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 $3000 \times$ g for 30 minutes at 10°C, whereby the plasma was separated from the cellular fraction by aid of a mechanical separator. $100\,\mu L$ 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, $300\,\mu L$ was eluted in elution reagent. After depletion protein concentration was measured by using RC-DC Protein assay kit (Bio-Rad Laboratories, Inc.)

2.4. Tryptic Digestion and iTRAQ Reagent Labelling. Equal amounts ($100\,\mu$g) 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 $2\,\mu L$ 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 $70\,\mu L$ 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
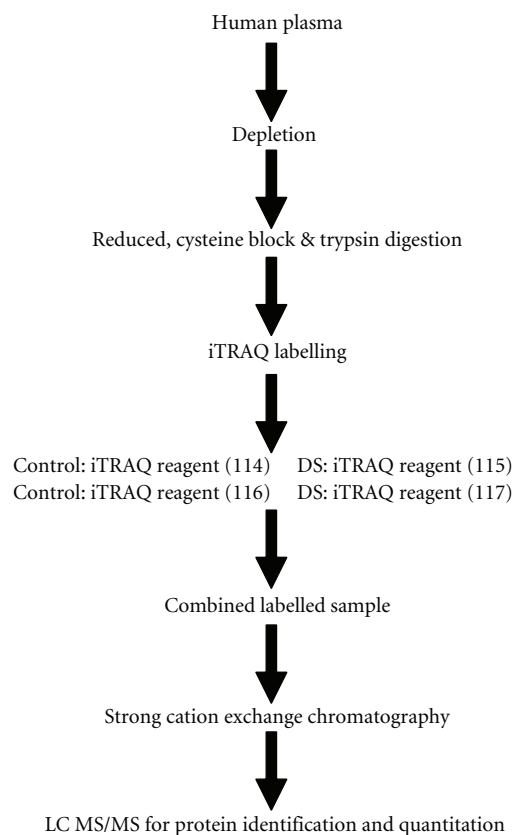
Human plasma

↓

Depletion

↓

Reduced, cysteine block & trypsin digestion

↓

iTRAQ labelling

↓

Control: iTRAQ reagent (114)   DS: iTRAQ reagent (115)
Control: iTRAQ reagent (116)   DS: iTRAQ reagent (117)

↓

Combined labelled sample

↓

Strong cation exchange chromatography

↓

LC MS/MS for protein identification and quantitation

FIGURE 1: Workflow for quantitative proteomics using iTRAQ reagent. Equal amounts of plasma protein ($100 \, \mu$g) 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 $200 \, \mu L$ 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$_2$PO$_4$, 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$_2$O, 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
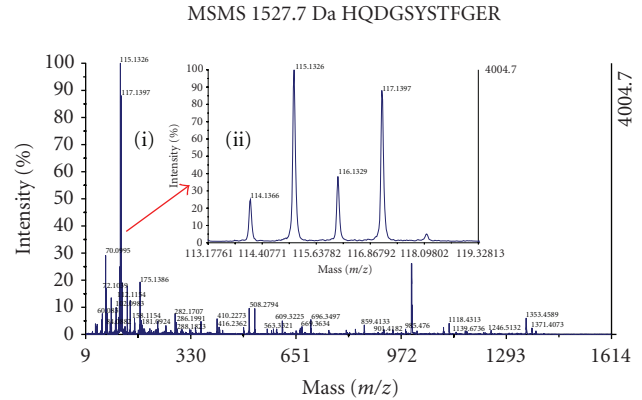

MSMS 1527.7 Da HQDGSYSTFGER

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.
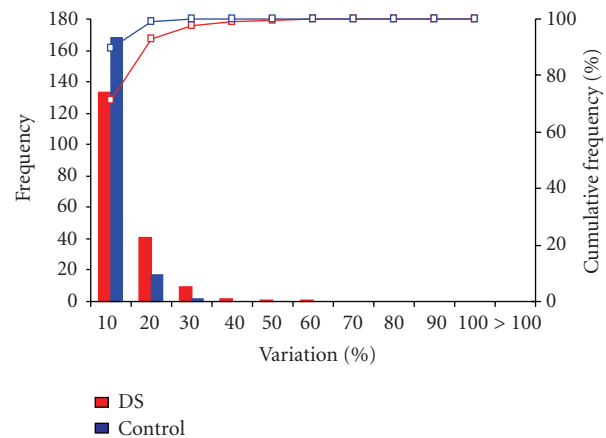

FIGURE 3: Frequency distribution (bars) from both DS and control replicates across different ranges of variation. The cumulative percentage (lines) is defined as the cumulative number of proteins falling within the defined variation range against the total number of protein.

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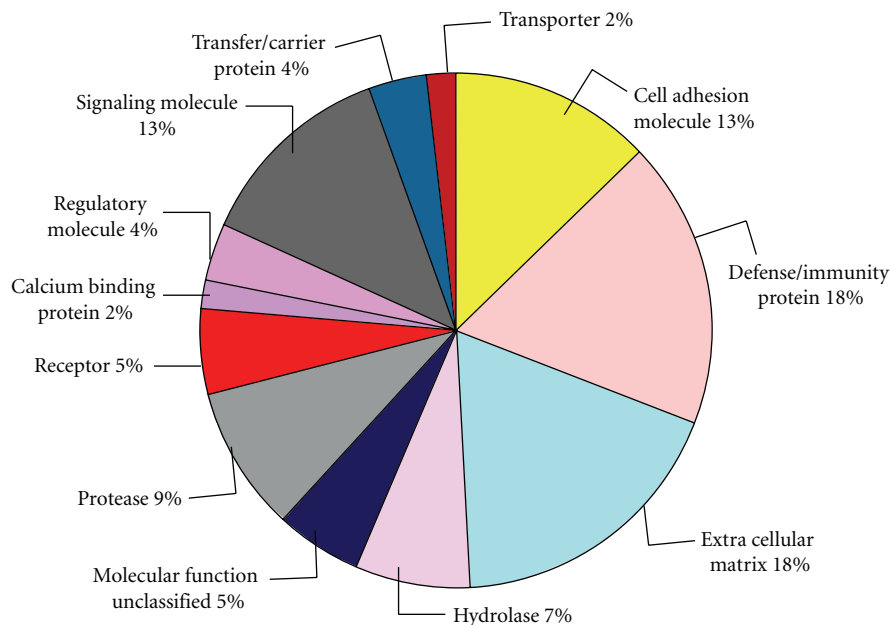
FIGURE 4: Number of plasma protein identified using iTRAQ reagent. In total, 235 proteins were identified. Shown above is the classification of these protein in different category based on molecular function.

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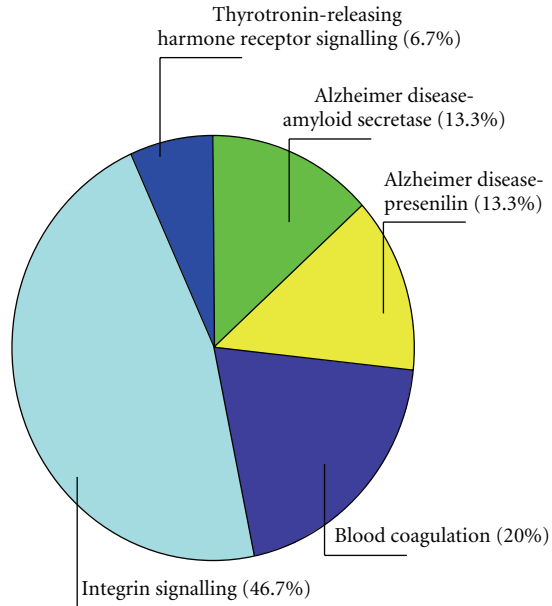
FIGURE 5: *PANTHER analysis for pathway*. In total, 28 proteins were identified as elevated and 22 proteins were under expressed. Shown above is the different signaling pathways hits by these protein.

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.

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