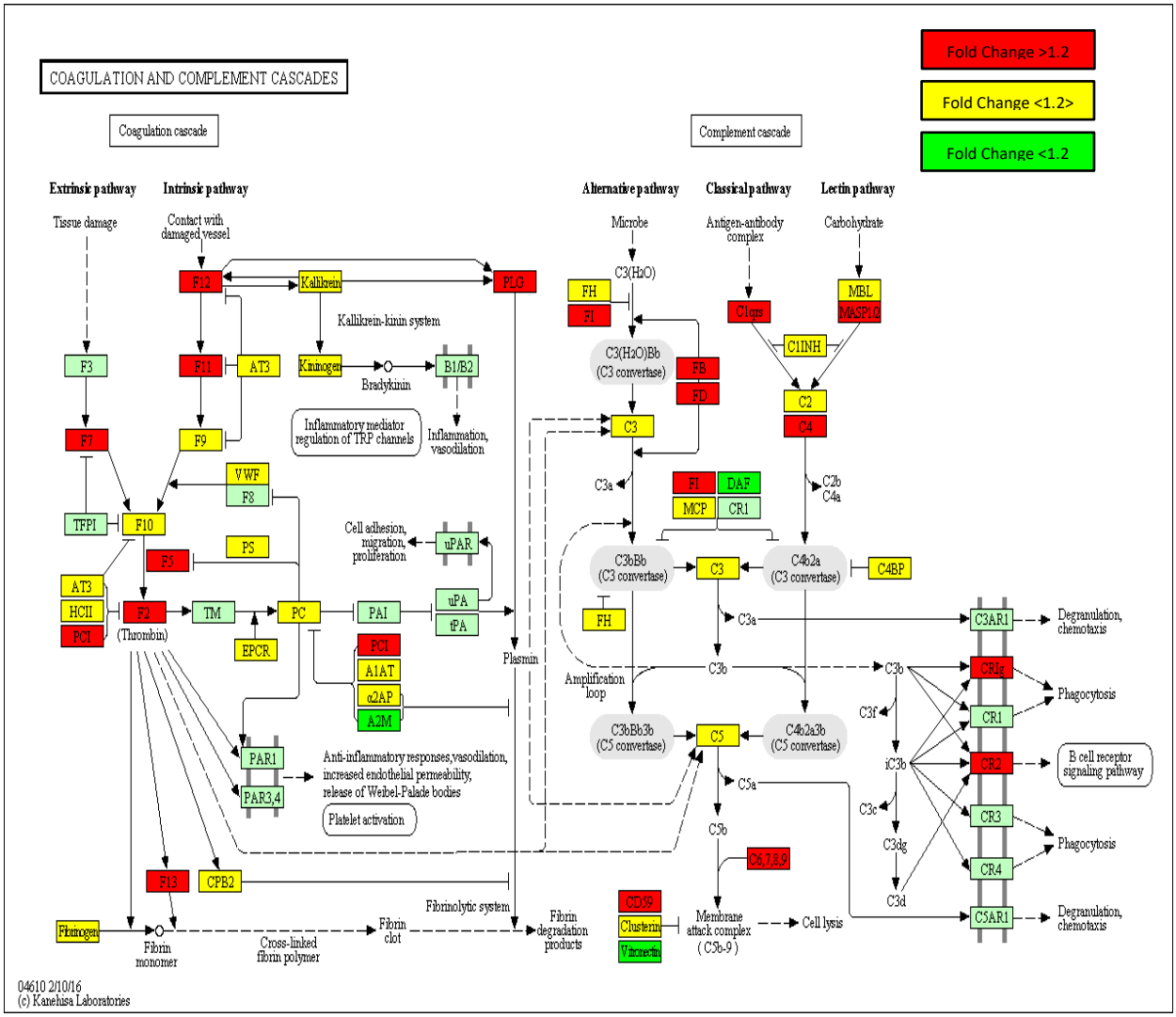


Figure 5.10: Coagulation and complement cascade, majority of coagulation factors identified from analysis, which were mainly up regulated in keeping with pro-coagulopathic effect on advanced pancreatic cancer

Extra Cellular Matrix (ECM) Receptor Interaction: Similarly, multiple proteins involved in the extracellular matrix were identified, with a mixed picture. This again

reflects that the majority of the extracellular matrix proteins are identified within the plasma. (Figure 5.11).

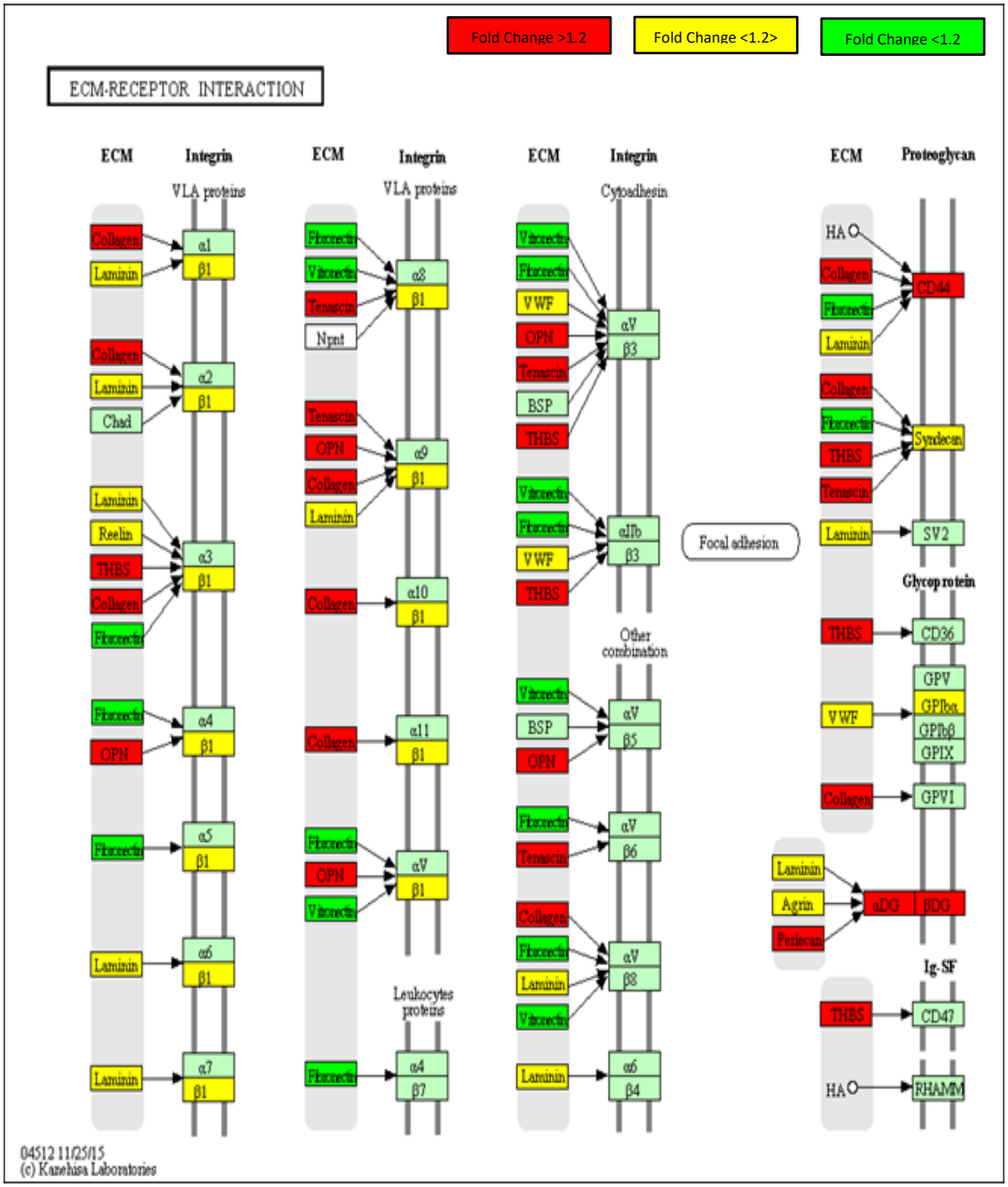


Figure 5.11: ECM-Receptor interaction, showing a large number of proteins identified from the extracellular matrix.

5.3.6 Correlation with Scaffold 4.7

The MSF file was reprocessed with Scaffold 4.7, under conditions of at least 95% protein probability, 95% peptide probability and minimum of two peptides per protein, corrected for the combined Quality Control (QC) samples. A total of 1047 proteins were discovered. (Section 9.6 Appendix Table 6). The associated fold change between treatment (n-3 fatty acids & gemcitabine) vs control (gemcitabine alone) was obtained, then compared to the associated protein fold changes from PD 2.1 and normalised for comparison. There were 627 matching proteins.

5.4 Discussion

This analysis has again shown that correcting for the effects of gemcitabine, n-3 fatty acid infusion has an anti-inflammatory effect in the plasma proteomic profile. Known markers for inflammation (example Haptoglobin, CRP and Serum Amyloid A 1 and 2 Proteins) all showed marked significant reduction in quantitative analysis. Using Gene Ontology enrichment, this was further confirmed with significant findings of multiple proteins related to biological processes involved in the anti-inflammatory process. This also correlates with the overall effect of decreased cytokine production and increased in the complement pathways.

This experiment yields 25 out of the 36 proteins involved in the coagulation cascade (Figure 5.10). As coagulation factors comprise a large proportion of proteins in plasma, this was to be expected. There was a quantitative increase in the coagulopathic factors identified. Other studies have further found that use of n-3 fatty acids has no effect on the coagulation status of patients in septic or cancer patients [125,126]. Therefore it is proposed that the increase in coagulopathic factors is secondary to the presence of advanced pancreatic cancer in these patients.

Epigenetic modification leading to translational changes in the chromatin is an exciting avenue in cancer research. The discovery phase (Section 4.3.5) already identified a potential mechanism of down regulation of histone bodies (H2) and upregulation of

methylation and deacetylation enzymes (Section 4.4). In this experiment, correcting for the use of gemcitabine, again it is shown that n-3 fatty acid infusion results in down regulation of Histone H2 and H4 proteins and up regulation of Histone-lysine N-methyltransferase 2C, a methylation enzyme. Recent work has postulated a diet supplemented with n-3 fatty acid may increase the expression of chemo-protective genes by histone modification in rat colon cancer models [127]. Huang *et al* further identified a potential mechanism of n-3 fatty acid preventing the decrease of DNA methylation in colorectal cancer rat models, which would fit with our findings [128]. To date, there has not been any documented work describing the epigenetic modification of n-3 fatty acid in patients with advanced pancreatic cancer.

As explained, the original clinical study contained only a single treatment arm of n-3 fatty acid and gemcitabine in patients with advanced pancreatic cancer [77]. By recruiting the control arm of patients with advanced pancreatic cancer treated only with gemcitabine, it was hoped to provide a comparison arm to elucidate the effect of n-3 fatty acid correcting for gemcitabine. However, ideally this would have been done at the start of the original study to minimise treatment and selection bias hence improving the strength of the clinical and proteomic results. Similarly, though with the addition of a control arm, as the treatment arm contained both n-3 fatty acids and gemcitabine, it is difficult to attribute the effects identified above solely due to the administration of n-3 fatty acids (it may have acted independently or synergistically with gemcitabine). The only way to elucidate this would have been to only have an arm of patients treated with n-3 fatty acids without gemcitabine, though this would be unethical as a treatment option in advanced pancreatic cancer.

The next chapter will outline efforts to verify some selected proteins of interest from this cohort of patients.

6 Results III: Verification with Selective Reaction Monitoring

6.1 Introduction

The previous chapters have shown the direct effects of n-3 fatty acid infusion in patients with APC include down regulation of the inflammatory processes on anti-inflammatory markers, modulation of cancer pathways including PI3k-AKT pathway and direct modulation pm epigenetic factors on histone bodies via methylation enzymes. This next chapter aims to highlight the efforts to verify these changes using Selective Reaction Monitoring (SRM).

SRM uses the unique capabilities of the Triple Quadrupole instrument (TQ) where the first and third quadrupoles act as filters to select predefined m/z values corresponding to the peptide ion and a specific fragment respectively, whilst the second quadrupole acts as the collision cell [59]. In filtering the ions in this highly selective manner, background noise is effectively eliminated, thus boosting signal to noise (s/n) significantly. SRM techniques have evolved and are able to measure peptides down to the low femtomoles concentrations in complex samples, for example in human plasma [129]. Many proteotypic peptides (peptides cleaved by trypsin) produce specific m/z ratios, which can be found within SRM atlas (http://www.srmatlas.org). These transitions can then be used to establish assays which allow quantification on the TQ.

6.2 Materials and Methods

6.2.1 Putative Protein Markers

Based on the findings that n-3 fatty acids have direct anti-inflammatory, anti-apoptotic growth factors, modulation on cancer pathways and epigenetic changes in advanced pancreatic cancer, a shortlist of 7 proteins and 15 peptides were selected. These were shortlisted from their strong fold changes (more or less than 1.5), or significant p values or postulated direct effect on cancer pathways (Pi3K-AKT and TGF-β). Due to pressures on time and limited resources, only 15 peptides were selected from a possible 8862 peptide groups. However, the above selection criteria identified statistically

significant peptides showing potential clinical relevance. The proteins are summarised in Table 6.1.

Uniprot Accession ID	Protein Name	Description
P0DJI8	Serum amyloid A-1 protein (SAA1)	SAA1 is a major acute phase protein, involved in various levels of the inflammatory process including cytokine production. Lower levels of SAA is associated with improved overall survival in advanced pancreatic cancer patients [130]. In our discovery phase, SAA1 was reduced <1.5 fold changes with p <0.001, with n-3 fatty acid infusion from baseline as well as corrected for gemcitabine.
P02741	C-reactive protein OS (CRP)	CRP is a widely used marker of inflammation used in clinical practice, found in plasma and is induced by the presence if interleukins 1 and 6. CRP was reduced with a fold change <2, p<0.05, with n-3 fatty acid infusion compared to baseline.
P08238	Heat shock protein HSP 90-beta (HSP90AB1)	HSP90AB1 is a molecular chaperone protein involved in cell cycle and signal transduction. Inhibitors of HSP90 are pro-apoptotic and anti-proliferative in pancreatic cancer cell line [122]. It is involved in the Pi3K-AKT and NF-KB pathways. HSP90 inhibitors also aid in gemcitabine resistance in pancreatic cancer

		cell lines [131]. We identified a reduction (Fold change <1.3) with a strong p<0.01 in our cohort receiving n-3 fatty acids compared to gemcitabine alone.
P07900	Heat shock protein HSP 90-alpha (HSP90AA1)	HSP90AA1 is molecularly very similar to HSP90AB1, with similar findings to above. We employed the use a generic peptide sequence coding for both HSP90AA1 and AB1 (peptide sequence ADLINNLGTIAK).
Q9BYW2	Histone-lysine N-methyltransferase (SETD2)	SETD2 is involved in methylation of histone bodies, modulation of chromatin structure hence is important in translational epigenetic modifications. Mutations in SETD2 is involved in 24% of pancreatic ductal adenocarcinomas [14]. Our cohort showed an upregulation of SETD2 with n-3 fatty acid infusion.
P41271	Neuroblastoma suppressor of tumorigenicity 1 (NBL1)	NBL1 is a tumour suppressor gene for neuroblastoma. High expression is associated with pancreatic adenocarcinoma [132]. In the TGF-β signalling pathway, NBL1 inhibits BMP which further inhibits TGF-β and SMAD signalling [133]. In our discovery phase, there was a moderate increase in NBL1 (FC >1.3) with n-3 fatty acid infusion.
Q8WUH2	Transforming growth factor-beta receptor-associated	TGFBRAP1 plays a direct role in the TGF-β pathway. It is associated with SMAD4 proteins, which is strongly associated with pancreatic adenocarcinoma. Our discovery phase identified strong upregulation (>2

	protein 1 (TGFBRAP1)	fold change, p<0.001) with n-3 fatty acid treatment, compared to baseline and gemcitabine.
Q9Y5Y7	Lymphatic vessel endothelial hyaluronic acid receptor 1 (LYVE1)	LYVE1 is an early marker of pancreatic adenocarcinoma [111]. Identification of this protein would strengthen confidence in the methodology.

Table 6.1: Shortlisted protein for SRM analysis and verification with description summarising protein choices.

6.2.2 Peptide Selection

From the shortlist of proteins (Table 6.1), two proteotypic peptides were selected for each protein based on their uniqueness to each protein (no isomers) which were also identified from PD 2.1 or Scaffold 4.7 with high confidence. Each peptide were examined on databases (https://www.proteomicsdb.org and https://blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure its proteotypicity and uniqueness to the particular protein. These peptide sequences were further examined on SRMAtlas (http://www.srmatlas.org) which lists the most optimum peptide ion and top three specific fragment ions.

Two peptide sequences were chosen, with standards obtained for each, all 15 peptides unlabelled for qualitative measurement and selected 9 heavy labelled for accurate quantitative analysis. 15 unlabelled peptides (purity: desalted, condition: dry powder) were acquired from GeneCust Europe Ltd (Ellange, Luxembourg). 9 heavy labelled peptides (^{13}C, ^{15}N for C-terminus of Lysine and Arginine, Purity >99%, condition: clear solution, concentration: 5000 fmol/µL in ~5%Acn/H_2O) were acquired from Pepscan Presto Ltd (Lelystad, The Netherlands). Peptide modification of Carbamidomethylation

of the free Cysteines. Table 6.2 lists the proteins with the corresponding peptides, modifications and standard labels.

Uniprot Accession ID	Protein ID	Sequence	Modifications	Sequence Length	Standard Label	Molecular Weight
P08238	HSP90AB1	ADLINNLGTIAK		12	Heavy & Unlabelled	1242.45
P08238	HSP90AB1	TLTLVDTGIGMTK		13	Heavy & Unlabelled	1349.62
P07900	HSP90AA1	HLEINPDHSIIETLR		15	Heavy & Unlabelled	1787.02
P0DJI8	SAA1	GPGGVWAAEAISDAR		15	Unlabelled only	1456.59
P0DJI8	SAA1	FFGHGAEDSLADQAANEWGR		20	Heavy & Unlabelled	2178.28
P02741	CRP	GYSIFSYATK		10	Heavy & Unlabelled	1136.28
P02741	CRP	ESDTSYVSLK		10	Unlabelled only	11.28.21

Q9BYW2	SETD2	ETEPLV SPHQDK		12	Heavy & Unlabelled	1379.5
Q9BYW2	SETD2	FLTALG NEK		9	Unlabelled only	992.15
P41271	NBL1	SAWCE AK	1xCarba midomet hyl [C4]	7	Unlabelled only	850.96
P41271	NBL1	LALFPD K		7	Heavy & Unlabelled	802.98
Q8WUH2	TGFBRAP1	NSFNPD DIINCLK	1xCarba midomet hyl [C11]	13	Heavy & Unlabelled	1549.74
Q8WUH2	TGFBRAP1	SGQLDV R		7	Unlabelled only	773.85
Q9Y5Y7	LYVE1	LLGLSL AGK		9	Unlabelled only	871.1
Q9Y5Y7	LYVE1	IMGITL VSK		9	Heavy & Unlabelled	961.24

Table 6.2: Selected peptides for analysis with modifications and standard labels.

6.2.3 Peptide Transitions

Peptide sequences were entered onto SRMAtlas (http://www.srmatlas.org), which listed the top three optimum transitions. These transitions used to establish a SRM assay for each peptide (please refer to Section 2.4.4 **Mass spectrometry on the XEVO Triple Quadrupole** for mass spectrometer details). A series of experiments to scan for optimum transitions, collision energy and retention time windows where 1000

femtomoles (fmol) of standards were injected (2 µL, 500 fmol/µL) in 50 minute runs was performed.

Figure 6.1(a) is an example of a chromatogram obtained from the injection of peptide sequence FFGHGAEDSLADQAANEWGR. On completion of these experiments, the optimised top three transitions, collision energy and retention time window were obtained for all 15 peptides (both labelled and unlabelled). Optimised conditions are outlined in Section 9.7 Appendix Table 7.

6.2.4 Peptide Standard Calibration Curve on Skyline 3.7

Using the optimum transition, collision energy and retention windows, calibration curves were constructed for each standard peptides. The molecular weights for all peptides were obtained, from which a series of dilution was performed to obtain known concentrations.

From the 15 unlabelled, light peptide standards (Genecust, Ellange Luxembourg), a calibration line was constructed spanning 4 orders of magnitude from 10 fmol/µL to 2500 fmol/µL. For the heavy labelled peptides (PepScan, Lelystad, Netherlands), 500 fmol/µL spikes were added to the unlabelled, light peptides apart from peptides FFGHGAEDSLADQAANEWGR, HLEINPDHSIIETLR and ETEPLVSPHQDK, where concentration prepared was 1000 fmol/µL (During the preliminary transition SRM optimisation phase, it was discovered that the retention windows for these peptides were similar to other peptides, and yielded low TIC detection levels, hence they were analysed separately). A volume of 2 µL was injected onto the TQ. All samples were made up in 3% ACN / 0.1% FA.

The heavy labelled peptides act as a constant marker to control for variations in recovery, matrix effect and ionisation[134]. Figure 6.1 (b) illustrates the appearance on Skyline 3.7, where the blue chromatogram corresponds to the internal heavy label (1000 fmol/µL) and the red chromatogram corresponds to the unlabelled standards (100 fmol/µL). The area under the curve measures the amount of peptide in question.

Triplicate injections were performed. Standard calibration curves were constructed using the different concentrations of known unlabelled and heavy labelled peptides, with the constant heavy labelled peptides acting as a control for normalisation. These were used to measure the unknown concentration in the patient cohort.

6.2.5 Patient Cohort

The same cohort of patients with advanced pancreatic cancer receiving one month treatment of n-3 fatty acids and gemcitabine (Treatment – G05, G13, G15, G19 and G21) vs one month of gemcitabine alone (Control – C02, C03, C04, C06 and C08), as per Section 5.2.1 were analysed using SRM. Intensely immunodepleted plasma samples (IgY14 + Supermix Seppro) with known concentration of 4 µg from each patient were combined per group (20µg of protein combined per group): Treatment vs Control.

Each group was subjected to Rapigest solubilisation, DTT reduction, IAA alkylation, endoproteinase digestion using Trypsin and finally TFA addition to break up the Rapigest detergent for MS and endoproteinase reactions (as detailed in Section 2.5.3). Samples were lyophilised and reconstituted with the heavy labelled peptide standards (as detailed in 6.2.4) in 20 µL vials of 3% ACN / 0.1% FA. This produced a final protein concentration of 1 µg/µL per group (Treatment vs Control). Triplicate injections were performed on the TQ and unknown peptide concentration were measured on Skyline 3.7 using the known standard calibration curves.

Graphpad Prism 7.02 (San Diego, USA) was used for statistical analysis to detect any significant differences.

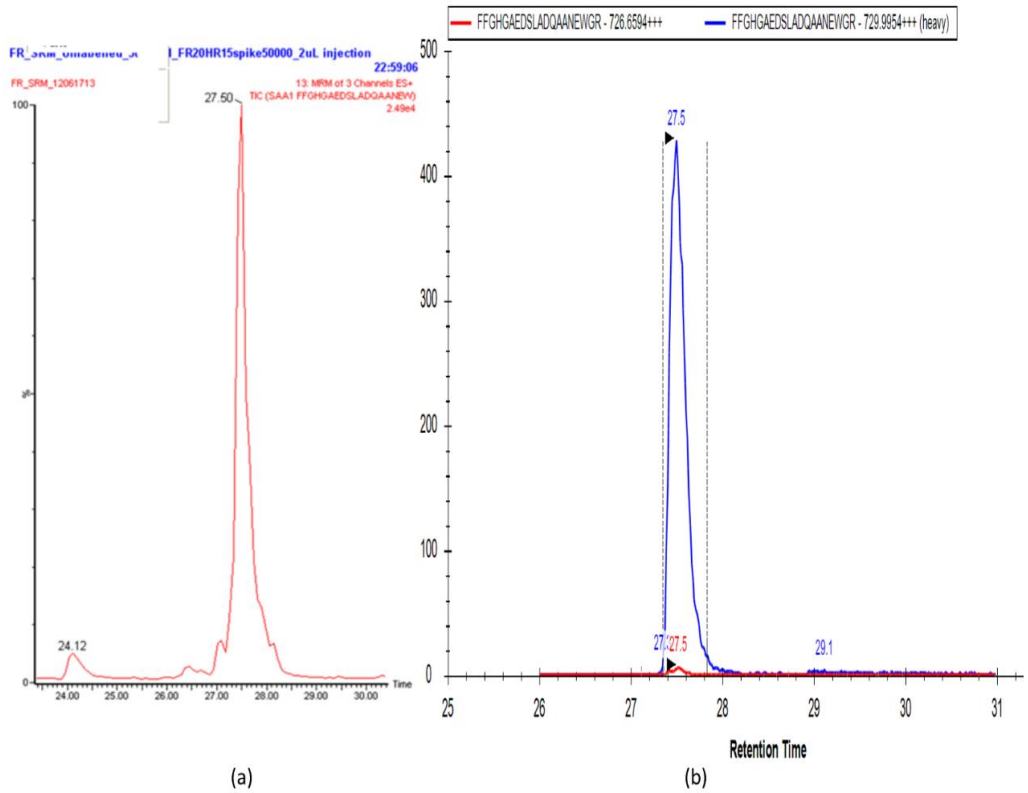


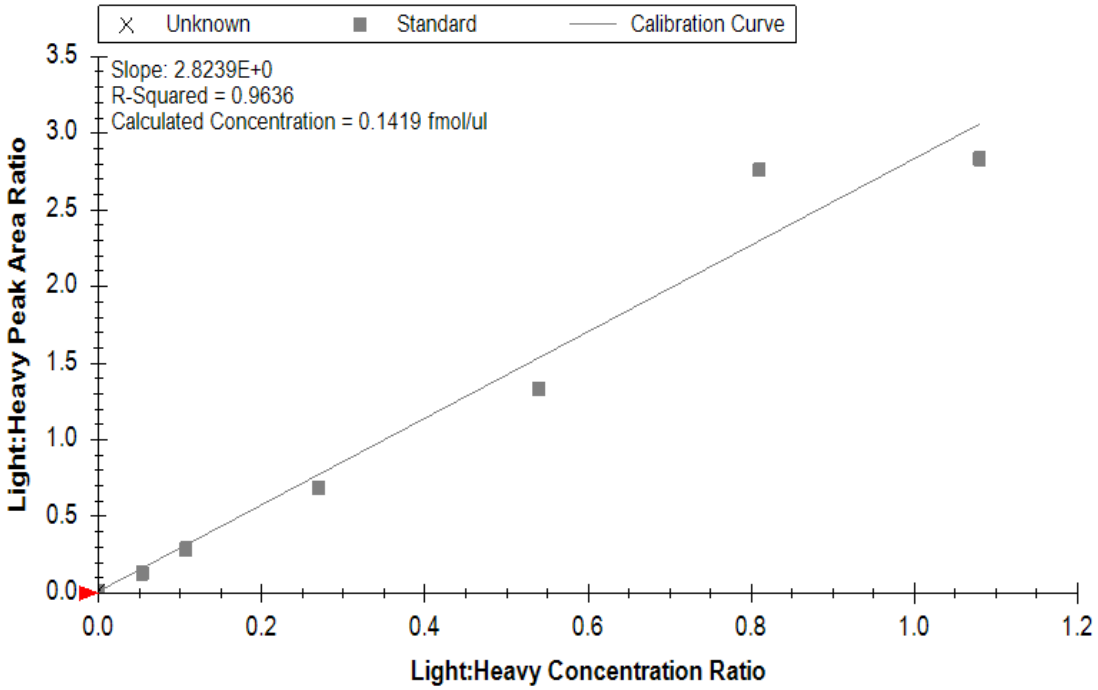
Figure 6.1: (a) Example of a chromatogram obtained for the peptide sequence FFGHGAEDSLADQAANEWGR, from TQ, viewed on MassLynx 4.1 (Waters, Milford USA), with a peak retention time at 27.50. (b) Skyline view of chromatogram when .raw files transferred into Skyline 3.7 – note two peaks, blue (heavy labelled) and red (unlabelled).

6.3 Results

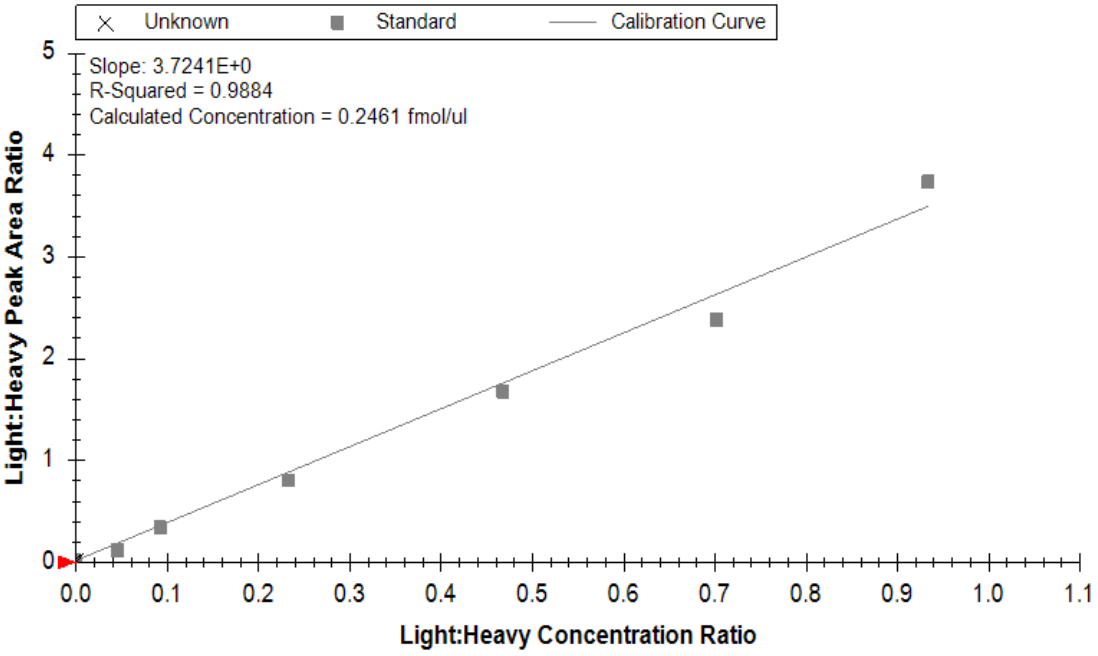
6.3.1 Peptide Calibration Curves and Sample Measurement

Peptide calibration curves were obtained for all 15 peptides, as shown in Figure 6.2. The R^2 measurement ranged from 0.9991 to 0.7263. From the patient cohort, 9 peptides were confidently identified and measurable. Peptide FLTALGNEK (SETD2), could not be reliably measured, as the calculated sample levels were too low, though qualitatively the chromatograms confirmed the presence of the peptide in the patient sample. This is illustrated in Figure 6.3 which shows a series of chromatograms from Skyline 3.7, from the top: (i) a low fmol/µL concentration standard, followed by the middle (ii)

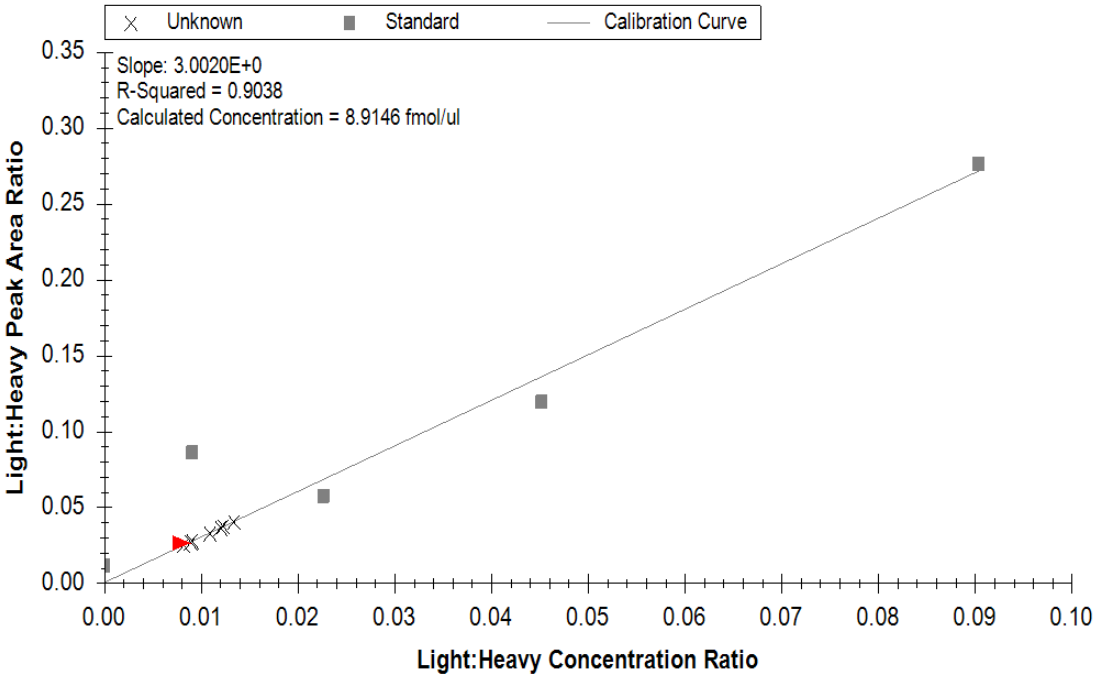
chromatogram at 0 fmol/µL and bottom (iii) showing a representative chromatogram from the patient cohort (either Treatment or Control).



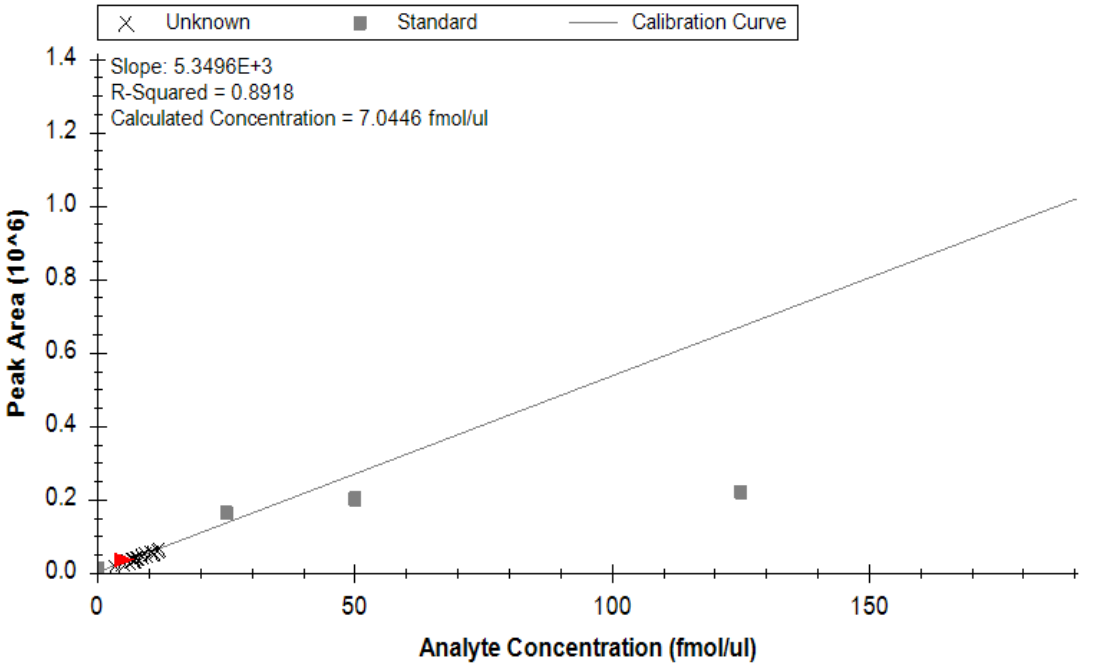
(a) Protein: HSP90AB1 Peptide: ADLINNLGTIAK



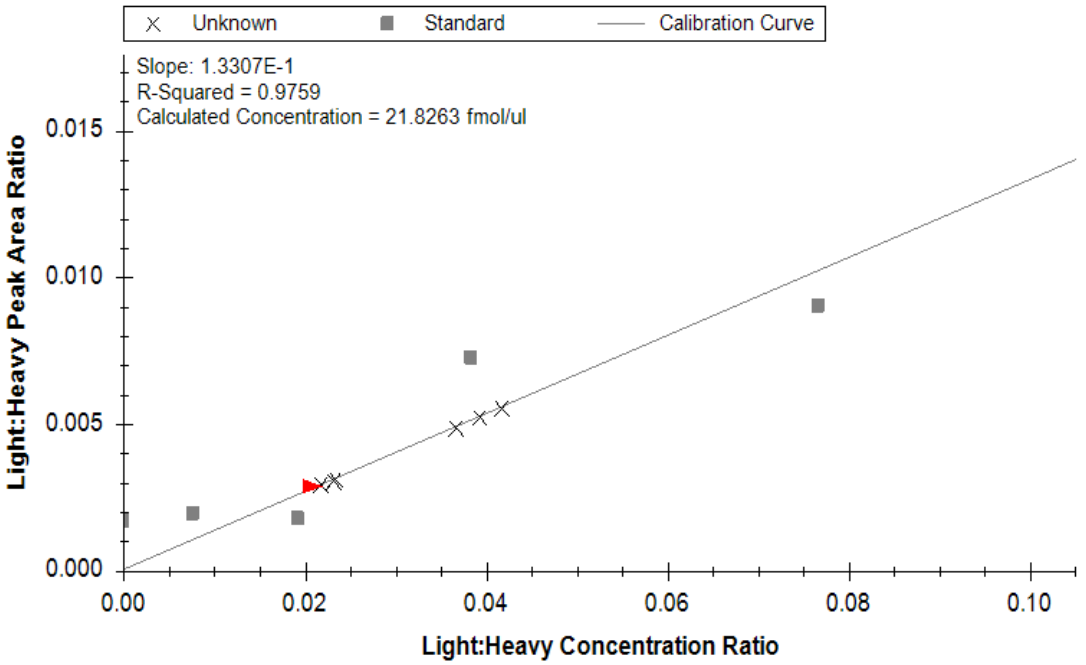
(b) Protein: HSP90AB1 Peptide: TLTLVDTGIGMTK



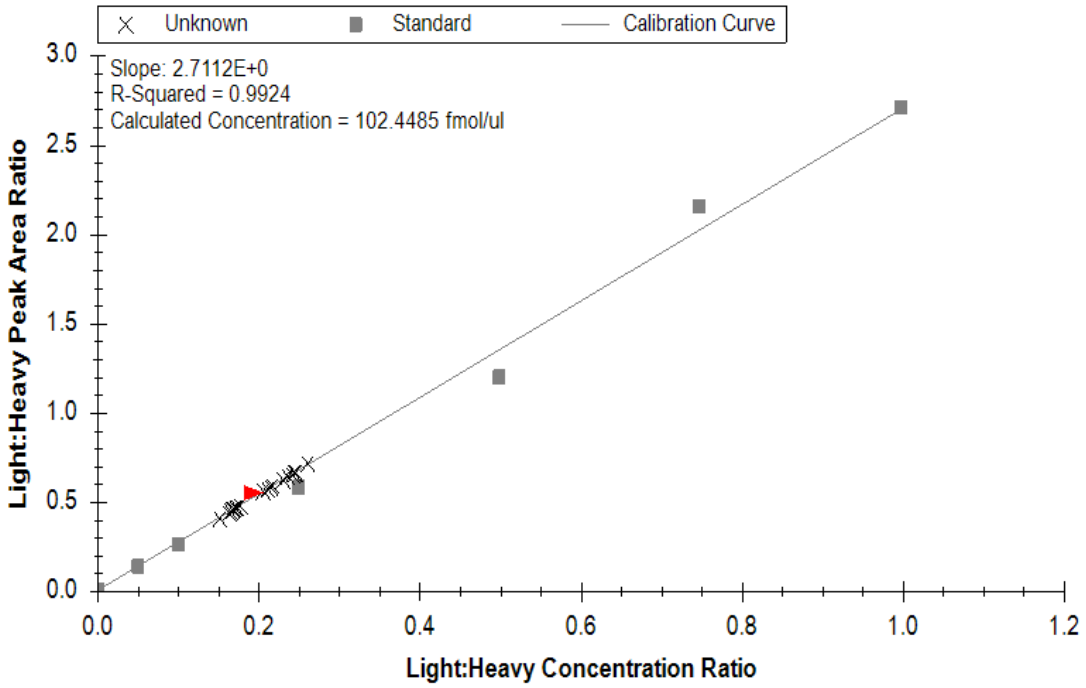
(c) Protein: HSP90AA1 Peptide: HLEINPDHSIIETLR



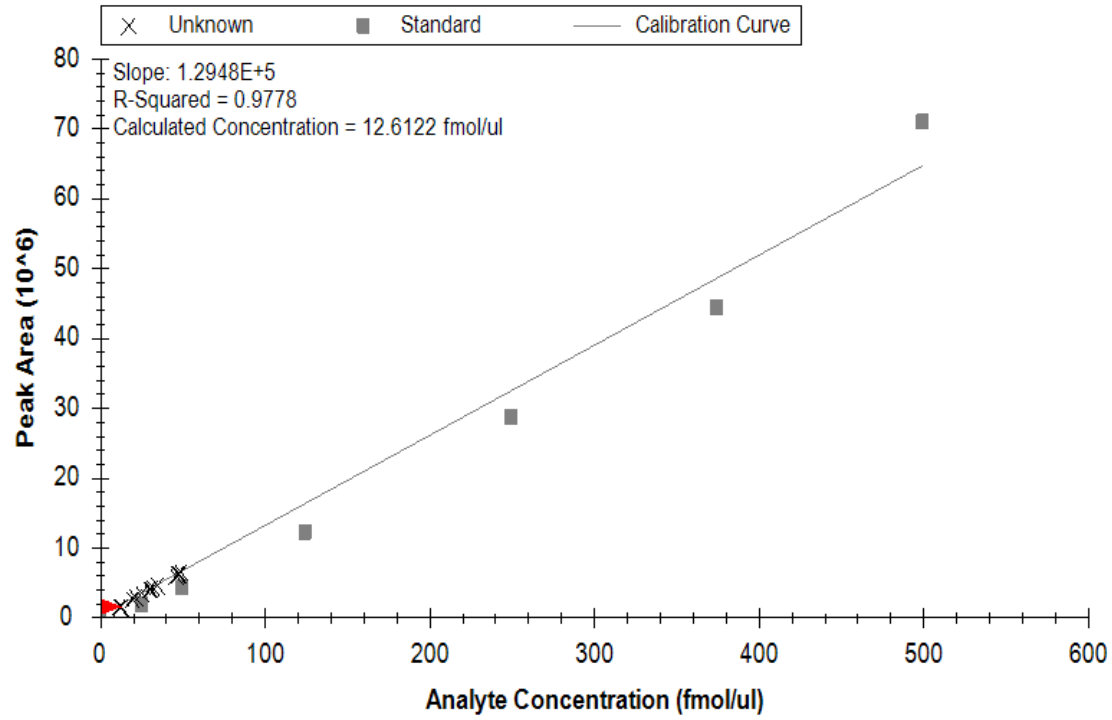
(d) Protein: SAA1 Peptide: GPGGVWAAEAISDAR



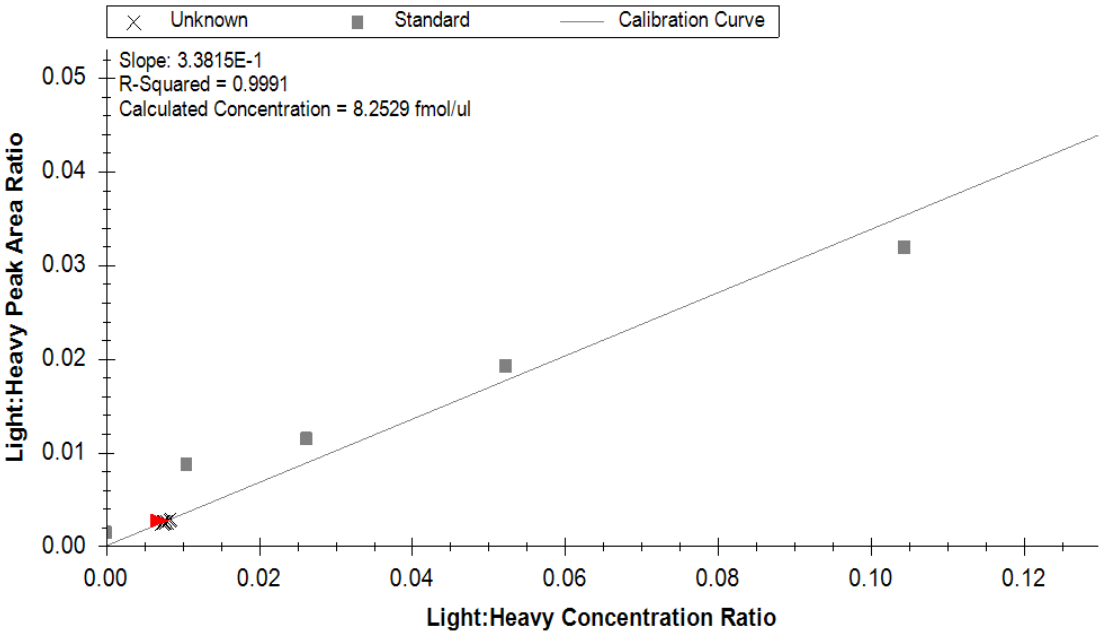
(e) Protein: SAA1 Peptide: FFGHGAEDSLADQAANEWGR



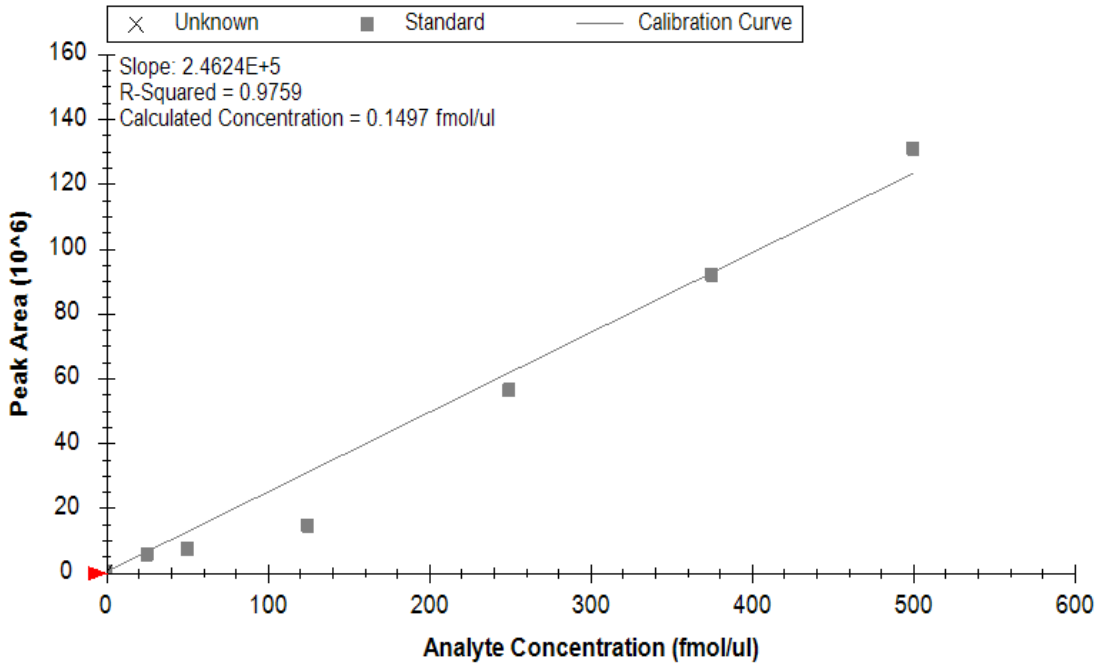
(f) Protein: CRP Peptide: GYSIFSYATK



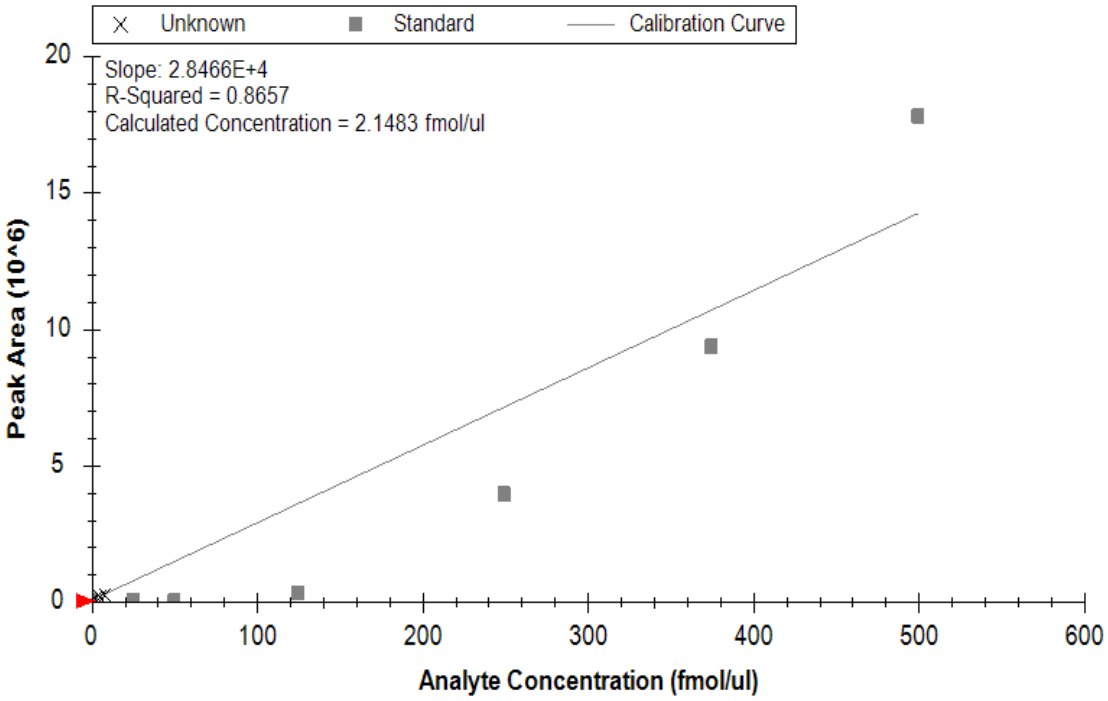
(g) Protein: CRP Peptide: ESDTSYVSLK



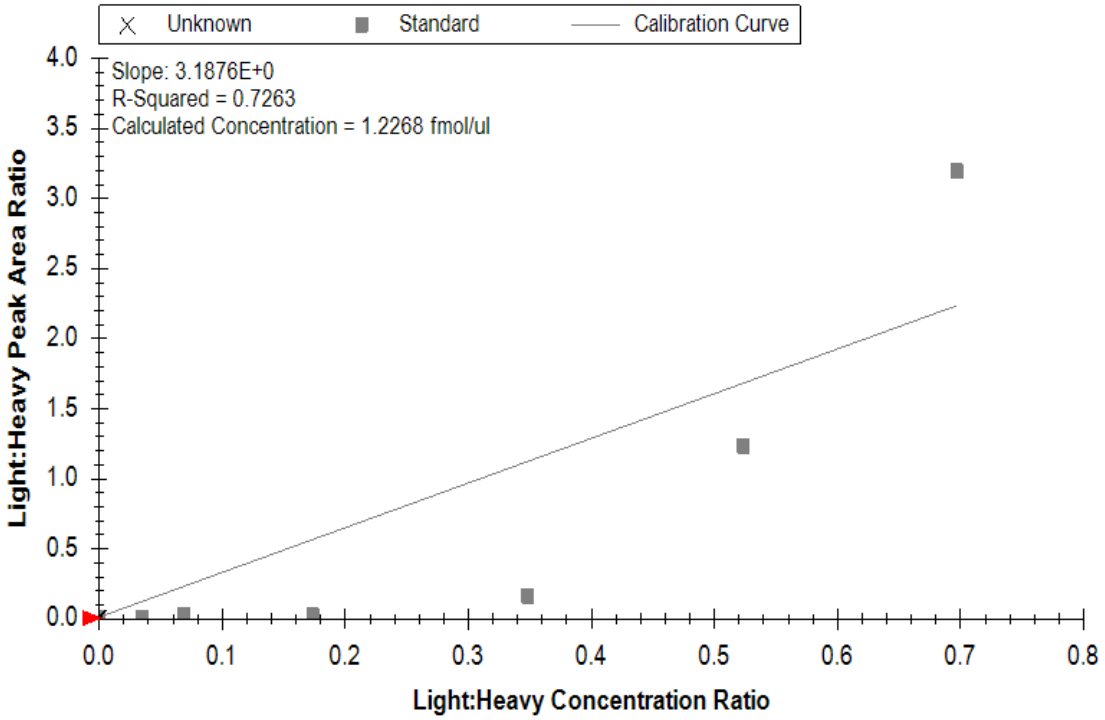
(h) Protein: SETD2 Peptide: ETEPLVSPHQDK



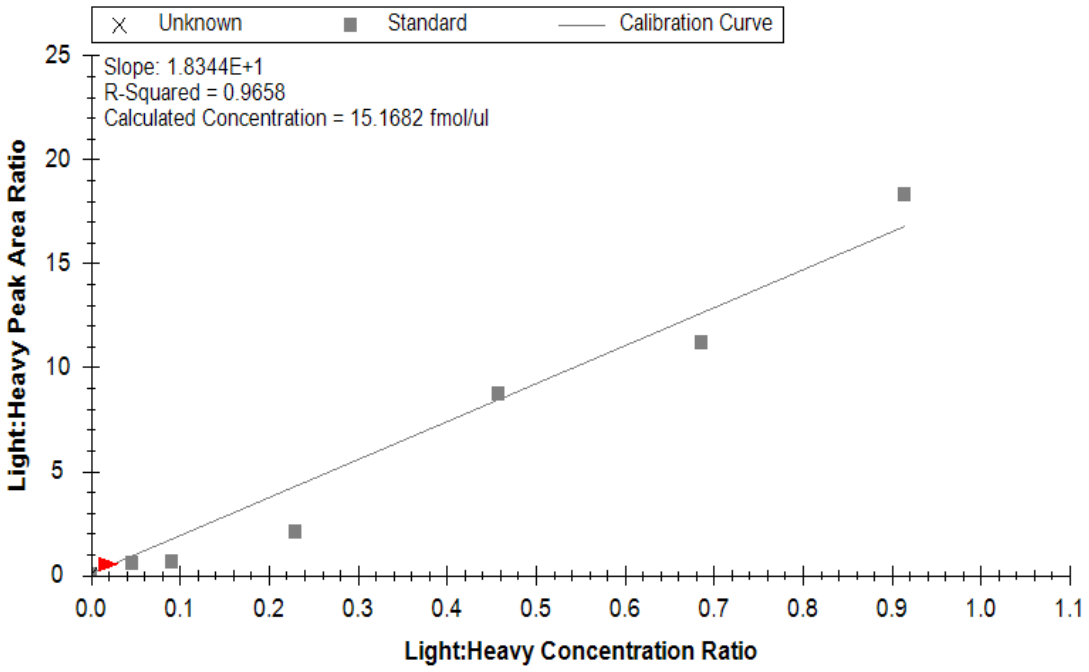
(i) Protein: SETD2 Peptide: FLTALGNEK



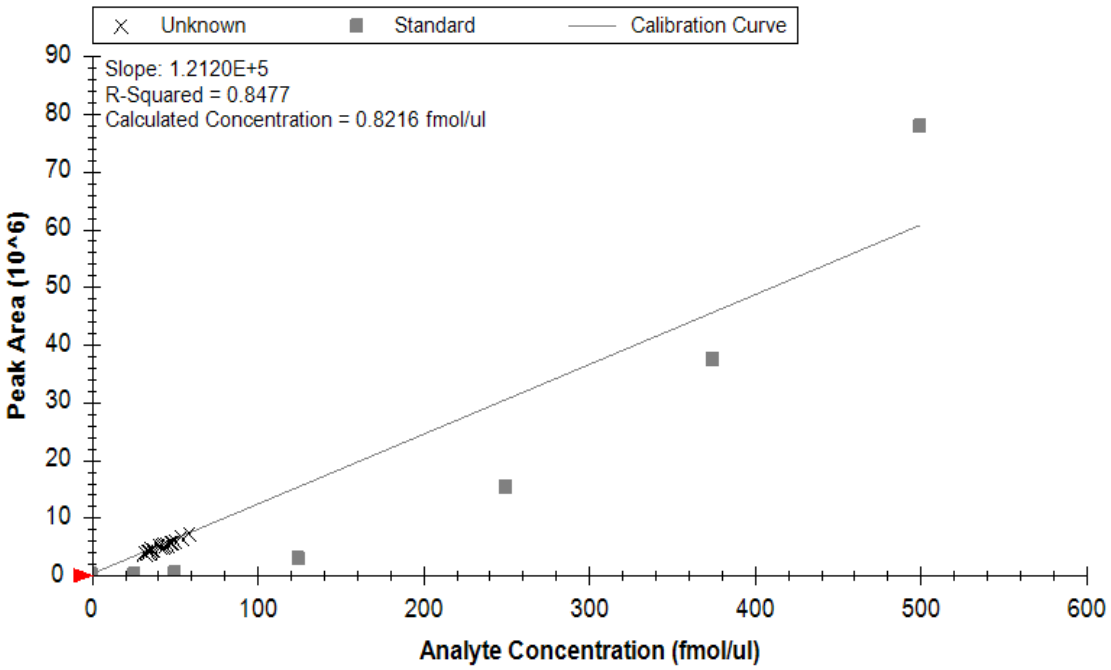
(j) Protein: NBL1 Peptide: SAWCEAK



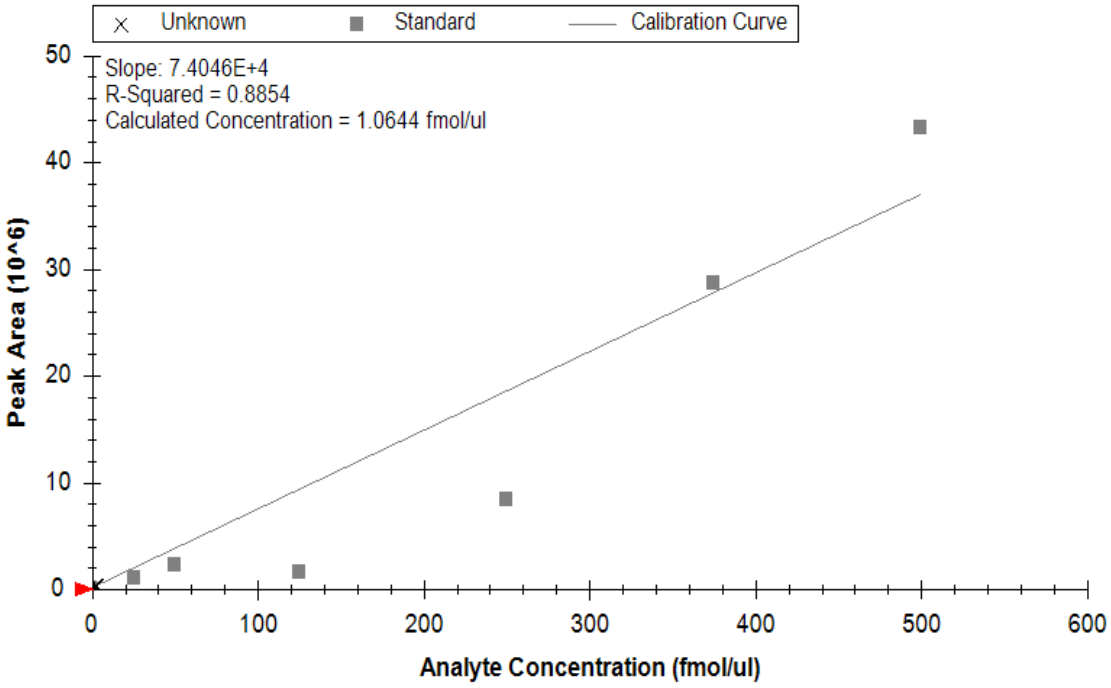
(k) Protein: NBL1 Peptide: LALFPDK



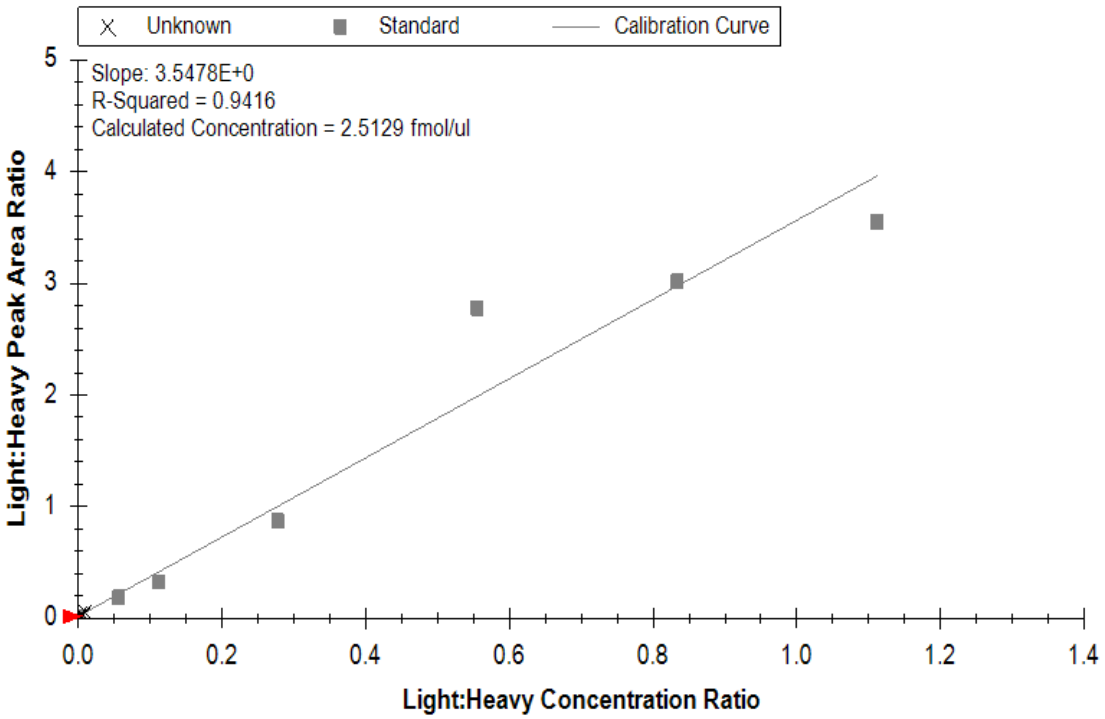
(l) Protein: TGFBRAP1 Peptide: NSFNPDDIINCLK



(m) Protein: TGRFBAP1 Peptide: SGQLDVR



(n) Protein: LYVE1 Peptide: LLGLSLAGK



(o) Protein: LYVE1 Peptide: IMGITLVSK

Figure 6.2: Calibration curves obtained for all 15 peptides with corresponding protein. x marks unknown concentration of peptides obtained from Treatment or Control group. Note the red arrows corresponds to a particular measurement of the peptide of interest which was measured at the time on Skyline.

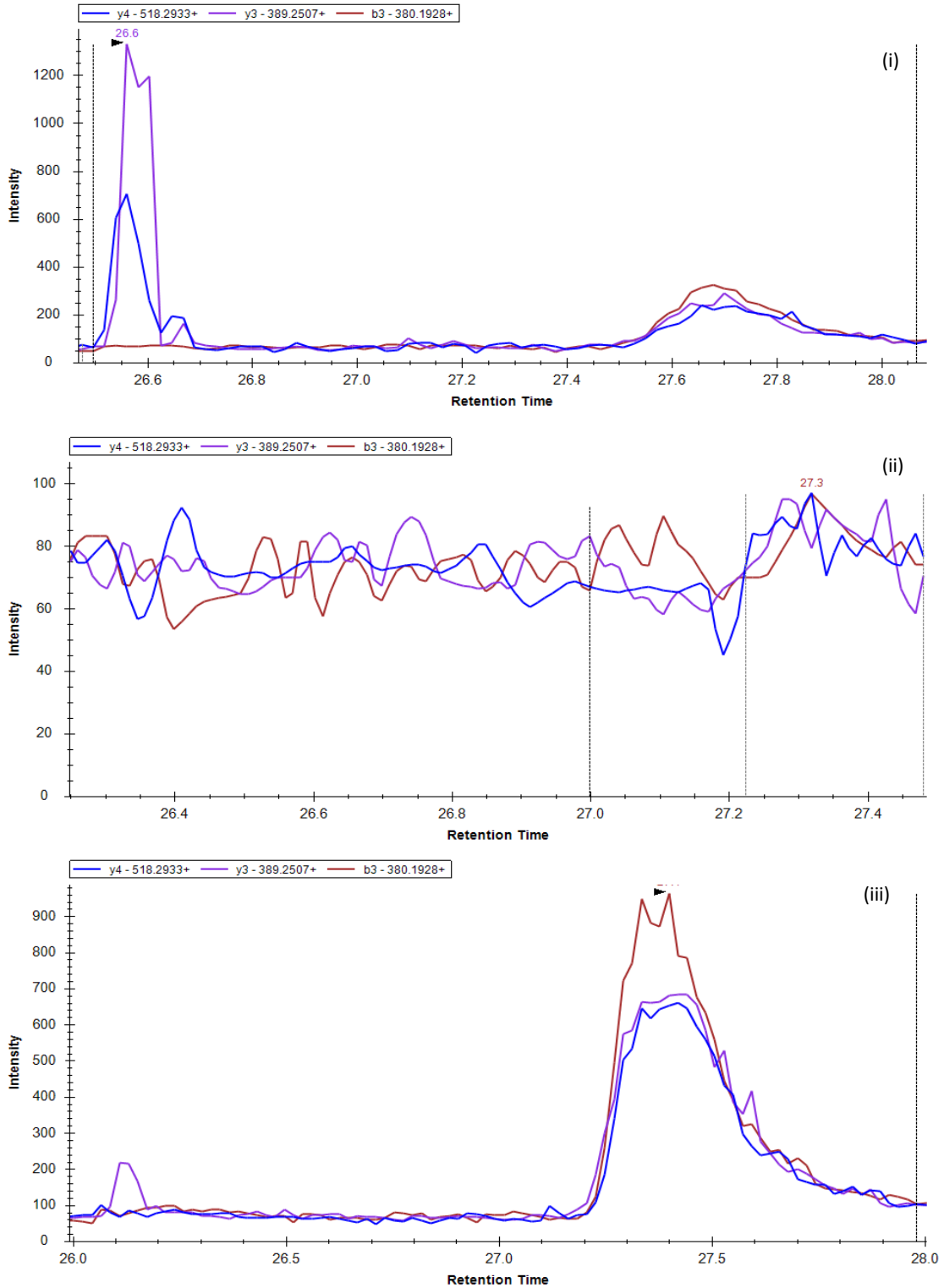


Figure 6.3 (a) Protein HSP90AA1: Peptide: HLEINPDHSIIETLR: i. 10 fmol/µl standard vs ii. 0 fmol/µl vs iii. Patient sample

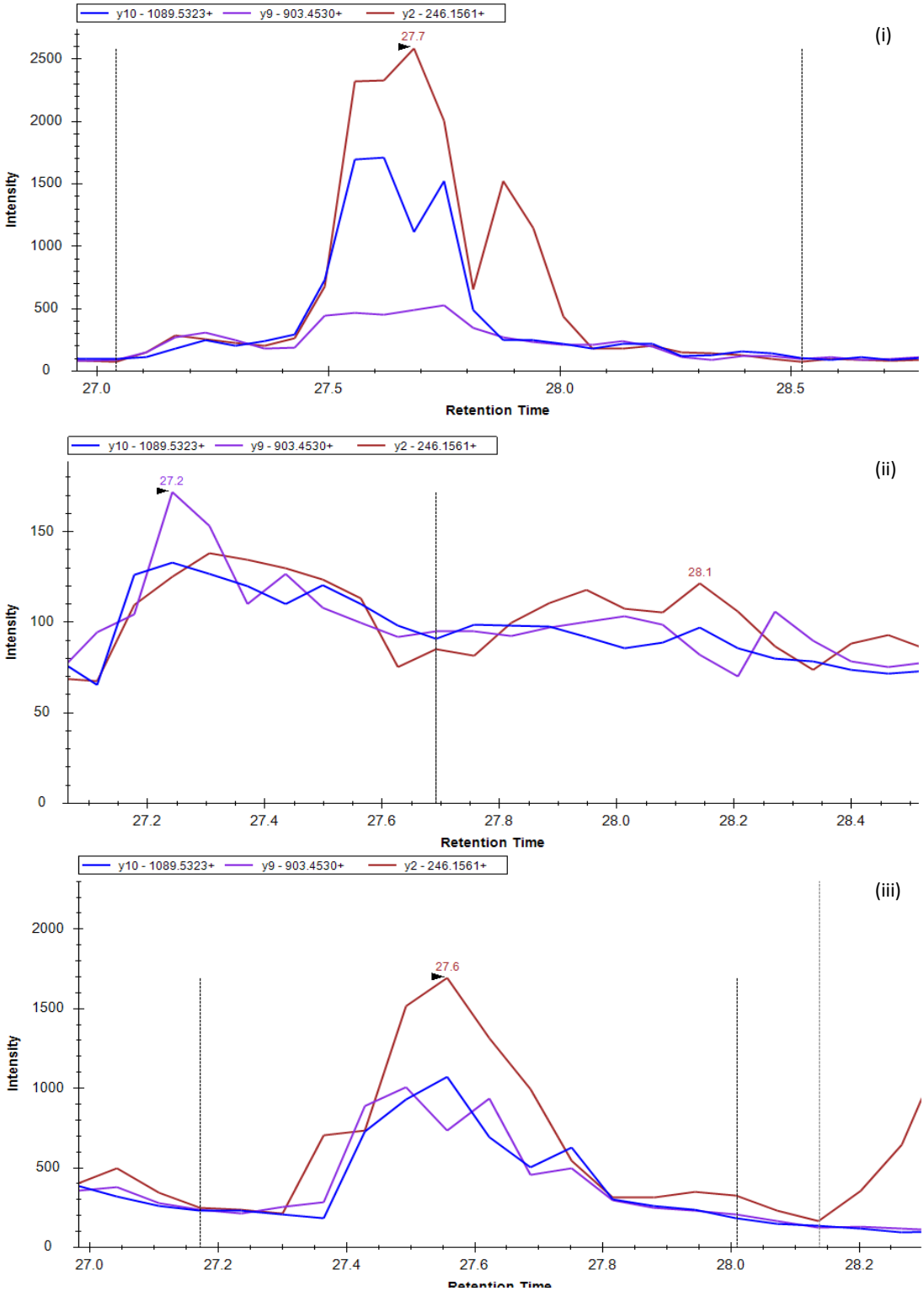
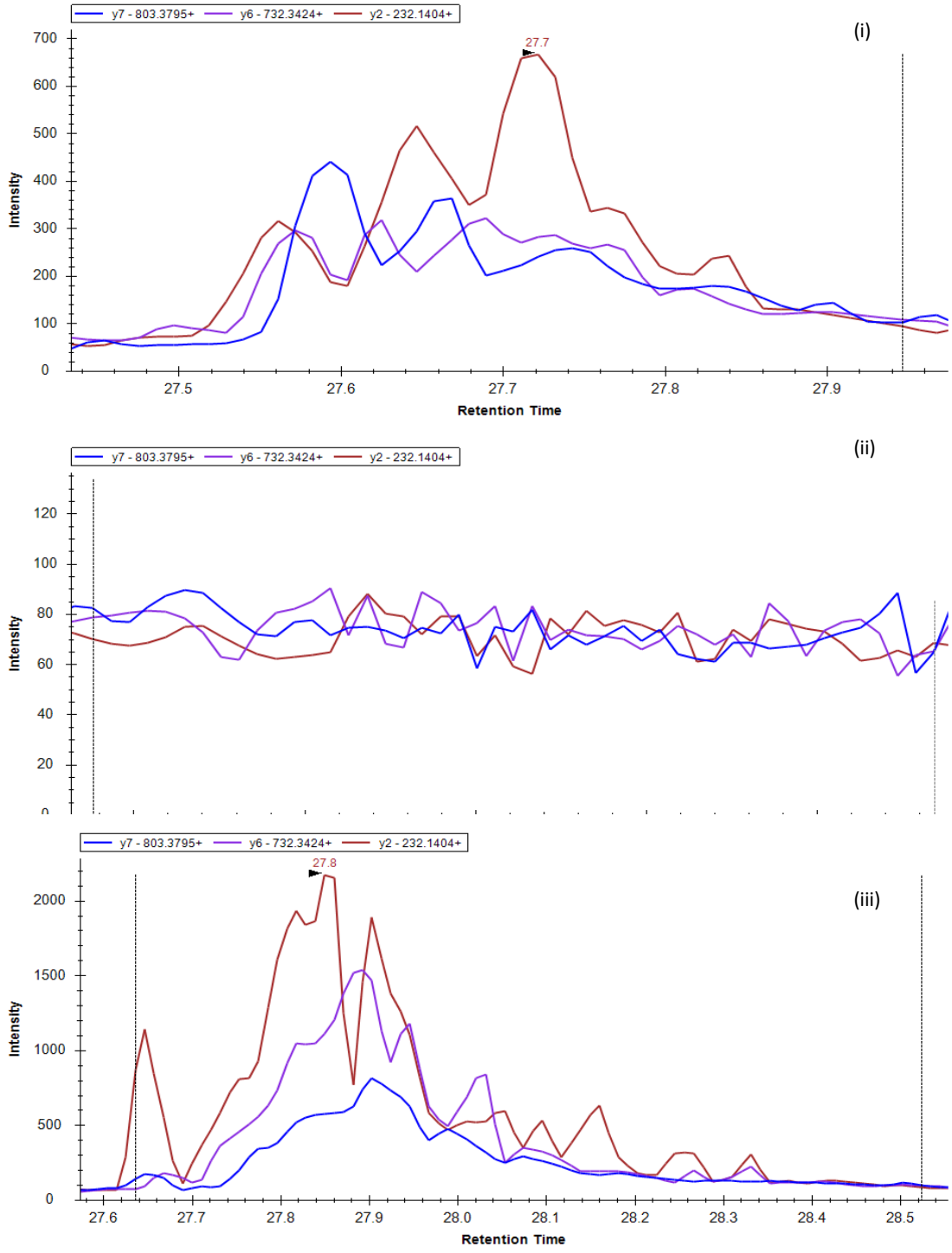


Figure 6.3 (b) Protein SAA1: Peptide: GPGGVWAAEAISDAR : i. 25 fmol/µl standard vs ii. 0 fmol/µl vs iii. Patient sample



(Figure 6.3 c) Protein SAA1: Peptide: FFGHGAEDSLADQAANEWGR : i. 10 fmol/µl standard vs ii. 0 fmol/µl vs iii. Patient sample

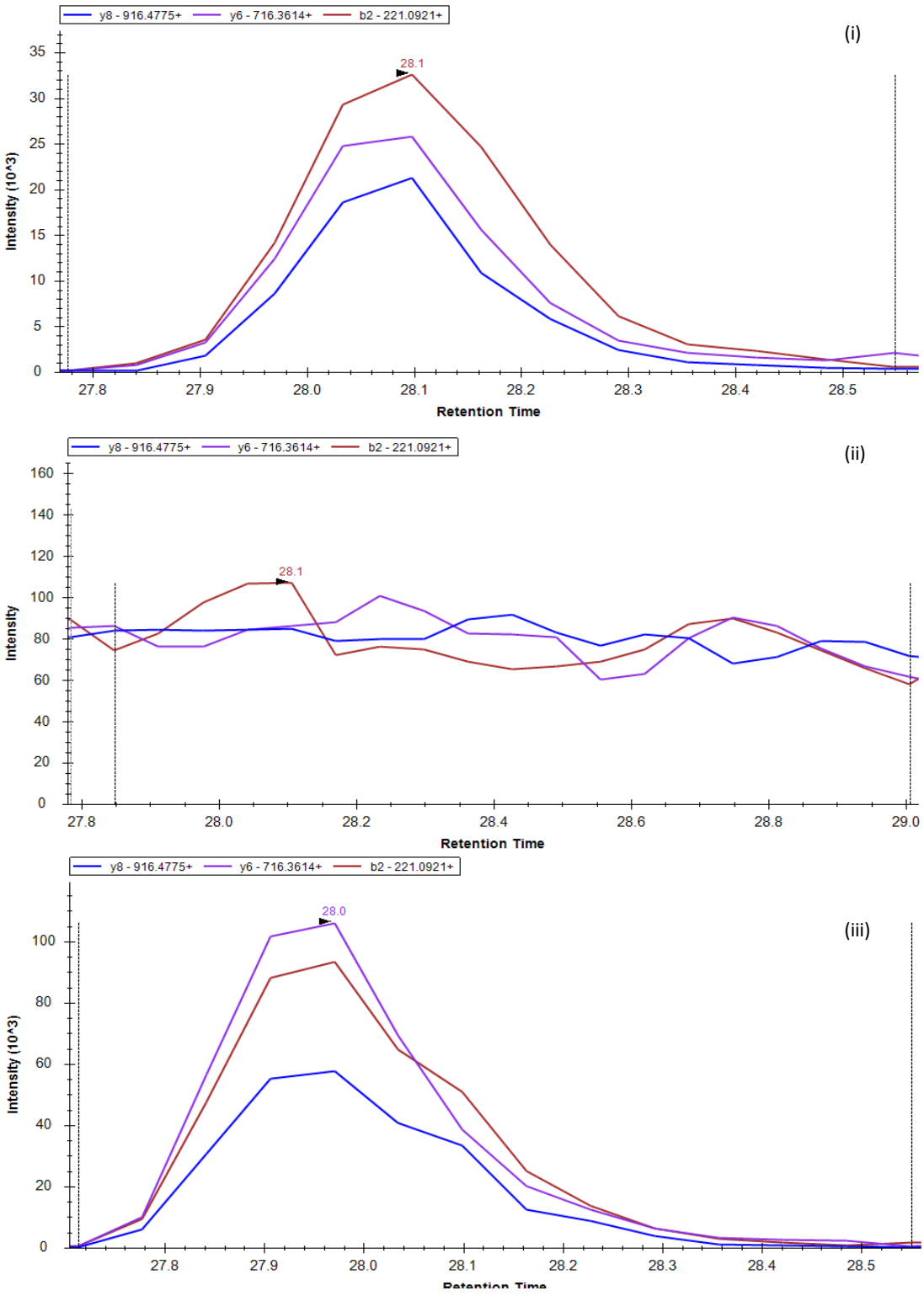


Figure 6.3 (d) Protein CRP: Peptide: GYSIFSYATK : i. 25 fmol/µl standard vs ii. 0 fmol/µl vs iii. Patient sample

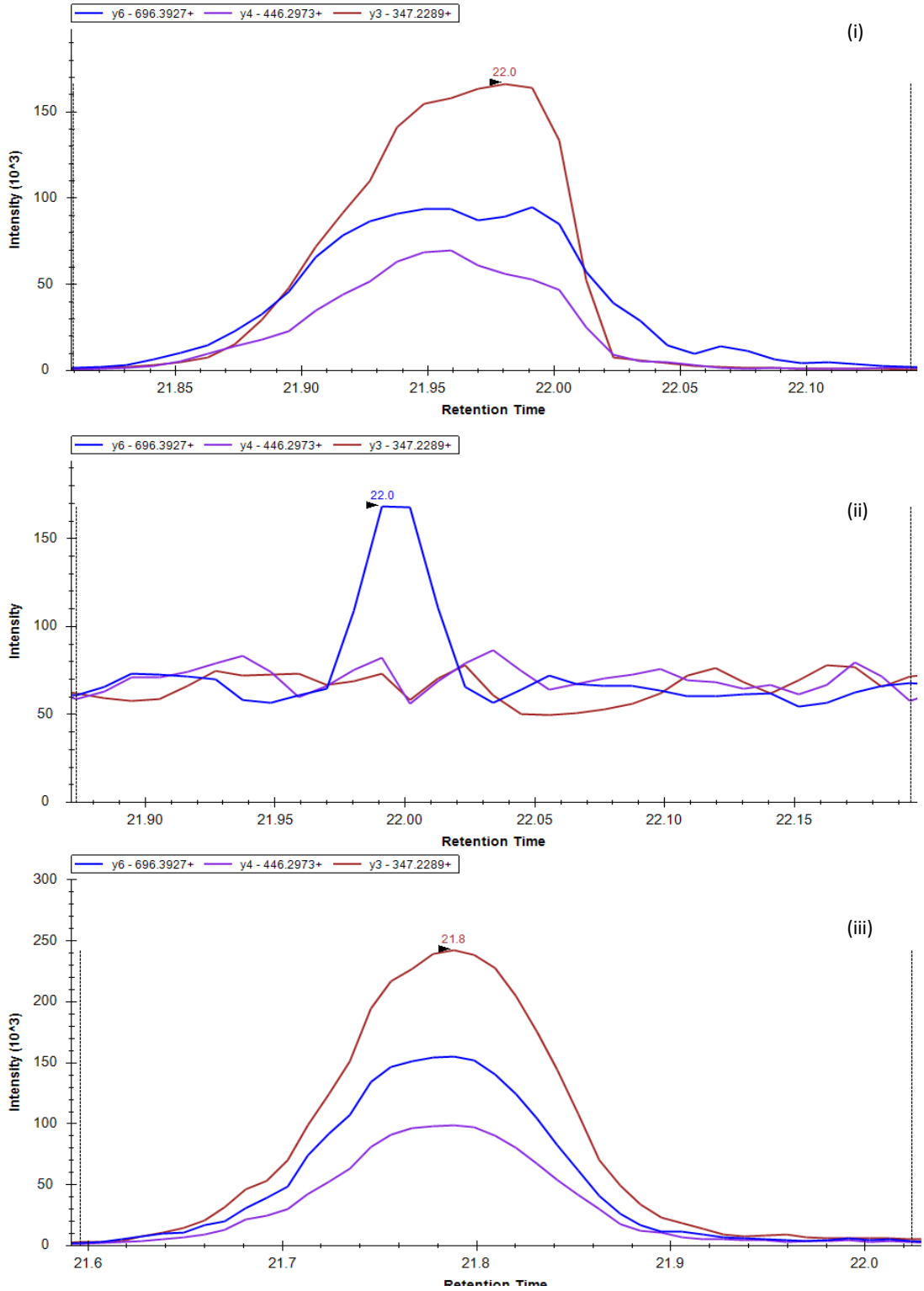


Figure 6.3 (e) Protein CRP: Peptide: ESDTSYVSLK : i. 25 fmol/µl standard vs ii. 0 fmol/µl vs iii. Patient sample

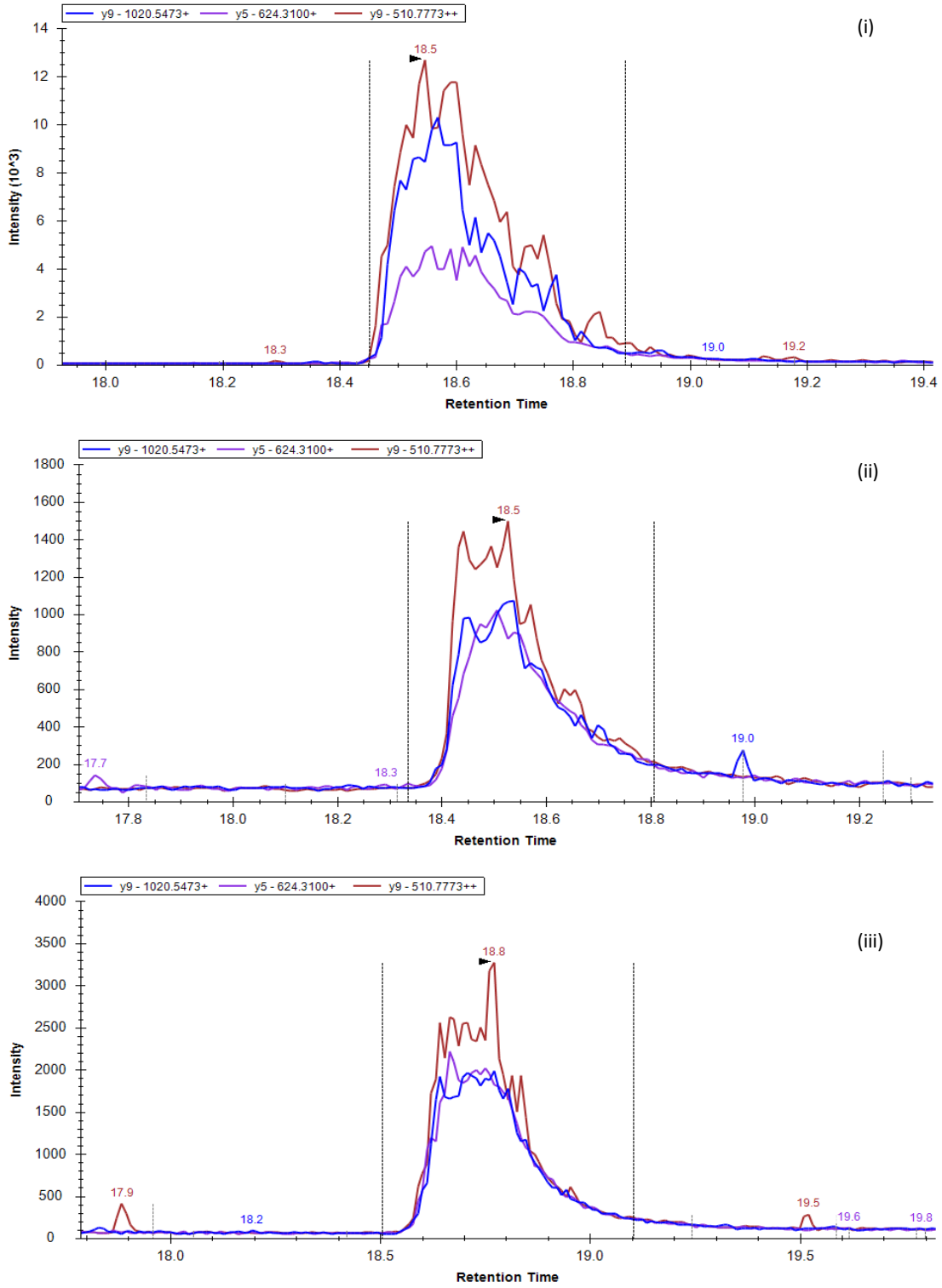


Figure 6.3 (f) Protein SETD2: Peptide: ETEPLVSPHQDK : i. 25 fmol/µl standard vs ii. 0 fmol/µl vs iii. Patient sample

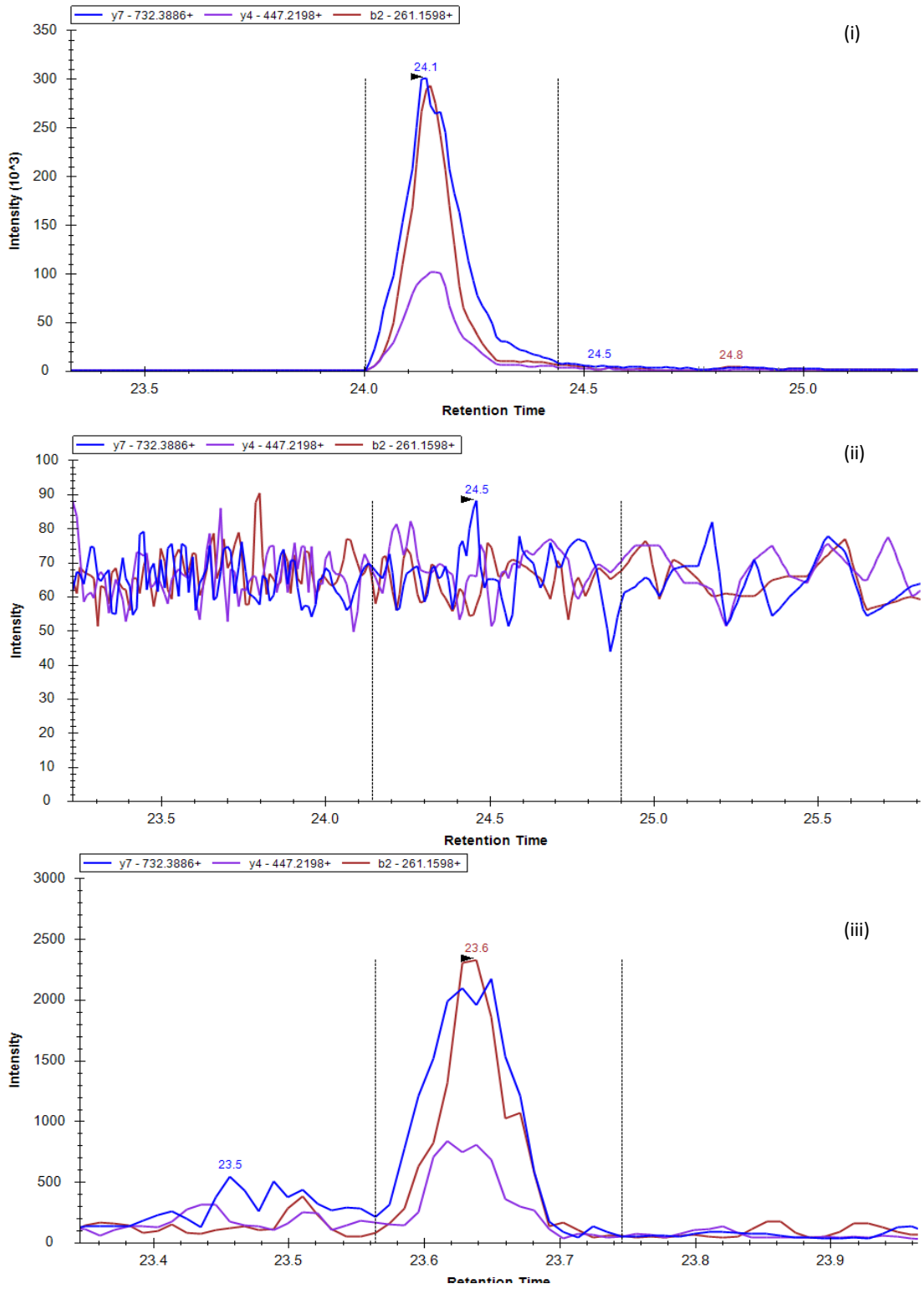


Figure 3.6 (g) Protein SETD2: Peptide: FLTALGNEK : i. 25 fmol/µl standard vs ii. 0 fmol/µl vs iii. Patient sample

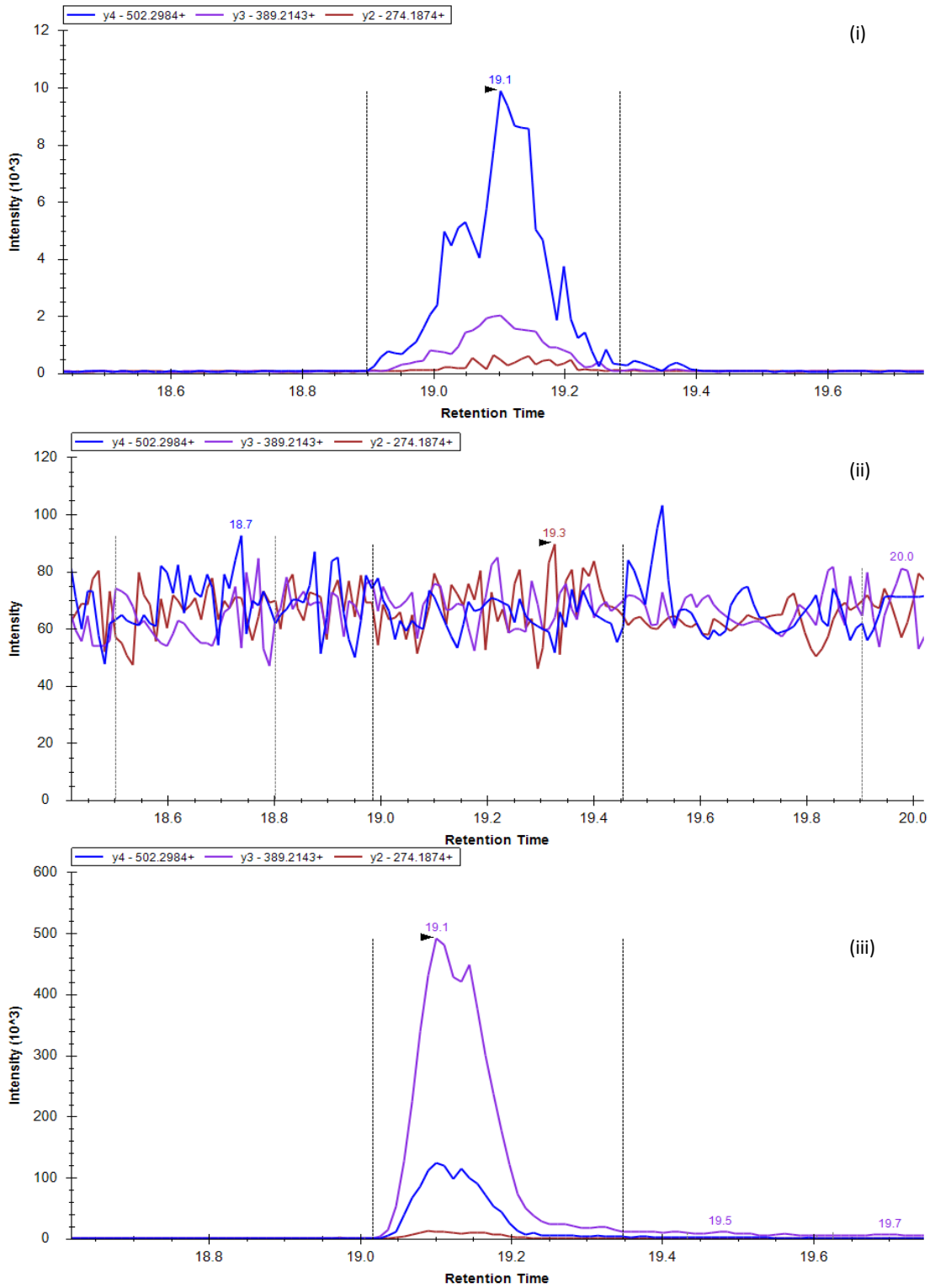


Figure 6.3 (h) Protein TGFBRAP1: Peptide: SGQLDVR : i. 25 fmol/µl standard vs ii. 0 fmol/µl vs iii. Patient sample

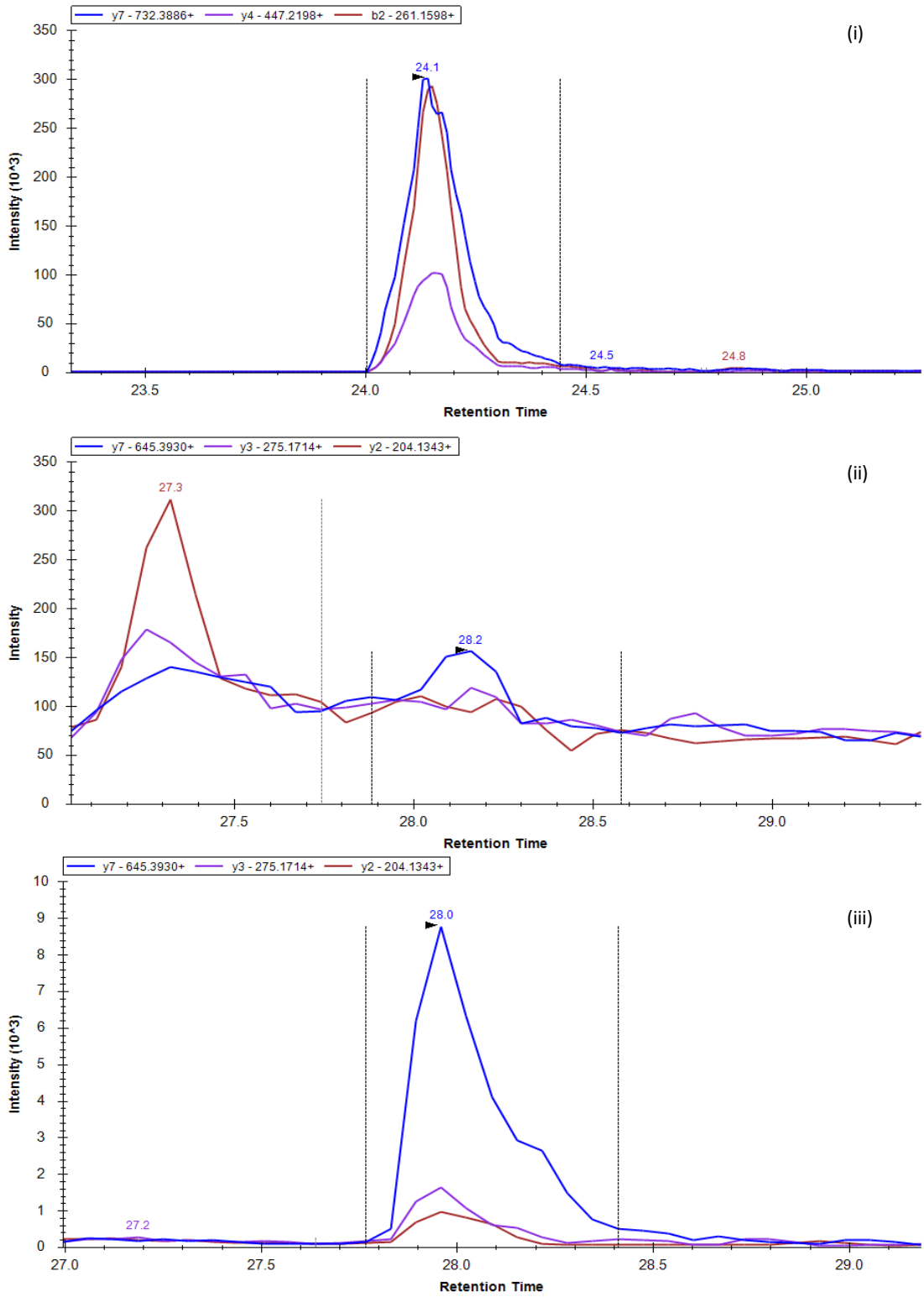


Figure 6.3 (i) Protein LYVE1: Peptide: LLGLSLAGK : i. 25 fmol/µl standard vs ii. 0 fmol/µl vs iii. Patient sample

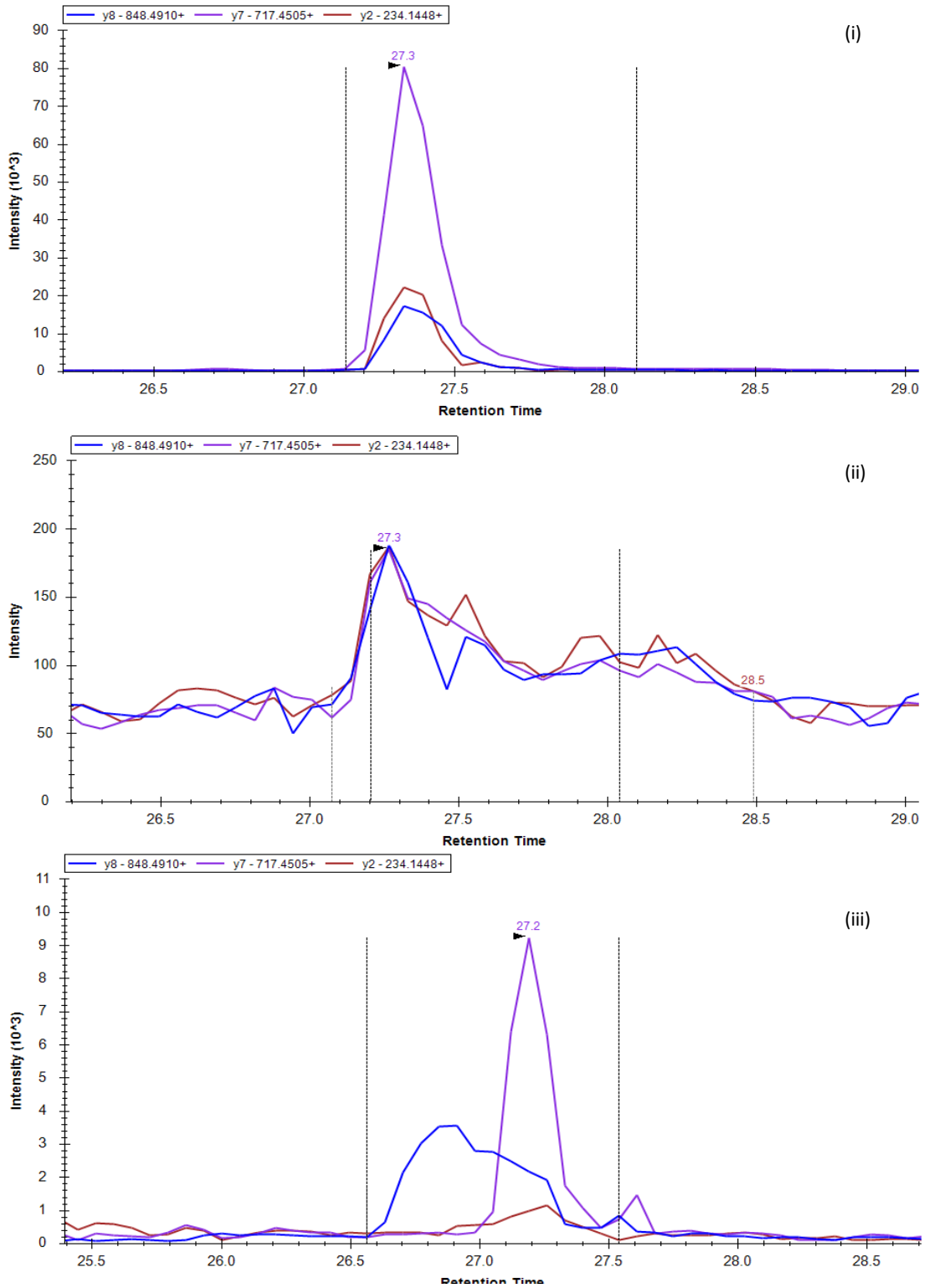


Figure 6.3 (j) Protein LYVE1: Peptide: IMGITLVSK : i. 25 fmol/µl standard vs ii. 0 fmol/µl vs iii. Patient sample

Figure 6.3: Series of chromatograms obtained from Skyline 3.7, showing the ten peptides identified from SRM experiment, with the top most showing a standard peptide at low fmol/µL (i), middle

chromatogram at 0 fmol/µL (ii) and bottom chromatogram of peptide identified from patient cohort (iii).

6.3.2 Peptide Measurements

From the calibration curves, the absolute peptide measurements were obtained and analysed on GraphPad Prism 7.02 (San Diego, USA). Table 6.3 and Figure 6.4 illustrates the results of the peptide differences between the Control Group versus the Treatment Group.

Protein: Peptide	Control A (fmol/ µL)	Control B (fmol/ µL)	Control C (fmol/ µL)	Fish Oil A (fmol/ µL)	Fish Oil B (fmol/ µL)	Fish Oil C (fmol/ µL)	P-value
HSP90A: HLEINPDHSIIETLR	10.90	12.31	13.41	8.91	9.11	8.91	0.012
SAA1: GPGGVWAAEAISDAR	11.17	10.40	12.84	4.61	4.05	7.58	0.009
SAA1: FFGHGAEDSLADQAANEWGR	36.70	41.66	39.22	21.83	23.15	23.41	<0.001
CRP: GYSIFSYATK	131.03	123.25	121.24	82.22	81.38	85.06	<0.001
CRP: ESDTSYVSLK	42.06	43.81	44.45	28.21	29.19	28.07	<0.001
SETD2: ETEPLVSPHQDK	7.37	7.29	7.56	8.47	8.25	7.93	0.009
TGFBRAP1: SGQLDVR	35.11	39.00	35.35	26.21	24.97	28.77	0.004

LYVE1: LLGLSLAGK	1.00	2.05	1.40	1.72	1.80	1.69	0.463
LYVE1: IMGITLVSK	2.93	3.10	3.16	3.11	2.51	2.51	0.174

Table 6.3: Peptide (with corresponding protein) measurement in triplicate injections comparing Control vs Treatment groups.

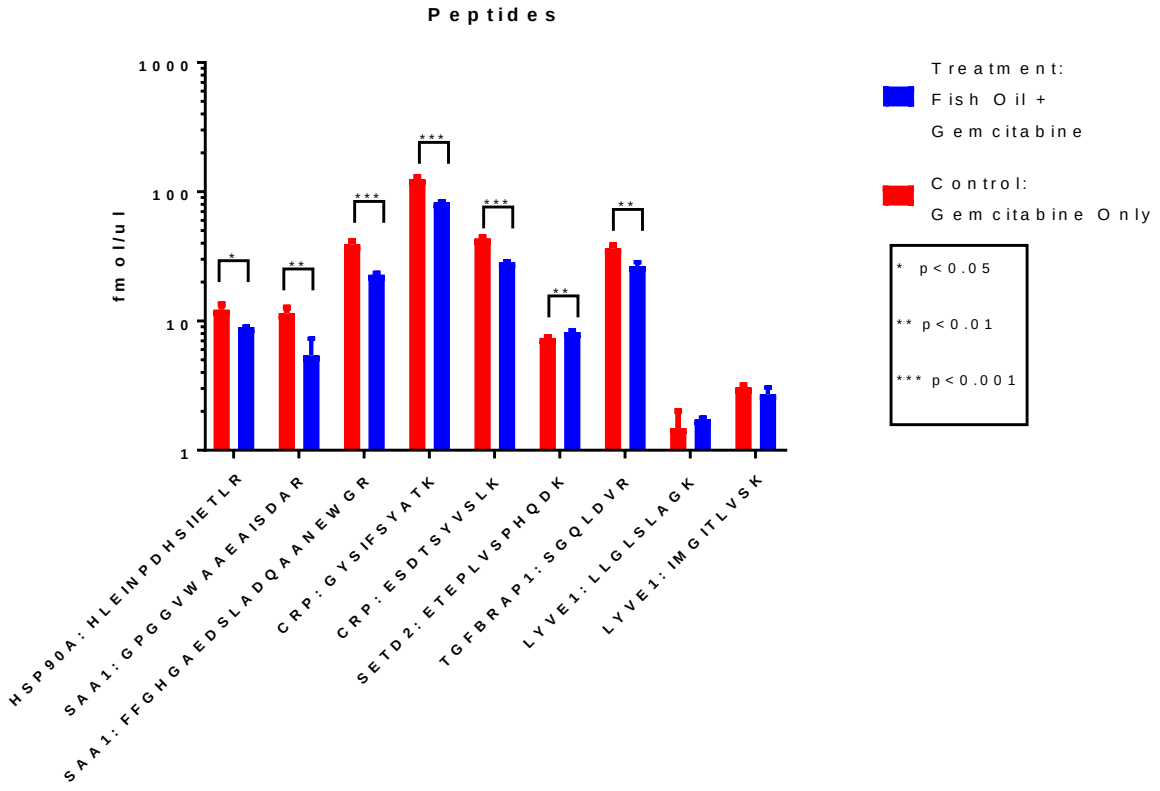


Figure 6.4: Bar chart showing differences in protein:peptide measurements between Control vs Treatment group with significant p values. Note Y-axis is logarithmic

6.4 Discussion

SRM analysis of advanced pancreatic cancer patients receiving n-3 fatty acids and gemcitabine versus gemcitabine alone show a strong reduction in the acute phase proteins Serum Amyloid A1 and C-Reactive Protein, in all four peptides (p value < 0.01). This confirms the previous findings from the discovery phase experiments, that

treatment with n-3 fatty acid reduces the inflammatory response, both from baseline changes and correcting for gemcitabine effect. Reducing levels of SAA is associated with improved overall survival in advanced pancreatic cancer patients[130]. A recent study found higher levels of SAA1 is associated with higher levels of cancer associated fibroblasts which leads to worsening survival [135].

The anti-inflammatory effect of n-3 fatty acid is well established, with a recent large meta-analysis of parenteral n-3 fatty acids in gastro-intestinal cancers showing a very strong reduction in CRP levels [136]. The SRM finding that CRP is strongly reduced in both specific peptides with a fold change < 1.5 ($p<0.001$) is similar to the previous discovery experiments (See Section 4.3.5 and 5.3.4). This further strengthens confidence in this proteomic method in analysing the effect of n-3 fatty acid in these patient plasma samples.

The methylation enzyme SETD2 (Peptide: ETEPLVSPHQDK) was up-regulated on SRM analysis, with a strong p value <0.01. As discussed, SETD2 is involved in methylation of chromatin which leads to epigenetic changes. Mutations in SETD2 is involved in 24% of Pancreatic Ductal Adenocarcinoma [14]. Levels of the second specific peptide to SETD2 (FLTALGNEK) were too low (<1 fmol/µL) for accurate analysis. However, on inspection of the chromatograms on Skyline 3.7, the chromatograms strongly suggest the presence of the peptide at low concentrations Figure 6.3 (g). Further experiments onto the effect of n-3 fatty acid on other methylation enzymes or histone bodies would be of interest, especially with the advent of next generation SRM TQ instruments which may yield better sensitivity and allow better quantitation of this peptide.

A single peptide sequence specific and unique to protein HSP90AA1 (HLEINPDHSIIETLR) showed a significant down regulation with n-3 fatty acid infusion. As postulated, down regulation of HSP90A leads to down regulation of the Pi3K-AKT pathway. This further leads to down regulation of other cancer related pathways, including NF-KB pathways. Inhibitors of HSP90 are pro-apoptotic and anti-

proliferative in a pancreatic cancer cell line [122]. Further work looking into other related proteins in the Pi3-AKT pathway, for example 14-3-3 proteins, would provide further insight into the overall effect of n-3 fatty acids on this pathway.

The peptide sequence SGQLDVR, is unique to the isoforms for protein TGFBRAP1 was down-regulated following treatment with n-3 fatty acid. This was an unexpected finding, as the discovery experiments had found up-regulation with treatment. The second peptide (NSFNPDDIINCLK) was not identified on SRM analysis. TGFBRAP1 is directly involved in the TGF-β pathway and associated with SMAD4 proteins. Reduction of these pathways is of benefit to the outcome in pancreatic adenocarcinoma. There may be a complex relationship between n-3 fatty acid and TGFBRAP1.

LYVE1 is an early and strong marker for pancreatic adenocarcinoma [111]. There was no significant difference in both peptides for LYVE1 (LLGLSLAGK and IMGITLVSK) between the treatment and control group. However, LYVE1 is not a prognostic marker for PDAC, hence no differences in concentrations is expected. Chromatograms from Skyline 3.7 in Figure 6.3 (i) and (j) clearly show the presence of these peptides in the patient cohort. This further strengthens the argument for proteomic methodology developed in being able to identify this protein in the low fmol/µL levels.

In summary, SRM has verified the anti-inflammatory effect of n-3 fatty acids in patients with APC. It has also uncovered potential new avenues of its direct anti-proliferative effect via established pancreatic cancer pathways and via epigenetic modifications. Further verification using more sensitive and modern TQ instrument might provide more reliable measurements to help answer and confirm these changes.

7 Discussion

7.1 Introduction

Despite advances in medical therapy which have improved survival in other solid organ cancers, advanced pancreatic adenocarcinoma carries the same poor prognosis from the 1970s with a median survival of less than 18 months even in the highest income countries in Europe [137]. Trials using n-3 fatty acid in multiple pre-clinical and clinical settings have shown promising avenues in treating APC (See Section 1.3). Consequently, the hypothesis was tested that the administration of n-3 fatty acids would yield protein changes in favour of improving outcomes in patients with APC.

7.2 High Definition Plasma Proteomics in Advanced Pancreatic Cancer

Plasma is a rich source for investigation of proteomic changes corresponding to the effect of intravenous n-3 fatty acid infusion in patients with APC receiving palliative gemcitabine chemotherapy. The advantages of plasma are that it is relatively non-invasive (venepuncture versus direct tissue biopsy or extraction of pancreatic fluid), quick to obtain, easy to store and provides a general overview of protein based mechanisms and pathways systemically.

The complexity of plasma can be overcome by the methods developed above (Refer to Sections 2 and 3): optimised immunodepletion of high and moderate abundant proteins (99%) using combined IgY14 + Supermix columns and high pH-Reverse Phase HPLC Fractionation (up to 400 µg injections, 30 fractions). The use of TMT-6plex labelling allows multiplexing of various samples into one experiment (e.g. 6 samples, combined to one before fractionation): this saves time in terms of LC-MS/MS analysis without compromising throughput. The optimisation of multiple, iterative search engines processing workflows in Protein Discoverer 2.1 has increased the yield and sensitivity of proteins identified. Finally, as the analysed LC-MS/MS samples are stored as raw files, when further advances in proteomic studies are identified, for example more proteins are discovered and added to the database, newer proteins relevant to pancreatic

cancer can be identified and validated in the future. The findings were further verified on Scaffold 4.7, with a yield of more than 1000 proteins per experiment.

This methodology identified 125 known pancreatic cancer protein markers as collated by Kim *et al* [108] , Oncomine [109], and Plasma Proteome Database (PPD, http://www.plasmaproteomedatabase.org) [110] (Refer to Section 4.3.2). Specifically two proteins (LYVE-1 and REG1A) and an isomer of TFF1 was also identified. The landmark study by Radon *et al*[111] identified a panel of three proteins (LYVE-1, REG1A and TFF1) as a biomarker panel for early detention of pancreatic adenocarcinoma. On SRM analysis, two specific proteotypic peptides for LYVE1 were identified at the low fmol/µL range (Section 6.3.2). This further boosts the confidence that this methodology and its results corroborate for analysis in APC.

As plasma was the choice of biological sample, the finding of the majority of the plasma proteins involved in coagulation and complement factors is unsurprising (Section 5.3.5). Twenty five of the 36 proteins involved in the coagulation cascade was measurably identified. This further strengthens confidence for the method in identifying high numbers of plasma proteins from a small sample size. Interestingly, the majority of the plasma coagulation proteins were upregulated, meaning a state of hyper-coagulopathy was induced following treatment of n-3 fatty acid. It is unclear if this is related to the pro-coagulopathic nature of advancing pancreatic adenocarcinoma or solely related to the administration of n-3 fatty acid.

One of the main proponent effects of n-3 fatty acid infusion is its strong anti-inflammatory properties. In both discovery phases (Sections 4 and 5) there was strong down regulation of various proteins in the acute phase reaction (CRP, Haptoglobin, Serum Amyloid A 1 and 2). This was further verified with SRM (Section 6) which showed significant down regulation of CRP and Serum Amyloid A 1 following infusion on n-3 fatty acid. This both strengthens the confidence in the methodology of high definition plasma proteomics to identify changes in APC and also confirms that n-3

fatty acid infusion has strong anti-inflammatory properties that have been proven in other studies.

7.3 Mechanistic Action of n-3 Fatty Acid

The biggest drawback from these experiments has been the inability to attribute all changes solely on n-3 fatty acid treatment. As has been described, the original study [77] only included a single arm of n-3 fatty acid and gemcitabine and the control arm of gemcitabine only arm was recruited after completion of the original study. Both factors would have introduced selection and treatment biases affecting the strength of the clinical and proteomic results. The treatment arm included both n-3 fatty acids and gemcitabine, an arm consisting of only n-3 fatty acid infusion without gemcitabine in advanced pancreatic cancer would have been ideal experimentally in order to elucidate its direct effects but unethical. However, based on the known anti-inflammatory, anti-apoptosis and anti-proliferation effects of n-3 fatty acids and the mode of action of gemcitabine to stop DNA synthesis, it is inferred that the action on these pathways are due to n-3 fatty acids either independently or in combination with gemcitabine.

The anti-inflammatory effects of n-3 fatty acids have been discussed and proven in this cohort. Reducing the inflammatory load is associated with increased survival in APC and various anti-inflammatory agents have been postulated to decrease the risk of pancreatic adenocarcinoma [138]. Cytokines have pro-tumorigenic effects and depress the immune response in pancreatic cancer cell lines [139]. Significant down regulation of cytokine production, including Interleukin-6 (as discovered in Section 4.3.5) is associated with improving outcomes in patients with APC [116]. The Complement Activation System (classical, lectin and alternative) were also found to be up-regulated following treatment. The finding of upregulation of the immune processes, including the innate immune response, is promising in terms of improving outcomes in patients with APC. This may be related to the direct effect of n-3 fatty acid in reducing cytokine production via its anti-inflammatory effect.

Upon infusion of n-3 fatty acid there is immediate uptake into the cells via the cell membrane. The findings of direct changes to the apolipoproteins in the plasma also reflects the immediate effect of n-3 fatty acids in these patients. Apolipoprotein remodelling leading to upregulation of HDLs and down regulation of LDLs and VLDLs (Section 4.3.4) is in keeping with the known effects of n-3 fatty acids in other settings[112].

Pancreatic adenocarcinoma progression is driven by uncontrolled cell proliferation and anti-apoptotic mechanisms. The use of n-3 fatty acid in this cohort has shown down regulation of cell proliferative markers, including Vascular Endothelial Growth Factor, specifically Nuclear Factor 2, Transforming Growth Factor Beta and via the Tumour Necrosis Factor pathway. Proteins involved in inducing apoptosis were up-regulated, again via the Tumour Necrosis Factor pathway and protein DJ-1 which is strongly associated with cellular apoptosis. Another set of proteins involved in promoting apoptosis are the Complement proteins (C3 and C4) and Annexin A1 proteins which were upregulated following n-3 fatty acid infusion. (Sections 4.3.4 and 5.3.3). On SRM analysis in the cohort, Transforming growth factor-beta receptor-associated protein 1 (TGFBRAP1) was down regulated following treatment (Section 6.3). This corresponds with the down regulation in TGF-β related cell proliferation, though will need further investigation to confirm the nature of the response.

Epigenetic modification following dietary intake of n-3 fatty acid includes changes to histone bodies and methylation of DNA [118]. Recent evidence has shown that administration of n-3 fatty acid has a direct effect on methylation of DNA in blood leucocytes [140,141]. In this cohort of APC, the administration of n-3 fatty acid has a direct effect on down regulation of histone bodies (H2 and H3) and up-regulation of de-acetylation and de-methylation enzymes (HDAC5, HDAC11 and SETD2, Sections 4.3.5 and 5.3.4). Mutations in SETD2 is a known genetic subtype of pancreatic adenocarcinoma. SRM analysis found upregulation of SETD2 following treatment of n-3 fatty acid (Section 6.3). To the best of our knowledge, this is the first reported finding of changes to histone de-acetylation and methylation of DNA in pancreatic

adenocarcinoma following n-3 fatty acid treatment. It is unclear what the exact mechanism of action and requires further investigation, specifically looking into DNA and histone body modifications.

Proteins involved in various cancer pathways were identified to be up or down regulated following treatment with n-3 fatty acid. Pathways with links to progression in pancreatic adenocarcinoma including WNT, NIK/NF-kappa β, ERBB signalling pathway and Epidermal growth factor receptor signalling pathways were down regulated following treatment. (Section 4.3.5). Specifically, the PI3k-AKT pathway is closely related to progression in pancreatic adenocarcinoma, with 2 proteins of interest with significant changes, namely HSP90 and 14-3-3 family of proteins. HSP90 inhibitors have been shown to inhibit pancreatic adenocarcinoma cell growth[122]. HSP90 have a direct role in promoting protein AKT, following infusion with n-3 fatty acid, there was down regulation of this important protein (Section 5.3.5). This was investigated further on SRM analysis, and on a single specific peptide (HLEINPDHSIIETLR) HSP90AA1 was confirmed to be down regulated significantly (Section 6.3.2). However, this needs to be taken with caution, as only a single peptide was identified on analysis (the second proteotypic peptide was not discovered in the SRM cohort analysis). Furthermore, multiple other proteins are involved in the PI3k-AKT pathway which may correct for the HSP90 findings. Future work would include direct measurement of the effect of n-3 fatty acid on AKT directly and also investigating other proponents of the PI3k-AKT pathway on SRM including the 14-3-3 protein family.

7.4 Limitations and Future Work

One limitation from this work is the small number of patients investigated. In total, 16 different patient samples were investigated on, from 13 individual patients (Three patients comparing baseline to treatment with n-3 fatty acid = 6 samples and Five patient sample following treatment with gemcitabine + n-3 fatty acid vs control arm of Five patients with gemcitabine alone = 10 samples). In an effort to overcome this, the methodology of a high throughput, high definition plasma proteomic analysis with

multiplexing was developed (Section 3). This successfully yielded high amounts of measurable proteins (>1000) and provided insights into the effect of n-3 fatty acid and gemcitabine in APC. Further patient pooling of samples would have been possible by combining more patients to each TMT channel. However, this would have been limited by the 60 µg of protein labelled per TMT-6 channel prior to fractionation. A larger fractionation column may mean a larger amount of protein could be loaded, but this would require further verification and costs.

Pooling is often a necessity in proteomic experiments but the actual consequence on true quantitative evaluation of individuals is eliminated and the assessment on measurement range across individuals is also reduced. This may increase the number of proteins identified, but reduce the measurable proteins for comparison. Commercial TMT kits are available up to 11 TMT-plex channels. Using TMT technology, individual patients could be labelled per channel and keeping a pooled Quality Control channel as standard, multiplexing experiments could be performed on a larger number of patients. However, the main drawbacks are the cost of the commercial TMT kits and the time taken for analysis on the Mass Spectrometer Instruments (a measure of time for experiments using the TMT-6 kit multiplexing experiment comparing Control vs Treatment in Section 5 was 8 weeks on the QExactive-Orbitrap Mass Spectrometer Instrument). Following on from MS analysis, protein identification on Proteomic Discoverer or Scaffold would also require excellent computing performance and time. A typical MSF file from a TMT-6 2 arm multiplex experiment require one week to complete processing on Proteomic Discoverer 2.1.

SRM analysis confirmed the presence of multiple proteins identified from earlier discovery experiments. Verification using SRM is a well-known robust technique to confirm and measure proteins of interest. However, this was completed on the same cohort from earlier discovery phases. Further corroboration could be done on different patients to measure those proteins of interest or using different validating methods.

Further questions have been raised following on from this work. The epigenetic effects of n-3 fatty acid and gemcitabine in pancreatic adenocarcinoma is worth looking into further by its influence on the Histone proteins, which potentially indicate effects on the DNA of these patients. There are also associated changes to proteins involved in methylation of DNA (SETD2) which is a known genomic subtype in pancreatic adenocarcinoma. Future work could involve exploring the effects of n-3 fatty acid specifically on other Histone bodies and other de-acetylation enzymes (such as HDAC 5 and 11) and the direct effect on the DNA using further genomic analysis.

Cancer pathways are invaluable to provide an understanding of the pathogenesis of cancer and explore ways to overcome it. The PI3k-AKT pathway is well established and the inhibition of HSP90 following n-3 fatty acid infusion reveals a direct effect on this pathway. Due to the complexity of the pathway however, other proteins changes need to be verified, for example the direct effect of n-3 fatty acid on the AKT levels. There is also the inhibitory effect of the 14-3-3 family of proteins which further inhibits cancer progression on this pathway, which was identified in the discovery stages. Future work investigating n-3 fatty acids and gemcitabine on the PI3k-AKT pathway is worthy of consideration.

8 Conclusion

The use of n-3 fatty acid as an adjuvant treatment together with gemcitabine in advanced pancreatic cancer has shown promising clinical benefits. Using advanced mass spectrometer based technology and developing novel methodology in plasma preparation and protein labelling, high definition proteomics has successfully delved deep into the proteome of plasma revealing new insights. Proteomic analysis following administration of n-3 fatty acid and gemcitabine has confirmed its known anti-inflammatory effect but also yielded new understanding of its anti-proliferative actions, direct effect on various cancer pathways and novel information on epigenetic modifications. This has aided in elucidating the mechanism of how this essential fatty acid may improve the outcome in unresectable pancreatic adenocarcinoma.

9 Appendix

9.1 Appendix Table 1:

In depth summary of published research of proteomic studies on fish oils.

Abbreviations: 2-DE: 2 dimensional electrophoresis, LC: Liquid Chromatography, MALDI: Matrix Assisted laser desorption/ionization, MS: Mass Spectrometry, SDS-PAGE: 1-Dimensional electrophoresis HPLC: High performance liquid chromatography, TOF: Time of flight, ESI: Electro Spray Ioniser.

9.2 Appendix Table 2:

In depth summary of published research of proteomic studies on fish oils.

Abbreviations: 2-DE: 2 dimensional electrophoresis, LC: Liquid Chromatography, MALDI: Matrix Assisted laser desorption/ionization, MS: Mass spectrometry, SDS-PAGE: 1-Dimensional electrophoresis, HPLC: High performance liquid chromatography, TOF: Time of flight, ESI: Electron Spray Ioniser.

9.3 Appendix Table 3:

Output from PD 2.1 comparing patients G03, G09 and G17 from baseline (treatment naïve) versus one cycle (one month) treatment with gemcitabine and n-3 fatty acid. Table detailing master protein list with accession numbers, protein names, coverage, number of peptides, number of peptide spectral matches (PSMs), unique peptides, TMT abundance levels, SequestHT HT scores, MS Amanda scores and MS PepSearch scores.

File name: FR_G030917_TMT_extHD_PD21_SN0_240217 master.xls 27/02/2017

(File password: 1234)

9.4 Appendix Table 4:

Output from Scaffold 4.7 comparing patients G03, G09 and G17 from baseline (treatment naïve) versus one cycle (one month) treatment with gemcitabine and n-3 fatty acid. Table detailing 1039 proteins identified with master protein name, accession number, log 2 fold changes, Mann-Whitney U test P values and TMT quantitation values.

File name: FR G030917 031116 Xtandem 95% pro 95% pep 2 pep.xls 27/07/2017

(File password: 1234)

9.5 Appendix Table 5:

Output from PD 2.1 comparing Control (APC with gemcitabine only, n-3 fatty acid naïve, Patients: C02, C03, C04, C06 and C08) versus Treatment (APC with n-3 fatty acid and gemcitabine treatment, Patients: G05, G13, G15, G19 and G21) at one cycle (1-month) of treatment (See Section 5.2). Table detailing master protein list with accession numbers, protein names, coverage, number of peptides, number of peptide spectral matches (PSMs), unique peptides, TMT abundance levels, SequestHT HT scores, MS Amanda scores and MS PepSearch scores.

File name: FR_FishOilVSGem_2402_SN0_v7 master.xls 28/02/2017

(File password: 1234)

9.6 Appendix Table 6:

Output from Scaffold 4.7 comparing Control (APC with gemcitabine only, n-3 fatty acid naïve, Patients: C02, C03, C04, C06 and C08) versus Treatment (APC with n-3 fatty acid and gemcitabine treatment, Patients: G05, G13, G15, G19 and G21) at one cycle (1-month) of treatment (See Section 5.2). Table detailing 1047 proteins identified

with master protein name, accession number, log 2 fold changes, Mann-Whitney U test P values and TMT quantitation values.

File name: FR Fish oil vs gem 020517 95% pro and pep 2 peps.xls 08/08/2018

(File password: 1234)

9.7 Appendix Table 7:

Optimised conditions for SRM analysis for identifying 15 shortlisted peptides (Heavy Labelled and Light unlabelled). Detailed are Peptide sequences, top three transitions (Precursor m/z and charge and Product m/z and Charge, collision energy and fragment ion.

File name: peptide transition list 3 peptides labelled unlabelled.xls 21/06/2017

(File password: 1234)

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