Identification of screening biomarkers for chromosomal anomalies and pregnancy-related disorder using quantitative plasma proteomics

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To

My Guru Sai Baba,

My Parents,

Abiraj

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1 Introduction: Prenatal screening, Invasive and Non-invasive Diagnosis.

Prenatal screening is carried out to detect pregnancies at risk for fetal anomalies, specifically chromosomal anomalies such as trisomy 21 (Down Syndrome)(figure 1), trisomy 18 (Edwards Syndrome) and trisomy 13 (Patau Syndrome) [1].

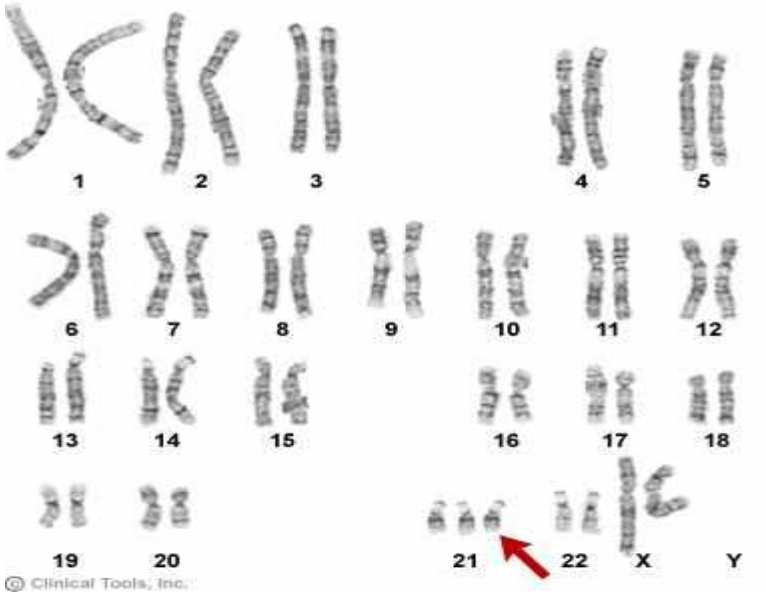

Figure 1. Karyotype from a female with Down syndrome. The red arrow indicates the triplication of chromosome 21. Image adapted from Clinical Tools, Inc.

Prenatal screening is a routine procedure in current obstetric practices. The first method introduced in the screening was advanced maternal age, as it was known for long time that advanced maternal age is associated with an increased risk of having a trisomic baby [2]. It has being shown that chances of having trisomy baby is about 2% in the women under 25 years, but it increase to 35% in women over 40 years (Figure.2) [3].

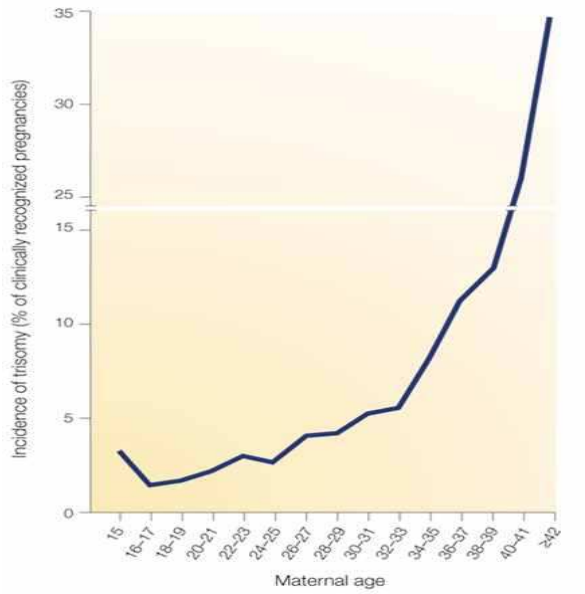

Figure 2. Maternal age and trisomy. This shows the maternal-age-specific estimates of trisomy among all clinically recognized human pregnancies. Graph adapted from Nature Reviews in Genetics, 2001 (Hassold T *et al*).

In the developed world, due to the change in demographics many women are older when having children, with many women above 35 years at the time of first pregnancy [4]. As discussed above, an advance in maternal age is one of the criteria for the prenatal screening. In a recent longitudinal [5] study carried out (1998-2008) on women in the UK, a 71% increase in the number of Down Syndrome (DS) cases was observed (Figure.3). As women at risk of carrying a fetus with a chromosomal anomaly, as identified by screening procedures, are encouraged to undergo an invasive prenatal diagnostic procedure, this will be a big health care burden.

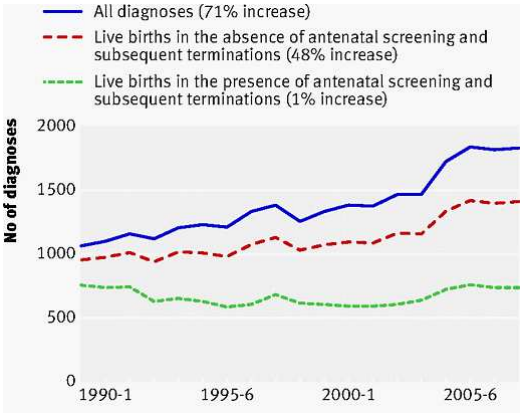

Figure 3. 71% increase in the number of pregnancies carrying a fetus with Down's syndrome in the period of 1990 till 2006. Graph adapted from BMJ, 2009 (Morris J K *et al*).

Current prenatal screening tests for the detection of pregnancies at risk of bearing a fetus with a chromosomal anomaly are carried out in 1st trimester (11-13 weeks of gestation). Hence, this procedure is also known as the 1st trimester screen. This non-invasive screening test largely relies on the ultrasound analysis of the nuchal fold of the fetus neck, also called nuchal translucency (NT), which is increased in DS fetuses. (Figure.4). If the nuchal fold is thicker then average, it is considered as a high risk pregnancy for a chromosomal anomaly. On its own, the NT screening test can detect approximately 75 % of DS fetuses, but is hampered by a high false positive rate (> 5%).

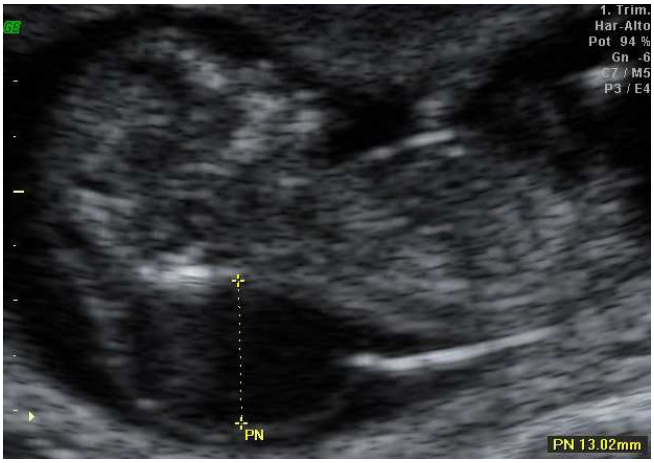

Figure 4. Nuchal Translucency Scan: Increased nuchal fold in the neck of DS fetus. Scan adapted from www.obgyn.net

In order to improve the accuracy of this test, current 1st trimester screens combine ultrasound NT measurements with maternal serum markers such as beta-hCG (Beta-Human Chorionic Gonadrotropin) and PAPP-A (Pregnancy-associated Plasma Protein A) for DS screening. These serum proteins are shown to be elevated in the maternal serum with DS pregnancy. This approach is known as the combined test, which yield a detection rate of approximately 80%, with significantly reduced false positive rate of around 5%.

The combined 1st trimester screening test is a great improvement over the previous 2nd trimester screen, which had a false positive rate (8-10%) and a detection rate of only 65%. Hence, the combined test in 1st trimester is routine practice for DS screening in many centres worldwide.

Those pregnancies which are screen positive are counselled to consider an invasive prenatal examination. Currently invasive procedure includes Chorionic Villous Sampling (CVS) or Amniocentesis, which are described in detail later (refer to section 1). The main drawback with 1st trimester screen is large number of false positive cases that are detected, and that these healthy pregnancies are unnecessarily subjected to an invasive prenatal diagnostic procedure. Since the risk of fetal loss calculated for invasive procedure is around 1%, this implies that a lot of fetuses are exposed to an unnecessary risk due to the current high false positive rate. In reality, this means that a large number of healthy babies are lost due to this problem.

In order to increase the accuracy of the 1st trimester screening test, a number of efforts have been undertaken to increase efficacy and accuracy of the current procedure. This includes the inclusion of a number of additional serum screening markers such as the inhibin, or ultrasonography for the fetal nasal bone. Unfortunately, these have not lead to the desired reduction in the FP rate.

Hence, it has become obvious that there is a need for additional biomarkers, which can add more to the sensitivity and specificity of current non-invasive screening procedure.

This aspect forms the major aim of this thesis.

1.1 Current invasive procedures for prenatal diagnosis.

1.1.1 Amniocentesis:

In this procedure amniotic fluid (AF) is obtained from the fetal amnion, which contains amniotic cells that can be used to determine whether a fetal chromosomal abnormality of developing fetus is present. The procedure is performed under ultrasound guidance, whereby a thin canulla is inserted into the amniotic sac and a volume of 15-20 ml of AF is collected using a standard syringe. The procedure is usually done after 16 weeks of pregnancy. Conventionally, the chromosomal analysis relies on karyotyping of cultured AF cells, a procedure which can take up to 2 weeks. In order to improve the speed of the diagnosis, many labs now offer direct fluorescent *in situ hybridization* (FISH) or QF-PCR (quantitative fluorescence polymerase chain reaction) of uncultured AF cells [6].

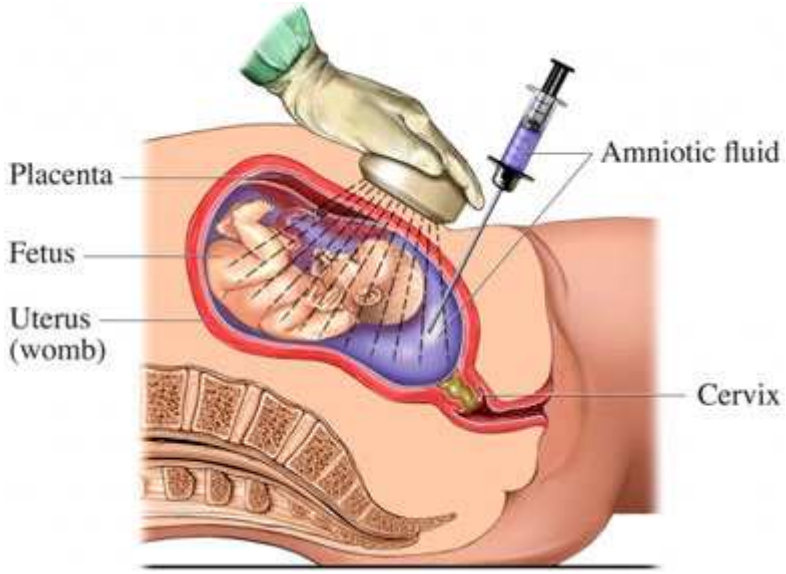

Figure 5.Transabdominal Amniocentesis procedure (2008 Nucleus Medical Art, Inc.)

1.1.2 Chorionic villus sampling

During Chorionic villous sampling (CVS), a sample from the villi of the placenta, as this material contains cells with the same genetic composition as the developing fetus. The advantage of CVS is that it can be done in first trimester. Hence, it is a method of choice for the karyotyping or molecular diagnosis of inherited disorders such as the hemoglobinopathies in early gestation. Akin to amniocentesis, it is performed under ultrasound guidance, either transabdominally or transcervically, to determine fetal position, placental location and amniotic fluid volume.

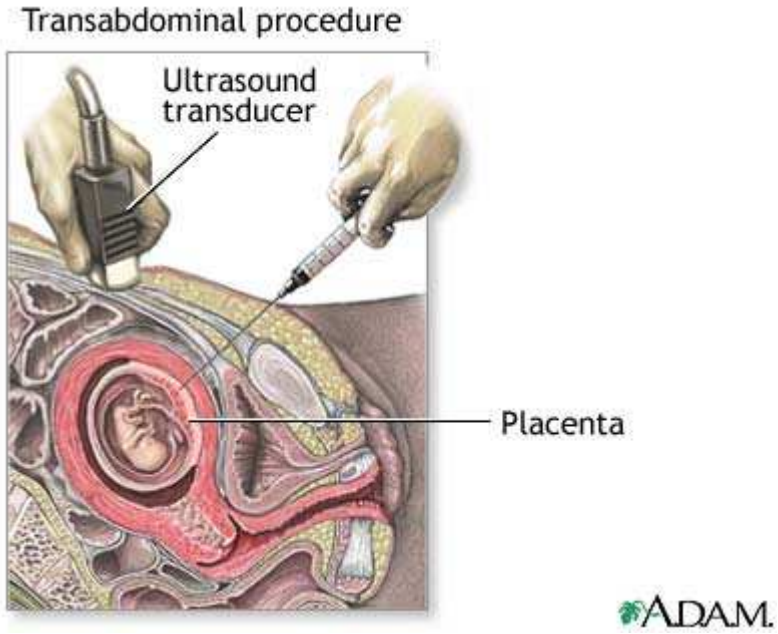

Figure 6. Transabdominal Chorionic Villous Sampling (Adapted from ADAM)

Due to the intricacy of the operation, CVS requires more skilled staff to perform it than AC. A confounder with CVS is confined placental mosaicism (CPM), which is one of the major problems in placental samples, as it can impose serious questions when the discrepancy between the chromosomal makeup of the cells in the placenta and the fetus are dissimilar. CPM is detected in approximately 1-2 % of ongoing pregnancies that are studied by CVS. There are also some indications that CVS may be more dangerous than amniocentesis. Other complications, which are common to AC includes infection, vaginal bleeding and uterine irritability.

1.2 Non-invasive prenatal diagnosis:

Due to the risk associated with invasive procedures, a large research effort has been expended to the development of risk free alternatives. Current results indicate that we may be approaching the long sought goal of Non-Invasive Prenatal Diagnosis (NIPD), whereby it will be possible to identify hereditary single gene disorders or a chromosomal abnormality in the growing fetus.

One of the routes explored for NIPD was via the enrichment of fetal cells, specifically erythroblasts, from maternal blood. After the enrichment, the putative fetal cells were examined by FISH analysis for the presence of a chromosomal anomaly. In the large a multi-centre NIFTY study it was concluded that although promising, the sensitivity and specificity was below the required clinical application.

A new avenue was provided by the detection of fetal cell-free DNA (cf-DNA) in maternal plasma or serum [7, 8]. The advantage of this approach is that cff-DNA is more abundant than fetal cells in maternal circulation. It is, however, hampered by the overwhelming presence of maternal cell free DNA.

In order to overcome this deficit, our laboratory examined for possible biochemical differences between maternal and fetal cf-DNA fragments. Here we made the striking observation that a difference in the size exists between the fetal and maternal cf-DNA fragments, in that the fetal fragments were smaller than maternal ones. We showed that this difference could be used to selectively enrich the fetal cf-DNA sequences, thereby permitting the detection of otherwise masked fetal loci, such as point-mutations or polymorphisms which could assist with the determination of fetal aneuploidies [9]. In order for this approach to become clinically practical, the enrichment procedure will need to be optimized and automated.

Other attempts to overcome this drawback rely on epigenetic differences between mother and

fetus, such as the *maspin* gene on chromosome 18 or the RASF1 gene [10]. Epigenetic approaches are currently hindered by inefficient methods such as bisulfite conversion, which leads to massive loss of epigenetically modified target DNA.

A further approach being explored is that of cell-free mRNA of placental origin, such as that of the *PLAC4* gene, which is located on chromosome 21. In a small pilot study, it has been suggested that it may be possible to detect DS by such means [11]. However, this approach is hampered by the instability of the placentally-derived mRNA, cost of processing and shipment requiring dry-ice.

These facets are discussed in an invited review we wrote on new developments for NIPD of chromosomal anomalies and single gene disorders (see publications) [12]. In this we also describe the new strategies, such a next generations sequencing and digital PCR, being explored in this field

1.3 Mass spectroscopy based proteomics

Proteomics is defined as the analysis of whole protein component of a tissue (e.g. brain), cell (e.g. yeast) or a body fluid (blood or urine). More precisely it involves the determination of identity of the protein present in the mixture and its relative and absolute quantity. Recently it is also used to identify protein modifications (e.g. phosphorylation, glycosylation). Applications of proteomics are very wide, with the major application in clinical research being applied for the better understanding of biological processes and disease state. Examples are, to find and validate new biomarkers (diagnostic and prognostic) or to understand the pharmacodynamics and pharmacokinetics of a drug compound.

A specific proteome is very dynamic and can provide lot of information on the expression pattern on normal vs. disease or control vs. treated. To study the proteome in its complexity, advanced tool are required, and in this context mass spectroscopy has emerged as a powerful technique for proteomic analysis.

A typical mass spectroscopy based proteomics workflow involves the digestion of protein in-gel or in liquid. Online or off-line fractionation of the peptides is performed by liquid chromatography (LC) and followed by ionization. The peptides are converted into ions and mass analysis is done on these ions, following which the mass to charge ratio is recorded. The resulting fragment masses is used to search of large protein databases search, resulting in the identification of the peptide and protein (Figure.7)

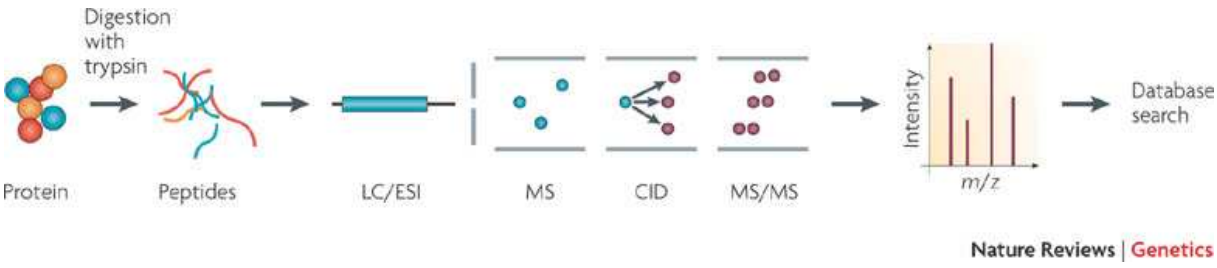

Figure 7. Typical workflow of mass spectroscopy based proteomics (adapted from Gstaiger M et al., Nature Review, Genetics 2009)

1.4 Plasma proteomics for new screening biomarkers

Plasma is an attractive entity for the proteomics studies. It contains different proteins from the various organs in high or low abundance. Many pathology or disease associated proteins are often present in plasma. Due to there low abundance, a number of different strategies have been developed to detect these in the plasma proteome, of which a few are discussed below.

1.4.1 Two-dimensional gel electrophoresis (2-DE)

Two-dimensional gel electrophoresis was introduced in 1975 by Klose and O'Farrell. 2-DE involves the separation of the protein in first dimension according to the isoelectric point (p*I*) by isoelectric focussing and in second dimension by molecular weight by running it on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After staining the gel and comparing it with the control gel, differential spots are located, excised and analyzed

using the mass spectroscopy. The major drawback of this strategy is that the resolution of the protein spots for plasma is poor. This is because the most abundant plasma proteins like albumin, IgG, IgM mask the low abundant proteins. The reproducibility and sensitivity is low and it is very difficult to use 2DE for quantitative analysis.

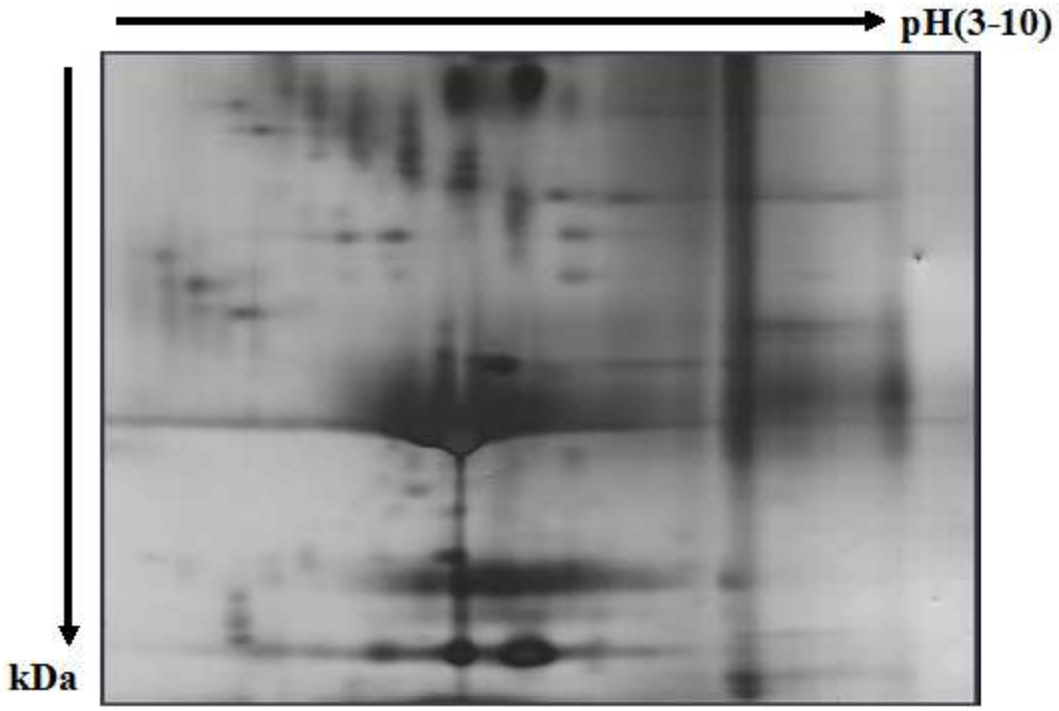

Figure 8. 2D gel for Human plasma, after the electrophoretic run gel was stained with sliver satin

1.4.2 2-D Differential Gel Electrophoresis (2D DIGE)

In traditional 2D gel electrophoresis the most preferred staining method is by Coomassie Brilliant Blue (CBB) or sliver stains. After staining intensity of the protein spot is used for relative quantitation when compare with the gel which is run in parallel. But CBB has poor detection sensitivity, where as sliver stain is not compatible with the down stream mass spectroscopy analysis. To over come this problem the proteins were label with fluorescent cyanine dye (cy2, cy3 and cy5) before the 2D separation. This method in know as 2-D differential gel electrophoresis (2D DIGE) to avoid the gel to gel variation. In same gel one can run control and experimental sample as well the internal standard. Internal standard is made by mixing equal amount of control and experimental sample and is used for the relative

quantitation (Figure.9). After the run the gel is scanned with the special Typhoon variable mode imager and DeCyder software is used for the differential analysis.

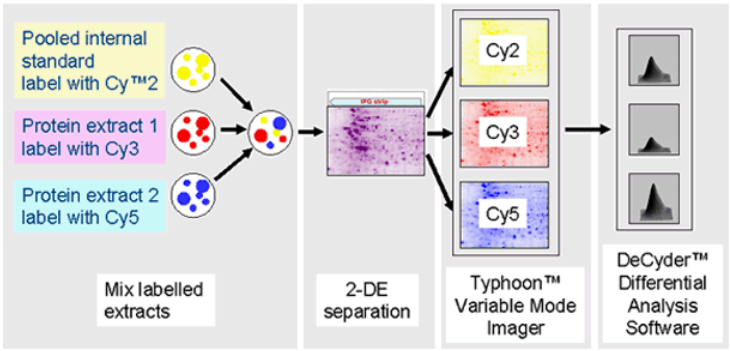

Figure 9. Workflow of 2D-DIGE (adapted from GE health care)

1.4.3 Isobaric Tagging for Relative and Absolute Quantitation (iTRAQ)

Over a decade MS has evolved as a powerful technique. This has lead to the development of shotgun proteomics, which is a useful tool as it bank ready quantification using special reagent and technique. Lately there are different techniques available for the labeling which enables the quantification of the protein like stable isotope labeling of amino acid in cell cultures (SILAC) [13, 14], isotopic-coded affinity tags (ICAT) and isobaric tags for relative and absolute quantitation (iTRAQ) [15]

iTRAQ is an isobaric chemical labeling approach currently the only technique capable of multiplexing up to eight different samples for relative quantification. 8-plex chemically identical iTRAQ reagents are available, named 114, 115, 116, 117, 118, 119, and 121 which have the same overall mass. Each label is composed of a peptide reactive group (NHS ester) and an isobaric tag of 145 Da that consists of a balancer group (carbonyl) and a reporter group (based on N-methyl piperazine), between the balancer and the reporter group is a fragmentation site (Figure.10). The peptide reactive group attaches specifically to free primary amino groups – N-termini and ε-amino groups of lysine residues [16].

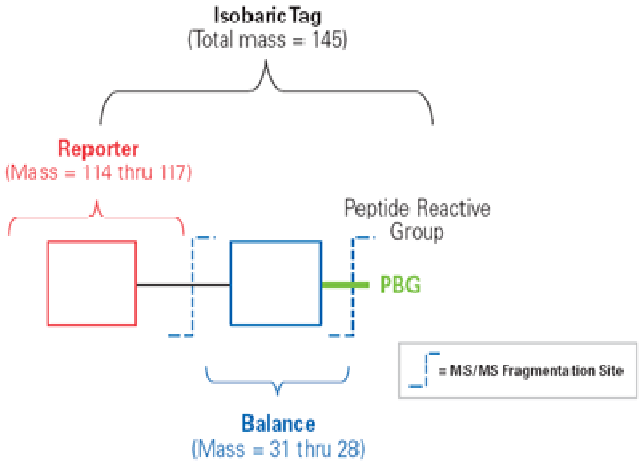

Figure 10. Schematic representation of the iTRAQ reagents (adapted from Applied Biosystems)

Each sample to be analyzed is tryptic digested and labeled with the single iTRAQ label after which sample are pooled for tandem mass analysis. The peptide product ion spectra is then used for the identification of the proteins and relative quantitation is derived from the peak intensities of the 8-plex iTRAQ reporter ions detected in the 114-121 m/z region of the fragmented ion spectra. Data acquired is always compared to a reference sample, and the quantity of each peptide is expressed as a ratio relative to the reference sample [17]. As the field of shotgun proteomics is evolving rapidly, it is likely to play a role in the detection of biomarkers. iTRAQ has been shown to permit very reliable quntitation of proteins in complex mixture such as plasma, serum or urine. As such it has been suggested to be a useful tool for the detection of biomarkers. Hence, it is likely to play a key role in this field.

1.5 Plasma proteomics for DS biomarker development

Only a limited number of studies have attempted to use proteomic approaches for the discovery of new biomarkers for pregnancies at-risk of carrying a fetus with DS. Two of these used 2-DE (see above) approaches [21, 22], while a further used a SELDI method [23]. Of these studies that by Nagalla and colleagues is the largest, having examined serum samples from 56 pregnant women. This study used samples collected in both the 1st and 2nd trimester of pregnancy, which were recruited as part of the NIH funded FASTER study, and largely made use of the fluorescent 2D-DIGE process. In their study, 18 proteins were found to be elevated in 1st trimester samples, which included members of the apoliprotein family, clusterin and proteins involved in skeletal development (tetranectin). None of the identified marker proteins attained any specificity in a blinded analysis of maternal serum samples.

The study by Kolialexi and colleagues [21] used traditional 2DE stained with Coomassie blue on 20 maternal plasma samples (8 cases, 12 controls, 16-18 weeks of pregnancy), by which means 8 candidate proteins were detected. Elevations were noted for apoliprotein E and serum amyloid P-component. In contrast to the study by Nagalla et al., [22] a down regulation for clusterin was noted.

The drawback or merits of the various approaches, as well as future development are discussed in detail in our review on this topic [24].

In our experimental analysis we determined that the use of isobaric tagging (iTRAQ) was the most promising for the quantitative analysis of changes in the maternal plasma proteome in DS affected pregnancies. This approach forms a major part of this thesis (refer to section 3 and 5).

1.6 Preeclampsia

Preeclampsia (PE) is a severe disorder of pregnancy and a major cause of fetal and maternal mortality. It occurs almost 3-5% of pregnancies in developed countries, and is considerably higher in less developed nations. The clinical symptoms include rapid hypertension in previously normotensive women, as well as excess protein in urine. Other symptoms may include oedema, severe headache and liver or kidney dysfunction [25].

The underlying aetiology of PE is unknown, but involves aberrant placentation. Frequently the only therapeutic option is delivery of the baby and removal of the placenta, upon which most cases with PE resolve. This, however, leads to the delivery of very premature fetus, which is frequently affected by growth retardation. A major clinical concern is that no reliable method exists to detect at-risk pregnancies, and hence, a many efforts are expended for the development of new screening biomarkers. It is hoped that these markers will facilitate better management of at-risk pregnancies, by permitting early therapeutic intervention [26].

Amongst the markers currently being explored are angiogenic factor like soluble fms-like tyrosine kinase-1 (sFLT), placental growth factor (PIGF), vascular endothelial growth factor (VEGF) [27] and endoglin which are produced by placenta, and whose abnormal expression are thought to play a role in the hypertensive symptoms. Unfortunately changes in these molecules only appear to occur in the 2nd trimester of pregnancy very shortly before onset of symptoms [28].

Apolioprotein E (ApoE) is a major constituent of very low-density lipoprotein. As it has been hypothesised that preeclampsia may developed because of abnormal lipid metabolism, leading to oxidative stress, ApoE may play a role in this cascade. In this context it has been

observed that women with PE have an abnormal lipid profile, which contributes to endothelial dysfunction [29].

Inhibin A and Activin A are placentally produced glycoproteins that have been reported as possible predictors for preeclampsia [30], although this may be limited to the 2nd trimester, where elevations in inhibin A have been noted. First trimester inhibin A analysis has been shown to have a low predictive value [31].

Pregnancy-associated plasma protein A (PAPP-A) is highly glycosylated protein and produced by the developing trophoblast. Lower levels of PAPP-A appear to be present in first trimester serum of pregnant women who subsequently develop PE, however, its specificity has been questioned as alterations are also observed in other pregnancy related conditions [32].

Hence, the quest continues for biomarkers that possess predictive value even in the 1st trimester of pregnancy.

One of the most promising of those currently being examined is placental protein 13 (PP13), a short diamer protein (32-kDa), which is highly expressed in placenta. During normal pregnancy PP13 expression gradually increases with the gestational age [33]. Of interest is that PP-13 expression is lower in women who subsequently develop preeclampsia when compared to healthy controls [34]. The efficacy of PP-13 as a screening marker was investigated in the EU funded Pregnancy project, in which our lab participated, and which funded a large part of this thesis.

1.6.1 Plasma Proteomics for new screening biomarkers for Preeclampsia

As discussed above, currently no reliable early screening marker exists for the prediction of preeclampsia. It is our hypothesis that as the placenta is in close contact with the maternal

circulation, and as preeclampsia is associated with placental alterations, that these could be detectable via analysis of the maternal plasma proteome. Such biomarkers could then be used for the early detection of at-risk pregnancies, and thereby could assist with better management of these. In our study we examined 1st trimester maternal plasma samples by quantitative proteomic analysis using isobaric tagging for the detection of putative biomarkers.

2 Analysis of Pre- and Post-Delivery Maternal Plasma Proteome using Free Flow Electrophoresis and Shotgun Proteomics

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2.1 Abstract

Objectives: To examine whether there is difference in pre- and post-delivery maternal plasma proteome by shotgun proteomics.

Methods: Blood samples were collected from pre- and 24 hr post- delivery sample. Plasma extracted from the sample was subjected to 2 different types of pre-fractionation techniques, namely immuno-depletion and free flow electrophoresis (FFE). Tryptic peptides were analysed using mass spectroscopy (LC-MS/MS).

Results: Eleven plasma proteins were detected in pre delivery sample, which were absent from the post delivery sample.

Conclusion: FFE and immuno-depletion are efficient system for pre-fractionation of maternal plasma samples to remove albumin, thereby permitting the detection of changes in the maternal plasma proteome which occur following delivery.

Keywords: Free Flow Electrophoresis, maternal plasma, shotgun proteomics

2.2 Introduction:

A successful pregnancy outcome depends on many different factors, which includes the physiologic implantation of the placenta. The placenta is a dynamic organ which increasingly nurtures the developing fetus with required nutrients and oxygen. It also helps to protect the fetus against potentially adverse maternal immune responses [35]. The placenta is the direct interface between the mother and developing fetus [36]. In this context it is noteworthy that a number of pregnancy related disorders, such as preeclampsia, preterm labour or even fetal aneuploidies, are associated with placental anomalies, which can contribute to the underlying pathology. Furthermore, there is continuous shedding of trophoblast-derived micro-particles, as well as liberation of cell-free nucleic acids and trophoblast deportation into the maternal circulation by the placenta during almost all stages of fetal development [37]. Hence, and analysis of the maternal plasma proteome for placenta-derived peptides may yield important biological clues regarding various physiological and pathological states of fetal development [38].

The aim of this study was to examine whether changes in the maternal plasma proteome occurred as a result of delivery, due to removal of the placenta. For this purpose we examined maternal plasma samples taken prior to and 24 hrs post removal of the placenta by before and shotgun proteomics. This analysis permitted s to ascertain whether we could detect placenta specific peptides and whether these were cleared from the maternal circulation post delivery.

In this study, we also attempted to optimize the method for the depletion of major abundant proteins in plasma, as these are a great hindrance when desiring to detect less prevalent peptides, such as those of fetal origin. We used the two commercially available depletion column systems and the recently described Free Flow Electrophoresis (FFE) system [39]. FFE essentially operates on the principal of isoelectric focusing (IEF). The usefulness of this

system is that it permits the clear separation of albumin from the remaining plasma proteins [40].

2.3 Material and Methods:

Urea was purchased from Serva (Heidelberg, Germany). Sodium hydroxide, sulphuric acid were purchased from Rield-de-Haen (St. Louis, Mo, USA). $_{DL}$-2-Aminobutyric acid, glycyl-glycine, ethanol-amine, 6-aminohexanoic acid, and N-acetylglycine were obtained from Fluka (St. Louis). HEPES, AMPSO, and acetic acid were purchased from Roth (Karlsruhe, Germany). ß-Alanin was obtained from Sigma-Aldrich (St. Louis). TAPS were purchased from CalBiochem (Darmstadt, Germany). Sequencing grade trypsin was purchased from Promega (Madison, WI, USA). FFE reagent, IEF prolyte Buffer2, IEF Prolyte Buffer 3-9, and 2-(-4 sulfophenylazol)1,8-dihydroxy-3,6-naphthalene di-sulfonic acid (SPADNS) were bought from BD Diagnostics (Munich, Germany). SepPak cartridges were purchased from Waters (Milford, MA, USA). Ultrafilteration spin columns (MWCO 3 kDa) were bought from Vivascience (Hannover, Germany).

2.4 Samples

This study was approved by the Institutional Ethical Board of the University Hospital Basel, Switzerland. Informed consent was taken from all the pregnant women who participated in the study. Blood for this study was collected from six low risk pregnant women with singleton pregnancies prior to and 24 hrs after an elective caesarean section. In this manner, the plasma proteome was not influenced by the effect of labour.

Characteristic	Case (n=6)
Maternal age, (y)	35.1±1.3
Gestational age, (wk)	38.8±0.8

Table 1. Clinical details of the pregnancy cases used for the study

2.5 Sample preparation

9 ml blood was drawn into BD P100 tubes (BD Diagnostics, Franklin Lake, NY, USA), which are specially designed for the proteomic analyses [39]. EDTA and protease inhibitor, present in the tube, prevent coagulation and stabilize plasma proteins. Tubes were centrifuged at 3,000x g for 30 minutes at 10ºC. Plasma was separated from maternal erythrocytes and leukocytes by a mechanical separator. Small 100µl aliquots of plasma were transferred in Eppendorf tubes and stored at -80ºC until further processing.

2.5.1 Immuno-depletion

To remove the most abundant proteins present in the maternal plasma samples, we made use of commercially available columns, which either depleted fourteen (MARS Human-14, Agilent) or twenty (Proteoprep 20, Sigma-Aldrich) of these proteins. Both systems were used according to the manufacturer's instructions.

2.5.2 Free Flow Electrophoresis (FFE)

Electrophoresis was performed in isoelectric focusing (IEF) [40] mode using a BD™ FFE system (BD diagnostics) [41]. In brief, all the media were prepared fresh for the run according to the manufacturer's instructions. The media used for the IEF run contained 8 M Urea. The counter flow media (inlet 1-3) was made by 8M urea with no prolyte addition. Anode stabilization buffer (inlets 1 and 2) consisted of 100 mM sulphuric acid, 50 mM acetic acid,

100 mM $_{DL}$-2aminobutytic acid and 30 mM glycyl-glycine; Depletion buffer 1 (inlet 3 and 5) contained 29% of prolyte buffer 2 (BD Diagnostics); depletion buffer 2 contained 17% prolyte buffer 2, 50 mM HEPES, and 42 mM 6-aminohexanoic acid; and cathodic stabilization buffer (inlets 6 and 7) 100 mM sodium hydroxide, 30 mM ethanolamine, and 300 mM ß-alanin; anode and cathode electrode buffer contained 100 mM sulphuric acid and sodium hydroxide respectively.

The instrument was operated in horizontal mode with a spacer of 0.4 mm and the filter paper of 0.6 mm thickness. During entire depletion electrophoretic run the temperature was maintained at 10ºC and the media flow rate was set at 60 ml/h, with a constant voltage of 700 V. Prior to electrophoresis, 500 ul of plasma was diluted ten times with the depletion buffer 2 and was introduced into the instrument from the sample port. After the fractionation, samples were collected in a 96 well plate. About 2 ml of each fraction was collected. A volume of 500 ul of the each FFE fraction 24-33 was reduced in 10 mM TCEP for 60 min at 25ºC and alkylated in the dark in 50 mM iodoacetic acid for the 60 min at 25ºC. The pH was adjusted to 7.8 with ammonium bicarbonate and the urea concentration was reduced to approximately 2 M using an ultra-filteration spin column Vivascience (Hannover, Germany).

2.5.3 Mass Spectroscopy analysis

The pooled FFE peptide fraction, as well as those from the immuno-depletion columns were digested with trypsin (100 ng) at 37ºC for 16 h. Sep Pak™ C18 RP cartridges were used to purify the peptides. The peptides were analysed by capillary liquid chromatography tandem MS (LC/MS/MS) using a 300SB C-18 trap column (0.3x50mm) (Agilent Technologies, Basel, Switzerland) connected to a 0.1 mm x 10 cm capillary separation column packed with Magic C18 (5 µm particle diameter). The capillary column was connected to an Orbitrap FT hybrid instrument (Thermo Finnigan, San Jose, CA, USA). A linear gradient from 2 to 60% solvent B (0.1% acetic acid and 80% acetonitrile in water) in solvent A (0.1% acetic acid and 2% acetonitrile in water) in 85 min was delivered with a Rheos 2200 pump (Flux Instruments,

Basel, Switzerland) at a flow of 100 µl/min. A pre-column split was used to reduce the flow to approximately 500 nl/min. 10 µl sample was injected with an autosampler, thermostated to 4°C onto the trap column for efficient desalting. The eluting peptides were ionized at 1.6 kV. The mass spectrometer was operated in a data-dependent fashion so that peptide ions were automatically selected for fragmentation by collision-induced dissociation (MS/MS) in the Orbitrap. The MS/MS spectra were then searched against the data bank using TurboSequest software [42].

2.6 Two-Dimensional Gel Electrophoresis

We performed the two dimensional electrophoresis on the immuno-depleted plasma, as well as FFE depleted pooled fractions (24-33) using an ISODALT system. In this run, ampholine pH 3-10 (Invitrogen) in the first dimension; and 11-19% linear acrlyamide gradient in the second dimension was used. After electrophoresis the gels were stained with silver stain, and then scanned by Pharmacia Image Scanner at 300 dpi, 16 bit [43].

2.7 Results

In this study we examined for differences in the maternal plasma proteome following deliver via the use of shotgun proteomics. Samples were obtained from 6 cases with singleton pregnancies, all of which were delivered by voluntary Caesarean section. Care was taken to maintain the uniformity in the collection time for all the samples.

In the analysis of un-depleted maternal plasma samples, a 2µl volume from a pre- and post-delivery was examined by 4-12% SDS-PAGE. Following silver staining, it became apparent that the presence of the most abundant proteins

Such as albumin, or immunoglobulins, totally masked the presence of other less abundant proteins (refer to Figure1).

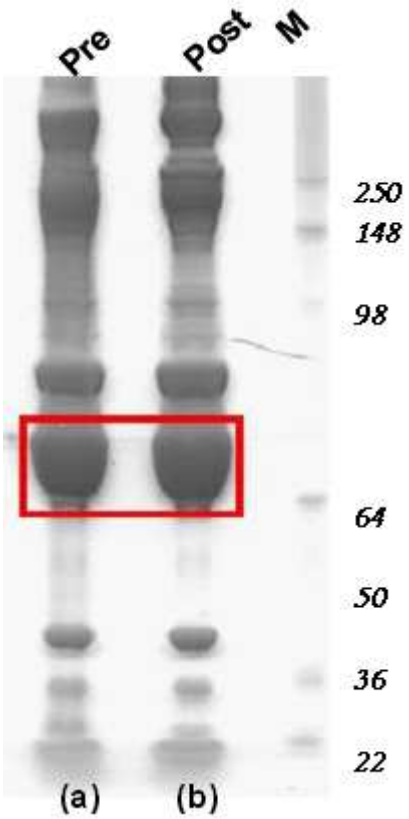

Figure 1. Undepleted maternal plasma from (a) pre and (b) post delivery. A volume of 2µl was loaded on the 4-12% SDS-PAGE. M represents the protein marker

In order to overcome this problem, we next examined whether we could deplete these abundant plasma proteins, which contribute more than 99% of the plasma proteome, by using commercially available immuno-affinity columns. We examined two different column systems which either have the ability to deplete fourteen (MARS Human-14, Agilent) or twenty (Proteoprep 20, Sigma-Aldrich) major plasma proteins (refer to Figure 2. (A) and (B) respectively).

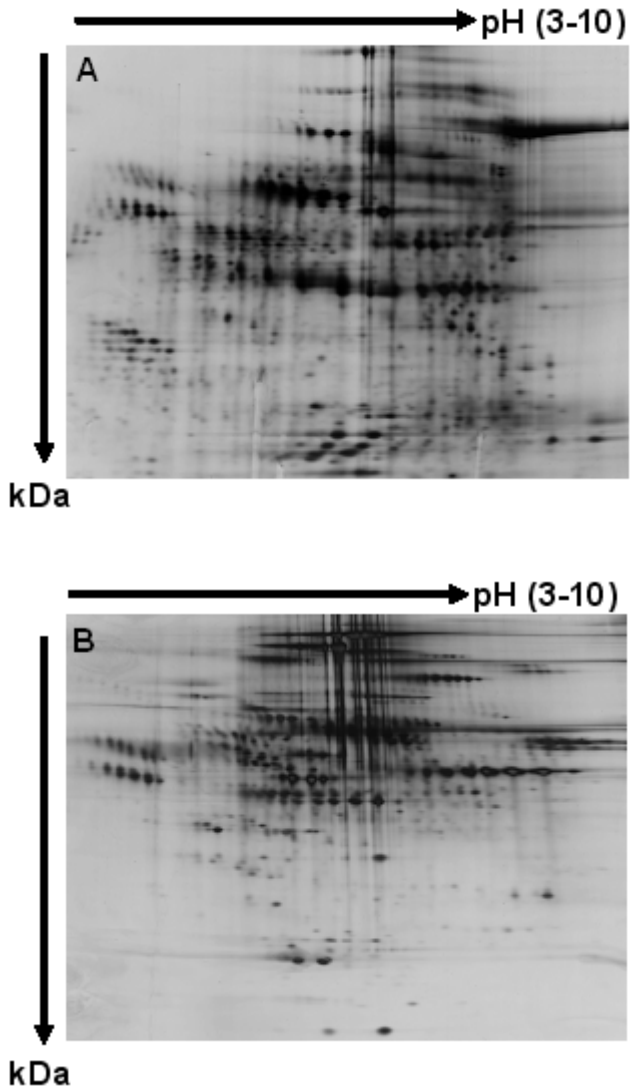

Figure 2. Two dimensional electrophoresis of depleted maternal plasma was preformed in the ISODALT system. After electrophoresis, the gels were sliver stained. (A) Depletion of fourteen major plasma protein.(MARS Human-14, Agilent). (B) Depletion of twenty major plasma proteins (Proteoprep 20, Sigma-Aldrich).

We determined that although these immuno-depletion columns did permit reliable removal of the most abundant plasma proteins, their use entailed a large number of washing steps which resulted in low recovery of the desired placenta-derived proteins.

In order to improve the recovery of these rare proteins species, we next examine the efficacy of a recently described Free Flow Electrophoresis system, which operates on the principal of isoelectric focussing (IEF) [44]. By the use of this technology, we were able to confine

albumin into certain fractions (numbers 37-40), as is illustrated in Figure 3. The system was used under denaturing conditions.

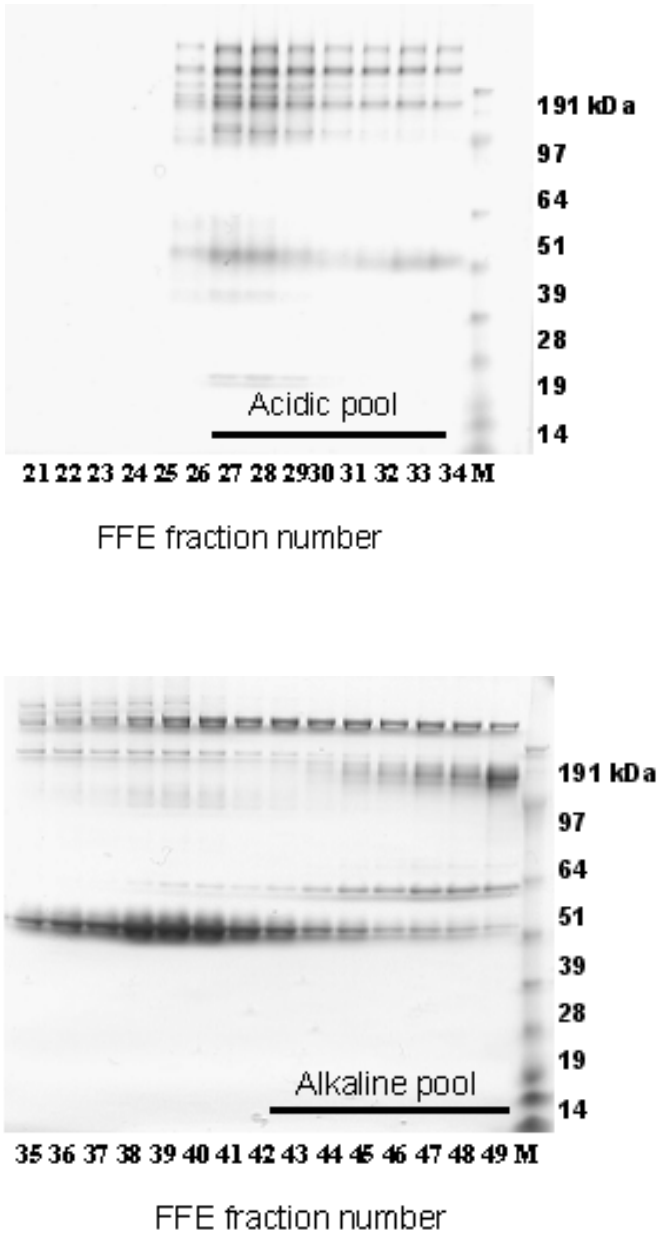

Figure 3. Separation of plasma using FFE protocol. SDS-PAGE (4-12%) analysis of the FFE fraction obtained using the depletion protocol. Proteins were visualized by silver staining. Albumin has a molecular mass of almost 50 kDa. A volume of 5 µl was loaded per FFE fraction. M indicates protein molecular weight marker.

After mass spectrometric analysis of acidic (fractions 26-33) and alkaline pool (fractions42-49), we were able to identify 51 unique proteins in pre- and 71 proteins in post-delivery samples. Of these, eleven of the proteins were present in the pre-delivery samples, which were absent in post-delivery samples (refer to Table 2).

Accession no.	Protein Name	Molecular function
P02671	Fibrinogen, alpha polypeptide isoform alpha-E	Signal transduction
P01031	Alpha-2-macroglobulin	Complement activity
P17931	Galectin 3	Transcriptional regulator
P01019	Angiotensinogen	Signal transduction
P04114	Apolipoprotein B-100	Signal for cellular binding
P02787	Transferrin	Transporter activity
P02746	C1q B-chain	Complement activity
P00736	Complement factor H	Complement system
P0C0L4	Complement component 4 binding protein	Complement system
P15814	Immunoglobulin lambda light chain VLJ region	Immuno response
Q96KN2	Carnosinase 1	Metalloproteinase activity

Table 2. List of proteins identified in pre-delivery maternal plasma, but absent 24 hour post-delivery.

2.8 Discussion:

In this pilot study set out to examine whether changes in the maternal plasma proteome were apparent following delivery (24 hr). In order to remove the most abundant proteins, such as albumin, which mask the rare placentally-derived proteins from the maternal plasma sample we tested two strategies. In the first we examined the use of two different commercially available immuno-depletion columns, that either remove 14 (MARS Human-14, Agilent) or twenty (Proteoprep 20, Sigma-Aldrich) of the major plasma proteins.

Our experience indicated that although though these immuno-depletion columns are convenient to use, they do not permit optimal recovery or rare plasma proteins due to a large number of washing steps.

In the second strategy we examined the use of a Free Flow Electrophoresis system. In our experience this system permits a more optimal recovery of rare plasma proteins, as it permits the effective removal of albumin, which constitutes almost 99% of the total plasma protein. Furthermore, this system permits the fractionation of large plasma volumes (up to 3ml/h), thereby permitting large-scale analyses.

A further advantage of the FFE system is that in the denaturing mode, it permits the recovery of proteins which normally bind to albumin, and which would as such be removed by methods relying on immuno-depletion. Consequently, it should permit the more optimal recovery of rare proteins that would be achieved when using immuno-depletion strategies. [45].

In our analysis of pre- and post delivery maternal plasma samples we were able to detect 11 proteins present in the former, but absent in the latter. Amongst the proteins we detected was angiotensinogen (P01019) [46], which plays a physiological role in the regulation of uteroplacental blood circulation. Alterations in the levels of this protein, as well as auto-antibodies directed against the receptor have been implicated in the underlying aetiology

leading to the development of preeclampsia Alpha-2-macroglobulin (P01031) is also noteworthy, as a number of studies have indicated that it may play possible role in pregnancy Two proteins known to be expressed in the placenta are galectin 3 (P17931) [47] and Apolipoprotein B-100 (P04114) [48]. Galectin 3 has been shown to be present in all trophoblastic lineages including villous cytrotrophoblast and extravillous trophoblasts. Apolipoprotein B-100 (P04114) [48] has been shown to be synthesized and secreted by the placenta. The detection of transferrin (P02787) [49] is also significant, as this is a very important iron transport protein, responsible for the transfer of iron from mother to the fetus. An elevation in the inflammatory response markers, such as C-reactive protein [50] and members of the immunoglobulin family such Ig lambda chain C region were present in pre-delivery samples, but absent in post-delivery samples, which could be indicative of an increased inflammatory response associated with the onset of labour towards the end of pregnancy.

3 Quantitative proteomic (iTRAQ) analysis of 1st trimester maternal plasma samples in pregnancies at risk for preeclampsia

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3.1 Abstract

A current major obstacle is that no reliable screening markers exist to detect pregnancies at risk for preeclampsia. Quantitative proteomic analysis employing isobaric labelling (iTRAQ) has been suggested to be suitable for the detection of potential plasma biomarkers, a feature we recently verified in analysis of pregnancies with Down syndrome foetuses. We have now examined whether this approach could yield biomarkers to screen pregnancies at risk for preeclampsia. In our study, we used maternal plasma samples obtained at 12 weeks of gestation, six from women who subsequently developed preeclampsia and six with uncomplicated deliveries.

In our analysis, we observed elevations in 10 proteins in the preeclampsia study group when compared to the healthy control group. These proteins included clusterin, fibrinogen, fibronectin, angiotensinogen, increased levels of which are known to be associated with preeclampsia. An elevation in the immune-modulatory molecule, galectin 3 binding protein, was also noted. Our pilot study, therefore, indicates that quantitative proteomic iTRAQ analysis could be a useful tool for the detection of new preeclampsia screening markers.

Key words: iTRAQ (Isobaric Tags for Relative and Absolute Quantitation), preeclampsia, maternal plasma, biomarker, screening.

3.2 Introduction

Preeclampsia is a major health concern, as it is a leading cause of fetal and maternal mortality and morbidity, worldwide. A major clinical deficit is that no reliable screening markers exist to detect at-risk pregnancies, thereby offering no opportunity for intervening therapies prior to the onset of symptoms. This is especially relevant for severe cases of early onset forms of the disorder, resulting in the delivery of premature babies, who are frequently affected by intra-uterine growth retardation.

It is widely accepted that the underlying aetiology of this enigmatic disorder, characterised by sudden hypertension in previously normotensive pregnant women, involves the placenta. In this context, the placentae of early onset preeclampsia are usually characterised by abnormalities in trophoblast differentiation, lack of modification of the maternal spiral arteries and ill-defined villous structure.

Recent studies have indicated that the clinical symptoms, such as hypertension and proteinuria, are largely brought about by alterations in the levels of angiogenic factors produced by the placenta such as placental growth factor (PIGF), vascular endothelial growth factor (VEGF), soluble Flt-1 and endoglin [51] Although alterations in the levels of these cytokines do precede the onset of clinical symptoms, it is not clear how effective they will be as screening markers, as these changes occur relatively late in gestation.

A considerable effort in prenatal medicine is being devoted to the development of efficacious first trimester screening tools, to detect pregnancies at risk for fetal aneuploidies, but also pregnancy-related disorders such as preeclampsia, as this would permit sufficient time for intervention. In this regard, quantitative changes at 11-14 weeks of pregnancy in proteins such as ADAM-12 [52], PP-13 (placental protein -13) [53, 54], pregnancy-associated plasma protein-A (PAPP-A) in combination with Doppler ultrasound, have been shown to posses some predictive value in detecting cases at risk for preeclampsia [32].

Since the aetiological cascade leading to the development of manifest preeclampsia is multi-factorial, it is very unlikely that a single molecule (or small group of analytes) will possess sufficient sensitivity and specificity. Hence, new strategies will need to be explored in order to increase the pool of biomarker candidates.

One such route is by proteomic analysis of the maternal plasma proteome. The underling rationale for such investigations is that as the placenta is in direct contact with the maternal circulation, proteins released by it should be detectable in maternal plasma. Since preeclampsia is associated with structural placental anomalies, the underlying alterations in protein expression should be reflected in plasma proteome. Consequently, this serve as a promising route for the detection of biomarkers indicative of abnormal placentation [55]

Consequently, a number of studies have been carried out under this premise, and have shown that alterations in protein expression are evident maternal serum or plasma of affected pregnancies. Unfortunately the validity of these studies is limited, as they were performed on samples obtained from cases with manifest symptoms, and furthermore, did not employ state-of-the-art quantitative approaches permitting precise assessment of the extent of up- or down-regulation of the newly discovered markers.

A further limitation in these studies is the extremely complex nature of the plasma / serum proteome, whereby low abundance proteins are masked by the preponderance of a small number of highly abundant plasma proteins. In addition, the high dynamic range of the plasma proteome precludes the use of conventional proteomic strategies employing gel electrophoresis, even when making use of fluorescent labelling e.g. DIGE (Difference Gel Electrophoresis). Hence, the identification of new biomarkers in plasma/serum by conventional proteomic approaches is not very feasible.

A method that has been proposed to largely overcome these deficits is isobaric labelling (iTRAQ: Isobaric Tags for Absolute and Relative Quantitation) coupled with MALDI TOF/TOF analysis. In this approach, the plasma peptides, following trypsin digestion, are

chemically labelled with a discrete set of isotopes via an amine-tagging reagent. These isotope tags permit ready discrimination by mass spectrometry, thereby permitting comparative quantification to a reference sample in a multiplex manner. To date, most applications have made use of commercial 4-plex reagents, but 8-plex reagents are becoming available. By the use of these different isobaric tags, it is possible to examine 4 or 8 different samples in a single mass spectrometric analysis.

Since this method has been shown to permit highly reproducible comparative assessment of tagged peptides, it has been suggested to be suitable for the discovery of biomarkers in complex body fluid PE such as plasma. In a recent study we have shown that the iTRAQ isobaric labelling approach may indeed be valid for the detection of biomarkers for pregnancy related conditions, such as fetuses with Down syndrome, in that we were able to discern quantitative alterations in known screening markers such as βhCG [56].

Confident by the validity of the iTRAQ methodology, we have now in a pilot experiment investigated whether this approach could be suitable for the detection of biomarkers useful for determining pregnancies at risk for preeclampsia. In this study we examined 12 maternal plasma samples obtained at 12 weeks of pregnancy, of which 6 cases subsequently developed preeclampsia, while 6 cases had uncomplicated deliveries.

Maternal plasma peptides were examined by 4-plex iTRAQ labelling in conjunction with a 4800 MALDI TOF/TOF analysis. Our results indicate that quantitative differences can be already being detected in the first trimester of pregnancy between pregnancies which subsequently developed preeclampsia and those which had normal healthy outcome.

3.3 Materials and Methods

3.3.1 Samples

Blood samples for this case-control proteome study were collected prospectively from pregnant women at approximately 12 weeks of gestation. In a retrospective manner, 6 samples from cases that subsequently developed preeclampsia were matched with 6 samples from pregnancies with normal healthy outcome (Table 1). This study was undertaken with the approval of the Institutional Ethical Board of the University Hospital, Basel, Switzerland and written informed consent was required in all instances.

(A)

	Control	PE
Maternal age (years)	35.1±1.3	35.1±6.8
Gestational age (weeks)	38.8±0.8	34.9±2.4
Systolic BP (mmHg)	122.5±14.2	188.8±18.6
Diastolic BP (mmHg)	73.6±7.0	111.8±14.1
Proteinuria (dipstick)	Negative	+++

(B)

	Control	PE
Gestational age (weeks)	12.4±1.2	12.3±1.1

Table1. Characteristic of study population. (A)Criteria used for pregnancy with or without preeclampsia for the longitudinal study. (B) Gestational window selected for the current study (n=6) who subsequently developed the preeclampsia

3.3.2 Sample preparation

As described previously, 9 ml blood was drawn into BD P100 tubes (BD Diagnostics, Franklin Lake, NY, USA), which are specially designed for proteomics experiments, in that the EDTA (Ethylenediaminetetraacetic acid) and protease inhibitor present in the tube prevent coagulation and stabilize the plasma proteome. Following phlebotomy the samples were centrifuged at 3,000x g for 30 minutes at 10ºC, whereby the plasma was separated from the cellular fraction by aid of a mechanical separator100µl aliquots were stored at -80ºC until further use. For an overview of the work-flow used in this analysis refer to Figure 1.

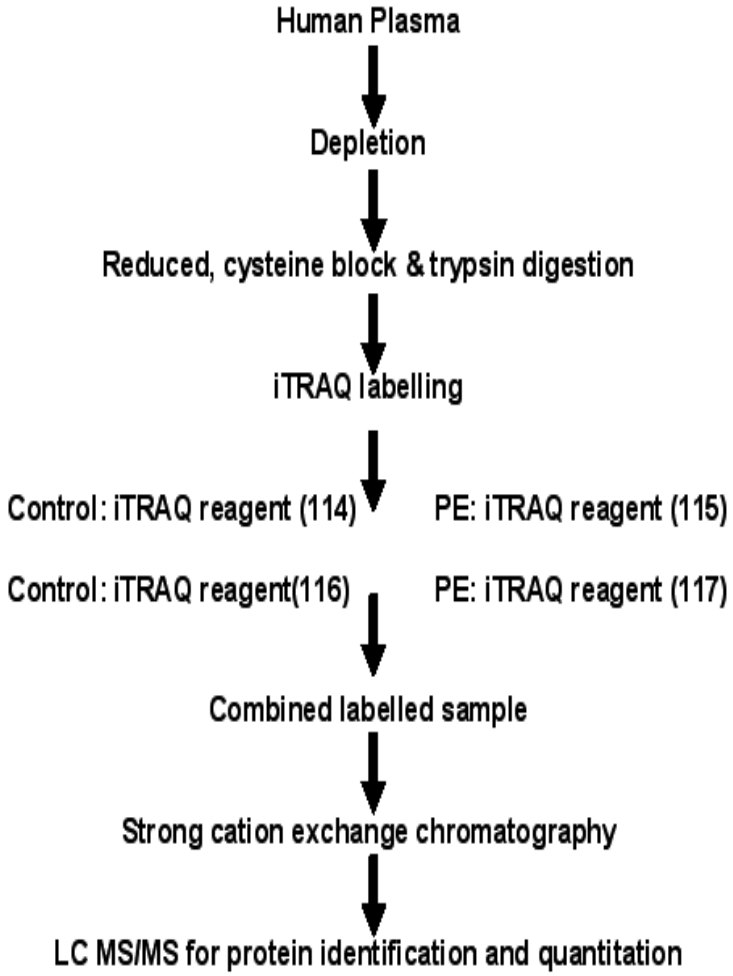

Figure 1: Workflow for quantitative proteomics using iTRAQ™ reagent. Equal amount of plasma protein (100 µg) from control and PE (n=6) were pooled separately and duplicated, controls were labeled with 114 and 116 iTRAQ™ label and DS were labeled with 115 and 117 iTRAQ™ label. The labeled samples were pooled and was subjected to a strong cation exchange chromatography to remove the excess label. Afterwards LC-MALDI MS/MS was performed for protein identification and quantification.

3.3.3 Immuno-depletion of High-Abundance Plasma Proteins

Highly abundant plasma proteins were depleted using ProteoMiner™Protein Enrichment Kit (Bio-Rad Laboratories, Inc.), as per the manufacturer's instructions. 1 ml of plasma was used for the depletion and after the whole procedure, 300 µl was eluted in elution reagent. After depletion protein concentration was measured by using RC-DC Protein assay kit (Bio-Rad Laboratories, Inc.)

3.3.4 Tryptic digestion and iTRAQ™ Reagent labelling

Equal amounts (100 µg) of depleted plasma protein from six of the PE cases and controls were pooled separately in duplicate for the iTRAQ labelling. These samples were denatured with 2% SPE in 500mM triethylammonium bicarbonate (TEAB) (Sigma-Aldrich) for 15 minutes at room temperature, following which they were reduced with 2µl of 50mM tri-(2-carboxyethyl) phosphine (TCEP) (Sigma-Aldrich) at 60°C for 1h and were then alkylated with 10mM s-Methylmethanethisulfonate (MMTS) for 10 minutes in room temperature. After alkylation, the proteins were digested overnight at 37ºC with 1U/µl trypsin (TPCK treated) (Applied Biosystems, Foster City, CA 94404, USA). Peptides were labelled with one unit of iTRAQ™ Reagent Multi-plex kit (Applied Biosystems, Foster City, CA 94404, USA) that was reconstituted in 70 µl of ethanol. iTRAQ labels 114, 116 were used separately for labelling the pooled duplicated control sample and 115, 117 were used separately to label the pooled duplicate preeclampsia samples. The iTRAQ labelling reagent solution was added to the digest and incubated for 1h at room temperature. To assess the accuracy of the ratiometric quantitation of iTRAQ reagent a split in signal was performed and the data was corrected as described in detail by Unwin and colleague [57].

3.3.5 Strong cation exchange chromatography (SCX)

Strong cation exchange chromatography was preformed to remove the excess iTRAQ reagent and interfering substances for the mass analysis. Dried peptides were resuspended in 200 ml of Buffer A and were loaded on Poly Sulfoethyl A Column (200 mm length x 4.6 id, 5 µm particle size, 200 Å pore size) on a BioLC HPLC unit (Dionex). Buffer A consisted of 10mM $KHPO_4$ and 25% acetonitrile, 500 mM KCl, pH 3.0, and Buffer B consisted of 10mM KH_2PO_4, 25% acetonitrile, and 500 mM KCl pH 3.0. The 60 min gradient comprised of 100% Buffer A for 5min, 5-30% Buffer B for 40 min, 30-100% Buffer B for 5min, 100% Buffer B for 5 min, and finally 100% Buffer A for 5 min. Ten fractions were collected using a Foxy Jr. Fraction Collector (Dionex). Subsequently, these fractions were pooled according to the chromatogram profile based on the peak intensity and the products dried in a vacuum concentrator, after which they were stored at -20ºC prior to mass spectrometric analysis.

3.3.6 Nano LC MALDI

The dried SCX iTRAQ-labeled peptides were dissolved in Buffer A which consist of 95% H_2O, 5% acetonitrile, 0.1 % TFA and were loaded on C18 trap column (1mm x 300um i.d. column) at 30 ul/min and separated on an analytical column (150 mm x 100 um i.d. column) at 500 nL/min using the LC-packing Ultimate system. The peptides were separated using a linearly increasing concentration of acetonitrile in Buffer B from 5% to 30% in 120 min, and from 30% to 60% in 40 min. The elute was mixed with matrix (2 mg/ml alpha-cyano-4-hydroxycinnamic acid in 80% acetonitrile, 0.1% TFA) at a flow rate of 800 nl/min and deposited on an Opti-TOF LC/MALDI (Applied Biosystems) plate in 10S fractions, using an automatic robot, (Probot,Dionex).

3.3.7 MS and MS/MS

The Mass spectrometer 4800 plus MALDI TOF/TOF™ Analyzer (Applied Biosystems) was set to perform data acquisition in positive ion mode. An MS condition of 1000 shots per spectrum was used. Monoisotopic precursor selection for MS/MS was done by automatic precursor selection using an interpretation method using the12 most intense peaks per spot with an MS/MS mode condition of 4000 laser shots per spectrum. Minimum peak width was one fraction and mass tolerance was 80 ppm. Adduct tolerance (m/z) +/- 0.003. MSMS was done with a gas pressure of $2x10^{-2}$ bar in the collision cell Air was used as collision gas.

Protein Identification and Database Searches:

Protein identification and quantification was done by using the ProteinPiolt™ software v2.0 (Applied Biosystems; MPE-Sciex). The search was performed against the Human database of UniProtKB/Swiss-Prot (Version 3.50) from the EBI website (www.ebi.ac.uk./IPI/IPIhelp.html) and concatenated target-decoy database search strategy was used to check the false positive rate [58] in our case it was found to be 0%, which boosted the reliability of our data.

3.3.8 Relative quantitation criteria:

The Paragon algorithm [17, 59] in ProteinPiolt v2.0 software was used as the default search program with digestion enzyme set as trypsin and methyl methanethiosulfonate as cysteine modification. The search also included the possibility of more than eighty biological modifications and amino acid substitution of up to two substitutions per peptide using the BLOSUM 62 matrix. Data was normalized for loading error by bias correction calculated with Progroup algorithm identified proteins with at least 95% confidence and with a ProtScore of 1.3, were reported. The results obtained from ProteinPilot software v2.0 software were exported to Microsoft Excel for the further analysis. The study was performed in double duplex manner, where PE samples were labelled with 115 and 117; control was labelled with 114 and 116. Peptides were selected based on the criteria defined in the protein pilot software,

which means all the peptides were included for quantitation with an exception for those without an iTRAQ modification or reporter ion, an area count less then 40 and peptides with p value less then 0.001 were excluded [60]. As described by Gan and colleague in their study on estimation of relative quantitative ratio from iTRAQ experiments, we also used only peptides above or equal to 70 % confidence level for the estimation of relative quantitation [61].

3.3.9 PANTHER analysis:

The PANTHER database was used to elucidate the molecular function, biological process and signalling pathway associated with each individual protein (http://panther.appliedbiosystems.com)

3.4 Results

Samples were obtained from 6 cases with subsequent preeclampsia and 6 samples from normal healthy deliveries. Care was taken to match both maternal and gestational age, to rule out any confounding influence of these two parameters.

Low abundant plasma proteins were enriched by using ProteoMiner™Protein Enrichment Kit. This was accomplished through the use of a large, highly diverse bead-based library of combinatorial peptide ligands. When plasma was applied to the beads, a small fraction of the high abundance proteins saturated their high affinity ligands and the excess high abundance proteins were washed away. In addition a very small amount of high abundance proteins and low abundance proteins were concentrated on their specific affinity ligands. This provides for a significant enrichment of medium and low abundant plasma proteins.

The samples were pooled separately in and duplicate in order to have more precise analytical replicate measurements. The iTRAQ analysis was done in double duplex style, the PE samples were labelled with iTRAQ 115 and 117 and the control samples with iTRAQ 114 and 116, using the work-flow illustrated in Figure.1.

Following tandem MS MS, and by focussing on iTRAQ reporter ions in low molecular mass range (114-117Da) for quantification, we identified 200 proteins with ≥ 95% confidence. However, after manually rechecking the MS/MS data thoroughly peak by peak, only 167 out of 200 proteins (83.5%) had a relative quantitation derived from the analysis of two or more peptides, while for 30 proteins, the quantitation was based on single peptide. For 3 proteins no quantitation could be ascertained by analysis using Protein Pilot. Figure.2 shows the MSMS spectrum of the precursor ([M+H] +, m/z 1259.67 Da). In low mass region the reporter ions, are seen while the where area under of the curve was used for quantification.

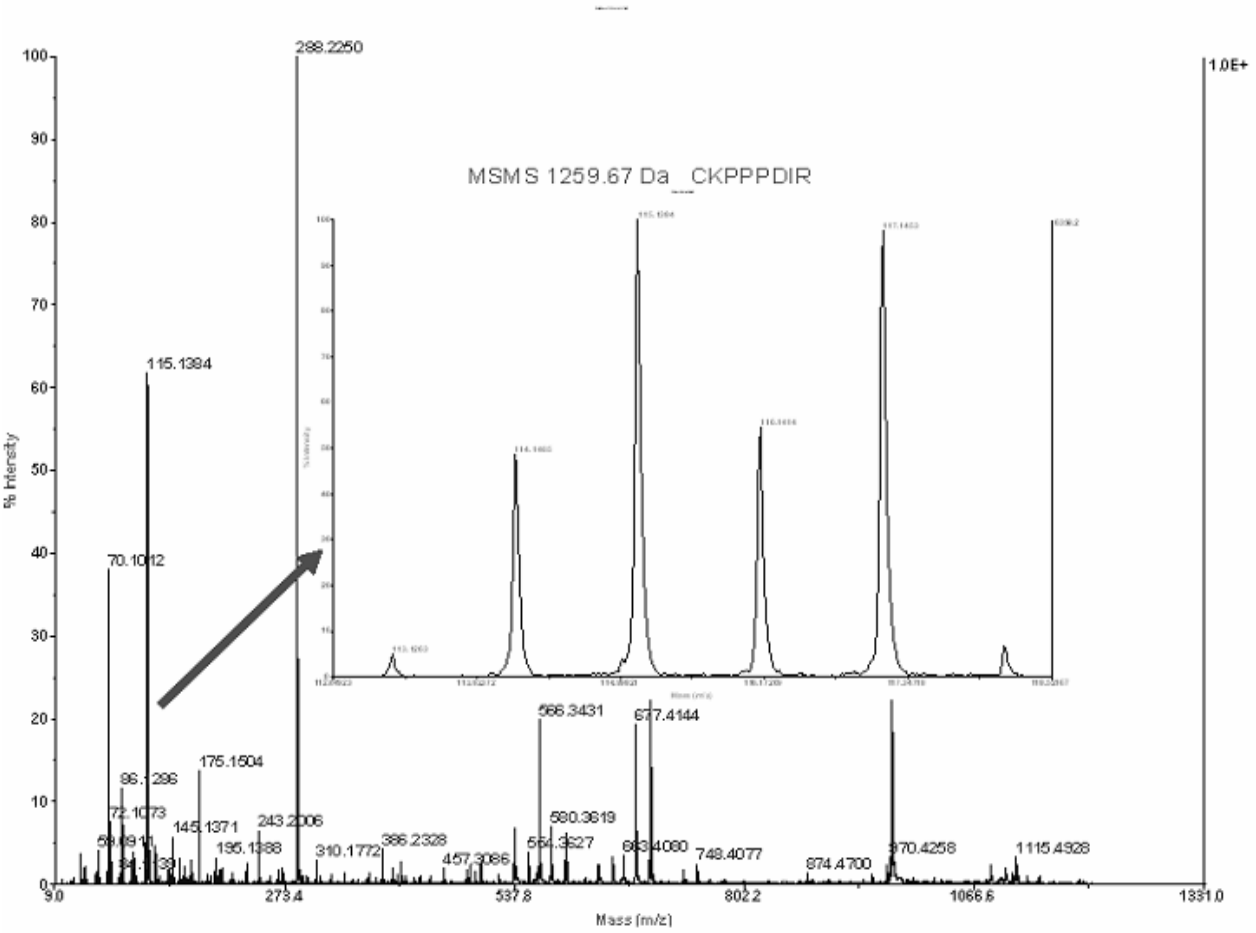

Figure 2. Components of the spectrum illustrated are (i) MSMS spectrum of the precursor ([M+H] +, m/z 1259.67 Da),(ii) low mass region showing the reporter ions used for quantitation. The peptide is labeled by isobaric tags at both the N terminus and C-terminal lysine side chain. The precursor ion and all the fragment ions therefore contain all four members of the tag set, but remain isobaric. The MSMS spectrum was obtained from the singly charged [M+H]+ peptide using a 4800 MALDI TOF-TOF analyzer

As we did the experiment in double duplex manner, PE (115 and 117) and control (114 and 116), it was possible to estimate the cut-off point for differentially expressed protein in our sample [60, 61]. Based on 167 relative abundant protein ratios from PE and control sample, an average variation of 4.4% (± 0.04) was measured. If the cut off was set at 5% average variance then only 75% of the proteins would fall with in this variation range, but. If the range was increased to 20% then about 94% of the protein falls in this variation range as illustrated in Figure.3. So the cut-off point in this experiment was set at 20% (±0.2). That means any

relative change in protein ratio below or above ± 1.2 fold was considered as differentially under or over expressed.

N	Acc. No	Protein Names	Biological Processes	Molecular Function
1	P02679	Fibrinogen Fragment D	Signal transduction	Calcium ion binding
2	P10909	Clusterin isoform 2	Compliment activation	Misfolded protein binding
3	P02647	Apolipoprotein A-I	Signal transduction	Receptor binding
4	P02751	Fibronectin	Angiogenesis	Collagen binding
5	P01019	Angiotensinogen	Blood vessel remodeling	Receptor binding
6	P09382	Galectin 3 binding	Apoptosis	Galactoside binding
7	P00750	Plasminogen	Proteolysis	Protein binding
8	P02787	Transferrin	Iron ion transporter	Ferric ion binding
9	P04003	C4beta binding	Compliment activation	Protein binding
10	P02790	Hemopexin	Heme transporter	Iron ion binding

Table 2. List of proteins unregulated in iTRAQ experiment

The functional distribution of these proteins is illustrated in Figure.3. For this interpretation, an analysis of 176 proteins was performed using the PANTHER classification system, which sorts the proteins into respective classes based on their biological process.

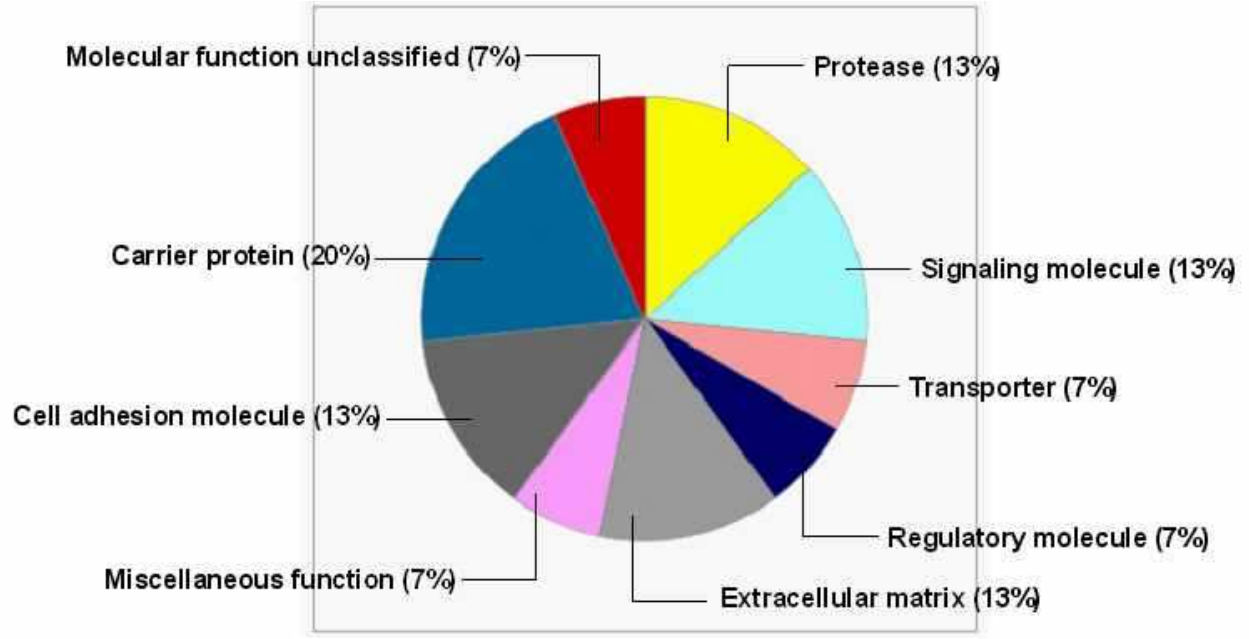

Figure 3. Number of plasma protein identified using iTRAQ reagent. In total, 167 proteins were identified. Shown above is the classification of these proteins in different category based on molecular function

It is of interest that three of the major groups involve cell adhesion molecules (13%), extra cellular matrix proteins (13%) and member of the protease family (13%), factors that are known to be aberrant in placental insufficiency apparent in preeclampsia. Further more large groups were found to involve signalling molecules (13%) and carrier proteins (20%).

A list of 10 of the most pronounced protein differences is provided in Table 2. A PANTHER sub-analysis for pathway association of these 10 proteins indicates that 33% are associated with blood coagulation, 33% with plasminogen activation, 17% with angiotensin II and 17% with integrin function shown in Figure 4.

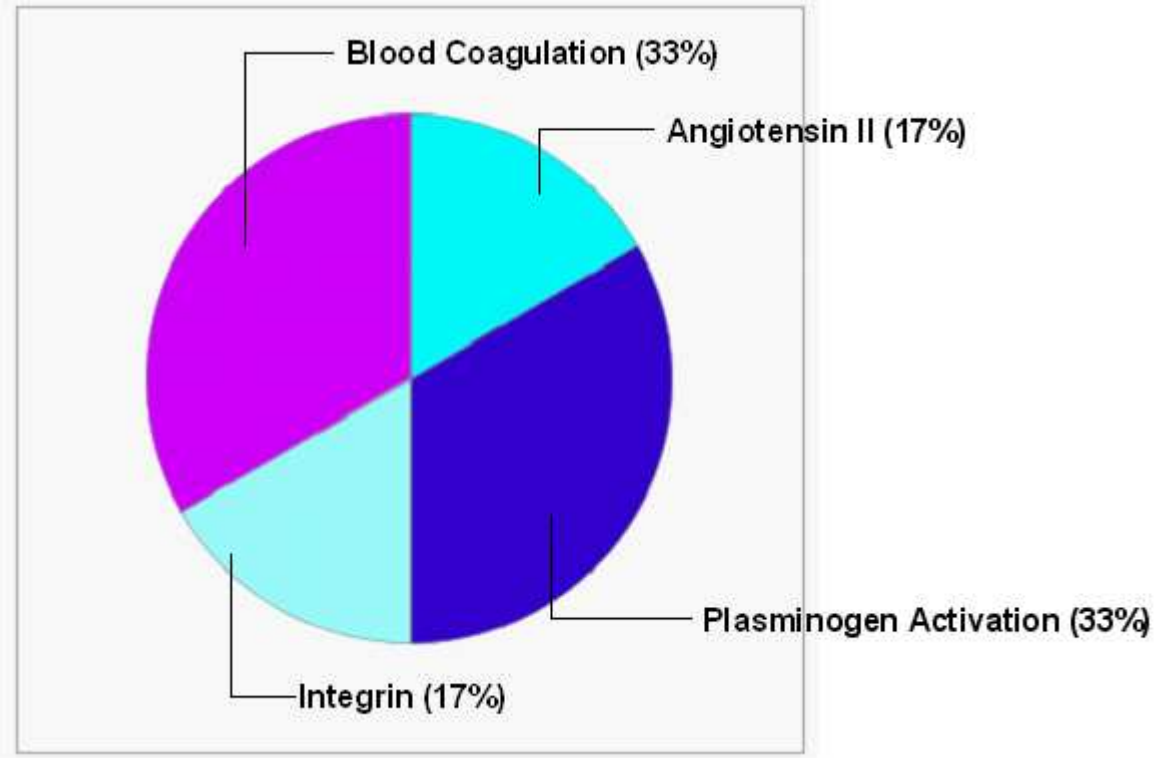

Figure 4. PANTHER analysis for pathway. In total, 10 proteins were identified as elevated. Shown above is the different signaling pathways hit by these protein.

3.5 Discussion:

The comparative quantitative assessment of serum or plasma proteins via iTRAQ isobaric labelling combined with MALDI-TOF/TOF analysis has suggested to be suitable for the detection of biomarkers. This is largely due to the highly reproducible nature of the system, which exhibits little run-to-run variation, determined after a lengthy comparison of pooled and individual of case and control samples, yielding a coefficient of variation of 11.7% [20].

In a previous proof-of- principle study, we examined whether this approach was suitable for the detection of screening markers that could be sued to screen for pregnancies with a Down syndrome fetus [56]. This analysis indicated that this approach may indeed be valid, as we were capable of detecting known screening markers, since βhCG was determined to amongst pool of elevated proteins.

In a preliminary proof-of-principle experiment, we have now assessed whether this method could be suitable for the detection of biomarkers useful for PE screening. From our small-scale preliminary evaluation we identified over 10 proteins whose concentration was altered in the plasma of pregnancies with a PE compared to those with normal pregnancies.

It is of interest that glycoprotein clustrine isoform 2 was found elevated in our study. In one of the recent study Blumenstein and colleague [62] has also seen the elevation in isoform of clusterin. Other protein like angiotensinogen, which is was reported in PE to be elevated. The presence of this important screening marker in our pool of elevated plasma proteins suggests that the strategy we have chosen for the identification of new biomarkers is functional and worthy of further pursuit.

That our assay is indeed detecting proteins of placental origin is illustrated by presence of galectin 3which is a glycoprotein and derive its importance in pregnancy by playing potential role in preventing the attack from maternal immune system on the developing fetus which can be seen as foreign allograft [63]. This protein was amongst our list of up-regulated proteins,

apolipoprotein E3 isoform. The altered production of apoE3 in PE might impair reverse cholesterol transport contributing to arterial damage [64].

Elevations in a number of inflammatory molecules C4 beta and fibronectin, most of which are probably of maternal origin, may be a reflection of the elevated release of placental debris which has been suggested to occur in pregnancies with PE. Williams and colleague has reported the elevation of and fibrinogen in PE cases [65].

In our follow up studies we would like to validate these putative biomarkers using immunoblot and Enzyme linked immunosorbent assay (ELISA). More recently Selected Reaction Monitoring (SRM) has evolved as a method of choice for validation of biomarkers using mass spectroscopy.

The increasing popularity of the iTRAQ approach due to its reproducibility and robustness, including studies for cancer or inflammatory autoimmune disorder specific biomarkers suggests that it will become the method of choice for future studies, until it is surpassed by a new technical development. As pregnancy represents a unique constellation, whereby a foreign being is supported and nourished by the host, it may serve as an ideal model for proteomic analyses, as any unique markers should ideally disappear post delivery. Furthermore, as very few specific biomarkers exist to assist with the screening of a number of pregnancy related disorders, especially preeclampsia or preterm labour, it is likely that this will become the focus of considerable research attention in the near future.

3.6 Conclusion:

In this report we conclude, isobaric labelling technique is a suitable approach for the quantitative detection of new screening biomarkers in the plasma of pregnancies with a PE compared to those with normal. In this preliminary proof-of-principle study, we were able to detect quantitatively under or over expressed proteins. In the future additional studies, using

larger sample sizes will be required to identify a panel of biomarkers which can be used in screening for PE pregnancies.

Acknowledgment:

We thank Vivian Kiefer for her technical assistance and Prof. E. Palmer and Dr. D. Huang for the proof reading of the manuscript. This study was supported by PREGENESYS (ref.no. 37244), Sixth Frame Work (FP6) grant.

4 Examination of the 1st trimester maternal plasma proteome by SELDI in pregnancies at risk for Preeclampsia

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4.1 Abstract

4.1.1 Background

Currently no reliable 1st trimester screening method exists to detect for pregnancies which will develop preeclampsia. This deficit makes it difficult to develop appropriate intervention strategies. As the underlying placental aetiology leading to the development of preeclampsia is thought to occur early during gestation, we hypothesised that such changes may already be evident in the maternal plasma proteome. Hence, such unique proteomic fingerprints could be used to distinguish between pregnancies with healthy deliveries form those which developed preeclampsia. For this purpose we investigated the use of Surface-Enhanced Laser Desorption/Ionization Time-of-Flight mass spectrometry (SELDI-TOF-MS) SELDI.

4.1.2 Results

Unique spectral profiles were generated using SELDI-TOF-MS.A total of 8 peaks corresponding to peptides and proteins in the range between 3.2 kDa and 22.2 kDa were identified from a set of 26,707 representing the whole spectral analysis (0 to 60 kDa), were identified which could discriminate pregnancies with normal deliveries to those which developed preeclampsia.

4.1.3 Conclusion

Maternal plasma proteome fingerprinting by SELDI-TOF MS may lead to the development of a set of markers which can be used in discern pregnancies at risk for preeclampsia.

4.2 Introduction

In the last two decades several biochemical and biophysical traits were identified which led to a considerable improvement in the screening of preeclampsia (PE). But there is no reliable screening biomarker for PE. So there is a need in improve both the sensitivity and specificity of current screening strategies. As considerable efforts has being invested in the discovery and development of further markers. Platforms based on proteomics [66] coupled with mass spectrometry (MS) techniques [21] is gaining considerable acceptance following the identification of different markers associated with several types of disease conditions such as ectopic pregnancies [67], detection chromosomal abnormalities [22, 23], ovarian cancer [68, 69], diabetic nephropathy [70] and inflammatory bowel disease [71]. The current practice of using single protein biomarkers will most likely give way to the use of multiplexed biomarkers, as they promise better sensitivity and specificity [72]. High-throughput platforms using MS permit profiling large numbers of samples in relatively shorter time mostly within the same day of admission. Using proteomics platform to mine deep into the plasma proteome has resulted in the identification of several target candidates for Down's syndrome [73, 74]. Moreover proteomic signatures can reduce problems with individual variability in peak detection [75]. We hypothesized that by using a combination of unique serum proteomic features (hydrophobic proteins) in protein profiles and differentially expressed proteins (hydrophilic proteins) could be employed to distinguish pregnancies with trisomy fetuses from control healthy conditions.

4.3 Materials and Methods

4.3.1 Samples

Blood samples for this case-control proteome study were collected prospectively from pregnant women at approximately 12 weeks of gestation. In a retrospective manner, 6

samples from cases that subsequently developed preeclampsia were matched with 6 samples from pregnancies with normal healthy outcome (Table 1). This study was undertaken with the approval of the Institutional Ethical Board of the University Hospital, Basel, Switzerland and written informed consent was required in all instances.

4.3.2 Sample preparation

As described previously, 9 ml blood was drawn into BD P100 tubes (BD Diagnostics, Franklin Lake, NY, USA), which are specially designed for proteomics experiments, in that the EDTA (Ethylenediaminetetraacetic acid) and protease inhibitor present in the tube prevent coagulation and stabilize the plasma proteome. Following phlebotomy the samples were centrifuged at 3,000x g for 30 minutes at 10ºC, whereby the plasma was separated from the cellular fraction by aid of a mechanical separator, 100µl aliquots were stored at -80ºC until further use.

(A)

	Control	PE
Maternal age (years)	35.1±1.3	35.1±6.8
Gestational age (weeks)	38.8±0.8	34.9±2.4
Systolic BP (mmHg)	122.5±14.2	188.8±18.6
Diastolic BP (mmHg)	73.6±7.0	111.8±14.1
Proteinuria (dipstick)	Negative	+++

(B)

	Control	PE
Gestational age (weeks)	12.4±1.2	12.3±1.1

Table 1. Characteristic of study population. (A)Criteria used for pregnancy with or without preeclampsia for the longitudinal study. (B) Gestational window selected for the current study (n=6) who subsequently developed the preeclampsia

4.3.3 Plasma protein profiling

Protein profiling was performed using the ProteinChip™ Biomarker System (Ciphergen Biosystems®, Freemont, CA, USA), a SELDI-TOF MS platform. We used H50 (C8 reversed phase/hydrophobic interaction chromatography) ProteinChip™ Array. Samples were applied to H50 protein chip arrays (Ciphergen, Fremont, CA, USA) according to the manufacturer's protocol. Saturated solution of sinapinic acid in 50% acetonitrile, 0.5% trifluoroacetic acid was applied twice to each spot on the array, with air drying between each application. To minimize bias, plasma samples from mothers who developed PE and those with normal outcome were assayed on the same chips. Plasma samples were analyzed using the Protein Biology System 2 SELDI-TOF mass spectrometer (Ciphergen Biosystems). Peptides and

proteins below the 60 kDa range were ionized on a α-cyano-4-hydroxy-cinnamic acid matrix. Known proteins were used for data calibration between experiments, and the mass accuracy was determined daily using the Ciphergen Broad Range molecular weight standards (C100-0001, Ciphergen Biosystems). Chips with plasma samples were analyzed under the following conditions: laser intensity 260V, detector sensitivity 10, mass focus 30 kDa, with molecular mass/charge (m/z) range from 0-60 kDa and mass optimization 3-60 kDa. Data were collected by averaging 65 laser shots per sample. These were exported as raw data (Ciphergen PBSII™ software), and used without modification for downstream bioinformatics analyses. Spectra were calibrated, baseline subtracted, and normalized. Qualified mass peaks (signal/noise >5; cluster mass window at 0.3%) within the m/z range of 2–60 kDa were selected automatically. Logarithmic transformation was applied to the peak intensity before analysis for biomarker discovery. After biomarker discovery, the quality and intensity readings of the selected peaks were manually reconfirmed from raw spectra.

4.3.4 Bioinformatics approach

We analyzed spectra of 6 control and 6 cases which developed PE. Each spectrum had 26,707 mass spectral components. To reduce non-specific peaks especially in the low mass range, we eliminated the first 5,989 components that all corresponded to masses < 3kDa. We manually inspected the remaining part of the spectra and selected 62 apparent peaks in the spectra with the aim to select few that could be used to classify PE cases and separate them from the control healthy cases. These 62 peaks served to generate initial feature set based on which the individual cases were analyzed.

4.3.5 Classification process and final selection of features

All classification tasks were done in the same fashion, distinguishing between cases which developed PE and those with normal deliveries, based on the Mahalanobis distance between the groups using the Lawrence and Solovyev algorithm [76]. We also searched for the minimum number of features and the small portion of the data in the training set, so as to achieve good generalization properties of the linear discriminate model. The process involved separation of each of the groups to the training part and the test part. The model is built using only the training data, and it is tested using the test data.

4.4 Results and Discussion:

Unique spectral profiles were generated using SELDI-TOF-MS method (Figure1). A total of 8 spectral peaks corresponding to peptides and proteins in the range between 3.2 kDa and 22.2 kDa were identified from a set of 26,707 features representing the whole spectra (0 to 60 kDa) (Table 1; Figure 1a & 1b).

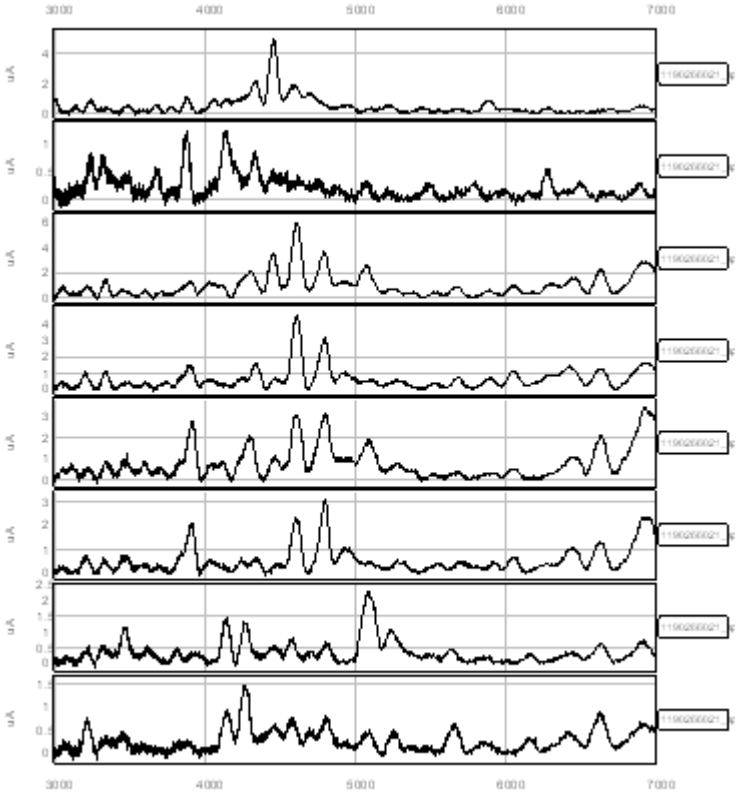

Figure 1a. Representation of peak identities corresponding to proteomic features identified using bioinformatics approach to discriminate healthy controls and PE cases that subsequently developed the preeclampsia.

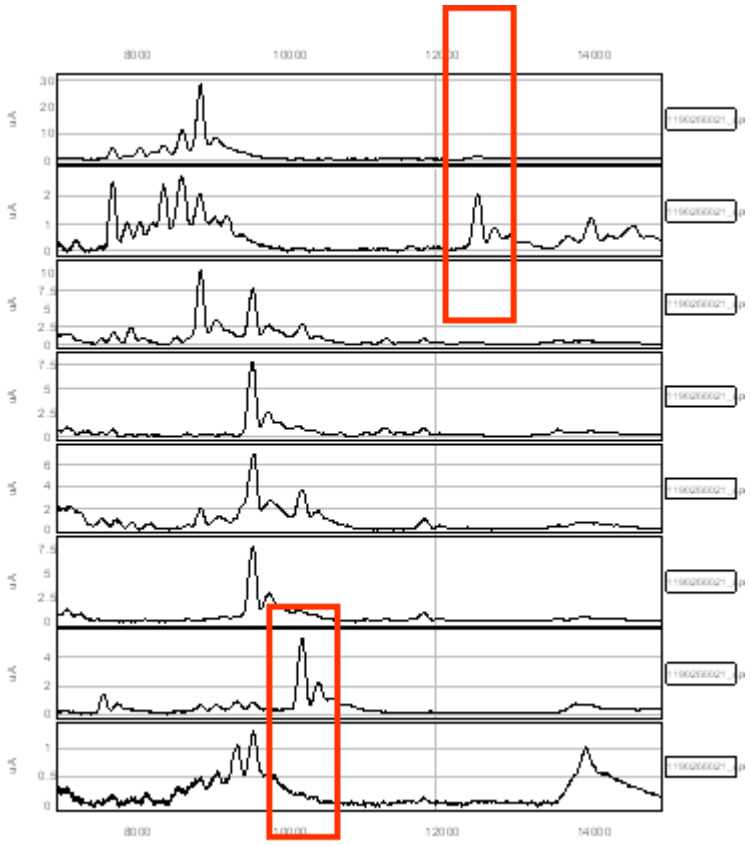

Figure 1b. Representation of peak identities corresponding to proteomic features identified using bioinformatics approach to discriminate healthy controls and PE cases that subsequently developed the preeclampsia

Eight features were identified which could discriminate all control pregnancies from the PE – before onset of PE.

	m/z Dalton								
Peaks	**7.7**	**8.4**	**8.9**	**9.2**	**10.2**	**12.6**	**13.9**	**22.2**	**IMAC-Cu**
Pre-eclapmsia	Y	Y	N	Y	**Y**	Y	Y	Y	
Control	N	N	Y	N	Y	N	N	N	

Table 2. Proteomic features identified to discriminate PE conditions from normal are based on protein profiling technique. Y is present and N is absent for peak.

Using SELDI platforms we tried to identify unique fingerprints of protein markers for pregnancies at risk for PE. Some studies have previously reported on the use of maternal serum [21, 23] and plasma [73]. The current study addresses these two important issues. Results from our study indicates that it might be possible to effectively screen for pregnancies at risk for PE using 2nd trimester maternal plasma samples and generating proteomic fingerprints by SELDI-TOF MS analysis.

Protein profiling is a powerful technique to look at several hundred proteins in a single spectrum [18].

Human serum is known to contain a complex mixture of different kinds of protein and peptides [77], and it is suggested that a success in distinguishing healthy person from a patient could be improved with the identification of unique features in protein profiles [78]. The number of distinct features of spectral information detected in the mass spectrum of a processed sample is a strong indicator of the information content of the signature. Recent studies have focussed on using the power of SELDI techniques to study amniotic fluid, cervical vaginal fluid for various disease conditions associated with the developing fetus such as inflammation, infection [79], and neonatal sepsis and also to investigate into the mechanisms of idiopathic preterm birth [80]. The same approach has been used to detect

ectopic pregnancies [67] and as well as Down syndrome [23]. Till now this approach has not been explored for prenatal screening in a large scale as well as in clinical settings. In the current analysis, the mass (mw) of the markers ranged between 3 kDa and 60 kDa with majority of the markers in the range less than 22 kDa.

4.5 Conclusions

In summary, maternal plasma proteomic profiling with SELDI may prove to be a useful tool for the screening of pregnancies at risk for PE. The accuracy of this approach, however, requires large scale verification before it can be considered for clinical use.

5 Publication

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Research Article

Quantitative Proteomics Analysis of Maternal Plasma in Down Syndrome Pregnancies Using Isobaric Tagging Reagent (iTRAQ)

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Currently no specific biomarkers exist for the screening of pregnancies at risk for down syndrome (DS). Since a quantitative proteomic approach with isobaric labelling (iTRAQ) has recently been suggested to be highly suitable for the discovery of novel plasma biomarkers, we have now used this method to examine for potential quantitative changes in the plasma proteome of the pregnancies bearing DS fetuses in comparison to normal healthy babies. In our study, we used plasma from six women with DS pregnancies and six with uncomplicated pregnancies care were taken to match cases and controls for gestational and maternal age, as these could be a confounder. In our quantitative proteomics analysis we were able to detect 178 proteins using iTRAQ labelling in conjunction with 4800 MALDI TOF/TOF. Amongst these we observed changes in βHCG, a known screening marker for DS, indicating that our assay was functional. We found a number of elevated proteins Ig lambda chain C region, serum amyloid P-component, amyloid beta A4, and under expressed proteins like gamma-actin and titin in DS pregnancies. These proteins are also found in the sera of patients with Alzheimer disease, which share similar pathologies of DS. Our study therefore indicates that the iTRAQ labelling approach may be indeed useful for the detection of novel biomarkers.

1. Introduction

Down syndrome (DS) is the most common chromosomal aneuploidy in live births and a leading cause of mental retardation. Prenatal detection of chromosomal anomalies, such as trisomy 21, in cases with DS relies on invasive practices such as amniocentesis or chorionic villi sampling. These procedures are associated with a risk of fetal loss or adverse pregnancy outcome [1]. Furthermore, it requires special facilities and highly trained staff. For this reason alternative strategies are sought, such as the noninvasive prenatal diagnosis of fetal genetic anomalies via the analysis of rare trafficking fetal cells [2] or cell-free fetal nucleic acids in maternal blood [3]. As this long sought goal has not yet reached clinical implementation [4], current clinical practice relies on a series of screening steps aimed at detecting at-risk pregnancies. In many centres the screening procedure for DS is carried out in the late first and early second trimester (11–14 weeks of gestation) and involves a combination of ultrasound and serum marker analysis [5]. Although the efficacy of this method has improved considerably, and is more accurate than previous second trimester screening approaches, it is hampered by the skill and precision of the ultrasonographer and external factors such as ethnicity or other factors, such as assisted reproductive technologies.

Hence, it is obvious that new tools are needed to improve current state of the art. One such strategy is to look for new potential biomarkers using noninvasive proteomic approaches. The rationale for this strategy is that placentae from fetuses with DS, especially the villi, are morphologically distinct from the placentae of euploid fetuses, a feature attributed to altered aberrant protein expression [6]. As the placenta, a large organ with rapid cell turn-over is in direct contact with the maternal circulation, proteins

Table 1: Patients with and without a down syndrome pregnancy at the time of screening $n = 6$.

	First trimester	
	DS	Control
Maternal age (years)	35.8 ± 4.3	35.1 ± 4.1
Gestational age (weeks)	12.4 ± 1.2	12.5 ± 1.1

released by it should be detectable in maternal plasma, and could consequently serve as promising markers for abnormal placentation involved in a number of pregnancy related disorders. In this regard, preliminary proteomic investigations have shown that quantitative alterations in protein expression are found to occur in the amniotic fluid [7, 8] and maternal serum of pregnancies with fetuses affected with down syndrome, and that these could serve as new potential biomarkers [9].

Unfortunately none of these studies used quantitative approaches of the type that permit precise assessment of the extent of up- or down-regulation of the proposed biomarkers. As such, it will be difficult to validate these in blinded studies of clinical serum or plasma samples, a crucial facet in determining their specificity [9–11].

These studies are further complicated by the complex nature of the plasma/serum proteome, whereby the majority of low abundance proteins are masked by the preponderance of a few highly abundant proteins. This high dynamic range [12] effectively precludes the use of more conventional proteomic strategies, such as those employing gel electrophoresis with or without or fluorescent labelling, for example, Difference Gel Electrophoresis (DIGE). This deficit renders the identification of potential biomarkers in plasma/serum by conventional comparative proteomic approaches very challenging.

For this reason an approach using Isobaric Tags for Absolute and Relative Quantitation (iTRAQ) has been proposed for the discovery of plasma biomarkers [13]. This chemical labelling method involves the stable incorporation of isotopes into an amine tagging reagent, which can be reliably detected by mass spectrometry, thereby permitting comparative quantitation in a multiplex manner. Currently 4-plex and 8-plex reagents are commercially available, which are used to label the protein samples of interest following trypsin digestion. The use of different isobaric tags implies that up to 4 or 8 different samples, one of which serves as a reference, can be examined simultaneously in a single mass spectrometric analysis. It is for this reason that the iTRAQ approach has been suggested to be suitable for the discovery of biomarkers in a wide range of body fluids and tissues, including plasma.

In order to examine whether this approach would be suitable for the detection of potential biomarkers for down syndrome, we performed a proof-of-principle experiment, in which we examined samples from 6 cases with down syndrome in comparison to 6 matching controls. In our study we have used a 4-plex iTRAQ labelling in conjunction with a 4800 MALDI TOF/TOF approach to examine plasma samples obtained in first trimester pregnancies. Our results indicate that quantitative differences can be detected between aneuploid samples and samples from euploid pregnancies and that such alteration may reflect upon changes known to occur in down syndrome.

2. Materials and Methods

2.1. Samples. Blood samples for this case-control proteome study were collected from six pregnant women carrying a DS fetus (11–13 weeks of gestation) and six pregnant women with normal euploid pregnancies. The samples were matched for maternal and gestational age (**Table 1**). This study was undertaken with the approval of the Institutional Ethical Board of the University Hospital, Basel, Switzerland and written informed consent was required in all instances.

2.2. Sample Preparation. 9 mL blood was drawn into BD P100 tubes (BD Diagnostics, Franklin Lake, NY, USA), which are specially designed for proteomics experiments, in that the Ethylenediaminetetraacetic acid (EDTA) and protease inhibitor present in the tube prevent coagulation and stabilize the plasma proteome. Following phlebotomy the samples were centrifuged at 3000× g for 30 minutes at 10°C, whereby the plasma was separated from the cellular fraction by aid of a mechanical separator. 100 μL aliquots were stored at −80°C until further use. For an overview of the work-flow used in this analysis refer to **Figure 1**.

2.3. Immunodepletion of High-Abundance Plasma Proteins. Highly abundant plasma proteins were depleted using ProteoMiner Protein Enrichment Kit (Bio-Rad Laboratories, Inc.) [14], as per the manufacturer's instructions. 1 mL of plasma was used for the depletion and after the whole procedure, 300 μL was eluted in elution reagent. After depletion protein concentration was measured by using RC-DC Protein assay kit (Bio-Rad Laboratories, Inc.)

2.4. Trypic Digestion and iTRAQ Reagent Labelling. Equal amounts (100 μg) of depleted plasma protein from six of the DS cases and controls were pooled separately in duplicate for the iTRAQ labelling. These samples were denatured with 2% SDS in 500 mM triethylammonium bicarbonate (TEAB) (Sigma-Aldrich) for 15 minutes at room temperature, following which they were reduced with 2 μL of 50 mM tri-(2-carboxyethyl) phosphine (TCEP) (Sigma-Aldrich) at 60°C for 1 hour and were then alkylated with 10 mM s-Methylmethanethisulfonate (MMTS) for 10 minutes in room temperature. After alkylation, the proteins were digested overnight at 37°C with 1 U/μL trypsin (TPCK treated) (Applied Biosystems, Foster City, CA 94404, USA). Peptides were labelled with one unit of iTRAQ Reagent Multi-plex kit (Applied Biosystems, Foster City, CA 94404, USA) that was reconstituted in 70 μL of ethanol. iTRAQ labels 114, 116 were used separately for labelling the pooled duplicated control sample and 115, 117 were used separately to label the pooled duplicate down syndrome samples. The iTRAQ labelling reagent solution was added to the digest and incubated for 1 hour at room temperature. To assess the

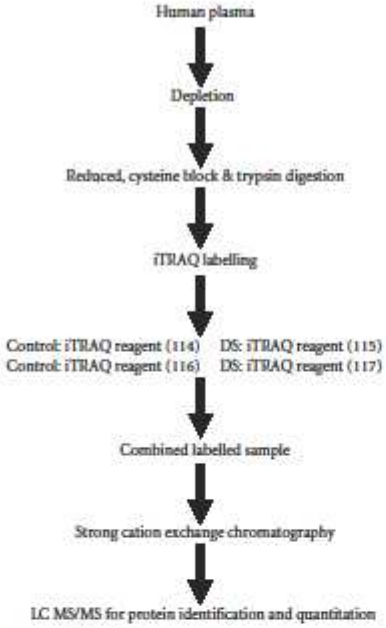

Figure 1: Workflow for quantitative proteomics using iTRAQ reagent. Equal amounts of plasma protein (100 μg) from control and DS ($n = 6$) were pooled separately and duplicated, controls were labeled with 114 and 116 iTRAQ label and DS was labeled with 115 and 117 iTRAQ label. The labeled samples were pooled and were subjected to a strong cation exchange chromatography to remove the excess label. Afterwards LC-MALDI MS/MS was performed for protein identification and quantification.

accuracy of the ratiometric quantitation of iTRAQ reagent a split in signal was performed and the data were corrected as described in detail by Unwin and colleague [15].

2.5. Strong Cation Exchange Chromatography (SCX). Strong cation exchange chromatography was preformed to remove the excess iTRAQ reagent and interfering substances for the mass analysis. Dried peptides were resuspended in 200 μL of Buffer A and were loaded on Poly Sulfoethyl A Column (200 mm length × 4.6 id, 5 μm particle size, 200 pore size) on a BioLC HPLC unit (Dionex). Buffer A consisted of 10 mM $KHPO_4$ and 25% acetonitrile, 500 mM KCl, pH 3.0, and Buffer B consisted of 10 mM KH_2PO_4, 25% acetonitrile, and 500 mM KCl pH 3.0. The 60 minutes gradient comprised of 100% Buffer A for 5 minutes, 5%–30% Buffer B for 40 minutes, 30%–100% Buffer B for 5 minutes, 100% Buffer B for 5 minutes, and finally 100% Buffer A for 5 minutes. Ten fractions were collected using a Foxy Jr. Fraction Collector (Dionex). Subsequently, these fractions were pooled according to the chromatogram profile based on the peak intensity and the products dried in a vacuum concentrator, after which they were stored at −20°C prior to mass spectrometric analysis.

2.6. Nano LC MALDI. The dried SCX iTRAQ-labeled peptides were dissolved in Buffer A which consist of 95% H_2O, 5% acetonitrile, 0.1% TFA and were loaded on C18 trap column (1 mm × 300 μm i.d. column) at 30 μL/minutes and separated on an analytical column (150 mm ×100 μm i.d. column) at 500 nL/min using the LC-packing Ultimate system. The peptides were separated using a linearly increasing concentration of acetonitrile in Buffer B from 5% to 30% in 120 minutes, and from 30% to 60% in 40 minutes. The elute was mixed with matrix (2 mg/mL alpha-cyano-4-hydroxycinnamic acid in 80% acetonitrile, and 0.1% TFA) at a flow rate of 800 nl/min and deposited on an Opti-TOF LC/MALDI (Applied Biosystems) plate in 10S fractions, using an automatic robot (Probot, Dionex).

2.7. MS and MSMS. The Mass spectrometer 4800 plus MALDI TOF/TOF Analyzer (Applied Biosystems) was set to perform data acquisition in positive ion mode. An MS condition of 1000 shots per spectrum was used. Monoisotopic precursor selection for MS/MS was done by automatic precursor selection using an interpretation method using the 12 most intense peaks per spot with an MS/MS mode condition of 4000 laser shots per spectrum. Minimum peak width was one fraction and mass tolerance was 80 ppm. Adduct tolerance is (m/z) ± 0.003. MSMS was done with a gas pressure of 2×10^{-2} bar in the collision cell. Air was used as collision gas.

2.8. Protein Identification and Database Searches. Protein identification and quantification was done by using the ProteinPiolt software v2.0 (Applied Biosystems; MDS-Sciex). The search was performed against the Human database of UniProtKB/Swiss-Prot (Version 3.50) from the EBI website (**http://www.ebi.ac.uk./IPI/IPIhelp.html**) and concatenated target-decoy database search strategy was used to check the false positive rate [16] in our case it was found to be 0%, which boosted the reliability of our data.

2.9. Relative Quantitation Criteria. The Paragon algorithm [17, 18] in ProteinPiolt v2.0 software was used as the default search program with digestion enzyme set as trypsin and methyl methanethiosulfonate as cysteine modification. The search also included the possibility of more than eighty biological modifications and amino acid substitution of up to two substitutions per peptide using the BLOSUM 62 matrix. Data were normalized for loading error by bias correction calculated with Progroup algorithm Identified proteins with at least 95% confidence and with a ProtScore of 1.3, were reported. The results obtained from ProteinPiolt software v2.0 software were exported to Microsoft Excel for the further analysis. The study was performed in double duplex manner, where DS samples were labelled with 115 and 117, control were labelled with 114 and 116. Peptides were selected based on the criteria defined in the protein pilot software, which means all the peptides were included for quantitation with

an exception for those without an iTRAQ modification or reporter ion, an area count less than 40 and peptides with P value less than .001 were excluded [19]. As described by Gan and colleague in their study on estimation of relative quantitative ratio from iTRAQ experiments, we also used only peptides above or equal to 70% confidence level for the estimation of relative quantitation [20].

2.10. PANTHER Analysis. The PANTHER database was used to elucidate the molecular function, biological process and signaling pathway associated with each individual protein (**http://panther.appliedbiosystems.com/**).

3. Results

Samples were obtained from 6 cases with a confirmed DS fetus and 6 samples from normal euploid singleton pregnancies. Care was taken to match both maternal and gestational age, to rule out any confounding influence of these two parameters.

Low abundant plasma proteins were enriched by using ProteoMiner Protein Enrichment Kit. This was accomplished through the use of a large, highly diverse bead-based library of combinatorial peptide ligands. When plasma was applied to the beads, a small fraction of the high abundance proteins saturated their high affinity ligands and the excess high abundance proteins were washed away. In addition a very small amount of high abundance proteins and low abundance proteins were concentrated on their specific affinity ligands. This provides for a significant enrichment of medium and low abundant plasma proteins.

The samples were pooled separately in and duplicate in order to have more precise analytical replicate measurements. The iTRAQ analysis was done in double duplex style, the DS samples were labelled with iTRAQ 115 and 117 and the control samples with iTRAQ 114 and 116, using the work-flow illustrated in **Figure 1**.

Following tandem MS MS, and by focussing on iTRAQ reporter ions in low molecular mass range (114–117 Da) for quantification, we identified 235 proteins with ≥95% confidence. However, after manually rechecking the MS/MS data thoroughly peak by peak, only 187 out of 235 proteins (78.5%) had a relative quantitation derived from the analysis of two or more peptides, while for 45 proteins, the quantitation was based on single peptide. For 3 proteins no quantitation could be ascertained by analysis using Protein Pilot. **Figure 2** shows the MSMS spectrum of the precursor ([M + H] +, m/z 1527.7 Da). In low-mass region the reporter ions, are seen while area under the curve was used for quantification.

As we did the experiment in double duplex manner, DS (115 and 117) and control (114 and 116), it was possible to estimate the cutoff point for differentially expressed protein in our sample [19, 20]. Based on 187 relative abundant protein ratios from DS and control sample, an average variation of 4.4% (±0.04) was measured. If the cutoff was set at 5% average variance then only 72% of the proteins would fall with in this variation range, but. If the range was

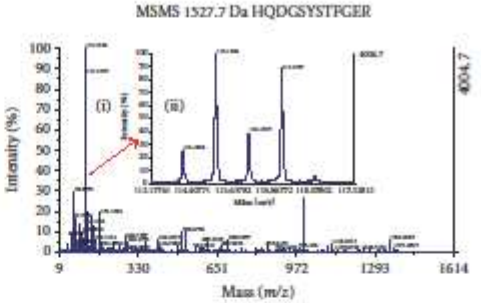

Figure 2: Components of the spectrum illustrated are (i) MSMS spectrum of the precursor ([M + H] +, m/z 1527.7 Da). (ii) low-mass region showing the reporter ions used for quantitation. The peptide is labeled by isobaric tags at both the N terminus and C-terminal lysine side chain. The precursor ion and all the fragment ions therefore contain all four members of the tag set, but remain isobaric. The MSMS spectrum was obtained from the singly charged [M + H] + peptide using a 4800 MALDI TOF-TOF analyzer.

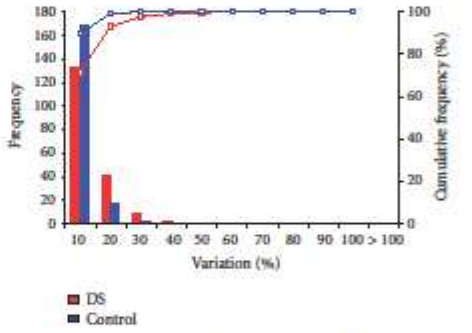

Figure 3: Frequency distribution (bars) from both DS and control replicates across different ranges of variation. The cumulative percentage (lines) is defined as the cumulative number of proteins falling within the defined variation range against the total number of protein.

increased to 20% then about 92% of the protein falls in this variation range as illustrated in **Figure 3**. So the cutoff point in this experiment was set at 20% (±0.2). That means any relative change in protein ratio below or above ±1.2 fold was considered as differentially under or over expressed.

The functional distribution of these proteins is illustrated in **Figure 4**. For this interpretation, an analysis of 235 proteins was performed using the PANTHER classification system, which sorts the proteins into respective classes based on their biological process.

It is of interest that two of the major groups involve cell adhesion molecules (13%) and extra cellular matrix proteins (18%) and member of the protease family, factors that are known to be aberrant in down syndrome. Further more large

Table 2: List of the protein identified as up-regulated from the iTRAQ experiment, indicating is the biological process and molecular function of these proteins. (P value. 001).

No.	Acc. number	% Cov	Protein name	Biological processes	Molecular functions
1	P20742	31.5	Pregnancy zone protein	Ligand-mediated signaling	Other cytokine
2	P04003	30	C4b-binding protein alpha chain	Complement-mediated immunity	Complement component
3	P01842	68.6	Ig lambda chain C regions	Immunity	Immunoglobulin
4	P01233	26.1	Choriogonadotropin subunit beta	mRNA transcription	Other signaling molecule
5	P43251	14.1	Biotinidase	Vitamin metabolism	Other hydrolase
6	P01834	84	Ig kappa chain C region	Immunity	Immunoglobulin
7	P01859	42	Ig gamma-2 chain C region	Immunity	Immunoglobulin
8	P01215	6	Glycoprotein hormones alpha chain	Protein targeting and localization	SNARE protein
9	P80108	16.5	Phosphatidylinositol-glycan	Intracellular signaling cascade	Lipase
10	P01860	53.8	Ig gamma-3 chain C region	B-cell- and antibody-mediated immunity	Immunoglobulin
11	Q9UGM5	27.5	Fetuin-B	Intracellular signaling cascade	Lipase
12	P35858	26	Insulin-like growth factor	Cell adhesion	Receptor
13	P26927	22.9	Hepatocyte growth factor-like protein	Ligand-mediated signaling	Growth factor
14	P02751	49.4	Fibronectin	Extracellular matrix protein	Cell adhesion molecule
15	P02743	54.3	Serum amyloid P-component	Amino acid biosynthesis	Synthase
16	P00751	37.7	Complement factor B	Proteolysis	Serine protease
17	P01031	42.6	Complement C5	Complement-mediated immunity	Complement component
18	P07358	36.5	Complement component C8 beta chain	Complement-mediated immunity	Complement component
19	P02790	67.3	Hemopexin	Vitamin/cofactor transport	Carrier protein
20	P00734	61.3	Prothrombin	Blood clotting	Serine protease
21	Q14624	54.8	Inter-alpha-trypsin inhibitor heavy chain H4	Proteolysis	Serine protease inhibitor
22	O00213	10.8	Amyloid beta A4	Other neuronal activity	Other signaling molecule
23	P08603	61.7	Complement factor H	Complement-mediated immunity	Complement component
24	P00738	53	Haptoglobin	Neurotransmitter release	Vesicle coat protein

Table 2: Continued.

No.	Acc. Number	% Cov	Protein name	Biological processes	Molecular functions
25	P22891	10.3	Vitamin K-dependent protein Z	Proteolysis	Serine protease
26	P22792	14.1	Carboxypeptidase N subunit 2	Cell surface receptor	Receptor
27	P07357	27.9	Complement component C8	Complement-mediated immunity	Complement component
28	P02741	8.9	C-reactive protein	Stress response	Defense and immunity

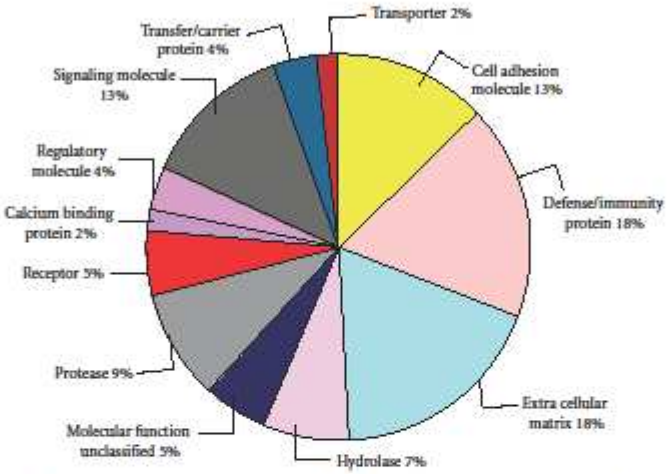

Figure 4: Number of plasma protein identified using iTRAQ reagent. In total, 235 proteins were identified. Shown above is the classification of these protein in different category based on molecular function.

groups were found to involve signalling molecules (13%) and 18% defense/immunity proteins.

Of the large group of significantly up-regulated proteins, it is highly noteworthy that this includes βHCG (Beta human chorionic gonadotropin), a subunit of the human chorionic gonadotropin protein known to be elevated in pregnancies [5] with DS fetuses, and which forms one of the backbone of current screening programs in the first trimester. This finding, therefore strongly suggests, that the iTRAQ method we have chosen appears to be able to identify relevant proteins (**Table 2**). In this group, elevations were also recorded for pregnancy zone protein (PZP) (P20742), thereby indicating that our assay is indeed detecting proteins of placental origin. Elevation of two members of the amyloid family (Serum amyloid P-component and Amyloid beta A4) was also observed, which could be a significant finding, as these proteins have been suggested to play a role in the dementia found to occur in adult DS patients. An elevation in the inflammatory response marker, C-reactive protein and members of the immunoglobulin family like Ig lambda chain C region was also noted, which could be indicative of an increased inflammatory response in DS pregnancies. phosphatidylinositol-glycan (P80108), Insulin-like growth factor (P35858) is involved in protein-protein interactions that result in protein complexes and hepatocyte growth factor (P26927) was also prominent amongst the list of the up-regulated proteins.

Amongst the group of down-regulated proteins (**Table 3**) were a number of molecules involved in cell adhesion and extracellular matrix including titin (Q8WZ42), basement membrane specific heparan sulfate proteoglycan (P98160), actin, cytoplasmic 2 (Gamma-actin) (P63261)and fibrinogen alpha chain (P02671). This observation could be important as changes in tissue elasticity are a hallmark of DS cases, and play significant role in their detection by ultrasound via the presence of an increased neck fold (nuchal translucency). peroxiredoxin 2 (PRDX2), an antioxidant enzyme, is under-expressed in DS fetal brain and dynein heavy chain 9, was also noted in our list of down regulated proteins.

The PANTHER database was used for the pathway analysis on the proteins which were shown to be over or under expressed in our study. It was interesting to note that

Table 3: List of the protein identified as down regulated from the iTRAQ experiment, indicating is the biological process and molecular function of these proteins. (P value .001).

No.	Acc. number	% Cov	Protein name	Biological processes	Molecular functions
1	P01009	32.8	Alpha-1-antitrypsin	Nerve-nerve synaptic transmission	Glutamate receptor
2	P02768	67	Serum albumin	Amino acid biosynthesis	Reductase
3	P63261	49.1	Actin, cytoplasmic 2 (Gamma-actin)	Cell structure	Actin and actin related protein
4	O95445	32.4	Apolipoprotein M (Apo-M)	Lipid transport	Plasma protein
5	P06727	67.7	Apolipoprotein A-IV	Lipid and fatty acid transport	Transporter
6	P02775	41.4	Platelet basic protein	Pyrimidine metabolism	Phosphorylase
7	P02647	85.8	Apolipoprotein A-1	Lipid and fatty acid transport	Transporter
8	P01023	39.8	Alpha-2-macroglobulin	Developmental processes	Serine/threonine kinase
9	P02787	58.9	Serotransferrin	Amino acid biosynthesis	Synthase
10	P04275	11.3	von Willebrand factor	Cell adhesion	Extracellular matrix glycoprotein
11	P02671	35.2	Fibrinogen alpha chain	Cell proliferation and differentiation	Extracellular matrix glycoprotein
12	P01024	29.6	Complement C3	Nerve-nerve synaptic transmission	Glutamate receptor
13	P98160	5.1	Heparan sulfate proteoglycan	Extracellular matrix	Cell adhesion mediated signal
14	P51884	33.4	Lumican	Receptor	Cell adhesion-mediated signaling
15	P04114	41.5	Apolipoprotein B-100	Lipid and fatty acid transport	Component of serum lipoproteins
16	Q8WZ42	9.5	Titin (EC 2.7.11.1) (Connectin)	Muscle contraction	Actin binding cytoskeletal protein
17	P08519	11	Apolipoprotein(a)	Cell proliferation and differentiation	Peptide hormone
18	P43652	47.7	Afamin	Transport	Other transfer/carrier protein
19	P06396	54.9	Gelsolin	Cell structure	Nonmotor actin binding protein
20	P02749	54.2	Beta-2-glycoprotein 1	Amino acid metabolism	Transaminase
21	Q9NYC9	4.8	Dynein heavy chain 9	Force generating protein	ATPase activity
22	P32119	4	Peroxiredoxin-2	Redox regulation	Eliminating peroxides

13.3% proteins identified in the DS sample correspond to proteins found in the Alzheimer disease-amyloid secretase and Alzheimer disease-presenilin pathways illustrated in **Figure 5**.This indicates that these pathways should be more closely studied in conjunction with DS. More than the 40% of the protein we identified in DS samples correspond to proteins in the Integrin signalling pathway. This might be due to the fact that cell adhesion molecules and extra cellular matrix protein represent 13% and 18% of the total proteins identified, respectively as illustrated in **Figure 4**.

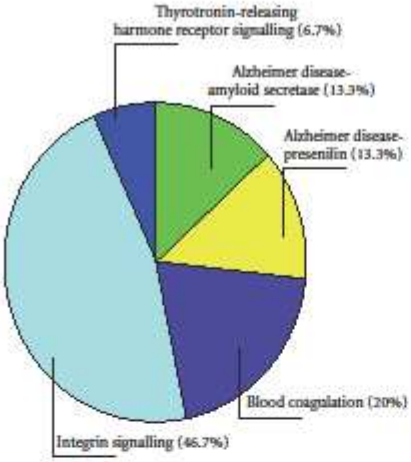

FIGURE 5: *PANTHER analysis for pathway*. In total, 28 proteins were identified as elevated and 22 proteins were under expressed. Shown above is the different signaling pathways hits by these protein.

4. Discussion

Quantitation of serum or plasma proteins via iTRAQ analysis has recently been suggested to be suitable for the detection of biomarkers, as the method is highly reproducible, with little run-to-run variation. This aspect, which is optimal to embark on such a "fishing-expedition", is actually quite surprising, granted the large number of individual steps in the work-flow. This conclusion was, however, derived at after a lengthy comparison involving three pools of case and control samples, as well as a number of individual samples, where a coefficient of variation of 11.7% was noted [21].

In a preliminary proof-of-principle experiment, we have now assessed whether this method could be suitable for the detection of biomarkers useful for DS screening. From our small-scale preliminary evaluation we identified over 200 proteins whose concentration was altered in the plasma of pregnancies with a DS fetus when compared to those with euploid fetuses.

It is of interest that βhCG is detected in the pool of proteins found to be elevated. This glycoprotein, which is produced early in pregnancy by the developing embryo and subsequently by the syncytiotrophoblast, forms the backbone of 1st and 2nd trimester screening strategies. In the 1st trimester, pregnancies at-risk of carrying a fetus affected by DS are identified on the basis of an almost 2 fold MoM (Multiples of the Median) elevation in βhCG. The presence of this important screening marker in our pool of elevated plasma proteins suggests that the strategy we have chosen for the identification of new biomarkers is functional and worthy of further pursuit.

In this context it is worth noting that this marker was not detected in two previous studies [9, 11] using proteomic technologies for the identification of protein markers for DS in maternal plasma or serum. This could be due to limitations in detection sensitivity of the 2D gel approach these studies had used.

That our assay is indeed detecting proteins of placental origin is illustrated by presence of pregnancy zone protein which is a glycoprotein and a proteinase inhibitor which derive its importance in pregnancy by playing potential role in preventing the attack from maternal immune system on the developing fetus which can be seen as foreign allograft [22].This protein was amongst our list of up-regulated proteins, which is similar to alpha 2-macroglobulin, is quantitatively the most important pregnancy-associated plasma protein.

Amongst the pool of up-regulated proteins were serum amyloid P-component and amyloid beta A4, which is encouraging as members of this family have found to be elevated in previous studies on pregnancies with DS fetuses, which may be a reflection of an altered gene expression pattern associated with DS-related dementia. It is also important to note that the amyloid precursor protein (APP) gene is located in DS region of chromosome 21. Teller and colleagues have shown that a relatively minor from of amyloid beta protein was present in the brain of DS affected pregnancy as early as in late 2nd trimester [23].

Elevations in a number of inflammatory molecules, most of which are probably of maternal origin, may be a reflection of the elevated release of placental debris which has been suggested to occur in pregnancies with DS fetuses. Phosphatidylinositol-glycan is expressed in the placental tissue [24], also involved in many cellular process and plays important role in several signal transduction pathways.

Of the down-regulated proteins like actin, gelsolin, heparan sulfate proteoglycan [25], and fibrinogen alpha chain, it is noteworthy that all these protein are involved in cell adhesion, extracellular matrix, cell structure or muscle contraction, since DS fetuses are known to exhibit connective tissue abnormalities [26], the most pronounced of which is the increased neck fold (nuchal translucency). Since adult DS patients are prone to skeletal muscle deficiency, our finding concerning reduced titin plasma levels is intriguing, as this very large protein plays an important role in muscle contraction. In one of recent study Du and colleague [27] have shown trophoblast expression of titin in first trimester placentae.

Only a limited number of studies have attempted to use proteomic approaches for the discovery of new biomarkers for pregnancies at-risk of carrying a fetus with DS, two of these used 2-DE approaches [9, 11], while a further used a SELDI method [10]. Study of the former studies that by Nagalla and colleagues is the largest, having examined serum samples from 56 pregnant women. This study used samples collected in both the 1st and 2nd trimester of pregnancy, which were recruited as part of the NIH funded FASTER study, and largely made use of the fluorescent 2D-DIGE process. In their study, 18 proteins were found to be elevated in 1st trimester samples, which included members of the apoliprotein family, clusterin and proteins involved in skeletal development (tetranectin). The study by

Kolialexi and colleagues [11] used traditional 2DE stained with Coomassie blue on 20 maternal plasma samples (8 cases, 12 controls, 16–18 weeks of pregnancy), by which means 8 candidate proteins were detected. Elevations were noted for apoliprotein E and serum amyloid P-component. In contrast to the study by Nagalla et al. [9] a down regulation for clusterin was noted.

In the study by Busch and colleagues [10] using a SELDI approach, traces were noted which differed between DS cases and controls. However, no attempt was made to discern what proteins were responsible for these altered patterns, nor was any detailed description provided of how they could be reproduced.

Other than common elevation in serum amyloid and complement component families, little commonality exists between our study and these studies. This may be due to a number of factors including, limited study size, time of sampling collection, sample processing and storage, as well as use of very different technical approaches. In our follow-up studies we would like to validate these putative biomarkers using immunoblot and Enzyme linked immunosorbent assay (ELISA). More recently Selected Reaction Monitoring (SRM) [28] has evolved as a method of choice for validation of biomarkers using mass spectroscopy.

The increasing popularity of the iTRAQ approach due to its reproducibility and robustness, including studies for cancer or inflammatory autoimmune disorder specific biomarkers suggests that it will become the method of choice for future studies, until it is surpassed by a new technical development. As pregnancy represents a unique constellation, whereby a foreign being is supported and nourished by the host, it may serve as an ideal model for proteomic analyses, as any unique markers should ideally disappear post delivery. Furthermore, as very few specific biomarkers exist to assist with the screening of a number of pregnancy-related disorders, especially preeclampsia or preterm labour, it is likely that this will become the focus of considerable research attention in the near future.

5. Conclusion

In this report we conclude that isobaric labelling technique is a suitable approach for the quantitative detection of new screening biomarkers in the plasma of pregnancies with a DS fetus compared to those with euploid fetuses. In this preliminary proof-of-principle study, we were able to detect quantitatively under- or over-expressed proteins. In the future additional studies, using larger sample sizes will be required to identify a panel of biomarkers which can be used in screening for DS pregnancies.

Acknowledgment

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References

[1] K. A. Eddleman, F. D. Malone, L. Sullivan, et al., "Pregnancy loss rates after midtrimester amniocentesis," *Obstetrics and Gynecology*, vol. 108, no. 5, pp. 1067–1072, 2006.

[2] E. Guetta, M. J. Simchen, K. Mammon-Daviko, et al., "Analysis of fetal blood cells in the maternal circulation: challenges, ongoing efforts, and potential solutions," *Stem Cells and Development*, vol. 13, no. 1, pp. 93–99, 2004.

[3] Y. M. D. Lo, "Recent advances in fetal nucleic acids in maternal plasma," *Journal of Histochemistry and Cytochemistry*, vol. 53, no. 3, pp. 293–296, 2005.

[4] R. D. Wilson, "Cell-free fetal DNA in the maternal circulation and its future uses in obstetrics," *Journal of Obstetrics and Gynaecology Canada*, vol. 27, no. 1, pp. 54–62, 2005.

[5] K. Spencer, "Aneuploidy screening in the first trimester," *American Journal of Medical Genetics, Part C: Seminars in Medical Genetics*, vol. 145, no. 1, pp. 18–32, 2007.

[6] R. Ricco, A. M. Dalena, T. Valente, et al., "Quantitative study of placental villi in trisomy by analytical morphometry," *Analytical and Quantitative Cytology and Histology*, vol. 31, no. 1, pp. 41–48, 2009.

[7] S.-J. Park, W.-G. Yoon, J.-S. Song, et al., "Proteome analysis of human amnion and amniotic fluid by two-dimensional electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry," *Proteomics*, vol. 6, no. 1, pp. 349–363, 2006.

[8] G. T. Tsangaris, A. Kolialexi, P. M. Karamessinis, et al., "The normal human amniotic fluid supernatant proteome," *In Vivo*, vol. 20, no. 4, pp. 479–490, 2006.

[9] S. R. Nagalla, J. A. Canick, T. Jacob, et al., "Proteomic analysis of maternal serum in down syndrome: identification of novel protein biomarkers," *Journal of Proteome Research*, vol. 6, no. 4, pp. 1245–1257, 2007.

[10] A. Busch, S. Michel, C. Hoppe, D. Driesch, U. Claussen, and F. Von Eggeling, "Proteome analysis of maternal serum samples for trisomy 21 pregnancies using proteinchip arrays and bioinformatics," *Journal of Histochemistry and Cytochemistry*, vol. 53, no. 3, pp. 341–343, 2005.

[11] A. Kolialexi, A. Mavrou, G. Spyrou, and G. T. Tsangaris, "Mass spectrometry-based proteomics in reproductive medicine," *Mass Spectrometry Reviews*, vol. 27, no. 6, pp. 624–634, 2008.

[12] N. L. Anderson and N. G. Anderson, "The human plasma proteome: history, character, and diagnostic prospects," *Molecular & Cellular Proteomics*, vol. 1, no. 11, pp. 845–867, 2002.

[13] P. L. Ross, Y. N. Huang, J. N. Marchese, et al., "Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents," *Molecular & Cellular Proteomics*, vol. 3, no. 12, pp. 1154–1169, 2004.

[14] P. G. Righetti, E. Boschetti, L. Lomas, and A. Citterio, "Protein equalizer technology : the quest for a "democratic proteome"," *Proteomics*, vol. 6, no. 14, pp. 3980–3992, 2006.

[15] R. D. Unwin, A. Pierce, R. B. Watson, D. W. Sternberg, and A. D. Whetton, "Quantitative proteomic analysis using isobaric protein tags enables rapid comparison of changes in transcript and protein levels in transformed cells," *Molecular & Cellular Proteomics*, vol. 4, no. 7, pp. 924–935, 2005.

[16] J. E. Elias and S. P. Gygi, "Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry," *Nature Methods*, vol. 4, no. 3, pp. 207–214, 2007.

[17] L. V. Shilov, S. L. Seymourt, A. A. Patel, et al., "The paragon algorithm, a next generation search engine that uses sequence temperature values sequence temperature values and feature

probabilities to identify peptides from tandem mass spectra," *Molecular & Cellular Proteomics*, vol. 6, no. 9, pp. 1638–1655, 2007.

[18] H. Mi, N. Guo, A. Kejariwal, and P. D. Thomas, "PANTHER version 6: protein sequence and function evolution data with expanded representation of biological pathways," *Nucleic Acids Research*, vol. 35, database issue, pp. D247–D252, 2007.

[19] A. Glen, C. S. Gan, F. C. Hamdy, et al., "iTRAQ-facilitated proteomic analysis of human prostate cancer cells identifies proteins associated with progression," *Journal of Proteome Research*, vol. 7, no. 3, pp. 897–907, 2008.

[20] C. S. Gan, P. K. Chong, T. K. Pham, and P. C. Wright, "Technical, experimental, and biological variations in isobaric tags for relative and absolute quantitation (iTRAQ)," *Journal of Proteome Research*, vol. 6, no. 2, pp. 821–827, 2007.

[21] X. Song, J. Bandow, and J. Sherman, "iTRAQ experimental design for plasma biomarker discovery," *Journal of Proteomic Research*, vol. 7, no. 7, pp. 2952–2958, 2008.

[22] E. L. Skornicka, N. Kiyatkina, M. C. Weber, M. L. Tykocinski, and P. H. Koo, "Pregnancy zone protein is a carrier and modulator of placental protein-14 in T-cell growth and cytokine production," *Cellular Immunology*, vol. 232, no. 1-2, pp. 144–156, 2004.

[23] J. K. Teller, C. Russo, L. M. DeBusk, et al., "Presence of soluble amyloid β-peptide precedes amyloid plaque formation in Down's syndrome," *Nature Medicine*, vol. 2, no. 1, pp. 93–95, 1996.

[24] A. M. Vinggaard, J. J. Provost, J. H. Exton, and H. S. Hansen, "Arf and RhoA regulate both the cytosolic and the membrane-bound phospholipase D from human placenta," *Cellular Signalling*, vol. 9, no. 2, pp. 189–196, 1997.

[25] R. Timpl and J. C. Brown, "Supramolecular assembly of basement membranes," *BioEssays*, vol. 18, no. 2, pp. 123–132, 1996.

[26] F. K. Wiseman, "Down syndrome—recent progress and future prospects," *Human Molecular Genetics*, vol. 18, no. R1, pp. R75–R83, 2009.

[27] M.-R. Du, W.-H. Zhou, F.-T. Yan, et al., "Cyclosporine A induces titin expression via MAPK/ERK signalling and improves proliferative and invasive potential of human trophoblast cells," *Human Reproduction*, vol. 22, no. 9, pp. 2528–2537, 2007.

[28] V. Lange, "Selected reaction monitoring for quantitative proteomics: a tutorial," *Molecular Systems Biology*, vol. 4, p. 222, 2008.

Review

EXPERT REVIEWS

Proteomic technologies for prenatal diagnostics: advances and challenges ahead

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Proteomics-based identification of biomarkers for fetal abnormalities in maternal plasma, amniotic fluid and reproductive fluids has made significant progress in the past 5 years. This is attributed mainly to advances in various technology platforms associated with mass spectrometry-based techniques. As these techniques are highly sensitive and require only small quantities of body fluids, it is hoped that they will pave the way for the development of effective noninvasive approaches, without subjecting the developing fetus to the same degree of harm as current invasive procedures (e.g., amniocentesis). It is possible that these developments will include same-day analyses, thereby permitting rapid intervention when necessary. To date, a host of body fluids, such as maternal serum and plasma, amniotic fluid, cervical fluid, vaginal fluid, urine, saliva or fetal material, such as placental trophoblast, fetal membranes or cord blood, have been used successfully in the quest to develop markers for a number of pregnancy-related pathologies. In the current review update we focus on the emergence of proteomics as a major platform technology in studying various types of fetal conditions and developing markers for pregnancy-related disorders, such fetal aneuploidy, preterm birth, preeclampsia, intra-amniotic infection and fetal stress. Should the development of these markers be successful, then it is to be envisaged that proteomic approaches will become standard of care for a number of disease conditions associated with feto–maternal health.

Keywords: amniotic fluid • biomarker • chromosomal aneuploidies fetal abnormalities • mass spectrometry • noninvasive prenatal diagnosis • prenatal diagnosis • proteomics

The economic divide between the developed and the developing world is narrowing fast. More than half of the world's population lives in developing countries, and the majority of births also take place there. However, with rapid development comes a changing set of demographics: working mothers, smaller families and later pregnancies. So, now more than ever, prenatal diagnosis is becoming an important issue for families around the world.

At the same time, the '-omics' era is offering new technological options for biomarker discovery and clinical diagnostics. Whether these technologies allow the identification of a single/panel of diagnostic markers that could be used on their own, or be part of an overall strategy to enhance accuracy and reduce false positives remains to be seen [1]. For example, in the antenatal detection of Down's syndrome (DS), it is likely that an -omics strategy could help to enhance screening detection beyond 95% and, more importantly, to reduce the false-positive rate to below 1%. We believe that this will generally be the case, except for a few single gene disorders.

The Human Genome Project was completed in 2003, and the impact of genomic medicine on prenatal diagnostics is only now becoming evident. Technologies such as array-comparative genomic hybridization are being explored as alternatives to conventional karyotyping [2], and cell-free nucleic acids circulating in maternal plasma are being exploited to diagnose several fetal genetic conditions noninvasively [3]. In this context it has been interesting to see the merging of new technologies and genomic strategies to address an important fetal genetic condition, DS (trisomy 21), in that the analysis of cell-free fetal nucleic acids by digital PCR [3–4] or high-throughput shotgun sequencing [5] may permit the noninvasive detection of this fetal disorder.

Even as the genome continues to unfold, the proteome is beginning to take center stage. After all, the protein is the execution arm of the

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cellular machinery. The situation has, however, become more complex as recent data have suggested that one gene can produce more than one protein product, and that single proteins can have many different functions [6]. We see the genomic and proteomic strategies not as mutually exclusive, but rather complementary strategies for the optimal detection the fetal malady. As a number of fetal medical and genetic conditions have already been studied using different proteomic platforms [7], we anticipate that many more will emerge shortly as the different technologies are used to focus on prenatal diagnosis, by both conventional invasive and novel noninvasive approaches.

In this review article, we explore novel proteomic strategies that have been used to study the fetal condition and focus on proteomic strategies that have been used successfully to date.

Clinical proteomics & fetal diagnosis

The term proteomics, coined by Marc Wilkins in 1994, indicates a large-scale characterization of the entire protein complement of body fluids, a cell type, tissue or of an entire organism [8]. The primary aim of clinical proteomics is to identify biomarkers for diagnosis and therapeutic intervention for various disease conditions by comparing the proteomic profiles or individual entities (proteins or peptides) of control and disease conditions, and differing physiological states. Different proteomics platforms have been successful in the assessment of clinical samples in feto–maternal medicine [9–10]. Although 2D gel electrophoresis (2DE) remains an important tool for protein identification, it is now being coupled with mass spectrometry (MS) analysis as a means to characterize complex milieus under study [11–12].

It is mostly accepted that the 'holy grail' for prenatal diagnosis is the detection of markers in maternal blood that reliably predict the occurrence of a fetal abnormality. This form of noninvasive prenatal diagnosis offers the opportunity to completely eliminate risk-associated procedures, such as amniocentesis and chorionic villus sampling [13]. In this context, proteomic approaches to detect fetal gene products in the maternal circulation may offer new opportunities to identify aberrant proteins that could prove to be key diagnostic markers for the disease in question. For instance, such proteomic markers might replace current screening markers in use for the screening of pregnancies at of risk of bearing an aneuploid fetus [13,14].

Advances in technology have led to a rapid increase in applications to a wide range of samples; from initial experiments using cell lines to more complex tissues and biological fluids now being assessed to establish changes in protein expression. A host of body fluids and tissues, such as maternal serum [10,15] and plasma [16], amniotic fluid [17], cervical–vaginal fluid [18–21], urine [22,23], saliva [24], trophoblast [25–26], placenta [27], fetal membranes [28], cord blood [29] and follicular fluid [30–32], has been examined to date. This expansion into clinical samples has not been without difficulties owing to the complexity and dynamic range in plasma and the difficulty of obtaining critical tissue biopsies. A number of these proteomic analyses have fostered the hope that this approach may lead to the identification of novel biomarkers for the detection of fetal aneuploidy. However, a major obstacle remains the large dynamic range of proteins or peptides in plasma, which necessitates the effective removal of abundant plasma proteins to allow analysis of the lower concentration analytes. In addition a number of other factors make this research very challenging, beginning with standardization of sample collection and consistent sample preparation, and continuing through the entire analytical process. Therefore, reproducible sample complexity-reduction methods, such as depletion or fractionation, are an essential first step in biomarker discovery experiments.

Conventional biochemical markers

Chromosomal aneuploidy & protein markers

Conventional prenatal diagnostic methods for the diagnosis of fetal aneuploidy rely on obtaining fetal material by invasive procedures, such as amniocentesis or chorion villus sampling, or in rare cases, fetal blood sampling, which are associated with a 1% risk of fetal loss. To minimize exposure to these invasive procedures, pregnant women are risk stratified by screening using a combination of ultrasonography and serum biochemical markers [33–36]. However, these screening methods generally target epiphenomena, such as nuchal translucency, which is associated with chromosomal anomalies. Despite this premise, this practice permits detection rates in excess of 90% for DS, Edward syndrome (trisomy 18) and Patau syndrome (trisomy 13) [35]. Some of the conventional markers used for screening fetal aneuploid conditions are described briefly in the subsequent sections.

Human α-fetoprotein

Human α-fetoprotein (AFP) is a tumor-associated fetal glycoprotein involved with both ontogenic and oncogenic growth [37]. This protein was first described in 1972 where elevated AFP levels in amniotic fluid were found to be associated with neural tube defects [38]. Presently, a vast biomedical literature has amassed concerning the use of human AFP during pregnancy as a biomarker in human maternal serum and amniotic fluid. Such studies have addressed the measurement of serum levels of AFP outside the normal levels in the sera of pregnant women; such values are indicative of intrauterine growth retardation of the developing fetus [39].

Inhibins as diagnostic markers in human reproduction

Inhibin A, the newest addition to second trimester serum screening, is an α-β-subunit hormone of placental origin, and is measured using a monoclonal two-site ELISA validated for use in prenatal screening. Inhibin A is a marker of dominant follicle and corpus luteum activity, and is decreased in polycystic ovary syndrome [40]. Inhibin A was also found to increase in gestational diseases, such as preeclampsia and fetal DS, and was found to decrease in women with declining ovarian function and correlates with female response to ovulation induction.

Triple test & quadruple test

A number of serum markers during the second trimester have been found to be associated with DS. The principal markers are AFP, hCG or its individual subunits (free α- and free β-hCG),

unconjugated estriol (uE) 3 and inhibin A. The screening modality will be dependent on the choice of markers, as well as whether or not ultrasound is used to estimate gestational age. Along with ultrasound scan, the estimated gestational age detection rate for a 5% false-positive rate is estimated to be 59% using the double test (AFP and hCG), 69% using the triple test (AFP, hCG and uE3) and 76% using the quadruple test (AFP, hCG, uE3 and inhibin A), all in combination with maternal age [41]. The multiple marker combination with the highest positive predictive value (PPV) currently available for DS is AFP, uE3, hCG and inhibin A, together with maternal age (quadruple marker test). With this combination, a detection rate of 80% with a 5% false-positive rate is reported [42]. Other well-studied trisomy conditions include Edward syndrome and Patau syndrome. ADAM12 (a disintegrin and metalloprotease 12) may be a useful addition to early screening for Edward syndrome alongside other chromosomal anomalies, particularly if biochemical screening can occur before 10 weeks [43]. ADAM12 maternal serum concentration in the early first trimester demonstrates reduced levels, while it is elevated during the second trimester approximately 16–18 weeks [44]. Placental protein (PAPP)-A levels were found to be reduced in Patau syndrome pregnancies in the second trimester, this isolated lower marker value is unlikely to be of value in screening for Patau syndrome in the second trimester [45]. The etiology of reduced levels of PAPP-A in cases of Patau syndrome may be similar to that in cases of Edward syndrome, but different from that in cases of DS since the temporal patterns in Patau syndrome and Edward syndrome are different from that in DS. PAPP-A and free β-hCG have been combined with the nuchal translucency in the combined first trimester screen.

Biomarkers for fetal diagnostics based on proteomics platforms

The subsequent sections describe recent research developments associated with proteomic interventions for various disease conditions associated with fetal development. Some of the most common disease conditions associated with the developing fetus, as well as associated complications in the maternal system, and the proteomics strategies used to identify biomarkers are described in Table 1.

Amniotic fluid

Amniotic fluid is an aqueous layer of fluid surrounding the fetus and is a significant contributor to fetal health. It constitutes a potential rich source of biomarkers for diagnosis of maternal and fetal disorders. Amniotic fluid protects the fetus from injury, helps in its mobility and stabilizes temperature. It is continually exposed to fetal waste products, skin and the cells [46]. Fetal products, such as fetal cells, lanugo, urine and phospholipids originating from the lungs, are deposited constantly in the amniotic fluid. The composition of the fluid changes over the course of gestation. Since amniotic fluid is constantly exposed to fetal materials, any change in the volume and composition of the amniotic fluid might be an indication of the metabolic or developmental status of the fetus at that particular point of time in its gestation [47,48]. Therefore, abnormalities in fetal development are reflected in the amniotic fluid in the form of nucleic acids (DNA and mRNA), metabolites, peptides, proteins, enzymes, lipids, cells and so forth [46]. Abnormal constituents in the amniotic fluid are amplified due to the difference in the gene expression [49–50], or could be associated with fetal infection which usually results in the preterm delivery [51]. Few systematic studies have been carried out on the changes in amniotic fluid composition starting from early gestation to term pregnancy, possibly for ethical reasons.

Monitoring gestational-dependent changes in the developing fetus

The human placenta produces a wide range of peptides and proteins throughout pregnancy. These placental proteins and peptides are associated with common gestational diseases, such as intrauterine growth retardation, preterm labor, preeclampsia, chromosomal disorders, gestational diabetes and trophoblastic disease [52]. In a recent study, human amniotic fluid has been subjected to comprehensive proteomic analysis to study gestational age-dependent changes [53]. The study used a host of molecular techniques, including gel-based 2D-liquid chromatography (LC)-MS/MS and 2D-DIGE on amniotic fluid, serum and cervico–vaginal fluid to study gestational-dependent changes. The results based on 2D-DIGE technique showed that among the first-, second- and third-trimester amniotic fluid samples, the maximal differences in the relative abundance of amniotic fluid proteins occur between the first and second trimesters. The study proposed that a systematic analysis of proteins present both in amniotic fluid and maternal serum could lead to the development of new noninvasive diagnostic procedures to monitor fetal status. The placenta has been described as a 'diary of intrauterine life' and has the potential to reflect many facets of this these processes. A systematic approach to this with clinical correlations, was recently reported by Redline [54]. The report has outlined a conceptual framework separating placental patterns of injury and maladaptation into three categories of lesions affecting the maternal and fetal vasculature (maldevelopment, obstruction and disruption) and two categories of inflammatory lesions (infectious and idiopathic). Maternal lipids were also found to be strong determinants of fetal environment and growth in pregnancies with gestational diabetes mellitus [55]. In well-controlled gestational diabetes mellitus pregnancies, maternal lipids were found to be strong predictors for fetal lipids and fetal growth. Infants with abnormal growth seem to be exposed to a distinct intrauterine environment compared with those with appropriate growth.

Markers for preeclampsia

Preeclampsia is a major cause of fetal and maternal morbidity and mortality [56]. It is an enigmatic disorder of unknown etiology, characterized by a sudden pathological elevation in maternal blood pressure, which if uncorrected, can lead to fits (eclampsia) and death [57–59]. Although most cases of preeclampsia occur close to term, the most severe forms occur in the late second/early third trimester and are associated with severe fetal growth retardation. As preeclampsia can only be resolved by delivery of the fetus and placenta, this

Table 1. Summary of different studies representing different body fluids used for biomarker discovery for fetal diagnostics and the different techniques used to identify these biomarkers.

Disease	Technique used	Protein markers	Ref.
Biomarkers from maternal blood			
Down syndrome	2D-GE, MALDI-TOF-MS Western blot	TTHY, CERU, AFAM, AMBP, APO-E, SAM); HRG and A1AT were upregulated and CLUS was downregulated	[30]
Down syndrome	2D-DIGE 2D-CE, MudPIT; LC/LC-MS/MS MALDI-TOF-MS peptide profiling	A2AP, ANT3, A2MG and ITIH4.3S A1AG and SAA complement system proteins B, C, and H Serum carrier proteins group, afamin and vitamin D Binding proteins are carriers for the fat-soluble vitamins E and D, Ceruloplasmin, TTHY	[15]
Preeclampsia	MALDI-TOF-MS	CLUS	[84]
Biomarkers from amniotic fluid			
Histologic chorioamnionitis	SELDI-TOF-MS	Defensins 2 and 1, calgranulins C and A	[52]
Down syndrome	MALDI-MS and nano-ESI-MS/MS	SFRS4; Q08170 only in DS fetuses AF Quantitative differences were detected for AMBP; P02760, CO1A1; P02452, CO3A1; P02461, CO5A1; P20908, and basement membrane-specific heparin sulfate PGBM; P98160 Four proteins; CO1A1, CO3A1, CO5A1 and PGBM, appeared as fragments	[17]
Intraamniotic inflammation	SELDI-TOF-MS	17 proteins were significantly overexpressed 5 of them were identified as human neutrophil protein 1-3, calgranulin A and B.	[51]
Neonatal sepsis	SELDI-TOF- MS	Neutrophil defensins 1 and 2 and calgranulins A and C	[85]
Fetal alcohol syndrome in C57BLV6	LC-MS/MS and MudRT	AFP	[86]
Rh– incompatibility	2DE and MALDI-TOF-MS	Albumin, serotransferrin, haptoglobin, AFP and immunoglobulin	[58]
Preeclampsia	SELDI-TOF-MS	Pro-Apo A-I and a functionally obscure peptide, SB842	[47]
Preeclampsia	2DE and MALDI-TOF-MS	Oxidized TTHY	[49]
Biomarkers from placental tissues			
Gestational disease		Placental peptides for intrauterine growth retardation, preterm labor, preeclampsia, chromosomal disorders, gestational diabetes and trophoblastic disease	[36]
Biomarkers from trophoblasts			
Preeclampsia	2D-PAGE and MALDI-TOF-MS	Disulfide isomerase precursor, endoplasmic reticulum resident protein, dihydrolipoyl dehydrogenase and TIM21-like protein	[87]

2DE: 2D gel electrophoresis; A1AG: α-1-acid-glycoprotein ; A1AT: α-1-antitrypsin precursor; A2AP: α-2-antiplasmin precursor; A2MG: α-2-macroglobulin precursor; AF: Amniotic fluid; AFAM: Afamin; AFP: α-fetoprotein ; AMBP: α-1-microglobulin; ANT3: Antithrombin-3; APO-E: Apolipoprotein E; CERU: Ceruloplasmin; CLUS: Clusterin; CO1a1: Collagen-α1 (I) chain; CO3A1: Collagen-α1 (III) chain; CO5A1: Collagen-α1 (V) chain d; CVF: Cervicovaginal fluid; DS: Down syndrome; HFF: Human follicular fluid; HRG: Histidine-rich glycoprotein; ITIH4: Inter-A trypsin inhibitor H4; LC: Liquid chromatography; MS: Mass spectrometry; PAGE: Polyacrylamide gel electrophoresis; SAA: Serum amyloid A; SAMP: Serum amyloid P-component; SFRS4: Splicing factor arginine/serine-rich 4; TTHY: Transthyretin.

Table 1. Summary of different studies representing different body fluids used for biomarker discovery for fetal diagnostics and the different techniques used to identify these biomarkers.

Disease	Technique used	Protein markers	Ref.
Biomarkers from human cervical–vaginal fluid			
Spontaneous preterm birth	LC/LC-MS/MS; MudRT; 2D-DIGE	Calgranulins, annexins, S100 calcium-binding protein A7 and epidermal fatty acid-binding protein were abundant in CVF and differentially expressed as were the serum proteins A1AT, A1AG, haptoglobin, serotransferrin, and vitamin D-binding protein	[34]
Intra-amniotic inflammation	SELDI-TOF-MS	17 proteins were significantly overexpressed Five of them were identified as human neutrophil protein 1-3, calgranulin A and B	[51]
Biomarkers from saliva			
Preterm premature rupture	Luminex technologies	Salivary matrix metalloproteinase 9	[54, 55]
Biomarkers from human follicular fluid			
HFF proteome from normo-ovulatory women	MALDI-TOF-MS	A large number of acute-phase proteins, including transferrin, CERU, afamin, hemopexin, haptoglobin and plasma amyloid protein, were identified in HFF in relatively high concentration supporting the hypothesis that mammalian ovulation can be compared with an inflammatory event Antioxidant enzymes such as catalase, superoxide dismutase, glutathione transferase, paraoxonase, heat-shock protein 27 and protein disulfide isomerase were detected Authors claim that this might be an indication that during maturation the human follicle is well protected against toxic injury due to oxidative stress	[58]
Cervicovaginal fluid and parturition	2D-GE	Blood transport proteins (albumin and transthyretin); structural protein (β-actin); proteins involved in fatty acid metabolism (fatty acid-binding protein and acetyl-CoA-binding protein); a calcium-binding protein (annexin III); an anti-inflammatory cytokine (IL-1 receptor antagonist); proteinase inhibitors (A1AT, monocyte/neutrophil elastase inhibitor, squamous cell carcinoma antigen-1 and cystatin A); and enzymes involved in oxidative stress defence (thioredoxin, peroxiredoxin 2, glutathione S-transferase P and copper,zinc superoxide dismutase)	[59]
Specific peptide patterns of follicular fluids at different growth stages	MALDI-TOF MS	Apo-A-I, collagen type IV, integrin	[60]
Recurrent spontaneous abortion	MALDI-TOF-MS, nano-LC MS/MS and Western blot analysis		[30]

2DE: 2D gel electrophoresis; A1AG: α-1-acid-glycoprotein; A1AT: α-1-antimypsin; A2AP: α-2-antiplasmin precursor; A2MG: α-2-macroglobulin precursor; AF: Amniotic fluid; AFAM: Afamin; AFP: α-fetoprotein; AMBP: α-1-microglobulin; ANTS: Antithrombin-3; APO-E: Apolipoprotein E; CERU: Ceruloplasmin; CLUS: Clusterin; COL1: Collagen-α1 (I) chain; COL4A1: Collagen-α1 (IV) chain; COL5A1: Collagen-α1 (V) chain; CVF: Cervicovaginal fluid; DS: Down syndrome; HFF: Human follicular fluid; HRG: Histidine-rich glycoprotein; ITIH4: Inter-α trypsin inhibitor H4; LC: Liquid chromatography; MS: Mass spectrometry; PAGE: Polyacrylamide gel electrophoresis; SAA: Serum amyloid A; SAMP: Serum amyloid P-component; SFRS4: Splicing factor arginine/serine-rich 4; TTHY: Transthyretin.

results in the delivery of a very premature sick baby. Preeclampsia also increases the risk of cardiovascular disease for the affected fetus in the future. Studies have shown that the association between abnormal uterine artery Doppler flow velocimetry, risk of preeclampsia, and indices of arterial structure and function. Women with abnormal uterine artery Doppler results may not only be at increased risk of developing subsequent preeclampsia but also of future cardiovascular disease [60].

A number of recent studies have identified that circulatory levels of angiogenic factors, such as VEGF, phosphatidylinositol glycan anchor biosynthesis, class F (*PIGF*) and the soluble form of their mutual receptor *Flt1*, as well as placentally derived soluble endoglin (sENG), are altered in those pregnancies that subsequently develop preeclampsia [61–62]. These findings have suggested that these molecules may serve as screening markers to detect at-risk pregnancies. Since the alterations in these angiogenic factors only occur relatively late in pregnancy, the search is continuing for markers whose behavior is altered earlier during gestation in such affected pregnancies. The reason being that early detection may aid in the development of effective therapeutic or preventative strategies. Once such factor is placental protein 13 (pp-13), where alterations occur in the first trimester in those pregnancies that subsequently develop preeclampsia [63]. Similar observations were made concerning the disintigrin/metalloproteinase ADAM-12 [64]. The most recent of these is by Buhimschi *et al.*, who examined urine samples from an at-risk cohort and compared these with a control group with healthy pregnancy outcomes [65]. They observed a unique proteomic profile in the study cohort of 19 women. This abnormal profile became readily apparent 10 weeks prior to onset of symptoms. MS/MS and *de novo* sequencing identified nonrandom cleavage products of albumin and SERPINA1. This approach is very promising and, by using urine, offers a noninvasive screening option. As mentioned, amniotic fluid (by being in direct contact with the fetus) is a highly interesting source of protein biomarkers. For this reason, Park and colleagues examined amniotic fluid samples by SELDI-TOF [66]. In their study they examined amniotic fluid samples from cases with preeclampsia, those with chronic hypertension and normal healthy controls. In their proteomic profiling experiments they identified two peaks that could be resolved by HPLC, SDS-polyacrylamide gel electrophoresis (PAGE) and tryptic digestion to be proapolipoprotein A-I and the peptide SBB#12. These two markers could be used to distinguish preeclampsia from chronic hypertension, or both preeclampsia and chronic hypertension from healthy controls. As placental hypoxia has been suggested to play a role in the etiology of preeclampsia, Ishioka *et al.* examined the influence aerobic/hypoxic conditions on the human choriocarcinoma cell line JAR [25]. Their experiments showed that that a number of apoptosis-specific proteins (Bcl-x, caspase-3 and -9, heat-shock protein [Hsp]70, PTEN, and Bag-1) are altered. Proteomic patterns approach were employed as a novel screening tool for preeclampsia [67]. Amniotic fluid might again prove to be an ideal source for detecting markers for diagnosing preeclampsia at an early stage. A recent report identified oxidized transthyretin (TTHY) in amniotic fluid as an early marker of preeclampsia [68]. TTHY is responsible for transporting both the thyroid hormone tyroxine and retinol-binding protein, and is present in the amniotic fluid of both preeclamptic and control women as a mixture of dimeric and post-translationally modified monomeric forms. Although the nature of these forms is similar in both groups, the preeclamptic women showed a significant increase in the amount of monomeric proteins with respect to the control group. The report concluded that TTHY monomeric forms are the results of different oxidizing reactions, and higher oxidative stress in preeclampsia is the major destabilizing factor of the TTHY functional dimeric form in preeclamptic women.

Intra-amniotic inflammation

Intrauterine infection results in intra-amniotic inflammation and has emerged as a common and important cause of preterm delivery adversely affecting normal fetal development. A third of all preterm births occur in mothers with microbial invasion of the amniotic cavity [69] and often result in fetal infection with the development of the fetal inflammatory response syndrome, a risk factor for the impending onset of labor, short-term neonatal complications and long-term handicaps, such as cerebral palsy and chronic lung disease. Proteomics analysis of cervical and amniotic fluid in women with intra-amniotic inflammation resulted in the identification of 17 proteins that were significantly overexpressed in amniotic fluid from intra-amniotic inflammation cases and occurred more often in women with preterm labor than those with rupture of membranes [70]. Five of these were identified as human neutrophil proteins 1–3, calgranulin A and B. Specific attempts were also made to study diseases such as histologic chorioamnionitis using proteomic platforms [71]. Four amniotic fluid proteomic biomarkers are characteristic of inflammation (defensins 2 and 1, calgranulins C and A). A proteomic fingerprint (Mass Restricted [MR] score) was generated from amniotic fluid using SELDI-TOF-MS. A MR score was created using four biomarkers to study the severity of intra-amniotic inflammation. Of the four biomarkers, calgranulin C showed the strongest relationship with presence of stage III chorioamnionitis, independent of race, amniocentesis-to-delivery interval and gestational age. The same approach was also used to detect diseases such as occult candidal chorioamnionitis [72].

Spontaneous preterm birth and premature rupture of the membranes

The evaluation of proteins as potential cervical–vaginal fluid biomarkers for noninvasive diagnosis of pregnancy-related disorders, such as spontaneous preterm birth (SPTB) and premature rupture of the membranes (PROM), has been reported recently. A recent study investigating SPTB identified 205 proteins in cervical–vaginal fluid, 28 of which exhibited significant differences in pairwise and progressive comparisons [73]. PROM is another complication that can result in fetal morbidity [28,74]. 2DE was used to generate protein profiles of plasma containing amniotic fluid proteins for PROM. Using a high-throughput approach for PROM biomarker discovery, Michel *et al.* studied both maternal plasma and amniotic fluid samples from the same patient at term of pregnancy and it was used to find specific amniotic fluid proteins as markers of PROM [16].

Detection of chromosomal abnormalities

Since chromosomal aneuploidy is one of the most common conditions associated with the developing fetus, it is not surprising that it is the subject of several reports using advanced genomics and proteomics techniques. In this context, current developments largely focus on applying various platform technologies to identify pertinent biomarkers in body fluids. This includes amniotic fluid, which constitutes a potentially rich source of biomarkers for the diagnosis of maternal and fetal disorders during pregnancy. A comprehensive 'proteomic profile' of amniotic fluid has huge potential for detecting several fetal disease specific markers, with the potential for carrying out prenatal diagnosis [75]. The availability of a normal amniotic fluid supernatant (AFS) proteome map is a valuable tool for the studying aberrant protein expression in affected pregnancies and serves as an important tool in the search for new markers. Towards this goal, a recent report by Tsangaris *et al.*, in 2005, reported a comprehensive study of amniotic fluid cells [76]. Analysis of the amniotic fluid cell extract resulted in the identification of 432 different gene products, of which the majority are enzymes, structural proteins, HSPs and proteins related to signal transduction. As proteins from a number of different cell types were found, this suggests that the amniotic fluid cell population may either be heterogeneous, originating from different fetal compartments, or still contains multipotent cells. The presence of these proteins could serve as markers for fetal diagnosis but may also assist with the identification of cells with stem cell characteristics. In a subsequent study, this group reported on the normal human amniotic fluid proteome [77]. The significance of this study lies in the fact that amniotic fluid samples are used routinely for prenatal diagnosis of a wide range of fetal abnormalities. Since proteomic analysis would require very small volumes of fluid, this could be performed in conjunction with other analyses. Owing to the speed with which these proteomic analysis can be performed, it is possible that they may supplant time-consuming, conventional karytotyping. Evidence for such a development was provided by Wang *et al.* who used a proteomic analysis of AFS as a rapid alternative for the detection of fetal aneuploidies [78]. In their examination they used proteomic profiles generated by MALDI-TOF-MS after fractionating samples with functionalized magnetic beads on 60 karyotypically normal and 20 aneuploid AFS samples. The two-step proteomics analysis of AFS with the C18 model, followed by the weak cation-exchange model, was able to detect aneuploid AFS at 3.3% disease prevalence rate with 100% senaitivity, 72–96% specificity, 11–50% PPV, and 100% negative predictive value. In another study Tsangaris *et al.* reported some proteins, such as splicing factor arginine/serine-rich 4 (SFRS4; Q08170), were present only in AFS from DS fetuses and completely absent in the control group [17]. Quantitative differences were also detected for α-1-microglobulin (AMBP; P02760), collagen-α1 (I) chain (CO1A1; P02452) collagen-α1 (III) chain (CO3A1; P02461), collagen-α1 (V) chain d (CO5A1; P20908), and basement membrane-specific heparin sulfate proteoglycan core protein (PGBM; P98160). These proteins were increased in cases of DS, whereas the protein IBP-1 (P08833) was decreased by 40% compared with chromosomally normal fetuses. Four proteins, CO1A1, CO3A1, CO5A1 and PGBM, appeared as fragments. As differentially expressed proteins were present in all pregnancies with DS tested, they may represent useful potential markers for prenatal diagnosis from amniotic fluid samples.

Another aneuploid condition subjected to proteomic analysis is Turner syndrome, which occurs in one in 2500 female births, and is caused by the complete or partial absence of one X chromosome. In an analysis of amniotic fluid from five second-trimester pregnancies with Turner syndrome fetuses and five control fetuses by 2DE, MALDI-TOF-MS and western blotting, increases in the levels of serotransferin, lumican, plasma retinol-binding protein and apolipoprotein (APO) A-I were detected in cases in Turner syndrome, while the levels of kininogen, prothrombin and APO A-IV were decreased [79].

It is not yet clear whether amniotic fluid proteins cross the placental barrier in order to enter the maternal circulation. Should this premise be true, it is possible that the differential pattern observed in these proteins in amniotic fluid may assist with the development of similar screening approaches using maternal plasma or serum.

As discussed previously, the real aim is the development of noninvasive strategies for the detection of abnormal fetuses, such as by the detection of protein markers in maternal plasma or serum. An important pioneering study in this regard was carried out recently by Nagalla *et al.* who set out to identify potential serum biomarkers for the detection of DS [15]. The study was carried out on first- and second-trimester maternal serum samples from pregnancies with DS fetuses and healthy gestational age-matched controls. A suite of advanced proteomic approaches, including 2D-DIGE, 2D-LC-chromatofocusing (CF), multidimensional protein identification technology (MudPIT; LC/LC-MS/MS), and MALDI-TOF-MS peptide profiling were used for the study. A total of 28 and 26 proteins were differentially present in first- and second-trimester samples, respectively. The majority of the identified proteins belonged to glycoproteins that might be associated with cellular differentiation and fetal growth. Analysis of MALDI-TOF-MS peptide profiles with pattern-recognition software led to the clear discrimination DS and controls in both trimesters, with an average detection rate of almost 96%. In another recent report using 2DE and MALDI-TOF-MS on maternal plasma from DS fetuses and matched controls, nine differentially expressed proteins were detected in the DS cases [10]. Of these, eight proteins, TTHY, ceruloplasmin (CERU), afamin (AFAM), α-1-microglobulin (AMBP), APO-E, serum amyloid P-component (SAMP), histidine-rich glycoprotein (HRG) and α-1-antitrypsin (A1AT) were upregulated, while one, clusterin, was downregulated. All nine proteins are known to be involved in fetal growth and development. Of these, APO-E, SAMP, AFAM and clusterin are associated with the DS phenotype. The proteins APO-E and SAMP were increased by 19 and 48%, respectively.

In summary, the above differentially expressed proteins have the potential to be developed as biomarkers for DS, providing opportunities for the development of new noninvasive screening and diagnostic strategies.

Proteomics techniques used for fetal diagnostics & screening

Major advances associated with clinical proteomics in recent years were based on MS platform [10]. Protein profiling using SELDI-TOF-MS as well as MALDI-TOF-MS techniques, coupled with novel algorithms for multiple protein identification and classification and clinical parameters (e.g., MR score) were demonstrated to diagnose disease conditions (Table 1). Multidimensional approaches using 2DE-based approaches and DIGE techniques, as well as shotgun proteomics approaches has yielded hundreds of candidate markers for fetal diagnostics in the last couple of years. The need for lesser sample quantity as well as the shorter turnaround time required for most of these techniques, not to mention the sensitivity and the accuracy, are in favor of these newer techniques to come with better biomarkers in the near future. The following section of this review article will focus on the major techniques used in the field of fetal diagnostics/screening. Application of these high-throughput methodologies has contributed to the comprehension of the underlying pathophysiologies and the successful identification of relevant protein biomarkers that can potentially change early diagnosis of abnormal fetal conditions.

Multidimensional proteomics approaches for fetal diagnostics & screening

This current section of the review focuses on the spectrum of techniques that various groups have used, as well as the nature of the biomarkers identified in the process for various fetal conditions. The current focus on proteomic technologies are on the following MS-based techniques such as fluorescence 2D-DIGE, cleavable isotope-coded affinity tags (cICAT) and isobaric tags for relative and absolute quantification (iTRAQ®) (Figure 1), using 2D gel- or LC-MALDI TOF/TOF. A comparative study of the three quantitative methods used frequently in proteomics, 2D-DIGE, cICAT and iTRAQ, could distinguish most of the differentially regulated proteins in controls and diseased conditions [80]. 2D-DIGE and cICAT are familiar techniques used in gel- and LC-based quantitative proteomics, respectively. iTRAQ is a new LC-based technique that is gradually gaining popularity. The following sections describe the different techniques used for a wide spectrum of diseases associated with fetal developments, pathogenesis and abnormal conditions associated with the developing fetus.

The basic technique to start with is 1D-SDS-PAGE, which requires the minimum infrastructure. However, if this technique is complemented with prefractionation approaches coupled with LC-MS, this could increase the number of proteins identified by several hundred-fold as was shown successfully in a recent report on the identification of proteins in human cervical–vaginal fluid [21]. Using a 'bottom-up' proteomic approach to characterize the protein repertoire of human cervical–vaginal fluid the authors used sample prefractionation methods, 1D-SDS-PAGE and strong cation-exchange chromatography, followed by LC-MS/MS and bioinformatics analysis. A total of 685 proteins were identified using this approach. Strong cation-exchange chromatography and prefractionation resulted in a larger number of proteins identified when compared with 1D-SDS-PAGE. It was interesting to find that extracellular or membrane proteins made up 30% of the proteins identified, according to genome ontology (GO) classifications and their presence in human cervical–vaginal fluid could probably be generated from the cells shed into the cervical–vaginal fluid. The study confirmed the presence of defense-related proteins, such as haptoglobin, defensins and lactoferrin; and identified new ones such as azurocidin and dermcidin. In addition, using this technology the group identified serine and cysteine proteases, including six members of the kallikrein family (KLKs 6, 7, 10, 11, 12 and 13). The same approach was also followed by Dasari *et al.* in 2007, where a multidimensional 2D-LC coupled with MS and GE-based protein separation and identification was used for understanding human parturition and pathologic conditions affecting pregnancy [18]. A total of 150 unique proteins were identified using multiple protein identification algorithms. Metabolism (32%) and immune response-related (22%) proteins are the major functional categories represented in the cervical–vaginal fluid proteome. A comparison of the cervical–vaginal fluid, serum and amniotic fluid proteomes showed that 77 proteins are unique to cervical–vaginal fluid, while 56 and 17 cervical–vaginal fluid proteins also occur in serum and amniotic fluid, respectively. This data set provides a foundation for evaluation of these proteins as potential cervical–vaginal fluid biomarkers for noninvasive diagnosis of pregnancy-related disorders, to develop new tests for the early, noninvasive positive prediction of SPTB. Recently the same group reported another combination of techniques to address markers associated with SPTB [73], using multidimensional LC/LC-MS/MS, MudPIT and 2D-DIGE to identify potential biomarkers of preterm labor (PTL) and SPTB. Following MudPIT analysis the authors reported 205 proteins in cervical–vaginal fluid, 28 of which exhibited significant differences in pairwise and progressive comparisons. Calgranulins, annexins, S100 calcium-binding protein A7 and epidermal fatty acid-binding protein were abundant in cervical–vaginal fluid and differentially present in PTL and SPTB samples, as were the serum proteins – α-1-antitrypsin – α1-acid glycoprotein, haptoglobin, serotransferrin and vitamin D-binding protein. 2D-DIGE identified 17 proteins that were significantly differentially present in PTL and SPTB. Immunoblotting with specific antibodies confirmed the differences and trends of selected markers. Further characterization and quantification of these markers in a larger cohort of subjects may provide the basis for new tests for the early, noninvasive positive prediction of SPTB.

Of the few reports emerging for utilizing similar multidimensional approaches on maternal plasma and serum, some have focused on the identification of makers for chromosomal aneuploidy of the developing fetus. A report by Nagalla *et al.* used multiple, complementary proteomic approaches, including 2D-DIGE, 2D-LC-CF, MudPIT; LC/LC-MS/MS and MALDI-TOF-MS peptide profiling [15]. In total, 28 and 26 proteins were differentially present in first- and second-trimester samples, respectively. Of these, 19 were specific for the first trimester and 16 for the second trimester, and ten were differentially present in

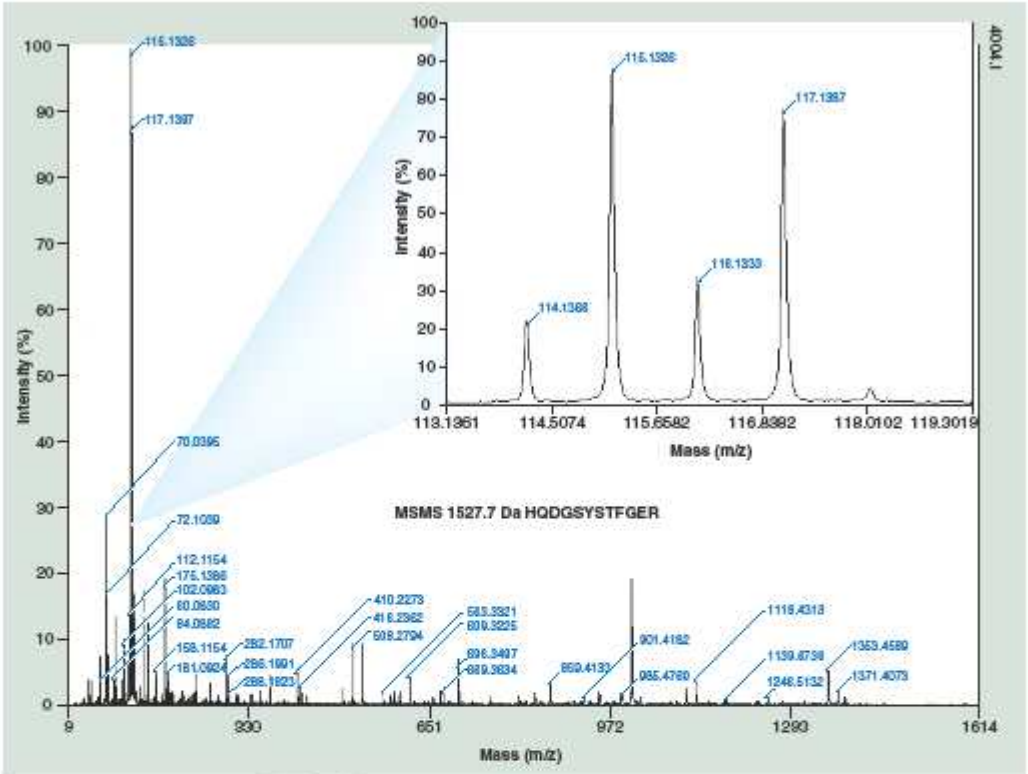

Figure 1. Quantitative identification of screening biomarkers for Down syndrome-carrying pregnancy using plasma with four-plex isobaric tag for relative and absolute-quantitation (ITRAQ™) method. ITRAQ reagents 114 and 116 labels were used for the labeling of the pooled and duplicated control samples (n = 6) and 115, 117 labels were used to label the pooled and duplicated Down syndrome samples (n = 6). In this figure, MS/MS of precursor ion of pregnancy zone protein was shown, which was found to be differentially expressed compared with normal, with an ITRAQ ratio of 115/114: 1.88 **(A)** MS/MS spectrum of the precursor ([M+H]+, m/z 1527.7 Da). **(B)** Low-mass region showing the reporter ions used for quantitation.
[Hahn S *et al.*; Unpublished Data].

both trimesters. Analysis of MALDI-TOF-MS peptide profiles with pattern-recognition software also discriminated DS and controls in both trimesters, with an average recognition capability approaching 96%. Most of the biomarkers identified are serum glycoproteins that may play a role in cellular differentiation and growth of fetus.

The 2D-DIGE technique was also used to study amniotic fluid samples [81]. This study was carried out to compare amniotic fluid in both normal and pathological situations. 2D-DIGE, Ettan DIGE as well as 2DE and silver staining followed by image analysis were used, and differentially expressed proteins were identified using MS. This approach was used to study electrophoregrams of normal amniotic fluid obtained at 17 weeks of gestation and at term, as well as amniotic fluid from fetuses presenting with congenital diaphragmatic hernia.

Premature rupture of the membranes was also studied using 2DE to generate protein profiles of plasma containing amniotic fluid proteins for PROM. More recently, a high-throughput approach was reported by Michel *et al.* for PROM study using maternal plasma and amniotic fluid samples from the same patient at term of pregnancy to find specific amniotic fluid proteins as markers of PROM [16]. Maternal plasma and the corresponding amniotic fluid were immunodepleted in order to remove the six most abundant proteins before the systematic analysis of their protein composition. The protein samples were then fractionated by isoelectric focusing Off-Gel electrophoresis (OGE), digested and analyzed with nano-LC-MS/MS separation, revealing a total of 73 and 69 proteins identified in maternal plasma and amniotic fluid samples, respectively. The proteins identified in amniotic fluid have been compared with those identified in the maternal plasma, as well as

with the reference human plasma protein list reported by Anderson *et al.* [82]. This comparison showed that 26 proteins were present exclusively in amniotic fluid and not in plasma, among which ten have already been described to be placenta- or pregnancy-specific. As a further validation of the method, plasma proteins fractionated by OGE and analyzed by nano-LC-MS/MS have been compared with the Swiss 2D-PAGE reference map by reconstructing a map that matches 2D gel and OGE experimental data. This representation demonstrates that 36 out of 49 reference proteins could be identified in both data sets, and that isoform shifts in pI are well conserved in the OGE data sets.

In an attempt to develop a comprehensive database of the amnion proteome, Cho *et al.* used two types of 2D LC/MS/MS as well as an LC-SDS-PAGE-LC-MS/MS [83]. A total of 16 amniotic fluid samples between gestational ages of 16 and 18 weeks from women carrying chromosomally normal fetuses were analyzed by one of the three fractionation methods, followed by a common reverse-phase LC-MS/MS step. Mascot® and The Global Proteome Machine engines were used to search the International Protein Index human database for peptide sequence identification. The list of proteins was generated by combining the results of both engines through the PeptideProphet® of Scaffold software. All identified proteins were combined to generate the amniotic fluid proteome, comprising 1026 unique gene matches or 842 non-redundant proteins. This list includes most of the currently used biomarkers for pregnancy-associated pathologic conditions, such as preterm delivery, intra-amniotic inflammation and chromosomal anomalies of the fetus. The subcellular localization, tissue expression, functions and networks of the amniotic fluid proteome were analyzed by various bioinformatic tools. These data will contribute to the better understanding of amniotic fluid function and to the discovery of novel biomarkers for prenatal diagnosis of fetal abnormalities. Another recent study by Park *et al.* in 2006, on human amnion and amniotic fluid at term, resulted in the identification of 92 soluble and 19 membrane proteins from amnion [84]; 35 proteins were identified from amniotic fluid. Calgranulin A and B were found in all patients infected with *Ureaplasma urealyticum*, although not in any of the patients without infection, indicating that they are potential markers of intrauterine infection. The identities of calgranulin A and B were confirmed by MALDI-TOF/TOF MS. The more standard method established workflow of 2DE, MALDI-TOF-MS, and western blot was also followed for identification of markers for Turner syndrome [79].

Shotgun proteomics

Over the last 10 years, MS has evolved as a powerful technique. This has led to the development of shotgun proteomics, which is a useful tool as a bank-ready quantification using special reagents and techniques. Lately, different techniques have become available for the labeling, which enables the quantification of the protein-like stable isotope labeling of amino acid in cell cultures (SILAC) [85–86], ICAT and iTRAQ [87].

Isobaric tag for relative and absolute-quantitation is an isobaric chemical labeling approach and currently the only technique capable of multiplexing up to eight different samples for relative quantification. Eight-plex chemically identical iTRAQ reagents are available, named 114, 115, 116, 117, 118, 119 and 121, which have the same overall mass. Each label is composed of a peptide reactive group (NHS ester) and an isobaric tag of 145 Da that consists of a balancer group (carbonyl) and a reporter group (based on N-methylpiperazine); between the balancer and the reporter group is a fragmentation site. The peptide reactive group attaches specifically to free primary amino groups – N-termini and ε-amino groups of lysine residues [88]. Each sample to be analyzed is tryptic digested and labeled with the single iTRAQ label, after which samples are pooled for tandem mass analysis. The peptide product ion spectra is then used for the identification of the proteins, and relative quantitation is derived from the peak intensities of the eight-plex iTRAQ reporter ions detected in the 114–121 m/z region of the fragmented ion spectra. Data acquired are always compared with a reference sample, and the quantity of each peptide is expressed as a ratio relative to the reference sample [89]. As the field of shotgun proteomics evolves, it is likely to play a role in increasing our understanding of pregnancy-related disorders. This is because most proteomic studies in the field were hindered by inaccurate methods for quantitation. This is likely to change as iTRAQ has a clear-cut advantage over other existing techniques in that it allows quantitation and multiplexing in a single experiment. To date, the iTRAQ technique has been used in a wide range of biological samples, such as cells and tissue, and body fluids such as serum and plasma [90–91]. Song *et al.* demonstrated that one can explicitly design the iTRAQ experiment for plasma biomarker discovery [92]. A shotgun proteomics approach showed reproducible identification of more than 40 proteins in vaginal fluid samples, such as plasma proteins, epithelial structural proteins and several immunoregulatory proteins, including some that had been linked previously to intra-amniotic infection [20]. However, this study using LC coupled with tandem MS, was carried out without prior fractionation. The study resulted in the identification of host defence proteins in vaginal fluid, suggesting that this technique may be useful for the future study of inflammation-related preterm birth.

Protein profiling using MALDI-TOF-MS

Wang *et al.* has described a two-step proteomics analysis of AFS with the C18 model, followed by the weak cation-exchange model [78]. This study could detect aneuploid AFS at a 3.3% disease prevalence rate with 100% sensitivity, 72–96% specificity, 11–50% PPV and 100% negative predictive value. Proteomics profiles generated by MALDI-TOF-MS after fractionating samples with functionalized magnetic beads were used for differentiating 60 normal karyotypic from 20 aneuploid AFS. A proteomic fingerprinting approach using MS coupled with a statistical classification method was used to improve diagnosis of aneuploidies, including trisomies 13, 18 and 21, in amniotic fluid samples [93]. Among the 208 expressed protein peaks, 40 differed significantly between aneuploid and non-aneuploid samples, with AUC diagnostic values ranging from 0.71 to 0.91. Hierarchical clustering, principal component analysis and support vector machine analysis were performed and two class predictor models were defined from the training set, which resulted in prediction accuracies of 92.3 and 96.43%, respectively, while an

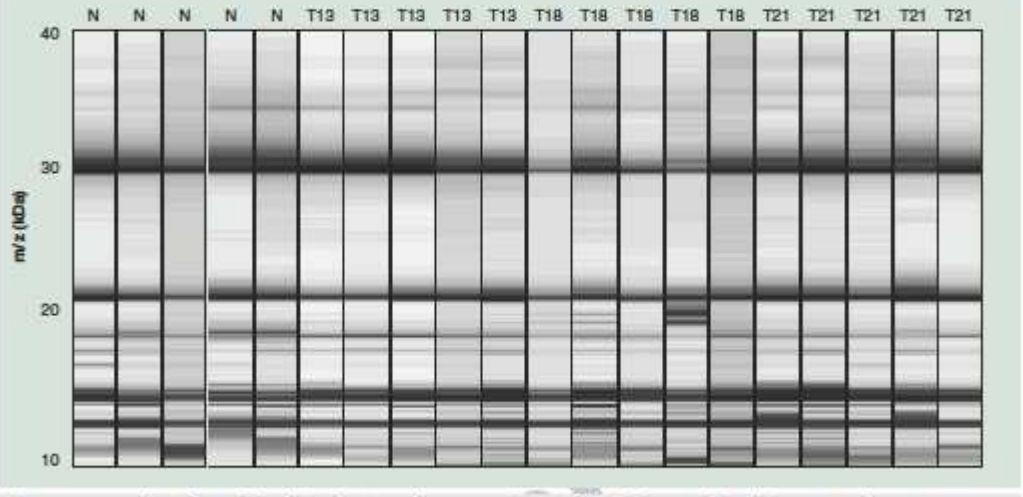

Figure 2. A snapshot of pseudogel view (maternal serum protein profile) of the normal and trisomy samples run on SELDI-TOF-MS machine. Only five representative samples from each category were selected for the graphical representation. The spectra present in the figure show common as well as unique peaks to each trisomy and normal samples. Protein peaks were shown as dark bands starting from 3 to 40 kDa. The majority of variation is observed in the low-molecular-weight region of less than 20 kDa. n: Control healthy; T13: Trisomy 13; T18: Trisomy 18; T21: Trisomy 21.
[Chooklani M *et al.*; Unpublished Data].

external and independent validation set showed 87.5 and 91.67%, respectively. Although this is a preliminary study, it will have wide-scale implications in reducing the time for obtaining result for chromosomal aneuploidies; one has to wait for approximately 14 days when performing karyotyping.

SELDI-TOF-MS & prenatal diagnosis

Clinical proteomics is an emerging field that will have a great impact on molecular diagnosis, identification of disease biomarkers, drug discovery and clinical trials in the postgenomic era. In recent years, SELDI-TOF-MS has been at the forefront of preferred techniques for nearly all disease conditions (Figure 2). The first report on the use of SELDI technology in clinical proteomics was reported for ovarian cancer by Petricoin's group in 2002 [94]. Following that landmark paper, this technique was used for diagnosing numerous disease conditions, including those associated with fetal development, such as intrauterine inflammation [70,95], histologic chorioamnionitis [71] and idiopathic preterm birth [51]. Although the technique was criticized for several shortcomings, it remains a favorite technique for many researchers due to its robustness and ease to perform as well as the well-established algorithms currently available to predict disease conditions. The following section describes the use of this technique on fetal diagnostic research. However, in order to enter clinical practice the technology has a long way to go and the short comings, such reproducibility and reliability, need to be established under stringent performance conditions.

Using SELDI-TOF-MS techniques and MR score, Buhimschi *et al.* used a novel algorithm to extract both clinically and biologically relevant biomarkers from proteomic SELDI tracings in amniotic fluid samples [95]. This study resulted in the identification in patients with intra-amniotic inflammation who deliver preterm, of a distinctive amniotic fluid proteomic profile of three or four of the following proteins: neutrophil defensins-1 and -2, and calgranulins A and C. Based on the presence or absence of these biomarkers, a MR score range was developed: 0 (all biomarker peaks absent) to 4 (all biomarker peaks present). A MR score of more than 2 was associated with imminent preterm delivery. This study was novel in the sense that it was able to predict an imminent preterm delivery in blinded testing (Stage 2). A MR score of more than 2 provided 100% specificity and sensitivity (95% CI: 100–100). In another similar study for the same condition, amniotic and cervical fluids were analyzed using SELDI-TOF-MS [70]. In total, 17 proteins were significantly overexpressed in amniotic fluid from intra-amniotic infection cases and more often in women with preterm labor than those with rupture of membranes. Five of these were identified as human neutrophil protein 1–3, calgranulin A and B.

The etiology of most preterm births still remains elusive, although recent advancements in proteomics have provided a novel perspective on several distinct pathogenetic mechanisms leading to preterm birth. A proteomic fingerprint was generated from fresh amniotic fluid using SELDI-TOF-MS in 286 consecutive samples retrieved from women who presented with signs or symptoms of preterm labor or preterm PROM [51]. The group reported the

use of a multidimensional proteomic analysis of amniotic fluid to identify pathways related to preterm birth in the absence of inflammation or bleeding. The major output of this study is the development of a novel profile (Q-profile) based on the presence of five SELDI peaks in the 10–12.5 kDa mass area. The study utilized identification-centered proteomics techniques (2D-DIGE, robotic tryptic digestion and MS) coupled with Protein Analysis Through Evolutionary Relationships (PANTHER) ontological classifications and identified amniotic fluids with a Q-profile, the differentially expressed proteins are involved primarily in non-inflammatory biological processes, such as protein metabolism, signal transduction and transport. More significantly, women displaying the Q-profile were found to be more likely to deliver preterm despite expectant management in the context of intact membranes and normal amniotic fluid clinical results. Proteomic profiling of amniotic fluid coupled with nonhierarchical bioinformatics algorithms identified a subgroup of patients at risk for preterm birth in the absence of intra-amniotic inflammation or bleeding, suggesting a novel pathogenetic pathway leading to preterm birth. The altered proteins may offer opportunities for therapeutic intervention and future drug development to prevent premature birth. SELDI technique was also used for diagnosing preeclampsia using proteins from amniotic fluid [66]. Using this technique it was found that it is possible to distinguish preeclampsia from chronic hypertension and normotensive controls. Based on only two biomarkers: peak X (17399.11 Da), which distinguished preeclampsia from normotensive controls, and peak Y (28023.34 Da), which distinguished preeclampsia and chronic hypertension from normotensive controls. Later, using HPLC coupled with SDS-PAGE MS, the peaks were matched to pro-APO-A-I (peak Y) and a functionally obscure peptide, SBB142 (peak X).

Further advances have focused on imaging MS to generate unique protein profiles that are characteristic of successful embryo implantation [96]. The urine proteome may develop into an easier material for noninvasive prenatal diagnosis [22,23]. Most changes detected in the serum proteome could be captured in the urine protein profiles. Similarly, saliva from pregnant mothers may prove to be a rich source of biomarkers for different fetal disease conditions, as was shown for salivary proteinase activity as a marker for PROM [24]. Using the Luminex platform, salivary matrix metalloproteinase 9 has been identified as a marker for preterm labor [97].

Biomarker validation

Validation of biomarkers is an important area for successful translation of proteomics discovery to clinical practice. Validation involves the use of stringent criteria being followed for proper trial design, sample collection and a sufficient number of patients. While using MS-based platforms, unbiased MS could be used for protein identification while targeted MS could be used to follow-up for validating prospective candidate biomarkers. More than one protein was often found for several disease conditions and these are present in different levels in body fluids. Using the above information on multiple biomarkers for the same disease condition, modeling networks as well as systems biology analysis of biological data sets could be developed for driving mathematical models to predict the disease, while published scientific literature could provide additional information in the modeling process. Large-scale validation using multicenter clinical trials will ensure the sensitivity and specificity required for of any marker to enter the market of prenatal diagnosis.

Expert commentary & five-year view: future directions for proteomics in fetal diagnostics/screening

Heterogeneity in the long run might prove to be a major hindrance for clinical proteomics. The current review has demonstrated that a large number of proteins were found to be altered during pregnancy-related disorders ranging from aneuploidy to severe disturbance in preeclampsia and preterm labor. In most diseases that were studied using advanced multidimensional techniques, several marker proteins were found to overlap for different disease conditions. The challenge hence would be to detect key candidate proteins through a combination approach (multiple biomarker panels as well as better algorithms using several approaches) to diagnose a particular disease accurately during the early first trimester of pregnancy. This approach will result in increased sensitivity and specificity of the tests developed using this approach. The candidate markers identified through several proteomics platforms should also be validated on higher numbers of patient samples through clinical trials, since the prevalence of these disease conditions in the normal population is very low. Technologies such as protein microarrays [98], whole-proteome scans and intact protein analysis systems could help to better understand the global fetal proteome environment. The future may hold for the development of panel or array of markers to address specific fetal disease conditions. The aforementioned proteomic platforms could ensure that specific biomarkers with higher sensitivity and specificity could be identified for diagnosing fetal abnormalities are at an early stage.

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Key Issues

- Clinical proteomics has made significant advances in the last 5 years.
- Most of the studies have focused on detection and characterizing protein biomarkers for chromosomal aneuploidy.
- Biological materials that have been used to identify biomarkers include maternal serum, amniotic fluid, cervical fluid, vaginal fluid, urine, saliva placental trophoblast, fetal membranes and cord blood.
- Most of the identified markers belong to high abundance proteins as well as immune response proteins.
- Future studies in this field will focus on the development of the panel of markers for disease conditions associated with pregnancy.

References

Papers of special note have been highlighted as:
• of interest
•• of considerable interest

1 Cuckle H. Time for total shift to first-trimester screening for Down's syndrome. *Lancet* 358(9294), 1658–1659 (2001).

2 Bi W, Breman AM, Venable SF *et al.* Rapid prenatal diagnosis using uncultured amniocytes and oligonucleotide array CGH. *Prenat. Diagn.* 28(10), 943–949 (2008).

3 Lo YMD, Lun FM, Chan KC *et al.* Digital PCR for the molecular detection of fetal chromosomal aneuploidy. *Proc. Natl Acad. Sci. USA* 104, 13116–13121 (2007).

4 Fan HC, Quake SR. Detection of aneuploidy with digital polymerase chain reaction. *Anal. Chem.* 79(19), 7576–7579 (2007).

5 Fan HC, Blumenfeld YJ, Chitkara U, Hudgins L, Quake SR. Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. *Proc. Natl Acad. Sci. USA* 105(42), 16266–16271 (2008).

6 Lian Y, Garner HR. Evidence for the regulation of alternative splicing via complementary DNA sequence repeats. *Bioinformatics* 21(8), 1358–1364 (2005)

7 Bahtiyar MO, Copel JA, Mahoney MJ, Buhimschi IA, Buhimschi CS. Proteomics: a novel methodology to complement prenatal diagnosis of chromosomal abnormalities and inherited human diseases. *Am. J. Perinatol.* 24(3), 167–181 (2007).

8 Wasinger VC, Cordwell SJ, Cerpa-Poljak A *et al.* Progress with gene-product mapping of the Mollicutes: *Mycoplasma genitalium*. *Electrophoresis* 7, 1090–1094 (1995).

9 Aldred S, Grant MM, Griffiths HR. The use of proteomics for the assessment of clinical samples in research. *Clin. Biochem.* 37(11), 943–952 (2004).

10 Kolialexi A, Tsangaris GT, Papantoniou N *et al.* Application of proteomics for the identification of differentially expressed protein markers for Down syndrome in maternal plasma. *Prenat. Diagn.* 28(8), 691–698 (2008).

11 Liotta L, Kohn EC, Petricoin EF. Clinical proteomics: personalized molecular medicine. *JAMA* 286, 2211–2214 (2001).

12 Lau AT, He QY, Chiu JF. Proteomic technology and its biomedical applications. *Acta Biochemica* 35, 965–975 (2005).

13 Avent ND, Plummer ZE, Madgett TE, Maddocks DG, Soothill PW. Post-genomics studies and their application to non-invasive prenatal diagnosis. *Semin. Fetal Neonatal Med.* 13(2), 91–98 (2008).

14 Shimizu C, Bryant-Greenwood P. Post-genome molecular diagnostics in obstetrics. *Curr. Opin. Obstet. Gynecol.* 16(2), 167–177 (2004).

15 Nagalla SR, Canick JA, Jacob T *et al.* Proteomic analysis of maternal serum in Down syndrome: identification of novel protein biomarkers. *J. Proteome Res.* 6(4), 1245–1257 (2007).

•• Elegant paper describing use of novel mass spectrometry techniques to study maternal serum for trisomy-specific markers.

16 Michel PE, Crettaz D, Morier P *et al.* Proteome analysis of human plasma and amniotic fluid by off-gel isoelectric focusing followed by nano-LC-MS/MS. *Electrophoresis* 27(5–6), 1169–1181 (2006).

17 Tsangaris GT, Karamessinis P, Kolialexi A *et al.* Proteomic analysis of amniotic fluid in pregnancies with Down syndrome. *Proteomics* 6(15), 4410–4419 (2006).

• One of the first papers to describe the comprehensive proteome analysis of amniotic fluid in pregnancies with Down syndrome.

18 Dasari S, Pereira L, Reddy AP *et al.* Comprehensive proteomic analysis of human cervical–vaginal fluid. *J. Proteome Res.* 6(4), 1258–1268 (2007).

•• Excellent paper describing comprehensive proteome analysis of cervical–vaginal fluid.

19 Gravett MG, Thomas A, Schneider KA *et al.* Proteomic analysis of cervical–vaginal fluid: identification of novel biomarkers for detection of intraamniotic infection. *J. Proteome Res.* 6(1), 89–96 (2007).

20 Klein LL, Jonscher KR, Heerwagen MJ, Gibbs RS, McManaman JL. Shotgun proteomic analysis of vaginal fluid from women in late pregnancy. *Reprod. Sci.* 15(3), 263–273 (2008).

•• Elegant paper describing shotgun proteomics to analyze vaginal fluid.

21 Shaw JL, Smith CR, Diamandis EP. Proteomic analysis of human cervico–vaginal fluid. *J. Proteome Res.* 6(7), 2859–2865 (2007).

22 Kronborg CS, Allen J, Vittinghus E, Knudsen UB. Pre-symptomatic increase in urine-orosomucoid excretion in pre-eclamptic women. *Acta Obstet. Gynecol. Scand.* 86(8), 930–937 (2007).

23 Decramer S, Gonzalez de Peredo A, Breuil B *et al.* Urine in clinical proteomics. *Mol. Cell. Proteomics* 7(10), 1850–1862 (2008).

24 Menon R, McIntyre JO, Matrisian LM, Fortunato SJ. Salivary proteinase activity: a potential biomarker for preterm premature rupture of the membranes. *Am. J. Obstet. Gynecol.* 194(6), 1609–1615 (2006).

25 Ishioka S, Ezaka Y, Umemura K, Hayashi T, Endo T, Saito T. Proteomic analysis of mechanisms of hypoxia-induced apoptosis in trophoblastic cells. *Int. J. Med. Sci.* 4(1), 36–44 (2006).

26 de Groot CJ, Steegers-Theunissen RP, Gitzel C, Steegers EA, Luider TM. Peptide patterns of laser dissected human trophoblasts analyzed by matrix-assisted laser desorption/ionisation-time of flight mass spectrometry. *Proteomics* 5(2), 597–607 (2005).

27 Liu AX, Jin F, Zhang WW, Zhou TH, Zhou CY, Yao WM. Proteomic analysis on the alteration of protein expression in the placental villous tissue of early pregnancy loss. *Biol. Reprod.* 75(3), 414–420 (2006).

28 Thadikkaran L, Crettaz D, Siegenthaler MA *et al.* The role of proteomics in the assessment of premature rupture of fetal membranes. *Clin. Chim. Acta* 360(1–2), 27–36 (2005).

29 Murphy VE, Johnson RF, Wang YC *et al.* The effect of maternal asthma on placental and cord blood protein profiles. *J. Soc. Gynecol. Investig.* 12(5), 349–355 (2005).

30 Kim YS, Kim MS, Lee SH *et al.* Proteomic analysis of recurrent spontaneous abortion: Identification of an inadequately expressed set of proteins in human follicular fluid. *Proteomics* 6(11), 3445–3454 (2006).

31 Hanrieder J, Nyakas A, Naessén T, Bergquist J. Proteomic analysis of human follicular fluid using an alternative bottom-up approach. *J. Proteome Res.* 7(1), 443–449 (2008).

32 Li L, Xing FQ, Chen SL, Sun L, Li H. Proteomics of follicular fluid in mature human follicles and antral follicles: a comparative study with laser desorption/ionization-time of flight-mass spectrometry. *Nan Fang Yi Ke Da Xue Xue Bao* 28(2), 275–278 (2008).

33 Nicolaides KH. Nuchal translucency and other first-trimester sonographic markers of chromosomal abnormalities. *Am. J. Obstet. Gynecol.* 191(1), 45–67 (2004).

34 Nicolaides KH. First-trimester screening for chromosomal abnormalities. *Semin. Perinatol.* 29(4), 190–194 (2005).

35 Spencer K. Aneuploidy screening in the first trimester. *Am. J. Med. Genet. C Semin. Med. Genet.* 145C(1), 18–32 (2007).

36 Flood K, Malone FD. Screening for fetal abnormalities with ultrasound. *Curr. Opin. Obstet. Gynecol.* 20(2), 139–145 (2008).

37 Mizejewski GJ. α-fetoprotein as a biologic response modifier: relevance to domain and subdomain structure. *Proc. Soc. Exp. Biol. Med.* 215, 333–362 (1997).

38 Brock DJ, Scrimgeour JB, Steven J, Barron L, Watt M. Maternal plasma α-fetoprotein screening for fetal neural tube defects. *Br. J. Obstet. Gynaecol.* 85(8), 575–581 (1978).

39 Bartha JL, Illanes S, González-Bugatto F, Abdel-Fattah SA, Mizejewski GJ, Soothill PW. Maternal serum transformed α-fetoprotein levels in women with intrauterine growth retardation. *Fetal Diagn. Ther.* 22(4), 294–298 (2007).

40 Tsigkou A, Luisi S, Reis FM, Petraglia F. Inhibins as diagnostic markers in human reproduction. *Adv. Clin. Chem.* 45, 1–29 (2008).

41 Wald NJ, Kennard A, Hackshaw A, McGuire A. Antenatal screening for Down's syndrome. *J. Med. Screen.* 4(4), 181–246 (1997).

42 Canick JA, MacRae AR. Second trimester serum markers. *Semin. Perinatol.* 29(4), 203–208 (2005).

43 Spencer K, Cowans NJ. ADAM12 as a marker of trisomy 18 in the first and second trimester of pregnancy. *J. Matern. Fetal Neonatal Med.* 20(9), 645–650 (2007).

44 Laigaard J, Cuckle H, Wewer UM, Christiansen M. Maternal serum ADAM12 levels in Down and Edwards' syndrome pregnancies at 9–12 weeks' gestation. *Prenat. Diagn.* 26(8), 689–691 (2006).

45 Spencer K, Crossley JA, Aitken DA, Nicolaides KH. Second-trimester levels of pregnancy-associated plasma protein-A and free β-hCG in pregnancies with trisomy 13. *Prenat. Diagn.* 25(5), 358–361 (2005).

46 Underwood MA, Gilbert WM, Sherman MP. Amniotic fluid: not just fetal urine anymore. *J. Perinatol.* 25(5), 341–348 (2005).

47 Schwartz M, Schwartz S, Wenk RE, Cohen M. Amniotic fluid and advances in prenatal diagnosis. *Clin. Lab. Med.* 5(2), 371–387 (1985).

48 Moore TR. Clinical assessment of amniotic fluid. *Clin. Obstet. Gynecol.* 40(2), 303–313 (1997).

49 Larrabee PB, Johnson KL, Lai C *et al.* Global gene expression analysis of the living human fetus using cell-free messenger RNA in amniotic fluid. *JAMA* 293(7), 836–842 (2005).

50 Nagy GR, Györffy B, Galamb O, Molnár B, Nagy B, Papp Z. Use of routinely collected amniotic fluid for whole-genome expression analysis of polygenic disorders. *Clin. Chem.* 52(11), 2013–2020 (2006).

51 Buhimschi IA, Zhao G, Rosenberg VA, Abdel-Razeq S, Thung S, Buhimschi CS. Multidimensional proteomics analysis of amniotic fluid to provide insight into the mechanisms of idiopathic preterm birth. *PLoS ONE* 3(4), e2049 (2008).

•• Elegant paper describing multidimensional proteome analysis of amniotic fluid for studying idiopathic preterm birth.

52 Page NM, Kemp CF, Butlin DJ, Lowry PJ. Placental peptides as markers of gestational disease. *Reproduction* 123(4), 487–495 (2002).

53 Michaels JE, Dasari S, Pereira L *et al.* Comprehensive proteomic analysis of the human amniotic fluid proteome: gestational age-dependent changes. *J. Proteome Res.* 6(4), 1277–1285 (2007).

54 Redline RW. Placental pathology: a systematic approach with clinical correlations. *Placenta* 29(Suppl. A), S86–S91 (2008).

55 Schaefer-Graf UM, Graf K, Kulbacka I *et al.* Maternal lipids as strong determinants of fetal environment and growth in pregnancies with gestational diabetes mellitus. *Diabetes Care* 31(9), 1858–1863 (2008).

56 Karumanchi SA, Lindheimer MD. Advances in the understanding of eclampsia. *Curr. Hypertens. Rep.* 10(4), 305–312 (2008).

57 Redman CW, Sargent IL. Latest advances in understanding preeclampsia. *Science* 308(5728), 1592–1594 (2005).

58 Mohaupt M. Molecular aspects of preeclampsia. *Mol. Aspects Med.* 28(2), 169–191 (2007).

59 Dechend R, Luft FC. Angiogenesis factors and preeclampsia. *Nat. Med.* 14(11), 1187–1188 (2008).

60 Anastasakis E, Paraskevas KI, Papamoniou N *et al.* Association between abnormal uterine artery Doppler flow velocimetry, risk of preeclampsia, and indices of arterial structure and function: a pilot study. *Angiology* 59(4), 493–499 (2008).

61 Rana S, Karumanchi SA, Levine RJ *et al.* Sequential changes in antiangiogenic factors in early pregnancy and risk of developing preeclampsia. *Hypertension* 50(1), 137–142 (2007).

62 Tjoa ML, Levine RJ, Karumanchi SA. Angiogenic factors and preeclampsia. *Front. Biosci.* 12, 2395–2402 (2007).

63 Huppertz B, Sammar M, Chefetz I, Neumaier-Wagner P, Bartz C, Meiri H. Longitudinal determination of serum placental protein 13 during development of preeclampsia. *Fetal Diagn. Ther.* 24(3), 230–236 (2008).

64 Spencer K, Cowans NJ, Stamatopoulou A. Maternal serum ADAM12s in the late first trimester of pregnancies with trisomy 21. *Prenat. Diagn.* 28(5), 422–424 (2008).

65 Buhimschi IA, Zhao G, Funai EF *et al.* Proteomic profiling of urine identifies specific fragments of SERPINA1 and albumin as biomarkers of preeclampsia. *Am. J. Obstet. Gynecol.* 199(5), 551.e1–e16 (2008).

66 Park JS, Oh KJ, Norwitz ER *et al.* Identification of proteomic biomarkers of preeclampsia in amniotic fluid using SELDI-TOF mass spectrometry. *Reprod. Sci.* 15(5), 457–468 (2008).

67 Myers J, Macleod M, Reed B, Harris N, Mires G, Baker P. Use of proteomic patterns as a novel screening tool in pre-eclampsia. *J. Obstet. Gynaecol.* 24(8), 873–874 (2004).

• One of the first papers to describe the use of proteomic patterns to study preeclampsia.

68 Vascotto C, Salzano AM, D'Ambrosio C *et al.* Oxidized transthyretin in amniotic fluid as an early marker of preeclampsia. *J. Proteome Res.* 6(1), 160–170 (2007).

69 Pettker CM, Buhimschi IA, Magloire LK, Sfakianaki AK, Hamar BD, Buhimschi CS. Value of placental microbial evaluation in diagnosing intraamniotic infection. *Obstet. Gynecol.* 109(3), 739–749 (2007).

70 Rhetsche U, Rosén A, Karlsson G *et al.* Proteomic analysis using protein chips to detect biomarkers in cervical and amniotic fluid in women with intraamniotic inflammation. *J. Proteome Res.* 4(6), 2236–2242 (2005).

71 Buhimschi IA, Zambrano E, Pettker CM *et al.* Using proteomic analysis of the human amniotic fluid to identify histologic chorioamnionitis. *Obstet. Gynecol.* 111(2 Pt 1), 403–412 (2008).

72 Crawford JT, Pereira L, Buckmaster J *et al.* Amniocentesis results and novel proteomic analysis in a case of occult candidal chorioamnionitis. *J. Matern. Fetal Neonatal Med.* 19(10), 667–670 (2006).

73 Pereira L, Reddy AP, Jacob T *et al.* Identification of novel protein biomarkers of preterm birth in human cervical–vaginal fluid. *J. Proteome Res.* 6(4), 1269–1276 (2007).

74 Vuadens F, Benay C, Crettaz D *et al.* Identification of biologic markers of the premature rupture of fetal membranes: proteomic approach. *Proteomics* 3(8), 1521–1525 (2003).

75 Hampton T. Comprehensive 'proteomic profile' of amniotic fluid may aid prenatal diagnosis. *JAMA* 298(15), 1751 (2007).

76 Tiangaris G, Weitzdörfer R, Pollak D, Lubec G, Fountoulakis M. The amniotic fluid cell proteome. *Electrophoresis* 26(6), 1168–1173 (2005).

77 Tsangaris GT, Kolialexi A, Karamessinis PM, Anagnostopoulos AK, Antsaklis A, Fountoulakis M. The normal human amniotic fluid supernatant proteome. *In Vivo* 20(4), 479–490 (2006).

78 Wang TH, Chang YL, Peng HH *et al.* Rapid detection of fetal aneuploidy using proteomics approaches on amniotic fluid supernatant. *Prenat. Diagn.* 25(7), 559–566 (2005).

79 Mavrou A, Anagnostopoulos AK, Kolialexi A *et al.* Proteomic analysis of amniotic fluid in pregnancies with Turner syndrome fetuses. *J. Proteome Res.* 7(5), 1862–1866 (2008).

80 Wu WW, Wang G, Baek SJ, Shen RF. Comparative study of three proteomic quantitative methods, DIGE, cICAT, and iTRAQ, using 2D gel- or LC-MALDI TOF/TOF. *J. Proteome Res.* 5(3), 651–658 (2006).

81 Queloz PA, Crettaz D, Thadikkaran L *et al.* Proteomic analyses of amniotic fluid: potential applications in health and diseases. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 850(1–2), 336–342 (2007).

82 Anderson NL, Polanski M, Pieper R *et al.* The human plasma proteome: a nonredundant list developed by combination of four separate sources. *Mol. Cell. Proteomics* 3(4), 311–326 (2004).

83 Cho CK, Shan SJ, Winsor EJ, Diamandis EP. Proteomics analysis of human amniotic fluid. *Mol. Cell. Proteomics* 6(8), 1406–1415 (2007).

** Elegant paper describing the amniotic fluid proteome.

84 Park SJ, Yoon WG, Song JS *et al.* Proteome analysis of human amnion and amniotic fluid by two-dimensional electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Proteomics* 6(1), 349–363 (2006).

85 Ong SE, Blagoev B, Kratchmarova I *et al.* Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol. Cell. Proteomics* 1(5), 376–386 (2002).

86 Mann M. Functional and quantitative proteomics using SILAC. *Nat. Rev. Mol. Cell. Biol.* 7(12), 952–958 (2006).

87 Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* 17(10), 994–999 (1999).

88 Ross PL, Huang YN, Marchese JN *et al.* Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Mol. Cell. Proteomics* 3(12), 1154–1169 (2004).

89 Shilov IV, Seymour SL, Patel AA *et al.* The Paragon Algorithm, a next generation search engine that uses sequence temperature values and feature probabilities to identify peptides from tandem mass spectra. *Mol. Cell. Proteomics* 6(9), 1638–1655 (2007).

90 Keshamouni VG, Michailidis G, Grasso CS *et al.* Differential protein expression profiling by iTRAQ-2DLC-MS/MS of lung cancer cells undergoing epithelial-mesenchymal transition reveals a migratory/invasive phenotype. *J. Proteome Res.* 5(5), 1143–1154 (2006).

91 DeSouza L, Diehl G, Rodrigues MJ *et al.* Search for cancer markers from endometrial tissues using differentially labeled tags iTRAQ and cICAT with multidimensional liquid chromatography and tandem mass spectrometry. *J. Proteome Res.* 4(2), 377–386 (2005).

92 Song X, Bandow J, Sherman J *et al.* iTRAQ experimental design for plasma biomarker discovery. *J. Proteome Res.* 7(7), 2952–2958 (2008).

* Excellent paper describing iTRAQ technology for studying plasma for biomarker discovery.

93 Mange A, Desmetz C, Bellet V, Molinari N, Maudelonde T, Solassol J. Proteomic profile determination of autosomal aneuploidies by mass spectrometry on amniotic fluids. *Proteome Sci.* 6, 1 (2008).

94 Petricoin EF, Ardekani AM, Hitt BA *et al.* Use of proteomic patterns in serum to identify ovarian cancer. *Lancet* 359(9306), 572–577 (2002).

95 Buhimschi IA, Christner R, Buhimschi CS. Proteomic biomarker analysis of amniotic fluid for identification of intraamniotic inflammation. *BJOG* 112(2), 173–181 (2005).

96 Burnum KE, Tranguch S, Mi D, Daikoku T, Dey SK, Caprioli RM. Imaging mass spectrometry reveals unique protein profiles during embryo implantation. *Endocrinology* 149(7), 3274–3278 (2008).

97 Yeast JD, Lu G. Biochemical markers for the prediction of preterm delivery. *Clin. Perinatol.* 34(4), 573–586 (2007).

98 Li D, Yang H, Zhang WH *et al.* A simple parallel analytical method of prenatal screening. *Gynecol. Obstet. Invest.* 62(4), 220–225 (2006).

99 Watanabe H, Hamada H, Yamada N *et al.* Proteome analysis reveals elevated serum levels of clusterin in patients with preeclampsia. *Proteomics* 4(2), 537–543 (2004).

100 Buhimschi CS, Buhimschi IA, Abdel-Razeq S *et al.* Proteomic biomarkers of intraamniotic inflammation: relationship with funisitis and early-onset sepsis in the premature neonate. *Pediatr. Res.* 61(3), 318–324 (2007).

101 Datta S, Turner D, Singh R, Ruest LB, Pierce WM Jr, Knudsen TB. Fetal alcohol syndrome (FAS) in C57BL/6 mice detected through proteomics screening of the amniotic fluid. *Birth Defects Res. A Clin. Mol. Teratol.* 82(4), 177–186 (2008).

102 Hassan MI, Kumar V, Singh TP, Yadav S. Proteomic analysis of human amniotic fluid from Rh(-) pregnancy. *Prenat. Diagn.* 28(2), 102–108 (2008).

103 Sun LZ, Yang NN, De W, Xiao YS. Proteomic analysis of proteins differentially expressed in preeclamptic trophoblasts. *Gynecol. Obstet. Invest.* 64(1), 17–23 (2007).

104 Angelucci S, Ciavardelli D, Di Giuseppe F. Proteome analysis of human follicular fluid. *Biochem. Biophys. Acta* 1764(11), 1775–1785 (2006).

105 Di Quinzio MK, Oliva K, Holdsworth SJ *et al.* Proteomic analysis and characterisation of human cervico–vaginal fluid proteins. *Aust. NZ J. Obstet. Gynaecol.* 47(1), 9–15 (2007).

106 Liu AX, Zhu YM, Luo Q *et al.* Specific peptide patterns of follicular fluids at different growth stages analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Biochem. Biophys. Acta* 1770(1), 29–38 (2007).

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Review

EXPERT REVIEWS

Noninvasive prenatal diagnosis of fetal aneuploidies and Mendelian disorders: new innovative strategies

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The application of recent technical developments, such as digital PCR or shot-gun sequencing, for the analysis of cell-free fetal DNA, have indicated that the long-sought goal of the noninvasive detection of Down syndrome may finally be attained. Although these methods are still cumbersome and not high-throughput, they provide a paradigm shift in prenatal diagnosis, as they could effectively pronounce the end of invasive procedures such as amniocentesis or chorionic villous sampling for the detection of such fetal anomalies. It, however, remains to be determined how suitable these approaches are for the detection of more subtle fetal genetic alterations, such as those involved in hereditary Mendelian disorders (e.g., thalassemia and cystic fibrosis). New technical developments such as microfluidics and reliable automated scanning microscopes have indicated that it may be possible to efficiently retrieve and examine circulating fetal cells. As these contain the entire genomic complement of the fetus, future developments may include the noninvasive determination of the fetal karyotype.

Keywords: cell-free fetal DNA/RNA • digital PCR • fetal cells • maternal blood • microfluidics • shot-gun sequencing

Although great strides have been made in the noninvasive determination of facile fetal genetic loci, such as Rhesus D in pregnancies at risk for hemolytic disease of the fetus and newborn, and fetal sex in pregnancies at risk for an X-linked disorder via the analysis of cell-free fetal DNA in maternal plasma/serum, the determination of more complex fetal genetic anomalies such as those involved in Down syndrome still rely on invasive procedures including amniocentesis of chorionic villous sampling [1,2]. Since these procedures pose a risk of fetal injury or loss, there is a need for safe efficacious alternatives.

A major driving force for the development of noninvasive prenatal diagnosis is the demographic change that has taken place in developed countries, whereby many pregnant women are over the age of 35 years, even for their first born. Since many couples elect only to have one child, they are naturally not keen to expose their long-desired baby to the risk of an invasive procedure.

A further complication is that despite incremental increases in the sensitivity and specificity of screening procedures for pregnancies bearing fetuses affected by chromosomal anomalies such as Down syndrome, there has been very little net change in the number of live births of such affected babies in the past decade in certain countries, in contrast to others where the rate of such affected births has been halved [3,4]. Hence, a method needs to be developed that can alter this current state of affairs.

What is required?

Currently, a large proportion of pregnant women would automatically be judged to be at an increased risk of bearing a fetus with a chromosomal anomaly due to advanced maternal age, thus the new system would need to be amenable to mass screening, akin to what is being undertaken using serum analytes in the first and second trimester of pregnancy. As such it should be:

- Simple and automatable
- Robust and high throughput
- Cost effective

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- Permit the easy shipping and storage of samples, as it is likely to be off-site from where the sample is taken

In short, this is quite a challenge!

What options exist?

Historically, rare trafficking fetal cells in maternal blood presented the first target for the obtaining of fetal genetic material in a noninvasive manner [5]. This strategy was first attempted in 1979 by Bianchi and colleagues in the laboratory of Herzenberg, the developer of flow cytometry [6]. Following a few high-profile case reports in the mid- to late 1980s [7,8], which indicated that this system may permit the detection of fetal aneuploidies via the analysis of enriched fetal cells by FISH, the NIH funded a large-scale study to test the feasibility of this approach [9,10]. To date, this study, termed NIFTY, is still the largest multicenter analysis of its type. Unfortunately, the goal chosen by NIFTY was too lofty for the technology available at the time, and the degree of sensitivity and specificity attained was way below that required for clinical application (Bianchi *et al.* [2002]). Some questions also remain unanswered as to whether the fetal erythroblast is indeed the most suitable target cell, as they have dense compact nucleus with apoptotic character and may be impervious to FISH analysis [11,12].

Towards the end of the NIFTY study, Dennis Lo and colleagues in Oxford, UK, made a startling discovery, by observing the presence of cell-free fetal DNA (cff-DNA) in the plasma and serum of pregnant women [13,14]. Not only was it easier to retrieve this material than to enrich for fetal cells, but it was much more abundant by a factor of 100- to 1000-fold [15]. As such, most attention in the past decade has been focused on the analysis of this new found analyte, and the quest for fetal cells has faded somewhat into obscurity [1].

Is there resurgent interest in fetal cells?

In 2008, two independent publications suggested that all interest had not been lost in the enrichment and detection of fetal cells [16,17]. What is perhaps most surprising about these reports is that they were made by private companies and not by publicly funded research groups. This implies that although many main stream research groups have largely given up hope on the use of fetal cells for noninvasive prenatal diagnosis, companies using private equity feel sufficiently motivated to follow this course as part of their business plan.

In the first of these reports, Seppo and colleagues at Ikonisys Inc., used an innovative automated scanning microscope system, for the rapid and simple detection of putative target cells, identified by fluorescent staining for fetal or embryonic hemoglobin molecules and FISH for the X and Y chromosomes [16]. The Ikonisys system is different to other previous approaches, in that here the microscope system is enclosed in an industrial box-like system, which includes a loader for 175 standard microscope slides. This has the advantage that the system can be placed anywhere in a standard diagnostic laboratory, without the need for dark room facilities, normally required for FISH analysis. This system can be linked to a central computer network, thereby permitting off-site data analysis. This also permits several machines to be run in parallel. As the system features a simple user friendly interface and standalone 24/7 operation, it is clear that Ikonisis have factored the requirements of routine diagnostic laboratory use into their design.

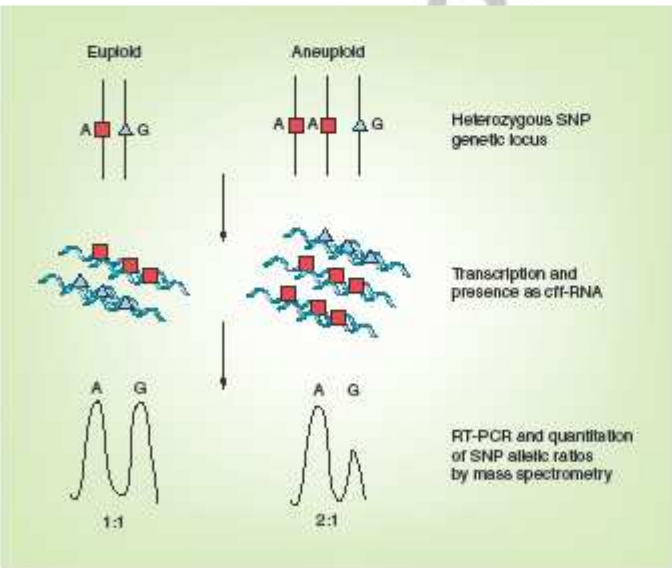

Figure 1. Mass spectrometry-based assay using placentally derived cell-free RNA for the determination of fetal aneuploidy.

In their first examination they analyzed whole blood samples for the presence of putative fetal erythroblast cells identified by XY-FISH, they determined that on average 3 XY-positive cells could be detected in samples taken in the first trimester of pregnancy, and about two such cells in second trimester samples. On average, close to 4 million individual cells were scanned per case, indicating that 0.4–0.8 fetal cells were present per 1 million maternal cells. This is in good agreement with previous assessments.

If the samples were prepared by standard density gradient centrifugation to remove the bulk of the erythrocytes, then the recovery of fetal cells was improved by between two- and three-fold – approximately two fetal cells per 1 million maternal cells. Unfortunately, a slight increase in the false-positive rate was also noted under such conditions.

It is of interest that the Ikonisys examination of more than 60 samples indicates that fetal cells could be reliably detected in

93% of cases, and that this was evident in both samples taken in the first and second trimesters of pregnancy. This pleasing result suggests that with a bit of optimization, this system may be suitable for use on all pregnancies. It also suggests that systems are now available that can reliably scan several million cells in a robust and automated manner for the presence of few or single target cells, indeed a quantum leap forward in this technological arena, as such systems were not available a few years ago.

What is not clear from this publication is the amount of time taken for the analysis of each case and how the issue for false-positives is to be addressed. What is, however, clear is that the system can be used for other diagnostic tasks, such as the detection of rare circulating tumor cells [18], thereby once again underscoring the similarity between these two fields.

In the second study, conducted by Huang and colleagues at Artemis Health Inc., use was made of a microfluidic filtration device to separate erythrocytes from other nucleated cells in maternal blood samples [17]. Similar systems have been examined previously; for instance the Nanos system in Singapore, with the use of a dielectrophoresis (DEP) microfluidic device [19]. DEP is the movement of neutral particles induced by polarization in an asymmetric electric field. Manipulation can be performed on cells based on the differences in their dielectrophoretic properties.

The device developed by Artemis appears to be very effective in depleting erythrocytes from the maternal blood sample (>99.9% efficacy) [17]. What is also remarkable is that they claim to be able to retrieve a large number of putative target cells, namely erythroblasts, which with an average recovery of 38 cells/ml maternal blood is almost 20 times better than what could previously be achieved using the most highly optimized magnetic cell separation (MACS) approach [20]. It is also almost double the number we have previously been able to recover using a soy bean lectin system [21].

The high recovery may stem, in part, from the effective clearance of erythrocytes without the concomitant loss of target cells, as is the case when using other approaches such a density gradient centrifugation. Although there are still a number of questions which remain to be addressed, including whether the recovered cells are fetal or maternal, the time taken for sample processing and the number of samples which can be processed in parallel, this development is to be lauded and its progress to be closely monitored.

Although it is estimated that we may be able to recover only one or two fetal cells per million maternal cells, accurate identification of the fetal origin of the cells using ε-globin would allow a pure population of fetal cells, and hence pure fetal genomic DNA, to be recovered [22,23]. This could form the basis for automated scanning and recognition systems [24,25].

Laser microdissection and pressure catapulting (LMPC) is a rapidly emerging technology for the isolation of single cells for genomic analysis [26–28]. One such device is the Zeiss PALM MicroBeam® system whereby integration of image analysis platforms fully automates screening, identification and cell capture for downstream applications, such as whole genome amplification, single-cell mRNA extraction, PCR-based technologies, and microarray analyses [26]. This system has been used effectively for the isolation of single fetal erythroblasts for subsequent analysis by single cell PCR [12]. It is not inconceivable that the very small numbers of fetal cells enriched from maternal blood may actually be sufficient for downstream analysis using the more modern single-cell analyses methods.

Consequently, these independent developments do strongly suggest that "fetal cells isn't dead yet" and that we are likely to see more commercially viable approaches appearing in the near future.

Problems besetting cell-free-DNA

Even though cff-DNA is much more abundant than trafficking fetal cells, it only makes up a small fraction of the total cf-DNA in maternal plasma (3–10%) [15]. This fraction becomes even smaller in serum, as here the amount of maternal cf-DNA increases by two- to three-fold due to dying cells releasing their nuclear DNA during the clotting procedure.

While the overt presence does not affect the analysis of facile fetal genetic loci completely absent form the maternal genome, such as the RHD gene in Rhesus d-negative pregnant women or the Y chromosome for fetal sex determination, it does become problematic when trying to discern fetal loci not so disparate from maternal ones [1,14]. The latter includes subtle alterations such as point mutations involved in Mendelian disorders such as the hemoglobinopathies or cystic fibrosis, or alterations in gene dosage, such as the presence of an additional chromosome or part thereof in fetal aneuploidies such as Down syndrome.

In these cases the large preponderance of maternal cf-DNA is problematic as it in essence excludes the possibility of performing these analyses. In order to overcome this problem several strategies have been explored.

Size-fractionation

In our examination of whether any physical differences existed between maternal and fetal cf-DNA fragments, we observed that fetal fragments were generally smaller in size than those of maternal origin (<300 bp vs >500 bp, respectively) [29]. This difference is probably attributable to differences in the apoptotic mechanisms involved in the release of cell-free fetal DNA by the syncytiotrophoblast, and that of maternal cf-DNA, which is largely of hemopoeitic origin, probably erythropoiesis. In this context, it is interesting to note that although the nuclear DNA in erythroblasts is cleaved prior to enucleation, this is not in the form of normal oligosomal apoptotic fragments, but rather in large megabase fragments which can only be resolved by pulse-gel electrophoresis [30]. This feature was also observed in our analysis of total cf-DNA fragments [29].

By exploiting this difference between maternal and fetal cf-DNA fragments, we were able to selectively enrich for fetal fragments using conventional agarose gel electrophoresis [29]. These primary experiments showed that the fetal cf-DNA fraction could be increased to over 30%, compared with less than 5% in the untreated sample. This increment permitted the detection of otherwise masked fetal loci, such as short-tandem

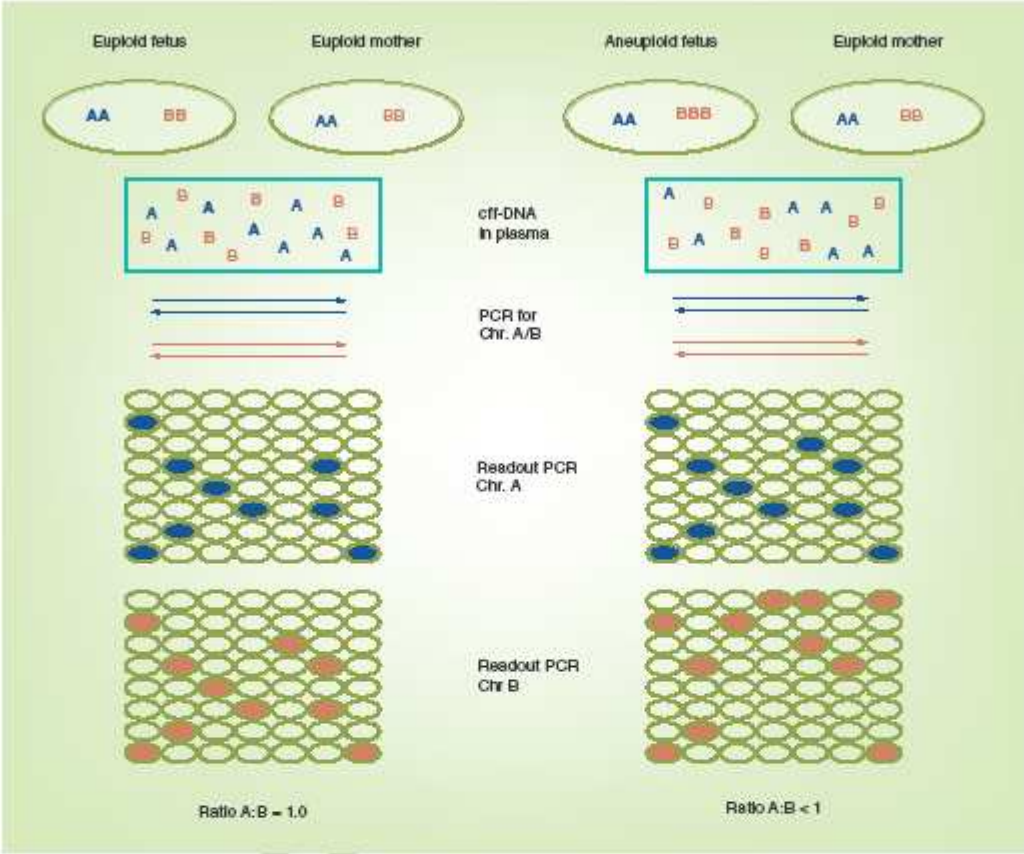

Figure 2. Digital PCR-based assay using cell-free fetal DNA for the determination of fetal aneuploidy.

repeats (STRs) or point mutations, such as those for achondroplasia or β-thalassemia [29,31,32]. Our analyses showed this feature held true for approaches using either real-time PCR or MALDI-TOF mass spectrometry [33,34].

The current problem with this approach is that no efficient method exists to perform the size-fractionation [35]. Although useful for proof-of-principle experiments, the agarose gel electrophoresis approach requires large volumes of cf-DNA (extracted from 10–20 ml plasma) and is associated with a large degree of loss (~50%). As such, it will only become viable once more efficient methods for this process emerge, perhaps via the use of microfluidic devices.

Epigenetic differences

An alternative method to discriminate between fetal and maternal cf-DNA sequences is to use epigenetic differences between these two DNA species [36,37]. An example of a gene sequence which is hypomethylated in the placenta and hypermethylated in maternal blood cells is the *maspin* gene promoter [36]. By the use of bisulfite conversion, the unmethylated fetal cytosine nucleotide is converted to Uralic (thymine), while the maternal methylated cytosine is left unchanged. The altered fetal allele can then be detected by mass spectrometry or real-time PCR. Since the *maspin* gene is located on chromosome 18, it was examined whether this approach could be used to detect fetal aneuploidies specific for this chromosome [38]. By targeting a SNP involving a methylated cytosine residue, it would be possible to use the epigenetic allele ratio to determine fetal ploidy. In their study, Tong and colleagues were indeed able to use this approach to discern trisomy 18 samples from euploid samples when using genomic placental DNA [38]. This distinction was, however, not absolute, and using the cut-off values proposed by the authors, three euploid samples would have been classified as being abnormal. Given this less than satisfactory state affairs

when examining total genomic DNA, it is questionable how effective this approach would be for the analysis of cff-DNA in maternal plasma.

A major problem besetting the use of epigenetically modified DNA sequences is the use of a bisulfite conversion step, a procedure that is very aggressive and destroys a vast amount of template DNA; a major problem when dealing with limiting input DNA quantities. As such alternative strategies need to be sought, perhaps such as the immunoprecipitation of methylated DNA sequences using antibodies specific for methylated cytosine residues [39].

Cell-free fetal mRNA

The discovery of CF mRNA species of placental origin in maternal plasma opened up the way for an alternative strategy to the analysis of cff-DNA [40,41]. The major advantage of cff-RNA over cff-DNA is that it is possible to select for placenta specific mRNA transcripts not expressed by any maternal tissues [42]. Hence, the analysis of cff-RNA is in essence very similar to the analysis of fetal genetic loci completely absent from the maternal genome (Rhesus D- or Y chromosome-specific sequences) as it is not hindered maternal background 'noise'.

In order to use such an approach for the determination of fetal ploidy, one hence has to select genes present on the chromosome of interest, for example chromosome 21, and then ensure that the gene is only expressed in the placenta and not by maternal tissues [43]. An example of such a gene is *PLAC4*. For the determination of chromosomal ploidy, an approach similar to that used for *maspin* gene in trisomy 18 can be taken, namely the use of mass spectrometry to determine allelic ratios of a SNP locus in the *PLAC4* gene [43].

Although the study conducted by Lo and colleagues was based on a small number of affected cases (n = 10), a remarkably clear difference between case and control samples (n = 56) was observed yielding a sensitivity of 90% and specificity of 96%. The current drawback of this approach is that the fetus needs to be heterozygous for the SNP allele being interrogated. As such, almost 100 cases had to be excluded from the aforementioned investigation. A further problem is the labile nature of mRNA, which requires complex immediate post-phlebotomy handling and processing. Furthermore storage and shipping needs to be carried out at -70°C or dry-ice, which is an additional burden. Regardless of these issues this approach has been seized upon by Sequenom Inc., which is exploring the commercial viability.

Digital PCR & its affiliates

Digital PCR differs from other quantitative PCR approaches such as real-time PCR in that the PCR reaction is allowed to proceed to its plateau and a simple 'yes or no' answer is used to monitor the presence or absence of input template [44]. In order to use this system in a quantitative manner it is necessary to monitor each PCR reaction individually. Although initial exploration of such an approach were made in 1997 by Kalinina [45] and colleagues and later optimized Vogelstein and Kinzler in 1999 [44], it was only with the introduction of microfluidic devices with several thousand individual reaction chambers, such as those developed by Fluidigm Inc., that 'digital PCR' came of age [46,47].

The use of this technology for the detection of fetal aneuploidies was published almost simultaneously by Fan and Quake [48] and the Hong Kong group of Dennis Lo [49]. In their examination Fan and Quake used a similar PCR assay to the one we had previously established for the detection of trisomy 21 by Taqman real-time PCR on pure fetal genetic material obtained by invasive practises, in which we compared the dosage of the amyloid gene on chromosome 21 to that of the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) on chromosome 12 [50]. In their analysis of pure fetal genetic material, they were similarly able to reliably discriminate between normal and aneuploid samples. In the Hong Kong, examination use was made both of a SNP locus and a gene dosage approach, obtaining results very analogous to those made by Fan and Quake.

What is of particular interest in these studies is that, they observed the ability to detect the presence of a fetal aneuploidy was still possible even if the fetal material only represented 10% of the total cf-DNA examined, provided that 4000 individual events were examined [48]. Hence, it may be possible to use this technology to detect fetal aneuploidies directly from cff-DNA in maternal plasma. Since subsequent studies using digital PCR have indicated that the amount of cff-DNA has been under estimated using real-time PCR, and may be as high as 10%, this facet could soon become a reality [51].

It is also likely that the digital PCR approach will be useful not only for the detection of fetal aneuploidies, but may also permit the noninvasive determination of single gene disorders such as the hemoglobinopathies [47,52]. Once again, it is evident that this technology may also be applicable to other clinical disciplines, such as oncology [53].

Although the current studies have focused on the use of microfluidic devices, such as those developed by Fluidigm Inc., it may be possible to use a somewhat 'cruder' approach termed 'beaming' for 'beads, emulsion, amplification and magnetics'. In this procedure developed by Diehl and colleagues, the input template DNA is first preamplified and then coupled to a streptavidin-coated bead via a primer containing a biotin tag [54]. The second round of PCR is then carried out in an emulsion phase, following which ration of the two DNA sequences of interest is monitored by flow cytometry using fluorescently labeled primers. By the use of such means, Diehl and colleagues have been able to monitor and quantify cancer-derived cf-DNA [55]. Although this method is technically more complex than straight-forward digital PCR, it does not require any expensive equipment or costly specialized microfluidic reaction chambers. It may, hence, emerge as an alternative for those with cash-strapped research budgets.

Shot-gun sequencing

Even though the digital PCR approach is quite promising, it is clear that the degree of accuracy required for the detection of a fetal aneuploidy is at the limit of current microfluidic devices, as they only have space for 12,000 individual reactions per chip

[47]. For this reason an alternative was sought that offered a higher degree of fidelity by examining more target molecules. Such a system was found in the Solexa/Illumina platform for shot-gun sequencing. In this system very short tags from the entire genome are amplified and sequenced. In their pioneering study, Fan and colleagues obtained an average of 10 million 25-bp sequence tags per sample, which contained an average of 65,000 tags specific for chromosome 21 [56]. By using these sequence tags for digital PCR-like measure, they were able to discern nine cases of trisomy 21 from nine euploid cases with 100% accuracy, in an analysis of cff-DNA. They were furthermore able to detect two cases of trisomy 18 and one case of trisomy 13. In a parallel report, Chiu and colleagues were able to correctly discern 14 cases of trisomy 21 from 28 normal cases [57]. These two pivotal studies, therefore, strongly suggest that shot-gun sequencing may be the most suitable approach for the noninvasive detection of fetal aneuploidies.

Other alternatives: urinary DNA?

Although the presence of cf-DNA in urine [58–60], other than that of kidney origin [61], has been a contentious issue, recent publication does suggest that 'transrenal DNA' may be another option for noninvasive prenatal diagnosis [62]. In this report by Shekhtman and colleagues, who are able to detect cff-DNA in maternal urine by using very short PCR amplicons (25–88 bp), observed that urinary cff-DNA fragments were very small, some specialized extraction and analytic procedures needed to be used in order to detect them reliably [62]. In their report they were able to detect male cff-DNA in 78 out of 82 samples form women pregnant with a male fetus. On the other hand, Y chromosome-specific signals were detected in 11 out of 91 samples where the pregnancy was with a female fetus. Hence, care needs to be taken to ensure that the maternal urine sample is not contaminated by male cells/DNA, probably as the result of intercourse.

In the analysis by the Hong Kong group of Dennis Lo, who pursued their investigations into cancer derived cf-DNA in urine [63] by examining bone marrow transplant recipients [64]. In this new study[65,66] they determined that both transplant-derived DNA and epithelial-like cells were present in recipient urine [64]. They furthermore concluded that the transplant-derived urinary DNA was derived from donor-derived stem cells, as these have settled in renal tubule niches.

As the persistence of transplacental trafficking of fetal cells with stem cell-like character has been reported on numerous occasions [67], and as these cell have been found to contribute to maternal tissues, especially in the capacity of tissue repair [68,69], it is unclear whether such cells could contribute to the phenomenon of transrenal cff-DNA.

Expert commentary & five-year view

Of all the systems tested to date shot-gun sequencing, used in a pseudodigital PCR mode, appears to offer the most reliable detection of fetal aneuploidies, with truly amazing discrimination between affected cases and normal controls. The current problem with this approach is time and money, in that the processing and data analysis of each sample is a lengthy and costly affair (~$700 to $1200 per case and only 16 samples/week/Illumina instrument). This revolutionary method, however, may offer the possibility of obtaining detailed karyotypic information by non-invasive means. It is, however, not clear if it will be useful for the analysis of Mendelian disorders, as the method relies on the analysis of a vast amount of genetic loci per chromosome targeted (in the order of 60,000 for chromosome 21), unlike the single/dual mutation involved in disorders such as the hemoglobinopathies.

The optimization of digital PCR approaches by the employment of microfluidic devices that permit in excess of 30,000 single PCR reactions on a single chip may offer a cheaper alternative to the complex shot-gun sequencing approach. A drawback of these analyses is that the amount of cff-DNA needs to be estimated quite accurately in order to ensure that the template concentration is in the narrow range required for efficient digital PCR analysis (<1 copy/reaction/well).

Even though the fetal mRNA approach has been propagated to a large extent by Sequenom, it is not clear how suitable this system is to wide-scale application due to processing and shipping issues, a major concern when dealing with a labile analyte such as mRNA. In this context it is worth noting that recent corporate statements from Sequenom indicate a possible move towards a DNA-based approach, perhaps involving epigenetic markers.

The resurgent interest in fetal cells suggests that these have not been buried, but are seriously being considered by commercial institutions. While it is unlikely that fetal cells will be offered for wide-spread screening, as could be done using cff-DNA/RNA, the ability to examine the entire fetal genome offers up a realm of possibilities which will not be possible via the cff-DNA route. As such, it is foreseeable that this route will be more restricted and costly.

As indicated earlier, it is likely that two strategies will emerge. In one instance, a quick and simple test will be offered which permits rapid screening for the most common fetal aneuploidies (e.g., chromosomes 13, 18 and 21). This is likely to occur using an approach developed by Sequenom that, by relying on its proprietary mass spectrometry technology, is geared for high-throughput analysis of several thousand samples per day. This could make the test cost effective, to the extent where it can compete with conventional screening approaches.

Even though digital PCR approaches are promising, and may be useful for the analysis of single-gene disorders, they currently require expensive equipment and analytic devices, with the microfluidic chambers used for each analysis still costing several hundred US$ a piece. Furthermore, the cff-DNA sample needs to be diluted in such manner that approximately 0.6 copies are present per reaction well, a tedious and time consuming exercise.

Shot-gun sequencing approaches are even more costly and time-consuming, facets which will restrict their use until a new generation of high-throughput devices becomes available. As discussed, the use of fetal cells is largely dependent on the speed of current progress in microfluidic enrichment tools, automated cell detection and retrieval, as well as analytic systems permitting whole-genome analysis from single or few cells.

As such, these latter options will be more costly and only offered to a select group, whilst the former employing high-throughput cff-DNA/DNA mass spectrometry-based strategies may end up replacing or complementing current screening procedures, such as ultrasound and concomitant serum analyte analysis.

As always, it pays to be aware of developments outside the immediate scope of this review, of which the most important is probably that of proteomics. Such approaches which may lead to the development of a new generation of highly specific screening markers, which could be so effective that other approaches become commercially unviable. Consequently, researchers and clinicians active in prenatal diagnosis need to be prepared for rapid changes and developments in the next decade.

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The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Key Issues

- Remarkable developments have occurred in the past 2–3 years, largely due to the use of 'digital PCR' and 'shot-gun sequencing'.
- Technical developments now have to focus on economic, robust, high-throughput processes.
- 'Fetal cells isn't dead yet', and may become a viable alternative via the use of microfluidics and automated scanning devices
- Unlike cf-DNA, fetal cells offer the possibility of examining the entire fetal genome
- New screening markers developed by proteomics may challenge some of the 'quick and dirty' approaches
- Detection of Mendelian disorders may remain complex, especially in cases where both parents share the same mutation.
- Urinary cf-DNA may become a new tool of interest.
- The use of cell-free fetal DNA and fetal cells may also serve as potential markers for the prediction of preeclampsia or preterm labor.

References

Papers of special note have been highlighted as:
• of interest
•• of considerable interest

1 Lo YM, Chiu RW. Prenatal diagnosis: progress through plasma nucleic acids. *Nat. Rev. Genet.* 8, 71–77 (2007).

2 Hahn S, Zhong XY, Holzgreve W. Recent progress in non-invasive prenatal diagnosis. *Semin. Fetal Neonatal Med.* 13, 57–62 (2008).

3 Ekelund CK, Jorgensen FS, Petersen OB, Sundberg K, Tabor A. Impact of a new national screening policy for Down's syndrome in Denmark: population based cohort study. *BMJ* 337, a2547 (2008).

•• Important report describing the impact of noninvasive screening techniques on the prenatal detection of Down syndrome in a highly developed healthcare system

4 Irving C, Basu A, Richmond S, Burn J, Wren C. Twenty-year trends in prevalence and survival of Down syndrome. *Eur. J. Hum. Genet.* 16, 1336–1340 (2008).

•• Important publication questioning the effectiveness of current screening approaches.

5 Hahn S, Sant R, Holzgreve W. Fetal cells in maternal blood: current and future perspectives. *Mol. Hum. Reprod.* 4, 515–521 (1998).

6 Herzenberg LA, Bianchi DW, Schroder J, Cann HM, Iverson GM. Fetal cells in the blood of pregnant women: detection and enrichment by fluorescence-activated cell sorting. *Proc. Natl Acad. Sci. USA* 76, 1453–1455 (1979).

•• First report indicating that fetal cells in maternal blood can be enriched.

7 Elias S, Price J, Dockter M *et al.* First trimester prenatal diagnosis of trisomy 21 in fetal cells from maternal blood. *Lancet* 340, 1033 (1992).

•• Pioneering report indicating that fetal cells in maternal blood can be enriched for and used for noninvasive prenatal diagnosis.

8 Bianchi DW, Mahr A, Zickwolf GK *et al.* Detection of fetal cells with 47,XY,+21 karyotype in maternal peripheral blood. *Hum. Genet.* 90, 368–370 (1992).

9 Bianchi DW, Simpson JL, Jackson LG *et al.* Fetal gender and aneuploidy detection using fetal cells in maternal blood: analysis of NIFTY I data. National Institute of Child Health and Development Fetal Cell Isolation Study. *Prenat. Diagn.* 22, 609–615 (2002).

10 Bianchi DW, Simpson JL, Jackson LG *et al.* Fetal cells in maternal blood: NIFTY clinical trial interim analysis. DM-STAT. NICHD fetal cell study (NIFTY) group. *Prenat. Diagn.* 19, 994–995 (1999).

11 Babochkina T, Mergenthaler S, De NG *et al.* Numerous erythroblasts in maternal blood are impervious to fluorescent *in situ* hybridization analysis, a feature related to a dense compact nucleus with apoptotic character. *Haematologica* 90, 740–745 (2005).

•• Erythroblast differentiation and enucleation influences genetic analysis.

12 Kolialexi A, Vrettou C, Traeger-Synodinos J *et al.* Noninvasive prenatal diagnosis of β-thalassaemia using individual fetal erythroblasts isolated from maternal blood after enrichment. *Prenat. Diagn.* (2007).

13 Lo YM, Corbetta N, Chamberlain PF *et al.* Presence of fetal DNA in maternal plasma and serum. *Lancet* 350, 485–487 (1997).

•• Pivotal discovery of cell-free fetal DNA in maternal circulation.

14 Hahn S, Holzgreve W. Prenatal diagnosis using fetal cells and cell-free fetal DNA in maternal blood: what is currently feasible? *Clin. Obstet. Gynecol.* 45, 649–656 (2002).

15 Lo YM, Tein MS, Lau TK *et al.* Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. *Am. J. Hum. Genet.* 62, 768–775 (1998).

16 Seppo A, Frisova V, Ichetovkin I *et al.* Detection of circulating fetal cells utilizing automated microscopy: potential for noninvasive prenatal diagnosis of chromosomal aneuploidies. *Prenat. Diagn.* 28, 815–821 (2008).

• Fetal cells can be detected solely by automated scanning device.

17 Huang R, Barber TA, Schmidt MA *et al.* A microfluidics approach for the isolation of nucleated red blood cells (NRBCs) from the peripheral blood of pregnant women. *Prenat. Diagn.* 28, 892–899 (2008).

18 Ntouroupi TG, Ashraf SQ, McGregor SB *et al.* Detection of circulating tumour cells in peripheral blood with an automated scanning fluorescence microscope. *Br. J. Cancer* 99, 789–795 (2008).

19 Xu G, Chan MB, Yang C *et al.* Design and fabrication of a microfluidic device for fetal cells dielectrophoretic properties characterization. *IPCS* 34, 1106–1111 (2006).

20 Hahn S, Kiefer V, Brombacher V, Troeger C, Holzgreve W. Fetal cells in maternal blood. An update from Basel. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 85, 101–104 (1999).

21 Babochkina T, Mergenthaler S, Lapaire O *et al.* Evaluation of a soybean lectin-based method for the enrichment of erythroblasts. *J. Histochem. Cytochem.* 53, 329–330 (2005).

22 Choolani M, O'Donoghue K, Talbert D *et al.* Characterization of first trimester fetal erythroblasts for non-invasive prenatal diagnosis. *Mol. Hum. Reprod.* 9, 227–235 (2003).

23 Choolani M, O'Donnell H, Campagnoli C *et al.* Simultaneous fetal cell identification and diagnosis by epsilon-globin chain immunophenotyping and chromosomal fluorescence *in situ* hybridization. *Blood* 98, 554–557 (2001).

24 Hennerbichler S, Kroisel PM, Zierler H *et al.* Fetal nucleated red blood cells in peripheral blood of pregnant women: detection and determination of location on a slide using laser-scanning cytometry. *Prenat. Diagn.* 23, 710–715 (2003).

25 Hennerbichler S, Mehes G, Schmied R *et al.* Detection and relocation of rare events. A comparative study using the laser scanning cytometer and the Metafer/RCDetect microscope scanning system. *J. Biochem. Biophys. Methods* 53, 109–115 (2002).

26 Burgemeister R. New aspects of laser microdissection in research and routine. *J. Histochem. Cytochem.* 53, 409–412 (2005).

27 Anoruo B, van OR, Mitchell J, Howells D. Isolating cells from non-sperm cellular mixtures using the PALM microlaser micro dissection system. *Forensic Sci. Int.* 173, 93–96 (2007).

28 Kuhn DE, Roy S, Radtke J, Khanna S, Sen CK. Laser microdissection and capture of pure cardiomyocytes and fibroblasts from infarcted heart regions: perceived hyperoxia induces p21 in peri-infarct myocytes. *Am. J. Physiol Heart Circ. Physiol* 292, H1245–H1253 (2007).

29 Li Y, Zimmermann B, Rusterholz C *et al.* Size separation of circulatory DNA in maternal plasma permits ready detection of fetal DNA polymorphisms. *Clin. Chem.* 50, 1002–1011 (2004).

• Cell-free fetal DNA fragments can be enriched on basis of size.

30 Hristoskova S, Holzgreve W, Hahn S, Rusterholz C. The chromatin of differentiating erythroblasts is cleaved into large size fragments independent of caspase activated DNase and apoptosis inducing factor. *J. Cell Physiol.* 213, 490–494 (2007).

31 Li Y, Holzgreve W, Page-Christiaens GC, Gille JJ, Hahn S. Improved prenatal detection of a fetal point mutation for achondroplasia by the use of size-fractionated circulatory DNA in maternal plasma – case report. *Prenat. Diagn.* 24, 896–898 (2004).

32 Li Y, Di NE, Vitucci A *et al.* Detection of paternally inherited fetal point mutations for β-thalassemia using size-fractionated cf-DNA in maternal plasma. *JAMA* 293, 843–849 (2005).

• Fetal DNA enrichment permits detection of point mutations.

33 Li Y, Page-Christiaens GC, Gille JJ, Holzgreve W, Hahn S. Non-invasive prenatal detection of achondroplasia in size-fractionated cf-DNA by MALDI-TOF MS assay. *Prenat. Diagn.* 27, 11–17 (2007).

34 Li Y, Wenzel F, Holzgreve W, Hahn S. Genotyping fetal paternally inherited SNPs by MALDI-TOF MS using cell-free fetal DNA in maternal plasma: influence of size fractionation. *Electrophoresis* 27, 3889–3896 (2006).

35 Li Y, Holzgreve W, Di Naro E, Vitucci A, Hahn S. Cell free DNA in maternal plasma: is it all a question of size? *Ann. NY Acad. Sci.* 5 (2006).

36 Chim SS, Tong YK, Chiu RW *et al.* Detection of the placental epigenetic signature of the maspin gene in maternal plasma. *Proc. Natl Acad. Sci. USA* 102, 14753–14758 (2005).

37 Chim SS, Jin S, Lee TY *et al.* Systematic search for placental DNA-methylation markers on chromosome 21: toward a maternal plasma-based epigenetic test for fetal trisomy 21. *Clin. Chem.* 54, 500–511 (2008).

38 Tong YK, Ding C, Chiu RW *et al.* Noninvasive prenatal detection of fetal trisomy 18 by epigenetic allelic ratio analysis in maternal plasma: Theoretical and empirical considerations. *Clin. Chem.* 52, 2194–2202 (2006).

39 Mohn F, Weber M, Schubeler D, Roloff TC. Methylated DNA immunoprecipitation (MeDIP). *Methods Mol. Biol.* 507, 55–64 (2009).

40 Ng EK, Tsui NB, Lau TK *et al.* mRNA of placental origin is readily detectable in maternal plasma. *Proc. Natl Acad. Sci. USA* 100, 4748–4753 (2003).

41 Chiu RW, Lui WB, Cheung MC *et al.* Time profile of appearance and disappearance of circulating placenta-derived mRNA in maternal plasma. *Clin. Chem.* 52, 313–316 (2006).

42 Tsui NB, Chim SS, Chiu RW *et al.* Systematic micro-array based identification of placental mRNA in maternal plasma: towards non-invasive prenatal gene expression profiling. *J. Med. Genet.* 41, 461–467 (2004).

43 Lo YM, Tsui NB, Chiu RW *et al.* Plasma placental RNA allelic ratio permits noninvasive prenatal chromosomal aneuploidy detection. *Nat. Med.* 13, 218–223 (2007).

•• Fetal mRNA analysis permits detection of Down syndrome.

44 Vogelstein B, Kinzler KW. Digital PCR. *Proc. Natl Acad. Sci. USA* 96, 9236–9241 (1999).

45 Kalinina O, Lebedeva I, Brown J, Silver J. Nanoliter scale PCR with TaqMan detection. *Nucleic Acids Res.* 25, 1999–2004 (1997).

46 Liu J, Hansen C, Quake SR. Solving the "world-to-chip" interface problem with a microfluidic matrix. *Anal. Chem.* 75, 4718–4723 (2003).

47 Zimmermann BG, Grill S, Holzgreve W *et al.* Digital PCR: a powerful new tool for noninvasive prenatal diagnosis? *Prenat. Diagn.* 28, 1087–1093 (2008).

48 Fan HC, Quake SR. Detection of aneuploidy with digital polymerase chain reaction. *Anal. Chem.* 79, 7576–7579 (2007).

49 Lo YM, Lun FM, Chan KC *et al.* Digital PCR for the molecular detection of fetal chromosomal aneuploidy. *Proc. Natl Acad. Sci. USA* 104, 13116–13121 (2007).

50 Zimmermann B, Holzgreve W, Wenzel F, Hahn S. Novel real-time quantitative PCR test for trisomy 21. *Clin. Chem.* 48, 362–363 (2002).

51 Lun FM, Chiu RW, Ian Chan KC *et al.* Microfluidics digital PCR reveals a higher than expected fraction of fetal DNA in maternal plasma. *Clin. Chem.* 54, 1664–1672 (2008).

52 Lun FM, Tsui NB, Chan KC *et al.* Noninvasive prenatal diagnosis of monogenic diseases by digital size selection and relative mutation dosage on DNA in maternal plasma. *Proc. Natl Acad. Sci. USA* 105, 19920–19925 (2008).

53 Yung TK, Chan KC, Mok TS *et al.* Single-molecule detection of epidermal growth factor receptor mutations in plasma by microfluidics digital PCR in non-small cell lung cancer patients. *Clin. Cancer Res.* 15, 2076–2084 (2009).

54 Diehl F, Li M, He Y *et al.* BEAMing: single-molecule PCR on microparticles in water-in-oil emulsions. *Nat. Methods* 3, 551–559 (2006).

55 Diehl F, Diaz LA, Jr. Digital quantification of mutant DNA in cancer patients. *Curr. Opin. Oncol.* 19, 36–42 (2007).

56 Fan HC, Blumenfeld YJ, Chitkara U, Hudgins L, Quake SR. Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. *Proc. Natl Acad. Sci. USA* 105, 16266–16271 (2008).

•• Shot-gun sequencing permits noninvasive detection of fetal aneuploidies.

57 Chiu RW, Chan KC, Gao Y *et al.* Noninvasive prenatal diagnosis of fetal chromosomal aneuploidy by massively parallel genomic sequencing of DNA in maternal plasma. *Proc. Natl Acad. Sci. USA* 105, 20458–20463 (2008).

•• Shot-gun sequencing permits noninvasive detection of fetal aneuploidies.

58 Li Y, Zhong XY, Kang A *et al.* Inability to detect cell free fetal DNA in the urine of normal pregnant women nor in those affected by preeclampsia associated HELLP syndrome. *J. Soc. Gynecol. Investig.* 10, 503–508 (2003).

59 Lo YM. Molecular testing of urine: catching DNA on the way out. *Clin. Chem.* 46, 1039–1040 (2000).

60 Botezatu I, Serdyuk O, Potapova G *et al.* Genetic analysis of DNA excreted in urine: a new approach for detecting specific genomic DNA sequences from cells dying in an organism. *Clin. Chem.* 46, 1078–1084 (2000).

61 Li Y, Hahn D, Zhong XY *et al.* Detection of donor-specific DNA polymorphisms in the urine of renal transplant recipients. *Clin. Chem.* 49, 655–658 (2003).

62 Shekhtman EM, Anne K, Melkonyan HS *et al.* Optimization of transrenal DNA analysis: detection of fetal DNA in maternal urine. *Clin. Chem.* 55, 723–729 (2009).

• Maternal urine contains short fragments of cell-free fetal DNA.

63 Chan KC, Leung SF, Yeung SW, Chan AT, Lo YM. Quantitative analysis of the transrenal excretion of circulating EBV DNA in nasopharyngeal carcinoma patients. *Clin. Cancer Res.* 14, 4809–4813 (2008).

64 Hung EC, Shing TK, Chim SS *et al.* Presence of donor-derived DNA and cells in the urine of sex-mismatched hematopoietic stem cell transplant recipients: implication for the transrenal hypothesis. *Clin. Chem.* 55, 715–722 (2009).

65 O'Donoghue K, Sultan HA, Al-Allaf FA *et al.* Microchimeric fetal cells cluster at sites of tissue injury in lung decades after pregnancy. *Reprod. Biomed. Online* 16, 382–390 (2008).

66 O'Donoghue K, Chan J, de la Fj *et al.* Microchimerism in female bone marrow and bone decades after fetal mesenchymal stem-cell trafficking in pregnancy. *Lancet* 364, 179–182 (2004).

67 Bianchi DW, Zickwolf GK, Weil GJ, Sylvester S, DeMaria MA. Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. *Proc. Natl Acad. Sci. USA* 93, 705–708 (1996).

68 Srivatsa B, Srivatsa S, Johnson KL *et al.* Microchimerism of presumed fetal origin in thyroid specimens from women: a case–control study. *Lancet* 358, 2034–2038 (2001).

69 Khosrotehrani K, Johnson KL, Cha DH, Salomon RN, Bianchi DW. Transfer of fetal cells with multilineage potential to maternal tissue. *JAMA* 292, 75–80 (2004).

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Brief Communications

Detection of Increased Amounts of Cell-Free Fetal DNA with Short PCR Amplicons

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AIM: A digital PCR approach has recently been suggested to detect greater amounts of cell-free fetal DNA in maternal plasma than conventional real-time quantitative PCR (qPCR). Because the digital qPCR approach uses shorter PCR amplicons than the real-time qPCR assay, we investigated whether a real-time qPCR assay appropriately modified for such short amplicons would improve the detection of cell-free fetal DNA.

METHOD: We developed a novel universal-template (UT) real-time qPCR assay that was specific for the *DYS14* sequence on Y chromosome and had a short amplicon size of 50 bp. We examined this "short" assay with 50 maternal plasma samples and compared the results with those for a conventional real-time qPCR assay of the same locus but with a longer amplicon (84 bp).

RESULTS: Qualitatively, both assays detected male cell-free fetal DNA with the same specificity and detection capability. Quantitatively, however, the new UT real-time qPCR assay for shorter amplicons detected, on average, almost 1.6-fold more cell-free fetal DNA than the conventional real-time qPCR assay with longer amplicons.

CONCLUSIONS: The use of short PCR amplicons improves the detection of cell-free fetal DNA. This feature may prove useful in attempts to detect cell-free fetal DNA under conditions in which the amount of template is low, such as in samples obtained early in pregnancy.

The analysis of cell-free fetal DNA in maternal serum and plasma is currently the method of choice for the noninvasive determination of fetal genetic traits *(1)*. Real-time quantitative PCR (qPCR)[4] is used for the majority of these analyses, because this method is amenable to automation, provides data in a real-time manner, and, by being a closed system, is less prone to contamination than conventional PCR methods with longer amplicons *(1, 2)*. Alternatives that are being explored and gaining in importance are mass spectrometry of primer-extended PCR products, digital PCR, and shotgun sequencing *(3, 4)*.

To date, clinical applications have centered largely on the rather facile detection of fetal genetic loci completely absent from the maternal genome, such as the determination of fetal sex in pregnancies at risk for X-linked disorders or the fetal Rhesus D genotype in pregnancies at risk for hemolytic disease of the fetus and newborn, because the detection of other, more subtle genetic differences between mother and fetus is rendered more complex because of the preponderance of maternal cell-free DNA sequences *(1, 2)*.

Because real-time qPCR also provides a quantitative answer, this approach has been used in a number of studies to determine the concentration of cell-free fetal DNA in maternal plasma samples. In general, these studies have indicated much higher concentrations of cell-free fetal DNA than those of rare circulating fetal cells, but they are still quite low, approximately 1%–3% early in pregnancy and progressing to approximately 5% at term. Through the use of this technology, measurements of increases in cell-free fetal DNA concentrations have also revealed a number of pregnancy-related conditions or disorders, including preeclampsia, pregnancies at risk for preeclampsia, preterm labor, and fetuses with certain aneuploidies, particularly trisomy 21.

Most of these studies have relied on the use of a real-time qPCR assay for the single-copy *SRY* (sex determining region Y) gene on the Y chromosome. Subsequent investigations have indicated that the accuracy of these quantitative (and qualitative) assessments is markedly improved through the use of a real-time qPCR assay for the multicopy *DYS14* sequence on the Y chromosome *(5)*. Consequently, such assays are now frequently used for the determination of fetal sex, especially for samples obtained early in pregnancy *(6)*.

A recent study with digital PCR, a procedure that individually monitors numerous PCR reactions, indicated that the concentration of cell-free fetal DNA may be greater, perhaps more than twice that previously surmised with the use of real-time qPCR *(7)*. Although absolute quantification by digital PCR is considerably more precise than analog real-time qPCR measure-

[4] Nonstandard abbreviations: qPCR, quantitative PCR; UT, universal template.

ments, there is a discrepancy between the 2 qPCR assays because the investigators used amplicons of differing lengths and targets. The amplicon size was 87 bp for the digital PCR assay, whereas it was 137 bp for the real-time qPCR assay. This feature might not have been relevant were it not for the observation that cell-free DNA is fragmented, probably into apoptotic nucleosomal fragments, and that fetal cell-free DNA fragments are generally smaller than those of maternal origin *(8, 9)*.

We therefore investigated this aspect in further detail. Conventional real-time qPCR assays have amplicon sizes that are longer, approximately 80–140 bp. We made use of another approach, a universal template (UT) for probe hybridization that is linked to the 5′ end of one of the PCR primers *(10)*. This approach permitted us to devise a new real-time qPCR assay with an amplicon size of only 50 bp for the *DYS14* locus.

This retrospective study used banked maternal plasma samples stored at −80 °C. All samples were analyzed in a blinded manner. For the determination of fetal sex, we obtained maternal blood samples from 51 pregnant women, 31 with a male fetus and 20 with a female fetus. Data are presented only for the women with a male fetus, of which 24 samples were from the first trimester (median gestational age, 12 + 4 weeks), 6 samples were from second-trimester pregnancies (median gestational age, 25 + 4 weeks), and 21 samples were from third-trimester pregnancies (median gestational age, 35 + 6 weeks). See Table 1 in the Data Supplement that accompanies the online version of this Brief Communication at http://www.clinchem.org/content/vol56/issue1. The Institutional Review Board of University Hospital, Basel, approved the study.

Plasma from maternal blood samples was processed and stored as described previously *(5, 8)*. Cell-free DNA was extracted from 500 μL plasma and eluted into 50 μL elution buffer with a commercially available manual column technology (High Pure PCR Template Preparation Kit; Roche) according to the manufacturer's instructions.

To detect and quantify cell-free fetal DNA, we used an Applied Biosystems ABI Prism 7000 Sequence Detection System with previously established real-time qPCR assays for the *DYS14* locus. The assays were either a real-time qPCR assay with conventional hydrolysis probes and a longer amplicon *(5)*, or the new UT-qPCR assay with a shorter amplicon (see the online Data Supplement for full details). All primers were synthesized by Microsynth, and PCR reagents were supplied by Eurogentec. The sequences of the primers and probes used for the short UT-qPCR assays are as follows: DYS-UT forward, aag ctc agt cat ttc cag gtg tgc gaa aGG GCC AAT GTT GTA TCC TTC TC (100 nmol/L final concentration); DYS-UT reverse, ACT AGA AAG GCC GAA GAA ACA CT (300 nmol/L); UT FAM-TAMRA probe, tcg cac acc tgg aaa tga ctg agc tt (200 nmol/L). The short UT sequence and the *DYS14*-specific sequence are indicated in lowercase and uppercase letters, respectively. The PCR cycling conditions were as follows: Uracil-*N*-glycosylase treatment at 50 °C for 2 min, polymerase activation at 95 °C for 10 min, and 45 cycles of 60 °C for 1 min, 72 °C for 45 s, and 95 °C for 15 s. Cell-free fetal DNA concentrations were expressed as genome equivalents per milliliter of maternal plasma. All samples were run in duplicate. Further details are provided in the supplemental figures and the Data File in the online Data Supplement.

For statistical analysis, we used the Wilcoxon signed rank test in SPSS for Windows (SPSS). Statistical significance was set at P values <0.05. Data were presented as a scatterplot of cell-free fetal DNA concentrations measured with the 2 assays in relation to gestational age.

We discerned no qualitative difference between the use of the longer-amplicon conventional assay and the new short-amplicon UT assay for determining fetal sex with Y chromosome–specific sequences (*DYS14*). All 31 male fetuses were detected correctly. There were no false-positive results among the 20 samples with female fetuses (data not shown). Despite these early results for diagnostic accuracy, we recommend delaying the use of the described short UT qPCR assay for the noninvasive determination of fetal sex until the assay has been validated and appropriate cutoff values have been ascertained *(5)*.

Quantitatively, the short-amplicon UT assay detected, on average, almost 1.6-fold more cell-free fetal DNA than the real-time qPCR assay with conventional hydrolysis probes and a longer amplicon (Fig. 1; Table 1 in the online Data Supplement), a difference that was statistically significant ($P < 0.001$). This observation held true for almost all of the 31 samples containing male cell-free fetal DNA. Our results indicate that the use of shorter amplicons in the real-time qPCR assay increases the number of cell-free fetal DNA molecules detected in maternal plasma. This nearly 1.6-fold increase in the detection of cell-free fetal DNA with the short-amplicon UT assay compared with the real-time qPCR assay with conventional hydrolysis probes and a longer amplicon (Fig. 1; Table 1 in the online Data Supplement) is very close to the improvement recently noted for a digital PCR approach over a conventional real-time qPCR assay with a longer amplicon *(7)*. This report *(7)* discussed the idea that this increase could be due to the more precise assessment of cell-free fetal DNA concentrations with digital PCR than with the analog real-time qPCR method. An aspect not addressed in detail was the issue of the different-sized PCR amplicons used in the experiment (87 bp for the

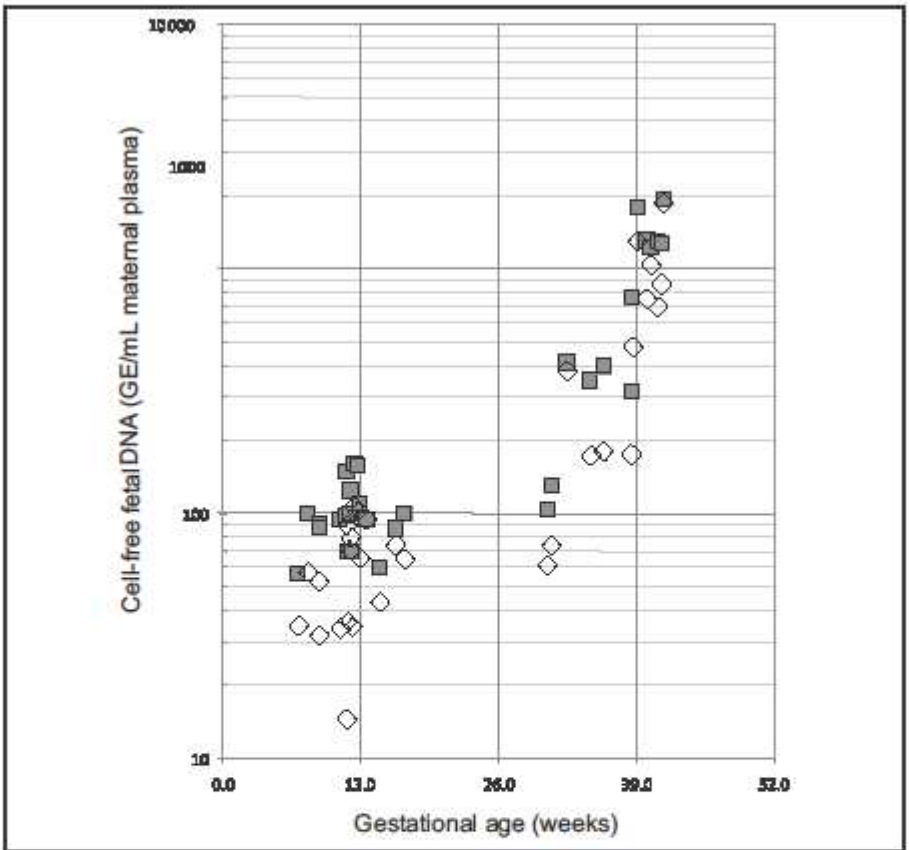

Fig. 1. Cell-free fetal DNA concentrations measured with a real-time qPCR assay with conventional hydrolysis probes and a longer amplicon or with a short-amplicon UT real-time qPCR assay.

The limit of detection for both assays was estimated as 4 genome equivalents (GE) per milliliter of maternal plasma. Indicated are results obtained with the novel short-amplicon UT qPCR assay (■) and the conventional qPCR assay with a longer amplicon (◇).

digital PCR assay and 137 bp for the real-time qPCR assay).

Amplicon length may be a salient issue, because cell-free DNA has previously been shown to be fragmented, with a ladder pattern of fragments reminiscent of patterns seen after oligosomal cleavage in apoptosis, and because cell-free fetal DNA molecules are generally smaller than comparable maternal molecules (*8*, *9*). These previous studies suggested that the majority of cell-free fetal DNA molecules are <300–500 bp; however, given that these studies were not very detailed in nature, it is possible that the majority of cell-free fetal DNA molecules may be even smaller, perhaps <200 bp. Because both the digital PCR study and our new study detected increased amounts of cell-free fetal DNA with shorter DNA amplicons, the combined data do suggest a that a substantial proportion of these molecules are smaller in size than what can be detected reliably in real-time qPCR assays with larger amplicons. This issue will need to be addressed further in a

more detailed analysis. The fact that greater quantities of cell-free fetal DNA are detected in PCR assays with short amplicons suggests that this approach may be useful to increase the sensitivity of detection in samples in which the amount of cell-free fetal DNA is limiting, such as in samples taken early in pregnancy (*6*).

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References

1. Lo YMD, Chiu RW. Prenatal diagnosis: progress through plasma nucleic acids. Nat Rev Genet 2007;8:71–7.
2. Hahn S, Chitty LS. Noninvasive prenatal diagnosis: current practice and future perspectives. Curr Opin Obstet Gynecol 2008;20:146–51.
3. Zimmermann BG, Grill S, Holzgrove W, Zhong XY, Jackson LG, Hahn S. Digital PCR: a powerful new tool for noninvasive prenatal diagnosis? Prenat Diagn 2008;28:1087–93.
4. Lo YMD, Chiu RWK. Next-generation sequencing of plasma/serum DNA: an emerging research and molecular diagnostic tool. Clin Chem 2009;55:607–8.
5. Zimmermann B, El-Shakhuh A, Nicolaides K, Holzgrove W, Hahn S. Optimized real-time quantitative PCR measurement of male fetal DNA in maternal plasma. Clin Chem 2005;51:1598–604.
6. Hahn S, Chitty LS. Non-invasive prenatal diagnosis: implications for antenatal diagnosis and the management of high-risk pregnancies. Semin Fetal Neonatal Med 2008;13:55–6.
7. Lun FMF, Chiu RWK, Chan KCA, Leung TY, Lau TK, YMD Lo. Microfluidics digital PCR reveals a higher than expected fraction of fetal DNA in maternal plasma. Clin Chem 2008;54:1664–72.
8. Chan KCA, Zhang J, Hui ABY, Wong N, Lau TK, Leung TN, et al. Size distributions of maternal and fetal DNA in maternal plasma. Clin Chem 2004;50:88–92.
9. Li Y, Zimmermann B, Rusterholz C, Kang A, Holzgrove W, Hahn S. Size separation of circulatory DNA in maternal plasma permits ready detection of fetal DNA polymorphisms. Clin Chem 2004;50:1002–11.
10. Zhang Y, Zhang D, Li W, Chan J, Peng Y, Cao W. A novel real-time quantitative PCR method using attached universal template probe. Nucleic Acids Res 2003;31:e123.

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6 Discussion and Outlook

In this thesis, I investigated the use of a number of proteomic techniques for the purpose of biomarker development to screen pregnancies at risk for DS or PE. Our hypothesis for this approach was that both DS and PE involve structural changes in the placenta, which should be evident in the maternal plasma proteome, since the placenta is in direct contact with the maternal circulation.

A major complication we faced when dealing with the plasma proteome, is the overwhelming presence of a discrete number of proteins, such as albumin, immunoglobulin, which mask less abundant protein species, especially those released in less prodigious quantities by the placenta. Strategies we took to overcome this issue included the use of a FFE device and commercial depletion columns. Although initially promising, the FFE approach had to be abandoned, due to the high concentrations of urea used in this procedure. The most successful approach proved to be the immuno depletion using ProteoMiner™Protein Enrichment Kit (Bio-Rad Laboratories, Inc.).

As DS and PE are unlikely to lead to the production of novel proteins, but rather be reflective of changes in levels of protein expression, we needed a method to reliably quantify changes in level of potential biomarkers. In our study we examined a number of approaches, including, shotgun MS, SELDI and iTRAQ labelling. iTRAQ labelling has recently been suggested to be the method of choice when considering biomarker development.

In our hands, this method was indeed shown to permit highly reproducible quantitation, and we could readily discern differences between the plasma proteome in cases with DS fetuses vs. controls, and well as in pregnancies at risk for PE and those with normal deliveries.

In our analysis of DS cases we were gratified to observe that we could detect known biomarker molecules such as bHCG, a protein which is at the core of current screening

approaches, we also detected a number of other proteins, which are reflective of the changes occurring in DS patients, such as loose skin associated with the enlarged nuchal fold.

Our analysis of 1st trimester samples of pregnancies at risk for PE proved more complex. Here we were able to detect a set of potential biomarkers, but not detect changes in new markers such as PP-13.This could be reflective of the small number of samples studied, or the very low levels of this molecule.

The research carried out in this thesis does however demonstrate that it is possible to mine the maternal plasma proteome for new biomarkers. Their verification will however need to be carried out in much larger studies, and using more convenient techniques such as selected reaction monitoring (SRM) lead into outlook.

During my thesis work, I used the fascinating method of isobaric labelling for protein quantitation. I will further address several projects which can be studied using quantitative proteomic approach. Our main emphasis will be on other chromosomal anomalies (trisomy 13, 18, etc) and pregnancy related disorders.

In the next leg of study, I will try to improvise the protocol for the better use of free flow electrophoresis (FFE) for the fractionation of tryptic peptides. We will also search for a more specific and highly sensitive method for the albumin fractionation. The concept of isobaric tagging is most promising for the fishing out the biomarkers from the complex sample like plasma. I would like to repeat it with bigger sample size of patients and control.

A major focus will be to design a validation of the list of putative markers we identified in our studies of DS and PE. This we will do by targeted proteomics approach. One of the best options available is the selected reaction monitoring (SRM) [81] which is a mass spectroscopy based quantitation. SRM is very rapidly developing tool for the MS based quantitation. It exploits the unique future of the triple quadrupole. The first and third quadrupole act as a filter to select the ion of defined m/z and the second quadrupole is a collision cell.

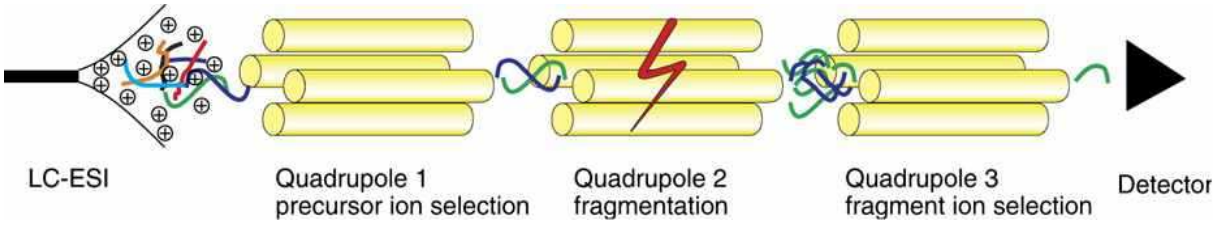

Figure 1. SRM analysis on triple quadrupole. In first quadrupole the ion of particular m/z are selected in second quadrupole the ions are fragmented and in third quadrupole specific ion are selected for the detection. (Adapted from V Lange *et al* MSB, 2008)

Picotti and colleagues [82],in there study on S *cerevisia* has shown the potential of SRM assay. They were able to detect and quantify proteins expressed to a concentration below 50 copies/cells. In there other study [83] they have generated and apply the SRM assay for all S *cerevisia* kinases and phosphatases.

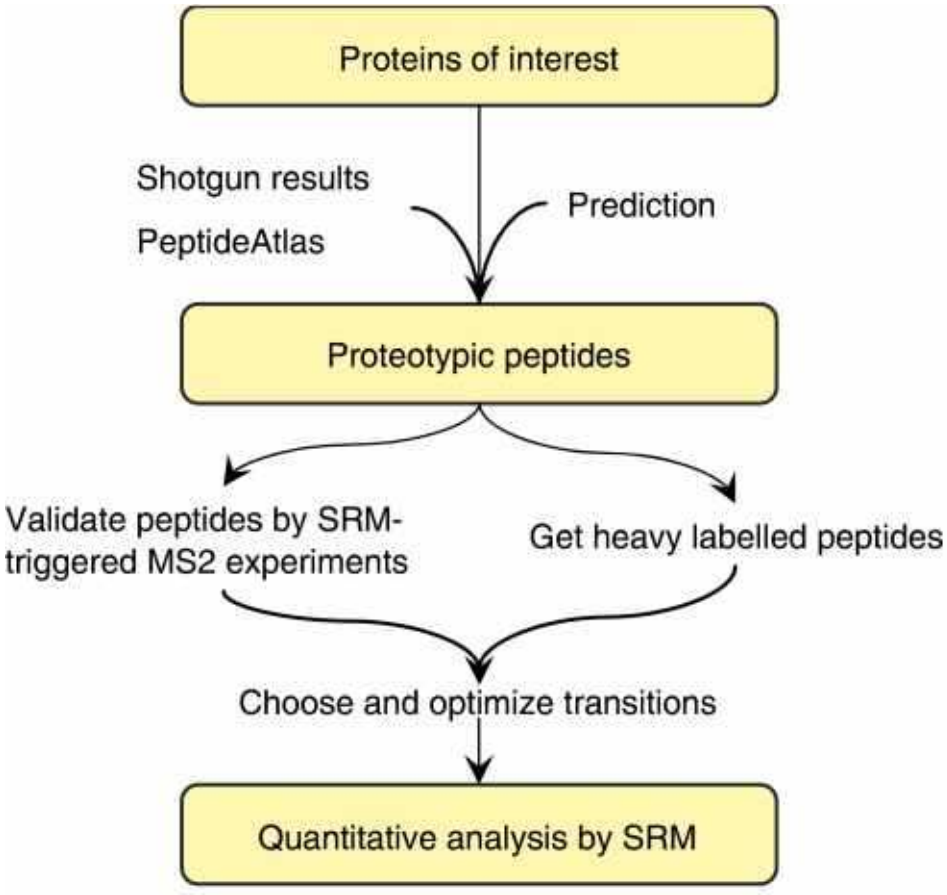

Figure 2. work flow for SRM assay (Adapted from V Lange *et al* MSB, 2008)

Major advantage of SRM is it can be used for the quantitation of the less abundant peptide and sample fractionation is not a prerequisite for these assays. The targeted SRM approach is the best suited method for validation of biomarks which are identified in shotgun proteomics experiment. As it need to performed on more samples and validated by antibodies-based assays, which needs lot of time and not cost-effective.

We have already started designing the SRM assay for the putative list of biomarks for Down syndrome. It will be of our interest that how these assays can be translated in clinic setting for the fast and reliable screening of DS and PE.

Urine is one of the most attractive biofluid in clinical proteomics. It is easy to collect and non-invasive. It contains significant amount of protein and peptid which are of clinical value. We are also indent to check the SRM assay in urine sample from DS and PE.

7 References

1. Nicolaides, K.H., *First-trimester screening for Down's syndrome.* N Engl J Med, 2004. 350(6): p. 619-21; author reply 619-21.

2. Penrose, L.S., The relative effects of paternal and maternal age in mongolism. 1933. J Genet, 2009. 88(1): p. 9-14.

3. Hassold, T. and D. Chiu, Maternal age-specific rates of numerical chromosome abnormalities with special reference to trisomy. Hum Genet, 1985. 70(1): p. 11-7.

4. Morton, N.E., et al., *Maternal age in trisomy.* Ann Hum Genet, 1988. 52(Pt 3): p. 227-35.

5. Morris, J.K. and E. Alberman, Trends in Down's syndrome live births and antenatal diagnoses in England and Wales from 1989 to 2008: analysis of data from the National Down Syndrome Cytogenetic Register. BMJ, 2009. 339: p. b3794.

6. Wilson, R.D., et al., Amended Canadian guideline for prenatal diagnosis (2005) change to 2005-techniques for prenatal diagnosis. J Obstet Gynaecol Can, 2005. 27(11): p. 1048-62.

7. Lo, Y.M., et al., *Presence of fetal DNA in maternal plasma and serum.* Lancet, 1997. 350(9076): p. 485-7.

8. Hahn, S. and W. Holzgreve, Fetal cells and cell-free fetal DNA in maternal blood: new insights into pre-eclampsia. Hum Reprod Update, 2002. 8(6): p. 501-8.

9. Li, Y., et al., Size separation of circulatory DNA in maternal plasma permits ready detection of fetal DNA polymorphisms. Clin Chem, 2004. 50(6): p. 1002-11.

10. Chim, S.S., et al., Detection of the placental epigenetic signature of the maspin gene in maternal plasma. Proc Natl Acad Sci U S A, 2005. 102(41): p. 14753-8.

11. Lo, Y.M., et al., Plasma placental RNA allelic ratio permits noninvasive prenatal chromosomal aneuploidy detection. Nat Med, 2007. 13(2): p. 218-23.

12. Hahn, S., et al., Noninvasive prenatal diagnosis of fetal aneuploidies and Mendelian disorders: new innovative strategies. Expert Rev Mol Diagn, 2009. 9(6): p. 613-21.

13. Ong, S.E., et al., Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol Cell Proteomics, 2002. 1(5): p. 376-86.

14. Mann, M., *Functional and quantitative proteomics using SILAC.* Nat Rev Mol Cell Biol, 2006. 7(12): p. 952-8.

15. Gygi, S.P., et al., Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat Biotechnol, 1999. 17(10): p. 994-9.

16. Ross, P.L., et al., Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. Mol Cell Proteomics, 2004. 3(12): p. 1154-69.

17. Shilov, I.V., et al., The Paragon Algorithm, a next generation search engine that uses sequence temperature values and feature probabilities to identify peptides from tandem mass spectra. Mol Cell Proteomics, 2007. 6(9): p. 1638-55.

18. Keshamouni, V.G., et al., Differential protein expression profiling by iTRAQ-2DLC-MS/MS of lung cancer cells undergoing epithelial-mesenchymal transition reveals a migratory/invasive phenotype. J Proteome Res, 2006. 5(5): p. 1143-54.

19. DeSouza, L., et al., Search for cancer markers from endometrial tissues using differentially labeled tags iTRAQ and cICAT with multidimensional liquid chromatography and tandem mass spectrometry. J Proteome Res, 2005. 4(2): p. 377-86.

20. Song, X., et al., *iTRAQ experimental design for plasma biomarker discovery.* J Proteome Res, 2008. 7(7): p. 2952-8.

21. Kolialexi, A., et al., *Mass spectrometry-based proteomics in reproductive medicine.* Mass Spectrom Rev, 2008. 27(6): p. 624-34.

22. Nagalla, S.R., et al., Proteomic analysis of maternal serum in down syndrome: identification of novel protein biomarkers. J Proteome Res, 2007. 6(4): p. 1245-57.

23. Busch, A., et al., Proteome analysis of maternal serum samples for trisomy 21 pregnancies using ProteinChip arrays and bioinformatics. J Histochem Cytochem, 2005. 53(3): p. 341-3.

24. Choolani, M., et al., Proteomic technologies for prenatal diagnostics: advances and challenges ahead. Expert Rev Proteomics, 2009. 6(1): p. 87-101.

25. Davison, J.M., et al., *New aspects in the pathophysiology of preeclampsia.* J Am Soc Nephrol, 2004. 15(9): p. 2440-8.

26. Meads, C.A., et al., Methods of prediction and prevention of pre-eclampsia: systematic reviews of accuracy and effectiveness literature with economic modelling. Health Technol Assess, 2008. 12(6): p. iii-iv, 1-270.

27. Venkatesha, S., et al., Lipocalin 2 antagonizes the proangiogenic action of ras in transformed cells. Mol Cancer Res, 2006. 4(11): p. 821-9.

28. Rana, S., et al., Sequential changes in antiangiogenic factors in early pregnancy and risk of developing preeclampsia. Hypertension, 2007. 50(1): p. 137-42.

29. Belo, L., et al., Apolipoprotein E and cholesteryl ester transfer protein polymorphisms in normal and preeclamptic pregnancies. Eur J Obstet Gynecol Reprod Biol, 2004. 112(1): p. 9-15.

30. Kim, S.Y., et al., Maternal serum and amniotic fluid inhibin A levels in women who subsequently develop severe preeclampsia. J Korean Med Sci, 2006. 21(3): p. 452-6.

31. Muttukrishna, S., et al., Uterine vein and maternal urinary levels of activin A and inhibin A in pre-eclampsia patients. Clin Endocrinol (Oxf), 2006. 64(4): p. 469-73.

32. Spencer, K., N.J. Cowans, and K.H. Nicolaides, Low levels of maternal serum PAPP-A in the first trimester and the risk of pre-eclampsia. Prenat Diagn, 2008. 28(1): p. 7-10.

33. Nicolaides, K.H., et al., A novel approach to first-trimester screening for early pre-eclampsia combining serum PP-13 and Doppler ultrasound. Ultrasound Obstet Gynecol, 2006. 27(1): p. 13-7.

34. Chafetz, I., et al., First-trimester placental protein 13 screening for preeclampsia and intrauterine growth restriction. Am J Obstet Gynecol, 2007. 197(1): p. 35 e1-7.

35. Robertson, S.A., Immune regulation of conception and embryo implantation-all about quality control? J Reprod Immunol. 85(1): p. 51-7.

36. Gellersen, B., I.A. Brosens, and J.J. Brosens, *Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives.* Semin Reprod Med, 2007. 25(6): p. 445-53.

37. Gupta, A.K., et al., A comparative study of the effect of three different syncytiotrophoblast micro-particles preparations on endothelial cells. Placenta, 2005. 26(1): p. 59-66.

38. Casals, E., et al., *First-trimester biochemical markers for Down syndrome.* Prenat Diagn, 1999. 19(1): p. 8-11.

39. Nissum, M. and A.L. Foucher, Analysis of human plasma proteins: a focus on sample collection and separation using free-flow electrophoresis. Expert Rev Proteomics, 2008. 5(4): p. 571-87.

40. Malmstrom, J., et al., Optimized peptide separation and identification for mass spectrometry based proteomics via free-flow electrophoresis. J Proteome Res, 2006. 5(9): p. 2241-9.

41. Moritz, R.L. and R.J. Simpson, Liquid-based free-flow electrophoresis-reversed-phase HPLC: a proteomic tool. Nat Methods, 2005. 2(11): p. 863-73.

42. Gatlin, C.L., et al., Automated identification of amino acid sequence variations in proteins by HPLC/microspray tandem mass spectrometry. Anal Chem, 2000. 72(4): p. 757-63.

43. Luzzati, A.L., E. Giacomini, and I. Lefkovits, Expression of antibody-forming cell clones in two-stage cultures: an attempt to separate proliferation and maturation events. Immunology, 1985. 56(4): p. 597-603.

44. Ouvry-Patat, S.A., et al., Free-flow electrophoresis for top-down proteomics by Fourier transform ion cyclotron resonance mass spectrometry. Proteomics, 2008. 8(14): p. 2798-808.

45. Nissum, M., et al., Two-dimensional separation of human plasma proteins using iterative free-flow electrophoresis. Proteomics, 2007. 7(23): p. 4218-27.

46. Anton, L., et al., Activation of local chorionic villi angiotensin II levels but not angiotensin (1-7) in preeclampsia. Hypertension, 2008. 51(4): p. 1066-72.

47. Jeschke, U., et al., Expression of galectin-1, -3 (gal-1, gal-3) and the Thomsen-Friedenreich (TF) antigen in normal, IUGR, preeclamptic and HELLP placentas. Placenta, 2007. 28(11-12): p. 1165-73.

48. Madsen, E.M., et al., Human placenta secretes apolipoprotein B-100-containing lipoproteins. J Biol Chem, 2004. 279(53): p. 55271-6.

49. Kralova, A., et al., Differential transferrin expression in placentae from normal and abnormal pregnancies: a pilot study. Reprod Biol Endocrinol, 2008. 6: p. 27.

50. Bulla, R., et al., Decidual endothelial cells express surface-bound C1q as a molecular bridge between endovascular trophoblast and decidual endothelium. Mol Immunol, 2008. 45(9): p. 2629-40.

51. Kim, S.Y., et al., Increased sFlt-1 to PlGF ratio in women who subsequently develop preeclampsia. J Korean Med Sci, 2007. 22(5): p. 873-7.

52. Spencer, K., N.J. Cowans, and A. Stamatopoulou, *ADAM12s in maternal serum as a potential marker of pre-eclampsia.* Prenat Diagn, 2008. 28(3): p. 212-6.

53. Akolekar, R., et al., Maternal serum placental protein 13 at 11-13 weeks of gestation in preeclampsia. Prenat Diagn, 2009. 29(12): p. 1103-8.

54. Khalil, A., et al., First trimester maternal serum placental protein 13 for the prediction of pre-eclampsia in women with a priori high risk. Prenat Diagn, 2009. 29(8): p. 781-9.

55. Norwitz, E.R., Defective implantation and placentation: laying the blueprint for pregnancy complications. Reprod Biomed Online, 2006. 13(4): p. 591-9.

56. Kolla, V., et al., Quantitative proteomics analysis of maternal plasma in Down syndrome pregnancies using isobaric tagging reagent (iTRAQ). J Biomed Biotechnol. 2010: p. 952047.

57. Unwin, R.D., et al., Quantitative proteomic analysis using isobaric protein tags enables rapid comparison of changes in transcript and protein levels in transformed cells. Mol Cell Proteomics, 2005. 4(7): p. 924-35.

58. Elias, J.E. and S.P. Gygi, Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. Nat Methods, 2007. 4(3): p. 207-14.

59. Mi, H., et al., PANTHER version 6: protein sequence and function evolution data with expanded representation of biological pathways. Nucleic Acids Res, 2007. 35(Database issue): p. D247-52.

60. Glen, A., et al., iTRAQ-facilitated proteomic analysis of human prostate cancer cells identifies proteins associated with progression. J Proteome Res, 2008. 7(3): p. 897-907.

61. Gan, C.S., et al., Technical, experimental, and biological variations in isobaric tags for relative and absolute quantitation (iTRAQ). J Proteome Res, 2007. 6(2): p. 821-7.

62. Blumenstein, M., et al., A proteomic approach identifies early pregnancy biomarkers for preeclampsia: novel linkages between a predisposition to preeclampsia and cardiovascular disease. Proteomics, 2009. 9(11): p. 2929-45.

63. Skornicka, E.L., et al., Pregnancy zone protein is a carrier and modulator of placental protein-14 in T-cell growth and cytokine production. Cell Immunol, 2004. 232(1-2): p. 144-56.

64. Atkinson, K.R., et al., An altered pattern of circulating apolipoprotein E3 isoforms is implicated in preeclampsia. J Lipid Res, 2009. 50(1): p. 71-80.

65. Williams, V.K., et al., Fibrinogen concentration and factor VIII activity in women with preeclampsia. Hypertens Pregnancy, 2007. 26(4): p. 415-21.

66. Rockett, J.C., Genomic and proteomic techniques applied to reproductive biology. Genome Biol, 2001. 2(9): p. REPORTS4020.

67. Gerton, G.L., et al., *A serum proteomics approach to the diagnosis of ectopic pregnancy.* Ann N Y Acad Sci, 2004. 1022: p. 306-16.

68. Rodland, K.D., Proteomics and cancer diagnosis: the potential of mass spectrometry. Clin Biochem, 2004. 37(7): p. 579-83.

69. Zhang, Z., et al., Three biomarkers identified from serum proteomic analysis for the detection of early stage ovarian cancer. Cancer Res, 2004. 64(16): p. 5882-90.

70. Otu, H.H., et al., Prediction of diabetic nephropathy using urine proteomic profiling 10 years prior to development of nephropathy. Diabetes Care, 2007. 30(3): p. 638-43.

71. Meuwis, M.A., et al., Biomarker discovery for inflammatory bowel disease, using proteomic serum profiling. Biochem Pharmacol, 2007. 73(9): p. 1422-33.

72. Johann, D.J., Jr., et al., *Clinical proteomics and biomarker discovery.* Ann N Y Acad Sci, 2004. 1022: p. 295-305.

73. Kolialexi, A., et al., Application of proteomics for the identification of differentially expressed protein markers for Down syndrome in maternal plasma. Prenat Diagn, 2008. 28(8): p. 691-8.

74. Krantz, D., et al., First-trimester maternal dried blood Down syndrome screening marker levels in early pregnancy loss. Prenat Diagn, 2006. 26(12): p. 1137-41.

75. Bajo, M., et al., Proteomic evaluation of intermediary metabolism enzyme proteins in fetal Down's syndrome cerebral cortex. Proteomics, 2002. 2(11): p. 1539-46.

76. Lawrence, C.B. and V.V. Solovyev, *Assignment of position-specific error probability to primary DNA sequence data.* Nucleic Acids Res, 1994. 22(7): p. 1272-80.

77. Adkins, J.N., et al., Toward a human blood serum proteome: analysis by multidimensional separation coupled with mass spectrometry. Mol Cell Proteomics, 2002. 1(12): p. 947-55.

78. Wagner, M., et al., Computational protein biomarker prediction: a case study for prostate cancer. BMC Bioinformatics, 2004. 5: p. 26.

79. Pettker, C.M., et al., Value of placental microbial evaluation in diagnosing intra-amniotic infection. Obstet Gynecol, 2007. 109(3): p. 739-49.

80. Buhimschi, C.S., et al., Proteomic profiling of the amniotic fluid to detect inflammation, infection, and neonatal sepsis. PLoS Med, 2007. 4(1): p. e18.

81. Lange, V., et al., Selected reaction monitoring for quantitative proteomics: a tutorial. Mol Syst Biol, 2008. 4: p. 222.

82. Picotti, P., et al., Full dynamic range proteome analysis of S. cerevisiae by targeted proteomics. Cell, 2009. 138(4): p. 795-806.

83. Picotti, P., et al., High-throughput generation of selected reaction-monitoring assays for proteins and proteomes. Nat Methods. 7(1): p. 43-6.

8 Curriculum vitae

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Switzerland

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PERSONAL DETAILS

Date of Birth	:	11th December 1978
Nationality	:	Indian
Language Proficiency	:	English, Hindi, Marathi, Telugu (Fluent)
		German (Basic)

EDUCATION AND REASEARCH EXPERIENCE

2006-till now **Ph.D. thesis in Molecular Medicine.**
Lab for Prenatal Medicine
Department of Biomedicine, University Hospital Basel, Switzerland
EU Research fellowship (FP6-Pregenesys project)

Project: "Early Identification of Screening Biomarker's for Pregnancy related disorder using Proteomics approach."
Supervisor: Prof. Hahn.

Degree expected: May 2010

2005-2006 **Scientific research assistant.**
Institute for Animal Pathology, Vet Swiss Faculty, University of Bern, Bern, Switzerland.

Project: "To investigate the role of Keratinocytes in Canine Atopic Dermatitis (AD)"
Supervisor: Prof. E. J. Muller

2003-2005 **Scientific research assistant.**
Centre for Cellular and Molecular Biology (CCMB), Hyderabad, India

Project: "To identify the nonconventional role of Heat Shock Factors (HSF)"
Supervisors: Dr. Usha Srinivas and Dr. A. S. Shreedhar.

Project: "Developing Molecular marker for Handigodu disease and Leigh's Syndrome"
Supervisor: Dr. G.R. Chandak.

2002-2003 **Advance Diploma**
Protein Modeling and Drug Design,
GVK Bioscience, Hyderabad, India

2000-2002 **Master degree (MSc.) in Biotechnology,**
Dept. Of Biotechnology, Amravati University, Amravati, India

Project: "Genetic Characterization of Multi Drug Resistant bacteria from Hospital waste"
Supervisors: Dr. Thakare

Ph.D. PROJECT

Project: "Identification of screening biomarker's for pregnancy related disorders using proteomics approach"
Down syndrome (DS) is the most common fetal chromosomal disorder in live births and a key cause of mental retardation. Current screening approaches are hampered by lack of specificity and a high false positive rate (>5%). Since the placenta is in direct contact with maternal circulation, we sought whether protein differences can be detected between affected and normal pregnancies.
Preeclampsia, is a severe pregnancy related disorder, is leading cause of fetal and maternal mortality. No marker exists for reliable identification of at risk pregnancies. As the DS placentae show structural alteration and PE involves placental dysfunction, we examined whether quantitative iTRAQ proteomics of plasma sample could be used differentially expressed proteins.

LABORATORY SKILLS AND TECHNICAL EXPERTISE

Mass spectrometry and Proteomics

- Quantitative Phosphoproteomics using SILAC approach
- Protein identification and characterization using nanoLC-MS/MS sequencing by Maldi-TOF/TOF (4800 from Applied Biosystems) and ESI-LTQ-Orbitrap (Thermo Fischer).
- Differential protein quantification by isobaric labeling approaches (iTRAQ)
- 2D-gel electrophoresis and image analysis
- Protein fractionation using Free Flow Electrophoresis (BD™ FFE system) in complex fluids such as plasma

Bioinformatics

- Mass spectrometry platform dedicated software: Analyst (Applied Biosystems), Xcalibur (Thermo Electron
- Protein identification software: Mascot (Matrix Science), Sequest (Bioworks from ThermoElectron) , on-line proteomics and sequence analysis tools.
- Bioinformatic tools for proteomic quantification: Protein Pilot
- Functional annotation tools : Ingenuity Pathway Analysis, Protein center, GoMiner, FatiGo, PANTHER
- Image analysis : Image Master 2D Platinum 5.0 (GE Healthcare), Quantity one (Biorad)

Protein and Peptide Biochemistry

- Protein and peptide separation using mono- and two-dimensional gel electrophoresis and liquid chromatography-based systems (RP, ion exchange, affinity).
- Protein proteolysis using endoproteolytic enzymes or chemical reagents in-gel or in- solution.

Biology

- Cell culture, ELISA, Western blot analysis, Immuno precipitation (IP).

COMMUNICATIONS

Talk **''**Biomarkers for Non-Invasive Prenatal Diagnosis and Pregnancy Related Disorders Using Quantitative Proteomics''. Swiss proteomics society annual meeting, ETH, Zurich, Switzerland, 2-4 December 2009

"Boimarker discovery Strategy for Trisomy 21 using Proteomics Tool" at Universitatklinikum , Gottingen, Germany, 24 October 2007

Conferences 2nd annual European Biomarkers Summit, Amsterdam, Netherlands, 4-5 September 2007 (poster presentation)

Workshop SAFE Practical Workshop on Non-Invasive Prenatal Diagnosis, Ronzano, Bologna, Italy, 24th -25th February, 2008

2nd EU-Summer school in Proteomic Basics 'Protein Identification-MassSpectrometry'. Kloster Neustift, (Brixen, South Tyrol, Italy), 13th-19th July 2008

SAFE Proteomics Workshop at University of the West of England, Bristol, England, 26th -28th January 2007

Posters **Varaprasad Kolla**, Wolfgang Holzgreve, Sinuhe Hahn
Biomarker discovery strategy for trisomy 21 using iTRAQ and 4800 plus MALDI TOF/TOF, European Biomarkers Summit, Amsterdam, 4-5 September 2007.

Course Faculty for 'Course in Prenatal Genetic Diagnosis' organised by European Cytogeneticists Association (ECA), Goldrain Castle-South Tyrol, Italy. 19th -24th September 2009

AWARDS AND ACHIEVEMENT

Registration and travel grant to attendant 2nd EU-Summer school in Proteomic Basics 'Protein Identification-MassSpectrometry'. Kloster Neustift, (Brixen, South Tyrol, Italy), 13th-19th July 2008.

PROFFESIONAL MEMBERSHIP

Swiss Proteomics Society (SPS)
BioValley, Basel

PUBLICATIONS

Kolla V, Jenö P, Moes S, Tercanli S, Lapaire O, Choolani M, Hahn S.
Quantitative proteomics analysis of maternal plasma in Down Syndrome pregnancies using isobaric tagging reagent (iTRAQ).
J Biomed Biotechnol. Volume 2010(2010) Article ID 952047.

Sikora A, Zimmermann BG, Rusterholz C, Birri D, **Kolla V**, Lapaire O., *et al*
Detection of Increased Amount of Cell-Free Fetal DNA with Short PCR Amplicons.
Clin Chem. 2010 Jan;56(1):136-8

Hahn S, Jackson LG, **Kolla V**, Mahyuddin AP, Choolani M.
Noninvasive prenatal diagnosis of fetal aneuploidies and Mendelian disorders: new innovative strategies.
Expert Rev Mol Diagn. 2009 Sep;9(6): 613-21. Review

Choolani M, Narasimhan K, **Kolla V**, Hahn S.
Proteomic Technologies for Prenatal Diagnostics: Advance and Challenges ahead
Expert Rev Proteomics. 2009 Fab;6(1):87-101. Review

Kolla V Prasad, Aftab Taiyab. D Jyothi, Usha K Srinivas, Amere S Sreedhar
Heat shock transcription factors regulate heat induced cell death in a rat histiocytoma.
J Biosci. 2007 Apr;32(3):585-93

The Indian Genome Variation database (IGVdb): a project overview Human Genetics. Hum Genet. 2005 Oct;118(1):1-11.

REFRENCES

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