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Targeted Mass Spectrometry for Plasma Glycoprotein Profiling in Pre-eclampsia

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Abstract

Pre-eclampsia is a common hypertensive disorder of pregnancy that substantially affects maternal and neonatal morbidity and mortality worldwide. Despite decades of research, the aetiology of the disease remains poorly understood, and the clinical management of it is hampered by the lack of reliable diagnostic tests and effective therapy. Several screening tests have been suggested for the prediction of pre-eclampsia; however, none possess the sufficient specificity and sensitivity. Moreover, multiple pathways are known to be involved in the pathogenesis of pre-eclampsia, so it is very unlikely that a single or a small group of biomarkers will accurately predict the disease. In this thesis, two separate targeted LC-MS/MS methods were developed and validated to quantify plasma glycoproteins in pre-eclampsia to increase the pool of pre-eclampsia biomarker candidates and explore new pathways associated with the disease. The first method was hypothesis-driven and aimed to quantify the oxidation level of the plasma glycoprotein angiotensinogen (AGT), which has been proposed to be involved in the increased blood pressure characteristic of pre-eclampsia. The second method was hypothesis-generating and aimed to detect glycoprotein fold changes between different disease conditions using a simple and cost-effective conventional LC-MS/MS workflow.

For both methods, a reproducible workflow for efficient glycoprotein/AGT extraction from human plasma was developed by coupling ConA lectin affinity chromatography with reversed-phase solid phase extraction fractionation (RP-SPE). Analysis of the enzymatically digested proteins was conducted using targeted LC-MS/MS working under the multiple reaction monitoring mode. For the quantification of the two distinct forms of AGT in the plasma (the sulphydryl-bridged oxidised form and the free thiol reduced form), a differential alkylation strategy was coupled with targeted LC-MS/MS to recognise and quantify the cysteine (Cys) peptides involved in the redox switch of AGT. The developed method enabled the reproducible detection of the two distinct forms of AGT in the plasma with CV% <15%, and confirmation of the identity of the differentially alkylated Cys peptides was supported by LC-MS/MS. Analysis of clinical plasma samples using the developed method showed a significantly higher level of the oxidised AGT in pre-eclamptic women compared to gestational age-matched normotensive controls (P=0.008), whilst maintaining a similar total AGT level in the plasma. The research findings indicate that the elevated level of oxidised AGT rather than its total level might be a contributing factor to the hypertension characteristic of

pre-eclampsia, and provide an extra line of evidence linking the oxidative state and the generation of reactive oxygen species with hypertension in pre-eclampsia.

In the second part of the research, 54 clinically relevant glycoproteins were selected to be profiled by label-free targeted LC-MS/MS. Measurement of the analytical precision of the method revealed acceptable CV values for the majority of the assays (median CV 11.8%). Analysis of plasma samples collected from early- and late-onset pre-eclamptic women using the developed glycoprotein profiling methodology successfully identified significant changes in the level of several proteins in pre-eclampsia. Two of them, apolipoprotein D and kallikrein, are reported for the first time to be altered in the plasma of pre-eclamptic women suggesting that they could be further evaluated as novel biomarkers. Some pre-eclampsia-relevant pathways and biological processes, including iron transport and metabolism, coagulation, and lipid metabolism and oxidative stress were found to be altered in the disease. Moreover, different glycoproteins were changed in early-onset compared to late-onset pre-eclampsia which might reflect different pathophysiological mechanisms. Additionally, the method was applied to identify any altered glycoproteins in plasma samples from women with polycystic ovary syndrome (PCOS). These were subsequently compared with those found to be altered in pre-eclampsia, resulting in the proposal of possible underlying pathophysiological mechanisms that may explain the reported association between the two conditions, such as hypofibrinolysis and thrombophilia and iron overload. Moreover, the study detected, for the first time, significant changes in the plasma levels of vitronectin and insulin growth factor acid labile subunit, suggesting that these may also be further appraised as potential biomarkers for the diagnosis of PCOS.

Taken together, the two targeted LC-MS/MS methods developed in this thesis provided relevant information regarding pre-eclampsia by identifying potential pre-eclampsia protein biomarkers. This sheds light on the different biochemical processes altered in the disease and points to possible pathophysiological mechanisms that might assist in explaining the link between PCOS and pre-eclampsia, all of which should improve the understanding of the molecular mechanisms of the disease. The present work offers information that may play a key role in improving the health care of women with pre-eclampsia and serves as a foundational cornerstone for future work.

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List of Abbreviation

ACE	Angiotensin converting enzymes
AGT	Angiotensinogen
CID	Collision-induced dissociation
ConA	Concanavalin A-Sepharose 4B
Cys	Cysteine
DDA	Data dependent acquisition
DTT	Dithiothreitol
1D, 2D-PAGE	one or two-dimensional polyacrylamide gel electrophoresis
ESI	Electrospray ionisation
FA	Formic acid
GE	Gel electrophoresis
IAM	Iodoacetamide
ICAT	Isotope-coded affinity tags
iTRAQ	Isobaric tag for relative and absolute quantitation
LC	Liquid chromatography
LIT	Linear ion trap
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
MALDI	Matrix-assisted laser desorption ionisation
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NEM	N-ethylmaleimide
OPLS-DA	Orthogonal partial least squares-discriminant analysis
PCA	Principle component analysis
PCOS	Polycystic ovary syndrome
PIGF	Placental growth factor
PTM	Post translational modification
QqQ	Triple quadrupole
RAS	Renin angiotensin system
RP-SPE	Reversed-phase solid phase extraction
SCX	Strong cation exchange
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography

SILAC	Stable isotope labelling by amino acids in cell culture
SIS	Stable isotope standards
S/N	Signal to noise ratio
SRM	Selected reaction monitoring
sFLT-1	soluble FMS-like tyrosine kinase
TFA	Trifluoroacetic acid
TIC	Total ion chromatogram
TMT	Tandem mass tags
TOF	Time of flight
UPLC	Ultra-performance liquid chromatography
VEGF	Vascular endothelial growth factor
VIP	Variable Importance for Projection
WB	Western blotting
WGA	Wheat germ agglutinin
XIC	Extracted ion chromatogram
1D, 2D-PAGE	One or two-dimensional polyacrylamide gel electrophoresis

Chapter One

General Introduction

1 General Introduction

1.1 The use of mass spectrometry for quantitative protein analysis

Proteins are essential biomolecules for virtually all processes within cells and are the most likely components to reflect the functional differences in gene expression. Therefore, quantitative proteomic studies, those that study the entire complement of proteins expressed by a cell, tissue, or organism, are extremely informative. They provide a useful window into a range of biological processes and allow the identification of differentially expressed proteins between samples (*e.g.* healthy versus disease-related samples) [1; 2]. However, the complexity of the biological samples and the need for a high-quality quantitative data represent significant analytical challenges in terms of sample preparation methods, instrumentation and data treatment, extraction and processing [3].

Mass Spectrometry (MS) is an indispensable analytical tool and a vital technology in the qualitative and quantitative analysis of proteins and their post-translational modifications (PTM), such as phosphorylation, glycosylation and disulphide linkage [4; 5]. During the last two decades, MS has moved into the front line of protein analysis research, and several well-established methods have been developed capable of analysing complex protein mixtures derived from blood plasma, cells and tissues. Moreover, the targeted MS approach has demonstrated that not only it can be practically applied for proteins quantification, but that it can also be a good alternative to ligand binding assays, as will be discussed later [6]. These advances in the MS analysis of proteins were achieved due to several factors: (1) the remarkable introduction of innovative soft ionisation techniques such as electrospray ionisation (ESI) [7; 8] and matrix-assisted laser desorption ionisation (MALDI) [9; 10], (2) the advances in the sensitivity, mass accuracy and the high-throughput capabilities of different mass analysers, and (3) the hyphenation of MS to different separation techniques [11].

In the following sections, the predominant approaches for sample preparation and MS-based methodologies applied for protein quantification will be discussed. Literature on the use of MS for the discovery of altered proteins that can act as potential biomarkers in pre-eclampsia, the main disease investigated in this thesis, is covered in Chapter Five.

1.1.1 Strategies for quantitative protein analysis

Two strategies have been commonly used for the quantitative analysis of proteins in complex mixtures. They both rely on coupling protein separation techniques with MS or tandem mass spectrometry (MS/MS) for subsequent identification and quantification of the separated protein or peptide species. The first strategy is a combination of one or two-dimensional polyacrylamide gel electrophoresis (1D, 2D-PAGE) with MS/MS [12; 13]. This approach has suffered from considerable limitations in the detection of low abundance proteins [14] and the separation of transmembrane proteins [15]. Moreover, the long processing and analysis time associated with 2D-PAGE-MS [16] narrowed its application in protein analysis. The second strategy is based on a more convenient automated liquid chromatography tandem MS (LC-MS/MS) analysis of peptides derived from complex protein mixtures, applying stable isotope labelling or label-free methodologies [17; 18]. In order to achieve a higher detection sensitivity with LC–MS/MS, the combination of microcapillary LC (µLC) or nanocapillary LC (nLC) with MS has been introduced [19].

1.1.2 Approaches in protein identification and quantification

Two approaches are widely applied for the identification and quantification of proteins; ‘bottom-up’ (also known as shotgun) and ‘top-down’ approaches (Figure 1-1). In the bottom-up approach, the whole proteome under investigation, or the protein of interest, is digested to generate peptide fragments that undergo chromatographic separation and analysis by MS or MS/MS techniques. The generated MS or MS/MS spectra will be used to search available databases (such as Mascot) for protein identification purposes using peptide mass fingerprint, or MS/MS ions search options. In the top–down approach, intact protein(s) of interest is/are isolated from a 1-D PAGE or 2-D PAGE, and the protein samples are analysed by MS without proteolysis. While the top-down approach has a higher ability to detect protein isoforms and PTM [16; 20], it is easier to obtain high mass measurement accuracy and to perform routine MS/MS experiments on peptides than on proteins due to the large molecular weight differences. Hence, bottom-up approach is the most commonly used workflow in protein analysis. However, the digestion step results in a large number of peptides that significantly increase sample complexity [21] and, therefore, places a high demand on the discriminating ability of the MS analysis.

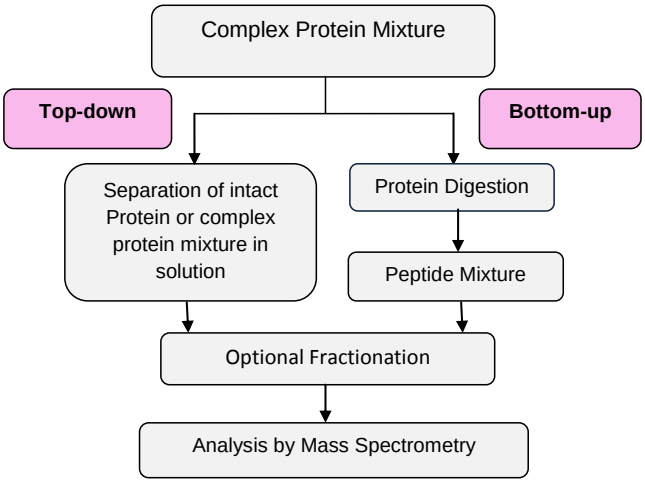


Figure 1-1: The two approaches followed in protein analysis by MS; bottom-up and top-down. In the bottom-up approach, the analysis will be carried out at the level of the peptides generated by enzymatic digestion. In the top-down approach, proteins will be extracted from complex biological samples and then analysed as intact proteins without proteolysis.

1.1.3 Sample preparation

One of the major challenges facing quantitative protein analysis by LC–MS/MS is the inherent complexity of protein biological samples. This in turn increased the awareness about the importance of appropriate sample treatment and simplification before reliable quantitative and qualitative analysis by MS take place. Typically, sample preparation for protein analysis involves selective enrichment of low abundance proteins or depletion of high abundance proteins, protein fractionation and digestion to simpler peptides [22; 23].

1.1.3.1 Human plasma

Despite its huge complexity and the wide range of protein concentrations, human plasma is still the preferred biofluid for quantitative protein profiling and identification of potential disease protein biomarkers. Plasma is a readily accessible biological fluid present in comparatively large quantities [24]. Moreover, changes in plasma proteins can reflect changes in organ function in disease states making it a potentially useful biofluid for the discovery of disease biomarkers. Plasma contains the most complex human-derived proteome, it is tremendously heterogeneous and has a dynamic range of protein concentrations exceeding ten orders of magnitude [24]. This wide dynamic range presents a barrier to the detection of medium and low abundance proteins. Anderson *et al.* [24] plotted the range abundance for 70 protein analytes in plasma. At the high

abundance end, there is plasma albumin (35 – 50 × 10^9 pg/ml) and at the low abundance end, interleukin 6 (normal range 0–5 pg/ml). These two clinically relevant proteins differ in plasma abundance by a factor of 10^{10}. A single protein, albumin, comprises around 55% of the plasma protein content, and approximately 20 highly abundant proteins account for greater than 95% of the total protein mass, making the detection and the quantification of lower abundant plasma protein extremely challenging (Figure 1-2).

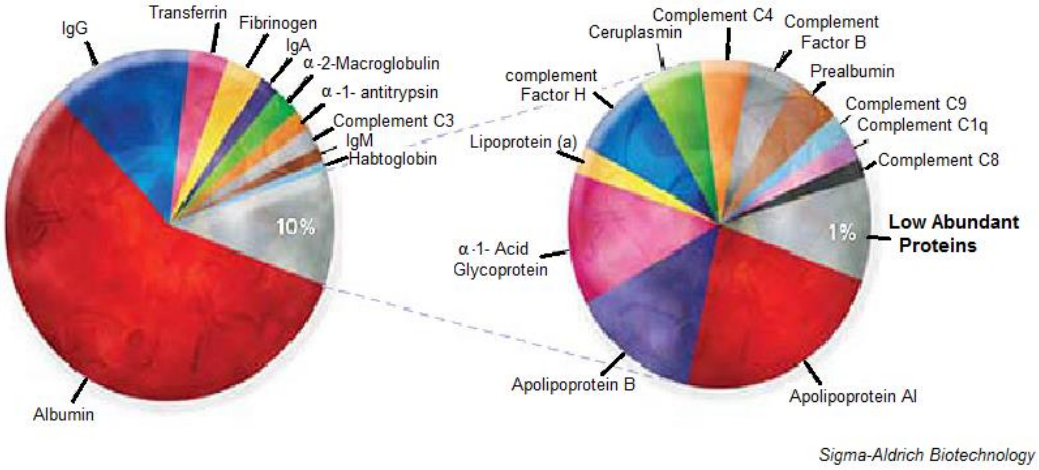


Figure 1-2: Abundance of plasma proteins. Albumin is the highest abundant plasma protein and comprises around 55% of the plasma protein content. Approximately 20 highly abundant proteins account for greater than 95% of the total protein mass, leaving small percentage for the majority of plasma proteins; moderately and, most importantly, low abundant proteins. Figure from Sigma-Aldrich [25].

1.1.3.2 Protein enrichment and extraction

Different strategies have been applied for the depletion of high abundance plasma proteins or the enrichment of target proteins in order to enhance the detection of lower abundance proteins in both shotgun and targeted proteomic analyses. Extraction processes aim to decrease sample complexity, and remove interfering contaminants that would compete for ionisation leading to ion suppression or poor yield of the desired ions in the MS instrument. However, these selective extraction methodologies can reduce the overall analytical throughput and can be a potential source of unwanted variability [11]. The most important selective extraction methodologies are briefly summarised below.

Immunoaffinity depletion and fractionation strategies

Immunoaffinity depletion columns containing immobilised antibodies are widely used to remove highly abundant plasma proteins, typically either 7, 12 or 14 major proteins [26; 27]. They offer good reproducibility, depending on the depletion column used, and allow

a 10- to 20-fold enrichment of low abundance proteins [28]. In addition, they enhance the sensitivity of targeted MS assay and improve the signal to noise ratio (S/N), limit of detection (LOD), limit of quantification (LOQ) and assay variability compared to the data obtained without depletion [29].

Besides immunoaffinity depletion, different fractionation methods have been commonly applied to improve low abundance proteins detection by concentrating different target analytes to specific fractions. This includes reversed phase LC [30], strong cation exchange (SCX), size exclusion chromatography (SEC) [31], isoelectric focusing [32; 33], and gel-based fractionation [34; 35]. Despite their advantages, immunoaffinity columns and fractionation strategies suffer from low sample throughput, and the addition of extra complexity and time to the overall assay [28]. Immunoaffinity depletion column, in particular, can be associated with high cost and poor sample recovery due either to nonspecific binding to the depletion column or non-covalent interactions with carrier proteins such as albumin. This unwanted cross-reactivity may result in the removal of clinically relevant proteins such as lipoproteins [36].

Affinity protein enrichment

Immunoaffinity capture of target proteins is likely the most effective method for sensitive detection of low abundance proteins in complex samples [37; 38]. However, the major drawback of this strategy is the lack of available antibodies for most new candidate biomarkers discovered, and the limited multiplexing power for quantifying a large number of target proteins in clinical samples as antibodies are needed for all analysed proteins [28].

Affinity peptide enrichment

An alternative for protein enrichment is to directly capture target peptides used for protein quantification by antipeptide antibodies. This strategy is used in targeted protein quantification and was introduced in 2004 by Anderson *et al*. and termed Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA) [39]. This approach provides potential for achieving high sensitivity detection, large-scale assay development and high throughput sample analysis [39]. However, it suffers from the high reagent cost (up to $5000/assay) and the long lead time for assay development (~24 weeks) [40].

Enrichment of sub-proteomes

Variable strategies for the extraction of different sub-proteomes, including different types of PTM (such as phosphorylated proteins and glycoproteins) are commonly used to reduce plasma complexity. For example, lectin affinity chromatography selectively purifies glycoproteins [41], while immobilised metal affinity chromatography and titanium oxide extract phosphorylated proteins and peptides [42]. Also, targeting peptides containing a specific amino acid can also be achieved as observed with isotope-coded affinity tags (ICAT) that selectively purify cysteine containing peptides and, therefore, decrease the complexity of the analysed sample [14].

1.1.3.3 Protein digestion

The extracted proteins are digested enzymatically using endoproteinases (trypsin, chymotrypsin) or chemically using cyanogen bromide to generate peptide fragments which are then subjected to MS analysis for identification (molecular mass and amino acid profile analysis) and quantification purposes. Protein digestion is carried out either in-gel or in-solution [43]. In the gel-based approach, proteins are separated by 1D or 2D PAGE, and the isolated protein bands undergo digestion. Whilst in the gel-free approach (also known as in-solution digestion), direct digestion of the proteins is achieved in solution. The generated peptides are separated by LC and analysed by MS, and proteins are identified by specialised database followed by proteins quantification and statistical treatment of the largely generated data by the application of suitable software.

Digestion is a crucial step in targeted quantitative protein analysis and can dramatically affect the reproducibility of protein assay. Protein digestion is discussed in more details in Chapter Two, Section 2.1.1.2.

1.1.4 Mass spectrometric instrumentation for quantitative protein analysis

MS is now recognised as a well-established analytical technique for qualitative protein analysis. The more challenging demands of quantifying proteins and peptides have encouraged further enhancement of the performance of MS instruments in terms of detection sensitivity, sample throughput, mass resolution and accuracy, and dynamic range [19].

In the section below some important aspects and advances in MS instrumentation that facilitate its use in quantitative protein analyses are described.

1.1.4.1 Soft ionisation techniques

With the introduction of soft ionisation techniques in the late 1980s [7; 9], protein analysis by MS has become feasible. The two common techniques used for the ionisation of large molecules and converting them into the gaseous state without excessive fragmentation are matrix-assisted laser desorption ionisation (MALDI) and electrospray ionisation (ESI).

Matrix-assisted laser desorption ionisation (MALDI)

MALDI is a widely-used analytical tool for the analysis of high molecular weight compounds such as peptides, proteins [44], and most other biomolecules (sugars and lipids) [45]. In MALDI analysis, the analyte is first mixed with a suitable matrix material, usually a UV-absorbing weak organic acid, and applied to a metal plate. After which, a pulsed laser irradiates the analyte–matrix mixture resulting in the vaporisation of the matrix carrying the analyte with it. The matrix causes, indirectly, the analyte to vaporise, and helps in its ionisation by acting as a proton donor and acceptor in positive and negative ionisation modes, respectively [46]. MALDI is often connected to a time of flight (TOF) analyser. MALDI-TOF-MS is a highly sensitive, fast and robust method requiring minimal sample pre-treatment as it tolerates the presence of contaminants such as salts and detergents, which make it suitable for the direct detection of proteins and peptides in biological samples [47; 48]. However, sample preparation and LC separation are decoupled from the MS analysis [49; 50], and larger peptides and proteins are detected with low sensitivity and may require >1 µmol/L concentrations for accurate measurements [51].

Electrospray ionisation (ESI)

ESI is the most widely used ionisation technique for the analysis of samples in liquid form [52]. It uses electrical energy to allow the transfer of ions from solution into the gaseous phase. Such process involves dispersal of a fine spray of charge droplets, followed by droplet size reduction through solvent evaporation and droplet fission, and finally, ion ejection from the highly charged droplets [52], as seen in Figure 1-3.

ESI is readily coupled to liquid-based separation tools like chromatography and capillary electrophoresis [53], and is generally connected to ion traps and triple quadrupole mass analysers for protein identification and quantification purposes [54]. ESI-MS requires more intensive sample purification steps than MALDI; the coupling of LC to ESI-MS can lead to ionisation suppression and irreproducible ionisation especially when different buffers are used as mobile phases. As a result, additives and salt concentration should be kept to a minimum [52].

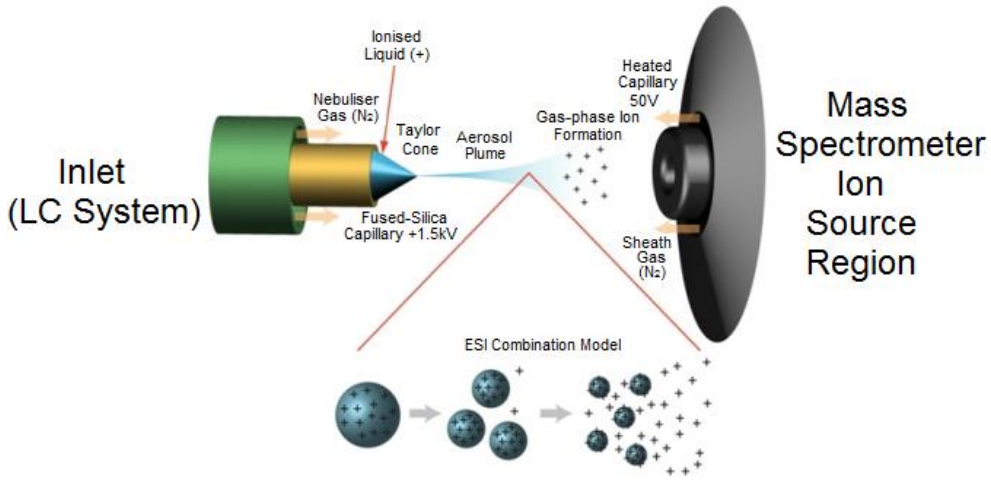


Figure 1-3: Mechanism of ion formation process in electrospray ionisation. A sample solution is passed through a capillary tube maintained at a high voltage (1.5 - 6.0 kV). A fine spray cone of highly charged droplets will be formed at the end of the capillary tube (known as Taylor cone), and the charged droplets pass down toward the mass spectrometer region by pressure and potential gradient. The high ESI-source temperature and the use of nitrogen drying gas lead to a continuous reduction in the size of the droplets by solvent evaporation. As a result, the charge density on the droplet surface increases until it reaches a critical point at which ions at the surface of the droplets will be ejected into the gaseous phase. The generated ions will be sampled by a skimmer cone then accelerated into the mass analyser for subsequent molecular mass and ion intensity measurement. The figure is taken from reference [55].

1.1.4.2 Mass analysers

The versatility of mass analysers, including several types of ion-traps (IT), time of flight (TOF) and quadrupole (Q) instruments encourage their use in the analysis of analytes in complex biological samples [56]. These mass analysers can stand alone or, in many cases be put together in tandem to benefit from the advantages of each one [53]. Currently, most of the studies involving protein analysis are performed on tandem mass spectrometers; typically MS/MS instruments are used. Table 1-1 summarises the main characteristics of the most commonly used tandem MS instruments [57].

Time of flight (TOF)

TOF analysers determine the mass to charge ratio (*m/z*) of an analyte ion from its flight time through a tube with a specified length that is under vacuum, and offers a good resolution (> 12,000 FWHM) and mass accuracy (low ppm range). To enhance the quantitative and qualitative analysis applications of TOF, it is hybridised to other analysers like quadrupole mass analyser (Q-TOF) [57].

Ion trap

In ion-trap analysers, ions are trapped and accumulated over time in a physical device. Ion-trap analysers are robust, offer fast data acquisition [57], are able to perform MS/MS (MS^2) and beyond, up to MS^{10} [58], and when used with data-dependent acquisition, they allow high throughput analyses [57]. However, they have relatively limited resolution and low ion trapping capacity [53]. Their sensitivity, resolution and dynamic range have been further enhanced with the development of linear ion trap (LIT) analysers. The implementation of LIT on triple quadrupole (Q-q-Q) instruments (the second analyser is substituted by a LIT) offers scanning capabilities including product and precursor ion scanning, Figure 1-4 [59].

Orbitrap

Orbitrap mass analyser was introduced into the market in 2005 and was the first mass analyser that separates ions in an oscillating electric field [60]. It offers high resolution (>100,000 FWHM) and mass accuracy (<3 ppm) capabilities which enable screening of the analytes with high selectivity, and accurate measurement of molecular mass for ions of the same nominal mass. But, it suffers from suboptimal sensitivity and limited linear range when compared to triple quadrupole instruments [58; 61].

Triple quadrupole

Triple quadrupole instruments can be considered the most sensitive MS instrument for quantitative analysis. They offer high throughput and allow the detection and quantification of analytes with a high degree of selectivity and sensitivity. Triple quadrupole work using a multiple reaction monitoring (MRM) scanning mode is discussed in more detail in Section 1.1.5.3; targeted protein quantification by MS.

Table 1-1: Characteristics and performances of commonly used MS instruments [57].

	IT-LIT	**Q-q-TOF**	**Q-q-Q**	**Q-q-LIT**	**Orbitrap**
Mass accuracy	Low	Good	Medium	Medium	High
Resolving Power	Low	Good	Low	Low	High
Sensitivity	Good	-----	High	High	Good
Dynamic Range	Low	Medium	High	High	Medium
ESI	Available	Available	Available	Available	Available
MALDI	Optional	Optional	-----	-----	-----
MS/MS capabilities	Available	Available	Available	Available	in hybrid
Identification	++	++	+	++	+++
Quantification	+	++	+++	+++	++
Detection of modification	+	+	-----	+++	with hybrid

+, ++, +++ indicate possible or moderate, good or high, and excellent or very high, respectively.
Q-q-Q, LIT, TOF refers to triple quadrupole, linear ion trap, time of flight mass analysers respectively.

Scanning techniques used in MS/MS

In general, four major scanning modes are routinely applied in MS/MS analysis (Figure 1-4); product ion scanning, precursor ion scanning, neutral loss scanning, and multiple reaction monitoring (MRM). In product ion scanning, specific peptides will undergo fragmentation and the amino acid sequences of these peptides are identified based on their fragment ion spectra [57]. Practically, a specific precursor ion is selected in the first mass analyser (MS1), then it undergoes fragmentation by collision-induced dissociation (CID) to generate product ions that are analysed by the second mass analyser (MS2) [58]. In precursor ion scanning, only one specific fragment ion is transmitted by MS2 to the detector. After that, MS1 is scanned to detect all precursor ions that generate this fragment. Usually, this scanning mode is applied for the detection of peptides that contain a specific functional group. For neutral loss scanning, both mass analysers are scanned simultaneously so that the mass difference of ions passing through MS1 and MS2 remains constant. The mass difference is related to a neutral fragment that is lost from a peptide ion in the collision cell such as the loss phosphoric acid from phosphorylated peptides [57].

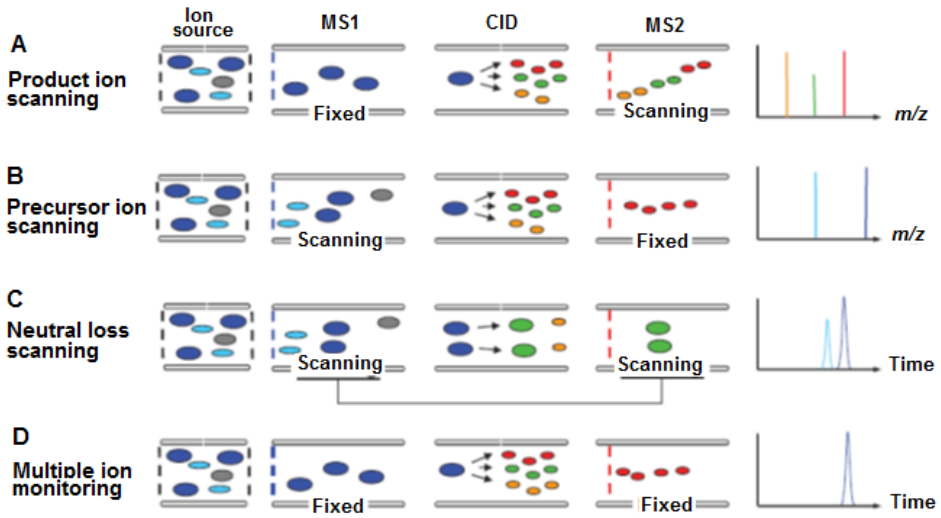


Figure 1-4: Multiple scan modes routinely used in MS/MS. A) In product ion scanning, a specific precursor ion is selected in MS1 and then undergoes fragmentation and all the resulting product ions will be analysed by MS2. B) In precursor ion scanning, only one specific fragment ion is selected by MS2 and then MS1 is scanned to detect all precursor ions that generate this fragment. C) For neutral loss scanning, both MS1 and MS2 are scanned simultaneously so that the mass difference of ions passing through both analysers remains constant. D) In ion monitoring techniques both mass analysers are set to a selected mass and molecules can be detected with higher selectivity and sensitivity by focusing the MS analysis time on compounds with specific masses and exclude all others. Figure 1-4 is from reference [57].

1.1.5 Protein quantification

Besides the advances in MS instruments, the developments in stable isotope labelling and label-free methodologies have enhanced protein quantification by MS. Proteins can be relatively or absolutely quantified applying the above two methodologies. For most biomedical and biomarker discovery studies, relative protein quantification to identify differentially altered proteins between the studied samples, generally healthy versus diseased samples, is the desired output of MS-based measurements. Absolute protein quantification strategies, widely achieved by targeted LC-MS/MS analysis, give an absolute measurement of protein abundance, and reflect more accurately functional proteins at the molecular level [62].

The below figure (Figure 1-5) represents the three main strategies used for protein quantification that will be discussed in the following sections.

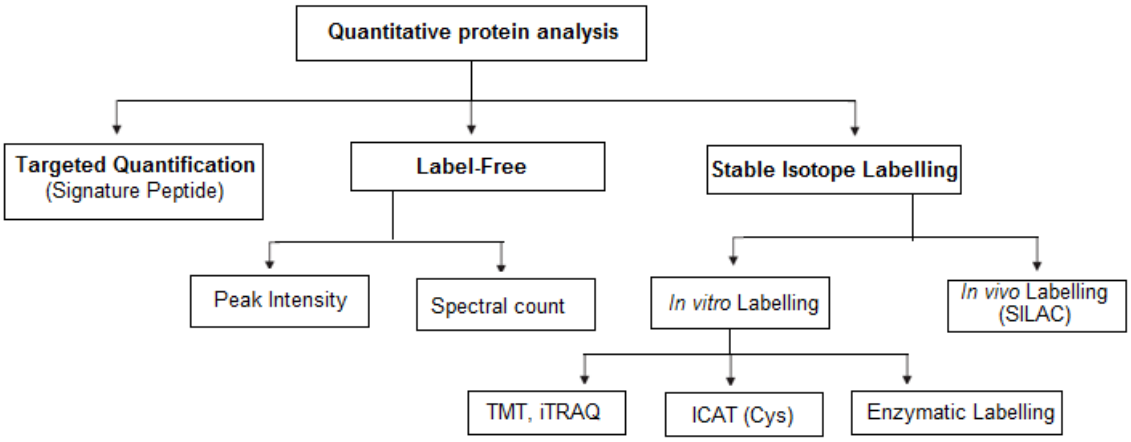


Figure 1-5: Main methodologies used for protein quantification. Stable isotope labelling and label-free strategies are often used in discovery proteomics screening experiments. Targeted proteomics is typically designed to quantify specific proteins with very high precision, sensitivity, specificity and throughput.

1.1.5.1 Isotope labelling methodologies

Stable isotope labelling involves proteins or peptides labelled with a stable isotope so that pairs of chemically identical analytes will have different stable isotope composition and, therefore, a distinguishable mass difference in MS. The ratio of signal intensities of the peptides will be compared between the light and the heavy isotope labelled samples to indicate the relative changes in the abundance of the analytes [53]. For accurate measurement, labelling should not affect the physicochemical properties of the peptides to ensure that the compared analytes behave almost identically in chromatographic and MS analysis [20].

Metabolic labelling

Stable isotope introduction into proteins during cell growth and division is the earliest possible point for isotope labelling. This can be accomplished by using heavy salts or amino acids through a method referred as metabolic labelling [63]. Metabolic labelling has become a well-known approach with the emergence of stable isotope labelling by amino acids in cell culture (SILAC) by Mann and co-workers in 2002 [64], (Figure 1-6, A). In SILAC, differently labelled samples will be combined at an early stage (before subsequent sample treatment steps) so both protein populations will experience the same experimental conditions. Such technique avoids variability of sample preparation, offers a good quality control and is generally considered the most accurate approach for quantitative analysis [65]. However, SILAC is practically difficult to be applied routinely,

expensive, time consuming [4] and can only be used with cultured cells and cannot be applied to tissues and body fluids [16].

Enzymatic labelling

In enzymatic labelling, proteins are digested with protease using ^{18}O-labeled water leading to the incorporation of ^{18}O at the carboxyl end of each peptide, and consequently a 2 Da mass shift per ^{18}O atom [66; 67]. However, full peptide labelling is rarely achieved and sometimes different peptides will be labelled at different rates which further complicate data analysis and quantification [68].

Chemical labelling

A chemical label probe is usually composed of a reactive group that modifies the active sites of all members of a given protein class, and a chemical tag for the detection or the isolation of the bound protein or peptides [3]. ICAT, isotope-coded affinity tags and iTRAQ, isobaric tag for relative and absolute quantitation (Figure 1-6, B and C respectively) are two commonly labelling techniques used in quantitative protein analysis. ICAT targets cysteine residues [3; 14] while iTRAQ targets N-termini and lysines of all peptides [69].

Isobaric tags, also known as tandem mass tags (TMT), have an identical mass but contain stable isotopes at specific positions within the tag, so upon their fragmentation in MS/MS mode, each tag will generate a reporter ion with a unique *m/z* used for quantification purposes [70]. TMT tagging is similar in principle to other isobaric isotope labelling approaches in that differentially labelled peptides are chemically identical, do not differ in mass and comigrate together during chromatographic separation. Therefore, one MS signal can be seen for each peptide pairs which reduces the spectrum complexity [70].

Chemical isotope tagging is currently the most commonly used labelling approach. Tags can be attached to specific functional groups which allow the isolation of selective peptides or proteins and reduce the complexity of the sample. [53]. However, chemical labelling can lead to unwanted side reactions that negatively influence the obtained measurements [4], and can be associated with higher variability than metabolic labelling since the samples to be compared are combined at a later stage [71]. Furthermore, trypsin does not cleave modified lysine residues of intact labelled proteins, resulting in significantly longer peptides that are typically more difficult to analyse by MS [4].

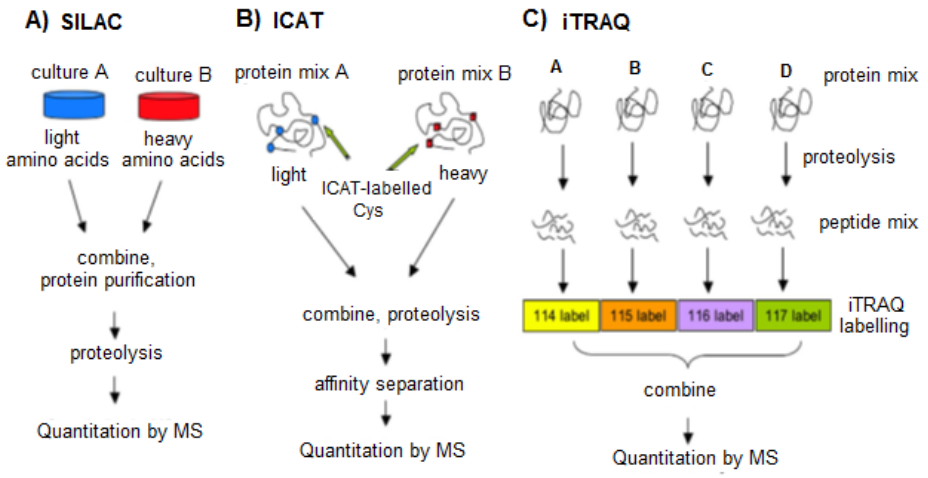


Figure 1-6: The three common approaches for stable isotope labelling in protein quantification. A) SILAC (metabolic labelling). Cells grow in isotopically enriched culture media and labelled cells are combined at an early stage prior to protein digestion and MS analysis. B) ICAT (chemical labelling) specifically labels cysteine residues which allows the extraction of only Cys containing peptides from the protein digest mixture. C) iTRAQ (chemical labelling) labels lysine residues after protein digestion and up to 8 different samples can be mixed and compared. Figure 1-6 is from reference [16].

1.1.5.2 Label-free methodology

Label-free quantification methods aim to compare two or more experiments without labelling. This is widely achieved by two strategies: peak intensity (precursor ion data) and identification frequency. In the peak intensity strategy, the intensity of each peptide signal (precursor ion), which belongs to a specific protein, in one experiment will be compared to the respective signal in one or more other experiments to give relative quantitative data. The ion chromatograms for every peptide are extracted from an LC-MS run, and their peak areas are integrated over the chromatographic time scale [72; 73].

The second strategy, identification frequency, estimates the relative difference in protein abundance based on either the number of identified peptides, precursor ions or MS/MS spectra (spectral count) for each protein [71]. Spectral count method counts and compares the number of MS/MS spectra that identify peptides of certain protein between samples [74]. It provides the highest correlation with relative protein abundance and is considered the best approach for relative label-free protein quantification [75].

When compared to isotope labelling, label-free is a faster, simpler, cheaper and more flexible and affordable technique. Also, an unlimited number of experiments can be compared in label-free strategy, while usually 2–8 experiments can be compared in stable isotope labelling techniques [4]. However, the label-free approach is associated

with higher variability in the obtained data which requires a careful control over the reproducibility of each step of the experiment to account for any experimental variations [16].

1.1.5.3 Targeted protein quantification

Targeted MS approaches are used to increase the precision, sensitivity specificity, dynamic range and throughput of quantitative biomolecule analysis [76; 77]. In this approach, signature peptides produced by the enzymatic digestion of protein mixture act as surrogates of the corresponding proteins and are used to infer the concentration or the relative abundance of the protein in the biological sample [29]. Multiple reaction monitoring (MRM) (also referred to as selected reaction monitoring (SRM)) is a targeted MS technique that was originally applied for the quantification of small molecules (*e.g.* drugs and metabolites) [78], but has emerged as a major technique for the precise quantification of targeted proteins [31; 36; 79; 80; 81].

Targeted precursor/product ions scan mode

Multiple reaction monitoring is an MS/MS scan mode that is unique to triple quadrupole (QqQ) MS instruments [82; 83] and is used most effectively in an LC system connected in-line to the ESI source of the mass spectrometer [29]. Two stages of mass filtering are used in MRM. In the first stage, molecular ions within a mass range around the mass of the signature peptide are selected in the first mass analyser (Q1) and are fragmented by CID with a neutral gas in a pressurised collision cell (Q2). In the second stage, one or several sequence-specific fragment ions (product ions) derived from the targeted peptide are selected by the second analyser (Q3). The fragment ions that reach the detector are counted over time, leading to a chromatogram with retention time and signal intensity as coordinates, Figure 1-4, D. Several precursor/product ion pairs, known as transitions, are repeatedly and rapidly measured yielding chromatographic peak for each transition. This multiplexing capability allows the simultaneous quantification of multiple analytes in a single LC-MS run and hence the term MRM [77; 84]. In addition, the number of analytes quantified per run can be increased by the use of retention time windows thus increasing the throughput of the assay and making it rapid enough for clinical applications [85]. Product ions MS/MS scan can also be acquired for the targeted peptide following an MRM scan when utilising a hybrid triple quadrupole linear ion trap instrument [86]. The predicted *y* and *b* ions from specific peptide fragmentation can be matched with the obtained MS/MS spectrum to further verify the identity of the candidate peptide.

Selection of signature peptide for protein quantification

The first step in targeted protein assay development is to identify the best signature peptide that will be used to infer the absolute or the relative abundance of the protein in the biological sample [29]. Selection of a peptide to represent a protein in an MRM assay is a crucial step that can dramatically affect the ultimate sensitivity and specificity of an assay. Several critical factors must be considered when selecting a surrogate peptide; 1) the peptide amino acid sequence must be unique to the target protein, composed of 7-16 amino acids in length, and preferably not containing any missed cleavage sites, 2) the peptide should be reproducibly detected in the proteolytic digests in every sample and between sample preparations, 3) the peptide should not contain amino acids that are susceptible to chemical modification (cysteine and methionine) unless it is the purpose of the analysis, and 4) should have high ionisation and fragmentation efficiencies [86; 87].

Signature peptides can be selected from the LC-MS of the pure form of the targeted protein digest or from online sources such as PeptideAtlas [88] and the Global Proteome Machine Database [89] that have a library of peptides reproducibly detected in previous MS-based protein analysis researches. For proteins not covered in these databases or having no available literature, computational software tools have been developed to predict best MS-observable peptides, this include ESP predictor [90] and PeptideSieve [91]. Selection of the most sensitive and specific fragment ions and the optimisation of the signal intensity of each peptide transitions and MS parameters are best achieved by using synthetic peptide standards [80; 85]. However, this can be excessively costly especially when large numbers of peptides are measured. For this purpose, software platforms have been developed to create candidate MRM assays *in silico* by generating transition lists and vendor-specific MS instrument parameters, such as Skyline software [92].

Absolute and relative quantification

The integrated peak areas of precursor/product ion transitions serve as the basis for a relative or absolute quantitative measurement in targeted MS analysis. Absolute measurement is achieved by using stable isotope labelled synthetic peptides that act as internal standards for each targeted peptide. These peptide standards are spiked immediately into samples following digestion, as their addition prior to digestion was found to generate unpredictable results. This will correct for subsequent analytical steps, mainly variations in instrument performance (*e.g.* variation in injection volume,

electrospray stability and LC solvent flow rate) [87]. As with stable isotope labelling methodologies previously mentioned, the labelled peptide standards co-elute with their respective unlabelled peptides generated from protein digestion and display an identical ionisation and fragmentation pattern. The mass shift in the precursor and product ions between the unlabelled peptide and the labelled peptide standard allows the differentiation between them [93].

The level of sensitivity that can be attained in targeted LC-MS/MS assay depends on several factors including the type of sample being analysed, MS instrument, and whether enrichment or fractionation techniques are conducted before analysis [93]. LC-MRM assays showed a LOD at the low attomole level and a high reproducibility even in complex samples as plasma [87; 94] and bacteria proteome [95]. The sensitivity of the assay can be enhanced by coupling microcapillary LC (μLC) or nanocapillary LC (nLC) with MS/MS, and/or by the addition of prefractionation steps to attain a LOQ in the low ng/mL range for targeted proteins in the plasma [96; 97].

Isotope labelled standard based assays exhibit the best performance characteristics of all targeted peptide measurements. Typically they have linearity over 4−5 orders of magnitude, and coefficient of variation measurement less than 10%. Due to the high cost of the synthetic peptide standards, label-free assays have emerged as an alternative approach for the relative quantification of protein levels between samples [93]. Label-free quantification is based on the raw integrated peak areas of the targeted peptides and does not apply isotopically labelled standards in its workflow. Therefore, it can be associated with higher analytical variability as no correction for the variation in the instrumental response is performed during data analysis [93]. However, Label-free is considered a cost-effective and affordable approach, and adequate reproducibility can be achieved when careful control is undertaken to minimise the technical variability through optimisation of every step in the analytical workflow [98].

Besides the relative and absolute quantification of protein in complex samples, targeted LC-MS/MS analysis is applied to study signalling pathways [99] and is considered the most specific means of site-specific quantitation of protein PTM such as phosphorylation [93; 100]. Additionally, targeted protein quantification is successfully used to validate biomarker candidates identified during discovery screening proteomics experiments. Therefore, it can act as an alternative to antibody-based assays typically used for biomarker verification [37; 101].

Advantages of targeted MS-based protein quantification over antibody-based assay

Antibody-based assays (ELISA: enzyme-linked immunosorbent assays) have been routinely used for protein quantification. They are highly sensitive, able to measure low protein concentration (where MS/MS may not achieve adequate sensitivity), require minimal sample preparation, and are available in convenient high throughput platforms [77]. However, antibodies may not be available for all proteins of interest, especially for new biomarker candidates, and the process of developing a new immunoassay is very expensive ($100K - $250K per biomarker candidate for a research assay) and time consuming (1-1.5 years) [102; 103]. Moreover, interference from homologous high abundance proteins present in the sample can affect the selectivity of immunoassay. Antibody-based assay also suffers from poor selectivity to discriminate between different protein isoforms and the process of developing a highly selective antibody for specific protein isoform is very challenging [98].

Targeted protein quantification by MS can offer several advantages over immunoassay methods. It has shorter assay development timelines, lower assay cost, improved reproducibility and precision, and of most importance, higher specificity that allows different structurally related protein isoforms to be selectively measured [104]. MS-based quantification is performed with low sample consumption (10-50 µL) and is highly multiplexed allowing the analysis of tens to hundreds of proteins in a single analysis with throughput capable of analysing hundreds of plasma samples with good precision [103]. Such an advantage is very important in biomarker verification studies since it allows testing large numbers of protein biomarker candidates emerging from discovery experiments to identify the best candidates to be taken forward for a further thorough clinical investigation [103].

1.2 Classification of hypertensive disorders of pregnancy

Over 280,000 women die each year from pregnancy-related causes with hypertensive diseases of pregnancy being one of the leading causes of maternal deaths. The International Society for the Study of Hypertension in Pregnancy (ISSHP) has published Consensus statements for the classification of hypertensive disorders of pregnancy [105], which divides hypertensive diseases of pregnancy into four main categories; chronic hypertension, gestational hypertension, pre-eclampsia (de novo or superimposed on chronic hypertension), and white coat hypertension (Table 1-2). In brief, chronic hypertension is hypertension diagnosed before gestational week 20 or de novo hypertension which fails to settle post-partum, while gestational hypertension refers to an increase in the blood pressure occurring after the 20th gestational week, without any of the abnormalities that define pre-eclampsia. Pre-eclampsia is defined as gestational hypertension characterised by proteinuria and/or maternal organ dysfunction, and white coat hypertension is recognised by having increased office and clinic blood pressure while having a normal blood pressure during the daytime.

Table 1-2: Classification of hypertensive diseases of pregnancy [105].

Disease	Description
Chronic hypertension	1- Increased BP before week 20 (or known to exist prior to pregnancy). 2- Hypertension persistent for more than 12 weeks after pregnancy.
Gestational hypertension	1- Transient hypertension appearing after 20 weeks gestation, without any of the abnormalities that define pre-eclampsia. 2- Confirmed by return to normal BP postpartum.
Pre-eclampsia	Hypertension developing after 20 weeks' gestation with the co-existence of one or more of the following new-onset conditions: 1. Proteinuria (at least 300 mg/24 hr, protein: creatinine ratio ≥ 30 mg/mmol or if neither are available dipstick analysis of ≥ +). 2. Other maternal organ dysfunction: - renal insufficiency (creatinine >90 µmol/L). - liver involvement (elevated transaminases and/or severe right upper quadrant or epigastric pain). - neurological complications (examples include eclampsia, altered mental status, blindness, stroke). - haematological complications (thrombocytopenia, haemolysis). 3. Uteroplacental dysfunction - fetal growth restriction
White coat hypertension	Increased office and clinic BP and normal daytime BP (a 24-hour blood pressure < 130/80 mmHg) measured with ambulatory blood pressure monitoring (ABPM).

Hypertension is defined as an increase in blood pressure, systolic BP ≥ 140 mmHg and/or diastolic BP ≥ 90 mmHg on 2 occasions 4-6 hours apart. BP refers to blood pressure

1.3 Pre-eclampsia

Pre-eclampsia is a multisystem pregnancy disorder characterised by hypertension and proteinuria developing after the 20th week of gestation [106]. It is one of the leading causes of maternal and fetal morbidity and mortality worldwide, especially in low-income and middle-income countries [107; 108]. Pre-eclampsia complicates 2-8% [109; 110] of pregnancies in the Western world and 5% of first-time mothers [109], and contributed to 10-15% of maternal deaths, of which 99% occur in the developing world [111].

Pre-eclampsia can be classified as mild (BP 140/90 to 149/99 mmHg), moderate (BP 150/100 to 159/109 mmHg) and severe (BP ≥ 160/110 mmHg) [112]. It may have an early-onset if occurring before 34 weeks' gestation, or late-onset if occurring after 34 weeks' gestation. Yet, it is unclear if the early- and late-onset of pre-eclampsia are the same disease or if they have different pathological mechanisms [113]. With regards to the disease outcome, pre-eclampsia can be associated with preterm or on term delivery, small for gestational age babies or appropriate birth weight for gestational age babies. The different outcomes of pre-eclampsia appear to have different consequences, particularly for the mother's future cardiovascular health [114].

1.3.1 Maternal risk factors

Accurate prediction of pre-eclampsia remains difficult. However, there are numbers of maternal risk factors that are known to increase the risk of developing pre-eclampsia. A woman's risk of developing pre-eclampsia triples with a family history of the disease or if she is pregnant with twins [115]. Moreover, previous pre-eclampsia is a high risk factor for developing pre-eclampsia; having pre-eclampsia in a first pregnancy increases the risk of pre-eclampsia in a second pregnancy almost seven times. Other pre-eclampsia risk factors are discussed briefly below.

Age

Women older than 40, whether they were primiparous or multiparous, had nearly twice the risk of developing pre-eclampsia [115; 116].

Ethnicity

Pre-eclampsia rates vary significantly around the world. A retrospective American cohort study (n=127,000), revealed higher rates of pre-eclampsia among African-American, and lower amongst Latina and Asian women compared to white women [117]. However, the

relation between developing pre-eclampsia and different ethnic populations remains uncertain [115].

Parity

Parity is defined as the number of times that a woman has given birth to a fetus with a gestational age of 24 weeks or more, regardless of the outcome [118]. Nulliparity, not given birth previously, has been shown to almost triple the risk of pre-eclampsia [115]. Nevertheless, the protective effect of having had a previous birth is lost when a subsequent pregnancy is conceived with a new partner, or when there is a long interval between pregnancies (≥ 10 years) [119]. The latter was more significantly associated with the risk of developing pre-eclampsia than changing the partner [120].

Obesity

Obesity before pregnancy or in early pregnancy is a well-known risk factor for developing pre-eclampsia [121]. In one cohort study, women with a body mass index (BMI) > 35 before pregnancy showed four times higher risk of having pre-eclampsia compared to matched controls with a BMI between 19-27 [122]. This is mainly related to the metabolic disturbances associated with marked obesity, and alteration of the plasma lipid profile that can lead to the development of dyslipidaemia; a condition known to be associated with pre-eclampsia [123].

Pre-existing medical conditions

Women with a history of diabetes, renal disease and pre-existing hypertension have an increased risk of developing pre-eclampsia [115; 124; 125]. Additionally, antiphospholipid syndrome, an autoimmune disorder with a hypercoagulable state caused by antiphospholipid antibodies, showed a significant increase in the risk of developing pre-eclampsia [2; 126].

1.3.2 Pathogenesis of pre-eclampsia

The aetiology of pre-eclampsia remains poorly understood [127]. However, the leading hypothesis strongly implicates disturbed placental function in early pregnancy. The pathology of pre-eclampsia is believed to occur in two main stages. The first stage begins in the poorly perfused placenta, while the second stage is associated with an abnormal maternal endothelial response that will lead to the hypertension and proteinuria that characterise the condition [128; 129].

1.3.2.1 First phase

The placenta plays a crucial role in the pathogenesis of pre-eclampsia. This is well-recognised since pre-eclampsia occurs only during pregnancy and resolves following the delivery of the placenta, and it can be developed in molar pregnancy (in which the placenta develops without a fetus) [129].

Normal placenta development requires extensive angiogenesis and effective remodelling of the spiral arteries, the primary blood supplier to the placenta. Remodelling transforms the arteries from low flow, highly resistant vessels into the high flow, low resistance vessels allowing for oxygen and nutrients supply necessary for the fetus development [130]. Impaired remodelling of the spiral arteries is considered a key factor in the pathogenesis of preeclampsia [131]. In pre-eclampsia, the spiral arteries remain narrow as a result of impaired trophoblasts differentiation (from epithelial to an endothelial phenotype) and diminished endovascular invasion by trophoblasts (Figure 1-7). This will lead to an inadequate remodelling of the uterine spiral arterioles and, therefore, restricted blood supply to the fetus [132]. The direct consequence of the failure to remodel the maternal arteries is that the developing placenta becomes severely hypoxic which results in placental ischaemia [133].

1.3.2.2 Second phase

The second phase of pre-eclampsia is characterised by exaggerated maternal endothelial activation and a pro-inflammatory state [134]. The abnormal placentation and placental hypoxia lead to oxidative stress, and the release of reactive oxygen species (such as superoxide, hydrogen peroxide and nitric oxide) and a variety of trophoblast debris and factors into the maternal circulation, such as the two antiangiogenic markers soluble endoglin and FMS-like tyrosine kinase 1 that will be discussed later [135]. The released factors with other mediators activate metabolic, inflammatory, and thrombotic responses, and lead to endothelial dysfunction and increased vascular reactivity culminating in the clinical signs and symptoms of pre-eclampsia typically occurring after the 20^{th} week of pregnancy (Figure 1-8) [129; 136].

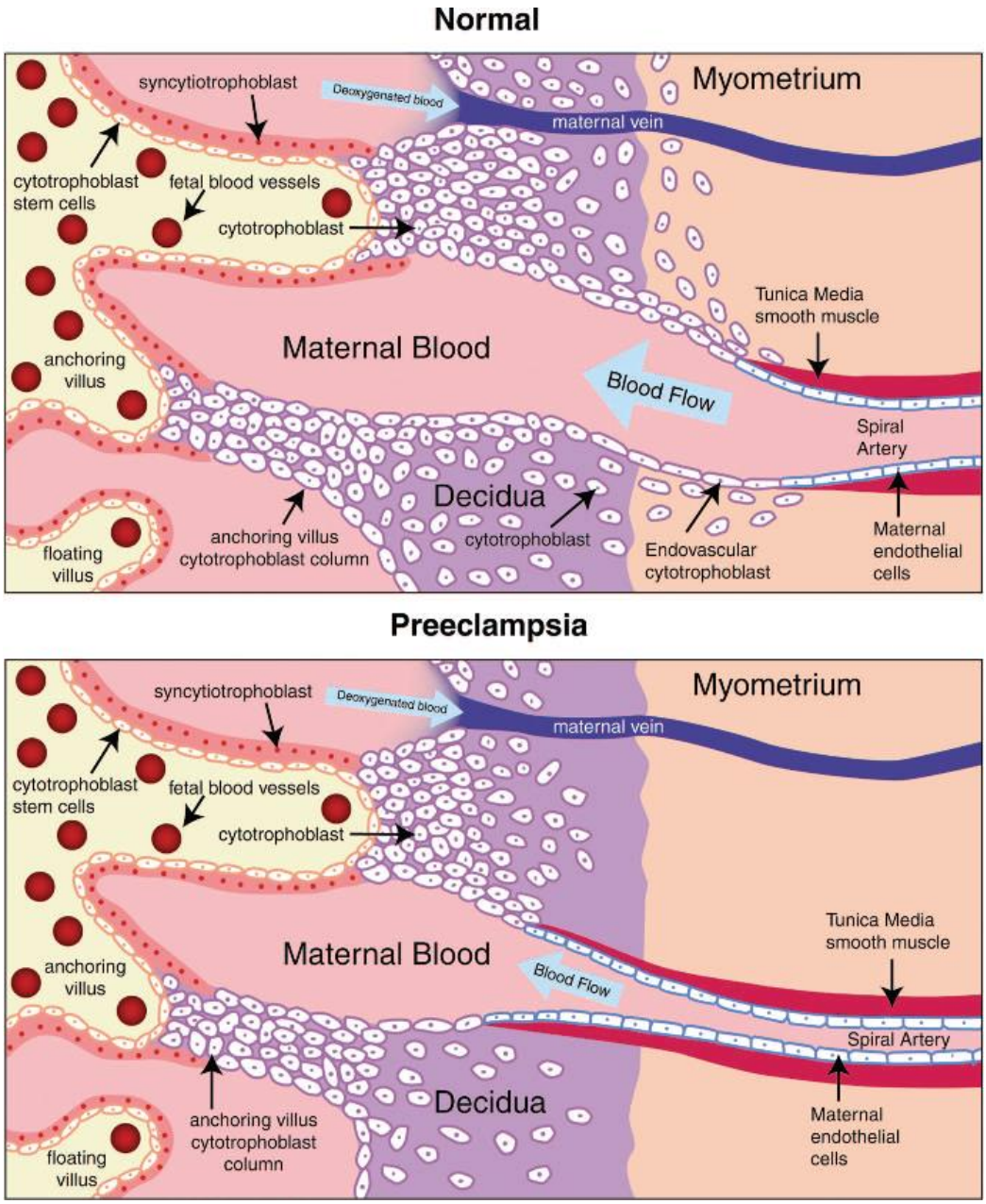


Figure 1-7: Abnormal placenta development in pre-eclampsia. In normal pregnancy (top), cytotrophoblast cells invade the maternal uterine wall (myometrium) and the decidual portion of the placenta spiral arteries (decidua) to induce remodelling of the spiral arteries and transform them from low flow, highly resistant vessels into high flow, low resistance vessels allowing for oxygen and nutrients supply necessary for the fetus development. In pre-eclampsia (bottom), impaired remodelling of the spiral artery is noticed. The cytotrophoblast invasion of the decidual portion of the spiral arteries and the myometrial segments is impaired and diminished due to impaired trophoblasts differentiation (from epithelial to an endothelial adhesion phenotype). As a result of this shallow invasion, the spiral arteries fail to be remodelled and remain as low flow, highly resistant vessels. This will restrict the blood supply to the fetus, and the developing placenta will suffer from hypoperfusion and ischemia. Figure 1-7 is taken from reference [130].

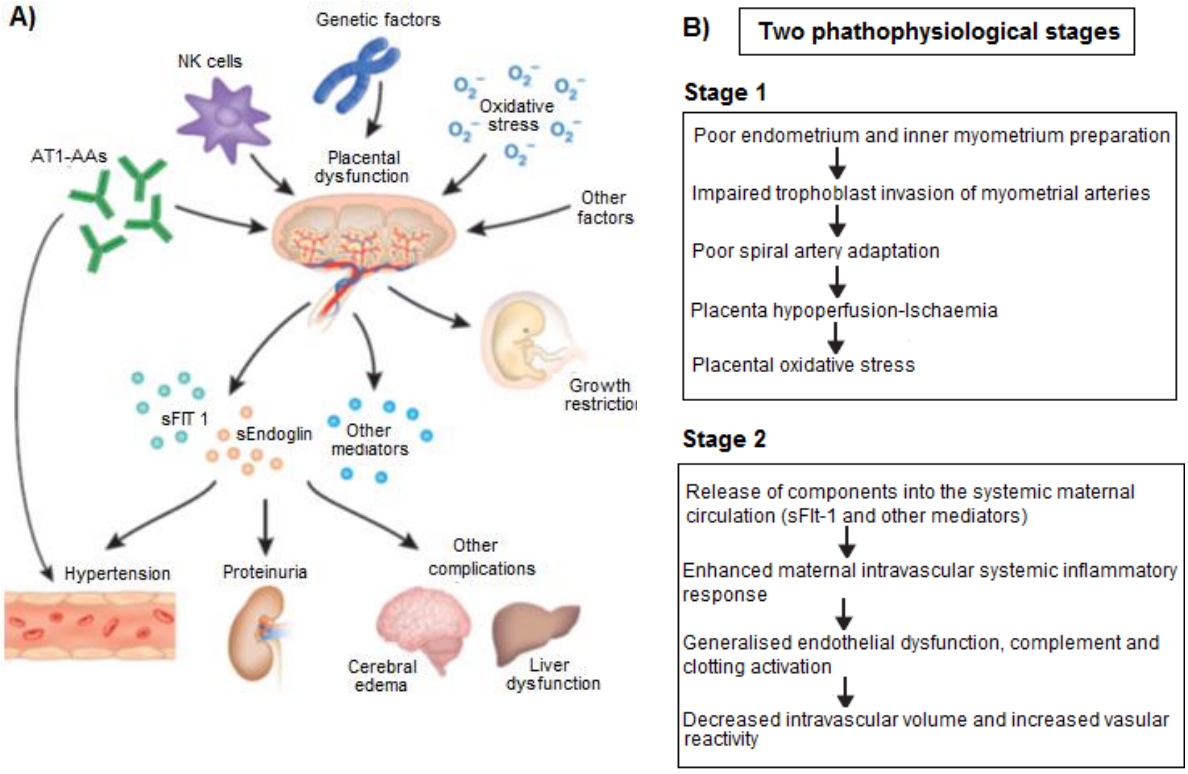


Figure 1-8: Development of pre-eclampsia, the two-stage model. A) Oxidative stress, genetic factors and other factors (AT1-AAs, NK cells) may cause placental dysfunction, leading to the release of antiangiogenic factors (such as sFlt1 and endoglin) and other inflammatory mediators to induce hypertension, proteinuria and other complication associated with pre-eclampsia. B) The two-stage model for describing the pathology of pre-eclampsia. The first stage is asymptomatic, characterised by abnormal placental development and the release of excessive amounts of placental materials into the maternal circulation. This leads to the second symptomatic stage characterised by endothelial dysfunction and activation of inflammatory responses. Figure A is from reference [137], and figure B is modified from reference [131]. AT1-AAs refer to angiotensin receptor autoantibodies. NK cells refer to natural killer cells and sFlt1 is soluble FMS-like tyrosine kinase.

1.3.3 Pre-eclampsia diagnosis (signs and symptoms)

The diagnostic criteria of pre-eclampsia are presented in Table 1-2. The basic features of pre-eclampsia are hypertension and proteinuria developing after the 20th week of gestation in women who were not previously known to be hypertensive. The clinical presentations of pre-eclampsia are varied [138]. Most women will remain asymptomatic until a late stage, which makes the identification of women likely to develop pre-eclampsia extremely difficult and contributes to the difficult and often delayed diagnosis [139]. Individual symptoms of pre-eclampsia such as headache, epigastric pain, visual disturbances, chest pain and dyspnoea do not adequately assess the severity of pre-eclampsia or predict adverse maternal outcomes and complications [140].

1.3.4 Complications of pre-eclampsia

Significant maternal morbidity is encountered in about 15% of women with severe pre-eclampsia [141]. Women with severe pre-eclampsia might present with symptoms such as headache, abdominal pain, visual disturbances (including blindness), epigastric pain, or/and nausea and vomiting [131]. They can also suffer multisystem complications [111; 131], as summarised in Table 1-3, including retinal detachment, cerebrovascular bleeding, and complications related to HELLP syndrome (haemolysis, elevated liver enzyme, low platelets syndrome) due to the excessive inflammation and endothelial damage associated with the disease. Furthermore, women might have disseminated intravascular coagulation or placenta-related complications, such as abruption. The more organ systems that are affected, the more maternal and perinatal complications arise [131]. Pre-eclampsia dose not only affect the mother. Besides the mother's multisystem morbidity, fetal complications have been associated with the disease, including growth restriction, prematurity-associated complications from preterm birth, and neonatal death [111; 131]. Additionally, children born to mothers with pre-eclampsia have a higher risk of bronchopulmonary dysplasia and cerebral palsy, as a result of preterm birth and being small for gestational age [142; 143]. Because of the heterogeneous complications and clinical presentations involved in severe pre-eclampsia, many other disorders should be considered before definitive diagnosis [131].

Cardiovascular risk after pre-eclampsia

Pre-eclampsia can have a subsequent effect on the mother's future health and quality of life. The association between pre-eclampsia and increased risk of developing cardiovascular diseases later in life was first made in 1964 [144]. Nowadays, there is overwhelming evidence of the higher risk of developing cardiovascular diseases for women who had hypertensive disorders during pregnancy [145; 146; 147; 148]. Two years after delivery, 30% of pre-eclamptic women had hypertension and 25% had metabolic syndrome [149; 150], which can prompt vascular endothelial dysfunction [151]. Recently, life style modifications after pre-eclampsia including smoking cessation, dietary habits and exercise were found to decrease the risk of cardiovascular disease by 4-13% [152]. A yearly follow-up of blood pressure, lipid profile, and blood glucose concentration has been recommended by the American Heart Association for women who had hypertension disorder during pregnancy [153]. However, intervention studies should be commenced before implementing such policies in clinical practice.

Table 1-3: Complications of pre-eclampsia [131].

System	Complication
Central nervous system	Eclampsia (occurrence of tonic-clonic seizures)
	Cerebral haemorrhage
	Cerebral oedema
	Cerebral systemic lupus erythematosus
	Metabolic disease
	Retinal oedema, retinal detachment
	Retinal arterial or venous thrombosis
	Cortical blindness
Cardiovascular system	Peripartum cardiomyopathy
	Myocardial infarction or ischaemia
Renal system	Renal tubular necrosis
	Lupus nephritis
	Acute and chronic glomerulonephritis
Respiratory system	Pulmonary oedema
	Pneumonia
Hepatic system	Haemolysis, elevated liver enzyme, low platelets (HELLP) syndrome
	Jaundice
	Hepatic rupture
	Acute pancreatitis
	Gastric ulcer
Haemostasis	Benign thrombocytopenia of pregnancy
	Septic or haemorrhagic shock
Vasculature	Phaeochromocytoma
	Hyperaldosteronism
Placenta	Placental abruption
	Placental infarction
Baby	Growth restriction
	Preterm delivery
	Death

1.3.5 Clinical management of pre-eclampsia

Despite decades of research into the condition, the only effective cure remains the delivery of the placenta. The decision to deliver is crucial and is mainly based on assessing the risk to the mother [131]. Novel therapies that target different aspects of pre-eclampsia pathogenesis are still in development [154]. Some drugs, such as dexamethasone, are in a clinical trial to assess their efficiency in decreasing post-partum maternal morbidity [155]. The current clinical intervention is mainly to control hypertension using antihypertensive drugs (*e.g.* nifedipine, intravenous hydralazine, labetalol) [156], and to manage pre-eclampsia organ complications (*e.g.* corticosteroids to improve laboratory parameters in HELLP) [157]. All pre-eclampsia complications should be managed in an inpatient setting.

1.3.6 Prevention of pre-eclampsia

Various interventions (pharmacological and non-pharmacological) have been assessed to evaluate their influence in reducing the risk of developing pre-eclampsia (Table 1-4). Aspirin has been extensively studied and is considered the drug of choice for the prevention of pre-eclampsia based on individual patient data meta-analysis that showed modest but significant benefits of aspirin in preventing pre-eclampsia [158]. Heparin and dalteparin, have also shown promising effects in women at high risk for pre-eclampsia, however, the studied populations were too small to draw definite conclusions [159; 160; 161]. Studies of calcium supplementation indicated that it is of most benefit when used in high-risk women with low dietary calcium intake [162; 163]. Dietary supplementation with vitamin C and vitamin E [164; 165], or magnesium [166] were less promising in reducing the risk of pre-eclampsia than calcium supplementation.

Generally, only aspirin and calcium supplementation in women with low dietary calcium intake are supported by robust evidence in reducing the risk of pre-eclampsia, all other interventions require further evaluation to prove the benefit of their recommendations for preventing pre-eclampsia.

Table 1-4: Interventions for the prevention of pre-eclampsia [131].

Intervention	Population
Pharmacological interventions	
Low-dose Aspirin	Women at risk of pre-eclampsia, gestational hypertension, or fetal growth restriction (week 8-16).
Low molecular weight heparin	Women at risk of placental dysfunction (ex. previous history of pre-eclampsia, placental abruption, or fetal growth restriction).
Non-pharmacological interventions	
Calcium	Women with low dietary calcium intake.
Vitamin C and E	Women at low, moderate, or high risk of pre-eclampsia.
Magnesium	Women at normal or high risk of pre-eclampsia.
L-arginine with antioxidants	Women at risk of pre-eclampsia.
Vitamin D and calcium	Women at any risk of pre-eclampsia.
Diet and lifestyle	Women at any risk of pre-eclampsia, women who are overweight or obese.

1.3.7 Prediction of pre-eclampsia

Predicting women that are at increased risk of developing pre-eclampsia is still challenging. Accurate early prediction of pre-eclampsia would facilitate intervention, closer monitoring, and preventive strategies before deterioration of the condition [131]. Clinicians rely mainly on the maternal risk factors, such as age, family history of pre-eclampsia and BMI to identify women at risk of developing the disease. Nevertheless, these risk factors can only predict 30% of women who develop pre-eclampsia [167], and they are not specific; millions of women worldwide have these risk factors but do not develop pre-eclampsia.

As a result, many screening tests (clinical and biomarkers) have been evaluated over the years for the prediction of pre-eclampsia. These have been comprehensively reviewed in a World Health Organisation (WHO) publication [168], and a summary of them is presented in Figure 1-9 and Table 1-5. The most promising clinical method and biomarkers according to the WHO publication are discussed below.

Utero-placental Doppler studies

Utero-placental Doppler ultrasound (uterine arteries and placental vessels) is a screening method used to evaluate impaired placental perfusion, associated with pre-eclampsia, either by direct visualisation (by detecting notching of the uterine artery), or by quantifying the waveform to determine vascular resistance (by measuring the blood flow velocity at peak systole and diastole) [169]. In a normal pregnancy, the reduction in the resistance of the spiral arteries as a result of remodelling will be associated with high diastolic velocity and loss of the diastolic “notch” at 20 to 24 weeks’ gestation, which can be detected by uterine artery Doppler studies [170]. In pre-eclampsia, however, the remodelling of the spiral arteries is impaired, and the increased pathological resistance to placental flow can be detected by Doppler studies of the maternal uterine vessels which in turn can help identifying women at risk of pre-eclampsia. A large comprehensive meta-analysis (reviewed 74 uterine artery Doppler studies including nearly 80,000 pregnant women) [171], revealed a poor predictive power for the majority of Doppler methods, but varied with the studied group and the severity of the disease. The authors concluded that Doppler studies were more accurate for the prediction of severe pre-eclampsia when performed in the second trimester while the positive predictive value in low-risk patients was not sufficient to recommend routine screening. As a result, attention has turned towards measuring various maternal biochemical markers that are

involved in the pathogenesis of pre-eclampsia (*e.g.* renal and endothelial dysfunction, and oxidative stress), to assess their role in predicting the disease.

Angiogenic proteins

Many researchers have focused their attention on factors related to angiogenesis; development of new blood vessel from the endothelium, which is a crucial process for normal placenta development [172]. Placental growth factor (PIGF) and vascular endothelial growth factor (VEGF) are two angiogenic growth factors produced by the placenta and have an important role in normal trophoblast differentiation and implantation [173]. The circulating levels of VEGF and PIGF have been shown to be lower in women who later developed pre-eclampsia compared to controls [174; 175; 176], and imbalance in the levels of the two factors has been suggested to be related to pre-eclampsia [130; 177].

Antiangiogenic proteins

Two of the most extensively studied antiangiogenic proteins that antagonise the effect of VEGF and PIGF are soluble FMS-like tyrosine kinase (sFLT-1) and soluble endoglin. sFLT-1, also known as soluble VEGF receptor 1, is produced by the placenta and binds to and neutralises the angiogenic actions of VEGF and PlGF [178]. Elevated maternal serum and placental levels of sFLT-1 have been detected in women with pre-eclampsia compared to controls [176; 179; 180]. Furthermore, the levels of sFLT-1 have been correlated with disease severity [181] and decreased markedly following delivery [182].
Consistent results were also observed with soluble endoglin [178]. Endoglin is a membrane glycoprotein that is highly expressed on the endothelial cell membranes and has a crucial role in angiogenesis [183]. Levine and colleagues [176] reported increased concentrations of circulating sFlt-1, and endoglin accompanied by decreased levels of circulating PlGF and VEGF detected five weeks before the onset of pre-eclampsia [176], which supports the importance of these key peptides in the prediction of pre-eclampsia.

Other markers with altered levels at or before the onset of pre-eclampsia are presented in Table 1-5. Figure 1-9 summarises the clinical tests that have been evaluated for the prediction of pre-eclampsia.

Despite the relatively long list of proposed screening tests and biomarkers for improving early and accurate prediction of pre-eclampsia, no single test has so far met the required specificity and sensitivity to allow its routine use as a predictive test in clinical practice

[131; 139]. However, combinations of different clinical tests have shown promising sensitivity and specificity for the early prediction of pre-eclampsia. One example is the improvement in the prediction of severe pre-eclampsia by combining serum placental protein 13 levels and uterine artery Doppler scanning [184]. Similarly, the ratios of antiangiogenic and angiogenic factors (ratio of sFlt-1/ placental growth factor) showed better discriminatory power than single analytes [185; 186].

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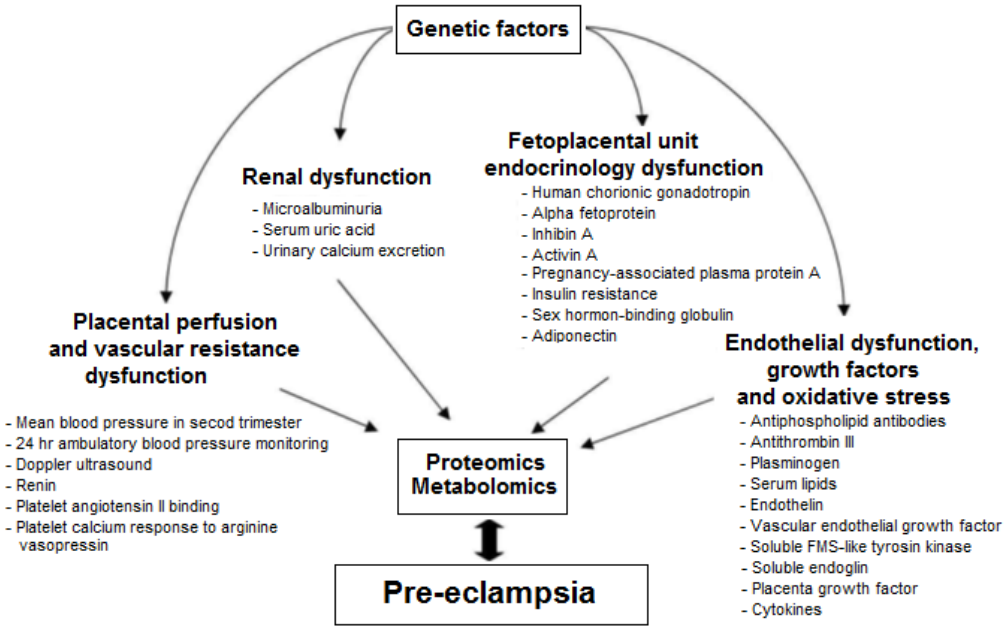


Figure 1-9: Biochemical markers of pre-eclampsia categorised into the four major groups involved in the pathogenesis of pre-eclampsia; placental hypoperfusion and impaired remodelling, oxidative stress and endothelial dysfunction, renal and endocrinology dysfunction. The production of these compounds depends mainly on genetic factors. However, the genome will not change during pregnancy related complications as preeclampsia. The dynamic metabolomics and proteomics studies can detect the changes in the levels of these molecules during pre-eclampsia and therefore will more accurately predict the risk of the disease than genomic studies. The figure is taken from the WHO publication, reference [168].

Table 1-5: Proteins with altered levels in pre-eclampsia.

Biomarker	Main function	Main finding in pre-eclampsia compared to control	Ref.
Placental Protein 13	Involved in placental implantation and maternal vascular remodelling	Low levels of PP-13 have been shown in the first trimester in women who later developed pre-eclampsia.	[187; 188]
Pregnancy-Associated Plasma Protein A	Cleavage of insulin-like growth factor binding proteins	Reduced first trimester serum levels of PAPP-A are associated with pre-eclampsia. Levels are also low in other complications of pregnancy.	[189]
Sex-hormone- binding globulin (SHBG)	Binds circulating oestrogens and testosterone. Production of SHBG is inhibited by insulin	- Reduced SHBG levels at first trimester in women who developed pre-eclampsia is an indication of insulin resistance associated with pre-eclampsia.	[190]
		- No significant difference in SHBG levels at weeks 10-14 between women who went on to develop pre-eclampsia compared to controls.	[191]
Adiponectin	- A protein hormone that modulates a number of metabolic processes, including glucose regulation and fatty acid oxidation. - Protein levels are inversely correlated with insulin resistance.	- Serum levels of adiponectin have been shown to correlate with soluble endoglin levels in women with pre-eclampsia.	[192]
		- Lowers adiponectin levels in the first trimester in women who subsequently developed pre-eclampsia were detected.	[193]
		- Serum adiponectin levels in the third trimester are higher in women with pre-eclampsia; physiological response to pre-eclampsia by improving insulin sensitivity.	[194]
Inhibin A and Activin A	Members of the transforming growth factor β family and are largely released by the foetoplacental unit during pregnancy.	- Approximately 10-fold increase in the levels of inhibin A and activin A have been noticed in women with severe pre-eclampsia.	[195]
		- Second trimester levels of activin A were elevated in women who developed pre-eclampsia later, inhibin A levels were not elevated.	[196]

1.3.8 The role of angiotensinogen in pre-eclampsia

Angiotensinogen (AGT), is another protein that has been linked to the increased blood pressure associated with pre-eclampsia [197]. AGT is a non-inhibitory member of the serpin family of protease inhibitors [198] and is expressed and secreted from the liver [199]. It is a moderately abundant plasma glycoprotein with a molecular weight of ~ 60 kDa (for the glycosylated protein) and 485 amino acids (Figure 1-10) according to the Swiss-Prot database (P01019).

MRKRAPQSEM APAGVSLRAT ILCLLAWAGL AAGDRVYIHP FHLVIHNEST **C**[18]EQLAKANAG
KPKDPTFIPA PIQAKTSPVD EKALQDQLVL VAAKLDTEDK LRAAMVGMLA NFLGFRIYGM
HSELWGVVHG ATVLSPTAVF GTLASLYLGA LDHTADRLQA ILGVPWKDKN **C**[138]TSRLDAHKV
LSALQAVQGL LVAQGRADSQ AQLLLSTVVG VFTAPGLHLK QPFVQGLALY TPVVLPRSLD
FTELDVAAEK IDRFMQAVTG WKTG**C**[232]SLMGA SVDSTLAFNT YVHFQGKMKG FSLLAEPQEF
WVDNSTSVSV PMLSGMGTFQ HWSDIQDNFS VTQVPFTESA **C**[308]LLLIQPHYA SDLDKVEGLT
FQQNSLNWMK KLSPRTIHLT MPQLVLQGSY DLQDLLAQAE LPAILHTELN LQKLSNDRIR
VGEVLNSIFF ELEADEREPT ESTQQLNKPE VLEVTLNRPF LFAVYDQSAT ALHFLGRVAN
PLSTA

Figure 1-10: Amino acids sequence of AGT protein obtained from Swiss-Prot database. The sequence starts from DRV…, the first 33 amino acids (smaller font) are for the signal peptide. The four cysteines are highlighted in bold red font.

AGT is an essential component of the renin-angiotensin-aldosterone system (RAAS); a hormone system that regulates blood pressure and fluid and electrolyte balance. The tail of AGT is cleaved by the action of renin, in response to lowered blood pressure, to yield the ten amino acids peptide angiotensin I which is further cleaved by angiotensin converting enzyme (ACE) to generate the physiologically active octapeptide angiotensin II, as demonstrated in Figure 1-11 [200]. Angiotensin II, in turn, causes vasoconstriction and a subsequent increase in blood pressure. Additionally, angiotensin II stimulates aldosterone release from the adrenal cortex resulting in sodium and water retention, which also contributes to the increase in blood pressure [201].

In pregnancy, it is already known that AGT is the rate-limiting component in the generation of angiotensin I, and hence angiotensin II [202]. Furthermore, there is increasing evidence indicating the expression of the RAS components in the placenta [203].

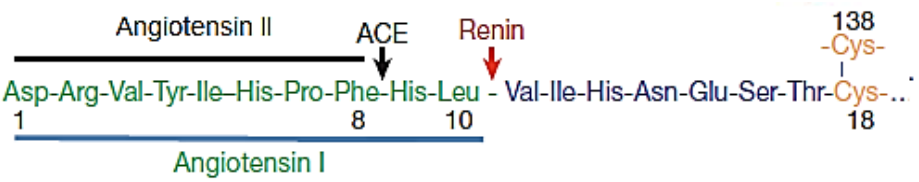


Figure 1-11: The renin-angiotensin system with the cleavage sites of renin and ACE within the tail of AGT. The tail of AGT is presented in blue with the terminal angiotensin I segment in green. The two key cysteine peptides connecting the amino tail to the body of AGT are shown in brown. AGT will be cleaved by renin to give angiotensin I which is further cleaved by angiotensin converting enzyme (ACE) releasing angiotensin II leading to increased blood pressure. Figure 1-12 is from reference [197].

The revealed crystal structure of AGT (Figure 1-12) shows that conformational rearrangement is needed to make the cleavage site in AGT accessible to renin and hence initiates the RAS [197]. This rearrangement takes place by displacement of the CD loop and the movement of the N-terminal peptide into the cleavage site of renin (Figure 1-12). Such rearrangement involves the disulphide bridge between cysteine (Cys) 18 and 138 which plays an important role in linking angiotensin I, in the tail of AGT, to the body of the molecule. The amino acids sequence of human AGT contains four cysteines (Figure 1-10) but the disulphide bridge between Cys18 and Cys138 is the one involved in renin binding rearrangement (Figure 1-11) and conserved in all species [204; 205]. Normally the ratio between the free thiol unbridged form (reduced form) to the sulphydryl-bridged form (oxidised form) of AGT is maintained in the circulation at 40:60 respectively. Renin will bind to the oxidised form of AGT with four-fold higher binding affinity leading to a four-fold increase in the release of angiotensin I and subsequently angiotensin II [197].

Based on the new information derived from the crystal structure of AGT, it was proposed that the oxidative stress associated with pre-eclampsia could enhance the conversion of AGT to its more active oxidised form resulting in an increase in the blood pressure related to pre-eclampsia. This was clearly noticed in the analysis of plasma samples from pre-eclamptic women, in which Western blot results revealed a decrease in the reduced form to 32 ± 6% while the matched normal pregnancies showed the consistent percentage of reduced form of AGT 40 ± 5% (Mean ± SD, P=0.004), [197]. Moreover, just recently Rahgozar *et al.* [206], observed a significant decrease in the relative proportion of the reduced AGT in the plasma of pre-eclamptic women as compared to healthy pregnant controls using ELISA.

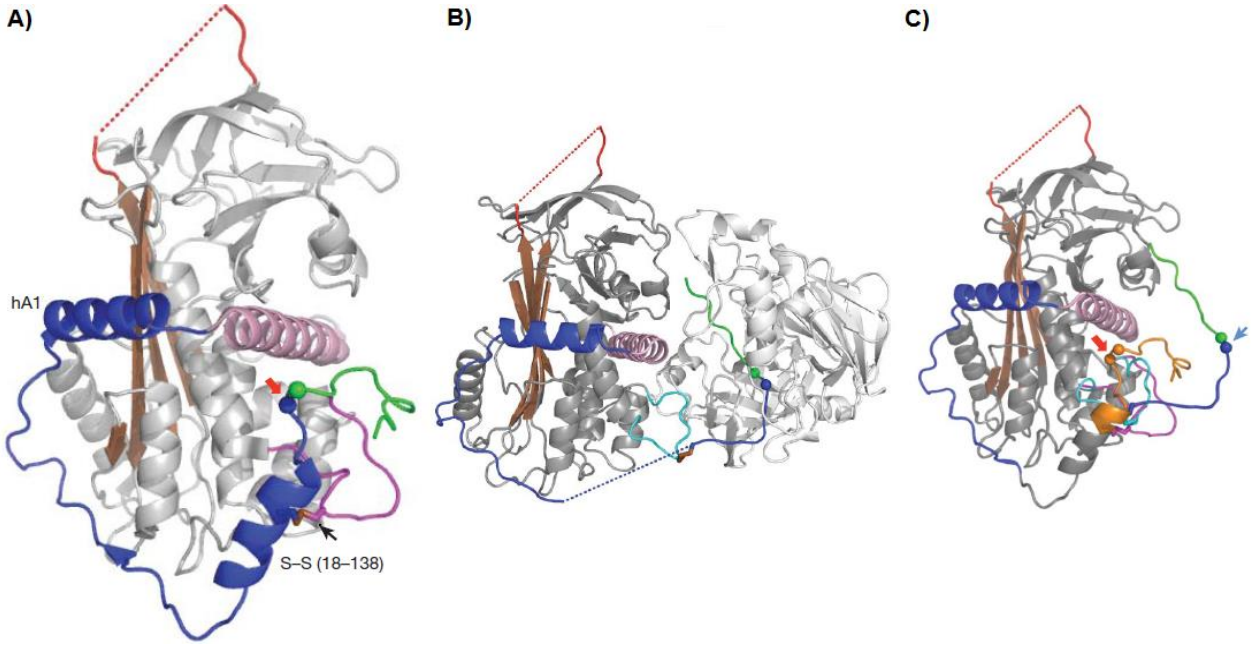


Figure 1-12: Angiotensinogen (AGT) and its complex with Renin. A) An image of AGT. The serpin template of AGT is shown in grey and helix A in purple with the A-sheet in brown, the unresolved reactive loop dotted in red, and the CD loop containing Cys 138 in magenta. The disulphide linkage between Cy138 and Cys18 is presented in brown. The terminal angiotensin I segment is in green with the renin-cleavage site shown as green and blue balls.
B) Angiotensin in the initiating complex with inactivated renin (left). C) Angiotensin superimposed on the unreacted form (brown, renin cleavage site red arrow) showing the displacement of the CD loop and the movement of the N-terminal peptide (visible to Cys 18, unresolved residues 19–30 blue dotted, renin-cleavage site blue arrow), into the active cleft of renin. Figure 1-6 is taken from reference [197]. The PDB code for the crystal structure of human AGT is 2WXW and the PDB code for the crystal structure of human AGT complexed with renin is 2X0B.

Only two studies have reported the involvement of the AGT redox switch in the pathology of pre-eclampsia [197; 206] and they both relied on antibody-based methods to quantify the reduced form of AGT in the plasma of pre-eclamptic women. Therefore, there is a need for a repeated clinical study that uses an entirely different quantification approach to evaluate and validate the previous findings.

1.4 General aims and objectives of the thesis

The majority of biomarkers investigated for the prediction of pre-eclampsia have been derived from the available knowledge of the biological processes involved in pre-eclampsia [139]. Pre-eclampsia is an asymptomatic multifactorial condition with unclear aetiology, so it is very unlikely that a single or a small group of biomarkers will possess the sufficient specificity and sensitivity to predict the disease. Moreover, no widely applicable, sensitive and specific screening test to help in the early diagnosis of pre-

eclampsia is currently available [131; 207]. Hence, there is a strong need for new strategies to increase the pool of pre-eclampsia biomarker candidates and to explore new pathways associated with the condition. Targeted protein analysis by MS has a great potential for the quantification of plasma proteins and post-translation modifications and therefore the discovery of disease biomarkers. The application of targeted LC-MS/MS method can increase the pool of potential pre-eclampsia protein biomarkers, and provide new insights into the underlying mechanisms associated with the pathophysiology of the disease.

This PhD research is best divided into two parts; the first part of the research aimed to develop a targeted LC-MS/MS method for the quantification of angiotensinogen (AGT) redox switch that has been linked recently to the pathogenesis of pre-eclampsia. The general objectives required to fulfil this aim were:

- Develop and validate a targeted LC-MS/MS method to allow the reproducible detection of the two AGT cysteine peptides, Cys18 and Cys138, involved in the redox switch of AGT from human plasma.
- Detect the two distinct forms of AGT; reduced and oxidised, in the human plasma using differential alkylation strategy coupled with targeted LC-MS/MS.
- Apply the method to plasma samples of pre-eclamptic women to quantify the oxidised and the reduced form of the protein.

The second part of the research aimed to develop a simple, affordable and cost-effective plasma glycoprotein profiling method that can be used to detect protein fold changes between different disease conditions. The general objectives required to fulfil this aim were:

- Develop a reproducible label-free targeted LC-MS/MS workflow for clinically relevant glycoproteins.
- Validate the method to confirm its biological applicability.
- Apply the method to identify potential protein biomarkers in;
    1) Early-onset pre-eclampsia.
    2) Late-onset pre-eclampsia.
    3) Polycystic ovary syndrome (PCOS).

Chapter Two

Analysis of Reduced and Oxidised Forms of Angiotensinogen in Human Plasma by LC-MS/MS

2 Analysis of Reduced and Oxidised Forms of Angiotensinogen in Human Plasma by LC-MS/MS

Summary

Angiotensinogen (AGT) is a critical protein in the renin-angiotensin system and has an important role in the pathogenesis of pre-eclampsia. The disulphide linkage between cysteine (Cys) 18 and 138 has a key role in the redox switch of AGT that modulates angiotensin release, and hence blood pressure, with an increased percentage of the oxidised form of AGT present in the plasma of pre-eclamptic women. There is currently no targeted, quantitative LC-MS/MS approach to detect and quantify the oxidised and reduced forms of AGT in human plasma. The aim of this chapter was to develop a targeted LC-MS/MS method for the reproducible detection of the key Cys peptides, 18 and 138, within the complex human plasma digest. These peptides will be used as signature peptides for the quantification of the oxidation level of AGT in the plasma of pre-eclamptic women in the forthcoming chapter.

Intensive work was undertaken to develop the methodology. Initially, different digestion protocols were evaluated using human recombinant AGT to allow maximal protein recovery and ensure reproducible alkylation and detection of the two Cys peptides. In-solution digestion with chymotrypsin provided a better sequence coverage (61.5%), and more reproducible detection of the two Cys peptides than the corresponding tryptic digest. Modification of the free Cys thiols with iodoacetamide (IAM) allowed the detection of the two Cys peptides while only Cys18 was reproducibly detected with NEM alkylation. Efficient deglycosylation of the sugar moiety linked to the asparagine residue in both Cys18 and Cys138 peptides sequences was achieved with PNGase F.

For the detection of the Cys peptides in human plasma, a new strategy for the selective extraction of AGT from human plasma was developed by coupling lectin-affinity chromatography with reversed phase-solid phase extraction fractionation (RP-SPR). A targeted LC-MS/MS method working under multiple reaction monitoring mode (MRM) was developed to detect the two target Cys peptides from plasma chymotryptic digest.

A reliable level for the overall recovery of AGT from the plasma was achieved (43% ± 2.0%). This enabled the reproducible detection of the two AGT Cys peptides in human plasma with acceptable analytical variability (CV ≤ 15%). Confirmation of the identity of the peptides was supported by LC-MS/MS data and the obtained spectra were comparable with their corresponding standards.

The reliable detection of the two key Cys peptides, involved in the redox switch of AGT, in the plasma provided a good level of confidence to move towards the detection and the quantification of the reduced and the oxidised forms of AGT in human plasma samples collected from pre-eclamptic patients.

2.1 Introduction

Mass spectrometry (MS) is recognised as a well-established analytical technique for protein identification, quantification and post-translational modification (PTM) characterisation. Disulphide linkage is one of the main protein modifications; cysteine thiol plays a central role in protein structure and redox signalling [208], and this importance is highlighted by its involvement in many pathological conditions involving oxidative stress [209], including neurodegenerative diseases [210] and pre-eclampsia [197]. The recently resolved crystal structure of angiotensinogen (AGT) revealed a key role for the disulphide linkage between Cys 18 and 138 in the redox switch of AGT that modulates angiotensin release, and hence blood pressure. An increased percentage of the oxidised form of AGT in the plasma of pre-eclamptic women has been reported [197] giving it an important role in the pathogenesis of pre-eclampsia, as discussed in Chapter One, Section 1.3.8. In this chapter a targeted LC-MS/MS method was developed to detect the two signature AGT Cys peptides in human plasma.

2.1.1 Preparation of protein samples for MS analysis in a bottom-up workflow

MS has been widely applied for the detection and quantification of different PTM such as glycosylation [41; 211], and thiol modifications [212]. However, there has been an increased awareness of the importance of appropriate sample treatment and preparation to provide reliable and high quality MS data. Therefore, great care should be taken in the sample handling procedure and each preparation step should be optimised, at both the protein and peptide level. Below, detailed consideration is given to Cys alkylation and protein digestion as they are crucial steps during sample preparation, particularly, in redox protein analysis, and have a high impact on the detection of the protein Cys peptides by MS.

2.1.1.1 Approaches for the alkylation of the free Cys thiol

With the increasing evidence of the importance of the Cys thiol, methods have been developed to detect, identify and quantify these modifications [208; 209]. The reactivity of Cys is mainly dependent on the ionisation of thiol to thiolate anion. Thiol groups in proteins have an average pK_a of *c*. 8.5, however, the pKa values can range from 2.5-12 [213]. This makes Cys considered as the most reactive nucleophile in the protein. Hence, free thiol alkylation is crucial to form a stable modified Cys residue that can be easily identified by MS [214]. Different strategies and probes to chemically modify free protein thiols are reported as presented in Figure 2-1. The used probes should have high reactivity to ensure reaction completion, and should not negatively interfere with the ionisation and fragmentation efficiency of the peptide [215]. Iodoacetamide (IAM) and N-ethylmaleimide (NEM) are two commonly used alkylating agents [216] with radio, fluorophore labelled and biotin-conjugated forms being commercially available [209]. Protein thiol modification with biotinylated and fluorophore tagged IAM or NEM have been measured and assessed by streptavidin blotting and florescent based methods respectively [217; 218]. Although the biotin thiol tagging approach has its own advantages such as the high affinity of biotin to streptavidin and the ability to purify biotinylated proteins, false positive results can be encountered from proteins having biotin covalently attached as an enzymatic cofactor [209]. Furthermore, low light conditions are required with fluorophore tagged alkylation strategy, and unless an antibody exists to the fluorophore itself the method cannot be applied [209].

Another common approach is to alkylate Cys peptides by an isotope labelling strategy. One example is isotope affinity coded tags (ICAT) which are widely used in quantitative proteomics [14]. The ICAT reagent consists of a reactive thiol group, usually iodoacetamide, a linker bearing either normal or deuterated hydrogen atoms (light and heavy ICAT respectively) and a biotin tag for affinity separation of the ICAT reacted peptides (Figure 2-1). This method assists in simplifying the LC-MS analysis by the enrichment and the isolation of peptides containing Cys. Moreover, two different conditions can be differentially labelled and compared by the use of the heavy and light versions of the ICAT reagent [14]. However, the first generation of ICAT suffered from non-specific binding, incomplete elution of Cys peptides, strong avidin peptide background, and a higher charge state for many peptides with complicated MS/MS spectra [219]. Improved versions of ICAT reagents were introduced by using Cys reactive beads with either photocleavable [220] or acid labile linkers [184]. Solid phase

isotope tagging provided a simpler and more efficient and sensitive approach to ICAT [220] in which Cys peptides could be eluted in high yield under mild conditions and simpler MS/MS spectra were obtained with these improved reagents [184].

Other thiol modifying reagents include iodoacetic acid, acrylamide; and 2 and 4-vinylpyridine. The latter were found to be effective and specific alkylating agents, with almost full thiol alkylation even in complex protein mixtures [221]. Different thiol reactive versions of dimedone carbon nucleophiles, 2-bromodimedone, 5-bromodimethyl barbituric acid and 2-bromo-1,3-cyclopentanedione were reported, and the corresponding carbon nucleophiles were described as effective probes for the MS identification of proteins sulphenic acid modification in a complex proteome from cellular extracts [215].

Although many chemicals can be used to alkylate free Cys, the decision of which compound or approach to utilise depends upon many factors, such as the aim of the methodology, the detection method applied and the Cys thiol alkylation chemistry [209].

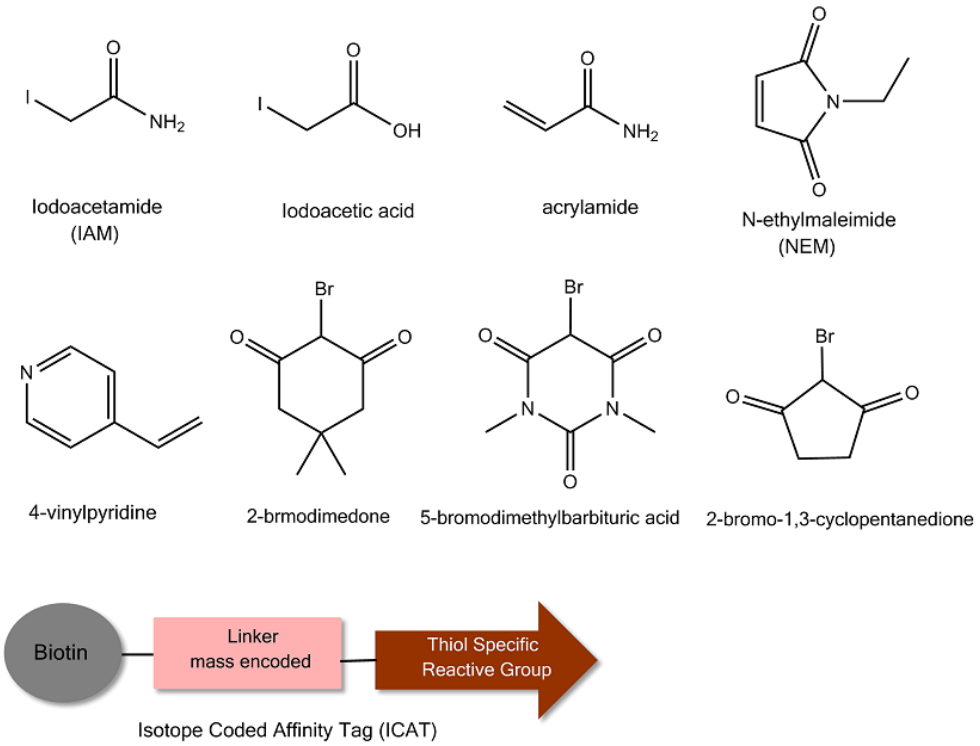


Figure 2-1: Different alkylating agents used to modify cysteine thiol in redox protein analysis. Iodoacetamide, NEM and ICAT (last reagent) are the most commonly used cysteine alkylating reagent.

2.1.1.2 Protein digestion

Digestion is a crucial step that can dramatically affect all subsequent stages in MS analysis and is often a major source of method variability [222]. Efficient digestion is of high importance, particularly in targeted proteomics, as signature peptides should be reproducibly detected for a successful MS analysis [87]. Although efficient digestion is crucial in relative protein quantification, it is the reproducibility of the digestion that is paramount, and any variation can significantly affect the MS data quality [222].

Two digestion protocols may be followed in the bottom-up approach; in-solution and in-gel digestion. Although the gel based strategy is a powerful tool to separate proteins in complex samples, generally, in-solution digestion is simpler in terms of sample handling, has higher throughput and is often more efficient [43]. Yet, both methods follow a common workflow for protein digestion (Figure 2-2).

Denaturation, reduction, alkylation and sample clean up

For efficient digestion, proteins are usually denatured, followed by breaking the disulphide bond and the alkylation of the free thiol. Upon the removal of the excess reagents, a proteolytic enzyme is added to release the peptides for LC-MS analysis. Protein denaturation will unfold the protein and disrupt its tertiary structure. Chaotropic agents such as urea are mainly used at this step. Heat and solvents can also be used. Proc *et al*. [223] examined 14 different combinations of denaturing conditions on the digestion efficiency of 45 moderately-high abundant plasma proteins; heat, chaotropic agents (urea, guanidine HCL), surfactants (SDS) and solvents (methanol, trifluoroethanol, acetonitrile). It was concluded that optimum digestion conditions are protein dependent, and no one digestion protocol is absolutely efficient for all proteins in one sample. However, it is the reproducibility of the digestion protocol that affects the quality of the results and therefore should be optimised [223].

After denaturation, disulphide bonds are reduced by 1,4-dithiothreitol (DTT) or tris (2-carboxyethyl)phosphine (TCEP) [216] and the free thiol are irreversibly modified with any of the alkylating agents mentioned previously. Generally in the bottom-up approach, reduction and alkylation of the disulphide bond are necessary to unfold the protein, and enhance the accessibility of the proteolytic enzyme and hence ensure maximal coverage of the protein [224]. Cys alkylation using three different alkylating agents: 4-vinylpyridine, acrylamide and IAM improved bovine serum albumin sequence coverage by approximately 30% when compared to reduced protein without alkylation [224].

Most of the reagents used in these steps are incompatible with MS, adding background noise to the spectra; therefore sample clean-up and desalting is essential to remove any excess. This can be undertaken prior to digestion using membrane filters or gel filtration, or afterwards either online by a trap column connected to the analytical column, or offline using spin column or commercially available tips with reversed phase chromatography [43].

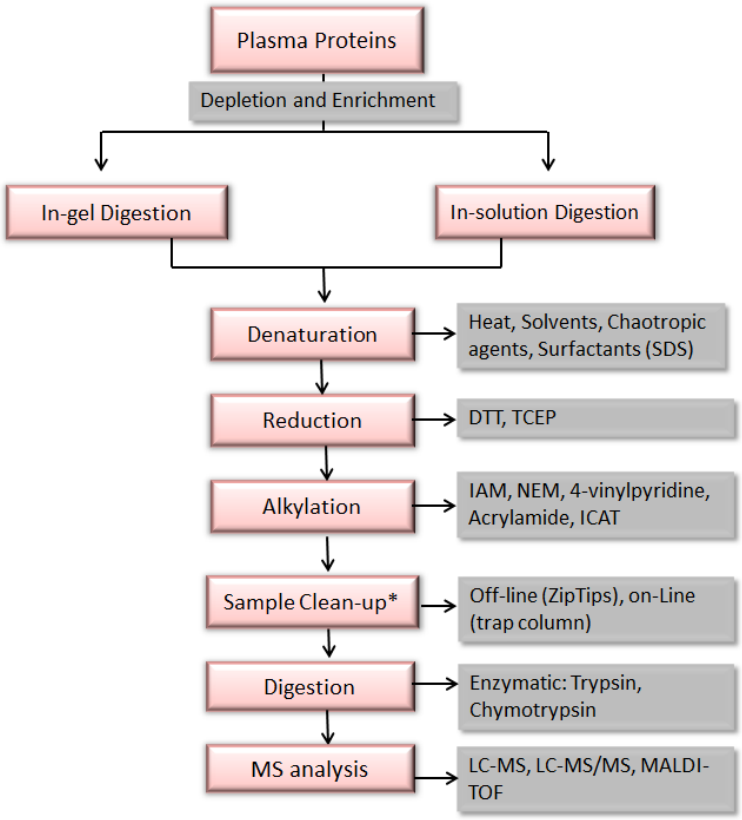


Figure 2-2: General steps in the bottom-up in-solution and in-gel digestion workflow. Both approaches include steps for the protein extraction from the biological sample followed by proteins denaturation, reduction, alkylation and digestion either in-gel or in-solution. The generated peptides are analysed using different MS approaches.

Enzymatic digestion

Enzymatic digestion is the classical approach for protein digestion. A wide range of proteolytic enzymes with different specificity, efficiency and optimum digestion conditions is available; however, trypsin is the gold standard in proteomics. It is highly specific, cost-effective and an autolysis resistant form is available [225]. Tryptic peptides usually produce high quality MS/MS spectra which increase the confidence in the peptide identification from the protein search database, and as such the inferred protein identity [226]. Although trypsin has many advantages, in cases involving the lack or

overabundance of trypsin cleavage sites in the protein sequence, or pH incompatibility, other proteolytic enzymes should be considered. Pepsin and chymotrypsin have lower specificity than trypsin; they may generate a large number of peptides that can complicate the MS analysis, nevertheless reliable digestion with both enzymes has been reported [227]. Chymotrypsin prefers neutral to slightly basic digestion conditions while pepsin is active under acidic conditions which makes it suitable for hydrogen/deuterium experiments [227]. Endoproteinases such as Arg-C, Asp-N, and Lys-C have high specificity as they are selective for a single amino acid residue, and are often used as an alternative to trypsin. They usually generate large peptides suitable for middle-down proteomics. Typically the choice of the proteolytic enzyme will depend mainly on the digestion conditions (pH, temperature) and the protein sequence [226].

Chemical digestion

Non-enzymatic digestion using diluted solutions of different chemicals may be selected as an alternative method. Examples include hydrochloric acid [228], acetic acid [229], formic acid [230], cyanogen bromide [231] and hydroxylamine [232].

As endoproteinases, these reagents are specific for a single residue and, therefore, suitable for use in middle-down proteomics [233]. Electrochemical oxidation can achieve protein proteolysis in minutes [234] with specificity to the low abundance amino acids tyrosine and tryptophan. Regardless, the size of the generated peptides should be compatible with most LC-MS instruments.

Multiple digestion

Multiple digestion strategies are achieved mainly by combining trypsin with other high or less specific enzymes to improve protein coverage [235; 236]. It is particularly useful in whole proteome studies where high sequence coverage will increase the confidence in protein identification, mainly from complex biological fluids [235]. Moreover, a combination of chemical and enzymatic digestion has been reported as an effective approach for the analysis of membrane proteins [237].

In conclusion, sample preparation methodology is increasingly recognised as a major source of high variability in protein quantification [94]. Therefore, great attention to details during method development should be undertaken, particularly, when developing a method to quantify the two redox forms of a protein in the plasma. This includes choosing an efficient alkylating agent, optimising a digestion protocol and developing a

protein extraction method that enable a reproducible detection of the signature Cys peptides used for the quantification.

AGT is a plasma protein that has been proposed to be linked to the increased blood pressure in pre-eclampsia through its conversion to the more active oxidised form. As a result, there is a need to develop a sensitive method to quantify the oxidation level of AGT in the plasma of pre-eclamptic women based on the quantification of the two signature Cys18 and Cys138 peptides involved in its redox switch. However, a method for the reproducible detection of these key peptides within the complex plasma digest should be established first before any quantification work takes place.

2.1.2 Aim of the chapter

The aim of the work presented in this chapter was to develop and optimise a targeted LC-MS/MS method to allow the reproducible detection of the two key AGT cysteine peptides, Cys18 and Cys138 following the digest of human plasma samples. These peptides were used as signature peptides for the relative quantification of the two protein forms in the plasma of pre-eclamptic women in the forthcoming chapter. In order to achieve this, the following objectives were addressed:

1) Development of a digestion protocol for reproducible detection and full alkylation of the key Cys peptides whilst maintaining high protein recovery.
2) Development and optimisation of a depletion method for the selective extraction of AGT from human plasma.
3) Development of a targeted LC-MS/MS method to reproducibly detect the two Cys peptides from the complex plasma digests.
4) Confirmation of the detected peptides identity by peptide fragmentation using LC-MS/MS.
5) Validation of the developed methodology using the coefficient of variation values.

2.2 Analytical strategy

As discussed previously, human plasma is a hugely complex biofluid with a wide dynamic range of protein concentrations [24]. Therefore, the method development strategy began by optimising a protocol for the detection of the key Cys peptides, using the simplest pure form of the protein; the unglycosylated human recombinant AGT. At this level, different strategies for protein reduction, alkylation, digestion and sample clean up were evaluated to achieve complete AGT digestion, and maximum protein recovery

with reliable detection of the modified Cys18 and Cys138 peptides. The protein bottom-up approach was followed, and both in-gel and in-solution digestions were examined, using trypsin and chymotrypsin as proteolytic enzymes and NEM and IAM as alkylating agents. The identity of the modified Cys peptides was confirmed by two MS techniques; a high resolution accurate MS with an orbitrap mass analyser, and LC-MS/MS, using an ion trap, to fragment the peptides and yield the distinctive MS/MS spectrum used for identification (Figure 2-4).

The suitability of the optimised digestion protocol was then tested on pure glycosylated human recombinant AGT; the actual form of the protein in the plasma, before applying the methodology on human plasma samples. With human plasma, a method for AGT enrichment was optimised, and a targeted LC-MS/MS method was developed to detect the key Cys peptides from the plasma digest. The identity of the detected peptides was confirmed and the variability of the method was validated (Figure 2-5).

2.3 Materials and Methods

2.3.1 Materials

Enzymes for AGT proteolysis, chymotrypsin sequencing grade and trypsin gold MS grade were purchased from Promega (Southampton, UK), while PNGase, glycerol free kit, (500,000 units/mL) for protein deglycosylation was acquired from New England Biolabs (Hertz, UK). Amicon ultra 0.5 mL centrifugal filter devices 10 kDa, calcium chloride, Concanavalin A-Sepharose 4B (ConA), Coomassie brilliant blue G-250, dithiothreitol (DTT), iodoacetamide (IAM), magnesium chloride, manganese chloride, methyl α-D mannopyranoside, MS grade trifluoroacetic acid (TFA) and formic acid, N-ethylmaleimide (NEM), sodium chloride and TRIS base were obtained from Sigma-Aldrich (Gillingham, UK). Acetonitrile optima LC-MS grade, ammonium hydrogen carbonate, NuPAGE LDS sample buffer, NuPAGE 4-12% Bis TRIS midi-gels, NuPAGE MES SDS running buffer were purchased from Fisher Scientific (Loughborough, UK). Urea ACS, 99.0-100.5% was bought from VWR International (Leicestershire, UK). Human serpin A8/angiotensinogen antibody was from R&D systems (Abingdon, UK). Custom peptide standards (Cys 18 peptide (HLVIHNESTC^{18}EQL), Cys 138 peptide (KDKNC^{138}TSRL) and AGT marker peptide (SVTQVPF)) were synthesised by Pierce Protein Biological Products, Thermo Fisher Scientific, UK.

Human recombinant AGT, 2.5 mg/mL in TRIS buffer (pH 7.4) and glycosylated AGT 3.4 mg/mL expressed in HEK293 cells (all cysteines are in the oxidised form), were kindly supplied by MRC group from Cambridge University (Prof. R. Carrell and Dr R. Read, Cambridge Institute for Medical Research).

2.3.2 Detection of Cys peptides from pure AGT digest

2.3.2.1 In-solution digestion of un-glycosylated human recombinant AGT

Twenty µL of 2.5 mg/mL AGT was denatured with 2 M urea and reduced with 10 µL 50 mM DTT to break down the disulphide linkage between cysteines. The free Cys were then alkylated with 10 µL of 200 mM IAM or NEM. All reactions were carried out at 37°C for 30 min in the dark in the upper well of 10 kDa Amicon ultra 0.5 centrifugal filter devices. After each reaction, the sample was washed twice with 400 µL of 50 mM ammonium hydrogen carbonate at 14000x *g* for 7 min. Prior to digestion, the resulting 40 µL protein solution was diluted to 100 µL with the same buffer, and 5 µL of 200 ng/µL chymotrypsin or trypsin (1:50 enzyme: protein *w/w* ratio) was added and digestion was carried out for 24 h in 37°C incubator. The enzymatic reaction was quenched with 1% formic acid and preceding LC-MS analysis the samples were centrifuged for 5 min at 11000x *g* and the 90% upper layer was transferred to HPLC analysis vials.

2.3.2.2 In-gel digestion of un-glycosylated human recombinant AGT

In-gel digestion was conducted according to the Shevchenko *et al.*, 1996 protocol [238]. Briefly, 5 µL AGT was diluted to 10 µL with 0.5 M DTT and sample buffer, heated for 5 min at 98°C then centrifuged for 1 min at 3000x *g*. Samples were loaded onto the sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE). The gel was run for 1 h applying 200 V. Upon completion, fixation was carried out for 30 min on a rocking platform (50% water: 40% methanol: 10% glacial acetic acid). The gel was washed twice and stained with Coomassie blue G-250 [239] for 3 h at RT with agitation, before washing for 2 h in ultrapure water. The stained protein bands were excised and destained overnight in washing buffer. The protein was reduced using 10 mM DTT and alkylated, in the dark, with 20 mM iodoacetamide. Each reaction was completed with agitation for 30 min at 37 °C. Bands were washed twice, dehydrated with 100% acetonitrile and allowed to dry. Trypsin or chymotrypsin (200 ng/µL) was added to the dehydrated gel piece to achieve 1:10; enzyme: protein weight ratio. Ammonium hydrogen carbonate buffer (50 mM) was added to the swollen gel before digestion was carried out for 24 hr at 37°C. Peptides

were extracted and excess solvent was evaporated. Samples were then stored at -20°C, and prior to LC-MS analysis, the extracts were diluted to a 50 µL volume with 0.1% formic acid in water.

2.3.2.3 Analysis of AGT digests by high resolution accurate MS (LC/MS)

An Orbitrap mass spectrometer coupled to HPLC (Thermo Exactive) from Thermo Scientific (Thermo Fisher Scientific, San Jose, CA, USA) operating with ESI running in the positive ion mode was used for the AGT digests analysis. Peptides were separated first by reversed-phase LC using C18 300 Å, 100 x 2.1 mm internal diameter, 3 µm particle size column from ACE (Scotland, UK), and eluted with a mobile phase of water and acetonitrile both with 0.1% formic acid (*v/v*). Organic phase linear gradient, starting with 20%, was applied for 6 min at 300 µL/min flow rate.

MS source parameters were optimised and tuned with a T piece in which 10 µL infusion of leucine enkephalin was mixed with 30% organic phase eluting from the column at 300 µL/min flow rate. Spectra were acquired in full MS scan in the range *m/z* 200–2000, with 25000 enhanced mass resolution and mass error less than 3 ppm when applying the optimised MS parameters (Table 2-1).

Peptides sequence and their corresponding *m/z*, for AGT (P01019, Swiss-Prot database) chymotryptic and tryptic digest, were predicted by *in silico* digestion using PeptideMass (ExPASy, PeptideMass, http://web.expasy.org/peptide_mass/) [240]. The predicted *m/z* for modified and unmodified Cys18 and Cys138 peptides were used to obtain the extracted ion chromatogram (XIC) of the corresponding masses from the total ion chromatogram (TIC) of AGT digest. The *m/z* for Cys peptides were predicted for single, double and triple charged peptides (Table 2-2).

Table 2-1: MS parameters applied in the accurate mass LC-MS method

MS Parameters	Value
Sheath gas flow rate	35
Aux gas flow rate	10
Sweep gas flow rate	3
Spray voltage (kV)	3
Capillary temperature (°C)	350
Capillary voltage (V)	29
Tune lense voltage (V)	105
Skimmer voltage (V)	18
Heater (°C)	300

Table 2-2: Predicted sequence and *m/z* of modified and unmodified Cys 18 and Cys 138 peptides from AGT chymotryptic digest

Peptide Sequence	Single Charge $[M+H]^+$ *m/z*			Double Charge $[M+2H]^{2+}$ *m/z*			Triple Charge $[M+3H]^{3+}$ *m/z*		
	Cys Unmodified	Cys Modified		Cys Unmodified	Cys Modified		Cys Unmodified	Cys Modified	
		NEM	IAM		NEM	IAM		**NEM**	**IAM**
HLVIHNESTC^{18}EQL	1522.7318	1647.7795	1579.7533	761.8695	**824.3936**	**790.3803**	508.2488	**549.9317**	527.2559
KDKNC^{138}TSRL	1064.5517	1189.5994	1121.5731	532.7795	**595.3036**	**561.2902**	355.5221	**397.205**	374.5292

* *m/z* value for unmodified and IAM modified peptides were predicted by *in silico* digestion with PeptideMass.
** Modification with NEM was calculated based on the monoisotopic mass of 125.0477 amu for NEM and 1.0078 amu for H^+.
*** Bold values refer to cysteine peptides detected by MS.

2.3.2.4 Fragmentation of modified Cys18 and Cys138 peptides by LC-MS/MS

An ion trap mass spectrometer coupled to UPLC (Thermo Fisher LTQ Velos) from Thermo Scientific (Thermo Fisher Scientific, San Jose, CA, USA), with ESI running in the positive ion mode was used to confirm the identity of the AGT peptides detected by accurate MS. Peptides were first separated by LC applying the same chromatographic conditions mentioned previously (Section 2.3.2.3). The MS/MS spectra for peptides of interest were acquired with one unit mass resolution and parent mass width ± 0.5 mass with MS2 set to *m/z* 200−1650.

Peptides are fragmented, generally, by CID using an inert gas. The weaker amide bonds of the backbone are the easiest to break to give the structurally informative sequence ions known as *y* and *b* ions, which contain the *C*- and *N*- terminus respectively [241; 242]. Potential sequence ions resulting from breaking down the C-C and/or C-N bonds may be produced; *a*, *x*, *c* and *z* ions, however, this usually requires higher energy (Figure 2-3). Less informative ions can also be produced by losing molecules like water and ammonia [241].

The freely-available fragment ion calculator website available from Proteomics Toolkit (http://db.systemsbiology.net:8080/proteomicsToolkit/FragIonServlet.html) was used to predict the *y*, *b* and *a* ions for the alkylated Cys peptides. The predicted values were matched with the obtained MS/MS spectrum to confirm peptides identity.

All data from LTQ Velos and Exactive were processed with Xcalibur 2.2 software (Thermo Fisher Scientific, San Jose, CA, USA).

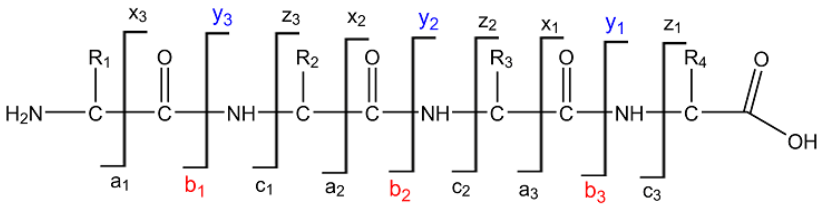


Figure 2-3: The nomenclature applied for ions generated from peptide fragmentation. The commonly produced *b* and *y* ions from breaking the peptide amide linkage are presented in red and blue respectively (based on reference [241]).

2.3.2.5 Analysis of AGT digests by LC-MS/MS and Mascot Search Engine

A full MS/MS scan for AGT digest was performed using an LTQ-Velos ion-trap MS (Section 2.3.2.4) and MS/MS spectra were acquired by data-dependent acquisition (DDA) in full scan mode (*m/z* 350−1650), with the three most intensive precursor ions dynamically selected and subjected to collision-induced dissociation (CID) performed at a normalised value of 35% (*m/z* 200−1650).

MSCONVERT provided by the ProteoWizard project (http://proteowizard.sourceforge.net/tools.shtml) was used to convert raw data files to Mascot generic format. MS/MS ion search option in Mascot search engine (version 2.3.01; Matrix Science Inc., London, UK) was selected to search against all *Homo sapiens* entries in the Swiss-Prot database. The peptide sequence coverage and protein score values were used to compare the two digestion approaches, in-solution and in-gel, performed with trypsin and chymotrypsin.

The search parameters were as follows: trypsin and chymotrypsin enzyme, one missed cleavage, peptide mass tolerance of 1.2 Da, MS/MS tolerance of 0.6 Da, fixed modification carbamidomethylation of cysteines, variable oxidation of methionines and peptide charge of 1+, 2+, and 3+. Decoy tool was utilised in all searches to have the significance threshold adjusted to obtain a global false discovery rate (FDR) at 1%.

2.3.2.6 Detection of modified Cys peptides from pure glycosylated human recombinant AGT

Glycosylated AGT was first deglycosylated with PNGase F kit according to the manufacturer's instructions. Briefly, AGT glycoprotein (3 µL) was combined with 1 µL of 10X glycoprotein denaturing buffer, and the reaction volume was adjusted to 10 µL with water, before heating to 100°C for 10 min. Two µL of 10X G7 reaction buffer, 2 µL 10% NP-40, 1 µL PNGase F and water were added to make a total reaction volume of 20 µL. The reaction was incubated at 37°C for 1 h. Following deglycosylation; protein reduction, alkylation with IAM and digestion with chymotrypsin was conducted as described previously (Section 2.3.2.1). The AGT digest was analysed using accurate mass LC-MS, as in Section 2.3.2.3, to detect the modified Cys peptides from the TIC of AGT digest.

Figure 2-4 demonstrates the workflow followed to develop a digestion protocol enables the reproducible detection of the two key Cys peptides using pure human recombinant AGT.

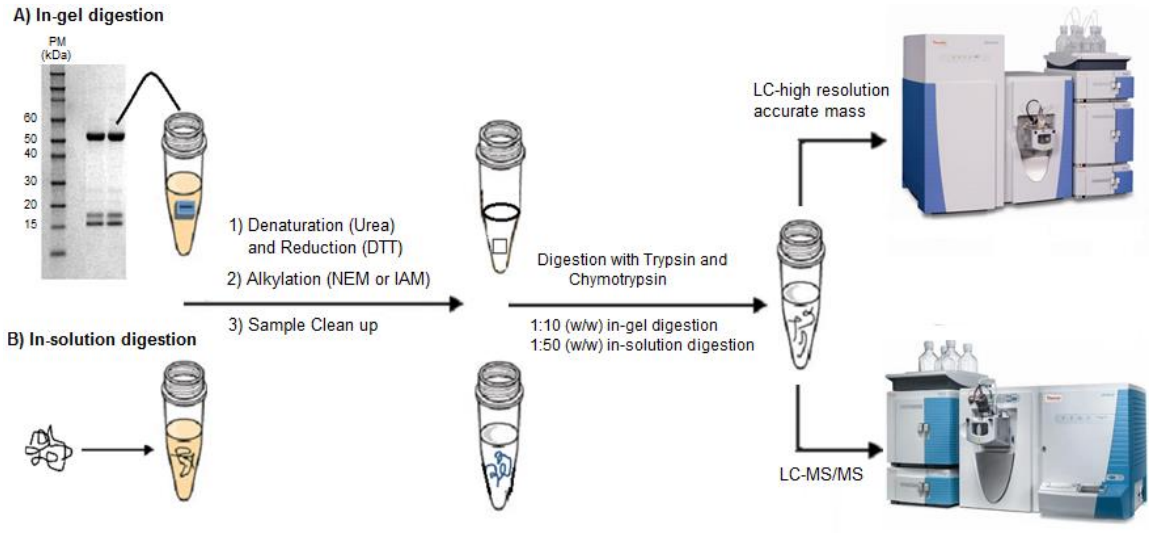


Figure 2-4: Optimisation of human AGT digestion using pure human recombinant AGT. The two strategies used in the bottom-up approach, in-gel and in-solution, were examined with two proteolytic enzymes, trypsin and chymotrypsin. The two key Cys peptides, Cys18 and Cys138 from AGT digest were modified with either NEM or IAM. The AGT digests were analysed by high resolution accurate mass and LC-MS/MS to compare the methodologies, and to confirm the identity and the alkylation of the two key peptides.

2.3.3 Detection of the key Cys peptides from human plasma chymotryptic digest

2.3.3.1 Selective enrichment of AGT with ConA Sepharose resin and reversed-phase solid phase extraction (RP-SPE)

Concanavalin (ConA) Sepharose resin slurry (100 µL) was placed into filter devices to provide 1:1 ratio of resin bed to sample volume, and conditioned with 3 x of 0.3 mL washing buffer (50 mM TRIS pH 7.4, 0.15 M NaCl, 1 mM $CaCl_2$, $MgCl_2$, $MnCl_2$). The plasma sample (50 µL), from healthy volunteers, was diluted in 250 µL washing buffer, loaded onto the activated resin and incubated at room temperature for 20 min with agitation. The resin was washed three times with the washing buffer to remove unbound proteins. Retained proteins were eluted with 1.2 mL elution buffer (0.40 M methyl α-D-mannopyranoside in washing buffer) for subsequent RP-SPE fractionation. The flow through the filter device was driven by centrifugation at 1000x *g* for 30 s in all steps.

Polymeric large-pore RP-SPE cartridge (IST ISOLUTE, PDVB, 1000 Å, 25 mg) was wetted with 1 mL of 90% acetonitrile with 0.1% TFA prior to conditioning with 1 mL of 0.1% TFA in water. The enriched glycoproteins sample was loaded onto the preconditioned SPE cartridges and washed with 1 mL 0.1% TFA. The retained proteins were sequentially eluted with 1 mL of 30, 35, 40, 43, 45, 48, 50, and 90% 0.1% TFA in acetonitrile, and the collected fractions (n=8) were dried using vacuum centrifuge. The

flow rate of the mobile phase through the RP-SPE cartridge was adjusted to 1 mL/min via a vacuum pump. ConA permits the enrichment of *N*-glycoproteins while RP-SPE fractionation adds another dimension to reduce sample complexity.

2.3.3.2 Monitoring AGT fractionation by Western blotting

The eight RP-SPE dried protein fractions were solubilised in NuPAGE sample buffer and 20% of the sample final volume was separated by SDS-PAGE run for 1 h applying 200 V. Proteins were transferred to a PVDF membrane using electro-blot module (Invitrogen) running at 30 V for 3 h. The membrane was incubated for 30 min with membrane blocker reagent (5% non-fat dried milk in TBST [10 mM TRIS with 150 mM NaCl and 0.05% (*v/v*) TWEEN 20, pH 7.4]), washed and incubated for 1 h with mouse anti-human serpin A8/angiotensinogen antibody (1:200 in blocker reagent). The membrane was washed 3 times, 10 min each, with TBST and incubated with horse-radish-peroxidase labelled anti-mouse IgG overnight (1:10,000 in blocker reagent). Finally, the membrane was washed, as before, and visualised by chemiluminescent detection reagents.

2.3.3.3 Chymotryptic digestion of plasma AGT enriched fraction

After ConA treatment, the dried RP-SPE protein fraction enriched with AGT was deglycosylated with PNGase F as described previously. Briefly, proteins were denatured with glycoprotein denaturing buffer and deglycosylation was carried out by adding 1 µL of PNGase F for each 40 µg proteins and the reaction was incubated for 1 h at 37 °C. Proteins were reduced with 50 mM DTT and alkylated with 200 mM IAM using 10 kDa Amicon ultra 0.5 centrifugal filter devices as described in Section 2.3.2.1. Chymotrypsin was added at an enzyme: protein ratio of 1:50 (*w/w*) and digestion was carried out at 37°C. To ensure complete digestion, a second aliquot of chymotrypsin (1:100 enzyme: protein *w/w* ratio) was added after 4 h and digestion proceeded at 37°C overnight. The reaction was quenched with formic acid and the samples were centrifuged for 5 min at 11000x *g* and the upper layer was transferred for LC-MS/MS analysis.

2.3.3.4 Transitions optimisation of AGT IAM modified Cys18 and Cys138 and AGT marker peptide

Unmodified and IAM alkylated Cys18, Cys138 and AGT marker standard synthetic peptides (2 µM) were infused at 10 µL/min flow rate to optimise the entrance and the declustering potentials for the three precursor ions, and to choose the best four MRM transitions per peptide. The collision energy and cell exit potential for each MRM channel

was optimised to maximise signal strength. Double charged precursor ions were selected for the two modified Cys peptides but single charge precursor ion for the marker peptide. Source gases and temperature were optimised with a T piece in which infusion of peptide standards was mixed with 0.1% FA acetonitrile (30%) eluting from the column at 100 µL/min flow rate. The optimised values for curtain gas, collision gas, GS1 and GS2 were 10, 12, 30 and 45 (arbitrary unit) respectively. The heated capillary temperature was maintained at 450°C and the ESI voltage was kept at 4200 V.

2.3.3.5 Detection of the key AGT peptides from plasma chymotryptic digest using targeted LC-MS/MS

Chymotrypsin digested peptides were analysed using a 4000 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) operating in positive ion mode. Peptides were first separated by a Shimadzu series 10AD VP LC system (Shimadzu, Columbia, MD) using C18 300 Å, 100 x 1mm id, 3 µm column from ACE (Scotland, UK) and a mobile phase of water and acetonitrile both with 0.1% FA. Organic phase gradient from 10% to 45% (*v/v*) was applied over 10 min at 100 µL/min flow rate. MRM transitions were monitored and acquired at unit resolution in both Q1 and Q3 to maximise specificity applying the optimised MS parameters mentioned above (Section 2.3.3.4). All data were processed by Analyst software 1.4.2.

To confirm the identity of the three monitored AGT peptides detected from plasma, samples were also analysed under similar chromatographic condition on an ion trap mass spectrometer coupled to HPLC (LTQ Velos) from Thermo Scientific (San Jose, CA). MS/MS spectra of the peptides of interest were acquired in positive ion MS data-dependent mode in a mass range of 200–1650 with one unit mass resolution, parent mass width ± 0.5 and retention time window 1 min. ESI source parameters were kept as follow: ion spray voltage 3 kV, capillary and source heater temperatures 275°C and 300 °C respectively and flow rate for the sheath, auxiliary and sweep gases were 30, 20 and 10 respectively.

2.3.4 Method Validation

Initial quantitative assessment of the developed LC-MS/MS was carried out by measuring the coefficient of variation (CV%) between six experiments for the three monitored peptides, IAM Cys18 and 138 peptides and the marker peptide, SVTQVPF. In both cases, CV values of ≤15% were considered acceptable, in accordance with the US-FDA bioanalytical method validation, guidance for industry, September 2013 [243].

The overall AGT recovery was calculated by comparing the peak area of MRM for the AGT marker peptide, SVTQVPF, from the plasma digest, with one obtained from SVTQVF peptide standard spiked into plasma digest at the expected concentration of endogenous AGT (based on the reported 45 µg/mL concentration of AGT in the plasma [244]). Recovery was calculated using the below equation:

$$\text{Recovery} = \frac{\text{Plasma AGT}}{\text{Plasma spiked with AGT peptide standard} - \text{Plasma AGT}} \text{ X } 100\%$$

Peptide standard could not be spiked directly into the plasma as it would be lost during sample preparation workflow. Consequently, the earliest point for spiking peptide standards in quantitative proteomics is either before or after the digestion step.

Figure 2-5 summarises the main workflow for the detection of AGT Cys peptides from the plasma chymotryptic digest.

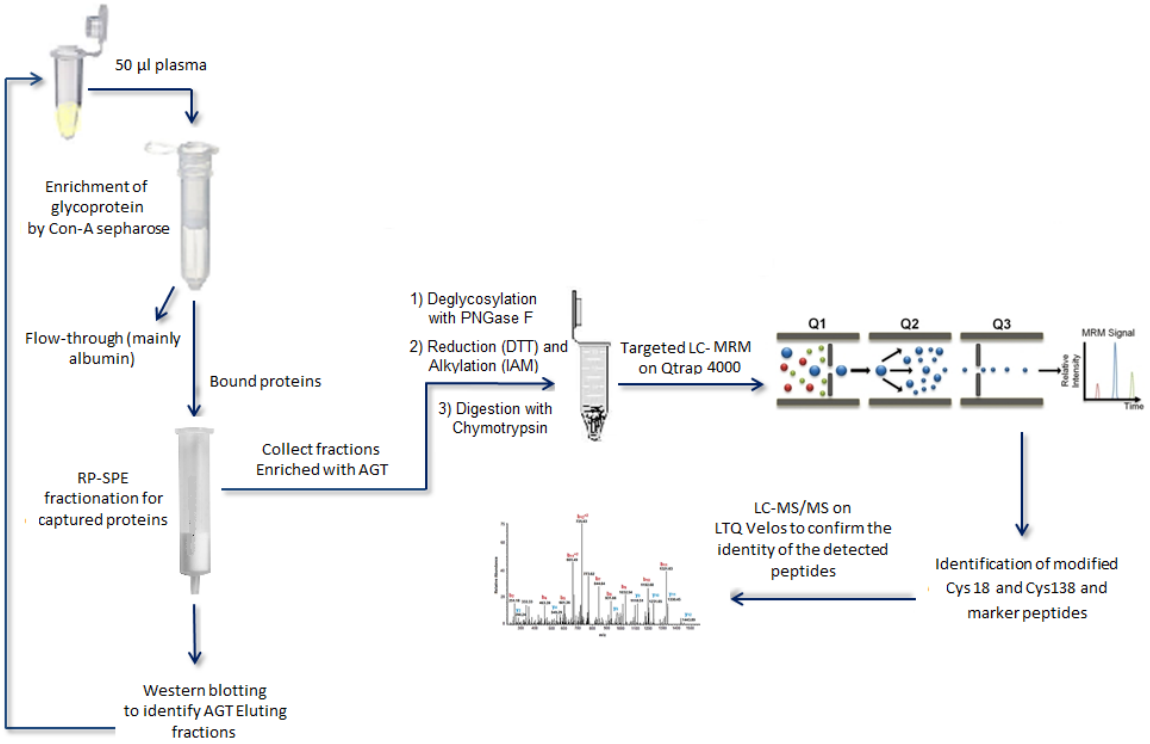


Figure 2-5: The workflow followed for the detection of the key AGT peptides from human plasma. Selective enrichment of AGT from human plasma was achieved using ConA Sepharose followed by fractionation with RP-SPE, applying eight step elutions of 0.1%TFA in acetonitrile, monitored by Western blotting. The dried, collected RP-SPE fraction, containing the highest percentage of AGT, was deglycosylated with PNGase F, reduced with DTT, alkylated with IAM and digested using chymotrypsin. A targeted microLC-MS/MS method working under MRM mode was developed to detect the presence of the two key Cys peptides, while LC-MS/MS full MS scan was conducted to confirm the identity of the detected peptides.

2.4 Results and Discussion

2.4.1 Experiments with unglycosylated recombinant AGT

2.4.1.1 Digestion optimisation

In-gel and in-solution digestion

The different digestion protocols were evaluated based on the peptide sequence coverage and the detection of the Cys peptides. Significant Mascot scores (scores > 56) for AGT were obtained with both digestion methods; in-gel and in-solution digestion (Table 2-3). However, better peptide sequence coverage, and reproducible detection of the two key AGT Cys peptides was achieved with in-solution digestion. Unsurprisingly enzymatic cleavage of AGT in the gel matrix suffered from lower coverage than in-solution digestion with both enzymes (trypsin and chymotrypsin) as noted in Table 2-3. Better coverage is usually attained with in-solution digestions as peptide extraction from gels is limited by diffusion kinetics [43]. Although gel electrophoresis MS is a successful approach widely used to identify and quantify proteins of interest, it has a number of limitations. The handling process of in-gel digestion involves many steps which may be determinant to the amount of protein recovered; alongside the time consuming and labour intensive nature [16]. In this instance, the key AGT Cys peptides could not be identified reliably even though a simple sample of pure protein was used, and so would become a greater challenge with human plasma, especially given the low signal intensities associated with these peptides. Particularly, with complex samples, the GE resolving power is not sufficient to separate proteins with similar molecular weight [43]. Taking into account the fact that AGT has a weight very close to albumin, the most abundant plasma protein, GE was unlikely to be the best approach for optimisation in this study. This is further supported by the findings of a previous study that could not achieve a successful detection of AGT using 2D in-gel digestion even after the depletion of the 14 high abundant plasma proteins using Seppro IgY14 [245]. AGT could only be detected when a second level of enrichment for low abundant proteins was added using Seppro IgY-SuperMix System [245].

Table 2-3: Peptide sequence coverage and protein score for in-solution and in-gel digestion of AGT with trypsin and chymotypsin using MS/MS search engine from Mascot.

Enzyme	Sequence coverage (%)[a]		Protein score[b]	
	In-solution Digestion[c]	In-gel Digestion	In-solution Digestion	In-gel Digestion
Chymotrypsin	61.5	32.0	151	90
Trypsin	31.0	7.5	301	85

[a] Peptide sequence coverage was recalculated to exclude the first 32 signal peptides in AGT sequence.
[b] Protein scores greater than 56 are significant ($p<0.05$).
[C] proteins denatured at 2M urea.

Chymotrypsin and trypsin

AGT in-solution chymotryptic digest resulted in a higher number of matched peptides, and a greater sequence coverage was achieved (61.5%) than with the corresponding tryptic digest (31%), Figure 2-6 and Table 2-3. Furthermore, the two key modified Cys18 and Cys138 peptides were consistently detected with chymotrypsin whilst only Cys138 was identified with trypsin (Figure 2-6). This result was also supported by high resolution accurate MS analysis (Section 2.4.1.2). Both trypsin and chymotrypsin enzymes are serine proteases but with different substrate specificity [246]. Trypsin has a well-defined specificity [247]; it cleaves mainly at the carboxyl end of the positively charged amino acids lysine (K) and arginine (R). These are relatively abundant and well distributed amino acids in human proteins. As a result, peptides with an average length of 14 amino acids carrying two positive charges and ideal for ionisation and MS analysis will be generated [226]. The trypsin cleavage site within the AGT sequence containing Cys138 produced a peptide satisfying the above criteria; therefore, Cys138 peptide could be detected from AGT tryptic digest. However, the modified Cys18 peptide, could not. For Cys18 peptides, trypsin cleavage site generated a peptide with 21 amino acids; which possibly render this peptide not readily amenable to MS analysis due to its large size, and consequently could not be detected (Figure 2-6, B). Chymotrypsin, however, acts on more peptide hydrolysing sites. It favours the aromatic amino acids phenylalanine (F), tyrosine (Y) and tryptophan (W) [248], and to a slower rate leucine (L) and methionine (M). Based on the distribution of the cleavage sites for both enzymes in the AGT sequence, digestion using chymotrypsin resulted in shorter peptides with the preferred mass range for MS analysis, and consequently higher sequence coverage and greater matched peptides were achieved. Of most importance, the reliable detection of the two Cys peptides was obtained with chymotrypsin. As a result, all AGT in-solution digestions were performed with chymotrypsin.

A) Chymotrypsin digestion

MRKRAPQSEM	APAGVSLRAT	ILCLLAWAGL	AAGD**RVYIHP**	F***HLVIHNEST***
C[18]EQLAKANAG	KPKDPTFIPA	PIQAKTSPVD	EKALQDQL**VL**	**VAAKLDTEDK**
LRAAMVGMLA	NFLGFRIY**GM**	**HSELWGVVHG**	**ATVL**SPTAVF	GTLASLYL**GA**
LDHTADRLQA	**ILGVPW*KDKN***	***C[138]TSRL*DAHKV**	**LSALQAVQGL**	**LVAQGRADSQ**
AQLLLSTVVG	**VFTAPGLHL**K	QPFVQGLALY	T**PVVLPRSLD**	**FTELDVAAEK**
IDRFMQAVTG	**WKTGCSL**MGA	SVDSTLAF**NT**	**YVHF**QGKMKG	FSL**LAEPQEF**
WVDNSTSVSV	PMLSGMGTF**Q**	**HWSDIQDNF**S	**VTQVPFTESA**	**CLLLIQPHY**A
SDLDKVEGLT	FQQNSLNWMK	KL**SPRTIHL**T	MPQLVLQGSY	DLQDLL**AQAE**
LPAILHTELN	LQKL**SNDRIR**	**VGEVLNSIFF**	**ELEADEREPT**	**ESTQQLNKPE**
VLEVTLNRPF	LF**AVYDQSAT**	**ALHFLGRVAN**	**PLSTA**	

B) Trypsin digestion

MRKRAPQSEM	APAGVSLRAT	ILCLLAWAGL	AAGD**RVYIHP**	FHLVIHNEST
C[18]EQLAK**ANAG**	**KPKDPTFIPA**	**PIQAKTSPVD**	**EKALQDQLVL**	**VAAKLDTEDK**
LRAAMVGMLA	NFLGFRIYGM	HSELWGVVHG	ATVLSPTAVF	GTLASLYLGA
LDHTADR**LQA**	**ILGVPW*KDKN***	***C[138]TSR*LDAHKV**	**LSALQAVQGL**	**LVAQGR**ADSQ
AQLLLSTVVG	VFTAPGLHLK	**QPFVQGLALY**	**TPVVLPRSLD**	**FTELDVAAEK**
IDR**FMQAVTG**	**WK**TGCSLMGA	SVDSTLAFNT	YVHFQGKMKG	FSLLAEPQEF
WVDNSTSVSV	PMLSGMGTFQ	HWSDIQDNFS	VTQVPFTESA	CLLLIQPHYA
SDLDKVEGLT	FQQNSLNWMK	KLSPRTIHLT	MPQLVLQGSY	DLQDLLAQAE
LPAILHTELN	LQKLSNDRIR	**VGEVLNSIFF**	**ELEADER**EPT	ESTQQLNKPE
VLEVTLNRPF	LFAVYDQSAT	ALHFLGR**VAN**	**PLSTA**	

Figure 2-6: AGT peptide sequence coverage from LC-MS/MS data analysed by Mascot for chymotrypsin digest (A) and trypsin digest (B). Matched peptides are shown in red. Detected Cys18 and Cys138 peptides are presented in italic red. Sequence starts from RVYI…. presented in bold black letter.

Sample clean-up

Minimal and incomplete digestion of AGT, with chymotrypsin, was observed when the excess reducing and alkylating agents were not removed prior to digestion (Figure 2-7, A). As with many other proteins, disulphide bridges maintain the tertiary structure and the functional activity of chymotrypsin, breaking this bond and the subsequent alkylation of the cysteinyl sulphydryl groups leads to conformational changes, and so affects chymotryptic activity [249]. Optimisation of the AGT digestion protocol revealed that removal of the excess reagents prior to digestion is crucial to ensure efficient protein digestion and MS compatibility. Consequently, gel filtration (by micro bio-spin 6 chromatography columns) and 10 kDa membrane filters were used to clean-up and to

wash out excess reagents. However, better protein recovery was noticed with 10 kDa filters, and complete AGT digestion was achieved rapidly with most of the protein proteolysis occurring within the first half hour of incubation (Figure 2-7, B). This allowed a more rapid analysis and eliminated the need for 24 h incubation as longer digestion times increase the possibilities of chymotrypsin autolysis.

Taken together, in-solution digestion using chymotrypsin and 10 kDa filters resulted in efficient protein digestion, best AGT sequence coverage, and enabled the reproducible detection of the key Cys peptides. Therefore, were encountered in all subsequent AGT experiments.

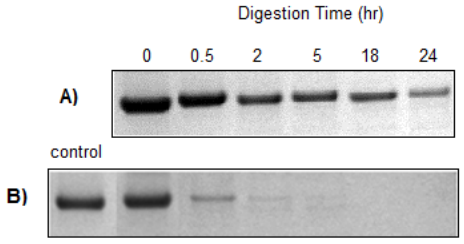


Figure 2-7: Coomassie blue stained 1D SDS-PAGE for the effect of 10 kDa filters on protein recovery and digestion. A) Excess reducing and alkylating reagents were not removed prior to digestion. B) 10 kDa membrane filter was used to wash excess DTT and alkylating agent prior to digestion. A sample of AGT with a similar amount of the protein to the one in the cleaned-up sample was used as a control to examine protein recovery from the filter. To monitor digestion, samples were taken (from A and B) prior to chymotrypsin digestion (0 h) and after 0.5, 2, 5, 18 and 24 h of incubation with the enzyme.

2.4.1.2 Alkylation of the Cys peptides with NEM and IAM

The Orbitrap Exactive mass analyser was used in the analysis of the AGT chymotryptic digest. It allows high mass accuracy and resolution, which results in the exact measurement of the molecular weight of ions of the same nominal mass [58]. Good signal intensity was obtained with the fast 6 min LC-MS run method (Figure 2-8, A). This allowed the analysis of larger numbers of samples and gave a much faster throughput to investigate the optimisation of the AGT Cys peptide detection with no loss in sensitivity. Most of the peptides were eluted between 25-45% organic phase and were multiple charged, double- and triple-charged, which is commonly noticed with ESI [250].

Two alkylating agents, NEM and IAM, were used to alkylate Cys free thiol. The predicted *m/z* of unmodified and modified Cys18 (HLVIHNEST**C**^{18}EQL) and Cys138

(KDKN**C**^{138}TSRL) peptides (Table 2-2), were used to extract the corresponding peptide ion signal from the total ion chromatogram (TIC) of AGT digests in the initial work. Thiol modification with both compounds was achieved and confirmed by accurate MS. With IAM modification, both alkylated Cys18 and to a lower intensity Cys138 peptides were readily detected from the TIC of AGT digest as double-charged ions (Figure 2-8, B and C respectively). Full alkylation was achieved as unmodified peptides could not be identified.

The two NEM modified Cys peptides, 18 and 138, were extracted as double- and triple-charged ions (Figure A2-1, B and C respectively in Appendices). However, Cys138 peptide was not reproducibly detected, and even when so, it was associated with low signal intensity. This may be due to the presence of Cys138 in the chymotryptic digest of NEM alkylated AGT in two peptide sequences; no missed cleavage (KDKN**C**^{138}TSRL) and one missed cleavage (KDKN**C**^{138}TSRLDAHKVL), which decreased the overall abundance of the fully digested Cys138 peptide.

Both Cys peptides modified with IAM were detected with a higher signal intensity and showed a higher MS response when compared to NEM modified ones (3.5 and 10 fold higher MS response for Cys 18 and 138 respectively, Figure 2-9). This pointed out that the release of NEM Cys peptides from AGT during digestion was not efficient as with IAM, possibly due to the non-specific cleavage with chymotrypsin which generated more than one peptide sequence with the same Cys amino acid. The difference was more pronounced with the Cys138 peptide which was detected with very low intensity with NEM. However, with both modifications, Cys 138 was associated with a lower mass response than Cys18 peptide (Figure 2-9). This difference in the signal intensity for peptides derived from the same protein can be explained by the fact that peptides vary in their ionisation efficiency, and, therefore, MS detection sensitivity would be based on their amino acid sequences [251].

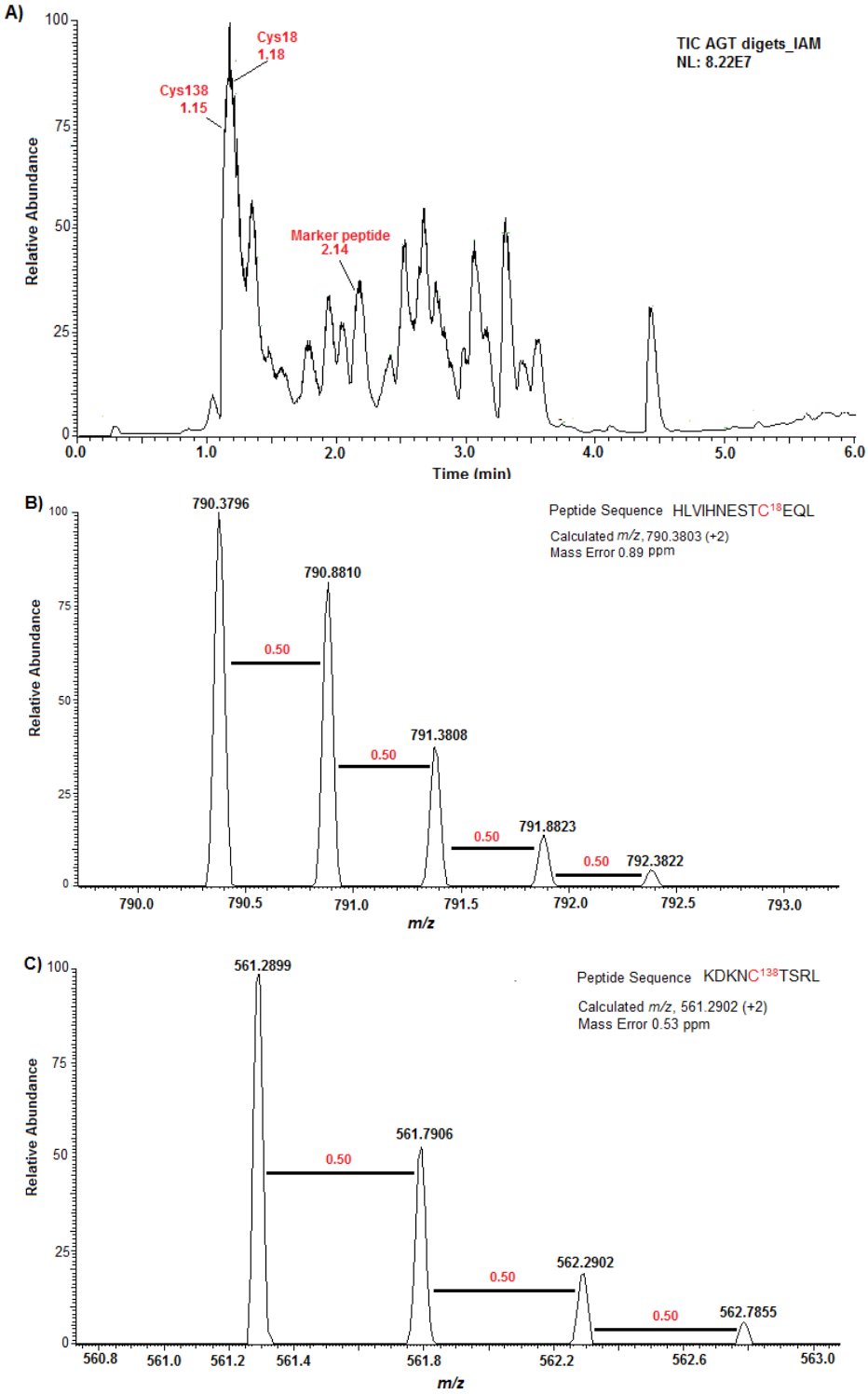


Figure 2-8: Analysis of human recombinant AGT chymotryptic digest by high resolution accurate mass. (A) Total ion chromatogram (TIC), (B) Ion signal of iodoacetamide alkylated Cys18 peptide extracted from the TIC and (C) Ion signal of iodoacetamide alkylated Cys138 extracted from the TIC.

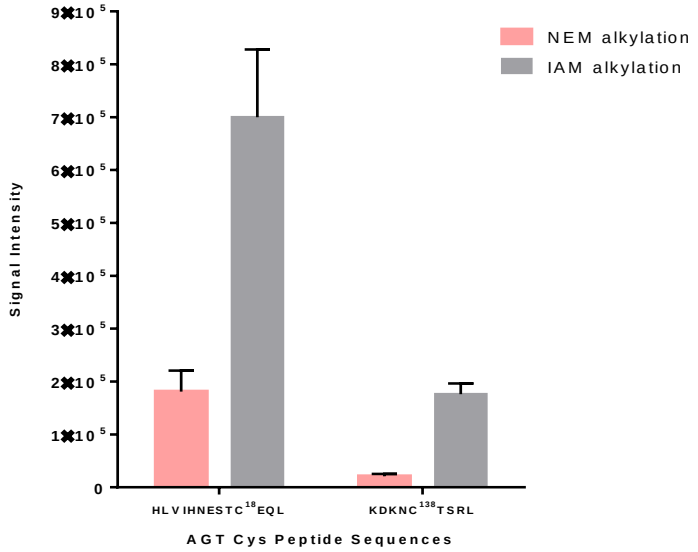


Figure 2-9: Signal intensities of the two Cys peptides alkylated with NEM and IAM, and extracted from AGT chymotryptic digest. IAM alkylation showed almost 3.5 and 10 fold increase in the signal intensity than the corresponding NEM alkylated Cys 18 and Cys 138 peptide respectively. With both modifications, Cys138 peptide showed lower signal intensity.

Regardless the signal intensity, full alkylation was observed with both NEM and IAM when used to alkylate AGT Cys peptides at 200 mM concentration under mild denaturing conditions (2 M urea), and the two IAM and NEM modified Cys peptides were detected with a mass error < 2 ppm (Table A2-1 in Appendices). Mass error was calculated based on the below equation:

$$\text{Mass Error (ppm)} = \frac{\text{Predicted } m/z - \text{Detected } m/z}{\text{Predicted } m/z} \times 10^6$$

Besides the high mass accuracy for the detected peptides, the correct charge state of the detected ions (Figure 2-8), and consistent retention time between different experiments were also evaluated to confirm the positive detection of the key peptides. These peptide structures were later confirmed by the use of synthetic peptide standards (Section 2.4.3.2).

Initially, covalent modification of the free thiol of Cys peptides after reduction, with either NEM or IAM, was necessary to have proper cleavage of the peptides by the proteolytic enzyme, and prevent the reformation of the disulphide linkage so that Cys peptides can be simply identified and quantified later by MS. Additionally, alkylation of the free thiol of the Cys peptides enhances the peptide MS signal as improvement in the peptides ionisation by modification of the Cys sulphydryl group has been reported [224]. Selection of an alkylating agent to modify the free reactive thiol of the Cys peptides is critical in the

MS measurement of protein abundance in different redox states [208]. In order to achieve reliable MS detection, the alkylating agent should have high reactivity to ensure reaction completion, and should not negatively interfere with the ionisation and fragmentation efficiency of the peptide [215]. IAM and NEM are routinely used in the modification of Cys thiol; however IAM is more frequently encountered in proteomics [252]. Previous studies have reported different effectiveness of NEM and IAM in modifying protein thiols. Reaction of NEM with thiols was found to be faster [212; 216], more efficient, and effective (at physiological pH) when compared with IAM [216; 251]. However, lower specificity (when used in excess) [251] and reactivity towards buried or partially exposed thiols have been observed with NEM by Zander *et al.* [253]. Another study has reported comparable efficiency and selectivity between IAM and NEM when used to study the redox state of the Cys peptides in oval albumin [254], whilst a higher number of IAM modified Cys peptides in BSA was identified when compared with NEM [215]. The previous studies indicate that no one alkylating agent is absolutely efficient for all proteins thiols modification and that the efficiency of alkylation of IAM and NEM can differ depending mainly on the studied protein and the chemistry of the Cys peptides. As a result, reagent selection will depend upon the experiment conditions and the study applied.

In the current work, IAM was more effective than NEM in the alkylation of the key AGT Cys peptides and consequently was associated with higher MS responses for the Cys peptides, as noticed in the AGT chymotryptic digest. Therefore, all subsequent AGT experiments were conducted using IAM as an alkylating agent.

2.4.1.3 The identification of AGT marker peptide

Beside the detected Cys peptides, a seven amino acids peptide, SVTQVPF, unique in sequence to AGT was identified from the AGT chymotryptic digest (Figure 2-10). It was detected as single-charged ion and with high signal intensity (higher than both Cys peptides) and mass accuracy, < 1 ppm. The peptide is ideal as a signature peptide for the absolute quantification of the total AGT in the plasma. Furthermore, it has a good ionisation property and does not contain amino acids which are susceptible to modification, such as methionine (M), asparagine (N), and cysteine (C), and so shall be referred to as the ‘AGT marker peptide’ henceforth.

The uniqueness of the three peptides, Cys 18, Cys138 and the marker peptide, to AGT protein was confirmed with PepServer from BioServer [255].

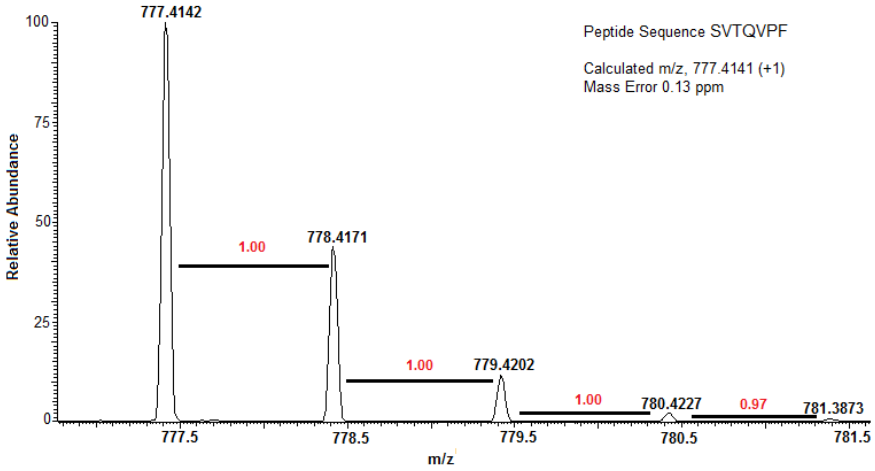


Figure 2-10: The ion signal of AGT marker peptide extracted from the TIC of AGT chymotryptic digest analysed by high resolution accurate mass LC/MS.

2.4.1.4 LC-MS/MS for modified Cys18, Cys138 and marker peptides

The predicted *y and b* ions from the fragmentation of the modified Cys peptides and the marker peptide were matched with the MS/MS spectra obtained, and the identity of the three peptides was confirmed (Figure 2-11). In IAM modified Cys18 MS/MS spectrum, most of the ions identified were *b* ions; whilst almost all of the predicted *y* ions were detected in IAM Cys138 spectrum. Limited fragmentation was observed with the marker peptide and ions produced by losing water molecules were detected. Many parameters can affect the fragmentation pattern of peptides, including peptide characteristics (size, charge, amino acid composition) but also the type and energy of the fragmentation, and the type of instrument used.

The same fragmentation patterns were obtained for Cys18 and the marker peptide from the NEM alkylated AGT digest. However, differentiated fragmentation for Cys138 could not be achieved mainly due to the low signal intensity (Figure A2-2 in Appendices).

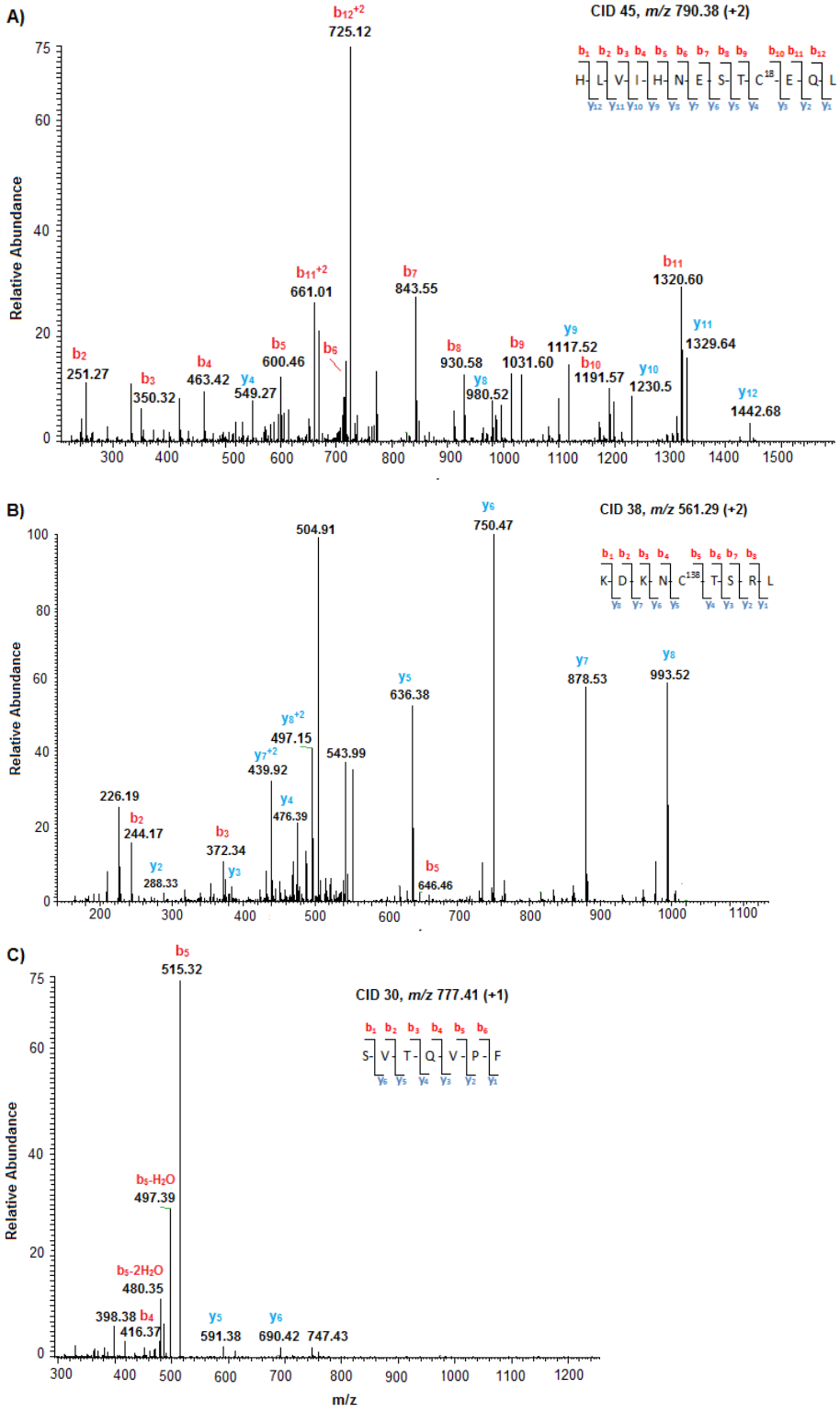


Figure 2-11: LC-MS/MS spectra of IAM modified Cys 18 (A), IAM modified Cys138 (B) and marker peptides (C) obtained from human recombinant AGT chymotryptic digest. The identity of the detected peptides was confirmed by matching the obtained spectrum with the predicted fragment ions (*b* and *y* ions mainly).

2.4.2 Experiments with glycosylated recombinant AGT

2.4.2.1 Detection of IAM modified Cys peptides and marker peptide

The three AGT peptides were detected in the TIC of the chymotryptic digest of glycosylated AGT with high mass accuracy when the optimised digestion protocol and the fast LC-MS method was applied (Figure 2-13). This step in the method development was critical as endogenous AGT in human plasma is also glycosylated.

AGT has four reported potential sites for glycosylation through the asparagine (Asn) moiety; (Asn^{14}, Asn^{137}, Asn^{271} and Asn^{295}) which give rise to the common *N*-linked type of glycosylation [256]. Both Cys peptides have a potential site for glycosylation through Asn (N) amino acids in their sequences; HLVIH**N**ESTC^{18}EQL and KDK**N**C^{138}TSRL. Consequently, effective deglycosylation was essential for two reasons. Firstly, to achieve efficient digestion and the generation of these key peptides as glycosylation can inhibit protease digestion. Secondly and more importantly, it is required to attain the correct peptide mass that will be subsequently used to extract the corresponding ion from the TIC. The deglycosylation enzyme used, PNGase F, is a widely applied effective enzyme for the cleavage of most types of *N*-linked oligosaccharides, even those with complex structures [257; 258]. However, under non-denaturing conditions and for complex samples, higher concentrations of PNGase F and longer incubation times are required [41]. Upon oligosaccharide cleavage, Asn is converted to aspartic acid (Asp) (Figure 2-12) resulting in almost 1 and 0.5 Da increase in the *m/z* of single- and double-charged peptides respectively [41]. This was observed in the XIC of the modified peptides (Figure 2-13); with the *m/z* for both peptides increasing as expected by 0.5 Da for double-charged ions (IAM Cys18: from 790.38 to 790.87 Da and IAM Cys138: from 561.29 to 561.78 Da).

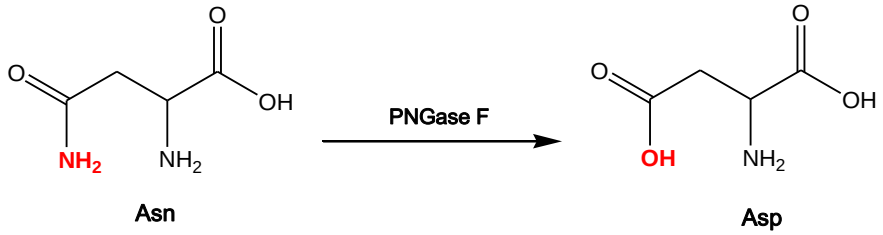


Figure 2-12: Conversion of asparagine to aspartic acid by PNGase F. The amine group (M.Wt16) of the asparagine amide will be converted to carboxyl group (M.Wt 17), resulting in 1 Da increase in the peptide mass.

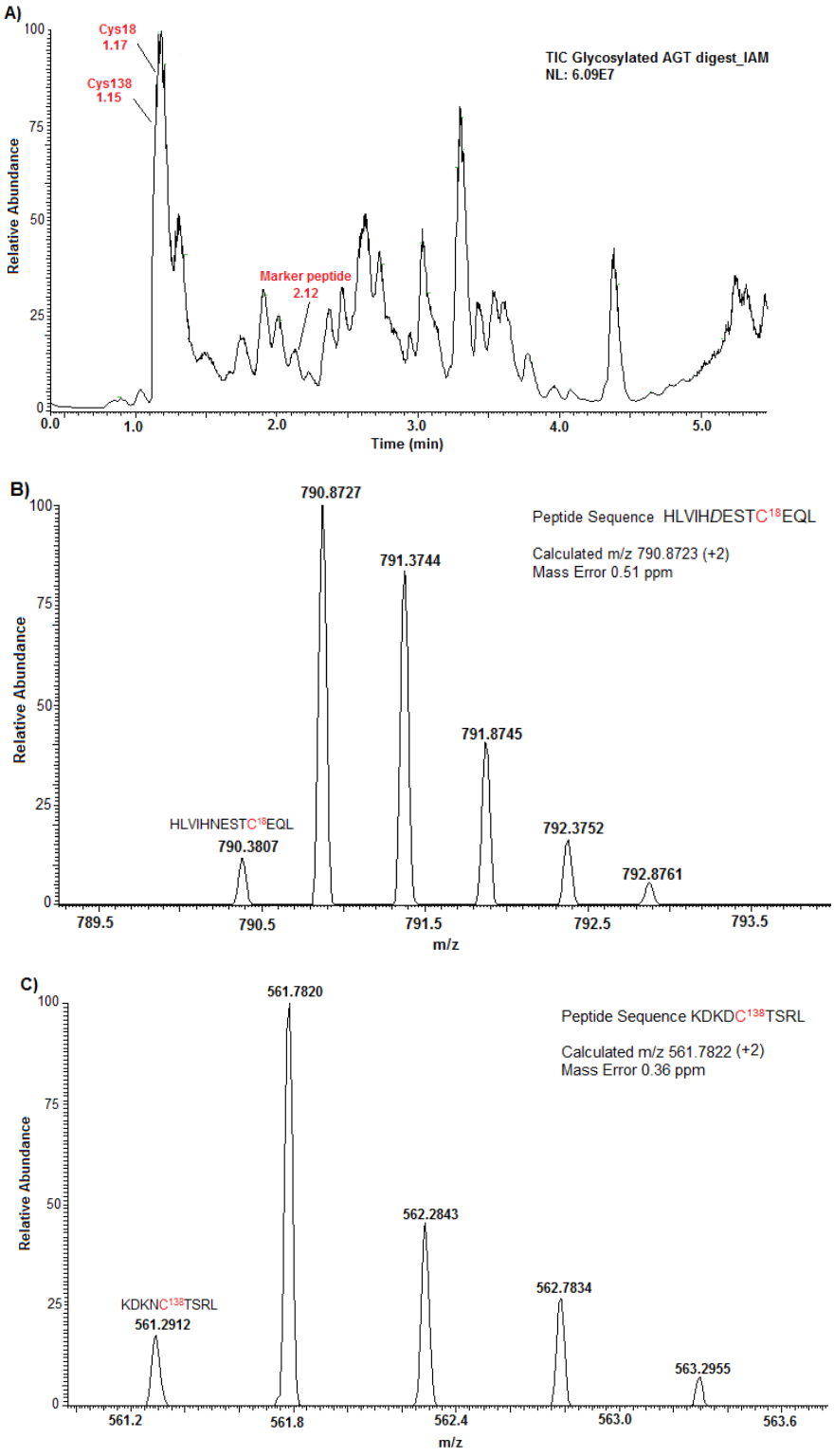


Figure 2-13: Analysis of glycosylated AGT chymotryptic digest by high resolution accurate mass. (A) TIC, (B) Ion signal of IAM alkylated Cys18 peptide extracted from the TIC and (C) ion signal of IAM alkylated Cys138 extracted from the TIC. Both modified Cys peptides were detected with mass error < 1%. The small signals at 790.38 and 561.29 in figure B and C respectively, refer to the unglycosylated form of the protein.

With high concentrations of AGT, a signal with a lower intensity corresponding to free *N*-residue in Cys18 and Cys138 peptides was noticed in the LC-MS spectra (Figure 2-13, B and C). This accounts for the lack of a glycan in the Asn residue since the detected ions have *m/z* similar to the corresponding peptides from unglycosylated AGT (Section 2.4.1.2). As Cys peptides were detected with much higher signal intensity and with high mass accuracy after deglycosylation, the obtained results indicate that Asn^{14} and Asn^{137} are actual glycosylation sites, with only a small percentage (< 10%) of both sites remaining unglycosylated.

Because deglycosylation is essential for the release of the key Cys peptides, complete deglycosylation of AGT was further confirmed using SDS-PAGE. A shift in the glycosylated AGT band from 60 to approximately 53 kDa was evident, and corresponding to that of the human recombinant unglycosylated AGT (Figure 2-14).

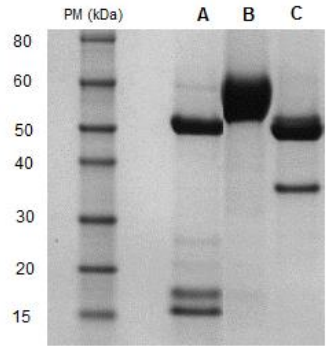


Figure 2-14: Coomassie blue stained 1D SDS-PAGE for human recombinant AGT treated with PNGase F. A, B and C represent unglycosylated AGT, glycosylated AGT, and glycosylated AGT after deglycosylation with PNGAse F. After deglycosylation, glycosylated AGT band at 60 kDa disappeared and a new band appears at the same mass of the unglycosylated AGT (around 53 kDa) indicating full deglycosylation was achieved. The band at 36 kDa refers to PNGase F.

2.4.3 Experiments with AGT in human plasma

As previously discussed, the complexity of human plasma makes the direct detection and quantification of specific peptides within the plasma proteome digest a very challenging analytical task, especially for low abundance proteins [28]. Although AGT is a moderately abundant plasma protein, direct detection of the modified key Cys peptides could not be achieved without protein extraction from the plasma. This was especially true in the case of Cys138, which had a lower signal intensity in comparison to other peptides derived from the same protein. Towards this end, a method for the selective enrichment of AGT followed by digestion of the sample using the optimised protocol was

developed, and a targeted LC-MS/MS method working on the MRM mode was used to detect the key Cys peptides as well as the marker peptide from the plasma digest.

2.4.3.1 Selective enrichment of AGT from human plasma by ConA/RP-SPE

AGT was first captured together with other *N*-glycoproteins by ConA as it could not be detected in the flow through. This indicates that AGT glycan has a high affinity for ConA. Similar results were obtained previously when three different lectins, with variable but partially overlapping binding profiles; ConA, WGA, LCA, together with boronic acid beads, were applied to capture plasma glycoproteins, where AGT was exclusively detected with ConA fraction [259]. This agrees with the results presented here and supports the conclusion that ConA is the best lectin to capture plasma AGT.

Further enrichment of AGT was achieved by differential fractionation of ConA bound glycoproteins using RP-SPE and applying eight gradients of the mobile phase. (ConA/RP-SPE workflow will be discussed in more detail in Chapter 4, Section 4.3.1). In identifying AGT eluting fractions, Western blotting (WB) revealed good recovery for AGT, and detection in more than one fraction with the majority eluting at 45% and 48% of the organic solvent (Figure 2-15). This is to be expected with glycoproteins in general due to the heterogeneity of the sugar moiety attached to the protein and the large number of potential glycosylation combinations. The same glycosylation site can be attached to different types and numbers of sugar moieties. Accordingly, different glycans can be linked to the protein creating diverse isoforms for the same protein, and giving them different hydrophilic/hydrophobic properties [41; 260]. Human AGT is known to be a heterogeneous glycoprotein with different glycosylation levels responsible for its heterogeneity [261]. So, the result of WB is considered consistent with glycoprotein heterogeneity and previous studies conducted on AGT glycosylation.

In the current work, fractionation of the extracted glycoproteins using eight gradients of the mobile phase was only required one time during the method development to identify AGT eluting fractions by Western blotting. Following the results of Western blotting (Figure 2-15), the majority of plasma AGT was collected applying only a two-step gradient of the mobile phase. In the first step, 40% mobile phase was used to elute proteins in the first three fractions where very minimal AGT was evident. In the second step, 50% organic solvent was used to collect the remaining proteins (in fractions 43, 45, 48 and 50%); which yielded the majority of the AGT.

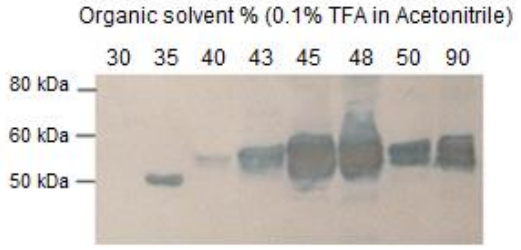


Figure 2-15: Western blotting for human AGT in the 8 RP-SPE fractions using mouse anti-human serpin A8/angiotensinogen antibody. Plasma glycoprotein were enriched with ConA then fractionated into 8 fractions by RP-SPE. Proteins were eluted sequentially with 8 gradients of 0.1%TFA in acetonitrile. Good recovery of plasma AGT is noticed with the highest amount of the protein eluted at 45 and 48%.

Reportedly, AGT has been detected directly from the plasma digest without applying any enrichment or depletion approaches [27; 79; 80; 87; 262]; however, different depletion strategies have significantly improved the detection limit and the reproducibility of the methods [27; 79]. AGT has been identified with other plasma glycoproteins by using multi-lectin approach [262; 263], and with commercial immune depletion columns, MARS-7 and MARS-14 [27], ProteoPrep20 [264] and Seppro® IgY12 coupled with multi-lectins [265]. However, it could not be detected using ProteoMiner, in which a combinatorial library of bead bound hexapeptides with different properties are used to bind and enrich low abundant proteins [264]. Although the previously mentioned enrichment strategies, especially immunoaffinity depletion columns, offer high reproducibility and improved in-depth analysis for moderately and low abundant proteins; they are generally associated with high costs which increase markedly with increased capacity volume and samples number. Besides, they require long processing times to deplete the highest abundant proteins and some of which have non-specific binding. AGT was described binding to MARS-7 and MARS-14 columns, and portions were eluted alongside the depleted proteins [27].

The methodology described herein, however, achieved a reasonably quick extraction of AGT, as only two-step gradient are required in RP-SPE to capture the majority of the ConA bound AGT. As a result, high recovery and specificity were achieved without reducing throughput, and a larger number of samples could be processed at a lower cost per sample compared with immunoaffinity depletion.

2.4.3.2 Detection of the AGT peptides by targeted LC-MS/MS

Standard synthetic peptides were used for MRM parameters optimisation and to identify the retention time for each peptide. For the Cys18 and Cys138 peptides, MRM parameters were optimised for IAM modified (Table 2-4) and unmodified peptides (Table A2-2, Appendices), and four transitions were monitored per peptide. By monitoring more than one product ion per peptide, and with the prior knowledge of the expected retention time, the possibility of false positive detection is reduced.

Table 2-4: Optimised MRM of the two IAM alkylated AGT Cys peptides and the identified marker peptide

Peptide sequence	Peptide mass	*m/z* (charge)	MS1/MS2	DP	CE	CXP
HLVIHDEST**C**^{18}EQL[a]	1579.75	790.8723 (+2)	790.9/725.3	80	36	17
			790.9/844.4	80	40	16
			790.9/1321.6	80	38	24
			790.9/661.3	80	40	15
KDKD**C**^{138}TSRL[a]	1121.57	561.7822 (+2)	561.8/751.3	65	38	15
			561.8/994.4	65	32	15
			561.8/879.5	65	34	15
			561.8/636.5	65	38	16
SVTQVPF (Marker Peptide)	776.41	777.4141 (+1)	777.4/515.3	75	32	15
			777.4/497.4	75	30	13
			777.4/480.3	75	38	13
			777.4/230.0	75	59	16

[a] Peptide Sequence after PNGase F treatment contains Asp formed by deglycosylation.
CE: collision energy, CXP: cell exit potential, DP: declustering potential, IAM: iodoacetamide, MS1/MS2: precursor/product ions.

The IAM modified Cys peptides, 18 and 138 were reproducibly detected with good signal intensity from the chymotryptic digest using the developed LC-MRM method. Figure 2-16 A and B represent the XIC of the best three MRM transitions for Cys18 and Cys138 respectively. The three transitions were detected at the same retention time, indicating that the product ions are from the same precursor. Higher signal intensity and better S/N ratio were observed with Cys18; however, the highest signal intensity was observed with the marker peptide MRM (Figure 2-16, C). This agrees with the previous findings from the pure form of the AGT, Section 2.4.1.2, and with the results obtained from the comparison between the three peptide standards; where the marker peptide showed ~13x higher peak area than Cys18 which in turn displayed ~12x higher response than Cys138 peptide (Figure A2-3 in Appendices).

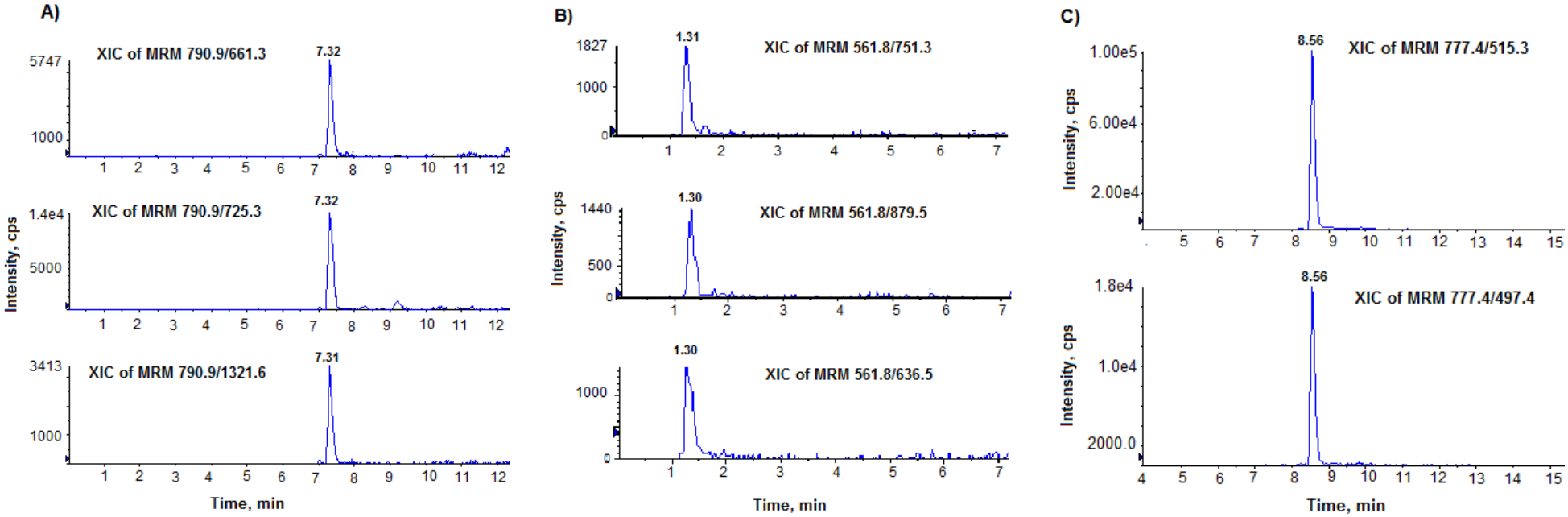


Figure 2-16: XIC of MRM for the best transitions of IAM modified Cys18, HLVIHDESTC18EQL (A), IAM modified Cys138, KDKDC138TSRL (B) and the marker peptide, SVTQVPF detected from plasma chymotryptic digest. All the transitions from the same peptide were eluted at the same retention time indicating that the product ions are from the same precursor. No other secondary peaks were detected in the XIC which indicate the specificity of the monitored transitions.

The different MS response among the three peptides is not unexpected. The relative ionisation and fragmentation of the peptide are dependent on multiple factors including the sequence, other co-eluting ions and the presence of PTM. This can lead to different MS responses even between peptides released from the same protein. In fact, peptides from the same protein have been reported to produce ion currents which differ by at least $1x10^3$ in LC-MS/MS experiment [79].

In the current work the coupling of the selective AGT enrichment with conventional LC-MS/MS (micro-flow was used) achieved shorter chromatographic run time (20 min) than nano-LC-system (hours) typically used in protein analysis by MS giving the method a higher throughput. Moreover, the method allowed the detection of Asn^{14} and Asn^{137} peptides, which were very challenging to be detected by previous studies using different approaches such as multi-lectin [263] and immune-affinity depletion coupled with glycoprotein extraction by hydrazide resin [266].

2.4.3.3 Confirmation of the detected peptide identity by LC-MS/MS

Even though MRM signals are highly analyte-specific, and the MRM transitions for each peptide were detected at the same retention time as the corresponding standard, the identity of the three peptides was further confirmed by LC-MS/MS analysis of the plasma digest. The acquired spectra were very similar to their corresponding standards (Figure 2-17). The amino acid sequence of generated peptides was confirmed using comprehensive *y*- and *b*-ion series for Cys18 (Figure 2-17, A), whilst most of the detected product ions were *y* ions for Cys138 (Figure 2-17, B). Poor fragmentation was noticed with the marker peptide, but still reliable with the standard (Figure 2-17, C). The selected product ions in MRM transitions for each peptide were apparent in the MS/MS spectra.

For Cys18 and Cys138 plasma MS/MS spectra, product ions with 1 Da higher *m/z* than the corresponding standard fragmentation (b_{11}, b_9 and y_{11} for Cys18 and y_8, y_7 and y_6 for Cys138) were observed. This is due to the conversion of Asn to Asp by deglycosylation with PNGase F. *m/z* were higher either by 1 or 0.5 Da for single and double charged fragments respectively. The shift in *m/z* only affected fragments with Asn in the sequence.

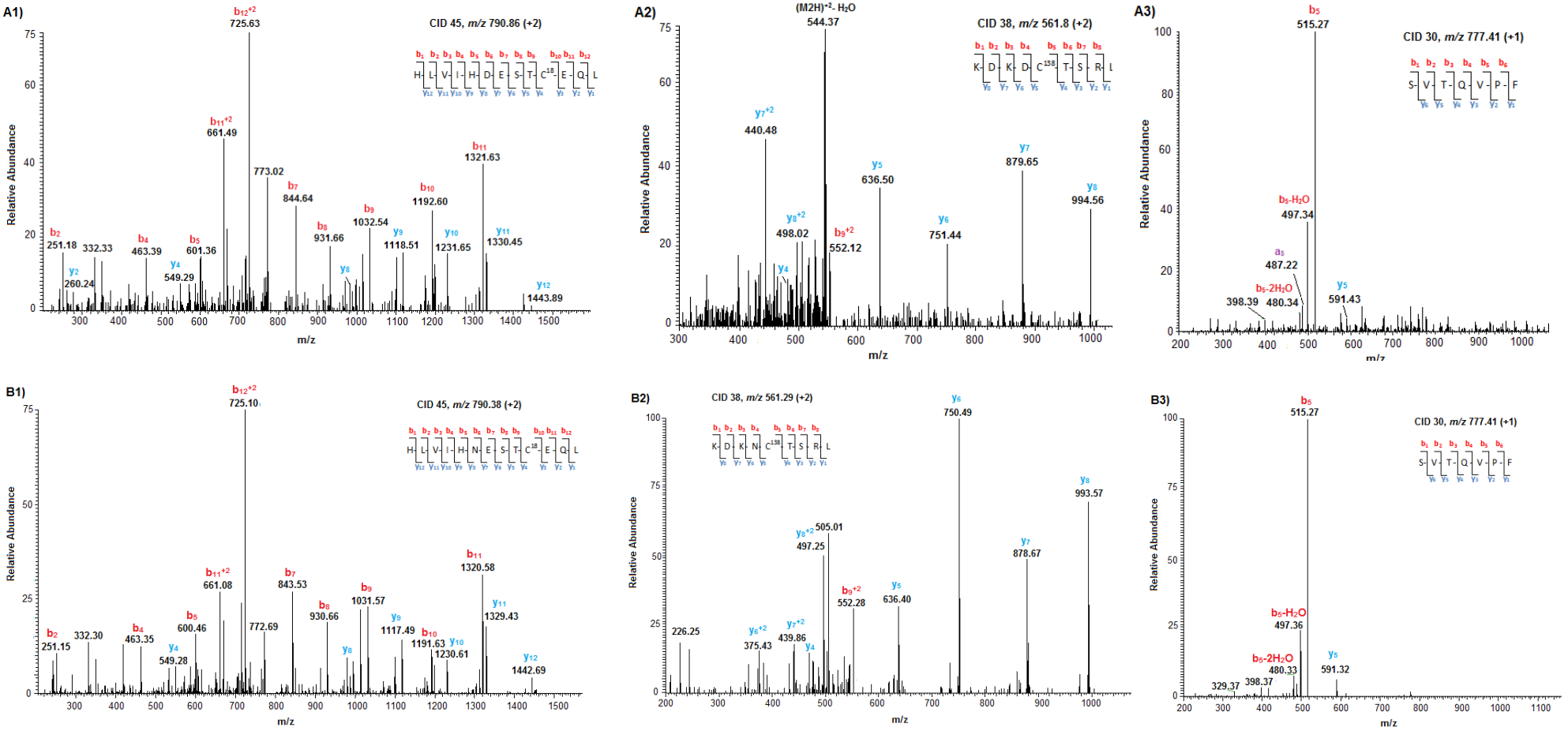


Figure 2-17: (Above) the average scan MS/MS spectra for the IAM modified Cys18, Cys138 and marker peptides detected from plasma chymotryptic digest (A1, A2 and A3 respectively). (Below) the average scan MS/MS spectra for the corresponding IAM modified Cys18, Cys138 and marker peptide standards (B1, B2 and B3). The acquired spectra were very similar to their corresponding standards confirming the identity of the AGT peptides detected in the human plasma.

2.4.4 Method validation

A reliable level of 43% (± 2.0%) for the overall recovery of AGT from the plasma, estimated by five experiments, was achieved which enables the reproducible detection of the three AGT peptides. AGT marker peptide and IAM modified Cys18 peptide showed acceptable analytical precision (CV < 15%), while modified Cys 138 peptide had a higher variability as shown in Table 2-5. As expected, the better the signal intensity and S/N ratio, the higher the analytical precision and the lower CV that can be achieved [79]. This was apparent with the CV values obtained from the three peptides. The marker peptide showed the best CV while Cys138 was the lowest. However, the sum of more than one transition brought the CV for Cys138 close to 17%.

Although Cys18 has one missed cleavage site in its sequence, the peptide was reproducibly detected with an acceptable CV of 10%. This indicates that peptides with missed cleavages do not seem to introduce an extra source of variability under reproducible digestion conditions. This is consistent with the findings of Chiva *et al.* [222], who reported the selection of missed cleavage peptide as a signature peptide for the relative quantification of proteins was not associated with higher method variability, and that they produced similar quantitative results (precision and accuracy) when compared to a peptide with no missed cleavage.

The promising CV achieved by coupling the new AGT extraction strategy with targeted LC-MS/MS demonstrates the high reproducibility of the developed methodology. It also provides the confidence necessary for the quantitative analysis of the AGT peptides in clinical samples, Chapter Three, where other validation parameters such as linearity will be investigated.

Table 2-5: Analytical precision for the three AGT peptides.

Peptide	Peptide transitions	Analytical precision[a]
Marker Peptide	777.4 / 515.3	8.1
IAM alkylated Cys18	790.9 / 725.3	10.3
IAM alkylated Cys138	Sum of three transitions[b]	17.2

[a] Presented as CV% from 6 experiments.
[b] The transitions are 561.8/751.3, 561.8/879.5, and 561.8/636.5

2.5 Conclusion

The purpose of this work was to develop a targeted LC-MS/MS method for the reproducible detection of the two signature AGT Cys peptides, Cys18 and Cys138. These two peptides are known to be involved in the AGT redox switch linked to pre-eclampsia [197] hence development of methods for their detection in human plasma samples is crucial. The current work represents the first MS-based method available for the redox measurements of AGT in the plasma. During method development, a close attention to details in the experimental design was necessary to lend credibility to the results obtained.

The presented study showed that in-solution digestion with chymotrypsin using IAM as an alkylating agent was associated with the best results regarding signal intensity, complete alkylation and reproducible detection of the signature Cys peptides. The method avoided the use of sizable costly alkylating reagents such as ICAT, TMT, biotinylated IAM generally used in redox protein analysis. These alkylating reagents can limit effective alkylation especially when used under non-denaturing conditions. Moreover, the use of targeted LC-MS/MS method working under the MRM mode provided a higher selectivity and sensitivity to detect the AGT signature peptides in the complex plasma digest than LC-MS workflow that is used in redox protein analysis, particularly with ICAT alkylating reagent. The coupling of a new two-step extraction strategy (ConA and RP-SPE) with a micro-scale LC-MS/MS gave the method a higher throughput in terms of sample preparation and MS run time compared to other reported studies that used immunodepletion enrichment and a nano-LC system to analyse proteins in biological samples.

The new targeted LC-MS/MS method developed in this chapter should enable the identification and the quantification of the two AGT forms (oxidised/reduced), reported to be involved in the pathogenesis of pre-eclampsia, in the plasma when used with differential alkylation approach. The importance of the demonstrated method could be seen in the acceptable analytical precisions that were achieved for the three AGT peptides, of most important are the Cys peptides which permitted the application of the methodology to clinical samples, as taken from pre-eclamptic women (Chapter Three).

In conclusion, the presented findings provide a good level of confidence to move towards the quantification of the reduced and oxidised forms of AGT in human plasma samples collected from pre-eclapmtic patients.

Chapter Three

Quantification of Plasma Angiotensinogen Redox Forms in a Case-control Study of Pre-eclampsia

3 Quantification of Plasma Angiotensinogen Redox Forms in a Case-control Study of Pre-eclampsia

Summary

The disulphide linkage between Cys 18 and 138 has a key role in the redox switch of AGT that modulates the release of angiotensin I, and hence production of angiotensin II, with consequential effects on blood pressure. An increased percentage of the oxidised form of AGT is present in the plasma of pre-eclamptic women. Currently, there is no quantitative LC-MS/MS approach to detect and quantify the sulphydryl-bridged (oxidised) and the free thiol (reduced) forms of AGT in human plasma. The aim of the work described in this chapter was to quantify the level of total AGT and the percentage of AGT oxidation in the plasma of pre-eclamptic women (n=17), normotensive matched pregnant controls (n=17), and healthy non-pregnant women (n=10) using a differential alkylation approach coupled with the targeted LC-MS/MS method developed in Chapter 2.

Plasma glycoproteins were enriched via ConA Sepharose followed by fractionation by RP-SPE. The dried collected fraction, containing the highest % of AGT, was deglycosylated with PNGase F followed by alkylation of the protein reduced form with iodoacetamide. The oxidised form of the protein was reduced first, then the free thiol was blocked with $^{13}C_2,D_2$-iodoacetamide and digestion was carried out using chymotrypsin. Targeted LC-MS/MS method was used to quantify the total and the oxidation plasma levels of AGT based on the quantification of the marker AGT and Cys18 peptides respectively.

The study revealed a significant increase in the level of AGT (~3.5 fold) in the maternal circulation compared to non-pregnant women and showed that patients with pre-eclampsia had significantly higher plasma level of oxidised AGT (70 ± 4.8) compared to matched normotensive pregnant controls (65.3 ± 3.6, P=0.008), whilst maintaining a similar total AGT level in the plasma. This provides further evidence that the conversion of the reduced form to its more active oxidised form might be a contributing factor to the hypertension characteristic of pre-eclampsia.

The findings of this study confirm the previous two reports that linked the AGT redox switch with the pathogenesis of pre-eclampsia, and provide an extra line of evidence linking the oxidative state and the generation of reactive oxygen species in the placenta with hypertension in pregnancy that leads on to the development of pre-eclampsia.

3.1 Introduction

3.1.1 Oxidative stress and protein modification

There is an increasing interest on the effect of reactive oxygen species (ROS) on modifying protein activity and function. ROS including superoxide ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), nitric oxide ($NO^{\cdot}$), and a variety of lipid peroxide electrophiles are generated by variable factors such as metabolic events, environmental processes, or ischemia, and can lead to disruption of the biological function of endogenous proteins [212]. Normally, the levels of ROS are tightly regulated by antioxidant defence mechanisms [254; 267], however, excessive generation of ROS and/or impaired antioxidant defences results in oxidative stress which is linked with the aging process and many diseases including cardiovascular disorders [268], cancer [269] and pre-eclampsia [270]. Excess ROS can directly affect membrane lipids and nucleic acids, but one of their major harmful effects is the aberrant modification of the thiol group of the cysteine (Cys) amino acid of proteins, which has detrimental consequences on protein activity [212; 267]. The rates and reaction products of the ROS modification of the reactive Cys residues are determined by the local environment of the Cys residue in the protein [271]. Upon exposure to ROS, a transient highly unstable compound, sulphenic acid (RSOH), will be formed. Sulphenic acid reacts further to produce disulphide or a more stable higher oxidation product such as sulphinic acid or sulphonic acid which are generally produced under severe oxidation conditions [271], (Figure 3-1). The formation of the disulphide bond is a reversible type of oxidation and plays a regulatory role while the remaining Cys modifications are irreversible and related to a damage of protein activity.

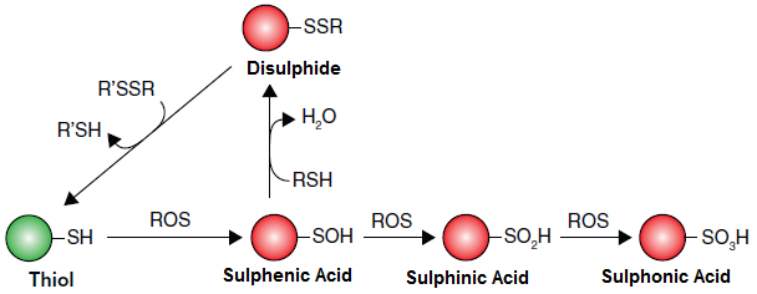


Figure 3-1: Reversible and irreversible cysteine modifications. Oxidation of Cys thiol (R-S^{-}) by ROS leads to the formation of unstable reactive sulphenic acid which can react with another Cys thiol to form a disulphide bond. This type of oxidative modification is reversible. Alternatively sulphenic acid may be oxidised to sulphinic acid, and under severe oxidising conditions, sulphonic acid will be formed. The last two oxidative modifications are irreversible *in vivo*. Figure 3-1 is taken from reference [213].

In either case, the identification of these sites and the quantitative assessment of their modification have become increasingly important since these modifications have large consequences on protein function and activity by affecting the catalytic activity or by altering the conformation of the protein. Qualitative and quantitative analysis of Cys modifications can improve the understanding of how proteins and pathways will respond to the oxidative stress associated with many diseases and, thereby, facilitate the discovery of new treatment possibilities for diseases in which the redox balance is compromised [272].

3.1.2 Approaches for the identification and quantification of protein oxidation status

Methods for the identification and quantification of oxidative post-translational modifications are developing rapidly. Several works have been implemented in the field of cysteine-based redox protein analysis, and many researchers have focused their attention on the effect of oxidative stress on different protein thiol modifications including reversible and irreversible reactions [213; 273; 274; 275]. Measuring the oxidation state of Cys is very challenging due to the high reactivity of this amino acid which requires thoughtful sample preparation to preserve the endogenous oxidation state, and specialised analytical approaches to detect and quantify the oxidation at the site-specific level. The advances in MS-based protein analysis techniques, including a range of variable redox analysis methodologies, and the availability of several Cys selective chemical probes (discussed in Section 2.1.1.1, Chapter Two) have considerably aided in redox protein analysis, and provided researchers with the tools and workflows necessary to assess thiol redox changes in complex biological systems [213; 273; 274; 275]. Nevertheless, there is no one workflow that fits all solutions and the limitations of each methodology must be considered.

For the purpose of this research, the most common approaches followed for the detection and quantification of reversible disulphide oxidation in proteins are described below.

3.1.2.1 Redox differential gel electrophoresis

Redox differential gel electrophoresis (GE) is a gel-based fluorescence approach that compares the relative levels of oxidation between two samples. Initially, each sample is alkylated with unlabelled NEM or iodoacetamide and then reduced and fluorescently labelled with either green Cy3- or red Cy5-maleimide to code each sample [276]. After

that, the samples are combined and analysed by 2D-GE. Protein spots with an equal level of oxidation will appear as yellow spots due to the equal signals of the green Cy3- and red Cy5-maleimide dyes, while protein spots with either green or red colour will reflect differentially oxidised proteins, which can be identified by in-gel digestion and MS analysis [276]. However, it is difficult to conclusively identify which protein is oxidised if more than one protein is identified per spot. Besides, 2D-GE suffers from limited dynamic range which can restrict its usefulness in redox proteomics [212].

3.1.2.2 Biotin-conjugated iodoacetamide

Biotin-conjugated iodoacetamide is one of the commonly used reagents to detect protein oxidation by selective labelling of Cys residues which are found in the reactive thiolate anion form at neutral pH under both normal and oxidative stress conditions [277]. For analysis, proteins are digested and the generated alkylated Cys peptides are enriched by biotin and analysed by MS to identify the reactive cysteines. Alternatively, intact proteins can be purified and the change in the oxidation of specific proteins between conditions can be assessed by Western blot [212]. In these experiments, cysteines that become oxidised after exposure to oxidant stress do not react with iodoacetamide and exhibit a decrease in biotin-conjugated iodoacetamide labelling corresponding to increased oxidation of the protein [254].

A potential limitation of this approach is the bulky nature of the biotin iodoacetamide reagent that may limit efficient alkylation and thus accurate quantification [277]. This limitation is also applicable to other methodologies that use sizable alkylating agents especially if alkylation is performed under non-denaturing conditions [212].

3.1.2.3 Isotope-coded affinity tags (ICAT) and OxICAT

As mentioned earlier (Section 2.1.1.1, Chapter Two), ICAT reagents are widely used in redox quantitative proteomics since they label protein cysteines in two sample conditions (*e.g.* one control and the other oxidant exposed) with two tags of different mass (9-^{12}C or 9-^{13}C atoms). The major disadvantage of the technique is that the modified cysteine in the protein is not tagged, and therefore not available for examination [251] so differences in the signal may reflect changes in protein abundance between samples rather than oxidation [212]. This is a limitation to all approaches that label only the free thiol of the protein.

To overcome this limitation, Leichert *et al.* [273] have developed the OxICAT method to quantify reversible oxidation of cysteine residues by differential alkylation of a single sample with both ICAT reagents, taking advantage of the fact that the stable isotope alkylated peptides have equivalent ionisation efficiencies by MS. In the OxIACT method, one reagent alkylates the free thiol of Cys, before reduction, to reflect the level of non-oxidised form of the protein and the second reagent alkylates after reduction to code for the oxidised form of the protein.

ICAT quantitation is mainly performed at the MS level which has a lower dynamic range than quantification at the MS/MS level, therefore, ICAT methodology has less potential to detect and accurately quantify the oxidation state of low abundance proteins. Additionally, ICAT quantitation has less ability to distinguish the oxidation status of individual Cys in peptides with two or more Cys [212].

3.1.2.4 Differential alkylation

Differential alkylation is the most common approach followed for the identification and quantification of reversible cysteine oxidations in proteins [216; 254]. It is a flexible technique that labels redox sensitive Cys before and after reduction using two different alkylating agents. In this approach, an initial irreversible alkylation of all free thiols with the first alkylating agent will be carried out to avoid any thiol–disulphide exchange reactions that can affect the assessment of the oxidation status. A subsequent reduction of the disulphide bond and modification of the free thiols with a second alkylating agent would uniquely identify the two protein states, Figure 3-2 [216]. Thiol-specific alkylation reagents can be conjugated to a wide variety of fluorophores and epitope tags for enrichment and detection of alkylated Cys. Moreover, stable-isotope labelled alkylating agents (*e.g.* d_5-NEM, d_4-IAM) are also available which enhance the use of the differential alkylation approach for redox protein detection and quantification [212].

For accurate quantitative comparisons of the two oxidation states of a protein by MS using the differential alkylation approach, the two alkylating agents must give differentially labelled Cys peptides with very similar physiochemical properties to ensure they have similar ionisation efficiencies and elute at the same retention time and therefore experience an identical environment [278]. This can be achieved by using chemically identical but isotopically different Cys alkylating agents to allow the two peptides to be mass distinguishable by MS. Consequently, the sum of the intensity of the

two alkylated peptides will equal the total amount of Cys, and the ratio of the oxidised signal to the total signal will be equivalent to the percent oxidation of the Cys [273].
In selecting suitable isotopically labelled reagents, cost, availability and the resulting mass differences are important factors. Deuterated reagents, in which ^{1}H atom is replaced with deuterium (D), are widely available at reasonable cost. However, the addition of multiple deuterium atoms can sufficiently reduce the polarity causing a shift in the retention time (the deuterated peptides will elute slightly earlier than the ^{1}H analogues). Normally, this problem is avoided with the use of ^{13}C labelled alkylating reagents, nevertheless, these reagents are generally more expensive [278].

To enhance the ability to detect redox changes in low abundance proteins, Held *et al.* [275] have developed an approach termed OxMRM, quantitative cysteine oxidation analysis by MRM. This approach combines differential alkylation of the sample with targeted MS/MS analysis using MRM technique and was successfully used to quantify the percent oxidation of targeted Cys from a cellular extract. OxMRM workflow can distinguish between the oxidation of two Cys within a peptide and can measure many Cys simultaneously. Moreover, the sensitivity of the OxMRM analysis enables the quantification of the redox status of low abundance proteins which present a challenge for quantification by other quantification approaches with a limited dynamic range, such as data-dependent acquisition MS or gel-based fluorescent techniques [212].

Examples of different studies that applied the approaches described above to identify and quantify the oxidation state of cysteine proteins are summarised in Table 3-1.

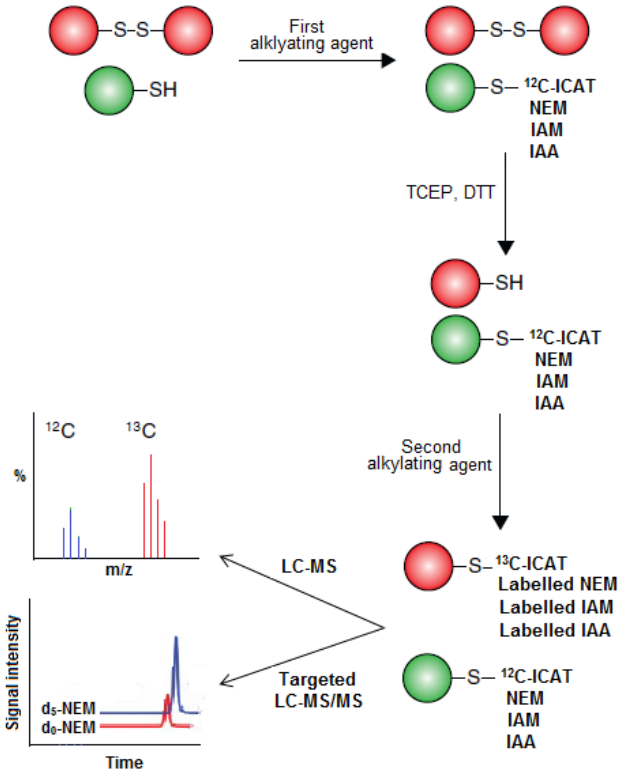


Figure 3-2: Differential alkylation approach. Initially irreversible alkylation of all free thiols with the first unlabeled alkylating agent (NEM, IAM, ICAT) will be carried out. A subsequent reduction of the disulphide bond will be performed with DTT or TCEP and the free thiols will be alkylated with the labelled version of the alkylating agent (d_5-NEM, d_4-IAM, ^{13}C-ICAT). The differentially alkylated Cys peptides representing the two protein redox status will be analysed either by LC-MS (mainly ICAT) or by targeted LC-MS/MS. Figure 3-2 modified from reference [213].
IAA: iodoacetic acid, IAM: iodoacetamide, ICAT: isotope-coded affinity tags, NEM: *N*-ethylmaleimide

Table 3-1: Selected studies that investigated cysteine oxidation status in pure or endogenous proteins using different alkylation approaches

Analayte	Approach followed	Brief description	Analysis	Ref.
Bovine serum albumin and oval albumin	Differential alkylation	NEM was used to alkylate the reduced Cys while IAM alkylation was performed on gel after DTT reduction using labelled form linked to a biotin tag.	MALDI-TOF	[254]
Human recombinant oestrogen receptor	Differential alkylation	Initial irreversible alkylation of all free thiols with [^{13}C2] bromoacetic acid followed by reduction of any disulphides and treatment with iodoacetic acid.	HPLC-MS	[278]
T lymphocyte cell line	Differential alkylation	Cells were disrupted in the presence of NEM to block the reduced thiol proteins and DTT was added to reduce the oxidised thiol proteins before labeling with 5-iodoacetamidofluorescein.	2D GE and Identification using MALDI-TOF	[218]
Mitochondria-enriched rat hearts	Redox differential gel electrophoresis	Measured the oxidation of 50 proteins most of which were differentially oxidised after treatment with hydrogen peroxide. Initial blocking step was achieved with NEM, followed by reduction and the subsequent Cy3 or Cy5 maleimide labelling.	2D-GE and identification by in-gel digestion and MS (MALDI-TOF-TOF)	[276]
E.coli	Redox differential gel electrophoresis	The overall cysteine oxidation state of ~100 *E. coli* proteins was determined per 2D-gel. IAM was used to label the free thiols under denaturing conditions and radiolabelled [^{14}C]-IAM, after reduction, has been used to identify oxidised proteins.	2D-GE and identification by in-gel digestion and MS	[279]
Caenorhabditis elegans	The OxICAT approach	42 cysteines with over a 1.5-fold increase in oxidation in *Caenorhabditis elegans* treated with hydrogen peroxide, representing ~20% of the total cysteines were detected in the sample.	LC-MS/MS	[280]
Human recombinant redox-regulated chaperone Hsp33	The OxICAT approach	The OxICAT approach was able to precisely quantify oxidative thiol modifications at distinct cysteines within the protein.	LC-MS/MS	
The 45 subunit Complex I of the electron transport (mouse model of parkinson's disease)	OxMRM: Quantitative Cys oxidation analysis by MRM	Quantified the reversible oxidation status of 34 cysteines in the 45 subunit complex I and discovered 6 of those to be oxidised in a mouse model of Parkinson's disease. d_5-NEM was used to alkylate the reduced thiol while oxidised cysteines were alkylated with d_0-NEM after reduction.	Targeted LC-MSMS	[281]
Cancer cell lines and primary fibroblasts	OxMRM: Quantitative Cys oxidation analysis by MRM	Quantified the percent oxidation of seven of the 10 cysteines of endogenous p53, which is a highly negatively regulated protein in both cancer cell lines and primary fibroblasts. d_0-NEM was used to alkylate the reduced thiol while oxidised cysteines were alkylated with d_5-NEM after reduction.	Targeted LC-MSMS	[275]
Rat liver mitochondria, Human fibroblasts	Organelle specific alkylation	Targeted labelling of mitochondrial thiols with (4-iodobutyl)triphenylphosphonium enables measurement of the thiol redox state of individual mitochondrial proteins during oxidative stress and cell death.	2D GE and Western blotting	[282]

3.2 Angiotensinogen redox switch in pre-eclampsia

The reversibility of some of the oxidative protein modifications, such as disulphide bond formation, makes them ideally suited to take on regulatory roles in protein function [272]. A disulphide bond is the main product of Cys oxidation that is formed as a consequence of increased exposure to ROS associated with oxidative stress. Oxidative stress, in turn, can initiate many common diseases and conditions (*e.g.* atherosclerosis and diabetes), or act as the basis for their complications (*e.g.* cancer and neurodegenerative disease) [268; 269; 283]. In pre-eclampsia, oxidative stress is mainly initiated as a consequence of impaired cytotropoblast invasion that leads in early pregnancy to inadequate placentation, and later to reduced perfusion of the placenta resulting in ischemia, described previously in Section 1.3.2, Chapter One. The oxidative state in pre-eclampsia is proposed to enhance the conversion of AGT in the maternal circulation in pre-eclampsia to its more active oxidised form by the formation of a disulphide-bridge between Cys18 and Cys 138 [197]. The oxidised form of AGT, compared to the free thiol form, interacts with renin with four-fold higher binding affinity resulting in an increased generation of angiotensin I and hence angiotensin II [197]. Using Western blotting or ELISA, previous studies have revealed a significant decrease in the level of the reduced form (free thiol) of AGT, reflecting a higher level of the oxidised form, in the plasma of pre-eclamptic women compared to controls [197; 206], which could be linked functionally to the increase in the blood pressure in pre-eclampsia [197].

Only two studies have reported the involvement of the AGT redox switch in the pathology of pre-eclampsia [197; 206] and they both relied on antibody-based methods to quantify the reduced form of AGT in the plasma of pre-eclamptic women. Therefore, there is a need for a repeated clinical study that uses an entirely different quantification approach to evaluate and validate the previous findings. A differential alkylation approach coupled with the developed targeted LC-MS/MS method should enable the detection and the quantification of the two distinct forms, the free thiol reduced form and the sulphydryl-bridged oxidised form of AGT in the plasma, with high sensitivity and selectivity.

3.2.1 Aim of the chapter

1) To detect the oxidised and the reduced forms of AGT in the plasma using the Cys peptides involved in the protein redox switch as signature peptides and applying a differential alkylation strategy.
2) To measure the concentration of total AGT protein in the plasma of pre-eclamptic women, matched controls and normal non-pregnant women.
3) To quantify the oxidation level of AGT in the plasma of pre-eclamptic women, matched controls and normal non-pregnant women.

3.3 Materials and Methods

3.3.1 Materials

Isotope labelled iodoacetamide $^{13}C_2,D_2$ (98 atom% D, 99 atom% ^{13}C) was purchased from Sigma-Aldrich (Gillingham, UK). All other materials are as described in Section 2.3.1, Chapter Two.

Peripheral blood samples from pre-eclamptic and age-matched normotensive pregnancies and healthy non-pregnant women were collected after obtaining fully informed written consent from each participant. Ethics permission for the sample collection and utilisation was approved by the Hospital Ethics Committee of the Nottingham University Hospitals (OG 090301, LREC2 Q2090312).

3.3.2 Patient recruitment

The study population consisted of 3 groups of women: 10 healthy non-pregnant women, 17 pre-eclamptic, and 17 gestational age-matched normotensive pregnant women. Pre-eclampsia was stringently defined and pregnant women in the pre-eclampsia group had (1) normal booking blood pressures (at 12-20 weeks), (2) subsequently developed blood pressures of ≥ 140 mmHg systolic or ≥ 90 mmHg diastolic on two occasions (minimum 24 h apart) and (3) had at least 1+ proteinuria (≥ 300 mg/L) using dipstick analysis [284], as per the guidelines of the American College of Obstetricians and Gynecologists for diagnosis of pre-eclampsia [285]. Subjects in the control group did not have any documented hypertensive problems throughout their pregnancy.

Maternal demographic and clinical characteristics of pre-eclampsia and control samples are covered in Table 3-2.

3.3.3 Sample collection and processing

Blood samples were collected into chilled tubes containing EDTA and centrifuged immediately after collection at 1000x *g* for 15 min at 4ºC. The separated plasma was then transferred to Eppendorf tubes in 0.5 mL aliquots, snap-frozen and stored at -80°C.

3.3.4 Sample preparation for LC-MS/MS analysis

Plasma glycoproteins were extracted using ConA Sepharose from the plasma (50 µL) of the three studied groups (pre-eclampsia, age-matched controls, and non-pregnant women) and then fractionated by RP-SPE, applying two-step elution using 40% and 50% of 0.1%TFA as described in Sections 2.3.3.1, Chapter Two. The dried collected RP-SPE fraction, containing the highest % of AGT, was reconstituted in 2 M urea (prepared in 50 mM Tris buffer, pH 7.4), then deglycosylated with PNGase F (1 µL enzyme for each 40 µg proteins) for 1.5 hr at 37°C followed by alkylation of the protein reduced form with 200 mM iodoacetamide. The oxidised form of the protein was reduced first with 50 mM DTT, and the free thiol was then blocked with $^{13}C_2$,D_2-iodoacetamide to achieve a 4 mass unit increase in the mass of singly charged Cys peptides and thus make the two AGT forms (reduced and oxidised) mass distinguishable. All reactions were conducted using 10 kDa Amicon ultra 0.5 centrifugal filter devices as described in Section 2.3.3.3, Chapter Two. Digestion was carried out using chymotrypsin at 37°C using enzyme: protein ratio of 1: 50 (*w/w*), and after 4 h a second aliquot of chymotrypsin (1:100 enzyme: protein *w/w* ratio) was added to ensure complete digestion, and digestion proceeded at 37°C overnight. After quenching the digestion reaction with 1% formic acid, NEM alkylated Cys18 peptide was added to the samples to achieve 0.2 µM final concentration and act as an internal standard. The samples were then centrifuged for 5 min at 11000x *g* and transferred for LC-MS/MS analysis.

3.3.5 Quantification of the total AGT and AGT oxidation levels in the plasma using targeted LC-MS/MS

Chymotrypsin digested peptides were first separated by a Shimadzu series 10AD VP LC system and analysed using a 4000 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer operating in positive ion mode as described in Section 2.3.3.5, Chapter Two. MRM transitions were monitored and acquired at unit resolution in both Q1 and Q3 for the following AGT signature peptides; (1) AGT marker peptide (SVTQVPF) which was used to infer the plasma level of total AGT (2) iodoacetamide alkylated Cys18 and

Cys138 peptides which correspond to the reduced form of plasma AGT and (3) $^{13}C_2$,D_2-iodoacetamide alkylated Cys18 and Cys138 peptides which correspond to the oxidised form of plasma AGT. The final optimised precursor/product ions transitions that were used to monitor the above peptides are presented in Table 3-3. All data were processed by Analyst software 1.4.2.

To confirm the identity of the differentially alkylated Cys peptides which represent the two distinct AGT forms detected in the plasma, samples were also analysed on an ion trap mass spectrometer coupled to HPLC (LTQ Velos) from Thermo Scientific (San Jose, CA). MS/MS spectra of the peptides of interest were acquired in positive ion MS data-dependent mode in a mass range of 200–1650 with one unit mass resolution, parent mass width ± 0.5 and retention time window 1 min as mentioned in Section 2.3.3.5, Chapter Two.

Quantification of total plasma AGT

The signal area of the AGT marker peptide was used to reflect the plasma total AGT. For relative measurement of plasma AGT, the ratio of AGT marker peptide signal area over the internal standard signal area was calculated and the mean of the peak area ratio was compared between the three studied groups to measure protein fold changes.

For absolute AGT measurement, marker peptide standard was spiked into the plasma chymotryptic digest to get the following final concentrations: 5, 10, 50, 100, 150, 200, 250, and 400 nM. The ratio between the marker peptide standard peak area and the internal standard peak area was plotted against the marker peptide standard concentrations. The calibration curve obtained was used to determine the concentration of AGT in the plasma.

Measurement of the oxidation level of plasma AGT

To provide a quantitative comparison of the oxidation level of AGT in the plasma, the level of Cys18 peptide oxidation was calculated and compared between the three conditions (pre-eclampsia, controls and non-pregnancy). The level of Cys18 peptide oxidation was calculated as the percentage of the peak area of the oxidised Cys18 peptide (alkylated with $^{13}C_2$,D_2-ioodacetamide) over the sum of the reduced Cys18 peptide (alkylated with $^{13}C_0$,D_0-iodoacetamide) and oxidised Cys18 peptide as shown below:

$$\text{Oxidised AGT \%} = \frac{\text{Oxidised Cys peptide } (^{13}C_2,D_2\text{-IAM})}{\text{Oxidised Cys peptide } (^{13}C_2,D_2\text{-IAM}) + \text{Reduced Cys peptide } (^{13}C_0,D_0\text{-IAM})} \text{ X } 100\%$$

3.3.6 Statistical Analysis

Descriptive subjects' characteristics of pre-eclampsia and control groups were compared using a two-tailed independent *t*-test for continuous parameters, and Pearson Chi-square for comparative analysis of categorical variables. A p-value ≤ 0.05 was considered statistically significant.

One way ANOVA (with post hoc Tukey HSD test if significant) was used to compare the plasma levels of the total and the oxidised AGT between the three studied groups. All data are presented as the mean ± standard deviation. Statistical analysis was performed with the Statistical Package for Social Sciences version 22.0 (SPPS Inc., Chicago, IL, USA) and GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA 92037 USA).

3.4 Results

3.4.1 Maternal demographic and clinical characteristics

Table 3-2 describes the maternal characteristics of the 34 pre-eclampsia cases and controls and the 10 non-pregnant women who participated in this study. All women included in the study were European except one African in the pregnant control group and one South East Asian in the pre-eclampsia group. None of the pregnant women in either group were taking medication other than paracetamol or prescribed antihypertensive agents. Non-pregnant women were not taking any medication, including oral contraceptives.

No significant differences were detected between pre-eclampsia and matched control groups with regards to blood pressure at booking, delivery method, maternal age, parity, and body mass index (BMI).

Both pregnancy groups conceived spontaneously and carried singleton pregnancies. The normal pregnancy controls had all delivered healthy babies without any pregnancy complications and gave birth to infants weighing >2500 g, delivered 38 weeks or later. Women in the pre-eclampsia group all had moderate to severe disease, without HELLP and had significantly higher blood pressure, lower gestational ages at delivery and lower infant birth weight than the control group (Table 3-2). All neonates from both pregnancy groups survived.

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Table 3-2: Maternal demographic and clinical characteristics of pre-eclampsia cases and their matched controls

Maternal Characteristics	Non-pregnant (*n=10*)	Controls (*n=17*)	Pre-eclampsia (*n=17*)
Age (years)	33.4± 11.3	29.3 ± 6.8	32.5 ± 6.0
BMI at booking (kg/m^2)	22.1 ± 1.8	25.6 ± 4.9	26.7 ± 5.8
Systolic blood pressure at booking (mm Hg)	-----	113.7 ± 11.4	122.6 ± 8.9
Diastolic blood pressure at booking (mm Hg)	-----	68.7 ± 10.6	73.6 ± 11.7
Systolic blood pressure outside labour (mm Hg)	-----	115.9 ± 4.4	158 ± 11.3*
Diastolic blood pressure outside labour (mm Hg)	-----	75.5 ± 2.8	97.9 ± 5.3*
Gestation age at delivery (weeks)	-----	39.7 ± 1.2	36.4 ± 4.3*
Caesarean section, n (%)	-----	3 (17.6%)	6 (35.3%)
Birthweight (kg)	-----	3.49 ± 0.43	2.78 ± 1.16*

Data are presented as mean ± SD except for caesarean sections (No. (percentage)).
* P< 0.05 between normotensive and pre-eclamptic pregnancies.

3.4.2 Detection of the oxidised and the reduced forms of AGT in the plasma

The two distinct redox forms of AGT were detected in the human plasma using the developed targeted LC-MS/MS workflow and applying a differential alkylation approach. Differentially alkylated Cys18 peptide and AGT marker peptides with their corresponding MS/MS spectra are presented in Figure 3-3 and Figure 3-4 respectively. The optimised transitions that were used to detect the $^{13}C_0,D_0$-iodoacetamide and $^{13}C_2,D_2$-iodoacetamide alkylated Cys peptides which corresponded to the reduced and the oxidised forms of AGT are presented in Table 3-3.

Alkylation with $^{13}C_2,D_2$-iodoacetamide introduced a 4 dalton increase in the mass of the singly charged Cys peptides (2 Da for doubly charged peptides) compared to the unlabelled alkylated peptide which made the two plasma AGT forms mass distinguishable when analysed using the developed targeted LC-MS/MS method. The differentially alkylated Cys peptides, that represent the oxidised and the reduced forms of plasma AGT, exhibited similar physiochemical properties and therefore were eluted at the same retention (Figure 3-3). This ensured that the two peptide forms experienced similar chemical environment upon analysis which is critical for accurate quantification.

The identity of the oxidised and the reduced Cys18 peptides was confirmed by MS/MS (Figure 3-3) and, as expected, an increase in the mass (4 Da for singly charged and 2 Da for doubly charged) of fragment ions that incorporate the modified cysteine amino acid in their sequence was noticed in the MS/MS spectrum of $^{13}C_2$,D_2-iodoacetamide alkylated Cys peptide (*e.g.* b_{11}^{+1} ion in Figure 3-3).

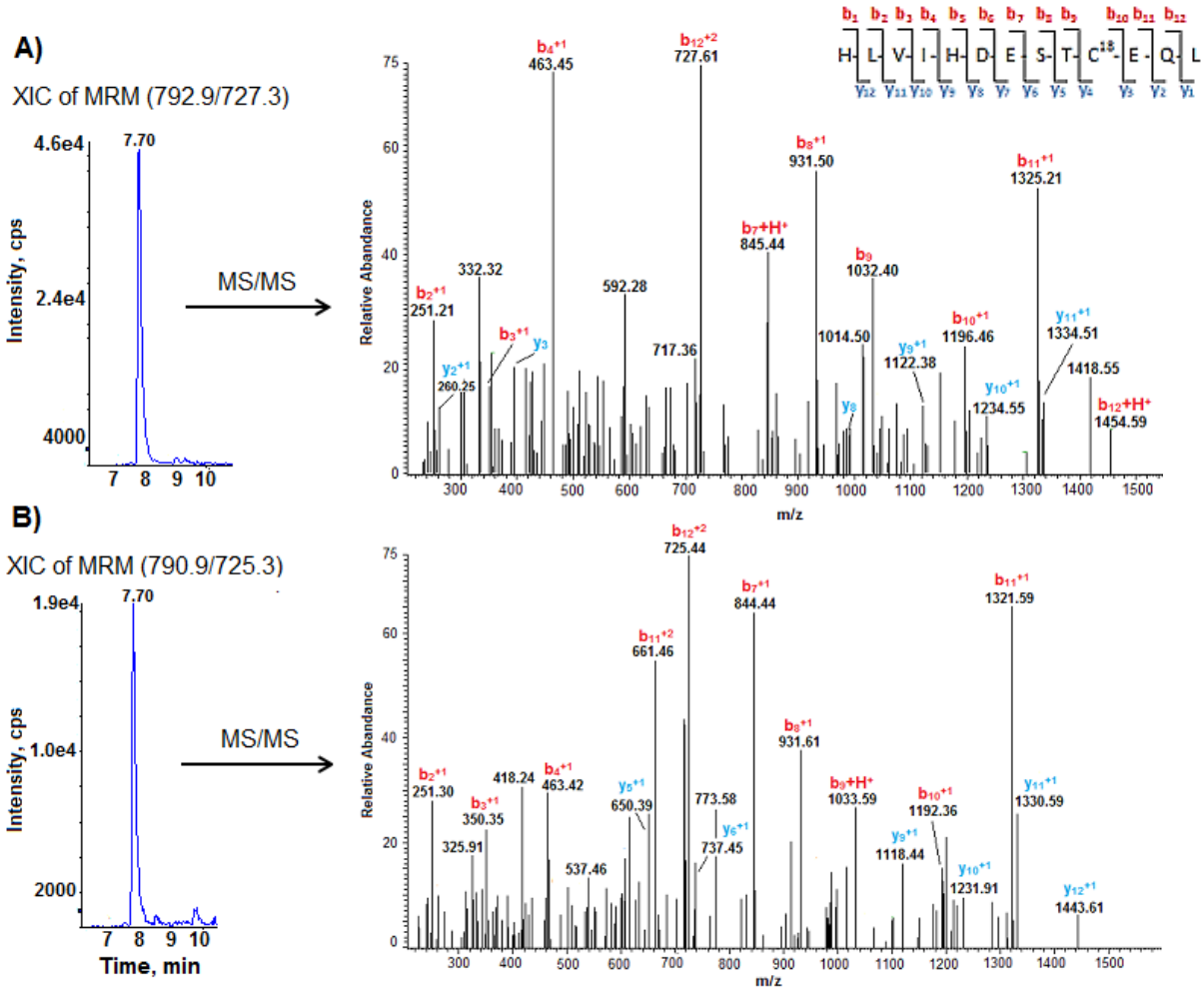


Figure 3-3: The XIC of MRM and the corresponding MS/MS spectra for the differentially alkylated Cys18 peptides detected in a typical pregnant women plasma digest. A) Cys18 peptide alkylated with isotope labelled $^{13}C_2$,D_2-iodoacetamide representing the oxidised form of AGT in the plasma. B) Cys18 peptide alkylated with unlabelled $^{13}C_0$,D_0-iodoacetamide representing the reduced form of AGT in the plasma. Peptides identity and differential alkylation were confirmed by MS/MS spectrum.

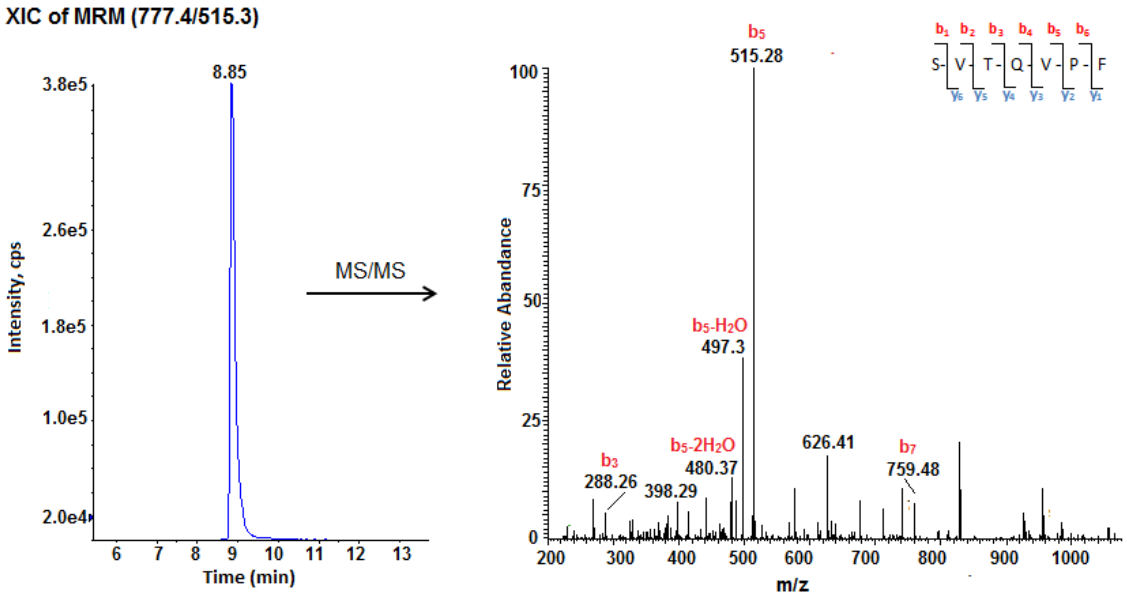


Figure 3-4: The XIC of MRM and the corresponding MS/MS spectra for AGT marker peptide detected in the plasma digest of a typical analysed sample from pregnant women. Peptide identity was confirmed by MS/MS spectrum.

Table 3-3: The optimised precursor/product ion transitions of iodoacetamide and isotope labelled iodoacetamide alkylated AGT Cys peptides and the identified marker peptide.

Peptide Sequence	Peptide mass	*m/z* (charge)	MS1/MS2	Product ion	DP	CE
HLVIHDEST**C**^{18}EQL[a] (IAM alkylated, Reduced form)	1579.75	790.8723 (+2)	790.9/725.3	b_{12}^{+2}	80	36
			790.9/844.4	b_7	80	40
			790.9/1321.6	b_{11}	80	38
			790.9/661.3	b_{11}^{+2}	80	40
HLVIHDEST**C**^{18}EQL[a] (Isotope labelled IAM, Oxidised form)	1583.75	792.8723 (+2)	792.9/727.3	b_{12}^{+2}	80	36
			792.9/844.4	b_7	80	40
			792.9/1325.6	b_{11}	80	38
			792.9/663.3	b_{11}^{+2}	80	40
KDKD**C**^{138}TSRL[a] (IAM alkylated, Reduced form)	1121.57	561.7822 (+2)	561.8/751.3	y_6	65	38
			561.8/994.4	y_8	65	32
			561.8/879.4	y_7	65	34
			561.8/636.5	y_5	65	38
KDKD**C**^{138}TSRL[a] (Isotope labelled IAM, Oxidised form)	1125.57	563.7822 (+2)	563.8/755.3	y_6	65	38
			563.8/998.4	y_8	65	32
			563.8/883.4	y_7	65	34
			563.8/640.5	y_5	65	38
SVTQVPF (AGT marker peptide)	776.41	777.4141 (+1)	777.4/515.3	b_5	75	32
			777.4/497.7	b_5-H_2O	75	30
			777.4/480.3	b_5-$2H_2O$	75	38

[a]Peptide Sequence after PNGase F treatment contains Asp (D) instead of the original Asn (N) due to deglycosylation.
CE: collision energy, DP: declustering potential, IAM: iodoacetamide, MS1/MS2: precursor/product ions.

Calculation of the variability of the method revealed an acceptable analytical precision (CV% below 15%) for the two differentially alkylated Cys 18 peptides and the AGT marker peptide (SVTQVPF), which make them suitable for quantitative protein measurement in clinical samples in line with the FDA guidelines for bioanalytical method validation [243] (Table 3-4). Consistent with the previous findings presented in Chapter Two, differentially alkylated Cys138 peptides were associated with low signal intensity, high variability and were not reproducibly detected between replicates (Table 3-4) and therefore were not suitable for the quantification of the redox forms of AGT in the plasma. The differentially alkylated Cys 18 peptides were used to infer the oxidation level of AGT while the marker peptide was used in the measurement of the total AGT in the plasma. The ratio between the marker AGT peptide standard peak area and the internal standard (NEM alkylated Cys18 peptide) peak area was plotted against the marker peptide standard concentrations. The calibration curve obtained was used to determine the concentration of AGT in the plasma. The marker peptide showed a linear response in the nano-molar range with a high correlation coefficient (R^2=0.992), Figure 3-5.

Table 3-4: The analytical precision of the developed targeted analysis for plasma AGT.

Protein	AGT peptide	Peptide transition	CV% (n=6)
Oxidised AGT	Cys18 peptide alkylated with $^{13}C_2$,D_2-IAM	792.9 / 727.3	11.1
	Cys138 peptide alkylated with $^{13}C_2$,D_2-IAM	Sum of three transitions*	18.7
Reduced AGT	Cys18 peptide alkylated with $^{13}C_0$,D_0-IAM	790.9 / 725.3	9.6
	Cys138 peptide alkylated with $^{13}C_0$,D_0-IAM	Sum of three transitions**	27.2
Total AGT	Marker Peptide	777.4 / 515.3	6.8

* The transitions are 563.8 / 640.6, 563.8 / 755.4 and 563.8 / 883.4
** The transitions are 561.8 / 636.5, 561.8 / 751.3 and 561.8 / 879.5

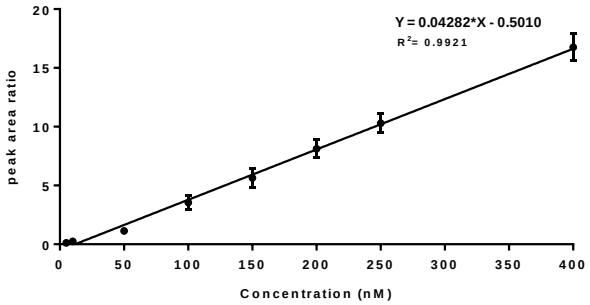


Figure 3-5: The linear response of the total AGT assay. Known quantities of AGT marker peptide standards were spiked in the plasma chymotryptic digest to generate a calibration curve from 5 to 400 nM. The ratio between the peak areas of the marker peptide standard and the internal standard (2 µM NEM alkylated Cys 18 peptide) was plotted against AGT marker peptide standard concentrations. The regression line of the marker AGT peptide showed a linear response extends to the low nM range with a correlation coefficient of R^2 =0.992. Error bars represent the SD of three technical replicates.

3.4.3 Measurement of AGT concentration in the plasma of normal and pregnant women (pre-eclampsia and controls)

Measurement of the plasma AGT level revealed a significantly higher plasma level of AGT in pregnancy compared to normal non-pregnant cases as noted in Figure 3-6. Pre-eclamptic women and age-matched controls showed respectively a 3.3 and a 3.5 fold increase in the level of circulating AGT compared to non-pregnant women while the mean level of AGT in pregnancy was comparable between the pre-eclampsia group and their matched controls and showed no statistically significant difference (P=0.784), (Figure 3-6).

The concentrations of plasma AGT were determined for the three studied groups after correcting for recovery (40%). Pregnant women showed AGT level in the micro-molar range with a mean plasma concentration of 1.80 ± 0.4 and 1.89 ± 0.6 µM for pre-eclampsia and matched controls respectively, whereas normal non-pregnant women had a plasma AGT concentration of 0.544 ± 0.2 µM (Table 3-5).

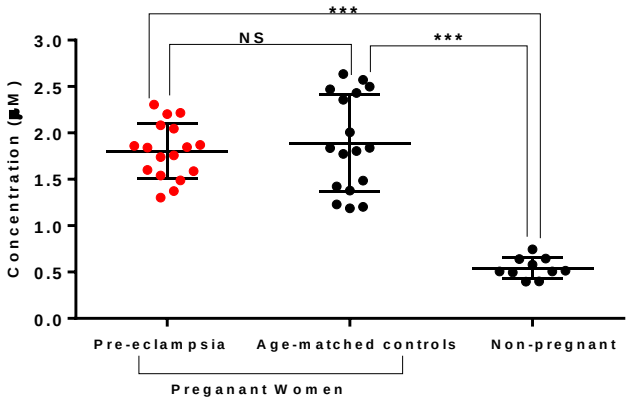


Figure 3-6: The change in the level of AGT in the plasma of pregnant women (pre-eclampsia and age-matched controls) and non-pregnant women. Error bars represent the mean ± SD of the ratio of the analyte signal area over the internal standard signal area.
*NS: non-significant difference, ***P<0.001.*

Table 3-5: AGT level in the plasma of the three studied groups

	Pre-eclampsia (n=17)	**Controls (n=17)**	**Non-pregnant (n=10)**
Total AGT (µM)	1.80 ± 0.36	1.89 ± 0.58	0.54 ± 0.17
Total AGT (µg/mL)*	108.7 ± 17.5	113.5 ± 31.2	32.7 ± 6.5
Oxidised AGT percentage (%)	70 ± 4.8	65.3 ± 3.6	64.7 ± 4.0

Data are presented as the mean ± standard deviation.
*Concentrations in µg/mL were calculated based on protein molecular weight of 60 kDa.

3.4.4 Measurement of the oxidation level of AGT in the plasma of pre-eclamptic, controls and normal non-pregnant women

Quantitation of the oxidation level of AGT in the plasma of pre-eclamptic, controls and normal non-pregnant women was determined using the differentially alkylated Cys18 peptide which is involved in the redox switch of the protein. Women with pre-eclampsia showed a higher percentage of oxidised AGT in their plasma (70.0 ± 4.8) when compared to controls (65 ± 3.6) and normal non-pregnant women (64.7 ± 4.0). Although the increase in the level of oxidised AGT in the plasma of pre-eclamptic women was relatively small it was still statistically significant (P= 0.008). Pregnant controls and non-pregnant women showed very similar levels of oxidised AGT in their plasma and no significant difference was detected (P= 0.942) as illustrated in Figure 3-7.

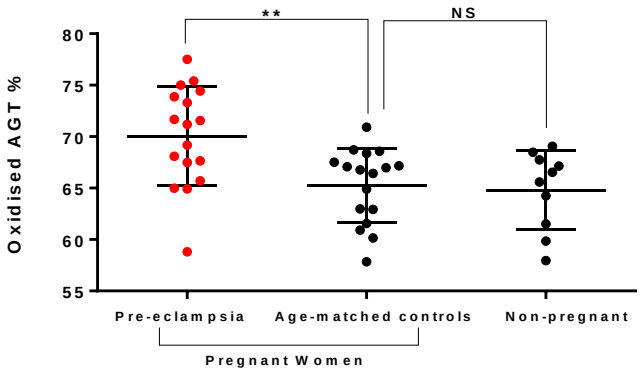


Figure 3-7: The level of AGT oxidation in the plasma of pre-eclamptic, controls and normal non-pregnant women. Error bars represent the mean ± SD of the percentage of oxidised AGT.
*NS: non-significant difference, **P=0.008*

3.5 Discussion

The current work represents the first study to use MS workflow to detect the oxidised and the reduced forms of AGT and quantify its oxidation level in the plasma of non-pregnant, normotensive and pre-eclamptic women using a differential alkylation approach coupled with targeted LC-MS/MS.

3.5.1 Total plasma AGT level

The study showed a significantly higher concentration of AGT in the plasma of normotensive (113.5 µg/mL) and pre-eclamptic pregnancies (108.7 µg/mL) compared to healthy non-pregnant cases (32.7 µg/mL), with a slightly lower plasma level of AGT in

pre-eclamptic women compared to their matched controls which was not statistically significant. The higher plasma concentration of AGT during pregnancy is presumably a result of the increase in the circulating oestrogen produced by the growing placenta during pregnancy which stimulates the synthesis of AGT by the liver and leads to an increase in the plasma angiotensin II and aldosterone levels, which would normally increase the blood pressure [286]. However, normotensive pregnant women are refractory to the vasopressor effects of angiotensin II due to a specifically-decreased sensitivity requiring a higher level of angiotensin II, than non-pregnant women, to achieve the same vasoconstriction response [286; 287]. Moreover, the higher plasma concentration of AGT in pregnancy can also be related to its involvement in the renin-angiotensin-aldosterone system (RAAS); a main regulator of blood pressure and fluid and electrolytes balance in the body (Section 1.3.8, Chapter One). During early normal pregnancy, the RAAS has been proposed to play an important role in the physiological remodelling of the spiral arteries and contribute to renal sodium retention, and hence plasma volume expansion, allowing sufficient placental perfusion, critical for the well-being of the mother and the fetus [288].

Previous observational studies have reported an overexpression of many components of the RAS, including AGT, both in the blood and placental tissues throughout normal pregnancy [287; 289; 290]. The plasma level of AGT was reported to be 3-5 times higher in pregnancy compared to non-pregnant women, which is consistent with the higher level (~3.5 fold) of AGT detected herein in the maternal circulation compared to normal cases. However, previous studies relied on radioimmunoassay to measure plasma AGT in pregnancy and expressed its concentration as the amount of angiotensin I released from a plasma sample in the presence of an excess of renal renin [291; 292], and hence the values reported could not be compared with the one measured in the current work. Nevertheless, the concentration of AGT reported herein in the plasma of normal non-pregnant women (~33 µg/mL) was close to the reported normal plasma concentration of AGT (45 µg/mL) [244; 293], increasing the confidence in the presented findings.

In this study, no significant change in the plasma total AGT level was detected in pre-eclampsia compared to normotensive controls. Although disturbances of the equilibrium in the RAS in the circulation have been reported in pre-eclampsia [290], previous studies showed no significant change in the plasma level of (total) AGT in pre-eclampsia compared to normotensive pregnancy [206; 294] which is in agreement with the current work findings.

3.5.2 The oxidation level of AGT in pre-eclampsia

In the present work the plasma levels of total AGT in pre-eclamptic women and normotensive pregnancies did not show a significant difference; however, a higher plasma level of the oxidised AGT was detected in pre-eclampsia (70.0 ± 4.8) compared to normotensive controls (65 ± 3.6) and normal cases (64.7 ± 4.0), that showed a comparable level of oxidised AGT. This finding indicates that a possible influence of AGT in the pathophysiology of pre-eclampsia could be related to the change in the redox state of the protein, directed towards the oxidised form, rather than the overall expression of AGT. The oxidised form of AGT compared to the free thiol reduced form preferentially interacts with renin resulting in an increase in the generation of angiotensin I [197]. The cleavage of AGT by the renin to angiotensin I is considered the rate-limiting step of the RAS cascade that ends normally with the generation of angiotensin II and, among other effects, an increase in the blood pressure [290].

The significant increase in the oxidation level of AGT in the plasma of pre-eclamptic women detected in this study confirms the only two previous reports [197; 206]. The plasma level of oxidised AGT detected herein in pre-eclampsia (70.0 ± 4.8) was comparable to that which was reported by Zhou and colleagues in the plasma of pre-eclamptic women using Western blotting (32 ± 6% reduced form which reflected 68% oxidised form) [197]. Zhou *et al.* [197] were the first to propose a link between the redox switch of AGT towards the more active oxidised form and pre-eclampsia after they resolved the crystal structure of the protein in 2010. Using Western blotting, they showed that AGT exists in a near 40:60 ratio of the reduced unbridged form to the oxidised sulphydryl-bridged form in the plasma of healthy individuals, independent of age and gender [197]. In pre-eclampsia, however, the same group noticed a significant decrease in the percentage of the plasma reduced form to 32 ± 6%. Moreover, just recently Rahgozar *et al.* [206], observed in a larger cohort (115 pre-eclamptic women and 55 matched controls) a significant decrease in the relative proportion of the reduced AGT, expressed as a percentage of that observed with an in-house standard, in the plasma of pre-eclamptic women (70.8 ± 29.5%) as compared to healthy pregnant controls (93 ± 24.9%) with no change in the plasma level of total AGT. They used biotinylated MPB alkylating agent to label the free thiol of plasma protein and quantitate the level of plasma free thiol AGT as a percentage of that observed in a pooled standard (derived from non-pregnant healthy volunteers) using ELISA [206].

The higher level of oxidised AGT in the plasma of pre-eclamptic women could be a result of the placental ischemia which is largely believed to be associated with the pathology of the disease. This oxidative stress state may be initiated as early as the time of placentation [295], and the subsequent generation and release of toxic factors (such as ROS) from the placenta into the maternal circulation could enhance the conformational change of AGT from the reduced form to the more active oxidised form and, therefore, contribute to the increase in the blood pressure in pre-eclampsia [197]. Unlike normotensive pregnant women who are known to have decreased vascular sensitivity to angiotensin II, pre-eclamptic women have been reported to exhibit increased sensitivity of the adrenal cortex and vascular system to angiotensin II which could further contribute to the increase in the blood pressure associated with pre-eclampsia [286]. Furthermore, angiotensin II itself can contribute to oxidative stress. Production of ROS (such as $O_2^{\cdot-}$) and modulation of redox-sensitive signalling pathways are involved in the intracellular signalling pathway(s) of angiotensin II. Under normal conditions, the rate of ROS generation is balanced by the rate of elimination through endogenous antioxidants defence mechanisms. However, with dysregulation of angiotensin II signalling (as in pre-eclampsia), this balance is tilted in favour of elevated ROS, which contributes to oxidative stress in angiotensin II-associated disorders, leading to endothelial dysfunction and vascular inflammation [296].

In this study, an MS-based workflow based on coupling a differential alkylation approach with targeted LC-MS/MS was used to quantify the Cys peptides involved in the redox switch of AGT. This is considered a more reliable quantitative methodology than the Western blotting, which is, at best, semi-quantitative, used by Zhou *et al*. [197]. Moreover, the quantitative method developed here enabled the detection of the two plasma forms of AGT and therefore the oxidation level of AGT in each sample was reported as a percentage of the sum of the reduced and oxidised AGT forms, which indicated that the change in the protein level is actually due to the change in its oxidation state rather than its total level. This is a more accurate approach than the one Rahgozar *et al.* [206] followed in which they developed an ELISA assay to detect the reduced form of the protein and reported the level of plasma free thiol AGT as a percentage of that observed in a pooled standard.

However, it should be pointed out that developing a methodology useful to recognise and quantify the redox state of the Cys residues in a protein is challenging and is complicated

by the possibility of the disruption of the original redox state of the protein during sample preparation due to the reversible nature of the disulphide linkage. In this context, the recognition of the original redox state requires the immediate freezing of the reduced form of the protein in the plasma by an irreversible reaction as alkylation. With the presented methodology, the high complexity of the plasma (~ 3.3 mg proteins /50 µL plasma), and the presence of sugar moiety attached to the asparagine residue in both Cys peptides retarded the effective alkylation directly from the plasma. Effective alkylation was achieved after protein enrichment and deglycosylation, therefore, the detected level of AGT oxidation may not accurately reflect the actual oxidation level of the protein in the plasma. However, the ratio of the oxidised to the reduced AGT detected in the current work in the plasma of healthy women (3.25:1.75) was very close to the original ratio reported by Zhou *et al*. [197] in the plasma of healthy individuals (3:2 oxidised to reduced), increasing the confidence in the research findings. Furthermore, to account for any artefacts that could affect the redox state of the protein during extraction procedure, fully reduced and oxidised human recombinant AGT were spiked in the plasma and were analysed parallel with the clinical samples to act as controls.

Regardless of this possible limitation of the presented methodology, the data presented here provide further evidence of the potential importance of AGT in the pathophysiology of pre-eclampsia, and corroborated previous observations showing that the higher plasma level of oxidised AGT in pre-eclampsia, rather than the total AGT, could be a main contributor to the increase in the blood pressure associated with the pathology of the disease. The measurement of the oxidation level of AGT in the plasma early in pregnancy might potentially help in the diagnosis of pre-eclampsia, particularly when used in conjunction with other proposed biomarkers of the disease (discussed in Section 1.3.7, Chapter one).

3.6 Conclusion

This is the first study that used MS workflow to detect and quantify the plasma level of AGT; a protein that has been proposed to be linked to the increase in the blood pressure in pre-eclampsia through its redox switch to the more active oxidised form. The central message from the presented findings confirms that patients with pre-eclampsia had significantly higher plasma level of sulphydryl-bridged oxidised AGT compared to matched normotensive pregnant controls, whilst maintaining a similar total AGT

concentration in the plasma. Therefore, the elevated level of oxidised AGT rather than its total level might be a contributing factor to the hypertension characteristic of pre-eclampsia.

Several studies have evaluated the activity of the renin-angiotensin system components in women with pre-eclampsia over the years but the focus was mainly on renin, angiotensin I and angiotensin II [287; 289; 297]. This has partly been due to the perception that renin would be the rate-limiting factor in the enzyme: substrate reaction in pregnancy, as it is in the non-pregnant state. However, in hyper-oestrogenic states, such as pregnancy, AGT becomes rate-limiting [202; 292]. Only two studies have evaluated the possible role of the influence of the redox switch between the two plasma forms of the renin substrate, AGT, in pre-eclamptic women. The current study confirms the previously proposed involvement of the AGT redox switch in pre-eclampsia using a novel LC-MS/MS method that enabled the detection and the quantification of the oxidised and the reduced forms of the protein in the plasma. Moreover, the presented findings provide an extra line of evidence linking the oxidative state and the generation of reactive oxygen species with hypertension in pre-eclampsia. The changes in the level of AGT oxidation remains to be evaluated at an earlier point of pregnancy (1st trimester) by further studies to evaluate the potential of using the measurement of the oxidised AGT level in the maternal circulation to predict women at risk of developing pre-eclampsia.

Chapter Four

Profiling of Plasma Glycoproteins by Label-free Targeted LC-MS/MS

4 Profiling of Plasma Glycoproteins by Label-free Targeted LC-MS/MS

Summary

Glycoproteins play a central role in diverse biological processes and are linked with many serious human diseases. Therefore, measurement of the changes in the glycoprotein levels has the potential to increase the pool of potential diseases biomarkers, and to give a better understanding of the biochemical pathology underlying these conditions. The aim of the work described in this chapter was to develop a simple, reproducible, and cost-effective analytical workflow with a high throughput potential that enables the reliable quantification of clinically relevant human plasma *N*-glycoproteins using conventional LC-MS/MS equipment.

Plasma *N*-glycoproteins were selectively extracted via lectin affinity chromatography (ConA) then fractionated by reversed-phase solid phase extraction (RP-SPE) into two fractions applying two gradients of the mobile phase. This enrichment strategy resulted in a good reproducibility and efficiency (71 ± 2.3 of the total plasma proteins were depleted). LC-MS/MS analysis of the tryptic digest of both fractions confidently identified 90 proteins involved in various biological processes such as coagulation/fibrinolysis, inflammation, and iron and lipid metabolism. The captured proteins had a dynamic range of concentrations covering four orders of magnitude. From the 90 identified proteins, 54 clinically relevant glycoproteins were selected for protein profiling by targeted LC-MS/MS working under MRM mode. Measurement of the analytical precision of the method revealed acceptable CV values for the majority of the assays with 45 glycoproteins assays showed CV ≤15% (median CV 11.8%, range 3.6-33%). Notably, the ability of the developed LC-MS/MS method to detect changes in glycoprotein composition was observed with the ability of the method to successfully measure the small difference in the glycoprotein levels of plasma treated with ammonium sulphate, when compared to controls, and to be capable at distinguishing between plasma and serum samples.

The developed methodology provides a reliable and simple approach for the rapid quantification of relevant glycoproteins in human plasma, and can be a cost-effective alternative to isotope labelled standards workflow when applied for relative protein quantification in clinical samples.

4.1 Introduction

4.1.1 The importance of protein glycosylation

Glycosylation plays a central role in protein folding and stability [298; 299], and has a great impact on protein interactions with diverse biological processes such as cell signalling pathways, cell division and immunological reactions [300]. Glycoproteins represent an interesting class of human plasma proteome, with more than 50% of the proteins being glycosylated [301], making glycosylation the most common post-translational modification [302]. Various glycoproteins have been identified as potential disease biomarkers and therapeutic targets in a range of severe human diseases [211; 303; 304; 305]. Key examples include Her2/neu in breast cancer [306], prostate-specific antigen in prostate cancer [307], and vascular cell adhesion molecule 1 in rheumatoid arthritis [305]. Moreover, many of the protein biomarkers currently utilised in clinical settings are glycoproteins such as α α-fetoprotein (hepatocellular carcinoma) and prolactin (prolactinomas) [304].

Therefore, measurement of glycoprotein profiles and the subsequent changes in diseased and non-diseased samples using simple accessible MS-workflow has the potential to increase the pool of biomarker candidates for several diseases, and to aid a better understanding of the biochemical pathologies underlying them in a wider context.

4.1.2 Types of protein glycosylation

Glycosylation introduces a considerable heterogeneity to the protein through the different size and types of oligosaccharides attached [308], which creates further complexity into the analysis of glycoproteins, over and above that of the native proteins. Typically the sugar moiety (the glycan) is attached to the protein either through asparagine (Asn) residues to give rise to the *N*-linked glycosylation, or through serine (Ser) or threonine (Thr) residues to produce *O*-linked glycosylation [302]; however, not all potential glycosylation sites in the protein would necessarily be linked to glycan moieties [309]. Generally, *N*-glycosides fall into the Asn-Xxx-Y sequence motif (Y is Ser or Thr or occasionally Cys, Xxx can be any amino acids except proline) [310], while no consensus in the primary amino acid sequence has been identified for *O*-glycosylation sites [304].

Most glycoprotein studies have focused on *N*-glycosylation which is predominant in extracellular proteins (*e.g.* secreted forms) and in different body fluid proteins (blood serum, cerebrospinal fluid, urine and saliva) [302]. Various techniques for *N*-glycoprotein

enrichment and analysis exist, including the availability of an efficient enzyme (PNGase F) for the deglycosylation and the release of the *N*- glycopeptide and glycan; and hence the identification of glycosylation sites [311]. Regarding *O*-glycosides, these appear to be more difficult to study, mainly due to the lack of a consensus sequence around the glycan attachment site, and the lack of an efficient enzyme to release the linked carbohydrate.

4.1.3 Glycoprotein analysis by MS

Mass spectrometry is a core technique in glycoprotein analysis, and a variety of glycoprotein enrichment techniques and MS analytical tools are available to improve the quality of the generated data. Typically, glycoprotein analysis methods from complex samples (such as plasma) share a common workflow (Figure 4-1) which includes: glycoprotein or glycopeptide enrichment, enzymatic digestion of glycoproteins to produce peptides or glycan mixtures, online or offline chromatography purification and separation, high resolution MS or tandem MS/MS analysis and finally, data interpretation [311].

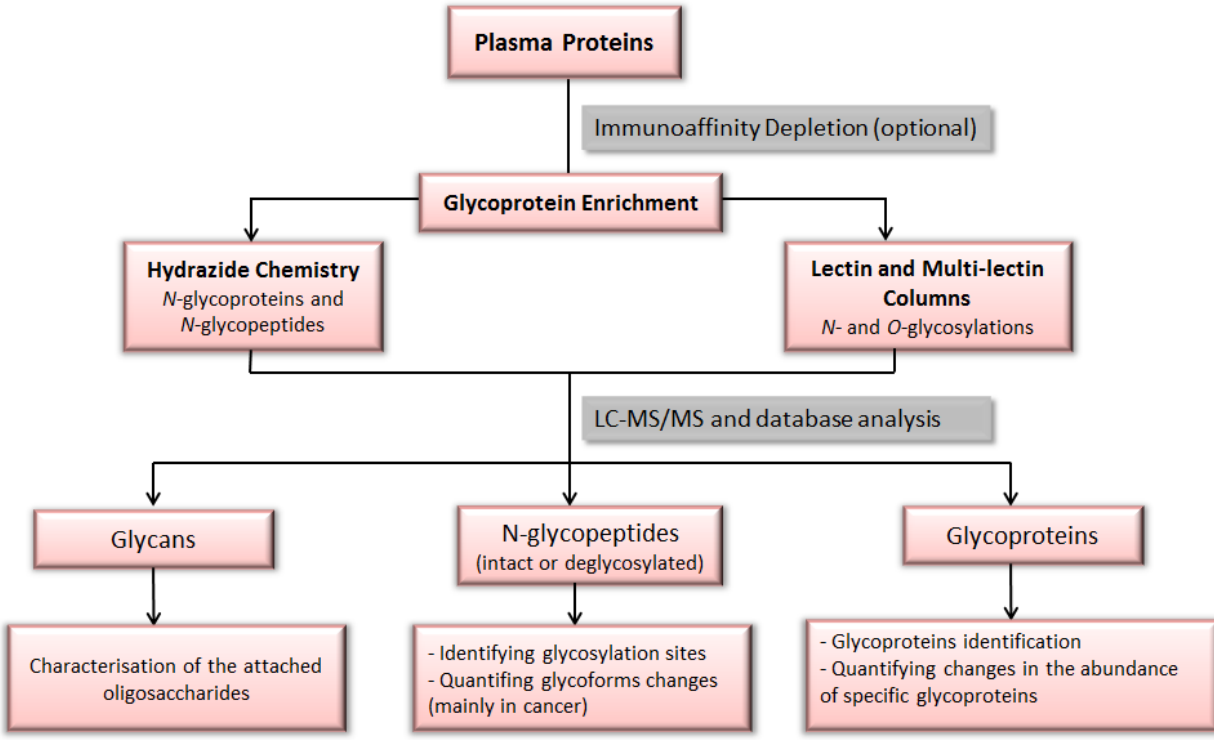


Figure 4-1: Schematic representation of the approaches used to analyse glycoproteins by MS. Glycoproteins are extracted from the plasma using either hydrazide chemistry or lectin affinity chromatography. Analysis using MS can be conducted using glycopeptides, non-glycopeptides or glycan to provide qualitative and quantitative data.

4.1.3.1 Glycoprotein enrichment

Different approaches are available for the selective extraction of glycoproteins, based mainly on lectin affinity chromatography or hydrazide chemistry. In the latter, *N*-glycoproteins are extracted by a chemical reaction between the hydrazide group of the immobilised bead and the dialdehyde group derived from the glycan moiety of the glycoprotein [302]. The captured *N*-glycoproteins are digested *in situ*, and non-glycosylated peptides or unbound peptides are washed away, while *N*-glycopeptides are released by PNGase F or acidic hydrolysis and analysed by LC-MS/MS [263]. Selective purification of *N*-glycopeptides rather than *N*-glycoproteins by hydrazide chemistry has also been reported [312].

Regarding lectin affinity, this has become a common and powerful tool for the selective capturing of various glycoproteins [211]. Lectins are proteins isolated from various plants and animal sources and possess a selective affinity for carbohydrate moieties [14]. This biospecific interaction between the lectins, immobilised to a solid support, and the glycan component of the glycoprotein is strong enough to enable the capture of these glycoproteins from a complex biological sample [309]. The unbound proteins are eluted with the washing buffer, and the extracted glycoproteins are displaced with a heptane sugar [313]. Different lectins with variable, but partially overlapping, binding profiles can recognise and bind to specific carbohydrate residues [41]. Commonly, two or more lectins, with complementary specificities for different glycosylation structures are used in multi-lectin affinity enrichment to provide a more comprehensive coverage for the glycoproteome of interest [314]. Concanavalin A (ConA) is a lectin which preferentially recognises mannosyl oligosaccharides, and to lesser extent glucosyl oligosaccharides, attached to proteins through asparagine (*N*-linked) residues. This type of carbohydrate is commonly present in a wide variety of serum glycoproteins, and as a result, ConA is amongst the most commonly used lectin for *N*-glycoprotein isolation [309]. Moreover, ConA is mainly used due to its ease of availability, low cost and relatively broad specificity and high affinity [315], which allows the isolation of the majority of the glycoproteins in the sample [316].

Both lectins and hydrazide chemistry strategies have reportedly demonstrated effective glycoprotein and glycopeptide extraction from complex biological samples such as human plasma [266; 317]. However, the lectin affinity approach has some advantages over hydrazide chemistry; it avoids potential chemical side reactions [314], provides a

better coverage for proteins with different glycosylation (multi-lectin strategy), and enables the isolation of specific protein glycoform using a particular lectin type [311].

Immunoaffinity depletion of the high abundance proteins has been coupled with both lectin [262] and hydrazide chemistry [266] to further improve the dynamic range, and enhance the detection of low abundance glycoproteins. Other less common approaches reported for glycoproteins and glycopeptides enrichment include boronic acid [318], size-exclusion chromatography [319], and hydrophilic interaction liquid chromatography (HILIC) for glycopeptides and glycan separation [320].

4.1.3.2 Approaches for glycoprotein analysis by MS

Identification of glycoproteins and glycosylation sites

Given their high sensitivity and selectivity, MS-based methods can identify and quantify glycoproteins [321], recognise glycosylation sites [322], and characterise the attached oligosaccharides [323], (Figure 4-1). Glycoproteins can be identified by the glyco- or non-glycopeptides generated from the enzymatic digestion of the extracted glycoproteins [302; 321; 324]. Glycopeptide-based identification has been a widely used strategy over the years; yet non-glycopeptides, which count for the majority of the glycoprotein sequences, are neglected in this approach [325]. Recent studies have demonstrated the feasibility of using non-glycopeptides for glycoprotein identification and quantification from different complex biological samples (including serum), and an overlap of the results between both strategies has been noticed, with more glycoproteins commonly identified using non-glycopeptide markers [324; 326]. Additionally, the larger number of generated non-glycopeptides should increase the confidence in identification especially for proteins with only one glycosylation site [324]. However, a disadvantage of using non-glycosylated peptides is that no information about the glycosylation sites and the attached glycans can be obtained [308; 327]. MS analysis at the glycopeptide level is a well-established technique for the identification of glycosylation sites. Typically, the glycan part is removed first and the free *N*-glycopeptide is subsequently analysed by MS using ion trap [302; 328], Orbitrap [329], Q/TOF [330], Fourier transform-ion cyclotron resonance [331] or MALDI-based TOF/TOF mass analysers [325]. *N*-glycosylation sites can be mapped using the consensus sequence of Asn-Xxx-Ser/Thr, and the 0.984 Da mass difference noticed with the conversion of Asn to Asp by the action of PNGase F [311]. Although this approach identifies the *N*-linked glycosylation sites, the information about the attached glycan is lost during sample preparation [308].

MS-based quantification methods

Following extraction, peptides and glycans released from glycoproteins can be used to obtain different quantitative information. Glycoprotein quantification focuses mainly on measuring the concentration of specific glycoproteins, absolutely or relatively, between different conditions or relative to other glycoproteins; such as the quantification of aberrant protein glycoforms involved in the pathogenesis of diseases as cancer [332]. Additionally, quantification of glycosylation expression level and monitoring the changes in the glycan profile attached to the glycoprotein is achieved by glycopeptides and glycan analysis (Table 4-1). Absolute or relative glycoproteomics quantification is achieved by adopting a number of stable isotope labelling (metabolic, enzymatic or chemical labelling) [333; 334; 335], or label-free (ion intensity or spectral count) methodologies [336; 337], as described in Section 1.1.5, Chapter One. Table 4-1 provides recent examples from the literature for quantitative glycoproteomics studies using labelled and label-free approaches.

Targeted glycoprotein quantification by LC-MS/MS, provides an improved sensitivity when compared to shotgun glycoproteomics workflows [304]. It is applied to target specific peptides of the glycoprotein (glycopeptides, deglycopeptides and non-glycopeptides), or the released glycan (Table 4-2) [332]. Such an approach can be used to precisely quantify the status of glycosylation sites and assess the glycosylation occupancy at the molecular level [311], and to measure the changes in the abundance of specific glycoproteins between individuals or the compared samples [332]. A targeted approach can also be used for relative or absolute protein quantification applying either stable isotope standards (SIS), reference-labelled peptides or label-free workflows [338]. Of the three, SIS is considered the gold standard method for absolute quantification [87; 94; 311]. Nevertheless, the high cost of the labelled peptide standards creates an obstacle for the routine use of this methodology, especially for large numbers of clinical samples and/or when a large number of glycoproteins require simultaneous monitoring [93]. The reference-labelled peptide strategy involves a single labelled peptide used as a reference for the normalisation of all peptides monitored, whilst in the label-free strategy, this is omitted and the raw peak areas of the monitored peptides are compared between different conditions [93]. Label-free methodology has the advantages of being cost-effective with the ability to measure a large number of proteins simultaneously [338], and is not limited by the number of samples for analysis [304]. However, it can only be used for relative quantification and is generally associated with higher variability [338].

Table 4-1: Examples of quantitative glycoproteomic studies using stable isotope labelling and label-free MS

Sample	Enrichment process	Signature peptides	Isotope labelling	Main finding	Ref.
Mouse serum	Hydrazide	Deglycopeptide	^{18}O	Identification and quantification of 224 *N*-glycopeptides representing 130 unique glycoproteins.	[339]
Cell extract	Lectin	Deglycopeptide	^{18}O	Identification of 250 glycoproteins, with 400 unique *N*-glycosylation sites.	[340]
Serum	Hydrazide	Deglycopeptide	Methyl tag	Quantification of the level of aberrant glycosylation between liver cirrhosis and hepatocellular carcinoma samples.	[341]
Serum	Hydrazide	Deglycopeptide	^{18}O	Quantification of 86 *N*-glycosylation sites occupancy and 56 glycopeptides showed significant changes between ovarian cancer and healthy individual.	[342]
Serum	Sepharose	Glycopeptide	^{18}O	Site-specific quantification of core fucosylated glycoprotein and recombinant human erythropoietin.	[333]
Plasma	Lectin	Non-glycopeptide	iTRAQ	Identification of aberrant glycoproteins from breast cancer plasma.	[335]
Plasma	Lectin	Non-glycopeptide	2-nitrobenze-nsulfenyl	Identification of 34 serum glycoproteins differentially expressed between lung cancer and healthy control.	[343]
Serum	HILIC	Glycan	Label-free	The expression of 44 oligosaccharides was found to be distinct in the pancreatic cancer serum.	[344]
Pure protein	HILIC	Glycopeptide	Label-free	Increased fucosylation of haptoglobin in liver disease with up to six fucoses associated with specific glycoforms of one glycopeptide.	[345]
Serum	Hydrazide	Deglycopeptide	Label-free	High-throughput profiling of differentially expressed glycoproteins in hepatocellular carcinoma.	[324]
Plasma	Hydrazide	Deglycopeptide	Label-free	Identification of eight hepatocellular carcinoma biomarker candidates.	[321]
Serum	Hydrazide	Deglycopeptide	Label-free	A total of 38 glycopeptides representing 22 proteins showed significant fold changes between non-small cell lung cancer and matched controls.	[346]
Serum	Lectin	Non-glycopeptide	Label-free	Identification of 26 candidate biomarkers for colorectal cancer.	[337]
Serum	Multi-lectin	Non-glycopeptide	Label-free	Identification of 38 serum biomarkers candidates for lung adenocarcinoma.	[347]
Stem cell lysate	Multi-lectin	Non-glycopeptide	Label-free	*N*-linked glycoproteins profiling of cancer stem cells to detect the change of glycosylation pattern upon drug treatment.	[348]

Table 4-2: Examples of targeted glycoproteins quantification by LC-MS/MS.

Sample	Enrichment process	Signature peptides	No. of quantified glycoproteins	Ref.
Mouse serum	Hydrazide	Glycopeptide	95 identified, 26 targeted	[349]
Plasma	Hydrazide	Deglycopeptide	5 targeted using SIS approach	[350]
Secretome	Phytohemagglutinin-L4 (L-PHA) lectin	Non-glycopeptide	5 targeted using SIS approach	[351]
Plasma	Lectin (AAL)	Non-glycopeptide	44 identified, 8 targeted using SIS approach	[352]
Plasma	Lectin (AAL)	Non-glycopeptide	12 targeted using SIS approach	[353]
Plasma	Multiple lectins	Non-glycopeptide	67 identified , 14 targeted using SIS approach	[354]
Plasma	L-PHA and immunoenrichment via anti-peptide antibody	Non-glycopeptide	Quantitation of a specific aberrant glycoform of tissue inhibitor of metalloproteinase 1	[96]
Plasma	Albumin and IgG depletion	Non-glycopeptide	17 targeted using label-free approach	[86]

In conclusion, plasma glycoproteins, especially *N*-glycoproteins, are involved in many biological processes and have been linked with important human diseases. They represent a vital subclass of plasma proteome for the discovery of biomarkers that can help in disease diagnosis and progression monitoring. Targeted LC-MS/MS offers a sensitive and specific tool to measure changes in glycoprotein levels and explore diseases pathways. However, most of the reported targeted glycoprotein profiling studies used the costly stable isotope standards (SIS) workflow and hence have only profiled a small number of glycoproteins (≤ 15). Therefore, there is a growing need to develop a simple, reproducible and cost-effective methodology that can be easily adapted to a standard LC-MS/MS system, with the ability to achieve high throughput profiling of a high number of clinically relevant plasma *N*-glycoproteins (≥ 50). This can be achieved by label-free targeted LC-MS/MS, which could be an affordable alternative to isotope labelled standards workflow when applied to relative protein quantification, especially when targeting large numbers of proteins.

4.1.4 Aim of the chapter

The aim of this work was to develop a simple, reproducible and economical analytical workflow for the reliable quantification of clinically relevant *N*-glycoproteins using conventional LC-MS/MS equipment. A series of objectives were formulated in order to achieve this aim:

1) Optimisation of a new simple protocol for the selective extraction of plasma *N*-glycoproteins by coupling lectin affinity chromatography with RP-SPE fractionation.
2) Identification of the captured proteins by LC-MS/MS and the Mascot search engine.
3) Development of a label-free targeted LC-MS/MS method for clinically relevant identified proteins.
4) Validation of the analytical precision of the developed method using the coefficient of variation values.
5) Confirming the biological applicability of the validated method by measuring protein fold changes in plasma treated with ammonium sulphate, and distinguishing plasma and serum protein profiles.

4.2 Materials and Methods

4.2.1 Materials

Ammonium sulphate and bicinchoninic acid protein assay kit (BCA) were purchased from Fisher Scientific (Loughborough, UK). All other materials are as described in Section 2.3.1, Chapter Two.

Control plasma was obtained by collecting 10 mL blood from a healthy volunteer into tubes containing anticoagulant. Immediately after collection and mixing, the samples were centrifuged at 1000x *g* for 15 min. For serum samples, blood was collected into BD Vacutainer® SST™ serum separation tubes, inverted several times, allowed 30 minutes clotting time, and centrifuged for 15 minutes at 1000x *g*. The separated plasma and serum were then divided into 0.5 mL aliquots in sterile low binding Eppendorf tubes and stored at -80°C for subsequent analysis.

4.2.2 Glycoprotein enrichment and fractionation using ConA Sepharose resin and RP-SPE

Plasma *N*-glycoproteins were extracted using ConA Sepharose resin as described in Section 2.3.3.1, Chapter 2. Briefly, plasma (50 µL) was diluted in 250 µL washing buffer and then loaded onto the activated resin. After 20 min of incubation, the unbound proteins were cleared, and the retained proteins were eluted with methyl α-D-mannopyranoside for subsequent RP-SPE fractionation. The captured glycoproteins were fractionated using eight gradients of 0.1% TFA acetonitrile in 0.1% TFA water; 30, 35, 40, 43, 45, 48, 50, and 90% (*v/v*). This gradient profile was the same as applied to fractionate extracted human Neuro-2A cells cytosolic proteins [355] since proteins are expected to be eluted from the RP-SPE column within a narrow range of the eluent, 30-50%.

The total amount of protein loaded onto the resin, the unbound and retained ConA proteins and proteins in RP-SPE collected fractions were quantified by BCA kit. A calibration curve was constructed using the following BSA concentration 25, 50, 100, 200, 300, 400, 500, 600, 800, 1000 and 1500 µg/mL. For each 25 µL of BSA standard and protein samples, 200 µL of the BCA working reagent [50:1 (*v/v*) parts of BCA reagent A: reagent B] was added and the reaction was incubated at 60°C for 15 min. Using 96 well plates, the absorbance was measured at 570 nm using MRX microplate reader from Dynex Technologies (Chantilly, VA, USA).

4.2.3 Digestion of RP-SPE protein fractions

Following ConA treatment, glycoproteins were collected by RP-SPE into two fractions; A and B representing glycoproteins eluted at 40% and 50% of the mobile phase respectively. Proteins in both fractions were denatured with 6 M urea and reduced with 50 mM DTT followed by alkylation with 200 mM iodoacetamide. Both reactions were performed in 10 kDa Amicon ultra 0.5 mL filter devices at 37°C for 30 min in the dark, and the reaction mixture was washed twice with 400 µL of 50 mM ammonium bicarbonate at 14000x *g* for 8 min to remove excess reagents. The resulting protein mixture was diluted to 100 µL with 50 mM ammonium bicarbonate and trypsin was added at an enzyme: protein ratio of 1:50 (*w/w*) and digestion was carried out at 37°C. To ensure complete digestion, a second aliquot of trypsin (1:100 enzyme: protein (*w/w*)) was added after 4 h, and digestion proceeded at 37°C overnight. The reaction was

quenched with 1% formic acid before centrifugation for 5 min at 11000x *g* and the upper layer was transferred to HPLC vials for LC-MS/MS analysis.

4.2.4 LC-MS/MS analysis of plasma proteins in RP-SPE fractions

HPLC coupled to a Thermo Fisher LTQ Velos ion trap mass spectrometer from Thermo Scientific operating in positive-ion ESI was used to analyse the tryptic peptide digests of the two RP-SPE fractions, A and B. Peptides were separated on a C18 300 Å, 100x2.1 mm internal diameter, 3 µm particle size column from ACE, using a mobile phase consisting of water as eluent A, and acetonitrile as eluent B, both solvents with 0.1% formic acid. Peptides were eluted with 10-45% (*v/v*) linear gradient of eluent B over 15 min at 300 µL/min flow rate. ESI source parameters were: ion spray voltage 3 kV, capillary and source heater temperatures 275°C and 300°C respectively and the flow rate for the sheath, auxiliary and sweep gases were 30, 20 and 10 arbitrary units respectively. MS/MS spectra were acquired by DDA in a full scan mode (*m/z* 350−1650), with the precursor ions dynamically selected and subjected to CID performed at a normalised value of 35%. The resulting data were analysed using Xcalibur 2.2 software.

4.2.5 Identification of glycoproteins collected in RP-SPE fractions

MSCONVERT provided by the ProteoWizard project (http://proteowizard.sourceforge.net/tools.shtml) was used to convert raw data files to Mascot generic format. MS/MS ion search option in Mascot search engine was selected to search against all *Homo sapiens* entries in the Swiss-Prot database for protein identification applying the following search parameters: trypsin enzyme, one missed cleavage, peptide mass tolerance of 1.2 Da, MS/MS tolerance of 0.6 Da, fixed modification carbamidomethylation of cysteines, variable oxidation of methionines and peptide charge of 1+, 2+, and 3+. Decoy tool was selected for all searches, to adjust the significance threshold for a global FDR at 1%.

4.2.6 Plasma glycoprotein profiling by targeted LC-MS/MS

Fifty-four glycoproteins with a high confidence of identity were selected for protein profiling by targeted LC-MS/MS. One signature peptide per protein was used in the assay with 1, 2 or 3 transition(s) per peptide being monitored.

Source gases and temperature were optimised using a 500 ng/mL five-peptide standard mixture (angiotensin II, Gly-Tyr, Leu encephalin, Met encephalin, Val-Tyr-Val, Sigma-

Aldrich). The optimised values for curtain gas, collision gas, GS1 and GS2 were 10, 9, 30 and 45 (arbitrary units) respectively. The heated capillary temperature was maintained at 450°C and the ESI voltage was kept at 4200 V.

Trypsin digested peptides were analysed using a Shimadzu series 10AD VP LC system coupled to a 4000 QTRAP mass spectrometer operating in a positive ion mode at 100 µL/min flow rate using a C18 300 Å, 100 x 1mm internal diameter, 3 µm column from ACE. The mobile phase and the chromatographic gradient were as described in section 4.2.4. MRM transitions were monitored and acquired at unit resolution in both Q1 and Q3 and were used to trigger enhanced product ion (EPI) scan, for the monitored signature peptides, in the linear ion trap of the QTRAP in the mass range *m/z* 100-1500. The MS/MS spectrum generated for each targeted peptide in the assay was used to confirm its identity.

The freely-available fragment ion calculator website available from Proteomics Toolkit (http://db.systemsbiology.net:8080/proteomicsToolkit/FragIonServlet.html) was used to predict the *y*, *b* and *a* ions for the targeted peptides, and the predicted values were matched with the obtained MS/MS spectra from the EPI experiment. All MRM and EPI data were processed with Analyst software 1.4.2.

4.2.7 Validation of the targeted LC-MS/MS method

Most of the selected signature peptides together with their MRM transitions were from the literature [79; 80; 86; 87]. The MS/MS data obtained from Mascot were used to choose signature peptides for the remaining proteins, either due to higher signal intensity, better peak shape, or absence of published transitions.

Although one signature peptide per protein was used in the final assay, for many proteins two or three signature peptides were evaluated initially (*e.g.* fibronectin, pigment epithelium derived factor), and the best one in terms of sensitivity, selectivity and peak shape was chosen. Qualitative assessment of the chosen peptides and transitions was carried out by monitoring more than one product ion per peptide. The resulting MS/MS spectra were manually inspected and the transition that yielded the best signal intensity, selectivity (no interferences from background matrix), and peak shape for integration was selected for subsequent validation. For most peptides one transition was used in the final assay, however monitoring two or three fragments was necessary for the remainders. Where possible, the highest intensity fragment ion (+1) with *m/z* greater than the precursor ion (+2) was selected to increase the sensitivity and selectivity of the assay.

MRM results from the above approaches were pooled, and a set of optimised 56 MRM channels that covered 39 and 33 proteins in fraction A and B respectively was obtained.

Quantitative assessment of the analytical precision of the developed methodology was evaluated by measuring the selected glycoproteins in six biological replicates of control human plasma and calculating the CV%. CV values of ≤ 15% were considered acceptable for quantitative bioanalytical measurements, in accordance with the US-FDA analytical procedures and methods validation for drugs and biologics, as published in September 2013 [243].

4.2.8 Application of the developed method to ammonium sulphate treated plasma

To confirm the ability of the profiling method to reproducibly detect small differences in glycoprotein abundance, human plasma samples were treated with ammonium sulphate; a common approach for the selective precipitation of high to moderate abundance plasma proteins. A low concentration of ammonium sulphate, 15% (*w/w*), was used to induce a small change in the level of a small percentage of the targeted plasma glycoproteins, and the applicability of the developed methodology to detect this small change in the glycoprotein levels, as a result of their precipitation, was assessed.

Solid ammonium sulphate (80 mg) was added slowly, in four additions, to 1 mL chilled plasma with stirring to achieve a final 15% (*w/w*) saturated level. Following complete dissolution of the ammonium sulphate, the plasma was centrifuged at 12000x *g* for 15 min, and 50 µL of the supernatant was taken for subsequent sample preparation steps. Plasma glycoproteins were extracted, fractionated, digested and analysed by the developed targeted LC-MS/MS method as described in Sections 4.2.2, 4.2.3, and 4.2.6. Relative protein fold changes of the 54 profiled glycoproteins were measured by comparing the average of the peak areas of each signature peptide between plasma controls and ammonium sulphate treated plasma samples (6 samples in each group).
To identify plasma proteins that were precipitated by ammonium sulphate, the protein pellet obtained after plasma centrifugation was resuspended in 50 mM ammonium bicarbonate, digested and analysed by LC-MS/MS as described in Sections 4.2.3, 4.2.4, and 4.2.5.
Figure 4-2 summarises the workflow followed in this chapter.

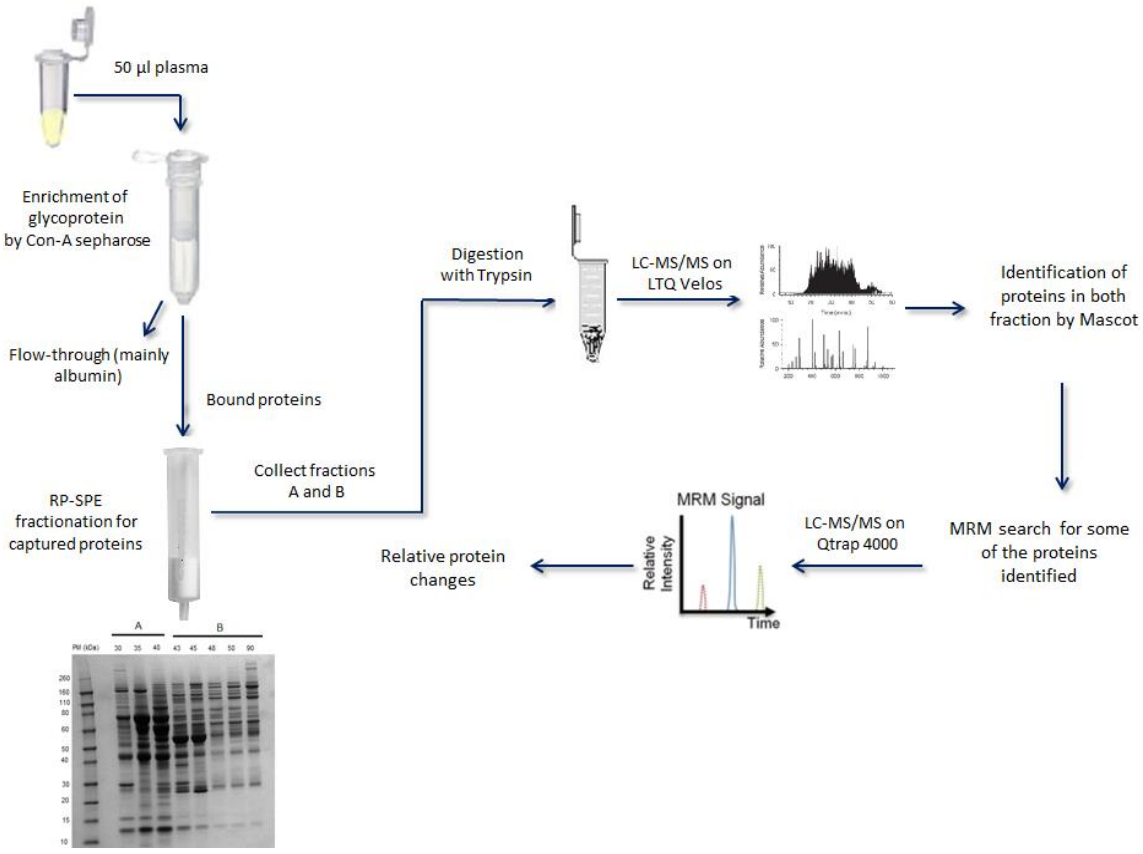


Figure 4-2: Schematic representation of the workflow in this chapter. Plasma glycoproteins were first enriched by ConA followed by RP-SPE fractionation. Tryptic protein digests of the two protein fractions; A and B representing proteins eluted at 40 and 50% respectively, were analysed by LC-MS/MS and proteins were identified by Mascot. Clinically relevant plasma glycoproteins (n=54) were targeted by MRM method and once validated the method was applied to measure protein fold changes in ammonium sulphate treated plasma.

4.3 Results and Discussion

4.3.1 Extraction of human plasma glycoproteins by ConA lectin resin and reversed-phase solid phase extraction (RP-SPE)

4.3.1.1 Extraction of glycoproteins by ConA

Following ConA enrichment, approximately 70% of the total plasma proteins (representing mainly non-glycosylated proteins) were depleted and 22% were captured by the ConA resin. The amount of protein in the ConA flow-through (depleted) and the ConA bound fractions were reproducible between replicates as noted in Table 4-3. The binding efficiency of ConA (as reflected by the percentage of the amount of proteins captured by ConA to the total amount of loaded protein) observed in this study (22%) was slightly lower than what was observed previously by Madera *et al*. (30%) [313].

Table 4-3: Total protein quantitation in ConA extraction experiment.

Sample	Amount of protein (µg)[a]	Recovery %[b]
Plasma	3320	100
Flow-through fraction	2376 ±78	71 ± 2.3
Bound fraction	713.6 ± 62	22 ± 1.9
- Fraction A	409.4 ± 20	12.3 ± 0.6
- Fraction B	148.8 ± 9	4.5 ± 0.3

[a] Protein quantification was carried out in triplicate by BCA quantification kit.
[b] All recoveries are expressed as the % of recovered proteins compared with 3320 µg of protein in 50 µL plasma. Results are shown as mean ± SD, n=5

The slightly lower binding efficiency noted here is mainly due to the higher amount of proteins initially loaded onto the ConA resin. However, similarly, Madera *et al.* [313] reported reproducible binding efficiency between replicates which supports the reliability of ConA as a selective extraction method for *N*-glycoprotein.

As expected, the major protein that was depleted with ConA affinity chromatography was the most abundant plasma protein albumin (seen as a large band at *c*. 65 kDa in the SDS-PAGE of the collected ConA flow-through and washes, (Figure 4-3, C). Albumin is a non-glycosylated protein which accounts for 50-60% of the total plasma protein, therefore, the efficient depletion of it is crucial as it should facilitate the identification of lower abundance proteins which otherwise would have been masked [24]. The same pattern of unbound proteins in the ConA flow-through noticed in this study (Figure 4-3, C) was also observed in the PAGE of previous ConA plasma glycoprotein isolation studies [262; 316].

4.3.1.2 Extraction of plasma protein by RP-SPE and ConA/PR-SPE

In the current work, a further separation of the ConA bound fraction was readily achieved with RP-SPE fractionation by eluting glycoproteins through the stepwise addition of an increasing percentage of an organic solvent (0.1% TFA in acetonitrile), Figure 4-3. SDS-PAGE separation of the eight RP-SPE fractions from whole plasma proteome and ConA captured proteins (Figure 4-3, A and B respectively) revealed distinguishable protein profiles between the two models, and demonstrated clear differences in the protein constituents of the fractions, providing evidence of successful sub-proteome and sub-glycoproteome fractionation. Comparison of the SDS-PAGE protein profiles of the RP-SPE fractions between the two models (plasma proteome and ConA captured proteins, Figure 4-3, A and B respectively) revealed additional protein bands, especially in the

higher mass range when ConA chromatography was coupled with RP-SPE fractionation. Moreover, some bands disappeared or were greatly diminished (*e.g.* albumin), while others were clearly more intense (*e.g.* transferrin bands at 75-80 kDa and eluted at 35 and 40%) in ConA treated fractions. These findings indicated that better enrichment and improved glycoproteins detection was achieved when ConA chromatography was coupled to RP-SPE, than with RP-SPE fractionation of the whole plasma alone. The precision of the two-step extraction strategy developed herein was confirmed by the reproducible SDS-PAGE protein profiles observed between the four biological replicates in the eight RP-SPE fractions, as seen in Figure 4-3, D.

The reproducible and successful fractionation observed herein has been previously reported when RP-SPE was applied to a range of partially fractionated complex proteome samples; rat plasma, human urine, and bacterial cell lysates, and when coupled with anion exchange separation [355]. This demonstrated reproducibility proved that RP-SPE, besides its application for sample purification, can also be effectively used for the fractionation and enrichment of plasma proteins [355; 356].
The developed two-step enrichment strategy (ConA coupled with PR-SPE) offers several advantages over lectin chromatography coupled with immunodepletion or extensive RP-HPLC fractionation methods to further reduce the complexity of the sample. It has a higher throughput (within minutes, instead of hours associated with immunoaffinity columns) [262; 266; 357], and it is not limited by the capacity, sample number or the high-cost as with immunodepletion columns [358]; it is limited only by the capacities of the employed devices. Moreover, it avoided the depletion of moderately abundant glycoproteins, such as protease inhibitors, and coagulation factors that might be a source of potential disease biomarkers [293; 359]. Similar to other enrichment tools, the collected RP-SPE fractions are compatible with down-stream analysis by gel electrophoresis and MS methods [355]. However, the method has limited ability to detect low abundant glycoproteins when compared to immunodepletion columns [262; 266].

Overall, sample preparation methodology is increasingly recognised as a major source of high variability in protein quantification [94]. The two-step extraction strategy developed in the current study provided a simple and reproducible protocol for efficient *N*-glycoprotein extraction, which is crucial to lower the overall variability of the analytical method.

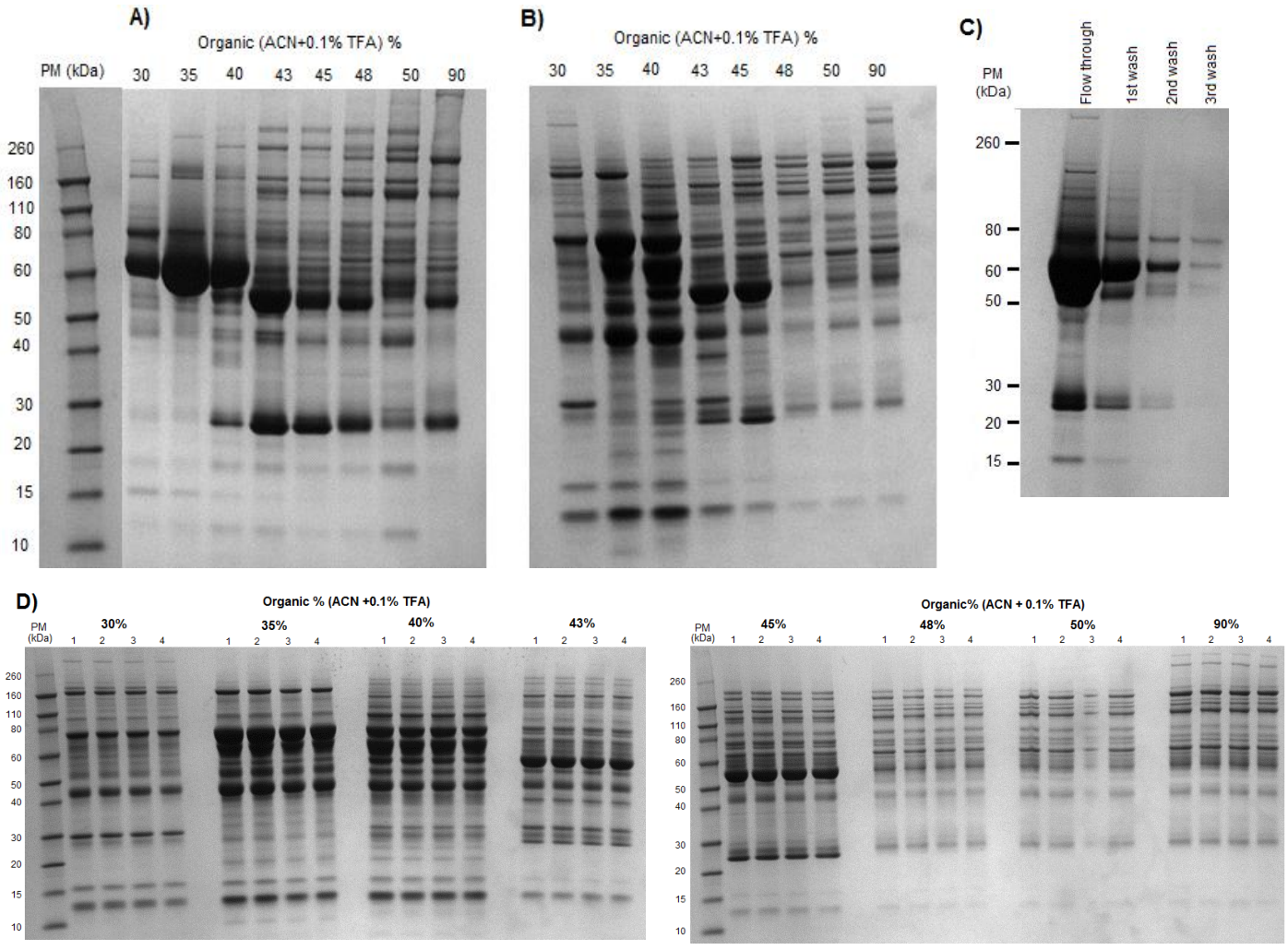


Figure 4-3: Coomassie blue stained 1D SDS-PAGE. A) Crude human plasma (25 µL) protein profiles in eight fractions eluted by increasing percentage of 0.1% TFA in acetonitrile using PR-SPE technique. The largest band in the gel is albumin, at around 65 kDa, with the majority of it eluting at 35%. B) Protein profiles of plasma (50 µL) treated with ConA affinity chromatography followed by RP-SPE fractionation for the captured proteins. 20% of the reconstituted fraction was loaded on all wells except for 35 and 40% wells in figure A where only 10% was loaded. C) ConA unbounded proteins eluted in the flow-through and the three resin washes. The main ConA unbound protein was albumin with the majority of it eluted in the flow-through and gradually decreased with the three washes. D) Proteins profiles across four human plasma replicates treated with ConA affinity chromatography followed by RP-SPE fractionation of the bounded proteins. Consistent protein profiles were observed between the four biological replicates in the eight fractions eluted by increasing % of 0.1% TFA in acetonitrile through PR-SPE technique. 20% of the reconstituted fraction was loaded on all wells.

4.3.2 Identification of plasma proteins eluted from RP-SPE by LC-MS/MS

In fraction A, 54 plasma proteins (379 matched peptides) were reproducibly identified while 62 proteins (521 matched peptides) were detected in fraction B, as shown in Table 4-4 and Table 4-5 respectively. Some proteins (*e.g.* afamin, apolipoprotein D, clusterin) were detected in both fractions due to the different hydrophilic/hydrophobic properties of their diverse glycoprotein isoforms [31]. The combined results from both fractions identified 90 plasma proteins with a total of 758 matched peptides ranging from, 65 matched peptides for complement C3 to 1 unique matched peptide for 16 proteins. To maximise the confidence of detection, FDR was set to 1% and those proteins not reliably identified between replicates (*e.g.* coagulation factor VII, vitamin K-dependent protein) were not included in the final list of the identified glycoproteins. Among the 90 proteins detected, 74 glycoproteins (82%) were identified with at least two unique different peptides per protein, which is widely recognised as being sufficient for positive confirmation of ID [324; 360]. The remaining 16 proteins were identified by one unique peptide, but were reproducibly detected and thus were included in the list and considered as positive identification. From these 16 proteins (8 of them were immunoglobulins, 2 haemoglobin subunits) only four were considered in the final profiling methodology: serum amyloid P-component, complement component C9, pigment epithelium derived factor and N-acetylmuramoyl-L-alanine amidase. The identity of the peptides used for the identification of these proteins was further verified by a separate more sensitive analysis using EPI scan triggered by MRM, as described in Section 4.2.6. The manual inspection of the obtained MS/MS peptide fragmentation spectra showed good S/N ratio, and the predicted *y* ion fragments were matched confirming the identity of these signature peptides (Figure 4-4). A similar strategy has been previously reported to confirm the identity of glycoproteins identified with a single matched peptide [314]. The researchers used a more sensitive instrument to acquire the MS/MS fragmentation spectra of these peptides, and to obtain signals with low noise levels. Generated fragment ion signals were then matched with the expected *b* or *y* ions and the identity of the peptides was confirmed, and therefore they considered the identified glycoproteins as positive identification [314].

Although not glycosylated, undepleted albumin was detected among the proteins captured by ConA herein. This has also been observed in glycoprotein extracted fractions in previous studies [262; 263], and can be either due to non-specific binding or

complex formation with glycosylated immunoglobulins. Apart from albumin and some unglycosylated proteins known to be associated with glycosylated proteins, such as the light chain of immunoglobulins, all other proteins captured by the lectin column were glycosylated proteins, which confirms the reported specificity of the ConA affinity column [262] and increases confidence in the identification of individual glycoproteins. The stringent criteria used here for the identification of glycoproteins, including the reproducibility between replicates, the number of matched peptides and the manual data validation contributed to the increased confidence in proteins' identity.

Table 4-4: Proteins identified in the tryptic plasma digest of fraction A by LC-MS/MS.

No.	Protein description[a]	Swiss Prot code[b]	Matched peptides	Sequence coverage	Protein Score[c]	Glycosylated protein[d]	Main function[e]
1	Serotransferrin	P02787	41	43	427	yes	Transport
2	Haptoglobin	P00738	21	35	179	yes	Captures free haemoglobin
3	Complement C3	P01024	20	14	208	yes	Immunity, inflammatory response, lipid metabolism
4	Complement factor H	P08603	20	20	220	yes	Immunity
5	Serum albumin	P02768	17	27	138	No	Regulation of the osmotic pressure of blood
6	Fibrinogen alpha chain	P02671	14	14	100	Yes	Blood coagulation
7	Fibrinogen gamma chain	P02679	14	25	97	Yes	Blood coagulation
8	Hemopexin	P02790	14	28	172	yes	Haem transporter
9	Fibrinogen beta chain	P02675	12	30	182	Yes	Blood coagulation
10	Prothrombin	P00734	11	23	189	yes	Blood coagulation
11	α-1-Betaglycoprotein	P04217	10	26	189	yes	Unknown function
12	α-2-Macroglobulin	P01023	10	8	156	yes	Enzyme inhibitor
13	Complement factor B	P00751	10	11	159	yes	Immunity
14	Complement factor I	P05156	8	16	127	yes	Immunity
15	Histidine-rich glycoprotein	P04196	8	18	114	Yes	Blood coagulation and haemostasis, angiogenesis
16	α-2-HS-glycoprotein	P02765	7	16	130	yes	Mineral balance
17	Antithrombin-III	P01008	7	13	120	yes	Blood coagulation
18	Ceruloplasmin	P00450	7	9	176	yes	Ion transport
19	Kininogen-1	P01042	7	14	158	Yes	Bradykinin is released from kininogen by plasma kallikrein
20	α-1-Antitrypsin	P01009	6	20	169	yes	Enzyme inhibitor
21	β-2-glycoprotein 1	P02749	6	23	180	yes	Binds to negatively charged substances such as heparin and phospholipids
22	Ig alpha-1 chain C region	P01876	6	19	148	yes	Immunity

Table 4-4: Continued

No.	Protein description[a]	Swiss Prot code[b]	Matched peptides	Sequence coverage	Protein score[c]	Glycosylated protein[d]	Main function[e]
23	Plasminogen (Plasminogen-like protein A)	Q15195	6	6	64	yes	Blood coagulation
24	Zinc-α-2-glycoprotein	P25311	6	13	78	yes	Stimulates lipid degradation
25	Afamin	P43652	5	7	100	yes	Transport
26	Complement C4-A	P0C0L4	5	2	126	yes	Immunity, inflammatory response
27	Complement component C6	P13671	5	3	150	yes	Immunity
28	Ig gamma-1 chain C region	P01857	5	13	112	yes	Immunity
29	Ig kappa chain C region	P01834	5	64	179	No	Immunity
30	Ig mu chain C region	P01872	5	13	161	yes	Immunity
31	Plasma kallikrein	P03952	5	9	146	Yes	Releases bradykinin from HMW kininogen and converts prorenin into renin
32	α-1-Acid glycoprotein	P02763	4	21	86	yes	Transport
33	C4b-Binding protein alpha chain	P04003	4	8	102	yes	Complement pathway
34	Clusterin	P10909	4	9	86	yes	Protein stabilisation and regulation of apoptotic process
35	Inter-α-trypsin inhibitor heavy chain H1	P19827	4	6	77	Yes	Enzyme inhibitor
36	Vitronectin	P04004	4	8	75	yes	Cell adhesion
37	Apolipoprotein D	P05090	3	20	66	yes	Transport
38	Complement C1r subcomponent	P00736	3	6	43	yes	Complement pathway
39	Complement C1s subcomponent	P09871	3	5	58	yes	Complement pathway
40	Complement component C8 alpha chain	P07357	3	5	52	yes	Immunity, Complement pathway
41	Ig gamma-2 chain C region	P01859	3	13	92	yes	Immunity
42	Ig lambda-1 chain C regions	P0CG04	3	28	62	No	Immunity
43	Immunoglobulin lambda-like polypeptide 5	B9A064	3	14	72	No	Immunity

Table 4-4: Continued

No.	Protein description[a]	Swiss Prot code[b]	Matched peptides	Sequence coverage	Protein score[c]	Glycosylated protein[d]	Main function[e]
44	Complement factor H-related protein 1	Q03591	2	5	66	yes	Immunity
45	Immunoglobulin J chain	P01591	2	11	80	yes	Immunity
46	Inter-α-trypsin inhibitor heavy chain H4	Q14624	2	2	84	Yes	Enzyme inhibitor
47	Lumican	P51884	2	7	47	Yes	Collagen binding
48	CD5 antigen-like	O43866	1	3	42	No	Immunity and may play role as inhibitor of apoptosis
49	Complement component C9	P02748	1	2	54	yes	Immunity, inflammatory response
50	Haemoglobin subunit alpha	P69905	1	10	51	yes	Oxygen transport
51	Haemoglobin subunit beta	P68871	1	8	54	yes	Oxygen transport
52	Protein AMBP	P02760	1	3	60	yes	Enzyme inhibitor
53	Plasma protease C1 inhibitor	P05155	1	2	57	Yes	Complement activation, blood coagulation, fibrinolysis
54	Serum amyloid P-component	P02743	1	4	47	Yes	Scavenger for nuclear material released from damaged circulating cells.

[a] Protein description in Mascot using Swiss-Prot database for searching.
[b] Swiss-Prot code (accession No.) for the proteins was added as a reference for the identification in UniProtKB/Swiss-Prot database.
[c] MASCOT score at FDR of 1%.
[d] According to SwissProt database.
[e] As described in Swiss-Prot database

Table 4-5: Proteins identified in the tryptic plasma digest of fraction B by LC-MS/MS.

No.	Protein description[a]	Swiss Prot code[b]	Matched peptides	Sequence coverage	Protein score[c]	Glycosylated protein[d]	Main function[e]
1	Complement C3	P01024	65	40	908	Yes	Immunity, inflammatory response
2	α-2-Macroglobulin	P01023	44	31	753	Yes	Enzyme inhibitor
3	α-1-Antitrypsin	P01009	29	52	392	Yes	Enzyme inhibitor
4	Complement C4-A	P0C0L4	22	20	413	Yes	Immunity, inflammatory response
5	Inter-α-trypsin inhibitor heavy chain H2	P19823	20	25	438	Yes	Enzyme inhibitor
6	Apolipoprotein A-I	P02647	20	53	280	Yes	Transport
7	Inter-α-trypsin inhibitor heavy chain H4	Q14624	19	27	354	Yes	Enzyme inhibitor
8	Haptoglobin	P00738	19	31	248	Yes	Captures free haemoglobin
9	Inter-α-trypsin inhibitor heavy chain H1	P19827	18	18	482	Yes	Enzyme inhibitor
10	α-1-Antichymotrypsin	P01011	16	29	228	Yes	Enzyme inhibitor
11	Plasma protease C1 inhibitor	P05155	16	22	181	Yes	Blood coagulation, fibrinolysis
12	Ig mu chain C region	P01871	16	31	164	Yes	Immunity
13	Antithrombin-III	P01008	15	36	284	Yes	Blood coagulation
14	Ceruloplasmin	P00450	15	22	271	Yes	Ion transport
15	Afamin	P43652	13	22	189	Yes	Transport
16	Serotransferrin	P02787	11	16	234	Yes	Transport
17	Complement C4-B	P0C0L5	10	12	305	Yes	Immunity, inflammatory response
18	Heparin cofactor 2	P05546	10	28	223	Yes	Enzyme inhibitor
19	Ig gamma-3 chain C region	P01860	9	18	97	Yes	Immunity
20	Ig alpha-1 chain C region	P01876	8	30	182	Yes	Immunity
21	α-2-antiplasmin	P08697	8	15	145	Yes	Enzyme inhibitor
22	Ig kappa chain C region	P01834	7	79	259	No	Immunity
23	Angiotensinogen	P01019	7	15	196	Yes	Hypertension
24	Fibronectin	P02751	7	4	90	Yes	Cell adhesion, angiogenesis

Table 4-5: Continued

No.	Protein description[a]	Swiss Prot code[b]	Matched peptides	Sequence coverage	Protein score[c]	Glycosylated protein[d]	Main function[e]
25	Ig gamma-2 chain C region	P01859	6	9	55	Yes	Immunity
26	Ig gamma-1 chain C region	P01857	5	17	170	Yes	Immunity
27	α-2-HS-glycoprotein	P02765	5	15	157	Yes	Mineral balance
28	Protein AMBP	P02760	5	24	139	Yes	Enzyme inhibitor
29	Serum paraoxonase/arylesterase 1	P27169	5	13	112	Yes	Metabolism
30	Immunoglobulin J chain	P01591	5	31	87	Yes	Immunity
31	Clusterin	P10909	4	10	109	Yes	Protein stabilisation and regulation of apoptotic process
32	Inter-α-trypsin inhibitor heavy chain H3	Q06033	4	4	63	Yes	Enzyme inhibitor
33	Ig alpha-2 chain C region	P01877	4	9	60	Yes	Immunity
34	Complement C1q subcomponent subunit B	P02746	4	18	38	Yes	Immunity, inflammatory response
35	Ig kappa chain V-III region SIE	P01620	3	39	115	No	Immunity
36	CD5 antigen-like	O43866	3	11	110	No	Immunity
37	Hemopexin	P02790	3	8	104	Yes	Haem transporter
38	Carboxypeptidase N subunit 2	P22792	3	6	100	Yes	Stabilises carboxypeptidase
39	Ig gamma-4 chain C region	P01861	3	14	88	Yes	Immunity
40	Kallistatin	P29622	3	7	71	Yes	Enzyme inhibitor
41	Corticosteroid-binding globulin	P08185	3	8	45	Yes	Transport
42	Apolipoprotein A-II	P02652	2	14	254	Yes	Transport
43	Insulin-like growth factor-binding protein complex acid labile subunit	P35858	2	4	118	Yes	Protein-protein interactions
44	Apolipoprotein D	P05090	2	10	114	Yes	Transport
45	Ig heavy chain V-III region BRO	P01766	2	15	98	No	Immunity
46	Vitronectin	P04004	2	5	79	Yes	Cell adhesion
47	Complement C5	P01031	2	1	71	Yes	Immunity, inflammatory response

Table 4-5: Continued

No.	Protein description[a]	Swiss Prot code[b]	Matched peptides	Sequence coverage	Protein score[c]	Glycosylated protein[d]	Main function[e]
48	Thyroxine-binding globulin	P05543	2	2	54	Yes	Transport
49	Ig lambda-2 chain C regions	P0CG05	2	17	39	No	Immunity
50	Ig kappa chain V-I region AG	P01593	1	16	95	No	Immunity
51	Ig kappa chain V-IV region Len	P01625	1	15	88	No	Immunity
52	Ig kappa chain V-I region CAR	P01596	1	16	81	No	Immunity
53	Ig heavy chain V-III region WEA	P01763	1	9	62	Yes	Immunity
54	Complement C1q subcomponent subunit C	P02747	1	4	56	Yes	Immunity, inflammatory response
55	Pigment epithelium-derived factor	P36955	1	3	54	Yes	Neurotrophic protein
56	Leucine-rich alpha-2-glycoprotein	P02750	1	4	47	Yes	Cell adhesion
57	Complement component C9	P02748	1	4	43	Yes	Immunity, inflammatory response
58	N-Acetylmuramoyl-L-alanine amidase	Q96PD5	1	2	42	Yes	Enzyme inhibitor
59	Ig heavy chain V-III region HIL	P01771	1	9	41	No	Immunity
60	Ig kappa chain V-II region TEW	P01617	1	21	38	No	Immunity
61	Ig kappa chain V-III region VG (Fragment)	P04433	1	7	34	No	Immunity
62	Ig kappa chain V-III region CLL	P04207	1	17	28	No	Immunity

[a] Protein description in Mascot using Swiss-Prot database for searching.
[b] Swiss-Prot code (accession No.) for the proteins was added as a reference for the identification in UniProtKB/Swiss-Prot database.
[c] MASCOT score at FDR of 1%.
[d] According to SwissProt database.
[e] As described in Swiss-Prot database

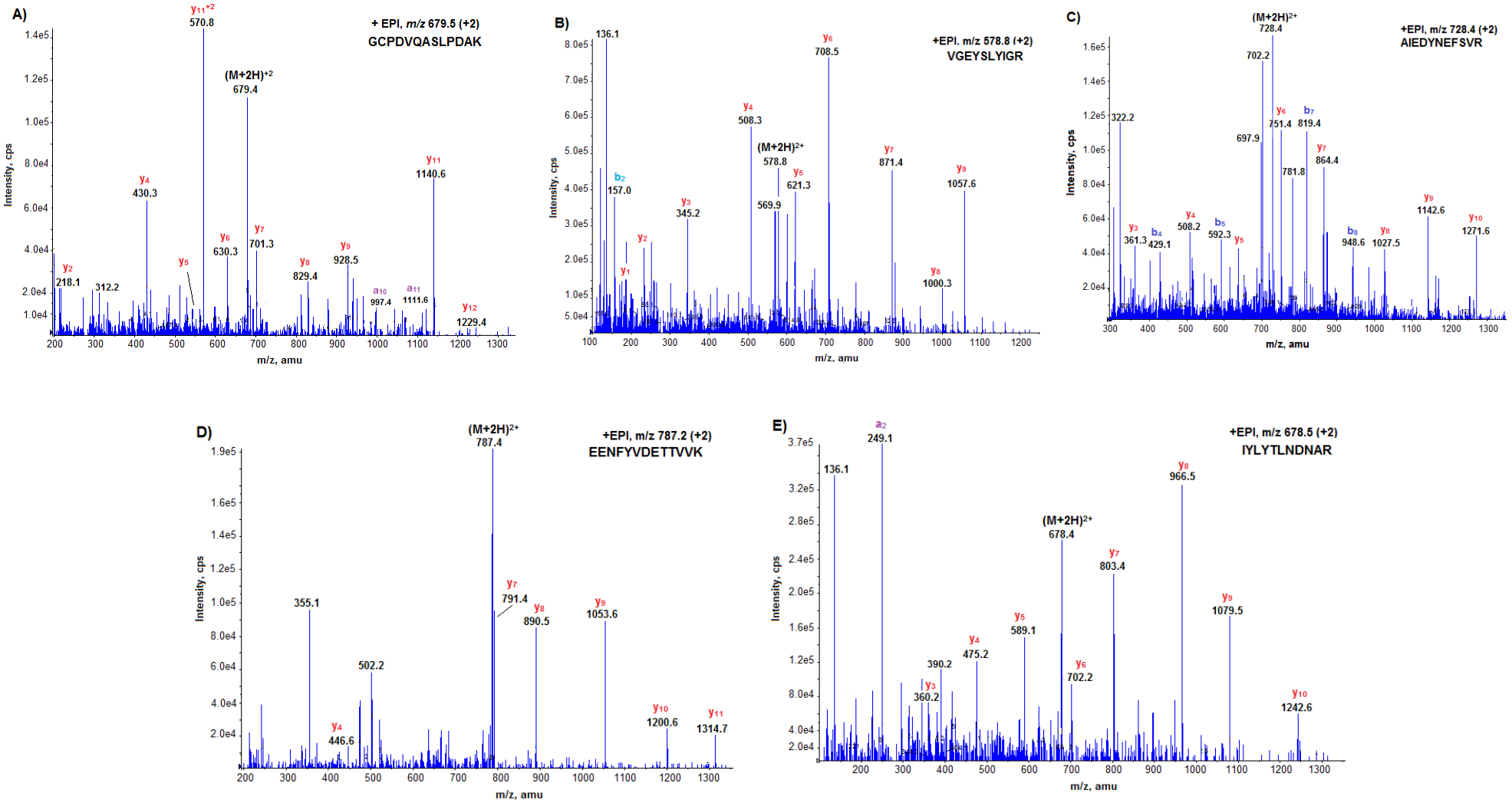


Figure 4-4: MS/MS spectra of selected signature peptides of low abandance glycoproteins of human plasma extract. The spectra were obtained by EPI scan triggered by MRM, and the predicted *y* and *b* ions for each peptide were matched with the obtained MS/MS spectrum A) N-acetylmuramoyl-L-alanine amidase B) Serum amyloid P-component C) Complement component 9 D) Corticosteroid-binding globulin E) Fibronectin. A, B and C proteins were reproducibly identified with only one peptide by Mascot.

The glycoproteins identified in the plasma fractions are reported to be involved in a range of biological processes (Table 4-4, Table 4-5 and Figure 4-5), and varied in concentration, with high to moderate abundance proteins dominating the list, as ConA enrichment was conducted without prior depletion of the naturally highly abundant proteins. The combined results from both fractions confidently identified plasma proteins ranging in concentration from the highly abundant immunoglobulin IgG (1.2×10^4 µg/mL, [361]) to the pigment epithelium-derived factor (5 µg/mL, [293]), covering four orders of magnitude. The range of glycoprotein concentrations observed is shown in Figure 4-6. This gain in the dynamic range obtained with the formulated two-step enrichment strategy, compared to whole plasma or ConA extraction alone protein analyses, enabled the identification of proteins that could not be detected either in the total plasma proteome or by ConA enrichment alone, including peptides from less abundant glycoproteins such as corticosteroid binding globulin, fibronectin, lumican and pigment epithelium-derived factor. Such result highlighted the efficiency of the method developed herein as a powerful tool for extracting a glycoprotein subproteome.

Compared to the presented method, extended dynamic range and improved coverage of low abundant glycoproteins (proteins in the low ng/mL concentration were detected), have been reported previously when immunodepletion was coupled with glycoprotein extraction technique [266]. However, as previously discussed, despite these advantages, immunodepletion columns will lead to the loss of potential protein biomarkers [359] and will add to the cost and the overall analysis time [358].

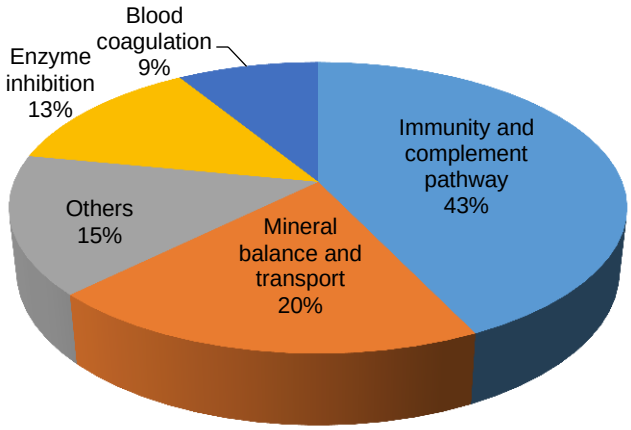


Figure 4-5: General classification of glycoproteins identified according to their main function as described in Swiss-Prot database. High percentages of immunoglobulins and complement factor involved in immunity responses were isolated. Enzyme inhibitors, especially protease inhibitors as α-1-antitrypsin, α-1-antichymotrypsin, and metal and lipids protein transporters were also detected in a good percentage. Proteins involved in coagulation like fibrinogen and prothrombin were detected in fraction A. The other detected proteins are involved in diverse biological processes as cell adhesion, protein–protein interaction and blood pressure.

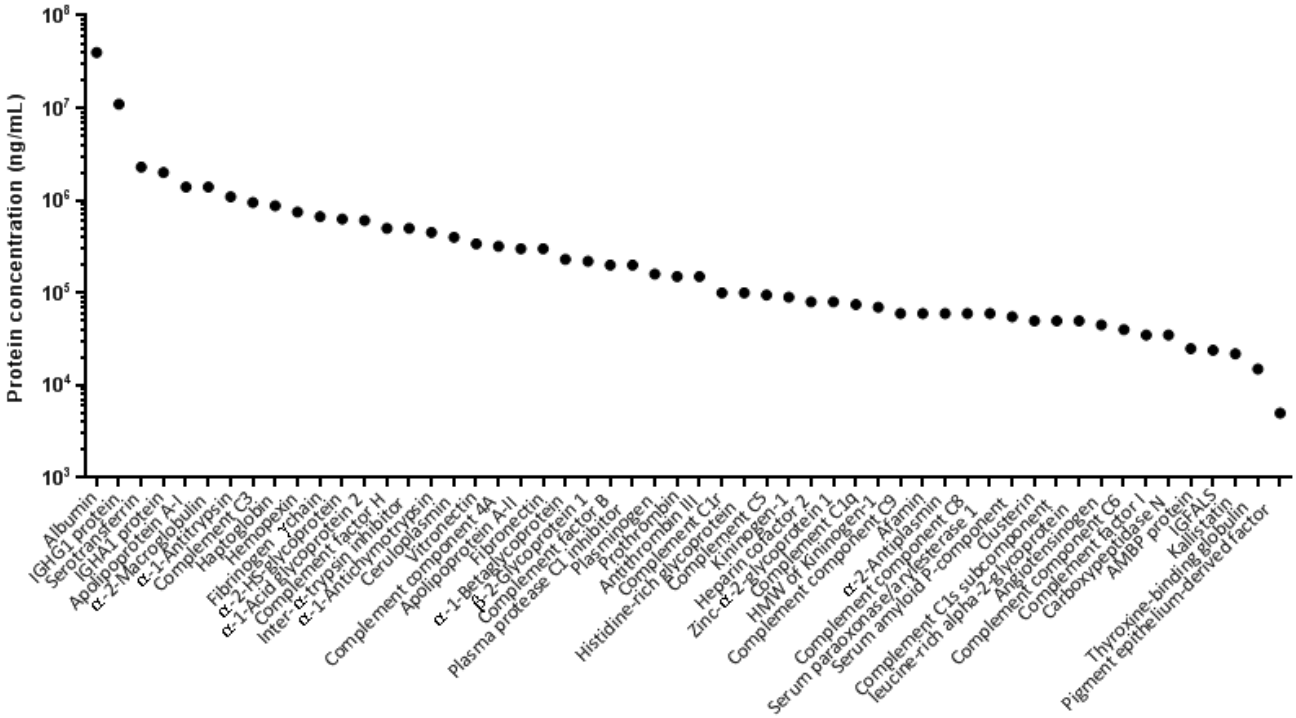


Figure 4-6: Concentration distribution of most of the detected glycoproteins expanding over four orders magnitude. From the immunoglobulins only IgG and IgA are presented. At the highest concentration are IgG, serotransferrin and apolipoprotein A-I and at the lowest end is pigment epithelium-derived factor. IGFBP refers to insulin-like growth factor binding protein. References of the presented protein concentrations are covered in Appendices, Table A4-1.

With regards to glycoprotein coverage, the list of the identified glycoproteins herein (Table 4-4 and Table 4-5) was comparable with what have been reported by previous studies that used ConA and multi-lectin enrichment methods [259; 262; 316], and with a higher number of identifications. The methodology demonstrated here was able to confidently identify 78 glycoproteins, while other multi-lectin glycoprotein extraction studies identified 64 [259] and 50 [262] glycoproteins. Moreover, this study has identified relevant glycoproteins which were not detected in a previous ConA enrichment study [354], including α-1-antichymotrypsin, pigment epithelium derived factor, corticosteroid binding globulin and vitronectin,

Another advantage of the current method is the use of non-glycopeptides in glycoprotein identification, which added to the improved throughput and analysis cost-effectiveness, as no deglycosylation steps were carried out. Additionally, the use of non-glycopeptides is well-known to yield better glycoprotein coverage and improved confidence in detection, when compared with glycopeptides-based identification [321; 324]. The present findings are in agreement with this, as larger numbers of glycoproteins were identified using non-glycopeptides herein (78 glycoproteins), compared to similar ConA glycoprotein extraction studies that were able to identify only 40 [321] and 49 [259] glycoproteins using *N*-glycopeptides.

4.3.3 Inclusion criteria for specific plasma glycoproteins

For the targeted MS analysis, 54 clinically relevant glycoproteins with high confidence of identity were selected to be monitored by MRM; 38 from fraction A and 33 from fraction B. Each protein was specifically detected by one signature peptide, and for most a single fragment ion was monitored, as shown in Table 4-6. The selection of suitable signature peptides for each glycoprotein together with their MRM transitions was conducted predominantly by reference to relevant scientific literature (as is cited in Table 4-6). It is worth mentioning that all of the targeted peptides had already been identified in Mascot searches during glycoprotein identification.

The sensitivity and specificity of a protein LC-MS/MS assay are highly dependent on the surrogate peptide and precursor/product ion selections [87]. Therefore, careful qualitative assessment of the chosen peptides and transitions in terms of reproducibility, signal intensity and peak shape was carried out (as described in Section 4.2.7). The MS/MS spectra for all targeted signature peptides confirmed their identities by matching most of

the predicted *y* ions with the obtained spectra. Examples of MS/MS spectra for selected peptides representing low abundance glycoproteins are shown in Figure 4-4.

Extracted ion chromatogram (XIC) for the targeted proteins in fractions A and B of a human plasma sample are presented in Figure 4-7 and Figure 4-8 respectively. The signal intensities varied between the glycoproteins, with the highest being for transferrin (~$1x10^6$) and α-1antitrypsin (~$8x10^5$), and the lowest for lumican (~$7x10^3$) and corticosteroid binding globulin (~$4.5x10^3$) in fractions A and B respectively. Signature peptides transitions were highly selective and only one peak per transition was detected in the XIC. However, a minor secondary peak, caused by the interference from the plasma matrix background, was noticed in the XIC of some peptides (such as ceruloplasmin); therefore, the chromatographic elution time of three transitions was used to assure the specific and reliable detection of the targeted peptide [32; 362]. Retention times for the individual signature peptide transitions were consistent throughout.

One important factor that can highly affect the reproducibility of MS-based quantitative protein analysis is ion suppression, as caused by interferences from complex plasma background. The presence of non- or less volatile components (*e.g.* salts, endogenous compounds) can decrease the efficiency of droplet formation or evaporation during ionisation and subsequently suppresses the signal of the analytes [363]. If these components co-elute with the peptide of interest, then the reproducibility of the peptide ionisation will be affected leading to poor precision. The presented sample preparation workflow (ConA/RP-SPE fractionation) greatly minimised the matrix effect by reducing the complexity of the plasma and removing many endogenous compounds that can cause ion suppression, such as metabolites and lipids. Additionally, the chromatographic separation of protein digest allowed peptides to be eluted at different retention times thus reducing the ion suppression effect. These tools culminated in the reproducible detection of the signature peptides signals with a signal to noise ratio ≥10. Assay precision will be discussed further in the following section.

Table 4-6 MRM transitions and CV values for the targeted glycoproteins.

No.	Protein	Fraction	Signature peptide sequence	MS1/MS2	Fragment ion	CV (%)	Reference
1	α-1- Acid glycoprotein	A	NWGLSVYADKPETTK	**570.3/273.1**	a2	12.8	[79]
2	α-1-Antichymotrypsin	B	ADLSGITGAR	**480.8/574.3** 480.8/661.4	y6 y7	13.4	[79], Mascot
3	α-1-Antitrypsin	A B	LSITGTYDLK	**556.0/797.4**	y7	11.7 10.0	[80; 86]
4	α-2-Antiplasmin	B	LGNQEPGGQTALK	**656.8/771.4**	y8	16.0	[87]
5	α-1-Betaglycoprotein	A	LETPDFQLFK	**619.3/243.1**	b2	11.5	[87]
6	α -2-HS glycoprotein	A B	FSVVYAK	**407.6/480.3** 407.6/579.5	y4 y5	7.2 14.8	Mascot
7	α-2-Macroglobulin	A B	LLIYAVLPTGDVIGDSAK	**615.7/530.3**	$y11^{+2}$	22.5 12.6	[87]
8	β-2-glycoprotein 1	A	ATVVYQGER	**511.8/652.3**	y5	11.9	[87]
9	Afamin	A B	DADPDTFFAK	**563.8/825.4**	y7	10.0 13.0	[87]
10	Albumin	A	LVNEVTEFAK	**575.3/937.5**	y8	14.4	[87]
11	Angiotensinogen	B	ALQDQLVLVAAK	**634.9/501.4**	y5	11.8	[87]
12	Antithrombin-III	A B	DDLYVSDAFHK	**437.2/704.3**	y6	11.0 9.7	[79; 87]
13	Apolipoprotein A-1	B	ATEHLSTLSEK	**405.9/363.2**	y3	9.5	[87]
14	Apolipoprotein A-II	B	SPELQAEAK	**486.8/443.2**	$y8^{+2}$	12.8	[87]
15	Apolipoprotein D	A B	NILTSNNIDVK	**615.8/1003.5** 615.8/890.5	y9 y8	13.1 11.2	[80]
16	C4b-Binding protein α-chain	A	WTPYQGCEALCCPEPK	**998.7/855.2**	$y14^{+2}$	20.8	Mascot
17	CD5 antigen-like	B	IWLDNVR	**458.4/616.4** 458.4/503.3	y5 y4	12.5	Mascot
18	Ceruloplasmin	A B	EYTDASFTNR	**602.3/695.3** 602.3/911.4	y6 y8	14.6 7.1	[79; 87]
19	Clusterin	A B	ASSIIDELFQDR	**697.5/565.3** 697.5/922.4	y4 y7	14.7 8.1	[86]
20	Complement C3	A B	TGLQEVEVK	**501.8/603.3**	y5	22.5 4.0	[87]
21	Complement C4-β-chain	A B	VGDTLNLNLR	**557.8/629.4** 557.8/843.5	y5 y7	13.4 11.0	[87]
22	Complement C5	B	TDAPDLPEENQAR	**728.3/584.8** 728.3/843.4	$y10^{+2}$ y7	14.6	Mascot
23	Complement C6	A	SEYGAALAWEK	**612.8/1008.6** 612.8/845.6 612.8/717.5	y9 y8 y6	15.4	Mascot
24	Complement C8 α-chain	A	MESLGITSR	**497.5/533.4**	y5	17.3	Mascot
25	Complement C9	A B	AIEDYINEFSVR	**728.5/1271.6** 728.5/1027.5	y10 y8	8.5 3.6	[79]
26	Complement factor B	A	EELLPAQDIK	**578.3/372.2**	b3	11.6	[87]
27	Complement factor H	A	SPDVINGSPISQK	**671.4/830.4**	y8	7.6	[87]
28	Complement factor I	A	GLETSLAECTFTK	**729.1/856.5** 729.1/785.6	y7 y6	12.8	Mascot
29	Corticosteroid binding globulin	B	EENFYVDETTVVK	**787.2/890.5** **787.2/791.4** 787.2/1053.5	y8 y7 y9	12.6	Mascot
30	Fibrinogen α-chain	A	GSESGIFTNTK	570.8/780.4 **570.8/610.3**	y7 y5	32.7	[79; 87]
31	Fibrinogen β-chain	A	QGFGNVATNTDGK	**654.8/706.3** 654.8/635.3	y7 y6	30.7	[79; 87]

Table 4-6: Continued

No.	Protein	Fraction	Signature peptide sequence	MS1/MS2	Fragment ion	CV (%)	Reference
32	Fibrinogen γ-chain	A	DTVQIHDITGK	**409.6/217.1** 409.5/533.3	b2 y5	30.8	[79; 87]
33	Fibronectin	B	IYLYTLNDNAR	**678.5/803.4** **678.5/966.4** 678.5/1079.5	y7 y8 y9	25.9	Mascot
34	Hepatoglobin β chain	A B	DIAPTLTLYVGK	**646.1/496.5**	$y9^{+2}$	15.2 6.7	Mascot
35	Hemopexin	A	NFPSPVDAAFR	**610.8/959.6**	y9	12.9	[79]
36	Histidin rich glycoprotein	A	GGEGTGYFVDFSVR	**746.2/869.6** 746.2/623.4	y7 y5	7.8	Mascot
37	Heparin cofactor II	B	TLEAQLTPR	**514.8/814.4** 514.8/685.4	y7 y6	7.1	[87]
38	Inter-α-trypsin inhibitor heavy chain (H1)	A B	AAISGENAGLVR	**579.4/902.5**	y9	10.2 23.7	[79]
39	Insulin-like growth factor-binding protein acid labile subunit	B	LAELPADALGPLQR	**732.6/1037.7** 732.6/570.4 732.6/894.6	y10 y5 b9	11.0	Mascot
40	Kallikrein	A	LSMDGSPTR	**482.6/632.4** **482.6/850.5** 482.6/763.5	y6 y8 y7	12.6	Mascot
41	Kallistatin	B	IAPANADFAFR	**596.8/540.3** 596.8/504.9 596.8/840.4	y4 $y9^{+2}$ y7	6.9	Mascot
42	Kininogen	A	TVGSDTFYSFK	**626.3/1051.5**	y9	11.8	[87]
43	Lumican	A	SLEDLQLTHNK	**649.5/853.5** **649.5/549.5** 649.5/612.4	Y7 $Y9^{+2}$ Y5	26.6	Mascot
44	N-acetylmuramoyl-L-alanine amidase	B	GCPDVQASLPDAK	**679.5/571.0** 679.5/430.3	$y11^{+2}$ y4	14.7	Mascot
45	Pigment epithelium derived factor	B	LAAAVSNFGYDLYR	**780.4/1134.6** 780.4/933.6 **780.4/1047.5**	y9 y7 y8	10.0	Mascot
46	Plasma protease C1 inhibitor	A B	LLDSLPSDTR	**558.8/575.3**	y5	19.2 6.9	[80]
47	Protein AMBP	A B	TVAACNLPIVR	**607.4/484.3**	y4	9.9 10.2	Mascot
48	Plasminogen	A	LFLEPTR	**438.3/615.3** 438.3/502.3	y5 y4	13.2	[79; 87]
49	Prothrombin	A	ETAASLLQAGYK	**626.3/879.5**	y8	13.2	[79; 87]
50	Serum-amyloid P-component	A	VGEYSLYIGR	**578.8/708.4** 578.8/508.3	y6 y4	11.7	[87]
51	Serum paraoxonase/aryleste-rase1	B	IQNILTEEPK	**592.8/943.5** 592.8/716.6	y8 y6	7.9	Mascot
52	Thyroxine-binding globulin	B	GWVDLFVPK	**530.8/ 244.1**	b2	15.1	Mascot
53	Transferrin	A B	SVIPSDGPSVACVK	**708.7/558.9**	$y11^{+2}$	12.6 5.6	Mascot
54	Vitronectin	A B	DVWGIEGPIDAAFTR	**823.9/947.5** 823.9/1076.6	y9 y10	12.9 13.2	[79]
55	Zinc-α-2-glycoprotein	A	EIPAWVPFDPAAQITK	**892.0/770.9**	$y14^{+2}$	7.5	[87]

* Validated transitions are presented in bold.
** Swiss-Prot codes (accession No.) for the proteins are presented in table 4-4 and 4-5.
*** MS1/MS2 represents precursor and product ions.

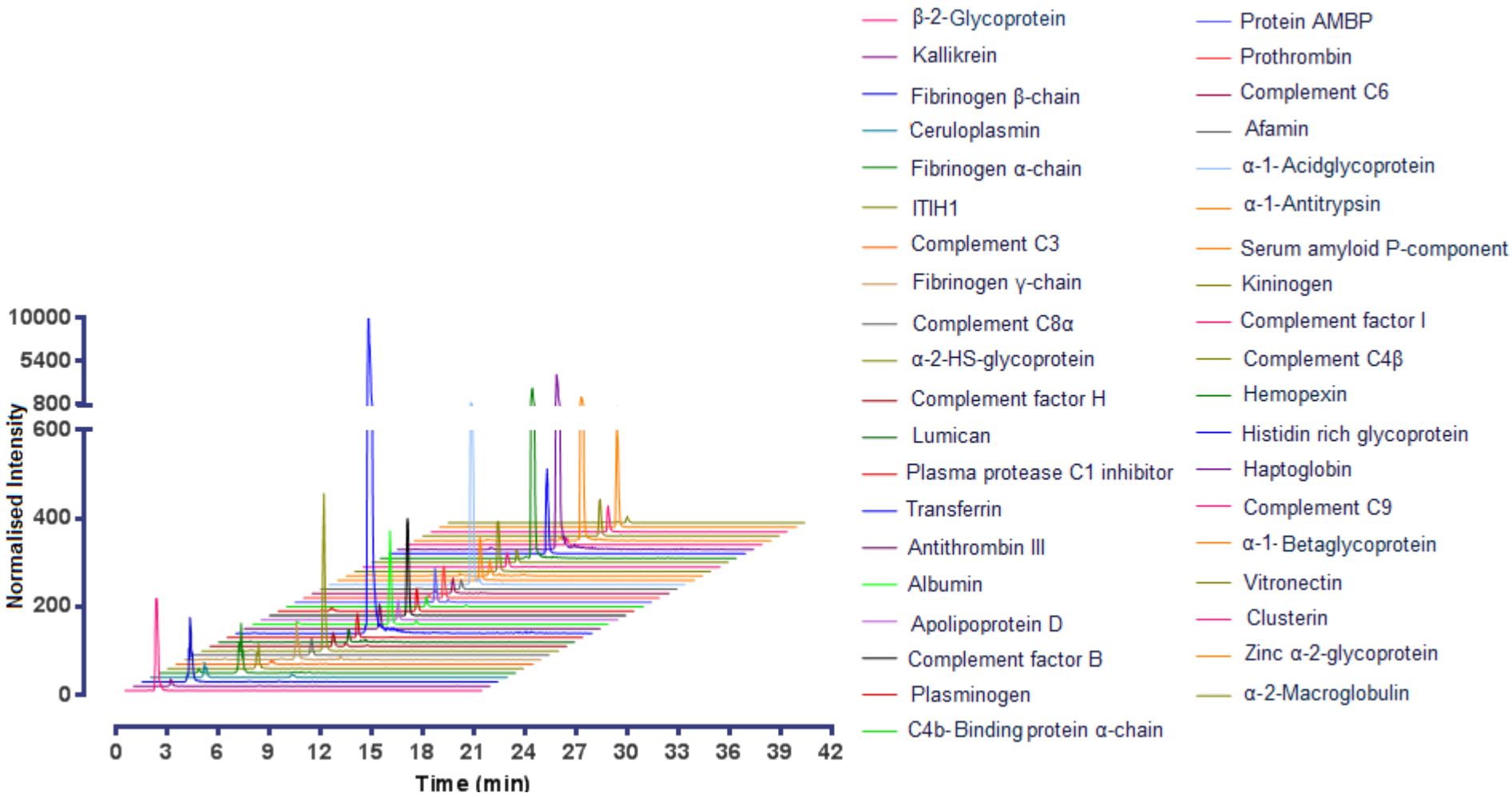


Figure 4-7: Extracted ion chromatogram of human plasma extract for 39 glycoproteins profiled in fraction A using targeted LC-MS/MS. The analysis time was 21 min and signal intensity for each protein was normalised to the protein with the highest signal intensity in the run; transferrin.
ITIH1 refers to inter-alpha-trypsin inhibitor heavy chain H1.

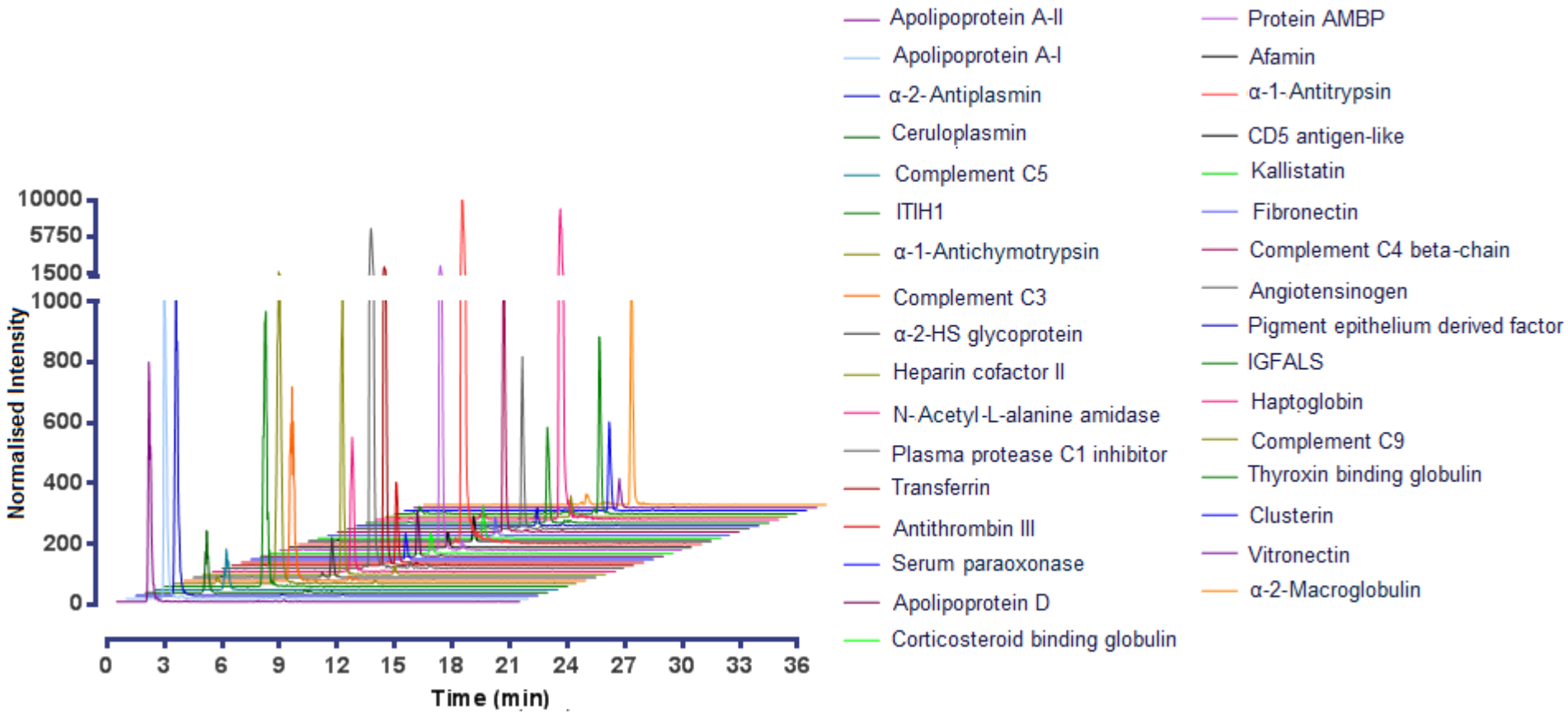


Figure 4-8: Extracted ion chromatogram of human plasma extract for 33 glycoproteins profiled in fraction B using targeted LC-MS/MS. The analysis time was 21 min and signal intensity for each protein was normalised to the protein with the highest signal intensity in the run; α-1-antitrypsin.
ITIH1 and IGFALS refer to inter-alpha-trypsin inhibitor heavy chain H1 and insulin like growth factor binding protein acid labile subunit respectively.

4.3.4 Validation of the targeted LC-MS/MS profiling method

4.3.4.1 Method validation using 50 µL plasma volumes

The assessment of the profiling method revealed acceptable (CV ≤ 15%) analytical precision for the majority of the targeted glycoproteins, reflecting high reproducibility in the developed analytical workflow. The coefficient of variation values (n=6) ranged from 3.6% for complement C9 to 33% for fibrinogen α chain. The median value for the 55 assays was 11.8%, and 83.5% of the MRM had a precision of ≤15% (of which 38% were ≤ 10%) as shown in Figure 4-9. The values for analytical precision achieved were within the acceptable limit for quantitative bioanalytical measurement as set by the FDA for the validation of biological samples [243], which supports the use of this method for in clinical sample analysis.

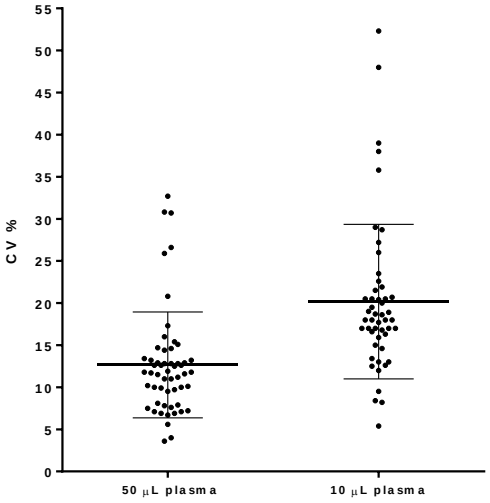
The peak areas obtained were generally in the order of > $1x10^4$, which appears to have contributed to the analytically acceptable CV values attained, as lower peak areas has been reported to be less likely to yield CVs below 10% [79]. Furthermore, Anderson *et al.* [79] also demonstrated that the sum of the peak areas for more than one transition improved the precision of the assay and reduced the CV value achieved. In agreement with those reported findings, the sum of two transitions for corticosteroid binding globulin signature peptide (detected with low signal intensity) achieved an overall peak area > $1x10^4$, and enhanced the CV value to 12.6% in the present study.

Most of the published targeted glycoprotein profiling methods applied stable isotope standards (SIS) strategies and resulted in expectedly lower CVs (≤ 15%, majority below 10%) [351; 352; 353; 354]. However, the overall number of targeted glycoproteins in these studies was limited to less than 15, mainly due to the high cost of the synthetic labelled peptide standards used in the workflow. The current method used the more cost-effective label-free approach and hence was able to profile a greater number of glycoproteins (n=54), many of them with CV values comparable to those from SIS studies [354], including 45 glycoproteins with CV ≤ 15%, of which 21 glycoproteins had CV ≤ 10%. An alternative previous study that used a label-free strategy and albumin/IgG depletion (instead of lectin chromatography) to decrease the complexity of plasma samples, reported a slight improvement in analytical precision and lower CV values (CV% 1.2–11.2%, median 6.3%) [86] compared to the present work. However, the study targeted only 18 glycoproteins which were all covered in the method developed here and had acceptable reproducibility (CV% ≤ 15%). Moreover, the current method showed

improved precision and lower CV values (median CV 11.8%) than previous studies which applied label-free targeted LC-MS/MS to quantify a different set of plasma proteins and reported higher median CVs; 18.9% [98] and 16.5% [338].

4.3.4.2 Method validation using reduced plasma volumes (10 µL)

The analytical precision substantially decreased for many glycoproteins when the plasma volume was decreased to 10 µL. The majority of the assays had CV values between 15-20%, and only four glycoproteins showed CVs ≤10% (α-2-HS glycoprotein α-1-antitrypsin, afamin, and angiotensinogen), as demonstrated in Figure 4-9. Moreover, five glycoproteins were not reliably detected (C4-binding protein α-chain, corticosteroid binding globulin, lumican, pigment epithelium derived factor and serum amyloid P-component), either due to their low abundance, insufficient recovery, or the low MS response for the selected surrogate peptides. However, a larger plasma volume of 50 µL was sufficient to achieve reliable detection and improved assay precision, and this volume was used for subsequent applications. Comparison between the CV values obtained from 50 and 10µL fractionated plasma for each protein is presented in Figure A4-1 in Appendices.



CV%	50 µL plasma	10 µL plasma
≤ 10	38% (21/55)	8% (4/50)
10-**15**	45.5% (25/55)	16% (8/50)
15-**20**	5.5% (3/55)	40% (20/50)
20-**30**	5.5% (3/55)	26% (13/50)
>30	5.5% (3/55)	10% (5/50)

Figure 4-9: Distribution of coefficient of variation for the proteins targeted in 50 and 10 µL human plasma extract obtained from 6 technical replicates. 55 plasma proteins were targted representing 54 clinically relevant glycoproteins and albumin. For proteins monitored in both fractons (A and B), the best CV value was presented. CV values for each protein in each fraction is presented in table 4-6. Error bars presented in the graph refers to the mean ± SD.

4.3.4.3 MRM transitions variability (run to run variability)

It is accepted that sample preparation (*e.g.* extraction and protein digestion) is the major contributor to the overall variation within a targeted LC-MS/MS workflow [98]. However, variation in the instrument performance can affect the reproducibility of an analytical assay in label-free workflows. To address this, replicate LC-MS/MS injections (n=6) were analysed and revealed an acceptable level of precision for the MRM transitions (median CV 7.1%). The majority of the MRM assays produced CV values of ≤ 10%, and all with CV ≤ 20% except for two glycoproteins (complement factor H and fibrinogen α-chain, as seen in Figure 4-10). These results highlighted the reproducibility of the LC-MRM transitions performance between runs, which together with the reproducible two-step extraction workflow allowed an analytical precision of ≤ 15% to be achieved for the majority of the glycoproteins in this study.

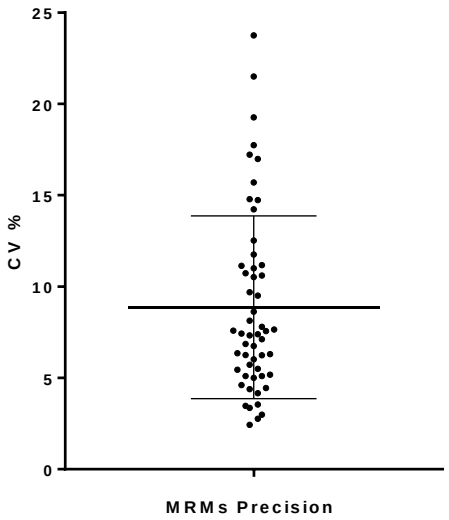


Figure 4-10: Scatter plot of the MRMs precision. Each data point represents the CV% for each targeted protein from 6 replicate injections of the same plasma extract. Almost 70% of the MRM transitions showed CV ≤10%. Error bars presented in the graph refers to the mean ± SD.

4.3.4.4 Label-free targeted LC-MS/MS workflow

The above results pointed out that an acceptable level of analytical precision can be obtained with label-free workflow. Here, this was achievable on account of the carefully optimised plasma fractionation approach, the stability of the LC-MS/MS system, and the use of micro-flow rate liquid chromatography. Conventional micro-flow LC-systems are known to be more robust, with more stable electrospray performance and a lower retention time shift when compared to the nano-LC commonly used to enhance the assay sensitivity [79]. Moreover, the use of the conventional micro-flow LC system improved the throughput of the developed method by reducing the LC-MS/MS analysis time to 20 min, instead of the 120 min typically required with nano-LC systems [86].
Similarly, Blankley *et al.* [98] observed adequate assay reproducibility (CV% 12.8-23.4) using a standard conventional micro-HPLC system and applying a label-free targeted LC-MS/MS workflow to relatively quantify pregnancy specific glycoproteins [98]. The same study also reported that when compared to nano-flow chromatography/ESI, the higher flow rate system caused a reduction in sensitivity but a more reproducible separation, with a more robust ionisation over a long period of time, and hence contributed to the acceptable precision observed for many of the assays [98].

The developed method here also demonstrates that a label-free workflow, when carefully designed, can achieve CV values comparable to those reported using SIS workflows. This highlights the capability of the targeted label-free method to be used as a cost-effective alternative to SIS workflows for relative protein quantification in clinical samples. A similar finding was reported by Zhang *et al.* [338] who detected significant changes in the levels of endogenous proteins in 10 pairs of human lung tumour and control tissues with both SIS and label-free methods, and concluded that targeted MS label-free method provided a cost-effective alternative to SIS [338].

4.3.5 Measuring small changes in glycoprotein composition in ammonium sulphate treated plasma

Ammonium sulphate precipitation was used as a model system to investigate the suitability of the developed LC-MS/MS method to measure the typically small changes in glycoprotein composition seen in biological/clinical samples, for example when comparing treated versus control groups.
As a result of ammonium sulphate precipitation, the levels of some glycoproteins in human plasma were altered compared with control plasma and these small, but

measurable changes, could readily be detected by the developed LC-MS/MS methodology. Changes in the abundance of proteins resulted in a clear differentiation between the two conditions as shown in Figure 4-11, A. Comparison with plasma controls revealed that the levels of eight proteins; fibrinogen α and β, β2 glycoprotein 1, plasminogen, haptoglobin, fibronectin, apolipoprotein A-1 and transferrin were significantly lower in ammonium sulphate treated plasma, (Figure 4-11, B). Fibrinogen α showed the highest change with a decrease of 2.6 fold change. Lower levels of fibrinogen γ and complement C3 were detected; however this change was not statistically significant, mainly due to the high variability between samples. As observed, the differentially altered glycoproteins were high or moderate abundance proteins, which supports the use of ammonium sulphate fractionation as an alternative, simple and cost-effective approach for the depletion of major plasma proteins [364]. Consistent with the above findings, a similar list of altered proteins has been reported previously using 20% ammonium sulphate plasma fractional precipitation [365], which increase the confidence in the presented results.

A full scan LC-MS/MS analysis of the tryptic digest of the 15% ammonium sulphate precipitate herein detected all of the altered glycoproteins, except β2 glycoprotein 1 (Table A4-2 in Appendices), which further confirmed the above results and highlighted the applicability of the method for relative protein quantifications.

Taken together, the above findings confirmed that the precision of the developed profiling method is sufficient to detect differences in the abundance of targeted glycoproteins between different conditions, as observed with ammonium sulphate treated plasma.

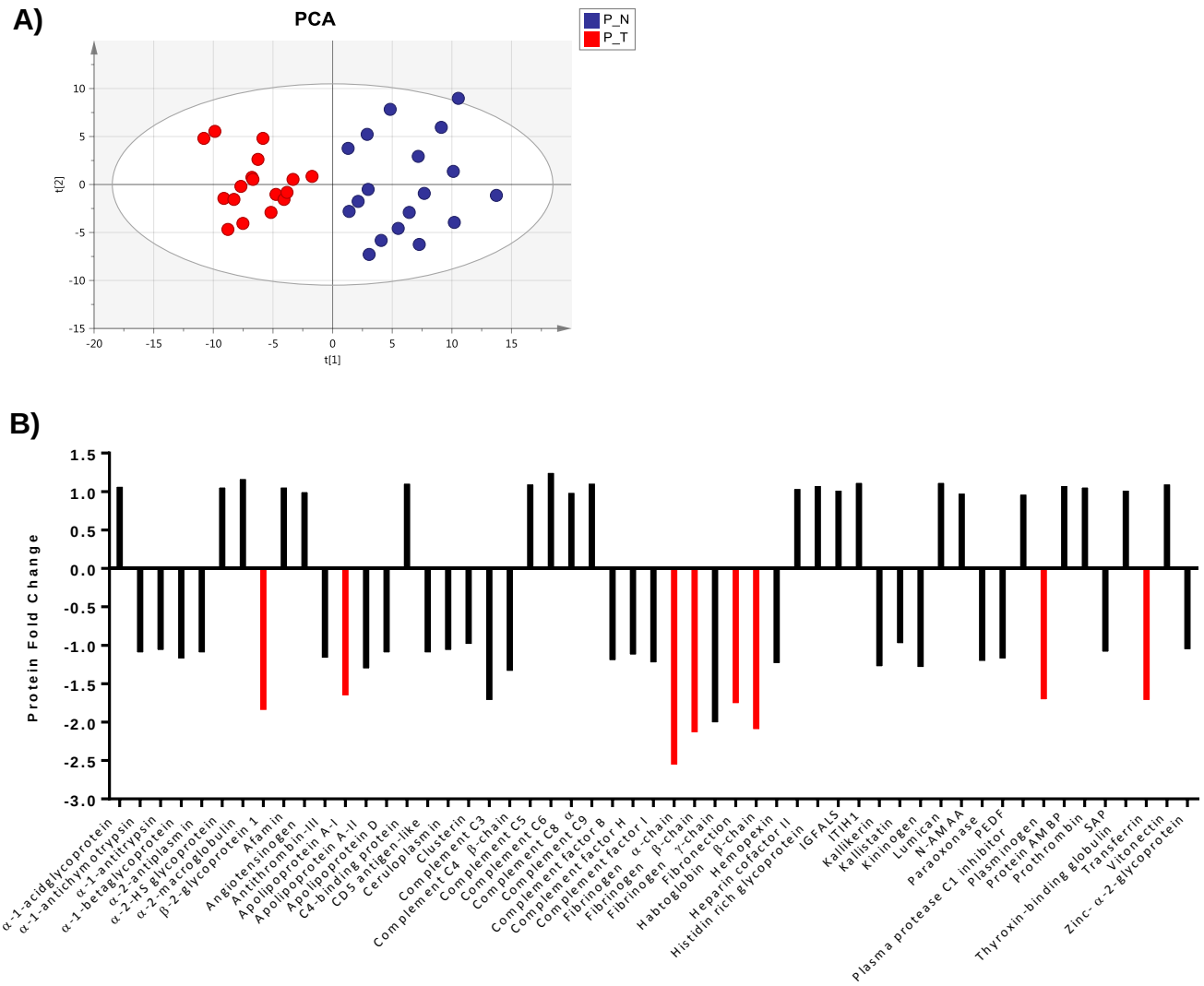


Figure 4-11: A) Principle component analysis (PCA) score plot of targeted LC−MS/S data sets for control normal plasma (blue colour) and ammonium sulphate treated plasma samples (red colour). Clear separation between the two groups is noticed as a result of the changes in the level of some of the targeted glycoproteins caused by ammonium sulphate precipitation. B) Protein fold changes between ammonium sulphate treated plasma and plasma control samples. Protein levels significantly altered are presented in red. Two-tailed independent *t*-test was used to calculate the p-value between the compared groups, p value ≤ 0.05 was considered statistically significant.
ITHI1: inter-alpha-trypsin inhibitor heavy chain H1, IGFALS: insulin like growth factor binding protein acid labile subunit, N-AMAA is N-acetylmuramoyl-L-alanine amidase, PEDF: pigment epithelium derived factor, SAP: serum amyloid P-component.

4.3.6 Monitoring the differences between plasma and serum glycoprotein composition

The ability of the developed method to distinguish between plasma and serum samples was investigated as an alternative test to determine the suitability of the method to detect changes in plasma glycoprotein profiles. As expected, the major difference observed was the absence of the coagulation factors fibrinogen α, β and γ from the serum, as they are expected to be removed during the clotting process during serum preparation (Figure 4-12, A). The other remaining targeted glycoproteins were detected in both serum and plasma with no statistically significant differences in their levels between the two biological fluids (Figure 4-12, B). This suggested that the serum preparation method maintains the level of all of the targeted plasma glycoproteins, with the exception of fibrinogens. Both plasma and serum are principal components of the blood. Serum resembles plasma in composition but lacks the coagulation factors; it is obtained by permitting the blood specimen to clot prior to centrifugation. Therefore, fibrinogen is converted to a fibrin clot [366]; and hence, it was not detected in the serum herein.

Similar results were obtained with a previous study that compared the glycoproteome of plasma and serum samples, using multi-lectin affinity chromatography, and revealed a close correlation between the two samples, with the absence of fibrinogen from the identified protein list of the serum, apparently as a result of the clotting process [314]. The analysis of serum and plasma samples using antibody suspension bead-array-based workflow also reported fibrinogen as the main plasma protein being depleted during serum preparation [367]. The agreement of the current findings with what have been reported by previous studies, supported the results obtained here and highlighted the ability of the developed method to detect differences in the level of glycoproteins when applied for relative protein quantification.

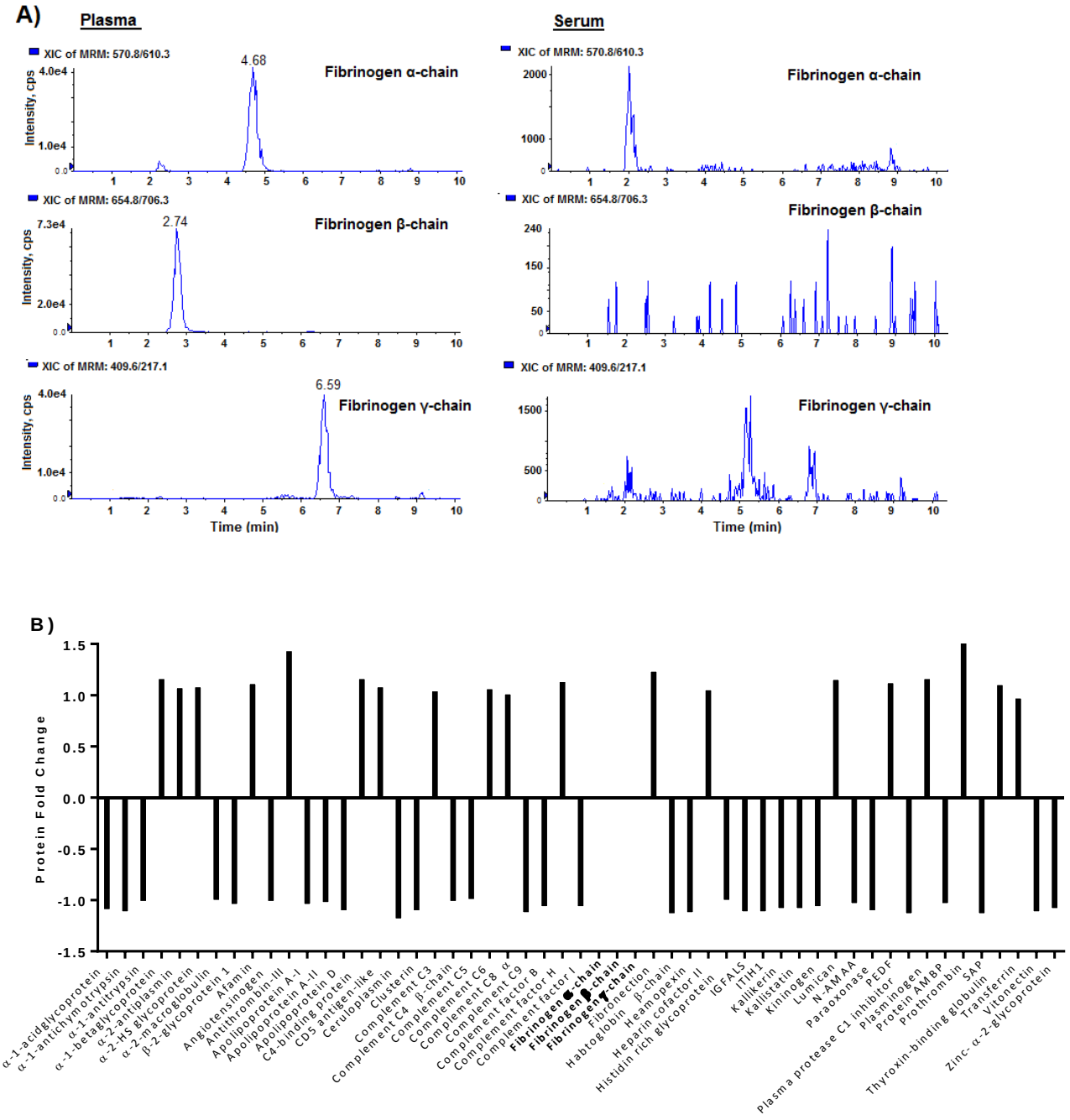


Figure 4-12: XIC of MRM for fibrinogen α, β and γ extracted from the plasma and serum tryptic digests analysed by the developed glycoprotein profiling method. B) Protein fold changes between plasma and serum samples. The coagulation factors fibrinogens α, β and γ (in bold) could only be detected in the plasma and were absent from the serum. All other targeted glycoproteins could be detected in both biological fluids with no statistically significant differences in their levels. Two-tailed independent *t*-test was used to calculate the p-value between the compared groups, p value ≤ 0.05 was considered statistically significant.
ITHI1: inter-alpha-trypsin inhibitor heavy chain H1, IGFALS: insulin like growth factor binding protein acid labile subunit, N-AMAA is N-acetylmuramoyl-L-alanine amidase, PEDF: pigment epithelium derived factor, SAP: serum amyloid P-component.

4.4 Conclusion

A label-free targeted methodology was used for the first time to profile selected ConA captured glycoproteins. The method provided a new, simple, and cost-effective workflow for the profiling of 54 clinically relevant human plasma glycoproteins involved in various biological processes. Many of these glycoproteins are associated with severe conditions, as with angiotensinogen and cardiovascular disease [368], and some have been identified and utilised as disease biomarkers as with afamin and ovarian cancer [369; 370], and pigment epithelium derived factor and Alzheimer's disease [371].

The developed method has a high throughput potential in terms of sample preparation since it avoided costly and time consuming stages such as immunodepletion enrichment, and complex deglycosylation, as used with many existing glycoprotein studies. The use of conventional micro-flow as opposed to a nano-LC system resulted in shortened and more stable chromatographic run times, while maintaining sensitivity enough for the detection, making the method more applicable for routine use when high throughput analysis is required.

The profiling method showed precision sufficient to detect a difference in the abundance of targeted glycoproteins between different conditions as observed with ammonium sulphate treated plasma. The acceptable analytical precision (CV% ≤ 15) achieved for most of the profiled glycoprotein assays highlighted the reproducibility of the method, and pointed out that label-free methodology can be a cost-effective affordable alternative to isotope labelled standards workflow when applied for relative protein quantification, especially when targeting a large number of proteins. Moreover, the observed CVs for the majority of the glycoproteins (85%) were within the acceptance limit for quantitative bioanalytical measurements (<10-15%) and were better than what is achieved with immunoblotting (CVs in the range from 20–40%) typically used for the validation of potential disease biomarkers. This showed the capability of label-free targeted LC-MS/MS to be used as an alternative tool for the verification of putative candidate biomarkers for disease states.

Although the focus of this study was on the quantitative analysis of ConA captured glycoproteins, the same workflow can be extended to analyse different glycoproteins captured by a variety of lectins. Additionally, the assay developed here did not require

special equipment for sample preparation and could be easily transferred to other laboratories for LC-MS/MS-based protein quantitation experiments.

In brief, the glycoprotein profiling method developed in this chapter provides a simple and cost-effective workflow, with high throughput potential, for the profiling of clinically relevant human plasma glycoproteins, using two-step fractionation coupled with conventional LC-MS/MS. The method showed an acceptable analytical precision for the majority of the assays which gives the confidence needed for the quantification of the glycoproteins in different disease conditions.

Chapter Five

Profiling of Plasma Glycoproteins in Pre-eclampsia

5 Profiling of Plasma Glycoproteins in Pre-eclampsia

Summary

Glycoproteins are involved in many biochemical processes which have relevance to the pathogenesis of pre-eclampsia, but no proteomic studies have been done focusing entirely on this class of proteins. The aim of this chapter was to profile 51 glycoproteins in blood plasma from women with early- (n=10) and late- (n=10) onset pre-eclampsia compared with age-matched normotensive controls using the developed LC-MS/MS method.

In early-onset pre-eclamptic women, the abundance of 25 glycoproteins was significantly altered in the plasma compared to gestational age-matched controls. Twenty-four of the changed glycoproteins showed an increased in level and one (corticosteroid binding globulin) showed a decrease. Among the up-regulated glycoproteins were zinc-α-2 glycoprotein, fibronectin, and clusterin. Significantly changed levels of apolipoprotein D and kallikrein were detected for the first time in the plasma of pre-eclamptic women, suggesting that these glycoproteins could be involved in the underlying metabolic pathology of pre-eclampsia and could be further investigated as new potential biomarkers. In late-onset pre-eclampsia compared to gestational age-matched controls, six glycoproteins were significantly changed. Five of the six altered glycoproteins, α-2-HS glycoprotein, clusterin, hemopexin, vitronectin, and transferrin were significantly up-regulated in the plasma of both early- and late-onset pre-eclampsia.

The identified differentially altered glycoproteins are involved in a number of pre-eclampsia-relevant pathways and biological processes, including immune response, coagulation, cell adhesion and lipid metabolism, supporting the multisystem nature of pre-eclampsia and the need for more than one biomarker to predict the disease. The study demonstrated the capability of the developed cost-effective targeted LC-MS/MS method for the clinically relevant profiling of glycoproteins as potential disease biomarkers.

5.1 Introduction

5.1.1 Pre-eclampsia

Pre-eclampsia is a multisystem pregnancy disorder characterised by hypertension and proteinuria developing after the 20th week of gestation [106]. It is one of the leading causes of maternal and fetal morbidity and mortality worldwide [107].

Despite the multiple risk factors associated with pre-eclampsia, the aetiology of the disease remains poorly understood [127]. Moreover, the disease remains asymptomatic until a late stage, making the identification of women likely to develop pre-eclampsia extremely difficult [139]. This leads to the late diagnosis of the condition, where the delivery of the fetus and placenta will be the only effective intervention [372]. (For disease risk factors, symptoms, complication, and pathogenesis see Chapter One).

5.1.2 Biomarkers in pre-eclampsia: current trend

Several biomarkers (discussed in Section 1.3.7, Chapter One) including serum FMS-like tyrosine kinase-1 and the two angiogenic growth factors, vascular endothelial and placental growth factors have been proposed for improving the identification of women at risk of pre-eclampsia [168; 173]. The candidate biomarkers were derived from the pathophysiological abnormalities associated with pre-eclampsia, such as placental and endothelial dysfunction and systemic inflammation [106; 373] in a hypothesis-driven approach. However, these markers lacked the sufficient consistency, sensitivity and specificity required to allow their routine application in clinical practice for the early prediction of the disease [106; 139]. Additionally, pre-eclampsia is a multifactorial disorder that involves several different pathophysiological mechanisms and pathways [374], and it is unlikely that a single or small group of biomarker(s) could be used to diagnose such a heterogeneous disorder. Hence, new strategies have emerged [207; 375].

Research attention has recently moved from the above hypothesis-driven approach, towards the unbiased hypothesis-generating strategy applying MS based systems biology approaches in order to investigate new pathways, and discover new pre-eclampsia candidate biomarkers [106; 376]. Proteomics is a very promising technique that allows the simultaneous analysis of hundreds of proteins, and enables the comparison of protein patterns between healthy and diseased groups to enhance the

identification of potential biomarkers, and shed light on the mechanism underlying the pathophysiology of the studied condition [106].

5.1.3 Literature on the discovery of potential pre-eclampsia biomarkers by proteomics studies

5.1.3.1 Investigated biological samples

Various MS techniques have been applied to identify new pre-eclampsia markers, and efforts have been directed to analyse maternal blood serum, plasma, urine, placental tissue and placental trophoblast cells (Table 5-1).

The origin of pre-eclampsia is recognised as lying in the placenta and altered proteins expression at the placental level suggests their role in the development of the disease [373]. The placenta predominantly consists of trophoblast cells that have an important role in adhesion and invasion during early pregnancy, and secretory role in late pregnancy. The analysis of the isolated trophoblast cells eliminates the placental tissue heterogeneity problem while providing a good source of altered proteins during disease progression [376].

The majority of the existing pre-eclampsia proteomics studies were performed using maternal plasma or serum [377]. Both plasma and serum are readily accessible and cheaply obtained biological fluids that can comprehensively reflect changes in organ function in disease states. The placenta is in direct contact with the maternal circulation. Hence, any disease-specific changes in placenta-derived proteins, or the release of proteins due to the interaction between the endothelium and systemic pathways, is expected to be reflected in the plasma proteins of women who subsequently develop pre-eclampsia [373; 378; 379].

Additionally, urine is a good biological fluid for the discovery of pre-eclampsia biomarkers as proteinuria is a major characteristic of pre-eclampsia, and renal pathology can be associated with the disease [377].

5.1.3.2 Summary of previous proteomic studies of pre-eclampsia

Previous studies of pre-eclampsia have involved the collection of maternal serum, plasma, and urine samples either at the onset of the disease (clinical pre-eclampsia) or at earlier weeks of gestation from women who subsequently developed pre-eclampsia (preclinical pre-eclampsia). These proteomic studies are summarised in Table 5-1. The majority of these studies compared pre-eclampsia samples (either clinical or preclinical) to gestational age-matched uncomplicated pregnancies to identify potential pre-

eclampsia biomarkers that could predict those women at risk of developing the disease [207; 378; 380; 381]. Fewer studies compared the protein profiles between clinical and preclinical pre-eclampsia [127], the early- (< 34 weeks of gestation) and late-onset pre-eclampsia (> 34 weeks of gestation) [375], or between the different disease classes (mild, moderate and severe) [127]. These comparisons will shed light on the mechanisms involved during disease progression and increase the understanding of the molecular mechanisms underlying different pre-eclampsia onsets and classes.

The proteomic methodologies of previous studies (summarised in Table 5-1) have used various MS techniques (*e.g.* MALDI, SELDI-MS, targeted and untargeted LC-MS/MS) to identify new candidates for the prediction of pre-eclampsia. They relied mainly on steps of immunodepletion and fractionation coupled with label-free [127; 382; 383] or label-based (particularly iTRAQ technique) shotgun strategies [207; 384], to detect differentially expressed proteins between the compared groups. A further validation for a small number of the discovered markers was conducted by targeted proteomics [382; 385] or biochemical assays, mainly Western blotting [98; 382], to ascertain the sensitivity and specificity of the proposed biomarkers [376]. The potential biomarkers listed in Table 5-1 were reported to be involved in a range of pathways, including haemostasis, immune response, coagulation, and lipid metabolism supporting the multisystem nature of pre-eclampsia. The abundance of specific proteins was differentially regulated in more than one biological site, such as pregnancy-specific β-1-glycoprotein 3 identified as a pre-eclampsia biomarker in both serum and trophoblast cells [376].

The previous studies investigated the whole plasma or serum proteome, and none of them had focused exclusively on plasma glycoproteins which are considered an important class for the discovery of potential diseases biomarkers including pre-eclampsia. Profiling plasma glycoproteins in pre-eclampsia is of high importance given the fact that many of the proteins that have been identified as consistently up- or down-regulated, through different studies, are glycoproteins, such as fibronectin [127; 207], fibrinogen α chain [207; 378; 381], and α-2-HS glycoprotein [382; 383; 386]. This highlights the involvement of these proteins in the pathology of pre-eclampsia. Moreover, various glycoproteins are involved in the different biochemical pathways and mechanisms generally recognised to be altered in pre-eclampsia such as lipid metabolism, coagulation/fibrinolysis, inflammatory responses and endothelium

dysfunction, reinforcing the importance of glycoproteins for the discovery of potential pre-eclampsia biomarkers.

To conclude, the pathogenesis of pre-eclampsia is complex with an interaction occurring between diverse biological processes. Despite decades of research, until now there is no biomarker-based screening test that accurately predicts pre-eclampsia in early pregnancy. MS proteomics studies have contributed to the identification of a large number of plasma protein biomarkers but, none of these studies have focused entirely on plasma glycoproteins, which are well-recognised as an interesting subclass of plasma proteome for the discovery of disease biomarkers. Furthermore, comparison of glycoproteins differentially expressed in the early- and the late-onset pre-eclampsia has not been evaluated before. The targeted LC-MS/MS method developed in this thesis covers a range of relevant plasma glycoproteins that can be linked to many of the underlying mechanisms and pathways associated with the pathophysiology of pre-eclampsia. This includes glycoproteins involved in inflammation, complement system (complement components and factors), coagulation, cell adhesion and tissue repair, oxidative stress and lipid metabolism and transport. The application of the developed glycoprotein profiling methodology to early- and late- onset pre-eclampsia samples can aid in the identification of significantly altered glycoproteins that can serve as potential pre-eclampsia biomarkers. This will enhance the understanding of the mechanisms and pathways associated with this disease, and assist in clarifying the molecular basis of the two pre-eclampsia onsets, by comparing the changes in the glycoproteins level in the early and the late stages of the condition.

Table 5-1: Literature for the application of MS techniques for the discovery of protein biomarkers in pre-eclampsia (PE).

Sample type	Time of sampling and Sample size	Main results	Selected protein markers	Main techniques used	Ref.
Plasma	Samples were obtained at week 12 of gestation from women who subsequently developed PE (n=6) and controls (n=6).	Elevation in 10 proteins (out of 64) in the PE group.	Clusterin, fibrinogen, fibronectin, angiotensinogen, hemepexin and galectin 3.	Immunodepletion, iTRAQ labelling and LC-MS/MS.	[207]
	Samples were taken at week 20 of gestation from women who later developed PE with: - Appropriate birth weight for gestational age baby (n=27), or -A small for gestational age baby (n=12), and healthy controls (n=57).	Identification of 39 differentially expressed proteins.	Fibrinogen α-chain and α-1-antichymotrypsin.	Combination of immunodepletion and 2D DIGE, LC-MS/MS Overexpression of in plasma prior to PE was confirmed by Immunoblots.	[378]
	Samples were collected between weeks 19-21 of gestation from women who later developed PE (n=100), and controls (n=200).	SRM assays were successfully developed for 51 out of the 76 selected proteins (64 discovery phase, 12 with a recognised association with PE). The 44 generated algorithms were then tested in an independent cohort (n=300) yielding 8 validated models. These 8 models detected 50% to 56% of PE cases in the training and validation sets; the detection rate for preterm PE cases was 80%.	Validated models combine insulin-like growth factor acid labile subunit, soluble endoglin, placental growth factor, serine peptidase inhibitor Kunitz type 1, melanoma cell adhesion molecule, selenoprotein P, and blood pressure. This study has identified insulin-like growth factor acid labile subunit as a novel candidate biomarker for PE.	MALDI-MS/MS used during discovery process. albumin and IgG depletion and LC-triple quadrupole MS for biomarkers verification and validation.	[385]
	Clinical PE (n=23), controls (n=23).	12 proteins were down-regulated while 23 were up-regulated in PE.	Among the up-regulated proteins: clusterin, Apo B, Insulin-like growth factor binding protein complex acid labile chain and pregnancy-specific glycoprotein 9. Among the down-regulated proteins: carboxypeptidease N catalytic chain, fibronectin and plasma protease C1 inhibitor.	Depletion of the 12 most abundant protein, iTRAQ labelling, SCX fractionation and LC-MS/MS.	[380]

Table 5-1: continued

Sample type	Time of sampling and sample size	Main results	Selected protein markers	Main techniques used	Ref.
Plasma	Discovery phase: plasma samples were taken at 15±1 weeks of gestation from women who developed early onset-PE (n=12) or had uncomplicated pregnancies (n =24).	502 proteins were identified and a total of 113 proteins altered in abundance.	Pregnancy-specific beta-1-glycoprotein 9 and platelet basic protein were subjected to further SRM-based verification.	Depleted plasma samples were iTRAQ labelled, fractionated using high pH RP-chromatography, and analysed by LC-MS/MS or MALDI TOF-TOF.	[98]
	For validation: early-onset PE (n=16), late-onset PE (n=42), and controls (n=42).	The SRM data correlated with a commercial ELISA.	Pregnancy specific glycoprotein and platelet basic protein.	Fractionation for depleted plasma samples and analysis by Label free targeted LC-SRM on triple quadrupole system and ELISA.	[98]
	Preclinical PE plasma samples were collected at week 20 of gestation from women later developed PE (n=6) and normotensive controls (n=6). Samples were taken from the same women after PE was diagnosed at weeks 33-36. PE (n=6), and normotensive women (n=6).	The 75 kDa single-chain vitronectin increased in PE, whereas the 65 kDa moiety of the 2 chain vitronectin molecule decreased compared to healthy controls.	Vitronectin and α-1-antichymotrypsin (SERPINA3).	Depletion of the six most abundant proteins, labelled samples were analysed by 2D GE LC-MS/MS.	[387]
Serum	Early-onset severe PE (n=11), controls (n=13).	The best differentiating signals were related to the protein transthyretin and its modified forms.	Transthyretin.	MALDI-MS.	[388]
	Clinical PE (n=31), controls (n=31) with two-thirds used as a training set and the other third as a testing set.	Detection of 52 peptides derived from 14 protein precursors with highly significant differences in expression between PE and control samples.	Fibrinogen α-chain, α-1-antitrypsin, apolipoprotein L1, inter-α trypsin inhibitor heavy chain H4, kininogen-1 and thymosin beta-4.	LC-MS, the peptidomic profiles were then analyzed by significance analysis of microarrays (SAM) and predictive analysis of microarrays (PAM).	[381]

Table 5-1: continued

Sample type	Time of sampling and sample size	Main results	Selected protein markers	Main techniques used	Ref.
Serum	Severe PE (n=8), controls (n=5).	62 proteins were differentially expressed. Among them, 27 proteins were up-regulated, whereas 35 proteins were down-regulated.	α-2-HS glycoprotein, insulin-like growth factor binding protein acid labile subunit, and alpha-1-microglobulin/bikunin (up-regulated) and retinol binding protein4 (down-regulated).	Depletion of six most abundant proteins, label-free LC-MS/MS quantification.	[382]
	Severe PE (n=5), controls (n=5).	Identification of 31 up-regulated proteins and 20 down-regulated.	Chorionic somatomammotropin hormone, fibulin-1, α-2-HS-glycoprotein, transthyretin and retinol-binding protein 4.	Peptide ligand library beads for capturing the low abundance proteins, and SDS gel label-free LC-MS/MS quantification.	[383]
	PE (n=32) and control (n=32) samples collected as following: PE: 24-34 weeks (n=15) >34 weeks (n=17) Control: 24-34 weeks (n=16) >34 weeks (n=16)	20 protein markers (from 22 proteins identified in PE [9 by proteomics and 13 by genomics studies]) were validated by ELISA. The PE biomarkers were not significantly different between early and late gestation in either PE or control sera.	Placental growth factor, Soluble fms-like tyrosine kinase, APO A-I, APO C-III, APO-E and haptoglobin.	Depletion of the top fourteen serum-abundant proteins, 2D DIGE and LC-MS/MS for identification. ELISA for validation.	[375]
	Clinical PE: patients with mild PE (n=30), severe PE (n=30), and controls (n=58). Preclinical PE: included149 women whose serum samples were collected at weeks 8-14. Women later developed mild PE (n= 30), severe PE (n=40), or remain healthy controls (n=79).	Clinical PE: the levels of 34 proteins were differentially expressed between women with and without PE identified. Preclinical PE: 38 proteins were differentially expressed between women who subsequently developed PE and women who remained normotensive.	Clinical PE: fibronectin, pappalysin-2, choriogonadotropinβ, ApoC-III, cystatin-C, vascular endothelial growth factor receptor-1, and endoglin were up-regulated in PE. Preclinical PE: placental, vascular, transport, matrix, and acute phase proteins.	2D-LC-MS/MS and label-free quantification.	[127]

Table 5-1: continued

Sample type	Time of sampling and sample size	Main results	Selected protein markers	Main techniques used	Ref.
Serum	Discovery phase: Clinical PE (n=6), controls (n=6). For validation: Clinical PE (n=80), controls (n=80).	Overexpressed spots were identified as clusterin.	Clusterin.	2D-PAGE and MALDI TOF/TOF for discovery. Validation by immunoassay methods	[389]
Urine	Samples from women who subsequently developed PE collected at the following gestational weeks - 12 to 16 (PE n=45, control=86), - 20 (PE n=50, control=49), - 28 (PE n=18, control= 17).	Identification of 10 biomarkers from specimens obtained at week 28 associated with future PE. The created model could not reliably detect PE before gestational week 28 and did not allow the early identification of women at risk for PE.	Fibrinogen α chain, collagen α chain and uromodulin fragments.	Capillary electrophoresis online coupled to microLC-MS/MS.	[139]
	Samples were collected at the time of disease onset: Clinical PE (n=88): mild PE (n=29), severe PE (n=31), and PE superimposed on chronic hypertension (n=28), controls (n=82).	Identification of two candidate biomarkers for PE, and for differentiation of preeclampsia from other hypertensive disorders of pregnancy.	Fragments of serpin peptidase inhibitor-1 and albumin.	SELDI-TOF-MS.	[390]
	Clinical PE (n=18): severe PE (n=11) and mild PE (n=7), normotensive controls (n=8).	4 biomarkers were able to discriminate patients with severe PE from mild PE and controls.	4 discriminatory protein peaks were identified at *m/z*: 4155, 6044, 6663, and 7971.	SELDI-TOF-MS.	[391]
	Clinical PE (n=10), gestational hypertension (n=10), and controls (n=10).	113 and 31 proteins were expressed differentially between PE and normal pregnant groups, and among the three groups respectively. The increased SERPINA 1 could discriminate PE from other hypertensive disorders.	Urinary angiotensinogen was down-regulated and SERBINA 1 was increased.	iTRAQ labeling coupled with 2D LC-MS/MS.	[384]

Table 5-1: continued

Sample type	Time of sampling and sample size	Main results	Selected protein markers	Main techniques used	Ref.
Placenta tissue	Clinical PE (n=30), controls (n=30).	Two proteins were differentially expressed.	Apolipoprotein 1 increased while tropomyosin -3 decreased.	2D GE and MALDI TOF/MS.	[392]
	Clinical PE (n=20), controls (n=20).	147 proteins were up-regulated and 24 were down-regulated in PE.	Endoglin, annexin A5, prothrombin and ceruloplasmin (up-regulated). Hemoglobin subunit alpha and haptoglobin (down-regulated).	ICAT labelling and UPLC-MS/MS. Validated by western blot.	[393]
	Clinical severe PE (n=5), controls (n=5).	17 spots were differently expressed in PE compared to normal placentas, 11 out of 17 spots were identified.	Chloride intracellular channel 3, apolipoprotein A-I, transthyretin and protein disulphide isomerase were up-regulated, while peroxiredoxin 2 and 3, Hsc 70, Cu/Zn-superoxide dismutase, and HSP gp96 were down-regulated.	2D-PAGE and MALDI TOF/TOF.	[394]
Cytotrophoblastic cells from placentas obtained at delivery	Clinical PE (n=4), controls (n=6).	Identification of 33 proteins able to discriminate between PE and controls when samples were pooled and 24 proteins when samples were individually analysed.	Factor XIII chain A.	LC-ESI-MS/MS.	[395]
	Clinical PE (n=6), controls (n=6).	11 proteins were decreased in PE. Only one protein was up-regulated.	α-2-HS glycoprotein, actin, Hsp70, ezrin, and glutatione S-transferase.	In-gel digestion and LC-MS/MS. Validated by Western blot.	[386]
	Clinical PE (n=8), controls (n=8).	13 differential expressed proteins in which 3 proteins were down-regulated and 10 proteins were up-regulated.	Signal transduction protein, molecular chaperone, cell skeleton proteins.	2D GE LC-MS/MS.	[396]
Amniotic fluid	Clinical PE (n=10), controls (n=10).	5 protein peaks were significantly differentially expressed.	Fragmented albumin and apolipoprotein A-I.	SELDI-MS.	[397]

5.1.4 Aim of the chapter

1) To identify potential pre-eclampsia glycoproteins biomarkers using the developed targeted LC-MS/MS glycoprotein profiling method. To achieve this aim, early-onset pre-eclampsia samples were compared with gestational age-matched controls.
2) To evaluate glycoproteins involved in the early- and late-onset pre-eclampsia by comparing the differentially expressed glycoproteins in each disease onset.

5.2 Materials and Methods

5.2.1 Materials

Maternal peripheral blood samples from pre-eclamptic women and the matched controls were collected by PhD student Katrin Sander, School of Medicine, University of Nottingham after obtaining fully informed written consent. Ethics approval for the sample collection and utilisation was granted by Derby Research Ethics Committee (REC Reference No. 09/H0401/90) to Dr. Raheela Khan, School of Medicine, University of Nottingham. The samples were collected at the Royal Derby Hospital between 2012 and 2015. All other materials are as described in Section 2.3.1, Chapter Two.

5.2.2 Patient recruitment

A total of 37 women were included in this study and were separated into four groups;
(1) early-onset pre-eclampsia group (before week 34 of gestation, n=10), and their (2) gestational age-matched normotensive controls (n=10), (3) late-onset pre-eclampsia group (after week 34 of gestation, n=10), and their (4) gestational age-matched normotensive controls (n=10). Three subjects were common in both control groups.
Pregnant women in the pre-eclampsia groups had (1) normal booking blood pressures (at 12-20 weeks), (2) subsequently developed blood pressures of ≥ 140 mmHg systolic or ≥ 90 mmHg diastolic on two occasions (minimum 24 h apart) and (3) had at least 1+ proteinuria (≥ 300 mg/L) using dipstick analysis [284], as per the recent guidelines of the American College of Obstetricians and Gynecologists for the diagnosis of pre-eclampsia [285]. Subjects in the control groups did not have any documented hypertensive problems throughout their pregnancy.
Pre-eclampsia and control samples were matched for a range of demographic and clinical parameters as covered in Table 5-2.

5.2.3 Sample collection and processing

Maternal blood samples were collected into BD Vacutainer EDTA blood collection tubes and centrifuged at 1000x *g* for 15 min at 4ºC. The separated plasma was then transferred to Eppendorf tubes in 0.5 mL aliquots, snap-frozen and stored at -80°C. Only samples that were processed within 1 h were included in the study.

5.2.4 Sample preparation for LC-MS/MS analysis

Plasma glycoproteins were extracted using ConA Sepharose resin and fractionated into two fractions by RP-SPE, A and B, representing glycoproteins eluted at 40% and 50% of the mobile phase respectively as described in Section 4.2.2, Chapter Four.

The dried collected glycoproteins fractions were then denatured, reduced, alkylated and digested with trypsin as described in Section 4.2.3, Chapter Four. Prior to LC-MS/MS analysis, a seven amino acids peptide internal standard (SVTQVPF) was added to achieve 0.1 µM final concentration. This peptide is unique to AGT and was reproducibly detected with good signal intensity from the AGT chymotryptic digest. As digestion was carried out with trypsin, the peptide was not detected in the TIC of the LC-MRM analysis and did not interfere with the analysis.

5.2.5 Glycoprotein profiling by targeted LC-MS/MS

Trypsin-digested peptides were analysed using the validated glycoprotein profiling method (Section 4.3.6, Chapter Four). The targeted signature peptides and their transitions were as described in Table 4-6, Section 4.3, Chapter Four.

The transitions monitored for the internal standard were 777.4/515.6 and 777.4/497.7

The ratio of the analyte signal area (endogenous peptide) over the internal standard signal area was calculated, and the mean of the peak area ratio of each glycoprotein was compared between early- and late-onset pre-eclampsia samples and their corresponding matched controls to measure protein fold changes.

5.2.6 Statistical analysis

Descriptive subject characteristics of pre-eclampsia and control groups were compared using Student *t*-test for continuous parameters, and Fisher's exact test or Pearson Chi-square for comparative analysis of categorical variables. A p-value of ≤ 0.05 was considered statistically significant.

Univariate analysis

A two-tailed independent *t*-test or Mann–Whitney *U*-test, depending on the distribution, was used to calculate the p-value between the pairwise compared groups using the normalised protein peak areas. The normal distribution of the data set was tested with Shapiro-Wilk test. Statistical adjustment for multiple comparison problem was performed using false discovery rate adjustments of Benjamini and Hochberg [398]. Adjusted p-value (q-value) ≤ 0.05 was considered statistically significant.

Statistical analysis was performed with the Statistical Package for Social Sciences version 22.0 (SPPS Inc., Chicago, IL, USA) and GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA 92037 USA).

Multivariate analysis

The normalised peak areas and the MS/MS transitions of the targeted glycoproteins were exported for multivariate analysis using SIMCAP+14 (Umetrics AB, Sweden). Principle component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) were used for modelling the differences between the pre-eclampsia cases and controls. The robustness of the created models was evaluated by monitoring the fitness of model (R^2Y) and predictive ability (Q^2) values. Models that yielded large R^2Y (close to 1) and Q^2 (> 0.5) values were considered good models. The glycoproteins that were responsible for the class separation between the compared groups in the OPLS-DA model were selected based on Variable Importance for Projection (VIP) score above 1.0, which is considered significant for the created OPLS-DA model [399].

5.3 Results

5.3.1 Maternal demographic and clinical characteristics

Maternal characteristics of pre-eclampsia cases and controls included in the study are presented in Table 5-2. No significant differences were detected between early- and late-onset pre-eclampsia and their respective gestational age-matched controls with regard to maternal age, ethnicity, smoking, previous pre-eclampsia, parity, gravida, twins pregnancy, body mass index (BMI) at booking and blood pressure before the 20th week of gestation.

Women in the early-onset pre-eclampsia group developed high protein urea and increased blood pressure between weeks 28-33 of gestation, and between weeks 34-40

in the late-onset pre-eclampsia group. Both groups showed significantly higher blood pressure at sampling when compared to their matched controls.

As expected, early-onset pre-eclamptic women delivered earlier (mean of 33.6 gestation week) with significantly lower infant birth weight (mean of 1714.2 g) as compared to normotensive matched pregnancies and women with late-onset pre-eclampsia. There were no significant differences in gestation at delivery and infant birth weight between the late onset pre-eclampsia group and its matched controls.

Table 5-2: Maternal demographic and clinical characteristics of pre-eclampsia cases and their matched controls.

Maternal characteristics	Early onset		Late onset	
	Pre-eclampsia, *n=10*	**Controls, *n=10***	**Pre-eclampsia, *n=10***	**Controls, *n=10***
Age (years)	30.2 (5.7)	28.0 (4.9)	30.2 (5.3)	29.5 (4.6)
Ethnicity				
- South Asian	1 (10%)	1 (10%)	0 (0%)	0 (0%)
- Other Non-European: Middle East	0 (0%)	0 (0%)	1 (10%)	0 (0%)
- (Saudi Arabia, Iran, Iraq……)				
- Southern and other European (white)	1 (10%)	0 (0%)	0 (0%)	0 (0%)
- United Kingdom	8 (80%)	9 (90%)	9 (90%)	10 (100%)
BMI at booking (kg/m^2)	25.5 (5.31)	25.7 (4.2)	26.9(5.2)	27.1 (6.1)
Smokers at sampling**				
- Yes	1 (11.1%)	2 (20%)	0 (0%)	1 (10%)
- No	8 (88.9%)	8 (80%)	9 (100%)	9 (90%)
Blood pressure at booking				
- Systolic (mm Hg)	114.6 (12.2)	111.8 (7.3)	113.9 (9.2)	112.3 (7.2)
- Diastolic (mm Hg)	71.2 (10.0)	66.5 (8.7)	69.20 (6.8)	64.0 (6.3)
Blood Pressure at sampling				
- Systolic (mm Hg)	**151.1 (15.4)**	**111.7 (9.8)**	**147.5 (8.8)**	**106.1 (8.7)**
- Diastolic (mm Hg)	**91.0 (11.4)**	**70.7 (11.1)**	**91.3 (9.6)**	**64.8 (8.3)**
Gestation at sampling (w)	32.0 (1.7)*****	31.4 (3.1)	37.3 (1.9)*****	36.9 (2.3)
Gestation at delivery (w)	**33.6 (2.7)***	**37.7 (3.1)**	37.6 (1.9)*****	38.2 (1.0)
Infant birth weight (g)	**1714.2 (694.9)***	**2807.1 (505.7)**	2724.0 (641.8)*****	3129.0 (455.8)
Previous pre-eclampsia**	2 (20%)	0 (100%)	0 from 8	1 from 6
Caesarean section	**10 (100%)**	**2 (22.2%)**	3 (30%)*****	4 (40%)

All data are presented as mean (SD) or number (% of total).
Significant differences between pre-eclampsia and respective normotensive controls are presented in bold (p ≤0.001).
*****Significant difference between early- and late-onset pre-eclampsia (p ≤0.001).
****** Missing information for some subjects.

5.3.2 Univariate analysis of pre-eclampsia and matched control samples

The application of the developed targeted LC-MS/MS method to identify potential biomarkers in pre-eclampsia revealed 25 plasma glycoproteins to be significantly altered in early-onset pre-eclampsia and six in late-onset pre-eclampsia compared to gestational age-matched controls. The glycoproteins changed in pre-eclampsia ranged in abundance from high (*e.g.* transferrin) to low (*e.g.* corticosteroid binding globulin) abundant proteins, and were involved in a range of different biological pathways. Two of the differentially expressed glycoproteins, apolipoprotein D, and kallikrein, are reported for the first time to be altered in the plasma of pre-eclamptic women suggesting that they have the potential to be evaluated as novel biomarkers.

5.3.2.1 Early-onset pre-eclampsia versus gestational age-matched controls

Univariate pairwise comparisons (with p-values adjustment) of glycoprotein peak areas between early-onset pre-eclampsia and control samples revealed 25 glycoproteins (out of 51 glycoproteins) significantly altered in the maternal plasma of pre-eclamptic women, as summarised in Table 5-3 and Figure 5-1.

Protein fold changes varied between the 25 differentially expressed glycoproteins and ranged from 1.3 to 2.5; 21 glycoproteins showed ≥ 1.5 fold change in their abundance including nine glycoproteins with ≥ 2 fold changes. The plasma level of all these glycoproteins was up-regulated in pre-eclampsia except for corticosteroid binding globulin which showed a 2.3 fold decrease in its circulating level in pre-eclamptic women. Fibronectin, involved in cell adhesion and vascular remodelling, and zinc-α-2 glycoprotein, involved in lipid metabolism, showed the highest increase in their plasma level in pre-eclampsia compared to controls and were detected with 2.5 and 2.4 fold changes respectively. Among the other up-regulated glycoproteins were kallikrein, serum amyloid P-component, α-2-HS glycoprotein, apolipoprotein D, plasminogen and different complement components and factors including complement C8 and complement factor H. Figure 5-2 compares the normalised peak areas of pre-eclampsia cases and controls for glycoproteins that showed ≥ 2 fold changes in their abundance.

With the exception of α-1-betaglycoprotein and zinc-α-2-glycoprotein, each of the remaining 23 glycoproteins altered in the plasma of pre-eclamptic women, showed functional interactions with at least one other glycoprotein (Figure 5-3) when examined using STRING (an online functional protein interaction network; http://string-db.org/); which pointed to the involvement of the glycoprotein in more than one pathway. Moreover, strong associations were clearly observed between certain glycoproteins such as complement components C6, C5 and C8; kininogen, plasminogen and kallikrein; and haptoglobin and hemopexin, indicating that they may be involved in a common pathway, which rationalises how the changes in the plasma level of these glycoproteins may relate to one another.

Table 5-3: Glycoprotein fold changes measured between the compared groups by the developed targeted LC-MS/MS profiling method. Glycoproteins are presented in alphabetical order.

		Fold changes	
No.	**Targeted Proteins**	**Early-onset PE versus matched controls**	**Late onset PE versus matched controls**
1	α-1-Acidglycoprotein	**1.8***	-1.2
2	α-1-Antichymotrypsin	1.1	-1.2
3	α-1-Antitrypsin	-1.7	**-2.5***
4	α-2-Antiplasmin	-1.2	-1.3
5	α-1-Betaglycoprotein	**2.2***	1.1
6	α-2-HS glycoprotein	**2.0***	**1.7***
7	α-2-Macroglobulin	-2.5	-1.1
8	β 2-Gglycoprotein 1	1.4	-1.1
9	Afamin	**1.5***	-1.1
10	Angiotensinogen	-1.4	1.1
11	Antithrombin-III	1.3	1.1
12	Apolipoprotein A-I	1.2	-1.2
13	Apolipoprotein A-II	1.0	1.2
14	Apolipoprotein D	**1.8***	1.2
15	C4-Binding protein α chain	**2.3***	1.0
16	CD5 antigin like	-1.1	-1.6
17	Ceruloplasmin	1.4	1.4
18	Clusterin	**1.8***	**1.5***
19	Complement C3	-1.1	-1.2
20	Complement C4 β- chain	-1.9	-1.7
21	Complement C5	**1.5***	-1.1
22	Complement C6	**1.5***	1.1
23	Complement C8 α-chain	**2.1***	1.1
24	Complement C9	1.1	-1.3
25	Complement factor B	1.4	-1.0
26	Complement factor H	**1.3***	1.1
27	Complement factor I	**1.9***	1.1
28	Corticosteroid binding globulin	**-2.3***	-1.3
29	Fibronectin	**2.5***	1.7
30	Haptoglobin β chain	**1.9***	-1.4
31	Hemopexin	**1.4***	**1.5***
32	Heparin cofactor II	-1.1	1.0
33	Histidin rich glycoprotein	1.5	-1.1
34	IGFALS	1.1	1.0
35	ITIH1	1.0	1.1
36	Kallikrein	**2.3***	-1.5
37	Kallistatin	1.1	1.1

Table 5-3: Continued

		Fold changes	
No.	**Targeted Proteins**	**Early-onset PE versus matched controls**	**Late onset PE versus matched controls**
38	Kininogen	**1.4**	-1.2
39	Lumican	1.6	2.0
40	N-Acetylmuramoyl-L-alanine amidase	1.2	1.0
41	Paraoxonase/arylesterase 1	1.1	1.0
42	Pigment epithelium derived factor	-1.2	-1.3
43	Plasma protease C1 inhibitor	1.3	-1.1
44	Plasminogen	**1.7**	-1.1
45	Protein AMBP	**1.4**	-1.1
46	Prothrombin	**1.5**	1.1
47	Serum-amyloid P-component	**2.1**	1.2
48	Thyroxin-binding globulin	-1.4	-1.1
49	Transferrin	**1.5**	**1.4***
50	Vitronectin	**1.8**	**1.5***
51	Zinc-α-2-glycoprotein	**2.4**	-1.1

*(bold) Significant changes in protein levels between pre-eclampsia and controls (p≤0.5 after FDR correction).
IGFALS refers to insulin-like growth factor-binding protein complex acid labile subunit, and ITIH1 refers to inter-α-trypsin inhibitor heavy chain 1.

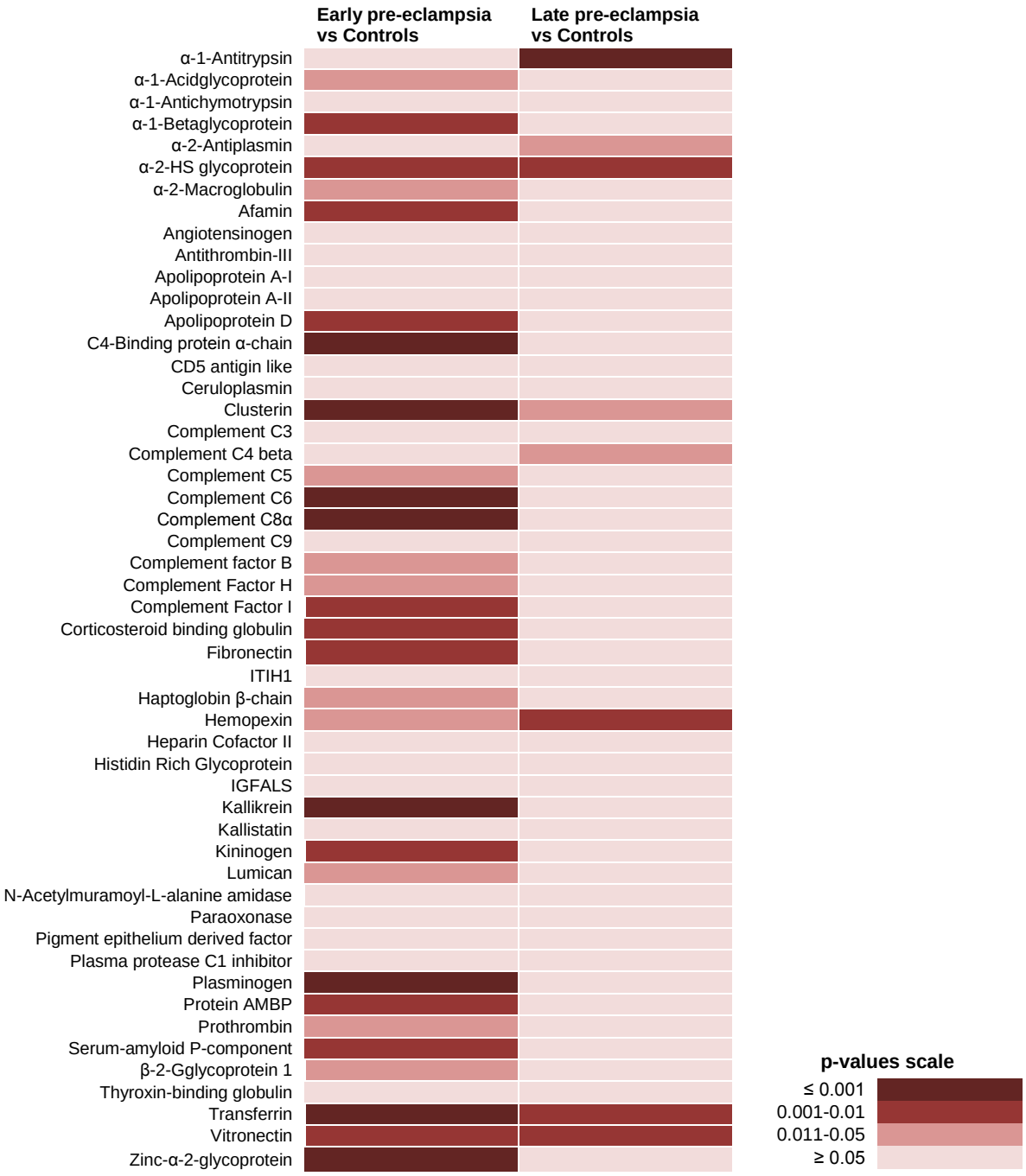


Figure 5-1: Heatmap of the p-values of glycoproteins level fold changes between early-onset pre-eclampsia and matched controls (first column form the left), and late-onset pre-eclampsia and its matched controls. The changes in the levels of α-2-macroglobulin, β-2-glycoprotein 1, complement factor B and lumican in early-onset pre-eclampsia compared to controls, and α-2-antiplasmin and complement C4 beta in late-onset pre-eclampsia compared to controls, were not significant after applying FDR correction.
IGFALS refers to insulin-like growth factor-binding protein complex acid labile subunit, and ITIH1 refers to inter-α-trypsin inhibitor heavy chain 1.

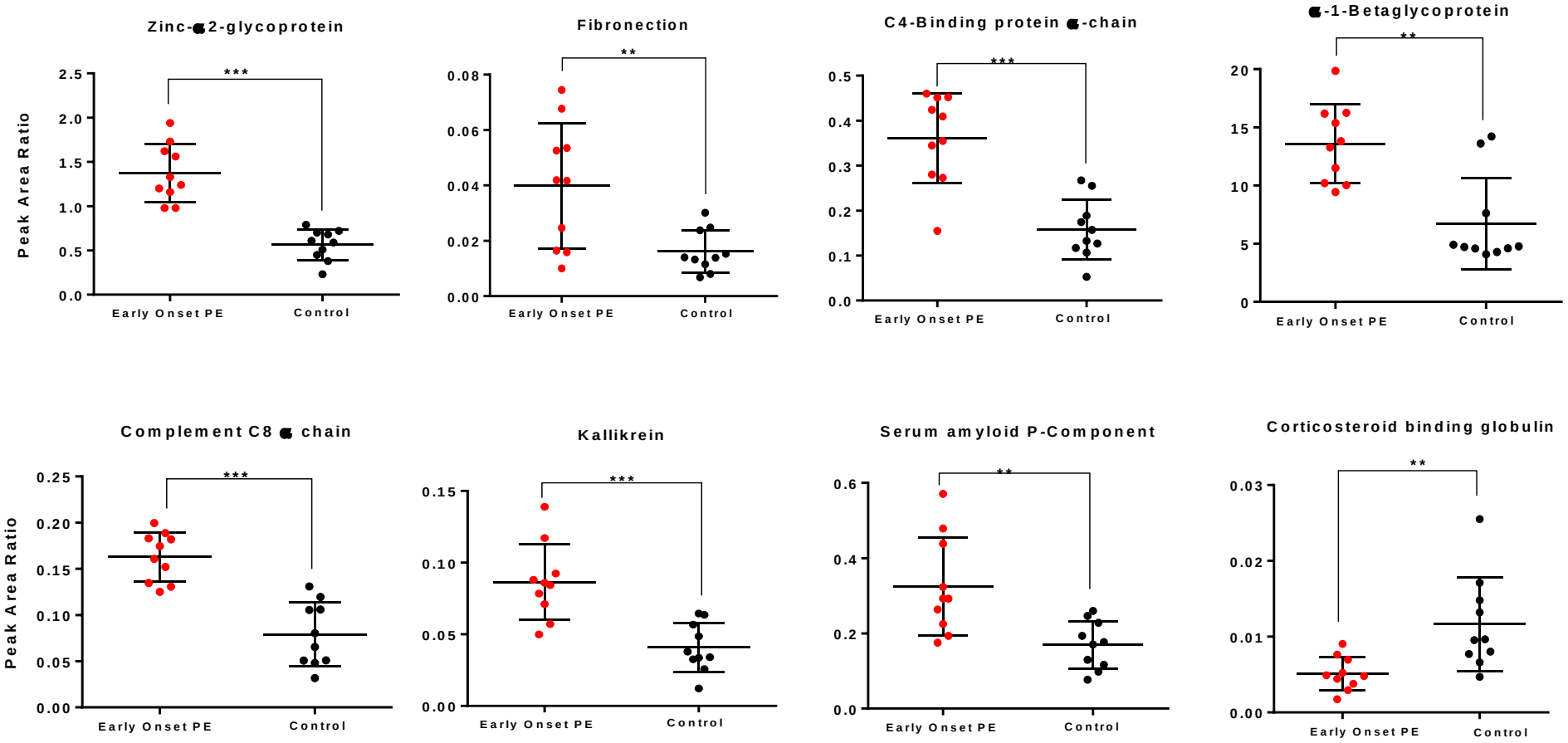


Figure 5-2: Glycoproteins that showed 2 or more fold changes in their abundance between early-onset pre-eclampsia plasma samples and gestational age-matched controls. Samples were analysed by label-free targeted LC-MS/MS method. Error bars represent the mean ± SD of the ratio of the analyte signal area over the internal standard signal area. The levels of all glycoproteins significantly increased except for corticosteroid binding globulin that showed a significant decrease in its level.
** p<0.05, ** p<0.01, *** P<0.001.*

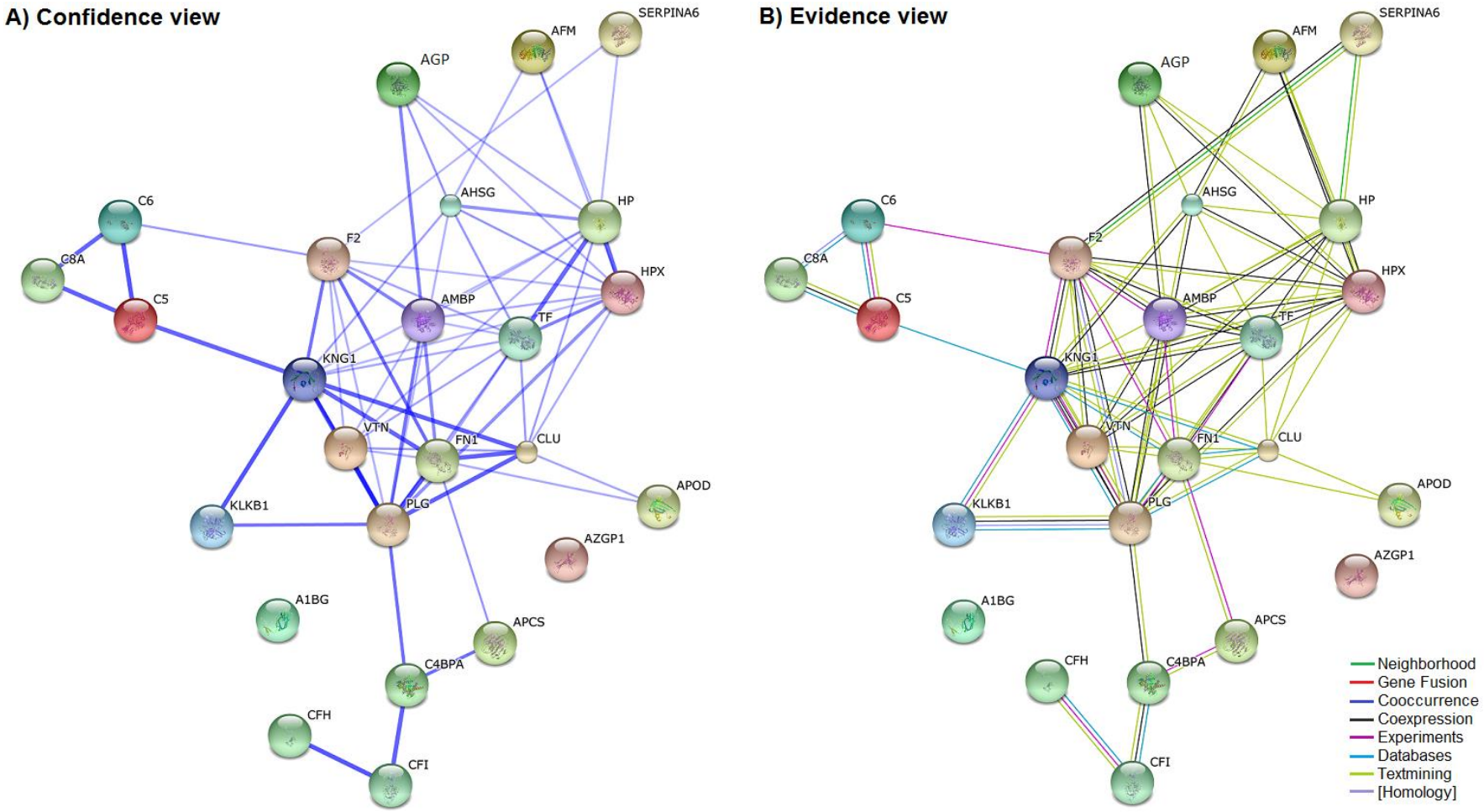


Figure 5-3: The functional interactions between glycoproteins differentially expressed in the plasma of pre-eclamptic women compared to controls detected using STRING. A) Confidence view; stronger associations are represented by thicker lines. B) Evidence view; different line colours represent the types of evidence for the association. Predicted functional links consist of up to eight lines; one colour for each type of evidence.
AGP: α-1-acidglycoprotein, AHSG: α-2-HS glycoprotein, AZGP1: zinc-α-2-glycoprotein, AFM: afamin, APOD: apolipoprotein D, APCS: amyloid p-component, AMBP: AMBP protein, C5: complement C5, C6: complement C6, C8A: complement C8-α, CFH: complement factor H, CFI: complement factor I, C4BPA: complement C4 β- chain, AFM: afamin, CLU: clusterin, FN1: fibronectin, F2: thrombin, HP: haptoglobin, HPX: hemopexin, KLKB1: kallikrein, KNG1: kininogen, PLG: plasminogen, SERPINA6: corticosteroid binding globulin, TR: transferrin and VTN: vitronectin.

5.3.2.2 Late-onset pre-eclampsia versus gestational age-matched controls

Univariate pairwise comparisons of glycoproteins between late-onset pre-eclampsia and control samples showed a different pattern of differentially expressed glycoproteins in late-onset compared to early-onset pre-eclampsia. The plasma level of the majority of the glycoproteins remained unchanged in late-onset pre-eclamptic women and the abundance of only six out of the 51 glycoproteins was significantly altered compared to gestational age-matched controls as presented in Table 5-3 and Figure 5-1. Five of the six altered glycoproteins, α-2-HS glycoprotein, clusterin, hemopexin, vitronectin, and transferrin, were detected with a significantly higher level in the plasma of both early- and late-onset pre-eclampsia compared to their gestation age-matched controls (Figure 5-4). The changes in the level of these glycoproteins in both disease onsets were comparable, and no significant changes in their abundance were detected between the early- and late-onset pre-eclampsia groups, except for α-2-HS glycoprotein which showed a significantly higher level in the early-onset group.

The sixth glycoprotein that was altered in late-onset pre-eclampsia was the highly abundant protease inhibitor α-1-antitrypsin, which showed a 2.5 fold decrease in its level in the plasma of late-onset pre-eclamptic women compared to matched controls. The plasma level of α-1-antitrypsin was lower in both early- and late-onset pre-eclampsia groups compared to normotensive matched controls; however, this decrease was statistically significant only in the late-onset of the disease as shown in Figure 5-4.

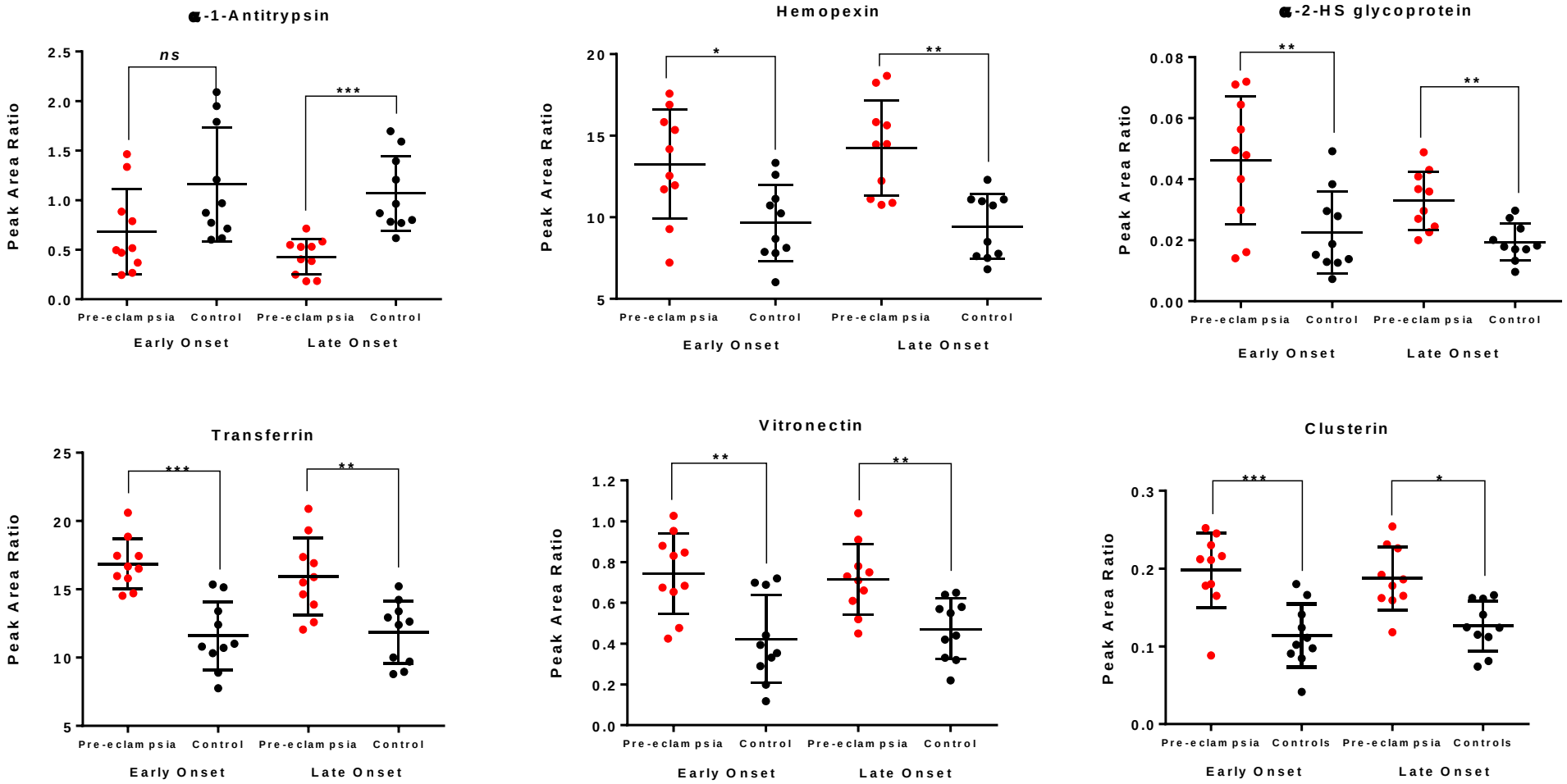


Figure 5-4: Proteins differentially expressed in early- and late-onset pre-eclampsia plasma samples compared to gestational age-matched controls. Samples were analysed by label-free targeted LC-MS/MS method. α-1-Antitrypsin was significantly down-regulated in late-onset, the level of other proteins were significantly changed in both pre-eclampsia conditions. Error bars represent the mean ± SD of the normalised peak areas.
** p<0.05, ** p<0.01, ***P<0.001*

5.3.3 Multivariate analysis of pre-eclampsia and matched control samples

Multivariate analysis is widely used in untargeted MS-based metabolomic and proteomic studies, that involve the analysis of treated and control sets of samples, to help in extracting metabolites or proteins that are different between the two groups from the highly complex data generated from these workflows. In a targeted analysis of proteins, as in this study, platform-dependent software provided with the MS instrument is satisfactory in processing the limited number of the targeted proteins and is the main source of the quantitative data obtained. Nevertheless, the performance of multivariate analysis on data derived from targeted protein analysis was investigated herein, and the results obtained were compared with univariate statistical analysis to check the suitability of multivariate analysis to be used when a limited number of variables are included (*c*. 50).

5.3.3.1 Early-onset pre-eclampsia versus gestational age-matched controls

A good separation was noticed between early-onset pre-eclampsia and matched control groups in the unsupervised PCA score plot that was used to give an unbiased overview of any possible trend or grouping in the samples datasets (Figure 5-5). The supervised OPLS-DA model resulted in a clear complete separation and clustering of the two groups noticed in the score plot (Figure 5-5), and yielded satisfactory fitness of the model value (R^2Y= 0.96) and predictive ability value (Q^2= 0.781).

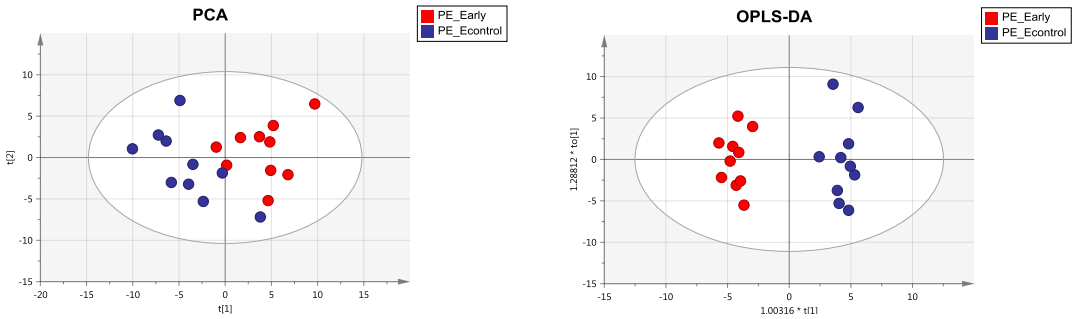


Figure 5-5: PCA and OPLS-DA score plots of early-onset pre-eclampsia samples (n=10) and their respective matched normotensive controls (n=10). Pre-eclampsia and control samples are presented in red and blue respectively.

Extraction of glycoproteins with a variable importance of projection (VIP) score >1 from the OPLS-DA model revealed 27 out of 51 glycoproteins contributed significantly to the clear separation noticed between pre-eclamptic women and matched control groups, (Table 5-4). The large number of glycoproteins altered in the plasma of early-onset pre-eclampsia compared to matched controls was the reason for the good separation observed in the PCA model between the two compared groups. Normally good separation using the PCA model is difficult to obtain with clinical samples due to the high biological variability.

All of the 27 glycoproteins identified by multivariate analysis showed significant differences between pre-eclamptic women and matched controls in the univariate analysis, except for complement factor B and β 2-glycoprotein 1. Zinc-α-2-glycoprotein showed the highest VIP score (1.52) and was at the top of the list of glycoproteins responsible for the separation in the OPLS-DA model. The same protein was detected with 2.4 fold increase in its plasma level in pre-eclamptic women compared to controls using univariate analysis. Moreover, C4-binding protein α chain, α-1-betaglycoprotein, complement C8α, corticosteroid binding globulin and kallikrein were among the top 10 glycoproteins with the highest VIP scores, and all of them showed > 2 fold change in their plasma level in pre-eclampsia using univariate analysis, indicating an agreement between univariate and multivariate results.

Table 5-4: Glycoproteins extracted from the OPLS-DA model with VIP score above 1.

Early-onset pre-eclampsia vs matched controls			Late-onset pre-eclampsia vs matched controls	
No.	**Glycoprotein**	**VIP score**	**Glycoprotein**	**VIP score**
1	Zinc-α-2-glycoprotein	1.52	α-1-Antitrypsin	2.02
2	Plasminogen	1.48	Clusterin	1.93
3	C4-Binding protein α chain	1.40	α-2-HS glycoprotein	1.90
4	α-1-Betaglycoprotein	1.39	Hemopexin	1.70
5	Complement C8α	1.38	Transferrin	1.67
6	Vitronectin	1.37	Ceruloplasmin	1.60
7	Corticosteroid binding globulin	1.33	Vitronectin	1.50
8	Kallikrein	1.33	CD5 antigin like	1.48
9	Transferrin	1.33	Fibronectin	1.45
10	Complement C6	1.31	Complement C4 beta	1.40
11	Serum-amyloid P-component	1.27	Protein AMBP	1.36
12	Kininogen	1.23	α-2-Antiplasmin	1.33
13	α-1-Acidglycoprotein	1.17	Complement C5	1.28
14	Clusterin	1.15	Apolipoprotein A-I	1.28
15	Complement Factor I	1.14	Kallistatin	1.17
16	Complement Factor H	1.13	α-1-Antichymotrypsin	1.17
17	Apolipoprotein D	1.12	Plasma protease C1 inhibitor	1.15
18	Fibronectin	1.10	Heparin cofactor II	1.10
19	Complement C5	1.09	Complement C3	1.08
20	Afamin	1.09	Apolipoprotein D	1.07
21	Haptoglobin β chain	1.06		
22	Complement factor B	1.05		
23	β 2-Gglycoprotein 1	1.05		
24	Prothrombin	1.04		
25	α-2-HS glycoprotein	1.03		
26	Protein AMBP	1.02		
27	Hemopexin	1.01		

5.3.3.2 Late-onset pre-eclampsia versus gestational age-matched controls

Multivariate analysis of the late-onset pre-eclampsia and matched control data sets showed no separation or clustering pattern between the two compared groups in the PCA score plot; however, clear separation was achieved with the supervised OPLS-DA model, as shown in Figure 5-6, and satisfactory goodness of fit and predictive ability values were achieved (0.903 and 0.644 respectively).

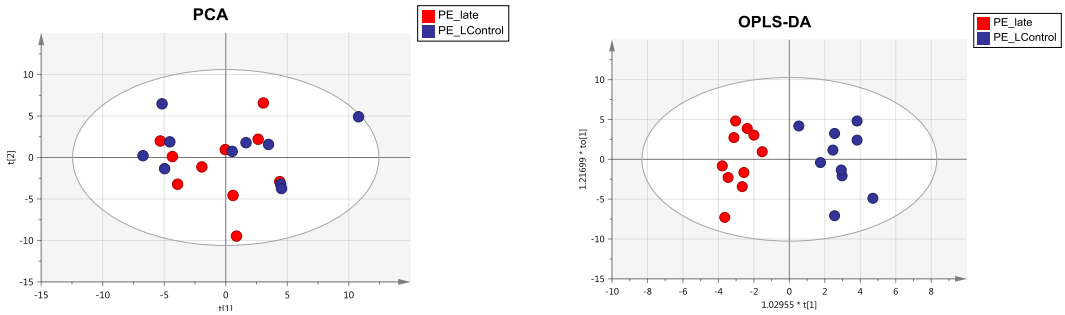


Figure 5-6: PCA and OPLS-DA score plots of late-onset pre-eclampsia samples (n=10) and their respective matched normotensive controls (n=10). Pre-eclampsia and control samples are presented in red and blue respectively.

Twenty glycoproteins had VIP score >1 (Table 5-4) and resulted in the separation noticed in the OPLS-DA model between the late-onset pre-eclamptic women and matched control groups. Alpha-1-antitrypsin showed the highest VIP score (2.02) in multivariate analysis and the highest significant change in its abundance in univariate analysis. Similarly, the remaining five glycoproteins that showed statistically significant differences when analysed using univariate analysis (α-2-HS glycoprotein, clusterin, hemopexin, vitronectin, and transferrin) were also amongst the top 10 glycoproteins with the highest VIP scores.

5.4 Discussion

This is the first study that has focused on detecting changes in the plasma level of glycoproteins in women with pre-eclampsia (early- and late-onset) compared with controls. Out of the 51 glycoproteins profiled in this study, 25 were altered with the early-onset and 6 with the late-onset pre-eclampsia compared to gestational age-matched controls. Two of them, apolipoprotein D and kallikrein, were identified for the first time to be changed in the plasma of women with pre-eclampsia.

Although monitoring protein changes in placental tissue samples is the optimal way to detect pathological changes and identify relevant biochemical pathways and mechanism underlying pre-eclampsia, this will always be limited by the invasive measures necessary to obtain the placental tissues, and the challenging sample processing, which make other more accessible biological fluids (*e.g.* plasma, serum, urine) more widely used for quantitative protein profiling and identification of potential disease biomarkers. In pre-eclampsia, local protein changes in placental tissue many not be reflected in plasma, but changes in placenta-derived proteins or circulating proteins due to the interaction between the endothelium and systemic pathways is expected to be reflected in plasma proteins, making plasma a clinically important biofluid for identifying potential biomarkers and different biochemical pathways underlying this condition.

5.4.1 The biochemical significance of the changed glycoproteins in pre-eclampsia

The differentially expressed glycoproteins between pre-eclampsia cases and controls, particularly the early-onset variant of the disease, were responses to placental triggers to protect mainly against endothelial damage, coagulation, and inflammation. The changed glycoproteins pointed towards the biological processes that were altered in pre-eclampsia including placenta endothelial dysfunction, complement cascade and immune activation, coagulation/fibrinolysis pathway, lipid transport and metabolism, haem scavenging, and inflammation responses. The identified pathways altered herein are well-known to be involved in the pathogenesis of pre-eclampsia [136; 378], in line with previous proteome data from the plasma of pre-eclamptic women [381; 383], and support the previously reported synergism of multiple pathways in the pathology of the condition [378].

The current study highlighted the participation of various pathways in pre-eclampsia is expected given the well-accepted multisystem and heterogeneous nature of the disease [107; 164], which provides evidence and agrees with the current trend that more than one biomarker is needed in order to identify this multifactorial pregnancy complication [127; 389].

Of interest, 11 out of the 25 significantly altered glycoproteins in early-onset pre-eclampsia herein overlapped with proteins complexed to high-density lipoprotein as identified in a previous study [400]. These proteins are α-1-acidglycoprotein, α-2-HS-glycoprotein, α-1-betaglycoprotein, Apo D, clusterin, complement C4 binding protein, hemopexin, haptoglobin, kininogen, transferrin, and vitronectin. This overlay provides

molecular evidence for the well-recognised association between having pre-eclampsia and the higher risk of developing cardiovascular disease [401], and suggests that these proteins might be involved in pathways that, when altered, can increase the risk of having cardiovascular diseases among women with pre-eclampsia.

The functional significance of the identified altered glycoproteins should enhance the understanding of the mechanisms and pathways associated with pre-eclampsia, and provide insights into the disease pathogenesis as will be discussed below. Of note, some of the altered glycoproteins participate in several biological processes but they were grouped according to their main function.

5.4.1.1 Glycoproteins involved in cell adhesion

In the current work, a higher level of fibronectin, a protein with cell adhesion and maternal endothelium repair functions, was detected in the plasma of pre-eclampsia cases compared to controls. Increased level of fibronectin indicated mainly the presence of endothelial dysfunction and injury [402]. Fibronectin is involved in a wide spectrum of processes linked to pre-eclampsia including coagulation, cell adhesion, platelet function, tissue repair, trophoblast invasion and vascular remodelling [403; 404]. The elevated plasma fibronectin level observed with pre-eclampsia herein is not entirely unexpected in light of the strong link between higher fibronectin level and pre-eclampsia recognised over a long period of time [402; 403; 405; 406; 407]. Increased levels of fibronectin have been detected previously not only in the plasma but also in placental tissues of pre-eclamptic women [376; 380; 383], which reflect the close involvement of the protein in the disease pathophysiology. Additionally, a significant increase in the plasma level of fibronectin has been previously reported in the first trimester in pre-eclamptic women [408], which suggests that fibronectin has the potential to act as a diagnostic marker of pre-eclampsia.

Recently, strong correlations have been observed between increased serum fibronectin level and important pregnancy outcomes including earlier delivery and decreased infant birth weight [408]. This might explain the detection of significant increase in the plasma level of fibronectin only with the early-onset but not the late-onset pre-eclampsia in this work. Early-onset pre-eclamptic women gave birth earlier to infants with significantly lower birth weight, where in the late-onset pre-eclampsia, women delivered almost at term with birth weight comparable to the uncomplicated pregnancy controls as observed in Table 5-2.

5.4.1.2 Glycoproteins involved in oxidative stress and lipid metabolism

There is increasing evidence that oxidative stress plays an important role in pre-eclampsia [389], (Figure 5-7). As discussed in Chapter One, Section 1.3.2, oxidative stress is mainly initiated as a consequence of impaired cytotropoblast invasion that leads to inadequate perfusion of the placenta cells and ischemia, resulting in the generation of reactive oxygen species (ROS) and abnormal lipid metabolism [389], that might contribute to the endothelial dysfunction in pre-eclampsia [123].

In the current work, three glycoproteins (clusterin, apolipoprotein D and zinc-α-2-glycoprotein) that are involved in lipid metabolism and have anti-oxidation function were increased in the plasma of pre-eclamptic women compared to controls. clusterin (also known as apolipoprotein J) was among the five glycoproteins that were significantly increased in the plasma of early- and late-onset pre-eclampsia in the present study. Clusterin is a multi-functional glycoprotein associated with lipid transport, cell adhesion, apoptosis, and angiogenesis regulation [409; 410]. Moreover, it is considered as a form of extracellular chaperone molecule, due to its ability to prevent stress-induced protein aggregation during oxidative stress associated diseases such as neurological disorders [410]. Therefore, its higher plasma level in pre-eclampsia perhaps reflects endothelial cell injury, oxidative stress or platelet activation associated with the disease [389].
Similarly, increased serum level of clusterin in pre-eclampsia has been detected previously [207; 382; 389; 409], and literature on clusterin highlighted higher plasma level prior to the onset of pre-eclampsia (at weeks 12 and 20 of gestation), in cases that subsequently developed pre-eclampsia [207; 378], suggesting that clusterin may serve as a predictive marker for the disease. Furthermore, clusterin has been reported to be up-regulated by the trophoblast and villous endothelial cells in the placental tissue in pre-eclampsia [411], pointing to a strong correlation between clusterin level and the pathophysiology of the disease.

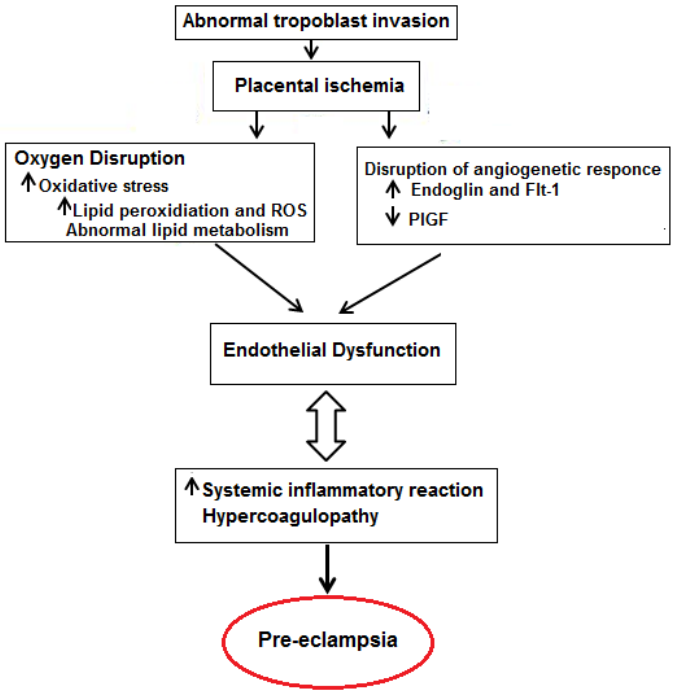


Figure 5-7: Oxidative stress involvement in pre-eclampsia. Defective placentation triggers a cascade of events including oxidative stress, angiogeneic imbalance and exaggerated inflammatory reaction which lead to endethelial dusfunction and eventually pre-eclampsia.

Apolipoprotein D (Apo D) plays an important role in lipid metabolism and transport, and showed a higher level in the plasma of early-onset pre-eclampsia herein. Apo D is an atypical apolipoprotein with structural similarity to the lipocalin family of proteins and is expressed widely in mammalian tissues including the placenta [412]. Besides its lipid transporting function [413], Apo D has been reported to have anti-oxidation and anti-stress functions by protecting against lipid peroxidation [412]. So, the increased abundance of Apo D in the plasma of pre-eclamptic women detected in this study may reflect a body defense mechanism against the higher level of generated ROS in pre-eclampsia. A higher level of Apo D has not been reported before in pre-eclampsia and the role of its up-regulation in the pathology of the disease remains inconclusive, and more work is needed to elucidate its involvement in the disease mechanism.

Zinc-α-2-glycoprotein is a lipid-mobilising adipokine [414], that regulates lipid metabolism by inhibiting lipogenesis and inducing lipolysis via a cyclic AMP-mediated system and through interaction with the β3-adrenoreceptor [415; 416]. Consequently, its higher circulating level in pre-eclamptic women compared to normotensive controls might explain the increased lipolytic activity and the up-regulation of free fatty acids noticed in pre-eclampsia and reported by previous studies [417; 418]. The serum level of other

adipokines including visfatin, chemerin, and adipocyte fatty acid-binding protein have been reported to be increased in pre-eclampsia by previous studies [419; 420; 421]. The finding of the current work supports the previously reported importance of lipid homeostasis regulators in the pathogenesis of pre-eclampsia [375; 422], and confirms the findings of a previous study that showed a higher concentration of zinc-α-2-glycoprotein in the serum of pre-eclampsia patients, as measured by ELISA assay [418].

5.4.1.3 Glycoproteins involved in complement pathway and inflammation

Higher levels of acute phase immune response glycoproteins (*e.g.* α-1 acid glycoprotein, haptoglobin, and serum amyloid P-component) and complement system components and factors were detected in the plasma of pre-eclampsia patients compared to controls in this work, reflecting an inflammatory state associated with the disease. Regulation of the complement system activation is crucial for uncomplicated pregnancy and normal placentation [423], however in pre-eclampsia complement system dysregulation and excessive complement activity have been observed in the plasma and placental tissues and have been suggested to contribute to the disease pathology by different related studies [383; 423; 424; 425]. Activation of the complement system pathways (classical, lectin and alternative) lead to opsonisation, release of pro-inflammatory anaphylatoxins, and generation of the membrane attack complex (MAC) to enhance the clearance of the placenta-derived microparticles or dead cells from the maternal circulation [378; 424], (Figure 5-8). This might explain the higher plasma levels of the terminal pathway MAC constituents, C8 and C6, detected in this study in pre-eclampsia cases compared to controls.

Besides complement components, the higher plasma levels of complement factor I and C4 binding protein α detected in the current study were also reported to be up-regulated prior to pre-eclampsia (at week 20) in women who later developed the disease [378], emphasising the involvement of the complement system throughout disease progression.

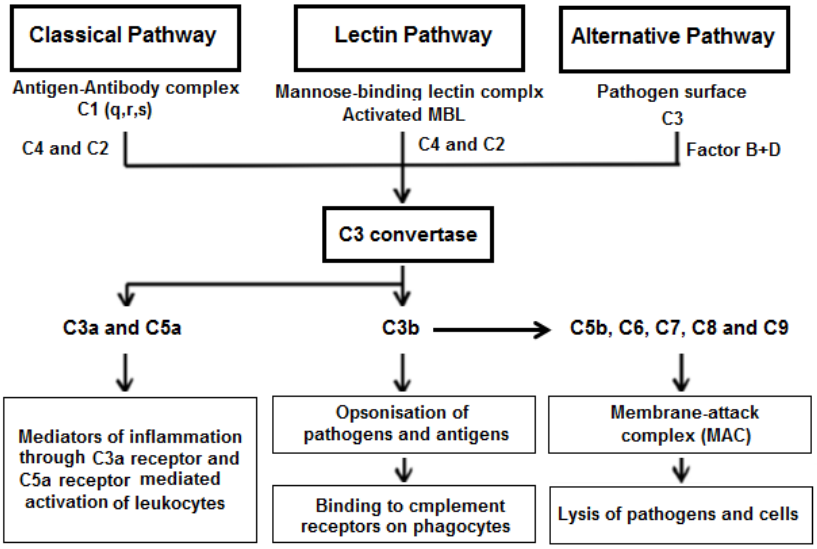


Figure 5-8: The three pathways of the complement system activation. Complement system can be activated through the classical (triggered by antibody or by direct binding of complement component C1 to the pathogen surface), lectin (triggered by mannan-binding lectin) and alternative pathways (triggered directly on pathogen surfaces). All activation pathways initiate the formation of C3 convertase which generates anaphylatoxins (C3a and C5a), opsonin C3b and membrane attack complex to end with inflammation, opsonisation and cell lysis. Figure 3-8 modified from reference [426].

Low plasma level of corticosteroid-binding globulin, a high affinity steroid-binding protein that transports glucocorticoids and progesterone [427], and mainly binds >80% of cortisol in human peripheral blood [428], was detected in pre-eclampsia compared to normotensive controls in the present work. The reduced level of circulating corticosteroid-binding globulin is possibly a consequence of either reduced synthesis induced by the circulating cytokines, or enhanced degradation as a result of inflammation [429] and metabolic abnormalities associated with hypertension in pregnancy [430]. The decreased level of corticosteroid-binding globulin leads to an increase in the level of free cortisol in the circulation, which in turn can contribute to the elevated blood pressure in pre-eclampsia [431]. Reduced plasma corticosteroid-binding globulin level in pre-eclampsia has been observed by previous studies [429; 430] that also reported a correlation between reduced plasma corticosteroid-binding globulin level and infant birthweight [430]. The previous finding might explain the detection of a significant reduction in plasma corticosteroid-binding globulin level in the early-onset but not the late-onset pre-eclampsia in the current study, since early-onset pre-eclamptic women gave birth to significantly lower infant birth weight as compared to women with late-onset pre-eclampsia (Table 5-2).

5.4.1.4 Glycoproteins involved in coagulation/fibrinolysis pathways

Alteration of the coagulation/fibrinolysis system in pre-eclampsia was observed in the current work as indicated by the higher levels of plasma prothrombin, plasminogen, kininogen, and kallikrein in pre-eclamptic women compared to controls (reported in Table 5-3). The higher level of prothrombin detected may suggest the activation of the coagulation cascade through activation of factor X (the common point in the intrinsic and extrinsic pathways), Figure 5-9, A. Factor Xa activates prothrombin to thrombin which in turn cleaves fibrinogen to insoluble fibrin, and aids in the cross linking of fibrin polymers to initiate clot formation and thus contribute to the well-recognised hypercoagulability state associated with pre-eclampsia [378; 432]. Moreover, the higher level of kallikrein detected herein might reflect activation of the intrinsic clotting cascade. Kallikrein activates factor XII which in turn ends with the activation of factor X and the conversion of prothrombin to thrombin (Figure 5-9, A). Factor XIIa will also hydrolyse more prekallikrein to kallikrein establishing a reciprocal activation cascade. This hypercoagulability condition leads to the activation of the fibrinolytic system by converting plasminogen into the active plasmin, by a variety of enzymes including kallikrein, which dissolves fibrin and fibrinogen in the blood clot as part of tissue repair and remodelling process [433]. Kallikrein, besides its engagement in clotting cascade and plasminogen activation, plays an important role in the renin-angiotensin system (Figure 5-9, B). Kallikrein mainly converts prorenin into renin which will further cleave angiotensinogen to generate angiotensin I, or it can cleave angiotensinogen directly to generate angiotensin I and initiates the renin angiotensin system cascade to end with an increase in the blood pressure in both cases [435; 436]. Also, plasminogen besides its involvement in fibrinolysis has other important roles related to inflammatory processes [434], so it is likely that the increase in its plasma level is secondary to the inflammation associated with pre-eclampsia.

The higher level of plasminogen has been previously reported not only in the plasma of pre-eclamptic women [207; 383], but also in the placental tissue and trophoblast cells [376] indicating a possible link between the production by the placenta and deposition into the circulation. The higher level of kallikrein in the plasma of pre-eclamptic women, detected for the first time in the current study, sheds light on a possible contribution of kallikrein in the hypertension that is a defining feature of pre-eclampsia and further study at early stage of pregnancy is therefore required to evaluate its applicability as a new diagnostic biomarker of pre-eclampsia.

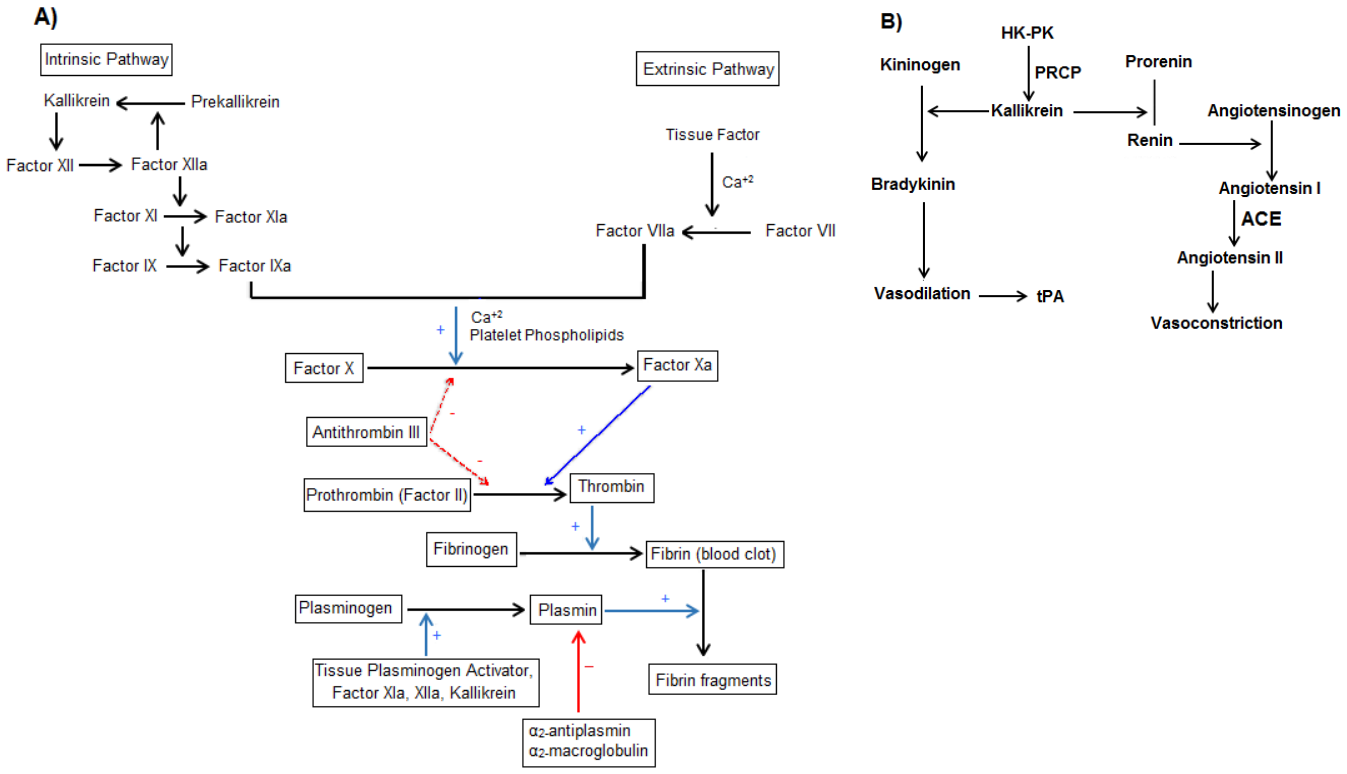


Figure 5-9: A) Simplified clotting and fibrinolysis cascade. Activation of both the intrinsic and the extrinsic coagulation pathways will lead to the activation of factor X, the common point in both pathways. Factor Xa activates prothrombin to thrombin which in turn converts fibrinogen to fibrin and initiates clot formation. To prevent hypercoagulability state, tissue plasminogen activator cleaves plasminogen to plasmin which then degrades the fibrin clots. B) Interaction of the plasma kallikrein with the renin angiotensin system. Kallikrein mainly converts prorenin into renin which will further cleave angiotensinogen to generate angiotensin I, and initiates the renin angiotensin system cascade. Kallikrein also acts upon kininogen leading to the release of bradykinin. HK: high molecular weight kininogen, PK: prekallikrein, PRCP: prolycarboxypeptidase, ACE: angiotensin converting enzyme, tPA: tissue plasminogen activator. Figure A and B were modified from references [437] and [438] respectively.

5.4.2 Early- and late-onset pre-eclampsia show different glycoprotein changes

Whilst the abundance of 25 glycoproteins was significantly altered in the plasma of early-onset pre-eclampsia, the abundance of only six glycoproteins was changed in the late-onset of the disease compared to the respective controls. Five of them (hemopexin, α-2-HS-glycoprotein, clusterin, transferrin, and vitronectin) were significantly increased in both pre-eclampsia onsets, and they represent proteins involved mainly in haem scavenging, lipid and iron transport and cell adhesion. Only hemopexin was previously reported to be up-regulated in both early- and late- onset pre-eclampsia [375]. As only limited literature is available on late-onset pre-eclampsia, the remaining glycoproteins were reported by other studies to be up-regulated prior to the development of pre-eclampsia [207; 375; 378; 387] or in the early stage of the condition [383].

The fact that the plasma level of several glycoproteins, involved in various system pathways, were differentially expressed in the early-onset pre-eclampsia compared to the late stage in the present work, may reflect different pathophysiological mechanisms. This supports the increasing evidence that the two pre-eclampsia onsets have different aetiologies and should be considered as a two different forms of the disease [439; 440; 441]. Early-onset pre-eclampsia is linked mainly to failed placental vascular remodelling, and is associated with adverse maternal and neonatal outcomes including low infant birth weight. Late-onset pre-eclampsia might be related to maternal constitutional factors and is characterised by low rate of fetal involvement, and more favourable perinatal outcomes [441; 442], as noted in Table 5-2. This probably contributed to the more pronounced involvement of diverse glycoproteins and biological processes in the early-onset compared to late-onset pre-eclampsia.

It is worth mentioning that the level of other glycoproteins might be altered in the late-onset pre-eclampsia but they were not profiled in the targeted approach followed here. Given the limited literature available, a more comprehensive discovery strategy applying shotgun proteomics may provide a better insight into the pathophysiological changes in the latent phase of the disease.

5.5 Conclusion

The developed targeted LC-MS/MS glycoprotein profiling method successfully identified significant changes in the level of glycoproteins in pre-eclampsia as compared to controls. Two of them, apolipoprotein D and kallikrein, are reported for the first time to be altered in the plasma of pre-eclamptic women suggesting that they could be further evaluated as new biomarkers. The effectiveness of the developed method using simple, cost-effective conventional LC-MS/MS workflow was demonstrated by the overlap of the remaining altered glycoproteins with those previously reported in the scientific literature, which increased the confidence in the presented findings. The value of the identified changed glycoproteins, particularly the two novel biomarker candidates, remained to be evaluated at earlier time points of pregnancy in a larger prospective cohort study, to address their potential clinical usefulness for the early prediction of pre-eclampsia.

Some of the glycoproteins that were detected with higher levels in the plasma of pre-eclamptic women have also been reported previously to be increased in pre-eclampsia placental tissues (*e.g.* fibronectin, plasminogen and clusterin) pointing to a strong

correlation between these proteins and the pathophysiology of the disease. This suggests that changes in protein expression at the placental level or in the maternal vasculature can be reflected as changes in the plasma proteins in pre-eclampsia.

The current study is one of very few studies that compared the protein profile of the early- and late-onset pre-eclampsia. Analysis of the sample sets revealed a higher number of significantly altered glycoproteins in the early onset of the disease, which may reflect different pathophysiological mechanisms at different stages of pre-eclampsia. The glycoproteins changed in the plasma of pre-eclampsia patients are involved in a range of specific biological processes and pathways including lipid and iron metabolism, coagulation, complement regulation, cell adhesion, oxidative stress and acute-phase responses. This complex pattern of changes indicates a synergism between the pathways involved in the pathogenesis of pre-eclampsia and emphasises the heterogeneous nature of this complicated pregnancy condition. The results outlined here point to the need for a combination of independent biomarkers, each representing a pathophysiological process, to provide suitable diagnosis, and of upmost importance, the prediction of women at risk of developing pre-eclampsia.

The presented study demonstrated the capability of the developed cost-effective targeted LC-MS/MS methodology for the clinically relevant profiling of glycoproteins as potential disease biomarkers and sheds light on important molecular mechanisms underlying pre-eclampsia, leading to several potential areas for future investigation.

Chapter Six

Profiling of Plasma Glycoproteins in Polycystic Ovary Syndrome

6 Profiling of Plasma Glycoproteins in Polycystic Ovary Syndrome (PCOS)

Summary

Polycystic ovary syndrome (PCOS) is a prevalent heterogeneous endocrine disorder with poorly understood aetiology. Glycoproteins are involved in many biochemical processes which have relevance to the pathogenesis of the disease, and although several proteomic studies have been reported in PCOS, none have focused on this class of proteins. The aim of this chapter was to profile 54 glycoproteins in blood plasma from women with PCOS (n=25) and controls (n=25) using the developed LC-MS/MS method to identify glycoproteins altered in PCOS that can act as biomarker candidates of the disease, and compare them with glycoproteins altered in pre-eclampsia (Chapter 5) to propose possible pathophysiological mechanisms that can explain the association between PCOS and pre-eclampsia reported in the literature.

The abundance of 16 glycoproteins was significantly altered in the plasma of women with PCOS compared to controls. All altered glycoproteins showed an increase in their levels except apolipoprotein A-I and apolipoprotein D which showed a decrease. Among the up-regulated glycoproteins were fibrinogen α and γ chains, plasminogen, complement C3, and transferrin. Significantly changed levels of vitronectin and insulin growth factor acid labile subunit were detected for the first time in the plasma of women with PCOS suggesting that they could be further investigated as new potential biomarkers. The differentially altered glycoproteins pointed to a high inflammatory state, perturbation in the fibrinolysis/coagulation and insulin growth factor systems, and iron overload among women with PCOS. Additionally, several factors that could contribute to the increased risk of developing cardiovascular diseases in women with PCOS were identified including, a lower circulating level of apolipoprotein A-I, which reflected a lower level of the cardiovascular protective lipoprotein HDL.

Seven of the 16 glycoproteins altered in PCOS were also found to be changed in pre-eclamptic women (afamin, apolipoprotein D, α-1-acidglycoprotein, haptoglobin β chain, plasminogen, transferrin and vitronectin). The common altered glycoproteins pointed to underlying pathophysiological mechanisms, such as hypofibrinolysis and thrombophilia and iron overload, that might be common to both conditions and, therefore, might provide a possible justification to the association between PCOS and pre-eclampsia.

In conclusion, the presented study identified potential PCOS glycoprotein biomarkers, shedding light on different biochemical processes altered in PCOS and pointing to possible pathophysiological mechanisms that might assist in explaining the link between PCOS and pre-eclampsia.

6.1 Introduction

6.1.1 Polycystic ovary syndrome

Polycystic ovary syndrome (PCOS) is a common endocrine disorder in women of reproductive age that can profoundly affect women psychological, social and reproductive life [443]. It is characterised by hyperandrogenism, ovulatory dysfunction (oligo-ovulation or anovulation), and morphologically abnormal ovaries with numerous small follicular cysts [443; 444]. The presence of two of the above three criteria fulfils a diagnosis of PCOS according to the Rotterdam consensus [445].

PCOS is a heterogeneous disorder and has many signs and features. Beside the previous three main characteristics assessed to confirm PCOS diagnosis, PCOS is associated with insulin resistance, dyslipidaemia, metabolic disturbances and increased risk of many pathological conditions such as type 2 diabetes [446].

The pathogenesis of the condition is complex and the cause of it is not fully understood, however, the focus has been towards a defect in the hypothalamic–pituitary gland, ovary and/or insulin sensitivity as possible pathological causes of the syndrome [443], as shown in Figure 6-1.

1.1.1.1 Risk factors

Several factors have been associated with increased risk of developing PCOS such as family history, obesity, and type 1 [447] and type 2 diabetes [448]. A history of excess weight gain often precedes the development of the clinical features of PCOS [449] and has shown to worsen the metabolic and the reproductive abnormalities in women with the syndrome [450]. The high prevalence of PCOS or its features among first-degree relatives suggests that genetic factors can contribute to the development of PCOS [451]. Moreover, a number of factors that are associated with an increased prevalence of PCOS have been identified in children [452] including high birth weight in girls born to overweight mothers, atypical central precocious puberty, obesity and metabolic syndrome [453].

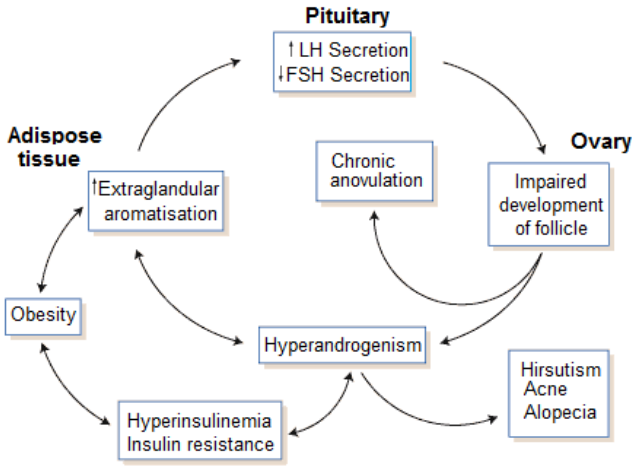


Figure 6-1: Pathophysiology of polycystic ovary syndrome. Impaired development of follicle, as a result of increased secretion of LH and decreased secretion of FSH from pituitary gland, will lead to menstrual irregularities and increased serum concentrations of androgen (resulting in hirsutism and acne). The hyperandrogenism state will lead to hyperinsulinemia which is a key feature of PCOS. On the other hand, hyperinsulinemia and insulin resistance (due to obesity mainly) can itself cause an increase in the androgen level in the circulation.
LH and FSH are luteinising hormone and follicle-stimulating hormone respectively, the figure is from reference [454].

6.1.1.1 Management of PCOS

Management strategies followed in PCOS aim mainly to treat the three characteristic symptoms of the disease; androgen-related symptoms, menstruation-related disorders and infertility [455]. Oral contraceptive pills and combined hormonal contraceptive are considered first-line for the treatment of androgen-related symptoms including hirsutism and acne [455; 456]. Low-dose combined hormonal contraceptives are also the primary recommended intervention to improve menstrual regularity in PCOS [455]. Women with PCOS and suffering from infertility caused by oligo-ovulation or anovulation might require ovulation induction or assisted reproductive technology to become pregnant [457], however, weight loss, particularly in overweight and obese women with PCOS, has shown to improve fertility [458]. The antidiabetic drugs, metformin and thiazolidinediones, are recommended to improve insulin resistance associated with PCOS and in turn, decrease the level of circulating androgen [455]. Apart from pharmacological treatments, following a healthy lifestyle has shown to reduce body weight and improve the metabolic and endocrine consequences of PCOS, resulting in decreased testosterone level and improved insulin resistance and fertility in women with PCOS [458].

6.1.1.2 Association of PCOS with other comorbidities

Oligo-ovulation or anovulation in women with PCOS is a major cause of infertility [457], and chronic anovulation can increase the risk of endometrial hyperplasia and carcinoma [455]. Aside from symptoms of androgen excess and reproductive consequences, PCOS can increase the risk of developing diabetes, cardiovascular diseases, and metabolic syndrome. Insulin resistance and hyperinsulinemia are common features in PCOS [456; 459] and therefore can substantially increase the risk of developing diabetes mellitus [460; 461; 462]. The reason behind the higher risk of developing cardiovascular diseases is related to the increase of several cardiovascular risk factors in women with PCOS including hyperlipidaemia, hyperandrogenaemia, hypertension, markers of a prothrombotic state, markers of inflammation [463], and impaired vascular and endothelial functions [464]. An increase in the prevalence of metabolic syndrome in women with PCOS has been reported [465] and is mainly a consequence of increased body weight, excess androgen, hyperlipidaemia, and insulin resistance and glucose intolerance associated with PCOS [443; 466].

6.1.1.3 Association of PCOS with pre-eclampsia

Evidence suggests that PCOS has a negative impact on pregnancy outcomes and increased risk of hypertensive diseases during pregnancy and preterm birth among PCOS affected women have been noticed [462; 467; 468]. In a large cohort study, a higher risk of pregnancy complications such as gestational diabetes, pre-eclampsia, preterm delivery were observed in 3787 women with PCOS compared with the matched controls [462]. Other studies have also reported a higher prevalence of pre-eclampsia among women with PCOS [446; 469; 470]; a systematic review performed in 2011 showed a four-fold increase in the risk of developing pre-eclampsia in pregnant women who are known to have PCOS when compared with controls [467].

Although the association between PCOS and pre-eclampsia has been documented, the pathophysiological mechanisms behind it are not fully understood. Nonetheless, some studies have pointed to the increased level of androgens in PCOS as a factor that might contribute to this association since higher circulating androgens level have been reported in pre-eclampsia [471; 472]. Furthermore, a relationship between hyperinsulinemia and hypertensive disorders during pregnancy has been reported [473], indicating that hyperinsulinemia and the subsequent development of diabetes in PCOS women could play an important role in increasing the risk of pre-eclampsia, especially as diabetes is

considered a strong predisposing risk factor for developing pre-eclampsia during pregnancy [122; 124; 131].

Whether the observed positive relationship between PCOS and pre-eclampsia arises from the above factors underlying the pathophysiology of PCOS or other factors remains to be elucidated by further studies [470].

6.1.2 Previous proteomics studies of PCOS

The application of an hypothesis-driven approach reliant on the previous knowledge of the role of specific protein in a certain signalling pathway, was unsuccessful in providing new insight on PCOS mechanisms or identifying potential biomarkers of the disease. PCOS is a heterogeneous disorder that involves different mechanisms, therefore, the shift from the previous hypothesis-driven approach towards hypothesis-generating approaches (*e.g.* genomics, metabolomics, and proteomics) prompted the identification of new candidate biomarkers whose participation in the pathogenesis of PCOS could not be suspected based on a previous knowledge [474].

Different proteomic platforms have been applied to biological fluids and tissues from patients with PCOS to discover altered proteins and new metabolic pathways associated with this complex disorder. A summary of selected studies that compared samples from women with PCOS with controls using different proteomic approaches is presented in Table 6-1. As it can be observed from the table, various samples have been utilised including serum, plasma, visceral adipose and ovarian tissue, ovarian granulosa cells and peripheral T lymphocytes. Although the study of ovarian tissues is more likely to identify differences that are more relevant to the pathogenesis of PCOS, tissues are generally difficult to obtain and analyse, and are not considered as a good biological sample for the discovery of biomarkers. Several studies used serum or plasma as these samples are clinically accessible, protein-rich and have a great potential for the discovery of potential PCOS biomarkers, as changes in the abundance of circulating proteins might reflect changes in organ function in women with PCOS.

Most of the proteomic studies applied gel-based techniques using mainly 2D-PAGE to allow for the efficient separation of proteins in the sample before the identification of the differentially altered proteins by MS using mainly MALDI-TOF.

The reported differentially expressed proteins in PCOS were found to participate in several biological processes including protein folding, carbohydrate and lipid metabolism, iron transport, regulation of fibrinolysis and thrombosis, and immune response and

inflammation. Many of these PCOS-relevant pathways are covered by the different glycoproteins monitored in the glycoprotein profiling methodology presented in this thesis and, therefore, the application of the developed method to PCOS may assist in identifying new potential PCOS biomarkers, and exploring different biochemical pathways that underline the disease.

In conclusion, PCOS is a heterogeneous prevalent endocrine disease that lessens the reproductive life of the affected woman and increases her risk of developing metabolic and cardiovascular diseases and pregnancy complications (*e.g.* pre-eclampsia) in the future. The current understanding of the underlying pathophysiology of PCOS is limited despite the conducted researches, and none of the reported proteomic studies have investigated plasma glycoproteins which are involved in many of the biochemical pathways that are related to the pathology of PCOS. Therefore, there is a need to explore plasma glycoproteins in PCOS to identify new candidate biomarkers and provide new PCOS insights which can improve the overall management of the disease.
Moreover, although the association between PCOS and the increased risk of developing pre-eclampsia is supported by the literature, there is still insufficient evidence of the factors that can attribute to the link between the two conditions, and no study has been performed were proteins altered in pre-eclampsia were investigated in women with PCOS. It can be hypothesised that the identification of glycoproteins altered in the plasma of the two disorders will possibly shed light and help in understanding the underlying pathophysiological mechanisms that link PCOS with pre-eclampsia.

Table 6-1: Summary of published studies comparing samples from women with polycystic ovary syndrome (PCOS) with healthy controls using proteomic approaches.

Biological sample	Sample size	Main proteins identified	Analytical method	Ref.
Plasma	PCOS= 12 Controls= 12	The levels of 4 proteins were differentially expressed, 2 were overexpressed (transferrin and κ-free light chain) and 2 were underexpressed (haptoglobin β-chain and α2-macroglobulin).	2D-DIGE, MALDI-TOF	[475]
Serum	PCOS with insulin resistance= 30 PCOS without insulin resistance= 30 Controls= 30	17 differential protein peaks were screened out compared with the controls. 16 of them were up-regulated, and one was down-regulated. 27 differential protein peaks were screened out compared with the controls. 17 of them were up-regulated, and 10 were down-regulated.	SELDI-TOF	[476]
Serum	PCOS= 12 Controls= 12	The levels of 4 proteins were differentially expressed, 3 were up-regulated (complement C4α3c and haptoglobin α- and β-chains) and one was down-regulated (complement C4γ).	2D-PAGE/RP-SPE, MALDI-TOF	[477]
Ovarian tissue	PCOS= 3 Controls= 3	The level of 69 proteins were altered in PCOS, 54 were up-regulated (including antithrombin III, fibrinogen, plasminogen related protein A) and 15 were down-regulated (including annexin A11, transgelin, macrophage capping protein).	2D-PAGE, MALDI-TOF-TOF	[478]
Ovarian granulosa cells	PCOS= 14 Controls= 11	15 protein spots were differentially expressed in PCOS patients, 5 were down-regulated (apolipoprotein A-I, annexin VI isoform 1, R-tubulin, chaperonin, protein disulphide isomerase A6) and 10 up-regulated (including *β*-tubulin, annexin 5, heat shock protein 90 kDa *β,* hypoxia upregulated 1 precursor). The down-regulated level of ApoA-1 was confirmed by Western blot.	2D-PAGE, LC-MS/MS	[479]
Visceral adipose tissue	PCOS= 10 Controls= 9	15 protein spots were differentially expressed in PCOS samples, 8 of them were identified by MS; 2 proteins were up-regulated (Annexin V, GSTM3) and 6 were down-regulated (albumin, ApoA-1, triosephosphate isomerase, actin, peroxiredoxin 2 isoform a, APMAP).	2D-DIGE, MALDI-TOF	[480]
Peripheral T cells	PCOS= 10 Controls= 10	The level of 11 proteins were altered in PCOS, 3 were up-regulated (superoxide dismutase, platelet basic protein, peroxiredoxin-1) and 8 were down-regulated (including cathepsin D, Alpha-enolase, cofilin-1, protein disulphide-isomerase A3).	2D-PAGE, MALDI-TOF	[481]

6.1.3 Aim of the chapter

1) To detect glycoproteins altered in PCOS compared to controls using the developed targeted LC-MS/MS glycoprotein profiling method, with a view to identify potential biomarkers of the disease.
2) To identify the underlying mechanisms that are associated with the pathogenesis of PCOS based on the biological function of the altered glycoproteins.
3) To identify differentially expressed glycoproteins common to both pre-eclampsia and PCOS which can reflect the underlying biochemical mechanisms that link the two diseases.

6.2 Materials and Methods

6.2.1 Materials

Blood samples were collected at the University Hospital Department of Obstetrics and Gynaecology at the Queen's Medical Centre Campus, Nottingham University Hospitals NHS Trust, Nottingham, after obtaining informed consent form. Ethics committee (Institutional Review Board) approval was obtained for this study from the UK East Midlands Health Research Authority, Research Ethics Committee (REC reference 10/H0408/69). All other materials are as described previously in Section 2.3.1, Chapter Two.

6.2.2 Patient Recruitment

Women with PCOS (n=25) and controls (n=25) aged between 18 and 40 years were included in this study. PCOS was diagnosed according to the Rotterdam consensus [445], and women with two or more of the following in the absence of other endocrine causes of oligo- and/or anovulation were included in the study: (1) oligo- and/or anovulation, (2) clinical and/or biochemical signs of hyperandrogenism and (3) polycystic ovary morphology on ultrasound scan.

Age-matched women with regular 21–35 day menstrual cycles, no evidence of PCOS on ultrasound, no evidence of hyperandrogenism and not currently using hormonal contraception were used as controls. Controls were identified from patients and female members of staff who volunteered to participate in the study.

Women with any of the following were excluded from the study: thyroid disease, current pregnancy, delivery or miscarriage occurring within the preceding 3 months, recent

surgery (within 3 months), history of myocardial infarction, use of aspirin or heparin, sex steroid therapy, a history of haematological disease, malignancy or liver disease, hyperprolactinaemia, a history of thrombosis and recent viral illness.

6.2.3 Sample collection and processing

Fasting blood samples were taken on any day of the menstrual cycles for the endocrine assays (testosterone, sex hormone binding globulin, luteinising hormone, follicle stimulating hormone, thyroid function tests, 17-hydroxyprogesterone, insulin, glucose, cholesterol, triglycerides and high-density lipoproteins) and glycoprotein profiling. Samples were collected in pre-chilled lithium heparin tubes and centrifuged within 30 min at 2000x *g* and 4ºC for 10 min. Plasma was separated and immediately stored at -80ºC until analysis.

6.2.4 Sample preparation for LC-MS/MS analysis

Glycoprotein extraction, digestion, profiling by the developed targeted LC-MS/MS method and data analysis to measure protein fold changes was performed as described with pre-eclampsia samples in Chapter Five.

6.2.5 Statistical Analysis

Descriptive subjects' characteristics of PCOS and control groups were compared using Student *t*-test for continuous parameters, or Pearson Chi-square for comparative analysis of categorical variables. A p-value ≤ 0.05 was considered statistically significant. Univariate and multivariate analyses were performed as described in Chapter Five.

6.3 Results

6.3.1 Demographic and clinical characteristics

The demographic and endocrine data characteristics of PCOS cases and controls included in the study are presented in Table 6-2.

There was no significant difference in the ages of both groups; however, women with PCOS had a significantly higher body mass index (BMI). Compared to controls, PCOS patients presented with significantly higher plasma testosterone, luteinising hormone (LH) and free androgen index, and lower level of sex hormone binding globulin (SHBG) as consistent with the diagnosis of PCOS.

No statistically significant differences between the two groups were observed in measurements including cholesterol and indexes of insulin resistance. Still, women with PCOS had higher triglycerides and lower high-density lipoprotein (HDL) levels compared to controls.

Table 6-2: Demographic and endocrine data comparing PCOS to controls.

Subject characteristics	**Controls (*n=25*)**	**PCOS (*n=25*)**	**p-value**
Ethnicity			0.392
- Caucasian	20 (80%)	16 (64%)	
- South Asian	3 (12%)	4 (16%)	
- Other	2 (8%)	3 (12%)	
Age (years)	29.1 (5.1)	29.8 (4)	0.60
BMI (kg/m^2)	26 (6.3)	31.2 (7.0)	0.008
LH (IU/L)	5 (2.1)	14.8 (16.6)	0.009
FSH (IU/L)	8.3 (3.2)	6.6 (3.8)	0.13
Testosterone (mmol/L)	1.2 (0.6)	1.9 (0.5)	<0.001
SHBG (nmol/L)	52.7 (26.1)	32.7 (13.6)	0.003
Free androgen index	3.6 (1.7)	7.0 (3.7)	<0.001
TSH (mlU/L)	2.0 (1.4)	1.8 (1.3)	0.551
FT4 (pmol/L)	15 (1.6)	15.2 (3.3)	0.775
17-OHP (nmol/L)	3.0 (2.1)	3.4 (1.5)	0.565
Fasting insulin (lU/mL)	6.4 (11.2)	9.5 (6.6)	0.273
Fasting glucose (mmol/L)	4.9 (0.6)	4.9 (0.5)	0.761
Insulin/glucose ratio	1.2 (1.6)	1.9 (1.3)	0.106
Cholesterol (mmol/L)	4.4 (0.8)	4.7 (1.1)	0.230
TG (mmol/L)	0.7 (0.2)	1.2 (0.5)	<0.001
HDL (mmol/L)	1.6 (0.3)	1.3 (0.3)	0.039

All data are presented as mean (SD) or number (% of total).
BMI: body mass index, SHBG: sex hormone binding globulin, FAI: free androgen index, LH: luteinising hormone, FSH: follicle stimulating hormone, TSH: thyroid stimulating hormone, FT4: free thyroxine, 17-OHP: 17-hydroxy progesterone, TG: triglycerides, HDL: high-density lipoproteins.

6.3.2 Analysis of PCOS and age-matched control samples by the developed glycoprotein profiling method

6.3.2.1 Univariate analysis

Univariate pairwise comparisons (with p-value adjustment) between women with PCOS and control samples revealed 16 glycoproteins (out of 54 glycoproteins) significantly altered in the plasma of women with PCOS compared to controls (Table 6-3 and Figure 6-2). Plasma protein fold changes varied between the differentially expressed glycoproteins and ranged from 1.3 to 1.7. All of the altered glycoproteins showed an increase in their plasma level in women with PCOS compared to controls except for apolipoprotein A-I (Apo A-I) and apolipoprotein D (Apo D) which displayed 1.5 and 1.4 fold decrease, respectively in their level in the plasma of affected women. Fibrinogen γ-chain, involved in the coagulation process, and complement C3, involved in immune responses, showed the highest increase in their plasma level in women with PCOS compared to controls and were detected with 1.8 and 1.7 fold changes respectively. Among the other up-regulated glycoproteins were ceruloplasmin, insulin-like growth factor-binding protein complex acid labile subunit (IGFALS), fibrinogen α-chain, plasminogen and vitronectin.

The changed glycoproteins in PCOS were mainly high to moderately abundant plasma proteins (*e.g.* fibrinogen, complement C3, Apo A-I), and were involved in a range of different biological pathways. Two of the differentially expressed glycoproteins, vitronectin and IGFALS are reported for the first time to be altered in the plasma of women with PCOS suggesting that they have the potential to be evaluated as new biomarkers.

With the exception of inter-α-trypsin inhibitor heavy chain 1 and kallistatin, each of the remaining 14 glycoproteins altered in the plasma of PCOS women showed functional interactions with at least one other glycoprotein (Figure 6-3) when examined using STRING (an online functional protein interaction network; http://string-db.org/), which pointed to the involvement of the glycoprotein in more than one pathway. Moreover, strong associations were clearly observed between certain glycoproteins such as fibrinogen α and γ chains; vitronectin and plasminogen; Apo A-I and Apo D; and transferrin and ceruloplasmin, indicating that they may be involved in a common pathway, which rationalises how the changes in the plasma level of these glycoproteins may relate to one another.

Table 6-3: Glycoprotein fold changes measured between the plasma samples form women with PCOS and matched controls using the developed targeted LC-MS/MS method. Glycoproteins are presented in alphabetical order.

No.	Targeted proteins	Fold changes	No.	Targeted proteins	Fold changes
1	α-1-Acidglycoprotein	**1.4***	28	Corticosteroid binding globulin	1.2
2	α-1-Antichymotrypsin	1.1	29	Fibrinogen α-chain	**1.5***
3	α-1-Antitrypsin	1.0	30	Fibrinogen β-chain	1.2
4	α-1-Antiplasmin	1.1	31	Fibrinogen γ-chain	**1.8***
5	α-1-Betaglycoprotein	1.1	32	Fibronectin	1.1
6	α-2-HS glycoprotein	-1.1	33	Haptoglobin β chain	**1.4***
7	α-2-Macroglobulin	-1.2	34	Hemopexin	1.1
8	β 2-Gglycoprotein 1	-1.1	35	Heparin cofactor II	1.2
9	Afamin	**1.3***	36	Histidin rich glycoprotein	1.2
10	Angiotensinogen	1.2	37	IGFALS	**1.4***
11	Antithrombin-III	1.2	38	ITIH1	**1.3***
12	Apolipoprotein A-I	**-1.5***	39	Kallikrein	1.1
13	Apolipoprotein A-II	-1.2	40	Kallistatin	**1.3***
14	Apolipoprotein D	**-1.4***	41	Kininogen	1.0
15	C4-binding protein α chain	1.2	42	Lumican	-1.1
16	CD5 antigin like	-1.1	43	N-Acetylmuramoyl-L-alanine amidase	1.2
17	Ceruloplasmin	**1.5***	44	Paraoxonase/arylesterase 1	1.1
18	Clusterin	1.2	45	Pigment epithelium derived factor	1.1
19	Complement C3	**1.7***	46	Plasma protease C1 inhibitor	1.2
20	Complement C4 β- chain	1.2	47	Plasminogen	**1.4***
21	Complement C5	1.2	48	Protein AMBP	-1.3
22	Complement C6	1.0	49	Prothrombin	1.1
23	Complement C8 α-chain	1.1	50	Serum-amyloid P-component	1.2
24	Complement C9	-1.2	51	Thyroxin-binding globulin	1.1
25	Complement factor B	**1.3***	52	Transferrin	**1.4***
26	Complement factor H	-1.0	53	Vitronectin	**1.4***
27	Complement factor I	1.1	54	Zinc-α-2-glycoprotein	-1.1

*(bold) Significant changes in protein levels between PCOS and controls ($p \leq 0.5$ after FDR correction).
IGFALS refers to insulin-like growth factor-binding protein complex acid labile subunit, and ITIH1 refers to inter-α-trypsin inhibitor heavy chain 1.

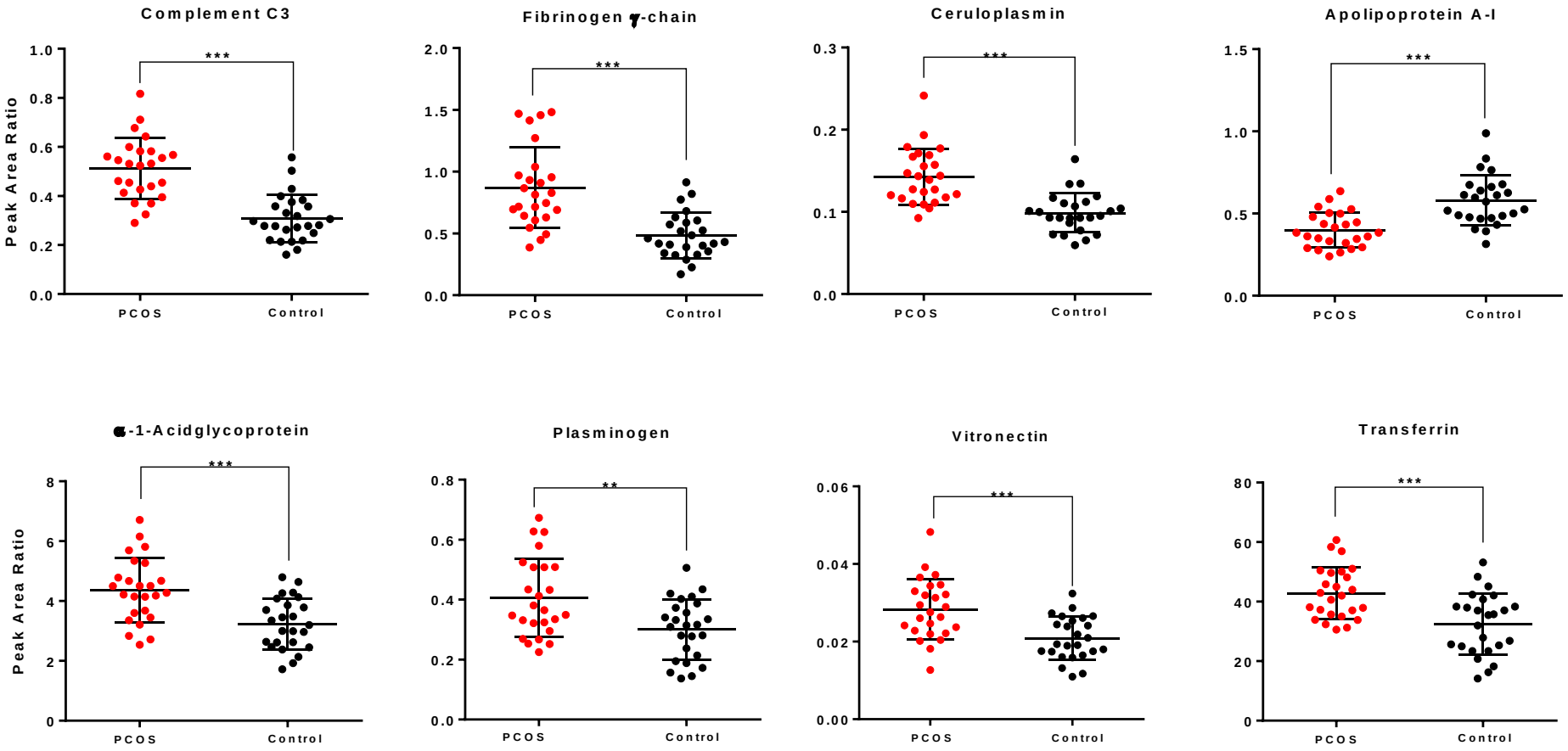


Figure 6-2: Glycoproteins that showed the highest change in their abundance between PCOS plasma samples and matched controls. Samples were analysed by the developed label-free targeted LC-MS/MS method. Error bars represent the mean ± SD of the ratio of the analyte signal area over the internal standard signal area. The levels of all glycoproteins significantly increased except for apolipoprotein A-I that showed a significant decrease in its level. ** p<0.05, ** p<0.01, ***P<0.001.*

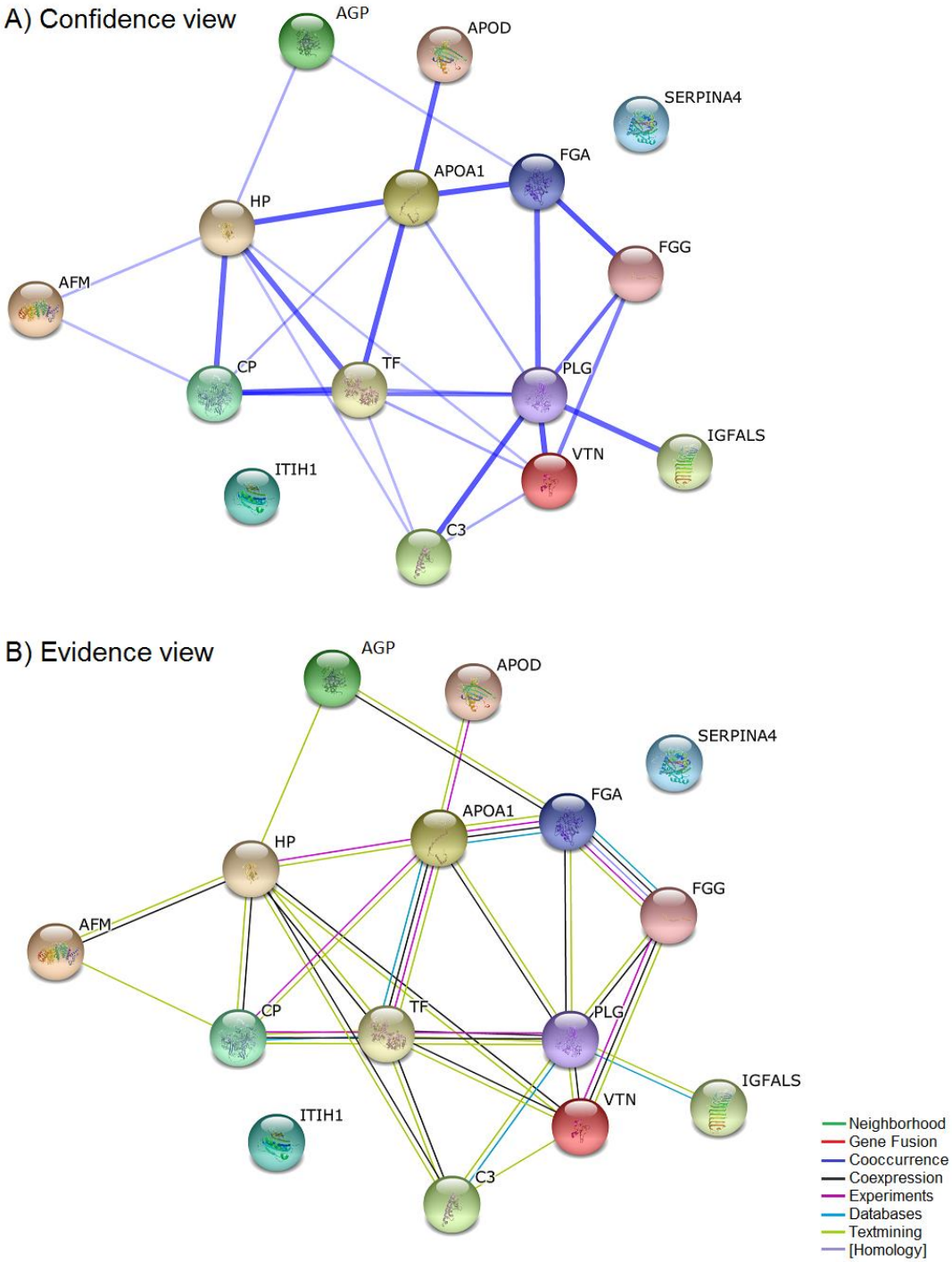


Figure 6-3: The functional interactions between glycoproteins differentially expressed in the plasma of PCOS women compared to controls detected using STRING. A) Confidence view; stronger associations are represented by thicker lines. B) Evidence view; different line colours represent the types of evidence for the association. Predicted functional links consist of up to eight lines; one colour for each type of evidence.
AGP: α-1-acidglycoprotein, AFM: afamin, APOA1: apolipoprotein A-1, APOD: apolipoprotein D, C3: complement C3, CP: ceruloplasmin, FGA: fibrinogen α-chain, FGG: fibrinogen γ-chain, HP: haptoglobin, IGFALS: insulin-like growth factor-binding protein complex acid labile subunit, ITIH1: inter-α-trypsin inhibitor heavy chain 1, PLG: plasminogen, SERPINA4: kallistatin, TR: transferrin and VTN: vitronectin.

6.3.2.2 Multivariate analysis

The performance of multivariate analysis on data derived from targeted protein analysis was investigated, and the results obtained from it were compared with univariate statistical analysis to check the suitability of multivariate analysis to be used when a limited number of variables are included (*c*. 55).

When multivariate analysis was performed to identify glycoproteins that were altered in plasma samples from women with PCOS compared with controls, clear separation and clustering of the two groups were achieved with the OPLS-DA model (Figure 6-4), and yielded a satisfactory fitness of the model (R^2Y=0.89) and predictive ability value (Q^2=0.727). Extraction of glycoproteins with a variable of projection (VIP) score >1 from the OPLS-DA model revealed 19 out of 54 glycoproteins contributed significantly to the separation noticed between PCOS and control samples (Table 6-4). All of the 19 glycoproteins identified by multivariate analysis showed significant differences between women with PCOS and controls in the univariate analysis except for heparin cofactor II, N-acetylmuramoyl-L-alanine amidase and complement C9. Complement C3 showed the highest VIP score (1.64) followed by ceruloplasmin (VIP=1.59) and fibrinogen γ-chain (VIP=1.57). The previous three glycoproteins also had the highest significant changes in their abundance when analysed using the targeted univariate analysis (Table 6-3) which demonstrated the strength of the created model and highlighted a good agreement between the univariate and multivariate results.

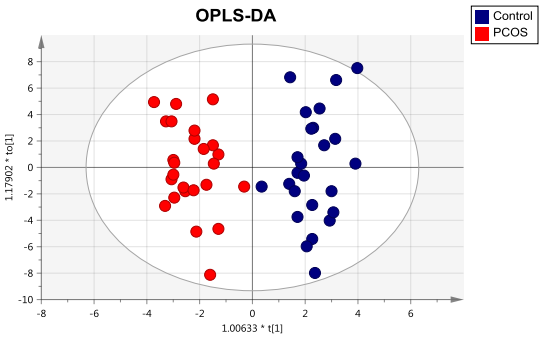


Figure 6-4: OPLS-DA score plot of PCOS samples (n=25) and their controls (n=25). PCOS and control samples are presented in red and blue respectively.

Table 6-4: Glycoproteins extracted from the OPLS-DA model with VIP score above 1.

No.	Glycoprotein	VIP score
1	Complement C3	1.64
2	Ceruloplasmin	1.59
3	Fibrinogen γ-chain	1.57
4	Apolipoprotein A-I	1.51
5	IGFALS	1.37
6	Transferrin	1.34
7	Vitronectin	1.33
8	α-1-Acidglycoprotein	1.27
9	Afamin	1.26
10	Complement factor B	1.25
11	Apolipoprotein D	1.22
12	Plasminogen	1.22
13	Inter-α-trypsin inhibitor heavy chain 1	1.16
14	Haptoglobin β chain	1.15
15	Kallistatin	1.14
16	Heparin cofactor II	1.07
17	N-Acetylmuramoyl-L-alanine amidase	1.03
18	Complement C9	1.02
19	Fibrinogen α-chain	1.02

IGFALS refers to insulin-like growth factor-binding protein complex acid labile subunit.

6.3.3 Plasma glycoproteins altered in both PCOS and pre-eclampsia

The 25 differentially expressed glycoproteins detected in the plasma of pre-eclamptic women (Chapter Five) were compared with the glycoproteins altered in the plasma of women with PCOS to identify glycoproteins with significant changes in their plasma level in both conditions.

The plasma level of seven glycoproteins was found to be changed, both in women with pre-eclampsia and PCOS, compared to their respective matched controls. Using the developed targeted LC-MS/MS glycoprotein profiling method, afamin, α-1-acidglycoprotein, haptoglobin β-chain, plasminogen, transferrin and vitronectin were all significantly increased in the plasma of pre-eclamptic women and women with PCOS, whereas the plasma level of Apo D was increased in pre-eclampsia and decreased in women with PCOS. The detected changes were more pronounced with pre-eclampsia and the above glycoproteins showed between 1.5 and 1.9 fold changes in their plasma

level compared to gestational age-matched controls, whilst the changes in the plasma level of the same proteins were between 1.3 and 1.4 fold changes in women with PCOS compared to controls.

Examining the interactions between the above seven glycoproteins using STRING highlighted a functional association between all the glycoproteins with the exception of Apo D which was not connected to any of the other altered glycoproteins as observed in Figure 6-5. The association was strong between plasminogen and vitronectin (involved in coagulation/fibrinolysis pathway), and between haptoglobin and transferrin (involved in iron transport and metabolism) suggesting that the changes in the plasma level of these glycoproteins are related to underlying biological mechanisms that might be common to both diseases.

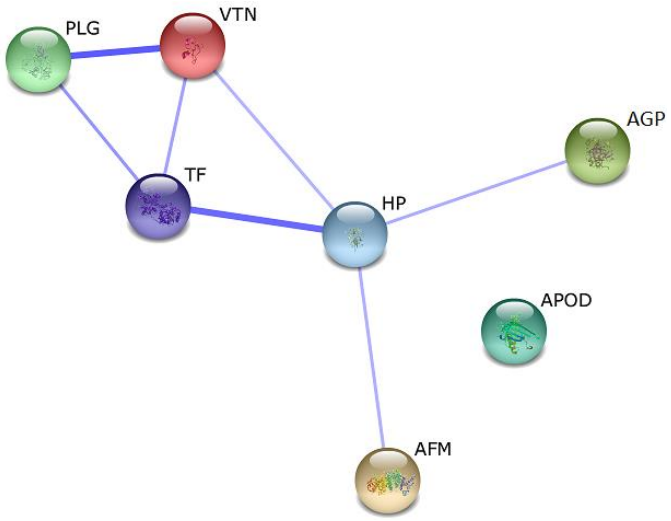


Figure 6-5: Protein interactions network of the seven glycoproteins altered in the plasma of both pre-eclampsia and PCOS from STRING software (confidence view). Stronger associations are represented by thicker lines, the types of evidence for the association included co-expression and textmining.
AGP: α-1-acidglycoprotein, AFM: afamin, APOD: apolipoprotein D, HP: haptoglobin, PLG: plasminogen, TR: transferrin and VTN: vitronectin.

6.4 Discussion

This is the first study that has focused on detecting changes in the level of plasma glycoproteins in women with PCOS compared with controls. The study identified 16 glycoproteins (out of 54) present in different abundances in women with PCOS compared to controls, two of them, vitronectin and insulin growth factor acid labile subunit (IGFALS), were identified for the first time to be changed in the plasma of women with PCOS. The altered glycoproteins are involved in different biological processes including lipid and iron transport, coagulation/fibrinolysis, inflammation, and insulin growth hormone system, which is consistent with the well-accepted and reported heterogeneous and complex nature of the disease, and in agreement with previous proteome data from the plasma of women with PCOS [474]. Although analysis of ovarian tissue samples from women with PCOS may reflect more accurately the underlying biochemical pathways and mechanisms of the disease, the invasive measures necessary to obtain tissue samples and the challenging sample processing make other more accessible biofluid, such as plasma, a more attractive option for the discovery of potential disease biomarkers [482]. Moreover, the interaction between the ovary endothelium and systemic pathways is expected to be reflected in plasma proteins which in turn can provide insights into the disease pathogenesis, and enhance the understanding of the pathways associated with PCOS.

In the following section, the functional significance of the identified changed glycoproteins in this study will be discussed.

6.4.1 The biochemical significance of the changed glycoproteins in PCOS

6.4.1.1 Glycoproteins involved in lipid and haem transport

Lipid metabolism and transport

The plasma levels of apolipoprotein A-I (Apo A-I) and apolipoprotein D (Apo D) were decreased in women with PCOS compared to controls. Apolipoproteins bind to lipid to form lipoproteins whose main function is to transport lipid through the circulation. Apolipoproteins, play a key role in maintaining the structural integrity of the lipoproteins and regulating lipid metabolism [483]. Apo A-I is the major component of the high-density lipoprotein (HDL) and participates in cholesterol metabolism by extracting free cholesterol from peripheral tissues, thus exerting a cardiovascular protective effect by preventing lipid accumulation in arterial walls. It has different biological functions that

include anti-inflammatory effects, inhibiting low-density lipoprotein (LDL) oxidation and scavenging toxic phospholipids, and improving endothelial health [483]. Apo D is present mainly in HDL and to a lower extent in LDL and is associated with anti-oxidation and anti-stress activities [412]. The lower plasma level of Apo A-I and Apo D detected herein in PCOS group compared to controls resulted in a significant decrease in the circulating HDL levels in PCOS patients as shown in Table 6-2. This suggests that lower circulating levels of Apo A-I and Apo D, particularly Apo A-I, might be possible factors for the increased risk of developing cardiovascular diseases in women with PCOS patients [484; 485; 486]. Lower circulating level of HDL in PCOS women compared to controls is one of the most common abnormalities observed in the lipid profile of PCOS patients, and has been previously reported in both obese [487] and lean women with PCOS [488].

In agreement with the decreased level of Apo A-I detected in this study, in a large cohort, Valkenburg *et al*. [486], reported a significantly lower plasma Apo A-I level in PCOS women (n=557) compared to controls (n=295) across the entire range of BMI when analysis was performed by immunoturbidimetric assay. Previous LC-MS/MS proteomic studies have also detected a decreased abundance of Apo A-I in visceral adipose tissue [480], ovarian tissue [478] and granulosa cells [479] samples obtained from women with PCOS, which indicates the important role Apo A-I plays in the pathology of the disease. However, a previous study has reported that the disturbances in the lipoproteins level can be associated more with the insulin abnormalities linked with PCOS than with endogenous androgens [489].

Iron transport

The increased plasma abundance of transferrin, ceruloplasmin and haptoglobin detected in women with PCOS compared to controls reflected mainly increased plasma iron level in PCOS, as the three glycoproteins are involved in iron transport and metabolism particularly, transferrin, which is the major iron transport protein in the blood. Ceruloplasmin assists in iron transport by oxidising Fe^{+2} into Fe^{+3} while haptoglobin forms soluble complexes with haemoglobin following haemolysis and liberation of free iron into the circulation [490]. Women with PCOS can present with iron overload as a consequence of reduced menstrual losses due to the oligomenorrhea and/or amenorrhea characteristics of the condition. Moreover, androgen excess, and/or PCOS associated abdominal adiposity, and/or insulin resistance can lead to a decrease in the serum concentrations of hepcidin, the main negative regulator of iron absorption that

lowers the plasma level of iron by decreasing its absorption in the small intestine and inhibiting its release from the macrophages [490; 491].

Increased plasma levels of transferrin, ceruloplasmin and haptoglobin have been reported before in PCOS. Insenser *et al*. [475] detected an increase in the plasma level of transferrin in patients with PCOS using an untargeted proteomic approach, and recently a higher level of ceruloplasmin in the serum of PCOS women has been reported [492], while two separate previous proteomic studies confirmed the involvement of haptoglobin in the pathogenesis of PCOS. The first study has reported a decreased plasma level of the two haptoglobin β-chain isoforms in PCOS and suggested that post-translational modifications of haptoglobin might contribute to haptoglobin dysfunction in PCOS [475], whilst the second study agreed with the presented findings and has reported an increased serum level of haptoglobin α and β chain in women with PCOS [477].

6.4.1.2 Glycoproteins related to coagulation/fibrinolysis pathway

Higher circulating levels of glycoproteins involved in the regulation of the fibrinolysis/coagulation processes were detected in the plasma of women with PCOS compared to controls in the present study, including fibrinogen α and γ, plasminogen and vitronectin. Fibrinogen is mainly synthesised by the hepatocytes upon the activation of the haemostatic system in response to inflammatory cytokines [493] and endothelium damage [494] to initiate fibrin formation and blood clotting. Plasminogen is converted by plasminogen activator to plasmin which in turn breaks down the formed fibrin in a process known as fibrinolysis to prevent hypercoagulability (Figure 6-6). Vitronectin plays a key role in haemostasis through its involvement in promoting cell adhesion and platelet aggregation. However, the main role of plasma vitronectin in regard to haemostasis might be its ability to bind, stabilise and activate plasminogen activator inhibitor (PAI-1); the major physiological inhibitor of tissue-type plasminogen activator (tPA) and the principal inhibitor of fibrinolysis which leads to enhanced thrombus formation [494; 495; 496] as illustrated in Figure 6-6.

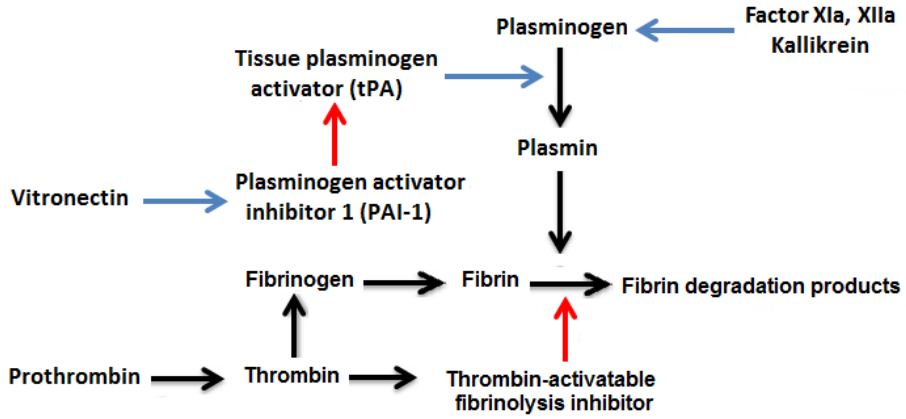


Figure 6-6: Simplified fibrinolysis process. Plasminogen is converted to plasmin by plasminogen activator. Plasmin in turn enhances the degradation of fibrin formed by the action of thrombin on fibrinogen to prevent blood clotting. Vitronectin activates plasminogen activator inhibitor which inhibits plasminogen activator and subsequently inhibits the conversion of plasminogen to plasmin which results in reduced fibrinolysis.
Blue arrows denote activation while red arrows denote inhibition.

The higher plasma levels of fibrinogen, plasminogen and vitronectin detected herein reflected mainly disturbances in the haemostatic system in women with PCOS, and a shift of the coagulation/fibrinolysis balance towards hypercoagulability state. Although not measured in this study, increased activity of circulating PAI-1 in women with PCOS has been detected by different previous studies [497; 498; 499]. Based on the findings of the current study it can be suggested that the higher plasma level of vitronectin in PCOS can contribute to the increased activity of PAI-1 reported in PCOS studies which will limit plasmin formation leading to increased level of plasminogen and decreased fibrinolytic response. This agrees with previous studies that have also reported hypofibrinolysis and abnormal fibrogenesis and thrombosis in PCOS [500; 501].

Fibrinogen is one of the most commonly studied fibrinolysis/coagulation markers in PCOS [478; 497; 502; 503], and previous studies have reported conflicting data about its plasma level in women with PCOS. Consistent with this study, a higher level of fibrinogen has been reported in the plasma of women with PCOS using different biochemical analysis tests such as coagulation analyser to measure its level [497; 502]. Moreover, an increased level of fibrinogen has also been detected in ovarian tissue in a previous proteomic study [478], which supports the close involvement of fibrinolysis/coagulation pathway in the underlying pathology of the disease and proves that protein changes in ovarian tissues can be reflected in the circulating plasma protein. However, other studies have reported a comparable plasma fibrinogen level between

PCOS and BMI-matched control groups [504; 505]. Although the higher fibrinogen level detected in PCOS in the present study may be explained by the greater BMI of women in the PCOS group compared to controls (Table 6-2), which can influence fibrinogen level, regression analysis showed higher correlation of plasma fibrinogen level with PCOS (R= 0.60) than with BMI (R= 0.37). Additionally, Manneras-Holm *et al.* [497] reported that higher fibrinogen level in PCOS group (n=74) compared to controls (n=31) remained significant even after adjustment for BMI.

With regards to plasminogen and vitronectin, plasminogen has been reported previously to be increased in the ovarian tissues of women with PCOS [478], whilst this appears to be the first study that reported a higher level of vitronectin in women with PCOS. This points toward the possibility of a new potential PCOS biomarker.

The current literature supports the increased risk of developing cardiovascular disease in women with PCOS [506; 507]; however, the mechanism underlying this association is not well-understood. Hyperglycemia, insulin resistance and dyslipidemia associated with PCOS have been suggested as potential factors for this association. The findings of the current work indicated that impaired coagulation/fibrinolytic pathway could also contribute to the development of cardiovascular diseases in PCOS as suppression of the fibrinolysis will lead to a hypercoagulability state that can increase the risk of having ischemic heart disease and a myocardial infarction in women with PCOS.

6.4.1.3 Glycoproteins related to complement pathway and inflammation

The presented study revealed a significant increase in the plasma level of acute phase proteins including α-1 acid glycoprotein, haptoglobin, ceruloplasmin, and the inflammatory cytokine marker complement C3, in patients with PCOS compared to controls, indicating an inflammatory state associated with PCOS. Chronic low-grade inflammation has emerged as a potential contributor to the pathogenesis of PCOS [508; 509; 510] which is supported by the current findings.

Complement C3 (C3) is a key protein of the innate immune system and the convergence point of the three complement pathways; classical, lectin and alternative, that elicit an inflammatory response [511]. Evidence is conflicting with respect to the association of C3 with PCOS as some researchers have reported elevated circulating C3 levels while others have not seen a significant difference in its level in women with PCOS. Consistent with the higher level of C3 detected herein, Yang *et al*. [512] observed a higher serum

C3 level in PCOS compared to age- and BMI-matched controls. Additionally, higher serum C3 in PCOS was also detected by Cheng *et al*. [511] using the rate nephelometry method [513]. On the other hand, Wu *et al.* [514] and Snyder *et al.* [511], both reported that C3 levels were elevated in women with PCOS compared to controls, however, this increase was not significant but was correlated with lipid parameters [514], traditional cardiovascular diseases risk factors and coronary artery calcium [511]. The authors concluded an increased risk of dyslipidemia and developing cardiovascular diseases in PCOS patients [514].

Elevated C3 level has been reported in adults with cardiovascular diseases (*e.g.* coronary heart disease) and has shown a significant correlation with their risk factors [515], Moreover, high C3 level was associated with tissue damage at the site of myocardial infarctions in a previous study [516]. Based on the well-established association of PCOS with the increased risk of developing cardiovascular diseases, the findings of this study suggest that C3 may be an inflammatory cardiovascular disease risk marker among women with PCOS, and that its higher plasma level in women with PCOS may prove beneficial in identifying PCOS women at higher risk of developing cardiovascular diseases.

6.4.1.4 Glycoproteins related to insulin resistance

Higher plasma level of insulin growth factor binding protein acid labile subunit (IGFALS) was detected in women with PCOS compared to controls. Production of IGFALS is stimulated by the growth hormone in order to bind to insulin-like growth factors (IGF-I and IGF-II) to increase their half-life and their vascular localisation [517].

Almost all IGF-1 circulates as a ternary complex composed of IGF-I, insulin-like growth factor binding protein (IGFBP-3 or IGFBP-1) and IGFALS, and so very little free IGF is detected in the circulation [518]. IGFALS has no direct IGF binding activity and appears to bind only to the IGF/IGFBP binary complex [519; 520]. This insulin growth factor (IGF) system has an important pathophysiological role across the spectrum of obesity, insulin resistance, and type 2 diabetes mellitus [517]. In PCOS, abnormalities in the IGF system (low circulating level of IGFBP and increased level of free IGF) as a result of stimulating growth hormone or insulin resistance, have been reported by several studies [521; 522; 523], and have been linked to the pathology of the disease. Abnormalities in the IGF system can lead to increase androgen synthesis in the ovary and, thus, contribute to the

hyperandrogenism state observed clinically in PCOS [522; 524; 525], as noted in Figure 6-7.

Increased circulating level of IGF and decreased level of IGFBP in PCOS have been reported in the literature [521; 522; 523]; however, no work has been conducted on the role of the third component of this ternary complex, IGFALS, in PCOS so far. Based on the finding of the previous studies, it is expected that the higher level of IGFALS, detected for the first time in the current work, reflected lower formation of the IGF/IGFBP binary complex due to lower IGFBP, which could contribute to the higher circulating level of free IGF-1 in PCOS reported previously [521; 522; 523]. This seems a plausible explanation given that patients with PCOS included in this study presented with a higher level of insulin (Table 6-2) which can contribute to abnormalities in IGF system.

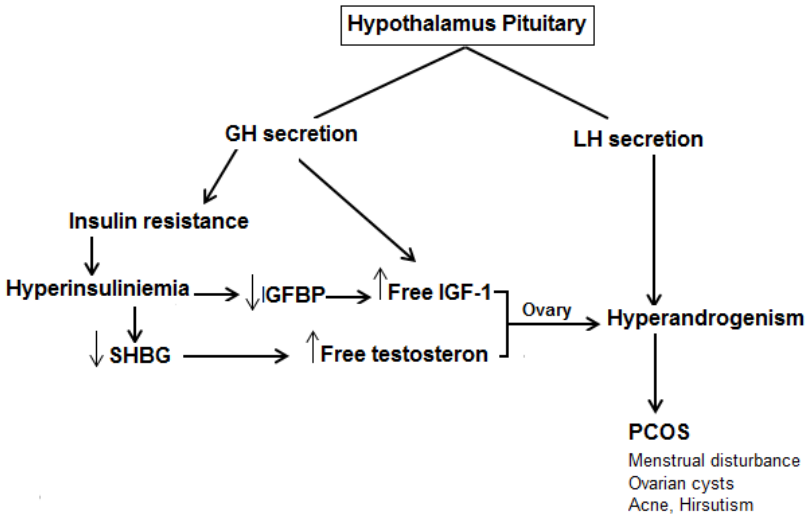


Figure 6-7: Pathways linking hyperinsulinemia and hyperandrogenemia as suggested by previous studies. Insulin or/ and increased level of LH can enhance ovarian androgen production. High level of insulin can increase androgen levels by reducing hepatic production of IGFBP and SHBG and thus elevates free IGF-I and testosterone levels.
GH: growth hormone, IGF-I: insulin-like growth factor I, IGFBP: insulin growth factor binding protein, LH, luteinising hormone, SHBG: sex hormone–binding globulin. Figure 6-7 is modified from reference [526].

6.4.2 Overlap of proteomics biomarkers between women with pre-eclampsia and PCOS

Seven common glycoproteins (afamin, Apo D, α-1-acidglycoprotein, haptoglobin β chain, plasminogen, transferrin and vitronectin) were altered in the plasma of women with pre-eclampsia and PCOS when compared with their respective controls in the presented work, which may assist in explaining the association between the two conditions. As observed in Figure 6-5, among the seven glycoproteins the strong functional interactions

between plasminogen and vitronectin, and transferrin and haptoglobin, suggest that they are involved in similar underlying biological mechanisms that might be common to both diseases.

Both vitronectin and plasminogen are central in regulating the fibrinolysis process, and their up-regulation, observed herein in both conditions, tilt the coagulation/fibrinolysis balance towards hypofibrinolysis state (discussed in Section 6.4.1.2 of this chapter) which agrees with the previously reported abnormal fibrogenesis and thrombosis in pre-eclampsia and PCOS [527]. Moreover, Glueck *et al.* [527] have reported that high plasminogen activator inhibitor activity, a protein activated by vitronectin and which inhibits fibrinolysis, can contribute to the placental alterations that occur in pre-eclampsia and recurrent miscarriages. Consequently, it can be suggested that the impaired expression of vitronectin and plasminogen in PCOS and the resulting hypofibrinolysis and thrombophilia may be a possible factor for the increased risk of spontaneous abortions (miscarriage) and developing pregnancy complications, such as pre-eclampsia, among women with PCOS [446; 469; 470].

In this study, two acute phase proteins involved in iron transport and metabolism, (transferrin and haptoglobin), showed a higher plasma level in both pre-eclampsia and PCOS compared to controls, which could be explained on the basis of the iron overload and the inflammatory constituent of the two conditions [490; 528]. As discussed earlier (Section 6.4.1.1), women with PCOS normally present with increased plasma level of iron as a consequence of reduced menstrual losses due to oligomenorrhea and/or amenorrhea characteristic of the condition. Whereas in pre-eclampsia injured red blood cells from ischemic placental tissue, and the resulting oxidative stress, may be a primary source of potentially toxic iron through the release of haemoglobin or haem into the circulation [528]. Based on the results of this work, it can be speculated that the high plasma level of iron in women with PCOS might be a factor in increasing their risk of having pre-eclampsia. As iron species act as catalytic transition metals, their high level can increase the chance of generating reactive oxygen species that likely exacerbate lipid peroxidation and promote damage to the vascular endothelium [528]. This in turn can contribute to the aetiology of pre-eclampsia.

It would appear that this is the first work that has studied the association between PCOS and pre-eclampsia through the identification of common proteins altered in the plasma of pre-eclamptic women and women with PCOS, using targeted LC-MS/MS workflow.

Several previous studies identified potential protein biomarkers in pregnant women with pre-eclampsia and in women with PCOS but they were studied as separate entities (Selected examples are presented in Table 5-1, Chapter 5 and Table 6-1 Chapter 6). Plasminogen, transferrin and haptoglobin have previously been reported to be involved in both diseases in separate PCOS and pre-eclampsia studies, whilst vitronectin has been reported to be increased in the plasma of pre-eclamptic women previously [375; 378; 383; 475; 477], but its higher level in the plasma of PCOS patients was identified for the first time herein. However, it is noteworthy that a recent systematic review of the overlap between altered proteins in PCOS and pre-eclampsia proteomic studies revealed the up-regulation of transferrin in both conditions [529].

The raised plasma levels of vitronectin, plasminogen, transferrin and haptoglobin detected by the current work in pre-eclamptic women and women with PCOS are of interest as these proteins could be potential biomarker candidates, used to identify women with PCOS who are at risk of developing pre-eclampsia in pregnancy. However, they need to be further evaluated in a larger longitudinal study before being used diagnostically.

6.5 Conclusion

This work constitutes one of the few proteomic studies reported in PCOS, as well as the first analysis to focus on plasma glycoproteins in PCOS. The study identified 16 glycoproteins with a significantly altered level in the plasma of women with PCOS compared to controls. Two of these altered proteins, vitronectin and insulin growth factor acid labile subunit, were identified for the first time suggesting that they could be further evaluated as new potential PCOS biomarkers. The overlap of the remaining differentially expressed proteins with the reported literature demonstrated the applicability of the method for the identification and verification of disease altered proteins using a simple, cost-effective, and conventional LC-MS/MS workflow.

The study identified a range of specific biological processes and pathways including inflammation, lipid and iron transport and metabolism, insulin growth factor system, and coagulation/fibrinolysis process that were impaired in PCOS. The alteration of these pathways pointed to a higher inflammatory state, iron overload, hypofibrinolysis and disturbances in the insulin growth factor system among women with PCOS compared to controls. This complex pattern of changes indicates a synergism between pathways

involved in the pathogenesis of PCOS and emphasises the heterogeneous nature of the condition. Moreover, the study identified several factors that could contribute to the increased risk of developing cardiovascular diseases among women with PCOS, supporting the general agreement that PCOS is associated with cardiovascular diseases. This included the lower circulating level of Apo A-I which reflected the lower level of the cardiovascular protective lipoprotein HDL, and impaired fibrinolysis process which can increase the risk of ischemic heart disease in women with PCOS.

The presented work identified, for the first time, seven glycoproteins (afamin, Apo D, α-1-acidglycoprotein, haptoglobin β chain, plasminogen, transferrin and vitronectin) altered in the plasma of both pre-eclamptic women and women with PCOS compared to controls. The altered glycoproteins shed light on possible molecular mechanisms including hypofibrinolysis and thrombophilia, and iron overload and the subsequent generation of oxygen species that can explain the previously reported increased risk of developing pre-eclampsia amongst women with PCOS.

The potential diagnostic value of the changed glycoproteins, particularly the two potential novel PCOS biomarkers and the common glycoproteins altered in PCOS and pre-eclampsia, remain to be further evaluated to address their potential clinical usefulness in the early prediction of PCOS, and to identify PCOS women at higher risk of developing pre-eclampsia. The presented results point to altered biological processes in PCOS and suggest possible pathophysiological mechanisms linking PCOS and pre-eclampsia which should serve as a foundational cornerstone for future work.

Chapter Seven

Conclusions and Future Work

7 Conclusions and Future Work

7.1 Conclusions

Pre-eclampsia is a pregnancy-specific syndrome of hypertension and proteinuria that substantially affects maternal and neonatal morbidity and mortality. Despite decades of research, the underlying pathogenic mechanisms of the disorder are not well understood, but are believed to be related to placental abnormalities such as placental ischemia that leads to the generation and the release of reactive oxygen species from the placenta into the maternal circulation. The clinical management of pre-eclampsia is hampered by the lack of reliable diagnostic tests and effective therapy, and the majority of biomarkers investigated for the prediction of the disease do not possess the sufficient specificity and sensitivity [139]. It is evident, therefore, that methods which could identify potential pre-eclampsia biomarkers and increase the understanding of the biochemical mechanisms associated with the condition, would have the potential to be of enormous clinical benefit. Pre-eclampsia is an asymptomatic multifactorial condition, so it is very unlikely that a single or a small group of biomarkers will accurately predict the disease.

In this thesis, two separate targeted LC-MS/MS methods were developed and validated to quantify plasma glycoproteins in pre-eclampsia to increase the pool of pre-eclampsia biomarker candidates and explore new pathways associated with the disease. The first method was hypothesis-driven and aimed to quantify the oxidation level of the plasma protein angiotensinogen (AGT); a protein that has been proposed to be involved in the increased blood pressure characteristic of pre-eclampsia through the change in its redox state, directed towards the more active oxidised form. The second method was hypothesis-generating and aimed to detect protein fold changes between different disease conditions using a simple and cost-effective conventional LC-MS/MS workflow that targeted 54 clinically relevant plasma glycoproteins.

7.1.1 Method development for plasma AGT and glycoprotein analysis

One of the main challenges facing plasma protein analysis is the huge complexity of the plasma, and the wide dynamic range of protein concentrations exceeding ten orders of magnitude. Therefore, depletion of the high abundant proteins is required to improve the detection and the quantification of proteins with lower abundance. Most of the methods reported in the literature rely on the use of immunoaffinity depletion columns or extensive

RP-HPLC fractionation to reduce the complexity of the sample. In this thesis, a simple and reproducible protocol for the efficient glycoprotein/AGT extraction was developed by coupling ConA lectin affinity chromatography with RP-SPE fractionation. This novel two-step extraction method was shown to have advantages over immunoaffinity columns typically used in MS-based protein analyses. It avoided the depletion of moderately abundant glycoproteins that might also be a source of potential disease biomarkers, had a high throughput (in minutes, instead of hours with immunoaffinity columns), and it was not limited by the capacity, sample number or the high-cost as with immunodepletion columns. Moreover, it did not require specialised equipment like HPLC and, consequently, could be easily transferred to other laboratories. Similar to other depletion tools, the collected RP-SPE fractions are compatible with down-stream analysis by gel electrophoresis and MS methods. One limitation of the current format of the developed method is its limited ability to measure and identify proteins of low abundance (low ng/mL concentration), some of which may have a diagnostic potential [262; 266].

Regarding MS analysis run time, both methods (AGT redox quantification and glycoprotein profiling) used a conventional mico-flow LC-MS/MS. The use of conventional LC-MS/MS as opposed to a nano-LC system has some advantages in ease of transfer to standard LC-MS/MS equipment and a shortened run times (20 min instead of hours with nano-LC systems). This makes the method more applicable for routine use when high throughput analysis is required, and more transferable to other laboratories that are not equipped with a special nano-LC system. However, a nano-LC system has a higher sensitivity to detect low abundance plasma proteins than micro-LC, and would clearly extend the range of glycoproteins detectable in plasma.

7.1.1.1 Quantification of AGT redox switch

The two previously studies that quantified the redox switch of AGT in the plasma of pre-eclamptic women used either Western blotting or ELISA [197; 206]. In this thesis an entirely new strategy based on coupling a differential alkylation approach with targeted LC-MS/MS was used to recognise and quantify the cysteine (Cys) peptides involved in the redox switch of AGT in the plasma. Developing such a method was challenging, and a close attention to details was necessary in the design of the sample preparation process to lend credibility to the results obtained. One particularly challenging aspect of the method development was optimising a digestion protocol that enables the reproducible detection of the AGT Cys peptides. This required investigating different

digestion approaches (in-gel and in-solution digestion), protocols and alkylating agents to choose the best workflow in terms of protein recovery, signal intensity, complete alkylation and reproducible detection of the signature Cys peptides.

The method developed in this thesis to quantify the oxidation level of AGT in the plasma had several advantages over the other two previously reported studies that used either Western blotting or ELISA [197; 206]; (1) The method avoided the use of sizable costly alkylating reagents such as ICAT, TMT, and biotinylated iodoacetamide, generally used in redox protein analysis. These reagents can limit effective alkylation especially when used under non-denaturing conditions. (2) The method avoided the use of high denaturing conditions (such as 8 M urea) that was used by a previous redox proteomic study to improve the first alkylation of the free thiols as urea under high concentration can facilitate the break-down of the disulphide linkage leading to inaccurate quantification (3). The method followed a rigorous sample preparation workflow that allowed efficient deglycosylation, alkylation and reproducible detection of the Cys peptides with CV% <15%. (4) The use of targeted LC-MS/MS approach gave the method the required sensitivity and selectivity to detect the signature peptides in the complex plasma digest. This approach is considered a more reliable quantitative methodology than the Western blotting (which is, at best, semi-quantitative) used by Zhou *et al*. [197] to quantify the redox switch of AGT. (5) The method enabled the reproducible detection of the two distinct forms of AGT (the sulphydryl-bridged oxidised form and the free thiol reduced form) in the plasma, and the identity of the differentially alkylated Cys peptides was confirmed by LC-MS/MS in a typical plasma sample. (6) The method measured the oxidation level of AGT as a percentage of the sum of the reduced and the oxidised AGT forms which indicated that the change in the protein level is actually due to the change in the oxidation level of the protein rather than its total level. This provided more accurate measurements than what has been reported by Rahgozar *et al.* [206] who developed an ELISA assay to detect only the reduced form of the protein and reported the level of plasma free thiol AGT as a percentage of that observed in a pooled standard.

However, the method had certain limitations that should be addressed including; (1) The method involved many steps that resulted in 43% overall protein recovery. Yet, the recovery was reproducible and allowed the reliable detection of the differentially alkylated Cys18 peptide and AGT marker peptide in the plasma digest. (2) The relatively long sample preparation workflow makes other quantitative methods as ELISA, which

requires minimal sample preparation, a more convenient high throughput platform for routine use. Nevertheless, the method answered the question under investigation which was the main purpose of developing it, and so far no ELISA assay that can detect the two protein forms has been developed. (3) Effective alkylation of the free thiol of the reduced form of AGT was achieved after protein enrichment and deglycosylation, therefore, the detected level of AGT oxidation may not accurately reflect the actual oxidation level of the protein in the plasma. However, the ratio of the oxidised to the reduced AGT detected in the current work in the plasma of healthy women (3.25:1.75) was comparable to the original ratio reported by Zhou *et al*. [197] in the plasma of healthy individuals (3:2 oxidised to reduced), increasing the confidence in the research findings.

7.1.1.2 Glycoprotein profiling methodology

Most of the previously reported targeted glycoprotein profiling methods used peptide synthetic standards in their workflow and as a result they profiled a small set of glycoproteins (≤ 15) due to the high cost of the synthetic peptides. The developed label-free plasma glycoprotein profiling methodology (discussed in Chapter Four) offered several advantages over previous glycoprotein profiling methodology including, the larger number of plasma glycoprotein profiled (54 glycoproteins) and the lower cost/analysis as it used a label-free workflow and avoided the use of costly and time consuming stages as immunodepletion enrichment and complex deglycosylation. Furthermore, the method had several strength points; (1) It achieved an acceptable analytical precision (CV% ≤ 15) for most of the profiled glycoprotein assays which gives the confidence needed for the rapid quantification of relevant plasma glycoproteins between different disease conditions, as observed with pre-eclampsia and PCOS (next section). (2) It could be a cost-effective affordable alternative to isotope labelled standards workflow when applied for relative protein quantification, especially when targeting a large number of proteins. (3) It has a better analytical precision (85% showed CV< 15%) than immunoblotting, which yields CVs in the range from 20–40%. This showed the capability of the label-free targeted LC-MS/MS to be used as an alternative tool for the verification of putative candidate biomarkers for disease states.

Numerous protein candidates have been discovered and reported previously with different quantitative proteomics workflows. However, few of the proposed protein markers were validated due to the high cost or the limited availability of antibody-based

assays used for validation, which increases the chances of false positive detection [103]. As noted with the developed methodology, targeted LC-MS/MS is highly specific, sensitive, can be multiplexed, customised, and provides a better reproducibility than western blotting commonly used for biomarkers validation in proteomic studies [87; 98; 101]. The finding of this thesis indicates that label-free targeted LC-MS/MS can be a reliable alternative for biomarker validation method (Western blotting), and a more economical approach for screening a large number of candidate biomarkers prior to setting up a more expensive isotope labelling targeted LC-MS/MS or ELISA assays for the most promising ones [378; 530].

However, the method had certain limitations including; (1) It only provides relative quantitative data of the change in the protein level between the studied conditions rather than absolute measurements as synthetic peptide standards were not included in the workflow. However, most of the biomarker discovery studies are interested in measuring fold changes in protein level rather than absolute concentrations. (2) The method was mainly able to profile moderately abundant plasma glycoproteins and only a few low abundant proteins were profiled.

7.1.2 The clinical application of the targeted LC-MS/MS glycoprotein profiling method

7.1.2.1 Quantification of plasma AGT redox forms in Pre-eclampsia

An MS-based workflow was used for the first time to quantify plasma total AGT and its redox forms in a case-control study of pre-eclampsia in this thesis. The study showed a significantly higher plasma level of sulphydryl-bridged oxidised AGT in pre-eclamptic women compared to gestational age-matched normotensive controls, whilst maintaining a similar total AGT level in the plasma. The presented findings indicate that the elevated level of oxidised AGT rather than its total level might be a contributing factor to the hypertension characteristic of pre-eclampsia, as oxidised AGT preferentially binds to renin to initiate the renin-angiotensin system cascade to result in an increase in the blood pressure. Moreover, the study confirms the previously proposed involvement of AGT redox switch in pre-eclampsia and provides an extra line of evidence linking the oxidative state and the generation of reactive oxygen species with hypertension in pre-eclampsia. Therefore, measurement of the plasma oxidation level of AGT may potentially be able to be used in conjunction with other proposed biomarkers of pre-eclampsia to provide a better diagnosis of this multifactorial disease.

7.1.2.2 Glycoprotein profiling in pre-eclampsia and PCOS

Numerous protein biomarker candidates have been discovered and reported with quantitative proteomics workflows. However, none has focused on plasma glycoproteins which are involved in many biochemical processes that have relevance to the pathogenesis of several diseases including pre-eclampsia and PCOS.

In chapter five, analysis of plasma samples collected from early- and late-onset pre-eclamptic women using the developed plasma glycoprotein profiling methodology successfully identified significant changes in the level of several proteins in pre-eclampsia compared to controls. Two of them, apolipoprotein D and kallikrein, are reported for the first time to be altered in the plasma of pre-eclamptic women suggesting that they could be further evaluated as novel biomarkers. The overlap of the remaining altered glycoproteins with those previously reported in the scientific literature increased the confidence in the presented findings, and further confirms their involvement in the pathology of the disease. Fibronectin, particularly, has been previously reported to be altered in the early stage of pregnancy suggesting it can be used as a diagnostic biomarker of the disease. Some pre-eclampsia-relevant pathways and biological processes, including iron transport and metabolism, immune response, coagulation/fibrinolysis, cell adhesion, and lipid metabolism and oxidative stress were found to be altered in the disease. This complex pattern of changes indicates a synergism between the pathways involved in the pathogenesis of pre-eclampsia, and emphasises the well-accepted heterogeneous and multisystem nature of the disease. This points to the need for a combination of independent biomarkers each representing a pathophysiological process to provide a reliable diagnosis of the condition.

The study was also one of the few studies that compared the plasma protein profile of the early- and the late-onset pre-eclampsia. Analysis of plasma samples from early- and late-onset pre-eclamptic women revealed a different profile of the altered glycoproteins at each state of the disease, which might reflect different pathophysiological mechanisms. This finding supports the increasing evidence that the two pre-eclampsia onsets have different aetiologies and should be considered as a two different forms of the disease [439; 440; 441].

Polycystic ovary syndrome (PCOS) is a prevalent heterogeneous endocrine disorder with poorly understood aetiology. The disease is well recognised to be associated with several disorders including cardiovascular diseases and pre-eclampsia. In Chapter Six, the glycoprotein profiling method was applied to analyse plasma samples from women with PCOS to identify glycoproteins altered in PCOS, and to compare them with glycoproteins altered in pre-eclampsia to propose possible pathophysiological mechanisms that can explain the reported association between the two conditions. The study identified 16 glycoproteins with a significantly altered level in the plasma of women with PCOS compared to controls; two of them, vitronectin and insulin growth factor acid labile subunit, were identified for the first time suggesting that they could be further evaluated as new potential PCOS biomarkers. The altered biological processes and pathways in PCOS denoted a higher inflammatory state, iron overload, hypofibrinolysis and disturbances in the insulin growth factor system with an increased risk of insulin resistance among women with PCOS compared to controls. The seven glycoproteins that were commonly changed in the plasma of pre-eclamptic women and women with PCOS pointed to underlying pathophysiological mechanisms, such as hypofibrinolysis and thrombophilia and iron overload, which might be common to both conditions and, therefore, might provide a possible justification to the association between PCOS and pre-eclampsia. Moreover, the lower circulating level of apolipoprotein A-I and the impaired fibrinolysis process detected in PCOS could contribute to the higher risk of developing cardiovascular diseases among women with PCOS which supports the association of PCOS with cardiovascular diseases.

The detected differences in the plasma level of some glycoproteins between women with PCOS and controls (such as fibrinogen) may be influenced by the higher BMI of PCOS patients compared to controls. The presence of obesity, which is characteristic of PCOS, is likely to confound the results of any study investigating PCOS. However, regression analysis showed a higher correlation of plasma fibrinogen level with PCOS than with BMI.

Although analysis of placental or ovarian tissue samples from women with pre-eclampsia or PCOS, respectively, might reflect more accurately the underlying biochemical pathways and mechanisms of the disease, the invasive measures necessary to obtain tissue samples and the challenging sample processing make other more accessible biofluid, such as plasma, a more attractive option for the discovery of potential disease biomarkers [482]. In this thesis, some of the glycoproteins that were altered in the

plasma of pre-eclamptic women or women with PCOS have been previously reported to be similarly changed in pre-eclampsia placental tissues (*e.g.* fibronectin, plasminogen and clusterin) and ovarian tissues (*e.g.* apolipoprotein A-I). This points to a strong correlation between these proteins and the pathophysiology of the disease, and suggests that interaction between the placenta and the ovary endothelium and systemic pathways could be reflected on plasma proteins. However, there is a need for future studies relevant to different clinical conditions (such as pre-eclampsia and PCOS) to consider the correlations between the relative expression levels of proteins in the placenta or ovarian tissues compared with the plasma/serum, to further determine whether changes in tissue expression are also manifest in the circulation.

In conclusion, there is clearly a high need for the development of analytical tools that assist in the diagnosis and the understanding of the aetiology of pre-eclampsia. The two targeted LC-MS/MS methods developed in this thesis provided relevant information to the disease. They identified potential pre-eclampsia protein biomarkers, shed light on different biochemical processes altered in the disease which led to an increased understanding of the molecular mechanisms underlying pre-eclampsia, pointed to possible pathophysiological mechanisms that might help in explaining the link between PCOS and pre-eclampsia, and opened several potential areas for future investigations.
The findings of the current research reflected a high level of oxidative stress in pre-eclampsia which should reinforce efforts to develop antioxidant-based therapeutic strategies. Moreover, the present work emphasises the need to use more than one biomarker (each representing a pathophysiological process) to provide an accurate diagnosis of pre-eclampsia. Given the multifactorial and the heterogeneous nature of pre-eclampsia, it is highly unlikely that one biomarker will have the specificity to distinguish pre-eclampsia from another disorder, and hence, a set of several biomarkers is often necessary as a determinant. At this stage, it is not recommended that the altered proteins identified in this study, particularly the novel ones, to be used as conclusive biomarkers of pre-eclampsia and PCOS. The present work, however, offers information that can play a key role in improving the health care of women with pre-eclampsia and serves as a foundational cornerstone for future work.

7.2 Future work and recommendations

Additional work which is beyond the scope of this thesis and would be beneficial includes; validation of some of the identified potential glycoprotein biomarkers in pre-eclampsia and PCOS using ELISA or isotope standards targeted LC-MS/MS, and a detailed analysis of protein-protein interactions and biochemical pathways for the biomarker candidates identified in the study. Analysis of the set of the identified potential protein biomarkers using an isotope peptide standards targeted LC-MS/MS workflow will also be beneficial to provide absolute measurements of the biomarker candidates' concentration in the plasma. This set of biomarkers can then be tested for their sensitivity and specificity (in a wider population if possible) to evaluate their potential to be translated into clinical practice.

In the analysis of plasma samples from early- (n=10) and late-onset pre-eclampsia (n=10), it would be advantageous to have a larger group of subjects to be analysed using the developed glycoprotein profiling methodology. Also, analysis of the altered proteins in the placental tissues (if expressed) can give better information on whether changes in tissue expression are also manifested in the circulation.

In pre-eclampsia, there is a need to identify early biomarkers that can identify women at risk of developing the disease before the onset of clinical symptoms. This will allow the health care professionals to take preventative measures. Therefore, it is recommended that the value of the identified changed glycoproteins in pre-eclampsia, particularly the novel biomarker candidates and the oxidation level of AGT, to be evaluated at an earlier stage of pregnancy in a larger prospective cohort study to address their potential clinical usefulness for the early prediction of pre-eclampsia. Additionally, it is recommended that the common proteins altered in PCOS and pre-eclampsia to be further evaluated to address their potential diagnostic value in identifying PCOS women at higher risk of developing pre-eclampsia. This can help in the early prediction of pre-eclampsia which would facilitate intervention, closer monitoring, and preventive strategies before the deterioration of the condition.

Finally, the developed glycoprotein profiling methodology has a wider application and could be applied to discover potential disease biomarkers and investigate biochemical pathways in different diseases.

Chapter Eight

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8 References

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Appendices

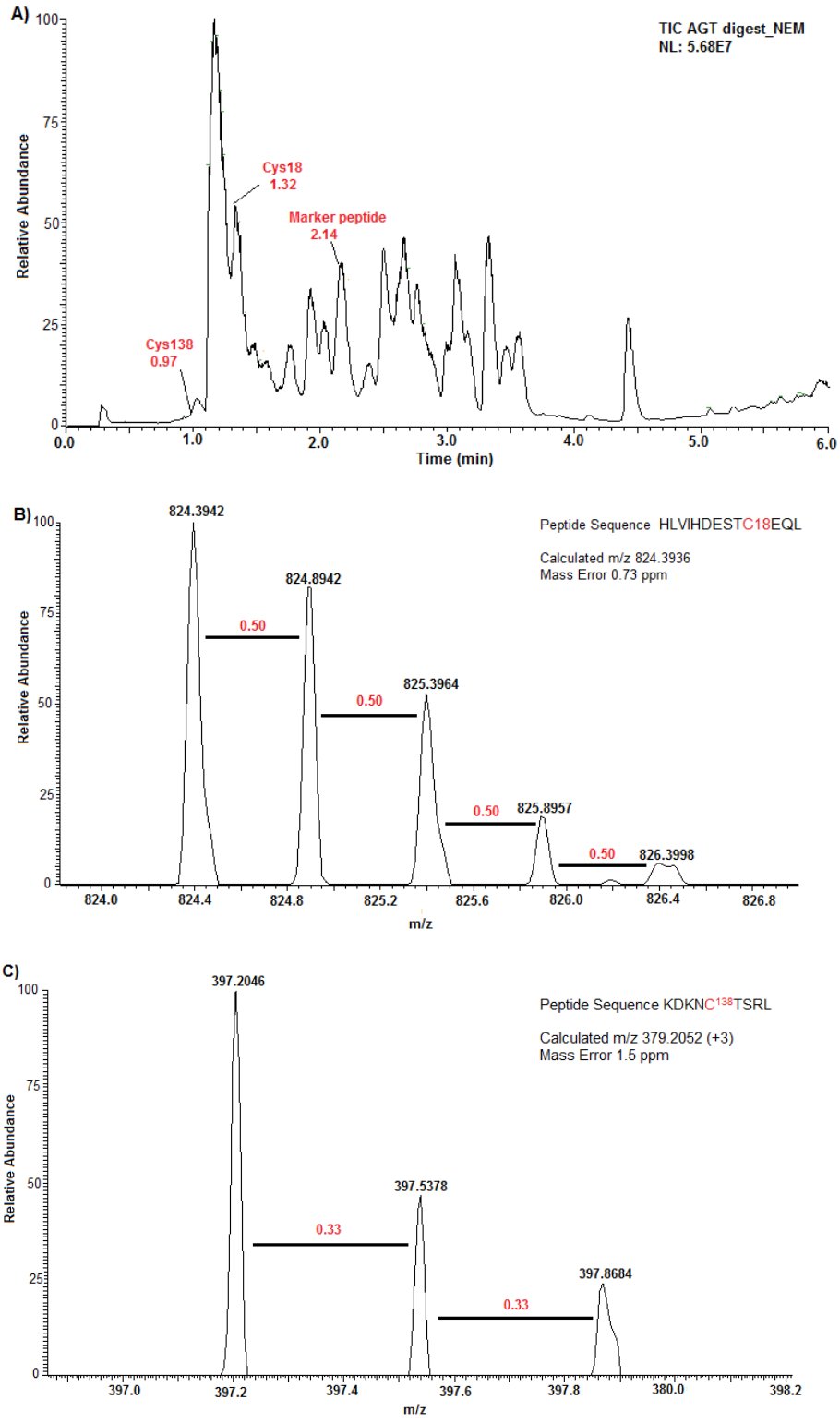


Figure A2-1: Analysis of NEM alkylated AGT chymotryptic digest (human recombinant unglycosylated AGT) by high resolution accurate mass. (A) TIC, (B) Ion signal of NEM alkylated Cys18 peptide extracted from the TIC and (C) Ion signal of NEM alkylated Cys138 peptide extracted from the TIC. The peptides were detected with high mass accuracy (mass error < 2 ppm) which confirm their identities

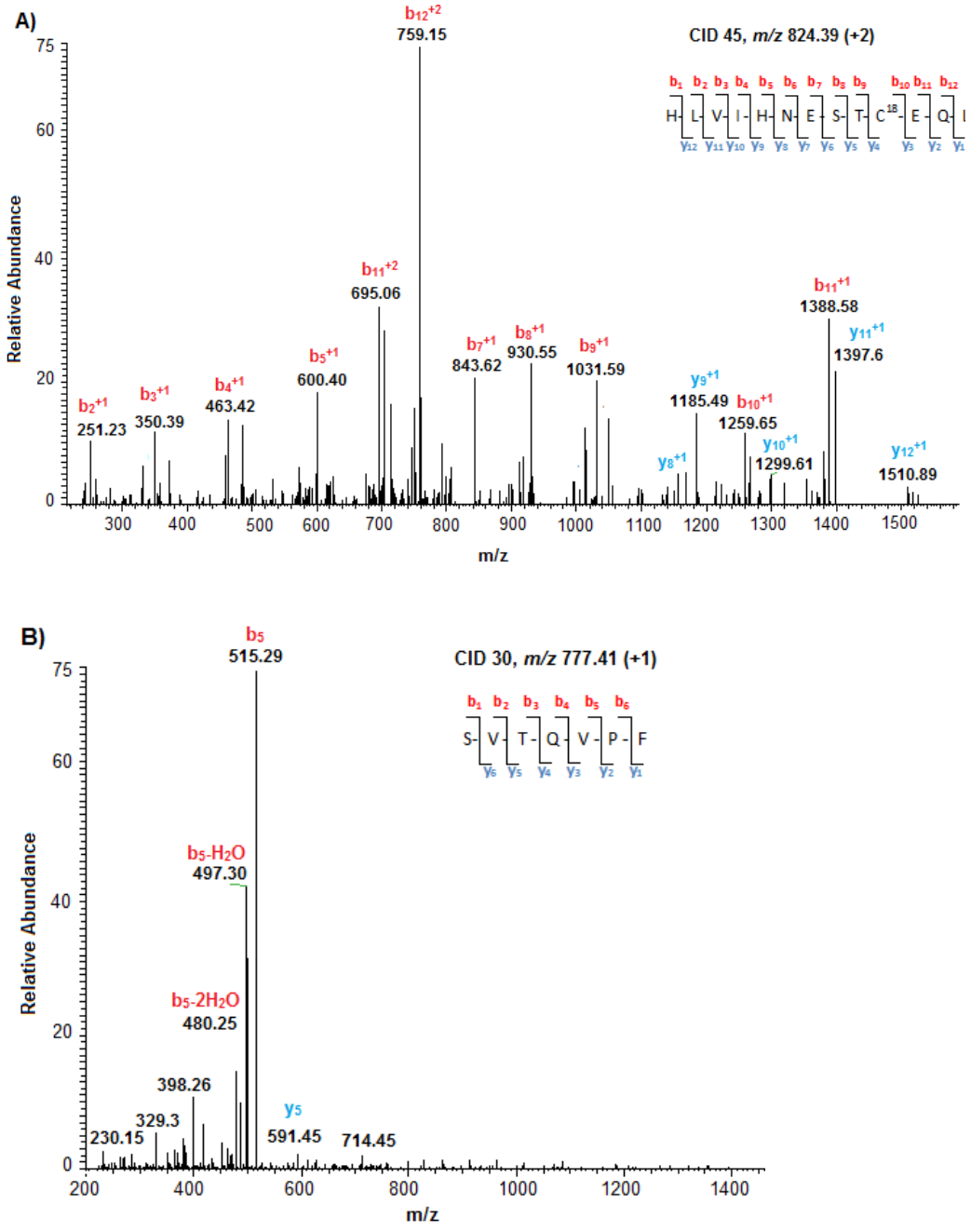


Figure A2-2: LC-MS/MS spectra of NEM modified Cys 18 (A) and AGT marker peptides (B) from human recombinant AGT chymotryptic digest. The peptides identity was confirmed by matching the expected fragment ions of each peptide with its generated spectrum.

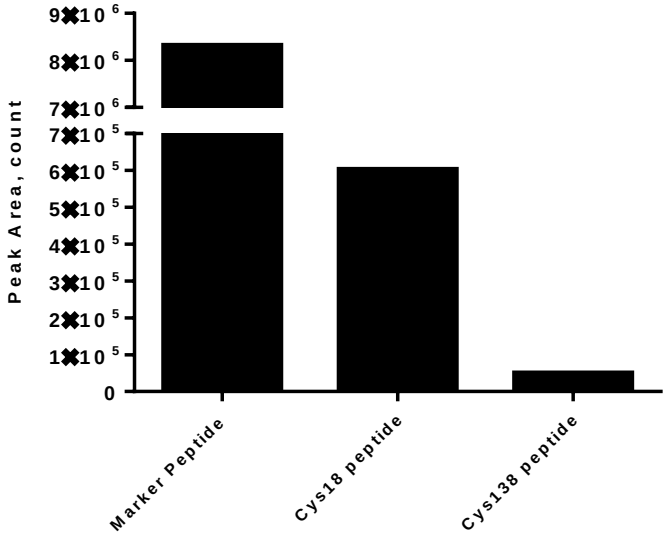


Figure A2-3: Different MS responses of 0.5 µM standards of marker peptide, iodoacetamide alkylated Cys18 and iodoacetamide alkylated Cys138 peptides analysed by targeted LC-MS/MS. AGT marker peptide showed the highest MS response while iodoacetamide alkylated Cys138 showed the lowest MS response.

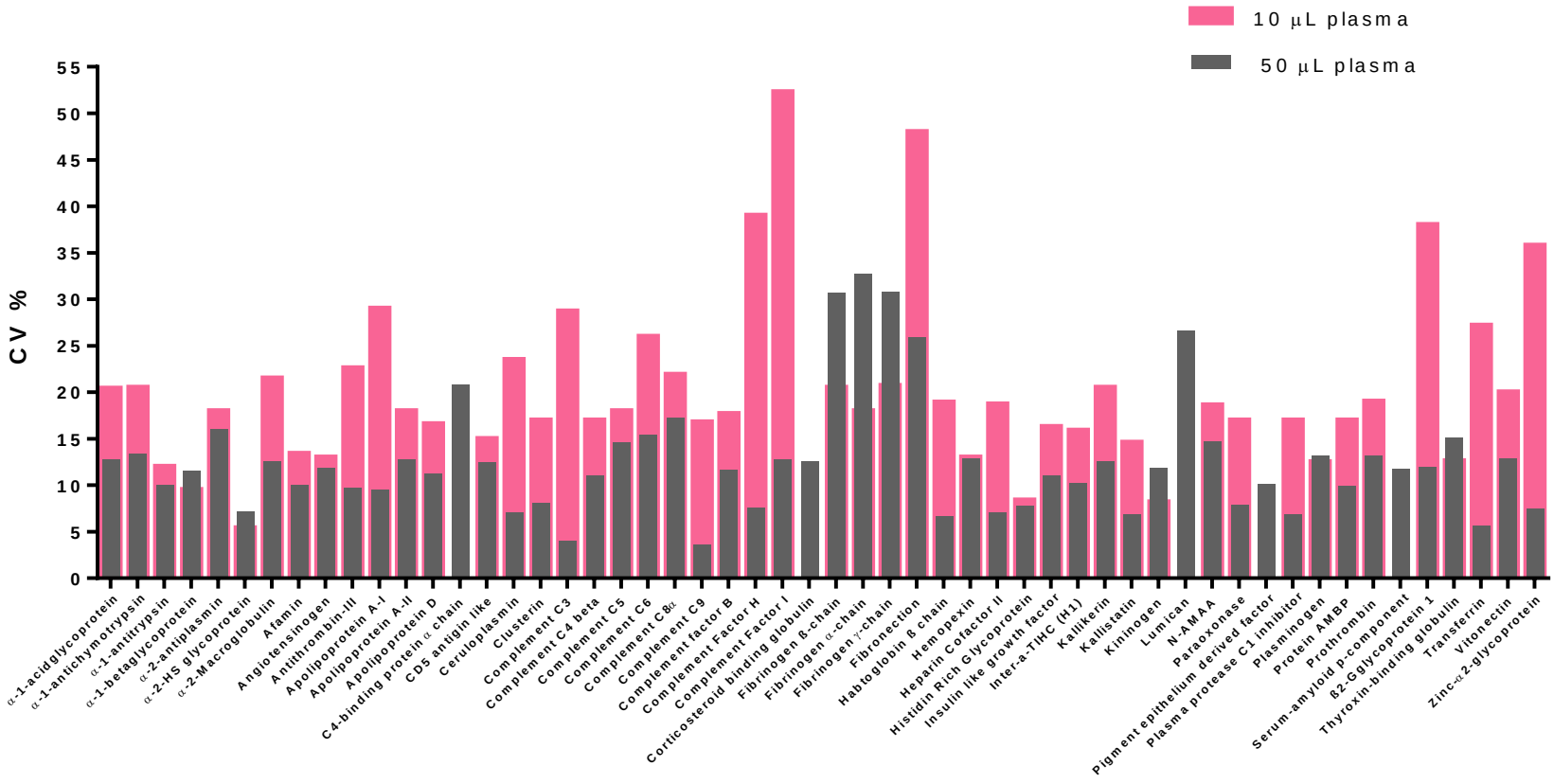


Figure A4-1: Comparison between the coefficient of variation values obtained from 50 and 10 µL fractionated plasma, represented by gray and pink bars respectively, for the plasma glycoproteins monitored by the targeted LC-MS/MS method developed in Chapter Four. Substantial increase in the variability of the method was noticed, for most proteins, when plasma volume was decreased to 10 µL.

Table A2-1: Predicted sequences and *m/z* of doubly charged modified Cys 18 and Cys 138 peptides with the mass error (ppm) for the corresponding peptides as detected (at different urea concentrations) from the AGT chymotryptic digests using high resolution accurate mass.

Peptide Sequence	Predicted *m/z*	Mass Error (ppm)			
		Without Urea	2 M Urea	4 M Urea	6 M Urea
HLVIHNESTC^{18}EQL (IAM modified)	790.3803	1.22 (0.76)	1.20 (0.53)	1.71 (0.53)	1.70 (0.40)
KDKNC^{138}TSRL (IAM modified)	561.2902	0.93 (0.37)	1.46 (1.06)	0.71 (0.40)	1.16 (0.65)
HLVIHNESTC^{18}EQL (NEM modified)	824.3936	0.80 (0.54)	0.65 (0.59)	1.03 (0.81)	0.61 (0.55)
KDKNC^{138}TSRL (NEM modified)	397.205*	0.71 (0.66)	1.95 (0.24)	1.10 (0.74)	1.10 (0.63)

* Predicted *m/z* for triply charged peptide
** Data is presented as mean (STD), n=6

Table A2-2: Optimised MRM for the unmodified and NEM modified AGT Cys 18 and 138 peptides

Peptide Sequence	Modification	Peptide mass	*m/z* (charge)	MS1/MS2	DP	CE
HLVIHDEST**C**[18]EQL	NEM	1647.78	824.8936 (+2)	824.9/759.4	85	40
				824.9/695.3	85	40
				824.9/1389.7	85	40
	No alkylation	1522.73	762.3695	792.4/844.6	85	38
				792.4/463.6	85	50
				792.4/696.8	85	40
KDKD**C**[138]TSRL	NEM	1189.58	397.506 (+3)	397.6/819.4	65	25
				397.6/476.3	65	30
				397.6/704.6	65	35
	No alkylation	1064.55	533.2795	533.3/468.9	60	28
				533.3/476.3	60	40
				533.3/694.3	60	35

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Table A4-1: Concentrations and references for proteins illustrated in Figure 4-6, Chapter Four

No.	Protein name	Concentration, ng/mL	Reference
1	Albumin	4.0E+07	1
2	IGHG1 protein	1.1E+07	2
3	Serotransferrin	2.3E+06	1
4	IGHA1 protein	2.0E+06	2
5	Apolipoprotein A-I	1.4E+06	1
6	Alpha-2-macroglobulin	1.4E+06	1
7	Alpha-1-antitrypsin	1.1E+06	1
8	Complement C3	9.5E+05	1
9	Haptoglobin	8.8E+05	1
10	Hemopexin	7.5E+05	1
11	Fibrinogen gamma chain	6.7E+05	1
12	Alpha-2-HS-glycoprotein	6.3E+05	3
13	Alpha-1-acid glycoprotein 2	6.1E+05	1
14	Complement factor H	5.0E+05	3
15	Inter-alpha-trypsin inhibitor	5.0E+05	2
16	Alpha-1-antichymotrypsin	4.5E+05	3
17	Ceruloplasmin	4.0E+05	3
18	Vitronectin	3.4E+05	3
19	Complement component 4A	3.2E+05	3
20	Apolipoprotein A-II	3.0E+05	1
21	Fibronectin	3.0E+05	4
22	Alpha-1B-glycoprotein	2.3E+05	3
23	Beta-2-glycoprotein 1	2.2E+05	3
24	Complement factor B	2.0E+05	2
25	Plasma protease C1 inhibitor	2.0E+05	3
26	Plasminogen	1.6E+05	3
27	Prothrombin	1.5E+05	3
28	Antithrombin III	1.5E+05	3
29	Complement C1r	1.0E+05	3
30	Histidine-rich glycoprotein	1.0E+05	3
31	Complement C5	9.5E+04	3
32	Kininogen-1	9.0E+04	3
33	Heparin cofactor 2	8.0E+04	3
34	alpha-2-glycoprotein 1, zinc	8.0E+04	3
35	Complement C1q	7.5E+04	3
36	HMW of Kininogen-1	7.0E+04	3

Table A4-1: Continued

No.	Protein name	Concentration, ng/mL	Reference
37	Complement component C9	6.0E+04	3
38	Afamin	6.0E+04	5
39	Alpha-2-antiplasmin	6.0E+04	3
40	Complement component C8	6.0E+04	2
41	Serum paraoxonase/arylesterase 1	6.0E+04	2
42	Serum amyloid P-component	5.5E+04	3
43	Clusterin	5.0E+04	2
44	Complement C1s subcomponent	5.0E+04	2
45	leucine-rich alpha-2-glycoprotein	5.0E+04	6
46	Angiotensinogen	4.5E+04	3
47	Complement component C6	4.0E+04	3
48	Complement factor I	3.5E+04	3
49	Carboxypeptidase N	3.5E+04	3
50	AMBP protein	2.5E+04	3
51	Insulin-like growth factor-binding protein complex acid labile chain	2.4E+04	3
52	Kallistatin	2.2E+04	7
53	Thyroxine-binding globulin	1.5E+04	3
54	Pigment epithelium-derived factor	5.0E+03	3

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Table A4-2: Plasma proteins precipitated by 15% (*w/w*) ammonium sulphate identified by LC-MS/MS analysis of the tryptic protein digest

No.	Protein Description	Matched peptides	Sequence coverage	Protein score
1	Alpha-1-acid glycoprotein	7	14	160
2	Alpha-2-macroglobulin	11	9	85
3	Apolipoprotein A-I	7	23	164
4	Complement C3	9	6	60
5	Complement C4-A	3	1	44
6	Fibrinogen alpha chain	44	31	266
7	Fibrinogen beta chain	35	56	421
8	Fibrinogen gamma chain	25	51	225
9	Fibronectin	38	14	405
10	Haptoglobin	7	15	101
11	Heparin cofactor 2	1	1	34
12	Histidine-rich glycoprotein	1	2	59
13	Ig alpha-1 chain C region	4	9	49
14	Ig gamma-1 chain C region	10	30	101
15	Ig kappa chain C region	4	32	108
16	Ig kappa chain V-III region SIE	1	16	51
17	Ig mu chain C region	6	13	62
1	Inter-alpha-trypsin inhibitor heavy chain H4	3	3	53
19	Plasminogen	11	16	95
20	Serotransferrin	9	14	139
21	Serum albumin	43	48	320