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LIST OF ABBREVIATIONS

SI unit prefixes are not included in the list below.

°C	degrees Celsius
1-D	one-dimensional
2-D	two-dimensional
2DE	two-dimensional gel electrophoresis
A	ampere(s)
A1BG	α-1B-glycoprotein
ANOVA	analysis of variation
ApoC2	apolipoprotein C-II
ApoE	apolipoprotein E
$ApoE_A$	apolipoprotein E, acidic isoform
$ApoE_B$	apolipoprotein E, basic isoform
BCA	bicinchoninic acid
BFA	brefeldin A
BSA	bovine serum albumin
C7BzO	3-(4-heptyl)phenyl-3-hydroxypropyl)dimethylammoniopropanesulfonate
cAMP	cyclic adenosine monophosphate
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHRP2	complement factor H-related protein 2
CI	confidence interval
CTB	cytotrophoblast
CV	coefficient of variation
Da	Dalton(s)
DIGE	difference gel electrophoresis
DLOO	double leave-one-out
DMEM	Dulbecco/Vogt Modified Eagle's Minimal Essential Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E-cadherin	epithelial cadherin
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionization
EVT	extravillous cytotrophoblast
FDR	false discovery rate
FSK	forskolin
FT-ICR	Fourier transform ion cyclotron resonance
g	gram(s)
GalNAc	*N*-acetyl galactosamine
Glc	glucose
GO	Gene Ontology
h	hour(s)
hCG	human chorionic gonadotropin
HCl	hydrochloric acid

HDL	high density lipoprotein
HELLP	hemolytic anemia, elevated liver enzyme activity, low platelet count syndrome
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	human leukocyte antigen
HPLC	high-performance liquid chromatography
HUPO	Human Proteome Organization
IAA	iodoacetamide
IATI	inter-α-trypsin inhibitor
ICAM	intercellular cell adhesion molecule
IFN	interferon γ
IgA	immunoglobulin A
IgG	immunoglobulin G
IHRP	inter-α-trypsin inhibitor family heavy chain-related protein
IMP5	ImageMaster™ 2D Platinum version 5.0
IMP6	ImageMaster™ 2D Platinum version 6.0.1
IPG	immobilized pH gradient
IS	internal standard
kDa	kilodaltons(s)
KIR	killer immunoglobulin-like receptor
L	liter(s)
LC	liquid chromatography
LC-MS	liquid chromatrography coupled with mass spectrometry
LC-MS/MS	liquid chromatography coupled with tandem mass spectrometry
LDH	lactate dehydrogenase
LDL	low density lipoprotein
MALDI	matrix-assisted laser desorption/ionization
MARS	Multiple Affinity Removal System
MBP	mannose-binding protein C
MES	2-(N-morpholino)ethanesulfonic acid
min	minute(s)
MMP	matrix metalloproteinase
mol	mole(s)
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MudPIT	multidimensional protein identification technology
MW	molecular weight
NCBI	National Center for Biotechnology Information
NK	natural killer cell
NL	nonlinear
NSC	nearest shrunken centroids
PAGE	polyacrylamide gel electrophoresis
PAI-1	plasminogen activator inhibitor-1
PAI-2	plasminogen activator inhibitor-2
PAPP-A	pregnancy-associated plasma protein A
PBS	phosphate-buffered saline

PBST	phosphate-buffered saline + Tween 20
PES	polyethersulfone
pI	isoelectric point
PlGF	placental growth factor
PLTP	phospholipid transfer protein
PP13	placental protein 13
PPP	Plasma Proteome Project
PSA	prostate-specific antigen
PVDF	polyvinylidene fluoride
RBP	retinol binding protein
RFE	recursive feature elimination
RNA	ribonucleic acid
s	second(s)
SALSA	Scoring Algorithm for Spectral Analysis
SAP	serum amyloid P-component
SCOPE	SCreening for Pregnancy Endpoints
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SELDI	surface-enhanced laser desorption/ionization
sFlt1	soluble fms-like tyrosine kinase 1
SFM	serum-free media
SGA	small for gestational age
SILAC	stable isotope labeling with amino acids in cell culture
SNP	single nucleotide polymorphism
STB	syncytiotrophoblast
STBM	syncytiotrophoblast microvillous membranes
TG	triglyceride
TGF-β	transforming growth factor-β
TIMP	tissue inhibitor of metalloproteinases
TNF-α	tumor necrosis factor-α
TOF	time-of-flight
Tris	2-amino-2-(hydroxymethyl)propane-1,3-diol
V	volts
V·h	volt hours
VCAM	vascular cell adhesion molecule
VE-cadherin	vascular epithelial cadherin
VEGF	vascular endothelial growth factor
VLDL	very low density lipoprotein
W	watts

CHAPTER 1 INTRODUCTION

1.1 Biomarker discovery

Biomarkers are specific representations of biological conditions including disease states. Physiological substances such as proteins and genetic markers, patterns of coordinated physiological changes as in gene expression patterns, and clinical observations like blood pressure or hair loss can constitute biomarkers. These markers have potential roles at every step of disease management: from prediction, early detection and diagnosis through to disease prognosis, monitoring progress, and evaluating response to therapy. Biomarkers are potentially useful adjuncts to clinical management as they can increase confidence in a diagnosis or treatment. Use of quantitative biomarker-based tests can be more objective than clinical assessment, as observer variation is minimized and quality assurance practices in the laboratory improve reproducibility and reliability. In addition to their direct use in tracking disease, biomarkers can provide targets for therapeutic intervention and advance knowledge about signaling pathways involved in disease.

The process of discovering a biomarker and validating its utility in clinical practice can be lengthy as several stages of research and development must be undertaken before a test based upon that marker can be released (Figure 1-1).

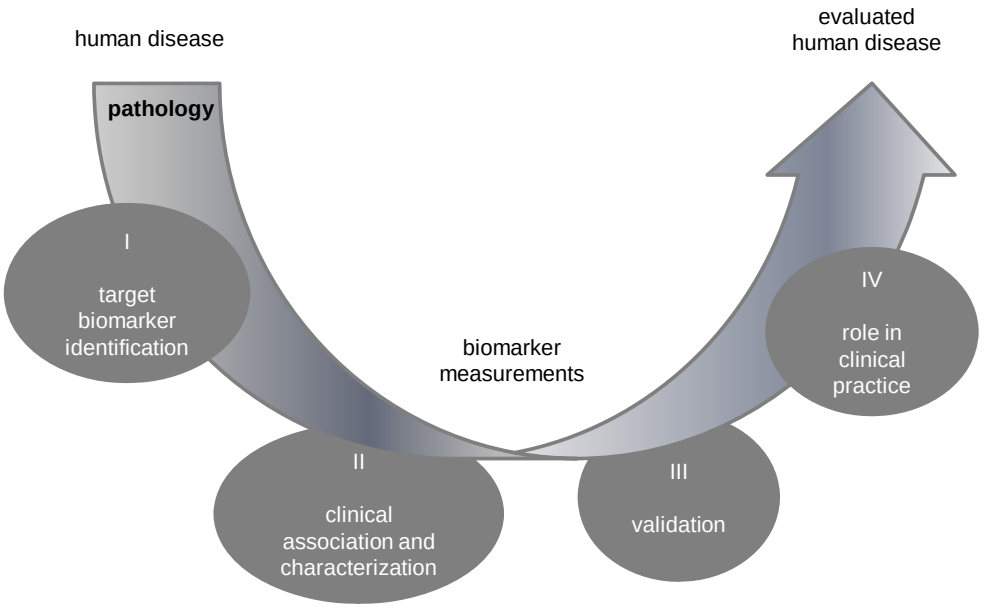

Figure 1-1: Biomarker discovery and validation spectrum

The relationship between biomarker discovery processes and the development/validation phases that follow. The context of this thesis is the first phase, target biomarker identification. Adapted from Marko-Varga *et al.* 2005, Figure 1.

In the pre-genomic era, biomarkers were identified serendipitously, or through targeted efforts with selection of marker candidates using knowledge of disease pathogenesis and biochemical pathway

involvement. The first pregnancy test specifically measuring human chorionic gonadotropin (hCG) dates from 1927 (Ascheim and Zondek 1927) and an immunoassay for this glycoprotein was developed in the 1960s (Wide and Gemzell 1960; Chard 1992). Changes in specific forms of the prostate-specific antigen (PSA) protein, discovered in 1979 through immunoprecipitation (Wang *et al.* 1979), have resulted in PSA being a well-recognized biomarker for prostate cancer (Sokoll and Chan 1997; Balk *et al.* 2003). The glycoprotein CA 125 may differentiate women with ovarian cancer from those with benign gynecological conditions; this protein was also discovered by immune-based approaches (Bast *et al.* 1983; Verheijen *et al.* 1999). Mutations in the *BRCA1* gene, discovered through gene-specific screening, are associated with increased incidence of breast and ovarian cancer (Miki *et al.* 1994; Narod and Foulkes 2004). In all cases, discovery of these biomarkers was enabled by prior knowledge of, and a specific hypothesis explaining, the medical conditions involved.

Recently, an untargeted approach not limited by *a priori* knowledge has gathered acceptance as an alternative to hypothesis-driven biomarker discovery efforts. This approach does not require prior knowledge of the target markers being sought, instead aiming to find differences in the biological states (*e.g.*, protein expression profiles) of healthy and diseased individuals. Numerous technologies can be used for unbiased biomarker discovery efforts. Wider studies of gene and protein expression, such as cDNA microarrays and high-throughput mass spectrometry (MS)-based proteomics, are examples of this type of approach.

Genomics approaches such as microarrays have always held promise for biomarker discovery, but these methods have disadvantages. Much of the remaining heterogeneity in the human genome is due to single-nucleotide polymorphisms (SNPs). Screening requires knowledge of specific candidate SNPs in genes of interest. The range of polymorphisms present can be large due to high variation between individuals, compounded by ethnic differences in SNP frequency which make translation to clinical use difficult. Polymorphisms may be identified from tissues of interest but may not be detectable in biofluids accessible to further testing, possibly due to discordance between levels of RNA transcription and protein translation (Anderson and Anderson 1998; Weinkauf *et al.* 2007) or simply absence of the protein of interest. Post-translational modifications to proteins, processing steps which are essential to their functions, also create diversity more substantial than that predicted by sequence modifications alone. Genomic approaches are unable to provide any information about these important protein modifications.

The Human Genome Project has shown that there are at least ten-fold more proteins in the human proteome than there are genes in the genome (Anderson and Anderson 2002). Consequently it is now well recognized that much of human biological diversity is generated from proteins and their modifications. Most tests used in clinical medicine today are protein based, except when diagnosing

specific genetic disorders. As a result, proteomics has gained considerable strength as an approach to characterize protein expression in tissues and bodily fluids in physiological and pathological conditions (Good *et al.* 2007). In particular, proteomics approaches are able to separate protein isoforms which contain different post-translational modifications. These modifications may alter protein function and can be of key importance in the pathology of disease (Krueger and Srivastava 2006), making them exciting targets for biomarker discovery research.

The following section will describe the growing field of proteomics and its application to biomarker discovery, introducing widely used gel- and MS-based methods. This is followed by a section on biomarker research focusing on the pregnancy complication preeclampsia using proteomic techniques. In the context of this thesis, the term 'biomarker' generally refers to protein biochemical markers reflecting disease states; the term 'marker' is used interchangeably.

1.2 Proteomics

Proteomics, broadly, is the study of proteins, and is akin to genomics as the study of DNA. Proteomic techniques examine the protein contents of their target sample, whether from intracellular extracts, isolated organelles, secreted proteins, whole tissues, or proteinaceous fluids like plasma. A major focus of proteomic research is the ability to compare proteomes using quantitative methods to detect differential expression of proteins between samples, commonly control or healthy samples versus treated or disease-related samples. The following sections will describe the predominant techniques for comparative proteomics, the bioinformatics methods used to extract significant quantitative information from these techniques, and selected applications of proteomic methods to biological systems in general and blood serum/plasma in particular.

1.2.1 Techniques

Comparative proteomic studies use gel-based and/or gel-free approaches to separate and identify proteins with differential expression. These methods are discussed in the following sections, with particular emphasis on two-dimensional (2-D) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the classical method used to compare protein expression patterns. A gel-based modification of the this technique which uses multiplexed fluorescent labeling is introduced, and complementary gel-free techniques based on MS approaches are also described.

1.2.1.1 Two-dimensional SDS-polyacrylamide gel electrophoresis (2DE)

The 2DE technique involves two orthogonal separation steps: first, isoelectric focusing uses charge differences to separate proteins according to their isoelectric points (pI); second, focused proteins are separated by mass using SDS-PAGE. The result is a two-dimensional map of protein separation with each form of a protein (isoform) represented by a spot. The area of a spot is a measure of the abundance of that isoform. This technique was first described by O'Farrell (1975) using a fragile and variable tube gel-based focusing step involving liquid carrier ampholytes to create the pH gradient. Later introduction of plastic-backed immobilized pH gradient (IPG) strips allowed standardization and increased reproducibility of pH gradients and enabled the development of high-resolution, high-throughput proteomics (Bjellqvist *et al.* 1982; Westermeier *et al.* 1983; Blomberg *et al.* 1995). Additional optimization of sample application through in-gel rehydration, in which the lyophilized IPG strip is reswelled passively within a solution containing a protein sample, enabled even greater resolution of proteins and larger sample loads on IPG strips (Rabilloud *et al.* 1994).

Like any proteomics technique, 2DE can separate only a subset of proteins from any particular sample. Early 2DE method development optimized the technique for separation of thousands of proteins with high resolution. 2DE is however limited to soluble proteins, and is known to separate hydrophobic, basic, and membrane proteins more poorly than other more soluble classes of proteins (Cordwell *et al.* 2000; Pedersen *et al.* 2003). Modifications of the initial solution used for sample solubilization, a highly denaturing mixture of chaotropes, zwitterionic or nonionic detergents, and reducing agents, can increase the solubility of these proteins (Rabilloud *et al.* 1997). In addition, basic proteins can be applied to IPG strips using paper bridge or cup loading methods which can increase their resolution in 2DE (Sabounchi-Schutt *et al.* 2000). The widespread use and varied modifications of 2DE techniques have increased its applicability to range of sample types.

Currently available protein detection technology enables 2DE to separate hundreds to a few thousand spots on a single gel (Righetti *et al.* 2003). Proteins are detected based on their abundance, with the lower concentration limit of detectable proteins dependent solely on the visualization technique used (Table 1-1). All methods will detect the most abundant proteins present in a sample; more sensitive methods detect less abundant proteins. Though not as sensitive as other methods, colorimetric stains based on Coomassie dyes are widely used since they stain to equilibrium with little hands-on time and are readily available. Silver nitrate staining remains very sensitive but has limited compatibility with downstream protein identification using MS. Recently, fluorescent stains have supplanted colorimetric stains as they are more linear with protein concentration, more sensitive, and MS-compatible (Patton 2002; Miller *et al.* 2006a; Harris *et al.* 2007).

Table 1-1: Detection limits of common post-electrophoresis 2-D gel stains

Protein stain	*Type*	*Detection limit (ng)*	*Linear range (orders of magnitude)*	*Reference*
Coomassie R-250	colorimetric	50–100	1–1.3	Fazekas de St. Groth *et al.* 1963
Colloidal Coomassie G-250	colorimetric	10–20	3	Neuhoff *et al.* 1985
Silver nitrate	colorimetric	1–5	2	Merril *et al.* 1979
SYPRO® Ruby	fluorescent	0.3–1	3	Berggren *et al.* 2002
Deep Purple®	fluorescent	< 1	4	Mackintosh *et al.* 2003

Adapted from Miller *et al.* 2006a.

Quantitative data can only be accurately derived from 2DE studies with computer assistance. 2-D gels are scanned after staining, generating digitized image files for computer-based spot analysis. Specialized software is used to quantitate protein expression on a spot-by-spot basis by defining spot area boundaries and matching spots between gel images, producing a dataset which allows comparison of a single spot's expression over a set of gels in a 2DE experiment. Early software packages required a great deal of user intervention which introduced subjectivity into the spot matching process (Appel *et al.* 1997; Wheelock and Buckpitt 2005; Wheelock and Goto 2006). Recent commercial software has automated the 2DE analysis process as much as possible to allow objective analysis by different operators (Mahon and Dupree 2001; Raman *et al.* 2002; Rosengren *et al.* 2003; Arora *et al.* 2005).

The major strength of 2DE-based analysis over other proteomic techniques is separation of intact proteins by isoform (Görg *et al.* 2004). Isoforms result from three main sources: single nucleotide polymorphisms within DNA, variant RNA derived through alternative splicing, and post-translational protein modification. Post-translational protein modifications include the addition of functional groups (*e.g.*, glycosylation or phosphorylation), changes in protein structure (*e.g.*, disulfide bridges or proteolytic cleavage), or changes in amino acid chemical properties (*e.g.*, deamidation). Any of these modifications can modify protein function. 2DE enables analysis of separate protein isoforms, which can increase knowledge about the relationship between these isoforms and biological states including diseases (Hunzinger *et al.* 2006). Dysregulation of protein isoforms may be more relevant to some pathologies than changes in total protein levels (Durand and Seta 2000), such as for aberrantly glycosylated isoforms of clusterin which are prevalent in serum from colorectal cancer patients (Rodriguez-Pineiro *et al.* 2006).

The ultimate goal of 2DE analysis is identification of the protein contained within a gel spot of interest. This is typically achieved using peptide MS techniques. Protein isoform spots of interest are excised from 2-D gels and subjected to proteolytic cleavage with trypsin to generate peptides in

solution. Peptides are ionized within a mass spectrometer to produce a measurement of their masses. A number of ionization methods are used; peptides can be co-crystallized with a matrix for matrix-assisted laser desorption/ionization (MALDI), or the liquid peptide mixture can be subjected to electrospray ionization (ESI). Liquid chromatography (LC)-MS enables high-performance liquid chromatography (HPLC) systems to be coupled to ESI MS, usually by the inclusion of a reversed-phase column eluting directly into the ionization source (Covey 1996). After ionization, peptides are detected within the spectrometer using time-of-flight (TOF), quadrupole, ion trap, or Fourier transform ion cyclotron resonance (FT-ICR) mass analyzers. Mass spectrometers are designated by their combination of ionization method and mass analyzer; for example, MALDI-TOF spectrometers are common. MS/MS methods refer to multiple peptide fragmentation steps occurring within the mass spectrometer, generally within a single run, and provide a greater level of detail about peptide amino acid sequences not available from single fragmentation approaches like MALDI-TOF. This sequence information can be key in identifying the specific protein isoform of interest.

Computational analysis is required to derive protein identifications from MS data. In general, for organisms with sequenced genomes, search algorithms are used to query the collected MS data against the organism's entire known protein complement from sequence databases such as those maintained by the National Center for Biotechnology Information (NCBI) or the UniProt consortium. To avoid computational bottlenecks, search algorithms accept parameters including phylogenetic restrictions (*e.g.*, to humans or mammals), specific information about the proteolytic digestion step used to generate the peptides, and details of the mass spectrometer including its ionization and mass analyzer methods and mass accuracy.

1.2.1.2 Difference gel electrophoresis

Difference gel electrophoresis (DIGE) is a modification of the 2DE technique using covalent fluorescent dyes to label up to three different samples for co-separation within a single gel. The most commonly used dyes, the cyanines Cy2, Cy3, and Cy5 (collectively branded as CyDyes), have an N-hydroxyl succinimidyl ester reactive group and selectively label the ε-amino group of a protein's lysine residues. Minimal labeling of a single lysine on approximately 3% of the available proteins is used to avoid deleterious effects on protein solubility experienced when the labeling reaction proceeds to completion. Dyes are size- and charge-matched to ensure co-migration to the same point on gels (Ünlü *et al.* 1997). In early DIGE experimentation, only pairs of samples labeled with Cy3 and Cy5 dyes were compared. Multiplexing samples in this manner reduced gel-to-gel variation substantially, with the DIGE process contributing approximately one-ninth of the inter-animal variation to a 2DE study of ten mouse liver homogenates (Tonge *et al.* 2001).

In recent years, the two-sample DIGE method described above was further developed to include labeling of an internal standard with Cy2 and co-separation of this standard with Cy3- and Cy5-labeled experimental samples. This standard is generated from a pool of all samples within one experiment, indicating the average protein expression over all samples. Use of the internal standard maximizes representation of all proteins present in the experiment (Alban *et al.* 2003). The internal standard can also be used in calculating the protein expression of all spots within the experiment as described below. The three-sample DIGE method is advantageous when compared to conventional 2DE as there is a significant further reduction in inter-gel variation.

The internal standard is a key component of both gel matching and spot quantitation using DeCyder™, a 2DE analysis software package designed specifically for DIGE (Gharbi *et al.* 2002). Within each gel, spots are detected first on the Cy2 internal standard image and then co-detected on Cy3 and Cy5 sample images. If any unique spots not present in the internal standard are detected on Cy3/Cy5 images, these spots are added to the Cy2 internal standard image. Matching between gels occurs using only the internal standard images which ensures highly accurate matches. Spot quantitation employs the internal standard as a normalization factor between images, with Cy3 or Cy5 sample volumes expressed as ratios of those spots versus the Cy2 spot from the same gel. These ratios can then be compared between gels as in conventional 2DE to yield a measure of spot expression changes over the experiment.

1.2.1.3 Gel-free proteomics using mass spectrometry

Gel-free methods for proteomics have gained acceptance as modern mass spectrometers have become both more affordable and more sensitive. LC-MS overcomes some limitations of 2DE in that proteins of all types can be analyzed, including basic and membrane proteins. Some of the dynamic range issues of 2DE apply to LC-MS, with even advanced spectrometers unable to identify low-abundance components in highly complex protein mixtures. Recent development of multidimensional separation technologies preceding MS analysis reduces the complexity of proteins examined in any single run. This approach is referred to as multidimensional protein identification technology (MudPIT) when it specifically employs 2-D LC before tandem MS (Washburn *et al.* 2001). The two dimensions of LC separation are usually strong cation exchange followed by reversed phase, sequentially separating proteins or peptides by their charge and hydrophobicity respectively.

The major limitation of LC-MS-based proteomics is the difficulty of precisely quantifying specific proteins between samples. Comparison and absolute quantitation require sample labeling with one of the following techniques: ICAT isotope tags (Gygi *et al.* 1999), iTRAQ isobaric tags (Ross *et al.* 2004), or $^{16}O/^{18}O$ isotope tags (Heller *et al.* 2003). Each of these tagging methods applies to a subset of

proteins studied; for example, ICAT labeling occurs at peptide cysteine residues, so peptides not containing cysteine will not be measured. Label-free protein quantitation using peptide intensities is becoming more frequently used, but requires computationally-intensive processing and tight reproducibility between LC runs (Wang *et al.* 2003a; Wiener *et al.* 2004; Old *et al.* 2005). Multiple reaction monitoring (MRM) is a technique for very accurate label-free MS quantitation of candidate proteins which has been recently applied to biomarkers (Anderson and Hunter 2006). MRM is not yet widely used as it requires the use of high-end triple-quadrupole mass spectrometers. The potential of this technique is in simultaneously measuring differential expression of multiple proteins. It is ideal for validation of existing biomarker sets, not for discovery of new markers, as it requires *a priori* information about the proteins being measured.

A significant number of biomarker discovery efforts have employed a proprietary type of MALDI-TOF MS called SELDI-TOF (surface-enhanced laser desorption/ionization). Using this method, complex protein samples react with SELDI chips containing any of a variety of surface chemistries (*e.g.*, cation and anion exchange, hydrophobic, metal-binding, or functionalized antibody or protein surfaces) to bind proteins according to their functional groups. The masses and intensities of the bound peptides/proteins are detected using a modified MALDI-TOF mass spectrometer. This method can be used for comparative proteomics but its quantitation is based upon peak intensities, providing only an estimate of a relative abundance change between samples. The major drawback of SELDI is that identification of proteins of interest is not possible as the biomarkers determined are comprised only of mass-to-charge patterns, though recent work using hybrid MS interfaces has led to on-chip identification of some small proteins (Caputo *et al.* 2003). This technique gained recognition after a serum proteomic study used SELDI patterns to diagnose ovarian cancer (Petricoin *et al.* 2002). However, numerous sources of bias were soon found within these results including variations in sample preparation and instrument analysis procedures (Baggerly *et al.* 2004). This led to widespread skepticism of SELDI specificity. Despite these major limitations, SELDI developed the concept of "pattern as a biomarker" in biomarker discovery studies. The emphasis now is not on pattern recognition in MS spectra, but on patterns of specific protein changes associated with disease states.

1.2.2 Statistical considerations for 2DE analysis

Proteomic analyses, like genomic methods, involve comparisons of a large number of factors from within a smaller number of samples. This contrasts with typical candidate-based approaches which compare a few factors from a large number of individuals. Many of the statistical and bioinformatic concepts developed for microarray analysis address this large number of comparisons. These concepts have only recently begun to be applied to proteomic research.

1.2.2.1 Power and sample size

Comparative 2DE studies have been criticized for their high gel-to-gel variability and the low numbers of experimental replicates (Valcu and Valcu 2007). These factors limit the statistical power of conventional 2DE approaches (Hunt *et al.* 2005). Addition of technical replicates does not increase power as much as additional biological samples (Horgan 2007). To detect significant protein changes between different groups, power analysis should be performed when planning comparative 2DE studies. Although these calculations may indicate that a large number of biological samples are required, conducting large 2DE studies is not always feasible due to the low throughput, labor-intensive nature of 2-D gel proteomics and the variation introduced through these experimental methods (Valcu and Valcu 2007).

1.2.2.2 Analysis of individual protein spots

Classical statistical approaches begin with a hypothesis and test the goodness of fit of the data to the hypothesis. When these classical approaches are used on proteomic data, the hypothesis is tested on each spot within the experimental dataset, usually between 500 and 1000 spots. The sheer number of comparisons leads to a high number of spots fitting the hypothesis by chance; that is, a large number of false positive spots with significant *p*-values. For example, in an experimental dataset of 500 to 1000 spots, significance testing at the 5% level ($p < 0.05$) would lead to 25 to 50 spots having significant changes originating purely by chance. These are termed Type I errors, identifying a significant difference where none exists.

Statistical techniques designed to correct for multiple comparisons can reduce the high Type I error rate, also called the false discovery rate (FDR). The most commonly used method is the Bonferroni correction which divides the desired *p*-value by the number of individual comparisons, thus raising the level of significance required for a result to be considered truly valid. However, this correction is very conservative, and other methods have evolved to perform similar corrections to the FDR without this level of strictness (Benjamini and Hochberg 1995; Broadhurst and Kell 2006).

Proteomic studies using 2-D gels generally identify proteins of interest by examining spots exhibiting the greatest fold change, or ratio of spot volume between diseases/experimental conditions. More recently, studies have found significant differences between conditions by applying univariate analysis (Alfonso *et al.* 2005). These approaches assume that protein differences of interest will be characterized by moderate up- or downregulation, or presence or absence, of individual protein isoform spots. A univariate approach may fail to uncover combinations of individual spots that

together allow discrimination of disease states. Single-spot approaches are also susceptible to influence by experimental artifacts (Li *et al.* 2002; Marengo *et al.* 2004).

1.2.2.3 Generation of multiprotein classifiers

In contrast to univariate methods, machine learning methods generally fit a hypothesis to data. These multivariate methods can be further separated into two categories: supervised methods, which utilize *a priori* knowledge about the group membership of the samples (healthy or disease), and unsupervised methods that do not use this knowledge.

The concept of classification of clinical samples using proteomic patterns is most frequently associated with MS-based studies in which a mass pattern classifies samples into disease or healthy groups (Anderson *et al.* 2003). However, this approach also has merit in 2DE proteomic studies (Broadhurst and Kell 2006). Few 2DE studies have analyzed experimental data using tools which detect patterns of smaller concomitant changes in groups of protein spots; those that do have used unsupervised algorithms that did not use information about the analyzed samples (Taylor and Giometti 1992; Dowsey *et al.* 2003; Anderson *et al.* 2007). Principal component analysis is one such multivariate method that has been used to reduce the dimensionality of 2DE data (Marengo *et al.* 2004; 2006), but as this is an unsupervised method, it is not appropriate for classifier generation (Sajda 2006). Supervised multivariate informatic techniques previously used in microarray analysis could be used to find groups of spots which classify gels according to the presence or absence of disease (Guyon *et al.* 2002; Meunier *et al.* 2005; Pan 2005; Rowell *et al.* 2005; Sajda 2006). This class of techniques has not yet been applied to the analysis of 2DE data.

1.2.3 Biological systems for proteomic studies

Nearly any biological model can be employed for proteomic studies. *In vitro*, both primary and immortalized cell lines are often studied under different experimental conditions. These systems are less variable than human samples but cannot be directly related to organ or whole body physiology.

Proteomic techniques can also be applied to tissue specimens from animal models or from humans themselves. Studies involving human tissues often make use of small biopsies obtained during surgical intervention. As such, these samples can represent the pathology of human disease, but are not directly applicable to most diagnostic tools. Blood perfuses every organ and is responsible for the majority of transport functions within the human body. As such, blood can reflect changes in organ function in disease states. Consequently, plasma, a readily accessible and cheaply obtained diagnostic specimen present in comparatively large quantities, has garnered a great deal of attention

from proteomic researchers. The remainder of this section will discuss methods for studying the plasma proteome.

1.2.3.1 Plasma proteomics

Plasma and serum have been the subject of numerous proteomic studies since the technique's introduction (O'Farrell 1975). Around twenty distinct plasma proteins were first identified using tube gel-based 2DE in 1977 (Anderson and Anderson 1977); fifteen years later, with the use of more reproducible IPG-based 2DE, double that number of proteins were identified by microsequencing (Hughes *et al.* 1992). Recently, chromatographic prefractionation and 2DE have increased this number to more than 300 distinct serum proteins from nearly 3700 spots (Pieper *et al.* 2003b). Identification of substantially more plasma proteins has been achieved by reducing the dynamic range of proteins present to better fit the range of protein concentrations detectable using standard 2DE analyses.

Within the last five years the Human Proteome Organization (HUPO) has undertaken a Plasma Proteome Project to more fully characterize the proteins present in plasma. This project's pilot phase initially compared a number of sample types and preparations using a wide variety of analytical methods, including both gel-based and gel-free approaches. Pilot phase results were released in 2005 and drew attention to the importance of standardized sample collection and to the effects of *ex vivo* specimen processing on the plasma proteome (Rai *et al.* 2005). Furthermore, it became evident that fractionation of the incredibly complex plasma proteome was essential in order to find proteins of interest below the level of high abundance plasma proteins such as albumin, immunoglobulins and transferrin (Omenn *et al.* 2005). These fractionation strategies are discussed in an upcoming section (Section 1.2.3.3).

1.2.3.1.1 Serum versus plasma

A perennial question in proteomic analysis of blood samples concerns the use of serum or plasma sample types. Serum and plasma are derived from whole blood that has been separated from the cellular component and, in the case of serum, from clotting factors. Serum is collected in a plain vessel without additives and allowed to clot *ex vivo* for anywhere between several minutes and a few hours before separation. Due to this clotting process, it has been noted that "sera, by nature, is an *ex vivo* artifact produced by the activation of the proteolytic coagulation cascade" (Marshall *et al.* 2003). In contrast, plasma is collected in the presence of anticoagulants which can include heparin, EDTA, or sodium citrate.

Following the release of results from the HUPO Plasma Proteome Project, the general consensus is that plasma may provide more consistency across multiple samples than serum as it is not subjected to the *ex vivo* clotting process by which serum is created. Plasma also does not contain additional proteases and peptides derived from the clotting process, which may contribute up to 40% of the serum proteome under 15 kilodaltons (kDa) (Rai *et al.* 2005). Plasma is not completely free of these *in vitro* artifacts. Clotting factors are still present in solution in plasma samples and may precipitate at low anticoagulant concentrations such as those occurring during sample preparation for downstream analyses (Drake *et al.* 2004).

Consistency in blood collection and specimen handling is consequently critical when preparing blood specimens for proteomic research. Since the advent of widespread serum/plasma proteomic studies, several groups including HUPO have reported on optimized sample collection and processing procedures to reduce experimental variability. For example, the use of protease inhibitors at the time of sample collection has been recommended (Hulmes *et al.* 2004; Rai *et al.* 2005), but is not always feasible; their cost may be prohibitive for large studies. Consistency in sample handling is the most essential factor for reproducible, precise results. For longitudinal studies, this consistency must be introduced early in the study design process.

1.2.3.1.2 The dynamic range challenge

Plasma may contain most, if not all, of the proteins present in the human body. Some proteins are present in very large amounts (>mg·mL^{-1}), including albumin, immunoglobulin G (IgG), transferrin, α-2-macroglobulin and fibrinogen (Figure 1-2). Plasma is also comprised of many very low abundance proteins (<pg·mL^{-1}) such as interleukins and interferons. This is a concentration difference of more than one billion-fold. When this dynamic range is combined with the myriad of sources of plasma proteins, plasma becomes one of the most complex samples to be analyzed using proteomics (Anderson *et al.* 2004).

Highly abundant plasma proteins prohibit detection of low-abundance proteins using 2DE. The 2DE technique itself can resolve proteins with concentrations differing by at most two to four orders of magnitude (Anderson and Anderson 2002). This concentration range is necessarily limited by the most abundant proteins present in the sample. In a raw plasma sample, the 2DE-analyzable range covers protein concentrations from 1 μg·mL^{-1} to 10 mg·mL^{-1} (*cf.* Figure 1-2, 1×10^{6} to 1×10^{10} pg·mL^{-1}). 2DE detection technologies have a different limit of detection which reaches as little as 1 ng of protein. A typical 2-D gel of unfractionated plasma would separate 250 μg of plasma. Using a total plasma protein concentration of 50 mg·mL^{-1}, this 250 μg gel load requires 5 μL of plasma. On this typical gel, a 1 ng spot would represent 1/250 000 of the protein present, reflecting a circulating

concentration of at most 200 ng·mL^{-1} (2×10^5 pg·mL^{-1}). A protein present at such a low concentration in raw plasma would not be resolved on a typical 2-D gel due to the limited concentration range analyzable by this method, even though the sensitive detection technologies could find such a spot if it were resolved. The increased theoretical sensitivity offered by the 1 ng detection method is lost due to the tendency of highly abundant proteins to overwhelm the 2DE image. Additional simplification of plasma samples prior to 2DE is necessary to extend the range of analyzable proteins to reach less abundant species.

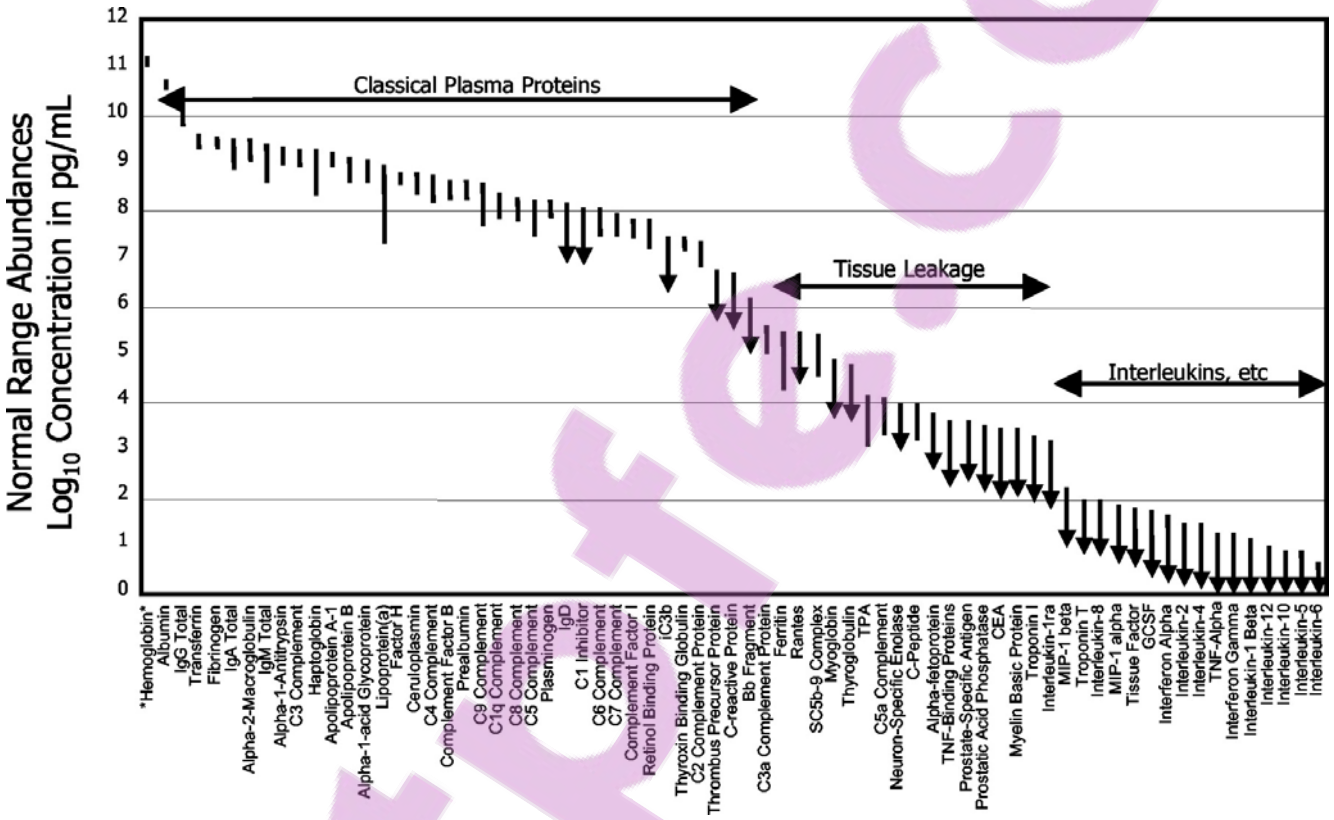

Figure 1-2: Concentration ranges of 70 plasma proteins

Diagram indicating the spectrum of protein concentrations at which 70 plasma proteins are present. Arrows indicate several broad categories of proteins including so-called classical plasma proteins, tissue leakage markers, and interleukins. Abundance is plotted on a log scale spanning 12 orders of magnitude. Reprinted from Anderson and Anderson 2002, Figure 3 (corrected in Anderson and Anderson 2003) with permission from the American Society for Biochemistry and Molecular Biology.

Addressing the dynamic range challenge in plasma/serum requires fractionation of the complex protein mixtures before 2DE. Although serum and plasma proteins can be fractionated by nearly any biophysical characteristic, the yields and reproducibility required for subsequent analyses have limited the methods in common use. Almost all proteomic research involving serum/plasma now specifically removes high-abundance proteins including albumin, IgG, and transferrin using immunoaffinity depletion approaches. Detailed information on this method can be found in Chapter 4.

1.2.3.1.3 Previous attempts to identify disease biomarkers in serum or plasma

Serum and plasma have been the subjects of intensive biomarker discovery efforts for many diverse disorders. Undepleted serum from patients with hepatitis B virus in immune-tolerant or immune-clearance phases was compared using 2DE, with differential expression in several abundant serum glycoproteins produced by the liver. Isoform pattern alterations were found for haptoglobin, apolipoproteins A-I and A-IV, α-1-antitrypsin, transthyretin, and DNA topoisomerase II, reflecting the involvement of these proteins in the hepatic disease and viral infection processes (He *et al.* 2003). 2DE has also been used to find differential protein expression in pooled serum from individuals with toxic oil syndrome, a complex disorder resulting from the illegal denaturation of rapeseed oil in Spain in 1981. Thirty-five protein differences were found in albumin-depleted pooled serum, including isoform pattern changes in haptoglobin. Haptoglobin is common to the two studies described above, which may be due to its involvement in inflammatory diseases (Quero *et al.* 2004).

DIGE has been applied to serum and plasma 2DE biomarker discovery studies as well. One elegant example compared depleted serum from patients with pancreatic cancer or benign pancreatic disease. Differential expression patterns of inter-α-trypsin inhibitor, α-1-anti-chymotrypsin, and apolipoprotein E spots were identified using DeCyder software. Concentration differences in these proteins were confirmed using western blot analysis of a separate set of serum samples. These three proteins were each relatively good biomarkers for discriminating between pancreatic cancer serum and control serum, but a model combining all three markers performed better than any individual marker (Yu *et al.* 2005). This example not only confirms the utility of 2DE and DIGE methods for serum/plasma biomarker discovery, but also underscores the importance of seeking combinations of markers rather than single proteins.

A portion of the research described in this thesis involves biomarker discovery in serum and plasma. A number of methods enabling comparison of serum and plasma proteomes were developed as part of this work. Immunoaffinity methods allowing depletion of highly abundant serum/plasma proteins were compared. Further development resulted in the successful separation of depleted serum proteins using 2DE. These methods were applied to a 2DE study of serum and to a study of plasma using DIGE, both of which compared samples from women with the pregnancy complication preeclampsia, which will be discussed further in the following section. Further details on these studies are provided in Chapters 4, 5, and 6.

1.3 Preeclampsia

The research described in this thesis focuses on proteomic biomarker discovery directed at the pregnancy disorder preeclampsia. The work is part of the SCOPE (SCreening fOr Pregnancy Endpoints) project, an international multicenter initiative to identify candidate biomarkers for this and other pregnancy disorders such as spontaneous preterm birth and small for gestational age (SGA) babies. The following sections will introduce the etiology of preeclampsia, specifically focus on pathophysiological changes associated with the disorder at the placental level, and discuss the maternal response to these placental changes. Previously characterized candidate markers for the diagnosis of preeclampsia will also be introduced.

1.3.1 Background

Preeclampsia is a complex multisystemic syndrome characterized by pregnancy-induced hypertension and proteinuria. In all cases, the only effective treatment is delivery of the baby or pregnancy termination. Delivery of the placenta leads to resolution of the clinical symptoms of preeclampsia, indicating that placental factors play a major role in the pathogenesis of the disease. This disorder is a leading cause of maternal mortality and morbidity (Geller *et al.* 2004; Khan *et al.* 2006), affecting approximately 3–5% of pregnant women (Baumwell and Karumanchi 2007; Magnussen *et al.* 2007). Maternal complications include disseminated coagulopathy, acute renal failure, liver impairment, pulmonary oedema, and seizures (eclampsia). Babies born to preeclamptic mothers may also be affected; a third are born preterm, 20% are growth restricted, and perinatal deaths are increased three- to ten-fold (Hauth *et al.* 2000; Sibai *et al.* 2005).

Development of preeclampsia is associated with increased maternal long-term health risks of later hypertension, stroke, and ischemic heart disease (Smith *et al.* 2001; Wikström *et al.* 2005; Bellamy *et al.* 2007). Women who had preeclampsia have evidence of the metabolic syndrome several years later, including insulin resistance, dyslipidemia, and higher blood pressure, body mass index and leptin levels (Gratacós *et al.* 2003; Forest *et al.* 2005; Girouard *et al.* 2007).

Preeclampsia is defined by gestational hypertension (new occurrence of high blood pressure after the 20th week of pregnancy) in combination with proteinuria (the presence of significant protein in the urine) or other multisystem complications (Brown *et al.* 2000a, 2000b). It is a spectrum of maternal responses to a pathological pregnancy rather than a single disease (Redman and Sargent 2005). It may present as a mild condition with asymptomatic hypertension and proteinuria through to a severe life threatening condition with multiorgan involvement, such as HELLP syndrome (hemolytic anemia, elevated liver enzyme activity, low platelet count) (Weinstein 1982).

Preeclampsia is increasingly classified into early-onset and late-onset disease subtypes, and/or as preeclampsia with or without SGA, according to the clinical phenotype (Groom *et al.* 2007; Oudejans *et al.* 2007).

Table 1-2: Early-onset versus late-onset subgroups of preeclampsia

	Early-onset	*Late-onset*
Gestational age of symptom onset	< 34 weeks	> 34 weeks
Relative frequency	30-40%	60-70%
Placental morphology	abnormal	normal
Etiology	placental ± maternal	maternal

Adapted from Oudejans *et al.* 2007.

Diagnosis of preeclampsia by the physician is complicated by the syndrome's heterogeneous symptoms, currently relying on the observation of a set of specific symptoms and signs. This process is similar to diagnosing a myocardial infarction on the basis of chest pain and associated cardiovascular signs. In this setting, the additional use of blood tests for markers that identify end organ complications (*e.g.*, troponins T and I) assists the clinician in distinguishing myocardial infarction from angina and other causes of chest pain (Jaffe 2007). Similarly, clinicians caring for women with the symptoms and signs of preeclampsia would be assisted if a biochemical test were available to confirm or refute the diagnosis. Additionally, elevated levels of troponins in patients with cardiac disease can also indicate poor cardiac prognoses (Sarko and Pollack 2002); given the linkages between preeclampsia and later cardiovascular and metabolic disorders, there is the potential for biochemical markers to identify women at increased risk of subsequent medical problems (Sattar *et al.* 1997; Girouard *et al.* 2007).

Though there is no curative agent for preeclampsia, antiplatelet agents including aspirin decrease the risk of the disorder (Askie *et al.* 2007; Duley *et al.* 2007). Calcium supplementation can also reduce both the risk and the severity of preeclampsia symptoms (Hofmeyr *et al.* 2006; Villar *et al.* 2006). Before propylaxis can be offered, women at increased risk of preeclampsia need to be identified. There are a number of widely recognized risk factors.

1.3.2 Risk factors

A number of risk factors contribute to an increased probability of development of preeclampsia (Duckitt and Harrington 2005). The majority of women who develop preeclampsia are nulliparous (Chesley 1984; Sibai *et al.* 2005). Risk is increased in women with multiple gestations (Coonrod *et al.* 1995), in women who have had preeclampsia in a previous pregnancy (Sibai *et al.* 2005), and with increasing maternal age (Zhang *et al.* 1997). Obesity is one of the most consistent risk factors,

associated with a two- to three-fold increased risk (O'Brien *et al.* 2003; Duckitt and Harrington 2005; LaCoursiere *et al.* 2005). Paradoxically, maternal smoking decreases the incidence of preeclampsia, although it does increase incidence of SGA (Cnattingius *et al.* 1997; North *et al.* 2000). Existing medical conditions, often with related pathophysiology, can predispose to preeclampsia. These include chronic hypertension, renal disease, diabetes (types 1 and 2) , systemic lupus erythematosus, and thrombophilias (Dekker and Sibai 2001; Clausen *et al.* 2005; Kupferminc 2005; Pabinger and Vormittag 2005).

Immunological interactions between parents play a key role in preeclampsia. From the mid nineties, it was recognized that the incidence of preeclampsia may decrease with increased duration of maternal exposure to paternal antigens prior to conception (Robillard *et al.* 1994; Einarsson *et al.* 2003). Preeclampsia was 17 times more common in women who cohabited with their child's father and used barrier contraceptive methods for less than four months compared to those who had been together more than 12 months (Einarsson *et al.* 2003). Changing paternity was thought to be associated with an increase in risk of preeclampsia, but more recently this has been questioned (Robillard *et al.* 1993; Lie *et al.* 1998; Skjaerven *et al.* 2002; Deen *et al.* 2006). Changing paternity interacts with birth interval, and a longer interval between pregnancies is also associated with an increased risk of preeclampsia (Skjaerven *et al.* 2002; Conde-Agudelo *et al.* 2007). After adjustment for birth interval, changing paternity was no longer associated with this increased risk (Trogstad *et al.* 2001; Skjaerven *et al.* 2002). Assisted reproduction data also support the importance of paternal antigen exposure in influencing the maternal risk of preeclampsia (Dekker and Robillard 2005). Preeclampsia is more common in women undergoing assisted reproduction using donated sperm, oocytes, or embryos (Wang *et al.* 2002a; Sibai *et al.* 2005). It is likely the maternal immunological response to the fetus plays a role in the development of abnormal placentation in preeclampsia (Section 1.3.3.3).

The increased frequency of preeclampsia in women whose mothers, sisters, and daughters suffered from preeclampsia strongly suggests a genetic component to the disorder (Cincotta and Brennecke 1998). This is also evident from population studies where offspring from a preeclamptic pregnancy were more likely to be involved in a preeclamptic pregnancy themselves as parents (Chesley and Cooper 1986; Esplin *et al.* 2001). Men who had previously fathered a pregnancy complicated by preeclampsia had an increased risk of a future partner developing the disorder (Lie *et al.* 1998). SNPs in multiple candidate genes have been associated with preeclampsia, but to date no universal susceptibility genes for preeclampsia have been identified (Sibai *et al.* 2005; Chappell and Morgan 2006; Goddard *et al.* 2007). Interaction between maternal and fetal genotypes is likely to be important (Goddard *et al.* 2007).

1.3.3 Pathophysiology

Preeclampsia involves numerous changes in both placental and maternal pathophysiology. It has been proposed that preeclampsia may result 1) when an abnormally functioning placenta incites a pathological response from an otherwise healthy mother ("placental" preeclampsia), 2) from an interaction of a normal placenta with a mother who is genetically or metabolically predisposed to microvascular disease ("maternal" preeclampsia), or 3) a combination of placental pathology with a maternal predisposition (Redman and Sargent 2005; Sibai *et al.* 2005; Oudejans *et al.* 2007). The following sections describe the roles of the placenta and the maternal vasculature in the disorder, as well as circulating factors linking these areas.

1.3.3.1 The placenta

Preeclampsia occurs in abdominal (extrauterine) pregnancy and hydatidiform mole (a nonviable fetus sustained by a developing placenta), indicating that the placenta, rather than the uterus and fetus, is fundamental to the syndrome (Chun *et al.* 1964; Redman 1991; Piering *et al.* 1993). Preeclampsia resolves after delivery of the placenta, supporting the importance of placental development in the disease (Brosens *et al.* 1977; Sibai *et al.* 2005).

The placenta is a transient organ formed during pregnancy which provides an exchange surface between the maternal bloodstream and the fetal circulation. Trophoblast cells, found only in the placenta, differentiate to fulfill specialized roles in placental biology (Figure 1-3). The placenta contains two types of chorionic villi made of trophoblasts: 1) anchoring villi, which attach the placenta to the uterine decidua and are the site of remodeling of the maternal spiral arteries, and 2) floating villi, where material exchange between the maternal and fetal circulations occurs (Myatt 2002).

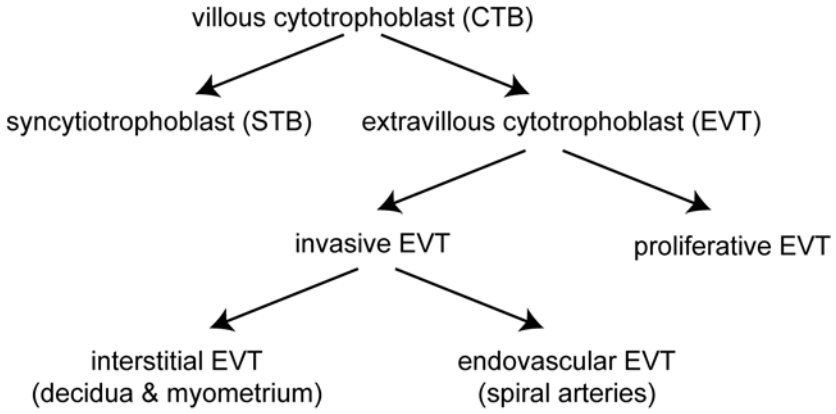

Figure 1-3: Trophoblast differentiation pathways

The differentiation pathways leading to formation of syncytiotrophoblast and extravillous trophoblast cells. Both floating and anchoring villi contain CTB and STB. EVT from anchoring villi cell columns continue to differentiate into invasive and proliferative EVT forms. Adapted from Myatt 2002.

Chorionic villous structures contain a stromal core with fetal blood vessels surrounded by a basement membrane and villous cytotrophoblast (CTB) progenitor cells. These chorionic villi differentiate into villous trees containing floating and anchoring villi. In both types of villi, CTB cells fuse to form a multinucleate syncytiotrophoblast (STB) which covers the villi in an epithelial layer (Figure 1-4). In floating villi the STB acts as the main site of hormone, nutrient, gas, and waste exchange with maternal blood (Hoshina *et al.* 1982; Pötgens *et al.* 2002). Columns of extravillous cytotrophoblasts (EVT) extend from the tips of anchoring villi into the decidual layer of the maternal uterine lining and are responsible for the physical connection between the placenta and the mother. Endovascular CTB also replace the endothelial lining of uterine spiral arteries, remodeling them into low-resistance vessels and providing STBs with greater access to maternal blood (McMaster *et al.* 2004). This remodeling process is a key step in early placentation and is vital to supplying maternal blood to the intervillous space, the location of maternal-fetal transport processes.

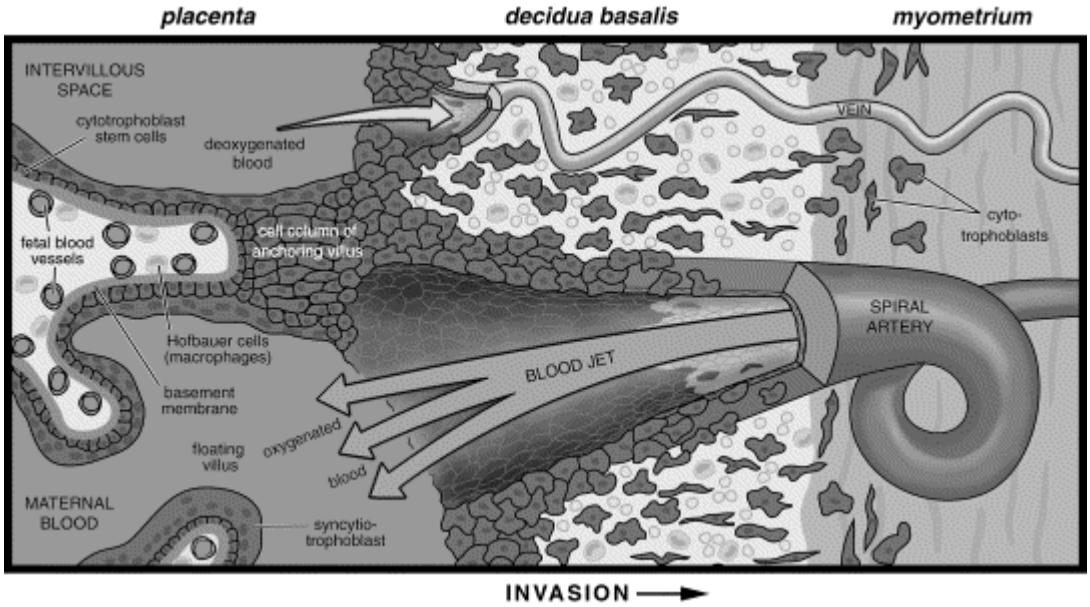

Figure 1-4: Placental invasion of uterine spiral arteries

Diagram illustrating the remodeling of a maternal spiral artery by extravillous CTB within an anchoring villus. A floating villus, not invading the decidua, is shown at the bottom left. Oxygenated blood perfuses the intervillous space, contacting syncytiotrophoblast cells on the outer layers of the villi and allowing nutrient, gas, and waste exchange and placental hormone secretion. Reprinted from Zhou *et al.* 2003, Figure 1, with permission from Elsevier.

In addition to its role in nutrient exchange, the placenta is also an endocrine organ. It secretes many pregnancy-related peptide and steroid hormones including hCG, estrogen, progesterone, and relaxin among others (Sherwood 1997). The placenta is integrated into the maternal bloodstream, and has such a major role in hormone secretion that most placentally-secreted hormones are detectable in maternal blood. These hormones can provide valuable information about placental function. Standard pregnancy tests, for example, measure hCG levels in blood and can be used to track the development of the placenta.

Cytotrophoblast differentiation processes leading to the invasion and remodeling of maternal spiral arteries are controlled in part by the oxygen tension surrounding the cells. In the first few weeks of pregnancy, spiral artery remodeling has not yet occurred so placental cells are exposed to a relatively hypoxic environment. Under these conditions CTB favor proliferation over differentiation. Once CTB reach and remodel the spiral arteries, blood flow to the intervillous space creates the circulatory link between the placenta and the maternal circulation by 10–12 weeks of gestation (James *et al.* 2006). With the establishment of this blood flow, the oxygen tension of the placental environment increases and the CTB phenotype changes to favor differentiation rather than proliferation (Genbacev *et al.* 1997; Robins *et al.* 2007).

1.3.3.2 Placental changes in preeclampsia

A primary hallmark of preeclampsia is the shallow invasion of maternal spiral arteries in the decidua by placental trophoblasts, which is termed abnormal or inadequate placentation (Brosens *et al.* 1972; Brosens *et al.* 1977). Placental bed biopsies from preeclamptic women have shown not only limited depth of spiral artery invasion but also a lack of arterial remodeling from high-resistance muscular structures to low-resistance vessels (Khong *et al.* 1986; Khong 2004). This inadequacy may be in part due to a failure of epithelial trophoblasts to adopt an endothelial phenotype when remodeling these arteries (Zhou *et al.* 1997b; Zhou *et al.* 1997a; McMaster *et al.* 2004). Acute atherosis, the relative obstruction of uterine spiral arteries by foam cell lesions, can be observed at the uteroplacental junctions in preeclampsia (Pijnenborg *et al.* 2006). These vascular changes resemble those seen in atherosclerosis.

In normal pregnancy, invasive trophoblasts express a specific series of cell adhesion molecules, including integrins that bind to extracellular matrix proteins including fibronectin, laminin and collagen (Damsky *et al.* 1994). In preeclampsia, there is disruption of the organised expression of integrins, cadherins, and vascular and platelet cell adhesion molecules by invasive trophoblasts (Lim *et al.* 1997; Zhou *et al.* 1997b; Zhou *et al.* 2002). It has been suggested that this may result from continued expression of epithelial cadherin (E-cadherin) and failure to express vascular epithelial cadherin (VE-cadherin), reduced expression of $\alpha 1/\beta 1$ integrin that binds to laminin, and decreased secretion of matrix metalloproteinase 9. Not all researchers have been able to reproduce these findings (Lyall 2006).

One mechanism that is likely to be important in the abnormal placentation seen in preeclampsia is the interaction between maternal decidual natural killer (NK) lymphocytes and the invading trophoblasts. NK cells have a role in the facilitation or inhibition of EVT migration and invasion into the decidua. EVTs express a unique combination of human leukocyte antigens (HLAs) including HLA-G, HLA-E and HLA-C. Killer immunoglobulin-like receptors (KIRs) on NK cells bind to HLA-C allotypes on the trophoblast, thereby modifying their expression of cytokines, metalloproteinases, and growth and angiogenic factors that then assist or inhibit invasion. Specific combinations of polymorphisms in the HLA-C and the KIR are associated with an increased risk of preeclampsia. Specifically, the combination of maternal KIR AA and fetal HLA-C2 genotypes occurs in 45% of preeclamptic women versus 27% of those with a healthy pregnancy (Hiby *et al.* 2004; Moffett and Hiby 2007).

When trophoblast invasion and spiral arterial remodeling are suboptimal, as occurs in preeclampsia, the placenta is exposed to ischemic reperfusion injury. The ischemic placenta releases a number of

factors into maternal circulation and may modify maternal white blood cells as they pass through the intervillous space, thereby activating inflammatory changes and triggering widespread damage to the vascular endothelium.

1.3.3.3 Placentally-derived factors in the etiology of the maternal syndrome

There are a number of potential mediators that acts as the link between placental disease and the maternal endothelial and inflammatory response seen in preeclampsia (Redman and Sargent 2005). The existence of one or several vasoactive factors, produced by the placenta and secreted into the maternal bloodstream, has been postulated (Rodgers *et al.* 1988). The STB, the main site of placental hormone secretion, may produce such a vasoactive factor and ciculate in the maternal vasculature (Hayman *et al.* 2001b; Myers *et al.* 2005a). The effects of preeclamptic plasma, presumably containing a vasoactive circulating factor, on endothelium has been assessed using cell-based (Brockelsby *et al.* 2000) and vessel-based (Hayman *et al.* 2000; Hayman *et al.* 2001a; Myers *et al.* 2005a) approaches. Myographic studies on isolated arteries have demonstrated attenuation of endothelial relaxation responses following exposure to preeclamptic plasma. To date, the circulating factor responsible for these effects has not been identified (Myers *et al.* 2005b).

Key candidate circulating factors include placentally-derived angiogenic factors. Soluble fms-like tyrosine kinase 1 (sFlt1) is an anti-angiogenic circulating receptor for placental growth factor (PlGF) and vascular endothelial growth factor (VEGF) thought to be derived from the placenta. Binding of sFlt1 to these growth factors neutralizes their pro-angiogenic actions (Venkatesha *et al.* 2006). sFlt1 was upregulated in placental tissue from women with preeclampsia analyzed using microarrays (Maynard *et al.* 2003). Its administration to pregnant rats causes hypertension, proteinuria, and glomerular endotheliosis, all characteristic signs of preeclampsia (Maynard *et al.* 2003), but does not cause hemolysis or thrombocytopenia, features of HELLP. Circulating levels of sFlt1 are increased in preeclamptic women up to five weeks before clinical symptoms have manifested (Levine *et al.* 2004a). Another placentally-derived angiogenic factor implicated in the development of preeclampsia is soluble endoglin (sEng). It is a co-receptor for TGF-β1 and β3 (Levine *et al.* 2006). Administration of sEng to pregnant rats also induces hypertension, and when combined with sFlt1 produces HELLP-like symptoms (Venkatesha *et al.* 2006). Circulating levels of sEng are also elevated prior to the onset of preeclampsia (Levine *et al.* 2006).

There is increased release of trophoblast debris from the placenta into the maternal circulation in preeclampsia. This debris contains cytotrophoblast cells, microparticles from STB membranes, cytokeratin fragments, and free fetal DNA which originate through processes of syncytial renewal (Knight *et al.* 1998; Zhong *et al.* 2002; Huppertz *et al.* 2003). Circulating microparticle levels are

elevated in preeclamptic women over those with a healthy pregnancy (Van Wijk *et al.* 2002), and there is some evidence that this elevation is more marked in early-onset versus late-onset preeclampsia (Goswami *et al.* 2006). Increased levels of fetal DNA have been detected in maternal circulation prior to the onset of preeclampsia and could be a predictive marker for this disorder (Zhong *et al.* 2002; Cotter *et al.* 2004; Farina *et al.* 2004; Levine *et al.* 2004b).

1.3.3.4 Maternal vascular and inflammatory responses

While its origins may lie in the placenta, preeclampsia manifests as a disorder of an "abnormal vascular response to placentation" (Sibai *et al.* 2005). Endothelial cell dysfunction, enhanced platelet aggregation and an intravascular inflammatory reaction occur. This results in increased vascular resistance, hypertension, capillary leakage and a coagulopathy (Sibai *et al.* 2005). Necrosis and hemorrhage may occur in the brain, liver, and adrenal glands as a result of the associated vascular changes (Roberts and Lain 2002).

Endothelial changes in the kidneys, termed "glomerular endotheliosis", which are seen with no other form of hypertension occur during preeclampsia (Pridjian and Puschett 2002). Markers of maternal endothelial damage are detectable in the blood of preeclamptic women including increased levels of fibronectin and endothelin, intercellular and vascular cell adhesion molecules (ICAM and VCAM), von Willenbrand factor, and plasminogen activator inhibitor-1 (PAI-1) (Stewart *et al.* 2007).

Activation of the inflammatory cascade is a normal part of pregnancy, but in preeclampsia there is an exaggeration of the inflammatory response to pregnancy (Sacks *et al.* 1998; Redman *et al.* 1999). This excessive inflammatory response may relate to the increased traffic of "foreign material" derived from the placenta or possibly represents an intrinsic inability of the mother to regulate the immune reponse to circulating debris (Redman and Sargent 2005; Meziani *et al.* 2006). Recent work by Germain *et al.* (2007) has shown that the increased level of syncytiotrophoblast microvillous membranes (STBM) in maternal blood in preeclampsia is associated with an increase in circulating inflammatory cytokines and interferon γ (IFN). This work also found that when compared to the non-pregnant state, normal pregnancy is associated with decreased circulating IFN levels and suppressed NK cell production of IFN. Germain *et al.* then showed that in preeclampsia, STBM stimulated monocytes to secrete inflammatory cytokines that then stimulated NK cell IFN production. In brief, this work showed that STBM could induce the systemic inflammatory changes seen in preeclampsia.

Preeclampsia is associated with increased circulating lipid peroxides and other factors indicating increased oxidative stress in the mother (Hubel 1997; Myatt and Cui 2004; Feinberg 2006).

Antioxidant defenses may be reduced in preeclamptic women as assessed by changes in serum levels of antioxidant markers (Kiilholma *et al.* 1984; Brophy *et al.* 1985; Chappell *et al.* 2002b; Atamer *et al.* 2005). The lipid profiles of preeclamptic women show increased levels of small, dense LDLs (low density lipoproteins) which are more susceptible to oxidative damage and peroxide formation (Walsh 2006). Oxidative stress may cause endothelial injuries similar to those seen in atherosclerosis, and this has been proposed as one possible cause for preeclampsia (Roberts and Lain 2002).

1.3.4 Biomarkers for preeclampsia

For over two decades, candidate markers for preeclampsia have been researched but as yet none have progressed into clinical management. Current preeclampsia biomarker discovery efforts can be broadly categorized into two groups: studies that seek markers for the disorder at the time of its clinical onset (*diagnostic* markers) and those that screen a population to identify prospective factors linked to an increased risk of developing the disorder (*screening* markers). Given the broad spectrum of placental and maternal involvement in the disorder, it is unlikely that changes in any single protein could account for or identify preeclampsia.

1.3.4.1 Diagnostic markers

At present the diagnosis of preeclampsia is based on the observation of symptoms and signs by clinicians. By the time of the disorder has manifested clinically, a large number of biochemical changes are taking place within a preeclamptic woman. Proteins with reproducible changes occurring at the time of preeclampsia undoubtedly reflect late pathogenic events, but may also be be the result of the underlying pathophysiological changes causing the disorder. These changes have the potential to be used as a diagnostic test.

Angiogenic factors have recently been investigated as potential diagnostic markers, specifically, the ratio of PlGF and its anti-angiogenic circulating receptor, sFlt1. PlGF may promote trophoblast migration, invasion, and proliferation (Thadhani *et al.* 2004a), and is present at lower levels in the placentas and blood of preeclamptic women compared to healthy pregnant women (Taylor *et al.* 2003; Thadhani *et al.* 2004a; Levine and Karumanchi 2005). Circulating levels of sFlt1 and the ratio of PlGF/sFlt1 are increased in preeclampsia (Levine *et al.* 2004a). The use of sFlt1/PlGF as a diagnostic or screening test is now being assessed in clinical trials (Salahuddin *et al.* 2007; Widmer *et al.* 2007; Wikström *et al.* 2007; RA North, personal communication). Recent research has suggested that combining measurement of sEng with the sFlt1/PlGF ratio may improve diagnostic performance (Levine *et al.* 2006; Venkatesha *et al.* 2006).

1.3.4.2 Screening markers

There is a need to not just diagnose preeclampsia, but to predict it in early pregnancy in order to identify women at risk who could be offered intervention to prevent the disorder. A number of biochemical changes in maternal blood are not only present once the disease is overt, but are also present before the onset of disease. Many of these changes are only present in the weeks leading up to diagnosis, but some are significantly different even before 20 weeks of gestation. Recently investigated screening markers for preeclampsia include factors related to angiogenesis, coagulation, lipids, placental hormones, cell adhesion, fetal DNA, inflammation, and growth factors.

Levels of circulating angiogenic factors, coagulation proteins, and lipids are affected by preeclampsia. Many studies have shown decreased levels of PlGF before 20 weeks of gestation in women who go on to develop the disorder (Taylor *et al.* 2003; Levine *et al.* 2004a; Bersinger and Odegard 2005). In contrast, sFlt1 levels are unchanged in early pregnancy in women with later preeclampsia (Levine *et al.* 2004a). Levels of the coagulation-related proteins PAI-1 and PAI-2 are increased and decreased respectively by 24 weeks of gestation in women with later preeclampsia (Chappell *et al.* 2002a; Clausen *et al.* 2002). Triglyceride levels are well-recognized as elevated during clinical preeclampsia, and this elevation is also observed in early pregnancy (Clausen *et al.* 2001; Chappell *et al.* 2002a). High density lipoproteins (HDL) are decreased before the onset of preeclampsia, whereas total cholesterol levels have been reported to be elevated, unchanged, and lowered in women destined to develop preeclampsia compared with healthy pregnant women (Gratacós *et al.* 1996; Chappell *et al.* 2002a; Enquobahrie *et al.* 2004).

Placental hormones are also present at changed levels prior to preeclampsia. Pregnancy-associated plasma protein A (PAPP-A) and inhibin A are placental hormones commonly assessed in early pregnancy as part of a screen for Down's syndrome. PAPP-A levels are reduced at 10-14 weeks of gestation in women from a general population who go on to develop preeclampsia (Bersinger and Odegard 2004; Dugoff *et al.* 2004). Inhibin A levels also show significant changes before 20 weeks of gestation, and have generally been found elevated prior to preeclampsia (Muttukrishna *et al.* 2000; Dugoff *et al.* 2005) though some studies have failed to find this (D'Anna *et al.* 2002). Activin A is a peptide related to inhibin A but with an opposing (enhancing) activity. Levels of activin A are generally elevated prior to preeclampsia when measured in nulliparous populations (Muttukrishna *et al.* 2000; Keelan *et al.* 2002), but not in populations of high-risk women (Blackburn *et al.* 2003; Ekbom *et al.* 2006). Levels of IGFBP-1, a growth factor-binding protein, are decreased at 16 weeks of gestation in women who later develop preeclampsia (Hietala *et al.* 2000).

Given the involvement of the maternal endothelium in the development of preeclampsia, markers of endothelial damage and inflammation have been studied as screening markers for the disorder. Cellular fibronectin, a marker of endothelial activation, is increased in preeclamptic women before 20 weeks of gestation (Chavarria *et al.* 2002). The cell adhesion molecules ICAM and VCAM, which also indicate endothelial damage, are also increased prior to preeclampsia (D'Anna *et al.* 2006), but the increased level of VCAM is not consistently found (Clausen *et al.* 2000). The soluble p55 receptor for tumor necrosis factor-α (TNF-α) is increased before 20 weeks of gestation in women who develop preeclampsia (Williams *et al.* 1999). Metabolic markers show changes in women destined to develop preeclampsia as well. Leptin levels are increased (Chappell *et al.* 2002a; Clausen *et al.* 2002; Ning *et al.* 2004) and both adiponectin (D'Anna *et al.* 2005; D'Anna *et al.* 2006; Odden *et al.* 2006) and sex hormone-binding globulin (Wolf *et al.* 2002; Thadhani *et al.* 2004b) decreased in early pregnancy in women with later preeclampsia.

Early detection of fetal DNA has also been assessed in relationship to development of preeclampsia. However, measurement of this DNA is complicated by the current inability to detect the DNA of female fetuses as it cannot be distinguished from that of the mother. Consequently measurement of the SRY locus present on the Y chromosome is used to assess circulating levels of male fetal DNA. These levels are increased before 20–25 weeks in preeclamptic women (Farina *et al.* 2004; Levine *et al.* 2004b).

Though a number of potential screening markers for preeclampsia are described here, differences in the populations studied, in study design/size/power, and in assay methods have all contributed to inter-study variation and inconsistencies in results. The absence of a robust, sensitive single screening marker is not surprising given the heterogeneity present in the pathophysiology of preeclampsia. As with diagnostic markers, it is unlikely that any single marker could predict the mixed presentations and potential causes of the disorder. It is now recognized that combinations of markers are needed to screen effectively for preeclampsia risk (Conde-Agudelo *et al.* 2004).

Recent studies have evaluated combinations of biomarkers for their ability to predict preeclampsia. The most common biomarker combinations already in use form part of a Down's syndrome screening set comprised of α-fetoprotein, estriol, inhibin A, and hCG. Combinations of abnormal levels of these placentally-derived factors have been associated with a greater risk of preeclampsia, but the occurrence of these biomarker combinations was extremely rare, leading to very low performance as a screening test (Dugoff *et al.* 2005). Many other studies examining Down's syndrome screening markers to predict preeclampsia have obtained samples retrospectively from biobanks established from poorly defined populations (Lambert-Messerlian *et al.* 2000; Zwahlen *et al.* 2007). The clinical relevance of these results is therefore difficult to interpret.

The combination of uterine artery Doppler ultrasonography, a measure of placental perfusion, with a modified quad screen measuring free hCG-β, PAPP-A, activin A, inhibin A, and PP13 has been investigated. The addition of some biomarkers, such as activin A and inhibin A, improved prediction compared with uterine artery Doppler alone (Spencer *et al.* 2006), whereas the addition of others, such as PP13, did not enhance prediction based on Doppler ultrasonography alone (Spencer *et al.* 2007). Several other biomarkers have been found to be altered in early pregnancy prior to the development of preeclampsia, but their usefulness in combinations as screening markers in larger populations has not been determined (Clausen *et al.* 2002; Levine *et al.* 2004b; Zwahlen *et al.* 2007).

To date, no biomarker combination has performed well enough for clinical application. Further research is necessary to identify additional combinations of markers that may predict the occurrence of preeclampsia. Suitably powered prospective studies in defined populations of women with well-characterized disease phenotypes are required for the validation of such biomarker combinations.

1.3.5 Previous proteomic biomarker discovery for preeclampsia

Proteomic techniques have recently been applied to discover novel associations of biomarkers with preeclampsia. The majority of these studies have used plasma collected at the time women presented with preeclampsia. Studies initially used SELDI and more recently 2DE approaches.

Preeclampsia has been the subject of three SELDI studies. The first study used SELDI to find five masses which discriminated women with late-onset preeclampsia from those with a healthy pregnancy using plasma samples collected at 26 weeks of gestation. Each of the five masses had a significantly increased intensity in samples from preeclamptic women compared to healthy pregnant women (Myers *et al.* 2004). A similar study of women with HELLP syndrome utilized plasma collected at the time of delivery compared to plasma collected from the same person several months after delivery. One mass was significantly changed between these samples and absent from healthy pregnant women when compared using cryodetector MS, a similar method to SELDI (Koy *et al.* 2005). Techniques used in these studies provided only masses of interest, not the molecular identities of these proteins.

Watanabe *et al.* (2004) described a 2DE study using undepleted serum from six women with early-onset preeclampsia and an SGA baby compared with serum from six healthy pregnant women. Upregulation was consistently observed in only a single spot from the preeclamptic samples, with no significance testing reported. MS identification of this spot was not performed and the spot was identified as clusterin based on its location on a published plasma 2DE map (Hoogland *et al.* 2004) and confirmed by 2-D western blot analysis. A significant increase in clusterin measured by

immunoassay was found in 80 women with preeclampsia compared with healthy pregnant women. The nature and size of the sample population and the lack of statistical evaluation of 2DE expression, along with the low likelihood of a single marker describing the complex preeclampsia pathophysiology, underscore the need for further 2DE studies to identify biomarkers of this disorder.

The cryodetector MS study of Koy *et al.*, discussed above, was extended by a recent 2DE study which identified the significantly changed mass as serum amyloid A, an acute phase protein (Heitner *et al.* 2006). This 2DE study found additional proteins differentially expressed in plasma from women with HELLP compared to healthy pregnant women, including inter-α-trypsin inhibitor heavy chain H4, kininogen 1, fibrinogen γ, transthyretin, and the α chains of haptoglobin alleles 1 and 2. The detection of haptoglobin chains in this 2DE study is not surprising in HELLP syndrome, where it is recognized that the free hemoglobin released during microangiopathic hemolysis is removed by circulating haptoglobins.

Proteomic studies on preeclampsia have also examined alternative fluids to serum/plasma. A SELDI study on cerebrospinal fluid from women with severe preeclampsia, mild preeclampsia, and healthy pregnant women found four masses whose presence discriminated severe preeclampsia from the other groups. All four masses were identified as hemoglobin α- and β-chains using MS identification of 1-D SDS-PAGE bands and on-chip antibody capture assays. An increase in free hemoglobin in the cerebrospinal fluid of preeclamptic women was confirmed using immunoassays (Norwitz *et al.* 2005). 2DE has also been used to examine amniotic fluid from preeclamptic women, in which oxidized transthyretin was present at higher levels than in the amniotic fluid of healthy pregnant women (Vascotto *et al.* 2007).

A few recent studies have used proteomics to identify differences in the proteins expressed within the preeclamptic placenta. Laser capture microdissection coupled with MS was used to compare villous trophoblast and villous stroma from preeclamptic and healthy pregnant women, with aberrant expression of calcyclin and choriomammotropin found in the preeclamptic placental samples (de Groot *et al.* 2007). A 2DE study of term placental tissue from preeclamptic and healthy women confirmed the similarities between preeclampsia and normal pregnancy, finding 80% of proteins present in both sample groups. Three stress-related proteins, heat shock protein 27, catalase, and glucose-regulated protein, were altered in 2-D gels from preeclamptic placentas and their dysregulation was confirmed using western blot analysis (Webster *et al.* 2007). A similar 2DE study on term placental tissue from women with preeclampsia and healthy pregnant women found alterations in proteins related to oxidative stress, including chaperonin 60, glutathione S-transferase, voltage-dependent anion channel 1, an endoplasmic reticulum protein, and cathepsin D (Kim *et al.*

2007). These placental proteins, if released into the maternal circulation, could also serve as biomarkers for preeclampsia.

Proteomic techniques have also been used to examine the protein expression of cultured placental cells to seek biomarkers for preeclampsia. Placental primary and explant culture models, as well as immortalized choriocarcinoma cell lines, have been widely used to study the trophoblast invasion and differentiation processes thought to be aberrant in preeclampsia (Lim *et al.* 1997; King *et al.* 2000; Frank *et al.* 2001). The intracellular proteomes of first trimester primary cytotrophoblasts, fibroblasts, and JEG-3 choriocarcinoma cells were compared using 2DE, leading to the identification of glycolytic and oxidative stress proteins (Hoang *et al.* 2001). Lysates from cultured cytotrophoblasts isolated from term placentas were compared before and after treatment with neurokinin B to investigate the role of this protein in preeclampsia (Sawicki *et al.* 2003). BeWo choriocarcinoma cells, in particular, adopt a STB-like phenotype after treatment with differentiation agents including forskolin (FSK) (Wice *et al.* 1990; Kudo *et al.* 2003a; Kudo *et al.* 2003c). As STBs are the main placental cell type responsible for hormone secretion, BeWo cells can provide a culture model of placental secretion processes. The intracellular proteomes of BeWo cells undergoing FSK-mediated differentiation were compared in two recent studies (Hu *et al.* 2007; Nampoothiri *et al.* 2007), but no research has yet examined the secreted proteome of this or any other placental cell type.

In addition to the serum- and plasma-based proteomic research discussed above and in Section 1.2.3.4, this thesis also describes application of proteomic techniques to characterize the proteins secreted by BeWo cells undergoing differentiation *in vitro*. Previous work in our laboratory has successfully used 2DE techniques to identify novel isoforms of adiponectin secreted during differentiation of 3T3-L1 cells, a preadipocyte cell line (Wang *et al.* 2002b). This strategy has been applied here to identify proteins involved in trophoblast differentiation and syncytialization in BeWo cells. In order to carry out proteomic studies on secreted proteins from BeWo cells, serum-free culture conditions were developed, and secreted proteins found in conditioned media using 2DE were compared between BeWo differentiation states. Further information on this research is presented in Chapters 2 and 3.

1.4 Project aims

This thesis is divided into two main areas of research to identify potential biomarkers for preeclampsia: 1) investigation of the secreted proteome in a trophoblast cell line as it undergoes syncytialization as a model for detecting potential biomarkers involved in placentation, and 2) 2-D gel-based proteomics of maternal serum and plasma in preeclampsia.

Specific aims were:

1. To investigate secreted proteomics of the BeWo line of trophoblast cells during syncytialization

    a. To develop a serum-free culture system for BeWo cells differentiated with FSK as a model to study the secreted proteome during syncytialization. (Chapter 2)

    b. To develop 2-D gel methods for the analysis of proteins secreted by BeWo cells. (Chapter 3)

    c. To identify secreted proteome changes associated with the differentiation process of BeWo cells. (Chapter 3)

2. To investigate the serum and plasma proteomes in preeclampsia

    a. To compare immunodepletion strategies to enable fractionation of the serum proteome prior to 2DE studies. (Chapter 4)

    b. To find differences in the serum proteomes of women with preeclampsia and healthy pregnant women using 2DE. (Chapter 5)

    c. To find differences in the plasma proteomes of preeclamptic women and healthy pregnant women using the DIGE method of multiplexed 2DE analysis. (Chapter 6)

Concluding thoughts on the significance and future directions of this PhD work form Chapter 7.

CHAPTER 2 SERUM-FREE CULTURE OF BEWO CELLS

2.1 Introduction

Proteins and other small molecules are continually secreted into maternal blood by the placenta throughout pregnancy. The STB, a fused multinucleate cell layer derived from villous cytotrophoblasts, is the main cell type responsible for secretion of placental hormones. STBs form the external covering of the placental villi and are in direct contact with maternal blood, as such being uniquely positioned to secrete hormones, growth factors, and other proteins into the circulation. For example, hCG is a glycoprotein secreted in large amounts mainly by the STB. Syncytialization, the differentiation process whereby cytotrophoblasts fuse into a multinucleate syncytium, is a key component of placentation. Analysis of changes in the secreted proteins associated with syncytialization may provide insight into key pathways involved in the placentation process. These proteins may be altered in abnormal placentation and as such have the potential to serve as biomarkers for preeclampsia where aberrant placentation plays a major role.

Analysis of proteins from a cell line pre- and post-differentiation allows comparison between "housekeeping" proteins expressed from pre-differentiated cells and biologically important proteins expressed as a result of differentiation. This approach has been used to compare the intracellular proteomes of mesenchymal stem cells (Sun *et al.* 2006), osteoblasts (Spreafico *et al.* 2006), maturing B cells (Salonen *et al.* 2006), and numerous carcinomas (*e.g.*, Juan *et al.* 2002). Study of the secreted proteome under differentiation conditions has enabled identification of novel isoforms of adiponectin from adipocytes derived from a preadipocyte cell line (Wang *et al.* 2002b). Comparative secreted proteomics strategies have also been used to identify differences in a myeloid cell line undergoing differentiation into osteoclasts (Kubota *et al.* 2003) and colon carcinoma lines with differing expression of SMAD4 (Volmer *et al.* 2004; Volmer *et al.* 2005). In the current study, this strategy has been applied to BeWo cells undergoing differentiation into an STB-like form.

Proteomic study of secreted proteins derived from placental cells requires culture in serum-free conditions. Primary culture of cytotrophoblasts and STBs isolated from human placenta has disadvantages for secreted proteomic studies. In villous explant cultures, the STB layer initially undergoes apoptosis and necrosis, subsequently regenerating in culture conditions but with increased fibroblast growth (Simán *et al.* 2001). Primary cultures of isolated cytotrophoblast cells require protein attachment matrices (*e.g.*, Matrigel or collagen) whose undefined compositions are not ideal for studies of endogenous proteins (Morrish *et al.* 1997). These matrices can release proteins into culture media which may complicate secreted proteome analysis. In addition to matrices, primary

trophoblast cultures also require supplementation with serum or its constituent proteins to maintain attachment and viability. Serum supplementation adds thousands of incompletely characterized proteins into culture media which can mask endogenous proteins. For effective proteomic study, cell culture media must be free of undefined protein additives. Therefore, a placental cell line model was used here in the development of serum-free viable cultures in order to study their secreted proteome.

The BeWo line of immortalized choriocarcinoma-derived cells has been used as an *in vitro* model for placental differentiation and secretion of hormones including hCG and placental lactogen (Ringler and Strauss 1990; Kudo *et al.* 2003a). BeWo cells form monolayers in culture without the use of adhesion-promoting matrices and have been successfully cultured in serum-free media (Taylor *et al.* 1991). Additionally these cells can at least partially differentiate into fused, STB-like cells in culture. This line has been extensively utilized to mimic cytotrophoblast differentiation *in vitro* (Mandl *et al.* 2002; Kudo *et al.* 2003c; Green *et al.* 2006) and is therefore an appropriate model system for studying the secreted proteome using 2-D gel electrophoresis (2DE).

BeWo cells differentiate into a fused multinucleate form in the presence of one of several agents, among them FSK, membrane-permeable analogs of cyclic AMP (cAMP), and methotrexate. FSK directly increases intracellular concentrations of cAMP by binding to a high affinity site on the catalytic subunit of adenylyl cyclase (Laurenza *et al.* 1989; Tesmer and Sprang 1998). Methotrexate acts through inhibition of dihydrofolate reductase leading to decreased purine, and consequently DNA, synthesis (Hussa and Pattillo 1972). FSK was selected for this study because *in vivo* trophoblast differentiation processes are known to involve cAMP-dependent protein kinases (Keryer *et al.* 1998). FSK was used in the sole report of serum-free BeWo differentiation (Taylor *et al.* 1991) and recent quantitative fusion studies have used this compound (Kudo *et al.* 2003a; 2003b; 2003c; 2004). During BeWo differentiation there is increased secretion of hCG into culture media consistent with a STB-like phenotype (Hoshina *et al.* 1985; Wice *et al.* 1990; Sibley *et al.* 1991). Histological changes indicative of STB-like differentiation have been reported in only 11% of cells with FSK treatment (Kudo *et al.* 2003a).

Conditioned media derived from primary trophoblast cultures or choriocarcinoma cell lines have not previously been used for proteomic analysis. However, two recent studies compared the intracellular proteomes of BeWo cells undergoing FSK-mediated differentiation: one study compared control cells versus FSK-treated cells (Nampoothiri *et al.* 2007), and the second compared FSK effects in normoxia and hypoxia (Hu *et al.* 2007). Other studies, discussed in the following chapter, have examined the proteomes of placental cytotrophoblast lysates. The exploration of BeWo culture and differentiation

conditions contained in this chapter leads to a novel field of placental research utilizing the secreted proteome, something which has not been examined previously.

Investigation of the secreted proteome of any cell type requires defined serum-free media. Serum is routinely used to supplement cells in culture but contains hundreds of proteins that can obscure endogenous proteins of interest on 2-D gels (Miller *et al.* 2006b) and can mask the effects of hormones (Barnes and Sato 1980). Serum-free culture of most cells requires protein additives to promote cell attachment and viability (Barnes and Sato 1980; Lim and Bodnar 2002); these can include albumin, insulin, transferrin, and other growth factors. Serum-free media with defined protein additives can be used, but the purity of each additive must be assessed using 2DE as even seemingly pure recombinant proteins may resolve as multiple charge isoforms on gels (Schlags *et al.* 2002).

High cell viability is an important requirement for secreted proteome studies. Cell culture inevitably results in low levels of cell death even in healthy, viable cultures. Maintenance of cells in the absence of serum's protective and stimulatory factors will result in a further decrease in viability (Barnes and Sato 1980). Unhealthy cultures may produce disproportionate amounts of proteins associated with apoptosis or necrosis, and lysed cells may contaminate conditioned media with intracellular proteins confounding studies of the secreted proteome. Therefore assessment of cell viability is necessary. Media levels of the cytosolic protein lactate dehydrogenase (LDH) can be used to assess cell viability in cytotrophoblasts (Legrand *et al.* 1992; Simán *et al.* 2001). LDH measurement has clear advantages over standard trypan blue methods, including detection of apoptotic and necrotic cells and of multinucleate cells not susceptible to trypsin dissociation, and was consequently employed in the current study.

Control conditions are also required to help identify proteins involved in the downstream effects of FSK treatment and syncytialization, not those released into the media during cell death. Brefeldin A (BFA) is a specific inhibitor of vesicle-mediated protein secretion which acts by inhibiting protein transport from the endoplasmic reticulum into the Golgi complex (Fujiwara *et al.* 1988). This inhibitor is used to confirm that protein secretion occurs through a vesicle-mediated pathway and is not associated with cell death (Lafon-Cazal *et al.* 2003). BFA has previously been used to examine hCG secretion from isolated cytotropoblast cells (Kovalevskaya *et al.* 2002) and to block protein secretion during a proteomic study of conditioned media from differentiating adipocytes (Wang *et al.* 2004).

My initial goal was to develop viable serum-free cultures of BeWo cells undergoing differentiation with FSK as a model to study the secreted proteome during syncytialization.

Specific aims were:

1. To determine that protein additives used for cell culture did not contain contaminants that would interfere with proteomic studies.

2. To optimize the FSK treatment dose and exposure time to maximize hCG secretion while maintaining cell viability in serum-containing and serum-free culture.

3. To identify the optimal time point to switch cells from serum-containing to serum-free culture.

4. To determine the effect of BFA treatment on BeWo cell hCG secretion and viability in serum-free conditions.

2.2 Methods

All chemicals and solvents were of analytical grade or higher. Supplier details for all materials are listed in Appendix A. Unless otherwise specified, or if not listed here, chemicals and solvents were sourced from BDH.

2.2.1 2DE of cell culture media additives

Three grades of bovine serum albumin (BSA) (ABGF IgG free, ABRE reduced endotoxin, and ABFF fatty acid-free) were generous gifts from ICPbio Ltd. Ten micrograms each of insulin, transferrin, and BSA grades ABGF, ABRE, and ABFF were dissolved in 125 µL of rehydration solution (8 M urea, 0.5% Triton X-100, 20 mM DTT, 0.5% IPG Buffer pH 3–10 NL, 0.002% bromophenol blue) and passively applied to 7 cm IPG strips covering a nonlinear (NL) pH 3–10 gradient (GE Healthcare). Strips were focused using a PROTEAN isoelectric focusing cell (Bio-Rad) at 20 °C using a maximum voltage of 8000 V for 10 000 V·h.

Focused IPG strips were reduced with equilibration solution (6 M urea, 20 mM Tris pH 8.8, 2% SDS, 20% glycerol) containing 1% dithiothreitol (DTT; GE Healthcare) for 15 min, followed by alkylation in equilibration solution containing 2.5% iodoacetamide (IAA; Sigma-Aldrich) for 15 min. Both incubations were performed with gentle agitation. Strips were then rinsed with Milli-Q H_2O and drained on damp filter paper before second-dimension electrophoresis.

Second-dimension separation used NuPAGE® 4–12% Bis-Tris minigels run with NuPAGE MES running buffer (Invitrogen); strips were positioned in the minigel wells using an overlay of 0.5% agarose in running buffer. Gels were run at a constant 200 V for 1 h.

Gels were stained with colloidal Coomassie according to the method of Neuhoff *et al.* (1985). Briefly, gels were agitated in colloidal Coomassie stain (0.1% Coomassie G-250, 34% methanol, 17% ammonium sulfate, 3% *ortho*-phosphoric acid) for 24 h at room temperature. Staining was performed in a sealed container to minimize methanol evaporation. After this time, stain was discarded and replaced with 1% acetic acid to enhance detection of faint spots. Incubation time with acetic acid solution varied between 24–96 h. Gels were scanned as 16-bit TIFF files on an ImageScanner II (GE Healthcare).

2.2.2 BeWo maintenance culture

BeWo cells obtained from the American Type Culture Collection were maintained as subconfluent monolayers in 75 cm^2 flasks in DMEM/Ham's F-12 1:1 media with HEPES (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS; Invitrogen). Cells were incubated in a humidified atmosphere of 95% air/5% CO_2 at 37 °C. Culture medium was replaced every two days. Cells were subcultured at a 1:3 ratio once or twice per week by the addition of TrypLE™ Express (Invitrogen) and were passaged no more than twenty times before being discarded. Cells were seeded at 2×10^4 cells·cm^{-2} and maintained in 400 µL·cm^{-2} of media during all experiments. All media changes entailed complete removal and replacement of culture media.

2.2.3 BeWo secretion and viability in serum-containing medium

In a pilot experiment (Figure 2-1A), BeWo cells were seeded into 12-well plates in serum-containing medium as described in Section 2.2.2. The medium was changed 24 h after plating and FSK (Sigma-Aldrich) was added to cells at 10, 25, 50, or 100 µM from a 50 mM stock in DMSO. DMSO alone was added to control cells at the largest volume used in the experimental conditions. Cells were treated with FSK for 72 h with media changes at 24, 36, 48, 54, 60, 66, and 72 h of treatment. Aliquots of media from each time point were submitted to an external laboratory for hCG and LDH quantitation as described in Section 2.2.5. This experiment was performed twice.

In a follow-up experiment (Figure 2-1B), BeWo cells were seeded into six-well plates in serum-containing media and grown until 50% confluence with media changes every 24 h. When cells were 50% confluent (about 72 h), 10 µM or 100 µM FSK or an equal volume of DMSO vehicle were added to cells. Cells were treated with FSK for 72 h with media changes every 12 h. Media aliquots from each 12 h time point were submitted for hCG and LDH quantitation as described in Section 2.2.5. This experiment was performed three times.

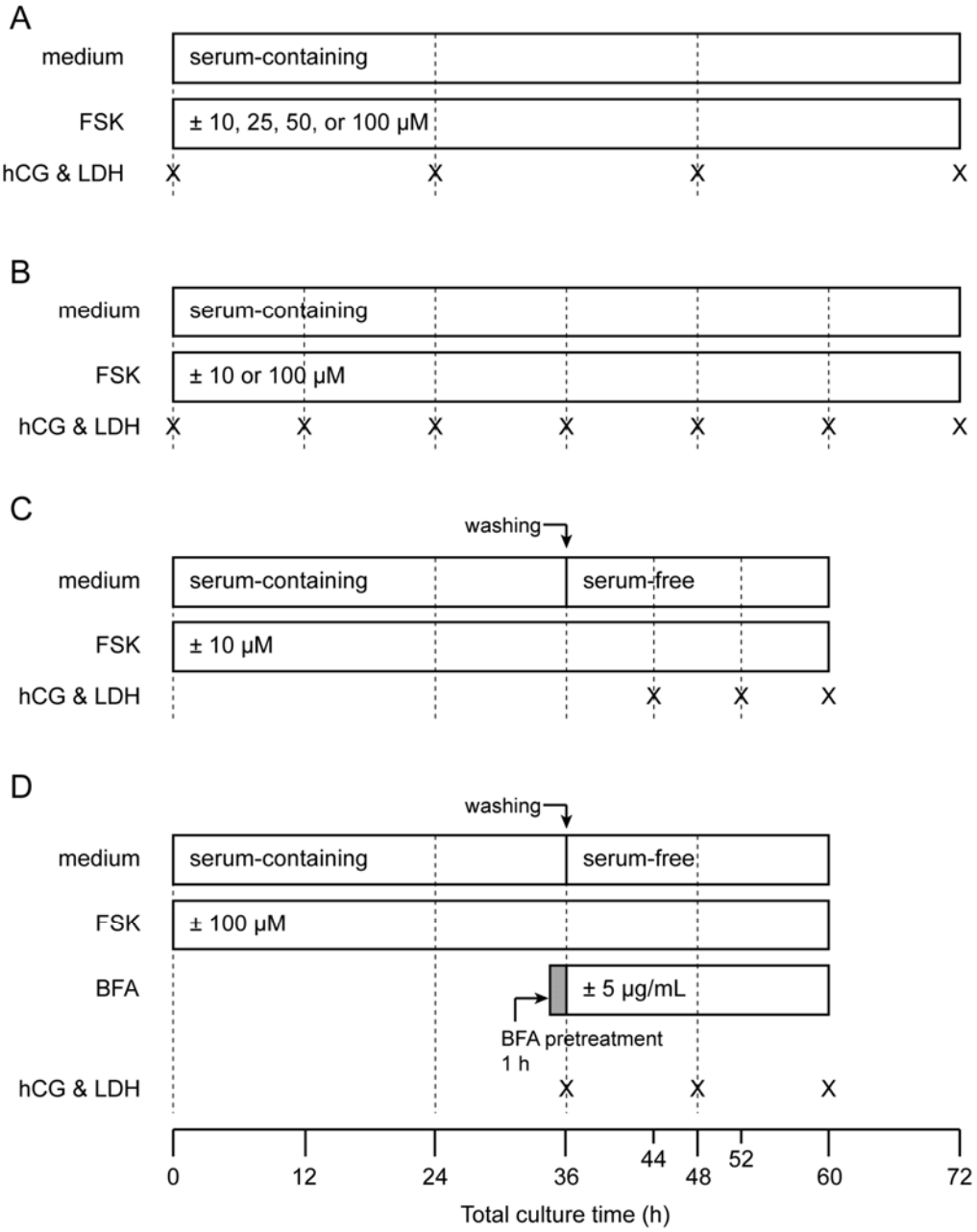

Figure 2-1: Experimental design for BeWo culture optimization

Timelines of BeWo culture conditions. Dotted lines indicate complete medium changes (collection and replacement) at least every 24 h. × indicates a time point at which medium was collected and submitted for hCG and LDH quantitation. (**A**), pilot experiments to optimize forskolin (FSK) dose in serum-containing media; (**B**), further experiments to determine FSK dose and time in serum-containing media; (**C-D**), design of serum-free media culture time optimization and supplementation experiments. The period of cell growth between plating and the commencement of FSK treatment is not shown but varied between 24–72 h as described in each experiment's methods.

2.2.4 BeWo secretion and viability in serum-free medium

In a pilot experiment (Figure 2-1C), BeWo cells were seeded into 12-well plates as described in Section 2.2.2. When cells were 50% confluent (about 72 h), 100 µM FSK or DMSO vehicle alone were added to media. Cells were treated with FSK for 60 h with media changes at least every 24 h. After 36 h of FSK

treatment, cells were washed three times with serum-free DMEM/Ham's F-12 medium alone and then incubated with serum-free medium containing 10 $\mu g \cdot mL^{-1}$ recombinant human insulin (Roche), MEM vitamin solution (Invitrogen), and either 0.5 $ng \cdot mL^{-1}$ ferrous sulfate (Sigma-Aldrich) or 5 $\mu g \cdot mL^{-1}$ human transferrin (Sigma-Aldrich). During serum-free culture, BeWo cells were treated with FSK for 24 h with media changes every 8 h. Aliquots of media from each 8 h time point were submitted to an external laboratory for hCG and LDH quantitation as described in Section 2.2.5. This experiment was performed twice.

In a follow-up experiment (Figure 2-1D), BeWo cells were seeded into 75 cm^2 flasks as described in Section 2.2.2 and were cultured and treated with FSK as described above. One hour prior to the switch to serum-free medium, a subset of FSK-treated cells were also pretreated with 5 $\mu g \cdot mL^{-1}$ BFA (Sigma-Aldrich) in methanol for 1 h, as used by Wang *et al.* (2004). An hour after BFA addition, cells were gently washed three times with serum-free medium and incubated with serum-free medium containing additives as above. The serum-free medium used in this experiment contained ferrous sulfate in place of human transferrin. Cells were maintained in serum-free media containing FSK, FSK and BFA, or vehicle alone for a further 24 h with a media change at 12 h. Conditioned media removed from BeWo cells after 12 h or 24 h of serum-free culture were centrifuged immediately at 2000 ×*g* for 10 min to pellet cellular debris. Media were then decanted and stored at −80 °C until prepared for proteomic analysis, with aliquots removed for hCG and LDH quantitation as described in Section 2.2.5. This experiment was performed five times.

2.2.5 Quantitation of hCG and LDH in conditioned media

Conditioned media samples were submitted to a reference laboratory contractor that was accredited to the International Organization for Standardization 15189 series standard. hCG was measured by an Abbott Architect autoanalyzer which detected total hCG-β, including free β subunits, by an immunoassay method. The limit of hCG-β assay sensitivity was 1.2 $mIU \cdot mL^{-1}$ and the uncertainty of measurement was 8.0% as calibrated against the World Health Organization 3rd International Standard 75/537. LDH was assayed by a modular analyzer (Roche) measuring the conversion of L-lactate to pyruvate colorimetrically. The range of the LDH assay was 5–1000 $U \cdot L^{-1}$ and the uncertainty of measurement was 4.5% as standardized manually against the original International Federation of Clinical Chemistry formulation.

2.2.6 Statistical analysis

All analyses were performed using GraphPad Prism version 4.03 for Windows (GraphPad Software). Within each set of experiments, results were compared using repeated measures two-way ANOVA with Bonferroni's *post-hoc* test. The two factors analyzed were culture time and treatment. Results were considered significant with $p < 0.05$.

2.3 Results

2.3.1 2DE determination of cell culture additive purity

Three common additives (human transferrin, recombinant human insulin, and BSA) were examined using 2DE to determine whether they would complicate 2-D gel patterns of BeWo conditioned media (Figure 2-2). The resulting proteome patterns were compared to pilot 2DE images of BeWo conditioned media (not shown) to identify additives which would obscure secreted proteins of interest. The complexity of 2-D gel patterns generated by albumin and transferrin caused these proteins to be excluded from use in future serum-free media for proteomic studies. Insulin's pattern was judged simple enough for this protein to be used as an additive. Replacement of transferrin with ferrous sulfate was investigated in culture (Section 2.3.3).

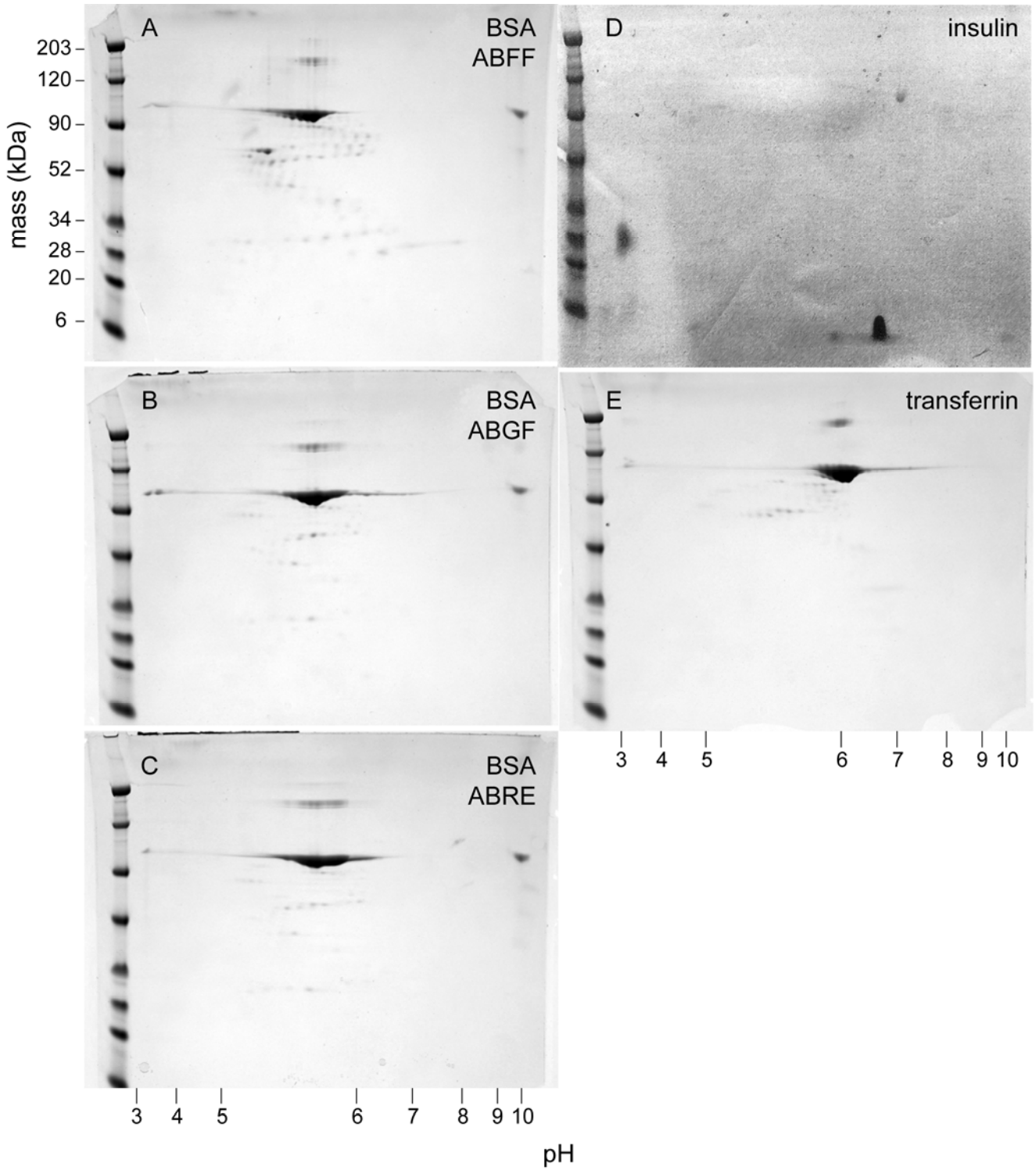

Figure 2-2: Proteome patterns of cell culture protein additives

Comparison of three grades of bovine serum albumin (BSA), human insulin, and human transferrin separated by 2DE. (**A**), 10 µg ABFF (fatty acid-free) BSA; (**B**), 10 µg ABGF (IgG-free) BSA; (**C**), 10 µg ABRE (reduced-endotoxin) BSA; (**D**), 10 µg recombinant human insulin; (**E**), 10 µg human transferrin. 2DE separation used pH 3–10 NL IPG strips and 4–12% acrylamide gels. Gels were stained with colloidal Coomassie stain.

2.3.2 Optimization of forskolin treatment regime

Pilot experiments (Figure 2-3A) showed that four concentrations of FSK (10, 25, 50, and 100 µM) had similar effects on BeWo hCG secretion and LDH release in serum-containing medium over 72 h. All concentrations of FSK produced significant increases in hCG secretion at 48 h and 72 h when compared to control cells (p<0.001 for all concentrations vs control at both time points; Figure 2-3B). At 48 h and 72 h, hCG secretion levels from cells treated with 10 µM or 100 µM FSK were significantly different from each other (p<0.001 at both time points).

Media levels of LDH released from 10, 25, 50, or 100 µM FSK-treated cells remained low, similar to control cells, until after 48 h but were significantly elevated in all FSK-treated cultures at 72 h (p<0.001 for all concentrations versus control; Figure 2-3C). This LDH increase occurred regardless of FSK concentration, indicating a reduction in cell viability by 72 h of treatment.

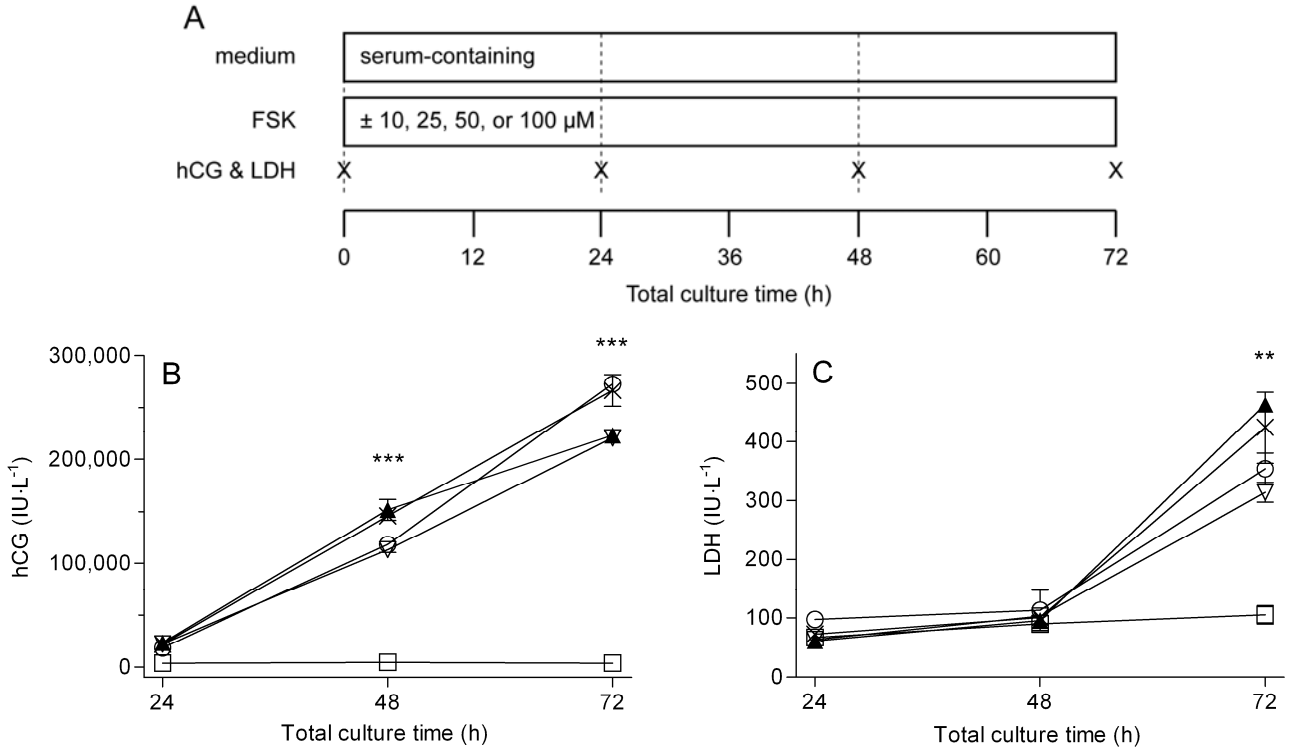

Figure 2-3: Effect of varying forskolin on BeWo hCG secretion and LDH release

(**A**) BeWo cells were cultured in serum-containing media containing varying concentrations of forskolin (FSK). hCG and LDH were measured from 500 µL media aliquots. (**B**) hCG secretion and (**C**) LDH release by BeWo cells stimulated with FSK for up to 72 h. □ control; ▲ 10 µM; ▽ 25 µM; × 50 µM; ○ 100 µM FSK. Data represent the mean ± SD from two experiments. *** p<0.001 and ** p<0.01 for FSK-treated versus control cells calculated using two-way ANOVA with Bonferroni's *post-hoc* test.

To further refine the optimal time to incubate cells in media, the pilot experiments above were repeated with 12 h sampling and only 10 or 100 μM FSK (Figure 2-4A). Minimal levels of hCG secretion were recorded at all time points in control BeWo cells cultured without FSK. Treatment with either dose of FSK significantly increased hCG secretion as compared to control cells beginning from 36 h (p<0.001 for both concentrations at all four time points; Figure 2-4B). There were significant increases in hCG secretion in cells treated with 100 μM versus 10 μM FSK at both 60 h and 72 h (p<0.001 at both time points).

An exceptionally high level of LDH was released by cells treated with 100 μM FSK for 12 h on this occasion (Figure 2-4D) but was not observed in any other similar experiments. After the initial 12 h, low LDH levels were found through the 60 h time point for 10 μM FSK treated cultures versus control cells, but LDH release was significantly higher in the 100 μM FSK group compared to controls starting at 60 h (p=0.007 at 60 h, p<0.001 at 72 h). At 72 h of FSK treatment LDH rose abruptly, especially in the group treated with 100 μM FSK. For this reason, further culture experiments extended to no more than 60 h total treatment time with 100 μM FSK.

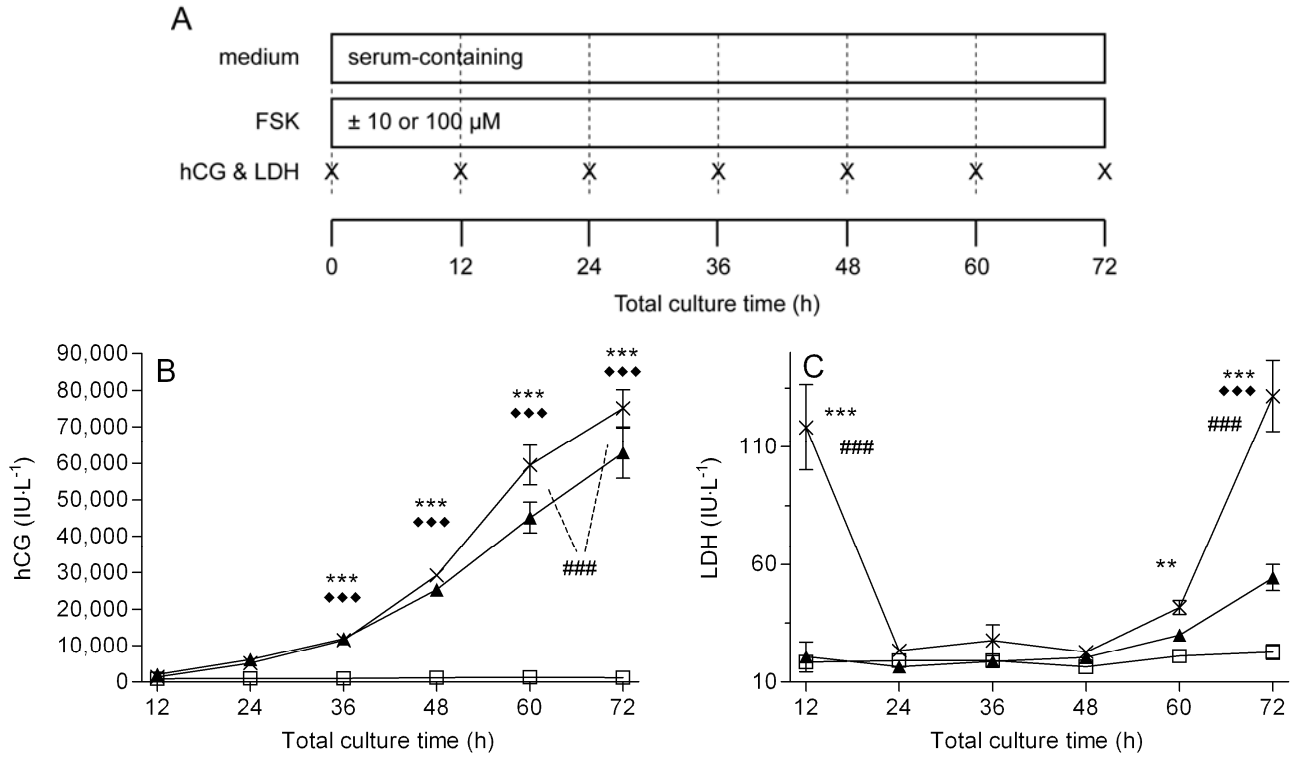

Figure 2-4: Effect of two forskolin concentrations on BeWo hCG secretion and LDH release

(**A**) BeWo cells were treated with 10 or 100 μM forskolin (FSK) in serum-containing media. hCG secretion and LDH release were measured from 1 mL media aliquots. (**B**) hCG secretion by BeWo cells treated with 10 or 100 μM FSK for up to 72 h. □, control; ▲, 10 μM; ×, 100 μM FSK (**C**) LDH released by BeWo cells treated with 10 or 100 μM FSK for up to 72 h. ** p<0.01 and *** p<0.001 for 100 μM FSK-treated cells versus control cells; ♦♦♦ p<0.001 for 10 μM FSK-treated cells versus control cells; ### p<0.001 for 10 μM versus 100 μM FSK-treated cells, calculated using two-way ANOVA with Bonferroni's *post-hoc* test. Data represent the mean ± SD from three experiments.

2.3.3 Secretion and cell viability in serum-free medium

Initial experiments described in Section 2.3.2 were conducted in serum-containing medium. The behavior of BeWo cells in serum-free medium containing insulin, transferrin, and vitamins was then determined over a 24 h period (Figure 2-5A). Under these conditions, BeWo cells displayed rising hCG secretion consistent with syncytialization, similar to that observed in serum-containing medium, without a marked increase in cell death (LDH release). Figure 2-5B and C demonstrate that viable BeWo cells with increasing secretion of hCG could be cultured in serum-free medium. FSK treatment over 24 h did not cause any significant differences in LDH release levels compared to control cells. Based on these results, cells were maintained in serum-free media for 24 h in future experiments.

Multiple isoforms of transferrin had been found on 2-D gels (Section 2.3.1), so ferrous sulfate ($FeSO_4$) was investigated as a replacement iron-carrying compound (Figure 2-5A). Replacement of transferrin with $FeSO_4$ caused no significant changes in hCG secretion from control or FSK-treated cells (Figure 2-5B). Iron supplementation also did not affect cell viability; LDH release from control or FSK-treated cells did not significantly change between iron sources (Figure 2-5C). As a result, $FeSO_4$ was used instead of transferrin in subsequent serum-free BeWo cultures.

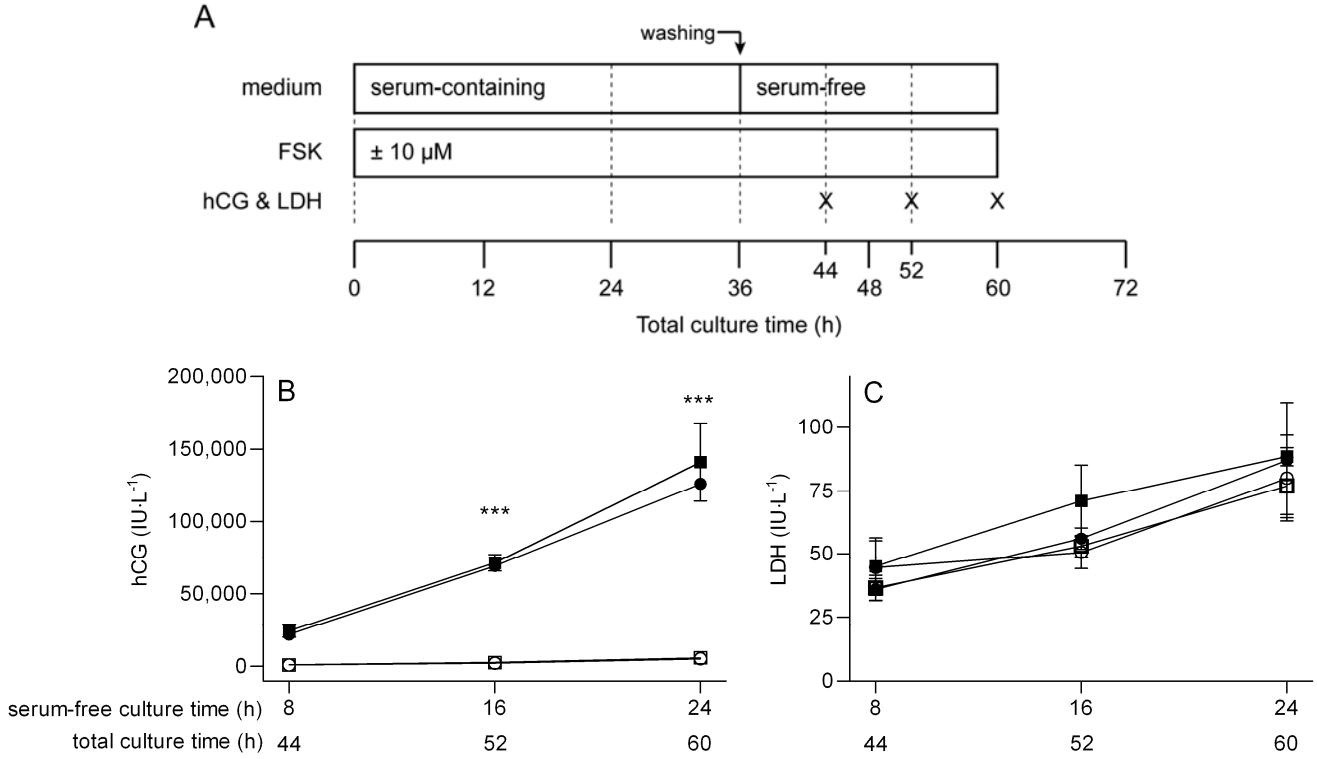

Figure 2-5: Effect of $FeSO_4$ on BeWo hCG secretion and LDH release in serum-free media

(**A**) BeWo cells were cultured in serum-free medium containing either human transferrin (□, ■) or $FeSO_4$ (○, ●) in the presence (filled symbols) or absence (open symbols) of 10 µM FSK for up to 24 h. (**B**) hCG secretion and (**C**) LDH release under these conditions. *** $p<0.001$ for FSK-treated versus control cells calculated using two-way ANOVA with Bonferroni's *post-hoc* test. Data represent the mean ± SD of two experiments.

2.3.4 Secretion time within serum-free media incubation

In order to monitor cell viability during serum-free culture, LDH release and hCG secretion were measured every 8 h in BeWo cultures treated with 100 μM FSK (Figure 2-6A). Secretion of hCG was not significantly different between FSK-treated cells cultured in serum-containing or serum-free conditions for 8 or 16 h, but hCG secretion at 24 h was significantly higher in serum-free than serum-containing cultures (p=0.006; Figure 2-6B). The effect of BFA treatment on hCG secretion in FSK-treated cells was marked after 16 h of culture, with significant hCG decreases noted at this time and at the later 24 h time point (p<0.001 at both time points).

LDH release levels from FSK-treated cells in serum-free culture were not significantly different from control cells at any time points, nor were levels significantly different between FSK-treated cultures maintained in serum-free or serum-containing conditions (Figure 2-6C). However, addition of BFA to FSK-treated cells did cause a significant increase in LDH release by 16 h (p<0.001 at both time points).

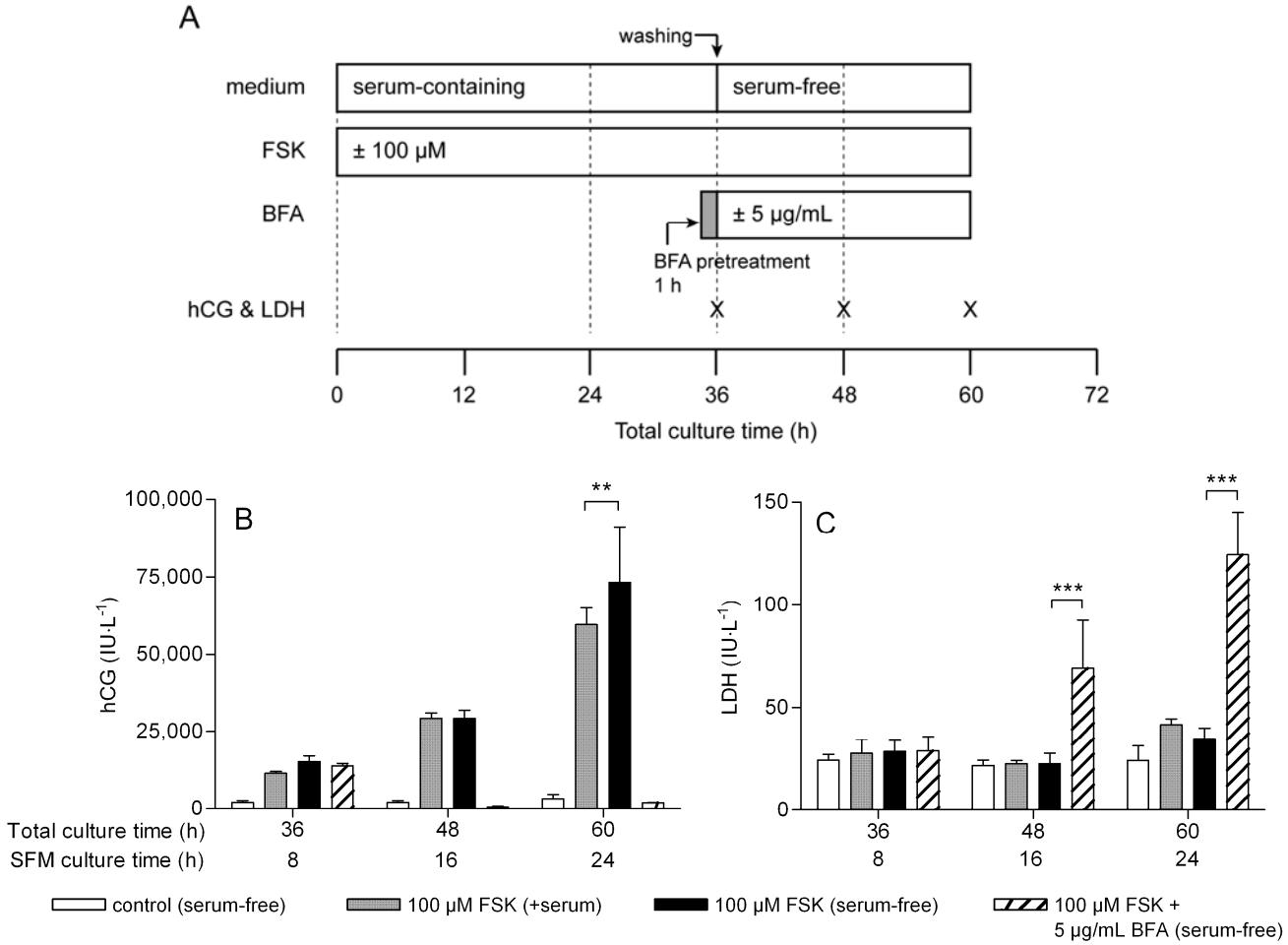

Figure 2-6: Effect of serum-free culture time on BeWo secretion and viability

(**A**) BeWo cells were cultured in serum-free media with or without 10 µM forskolin (FSK) and 5 µg·mL^{-1} brefeldin A (BFA) for up to 24 h. (**B**) hCG secretion and (**C**) LDH release into SFM over 24 h. Control cells (open bars), FSK-treated cells (solid bars), and FSK and BFA-treated cells (striped bars) are shown. hCG and LDH levels from similar serum-containing cultures (Figure 2-4; gray bars) are included. ** p<0.01 and *** p<0.001, calculated using two-way ANOVA with Bonferroni's *post-hoc* test. Data represent the mean ± SD of three (serum-containing) or five (serum-free) experiments.

2.4 Discussion

In this chapter, culture conditions were determined for the treatment of BeWo cells with FSK and the production of serum-free conditioned media from these cultures for proteomic analysis. Initial experiments determined the suitability of serum-free medium protein additives for 2DE studies. Analysis of insulin, transferrin, and three grades of BSA using 2DE showed that only insulin was an acceptable additive for further proteomic studies of BeWo conditioned media due to its favorable position at the edge of 2-D gels. Insulin was also available as a recombinant protein which ensured that its purity was well-characterized. Transferrin and BSA are both higher mass proteins derived from crude preparations which focused within the middle of the broad pH 3–10 range examined here. Transferrin is regarded as an essential media additive due to its iron-carrying property (Barnes and

Sato 1980), but can be replaced by ferrous sulfate (Keenan and Clynes 1996), as verified here. The BeWo cells cultured here grew equally well in serum-free media formulations containing either human transferrin or ferrous sulfate. This result suggests that ferrous sulfate can be used to replace transferrin in other serum-free cell culture systems.

Protein additives were analyzed by 2DE using 10 μg of total protein, the amount present in 1 mL of serum-free culture medium. However, BeWo cells were maintained in 75 cm^2 flasks in 30 mL of serum-free medium. The entire 30 mL of serum-free medium was used for future proteomic analysis, increasing the amount of exogenous protein by 30-fold. Therefore, culture media additives represent a significant proportion of the protein analyzable by the 2-D gel method employed here. This highlights the importance of carefully evaluating exogenous protein additives when culture media is used for secreted proteomics.

Differentiation of BeWo cells into a STB-like state following exposure to FSK was demonstrated by increased secretion of hCG. The treatment dose and exposure time of FSK were optimized to maximize hCG secretion while maintaining cell viability, first in serum-containing and then in serum-free culture conditions. In the presence of serum, FSK treatment caused significant increases in hCG secretion at 48 and 72 h of incubation, with a 100 μM dose raising hCG significantly more than a 10 μM dose. These results indicated that modulation of FSK treatment conditions was able to maximize hCG secretion. Consequently, the percentage of STB-like BeWo cells in culture may have been increased by treatment with 100 μM versus 10 μM FSK. This confirms earlier work by Wice *et al.* (1990) where 0.1 μM, 1 μM, 10 μM, or 100 μM FSK were applied to BeWo b30 cells in serum-containing culture, and associated rises in intracellular cAMP, secreted cAMP, and cell fusion levels were observed with increasing FSK doses.

Further refinement of the exposure time to FSK required assessment of cell viability. The release of LDH, an intracellular enzyme, into culture media can indicate not only dead cells but also those that have lysed, which is an advantage over trypan blue exclusion assays (Legrand *et al.* 1992). LDH measurements can also be performed on cultures of multinucleate cells like STBs, whereas trypan blue is only effective on single cells that respond to dissociation by trypsin (Burres and Cass 1986; Wice *et al.* 1990). In this study, FSK incubation in serum-containing cultures caused a significant increase in LDH release over control cell levels at 60 h of incubation, with a further substantial rise in LDH levels at 72 h of incubation. Treatment with 100 μM FSK raised LDH to higher levels, and more quickly (beginning from 60 h versus 72 h), than did 10 μM FSK treatment. BeWo cell treatment with FSK for greater than 60 h in serum-containing culture may be harmful to cells. These viability results, in conjunction with the significant increases in hCG secretion described above, enabled the

experimental timeline to be limited to 60 h of treatment with 100 μM FSK. The higher concentration of FSK was used as it was likely to lead to a greater percentage of differentiated, syncytia-like BeWo cells.

Cell secretion and viability experiments were then conducted in up to 24 h of serum-free culture to provide final refinement of the culture conditions necessary for secreted proteomic analysis of conditioned medium. These serum-free medium incubations began at 36 h of FSK treatment to ensure that cells were not exposed to FSK for more than 60 h total. First, the effect of serum-free medium supplementation with transferrin or ferrous sulfate was determined. Replacing transferrin with ferrous sulfate affected neither hCG secretion nor cell viability, so this non-protein additive was used in addition to insulin and vitamin supplements.

In this final formulation of serum-free medium, FSK treatment caused the same significant differences in hCG secretion compared to control cells at 36, 48, and 60 h of treatment. There were no significant differences in FSK secretion between serum-containing and serum-free culture conditions except at 60 h where serum-free secretion levels were even higher than those in serum. Serum-free culture itself does not inhibit the differentiation of FSK-treated BeWo cells. No changes in cell viability were observed during this period regardless of control or FSK treatment or culture time up to 60 h of total treatment. This is an improvement over the initial FSK treatment timecourse in serum-containing medium, in which LDH release levels began to rise significantly at 60 h of treatment. The cause of this improvement is unknown; the wash conditions used between serum-containing and serum-free culture steps may have improved the viability of BeWo cells treated with FSK.

Half of the BeWo cell serum-free cultures treated in this manner with FSK were also incubated with BFA, an inhibitor of vesicular protein secretion. FSK-enhanced hCG secretion was attenuated to control levels at 16 h and 24 h of serum-free culture by the addition of BFA. Specific inhibition of protein secretion by addition of BFA, which disrupts the Golgi apparatus and therefore interferes with protein secretion by the classical, vesicle-mediated pathway (Lafon-Cazal *et al.* 2003), attenuated FSK-activated hCG secretion to the level of control cells.

As above (in final serum-free medium), the various culture conditions including serum-free control, FSK-treated, FSK+BFA treated, and serum-containing FSK, showed no significant differences in cell viability as measured by LDH release until the effects of BFA became evident at 48 h and 60 h of FSK treatment. In these cases, the level of LDH release increased significantly during treatment with BFA. This increase indicates that disruption of the Golgi apparatus proves toxic to cells when they are stimulated with FSK in serum-free medium. Undoubtedly, some of this process of toxicity is due not

only to the inhibition of protein secretion *per se*, but also to its occurrence in a serum-free medium. Use of BFA on FSK-treated cells in serum-containing medium would have helped determine which portions of the observed rise in LDH release were due to the BFA, and which were due to serum-free conditions. In addition, it is expected that the decreased viability observed in these FSK and BFA-treated cells is partly due to increased protein secretion caused by the activation of cAMP by FSK. Use of BFA on vehicle-treated control cells is expected to cause a less significant or insignificant increase in observed LDH release (decrease in cell viability).

Specific culture conditions and times for BeWo serum-free differentiation were identified through the experiments described in this chapter. A stringent wash step was used between the initial culture with serum-containing medium and the final period of serum-free culture. Pilot 2DE experiments using media from the entire 24 h serum-free incubation period showed that much of the protein in BeWo conditioned medium was of bovine origin (data not shown). A recent report confirms the presence of high concentrations of bovine serum proteins in conditioned media used for proteomic analysis; there, bovine proteins were mitigated only by stringent washing before serum-free media incubation (Pellitteri-Hahn *et al.* 2006). Regardless of the wash stringency, the presence of a small amount of bovine-derived protein in media from cells previously cultured with bovine serum is inevitable. This is due in part to the intracellular processing of exogenous proteins derived from serum-supplemented media. To decrease the occurrence of serum-related bovine proteins in the serum-free conditioned media, the 24 h serum-free incubation period was broken into two 12 h periods. The medium from the first 12 h period was assumed to contain the majority of released bovine protein products. Apoptosis-related proteins generated by BeWo cells from the acute switch to serum-free culture would also be present in this initial medium fraction. Further proteomic studies (Chapter 3) consequently used media only from the second 12 h period.

Increased hCG secretion is a key indicator of syncytialization and trophoblast differentiation. The current study demonstrates highly increased hCG secretion in BeWo cells treated with FSK. This indicates that at least partial syncytialization occurs in culture. FSK treatment is known to cause differentiation of BeWo cells into a fused STB-like state at a low frequency (Kudo *et al.* 2003a). Subjective measurement of this process uses desmosomal staining to indicate cell-cell junctions (Douglas and King 1990). Recently, a flow cytometry-based quantitation strategy measured 10% syncytialization in BeWo cells transfected with nuclear or cytoplasmic dyes, mixed in culture, and treated with 100 μM FSK for 48 h (Kudo *et al.* 2003a). A similar approach using fluorescence microscopy to measure mixtures of cytoplasmic dyes reported 11% fusion in BeWo cells treated with 50 μM FSK for 42 h (Borges *et al.* 2003). Use of these quantitative methods, which were unknown at

the time this project began, could further characterize the effects of these serum-free culture conditions on BeWo syncytialization processes.

This study produced a set of culture conditions optimized for producing serum-free medium from BeWo cells treated with FSK. Previous research into the differentiation of BeWo cells primarily used serum-containing conditions for cell culture (Wice *et al.* 1990; Hohn *et al.* 1998; Hohn *et al.* 2000); only one prior report examined the effects of differentiation agents on BeWo cells under serum-free conditions (Taylor *et al.* 1991). The serum-free media formulation used in the current study was initially modeled on the published methods of Taylor *et al.* (1991) wherein insulin and transferrin were added to serum-free basal media. The results presented in this chapter extend that work by using ferrous sulfate in place of transferrin. In addition, the prior study used FSK treatment in serum-free medium for up to 48 h; the current study extended the FSK treatment time up to 60–72 h, confirming that treatment continues to enhance hCG secretion levels over the latter time period.

The initial work on FSK treatment conditions and times in serum-containing culture parallels earlier work by Wice *et al.* (1990) who reported that BeWo cell fusion was directly proportional to FSK concentration and to intracellular and secreted levels of cAMP. The research presented in this chapter necessarily builds upon the increases in fusion reported by Wice, but also furthers that work by finding that serum-free culture by itself caused minor, if any, changes in hCG secretion during FSK treatment.

While the culture conditions determined in this study are useful for further proteomic analysis, they are limited mainly by the decreases in cell viability observed after 60–72 h of FSK treatment. FSK treatment cannot extend indefinitely without consequences leading to decreased cell viability. This may be due to other downstream effects of a chronically increased intracellular concentration of cAMP which is undoubtedly driving additional cellular processes besides hCG secretion. In addition, in one case LDH release was aberrantly increased at 12 h of treatment with 100 μM FSK (Figure 2-4D), although this result was not reproduced in any other similar experiments.

In summary, BeWo cells were cultured for secreted proteomic studies of differentiation with 100 μM FSK for a maximum of 60 h with the last 24 h of this period being in serum-free media. Appropriate supplements were used to maintain cell viability under these conditions. Medium from the final 12 h of serum-free culture was used for the resulting proteomic studies of BeWo-secreted proteins discussed in the following chapter.

CHAPTER 3 SECRETED PROTEOMIC ANALYSIS OF BEWO DIFFERENTIATION

3.1 Introduction

The cytotrophoblast-STB differentiation process can be modeled *in vitro* by BeWo choriocarcinoma cells treated with FSK. Though basic serum-free conditions have been previously used for this model (Taylor *et al.* 1991), additional optimization of strictly-defined serum-free culture conditions was necessary before secreted proteomic studies could be performed. The previous chapter describes development of suitable culture conditions. This chapter focuses on the proteomic analysis of secreted proteins from BeWo conditioned media.

Proteomic analysis of cultured cells is a useful tool for protein identification and comparison, but the majority of studies to date have focused only on intracellular lysate proteomes. Only a few 2DE studies have examined secreted proteins from conditioned media. Discovery studies cataloging secreted proteins from keratinocytes were among the first in this area (Katz and Taichman 1999; Ahmed *et al.* 2001). More recently, fibroblast secreted proteins have been cataloged (Boraldi *et al.* 2003; Pflieger *et al.* 2006; Shimmura *et al.* 2006). Comparative studies of secreted proteins have involved human colon carcinoma cells expressing or deficient in the *SMAD4* tumor suppressor gene (Volmer *et al.* 2004; Volmer *et al.* 2005). Although most comparative proteomic studies focusing on cell differentiation have used intracellular lysate samples, the secreted proteomics approach is well-suited for examining differentiation processes and has been applied to adipocyte (Wang *et al.* 2002b; Wang *et al.* 2004; Zvonic *et al.* 2007) and osteoclast differentiation (Kubota *et al.* 2003).

An extension of secreted proteomic analysis techniques uses metabolic labeling with a radioisotope to restrict detected proteins to true secretion products as much as possible (Zwickl *et al.* 2005), though labeled proteins could also be released into culture medium from cell lysis or other non-secretory pathways. This radiolabeling technique may be adapted from stable isotope labeling with amino acids in cell culture (SILAC) methods previously used for comparative intracellular proteomics. In SILAC, cultures are supplemented with different isotopically-enriched media, mixed together, and the relative abundance of any single protein is measured within a mass spectrometer (Ong *et al.* 2002; Ong *et al.* 2003). The SILAC method has since been successfully applied to secreted proteomics (An *et al.* 2006).

Cell lines and primary cells of placental origin have been the targets of several proteomic studies. The first investigation of mitochondrial proteomics used placental tissue as a source of mitochondria

(Rabilloud *et al.* 1998). The proteomes of lysates from cultured human first-trimester cytotrophoblasts, placental fibroblasts, and JEG-3 choriocarcinoma cells were compared by Hoang *et al.* (2001). A more recent report by Sawicki *et al.* (2003) examined the proteome of cultured term cytotrophoblasts before and after treatment with neurokinin B to investigate its role in preeclampsia. Several placental research reviews have championed the use of proteomic techniques for studying pregnancy complications and preeclampsia (Pritlove *et al.* 2004; Shankar *et al.* 2004; Shankar *et al.* 2005; Nelson *et al.* 2006; Rice *et al.* 2006). A recent report compared the intracellular proteomes of normal term and preeclamptic preterm placentas, finding differences in several heat shock proteins (Webster *et al.* 2007). Even more recently, the intracellular proteomes of BeWo cells undergoing FSK-mediated differentiation were compared in two separate studies, one examining control cells versus FSK-treated cells (Nampoothiri *et al.* 2007) and a second comparing FSK effects in normoxia and hypoxia (Hu *et al.* 2007). All of these previous placental proteomic studies were based on intracellular extracts, whereas the present study is the first to investigate the secreted proteome of any placental cell type.

In order to carry out secreted proteomic studies, a defined culture model was developed to differentiate BeWo cells in a manner suitable for further proteomic analysis. This involved determining the optimum dose and time of FSK treatment, the length of serum-free culture, and assessing defined additives for their compatibility with 2-D gel analysis (described in the previous chapter). In a series of pilot experiments, optimal conditions were determined to successfully study the proteins secreted by BeWo cells while limiting sources of exogenous protein contamination by culture media additives and by intracellular proteins released from cell death processes.

The goal of this study was to compare the secreted proteomes of control (undifferentiated) versus FSK-treated (differentiated) BeWo cells to identify proteins involved in the downstream effects of FSK treatment and biochemical differentiation. However, even under the above optimized culture conditions, some cell death is inevitable in culture. In order to determine which proteins result from cell lysis alone, a protein secretion inhibitor was used in some FSK-treated cultures. BFA is a specific inhibitor of classical protein secretion along vesicle-mediated pathways; this identifies proteins present as a result of cell lysis and not secretion. This compound has been used to control protein secretion in secreted proteome studies of astrocyte differentiation (Lafon-Cazal *et al.* 2003) and adipocyte differentiation (Wang *et al.* 2004). In this study, therefore, secreted proteome maps were compared from FSK-treated, control (vehicle-treated), and FSK+BFA-treated BeWo cells.

The main aim of this research was to identify proteins secreted during the BeWo syncytialization process.

Specific aims were:

1. To establish 2-D gel methods suitable for analysis of conditioned media samples.

2. To identify proteins secreted from differentiated (FSK-treated) cells, but not from secretion-inhibited (FSK+BFA-treated) or undifferentiated (control) cells.

3.2 Methods

All chemicals and solvents were of analytical grade or higher. Supplier details for all materials are listed in Appendix A. Unless otherwise specified, or if not listed here, chemicals and solvents were sourced from BDH.

3.2.1 Production of serum-free BeWo conditioned media for 2DE

BeWo cells were cultured as outlined in Figure 3-1 and as previously described in Section 2.2.4. Briefly, cells were seeded into 75 cm^2 flasks at 2×10^4 cells·cm^{-2} and cultured in medium containing 10% fetal calf serum until 50% confluent, about 72 h. At this time, cells were treated with 100 μM FSK (Sigma-Aldrich) or DMSO for 36 h. Cells were then washed and cultured in serum-free medium containing recombinant human insulin, a vitamin solution, and ferrous sulfate for two 12 h periods. A subset of FSK-treated cells were also treated with 5 μg·mL^{-1} BFA (Sigma-Aldrich) in methanol for 1 h prior to the switch to serum-free medium and for the duration of serum-free culture (as used by Wang *et al.* 2004). Conditioned media removed from BeWo cells after the second 12 h serum-free culture period were centrifuged immediately at 2000 ×*g* for 10 min to pellet cellular debris. Media were then decanted and stored at −80 °C until prepared for proteomic analysis, with aliquots removed for hCG and LDH quantitation as previously described in Section 2.2.5.

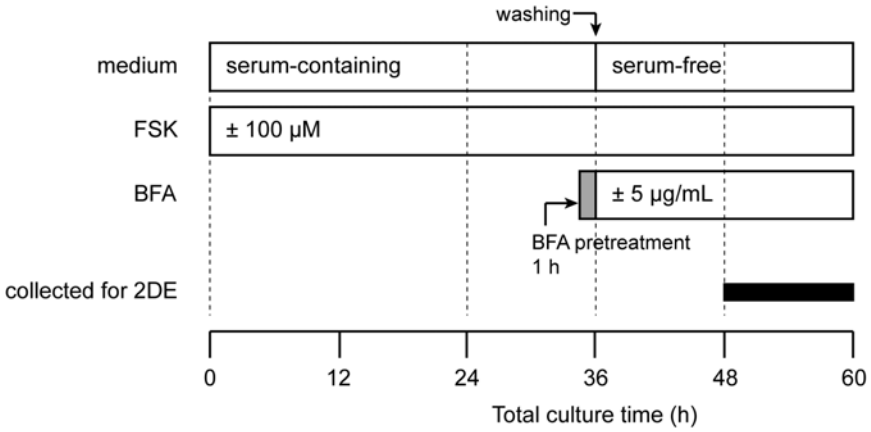

Figure 3-1: BeWo culture timeline for conditioned media collection

Cell culture conditions for production of serum-free media for proteomic analysis. Media were collected from cells treated with forskolin (FSK) only, FSK+BFA, and DMSO only from the 48–60 h time period above (black bar).

3.2.2 Protein purification from culture media

Crude protein was concentrated from 15 mL culture media using Vivaspin 20 centrifugal concentrators with a 5 kDa molecular weight (MW) cutoff (Vivascience). Media were centrifuged in the concentrators (3700 ×*g*, 1 h, 4 °C). This reduced sample volume by more than 20-fold. A total of 30 mL culture media were processed in this manner from each flask of BeWo cells.

A series of in-concentrator wash steps were used to desalt the concentrated protein sample. After the initial concentration step described above, one volume (~20 mL) of Milli-Q H_2O was added directly to the concentrator retentate and the rediluted mixture was then centrifuged for 90 min as above. This buffer exchange process was repeated at least twice more with the final spin extending for 120–180 min. These spins reduced the exchanged concentrate volume as much as possible, usually to less than 200 µL. Aqueous concentrates were then reduced to less than 100 µL with vacuum centrifugation and were mixed with 100 µL of a solubilization solution (8 M urea, 1% C7BzO, 1% CHAPS, 50 mM DTT) before storage at −20 °C.

In a subset of the purified samples, the conductivity of aqueous conditioned media concentrates was measured using a MeterLab CDM 210 conductivity meter (Radiometer Analytical SAS). This measurement verified that the concentration of salts present in culture medium was substantially reduced, thereby preventing their interference with isoelectric focusing.

3.2.3 Protein quantitation

Protein yields from BeWo media concentration and desalting steps were monitored using the BCA protein assay (Pierce). The 2-D Quant Kit (GE Healthcare), a reverse biuret protein assay specifically optimized for use with samples in strongly denaturing solutions, was used to quantify all protein samples before 2DE. Both assays were performed according to the manufacturer's instructions.

3.2.4 2DE of BeWo conditioned media

Initial experiments used variations of the rehydration solution composition and centrifugation step applied to conditioned media samples, with the final selection being formulation E (Table 3-1).

Table 3-1: Rehydration solution mixtures tested with BeWo conditioned media proteins

RHS formulation[a]	*Denaturant*	*Detergent*	*Other preparation*
A	8 M urea	1% C7BzO	none
B	9 M urea	1% C7BzO, 1% CHAPS	none
C	8 M urea	1% C7BzO, 1% CHAPS	10 min centrifugation at 20 800 ×*g* before rehydration
D	8 M urea	0.5% Triton X-100	10 min centrifugation
E	7 M urea, 2 M thiourea	0.5% Triton X-100	10 min centrifugation

[a] In addition to these ingredients, all rehydration solutions also contained 60 mM DTT, 0.5% IPG Buffer pH 3–11, and 0.002% bromophenol blue.

For first-dimension separations, 150 µg of each sample was diluted to 200 µL in final rehydration solution (7 M urea, 2 M thiourea, 0.5% Triton X-100, 60 mM DTT, 0.5% IPG Buffer pH 3–11, and 0.002% bromophenol blue). Diluted samples were centrifuged at 20 800 ×*g* for 10 min before strip application. First-dimension separations used 11 cm IPG strips covering a pH 3–11 nonlinear gradient (GE Healthcare). Focusing was performed using the Multiphor II flatbed electrophoresis system (GE Healthcare). Strips were focused using the protocol shown in Table 3-2 and were stored at −80 °C after focusing was completed.

Table 3-2: Isoelectric focusing parameters for BeWo conditioned media samples

Focusing phase	*Voltage (V)*[a]	*Time (h)*	*Purpose*
1	500	0:01	voltage gradient increasing to 500 V
2	500	5:00	initial focusing
3	3500	5:00	voltage gradient increasing to 3500 V
4	3500	12:00	extended focusing
5	100	0:01	voltage gradient decreasing to 100 V
6	100	(hold)	hold step when focusing is complete

[a] All steps were carried out at 1 mA, 5 W, and 20 °C.

Focused IPG strips were reduced with equilibration solution (6 M urea, 20 mM Tris pH 8.8, 2% SDS, 20% glycerol) containing 1% DTT (GE Healthcare) for 15 min, followed by alkylation in equilibration solution containing 2.5% acrylamide (GE Healthcare) for 15 min. Both incubations were performed with gentle agitation. Strips were rinsed with Milli-Q H_2O and drained on damp filter paper before second-dimension electrophoresis.

Second-dimension separations used Criterion 8–16% Tris-HCl midigels (Bio-Rad) run with Novex® Tris-glycine SDS running buffer (Invitrogen). Gels were run at a constant 200 V for 1 h. After electrophoresis, gels were fixed for 2×30 min in fix/wash solution (10% ethanol, 7% acetic acid) with gentle agitation prior to staining with SYPRO Ruby stain (Invitrogen) overnight at room temperature in the dark. The next day, gels were washed in fix/wash solution for at least 1 h and briefly rinsed with H_2O before imaging. Imaging was performed using an FLA–2000 phosphorimager (Fuji) with excitation at 473 nm and a 580 nm high-pass emission filter. Gels were imaged with a 50–100 µm pixel size and F1000 (maximum) resolution.

3.2.5 Image analysis

Images were analyzed with ImageMaster™ 2D Platinum version 6.0.1 software (IMP6; GE Healthcare). Briefly, analysis included spot detection and matching. After spots were detected on all gels using the same parameters, gels were matched together in a hierarchical structure as shown in Figure 3-2. The hierarchical structure is advantageous due to its ability to match whole populations of gels together without using an arbitrary reference gel; this is in contrast to typical flat structures used in previous ImageMaster versions and in other 2-D gel analysis software products. Hierarchical matching enabled all gels created from the FSK-treated and BFA-treated media to be compared and spots present only as a result of FSK treatment to be easily identified. These spots were then compared to all gels generated from control media.

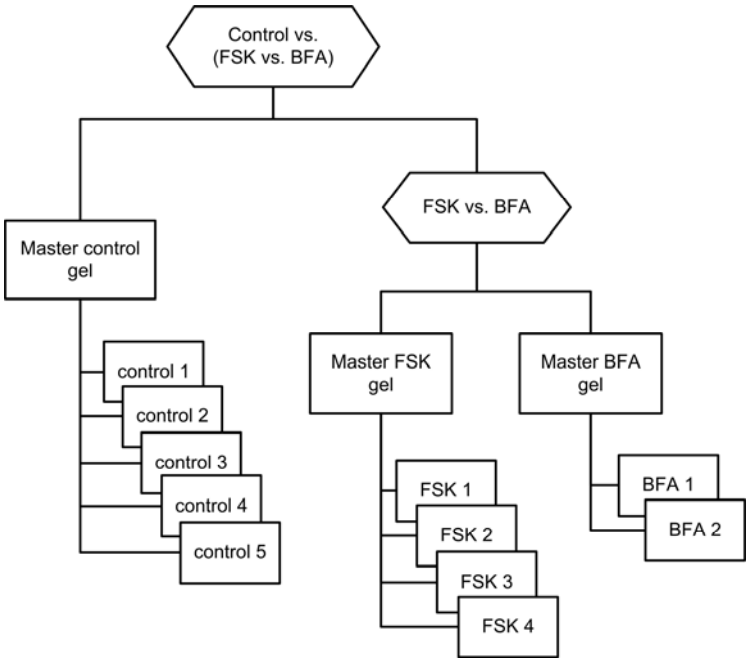

Figure 3-2: Hierarchical matching of BeWo 2-D gels using ImageMaster Platinum

Gel matching hierarchy employed in ImageMaster Platinum software to match control, FSK-treated, and FSK+BFA-treated gel images together.

After hierarchical matching, data were exported from the entire set of gels as the percentage volume of each spot on each gel. This dataset was analyzed using two different approaches:

1. Identify spots present only on all four FSK-treated gels, but absent from all control or BFA-treated gels ("unique spots").

2. Identify spots significantly upregulated on FSK-treated gels compared to control gels, but absent or present at a significantly lower level on BFA-treated gels ("upregulated spots"). Upregulation was determined using Student's *t* test (Section 3.2.6).

Spots of interest from both analysis approaches passed through the same filtering strategy (Figure 3-3). Briefly, spots positively identified as hCG by comparison with known hCG gel patterns were removed, as identification of hCG was not a goal of this study. Spots from either dataset which showed any expression in FSK+BFA-treated media (even if lower than in media treated with FSK alone) were removed as these proteins may indicate cell death processes triggered by the inhibition of protein secretion. Finally, spots whose differential regulation resulted from a technical problem on the gels (*e.g.*, streaks, smears, edge effects) were removed.

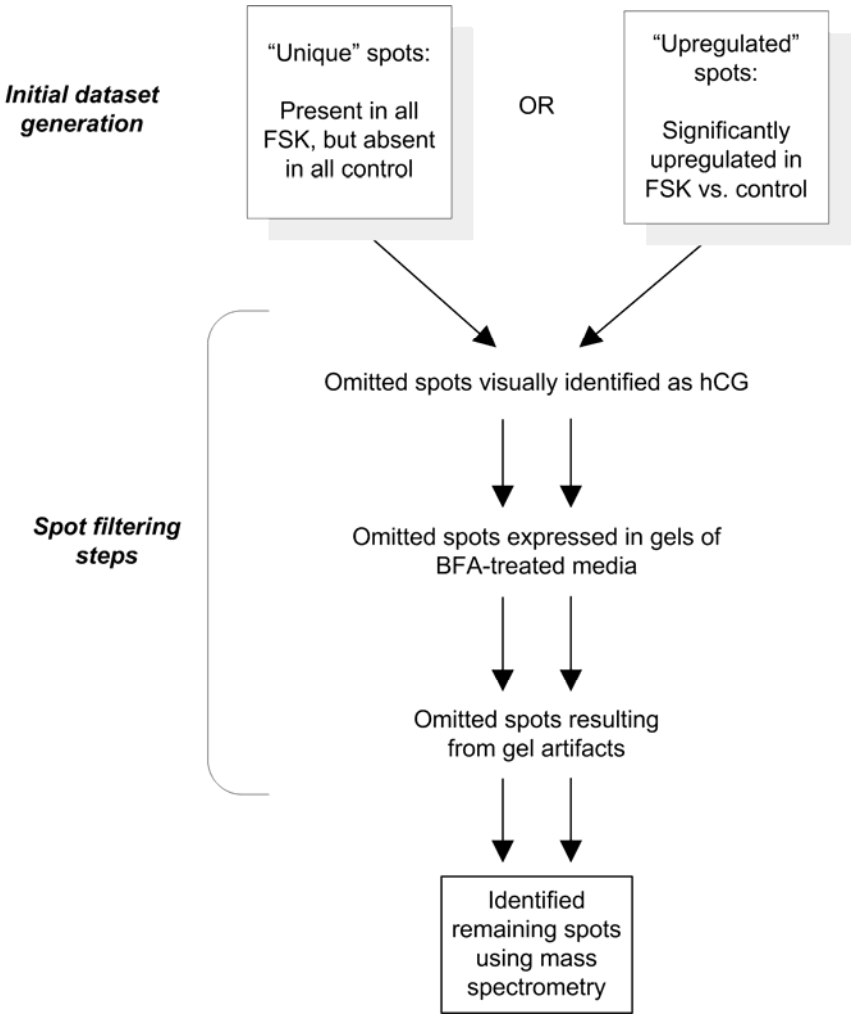

Figure 3-3: Filtering strategy applied to BeWo 2DE spot datasets before identification

After spot detection and hierarchical matching of all gels using ImageMaster Platinum software, unique and upregulated spot datasets are generated from 2DE data and dataset spots were filtered through the steps shown above.

Fold changes were calculated from the median volumes of matched spots as (FSK median volume)/(control median volume). Positive fold changes indicate upregulation in FSK-treated samples and negative fold changes indicate downregulation in these samples.

3.2.6 Statistical analysis

Statistical tests described in this chapter were performed by Mr SH Wu (The University of Auckland). Spots overexpressed in FSK-treated gels compared to control gels were determined using a one-tailed Student's *t* test with a significance level of $p<0.05$. To account for multiple comparisons, *p*-values were adjusted by the FDR controlling method of Benjamini and Hochberg (1995).

3.2.7 Protein identification

Spots of interest were excised from gels using OneTouch spot pickers and were stored at −80 °C until tryptic digestion. Gel spots were digested with Trypsin Gold (Promega) according to published methods (Hardt *et al.* 2005) with the following modifications: after digestion, peptides were recovered by one or two extractions with 50% acetonitrile/5% formic acid, and the digestion supernatant and extractions were combined and reduced to approximately 12 µL volume by vacuum centrifugation. Tryptic peptides were submitted for LC-MS/MS analysis on a QSTAR XL ESI-qTOF (Applied Biosciences) at the Maurice Wilkins Centre for Molecular Biodiscovery, The University of Auckland. LC-MS/MS analysis was performed by Mr M Middleditch.

Protein identification reporting followed recently published guidelines (Bradshaw *et al.* 2006). Tandem MS/MS data were extracted from raw spectra using Mascot Distiller (Matrix Science). Data were searched against the Swiss-Prot database (version 52.2, date 14 April 2007) using the Mascot search engine v2.2.0 (Matrix Science) with the following parameters: Taxonomy: human (or mammals as below), semitrypsin cleavage with up to 1 missed cleavage allowed, fixed modification: propionamide (of cysteines), variable modification: oxidation (of methionines), mass tolerances ±0.1 Da, peptide charges 2+ and 3+. Positive identifications reported here had at least three unique peptides match the database entry.

Searches within mammalian sequences confirmed that identified proteins were not of bovine origin. Mammalian search results were simplified using the "only bold red" option which displays peptide matches within their best unique protein match only. In cases where multiple homologous proteins were identified within the same spot, manual validation of MS/MS peptide matches was used to confirm the homolog present where possible.

3.3 Results

3.3.1 Preparation of conditioned media proteins for proteomic analysis

Conditioned media proteins were concentrated and desalted with water washes in preparation for proteomic analysis. Protein concentrations of conditioned media were monitored throughout concentration and desalting steps. Minimal loss of protein was observed after the initial concentration step which removed proteins < 5 kDa. Subsequent water wash steps also caused no protein losses. The amount of protein detected in concentrated media from FSK-treated cells was greater than that of control cells, and both were greater than media alone (data not shown). A series of water wash steps were used to reduce the salt concentration of conditioned media to allow

proteomic analysis. Conductivity of samples decreased substantially after the initial concentration and first wash steps and was near zero after a second wash step (Figure 3-4).

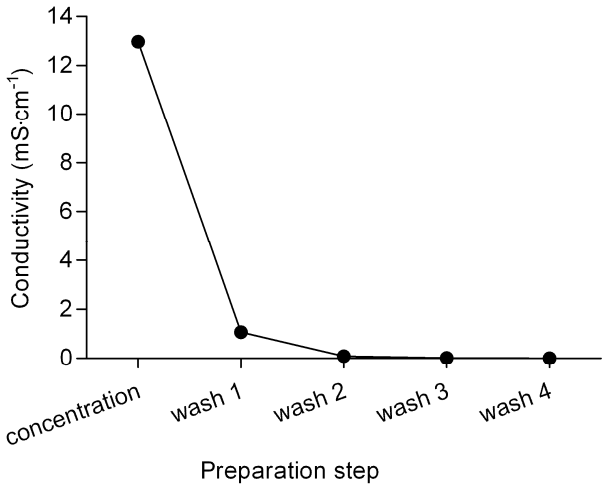

Figure 3-4: Conductivity of conditioned media following centrifugal washing

Conductivity of washed BeWo conditioned media following concentration and water washing. Total conductivity was measured after initial concentration of media and after four washes of ~20 mL H_2O each. Data represent the mean ± SD of one experiment performed in triplicate. Error bars are so small at this scale that they are not visible beneath the data points.

3.3.2 Optimization of rehydration solution components for BeWo media proteins

Optimal resolution of proteins on 2-D gels depends greatly on the composition of the rehydration buffer used, with variations in denaturant and detergent common between various sample types. Several formulations of rehydration solution (detailed in Table 3-1, page 53) were trialed before the final constituents for this study were determined. An initial formulation of rehydration solution (Formulation A) produced good resolution on 2-D gels (Figure 3-5A), but increased solubility of proteins was desired. Formulation B added CHAPS as a second detergent, which substantially increased the amount of protein visible on 2-D gels (Figure 3-5B). However these gels showed undesirable vertical streaking near their bottom edges. Streaking was alleviated by adding a centrifugation step (Formulation C), but this step also reduced the amount of protein visible on gels (Figure 3-5C). Protein solubility was increased by changing to Formulation D where the detergent used was Triton X-100 rather than CHAPS (Figure 3-5D). Results were further enhanced by using Formulation E, containing a combination of urea and thiourea (Figure 3-5E). In particular, thiourea increased the resolution of high MW and high abundance proteins and was used for further BeWo 2DE analyses.

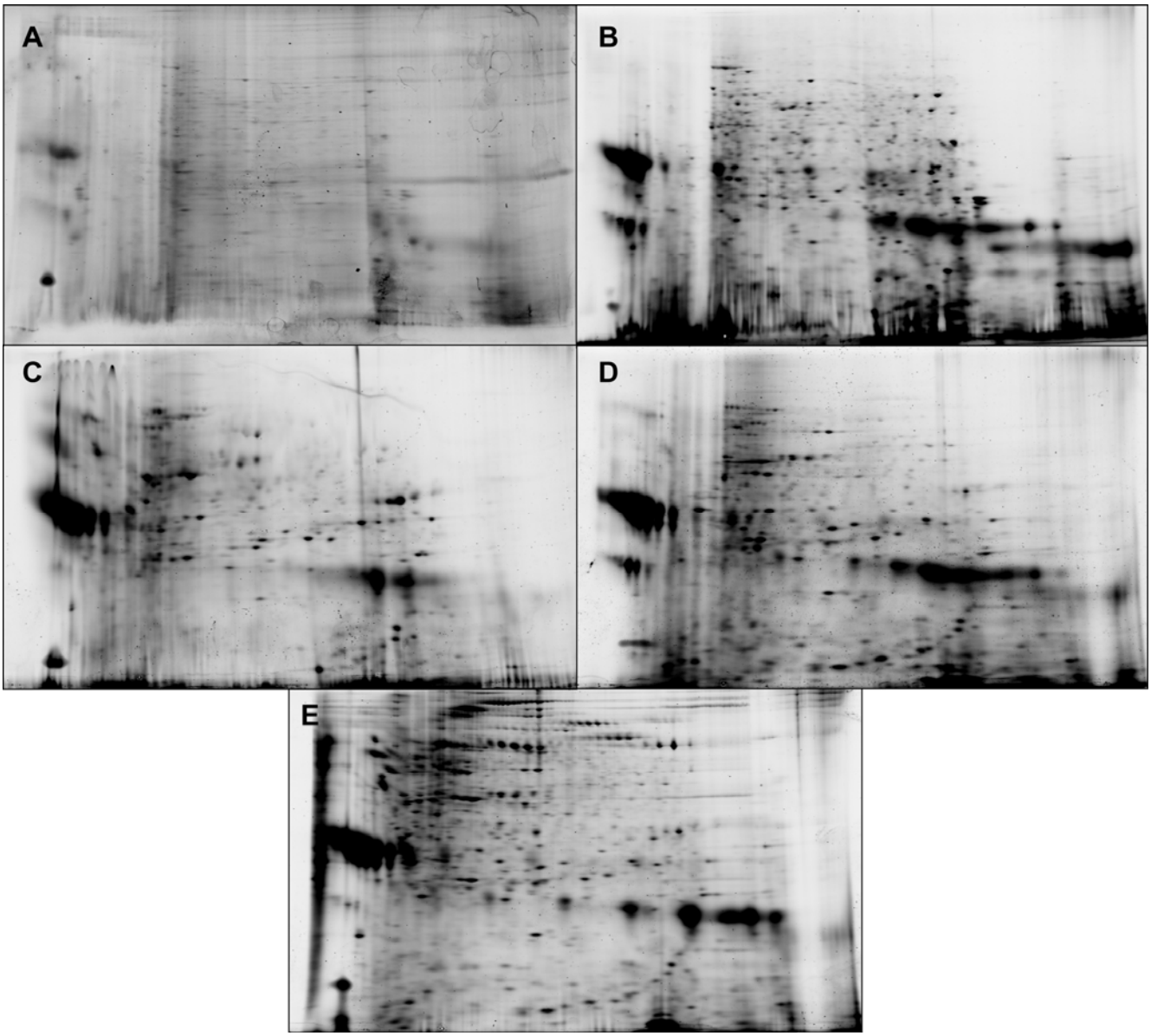

Figure 3-5: Effect of rehydration solution composition on media proteome patterns

2DE comparison of proteome patterns from conditioned media samples where rehydration solution (RHS) components varied (detailed in Table 3-1). RHS components included: (**A**), 8 M urea and 1% C7BzO; (**B**), 9 M urea, 1% C7BzO, and 1% CHAPS; (**C**), as in B with 8 M urea and 10 min centrifugation before rehydration; (**D**), 8 M urea, 0.5% Triton X-100, and 10 min centrifugation; (**E**), as in D with 7 M urea and 2 M thiourea.

3.3.3 2DE analysis of secreted proteins from BeWo serum-free medium

Secreted proteins isolated from BeWo conditioned serum-free medium were analyzed using 2DE. Three sample types (control, FSK-treated, and FSK+BFA-treated) were compared using a hierarchical matching strategy (Section 3.2.5). Two groups of results were sought: spots present only in FSK-treated gels and no others ("unique" spots), and spots significantly upregulated in FSK-treated gels versus control gels ("upregulated" spots). Though significantly upregulated spots were found using a one-tailed Student's *t* test, none remained significant after FDR correction for multiple comparisons. Consequently, the spot filtering strategy described in Section 3.2.5 was applied. The resulting filtered datasets contained eight unique spots and one upregulated spot (Table 3-3).

Table 3-3: Results of BeWo spot dataset filtration strategy

Filtration step	*Number of spots in "unique" dataset*	*Number of spots in "upregulated" dataset*
(initial number passing presence/absence or *t* test)	26	11
spots identified as hCG	3	7
spots present in FSK+BFA media	10	3
spots resulting from technical artifacts	5	none
spots in remaining filtered dataset	**8**	**1**

The upregulated spot, number 841, was upregulated 1.6-fold from FSK-treated cells on average when compared to control cells (p=0.04). The nine final spots are shown in Figure 3-6. Scatter plots of spot distribution in the three sample types are shown in Figure 3-7.

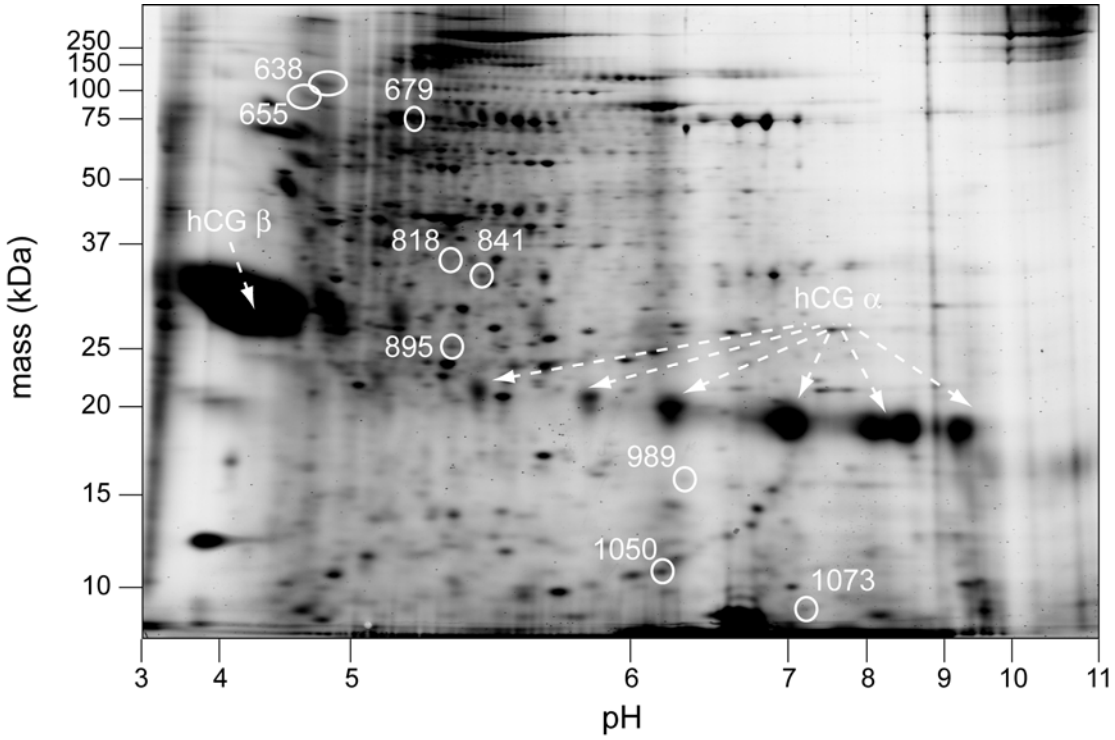

Figure 3-6: Representative map of the BeWo secreted proteome with forskolin treatment

Conditioned media proteins from BeWo cells cultured in serum-free medium containing 100 μM FSK. Spots resulting from dataset filtration are circled. Specific spot patterns corresponding to the α and β subunits of hCG are shown by the dotted arrows. 150 μg of protein were focused over pH 3–11 NL, separated on Criterion 8–16% Tris-HCl gels, and stained with SYPRO Ruby.

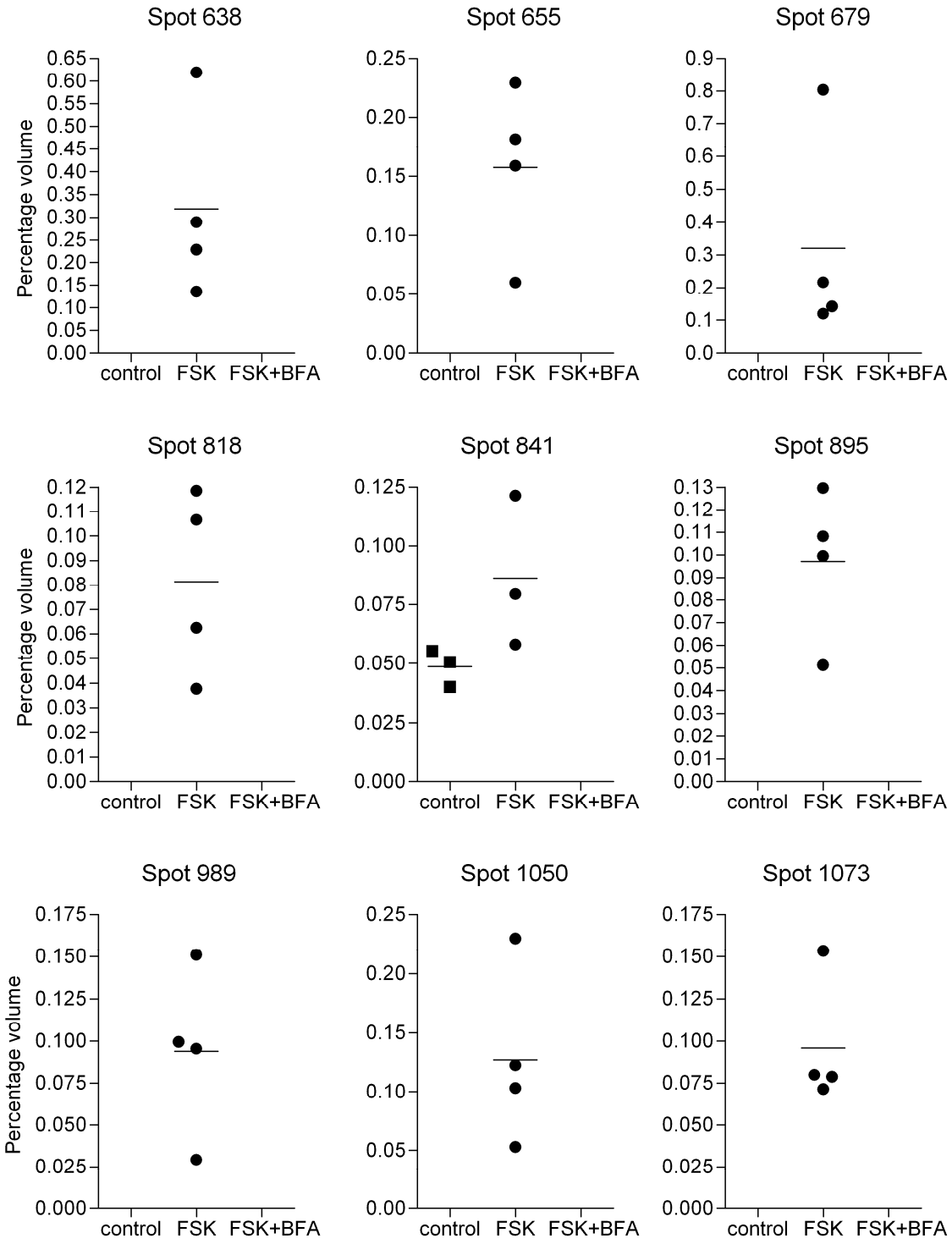

Figure 3-7: Distribution of 2DE spot volumes from BeWo secreted proteins

The percentage volumes of individual spots are shown from gels containing control (■), FSK (●), or FSK+BFA (▲; none present) samples. Each point represents the percentage volume from a single gel. Hortizontal lines indicate the mean percentage volume within each group. Eight spots were present only in media from FSK-treated cells and not in control media or FSK+BFA media. Secretion of spot 841 was significantly upregulated from FSK-treated versus control cells (1.6-fold; p=0.04) and this spot was not expressed by FSK+BFA-treated cells.

3.3.4 Identification of protein spots

Protein spots passing the spot filtering strategy were identified using MS (Table 3-4). Most spots contained multiple valid protein identifications spanning a large range of MW. No protein matches were found for spots 989 and 1050 which contained at least three unique peptides.

The species source of each protein was assessed by comparing protein matches resulting from database searches of human sequences versus those from mammalian sequences. Five proteins (heat shock protein HSP 90-α, heat shock 70 kDa protein, β-actin, tubulin β chain, and ubiquitin) matched equally well to homologous human and bovine proteins, so the bovine origin of these proteins in human samples could not be ruled out. One additional bovine protein, BSA, was definitively identified from mammalian searches. These bovine or suspected bovine proteins were not examined further at this stage; only proteins with confirmed matches to human sequences, with no higher-significance matching to homologous bovine proteins, were studied further.

Table 3-4: Proteins identified from BeWo conditioned media 2DE spots

Spot number (Dataset)	*Protein*[a]	*Accession*	*Sequence coverage*[bc] *(%)*	*Peptides (unique)*[b]	*Calculated MW*[d] *(kDa)*	*Gel MW (kDa)*	*Calculated pI*[d]	*Gel pI*
638 (unique)	desmoglein-2	Q14126	13	15 (13)	117	80	4.93	4.9
	VE-cadherin	P33151	13	10 (8)	83		5.01	
	phospholipid transfer protein	P55058	24	14 (11)	53		6.53	
655 (unique)	VE-cadherin	P33151	11	7 (7)	83	70	5.01	4.8
	phospholipid transfer protein	P55058	17	7 (7)	53		6.53	
	desmoglein-2	Q14126	3	3 (3)	117		4.93	
	proactivator polypeptide precursor	P07602	6	3 (3)	58 (precursor)[e]		5.06 (precursor)[e]	
679 (unique)	GRP 78 (heat shock 70 kDa protein 5)	P11021	44	39 (23)	70	65	5.01	5.2
	matrix metalloproteinase 2	P08253	20	14 (13)	62		5.02	
	plastin-2	P13796	25	15 (14)	70		5.20	
	protein disulfide-isomerase A4	P13667	17	9 (9)	71		4.89	
	α-1B-glycoprotein	P04217	16	7 (7)	52		5.65	
	ceruloplasmin	P00450	6	6 (6)	120		5.41	
	phospholipid transfer protein	P55058	10	5 (4)	53		6.53	
818 (unique)	tubulin α-1B chain	P68363	15	5 (5)	50	40	4.94	6.2
	heat shock protein HSP 90-β	P08238	6	4 (4)	83		4.97	
	L-lactate dehydrogenase B chain	P07195	11	3 (3)	37		5.72	
841 (upregulated) 1.6-fold	tubulin α-1B chain	P68363	19	7 (7)	50	34	4.94	5.3
	L-lactate dehydrogenase B chain	P07195	27	9 (8)	37		5.72	
	F-actin capping protein subunit α-1	P52907	19	4 (4)	33		5.45	

Spot number (Dataset)	*Protein*[a]	*Accession*	*Sequence coverage*[bc] *(%)*	*Peptides (unique)*[b]	*Calculated MW*[d] *(kDa)*	*Gel MW (kDa)*	*Calculated pI*[d]	*Gel pI*
895 (unique)	protein disulfide isomerase A3	P30101	30	13 (13)	54	24	5.61	5.4
	sulfhydryl oxidase 1	O00391	5	4 (4)	80		9.05	
1073 (unique)	fructose-bisphosphate aldolase A	P04075	13	4 (4)	39	9	8.39	7.2

[a] Proteins are listed within each gel spot number in descending order of Mascot match score. Database search result data are provided in Appendix B.1.

[b] Results are from searches of human sequences from the Swiss-Prot database. Identical searches of mammalian sequences confirmed that these proteins were not of bovine origin. Proteins matching equally well to human and bovine sequences are not shown.

[c] Sequence coverage was calculated as part of the Mascot search process using the entire chain of the protein's Swiss-Prot database entry.

[d] MW and pI were calculated on Swiss-Prot entries from the main chain or appropriate chain only using the Compute MW/pI tool (http://www.expasy.org/tools/pi_tool.html).

[e] MW and pI were calculated on the entire proactivator polypeptide precursor sequence as the peptides matched covered both a propeptide and small chain at different locations within the precursor.

3.4 Discussion

The results presented in this chapter represent the first proteomic study of secreted proteins from any placental cell type. This research elucidated not only the conditions necessary for the analysis of a secreted proteome using 2DE but also gave insights into paracrine processes involved in BeWo differentiation.

Initial experiments validated the methods used to generate 2-D gels of BeWo conditioned media. Protein samples used for 2-D gels must have very low conductivity to enable focusing of proteins at their pI, a process which uses thousands of volts of electricity. Solution conditions for these protein samples must have an extremely low concentration of salts. Secreted proteins were purified for 2DE using a centrifugal washing method which reduced the conductivity of samples to near zero within two washes. This is an extremely efficient method for preparing samples for proteomic analysis.

Following the successful cleanup of media samples for proteomic analysis, they were diluted with a rehydration solution containing chaotropic agents to denature proteins, detergents to improve solubility, and other components. Changes in detergent composition are largely responsible for variability in protein resolution on 2-D gels. Nonionic or zwitterionic detergents such as Triton X-100 and CHAPS solubilize proteins without affecting their charge during isoelectric focusing. C7BzO is a newer nonionic detergent reported to give better resolution in the basic pH range than CHAPS (Tastet *et al.* 2003; Maserti *et al.* 2007). Iterative formulation of the rehydration solution used in this study used three detergents: C7BzO, CHAPS, and Triton X-100. Samples also benefitted from centrifugation to remove contaminating particulates. A combination of urea and thiourea was also used as a chaotropic agent. Thiourea has long been reported to enhance sample resolution in 2DE and specifically to solubilize membrane proteins (Rabilloud *et al.* 1997; Molloy *et al.* 1998). More recently, thiourea has been used in a 2DE study of human serum in toxic oil syndrome (Quero *et al.* 2004). The rehydration solution composition used here is likely to work well for future 2DE studies of secreted proteins.

As with any proteomics experiment, a large amount of data was generated in this study. A key concept in this thesis is the filtering, or simplification, of this large dataset to produce meaningful results related to the experiment's aims. This study sought secreted proteins related to BeWo differentiation with FSK treatment, but with a specific focus on proteins upregulated in or unique to FSK-treated BeWo cells. Consequently, a data filtering strategy was applied to narrow the list of proteins of interest to these unique or upregulated categories. The 2-D gel data produced here could

alternatively have focused on proteins upregulated by control cells, or on proteins found in media from BFA-treated cells indicating apoptosis. These are avenues for further study.

Seventeen proteins were identified from the nine gel spots analyzed in this study. Six of these proteins belong within the extracellular space as identified in the Gene Ontology Annotation database (Table 3-5). These proteins are presumed to be secretion products and are discussed in more detail below. Proteins with functions obviously related to intracellular processes (*e.g.*, F-actin capping protein) are not discussed further. These proteins may result from contamination of culture media by lysis products. While their presence or upregulation in FSK-treated cells is probably related to the process of syncytialization, they are not the primary focus of this secreted proteomics study.

Table 3-5: Cellular location and function of proteins identified from forskolin-treated BeWo cells

Protein	*Spot number(s)*	*Cellular compartment (GO)*[a]	*Biological process (GO)*[a]
α-1B-glycoprotein	679	extracellular space	unknown
ceruloplasmin	679	extracellular space	iron ion homeostasis
desmoglein-2	638, 655	intercellular junctions	cell-cell adhesion
F-actin capping protein subunit α-1	841	cytoskeleton	cell motility
fructose-bisphosphate aldolase A	1073	cytoplasm	glycolysis; fructose metabolism
GRP 78 (heat shock 70 kDa protein 5)	679	cell surface; endoplasmic reticulum	anti-apoptosis
heat shock protein HSP 90-β	818	cytoplasm	signal transduction; mitochondrial transport
L-lactate dehydrogenase B chain	818, 841	cytoplasm	glycolysis
matrix metalloproteinase 2	679	extracellular space	proteolysis and peptidolysis
phospholipid transfer protein	638, 655, 679	extracellular space	lipid metabolism
plastin-2	679	cytoplasm	actin filament bundle formation
proactivator polypeptide precursor	655	extracellular space	lipid transport
protein disulfide isomerase A3	895	endoplasmic reticulum	protein retention
protein disulfide-isomerase A4	679	endoplasmic reticulum	protein secretion
sulfhydryl oxidase 1	895	cell membrane	regulation of cell growth
tubulin α-1B chain	818, 841	microtubules	microtubule-based movement
VE-cadherin	638, 655	extracellular space; intercellular junctions	cell-cell adhesion; negatively regulates cell growth

Shaded rows indicate proteins with known extracellular expression.

[a] Cellular compartment and biological process information was obtained from the Gene Ontology Annotation database (http://www.ebi.ac.uk/GOA/).

3.4.1 α-1B-glycoprotein

α-1B-glycoprotein (A1BG) is a member of the immunoglobulin superfamily (Ishioka *et al.* 1986; Udby *et al.* 2004) with no known function. An opossum homolog of A1BG, oprin, is a metalloproteinase inhibitor with functional similarity to the tissue inhibitor of metalloproteinases (TIMP) family of proteins (Catanese and Kress 1992; Kreunin *et al.* 2007). Placental cytotrophoblasts secrete matrix metalloproteinases (MMPs) to assist their invasion into the decidua; TIMP proteins inhibit this metalloproteinase activity and restrict invasion (Bischof *et al.* 2000). In addition, choriocarcinoma invasion and differentiation (syncytialization) are inversely related (Hohn *et al.* 1998). Secretion of a TIMP-like protein by syncytalized BeWo cells would inhibit invasion and promote differentiation, realized as syncytialization in this cell culture model. Further study of the function of A1BG within the BeWo cell model could confirm this role.

The culture conditions used in this study may affect the regulation of A1BG as culture in bovine serum has an inhibitory effect on the expression of an MMP protein, MMP-2, in BeWo cells (Mandl *et al.* 2002). The increase observed here in expression of a TIMP-like protein is realistic regardless of serum culture or syncytialization, or both, causing the reduced matrix metalloproteinase levels which may contribute to TIMP upregulation.

3.4.2 Matrix metalloproteinase 2

MMP secretion is well-correlated with cytotrophoblastic invasion potential, a necessary step along the invasive CTB differentiation pathway and one responsible for ensuring placental attachment to the maternal decidua. MMP-2 is one of two collagenases expressed in placental cells, the other being MMP-9. BeWo cells only produce MMP-2 and are unable to invade in an *in vitro* invasion assay, while other choriocarcinoma cell lines producing both MMP-2 and MMP-9 can invade (Morgan *et al.* 1998). While the MMP-2 upregulation observed here could suggest that BeWo cells are becoming more invasive, culture in bovine serum also inhibits MMP-2 expression in BeWo cells (Mandl *et al.* 2002). It is possible that the MMP-2 expression observed here in FSK-treated cells may reflect the different responses of control and FSK-treated BeWo cells to the removal of inhibitory bovine serum. The observation of both a matrix metalloproteinase and a TIMP-like protein (A1BG) within this study leaves the precise roles of these proteins unclear. However, the involvement of both types of proteins does support the important role of collagenases and their inhibitors in the syncytialization of BeWo cells.

3.4.3 Ceruloplasmin

The role of ceruloplasmin in syncytialization has not been studied, but its dysregulation may be implicated in the biology of preeclampsia (Aksoy *et al.* 2003; Serdar *et al.* 2006). Ceruloplasmin is the main plasma protein responsible for binding copper. This protein also has ferroxidase activity, oxidizing iron(II) to iron(III), an essential step before iron(III) is loaded onto transferrin (Healy and Tipton 2007). Ceruloplasmin therefore plays a key role in the regulation of iron homeostasis and has an antioxidant effect by helping to prevent lipid oxidation catalyzed by metal ions (Engin-Üstün *et al.* 2005). Expression of ceruloplasmin can be induced by inflammatory mediators and repressed through translational control when iron is deficient (Mazumder *et al.* 2006). Studies have found serum concentrations of ceruloplasmin increased (Griffin 1983; Vitoratos *et al.* 1999; Aksoy *et al.* 2003; Engin-Üstün *et al.* 2005; Serdar *et al.* 2006) or unchanged (Hubel *et al.* 1996) in preeclamptic women. It is unknown whether these increases are merely symptoms of inflammatory processes at work in clinical preeclampsia or whether they reflect a facet of the causative process.

No studies have yet examined the relationship of ceruloplasmin to syncytialization. The fetus is entirely dependent upon maternal iron which is transferred to the fetal circulation through the STBs and CTBs (Fuchs and Ellinger 2004). This suggests that placentally derived ceruloplasmin should be intimately involved in the iron transport process. Ceruloplasmin could be secreted by STBs basolaterally (into cytotrophoblasts) rather than apically (into maternal circulation) as part of this process. Further studies specifically focused on ceruloplasmin rather than iron transport are necessary to discover the finer details of placental iron transport. It should be remembered that the culture conditions used in this study incorporated direct iron supplementation through the addition of ferrous sulfate. The possibility that ceruloplasmin upregulation is in response to this stimulus cannot be ruled out, although the detection of the spot 679 isoform of ceruloplasmin from FSK-treated culture media alone suggests that FSK-treated BeWo cells may additionally modulate iron homeostasis in culture.

3.4.4 Phospholipid transfer protein

As its name suggests, phospholipid transfer protein (PLTP) transfers phospholipids between lipoproteins and cells. Transfer of phospholipids facilitates interconversion of chylomicrons and very low density lipoproteins (VLDL) into HDL, regulating plasma HDL levels (Setälä *et al.* 2007). PLTP also accelerates the removal of α-tocopherol (vitamin E) from atherogenic lipoproteins (Jiang *et al.* 2002). Dysfunctional vascular endothelium and lipid oxidation are key processes implicated in preeclampsia, and PLTP is involved in both processes: the vitamin E transported by PLTP can be

incorporated into the endothelial wall and may prevent the vessel dysfunction caused by oxidized LDL (Desrumaux *et al.* 1999). In the placenta, cell surface phospholipids are involved in the mechanism of syncytial fusion (Pötgens *et al.* 2002). The expression of PLTP observed here in FSK-treated BeWo cells may be involved in the STB-like fusion events known to occur in this culture system.

3.4.5 Proactivator polypeptide precursor

The proactivator polypeptide precursor, also called prosaposin, encodes four saposin or sphingolipid activator proteins found in lysosomes (Chataway *et al.* 1998). No studies have directly investigated the role of these proteins in the placenta or in preeclampsia. Both prosaposin and the saposins participate in the lysosomal degradation of sphingolipids (Vaccaro *et al.* 1999). As these lipid complexes are key components of the cellular plasma membrane's lipid bilayer, it is possible that prosaposin is present in FSK-treated BeWo cells in conjunction with syncytial fusion. Fusion may incorporate controlled breakdown of the plasma membrane at cell-cell junctions. Further studies are necessary to uncover information about the participation of saposins in placental biology.

3.4.6 VE-cadherin

Vascular endothelial cadherin, like its epithelial counterpart E-cadherin, is a cell adhesion protein with documented changes in its expression patterns during pregnancy and in preeclampsia (Zhou *et al.* 1997b). During cytotrophoblast differentiation, the expression of VE-cadherin is almost inversely related to that of E-cadherin (Zhou *et al.* 1997a). E-cadherin expression reduces markedly in both cytotrophoblasts and BeWo cells during syncytialization (Coutifaris *et al.* 1991). Staining for E-cadherin has been used as a qualitative measure of trophoblast differentiation, with a loss of E-cadherin staining evident during cell fusion (Al-Nasiry *et al.* 2006). Downregulation of E-cadherin, and its inverse relationship with VE-cadherin, may explain the presence of VE-cadherin in the FSK-treated BeWo cells used in this study.

3.4.7 Additional considerations

Secreted proteomic studies face three main challenges: 1) secreted proteins may be present in media at very low concentrations, 2) media can be contaminated by cell lysis products including intracellular proteins, and 3) exogenous proteins such as bovine serum can further contaminate media. The first of these challenges is common to any proteomic analysis and has been discussed earlier (see Chapter 1). Culture conditions developed in Chapter 2 attempted to reduce media

contamination by both intracellular and serum proteins. The proteins identified in the current study were present only, or at significantly increased levels, in FSK-treated cultures and were not found in cells additionally treated with BFA. This lessens the likelihood that these proteins were derived from cell lysis. However, the results obtained earlier in this chapter did include bovine-derived proteins and proteins of obviously intracellular origin, indicating that these challenges were not entirely overcome. A recent report of murine macrophage secreted proteomics (Chevallet *et al.* 2007), despite incorporating careful culture conditions and wash steps similar to those used here, found a similarly high incidence of bovine and intracellular proteins. Use of heterologous serum (*i.e.*, bovine serum for human cultures) has allowed differentiation of some protein origins within the current study, but in other cases no species-specific peptides were available to differentiate between human and bovine proteins. Bovine proteins may not be able to be removed completely even with extensive washing (Miller *et al.* 2006b).

As observed in Chapter 2, the use of serum-free culture conditions inherently decreases cell viability slightly as assessed by increased LDH release into media. For this reason, Chevallet *et al.* (2007) admit that comparing secreted proteomes especially between different cell types may not control for increased cell lysis; they suggest that alternative, more appropriate methods for comparison of secreted proteomes have yet to be developed. This is particularly true in cases where secreted proteins are compared between multiple cell types which have different levels of viability. In this instance, the similar culture conditions used cannot entirely control for variations in cell lysis and intracellular protein contamination. Treatment of cultures with BFA to inhibit protein secretion, as in this study, may help distinguish lysis products from truly secreted proteins. However, BFA itself also compromises cell viability which is disadvantageous. The extent of decreased cell viability in the present BeWo culture system as measured by LDH release was not high until the extreme end of the culture period (see Chapter 2), so the high proportion of intracellularly-derived proteins identified here was unexpected.

Multiple proteins were identified from each of the 2-D gel spots examined in this study. It is common to assume in 2DE analysis that only a single protein isoform is present (or identifiable) within a single gel spot. If multiple proteins are present, and differential regulation of at least one protein results in apparent changes on gel images, which protein is responsible? The phenomenon of protein co-identification, where multiple proteins are present and identified as significant within a single gel spot, has not been well discussed in 2DE literature. Some researchers agree that it exists and state that it confounds attempts to perform quantitative analyses (Campostrini *et al.* 2005) while others suggest that the phenomenon does not detract from the power of 2DE to provide meaningful

quantitative comparisons (Hunsucker and Duncan 2006). The large amount of protein data derived from MS analysis of each gel spot in the current study required its own dataset filtering method, similar to the large volume of 2-D gel data initially leading to those spots. Filtering techniques used here included species-specific searches and classification of the cellular compartment of each identified protein.

Current 2DE techniques do not allow precise analysis of comigrating spots; other validation techniques must be applied to confirm the presence of and quantitative changes in proteins of interest. Specific immunodetection in western blot analysis provides a semi-quantitative assessment of protein changes. More precise quantitation techniques could include label-free quantitation approaches utilizing MS to compare a number of samples, but requiring specialized methods and software (Old *et al.* 2005). MRM, a promising new MS quantitation technique, allows multiplexed measurement of the abundance of any key peptide signals from within proteins of interest, and may replace some immunoassay measurements (Anderson and Hunter 2006). Unfortunately, MRM techniques require triple quadrupole mass spectrometers not yet readily available.

As discussed in Chapter 2, even the most efficient FSK treatment conditions encourage only about 10% of cells to form syncytia. The serum-free conditions developed in this thesis were entirely appropriate for proteomic study and led to the observation of large differences in protein secretion (*e.g.*, hCG) but it is unlikely that smaller secretion changes between undifferentiated and differentiated BeWo cells would be robustly detected using these proteomic methods. Extension of the BeWo study to a primary placental cell culture model of STB formation could lead to further insights into this important physiological process, although the fibroblast contamination usually observed in these primary cultures would require management to allow true representation of the STB secreted proteome.

3.4.8 Conclusions

The six proteins identified in this study have diverse roles, but do have plausible linkages to the syncytialization process taking place in differentiating BeWo cells. PLTP is involved in the movement of cell membrane components, similar to the role of the proactivator polypeptide precursor in degradation of the cell membrane. Along with VE-cadherin, which is involved in cell fusion, these proteins illustrate the biochemical actions behind the process of FSK-induced cell fusion. FSK treatment encourages BeWo cells to differentiate rather than to invade the extracellular matrix environment, so secretion of the TIMP-like protein A1BG may help prevent this invasive process. The opposing, invasive action of MMP-2 and its sensitivity to culture environments containing bovine

serum could indicate that the presence of MMP-2 is artifactual. Similarly, the role of ceruloplasmin in syncytlialization is unclear, but specific isoforms of this protein may be secreted by BeWo cells in response to the iron-rich conditions of serum-free culture established here.

This study was useful for its systematic development of methods for the analysis of proteins secreted by cultured BeWo cells. These methods can be applied to any other culture system requiring similar conditions, and as such they will be useful to researchers performing analyses of secreted proteins. It was evident from this study that secreted proteins can be efficiently concentrated and desalted from BeWo conditioned media using centrifugal ultrafiltration and resolved using the 2DE methods developed here. The identification of several proteins with putative involvement in FSK-induced syncytialization has provided a glimpse into possible mechanisms involved in BeWo differentiation processes which will require further validation.

CHAPTER 4 COMPARISON OF SERUM IMMUNODEPLETION METHODS FOR PROTEOMIC ANALYSIS

4.1 Introduction

Plasma and its derivative, serum, are important sources of biomarkers. All tissues release proteins into circulating blood which makes plasma a valuable resource for discovering biomarkers related to disease states. The ease of sampling plasma and its routine clinical use give this fluid a position of premium importance in biomarker discovery research. However, the pervasiveness of plasma gives its proteome a complex nature. Identification of biomarkers in the plasma proteome is hampered by this complexity and the huge range of protein concentrations present, covering eleven orders of magnitude from $mg \cdot mL^{-1}$ to $pg \cdot mL^{-1}$. Current biomarkers can lie in the lower end of this dynamic range (Anderson and Anderson 2002). For example, detection of cardiac troponin T at levels greater than 100 $pg \cdot mL^{-1}$ can indicate acute cardiac disease (Jaffe *et al.* 2006). Proteomic techniques can assess only a limited number of proteins from the very complex plasma/serum proteome (Anderson and Anderson 2002). Simplification of this complexity is, therefore, an essential step before biomarkers for specific diseases can be identified using proteomic techniques.

Pre-fractionation of the plasma proteome into sub-proteomes can be achieved by depletion of high-abundance proteins or separation by nearly any biophysical characteristic. Protein characteristics employed for separation include pI, mass, hydrophobicity, and metal-binding ability among others (Lescuyer *et al.* 2004). Fractionation by liquid-phase isoelectric focusing cleanly separates proteins by their charges but requires very large volumes of plasma (Herbert and Righetti 2000). Bead-based separations by hydrophobicity or metal-binding ability require less volume but are hampered by low protein yields insufficient for 2-D gel analysis (Villanueva *et al.* 2004). The ability of these methods to be quantitatively applied to differential proteomic analyses, including those based on 2-D gel electrophoresis, is a critical step in the discovery of biomarkers.

Depletion of high-abundance proteins has been attempted using mass-based techniques such as the separation of plasma proteins under 30 kDa. This approach removes the majority of high-abundance proteins (Harper *et al.* 2004) but inherently eliminates other large proteins that may be of interest as biomarkers (Tirumalai *et al.* 2003). Further, suboptimal removal of albumin and imprecise separation of size fractions around the chosen MW cut-off have been reported with this technique (Georgiou *et al.* 2001). This has led to a preferred depletion approach which specifically removes high-abundance proteins using immunoaffinity-based methods.

Six highly abundant proteins make up 85–90% of the serum/plasma proteome: albumin, transferrin, IgG, immunoglobulin A (IgA), α-1-antitrypsin, and haptoglobin (Anderson and Anderson 2002; Chromy *et al.* 2004). The presence of these proteins in such large amounts obscures lower-abundance proteins in 2DE analyses (Fountoulakis *et al.* 2004). Current 2DE approaches can analyze protein concentrations two to four orders of magnitude lower than the most abundant proteins present. Depletion of albumin and other highly abundant serum/plasma proteins with immunoaffinity-based methods can add as much as two orders of magnitude to 2DE analysis (Anderson and Anderson 2002; Righetti *et al.* 2005). For example, PlGF is a low-abundance serum protein present at 90 $pg \cdot mL^{-1}$ in preeclamptic women and 140 $pg \cdot mL^{-1}$ in healthy pregnant women (Levine *et al.* 2004a). Depletion of high-abundance proteins would allow PlGF to be visualized using contemporary 2-D gel techniques. Concentration by two orders of magnitude after depletion of abundant proteins would raise the concentration of PlGF to at least 9 $ng \cdot mL^{-1}$, and gel separation of 60 µL of serum would make this protein present at around 0.5 ng after depletion. The most sensitive protein stains currently available can detect as little as 0.25–1 ng of protein. Specific depletion of highly abundant proteins is an effective method to study a larger range of biomarkers in serum/plasma by 2DE than would otherwise be accessible.

In the early 2000s, commercial depletion solutions were limited to removal of albumin and/or IgG. Cibacron Blue dye was commonly used to bind albumin (Gianazza and Arnaud 1982) but also binds other proteins containing nucleotide-binding domains (Lollo *et al.* 1999; Ahmed *et al.* 2003; Zhang *et al.* 2004a). Removal of IgG with Protein G-based columns (Greenough *et al.* 2004) also removes autoantibodies and their antigens, which are markers of pathological conditions (Zhang *et al.* 2004a). This led to the development of single component, and later multicomponent, immunoaffinity subtraction protocols based on the specific binding of abundant serum proteins by polyclonal antibody mixtures. Both custom-made (Pieper *et al.* 2003a; Wang *et al.* 2003b) and commercially available multicomponent antibody resins (Hinerfeld *et al.* 2004; Huang *et al.* 2005), including an Albumin and IgG Removal Kit released by GE Healthcare, have been used for specific and effective removal of high-abundance serum/plasma proteins.

One of the first commercial multicomponent immunoaffinity depletion systems targeting more than two proteins, the Multiple Affinity Removal System (MARS), was released by Agilent in late 2003. The MARS system provides mixed depletion resin in a column format for one-step chromatographic removal of the six most abundant serum proteins: albumin, IgG, IgA, transferrin, α-1-antitrypsin, and haptoglobin. This system was trialed by the Plasma Proteome Project of HUPO and its preliminary

results indicated that MARS was an excellent depletion method for proteomic studies (G Omenn, personal communication; Omenn *et al.* 2005).

The aim of this investigation was to compare two antibody-based depletion approaches, a MARS column and a similar method removing only albumin and IgG, to determine:

1. Which method most effectively removes high-abundance proteins from serum samples, and

2. Which method most improved the visualization of lower-abundance proteins from serum.

4.2 Methods

4.2.1 Study population

This analysis used specimens from a case-control 2DE study of nulliparous women recruited in late pregnancy. The Auckland Ethics Committee approved these studies (AKX/02/00/157) and written informed consent was obtained from all women.

4.2.2 Human serum specimens

Blood samples were collected by venipuncture into 6 mL plain tubes and allowed to clot on ice. Samples were centrifuged at 2400 ×*g* for 10 min at 4 °C to separate serum from cells. Serum was then transferred into a new tube and centrifuged a second time at 3200 ×*g* for 15 min at 4 °C to ensure removal of all cells. The platelet-poor serum was stored in 250 µL aliquots at −80 °C within 3 h of collection.

4.2.3 Depletion of albumin and IgG using a spin column

Serum was depleted of albumin and IgG using the Albumin and IgG Removal Kit (GE Healthcare) according to the manufacturer's instructions. Unless otherwise noted, all steps were performed at room temperature.

A 10× stock solution of Complete Mini EDTA-free protease inhibitor (Roche) was made by addition of one tablet to 1 mL H_2O. Ten microliters of 10× protease inhibitor stock were added to 90 µL of thawed serum. Twenty microliters of serum containing protease inhibitor were added to 750 µL of antibody slurry and mixed for 30 min on a rotary shaker at 300 rpm. The serum-antibody mixture was added to a microspin column and centrifuged at 6500 ×*g* for 5 min. The resulting filtrate was divided in half, precipitated by addition of four volumes of ice-cold acetone, and stored at −20 °C for

at least 2 h. After precipitation, proteins were pelleted by centrifugation at 13 000 ×*g* for 5 min at 4 °C. Pellets were allowed to air dry before being resuspended in rehydration solution (9 M urea, 2% CHAPS, 60 mM DTT, 0.5% IPG Buffer 3–10 NL, and 0.002% bromophenol blue) and the halves recombined.

Protein concentrations were assayed before 2DE using the 2-D Quant Kit (GE Healthcare), a reverse biuret protein assay, according to the manufacturer's instructions.

4.2.4 Depletion of six proteins using the Multiple Affinity Removal System

4.2.4.1 Chromatography

The Multiple Affinity Removal System (MARS; Agilent) was used for immunodepletion of the six most abundant serum proteins: albumin, transferrin, haptoglobin, α-1-antitrypsin, IgG, and IgA. Depletion was performed according to the manufacturer's instructions using their proprietary Buffer A (a neutral-pH phosphate buffer) and Buffer B (a low-pH urea buffer).

The MARS column used was 4.6×50 mm, with a binding capacity of 16 µL native serum. The LC instrument (Applied Biosystems) consisted of a 140B Solvent Delivery System, a 785A Programmable Absorbance Detector set at 280 nm, and a 112A Oven/Injector set at ambient temperature with a 100 µL loop. A portable stripchart recorder (Yokogawa) was used. For all steps, the maximum pressure was 1700 psi and the minimum pressure target was 300 psi. Blank runs where only Buffer A was injected were performed at the beginning of each day.

A 10× stock solution of Complete Mini EDTA-free protease inhibitor (Roche) was made by addition of one tablet to 1 mL Buffer A. Sixty microliters of serum were diluted 1:5 in Buffer A to give a 300 µL volume, to which 33 µL of 10× protease inhibitor was then added. The diluted serum and protease inhibitor mixture was centrifuged using 0.2 µm spin filters (Agilent) to remove any particulates.

Seventy-five microliters of diluted serum mixture were injected into the loop at the commencement of the LC program (Table 4-1). This LC procedure was recommended by Agilent and modified for the Applied Biosystems LC instrument. Each single depletion run spanned LC programs 1 and 2, run in series. Flowthrough fractions were pooled from four replicate runs per sample to provide enough depleted protein for 2DE analysis.

Table 4-1: Liquid chromatography program for MARS depletion of serum samples

Program step	*Time (min)*	*Buffer B (%)*	*Flow rate (µL·min^{-1})*	*Loop status*	*Purpose*
LC program 1					
Pressurization	-	50	2000		
(to equilibration)	0.2	0	1000	closed	
Equilibration	6.0	0	1000	closed	column equilibration
Step 1	0.1	0	250	closed	
Step 2	0.5	0	250	open	injection of serum
Step 3	3.3	0	250	closed	
Step 4	12.5	0	250	closed	flowthrough fraction
Step 5	12.6	100	1000	closed	
Step 6	16.6	100	1000	closed	elution fraction
LC program 2					
Pressurization	-	50	2000		
(to equilibration)	0.2	0	2000	closed	
Equilibration	1.0	0	2000	closed	
Step 1	3.5	0	2000	closed	column regeneration

4.2.4.2 Preparation of depleted serum for 2DE

For each depleted serum sample, four replicate flowthrough fractions containing depleted serum proteins were combined using Vivaspin 4 centrifugal concentrators with a 5 kDa MW cutoff. All four flowthrough fractions were centrifuged in a single concentrator at 3700 ×*g* for 30 min at 18 °C to reduce their volumes by more than four-fold (Table 4-2). Concentrated protein was exchanged first into Milli-Q H_2O, and then into solubilization solution (9 M urea, 2% CHAPS, 60 mM DTT). Washes were repeated three times with each solution with centrifuging at 3700 ×*g* for 60–90 min per wash at 18 °C. After the third wash with solubilization solution, proteins were concentrated to approximately 300 µL and removed for quantitation.

Table 4-2: Preparation conditions for depleted serum samples

Step number	*Diluent*	*Centrifugation time (min)*
1	none – concentration only	30
2	H_2O	60
3	H_2O	60
4	H_2O	60
5	solubilization solution	90
6	solubilization solution	90
7	solubilization solution	90
8	none – concentration only	30

Protein concentrations were assayed using the 2D Quant Kit (GE Healthcare) according to the manufacturer's instructions. Depleted serum proteins in solubilization solution were stored at −80 °C until required for 2DE.

4.2.5 2DE of depleted serum proteins

For first-dimension separations, 150 or 250 µg of each depleted serum protein sample was diluted to 340 µL in rehydration solution (9 M urea, 2% CHAPS, 60 mM DTT, 0.5% IPG Buffer pH 4–7 or pH 3–10 NL, 0.002% bromophenol blue). Diluted samples were used to passively rehydrate 18 cm IPG strips covering nonlinear pH 3–10 or linear pH 4–7 gradients. Strips were then focused on a PROTEAN isoelectric focusing cell (Bio-Rad) at 20 °C and a maximum voltage of 10 000 V for 60 000 V·h.

Focused IPG strips were reduced with equilibration solution (6 M urea, 20 mM Tris pH 8.8, 2% SDS, 20% glycerol) containing 1% DTT (GE Healthcare) for 15 min, followed by alkylation in equilibration solution containing 2.5% IAA (Sigma-Aldrich) for 15 min. Both incubations were performed with gentle agitation. Strips were then rinsed with Milli-Q H_2O and drained on damp filter paper before second-dimension electrophoresis.

Second-dimension separation was carried out on a Multiphor II flatbed electrophoresis system using ExcelGel XL 12–14% gradient polyacrylamide gels and ExcelGel SDS buffer strips (GE Healthcare). Gels were run using the parameters listed in Table 4-3. After phase one, the IPG strip and electrode wicks were removed from the electrophoresis system. After phase two, the anodic buffer strip was moved to the previous location of the IPG strip.

Table 4-3: Electrophoretic parameters for serum 2DE

Electrophoresis phase	*Voltage (V)*	*Current (mA)**	*Power (W)**	*Time (h)*
1	1000	20	40	0:45
2	1000	40	40	0:05
3	1000	40	40	2:40

[a] Current and power settings listed here are for a single gel. When two gels were run together, the current and power settings only were doubled – voltage and time remained as stated.

After electrophoresis, gels were fixed for 2×30 min in fix/wash solution (10% ethanol, 7% acetic acid) with gentle agitation prior to staining with SYPRO Ruby stain (Invitrogen) overnight at room temperature in the dark. The next day, gels were washed in fix/wash solution for at least 1 h and briefly rinsed with H_2O before imaging. Imaging was performed using an FLA–2000 phosphorimager (Fuji) with excitation at 473 nm and a 580 nm high-pass emission filter. Gels were imaged with pixel sizes of 50 or 100 µm and F1000 (maximum) resolution.

4.3 Results

4.3.1 Depletion of albumin and IgG using a spin column

The spin column-based method for removal of albumin and IgG yielded varying amounts of depleted protein from each depletion round of 20 µL serum (Table 4-4). Immunoaffinity resin provided with this kit was not reusable. A typical 2-D gel image of 250 µg depleted serum protein produced using this method is dominated by high-abundance transferrin, α-1-antitrypsin, haptoglobin, and some remaining IgG not fully depleted by the kit (Figure 4-1). The incomplete depletion of IgG is notable.

Table 4-4: Protein yields from serum depletion methods

Method	*Number of samples*	*Mean protein [a] (µg)*	*Median protein (µg)*	*Range (µg)*	*% CV*
Albumin and IgG Removal Kit	23	233 (77)	245	60–366	32.9%
Multiple Affinity Removal System (MARS)[b]	4	190 (16)	196	167–202	8.3%

[a] Mean protein values are given with SD.

[b] Four to five replicate runs were performed per MARS sample, with flowthrough fractions pooled before quantitation; protein yields are shown here per run.

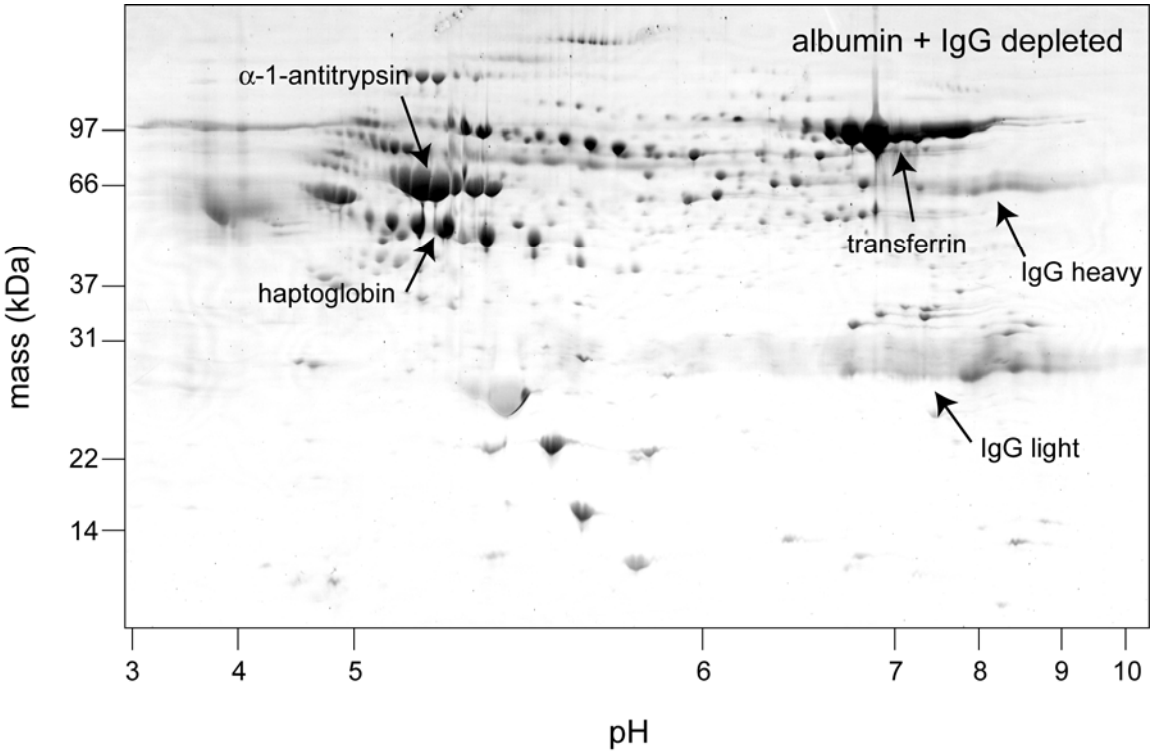

Figure 4-1: Effect of albumin and IgG depletion on 2DE protein separation

Representative 2-D gel image of 250 µg of serum depleted with the Albumin and IgG Removal Kit and separated over a pH 3–10 nonlinear gradient. Protein identifications are based on comparison with published plasma 2DE maps (Golaz *et al.* 1993).

4.3.2 Depletion of six proteins using a MARS column

The chromatograph of a typical chromatography run using a MARS column clearly shows separation of the initial flowthrough fraction containing depleted serum proteins and the later elution fraction containing the six bound antigens (Figure 4-2). The flowthrough fraction from each depletion run yielded approximately 190 µg of protein from the 15 µL of raw serum used (Table 4-4). Flowthrough fractions were pooled from four replicate runs per sample to provide enough depleted protein for 2DE analysis.

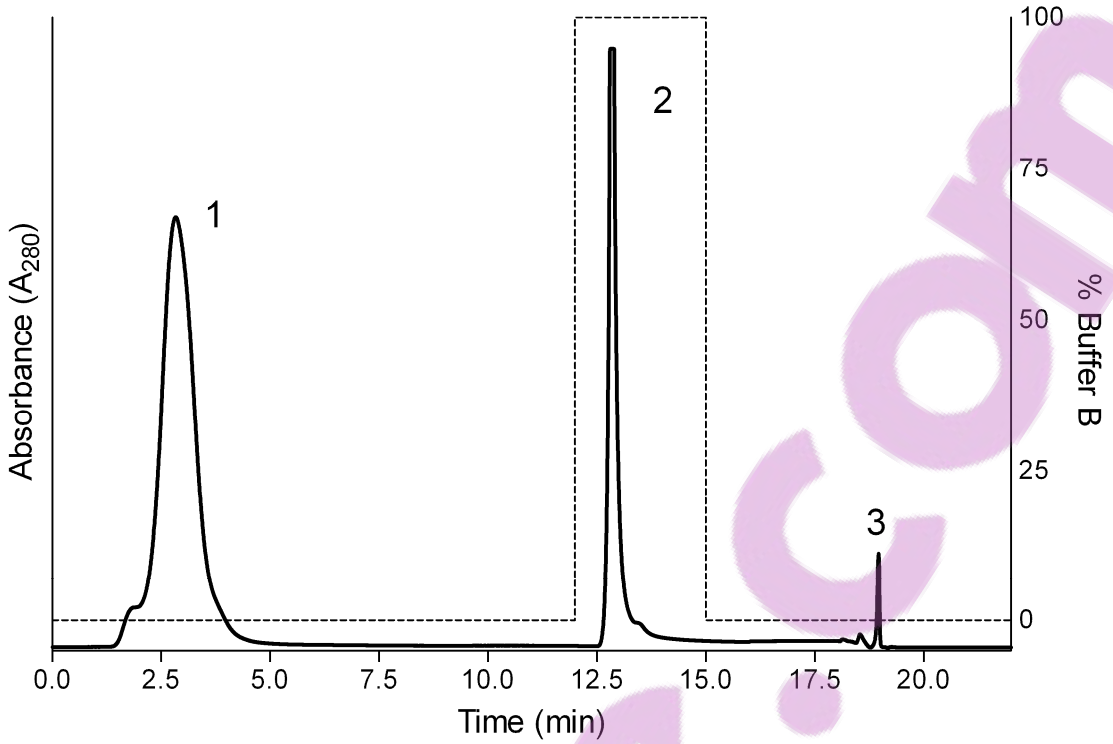

Figure 4-2: Chromatogram from MARS depletion of high-abundance serum proteins

Representative chromatogram from HPLC-based depletion of serum and plasma samples using a MARS system (Agilent). Peak 1 is the flowthrough fraction containing enriched, depleted proteins. Peak 2 represents the elution of bound high-abundance proteins from the MARS column. Peak 3 is a buffer miscibility peak and was ignored.

Depletion efficiency was judged by visualizing proteins in the flowthrough and elution fractions on 2-D gels (Figure 4-3). Most high-abundance proteins were absent from the enriched low-abundance protein flowthrough fraction (Figure 4-3B). However, significant amounts of proteins corresponding to the positions of α-1-antitrypsin and haptoglobin remained in the depleted protein mixtures. Notably, almost all proteins remaining after MARS depletion focused between pH 4–7, with no detectable proteins having pIs between pH 7–10. Therefore, further isoelectric focusing steps in the 2DE process used the pH 4–7 range.

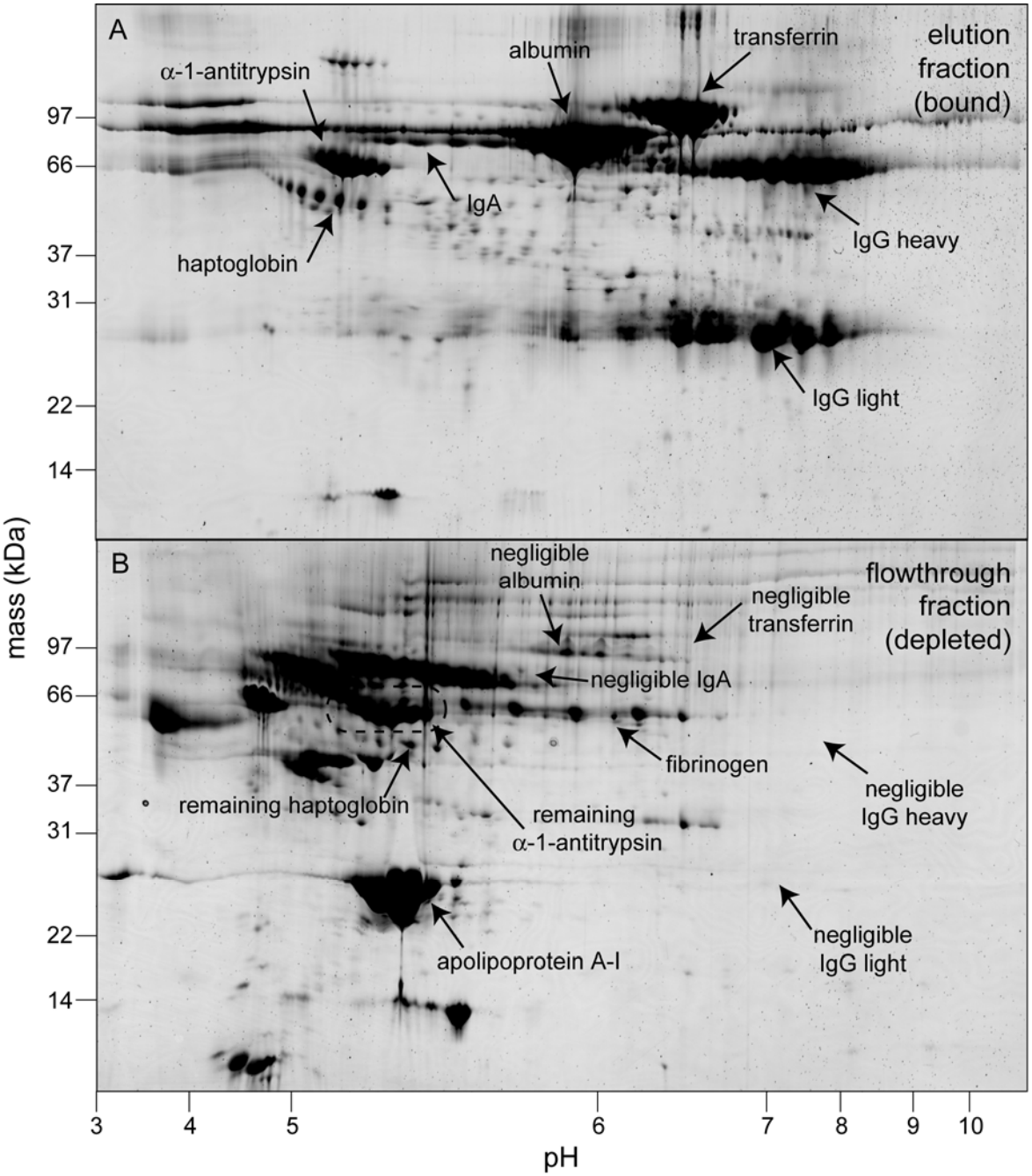

Figure 4-3: Comparison of MARS-depleted versus retained serum proteins using 2DE

2-D gels showing the effect of abundant serum protein depletion. Two hundred and fifty micrograms of (**A**) bound high-abundance proteins and (**B**) flowthrough low-abundance proteins were separated over a nonlinear gradient covering pH 3–10. Protein identifications are based on comparison with published plasma 2DE maps (Golaz *et al.* 1993) and with maps published in Agilent application note #5989–0265.

Serum samples depleted of high-abundance proteins using the Albumin and IgG Removal Kit and a MARS column were compared using 2DE (Figure 4-4). A number of differences between the two methods were apparent. Depletion of albumin and IgG using spin columns produced a well-resolved gel image (Figure 4-4A), although these gels were dominated by abundant transferrin and α-1-antitrypsin as well as visible amounts of IgG remaining in the samples after specific depletion. Depletion of six abundant serum proteins using a MARS column produced a much different image

(Figure 4-4B). In the MARS image, the abundant transferrin and α-1-antitrypsin have been removed, as well as traces of IgG which remained visible following Albumin and IgG Removal Kit depletion. No proteins are visible at pIs > 6.6, and very few are found at pIs < 4. Consequently, samples depleted by MARS were focused over a narrower pH 4–7 range. Loading only 150 µg of MARS-depleted serum instead of 250 µg enabled generation of a high-resolution 2DE image (Figure 4-4C) containing a substantially greater number of spots than albumin and IgG-depleted serum (*cf.* Figure 4-4 panels C and A). Of note, many additional spots are seen in the mass range below 50 kDa.

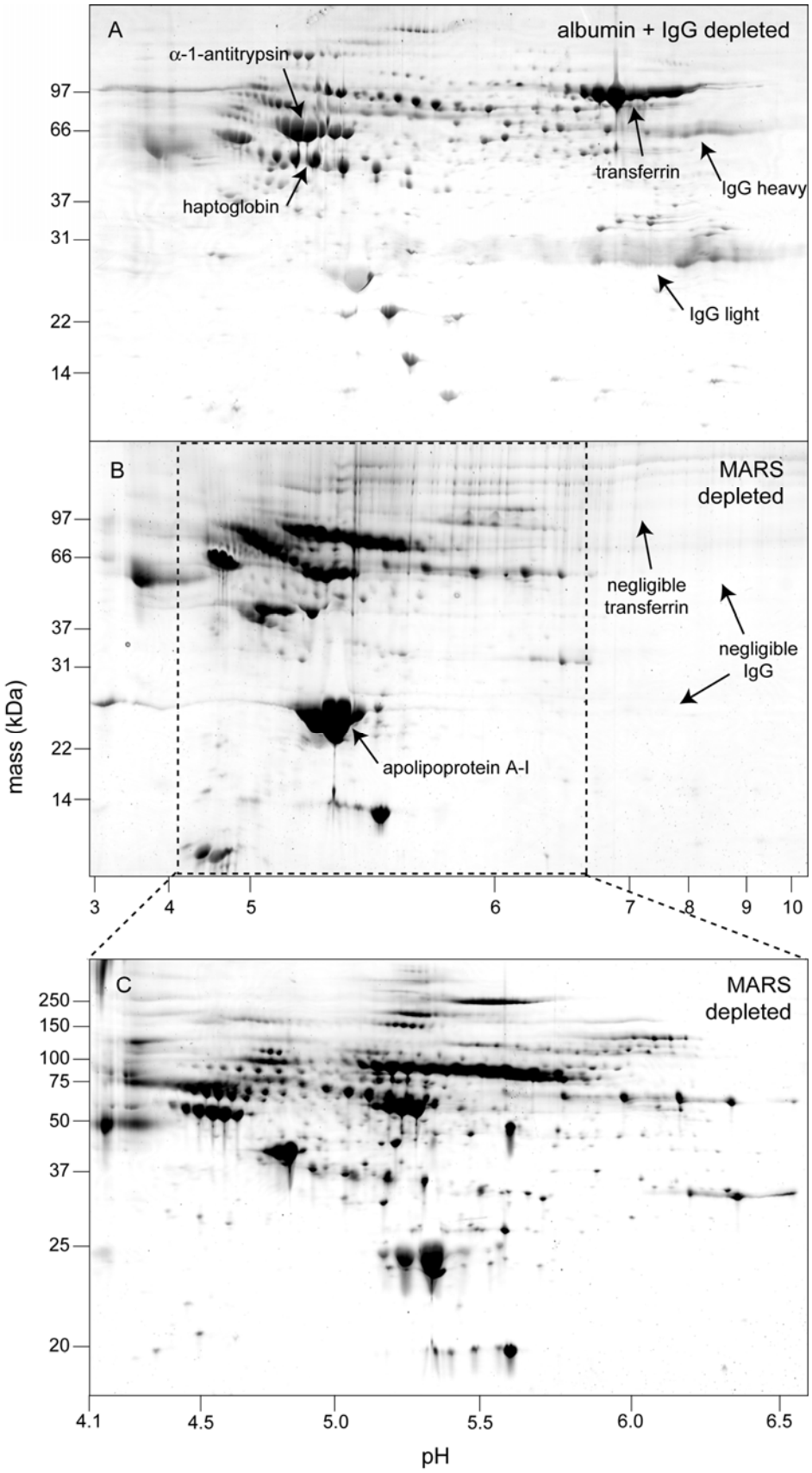

Figure 4-4: 2DE comparison of two serum depletion methods

Representative 2-D gel images of depleted serum. Two hundred and fifty micrograms of serum depleted with an Albumin and IgG Removal Kit (**A**) and a Multiple Affinity Removal System (MARS; **B**) were separated over a nonlinear pH 3–10 range. (**C**) MARS-depleted serum (150 µg) was separated over pH 4–7.

4.4 Discussion

Depletion of the most abundant serum/plasma proteins using immunoaffinity-based methods increases the number of spots detected in 2DE studies (Björhall *et al.* 2005; Zolotarjova *et al.* 2005) and enriches lower-abundance proteins (Pieper *et al.* 2003b; Anderson *et al.* 2004). Comparison of a MARS column and an Albumin and IgG Removal Kit revealed that the MARS system depletes more proteins with less variability between samples.

No depletion method entirely removes its target proteins. Although both methods examined here removed albumin and IgG, depletion of these proteins by the Albumin and IgG Removal Kit was incomplete, with notable amounts of IgG still present in post-depletion samples. The MARS depletion method was more efficient at removing these proteins than the Albumin and IgG Removal Kit. Some α-1-antitrypsin and haptoglobin remained in the samples following MARS depletion. α-1-antitrypsin is upregulated in serum during pregnancy, rising between 1.2- to 1.4-fold throughout pregnancy (Lisowska-Myjak *et al.* 2001), so the excess α-1-antitrypsin observed here suggests that its concentration in serum from pregnant women exceeds the binding capacity of the MARS column. Agilent confirmed that MARS columns were designed to bind their target proteins at their normal non-pregnant physiological concentrations, and that highly upregulated proteins may not be fully depleted (WC Barrett, personal communication).

MARS enables better visualization of lower-abundance proteins by depleting a larger number of proteins than the Albumin and IgG Removal Kit. The number of small proteins (<50 kDa) present on gels in this study was notably increased after MARS depletion compared to Albumin and IgG Removal Kit depletion. The removal of several additional high-abundance proteins by MARS led to the ability to increase loading of lower-abundance proteins onto gels. This resulted in the appearance of previously undetectable low MW proteins. These results are in agreement with other groups who have examined MARS depletion since the introduction of this system in 2003 (Chromy *et al.* 2004; Echan *et al.* 2005). Echan *et al.* (2005) compared several depletion systems using 1-D and 2-D gels. The MARS column was compared with a similar immunoaffinity-based albumin and IgG depletion column commercialized by Sigma-Aldrich, as well as a dye-based albumin depletion column and a MARS product available in spin-column format. Similar to these results, Echan *et al.* found a MARS column more effective at high-abundance protein removal than either the dye-based or two-protein depletion column.

The reusability of the MARS system for up to 200 runs per column makes its use more efficient than the single-use Albumin and IgG Removal Kit. Recent methods to deplete up to twelve of the most

abundant proteins using IgY antibodies are even more effective than MARS depletion (Hinerfeld *et al.* 2004; Huang *et al.* 2005), but were not available at the time of this study. Consequently, the MARS depletion system was judged to be more efficient than the Albumin and IgG Removal Kit and was the preferred method used in the studies described in the following two chapters.

Gel examination of proteins from the MARS bound fraction has confirmed the column's specificity with very few untargeted proteins detected (Echan *et al.* 2005). A shotgun MS analysis of the MARS bound fraction from cerebrospinal fluid identified only proteins specifically depleted by the column (Maccarrone *et al.* 2004). DIGE has also been used to directly compare the effects of MARS depletion within a single gel, finding none of the six depletion target proteins visible in depleted samples (Chromy *et al.* 2004). These published results agree well with the results of the current study.

Criticism of immunodepletion techniques cites the possible removal of proteins of interest along with high-abundance proteins such as albumin. Albumin is a carrier protein able to bind and transport other proteins, and its depletion could unwittingly remove those potential biomarkers (the albumin "sponge effect"; Zolotarjova *et al.* 2005). This is an inevitable side effect of any immunodepletion method. Loss of protein associated with abundant carrier proteins with MARS cannot be easily overcome, and should be considered when interpreting results from serum/plasma studies utilizing this depletion method.

Selection of the most appropriate serum/plasma prefractionation strategies for a study depends on the hypothesis being tested. The comparison of depletion methods described here permitted selection of the efficient and specific MARS technique for further studies of lower-abundance biomarker candidates. This method was then applied in 2DE studies of serum and plasma described respectively in Chapters 5 and 6.

CHAPTER 5 COMPARATIVE PROTEOMICS OF PREECLAMPTIC SERUM USING 2DE

5.1 Introduction

Preeclampsia is a multisystem disorder occurring in mid to late pregnancy which is associated with serious maternal and fetal morbidity and increased perinatal mortality. If untreated, kidney and liver function can be compromised and eclamptic seizures may occur. Inadequate placentation is a key causative factor in the pathophysiology of the disorder. Proteins are continually secreted into maternal blood by the placenta throughout pregnancy, and as these proteins may be altered in abnormal placentation they may serve as biomarkers for the disorder.

The complex biology of preeclampsia makes it highly unlikely the condition will be predicted or diagnosed by a single biomarker. Use of proteomic technologies to discover sets of proteins that are dysregulated in disease is a potentially fruitful approach to identify novel diagnostic or screening tests for preeclampsia. 2DE-based approaches have been used to examine serum and plasma in a number of different diseases to discover biomarkers. The combination of high levels of heat shock protein 27 and low levels of 14-3-3 sigma has been identified as a potential biomarker for breast cancer (Rui *et al.* 2003). Isoforms of apolipoprotein A-I and a C-terminal fragment of complement C3 were found to be biomarkers for hepatocellular carcinoma in hepatitis B patients (Steel *et al.* 2003). 2DE of serum or plasma may, therefore, offer a potential route to determine sets of proteins associated with preeclampsia.

Application of serum/plasma 2DE proteomic techniques to the study of preeclampsia has been reported (Watanabe *et al.* 2004; Heitner *et al.* 2006). In 2004, Watanabe and co-workers used 2DE to identify upregulation of clusterin in serum from women with early-onset preeclampsia and fetal growth restriction compared to healthy pregnant women (Watanabe *et al.* 2004). A more recent 2DE study of plasma from women with HELLP syndrome identified significant changes in six proteins, including striking upregulation of serum amyloid A (Heitner *et al.* 2006). While these studies indicate the potential of proteomic approaches to investigate preeclampsia, only small numbers of women with severe early-onset disease were included. Such women may have received steroids to promote fetal lung maturation (Fuchisawa *et al.* 2004). Steroid treatment modifies insulin resistance and is very likely to modify the serum proteome. The impact of any treatment with steroids prior to specimen collection in these studies is unclear. The significance of the results reported in these 2DE studies is limited by the lack of statistical analysis (Watanabe *et al.* 2004) and by the use of only

univariate methods not adjusted for multiple comparisons (Heitner *et al.* 2006). Neither applied multivariate methods to their 2DE analysis (Broadhurst and Kell 2006).

Bioinformatic methods which select sets of "classifier" proteins distinguishing preeclamptic women from healthy pregnant women may be applied to identify combinations of potential biomarkers (Sajda 2006). However, these have not been previously used in 2DE studies of preeclampsia. Accordingly, further study of the serum proteome is indicated within a more representative population of women with preeclampsia who have not received steroids.

The main aim of this study was to use 2DE to identify serum proteins that enabled classification of women into two groups: those with a healthy pregnancy or those with preeclampsia. Specific aims were:

1. To identify individual serum proteins differentially expressed between women in late pregnancy with preeclampsia and healthy pregnant women, and

2. To identify clusters of differentially expressed serum proteins associated with preeclampsia.

5.2 Methods

5.2.1 Study population

A case-control study was conducted in nulliparous women recruited in late pregnancy. The Auckland Ethics Committee approved these studies (AKX/02/00/157) and written informed consent was obtained from all women. Cases were women with preeclampsia from whom a blood sample was obtained between 36–38 weeks of gestation. Preeclampsia was defined according to the guidelines of the Australasian Society for the Study of Hypertension in Pregnancy (Brown *et al.* 2000a, 2000b). Severe preeclampsia was defined as the presence of one or more multisystem complications including: coagulopathy, microangiopathic haemolysis, liver impairment, acute renal insufficiency, imminent eclampsia, or eclampsia. Controls were healthy pregnant women, gestational age matched to a case at sampling, who remained normotensive and delivered a healthy baby with a birthweight in greater than the 10th customized centile (adjusted for infant sex and maternal ethnicity, height, and weight) as calculated at http://www.gestation.net. SGA babies had a birthweight less than the 10th customized centile.

5.2.2 Human serum and plasma specimens

For serum, blood samples were collected by venipuncture into plain tubes, allowed to clot on ice, and centrifuged (2400 ×*g*, 10 min, 4 °C). Serum was transferred into a new tube and centrifuged a second time at 3200 ×*g* for 15 min at 4 °C to ensure removal of all cellular material. Serum was removed and stored at −80 °C in 250 µL aliquots within 3 h of collection.

For plasma, blood samples were collected by venipuncture into EDTA-containing tubes and centrifuged at 2400 ×*g* for 10 min at 4 °C. Plasma was removed and stored at −80 °C in 250 µL aliquots within 3 h of collection.

5.2.3 Depletion of abundant serum proteins and sample preparation for 2DE

Immunodepletion of six abundant serum proteins (albumin, transferrin, haptoglobin, antitrypsin, IgG, and IgA) was performed using the Multiple Affinity Removal System (MARS) as described in Section 4.2.4. Specimens were depleted in the presence of a protease inhibitor cocktail, and depleted proteins were exchanged into a solubilization buffer (9 M urea, 2% CHAPS, 60 mM DTT) using centrifugal concentrators. Protein concentrations were measured from duplicate 5 µL volumes of each sample using the 2-D Quant Kit (GE Healthcare) according to the manufacturer's instructions.

5.2.4 2DE of depleted serum proteins

Depleted serum protein samples were separated according to their pIs and MWs using two-dimensional gel electrophoresis as described in Section 4.2.5. Briefly, 150 µg samples were diluted to 340 µL in rehydration solution and separated along 18 cm IPG strips covering pH 4–7. Focused IPG strips were equilibrated, and then separated in the second dimension using ExcelGel XL 12–14% gradient polyacrylamide gels. After electrophoresis, gels were stained with SYPRO Ruby (Invitrogen) overnight and imaged using an FLA–2000 phosphorimager (Fuji) with excitation at 473 nm and a 580 nm high-pass emission filter. Gels were imaged with a 50–100 µm pixel size and F1000 (maximum) resolution.

5.2.5 Image analysis

Digitized 2DE images were analyzed using ImageMaster™ 2D Platinum v5.0 (IMP5; GE Healthcare). This process maximized the matching of spots across all gel images through five steps: (1) spot detection; (2) generation of a five-gel subset for precise matching; (3) matching of the five-gel subset

to remaining gels to produce a network of matched spots; (4) synthetic gel creation from the matched spot network, and (5) matching of all gels to the synthetic gel.

Spot detection on serum 2-D images proceeded through two stages. First, detection parameters were optimized to find the maximum number of spots on any single gel within the experiment. As the number of spots on different gels varied from the maximum spot number (n=539 spots) by more than 10%, detection parameters were adjusted individually to detect the 539 ± 10% spots in each gel image. These steps ensured that all gels of depleted serum contained approximately the same number of spots, optimizing the probability of accurate matching between gels.

Following visual inspection of serum 2-D gel images, a subset of five gels with minimal artifacts (smears) and maximal spot resolution were selected (four from preeclamptic women and one from a healthy pregnant woman). Each of these five gels was in turn matched to the 23 remaining gels in the experiment. This step created a network of matched spots that maximized inter-gel matching.

A synthetic gel, defined as a reference gel incorporating merged spots from multiple gels onto a blank image, was generated within IMP5. Spots were included on the synthetic gel only if present in at least three of the five subset gels, that is, at least three networks of matched spots. All 24 gels were then matched to the synthetic reference gel. The median matching percentage was 68% (range 53%-84%).

Data were exported from IMP5 in a table listing the percentage volume of each matched spot in each gel. Percentage volume was used rather than raw spot volumes to minimize inter-gel differences from staining intensities or electrophoretic variations. Data were then analyzed by a bioinformatics team using the methods described in Section 5.2.9.2.

Fold changes are presented as the (arithmetic) mean percentage volume for a spot in preeclamptic samples divided by the mean percentage volume for that spot in healthy pregnant samples. Positive fold changes indicate upregulation in preeclamptic samples and negative fold changes indicate downregulation in these samples.

5.2.6 Selection criteria for spots of interest

Spots found as useful discriminators by at least one multivariate classification method (as described in Section 5.2.9.2) were tabulated. These spots were assessed for technical variation by examination of raw gel images and those that did not appear to result from artifactual variation were analyzed using LC-MS/MS.

5.2.7 Protein identification

Spots of interest were excised from gels, digested with trypsin, and analyzed by LC-MS/MS as previously described in Section 3.2.7. Protein identification reporting followed recently published guidelines (Bradshaw *et al.* 2006). Tandem MS/MS data were extracted from raw spectra using Mascot Distiller (Matrix Science). Data were searched against the Swiss-Prot database (version 52.2, date 14 April 2007) using the Mascot search engine v2.2.0 (Matrix Science) with the following parameters: Taxonomy: human, semitrypsin cleavage with up to 1 missed cleavage allowed, fixed modification: carbamidomethylation (of cysteines), variable modification: oxidation (of methionines), mass tolerances ±0.1 Da, peptide charges 2+ and 3+. Positive identifications reported here had at least two unique peptides match the database entry.

5.2.7.1 Analysis of post-translational modifications

Tryptic peptides of spots corresponding to apolipoprotein E isoforms were submitted for LC-MS/MS analysis on an LTQ ESI-trap FT-ICR (Thermo Electron) at the Maurice Wilkins Centre for Molecular Biodiscovery, The University of Auckland. LC-MS/MS analysis was performed by Dr DR Greenwood. The resulting data were searched for post-translational modifications using the SALSA algorithm (Hansen *et al.* 2001; Badghisi and Liebler 2002; Liebler *et al.* 2002) by Dr JM Cooney of the Horticultural and Food Research Institute of New Zealand Ltd.

5.2.8 Validation of differentially expressed proteins

5.2.8.1 2-D western blot analysis

Serum was depleted of abundant proteins and the resulting depleted protein samples were quantitated as previously described (Section 4.2). Two hundred micrograms of depleted serum protein were focused using 7 cm IPG strips covering pH 4–7 (Bio-Rad); IPG strips were focused using Multiphor II flatbed electrophoresis system (GE Healthcare) using the program provided in Table 5-1. After equilibration as described in Section 3.2.4, strips were separated in the second dimension on NuPAGE 4–12% Bis-Tris minigels (Invitrogen) at 200 V for 1 h.

Table 5-1: Isoelectric focusing parameters for depleted serum samples

Focusing phase	*Voltage (V)*[a]	*Time (h)*	*Purpose*
1	250	0:01	increase voltage to 250 V
2	250	2:00	initial focusing
3	3500	2:00	increase voltage to 3500 V
4	3500	6:00	extended focusing
5	100	0:01	decrease voltage to 100 V
6	100	(hold)	hold step when focusing has finished

[a] All steps were carried out at 1 mA, 5 W, and 20 °C.

SeeBlue® Plus2 pre-stained protein standard and MagicMark™ XP western blot standard (both Invitrogen) were run on all gels to assess transfer efficiency and protein MW respectively. Gels were blotted onto Immobilon-FL low fluorescence PVDF membranes (Millipore) using the XCell SureCell blotter (Invitrogen) according to the manufacturer's instructions for Bis-Tris gel chemistry; transfer was carried out at 30 V for 90 min.

Unless otherwise noted, all membrane treatment steps were carried out at room temperature and with agitation on a rocker. PBST (phosphate-buffered saline (PBS; Sigma-Aldrich) containing 0.1% Tween 20) was used for all blocking, antibody dilution, and wash steps. Immediately after blotting, membranes were blocked with 5% BSA in PBST overnight at 4 °C. The next morning, blots were briefly rinsed with PBST and incubated for 1 h with a goat anti-human apolipoprotein E polyclonal primary antibody (Abcam) diluted 1:5000 in PBST containing 1% BSA. Membranes were rinsed briefly in Milli-Q H_2O, washed for 3×10 min, and incubated for 1 h with a rabbit anti-goat Qdot® 655-conjugated secondary antibody (Invitrogen) diluted 1:1000 in PBST containing 1% BSA. Membranes were rinsed briefly with Milli-Q H_2O, washed for 3×10 min in PBST, and finally washed for 3×10 min with PBS alone. Blots were directly imaged on a Typhoon 9410 variable mode imager (GE Healthcare) using 457 nm excitation, a 670 nm ± 30 nm bandpass emission filter, and a photomultiplier tube voltage of 550 V.

5.2.8.2 Multiplexed immunoassays

A LINCOplex immunoassay (Millipore) was performed to simultaneously measure plasma concentrations of apolipoprotein E and apolipoprotein C-II. Plasma samples were diluted 1:5000 in assay buffer and were assayed according to the manufacturer's instructions. Assays were read on a Luminex 100 instrument (Luminex). Assistance with the instrument was given by Ms R Sutherland, Auckland Cancer Society Research Centre, The University of Auckland.

5.2.9 Statistical analysis

5.2.9.1 Clinical data

All analyses were performed using GraphPad Prism version 4.03 for Windows (GraphPad Software) using a significance level of $p<0.05$. Continuous data from preeclamptic and healthy pregnant women were compared using a two-tailed Student's *t* test. Categorical data were compared using Fisher's exact test.

5.2.9.2 Bioinformatic analysis of 2DE data

All bioinformatic analyses described in the following section were performed by collaborators Dr MA Black (Bioinformed, Ltd.), Dr N Kasabov and Mr P Hwang (both Auckland University of Technology), Dr RE Kates (Technical University of Munich, Germany), and Mr SH Wu (The University of Auckland).

For univariate analysis, raw spot data were logged and centered. Spot percentage volumes cannot be less than zero, leading to an inherently skewed distribution of spot volumes. A $\log_2$ transformation was applied to raw spot volume data to reduce this skewness. Boxplots were used to verify, before and after transformation, that no gel data were outliers. Logged data were then normalized by centering each gel at zero using median subtraction, with boxplots again used to view the data spread across all gels.

For multivariate analysis, raw spot data were first imputed to fill in missing values. 2DE experiments use protein stains which have a finite limit of detection. Spots with volumes lower than this limit and absent spots appear in analyses as "missing" spots (Ahmad *et al.* 2006; Krogh *et al.* 2007). These spots totaled approximately 32% of the data from the set of 24 gels. For multivariate analysis, each missing spot (a spot undetected in at least one gel but found in at least one other gel) was assigned the minimum non-zero percentage volume for that spot from all 24 gels. Data were then logged and centered as described above for univariate data. These imputed, log-transformed, centered data were used for all multivariate analysis methods except for neural networks.

5.2.9.2.1 Student's t test

A two-sample Student's *t* test was used to determine whether any individual spots had significantly different expression between healthy and preeclamptic samples. To account for multiple

comparisons, *p*-values were adjusted by the FDR controlling method of Benjamini and Hochberg (1995).

5.2.9.2.2 Nearest shrunken centroids

The nearest shrunken centroids (NSC) approach uses a variant of discriminant analysis to classify samples. The NSC method uses the t-like statistic of Tusher et al. (2001) to rank the spots according to the degree of separation exhibited between samples from preeclamptic and healthy pregnant women. These scores are shrunk towards zero using a parameter that eliminates uninformative spots from the analysis. The degree of shrinkage is varied by changing the size of the parameter; this optimizes the number of spots in the predictor based on the performance of the discriminant analysis-based classifier in a subset of data used in cross-validation. This method was used to analyze transformed 2DE data to produce a set of spots considered to be a useful classifier.

A misclassification rate was then calculated from the full dataset using the classifier. Bias in this misclassification rate was estimated using bootstrap analysis (Efron and Tibshirani 1993). In each of 1000 iterations, a subset of 20 gels (ten each from preeclamptic and healthy pregnant women) were used in cross-validation as described above to produce an NSC classifier. This classifier was used to predict the origin (from a preeclamptic or healthy pregnant woman) of each gel in the 20-gel subgroup, as well as the classes of the remaining four gels (which were not used to construct the model). The difference between the two classification rates (for the subgroup of 20, and for the remaining four) provides an estimate of the bias present when a classifier predicts the classes of the samples on which it is trained. This process was repeated 1000 times, with the average of the differences in classification rates used as a bias estimate. This estimate was then subtracted from the classification rate obtained in the original NSC analysis to produce a bias-corrected estimate of performance. The parametric confidence interval around this estimate was calculated based on a binomial likelihood function with $n = 24$ and number correctly classified equal to 24×(the uncorrected misclassification rate).

5.2.9.2.3 Recursive feature elimination

The recursive feature elimination (RFE) method of Guyon et al. (2002), with the modification of Ambroise & McLachlan (2002), uses an approach based on support vector machine methodology (Cortes and Vapnik 1995) coupled with an iterative method for variable selection. An initial support vector machine model was constructed based on data from all spots, and spots were retained in the model based on their importance in the predictive process as assessed by their model coefficient. Ten

iterative steps each removed the least informative 50% of spots from the analysis and constructed an additional model, repeating until a single spot remained. At each iteration the predictive ability of the model was assessed with cross-validation.

5.2.9.2.4 Double leave-one-out analysis

This method used a modification of standard leave-one-out cross-validation to select spots with large fold changes in an unbiased manner. Two layers of cross-validation were used. The inner layer was used to select spots and model parameters to form a classifier and the outer layer was used to obtain performance estimates from the classifiers. Model creation entailed a pre-filtering step in which spots were chosen for inclusion only if at least 50% of the spot volumes occurred within a range outside of −1 to 1. A Mann-Whitney *U* test was then used to rank spots passing the filter. Models were constructed from the top 100 spots passing the Mann-Whitney *U* test. The model giving the best classification rate within the inner cross-validation layer was used to generate unbiased performance estimates in each step of the outer cross-validation layer. This final model consisted of a list of spots able to discriminate between gels from preeclamptic and healthy pregnant samples.

5.2.9.2.5 Evolving connectionist functions

An evolving connectionist function (Kasabov 2003) model was used in conjunction with 24-fold leave-one-out cross-validation to produce a classifier able to distinguish between samples from preeclamptic women and healthy pregnant women. In each of the 24 iterations of the cross-validation process, spots were ranked by the absolute value of their signal-to-noise ratio, with the top x spots (with x varied from 1 to 50) used to construct a model in the training set of 23 gels and predict the group (from a preeclamptic or a healthy pregnant woman) of the left-out gel. The number of spots used in the final model was chosen as the number of spots that achieved the best overall cross-validated classification rate. This final evolving connectionist function model is constructed using the full data set. This technique generated a cluster of spots able to classify gels by disease state, that is, preeclamptic or healthy pregnant.

5.2.9.2.6 Neural networks

For neural network analysis, percentage volumes for each spot were ranked among gels using a fractional rank between 0 and 1. Unmatched or missing spots corresponded to the lowest rank below all present spot ranks.

Bootstrap analysis (Efron and Tibshirani 1993) to assess discriminatory power for gestation-paired subjects over 50 000 replications produced a set of candidate spots for neural network analysis ranked by bootstrap *p*-value. Top spots from this analysis were used for multivariate nonlinear learning in 24 neural networks as described below.

Neural networks were trained and tested using a likelihood-based complexity reduction (pruning) method (Kates *et al.* 2001) and 12-fold cross-validation. This pruning method removed extraneous connectors that did not improve prediction quality. The neural network was trained by adjusting individual parameters to maximize correct prediction of disease status while pruning out spots which did not add information to the prediction model. Following training, the spots remaining in the neural network models were ranked by frequency of appearance in the models.

5.2.9.3 Analysis of multiplexed immunoassay data

All analyses were performed using GraphPad Prism version 4.03 for Windows (GraphPad Software) using a significance level of $p<0.05$. The standards from each analyte were fitted to the four-parameter logistic equation using nonlinear regression. Analyte concentrations from each plasma sample were then calculated from standard curves. Comparisons between preeclamptic and healthy sample groups were performed using a one-tailed Student's *t* test or a one-tailed Mann-Whitney *U* test. Unless otherwise noted, data are given as mean ± SD.

5.2.9.4 Power calculations

Power calculations based on serum 2DE data were performed by Dr MA Black (Bioinformed, Ltd.) to estimate the number of samples required for follow-up studies (Moore and McCabe 2006). A significance level of $\alpha=0.05$ and a power of $\beta=0.80$ were used.

5.3 Results

5.3.1 Maternal and fetal outcomes

Twelve women with preeclampsia and 12 healthy pregnant women were studied in the 2DE experiment. An additional six women were later added to each group for a validation study using a multiplexed immunoassay. Maternal baseline characteristics and maternal and fetal outcome data for both groups are show in Table 5-2.

Table 5-2: Maternal and neonatal clinical characteristics from serum 2DE and immunoassay study participants

	2DE study			immunoassay study		
	Preeclamptic (n=12)	***Healthy pregnant (n=12)***	**p-*value***	***Preeclamptic (n=18)***	***Healthy pregnant (n=18)***	**p-*value***
Maternal characteristics						
Age (years)	31.4 (4.2)	31.8 (3.4)	0.83	31.5 (3.5)	31.4 (3.3)	0.96
Ethnicity						
Caucasian	7	8		12	14	
Maori or Pacific Islander	3	0	0.20	4	0	0.11
Other	2	4		2	4	
Body mass index at booking ($kg \cdot m^{-2}$)	26.4 (4.1)	23.2 (4.4)	0.09	25.5 (3.8)	24.1 (4.5)	0.31
Gestational age at first visit (weeks)	12.2 (2.7)	11.9 (4.1)	0.88	13.8 (2.6)	13.7 (3.2)	0.95
Systolic blood pressure at <20 weeks (mm Hg)	114 (14)	116 (12)	0.73	115 (12)	113 (12)	0.47
Diastolic blood pressure at <20 weeks (mm Hg)	70 (11)	63 (7)	0.09	71 (9)	64 (7)	0.01
Gestational age at sampling (weeks)	37.6 (0.8)	37.3 (1.0)	0.38	36.7 (1.9)	36.4 (1.8)	0.61
Systolic blood pressure at sampling (mm Hg)	138 (14)	116 (9)	0.0001	139 (14)	113 (9)	<0.0001
Diastolic blood pressure at sampling (mm Hg)	94 (11)	72 (7)	<0.0001	94 (10)	71 (7)	<0.0001
Maximum systolic blood pressure (mm Hg)	165 (19)	125 (7)	<0.0001	162 (17)	125 (8)	<0.0001
Maximum diastolic blood pressure (mm Hg)	106 (6)	77 (8)	<0.0001	104 (7)	76 (8)	<0.0001
Severe preeclampsia	2 (16.6%)†	0		6 (33.3%)‡	0	
Fetal characteristics						
Gestational age at delivery (weeks)	38.1 (0.6)	40.1 (1.5)	0.0003	37.4 (1.5)	40.3 (1.3)	<0.0001
Infant birthweight (g)	2943 (340)	3628 (329)	<0.0001	2854 (478)	3680 (359)	<0.0001
Small for gestational age (SGA)	4 (33.3%)	0		5 (27.7%)	0	
Perinatal deaths	0	0		0	0	

Values are reported as mean (SD) or n (%) unless otherwise noted. Multisystem complications indicative of severe preeclampsia were † imminent eclampsia (n=1) or HELLP (n=1), or ‡ imminent eclampsia (n=2), HELLP (n=2), or acute renal insufficiency (n=2).

5.3.2 Univariate statistical analysis of serum 2DE data

Boxplots indicated that no gels were extreme outliers and that the spread of logged data was relatively uniform. The data from each gel were centered around zero by subtraction of the gel median spot volume (Figure 5-1). At no point do any gels appear to be extreme outliers when compared to the rest of the gel population.

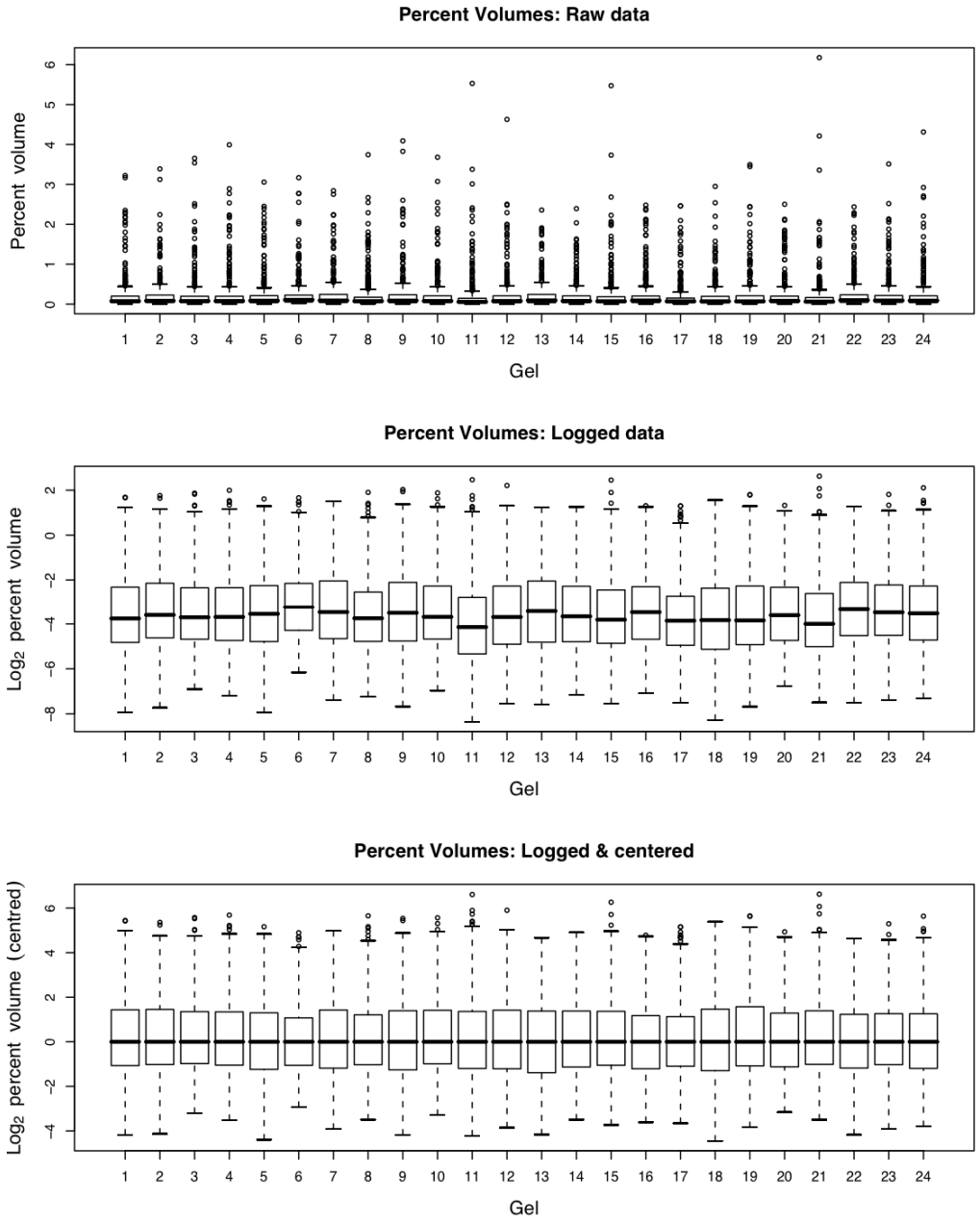

Figure 5-1: Plots of 2DE data during transformation for multivariate analysis

Scatter- and boxplots of percentage volume data from 24 2-D gels of depleted serum assessed their spread before multivariate analysis was performed. (**Top**), scatter plots of raw percentage volumes for all matched spots within each gel. (**Middle**), boxplots showing the mean, upper and lower quartiles, and spread of the logged percentage volumes over each gel. (**Bottom**), effect of centering logged data around the median value of each gel.

Twenty-three spots that were significantly up- or downregulated (unadjusted $p<0.05$) in samples from preeclamptic women are shown in Table 5-3. After applying FDR correction for multiple comparisons, none remained significant.

Table 5-3: Results from univariate statistical analysis of serum 2DE data

Spot number	*Student's* **t** *test* **p-***value uncorrected*	*Student's* **t** *test* **p-***value FDR*[a]	*Number of gels from preeclamptic samples where spot is present*	*Number of gels from healthy pregnant samples where spot is present*	*Fold change (preeclamptic /healthy pregnant)*
20	0.006	0.73	4	5	1.5
28	0.003	0.73	11	8	2.5
35	0.021	0.91	10	11	1.4
36	0.028	0.94	7	7	2.1
98	0.035	0.94	3	3	-2.9
128	0.041	0.94	12	12	-1.3
129	0.018	0.91	11	11	-1.4
145	0.001	0.51	9	7	-2.2
153	0.020	0.91	8	7	-2.2
165	0.032	0.94	10	9	-1.9
168	0.007	0.73	11	12	-1.7
194	0.040	0.94	10	12	-2.5
220	0.011	0.91	8	8	-2.3
222	0.021	0.91	5	7	-2.7
287	0.017	0.91	7	9	-2.5
288	0.007	0.73	12	12	-1.4
304	0.046	0.94	12	10	-1.7
447	0.020	0.91	6	6	-1.7
450	0.042	0.94	9	8	-1.8
471	0.027	0.94	2	3	-2.6
481	0.045	0.94	4	4	2.1
488	0.034	0.94	8	10	1.5
508	0.037	0.94	6	6	-1.3

[a] FDR = false discovery rate correction for multiple comparisons.

5.3.3 Multivariate statistical analysis of serum 2DE data

Sixteen spots were identified in one or more of the multivariate analysis techniques applied to identify sets of classifier spots (Table 5-4). Spots were assessed for artifactual variation by the presence of irregularly bordered, diffuse matched spots or of streak patterns on gel images. Five spots (56, 68, 71, 510, and 517) were excluded due to this artifactual variation. Of the remaining spots, five (30, 45, 128, 160, and 401) were excluded as they were only identified by one of the five multivariate analysis methods. The final six spots of interest are highlighted in Table 5-4 and shown on gel images in Figure 5-2. Scatter plots of individual spot volumes are shown in Figure 5-3.

Table 5-4: Results from multivariate analysis of serum 2DE data

Spot number	*Methods*					*Number of gels from preeclamptic samples where spot is present*	*Number of gels from healthy pregnant samples where spot is present*	*Fold change (preeclamptic /healthy pregnant)*	*Reason for exclusion*
	Nearest shrunken centroids (top 6)[a]	*Recursive feature elimination (appearing ≥ 4x)*[b]	*Double leave-one-out (top 5)*[a]	*Neural networks, matched (top 10)*[a]	*Feature selection using evolving connectionist functions*[b]				
28	1	x	1	8	x	11	8	2.5	
30				9		11	5	-1.1	found by one method only
45			4			7	3	1.5	found by one method only
56		x				8	1	1.9	artifact
68	2	x	2	5	x	11	6	1.2	artifact
71				2		9	11	-1.6	artifact
125		x	5			12	11	2.1	
128				10		12	12	-1.3	found by one method only
160				3		10	6	1.2	found by one method only
161	4	x		1	x	11	3	2.0	
168		x		6	x	11	12	-1.7	
194	6	x				10	12	-2.5	
401				4		1	8	1.6	found by one method only
428	5	x			x	1	7	-3.6	
510		x	3			8	3	1.5	artifact
517	3			7		9	4	5.9	artifact

[a] Method produced a ranked list of spots; numbers denote ranks.

[b] Method produced an unranked list of spots.

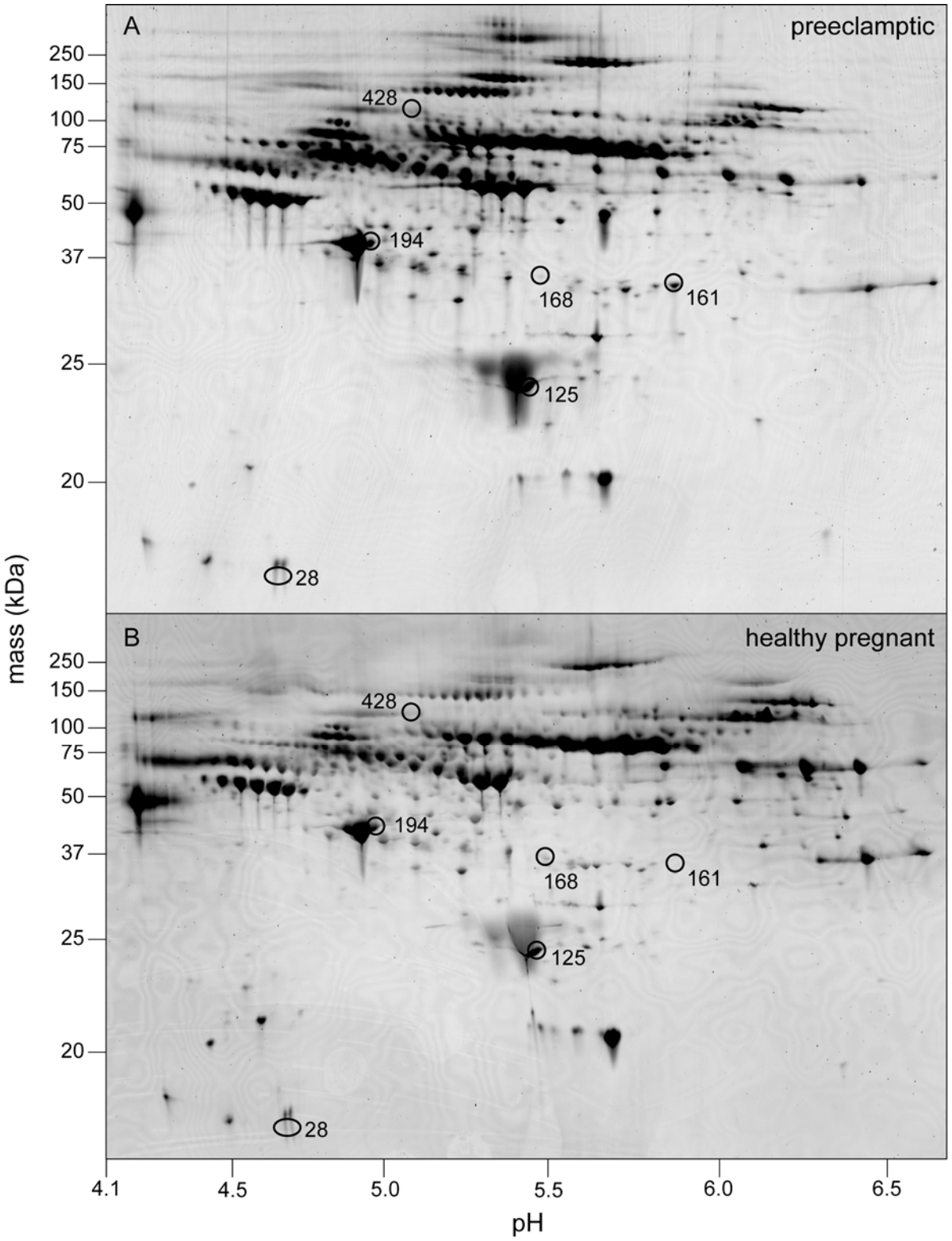

Figure 5-2: Locations of collected spot results from multivariate analysis of serum 2DE

Spots identified following multivariate analysis of serum 2DE data (as shown in Table 5-4) are indicated on representative 2DE images. (**Top**), gel from a preeclamptic sample at 36.9 weeks of gestation; (**bottom**), gel from a healthy pregnant sample at 36.1 weeks of gestation.

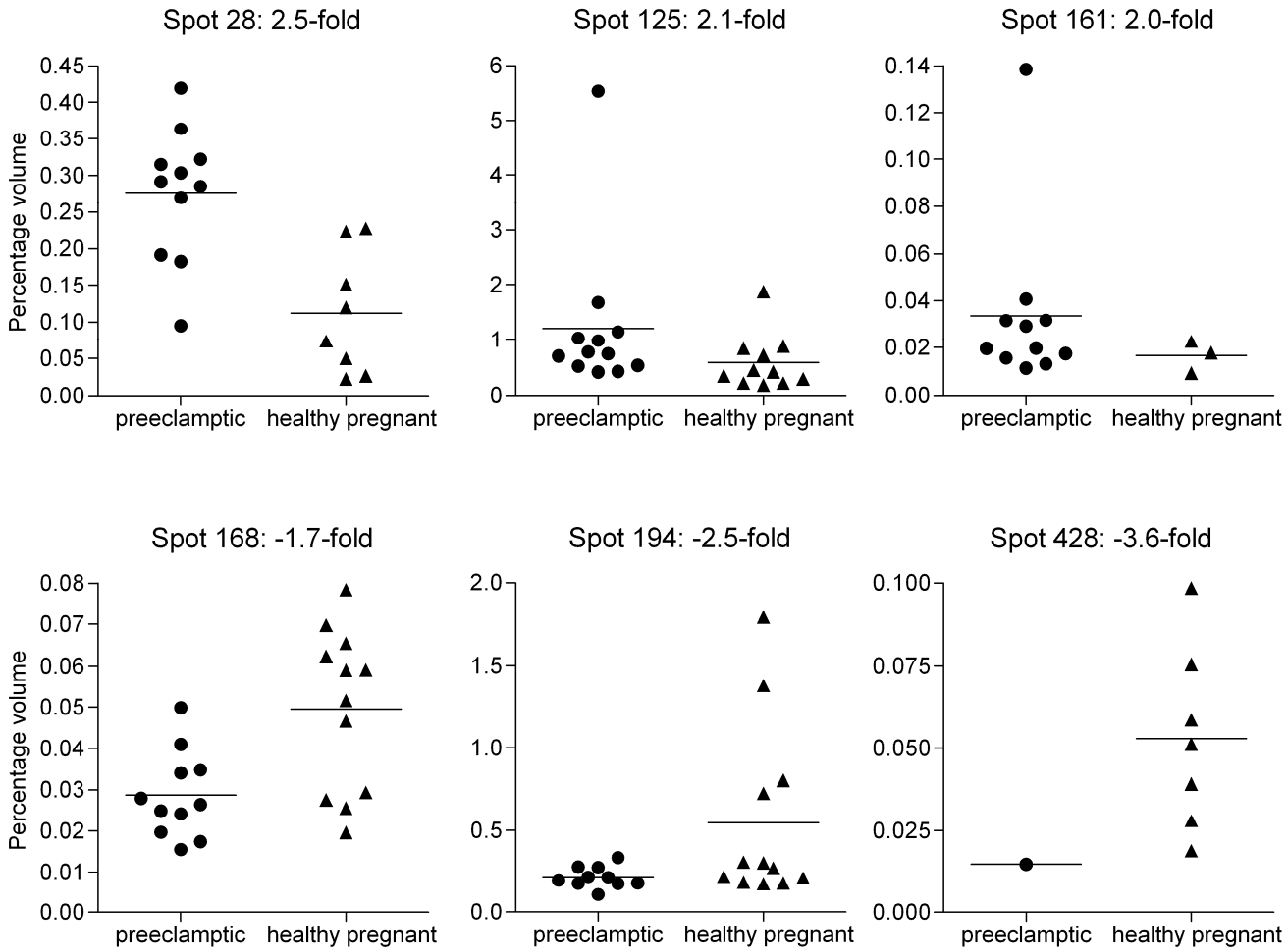

Figure 5-3: Distribution of 2DE spot volumes from candidate serum markers

The raw spot percentage volumes from individual serum 2DE spots are shown from gels containing samples from preeclamptic (●) or healthy pregnant (▲) women. Each point represents the percentage volume from a single gel. Hortizontal lines indicate the mean percentage volume within each group. Fold changes (as preeclamptic versus healthy pregnant) are provided for each spot.

The nearest shrunken centroids method correctly classified 75% of gels. This classification rate was corrected for bias using permutation analysis, generating a corrected classification rate of 63% (95% CI 43%–80%).

5.3.4 Identification of protein spots

The six serum protein spots of interest were identified using tandem LC-MS/MS as apolipoprotein E (ApoE; two isoforms), apolipoprotein C-II, plasma retinol-binding protein, a C-terminal fragment of the α′ chain of complement C3c, and heavy chain H4 of inter-α-trypsin inhibitor (Table 5-5). Except for inter-α-trypsin inhibitor, these proteins have well-correlated estimated and calculated MW, supporting their identifications.

The specific fragment of complement C3c present on gels was identified through mapping peptides identified from LC-MS/MS to the complement C3 sequence. Database searches matched spot 194 to the complement C3 α chain, which has a mass of 113 kDa compared to the estimated mass of 40 kDa observed on gels. The fragmentation of C3 *in vivo* is a multi-stage process of proteolytic cleavage (Figure 5-4). Further investigation of the region of LC-MS/MS peptide data specifically localized the matched sequences to an unnamed C-terminal fragment which is a component of the C3c protein. This C-terminal fragment spans residues 1321–1663 of the C3 sequence and has a predicted MW of 39.5 kDa and pI of 4.8, correlating very closely with the observed position of this spot on 2-D gel images (*cf*. Figure 5-2).

Table 5-5: Proteins identified from serum 2DE classifier spots

Spot number	*Fold change (preeclamptic /healthy pregnant)*	*Protein*[a]	*Accession*	*Sequence coverage*[b] *(%)*	*Peptides (unique)*	*Calculated MW (kDa)*[c]	*Gel MW (kDa)*	*Calculated pI*[c]	*Gel pI*
28	2.5	apolipoprotein C-II	P02655	71	8 (6)	8.9	10	4.66	4.5
125	2.1	plasma retinol-binding protein	P02753	56	11 (8)	21.1	24	5.27	5.2
161	2.0	apolipoprotein E (basic)	P02649	40	11 (10)	34.2	34	5.52	5.7
168	-1.7	apolipoprotein E (acidic)	P02649	21	5 (5)	34.2	35	5.52	5.2
194	-2.5	complement C3c, C-terminal fragment of α′ chain[d]	P01024	29[d]	8 (7)	39.5	40	4.79	4.7
428	-3.6	inter-α-trypsin inhibitor, heavy chain H4	Q14624	17	15 (14)	70.6	90	5.92	4.9

[a] Database search result data are provided in Appendix B.2.

[b] Sequence coverage was calculated as part of the Mascot search process using the entire chain of the protein's Swiss-Prot database entry.

[c] MW and pI were calculated on Swiss-Prot entries from the main chain or appropriate chain only using the Compute MW/pI tool (http://www.expasy.org/tools/pi_tool.html).

[d] Complement C3 is a multi-chain protein derived from a single polypeptide precursor; the percentage of sequence coverage is determined over the C3c C-terminal fragment only.

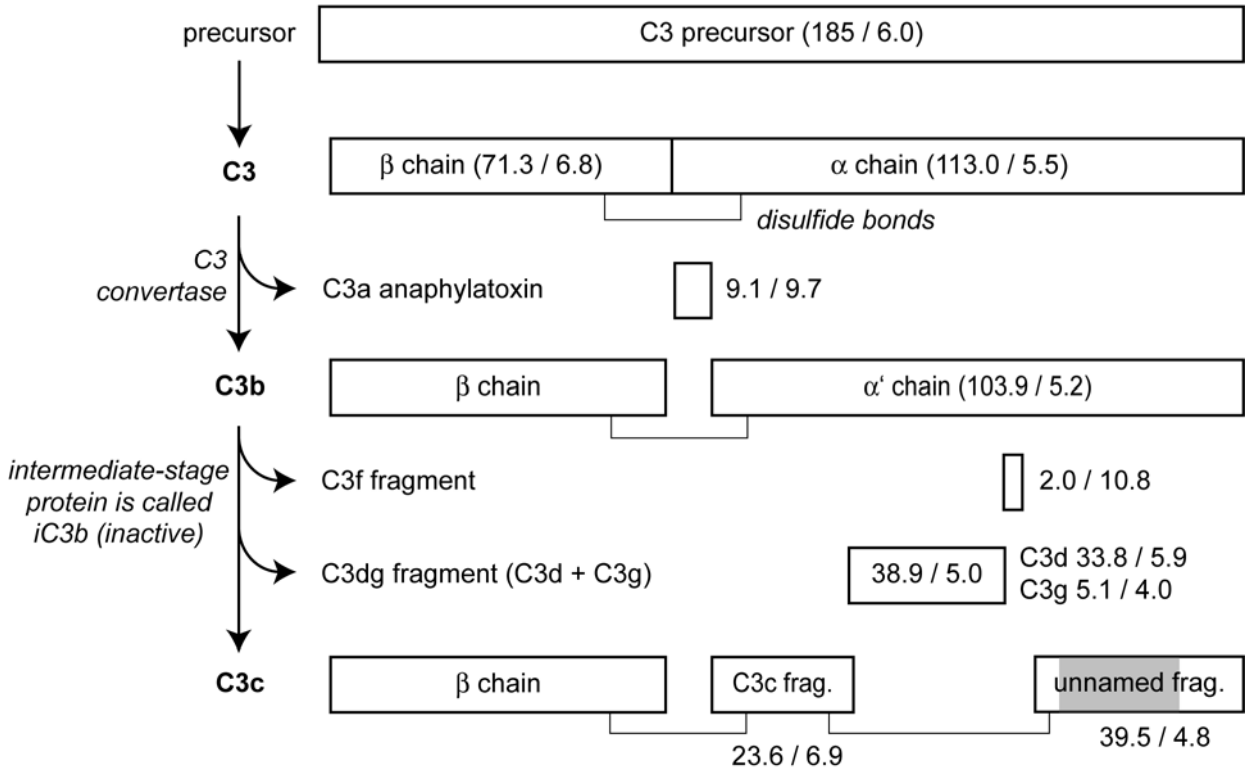

Figure 5-4: Complement C3 cleavage and fragment identification

Proteolytic cleavage pathway producing complement C3 proteins and fragments. MWs and pIs for each protein and fragment are noted as X kDa / pI X; these values were calculated from the Swiss-Prot database entry for C3 (accession P01024) using the Compute MW/pI tool (http://www.expasy.org/tools/pi_tool.html). Shading indicates the sole region of LC-MS/MS peptide sequence coverage. Adapted from Janssen *et al.* 2005, Figure 1C.

5.3.5 Investigation of apolipoprotein E isoforms

Spot 161, the basic isoform of ApoE ($ApoE_B$) present at pH 5.7, was increased two-fold on average in preeclamptic samples. $ApoE_A$, the acidic isoform at pH 5.2 as spot 168, was on average 1.7-fold lower in preeclamptic samples than in healthy pregnant samples. Three-dimensional representations of the $ApoE_A$ and $ApoE_B$ spot volumes are shown in Figure 5-5. $ApoE_B$ was detected in only three healthy pregnant samples versus 11 preeclamptic samples. $ApoE_A$ was also present 11 preeclamptic samples and in all 12 healthy pregnant samples.

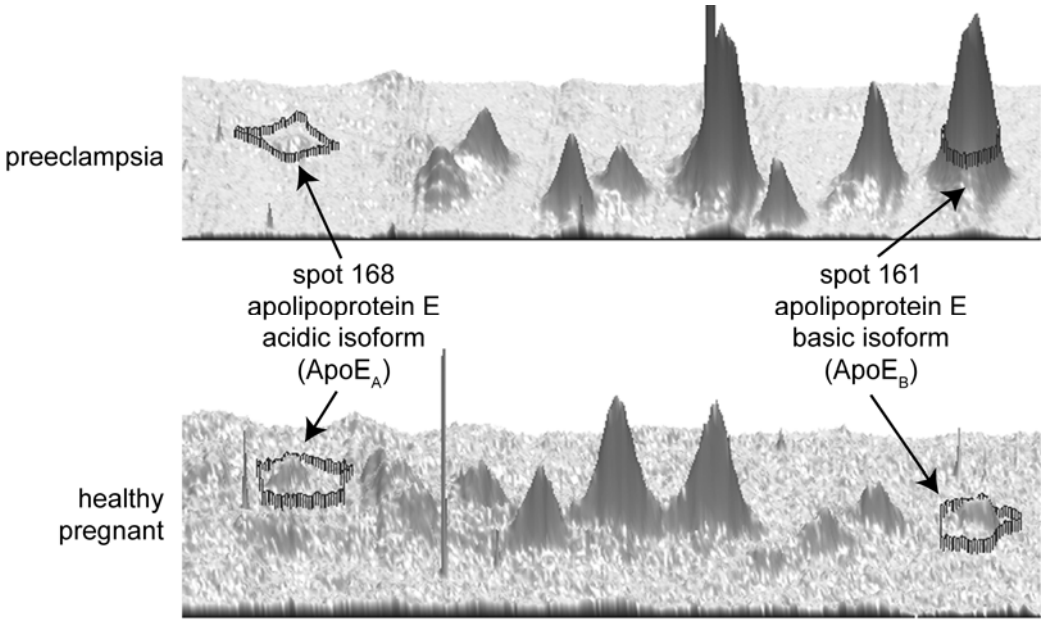

Figure 5-5: Three-dimensional spot volumes of apolipoprotein E isoforms

Spot volumes from gels containing (**top**) a preeclamptic and (**bottom**) a healthy pregnant serum sample represented as 3-D images derived from 2-D gel data. Images were generated using ImageMaster Platinum software. Boundaries of spots 168 and 161 are indicated as detected by the software. The tall vertical line on the bottom image shows a speckle resulting from SYPRO Ruby staining.

Further investigation of depleted serum from preeclamptic and healthy pregnant women using 2-D western blot analysis specifically identified of a number of ApoE isoforms (Figure 5-6). An isoform at the basic end of the ApoE spot "train" is present at higher abundance within preeclamptic serum samples than those from healthy pregnant women. The pI of this spot corresponds well to the $ApoE_B$ isoform identified on 2-D gels. The $ApoE_A$ isoform may be one of several spots near the acidic, higher mass end of the ApoE spot train. There appear to be a smaller number or a lower abundance of acidic ApoE isoforms present in preeclamptic samples than in samples from healthy pregnant women.

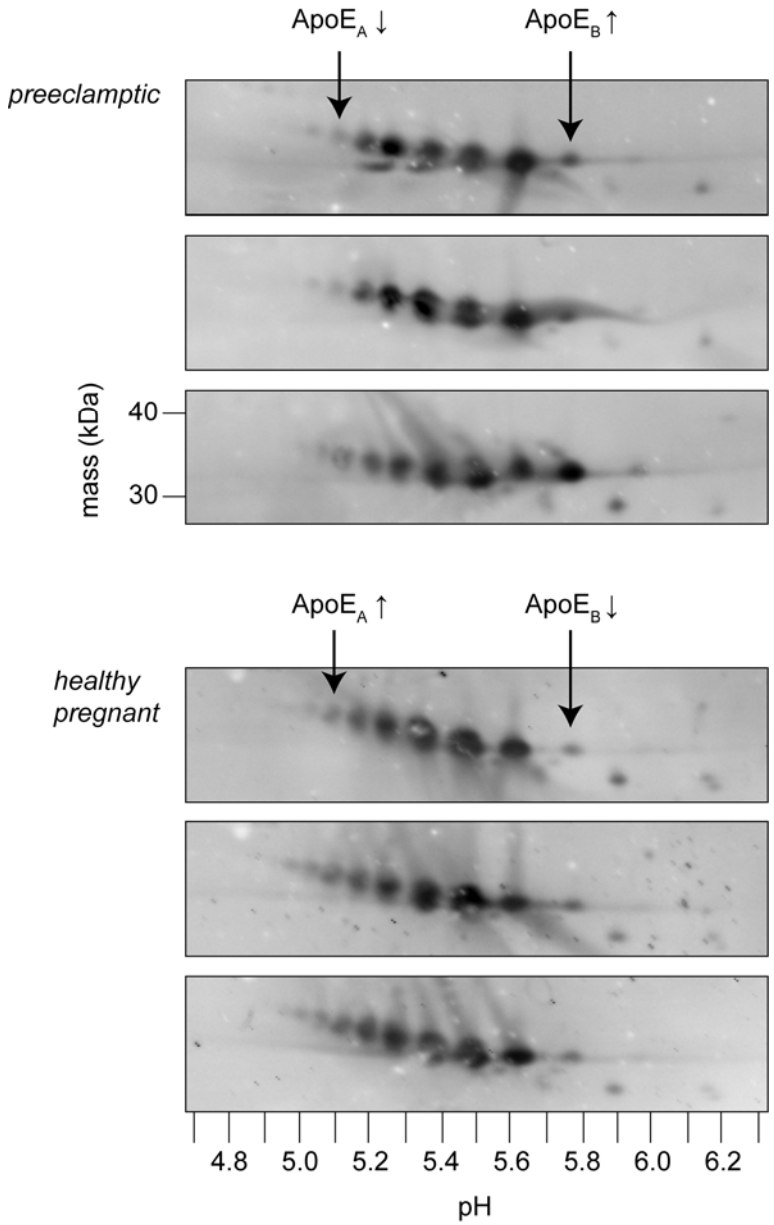

Figure 5-6: Specific detection of apolipoprotein E isoforms using 2-D western blot analysis

2-D western blot analysis of 200 µg depleted serum from three preeclamptic (**top**) and three healthy pregnant women (**bottom**). The apparent pIs of the ApoE$_A$ and ApoE$_B$ spots identified from 2DE studies, and the directions of their 2DE fold changes, are indicated with arrows above each group of blots.

As the 2-D western blot analysis confirmed the isoform changes observed on 2-D gels, the post-translational modifications responsible for the ApoE$_A$ and ApoE$_B$ isoforms were investigated with MS using the SALSA algorithm. Spot 168 (ApoE$_A$) was found to be *O*-glycosylated with a linkage comprising Thr194–*N*-acetyl hexosamine–hexose–sialic acid (Figure 5-7). Repeated analysis of peptides from spot 161 (ApoE$_B$) confirmed the identity of the protein but no peptides were detected that covered the protein's glycosylation site, so no information was available about the glycosylation state of this isoform.

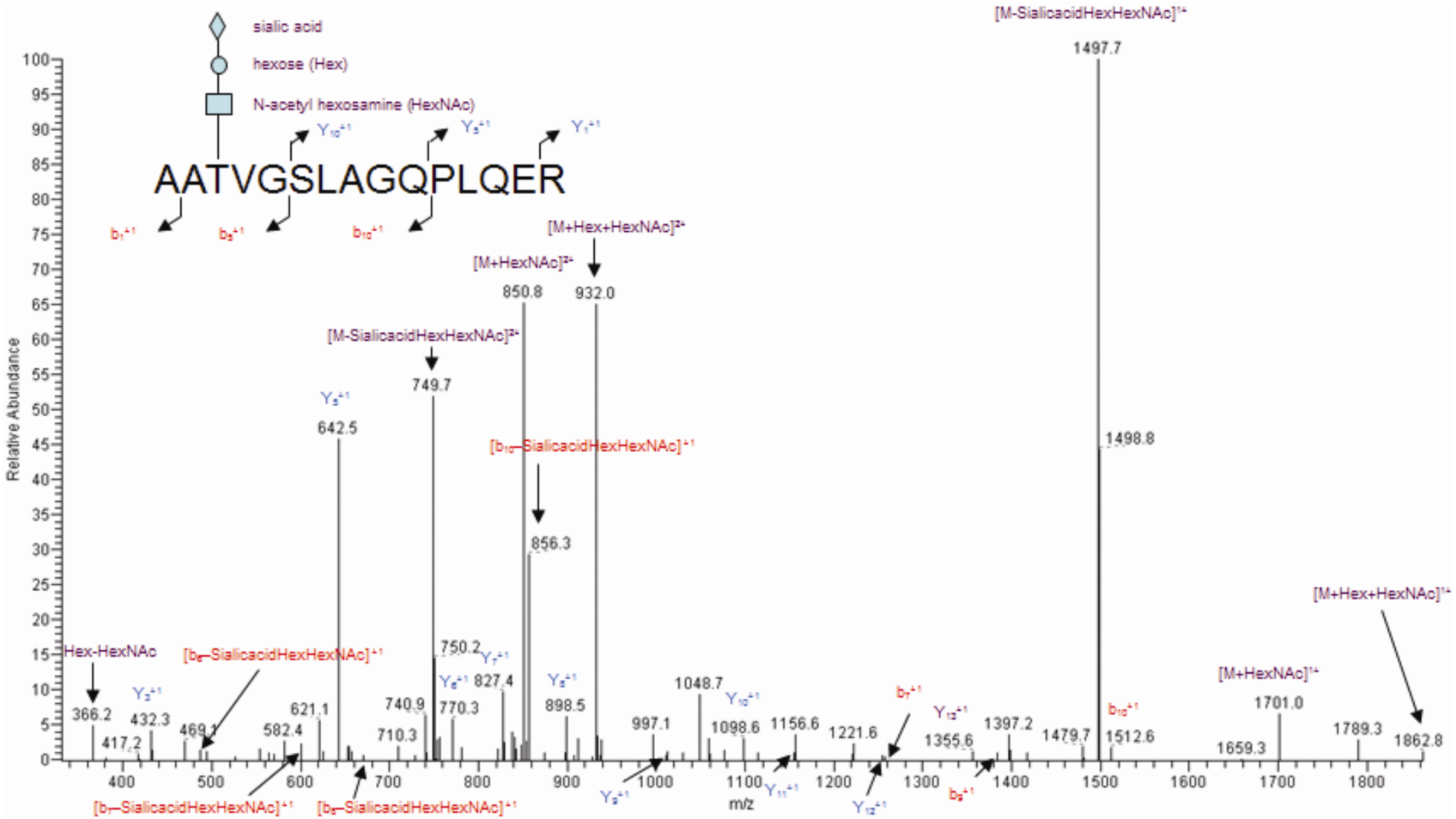

Figure 5-7: *O*-glycosylation pattern of ApoE$_A$ determined by SALSA analysis

Structure of ApoE$_A$ glycosylation as determined by interrogation of MS/MS spectra with the SALSA algorithm. The MS/MS spectrum shown corresponds to the AATVGSLAGQPLQER tryptic peptide containing the *O*-glycosylation site at threonine. Peptide fragments containing glycosylation patterns are annotated on the spectrum. The resulting carbohydrate structure is shown in the inset diagram at the upper left.

5.3.6 Measurement of plasma apolipoprotein E concentration

The plasma concentration of ApoE from preeclamptic women was 86.76 ± 17.92 µg·mL^{-1} and was 88.72 ± 20.95 µg·mL^{-1} in healthy pregnant women (Figure 5-8). No significant differences were detected between healthy and preeclamptic samples (p=0.38).

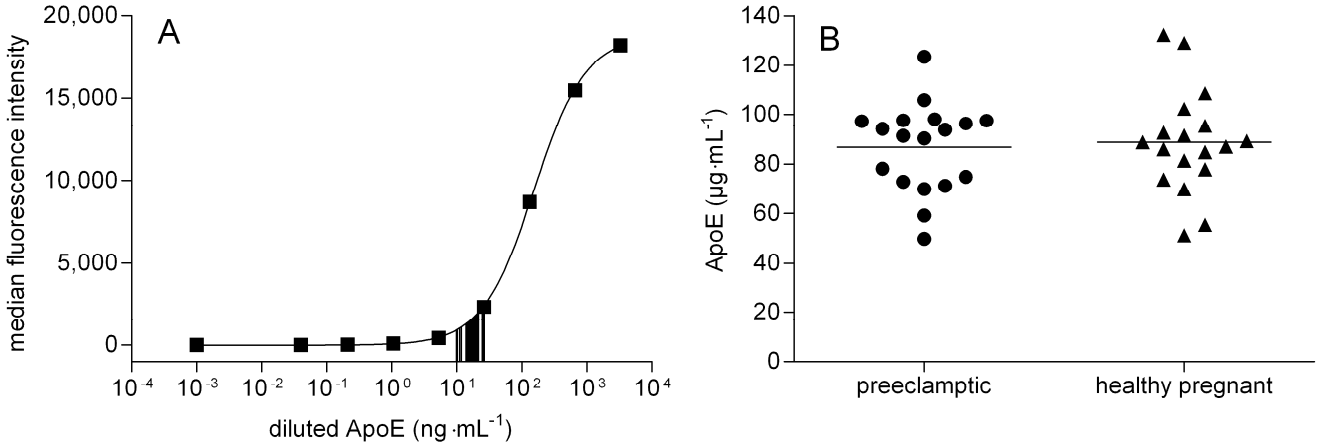

Figure 5-8: Immunoassay for apolipoprotein E in plasma samples

Multiplexed fluorescent immunoassay of total plasma ApoE in 24 samples from serum 2DE studies, plus an additional 12 plasma samples from the time of preeclampsia onset. Plasma samples were diluted 1:5000 before analysis. (**A**), standard curve with vertical lines indicating the mean concentrations of samples measured. (**B**), scatter plots showing distribution of ApoE concentrations in preeclamptic (●) or healthy pregnant (▲) plasma samples. Horizontal lines indicate the mean of each group.

5.3.7 Measurement of plasma apolipoprotein C-II concentration

Serum 2DE showed that apolipoprotein C-II (ApoC2) was upregulated an average of 2.5-fold in preeclamptic samples compared to samples from healthy pregnant women. The concentration of ApoC2 in preeclamptic plasma was 103.2 ± 23.6 µg·mL^{-1}, and was 89.5 ± 30.7 µg·mL^{-1} in healthy pregnant plasma samples (Figure 5-9). The difference between groups was not significant (Student's t: p=0.42). An outlier in the healthy pregnant samples (411 µg·mL^{-1}, with the next highest value being 157 µg·mL^{-1}) did not significantly skew the data (Mann-Whitney U: p=0.08). After exclusion of the outlier, the difference between groups remained insignificant (Student's t: p=0.07).

Given the p-value observed from these immunoassays and the two groups of 18 women studied, the power of the ApoC2 immunoassay to detect significant differences was estimated. Spot 28 was increased an average of 2.5-fold on 2-D gel images from preeclamptic samples. Based on the 2DE data, the lower bound of the 95% confidence interval for this fold change was 1.46-fold. The fold change in total ApoC2 observed in immunoassays was 1.15-fold. Assuming that the 1.46-fold change minimum observed from gel data is the true minimum fold increase in total ApoC2 in preeclampsia,

and assuming the mean total ApoC2 concentration in healthy pregnant samples is 89.5 $\mu g \cdot mL^{-1}$, only 10 samples per group are required to detect a 95% significant result ($\alpha = 0.05$) using a one-tailed Student's *t* test with 80% power. This indicates that the LINCOplex immunoassay for ApoC2 was sufficiently powered to detect the expected differences based on 2DE data with the sample size used.

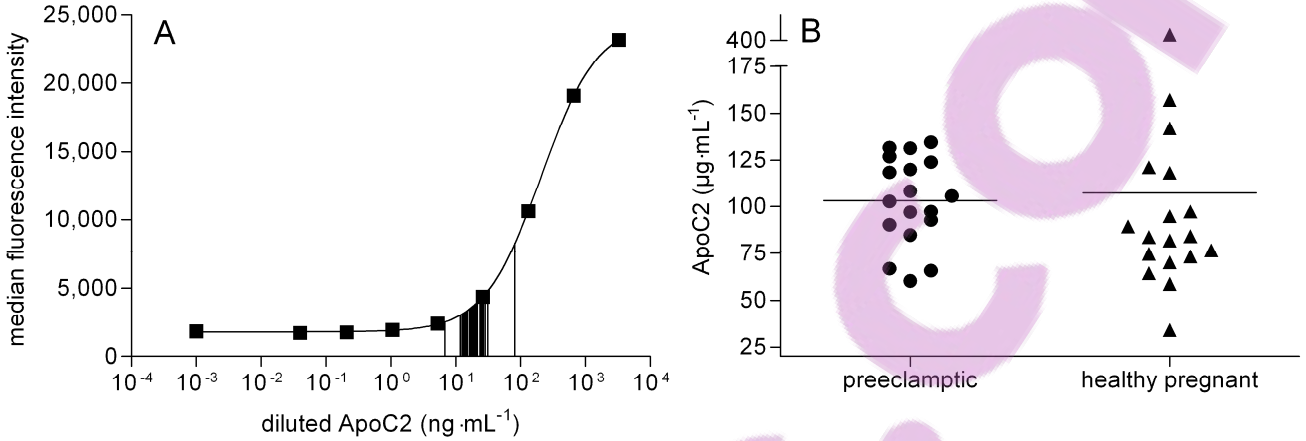

Figure 5-9: Immunoassay for apolipoprotein C-II in plasma samples

Multiplexed fluorescent immunoassay of total plasma ApoC2 in 24 samples from serum 2DE studies plus an additional 12 plasma samples from the time of preeclampsia onset. Plasma samples were diluted 1:5000 before analysis. (**A**), standard curve with vertical lines indicating the mean concentrations of samples measured. (**B**), distribution of ApoC2 concentrations in preeclamptic (●) and healthy pregnant (▲) plasma samples. Horizontal lines indicate the mean of each data group.

5.3.8 Power calculations based on serum 2DE data

Power curves shown in Figure 5-10 indicate that a 24-gel study performed using the techniques described in this chapter has 80% power to identify only 10% of the two-fold differences present between sample groups (*i.e.*, of spots exhibiting a two-fold difference, the method detected those with standard deviations below the 10th percentile). For future 2DE studies to detect even 50% of spots with a two-fold up- or down-regulation with 80% power, between 60 and 72 total samples would be required.

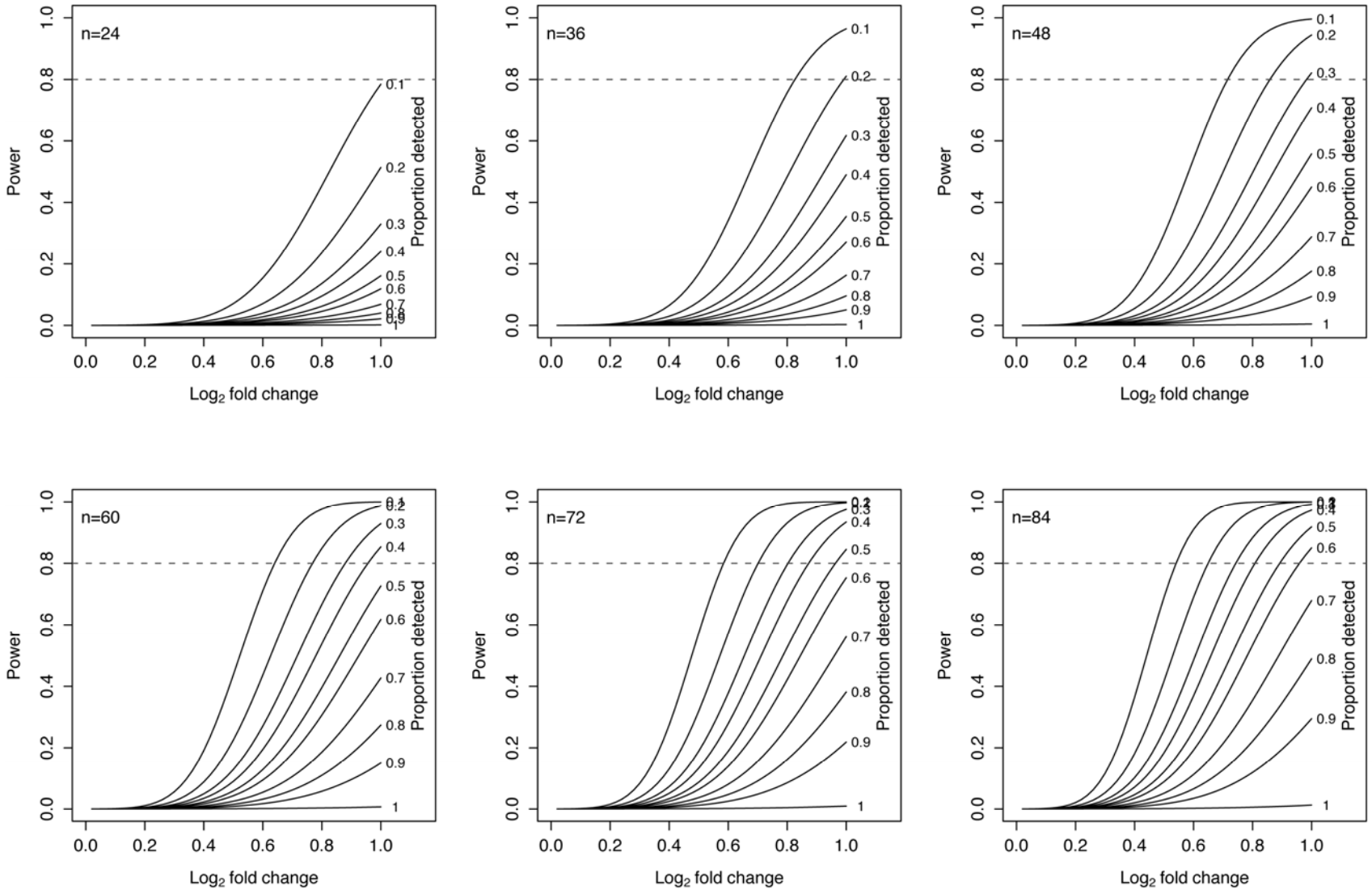

Figure 5-10: Power curves from serum 2DE data

Series of power curves calculated for different numbers of samples used in a 2DE experiment based on the data presented in this chapter. Graphs correspond to (left to right, top to bottom) 24, 36, 48, 60, 72, and 84 total samples. X-axes show effect size as the log detectable difference; 1.0 indicates a two-fold difference. Y-axes show power, or the percentage of detectable changes. Curve series within each graph differ by the percentage of spots detectable with the given power and effect size.

5.4 Discussion

The present study reports for the first time the comparison of serum proteomes from women with late-onset preeclampsia and healthy pregnant women in late pregnancy. Six spots representing five proteins were identified from 2-D gels following classifier analysis using supervised multivariate bioinformatic analysis methods. Previous proteomic studies of serum and plasma from preeclamptic women have included specific preeclampsia subgroups (HELLP; Heitner *et al.* 2006) or early-onset preeclampsia overlaid with SGA (Watanabe *et al.* 2004). The current study involved women in a narrow gestational age range (36–38 weeks) in late pregnancy.

Additionally, this study is novel due to its use of supervised multivariate classification methods on a 2-D gel dataset. A variety of classification methods were used in this study, many of which have previously been applied to microarray and clinical analyses. Comparison of the results from each classifier identified a subset of proteins found using more than one method which were subsequently identified from gels using LC-MS/MS. The five proteins found through this study were apolipoprotein E (acidic $ApoE_A$ and basic $ApoE_B$ isoforms), apolipoprotein C2, a C-terminal fragment of complement factor C3, plasma retinol-binding protein, and inter-α-trypsin inhibitor heavy chain H4.

5.4.1 Apolipoprotein E

We report for the first time that a basic isoform of ApoE ($ApoE_B$) is upregulated and an acidic isoform ($ApoE_A$) downregulated in preeclampsia. There are no published data on changes to ApoE isoforms in pregnancy or in preeclampsia. Total ApoE levels in plasma were not different between women with preeclampsia and gestation matched healthy pregnant women. This result is consistent with Francoual *et al.* (1999) who also found total ApoE levels were unchanged in preeclampsia, whereas Chalas *et al.* (2002) reported levels that were just significantly elevated in preeclamptic samples (5.0 ± 2.0 SD) compared to healthy pregnant samples (4.0 ± 1.7 SD), with p=0.049.

5.4.1.1 Role

ApoE is a 299 amino acid polypeptide synthesized primarily by the liver, but is also produced by trophoblasts during pregnancy and assists in the uptake of maternal lipids (Rindler *et al.* 1991; Francoual *et al.* 1999). It plays a key role in the transportation and release of fatty acids and cholesterol to the hepatocytes and many other cell types. ApoE is an integral component of several lipoprotein complexes including chylomicrons, VLDL, and to a lesser extent HDL. It mediates the

removal of lipids from within these complexes by binding to two receptors: the LDL receptor, and the LDL-related receptor. Chylomicrons, the lipoprotein particles of largest diameter, are synthesized in the small intestine and transport dietary fats (mainly triglycerides) through the bloodstream to the liver. VLDL have a smaller diameter and are synthesized in the liver from endogenous triglycerides and cholesterol, carrying these lipids to peripheral cells and tissues. LDL are smaller than VLDL and carry the majority of circulating cholesterol. HDL, the smallest diameter particle class, are mainly used to return excess cholesterol from tissues back to the liver for recycling or disposal. ApoE is encoded by the *APOE* gene, found on the long arm of chromosome 19 (Lusis *et al.* 1986), and has three allelic variants (ε2, ε3, ε4; Zannis and Breslow 1981). These variants differ by single amino acid substitutions between Cys and Arg at positions 112 and 158 (Weisgraber *et al.* 1981; Mabile *et al.* 2003) which cause slight structural modifications of the E2 and E4 proteins and important changes in lipid metabolism (Hatters *et al.* 2006).

It is well recognized that preeclampsia is associated with a number of alterations in plasma lipid levels compared to healthy pregnancy (van den Elzen *et al.* 1996; Sattar *et al.* 1997; Koçyigit *et al.* 2004). Typically preeclampsia is associated with increased triglycerides and VLDL, and reduced HDL, compared to normal pregnancy (Francoual *et al.* 1999; Cekmen *et al.* 2003; Winkler *et al.* 2003; Koçyigit *et al.* 2004; Bayhan *et al.* 2005; Uzun *et al.* 2005). Total LDL is generally unchanged or increased, but there is an increase in small, dense LDL particles and oxidized LDL (Sattar *et al.* 1997; Ogura *et al.* 2002; Cekmen *et al.* 2003; Belo *et al.* 2004; Koçyigit *et al.* 2004; Bayhan *et al.* 2005; Uzun *et al.* 2005). Small dense LDL particles oxidize more readily, and oxidized LDL can cause endothelial injury and platelet aggregation (Li and Mehta 2003). These changes may contribute to atherosis, an atherosclerosis-like foam cell lesion present in the uteroplacental arteries of preeclamptic women (Hubel 1997; Sattar *et al.* 1997).

Alteration in the genotypes of ApoE results in changes in circulating lipid levels in pregnancy (Belo *et al.* 2004). The *APOE* ε3 allele is the most common and is considered the "normal" benchmark for ApoE functions. The ε4 allele encodes ApoE4 which binds to receptors normally, and is associated with decreased ApoE and triglyceride levels and increased cholesterol, VLDL, LDL, and HDL levels (Mahley and Rall 2000). The ε2 allele encodes ApoE2 with drastically reduced affinity for LDL receptors (Weisgraber *et al.* 1982) and homozygosity in this allele predisposes to type III hyperlipoproteinemia (Mahley and Rall 2000). *APOE* ε2 is also associated with high circulating levels of ApoE, triglycerides, and VLDL, and decreased cholesterol, HDL, and LDL levels. This pattern is similar to that seen in preeclampsia. Although a linkage between the *APOE* ε2 allele and

preeclampsia was suggested by an initial study (Nagy *et al.* 1998), this failed to be confirmed by subsequent analyses (Chikosi *et al.* 2000; Makkonen *et al.* 2001; Francoual *et al.* 2002; Belo *et al.* 2004).

The different alleles for ApoE result in changes in the pI of ApoE, with the ApoE2 pI being more acidic than that of ApoE3 (Børresen and Berg 1981; Zannis *et al.* 1982). This shift is in the opposite direction to that observed in preeclampsia in our 2-D western blot analysis; therefore, ApoE2 is unlikely to explain the isoform changes seen in our study, which are more likely due to a specific alteration in the glycoslyation of ApoE.

5.4.1.2 Post-translational modification

ApoE is synthesized by hepatocytes as a heavily glycosylated protein containing a large number of sialic acid groups, but is processed by extracellular desialyation to an ~80% unglycosylated form (Zannis *et al.* 1984; Zannis *et al.* 1986). Plasma ApoE is found as a heterogeneous mixture of charge and mass isoforms on 2-D gel images (Børresen and Berg 1981; Zannis and Breslow 1981). The charge changes seen in ApoE are due to a combination of allelic variation in the *APOE* gene and post-translational protein modification (Zannis and Breslow 1980, 1981).

SALSA analysis of $ApoE_A$ identified *O*-glycosylation at Thr194 with an *N*-acetylhexosamine–hexose–sialic acid structure. This is consistent with the recognized *O*-glycosylation site at Thr194 (Wernette-Hammond *et al.* 1989) and with the known carbohydrate structure incorporating an *N*-acetylgalactosamine–hexose–$(\text{sialic acid})^n$ moiety (Zannis and Breslow 1981; Mancone *et al.* 2007). Further determination of hexose and hexosamine enantiomers is not possible from mass-based methods such as SALSA, but *O*-glycosylation of mammalian proteins almost always involves the direct linkage of *N*-acetylgalactosamine (GalNAc) to the Ser/Thr (van den Steen *et al.* 1998). Previous analysis of the carbohydrate content of ApoE showed that glucose (Glc) is present (Wernette-Hammond *et al.* 1989). Consequently, the glycosylation pattern of $ApoE_A$ probably corresponds to Thr194–GalNAc–Glc–sialic acid. We were unable to identify the glycosylation state of $ApoE_B$ by SALSA analysis. Its position as the most basic ApoE isoform present indicates it is likely deglycosylated. This conclusion corresponds well with other 2DE analyses of ApoE or LDL (Børresen and Berg 1981; Zannis and Breslow 1981; Utermann *et al.* 1982; Sprecher *et al.* 1984; Mancone *et al.* 2007).

More than twenty years after its glycosylation was discovered, the relationship between ApoE's glycosylation state and its function is unclear. Production of deglycosylated ApoE from cultured mammalian cells unable to incorporate GalNAc did not modify the synthesis or secretion of ApoE

compared to normal cells (Wernette-Hammond *et al.* 1989; Zanni *et al.* 1989). Production of ApoE in bacteria, which do not glycosylate eukaryotic proteins, did not affect its binding to LDL receptors and LDL-related receptors in culture when compared to plasma-derived ApoE (Vogel *et al.* 1985), but the impact of glycoslyation on receptor binding in other tissues has not been reported. Very little is known about the impact of glycoslyation on the three-dimensional structure of ApoE, and therefore potentially on its function. Recently, deglycosylated ApoE was found to form a horseshoe shape when in a complex with a synthetic lipid particle (Peters-Libeu *et al.* 2006). However there is no information yet on how glycoslyation modifies the structure of this complex (KH Weisgraber, personal communication). The glycosylation site is present in a hinge region of the protein which has unknown function and is susceptible to proteolysis (Wetterau *et al.* 1988). The circulation of ApoE in blood in a mostly deglycosylated form suggests that ApoE functions in this form. At this stage, the glycosylated form of ApoE serves an unknown purpose unrelated to receptor or lipid binding functions.

Serum 2DE results indicated here that $ApoE_B$ was upregulated, and $ApoE_A$ downregulated, in preeclampsia, suggesting that the glycosylated forms of $ApoE_A$ seen in normal pregnancy have been altered. Although dyslipidemia is a recognised feature of preeclampsia, the impact of altered ApoE glycosylation in preeclampsia on lipid metabolism remains unclear. Alterations in the glycoslyation of hCG and transferrin have also been reported in preeclampsia (Wu *et al.* 2003). Decreased levels of bioactive (glycosylated) hCG and a reduction in hyperglycosylated hCG have been observed in preeclamptic serum and urine (Casart *et al.* 2001; Bahado-Singh *et al.* 2002). In addition, diabetes is associated with an increased risk of preeclampsia, and the importance of protein glycoslyation in the development of microvascular complications in this condition is well recognized (Huebschmann *et al.* 2006; Fülöp *et al.* 2007). The alteration in ApoE glycosylation observed here may well be part of more widespread dysregulation of protein glycosylation that may contribute to the pathogenesis of preeclampsia.

5.4.2 Apolipoprotein C-II

An isoform of ApoC2 was upregulated in the serum of women with preeclampsia. ApoC2 is a very small protein of only 79 amino acids encoded by a gene present within the *APOE* gene cluster on chromosome 19. It is an essential cofactor of lipoprotein lipase, an enzyme which hydrolyzes triglycerides from chylomicrons and VLDL into free fatty acids and glycerol for transport into tissues (Wang 1991; Kingsbury and Bondy 2003). ApoC2 fulfills this role in lipid transport in concert with ApoE. Chylomicrons and VLDL form complexes with both ApoC2 and ApoE after synthesis. ApoC2 is required for removal of triglycerides from these complexes, which then exposes the LDL receptor

binding site of ApoE and enables hepatic uptake of delipidated chylomicron and VLDL remnants (Havel and Kane 2001).

As with ApoE, ApoC2 is polymorphic in plasma and is secreted as a glycosylated preprotein, though the glycosylation site and mechanism are not known. This preprotein is subsequently cleaved into a mature form that is present only in small amounts in plasma (Scanu *et al.* 1984; Fojo *et al.* 1986). Consequently, several isoforms of ApoC2 can be separated using 2DE (Sprecher *et al.* 1984; Hussain and Zannis 1990). The location of the ApoC2 isoform identified in the present study appears most similar to the that of the mature protein which is presumed to be deglycosylated (Sprecher *et al.* 1984; Fojo *et al.* 1986). Upregulation of this deglycosylated ApoC2 isoform observed in serum from preeclamptic women supports an enhancement in the deglycosylation process in general in this disorder. Unlike ApoE, however, the glycosylation state of ApoC2 has not yet been fully related to the protein's active form.

The present study is the first report to indicate ApoC2's involvement in preeclampsia. One previous study (Chalas *et al.* 2002) failed to find significant differences in ApoC2 levels between preeclamptic women around 30 weeks of gestation and healthy pregnant women around 34 weeks of gestation. Fluorescent immunoassays performed in the present study confirmed that total ApoC2 was present at similar levels in plasma from preeclamptic and healthy pregnant women. It is possible that the increased detection on 2-D gels of a putatively deglycosylated isoform of ApoC2 may simply indicate an increased circulating level of this isoform without a global change in ApoC2 concentration. This could occur through the model postulated in Section 5.4.1.2, where the balance of glycosylated/deglycosylated apolipoprotein isoforms is changed in preeclampsia but the total circulating levels of these proteins are not significantly affected.

5.4.3 Complement C3c

One isoform of a C-terminal fragment of complement C3 was downregulated in the serum of preeclamptic women in this study. Complement C3 is centrally involved in the activation of the complement system, a network of proteins involved in pathogen clearance. C3 comprises α and β chains derived by proteolytic cleavage from a single precursor protein and processed into the active fragment C3a (77 amino acids) and the inactive C3b and C3c forms (*cf.* Figure 5-4). C3c is a major cleavage product incorporating the remaining uncleaved portion of C3 β and two fragments representing the N-terminal and C-terminal portions of the C3 α′ chain (Davis *et al.* 1984; Janssen *et al.* 2005). The C-terminal fragment of complement C3c observed here may be a downstream product of the complement activation process which produces the active mediators C3a and C5a. This specific

C-terminal C3c fragment has been observed in a 2DE study of serum from patients with hepatitis B virus; its presence was attributed to the inflammatory processes occurring within infected individuals (Steel *et al.* 2003).

The role of the complement system in the pathophysiology of preeclampsia has been discussed for more than 70 years, though there is a lack of evidence showing that the system is activated in this disorder. Strict control of the complement system is required in healthy pregnancy to prevent fetal rejection, and invading CTBs express proteins which inhibit complement activation (reviewed in Girardi *et al.* 2006). The complement system is thought to be tied to the generalized vascular inflammation occurring in preeclampsia (Haeger 1993; Redman *et al.* 1999), and to the maternal immune adaptation required to host the semi-allogenic fetus. Perhaps in conjunction with impaired vascular remodeling, plasma levels of complement proteins including C3a are increased in women with preeclampsia compared with healthy pregnant women (Abramson and Buyon 1992; Haeger *et al.* 1992). This supports the theory that the complement system is activated in preeclampsia.

The downregulation of an isoform of a C3 proteolytic cleavage product in preeclamptic samples observed in this study seems incongruous with reports of increased total C3a expression in preeclampsia. However, C3 cleavage into C3a and C3b frees additional C3b protein to act as a cofactor for Factor H and properdin proteins further involved in complement activation. Only after C3b is used in this manner is it inactivated into iC3b and C3c forms (*cf.* Figure 5-4; Walport 2001). If complement activation in preeclampsia leads to additional C3a expression, then excess C3b may be utilized elsewhere in the complement cascade, and lower levels of C3c would be detected. Regardless of the mechanism, further quantitative studies are necessary to determine the extent of total complement C3 regulation and degradation in preeclampsia.

5.4.4 Inter-α-trypsin inhibitor heavy chain H4

An isoform of inter-α-trypsin inhibitor (IATI) heavy chain H4 was present on 2-D gels from only one preeclamptic and seven healthy pregnant serum samples. The volume of this spot varied widely within the healthy pregnant samples; this suggests the spot's differential expression may not be reproducible.

IATI heavy chain H4 is also called inter-α-trypsin inhibitor family heavy chain-related protein (IHRP) (Choi-Miura *et al.* 1995; Nishimura *et al.* 1995; Saguchi *et al.* 1995), which more accurately describes the relationship between this protein and the other three heavy chains of IATI. IATI heavy chains 1 and 2 form a complex with the IATI light chain, also called bikunin. IATI heavy chain 3 can form a similar

complex with bikunin. IHRP does not contain a bikunin binding site (Saguchi *et al.* 1995; Josic *et al.* 2006). IHRP is glycosylated through both *N*- and *O*-linkages (Choi-Miura *et al.* 1995).

Althought the precise function of IHRP is unknown, it is a substrate for the plasma peptidase kallikrein (Nishimura *et al.* 1995) which is involved in activation of coagulation, fibrinolysis, and neutrophil responses (Moreau *et al.* 2005). Kallikrein is directly responsible for the production of bradykinin, a vasodilatory nonapeptide, from high MW kininogen, and the kallikrein-kinin system is closely connected to the renin-angiotensin system (Schmaier 2003). These systems and their wide-ranging effects have been implicated in the pathophysiology of preeclampsia (Shah 2005). Urinary levels of kallikrein may predict the occurrence of preeclampsia in combination with other markers (Conde-Agudelo *et al.* 2004). The expression of IRHP has not been specifically determined in preeclampsia, but IATI was significantly upregulated in serum from healthy pregnant women undergoing labor (Karmowski *et al.* 2001).

Despite the lack of information about the function of IHRP, this protein has recently been identified as a candidate biomarker for several disorders. IHRP was found in a plasma biomarker set for HELLP syndrome; the protein was upregulated in HELLP plasma compared to healthy pregnant plasma (Heitner *et al.* 2006). Expression of a cleavage product of IHRP was upregulated in early stage ovarian cancer (Zhang *et al.* 2004b) but peptides derived from this protein were absent in melanoma and breast cancer samples (Caputo *et al.* 2005). IHRP was also upregulated in the serum of patients with pancreatic cancer (Yu *et al.* 2005) and lung cancers (Okano *et al.* 2006; Heo *et al.* 2007). Despite these numerous identifications of IHRP as a disease marker, very little is understood about its function and physiological role. Consequently, the precise involvement of IHRP in preeclampsia is unclear. Further investigation of the role of IHRP is necessary to elucidate mechanisms behind the disappearance of an IHRP isoform from preeclamptic serum samples, and its identification in healthy pregnant serum in this study.

5.4.5 Plasma retinol binding protein

Upregulation of an isoform of plasma retinol binding protein (RBP) was identified in this study. RBP is an 183 amino acid protein primarily responsible for the transport of circulating retinol throughout the body (Newcomer and Ong 2000). As it is smaller than the glomerular filtration cutoff, circulating RBP is bound to the much larger transthyretin protein (Newcomer and Ong 2000). Until recently, retinol transport was the only known function of RBP; however, upregulation of this protein may also contribute to systemic insulin resistance (Yang *et al.* 2005).

Levels of RBP have not been measured in women with preeclampsia. A recent study measuring free- and transthyretin-bound RBP in Nepalese women late in pregnancy concluded that women with sufficient retinol (vitamin A) concentrations had higher concentrations of RBP (Sankaranarayanan *et al.* 2005). A few studies have examined the plasma concentrations of retinol itself in pregnant women with or without preeclampsia, although these have been conducted on African (Ziari *et al.* 1996) or South American populations (Zhang *et al.* 2001). Both studies showed decreases in plasma retinol concentrations with preeclampsia compared to healthy pregnancy. One further study of Zimbabwean women demonstrated a small but significant increase in plasma retinol in preeclamptic women (Williams *et al.* 2003). The upregulation of RBP in preeclamptic women observed in the current 2DE study could be responsible for the observed decreases in retinol concentrations reported in the two earlier studies. In addition, RBP was recently found to be upregulated in the plasma of lung cancer patients (Okano *et al.* 2006).

Transthyretin, involved in the transport of RBP, has a less tenuous link to preeclampsia. Monomeric transthyretin is present at higher levels in the amniotic fluid of women who later developed preeclampsia than healthy pregnant women (Vascotto *et al.* 2007). Transthyretin was also identified as a marker for HELLP in a plasma 2DE study (Heitner *et al.* 2006). While transthyretin was not identified as a candidate biomarker in this study, the involvement of the retinol transport system in preeclampsia seems clear, although its precise mechanism is yet to be understood.

Interestingly, there is some evidence to suggest a role for retinol in post-translational protein glycosylation. Chan and Wolf (1987) found that the transfer of oligosaccharides to protein acceptors was vastly diminished in a vitamin A-deficient *in vitro* system. A later study of vitamin A-deficient rat livers (Tauber *et al.* 1992) was not able to reproduce this result. However, recently retinoic acid (a form of retinol) was found to increase expression of an essential gene in the formation of galactose-containing glycoproteins and glycolipids (Pai *et al.* 2007). This result may connect the increase in RBP (potentially leading to decreased free retinol, and subsequently decreased glycosylation) with the upregulated deglycosylated apolipoproteins observed in this study. If this linkage is correct, the identification of RBP here could further support the idea that development of preeclampsia may involve a dysregulation of protein glycosylation.

5.4.6 Additional considerations

Many of the candidate biomarker spots identified in this chapter had extremely low spot volumes, suggesting that the proteins are present in amounts close to the lower limit of 2DE sensitivity. Comparison of these low spot volumes could result in aberrantly high fold change values. The

method used to calculate fold changes employed the arithmetic means of observed spot volumes, which can be heavily affected by outlier values. Other analysis software used in the DIGE modification of 2DE uses geometric means which are less susceptible to influence by outliers. However fold changes calculated from geometric means are not yet widely used in published reports of 2DE studies.

Power analysis conducted after the conclusion of the 2DE study showed the high variability of the 2DE techniques used here. Detailed information on the power of 2DE analyses was unavailable at the time this study began, although a later study indicated that at least 10 samples per group were necessary to obtain 80% power of finding two-fold differences at the 5% significance level (Hunt *et al.* 2005). In comparison, power calculations performed from the data in this 2DE study resulted in between 60 and 72 total samples required to find even 50% of two-fold differences with 80% power at the 5% significance level. This result does not invalidate the 2DE study and classification analyses, but does indicate that less variable techniques could have resulted in a larger number of more significant biomarker candidates.

5.4.7 Conclusions

The serum 2DE study described in this chapter clearly demonstrates the utility of serum proteomics for biomarker discovery. Five candidate proteins were identified after numerous novel statistical analyses and these proteins have potent linkages to the biology of preeclampsia. Several of these proteins are glycosylated, and this research suggests that the regulation of their glycosylation may be deficient in preeclampsia. However, given the low power of the present 2DE study, a follow-up proteomic study was conducted using the less variable DIGE technique and is described in the following chapter.

CHAPTER 6 COMPARATIVE PROTEOMICS OF PREECLAMPTIC PLASMA USING DIGE

6.1 Introduction

The previous chapter described a serum proteomics study of preeclamptic and healthy pregnant women using 2DE performed using a low-throughput electrophoresis method. Power calculations indicated that this previous study was likely to detect only a small proportion of two-fold changes in protein expression. Others have also reported relatively high inter-gel variation in 2DE analysis which compounds biological variation and limits the power to observe differences between experimental sets (Hunt *et al.* 2005). Power calculations determined that detecting spots with two-fold changes in serum protein expression between preeclamptic women and healthy pregnant women would require between 60–72 gels, which is not feasible using conventional 2DE methods. A potential solution is difference gel electrophoresis (DIGE), which, through the co-separation of an internal standard and two multiplexed samples on one gel, significantly increases power to detect differentially expressed proteins.

DIGE is a modification of 2DE in which up to three different samples are each labeled with a fluorescent CyDye, mixed together, and run in a single 2-D gel (Ünlü *et al.* 1997). The first validation of this technique compared liver homogenates from acetaminophen-treated mice using a two-color labeling approach, revealing that inter-animal variability in the inbred mouse population used was nine times as high as variation contributed by the DIGE technique (Tonge *et al.* 2001). This result indicated that DIGE reduces inter-gel (technical) variability, enabling biological variability to be more readily assessed. In an additional study of variation between technical replicates of a single serum sample using DIGE, over half of the spots that matched well (*i.e.*, in eight of 12 gels) had abundances which varied by less than 10% (Corzett *et al.* 2006).

The major reason for improved reproducibility and accurate matching in DIGE analysis is the addition of a pooled internal standard. This standard is labeled with Cy2 and included on every gel, where it is mixed and co-separated with Cy3- and Cy5-labeled experimental samples. The internal standard is created from a pool of the samples used in the experimental set, and as such acts as an average of the protein expression over all samples. Therefore, comparison of protein expression from individual samples with the "average" expression in the internal standard allows calculation of relative protein abundance ratios. DIGE-specific image analysis software exploits the commonality of the internal standard in both intra-gel (sample 1 versus sample 2) and inter-gel matching which improves the quality of matches over conventional 2DE. Use of the internal standard provides

substantially more accurate calculation of matched spot volume ratios than DIGE experiments not incorporating the standard (Alban *et al.* 2003).

The aim of this study was to determine differences in the late pregnancy plasma proteome between women with preeclampsia and healthy pregnant women using DIGE.

6.2 Methods

6.2.1 Study population

A case-control study was conducted in nulliparous women recruited in late pregnancy. The Auckland Ethics Committee approved these studies (AKX/02/00/157) and written informed consent was obtained from all women. Cases were women with preeclampsia from whom a blood sample was obtained between 36–38 weeks of gestation. Preeclampsia was defined according to the guidelines of the Australasian Society for the Study of Hypertension in Pregnancy (Brown *et al.* 2000a, 2000b). Severe preeclampsia was defined as the presence of one or more multisystem complications including: coagulopathy, microangiopathic haemolysis, liver impairment, acute renal insufficiency, imminent eclampsia, or eclampsia. Controls were healthy pregnant women, gestational age matched to a case at sampling, who remained normotensive and delivered a healthy baby with a birthweight in greater than the 10th customized centile (adjusted for infant sex and maternal ethnicity, height, and weight) as calculated at http://www.gestation.net. SGA babies had a birthweight less than the 10th customized centile.

6.2.2 Human plasma specimens

Blood samples were collected by venipuncture into EDTA-containing tubes and centrifuged at 2400 ×*g* for 10 min at 4 °C. Plasma was removed and stored at −80 °C in 250 µL aliquots within 3 h of collection.

6.2.3 Depletion of abundant plasma proteins

Immunodepletion of six abundant serum proteins (albumin, transferrin, haptoglobin, antitrypsin, IgG, and IgA) was performed using a MARS (Agilent) high-capacity 4.6×100 mm column according to the manufacturer's instructions. The MARS column was connected to a 600E LC system (Waters) with a microUVIS20 detector (Carlo Erba) and a portable stripchart recorder (Yokogawa). Plasma depletions were performed by Dr KL Choi.

Plasma specimens (360 µL) were diluted in Buffer A (Agilent) containing Complete Mini EDTA-free protease inhibitor (Roche). Samples were then injected onto the LC system 30 s after the program had begun. The flowthrough fraction was collected between 2.5–6.0 min and the bound fraction (eluted with Buffer B (Agilent)) was not collected. A single chromatographic run per sample provided enough depleted plasma for 2DE analysis.

Table 6-1: Liquid chromatography program for MARS plasma depletion

Program step	*Time (min)*	*Buffer B (%)*	*Flow rate (mL·min^{-1})*
1	0.00	0	0.5
2	10.00	0	0.5
3	10.01	100	1.0
4	17.00	100	1.0
5	17.01	0	1.0
6	28.00	0	1.0

Depleted proteins were exchanged into a solubilization buffer (7 M urea, 2 M thiourea, 2% CHAPS) using Vivaspin 4 centrifugal concentrators with a 5 kDa MW cutoff using the steps detailed in Table 6-2. Flowthrough fractions were centrifuged in a single concentrator at 3700 ×*g* for 30 min at 18 °C to reduce their volumes by more than four-fold. Concentrated protein was exchanged first into Milli-Q H_2O, and then into solubilization solution (9 M urea, 2% CHAPS, 60 mM DTT) by washing three times with each solution while centrifuging at 3700 ×*g* for 60–90 min per wash at 18 °C. After the third wash with solubilization solution, proteins were concentrated to approximately 300 µL and removed for quantitation.

Table 6-2: Preparation conditions for depleted plasma samples

Step number	*Diluent*	*Centrifugation time (min)*
1	none – concentration only	30
2	H_2O	60
3	H_2O	60
4	H_2O	60
5	solubilization solution	90
6	solubilization solution	90
7	solubilization solution	90
8	none – concentration only	30

Protein concentrations were assayed using the 2D Quant Kit (GE Healthcare) according to the manufacturer's instructions. Depleted serum proteins in solubilization solution were stored at −80 °C until required for 2DE.

6.2.4 DIGE of depleted plasma proteins

6.2.4.1 Experiment design

The DIGE method allows two different samples and an internal standard to be differentially labeled with fluorescent Cy dyes, mixed together, and separated on the same 2DE gel. An overview of the experimental procedure is shown in Figure 6-1.

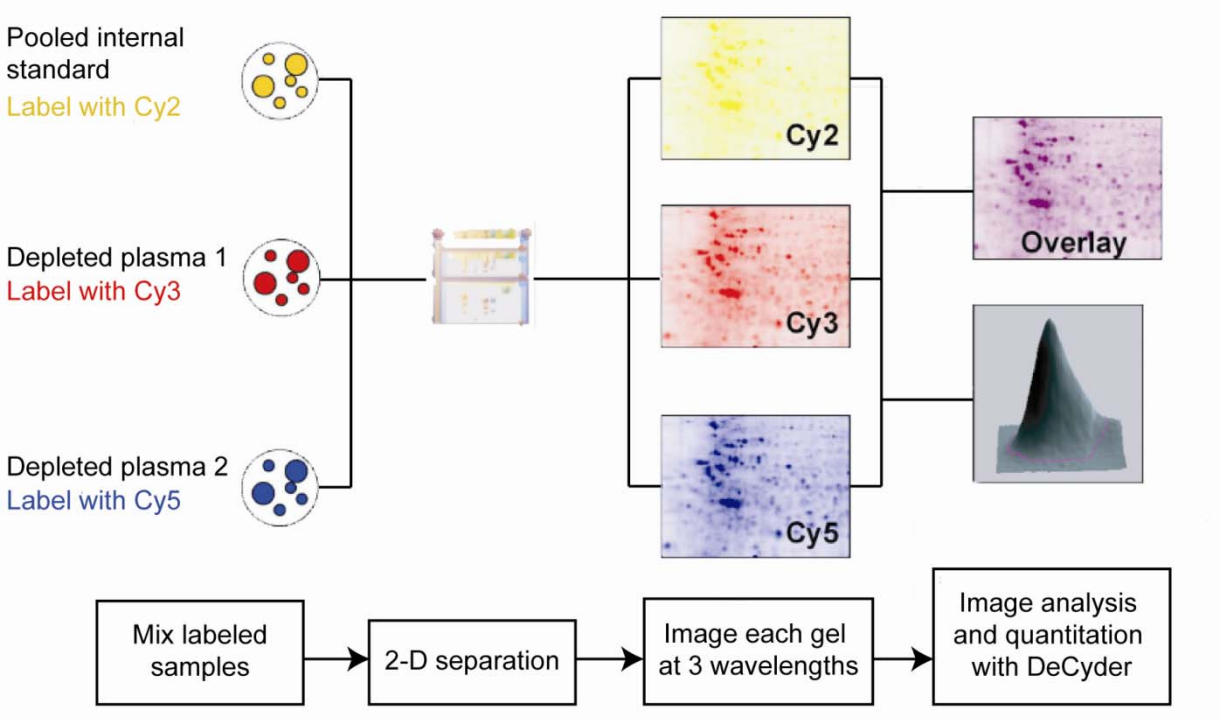

Figure 6-1: DIGE experiment overview

Schematic showing a typical DIGE workflow. From left, two plasma samples and a pooled internal standard are labeled with Cy2, Cy3, or Cy5, mixed together, and co-separated on a single 2-D gel. Variable wavelength imaging produces maps of each labeled sample which are analyzed using DeCyder software. Adapted from the DIGE System user manual, GE Healthcare #18–1173–17 AA, Figure 1–1.

The internal standard was created by mixing together 100 µg of twelve representative samples of depleted plasma from the 24 samples used in the experiment (Table 6-3). To ensure that the effects of differential dye binding did not introduce bias, half of the samples in each group (preeclamptic and healthy pregnant) were labeled with Cy3, and the other half were labeled with Cy5. This design was recommended by the DIGE CyDye manufacturer (GE Healthcare).

Table 6-3: Experiment design for plasma DIGE

Gel	*Cy3 sample type (50 µg)*	*Gestation (weeks)*	*Cy5 sample type (50 µg)*	*Gestation (weeks)*	*Cy2 standard (50 µg)*
1	healthy pregnant	35.6	preeclamptic	35.7	pooled internal standard[a]
2	preeclamptic	36.3	healthy pregnant	36.0	pooled internal standard
3	healthy pregnant	35.9	preeclamptic	36.4	pooled internal standard
4	preeclamptic	36.9	healthy pregnant	36.1	pooled internal standard
5	healthy pregnant	36.3	preeclamptic	36.9	pooled internal standard
6	preeclamptic	37.0	healthy pregnant	37.0	pooled internal standard
7	healthy pregnant	36.7	preeclamptic	37.1	pooled internal standard
8	preeclamptic	37.4	healthy pregnant	37.0	pooled internal standard
9	healthy pregnant	37.9	preeclamptic	38.3	pooled internal standard
10	preeclamptic	38.3	healthy pregnant	38.3	pooled internal standard
11	healthy pregnant	38.0	preeclamptic	38.7	pooled internal standard
12	preeclamptic	38.9	healthy pregnant	39.0	pooled internal standard

[a] The internal standard was comprised of equal amounts of protein from the shaded samples.

6.2.4.2 CyDye labeling

Tris (10 mM) was added to each of the 36 samples shown in Table 6-3 to maintain reaction pH between pH 8–9; this was checked using narrow-range indicator paper (Whatman).

Twelve 50 µg aliquots of pooled internal standard were each labeled by incubating with 200 pmol Cy2 on ice in the dark for 30 min. An additional 50 µg of each individual sample was labeled by incubating with 200 pmol of either Cy3 or Cy5 in the same manner as shown in Table 6-3. The labeling reaction was stopped by adding 1 µL of 10 mM lysine into each tube. Samples were rested on ice in the dark for 10 min and were subsequently mixed together for 2DE as shown in Table 6-3.

6.2.4.3 2DE of CyDye-labeled samples

All of the following steps were carried out in the dark.

An equal volume of 2× rehydration solution (7 M urea, 2 M thiourea, 2% C7BzO, 2% IPG Buffer pH 4–7, 130 mM DTT, and 0.002% bromophenol blue) was added to each of the twelve combined samples. The total volume of each combined sample was then adjusted to 200 µL using standard rehydration solution (7 M urea, 2 M thiourea, 1% C7BzO, 1% IPG Buffer pH 4–7, 65 mM DTT, and 0.002% bromophenol blue). Each 200 µL mixture was used to passively rehydrate an 11 cm pH 4–7 IPG strip (GE Healthcare) overnight. Focusing was performed using the Multiphor II flatbed

electrophoresis system (GE Healthcare). Strips were focused using the protocol shown in Table 3-2 and were stored at −80 °C after focusing was completed.

Table 6-4: Isoelectric focusing parameters for depleted plasma samples

Focusing phase	*Voltage (V)*[a]	*Time (h)*	*Purpose*
1	500	0:01	voltage gradient increasing to 500 V
2	500	5:00	initial focusing
3	3500	5:00	voltage gradient increasing to 3500 V
4	3500	12:00	extended focusing
5	100	0:01	voltage gradient decreasing to 100 V
6	100	(hold)	hold step when focusing is complete

[a] All steps were carried out at 1 mA, 5 W, and 20 °C.

Focused IPG strips were reduced with equilibration solution (6 M urea, 20 mM Tris pH 8.8, 2% SDS, 20% glycerol) containing 1% DTT (GE Healthcare) for 15 min, followed by alkylation in equilibration solution containing 2.5% acrylamide (GE Healthcare) for 15 min. Both incubations were performed with gentle agitation. Strips were rinsed with Milli-Q H_2O and drained on damp filter paper before second-dimension electrophoresis.

Second-dimension separations used Criterion 8–16% Tris-HCl midigels (Bio-Rad) run with Novex Tris-glycine SDS running buffer (Invitrogen). Gels were run at a constant 180 mA for 30 min followed by 360 mA for 95 min. Immediately after electrophoresis, DIGE gels were imaged using a Typhoon 9410 variable mode imager (GE Healthcare). Gels were scanned using normal sensitivity. Prescans were used to optimize the photomultiplier tube voltage to ensure that spots were not saturated; gels were imaged using 465 V for the Cy2 channel, 460 V for Cy3, and 470 V for Cy5. Gel images were cropped to the same size using ImageQuant version 5.2 (GE Healthcare).

6.2.5 Image analysis

DIGE images consisting of sets of Cy2, Cy3, and Cy5 images were analyzed using DeCyder version 6.5.14.1 for Windows (GE Healthcare) according to the manufacturer's instructions.

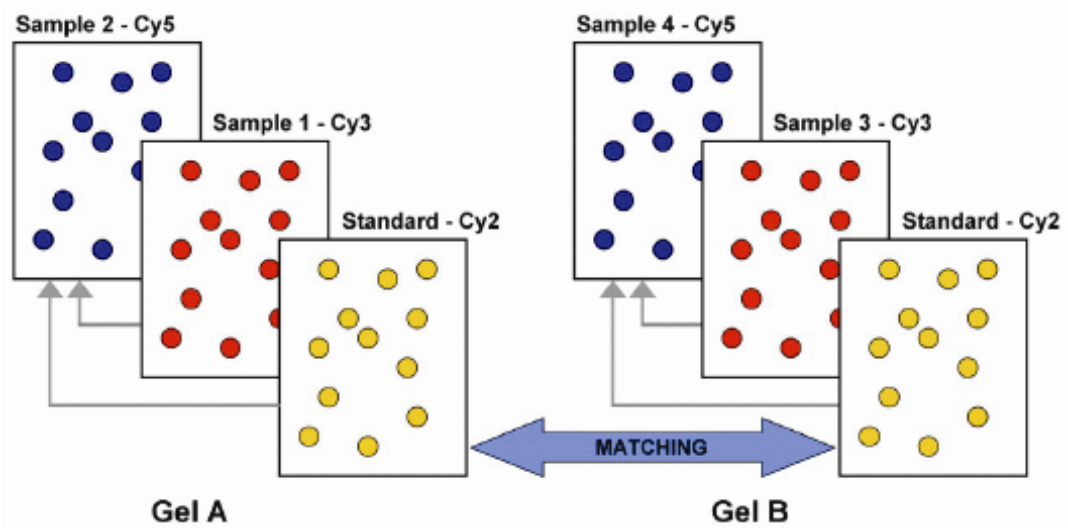

Figure 6-2: DIGE gel matching in DeCyder using a pooled internal standard

Diagram showing the process for DIGE gel matching within DeCyder software. Cy2 internal standard images are first matched to the Cy3 and Cy5 sample images within single gels. Inter-gel matching is performed by aligning only the Cy2 spot maps, as each Cy2 map should contain the same spots. Reprinted from the DIGE system user manual, GE Healthcare #18–1173–17 AA, Figure 2–5.

Briefly, spot detection and gel matching were automated using existing batch processing methods in DeCyder. The estimated number of spots was set to 10 000 and detected spots were filtered using a volume cutoff filter of 26 000 determined by calculating the percentage of genuine spots versus artifactual spots in every 1000 volume units from 10 000 to 31 000. At 26 000, the percentage of all spots that were genuine was 75%. Data were exported from DeCyder as Cy3/Cy2 and Ch5/Cy2 volume ratios for all matched spots on each gel.

Fold changes were calculated by DeCyder as the (arithmetic) mean volume ratio for a spot in preeclamptic samples divided by the mean volume ratio for that spot in healthy pregnant samples. Positive fold changes indicate upregulation in preeclamptic samples and negative fold changes indicate downregulation in these samples.

6.2.6 Preparative 2DE and protein identification

CyDye labeling of protein samples during the DIGE process introduces a small mass shift to each labeled spot. This shift can reduce the amount of protein present in a spot picked for subsequent MS analysis. Consequently, separate preparative 2-D gels, loaded with 300 µg of a single depleted plasma sample, were run as in Section 6.2.4.3 and were used for protein identification by LC-MS/MS.

Preparative gels were stained with new SYPRO Ruby as described in previous chapters. Briefly, gels were fixed for 2×30 min in fix/wash solution (10% ethanol, 7% acetic acid) and were stained overnight with new SYPRO Ruby stain. The next day, gels were washed in fix/wash solution for at

least 1 h and briefly rinsed with H_2O before imaging. Gels were imaged using an FLA–2000 phosphorimager (Fuji) with excitation at 473 nm and a 580 nm high-pass emission filter. Gels were imaged with a 50 μm pixel size and F1000 (maximum) resolution.

Spots of interest were excised from gels and stored at −80 °C until tryptic digestion. Gel spots were digested with Trypsin Gold (Promega) according to published methods (Hardt *et al.* 2005) with the following modifications: after digestion, peptides were recovered by one or two extractions with 50% acetonitrile/5% formic acid, and the digestion supernatant and extractions were combined and reduced to approximately 12 μL volume by vacuum centrifugation. Tryptic peptides were submitted for LC-MS/MS analysis on a QSTAR XL ESI-qTOF (Applied Biosciences) at the Maurice Wilkins Centre for Molecular Biodiscovery, The University of Auckland. LC-MS/MS analysis was performed by Mr M Middleditch.

Protein identification reporting followed recently published guidelines (Bradshaw *et al.* 2006). Tandem MS/MS data were extracted from raw spectra using Mascot Distiller (Matrix Science). Data were searched against the Swiss-Prot database (version 52.2, date 14 April 2007) using the Mascot search engine v2.2.0 (Matrix Science) with the following parameters: Taxonomy: human, semitrypsin cleavage with up to 1 missed cleavage allowed, fixed modification: propionamide (of cysteines), variable modification: oxidation (of methionines), mass tolerances ±0.1 Da, peptide charges 2+ and 3+. Positive identifications reported here had at least three unique peptides match the database entry.

6.2.7 Statistical analysis

6.2.7.1 Clinical data

All analyses were performed using GraphPad Prism version 4.03 for Windows (GraphPad Software) using a significance level of $p<0.05$. Continuous data from preeclamptic and healthy pregnant women were compared using two-tailed Student's *t* tests. Categorical data were compared using Fisher's exact test.

6.2.7.2 Bioinformatic analysis of DIGE data

All bioinformatic analyses described in the following section were performed by collaborators Dr MA Black (Bioinformed, Ltd.) and Mr SH Wu (The University of Auckland).

DeCyder data represents downregulated expression as negative ratios of sample volume over internal standard volume. For statistical analysis, data were transformed to a standard log-intensity

scale. First, negative ratios were transformed into positive values between 0 and 1 by taking the inverse of their absolute values. Subsequently data were logged and centered around the median of each gel to reduce noise as for serum data (Section 5.2.9.2).

6.2.7.2.1 Mann-Whitney U test

Transformed data were analyzed with the Mann-Whitney *U* test to assess differences between the preeclamptic and healthy pregnant specimen groups. Tests were performed with and without correction for multiple comparisons using the FDR controlling method of Benjamini and Hochberg (1995).

6.2.7.2.2 Limma analysis

The modified *t*-statistic from the limma package (Smyth 2004, 2005) was used to analyze transformed data. Limma tests were performed with and without FDR correction procedures for multiple comparisons as described above.

6.2.7.2.3 Nearest shrunken centroids

After DeCyder data had been transformed to a standard log-intensity scale as in Section 6.2.7.2, each missing spot (a spot undetected in at least one gel but found in at least one other gel) was imputed by assigning the median value for that spot from all gels. Data were then logged and median-centered as described in Section 6.2.7.2. The NSC method of classifier generation, and bias estimation of the NSC misclassification rate, were performed as in the previous serum study (Section 5.2.9.2.2).

Briefly, this method was used to analyze transformed 2DE data to produce a set of spots considered to be a useful classifier. A misclassification rate was then calculated from the full dataset using the classifier. Bias in this misclassification rate was estimated using bootstrap analysis (Efron and Tibshirani 1993). In each of 1000 iterations, a subset of 20 gels (ten each from preeclamptic and healthy pregnant samples) were used in cross-validation as described above to produce an NSC classifier. This classifier was used to predict the origin (a preeclamptic or healthy pregnant sample) of each gel in the 20-gel subgroup, as well as of the remaining four gels (which were not used to construct the model). The difference between the two classification rates (for the subgroup of 20, and for the remaining four) provides an estimate of the bias present when a classifier predicts the classes of the samples on which it is trained, also called overtraining. This process was repeated 1000 times, with the average of the differences in classification rates used as a bias estimate. This estimate was

then subtracted from the classification rate obtained in the original NSC analysis to produce a bias-corrected estimate of performance.

6.2.7.3 Power calculations

Retrospective power calculations were performed by Dr MA Black (Bioinformed, Ltd.) to determine the capability of this DIGE study to identify significant differences in the plasma proteomes of women with preeclampsia and healthy pregnant women (Moore and McCabe 2006). A significance level of α=0.05 and a power of β=0.80 were used.

6.3 Results

6.3.1 Maternal and fetal outcomes

As with the previous serum 2DE study, 12 women with preeclampsia and 12 healthy pregnant women were studied in the DIGE experiment. Maternal baseline characteristics and maternal and neonatal outcome data for both groups are shown in Table 6-5.

6.3.2 Statistical analysis of plasma DIGE data

A combination of rank-based (Mann-Whitney *U* test) and parametric (limma *t* test) univariate statistical methods identified 63 spots which had significantly different (p<0.05) expression ratios between preeclamptic and healthy pregnant groups (Table 6-6). After FDR correction for multiple comparisons, none of these spots remained significant. NSC analysis produced a classifier containing four spots: 355, 356, 521, and 569, which correctly classified 21 of 24 gels (a classification rate of 87.5%, reduced to 80% after bias correction). The four spots in the classifier were all ranked within the top six results from limma analysis (Table 6-7). The remaining spots ranked highly from limma analysis but not selected by the NSC classifier were numbers 31 and 830. The locations of the four NSC spots on a gel image are shown in Figure 6-3, and the distributions of sample/Cy2 ratios (standardized abundance ratios) for each of these spots are shown in Figure 6-4.

Table 6-5: Maternal and neonatal clinical characteristics from plasma DIGE study participants

	Preeclamptic (n=12)	*Healthy pregnant (n=12)*	p-*value*
Maternal characteristics			
Age (years)	30.2 (5.0)	31.6 (3.6)	0.43
Ethnicity			
Caucasian	7	9	
Pacific Islander	4	0	0.10
Other or not given	1	3	
Body mass index at booking ($kg \cdot m^{-2}$)	26.8 (4.0)	25.2 (5.1)	0.39
Gestational age at first visit (weeks)	13.8 (2.7)	13.6 (3.3)	0.91
Systolic blood pressure at < 20 weeks (mm Hg)	111 (15)	112 (11)	0.84
Diastolic blood pressure at < 20 weeks (mm Hg)	67 (10)	63 (7)	0.33
Gestational age at sampling (weeks)	37.3 (1.0)	37.0 (1.1)	0.44
Systolic blood pressure at sampling (mm Hg)	137 (9)	113 (12)	<0.0001
Diastolic blood pressure at sampling (mm Hg)	95 (8)	70 (8)	<0.0001
Maximum systolic blood pressure (mm Hg)	155 (13)	127 (8)	<0.0001
Maximum diastolic blood pressure (mm Hg)	103 (6)	77 (9)	<0.0001
Proteinuria: ≥ 0.3 g per 24 h or ≥ 2+ on dipstick or protein/creatinine ratio ≥ 30	12 (100%)	0	
24 h urinary protein (g per 24 h; median, range; n=9)	1.05 (0.39–8.94)	0	
Severe preeclampsia[a]	3 (25%)	0	
Fetal outcomes			
Gestational age at delivery (weeks)	37.8 (0.8)	40.2 (1.4)	<0.0001
Infant birthweight (g)	3107 (333)	3671 (399)	0.0001
Small for gestational age (SGA)	2 (17%)	0	
Perinatal deaths	0	0	

Values are reported as mean (SD) or n (%) unless otherwise noted.

[a] Severe preeclampsia included HELLP (n=1) or acute renal insufficiency (n=2).

Table 6-6: Results from uncorrected univariate statistical analysis of plasma DIGE data

Spot number	*Mann-Whitney* **U** *test* **p**-*value uncorrected*	*Limma* **t** *test* **p**-*value uncorrected*	*Number of gels from preeclamptic samples where spot is present*	*Number of gels from healthy pregnant samples where spot is present*	*Fold change (preeclamptic /healthy pregnant)*
31	0.002	0.001	8	8	1.5
33	0.06	0.04	10	10	1.3
46	0.02	0.07	8	8	1.1
203	0.04	0.05	12	12	1.2
221	0.01	0.03	12	12	-1.2
240	0.05	0.03	10	10	-1.4
241	0.09	0.02	12	12	1.4
242	0.07	0.05	11	11	1.3
250	0.03	0.11	12	12	-1.1
257	0.02	0.06	12	12	1.3
261	0.01	0.03	12	12	1.4
262	0.02	0.09	12	12	1.3
263	0.001	0.002	12	12	-1.3
264	0.002	0.004	12	12	-1.2
265	0.003	0.01	12	12	-1.2
267	0.01	0.03	12	12	1.3
280	0.05	0.04	12	12	-1.4
284	0.02	0.01	12	12	-1.4
290	0.03	0.02	12	12	-1.3
297	0.004	0.001	7	7	-1.6
298	0.04	0.03	12	12	-1.2
300	0.01	0.01	12	12	-1.3
317	0.03	0.11	12	12	1.2
325	0.05	0.05	8	8	1.2
344	0.06	0.04	12	12	1.2
345	0.02	0.17	12	12	-1.6
347	0.01	0.01	12	12	1.2
355	0.001	0.0001	12	12	1.9
356	0.0005	0.001	12	12	1.9
374	0.02	0.02	12	12	1.2
394	0.01	0.02	9	9	-1.5
395	0.04	0.13	10	10	1.2
398	0.03	0.10	12	12	1.2

Spot number	***Mann-Whitney* U *test* p-*value uncorrected***	***Limma* t *test* p-*value uncorrected***	***Number of gels from preeclamptic samples where spot is present***	***Number of gels from healthy pregnant samples where spot is present***	***Fold change (preeclamptic /healthy pregnant)***
425	0.01	0.01	12	12	-1.6
429	0.03	0.02	12	12	-1.7
431	0.08	0.03	11	11	-1.5
460	0.04	0.01	9	9	-1.7
466	0.07	0.04	12	12	-1.5
467	0.03	0.02	12	12	-1.4
501	0.01	0.01	11	11	-1.8
521	0.03	0.001	12	12	4.7
522	0.01	0.002	12	12	-1.4
523	0.02	0.01	8	8	1.4
526	0.03	0.02	9	9	1.2
545	0.33	0.02	2	2	2.0
553	0.03	0.07	4	4	1.3
558	0.02	0.003	5	5	-2.7
559	0.02	0.03	12	12	1.2
563	0.02	0.01	11	11	1.2
565	0.02	0.03	12	12	1.2
569	0.0004	0.0001	12	12	1.9
576	0.01	0.002	11	11	1.4
618	0.06	0.05	5	5	-2.2
718	0.06	0.02	5	5	-1.5
745	0.10	0.01	3	3	-1.6
747	0.04	0.04	12	12	-1.2
749	0.04	0.02	12	12	-1.2
768	0.02	0.03	11	11	-1.2
815	0.02	0.03	11	11	1.2
830	0.0004	0.0002	11	11	1.4
850	0.04	0.06	10	10	1.2
885	0.01	0.03	10	10	1.3
898	0.08	0.02	9	9	1.3
	49	52	total number of significant (p<0.05) spots		

Table 6-7: Spots resulting from nearest shrunken centroid analysis of plasma DIGE data

Spot number	*Mann-Whitney* **U** *test* **p**-*value uncorrected*	*Limma* **t** *test* **p**-*value uncorrected*	*Limma* **t** *test* **p**-*value rank*	*Mann-Whitney* **U** *test* **p**-*value FDR* [a]	*Limma* **t** *test* **p**-*value FDR* [a]	*Nearest shrunken centroids classifier*	*Number of gels from preeclamptic samples where spot is present*	*Number of gels from healthy pregnant samples where spot is present*	*Fold change (preeclamptic /healthy pregnant)*
355	0.001	0.0001	2	0.27	0.07	present	12	12	1.9
356	0.0005	0.001	5	0.15	0.11	present	12	12	1.9
521	0.028	0.001	6	0.68	0.18	present	12	12	4.7
569	0.0004	0.0001	1	0.15	0.07	present	12	12	1.9

[a] FDR = false discovery rate correction for multiple comparisons.

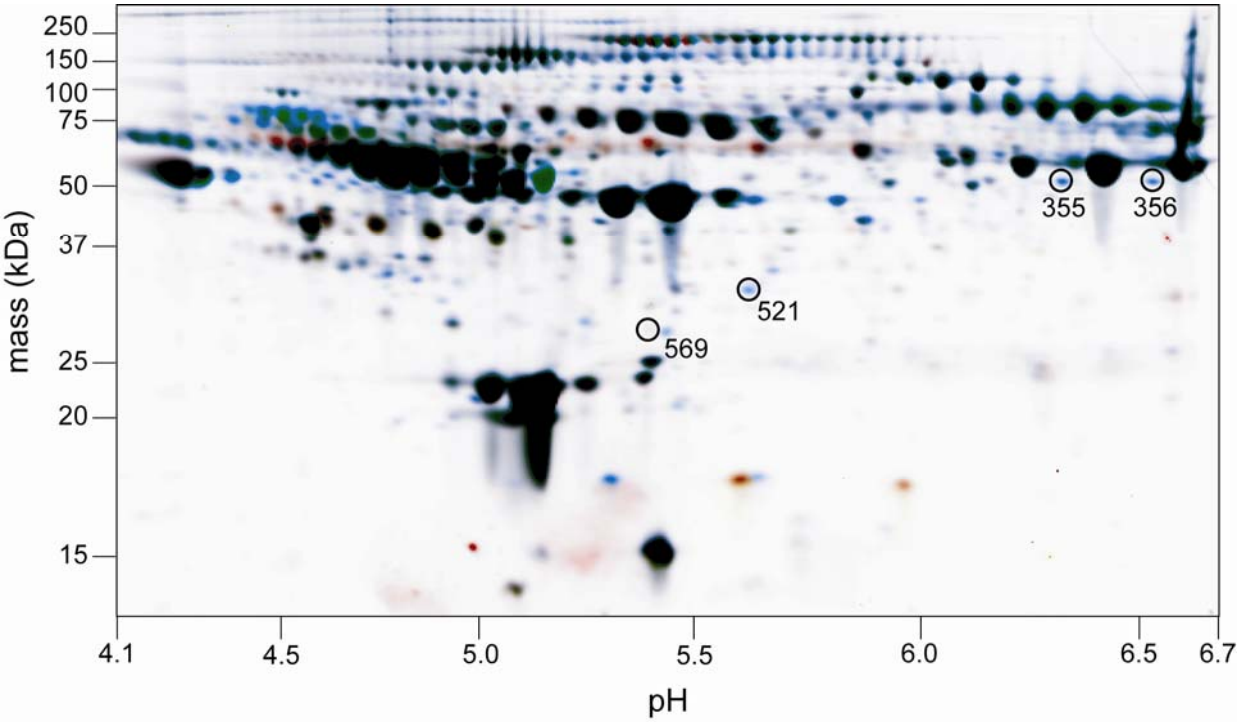

Figure 6-3: Locations of spot results from corrected statistical analysis of DIGE data

Spots resulting from informatic analysis of DIGE data (as highlighted in Table 6-7) are indicated on a representative overlaid image of a single DIGE gel imaged in three channels. The gel contains 50 µg pooled internal standard (Cy2, yellow) and 50 µg each of depleted plasma from a preeclamptic sample at 35.7 weeks of gestation (Cy5, blue) and a healthy pregnant sample at 35.6 weeks of gestation (Cy3, red). Areas of color represent differentially expressed proteins and black areas show spot coincidence.

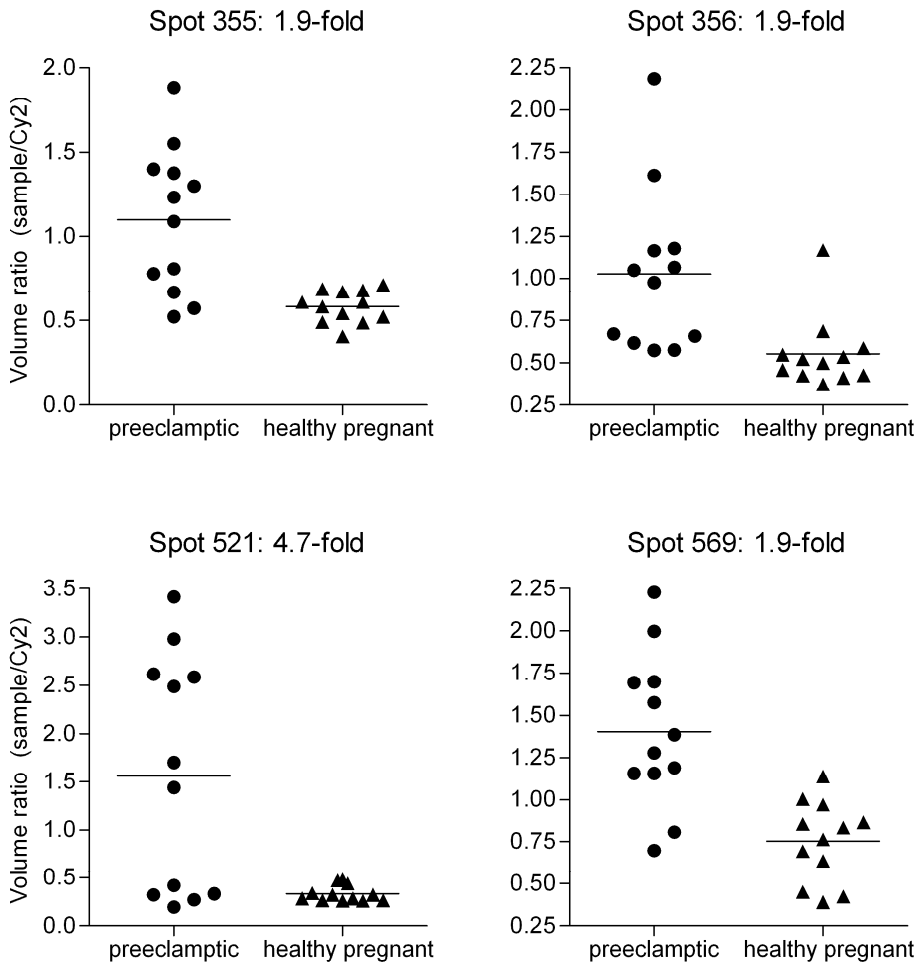

Figure 6-4: Distribution of DIGE spot volume ratios from candidate plasma markers

The spot volume ratios from individual plasma DIGE spots are shown from gels containing samples from preeclamptic (●) or healthy pregnant (▲) women. Each point represents the spot volume ratio from a single sample. Hortizontal lines indicate the mean percentage volume within each group. Fold changes (as preeclamptic versus healthy pregnant) are provided for each spot.

6.3.3 Protein identification

The four plasma protein spots of interest in Table 6-7 were identified from higher-load preparative gels using tandem LC-MS/MS (Table 6-8). With the exception of spot 521, each of the spots contained more than one protein. Although protein abundance does not correlate with the number of peptides observed in a mass spectrometer, two of the four protein mixtures contain a protein with the largest number of peptides and a high percentage of sequence coverage that matches very well with gel position data (Table 6-8). These proteins are likely to be the major components of the excised spots. In the case of spot 569, transthyretin was identified with high sequence coverage, but the predominant protein species contributing to the differential expression of this spot is unclear.

6.3.4 Power calculations based on plasma DIGE data

Power curves shown in Figure 6-5 indicate that the DIGE study performed here using a total of 24 samples had 80% power to detect approximately 75% of the two-fold differences present between preeclamptic and healthy sample groups (*i.e.*, of spots exhibiting a two-fold difference, the method detected those with standard deviations below the 75th percentile).

Table 6-8: Proteins identified from plasma DIGE spots

Spot number	*Fold change (preeclamptic /healthy pregnant)*	*Protein*[a]	*Accession*	*Sequence coverage*[b] *(%)*	*Peptides (unique)*	*Calculated MW (kDa)*[c]	*Gel MW (kDa)*	*Calculated pI*[c]	*Gel pI*
355	1.9	fibrinogen, β chain	P02675	59	39 (22)	50.7	50	7.95	6.1
		β-2-glycoprotein 1	P02749	45	14 (10)	36.2		8.37	
		transferrin	P02787	25	12 (12)	75.1		6.70	
		hemopexin	P02790	30	10 (8)	49.3		6.43	
		fibrinogen, α chain	P02671	16	12 (10)	91.3		5.79	
356	1.9	fibrinogen, β chain	P02675	64	36 (20)	50.7	50	7.95	6.3
		fibrinogen, α chain	P02671	25	20 (15)	91.3		5.79	
		fibrinogen, γ chain	P02679	19	5 (5)	48.5		5.24	
		complement C3 (peptides map to the β chain)[d]	P01024	14 v. β chain[d]	5 (5)	71.3[d]		6.82[d]	
521	4.7	apolipoprotein E	P02649	47	14 (12)	34.2	34	5.52	5.6
569	1.9	transthyretin	P02766	64	10 (5)	13.7	28	5.35	5.4
		mannose-binding protein C	P11226	31	7 (5)	24.0		5.40	
		serum amyloid P component	P02743	24	5 (5)	23.2		6.12	
		complement H-related protein 2	P36980	34	7 (7)	28.7		5.80	
		fibrinogen, γ chain	P02679	17	6 (5)	48.5		5.24	
		apolipoprotein E	P02649	24	5 (5)	34.2		5.52	

Shading indicates the likely major component protein within each spot based upon high sequence coverage and MW similarities.

[a] Proteins are listed within each gel spot number in descending order of Mascot match score (database search result data are provided in Appendix B.3).

[b] Sequence coverage was calculated as part of the Mascot search process using the entire chain of the protein's Swiss-Prot database entry.

[c] MW and pI were calculated on Swiss-Prot entries from the main chain or appropriate chain only using the Compute MW/pI tool (http://www.expasy.org/tools/pi_tool.html).

[d] Complement C3 is a multi-chain protein derived from a single polypeptide precursor; sequence coverage, MW, and pI were calculated from the C3 β chain only.

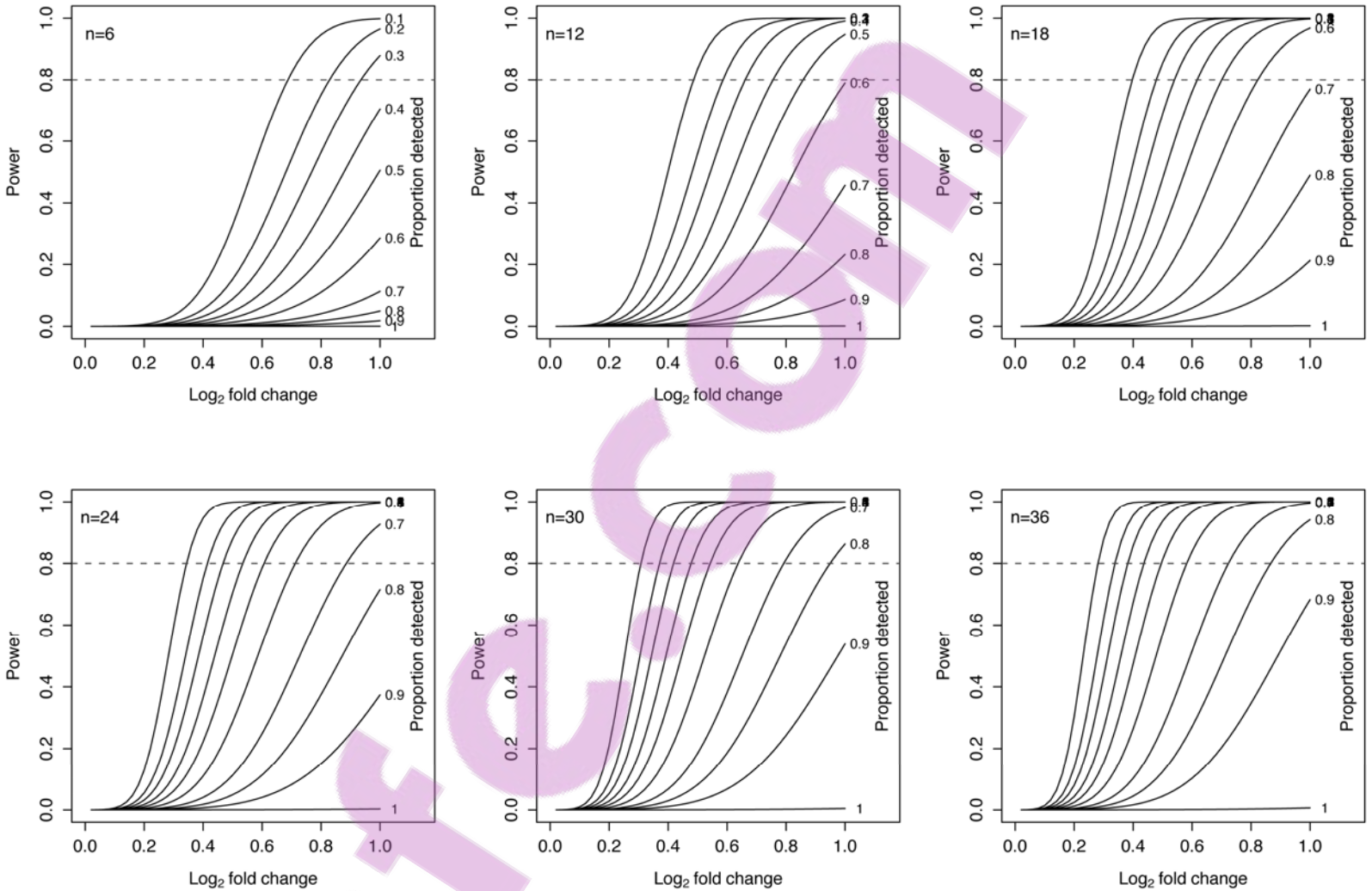

Figure 6-5: Power curves calculated from DIGE data

Series of power curves calculated for different numbers of samples used in a DIGE experiment based on the data presented in this chapter. Graphs correspond to (left to right, top to bottom) 6, 12, 18, 24, 30, and 36 total samples. X-axes show effect size as the detectable difference in $\log_2$(fold change); 1.0 indicates a two-fold difference. Y-axes show power, or the percentage of detectable changes. Curve series within each graph differ by percentages of spots detectable with the given power and effect size.

6.4 Discussion

This chapter describes a novel comparison using DIGE of plasma from preeclamptic women versus healthy pregnant women. This study is the first reported use of the DIGE method in any preeclampsia-related research project. As in the previous serum 2DE study, a supervised multivariate classification method was applied here to identify a set of DIGE gel spots that correctly classified samples into preeclamptic or healthy pregnant groups. The study identified a set of six proteins from four spots that were upregulated in the plasma of women with preeclampsia compared to healthy pregnant women. These proteins included fibrinogen β, ApoE, and a combination of transthyretin, mannose-binding protein C, serum amyloid P, complement H-related protein 2, and fibrinogen γ. The functions of these proteins are closely related to those identified from the serum 2DE study described in Chapter 5. Dysregulation of ApoE was identified in the previous study, along with RBP (a cofactor for transthyretin) and complement C3 (a key player in the complement system, as is mannose-binding protein C).

The statistical analysis techniques used in this study incorporated both nonparametric and parametric univariate testing methods. These methods identified different subsets of protein spots. The Mann-Whitney *U* test found significant differences between populations for spots that were present in many gels, but had small fold changes of generally less than 1.5-fold. In contrast, the limma *t* test found most spots as significant when they had higher fold changes of generally more than 1.3-fold, but may not have been present in all gels within the experiment. The limma *t* test differs from the more standard Student's *t* test by utilizing an estimate of the expected variance for an individual spot based on all spots in the experiment. This test has been previously used in microarray analysis (Smyth 2004) and in a previous DIGE experiment (Fodor *et al.* 2005). Interestingly, the NSC classifier generation method applied here corresponded well with the limma *t* test, with the four spots identified by NSC found within the top six limma results.

The power of this DIGE study was high, with 80% power to detect 75% of two-fold differences between sample groups. This high power stems from the low technical variation of this study. DIGE analysis reduces variation between samples by including a pooled internal standard which allows spot volumes and abundance ratios to be determined more accurately than when the standard is absent (Alban *et al.* 2003). In this study, inter-gel variation was further reduced by performing second dimension separation of all 12 gels within a single run in a single electrophoresis unit. The importance of minimizing this inter-gel variation was recently demonstrated by Eravci *et al.* (2007). In that study, the use of high-throughput conventional 2DE equipment capable of processing 24 gels

simultaneously resulted in an increased number of spots with significant differential regulation when compared to the low-throughput equipment used in the serum 2DE study described in Chapter 5.

In this study, multiple proteins were identified within three of the four gel spots comprising the NSC classifier. Issues around identification of multiple proteins within a single spot, and potential validation techniques to verify the relative abundance of each protein, have been discussed at length in Section 3.4.7 (page 74). In two of these three spots (spots 355 and 356), the top protein match from the protein identification database search process was clearly the predominant protein present in the spot. This confidence in identification stems from the high percentage of the protein's sequence with peptides successfully detected and matched (high sequence coverage percentage) and from concordance between the predicted protein MW and that observed on the 2-D gel image. Spots 355 and 356, both corresponding to fibrinogen β chain isoforms, had apparent MWs which matched the calculated mass of this protein. The third spot (spot 569) was primarily identified as transthyretin. However, the MW of transthyretin does not correspond to the MW of spot 569 on the DIGE gel. The other components of this spot, mannose-binding protein C, serum amyloid P, complement H-related protein 2, fibrinogen γ, and ApoE, could also contribute to the differential expression observed in this spot.

6.4.1 Apolipoprotein E

An isoform of ApoE was upregulated in plasma from women with preeclampsia. ApoE, a protein intimately involved in lipid transport and clearance from circulation, was also identified in the serum 2DE study described in Chapter 5. In preeclamptic serum (Chapter 5), two differentially glycosylated isoforms of ApoE were identified by 2DE and validated using 2-D western blot analysis. Visual comparison of the pIs of the serum ApoE isoforms and the isoform identified here in preeclamptic plasma suggests that the plasma ApoE isoform is similar to the more basic serum isoform ($ApoE_B$), proposed to be a deglycosylated circulating form of the protein. Changes in total ApoE levels have not been reproducibly associated with preeclampsia. The upregulation of this deglycosylated ApoE isoform in the studies described here, along with the upregulation of other deglycosylated proteins and of proteins involved in the transport of retinol (which may be required for glycosylation processes to occur) observed in the serum 2DE study, further suggests that glycosylation mechanisms could be dysregulated in women with preeclampsia.

6.4.2 Fibrinogen

Two isoforms of the β chain of fibrinogen were identified as upregulated in the plasma of preeclamptic women. Fibrinogen is a hexameric glycoprotein comprised of two trimers, each of which contains three chains: α, β, and γ. This protein plays a vital role in the biology of coagulation and in maintaining pregnancy (Iwaki and Castellino 2005). Fibrinogen is the zymogen (inactive precursor) of fibrin, the fibrillar protein which polymerizes as part of clot formation during coagulation. The link between the endothelial activation and coagulopathy of preeclampsia is well established (Roberts *et al.* 1989; Hayman *et al.* 1999). Even though circulating levels of fibrinogen increase two-fold during the course of a healthy pregnancy (van Buul *et al.* 1995), they are significantly increased further in preeclampsia (Manten *et al.* 2003; Üstün *et al.* 2005; Williams *et al.* 2007). In addition, fibrinogen γ was recently identified in a 2DE study comparing plasma from women with HELLP syndrome and healthy pregnant women (Heitner *et al.* 2006).

The molecular differences between the fibrinogen β isoforms identified here could help identify their roles in preeclampsia. Fibrinogen β has an *N*-glycosylation site at Asp346, though the glycosylation states of the isoforms identified in this study are not known. Aberrant glycosylation variants of other proteins have been associated with colorectal cancer (Rodriguez-Pineiro *et al.* 2006) and a phosphorylated isoform of the fibrinogen α chain was found to be upregulated in the plasma of ovarian cancer patients (Ogata *et al.* 2006). Further investigation of the post-translational modifications resulting in the fibrinogen β isoforms of spots 355 and 356 could uncover a novel role for this protein in preeclampsia. Regardless of the post-translational modification state(s) of this protein, its strong involvement in coagulation and hemostasis and its measured upregulation in preeclampsia in previous studies underline its importance to this disease state.

6.4.3 Proteins co-identified from spot 569

6.4.3.1 Transthyretin

Transthyretin was a component of spot 569, whose expression was upregulated in plasma from women with preeclampsia. Transthyretin functions as a transport protein for the thyroid hormone thyroxine and also forms a complex with RBP to transport retinol (vitamin A). RBP was upregulated in the serum 2DE study described in Chapter 5, and its relationship to preeclampsia was discussed in Section 5.4.5 (page 121). In brief, circulating levels of RBP have not been previously measured in women with preeclampsia, but there is some evidence to suggest that plasma levels of retinol are decreased in this disorder. Increased concentrations of RBP, the transport protein for retinol, could

explain this decrease. The upregulation of transthyretin observed here further implicates these proteins in the biology of preeclampsia. Transthyretin was recently identified from plasma from HELLP patients using 2DE, though its specific relationship to HELLP and preeclampsia was not discussed (Heitner *et al.* 2006).

Transthyretin circulates both as a homotetramer and as a spectrum of monomeric forms differing in their oxidation states (Pettersson *et al.* 1987). Interestingly, increased amounts of oxidized monomeric forms of transthyretin were recently found in the amniotic fluid of second trimester pregnant women who later developed preeclampsia (Vascotto *et al.* 2007). The MW of the transthyretin form found in spot 569 in the current study is not consistent with the transthyretin forms identified by Vascotto *et al.* but the similar upregulation of the protein may indicate the utility of this protein as a preeclampsia biomarker. The involvement of the retinol transport system in preeclampsia has not been well studied, and is an area which should be explored further.

6.4.3.2 Mannose-binding protein C

Mannose-binding protein C (MBP), also named mannose- or mannan-binding lectin, was present in an upregulated spot identified from the plasma of preeclamptic women. MBP is an oligomeric O-linked glycoprotein involved in activation of the innate immune response through the complement system (Kilpatrick 2003). MBP functions in a complementary manner with the classical and alternative complement pathways. This protein recognizes carbohydrate moieties on the surface of microorganisms and in turn associates with specific serine proteases which activate components of the complement system, possibly including complement C3 (Walport 2001). In addition, MBP can also recognize altered host material such as apoptotic cells (Presanis *et al.* 2003; Kravitz *et al.* 2005). The MBP gene has several variant alleles (discussed below) which may be associated with alterations in the circulating levels of the MBP protein.

Given the central role of the immune system in pregnancy maintenance, MBP levels have been measured throughout pregnancy in several longitudinal studies. These studies show that MBP levels increase with advancing gestation (Kilpatrick 2000; van de Geijn *et al.* 2007a). Few studies have examined MBP levels in women with preeclampsia. One early study measured MBP levels in serum from women with preeclampsia, but did not compare them to healthy pregnant women; this study found a relative decrease in MBP concentrations in women with recurrent preeclampsia compared to primiparous preeclampsia (Kilpatrick 1996). A study of decidual tissue from women with preeclampsia found that phagocytotic macrophages expressing a cell surface receptor for mannose were nearly nonexistent, indicating that the migration of these macrophages may be compromised in

this disorder (Burk *et al.* 2001). A lack of mannose-binding receptors competing for circulating mannose-containing species could result in increased MBP concentrations as observed in the present study.

Two recent studies examining genetic polymorphisms of the MBP gene in women with preeclampsia have found conflicting results showing that the roles of these genotypes are unclear. One study found that a variant leading to lower circulating MBP levels was more prevalent in women who did not develop preeclampsia as compared to healthy pregnant women (Sziller *et al.* 2007). Another study found that the same variant, leading to lower levels of MBP, was associated with preeclampsia (van de Geijn *et al.* 2007b). Neither study related their genotyped variants to measured concentrations of MBP in the women studied. In theory, increased levels of MBP in preeclamptic women could lead to increased activation of the complement system and higher levels of maternal response to trophoblast invasion of the uterine spiral arteries. This theory proposes that increased maternal damage to trophoblasts mediated by the immune response could lead to placental hypoxia and the subsequent symptoms of preeclampsia (Sziller *et al.* 2007). The upregulation of an MBP isoform observed in the current study supports this recent theory and suggests that lower relative levels of MBP could protect against preeclampsia, as was purported in the first of the two recent studies.

6.4.3.3 Serum amyloid P component

Serum amyloid P component (SAP) was a component of spot 569, upregulated in plasma from women with preeclampsia in comparison with healthy pregnant women. SAP, a glycoprotein, is a member of the pentraxin protein family and is involved in innate immunity as well as acute-phase responses to inflammation. SAP is able to bind complement factor C1q and activate the classical complement pathway (Biro *et al.* 2007). This protein has not been extensively studied in human pregnancy or preeclampsia. Tissue-based studies have shown that SAP abundance in the placenta increases with gestation (Khan and Walker 1985). Recently, one study measured plasma levels of cell-derived microparticles (released from activated or apoptotic cells) which were bound to SAP using flow cytometry. Levels of these SAP-bound microparticles were compared using plasma from nonpregnant, healthy pregnant (31 weeks of gestation), and preeclamptic women (29 weeks of gestation). SAP-bound microparticles were present in greater numbers in the plasma of healthy pregnant women versus nonpregnant women, but neither group had a significantly different number of microparticles than preeclamptic women (Biro *et al.* 2007). At this time, further studies are required to clarify the involvement of SAP in pregnancy and/or preeclampsia. The upregulation of SAP observed in this study could reflect the maternal inflammatory response to the later clinical symptoms of preeclampsia. SAP may also be involved in activation of the maternal complement

system, observed through the similar identification of complement-related proteins MBP and C3c in this and the previous study.

6.4.3.4 Complement H-related protein 2

Complement factor H-related protein 2 (CHRP2) was a component of spot 569, upregulated in the preeclamptic plasma samples used in the current study. CHRP2 is structurally and antigenically related to complement factor H, a protein with key involvement in host protection from the immune effects of the complement pathway (Zipfel and Skerka 1994; Kirkitadze and Barlow 2001). Factor H binds to activated complement C3b and inactivates it to form iC3b (*cf.* Figure 5-4 and Section 5.4.3; Kirkitadze and Barlow 2001). Though the precise function of CHRP2 is unknown, its clear similarity to complement factor H implicates this molecule in the complement system. The involvement of the complement system in the pathophysiology of preeclampsia has been discussed in Section 5.4.3 (page 119) and earlier in this chapter (Section 6.4.3.2). It is likely that the complement system becomes activated as part of the vascular inflammatory processes occurring in this disorder (Haeger 1993; Redman *et al.* 1999). The consequent upregulation of a protein related to complement factor H, and of other complement-related proteins identified in this and the previous study, is consistent with complement activation in preeclampsia.

6.4.4 Conclusions

This novel study identified six proteins of interest in the plasma of preeclamptic women using DIGE, a method which was more powerful than that used in the serum 2DE study in Chapter 5. The six proteins found in the current study extend the serum 2DE results by confirming the involvement of ApoE, the retinol transport system (transthyretin), and the complement system (MBP, SAP, CHRP2) in the pathophysiology of and maternal response to preeclampsia. In addition, fibrinogen was identified in this study, which has strong linkages to both the coagulopathy of preeclampsia and the maintenance of hemostasis essential in the disorder. Again, as with the serum 2DE study, the post-translational modifications of these six proteins may modulate their involvement in the disease process in preeclampsia. The most striking result in this chapter is the confirmation of deglycosylated ApoE as a protein of interest in preeclampsia, something which has not been found in any published result.

CHAPTER 7 SIGNIFICANCE AND FUTURE DIRECTIONS

The aim of this thesis was to develop and apply proteomic methods to discover biomarker proteins related to the pregnancy disorder preeclampsia. Several proteins with novel linkages to this disorder were identified during studies of serum and plasma from women with preeclampsia in late pregnancy. In addition, a unique serum-free culture system was devised for BeWo choriocarcinoma cells, and the effects of forskolin on these cells were examined using secreted proteomics. The impact of these results and ideas for further studies are discussed below.

The proteins identified from proteomic analyses of BeWo conditioned medium, serum, and plasma fall into six broad functional categories: lipid transport, the complement system, retinol transport, cell adhesion and invasion, coagulopathy, and copper transport. The existing linkages between these proteins and the pathophysiology of preeclampsia have been discussed within previous chapters. The identification of more than one protein from most of these functional categories strengthens their relationships to preeclampsia and offers reassurance that these proteins are potential biomarker candidates for this disorder.

The goal of the SCOPE project, of which this thesis research is a part, is the development of screening tests for preeclampsia and other major pregnancy complications (including SGA and spontaneous preterm birth). The serum/plasma work in this thesis used samples from late pregnancy, well after such a screening test would be applied. Similarly designed proteomic studies using early pregnancy plasma samples were conducted in parallel with this thesis research by other scientists in the SCOPE project. Notably, some of the proteins shown to be linked to preeclampsia in late pregnancy have also been identified as changing in early pregnancy (M Blumenstein, personal communication). Future work will extend these studies to a greater number of serum/plasma samples within the SCOPE project biobank, a large collection of early and late pregnancy samples from nulliparous women in Australasia, North America, and Europe.

7.1 Protein glycosylation

A particular strength of the 2DE technique employed throughout this thesis is its resolution of protein isoforms including those arising by post-translational modification. This led to one of the key results of this research: the detection of two differentially glycosylated isoforms of ApoE whose expression levels changed between women with preeclampsia and healthy pregnant women. Glycosylation is a key protein modification that can modulate protein function and physicochemical properties (Morelle *et al.* 2006). It has been estimated that over 50% of all proteins are glycosylated (Apweiler *et al.* 1999),

making this one of the most common post-translational modifications. It is therefore not surprising that so many proteins identified in this thesis research are glycoproteins.

The high frequency and importance of protein post-translational modifications have led to a targeted focus by the biofluid proteomics community on glycoproteomics, a specific subset of proteomic techniques applied to glycosylated proteins. Comparative studies of serum from patients with pancreatic cancer (Zhao *et al.* 2006) or prostate cancer (Kyselova *et al.* 2007) have identified glycosylation-specific protein changes associated with these diseases. Although proteomic analysis has become commonplace in biochemical laboratories, the application of glycoproteomics to the discovery of biomarkers is still in its infancy. Significant improvements in technology will certainly advance this field rapidly in the coming years.

The expression of differentially glycosylated isoforms of ApoE in preeclampsia was unexpected. The full significance of this result will not be known until studies are undertaken to uncover the role of glycosylation on ApoE function and its physiological role in preeclampsia. This examination of ApoE isoforms in preeclampsia is a completely novel and promising line of research. Confirmation of the post-translational modifications of ApoE in serum and plasma from preeclamptic women would require purification and analysis of the individual isoforms. A first step could involve immunoprecipitation of larger amounts of ApoE from serum/plasma. Separation of these ApoE proteins into differently charged isoforms could employ, for example, chromatofocusing LC techniques. The glycosylation moieties on these intact protein isoforms could be analyzed directly using FT-ICR MS approaches. Alternatively, tryptic digestion of purified ApoE isoforms would allow a peptide-based MS approach similar to that used to characterize haptoglobin glycosylation from the sera of prostate cancer patients (Fujimura *et al.* 2008). In-gel reductive β-elimination is another recently developed technique which may assist in removal of the *O*-linked carbohydrate groups from protein/peptide molecules before MS analysis (Taylor *et al.* 2006), thus providing aditional information about the carbohydrate structure of each ApoE isoform. The disadvantage of all of these approaches is the requirement for relatively pure preparations of separate ApoE isoforms, which may require specific chromatography methods to be developed.

MS quantification methods such as MRM may also be able to measure the abundance of glycosylated peptides directly within serum/plasma samples. MRM requires only a knowledge of the expected mass of the peptide of interest and of one or more of its MS/MS fragments. This technique has enormous potential to revolutionize biomarker measurement techniques. However, the ApoE peptide containing the protein's glycosylation site could ionize poorly in the mass spectrometer, making absolute concentration measurement exceedingly difficult. Further trials confirming the

behaviour of glycosylated ApoE peptides in an MRM-capable mass spectrometer are necessary before this method can be pursued as a analytical technique.

7.2 BeWo differentiation

The secreted proteome of BeWo cells treated with forskolin was examined in this study, and this is the first such research involving any placental cell type. This research is also the first to develop serum-free culture of BeWo cells under conditions compatible with proteomic analysis. The protein synthesis inhibitor brefeldin A was used in BeWo cultures as a control for intracellular proteins contaminating the secreted proteome of these cells. Towards the end of the culture timeline, BFA appeared to decrease cell viability. Alternative controls could increase the usefulness of the serum free culture method. These controls might include comparison of secreted proteins with those found in the cell lysates, which would more stringently separate extracellular from intracellular proteins.

The BeWo model of placental differentiation developed in this research is useful for the analysis of cellular responses during syncytialization. The proteins identified in the BeWo secreted proteomic study showed linkages to the membrane fusion events and differentiation processes occurring during FSK treatment of these cells. However, BeWo cells are a carcinoma-derived model which can represent only aspects of the *in vivo* CTB-STB differentiation process. As previously discussed in Chapter 3, *in vitro* studies of the secreted proteome from isolated CTB or explant cultures are a natural extension of this research. Such studies may provide additional information about the group of secreted proteins identified here from BeWo cells. Additional permutations of the BeWo culture system could also be useful for future comparative secreted proteomic studies. These permutations could involve, for example, specific inhibitors of glycosylation, agonists of protein secretion processes, or alternative differentiation agents.

7.3 Implications for other biomarker discovery efforts

Several of the proteins identified in this thesis are highly abundant serum/plasma proteins. For example, fibrinogen, found in the DIGE study described in Chapter 6, is abundant in plasma and has been targeted in newer versions of the MARS depletion system. Fibrinogen and similar abundant protein species have been assumed to play only minor roles as biomarkers due to their high circulating concentrations. However, post-translational modification changes in even abundant proteins are now recognized as potential disease biomarkers. Circulating levels of specific glycoforms of haptoglobin (a depletion target of the MARS system used in this thesis) have been recently correlated with hepatocellular carcinoma (Ang *et al.* 2006) and lung cancers (Heo *et al.* 2007;

Hoagland *et al.* 2007). Specific carbohydrate chains have been identified on haptoglobin isoforms from the sera of men with prostate cancer compared to healthy men (Fujimura *et al.* 2008). Acute-phase proteins and IgG are differentially glycosylated in ovarian cancer serum (Saldova *et al.* 2007). These examples illustrate the importance of studying changes in abundant or "classical" plasma proteins when seeking specific biomarkers. Sample fractionation techniques such as lectin chromatography produce different protein subsets which may help identify the roles of abundant proteins and their post-translational modifications as disease biomarkers.

The 2DE approach employed throughout this thesis is one of the oldest proteomic techniques and has a proven robustness for varied quantitative analyses. Nevertheless, comparison of the serum 2DE and plasma DIGE studies performed here illustrates the need for controlled experimental conditions (such as multiplexed electrophoresis of multiple samples within the same gel) to reduce variability and increase statistical power as much as possible. Newer electrophoresis equipment such as the twelve-gel cell used in the DIGE study can separate many more samples in one run than ever performed previously, further reducing the variability of this analysis. This technology has limits, however, as separation of more than 24 gels in an experiment has proven unfeasible (M Blumenstein, personal communication). MS-based proteomic approaches do not suffer from the same type of technical limitation and could theoretically compare hundreds of samples within an experiment, though at a substantial cost. Future proteomic studies illustrating quantitative changes between sample groups will probably use a MS-based rather than gel-based approach for at least a portion of the experimental work.

The identification of multiple proteins from within the majority of 2-D gel spots analyzed in this thesis underscores the importance of the spot co-migration phenomenon. In this phenomenon, isoforms from multiple protein species can be detected by MS within what appear to be single spots. This issue results from the sensitivity of the MS methods and the large dynamic range of protein concentrations found in serum/plasma (Campostrini *et al.* 2005; Hunsucker and Duncan 2006). Despite careful manual filtering, the predominant protein components of some of the co-migrating spots in this thesis were not able to be determined. Label-free MS quantitation techniques could be applied to future identification of 2DE spots to identify the major components of these protein mixtures.

Another theme recurrent throughout this thesis has been the need to filter the vast datasets gathered from each proteomic experiment. Simplification of these datasets through logical filtering of data is analogous to reducing the complexity of the serum/plasma proteome through immunodepletion. In this research, the data filtration process involved biostatistical analysis using univariate and

multivariate methods followed by removal of artifacts by assessment of each spot's appearance on 2-D gels. The variety of multivariate statistical approaches applied here, especially in the serum 2DE study described in Chapter 5, showed the power of these methods to repeatedly identify protein spots as classifiers. These methods have not been previously applied to proteomic analysis, and their use will likely become more prevalent.

High-abundance protein isoforms, including the glycoforms of haptoglobin described above, have been identified as biomarkers for diverse diseases. This raises the concern that these abundant protein isoforms may not distinguish people with disease from healthy individuals within a general population. Any biomarker (or combination of markers) derived from a specific population should be first validated in that population (*e.g.*, women at high risk of developing preeclampsia) before being extended to a more general group (*e.g.*, all first-time pregnant women). Consequently, a future direction from this thesis research is the validation of the proteins found here in a separate, larger group of serum/plasma samples from pregnant women.

7.4 Biomarker validation context

This research addresses the initial portion of the process by which new biomarkers are discovered, validated, and transformed into clinical tests (*cf.* Figure 1-1, page 1). Upcoming steps involve the validation of protein differences within successively larger sample sets and a clear continuing association of these proteins with clinical states. The use of protein isoforms as biomarkers is an important concept resulting from this thesis. Consequently, validation of these isoforms should entail measurement of the specific protein forms observed, not merely quantification of total protein concentrations. Care must be taken to isolate these specific isoforms to facilitate antibody production for immunoassays. This could be a time-consuming and arduous task given the similarities of the ApoE glycosylation isoforms examined in this thesis. As discussed previously (Section 7.1), the MRM technique for MS-based multiplexed quantitation may offer a more straightforward path to biomarker validation which does not require the isolation of differently glycosylated protein species. Though the dynamic range of the MRM approach is limited when compared to the full range of plasma protein concentrations, this technique can reliably measure multiple plasma proteins present at the levels typically found in a 2DE experiment (Anderson and Hunter 2006). Further research will confirm whether MRM can measure the specific protein isoforms identified in this study.

Preeclampsia affects a substantial number of pregnant women who may have no idea early in pregnancy that they could be at risk of serious complications. The research carried out in this thesis is

a step towards a screening test which could inform pregnant women about their chances of being affected by preeclampsia.

Appendix A MATERIALS SOURCES

Any materials not listed in this table were sourced from BDH.

Item	*Source*
2-D Quant Kit	GE Healthcare #80–6483–56
acrylamide	GE Healthcare #US75820
Albumin and IgG Removal Kit	GE Healthcare #RPN6300
antibody, goat anti-human apolipoprotein E, polyclonal	Abcam #AB7620–100
antibody, rabbit anti-goat IgG, Qdot® 655-conjugated secondary	Invitrogen #Q11821MP
BCA protein assay	Pierce #23227
BeWo cells, obtained at passage 194	American Type Culture Collection #CCL–98
brefeldin A (BFA)	Sigma-Aldrich #15870
BSA (used for 2-D western blot analysis)	Sigma-Aldrich #A7030
BSA, fatty acid free (used for 2DE)	ICP Bio Ltd. #ABFF
BSA, IgG free (used for 2DE)	ICP Bio Ltd. #ABGF
BSA, reduced endotoxin (used for 2DE)	ICP Bio Ltd. #ABRE
Buffer A (proprietary phosphate buffer)	Agilent #5185–5987
Buffer B (proprietary urea buffer)	Agilent #5185–5988
C7BzO detergent	Sigma-Aldrich #C0856
CHAPS, BioChemica grade	AppliChem #A1099
Complete Mini EDTA-free protease inhibitor	Roche #1–836–170
Criterion 8–16% Tris-HCl midigels, IPG+1 comb	Bio-Rad #345–0105
DMEM/Ham's F-12 1:1 media with 15 mM HEPES	Invitrogen #12500–039
DTT, PlusOne grade	GE Healthcare #US15397
electrode wicks	Bio-Rad #165–4071
ExcelGel SDS buffer strips	GE Healthcare #17–1342–01
ExcelGel XL 12–14% gradient gels	GE Healthcare #17–1236–01
ferrous sulfate, $FeSO_4$	Sigma-Aldrich #F8633
fetal calf serum, New Zealand origin	Invitrogen #10091–148, lots 280497, 546891
forskolin	Sigma-Aldrich #F6886
IAA, SigmaUltra grade	Sigma-Aldrich #I1149
Immobilon-FL low fluorescence PVDF blotting membrane	Millipore #IPFL20200
insulin, human recombinant	Roche #1376497
IPG Buffer, pH 3–10 NL	GE Healthcare #17–6000–88
IPG Buffer, pH 3–11 NL	GE Healthcare #17–6004–40
IPG Buffer, pH 4–7	GE Healthcare #17–6000–86
IPG strips, 7 cm, pH 3–10 NL	GE Healthcare #17–6001–12

Item	*Source*
IPG strips, 11 cm, pH 3–11 NL	GE Healthcare #17–6003–74
IPG strips, 11 cm, pH 4–7	GE Healthcare #18–1016–60
IPG strips, 18 cm, pH 4–7	GE Healthcare #17–1233–01
LINCOplex kit, human apolipoproteins C-II and E	Millipore #APO–62K
MagicMark™ XP western blot protein standard	Invitrogen #LC5602
MARS Buffer A	Agilent #5185-5987
MARS Buffer B	Agilent #5185-5988
MARS column, 4.6×50 mm	Agilent #5185–5984
MARS column, high capacity, 4.6×100 mm	Agilent #5188–5333
MEM vitamin solution	Invitrogen #11120–052
Novex® Tris-glycine SDS running buffer	Invitrogen #LC2675–5
NuPAGE® 4–12% Bis-Tris minigels, 2D well	Invitrogen #NP0330
NuPAGE® MES running buffer	Invitrogen #NP0002
NuPAGE® transfer buffer	Invitrogen #NP0006
OneTouch spot picker, 1.5 mm	The Gel Company #P2D1.5
OneTouch spot picker, 3.0 mm	The Gel Company #P2D3.0
SeeBlue® Plus2 pre-stained protein standard	Invitrogen #LC5925
spin filters, 0.2 µm	Agilent #5185–5990
SYPRO® Ruby stain	Invitrogen #S12000
thiourea, ACS grade	Sigma-Aldrich #T8656
transferrin, human	Sigma-Aldrich #T1147
Tris, PlusOne grade	GE Healthcare #17–1321–01
TrypLE™ Express, without phenol red	Invitrogen #12604–021
Trypsin Gold, MS grade	Promega #V5280
urea, PlusOne grade	GE Healthcare #US75826
Vivaspin 4 concentrators, PES membrane, 5 kDa MWCO	Vivascience #VS0413
Vivaspin 20 concentrators, PES membrane, 5 kDa MWCO	Vivascience #VS2011

Appendix B MASS SPECTROMETRY DATABASE SEARCH RESULTS

Details of the extraction of MS/MS data from raw mass spectra, and of database searches using the Mascot search engine, are provided in the methods of Chapters 3, 5, and 6. Result data from Mascot searches of the Swiss-Prot database are provided in sections B.1, B.2, and B.3, respectively. These results include the following information from Swiss-Prot (Apweiler *et al.* 2004):

- The protein's *accession number* and *name*.
- The *mass of the protein precursor*, in Daltons. In most cases the native protein's mass will be smaller due to cleavage of signal peptides or encoding of multiple proteins by a single chain. Mass may be increased by addition of post-translational modifications (*e.g.*, phosphorylation, glycosylation, acetylation).
- The *protein score*, also called its Mascot score, reflects the ion scores of its matched peptides (not counting duplicate matches where a peptide is observed more than once). Larger protein scores indicate proteins with a larger number of peptide matches. This is accepted as a standard measure of the accuracy of a protein match for all but very large datasets (*e.g.*, from MudPIT experiments).
- The *observed mass of the peptide*, in Daltons, is derived from the MS dataset. This mass is calculated from the peptide's m/z (mass to charge) value directly measured within the mass spectrometer and from its *charge* (Z).
- The *error*, in Daltons, between the observed peptide mass (above) and the calculated mass of the matched peptide in the database.
- The *sequence* of the matched peptide, including bracketing residues delineated by periods, or a dash indicating the protein terminus.
- The presence of any variable *modifications* to the peptide. For all database searches included in this thesis, variable oxidation of methionine residues was used as a search term.
- The number of *missed enzyme cleavage sites* present in the matched peptide. Data in this thesis were searched using the "semitrypsin" enzyme (permitting one end of the peptide to omit a tryptic cleavage site) with one additional missed cleavage site allowed within the peptide sequence.

- The *ion score for each peptide*, a similar measure to the protein score described above. This score is a measure of the probability that the database match between the observed experimental data and the database sequence is random. Higher ion scores indicate increasingly strong matches between peptide MS/MS sequence data and database sequences, and can indicate good-quality MS/MS spectra. More information on this score and its use in result interpretation is found at the Mascot website, http://www.matrixscience.com/help/interpretation_help.html).

- The *expectation value* for the peptide match. This is an alternative measure of the probability referred to in the peptide ion score description above. This value, like a *p*-value, reflects the frequency of obtaining an equal or higher-scoring peptide match by chance alone. Lower peptide expectation values indicate more significant results.

B.1 Results from BeWo secreted proteomics study (Chapter 3)

All results presented below are from searches of human sequences from within the Swiss-Prot database. Additional searches of mammalian sequences were undertaken to confirm the species origin of each significant protein match.

Table B-1: Database search results for BeWo 2DE spot 638

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
Q14126 Desmoglein-2 precursor (HDGC) - Homo sapiens (Human)	123232	258	862.4291	2	0.0106	K.LPDFESR.Y		0	32.42	0.43
			1055.6468	2	0.0089	K.YKPTPIPIK.V		0	20.09	2.9
			1068.534	2	0.014	K.IHSDLAEER.G		0	38.71	0.08
			1096.6532	2	0.0139	R.AWITAPVALR.E		0	26.24	0.7
			1161.61	2	0.007	K.IVAISEDYPR.K		0	57.93	0.00096
			1218.6696	2	0.0088	K.NLDFSVIVANK.A		0	66.89	0.0001
			1267.7107	2	0.0223	R.GNNVEKPLELR.I		0	41.58	0.026
			1564.8518	2	0.0228	K.GITEPPFGIFVFNK.D		0	35.04	0.11
			1569.7732	2	0.021	K.DTGEIYTTSVTLDR.E		0	75.59	1.30E-05
			1580.8466	2	0.0056	R.ILDVNDNIPVVENK.V		0	44.47	0.013
			1591.7919	2	0.019	R.DNWISVDSVTSEIK.L		0	32.39	0.27
			1848.9726	2	-0.03	R.IVSLEPAYPPVFYLNK.D		0	20.75	2.7
			1849.0279	3	0.0254	R.IVSLEPAYPPVFYLNK.D		0	12.02	13
			1959.9706	2	0.0135	K.VLEGMVEENQVNVEVTR.I	Oxidation (M)	0	83.62	1.60E-06
			1959.9781	3	0.0209	K.VLEGMVEENQVNVEVTR.I	Oxidation (M)	0	47.91	0.0059
P33151 Cadherin-5 precursor (Vascular endothelial-cadherin) (VE-cadherin) (7B4 antigen) (CD144 antigen) - Homo sapiens (Human)	87888	234	977.5227	2	0.0046	R.YEIVVEAR.D		0	40.57	0.057
			1170.5372	2	0.0067	K.EYFAIDNSGR.I		0	34.42	0.19

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
			1225.6069	2	0.009	K.YTFVVPEDTR.V		0	38.39	0.079
			1341.7229	2	0.0089	F.IVEATDPTIDLR.Y		0	11.7	31
			1420.6969	2	0.0135	R.VDAETGDVFAIER.L		0	86.92	1.10E-06
			1575.8219	2	0.0075	Y.SFIVEATDPTIDLR.Y		0	12.25	26
			1637.7983	2	0.0198	K.DTGENLETPSSFTIK.V		0	52.25	0.0026
			1682.9181	3	0.0156	K.KPLIGTVLAMDPDAAR.H	Oxidation (M)	0	73.97	1.20E-05
			1682.9248	2	0.0222	K.KPLIGTVLAMDPDAAR.H	Oxidation (M)	0	12.73	15
			1782.9207	3	0.012	K.VHFLPVVISDNGMPSR.T	Oxidation (M)	0	72.59	2.00E-05
P55058 Phospholipid transfer protein precursor (Lipid transfer protein II) - Homo sapiens (Human)	54989	188	744.4232	2	0.0102	R.TQLDLR.R		0	25.32	2.7
			774.435	2	0.0115	R.TGLELSR.D		0	41.34	0.063
			1036.5461	2	0.012	R.GAFFPLTER.N		0	46.44	0.013
			1081.5137	2	0.0122	R.MHAAFGGTFK.K	Oxidation (M)	0	32.21	0.34
			1196.7393	2	0.0264	R.AGALQLLLVGDK.V		0	54.21	0.00065
			1223.6292	2	-0.004	K.VPHDLDMLLR.A	Oxidation (M)	0	25.95	1.4
			1326.6518	2	0.0103	R.AVEPQLQEEER.M		0	54.16	0.0021
			1582.7191	2	0.0258	K.DPVASTSNLDMDFR.G	Oxidation (M)	0	44.64	0.013
			1651.7975	2	0.0059	K.VYDFLSTFITSGMR.F	Oxidation (M)	0	43.81	0.018
			1651.8186	3	0.027	K.VYDFLSTFITSGMR.F	Oxidation (M)	0	36.94	0.09
			1679.8342	3	0.0297	K.TMLQIGVMPMLNER.T	3 Oxidation (M)	0	35.75	0.12
			1679.8361	2	0.0317	K.TMLQIGVMPMLNER.T	3 Oxidation (M)	0	12.22	26
			1815.9739	3	0.0121	R.FLEQELETITIPDLR.G		0	41.31	0.022
			1815.9889	2	0.0271	R.FLEQELETITIPDLR.G		0	63.37	0.00012

Table B-2: Database search results for BeWo 2DE spot 655

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
P33151 Cadherin-5 precursor (Vascular endothelial-cadherin) (VE-cadherin) (7B4 antigen) (CD144 antigen) - Homo sapiens (Human)	87888	178	977.5477	2	0.0296	R.YEIVVEAR.D		0	51.37	0.0041
			1170.5548	2	0.0243	K.EYFAIDNSGR.I		0	37.88	0.091
			1225.6232	2	0.0252	K.YTFVVPEDTR.V		0	34.34	0.2
			1420.7342	2	0.0507	R.VDAETGDVFAIER.L		0	89.33	5.80E-07
			1637.8095	2	0.0311	K.DTGENLETPSSFTIK.V		0	22.31	2.6
			1682.9465	3	0.044	K.KPLIGTVLAMDPDAAR.H	Oxidation (M)	0	61.34	0.00017
			1782.9579	3	0.0492	K.VHFLPVVISDNGMPSR.T	Oxidation (M)	0	45.9	0.0075
P55058 Phospholipid transfer protein precursor (Lipid transfer protein II) - Homo sapiens (Human)	54989	138	1036.5692	2	0.035	R.GAFFPLTER.N		0	35.63	0.14
			1196.7294	2	0.0165	R.AGALQLLLVGDK.V		0	56.88	0.00041
			1223.6777	2	0.0445	K.VPHDLDMLLR.A	Oxidation (M)	0	12.56	24
			1326.6673	2	0.0258	R.AVEPQLQEEER.M		0	61.02	0.00043
			1651.8477	2	0.0561	K.VYDFLSTFITSGMR.F	Oxidation (M)	0	26.57	0.94
			1679.8497	2	0.0452	K.TMLQIGVMPMLNER.T	3 Oxidation (M)	0	52.97	0.0022
			1816.0254	2	0.0635	R.FLEQELETITIPDLR.G		0	56.62	0.00038
Q14126 Desmoglein-2 precursor (HDGC) - Homo sapiens (Human)	123232	70	1161.6323	2	0.0293	K.IVAISEDYPR.K		0	69.76	5.80E-05
			1580.8833	2	0.0423	R.ILDVNDNIPVVENK.V		0	10.44	24
			1849.0467	2	0.0442	R.IVSLEPAYPPVFYLNK.D		0	9.33	19

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
P07602 Proactivator polypeptide precursor [Contains: Saposin A (Protein A); Saposin B-Val; Saposin B (Sphingolipid activator protein 1) (SAP-1) (Cerebroside sulfate activator) (CSAct) (Dispersin) (Sulfatide/GM1 activator); Saposin C…] – Homo sapiens (Human)	60347	69	1013.6004	2	0.0247	K.QEILAALEK.G		0	66.42	0.0001
			1275.6853	2	0.065	K.EMPMQTLVPAK.V	2 Oxidation (M)	0	22.19	3.2
			1562.0025	3	0.0582	K.NVIPALELVEPIKK.H		1	32.71	0.01

Table B-3: Database search results for BeWo 2DE spot 679

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
P11021 78 kDa glucose-regulated protein precursor (GRP 78) (Heat shock 70 kDa protein 5) (Immunoglobulin heavy chain-binding protein) (BiP) (Endoplasmic reticulum lumenal Ca(2+)-binding protein grp78) - Homo sapiens (Human)	72431	714	771.3887	2	0.0124	T.PEEIER.M		0	56.43	0.0018
			917.4886	2	0.018	K.VLEDSDLK.K		0	47.97	0.012
			918.4821	2	0.0187	R.VMEHFIK.L	Oxidation (M)	0	26.64	1.7
			980.5035	2	0.0221	K.ETAEAYLGK.K		0	40.21	0.06
			985.5352	2	0.0272	R.LTPEEIER.M		0	41.53	0.046
			1045.6023	2	0.0368	K.VLEDSDLKK.S		1	36.91	0.11
			1073.5629	2	0.0163	K.ITITNDQNR.L		0	34.79	0.22
			1073.5703	2	0.0238	K.ITITNDQNR.L		0	52.99	0.0033
			1091.5843	2	0.0232	V.YEGERPLTK.D		0	30.04	0.61
			1114.6318	2	0.0336	Y.IQVDIGGGQTK.T		0	39.8	0.052
			1190.6492	2	0.0197	K.VYEGERPLTK.D		0	21.66	3.6

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
			1190.6583	3	0.0287	K.VYEGERPLTK.D		0	10.9	40
			1227.6502	2	0.0295	R.VEIIANDQGNR.I		0	62.12	0.00034
			1232.647	2	0.0287	K.DAGTIAGLNVMR.I	Oxidation (M)	0	59.13	0.00072
			1255.6305	2	0.0187	K.MKETAEAYLGK.K	Oxidation (M)	1	48.82	0.0072
			1255.6405	3	0.0287	K.MKETAEAYLGK.K	Oxidation (M)	1	28.92	0.69
			1315.6627	2	0.0332	R.NELESYAYSLK.N		0	41.14	0.043
			1320.7273	2	0.0314	L.TLGIETVGGVMTK.L	Oxidation (M)	0	23.99	1.7
			1328.6401	2	0.033	K.FEELNMDLFR.S	Oxidation (M)	0	74.87	1.80E-05
			1396.8165	2	0.0352	K.ELEEIVQPIISK.L		0	42.28	0.013
			1429.7186	2	0.0348	R.TWNDPSVQQDIK.F		0	50.59	0.0045
			1459.7826	2	0.0307	K.SDIDEIVLVGGSTR.I		0	61.03	0.00035
			1527.7638	2	0.0246	R.AKFEELNMDLFR.S	Oxidation (M)	1	48.66	0.0064
			1527.7829	3	0.0438	R.AKFEELNMDLFR.S	Oxidation (M)	1	44.14	0.018
			1551.8121	2	0.0267	K.TFAPEEISAMVLTK.M	Oxidation (M)	0	55.82	0.0011
			1565.8075	2	0.035	R.ITPSYVAFTPEGER.L		0	56.54	0.00097
			1565.8208	3	0.0482	R.ITPSYVAFTPEGER.L		0	35.12	0.13
			1587.8859	3	0.0391	K.KSDIDEIVLVGGSTR.I		1	60.31	0.00027
			1603.8968	3	0.0398	K.TKPYIQVDIGGGQTK.T		0	25.46	0.76
			1603.8982	2	0.0412	K.TKPYIQVDIGGGQTK.T		0	13.38	12
			1658.9166	2	0.0287	R.IINEPTAAAIAYGLDK.R		0	67.68	5.00E-05
			1658.9293	3	0.0414	R.IINEPTAAAIAYGLDK.R		0	60.03	0.00026
			1676.8332	2	0.0327	K.NQLTSNPENTVFDAK.R		0	75.95	1.10E-05
			1815.0414	3	0.0524	R.IINEPTAAAIAYGLDKR.E		1	75.42	3.90E-06
			1835.9676	2	0.0411	K.SQIFSTASDNQPTVTIK.V		0	52.34	0.0019
			1835.9721	3	0.0455	K.SQIFSTASDNQPTVTIK.V		0	33.3	0.15
			1887.0198	3	0.0559	K.VTHAVVTVPAYFNDAQR.Q		0	51.97	0.0017
			1933.0518	3	0.046	K.DNHLLGTFDLTGIPPAPR.G		0	63.67	9.90E-05
			1973.9424	3	0.0418	K.IEWLESHQDADIEDFK.A		0	51.35	0.0027

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
P08253 72 kDa type IV collagenase precursor (EC 3.4.24.24) (72 kDa gelatinase) (Matrix metalloproteinase-2) (MMP-2) (Gelatinase A) (TBE-1) - Homo sapiens (Human)	75184	267	724.4674	2	0.0191	A.APSPIIK.F		0	27.01	0.54
			806.3825	2	0.0192	K.MDPGFPK.L	Oxidation (M)	0	22.12	4.6
			829.4507	2	0.0173	K.FPGDVAPK.T		0	46.6	0.017
			886.4777	2	0.0188	R.GEIFFFK.D		0	26.19	1.8
			913.4843	2	0.0298	K.TYIFAGDK.F		0	33.87	0.27
			968.5396	2	0.0276	K.FPFLFNGK.E		0	36.24	0.12
			1036.5281	2	0.0303	R.VDAAFNWSK.N		0	56.06	0.0015
			1373.749	2	0.0187	K.QDIVFDGIAQIR.G		0	62.75	0.00024
			1390.6967	2	0.0351	K.IDAVYEAPQEEK.A		0	39.52	0.061
			1391.8105	2	0.0332	K.PLTSLGLPPDVQR.V		0	72.51	1.30E-05
			1417.77	2	0.0345	R.AFQVWSDVTPLR.F		0	66.91	8.80E-05
			1602.7883	3	0.0422	R.IHDGEADIMINFGR.W	Oxidation (M)	0	61.92	0.00031
			1621.7039	2	0.036	R.FQGTSYDSCTTEGR.T		0	74.82	9.50E-06
			1837.0509	3	0.0411	R.GYPKPLTSLGLPPDVQR.V		0	26.69	0.29
P08238 Heat shock protein HSP 90-beta (HSP 84) (HSP 90) - Homo sapiens (Human) Note: these data matched equally well to a bovine protein, and were excluded from further analysis.	83638	219	1038.5156	2	0.0287	R.YESLTDPSK.L		0	50.01	0.0059
			1150.582	2	0.0314	K.YIDQEELNK.T		0	38.05	0.094
			1193.6709	2	0.0305	K.IDIIPNPQER.T		0	37.5	0.073
			1241.7212	2	0.0233	K.ADLINNLGTIAK.S		0	41.76	0.023
			1248.6365	2	0.0267	K.EQVANSAFVER.V		0	64.61	0.0002
			1274.6659	2	0.0305	R.ELISNASDALDK.I		0	66.86	0.00012
			1310.5982	2	0.0356	K.EDQTEYLEER.R		0	79.98	4.60E-06
			1364.7572	2	0.035	R.TLTLVDTGIGMTK.A	Oxidation (M)	0	42.14	0.023
			1512.809	2	0.0306	R.GVVDSEDLPLNISR.E		0	65.91	0.0001
			1807.9893	3	0.0384	K.HSQFIGYPITLYLEK.E		0	28.21	0.37

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
P13796 Plastin-2 (L-plastin) (Lymphocyte cytosolic protein 1) (LCP-1) (LC64P) - Homo sapiens (Human)	70955	216	993.5334	2	0.0203	K.IGNFSTDIK.D		0	50.4	0.0054
			997.5389	2	0.0269	R.ISFDEFIK.I		0	31.09	0.42
			1011.6368	2	0.0292	R.NEALIALLR.E		0	30.59	0.18
			1042.6032	2	0.0373	N.ILEEIGGGQK.V		0	53.59	0.0022
			1068.6428	2	0.0249	K.LSPEELLLR.W		0	55.76	0.00082
			1134.6306	2	0.0272	R.QFVTATDVVR.G		0	68.38	7.20E-05
			1153.6108	2	0.0188	K.YAFVNWINK.A		0	15.41	15
			1165.5678	2	0.0506	R.EGESLEDLMK.L	Oxidation (M)	0	46.1	0.014
			1404.8288	2	0.041	K.LNLAFIANLFNR.Y		0	21.51	1.3
			1517.7647	2	0.0251	K.MINLSVPDTIDER.T	Oxidation (M)	0	97.28	9.30E-08
			1518.7633	2	0.0567	V.MTVFACLMGKGMK.R	2 Oxidation (M)	1	4.23	1.90E+02
			1533.8386	2	0.0348	R.YTLNILEEIGGGQK.V		0	29.01	0.44
			1584.8807	2	0.0408	R.VYALPEDLVEVNPK.M		0	21.96	1.8
			1674.8763	2	0.0437	K.FSLVGIGGQDLNEGNR.T		0	59.66	0.00043
			1798.9659	2	0.0445	K.VNDDIIVNWVNETLR.E		0	19.62	3.2
P07900 Heat shock protein HSP 90-alpha (HSP 86) (Renal carcinoma antigen NY-REN-38) - Homo sapiens (Human) Note: these data matched equally well to a bovine protein, and were excluded from further analysis.	85104	188	1038.5156	2	0.0287	R.YESLTDPSK.L		0	50.01	0.0059
			1150.582	2	0.0314	K.YIDQEELNK.T		0	38.05	0.094
			1234.6325	2	0.0383	K.DQVANSAFVER.L		0	52.58	0.0032
			1241.7212	2	0.0233	K.ADLINNLGTIAK.S		0	41.76	0.023
			1290.6704	2	0.0401	R.ELISNSSDALDK.I		0	61.82	0.00037
			1310.5982	2	0.0356	K.EDQTEYLEER.R		0	79.98	4.60E-06
			1364.7572	2	0.035	R.TLTIVDTGIGMTK.A	Oxidation (M)	0	42.14	0.023
			1512.809	2	0.0306	R.GVVDSEDLPLNISR.E		0	65.91	0.0001
			1778.0129	3	0.0726	K.HSQFIGYPITLFVEK.E		0	1.01	1.30E+02

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
P13667 Protein disulfide-isomerase A4 precursor (EC 5.3.4.1) (Protein ERp-72) (ERp72) - Homo sapiens (Human)	73313	138	1010.5028	2	0.0319	K.QFAPEYEK.I		0	33.41	0.27
			1125.5933	2	0.0227	R.FDVSGYPTIK.I		0	48.82	0.0073
			1132.5913	2	0.0301	K.VDATAETDLAK.R		0	32.55	0.35
			1189.6581	2	0.0279	K.IDATSASVLASR.F		0	78.49	7.20E-06
			1306.5783	2	0.0324	K.MDATANDVPSDR.Y	Oxidation (M)	0	56.99	0.0009
			1457.7972	2	0.0424	K.VSQGQLVVMQPEK.F	Oxidation (M)	0	5.59	1.10E+02
			1626.8266	2	0.0336	K.VEGFPTIYFAPSGDK.K		0	15.75	12
			1701.7731	2	0.0539	K.FAMEPEEFDSDTLR.E	Oxidation (M)	0	31.84	0.23
			1765.8025	3	0.0414	R.SHMMDVQGSTQDSAIK.D	2 Oxidation (M)	0	31.35	0.25
P11142 Heat shock cognate 71 kDa protein (Heat shock 70 kDa protein 8) - Homo sapiens (Human)	71138	128	1227.6502	2	0.0295	K.VEIIANDQGNR.T		0	62.12	0.00034
			1252.6315	2	0.0228	R.FEELNADLFR.G		0	27.62	0.94
Note: these data matched equally well to a			1658.9166	2	0.0287	R.IINEPTAAAIAYGLDK.K		0	67.68	5.00E-05
bovine protein, and were excluded from			1658.9293	3	0.0414	R.IINEPTAAAIAYGLDK.K		0	60.03	0.00026
further analysis.			1664.8139	2	0.0311	K.NQVAMNPTNTVFDAK.R	Oxidation (M)	0	7.89	72
P04217 Alpha-1B-glycoprotein precursor (Alpha-1-B glycoprotein) - Homo sapiens (Human)	54949	108	869.5473	2	0.0251	K.LLELTGPK.S		0	47.33	0.006
			1087.6041	2	0.0267	R.ATWSGAVLAGR.D		0	45.29	0.017
			1236.6683	2	0.0293	R.LETPDFQLFK.N		0	36.46	0.11
			1263.6748	2	0.0289	R.SGLSTGWTQLSK.L		0	63.93	0.00021
			1371.7135	2	0.024	K.HQFLLTGDTQGR.Y		0	32.31	0.3
			1658.8684	2	0.0347	R.CEGPIPDVTFELLR.E		0	17.3	7.5
			1673.9263	3	0.0526	K.NGVAQEPVHLDSPAIK.H		0	38.51	0.038

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
P00450 Ceruloplasmin precursor (EC 1.16.3.1) (Ferroxidase) - Homo sapiens (Human)	123193	105	1189.5555	2	0.023	K.MYYSAVDPTK.D	Oxidation (M)	0	49.03	0.0073
			1202.554	2	0.0336	R.EYTDASFTNR.K		0	39.01	0.07
			1206.6669	2	0.035	K.DIFTGLIGPMK.I	Oxidation (M)	0	33.51	0.21
			1370.7882	2	0.0324	K.GAYPLSIEPIGVR.F		0	37.05	0.059
			1518.7633	2	0.0279	K.ALYLQYTDETFR.T		0	46.34	0.012
			1911.171	3	0.0476	R.TTIEKPVWLGFLGPIIK.A		0	6.43	6.2
P55058 Phospholipid transfer protein precursor (Lipid transfer protein II) - Homo sapiens (Human)	54989	97	1196.7505	2	0.0376	R.AGALQLLLVGDK.V		0	43.03	0.007
			1326.6871	2	0.0456	R.AVEPQLQEEER.M		0	63.6	0.00023
			1651.8199	2	0.0283	K.VYDFLSTFITSGMR.F	Oxidation (M)	0	43.25	0.021
			1816.0065	2	0.0447	R.FLEQELETITIPDLR.G		0	34.3	0.082
			1816.0135	3	0.0517	R.FLEQELETITIPDLR.G		0	20.62	1.7
P17066 Heat shock 70 kDa protein 6 (Heat shock 70 kDa protein B~) - Homo sapiens (Human)	71553	70	980.5035	2	0.0584	K.ETAEAYLGQ.P		0	40.21	0.06
			1227.6502	2	0.0295	R.VEILANDQGNR.T		0	62.12	0.00034
Note: these data matched equally well to a			1255.6305	2	0.0551	K.MKETAEAYLGQ.P	Oxidation (M)	1	35.91	0.14
bovine protein, and were excluded from			1255.6405	3	0.0651	K.MKETAEAYLGQ.P	Oxidation (M)	1	19.73	5.7
further analysis.			1328.6401	2	0.033	R.FEELCSDLFR.S		0	26.89	1.1

Table B-4: Database search results for BeWo 2DE spot 818

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
P68363 Tubulin alpha-1B chain (Tubulin alpha-ubiquitous chain) (Alpha-tubulin ubiquitous) (Tubulin K-alpha-1) - Homo sapiens (Human)	50972	93	1084.6522	2	0.0394	K.EIIDLVLDR.I		0	45.36	0.0097
			1486.9458	2	0.0739	R.LISQIVSSITASLR.F		0	65.18	1.10E-05
			1700.9595	2	0.061	R.AVFVDLEPTVIDEVR.T		0	39.68	0.022
			1717.9297	3	0.055	R.NLDIERPTYTNLNR.L		0	23.85	1.3
			1756.027	3	0.0711	R.IHFPLATYAPVISAEK.A		0	29.11	0.14
P07437 Tubulin beta chain (Tubulin beta-5 chain) - Homo sapiens (Human)	50207	84	1129.6342	2	0.0462	R.FPGQLNADLR.K		0	47.37	0.0096
			1158.6714	2	0.0495	K.LAVNMVPFPR.L	Oxidation (M)	0	36.27	0.099
Note: these data matched equally well to a bovine protein, and were excluded from further analysis.			1300.6773	2	0.0474	R.ISVYYNEATGGK.Y		0	40.43	0.049
			1334.7516	2	0.0612	R.IMNTFSVVPSPK.V	Oxidation (M)	0	29.33	0.41
			1630.8877	2	0.0641	R.AILVDLEPGTMDSVR.S	Oxidation (M)	0	28.97	0.41
			1958.0523	3	0.0777	K.GHYTEGAELVDSVLDVVR.K		0	47.1	0.0051
P60709 Actin, cytoplasmic 1 (Beta-actin) - Homo sapiens (Human)	42136	83	1013.5116	2	0.0377	R.DLTDYLMK.I	Oxidation (M)	0	24.28	2.4
			1131.5578	2	0.0381	R.GYSFTTTAER.E		0	38.25	0.095
Note: these data matched equally well to a bovine protein, and were excluded from further analysis.			1176.6501	2	0.0441	K.EITALAPSTMK.I	Oxidation (M)	0	44.38	0.018
			1789.9431	2	0.0585	K.SYELPDGQVITIGNER.F		0	39.9	0.035
			1953.1512	3	0.0941	R.VAPEEHPVLLTEAPLNPK.A		0	26.88	0.13
P68371 Tubulin beta-2C chain (Tubulin beta-2 chain) - Homo sapiens (Human)	50367	72	1129.6342	2	0.0462	R.FPGQLNADLR.K		0	47.37	0.0096
Note: these data matched equally well to a bovine protein, and were excluded from further analysis.			1158.6714	2	0.0495	K.LAVNMVPFPR.L	Oxidation (M)	0	36.27	0.099
			1334.7516	2	0.0612	R.IMNTFSVVPSPK.V	Oxidation (M)	0	29.33	0.41
			1616.8588	2	0.0508	R.AVLVDLEPGTMDSVR.S	Oxidation (M)	0	18.76	5.3

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
			1958.0523	3	0.0777	K.GHYTEGAELVDSVLDVVR.K		0	47.1	0.0051
P08238 Heat shock protein HSP 90-beta (HSP 84) (HSP 90) - Homo sapiens (Human)	83638	67	1159.6095	2	0.0335	K.SIYYITGESK.E		0	50.37	0.0057
			1248.6536	2	0.0437	K.EQVANSAFVER.V		0	50.34	0.0051
			1415.6744	2	0.044	K.EGLELPEDEEEK.K		0	23.75	2.2
			1512.826	2	0.0476	R.GVVDSEDLPLNISR.E		0	19.2	4.3
Q9NY65 Tubulin alpha-8 chain (Alpha-tubulin 8) - Homo sapiens (Human)	50914	64	1486.9458	2	0.0739	R.LISQIVSSITASLR.F		0	65.18	1.10E-05
Note: these data matched equally well to a			1700.9595	2	0.094	R.AVMIDLEPTVVDEVR.A	Oxidation (M)	0	11.24	16
bovine protein, and were excluded.			1717.9297	3	0.055	R.NLDIERPTYTNLNR.L		0	23.85	1.3
P07195 L-lactate dehydrogenase B chain (EC 1.1.1.27) (LDH-B) (LDH heart subunit) (LDH-H) (Renal carcinoma antigen NY-REN-46) - Homo sapiens (Human)	36970	57	958.5854	2	0.0407	R.GLTSVINQK.L		0	10.34	45
			1282.6975	2	0.0497	K.MVVESAYEVIK.L	Oxidation (M)	0	35.61	0.13
			1628.9199	2	0.069	K.SLADELALVDVLEDK.L		0	47.74	0.004
			1693.9665	2	0.0779	K.LIAPVAEEEATVPNNK.I		0	23.2	0.86

Table B-5: Database search results for BeWo 2DE spot 841

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
P07437 Tubulin beta chain (Tubulin beta-5 chain) - Homo sapiens (Human)	50207	229	1076.5571	2	0.032	K.IREEYPDR.I		1	40.79	0.054
			1129.6343	2	0.0463	R.FPGQLNADLR.K		0	51.53	0.0037
Note: these data matched equally well to a			1158.6624	2	0.0405	K.LAVNMVPFPR.L	Oxidation (M)	0	54.34	0.0017
bovine protein, and were excluded from			1291.6987	2	0.0563	R.LHFFMPGFAPL.T	Oxidation (M)	0	21.91	3.1

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
further analysis.			1334.7369	2	0.0465	R.IMNTFSVVPSPK.V	Oxidation (M)	0	77.62	7.00E-06
			1550.8431	2	0.0457	H.SLGGGTGSGMGTLLISK.I	Oxidation (M)	0	10.94	29
			1630.8716	2	0.0479	R.AILVDLEPGTMDSVR.S	Oxidation (M)	0	67.61	6.40E-05
			1635.8864	3	0.0632	R.LHFFMPGFAPLTSR.G	Oxidation (M)	0	22.57	1.8
			1658.9513	3	0.0633	R.ALTVPELTQQVFDAK.N		0	13.35	8.9
			1658.9517	2	0.0638	R.ALTVPELTQQVFDAK.N		0	25.46	0.53
			1958.0228	2	0.0483	K.GHYTEGAELVDSVLDVVR.K		0	15.97	8
			1958.0433	3	0.0687	K.GHYTEGAELVDSVLDVVR.K		0	81.88	1.80E-06
Q13509 Tubulin beta-3 chain (Tubulin beta-III) (Tubulin beta-4) - Homo sapiens (Human)	50969	216	1129.6343	2	0.0463	R.FPGQLNADLR.K		0	51.53	0.0037
			1158.6624	2	0.0405	K.LAVNMVPFPR.L	Oxidation (M)	0	54.34	0.0017
Note: these data matched equally well to a bovine protein, and were excluded from further analysis.			1291.6987	2	0.0563	R.LHFFMPGFAPL.T	Oxidation (M)	0	21.91	3.1
			1334.7369	2	0.0465	R.IMNTFSVVPSPK.V	Oxidation (M)	0	77.62	7.00E-06
			1550.8431	2	0.0457	H.SLGGGTGSGMGTLLISK.V	Oxidation (M)	0	10.94	29
			1630.8716	2	0.0479	R.AILVDLEPGTMDSVR.S	Oxidation (M)	0	67.61	6.40E-05
			1706.9095	2	0.0546	R.ALTVPELTQQMFDAK.N	Oxidation (M)	0	11.08	27
			1958.0228	2	0.0483	K.GHYTEGAELVDSVLDVVR.K		0	15.97	8
			1958.0433	3	0.0687	K.GHYTEGAELVDSVLDVVR.K		0	81.88	1.80E-06
P68371 Tubulin beta-2C chain (Tubulin beta-2 chain) - Homo sapiens (Human)	50367	209	1076.5571	2	0.032	K.IREEYPDR.I		1	40.79	0.054
			1129.6343	2	0.0463	R.FPGQLNADLR.K		0	51.53	0.0037
			1158.6624	2	0.0405	K.LAVNMVPFPR.L	Oxidation (M)	0	54.34	0.0017
Note: these data matched equally well to a bovine protein, and were excluded from further analysis.			1291.6987	2	0.0563	R.LHFFMPGFAPL.T	Oxidation (M)	0	21.91	3.1
			1334.7369	2	0.0465	R.IMNTFSVVPSPK.V	Oxidation (M)	0	77.62	7.00E-06
			1550.8431	2	0.0457	H.SLGGGTGSGMGTLLISK.I	Oxidation (M)	0	10.94	29
			1616.8623	2	0.0543	R.AVLVDLEPGTMDSVR.S	Oxidation (M)	0	58.79	0.00052
			1635.8864	3	0.0632	R.LHFFMPGFAPLTSR.G	Oxidation (M)	0	22.57	1.8

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
			1706.9095	2	0.0546	R.ALTVPELTQQMFDAK.N	Oxidation (M)	0	11.08	27
			1958.0228	2	0.0483	K.GHYTEGAELVDSVLDVVR.K		0	15.97	8
			1958.0433	3	0.0687	K.GHYTEGAELVDSVLDVVR.K		0	81.88	1.80E-06
P60709 Actin, cytoplasmic 1 (Beta-actin) - Homo sapiens (Human)	42136	170	794.4903	2	0.0253	K.IIAPPER.K		0	39.74	0.04
			1013.5121	2	0.0382	R.DLTDYLMK.I	Oxidation (M)	0	15.05	20
Note: these data matched equally well to a bovine protein, and were excluded from further analysis.			1131.5597	2	0.0401	R.GYSFTTTAER.E		0	42.9	0.033
			1176.6468	2	0.0408	K.EITALAPSTMK.I	Oxidation (M)	0	40.7	0.044
			1515.7478	3	0.0525	K.QEYDESGPSIVHR.K		0	96.84	1.00E-07
			1789.9363	2	0.0517	K.SYELPDGQVITIGNER.F		0	74.1	1.40E-05
			1953.1162	3	0.0591	R.VAPEEHPVLLTEAPLNPK.A		0	14.62	4
			1953.1215	2	0.0644	R.VAPEEHPVLLTEAPLNPK.A		0	15.58	2.9
Q99867 Tubulin beta-4q chain - Homo sapiens (Human)	49043	109	1076.5571	2	0.032	K.IREEYPDR.I		1	40.79	0.054
			1129.6343	2	0.0463	R.FPGQLNADLR.K		0	51.53	0.0037
Note: these data matched equally well to a bovine protein, and were excluded from further analysis.			1158.6624	2	0.0405	K.LAVNMVPFPR.L	Oxidation (M)	0	54.34	0.0017
			1291.6987	2	0.0563	R.LHFFMPGFAPL.T	Oxidation (M)	0	21.91	3.1
			1550.8431	2	0.0457	H.SLGGGTGSGMGTLLLSK.I	Oxidation (M)	0	10.94	29
			1616.8623	2	0.0543	R.AVLVDLEPGTMDSVR.S	Oxidation (M)	0	58.79	0.00052
P68363 Tubulin alpha-1B chain (Tubulin alpha-ubiquitous chain) (Alpha-tubulin ubiquitous) (Tubulin K-alpha-1) - Homo sapiens (Human)	50972	101	1045.6239	2	0.0318	L.LYRGDVVPK.D		1	12.51	23
			1084.6405	2	0.0277	K.EIIDLVLDR.I		0	42.98	0.019
			1409.8129	2	0.0462	R.QLFHPEQLITGK.E		0	35.88	0.062
			1486.9388	2	0.067	R.LISQIVSSITASLR.F		0	36.89	0.0096
			1700.9639	2	0.0654	R.AVFVDLEPTVIDEVR.T		0	65.14	6.00E-05

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
			1717.9171	3	0.0424	R.NLDIERPTYTNLNR.L		0	29.92	0.35
			1756.0401	3	0.0842	R.IHFPLATYAPVISAEK.A		0	22.96	0.47
P07195 L-lactate dehydrogenase B chain (EC 1.1.1.27) (LDH-B) (LDH heart subunit) (LDH-H) (Renal carcinoma antigen NY-REN-46) - Homo sapiens (Human)	36970	87	956.6398	2	0.0339	K.FIIPQIVK.Y		0	28.62	0.26
			958.5638	2	0.0191	R.GLTSVINQK.L		0	46.58	0.013
			1026.5898	2	0.0366	R.IHPVSTMVK.G	Oxidation (M)	0	23.11	2.2
			1157.6667	2	0.0376	K.LKDDEVAQLK.K		1	29.37	0.51
			1282.6913	2	0.0435	K.MVVESAYEVIK.L	Oxidation (M)	0	42.91	0.024
			1509.8148	3	0.0473	K.IVADKDYSVTANSK.I		1	26.2	0.91
			1628.8991	2	0.0482	K.SLADELALVDVLEDK.L		0	52.03	0.0019
			1693.9466	3	0.058	K.LIAPVAEEEATVPNNK.I		0	14.44	8.3
			1693.953	2	0.0644	K.LIAPVAEEEATVPNNK.I		0	29.36	0.25
P52907 F-actin capping protein subunit alpha-1 (CapZ alpha-1) - Homo sapiens (Human)	33115	71	1196.7351	2	0.0474	R.LLLNNDNLLR.E		0	67.87	2.90E-05
			1541.7298	2	0.0453	K.EASDPQPEEADGGLK.S		0	30.3	0.41
			1569.9802	2	0.0671	K.FTITPPTAQVVGVLK.I		0	21.34	0.33
			1704.8881	2	0.0714	K.DVQDSLTVSNEAQTAK.E		0	26.63	0.85

Table B-6: Database search results for BeWo 2DE spot 895

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
P30101 Protein disulfide-isomerase A3 precursor (EC 5.3.4.1) (Disulfide isomerase ER-60) (ERp60) (58 kDa microsomal protein) (p58) (ERp57) (58 kDa glucose-regulated protein) - Homo sapiens (Human)	57244	252	994.5796	2	0.0236	K.QAGPASVPLR.T		0	62.06	0.00024
			1039.6007	2	0.0345	R.TADGIVSHLK.K		0	50.68	0.0035
			1083.5896	2	0.0296	K.YGVSGYPTLK.I		0	36.1	0.13
			1187.5626	2	0.0345	K.FVMQEEFSR.D	Oxidation (M)	0	56.78	0.0012
			1190.6376	2	0.0445	R.LAPEYEAAATR.L		0	62.32	0.00033
			1235.5413	2	0.0359	R.DGEEAGAYDGPR.T		0	63.18	0.00021
			1340.7132	2	0.0367	R.GFPTIYFSPANK.K		0	54.8	0.0016
			1358.6872	2	0.0366	R.FLQDYFDGNLK.R		0	18.09	8.6
			1367.6907	2	0.0338	K.SEPIPESNDGPVK.V		0	25.44	1.5
			1369.7131	2	0.0253	R.ELSDFISYLQR.E		0	28.33	0.73
			1638.7792	2	0.0419	A.SDVLELTDDNFESR.I		0	70.14	4.10E-05
			1679.7837	2	0.0376	K.MDATANDVPSPYEVR.G	Oxidation (M)	0	24.29	1.5
			1831.9467	2	0.0516	K.VVVAENFDEIVNNENK.D		0	28.27	0.55
P60709 Actin, cytoplasmic 1 (Beta-actin) - Homo sapiens (Human) Note: these data matched equally well to a bovine protein, and were excluded from further analysis.	42136	122	975.4796	2	0.0386	K.AGFAGDDAPR.A		0	39.54	0.081
			1013.5041	2	0.0302	R.DLTDYLMK.I	Oxidation (M)	0	26.01	1.6
			1131.5518	2	0.0321	R.GYSFTTTAER.E		0	50.3	0.0059
			1176.6402	2	0.0342	K.EITALAPSTMK.I	Oxidation (M)	0	36.67	0.12
			1515.7305	3	0.0351	K.QEYDESGPSIVHR.K		0	51.41	0.0036
			1789.9245	2	0.0398	K.SYELPDGQVITIGNER.F		0	47.27	0.0071
			1953.1078	3	0.0507	R.VAPEEHPVLLTEAPLNPK.A		0	25.13	0.4
P02768 Serum albumin precursor - Homo sapiens (Human)	71808	59	1296.7774	2	0.0485	K.KVPQVSTPTLVE.V		1	20.28	1.8

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
Note: these data were a better match to BSA,			1638.9651	2	0.0346	K.KVPQVSTPTLVEVSR.N		1	27.02	0.24
and were excluded from further analysis.			1638.9686	3	0.0381	K.KVPQVSTPTLVEVSR.N		1	58.96	0.00015
O00391 Sulfhydryl oxidase 1 precursor (EC 1.8.3.2) (Quiescin Q6) (hQSOX) - Homo sapiens (Human)	83520	51	1113.6533	2	0.0391	R.VLNTEANVVR.K		0	32.67	0.21
			1195.625	2	0.0376	K.LEEIDGFFAR.N		0	53.33	0.0024
			1261.6707	2	0.0252	R.DFNIPGFPTVR.F		0	30.19	0.54
			1481.7764	2	0.0363	R.NNEEYLALIFEK.G		0	11.54	31

Table B-7: Database search results for BeWo 2DE spot 1073

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
P04075 Fructose-bisphosphate aldolase A (EC 4.1.2.13) (Muscle-type aldolase) (Lung cancer antigen NY-LU-1) - Homo sapiens (Human)	39964	98	1043.6084	2	0.0473	R.QLLLTADDR.V		0	50.7	0.0042
			1331.7394	2	0.0462	K.GILAADESTGSIAK.R		0	58.06	0.00066
			1433.7624	2	0.0434	M.PYQYPALTPEQK.K		0	32.58	0.25
			1690.9172	3	0.0824	K.FSHEEIAMATVTALR.R	Oxidation (M)	0	54.1	0.0012
P62988 Ubiquitin - Homo sapiens (Human)	8560	85	1066.6504	2	0.0369	K.ESTLHLVLR.L		0	64.13	9.00E-05
Note: these data matched equally well to a			1080.5807	2	0.0356	R.TLSDYNIQK.E		0	38.09	0.08
bovine protein, and were excluded.			1786.9886	2	0.0685	K.TITLEVEPSDTIENVK.A		0	44.09	0.009

B.2 Results from serum 2DE study (Chapter 5)

Table B-8: Database search results for serum 2DE spot 28

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
P02655 Apolipoprotein C-II precursor (Apo-CII) (ApoC-II) - Homo sapiens (Human)	11277	385	1034.5057	2	-0.0227	K.TYLPAVDEK.L		0	39.65	0.07
			1036.4974	2	-0.0215	K.TAAQNLYEK.T		0	34.52	0.22
			1285.5534	2	-0.0292	K.ESLSSYWESAK.T		0	57.21	0.00069
			1419.7641	2	-0.0332	Y.TGIFTDQVLSVLK.G		0	65.32	0.00013
			1770.9099	2	-0.0305	M.STYTGIFTDQVLSVLK.G		0	115.78	1.00E-09
			2218.026	3	-0.0316	G.TQQPQQDEMPSPTFLTQVK.E	Oxidation (M)	0	67.36	4.90E-05
			2248.0972	3	-0.0325	K.STAAMSTYTGIFTDQVLSVLK.G	Oxidation (M)	0	125.11	9.40E-11
			2563.1551	3	-0.0813	K.STAAMSTYTGIFTDQVLSVLKGEE.-	Oxidation (M)	1	66.69	3.80E-05

Table B-9: Database search results for serum 2DE spot 125

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
P02753 Plasma retinol-binding protein precursor (PRBP) (RBP) - Homo sapiens (Human)	23337	160	1017.4823	2	-0.009	R.QEELCLAR.Q		0	59.24	0.00081
			1164.5825	2	0.005	R.DPNGLPPEAQK.I		0	16.71	13
			1176.538	2	0.0106	R.FSGTWYAMAK.K	Oxidation (M)	0	51.26	0.0042
			1197.6177	2	-0.0006	K.YWGVASFLQK.G		0	46.86	0.011
			1302.6074	2	-0.0065	R.LIVHNGYCDGR.S		0	25.03	1.7
			1827.7352	2	0.0128	K.GNDDHWIVDTDYDTY.A		0	21	0.89
			2063.9648	2	0.0026	R.LLNLDGTCADSYSFVFSR.D		0	42.35	0.018
			2288.9418	2	-0.008	K.GNDDHWIVDTDYDTYAVQY.S		0	18.75	1.1

	2627.1323	3	-0.0197	R.LLNNWDVCADMVGTFTDTEDPAK.F	Oxidation (M)	0	33.66	0.047
	2627.1412	2	-0.0108	R.LLNNWDVCADMVGTFTDTEDPAK.F	Oxidation (M)	0	12.45	6.8
	2692.0909	3	-0.0227	K.GNDDHWIVDTDYDTYAVQYSCR.L		0	53.64	0.00018

Table B-10: Database search results for serum 2DE spot 161

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
P02649 Apolipoprotein E precursor (Apo-E) - Homo sapiens (Human)	36246	305	947.4705	2	-0.0484	R.LAVYQAGAR.E		0	35.06	0.22
			967.5022	2	-0.0429	R.LGPLVEQGR.V		0	66.15	0.00014
			1032.4886	2	-0.0466	R.LQAEAFQAR.L		0	56.82	0.0014
			1113.5342	2	-0.0436	K.LEEQAQQIR.L		0	38.12	0.09
			1312.6543	2	-0.0555	R.AKLEEQAQQIR.L		1	69.89	5.50E-05
			1496.7318	2	-0.0629	R.AATVGSLAGQPLQER.A		0	63.82	0.0002
			1551.6453	2	-0.0574	K.SWFEPLVEDMQR.Q	Oxidation (M)	0	32.24	0.14
			1619.706	2	-0.0844	K.VQAAVGTSAAPVPSDNH.-		0	73.25	1.50E-05
			1662.7164	2	-0.0718	R.GEVQAMLGQSTEELR.V	Oxidation (M)	0	75.77	7.10E-06
			1729.7561	2	-0.0809	K.SELEEQLTPVAEETR.A		0	95.33	7.80E-08
			1752.8139	3	-0.0754	A.KVEQAVETEPEPELR.Q		1	15.38	10

Table B-11: Database search results for serum 2DE spot 168

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
P02649 Apolipoprotein E precursor (Apo-E) - Homo sapiens (Human)	36246	181	967.5679	2	0.0229	R.LGPLVEQGR.V		0	20.53	3.4
			1551.7274	2	0.0246	K.SWFEPLVEDMQR.Q	Oxidation (M)	0	23.23	2.1
			1619.8286	2	0.0383	K.VQAAVGTSAAPVPSDNH.-		0	35.1	0.14
			1662.8118	2	0.0235	R.GEVQAMLGQSTEELR.V	Oxidation (M)	0	48.69	0.0061
			1729.8773	2	0.0403	K.SELEEQLTPVAEETR.A		0	53.66	0.0017

Table B-12: Database search results for serum 2DE spot 194

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
P01024 Complement C3 precursor - Homo sapiens (Human)	188569	224	1091.6663	2	0.0436	R.NTLIIYLDK.V		0	53.5	0.001
			1170.5913	2	0.0322	K.GQGTLSVVTMY.H	Oxidation (M)	0	7.98	95
			1265.6518	2	0.0411	K.NTMILEICTR.Y	Oxidation (M)	0	38.45	0.076
			1299.6348	2	0.0543	K.ACEPGVDYVYK.T		0	2.38	3.20E+02
			1506.7982	3	0.0481	K.GQGTLSVVTMYHAK.A	Oxidation (M)	0	25.14	1.3
			1666.7909	2	0.0612	K.VYAYYNLEESCTR.F		0	69.98	4.20E-05
			1841.0447	3	0.0612	K.VHQYFNVELIQPGAVK.V		0	22.13	0.95
			2835.3018	3	0.0881	R.GDQDATMSILDISMMTGFAPDTDDLK.Q	3 Oxidation (M)	0	12.69	8.6

Table B-13: Database search results for serum 2DE spot 428

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
Q14624 Inter-alpha-trypsin inhibitor heavy chain H4 precursor (ITI heavy chain H4) (Inter-alpha-inhibitor heavy chain 4) (Inter-alpha-trypsin inhibitor family heavy chain-related protein) (IHRP) (Plasma kallikrein sensitive glycoprotein 120) (PK-120) (GP120) - Homo sapiens (Human)	103489	125	927.4852	2	-0.0173	K.ILDDLSPR.D		0	46.62	0.014
			932.5233	2	-0.0099	K.NVVFVIDK.S		0	26.89	1.3
			966.5682	2	-0.0292	K.VRPQQLVK.H		0	26.45	0.84
			998.5246	2	-0.0263	K.LALDNGGLAR.R		0	45.93	0.015
			1016.5246	2	-0.0157	R.FAHTVVTSR.V		0	32.72	0.37
			1046.6205	2	-0.017	R.LGVYELLLK.V		0	56.84	0.00081
			1143.5767	2	-0.0369	R.GPDVLTATVSGK.L		0	61.5	0.00045
			1271.551	2	-0.0247	K.YIFHNFMER.L	Oxidation (M)	0	22.81	1.9
			1306.6262	2	-0.0255	K.AEAQAQYSAAVAK.G		0	45.83	0.015
			1451.7079	2	-0.0177	K.NGIDIYSLTVDSR.V		0	36.68	0.11
			1721.7834	2	-0.046	R.ANTVQEATFQMELPK.K	Oxidation (M)	0	35.18	0.11
			1763.808	2	-0.0432	R.NMEQFQVSVSVAPNAK.I	Oxidation (M)	0	31.43	0.25
			1810.9042	3	-0.0383	K.SPEQQETVLDGNLIIR.Y		0	30.94	0.32
			1810.9197	2	-0.0228	K.SPEQQETVLDGNLIIR.Y		0	36.9	0.078
			2284.1161	3	-0.0286	F.STEATQWRPSLVPASAENVNK.A		0	8.77	39

B.3 Results from plasma DIGE study (Chapter 6)

Table B-14: Database search results for plasma DIGE spot 355

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
P02675 Fibrinogen beta chain precursor [Contains: Fibrinopeptide B] - Homo sapiens (Human)	56745	1036	843.4755	2	-0.0059	R.SILENLR.S		0	32.83	0.39
			850.4545	2	-0.0004	K.YQISVNK.Y		0	50.13	0.0061
			901.3794	2	0.0042	K.GSWYSMR.K	Oxidation (M)	0	21.55	3.4
			903.5025	2	0.0059	K.IRPFFPQ.Q		0	34.97	0.22
			961.4554	2	0.0015	K.ECEEIIR.K		0	43.79	0.029
			979.4405	2	0.0046	R.QDGSVDFGR.K		0	38.67	0.076
			1031.5549	2	-0.0004	K.IRPFFPQQ.-		0	36.24	0.16
			1167.5984	2	-0.0052	R.PAPPPISGGGYR.A		0	58.66	0.00075
			1238.507	2	-0.0035	K.EDGGGWWYNR.C		0	62.36	0.00017
			1274.5523	2	-0.0004	R.DNDGWLTSDPR.K		0	56.2	0.00097
			1534.7189	2	0.0025	K.AHYGGFTVQNEANK.Y		0	60.03	0.00046
			1534.7435	3	0.027	K.AHYGGFTVQNEANK.Y		0	57.5	0.00087
			1559.6978	3	0.0151	K.HGTDDGVVWMNWK.G	Oxidation (M)	0	37.53	0.063
			1616.6818	2	0.0008	K.LESDVSAQMEYCR.T	Oxidation (M)	0	102.29	1.30E-08
			1645.8233	2	0.0065	R.TPCTVSCNIPVVSGK.E		0	121.08	3.60E-10
			1667.7212	2	0.0133	R.YYWGGQYTWDMAK.H		0	61.83	0.00018
			1683.7024	2	-0.0003	R.YYWGGQYTWDMAK.H	Oxidation (M)	0	53.95	0.00076
			1690.8008	2	0.0062	R.MGPTELLIEMEDWK.G		0	42.59	0.024
			1706.7877	2	-0.0018	R.MGPTELLIEMEDWK.G	Oxidation (M)	0	28.38	0.56
			1706.8036	3	0.0141	R.MGPTELLIEMEDWK.G	Oxidation (M)	0	45.3	0.012
			1706.8137	2	0.0242	R.MGPTELLIEMEDWK.G	Oxidation (M)	0	49.38	0.0049
			1720.9741	3	0.0157	C.QLQEALLQQERPIR.N		0	26.47	0.44
			1722.7973	2	0.0129	R.MGPTELLIEMEDWK.G	2 Oxidation (M)	0	77.52	6.90E-06

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
			1767.7721	2	-0.0078	K.DNENVVNEYSSELEK.H		0	42.5	0.015
			1767.7973	3	0.0174	K.DNENVVNEYSSELEK.H		0	41.54	0.022
			1798.8257	2	0.0273	K.NYCGLPGEYWLGNDK.I		0	57.53	0.00059
			1851.8044	3	0.0124	R.TMTIHNGMFFSTYDR.D	2 Oxidation (M)	0	80.43	2.20E-06
			1950.0208	3	0.0249	R.EEAPSLRPAPPPISGGGYR.A		0	71.07	2.40E-05
			1983.942	3	-0.0039	R.KGGETSEMYLIQPDSSVK.P	Oxidation (M)	1	43.15	0.017
			2106.1294	4	0.0324	K.REEAPSLRPAPPPISGGGYR.A		1	50.78	0.0018
			2126.0722	3	-0.0034	K.HQLYIDETVNSNIPTNLR.V		0	77.71	5.40E-06
			2126.0722	3	-0.0034	K.HQLYIDETVNSNIPTNLR.V		0	39.79	0.033
			2126.0722	3	-0.0034	K.HQLYIDETVNSNIPTNLR.V		0	25.25	0.95
			2126.077	2	0.0014	K.HQLYIDETVNSNIPTNLR.V		0	88.15	4.80E-07
			2272.0807	3	0.0126	K.GGETSEMYLIQPDSSVKPYR.V	Oxidation (M)	0	83.04	1.40E-06
			2400.1827	4	0.0196	R.KGGETSEMYLIQPDSSVKPYR.V	Oxidation (M)	1	62.22	0.00016
			2400.2086	3	0.0455	R.KGGETSEMYLIQPDSSVKPYR.V	Oxidation (M)	1	67.65	4.40E-05
			3166.5189	3	0.0425	R.NSVDELNNNVEAVSQTSSSSFQYMYLLK.D		0	17.4	2.8
			3182.4022	3	-0.0692	R.NSVDELNNNVEAVSQTSSSSFQYMYLLK.D	Oxidation (M)	0	4.79	26
P02749 Beta-2-glycoprotein 1 precursor (Beta-2-glycoprotein I) (Apolipoprotein H) (Apo-H) (B2GPI) (Beta(2)GPI) (Activated protein C-binding protein) (APC inhibitor) (Anticardiolipin cofactor) - Homo sapiens (Human)	39907	225	800.3996	2	0.0105	K.VSFFCK.N		0	33.48	0.35
			998.5687	2	0.0178	F.AGILENGAVR.Y		0	79.9	5.10E-06
			1103.5498	2	0.0098	K.EHSSLAFWK.T		0	36.23	0.16
			1141.6602	2	0.0107	L.TGLWPINTLK.C		0	35.39	0.12
			1481.6889	2	0.0174	R.YTTFEYPNTISF.S		0	8.63	62
			1736.7782	2	0.0179	K.TFYEPGEEITYSCK.P		0	66.09	8.00E-05
			1785.9827	2	-0.0024	K.FICPLTGLWPINTLK.C		0	72.01	1.60E-05
			1914.1263	3	0.0462	R.KFICPLTGLWPINTLK.C		1	13.78	2.9

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
			1927.0262	3	0.0137	R.TCPKPDDLPFSTVVPLK.T		0	40.41	0.026
			2112.9543	2	0.02	K.CSYTEDAQCIDGTIEVPK.C		0	36.41	0.057
			2396.1231	3	0.0237	K.TFYEPGEEITYSCKPGYVSR.G		0	47.77	0.0042
			2453.2153	3	0.0315	K.CPFPSRPDNGFVNYPAKPTLY.Y		0	7.05	51
			2744.3527	4	0.0106	K.CPFPSRPDNGFVNYPAKPTLYYK.D		0	18.29	3.1
			2846.5064	3	0.0201	K.WSPELPVCAPIICPPPSIPTFATLR.V		0	23.73	0.51
P02787 Serotransferrin precursor (Transferrin) (Siderophilin) (Beta-1-metal-binding globulin) - Homo sapiens (Human)	79841	221	999.506	2	0.0147	K.YLGEEYVK.A		0	41	0.053
			1248.6065	2	0.0079	K.SASDLTWDNLK.G		0	72.52	3.30E-05
			1282.5597	2	-0.0022	K.EGYYGYTGAFR.C		0	65.2	0.00012
			1293.5852	2	0.0192	K.CDEWSVNSVGK.I		0	48.95	0.0062
			1367.6471	2	0.008	K.DYELLCLDGTR.K		0	66.77	0.0001
			1428.7315	2	0.0032	K.SVIPSDGPSVACVK.K		0	38.82	0.066
			1493.7314	2	0.009	K.MYLGYEYVTAIR.N	Oxidation (M)	0	44.12	0.019
			1592.8056	2	0.0035	R.TAGWNIPMGLLYNK.I	Oxidation (M)	0	35.14	0.14
			1628.8264	2	0.0178	K.EDPQTFYYAVAVVK.K		0	38.12	0.069
			2069.0386	3	0.0169	K.EDLIWELLNQAQEHFGK.D		0	38.07	0.051
			2184.0968	3	-0.0069	R.SAGWNIPIGLLYCDLPEPR.K		0	23.83	1.3
			3953.1013	4	0.0915	R.AIAANEADAVTLDAGLVYDAYLAP NNLKPVVAEFYGSK.E		0	28.06	0.068
P02790 Hemopexin precursor (Beta-1B-glycoprotein) - Homo sapiens (Human)	52567	210	972.561	2	0.0177	R.LWWLDLK.S		0	27.12	1.2
			1128.6519	2	0.0075	R.RLWWLDLK.S		1	29.95	0.46
			1499.6855	2	0.0099	R.EWFWDLATGTMK.E	Oxidation (M)	0	52.92	0.0021
			1725.7828	2	0.0048	R.GECQAEGVLFFQGDR.E		0	70.47	3.00E-05
			1770.9821	2	-0.01	K.LYLVQGTQVYVFLTK.G		0	53.68	0.001
			1771.0149	3	0.0228	K.LYLVQGTQVYVFLTK.G		0	26.85	0.32

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
			1836.9063	3	0.0268	K.SGAQATWTELPWPHEK.V		0	28.97	0.5
			2377.1788	3	0.0052	K.LLQDEFPGIPSPLDAAVECHR.G		0	72.25	1.60E-05
			2511.2497	3	0.0069	K.EVGTPHGIILDSVDAAFICPGSSR.L		0	46.22	0.0057
			3218.5199	4	0.0564	R.DGWHSWPIAHQWPQGPSAV DAAFSWEEK.L		0	46.46	0.0033
P02671 Fibrinogen alpha chain precursor [Contains: Fibrinopeptide A] - Homo sapiens (Human)	95838	204	1027.506	2	0.0086	K.NSLFEYQK.N		0	40.46	0.056
			1099.6341	2	0.0104	K.RLEVDIDIK.I		1	51.39	0.0034
			1105.6676	2	0.0068	K.VQHIQLLQK.N		0	41.92	0.017
			1139.5509	2	0.005	R.GSESGIFTNTK.E		0	52.08	0.0035
			1456.7761	3	0.0012	K.MKPVPDLVPGNFK.S	Oxidation (M)	0	22	2.8
			1491.7088	2	0.0343	K.TFPGFFSPMLGEF.V	Oxidation (M)	0	1.57	3.40E+02
			1516.7238	3	-0.0066	R.MELERPGGNEITR.G	Oxidation (M)	0	42.66	0.026
			1519.7324	2	0.0057	K.GLIDEVNQDFTNR.I		0	66.47	0.00011
			1644.81	3	-0.0041	K.DSHSLTTNIMEILR.G	Oxidation (M)	0	63.42	0.00021
			2280.0412	3	0.0003	K.TFPGFFSPMLGEFVSETESR.G	Oxidation (M)	0	48.23	0.0034
			2280.0859	2	0.045	K.TFPGFFSPMLGEFVSETESR.G	Oxidation (M)	0	29.2	0.33
			3157.4419	3	0.0352	K.EVVTSEDGSDCPEAMDLGTLSGIG TLDGFR.H	Oxidation (M)	0	44.66	0.0041

Table B-15: Database search results for plasma DIGE spot 356

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
P02675 Fibrinogen beta chain precursor - Homo sapiens (Human)	56745	928	843.4755	2	-0.0059	R.SILENLR.S		0	37.19	0.14
			850.4545	2	-0.0004	K.YQISVNK.Y		0	44.14	0.024
			903.5025	2	0.0059	K.IRPFFPQ.Q		0	34.46	0.25
			1031.555	2	-0.0004	K.IRPFFPQQ.-		0	36.36	0.16
			1167.577	2	-0.0268	R.PAPPPISGGGYR.A		0	70.32	5.20E-05
			1238.485	2	-0.0257	K.EDGGGWWYNR.C		0	65.45	5.70E-05
			1274.53	2	-0.023	R.DNDGWLTSDPR.K		0	66.42	7.10E-05
			1307.599	2	-0.0119	K.QGFGNVATNTDGK.N		0	87.52	9.00E-07
			1383.603	2	-0.0029	G.VNDNEEGFFSAR.G		0	34.89	0.12
			1534.694	2	-0.0222	K.AHYGGFTVQNEANK.Y		0	56.48	0.00092
			1534.713	3	-0.0032	K.AHYGGFTVQNEANK.Y		0	53.36	0.0021
			1559.667	3	-0.0154	K.HGTDDGVVWMNWK.G	Oxidation (M)	0	39.7	0.027
			1559.677	2	-0.0058	K.HGTDDGVVWMNWK.G	Oxidation (M)	0	33.91	0.12
			1616.682	2	0.0008	K.LESDVSAQMEYCR.T	Oxidation (M)	0	55.52	0.00061
			1645.798	2	-0.0191	R.TPCTVSCNIPVVSGK.E		0	112.34	2.60E-09
			1683.676	3	-0.0273	R.YYWGGQYTWDMAK.H	Oxidation (M)	0	3.51	57
			1683.677	2	-0.0262	R.YYWGGQYTWDMAK.H	Oxidation (M)	0	49.91	0.0013
			1706.788	2	-0.0018	R.MGPTELLIEMEDWK.G	Oxidation (M)	0	32.01	0.24
			1722.77	3	-0.0149	R.MGPTELLIEMEDWK.G	2 Oxidation (M)	0	34.46	0.12
			1722.771	2	-0.0133	R.MGPTELLIEMEDWK.G	2 Oxidation (M)	0	59.9	0.00034
			1767.772	2	-0.0078	K.DNENVVNEYSSELEK.H		0	63.78	0.00011
			1798.772	2	-0.0262	K.NYCGLPGEYWLGNDK.I		0	69.24	2.50E-05
			1851.77	2	-0.0221	R.TMTIHNGMFFSTYDR.D	2 Oxidation (M)	0	44.07	0.0062
			1851.771	3	-0.0209	R.TMTIHNGMFFSTYDR.D	2 Oxidation (M)	0	92.57	8.90E-08
			1949.987	3	-0.0092	R.EEAPSLRPAPPPISGGGYR.A		0	63.43	0.00016

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
			2106.088	4	-0.0086	K.REEAPSLRPAPPPISGGGYR.A		1	48.15	0.0044
			2126.048	2	-0.0277	K.HQLYIDETVNSNIPTNLR.V		0	67.62	5.70E-05
			2126.072	3	-0.0034	K.HQLYIDETVNSNIPTNLR.V		0	74.82	1.00E-05
			2272.044	3	-0.0243	K.GGETSEMYLIQPDSSVKPYR.V	Oxidation (M)	0	82.47	1.40E-06
			2272.053	2	-0.0149	K.GGETSEMYLIQPDSSVKPYR.V	Oxidation (M)	0	14.97	8.3
			2400.133	3	-0.0302	R.KGGETSEMYLIQPDSSVKPYR.V	Oxidation (M)	1	67.49	4.40E-05
			2400.139	4	-0.0241	R.KGGETSEMYLIQPDSSVKPYR.V	Oxidation (M)	1	37.92	0.041
			3166.432	3	-0.0445	R.NSVDELNNNVEAVSQTSSSSFQYMYLLK.D		0	12.27	6.6
			3182.44	4	-0.031	R.NSVDELNNNVEAVSQTSSSSFQYMYLLK.D	Oxidation (M)	0	10.29	9.9
			3758.782	4	-0.0362	R.KAPDAGGCLHADPDLGVLCPT GCQLQEALLQQER.P		1	65.1	3.10E-05
			3996.904	4	-0.0568	K.APDAGGCLHADPDLGVLCPT GCQLQEALLQQERPIR.N		0	14.31	3.3
P02671 Fibrinogen alpha chain precursor - Homo sapiens (Human)	95838	495	1027.506	2	0.0086	K.NSLFEYQK.N		0	33.39	0.29
			1099.613	2	-0.0105	K.RLEVDIDIK.I		1	52.52	0.0031
			1105.647	2	-0.0141	K.VQHIQLLQK.N		0	31.29	0.27
			1139.53	2	-0.0163	R.GSESGIFTNTK.E		0	63.78	0.00022
			1456.763	2	-0.0122	K.MKPVPDLVPGNFK.S	Oxidation (M)	0	43.67	0.02
			1456.776	3	0.0012	K.MKPVPDLVPGNFK.S	Oxidation (M)	0	23.1	2.2
			1491.66	2	-0.0144	K.TFPGFFSPMLGEF.V	Oxidation (M)	0	4.05	1.50E+02
			1508.669	3	-0.0304	R.EVDLKDYEDQQK.Q		1	34.9	0.12
			1516.724	3	-0.0066	R.MELERPGGNEITR.G	Oxidation (M)	0	45.24	0.014
			1519.705	3	-0.0221	K.GLIDEVNQDFTNR.I		0	66.67	9.60E-05
			1519.708	2	-0.0189	K.GLIDEVNQDFTNR.I		0	77.16	8.70E-06
			1535.683	2	-0.0025	T.ADSGEGDFLAEGGGVR.G		0	76.93	7.30E-06
			1636.735	3	-0.0245	K.ESSSHHPGIAEFPSR.G		0	26.76	0.74
			1644.8	2	-0.0142	K.DSHSLTTNIMEILR.G	Oxidation (M)	0	54.22	0.0017

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
			1644.81	3	-0.0041	K.DSHSLTTNIMEILR.G	Oxidation (M)	0	73.33	2.10E-05
			1885.838	3	-0.0325	R.HRHPDEAAFFDTASTGK.T		1	53.02	0.0013
			2258.991	3	-0.0283	R.PNNPDWGTFEEVSGNVSPGTR.R		0	53.6	0.00077
			2280.026	2	-0.0152	K.TFPGFFSPMLGEFVSETESR.G	Oxidation (M)	0	71.08	1.60E-05
			2280.041	3	0.0003	K.TFPGFFSPMLGEFVSETESR.G	Oxidation (M)	0	65.18	6.90E-05
			3157.399	3	-0.0082	K.EVVTSEDGSDCPEAMDLGTLSGIGTLDGFR.H	Oxidation (M)	0	76.6	1.90E-06
P02679 Fibrinogen gamma chain precursor - Homo sapiens (Human)	52260	108	1341.714	2	-0.015	K.IHLISTQSAIPY.A		0	67.76	8.10E-05
			1512.708	2	-0.0126	R.YLQEIYNSNNQK.I		0	33.94	0.17
			2205.994	3	-0.0395	K.EGFGHLSPTGTTEFWLGNEK.I		0	52.23	0.0013
			2444.08	3	-0.0231	R.FGSYCPTTCGIADFLSTYQTK.V		0	19.62	1.7
			2535.229	3	-0.0238	K.AIQLTYNPDESSKPNMIDAATLK.S	Oxidation (M)	0	2.3	1.50E+02
P01024 Complement C3 precursor - Homo sapiens (Human)	188948	78	1654.817	2	-0.0435	R.TVMVNIENPEGIPVK.Q	Oxidation (M)	0	29.9	0.45
			1871.038	3	0.0113	R.TELRPGETLNVNFLLR.M		0	42.79	0.011
			1877.942	2	-0.0242	K.EYVLPSFEVIVEPTEK.F		0	22.46	2.2
			2443.278	3	-0.0221	R.EPGQDLVVLPLSITTDFIPSFR.L		0	39.47	0.022
			2780.261	3	-0.037	K.YFKPGMPFDLMVFVTNPDGSPAYR.V	2 Oxidation (M)	0	39.66	0.017

Table B-16: Database search results for plasma DIGE spot 521

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
P02649 Apolipoprotein E precursor (Apo-E) - Homo sapiens (Human)	36274	380	843.4594	2	-0.0008	R.WELALGR.F		0	24.02	3.1
			898.4275	2	-0.0063	R.FWDYLR.W		0	32.17	0.35
			947.5172	2	-0.0017	R.LAVYQAGAR.E		0	49.44	0.0076
			967.5296	2	-0.0155	R.LGPLVEQGR.V		0	57.65	0.00088
			1032.5258	2	-0.0094	R.LQAEAFQAR.L		0	54.32	0.0026
			1496.7907	2	-0.0039	R.AATVGSLAGQPLQER.A		0	61.62	0.00029
			1551.7009	2	-0.0019	K.SWFEPLVEDMQR.Q	Oxidation (M)	0	65.58	0.00011
			1619.7853	2	-0.005	K.VQAAVGTSAAPVPSDNH.-		0	58.65	0.00062
			1662.778	3	-0.0102	R.GEVQAMLGQSTEELR.V	Oxidation (M)	0	49.24	0.005
			1662.7869	2	-0.0014	R.GEVQAMLGQSTEELR.V	Oxidation (M)	0	90.21	4.20E-07
			1729.8249	3	-0.0121	K.SELEEQLTPVAEETR.A		0	39.05	0.051
			1729.826	2	-0.011	K.SELEEQLTPVAEETR.A		0	78.67	5.60E-06
			1752.8775	3	-0.0119	A.KVEQAVETEPEPELR.Q		1	54.03	0.0016
			2729.3888	3	0.0016	R.WVQTLSEQVQEELLSSQVTQELR.A		0	94	8.00E-08

Table B-17: Database search results for plasma DIGE spot 569

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
P02766 Transthyretin precursor (Prealbumin) (TBPA) (TTR) (ATTR) - Homo sapiens (Human)	16019	328	1266.6598	2	0.0142	Y.SYSTTAVVTNPK.E		0	67.71	8.60E-05
			1266.7047	2	0.0075	R.RYTIAALLSPY.S		1	16.3	9.1
			1365.7582	2	0.0065	R.GSPAINVAVHVFR.K		0	57.7	0.00061
			1393.6159	2	0.0009	K.AADDTWEPFASGK.T		0	74.21	1.50E-05
			2359.21	3	-0.0211	R.YTIAALLSPYSYSTTAVVTNPK.E		0	42.69	0.013
			2359.2303	2	-0.0008	R.YTIAALLSPYSYSTTAVVTNPK.E		0	14.61	7.7
			2450.1937	3	-0.0042	K.ALGISPFHEHAEVVFTANDSGPR.R		0	46.34	0.006
			2454.1336	3	-0.0102	K.TSESGELHGLTTEEEFVEGIYK.V		0	102.29	1.30E-08
			2488.2321	3	-0.0416	R.YTIAALLSPYSYSTTAVVTNPKE.-		1	39.22	0.03
			2515.3546	3	0.0223	R.RYTIAALLSPYSYSTTAVVTNPK.E		1	70.21	1.30E-05
P11226 Mannose-binding protein C precursor (MBP-C) (MBP1) (Mannan-binding protein) (Mannose-binding lectin) - Homo sapiens (Human)	26624	182	950.5301	2	0.0076	K.WLTFSLGK.Q		0	35.28	0.17
			975.5148	2	0.0011	K.FQASVATPR.N		0	67.54	0.00013
			1250.5911	2	-0.0119	K.EEAFLGITDEK.T		0	65.78	0.00015
			1290.5694	2	0.0007	K.SPDGDSSLAASER.K		0	83.25	2.10E-06
			1335.6443	2	0.0024	K.TEGQFVDLTGNR.L		0	55.24	0.0016
			2568.2391	3	0.0047	K.EEAFLGITDEKTEGQFVDLTGNR.L		1	36.12	0.059
			2865.3287	3	0.016	R.LTYTNWNEGEPNNAGSDEDCVLLLK.N		0	4.5	60
P02743 Serum amyloid P-component precursor (SAP) (9.5S alpha-1-glycoprotein) - Homo sapiens (Human)	25513	153	992.5166	2	-0.0012	R.DNELLVYK.E		0	39.88	0.07
			1155.5793	2	-0.0131	R.VGEYSLYIGR.H		0	77.21	1.10E-05
			1285.7696	2	-0.0102	R.GYVIIKPLVWV.-		0	17.75	4.1

Protein accession and name	*Protein precursor mass (Da)*	*Protein score*	*Observed peptide mass (Da)*	*Peptide Z (charge)*	*Match error (Da)*	*Peptide sequence*	*Modification*	*Number of missed cleavages*	*Peptide ion score*	*Peptide expect value*
			1392.6741	2	-0.0144	K.IVLGQEQDSYGGK.F		0	80.39	4.80E-06
			1405.651	2	-0.0117	R.AYSLFSYNTQGR.D		0	62.2	0.0003
P36980 Complement factor H-related protein 2 precursor (FHR-2) (H factor-like protein 2) (H factor-like 3) (DDESK59) - Homo sapiens (Human)	31767	119	1137.5286	2	-0.009	K.LVYPSCEEK.-		0	41.67	0.037
			1173.4692	2	-0.0143	G.EAMFCDFPK.I	Oxidation (M)	0	15.22	7.2
			1180.5899	2	0.01	R.TGDIVEFVCK.S		0	45.76	0.015
			1329.6342	2	-0.0223	K.INHGILYDEEK.Y		0	68.08	8.40E-05
			1488.6895	2	-0.0023	R.ITCAEEGWSPTPK.C		0	53.84	0.0019
			1764.8034	2	-0.0062	K.CLDPCVISQEIMEK.Y	Oxidation (M)	0	34.46	0.12
			3136.4284	3	-0.0244	K.YKPFSQVPTGEVFYYSCEYNFVSPSK.S		0	33.02	0.062
P02679 Fibrinogen gamma chain precursor - Homo sapiens (Human)	52260	114	1341.7142	2	-0.015	K.IHLISTQSAIPY.A		0	44.21	0.018
			1490.7345	2	-0.002	K.YEASILTHDSSIR.Y		0	17.78	8.8
			1490.7435	3	0.007	K.YEASILTHDSSIR.Y		0	55.82	0.0014
			1512.7082	2	-0.0126	R.YLQEIYNSNNQK.I		0	33.66	0.19
			2206.0299	3	-0.0032	K.EGFGHLSPTGTTEFWLGNEK.I		0	48.53	0.0038
			2535.2677	3	0.0151	K.AIQLTYNPDESSKPNMIDAATLK.S	Oxidation (M)	0	36.3	0.056
P02649 Apolipoprotein E precursor (Apo-E) - Homo sapiens (Human)	36274	43	1032.5278	2	-0.0074	R.LQAEAFQAR.L		0	34.76	0.23
			1496.8046	2	0.0099	R.AATVGSLAGQPLQER.A		0	21.17	3
			1619.7772	2	-0.0132	K.VQAAVGTSAAPVPSDNH.-		0	36.33	0.11
			1729.8201	2	-0.0169	K.SELEEQLTPVAEETR.A		0	27.02	0.8
			2729.3828	3	-0.0044	R.WVQTLSEQVQEELLSSQVTQELR.A		0	29.81	0.22

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