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Investigation into Inter-alpha Trypsin Inhibitor Heavy Chain 4 (ITIH4) as a biomarker for prenatal screening of Down syndrome using maternal plasma samples

Jessica Elliott-Friend

Project Advisor: Neil Avent, School of Biomedical & Biological Sciences, University of Plymouth, Drake Circus, Plymouth, PL4 8AA

Abstract

Background: Down Syndrome (DS) affects 3 per 1000 births and is a significant cause of fetal loss. Currently screening tests detect ~85% of affected pregnancies, with a 5% false-positive rate, whilst diagnosis requires invasive tests such as amniocentesis which is associated with a ~1% risk of miscarriage. More effective screening methods, or Non Invasive Prenatal Diagnosis (NIPD) methods, are required to reduce the need for invasive diagnostic tests.
Aim: This study aims to investigate inter-alpha trypsin inhibitor heavy chain 4 (ITIH4) as a potential biomarker in DS pregnancies.
Method: Samples include 8 non-pregnant controls and 7 pregnant women known to be carrying a DS fetus. 1D Western Blot analysis was carried out on all plasma samples, and 2D Western blot (2-DE) was subsequently carried out on samples of interest. Real-time PCR was undertaken to determine the sex of the fetuses.
Results: 125kDa ITIH4 was identified in all samples using Western Blot analysis. ITIH4 is cleaved by kallikrein into a 35kDa fragment and a 100kDa fragment which is rapidly cleaved to yield a 70kDa fragment. Results for DS samples KC05 and KC06 suggested only the 35kDa fragment was present, whilst all other samples demonstrated the presence of the full 125kDa ITIH4 protein, as well as the presence of the 35kDa fragment in some cases. DS samples KC01 and KC05 underwent 2D Western blot. Results for KC05 again only presented the 35kDa fragment, whilst KC01 presented both the fragment and full ITIH4 protein in contrast to the previous 1D Western Blot which had only demonstrated the presence of the full ITIH4 protein. All DS fetal genders were successfully identified.
Conclusion: ITIH4 was successfully identified using the 1D and 2D Western Blot, in all samples. Reasons for the presence of different fragment of ITIH4 in different plasma samples remain unclear and require further investigation. Further research needs to be undertaken to compare euploid samples with known trisomy samples on a larger scale to identify whether ITIH4 could be an effective biomarker in DS screening.

Introduction

Down Syndrome (DS) occurs in 1-3 per 1000 births (including terminations) [1]. As 25% of DS foetuses in the second trimester do not survive to term, the occurrence is significantly higher, with an even greater loss from the first trimester [2]. Currently, prenatal screening tests for DS involve assessing the levels of a combination of maternal serum markers, including human chorionic gonadotropin (hCG), pregnancy-associated plasma protein-A (PAPP-A) [3], alpha-fetoprotein (AFP)[4], inhibin A [5] and unconjugated estriol (uE3) [6] and measuring fetal nuchal translucency thickness using ultrasonography at 10-14 weeks gestation[3]. These methods have a detection rate of 80-87% and an associated 5% false-positive rate[7]. However, these methods are not diagnostic and a positive test means parents will be offered invasive procedures including amniocentesis after 15 weeks gestation or chorionic villus sampling (CVS) between 11 to 14 weeks gestation [8]. These invasive procedures are associated with an ~1% risk of miscarriage[9], potentially to a healthy fetus falsely diagnosed as at risk through current screening methods.

Noninvasive Prenatal Diagnosis

The risks associated with the invasive procedures described above highlights the need for early Noninvasive prenatal diagnosis (NIPD) that can be offered routinely as part of antenatal care [10]. After free fetal DNA (ffDNA) was shown to be present in maternal plasma and serum by Lo *et al.,*[11] using real-time PCR, and through advances in molecular techniques, the reality of using NIPD routinely is getting closer [10]. Nucleated red blood cells (NRBC), trophoblasts and CD34+ hematopoietic progenitors have all been proven to be present within the maternal circulation, however, their application for clinical testing has been limited due to difficulties in reproducible techniques [12]. The methods for isolation of fetal cells, including Magnetic-based separation systems (MACS) and flow-sorting separation systems (FACS) also need to be improved before fetal cell analysis can have clinical applications [13].

Dhallan *et al*., [14] identified two out of three DS samples, with fetal DNA distinguished from maternal DNA through single nucleotide polymorphisms (SNPs). Fetal DNA was enriched using formaldehyde [15][14]. However, there is a significant amount of controversy surrounding the efficacy of this method [16][17][18]. Nonetheless, Lo *et al.,* [19] was able to use PCR amplification to determine the ratio of SNPs in *PLAC4* mRNA (expressed by the placenta and transcribed from chromosome 21). From this it was possible to determine chromosome dosage (Figure 1) and determine DS in 90% of affected heterozygous fetuses. Fan *et al.*, [20] sequenced ffDNA using high-throughput shotgun sequencing direcly from maternal plasma. Through this it was possible to measure the over and underrepresentaiton of the chromosomes in the fetus, this enabled 100% of DS fetuses to be detected. A significant advantage of these two methods is that they are polymorphism independent, unlike previous work by Dhallan *et al.,* [14]. However, currently these methods have only been tested on a small scale. Chiu, et al., [21] has recently begun large scale validity trials that have used multiplexed maternal plasma DNA sequencing analysis to detect trisomy 21 fetuses with 97.9% specificity and 100% sensitivity, suggesting the suitability for them to be used clinically with the potential to reduce the need to invasive diagnostic procedures by 98%.

DS Biomarkers

Whilst other methods involving ffDNA are improving, the use of biomarkers is still the current clinical method and therefore identifying proteins that are differentially or preferentially expressed in a disorder such as DS is useful for the development of rapid, sensitive and potentially diagnostic assays[22]. A complex interaction and balance of intracellular and extracellular signals affects the progression of pregnancy through to delivery, and it is thought that fetal abnormalities such as DS are able to disturb this balance [23]. Nagalla et al., [24] highlighted differences between protein abundance in DS serum samples when compared to the controls, within the first and second trimester. A long term goal of the Special Non-Invasive Advances in Fetal and Neonatal Evaluation (SAFE) Network of Excellence (NoE) is to identify a panel of biomarkers that are more informative than current DS markers, therefore increasing the detection rate and decreasing the need for invasive diagnostic tests. With the advances in proteomic techniques there is an opportunity to identify such biomarkers and improve prenatal screening or to potentially find diagnostic markers [10]. Michel *et al.,* [25] identified 36/49 proteins from both amniotic fluid (AF) and maternal plasma, demonstrating that noninvasive methods of acquiring samples could become as useful as those obtained through invasive means and without the risks of unneccesary miscarriage. However, as described above current screening methods use serum and there is variation in the use of either plasma or serum across many studies involved in biomarker detection. Whilst serum has cells and clotting factors removed through clot formation, leaving just proteins and other molecules representative of the whole body system, plasma includes cellular material and clotting is prevented through the presence of anticoagulants such as EDTA [26].

Several studies have used proteomic analysis on both serum and plasma to identify differentially expressed protein markers in DS. Kolialexi, et al., [27] analysed second trimester plasma samples using two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) to identify nine differentially expressed proteins. Whilst Tsangaris, et al., [28] identified seven proteins as differentially expressed in the second trimester of DS pregnancies compared with the control, again using 2-DE and MALDI-MS. However, this was carried out on amniotic fluid and therefore does not fulfil the role of a NIPD method, nonetheless it could be used to highlight potential biomarkers to investigate within maternal blood.

Nagalla *et al.,* [24] investigated the presence of biomarkers in maternal serum for both the first and second trimester, which could have a significant role in future early DS screening programmes. Fluorescence 2-D gel electrophoresis (2D-DIGE), 2-D liquid chromatography-chromatofocusing (2D-CF) and MALDI-TOF-MS were used to identify over 50 proteins that were differentially expressed betweem DS and control pregnancies. Kolla *et al.*, [29] used a more novel approach involving isobaric labelling (iTRAQ) in conjunction with mass spectrometry using MALDI TOF/TOF with plasma samples from the first trimester. This again, identified approximately 50 proteins that were either up- or down- regulated in DS pregnancies. However, these were not all in conjunction with Nagalla et al [24]. These studies have all highlighted the ability to identify potential biomarkers, however, more large scale studies need to be carryed out to identify whether they could be used clinically. Nonetheless, Kolla *et al.,* [29] and Nagalla *et al.,* [24] identified inter-alpha trypsin inhibitor heavy chain 4 (ITIH4) as being upregulated in DS pregnancies, with Nagalla *et al* [24] highlighting a

significant increase in DS pregnancies in both the first and second trimester. In contrast, Heywood *et al.,* (Unpublished data) only found ITIH4 to be up-regulated in the second trimester using 2-DE on maternal plasma samples. Following evidence from these three studies, and the contrast in results, ITIH4 was identified as the potential biomarker that would be investigated within this study.

ITIH4

ITIH4, a 120kDa plasma protein [30], has been identified as an acute-phase protein (APP) in humans and a member of the plasma protease inhibitor family [31], which is subject to cleavage by kallikrein [32]. Plasma kallikrein is synthesised in the liver as plasma prekallikrein, before being secreted into the blood and converting into plasma kallikrein [33] by factor XIIa [34]. When activated kallikrein, a serine protease, initiates the plasma kallikrein/kinin system [35] Cleavage of ITIH4 yields a 100kDa and 35kDa fragment, with the 100kDa fragment rapidly cleaved to 70kDa [30] (Figure 2).

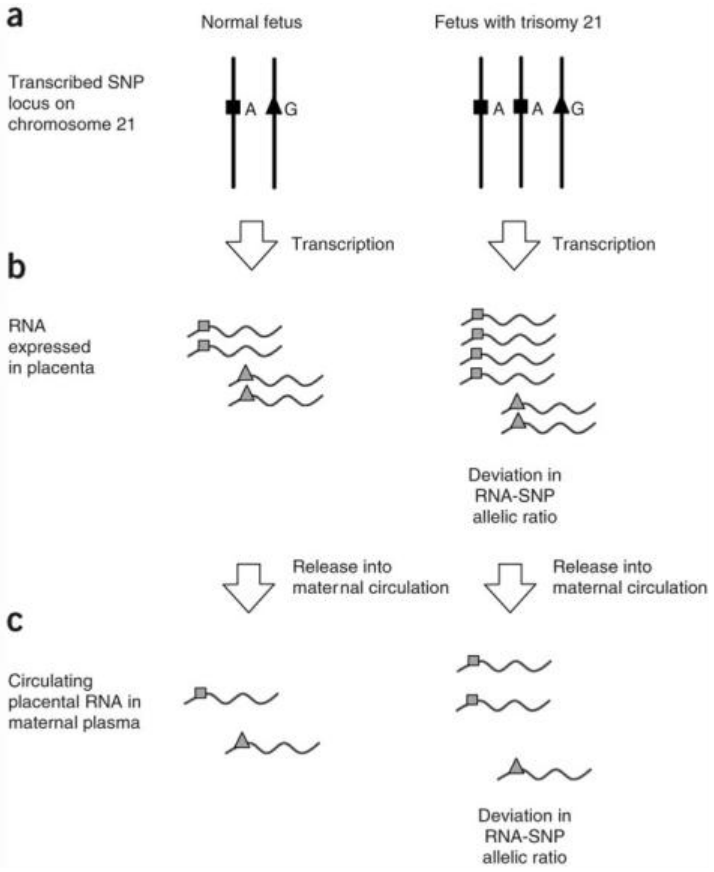

Figure 1. Chromosome dosage. Method of identifying the differences between normal and DS foetuses who are heterozygous at the SNP locus of *PLAC4,* which is transcribed by chromosome 21 and expressed from the placenta. PCR amplification was used to determine the ratio of SNPs in *PLAC4.* As the ratio of two alleles in a heterozygous euploid fetus would be 1:1 whereas in a trisomic fetus the ratio would be either 1:2 or 2:1. Using maternal plasma this ratio could be determined and the number of copies of chromosome 21 could be identified noninvasively [19].

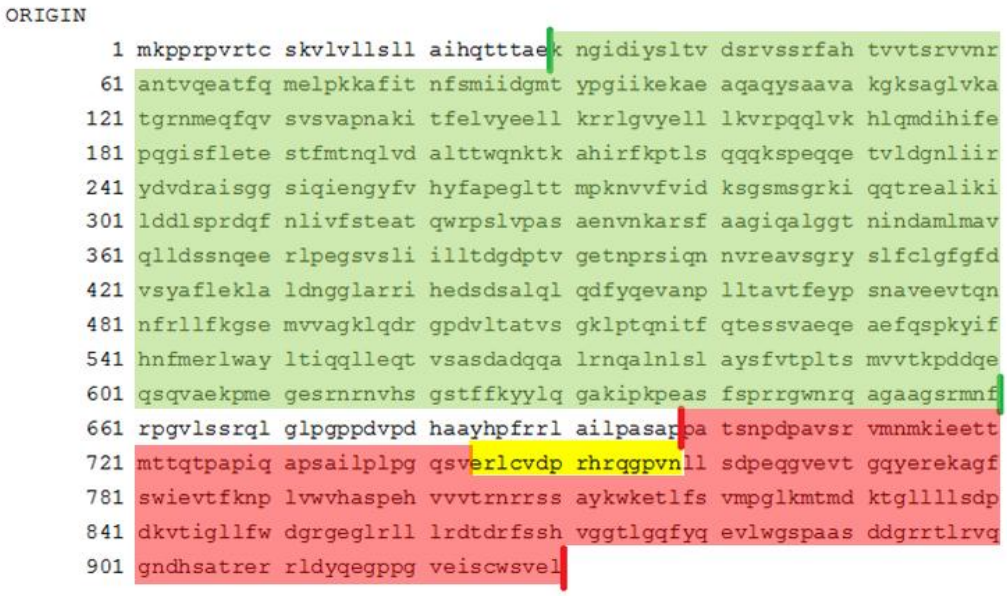

Figure 2. Amino Acid Sequence of Human ITIH4. 70kDa fragment highlighted in green and the red section highlights the 35kDa fragment. Primary goat antibody (Abcam Ab92338) binds to region within 35kDa fragment that is highlighted in yellow (See materials and methods). (NCBI: NP_002209.2, Uniprot: Q14624)

Aims & Objectives

Ideally, biomarkers could be identified with an increased detection rate and decreased false-positive rate using methods including 2-DE, that improve the ability of screening programmes to identify DS pregnancies without the need for invasive procedures. Within this study, the presence of ITIH4 in DS pregnancies will be tested through the use of 1D Western Blot and 2D Western Blot (2-DE) analysis. In pigs ITIH4 expression increases in the endometrium in early pregnancy, with suggestions of a protective role as an APP for the uterus against an inflammatory response due to the conceptus attachment [32]. Whilst no research has been carried out on humans it suggests a possible role for this protein during pregnancy, and with the evidence above [24][29][Heywood *et al.,* (Unpublished data)], a possible role as a prenatal screening marker for DS.

Materials and Methods

Samples

Eight control blood samples (assumed to be non-pregnant) were obtained from the National Health Service Blood and Transplant (Filton, Bristol, U.K) with informed donor consent. Seven maternal blood samples, from pregnant females carrying a DS fetus, were obtained with informed consent from St Michael's Hospital, Bristol, U.K. Maternal and gestational ages are shown in Table 1.

Table 1. Maternal and gestational age for DS pregnancy samples. *N=7.*

Sample	Maternal Age (years)	Gestational Age (weeks)
KC01	33	35
KC02	40	12+1
KC03	42	14+5
KC05	41	13+1
KC06	41	16
KC09	No information	No information
KC06B	36	19+6

Sample Preparation
Control blood samples were centrifuged at 3500rpm for 5 mins. The plasma was then removed and centrifuged again at 15000rpm for 15 mins at 4˚c. At least one aliquot from each sample had protease inhibitor cocktail (Sigma-Aldrich, Dorset, U.K.) added. Fractions were stored at -80˚c before analysis. Plasma from the DS samples was obtained through centrifugation of the blood samples for 10mins at 4700rpm and followed by 15mins at 15500rpm. At least one aliquot from each sample had protease inhibitor cocktail added. Fractions were stored at -80˚c before analysis.

Protein assay
Protein concentrations determined using 'Quick Start' Bradford Protein protocol (Bradford (1976) Anal. Biochem. 72:248). Bradford assay reagent (Coomassie Blue G-250 100mg, Ethanol 50ml, Orthophosphoric acid 100ml, dH_2O 850ml)(BioRad, Hertfordshire, U.K) used as blank with bovine serum albumin (BSA) for standard concentrations, 0.2-1.4mg/ml to form a standard curve. The absorbance of the DS plasma samples (1:100) were determined using the NanoVue Spectrophotometer, from this the concentration (mg/ml) of each sample was determined from the standard curve.

Western Blot
Preparation of 10% SDS-Polyacrylamide gel and gel run as per manufacturer's instructions (Mini-PROTEAN Tetra Cell BioRad), 1x SDS-PAGE (BioRad) running buffer used. Gels were run at 200V for ~50minutes. Samples were heated at 100˚C for 5 minutes prior to being added to the well, with samples kept on ice at other times. 18.5µg of protein from DS samples were added to each well (see Table 2 for volume of sample added to each well) and 10µl of marker (HyperPAGE, Bioline) was added to each gel. Control samples added to Gel A (Figure 3) were 3µl plasma with 9µl of sample buffer. Control samples added to Gel B (Figure 3) were 10µl of plasma + 20µl HBSS, with 3µl of this mix added to 9µl sample buffer which demonstrates a much clearer results than that obtained through no dilution. The proteins were then

blotted to an Immobilon^{TM} Transfer membrane (Millipore, Co Durham, U.K). The membrane was prepared by soaking in methanol for 15seconds before being transferred into H_2O for 2 minutes. The filter paper and membranes were then soaked in blotting buffer. The blot was carried out using BioRad 'Criterion' Blotter as per manufacturer's instructions. 1l Blotting buffer (14.4g 192mM glycine, 3.03g 25mM, 200ml 20% Methanol) was pre-cooled prior to transfer. Gels were blotted at 100v for ~35minutes before being blocked in 5% non-fat dried milk (Marvel) in 0.05% Tris Buffered Saline (TBS) Tween 20 with gentle agitation for 1-2hrs. ITIH4 goat primary antibody (Abcam Ab92338) was then made up to a 1 in 500 dilution in 5% Marvel, and kept on the membrane with gentle agitation at 4˚C overnight (See Figure 1 for binding site of antibody). After overnight incubation the membranes were washed in TBS Tween 20 before ITIH4 Rabbit anti-goat secondary antibody (Abcam Ab6741) was added (1 in 5000 dilution in TBS Tween), with gentle agitation for 2 hours. Washed with TBS Tween 20, before a final wash with 1xTBS.

Table 2. Concentration and volume of each maternal plasma sample to ensure equalised amounts of protein was added to each well (18.5µg)

Maternal Plasma Samples	Concentration of samples after 1:10 dilution (mg/ml)	Volume added (µl)
KC01	3.9	9.5
KC02	2.3	16
KC03	5.6	6.6
KC05	7.4	5
KC06	5.5	6.7
KC06B	5	7.9
KC09	4.7	7.4

Horseradish Peroxidase Blots

Developer prepared as to manufacturer's instructions (BioRad). 30ml of 1x HRP colour development buffer. 180µl HRP colour reagent B and 6ml of HRP colour reagent A (stored -20˚C) and kept in the dark. The developer was added to the membrane for ~15seconds and the reaction was stopped in H_2O for 10 minutes.

ECL Colour Development

0.1ml/cm^2 of ECL developer required from Chemiluminescence detection kit for HRP (EZ-ECL) (Geneflow, Staffordshire, U.K). Developer was incubated on the membrane for 2 minutes before images were captured, using the UVP EC3 Imaging system (Cambridge, U.K). Images were exposed for maximum of 25 seconds.

Albumin/IgG Depletion

KC01 and KC05 plasma samples were depleted of albumin/IgG using the Qproteome Albumin/IgG Depletion kit (Qiagen, Sussex, U.K) as per manufacturer's

instructions. Samples were diluted with phosphate buffered saline (PBS) then placed into the spin columns, which had been previously equilibrated with dilution buffer (50mM Tris·Cl; 4% (w/v) CHAPS; 200mM Urea, pH 7.5) suitable for 2-D PAGE analysis. The samples were shaken and incubated on at end-over-end shaker at room temperature for 5minutes. The flow-through, containing the depleted samples, from the spin columns were then collected by pulse centrifugation. The remaining protein within the spin column were collected by washing through with PBS and centrifugation as before.

Two-Dimensional Western Blot (2-DE)

Contaminants from the depleted samples were removed using 2'D Clean-Up kit (GE Healthcare, Little Chalfont, U.K.). 300µl of precipitant added to 100µl of depleted sample and vortexed for ~15seconds. The samples were then placed on ice for 15 minutes before 300µl of co-precipitant was added and vortexed again. The samples were then centrifuged for 5 minutes, 13000rpm, 4˚c, and the supernatant removed. 40µl of co-precipitant was added on top of the pellet and placed on ice for 5 minutes, before repeating centrifugation as previously described. De-ionised water was added to the pellet and vortexed, resuspending the protein, before 1ml of wash buffer and 5µl of wash additive was added. The sample was stored at -20˚c for 50minutes, with the samples vortexed at 10 minute intervals, before repeating centrifugation. The supernatant was then removed and rehydration buffer (7M urea, 2M thirourea, 2% CHAPS) added to each pellet. The sample was then vortexed before being placed at -20˚C overnight. 11cm IPG strips (pH 5-8) (GE Healthcare) rehydrated using rehydration solution (7M urea, 2M thirourea, 2% CHAPS, 0.002% Bromophenol blue, 1% IPG Buffer pH3-10 (BioLyte, BioRad), 0.28% DTT) and subjected to isoelectric focusing, for 35-40k Volt hours, using 1x XT-Mops (BioRad) running buffer. Next, each strip was equilibrated with gentle agitation for 15 minutes with DTT equilibration buffer (Equilibration buffer (50mM Tris.HCl pH6.8, 6M Urea, 30% Glycerol, 5% SDS, bromophenol blue) + 0.02% DTT) before being rinsed with H_2O. Then IAA equilibration buffer (Equilibration buffer + 0.0125% IAA) was added with gentle agitation for 15 minutes, as IAA is sensitive to light the strips were kept in the dark. IPG strips placed into precast SDS-PAGE gels (4-12% gradient)(BioRad) to undergo second dimension electrophoresis, (0.5W/gel, 40mA/gel, 120V max for 1.5hrs. 4W/gel for 2.25hrs and 0.5W/gel for 0.25hrs). Either the gels underwent Western blot, which was carried out as previous method described, or gels were stained. When stained, the gels were firstly fixed in 10% Methanol/7% acetic acid for 10-30 minutes. Next, the coomassie stain (0.1%, made up according to manufacturer's instructions)(PhastGel^{tm} Blue R)(GE Healthcare) was added, before they were destained in 25% methanol/10% acetic acid for one minute. Finally, the gels were destained overnight in 25% methanol. All images were gained using UVP EC3 Imaging system.

MS Profiling to determine identity of spots of interest

Spots of interest (Figure 12) were obtained from the KC01 coomassie stained gel and sent for MS profiling by Dr Kate Heesom, Direct Proteomic Lab (Department Of Biochemistry, University of Bristol).

Sex Determination - DNA Extraction and Real-Time PCR

DNA extraction carried out using QIAamp Circulating Nucleic Acid kit (Qiagen) for 1ml plasma samples by Dr Tracey Madgett. Real-time PCR carried out using qPCR guide (Eurogentec, Hampshire, U.K) as per manufacturer's instructions. Y

chromosome specific *DYS14* (Zimmermann *et al*., 2005) and *SRY* (Lo *et al.,* 1998) used, alongside *HBB* (Lo *et al.,* 1998), as positive control and H_2O as negative control. All were HPLC purified and the PCR was carried out on the StepOne Plus (Applied Biosystems, California, USA). The PCR primers and probes(Table 3) (Eurofins MWG Operon, London, U.K) were in different concentrations (Table 4). 5µl of DNA was used for each well. The conditions for the PCR were 50˚C for 2 minutes, followed by 95˚C for 10 minutes and 50 cycles of 95˚C for 15 seconds and 60˚C for 1 minute.

Table 3. Primers and probes (Eurofins MWG Operon) used to determine the sex of the fetus within the maternal plasma samples.

Forward Primer	
DYS14	5'- GGG CCA ATG TTG TAT CCT TCT C-3'
SRY	5' -TGG CGA TTA AGT CAA ATT CGC-3'
HBB	5' -GTG CAC CTG ACT CCT GAG GAG A-3'
Reverse Primer	
DYS14	5'- GCC CAT CGG TCA CTT ACA CTT C-3'
SRY	5' –CCC CCT AGT ACC CTG ACA ATG TAT T-3'
HBB	5' -CTT TGA TAC CAA CCT GCC CAG-3'
Probe	
DYS14	5' -(FAM)TCT AGT GGA GAG GTG CTG(BHQ1)-3'
SRY	5' -(YY)AGC AGT AGA GCA GCT AGG GAG GCA GA(BHQ1)-3'
HBB	5' -(FAM)AAG GTG AAC GTG GAT GAA GTT GGT GG(BHQ1)-3'

Table 4. Concentration (nM) of PCR Mixes used in for foetal gender determination on the real-time PCR

	PCR Mix Concentration (nM)	
	Primer	**Probe**
DYS14	**300**	**300**
SRY	**300**	**300**
HBB	**250**	**100**

Results

ITIH4 was identified as a potential new DS screening and/or diagnostic biomarker in several proteomic studies [24][29][Heywood, unpublished data]. Eight control plasma samples and seven maternal plasma samples, known to be carrying a DS fetus, underwent 1D Western blot analysis to determine the presence and expression of ITIH4. Information on gestational age and fetal sex were all determined to aid in analysis of the results.

Western blot analysis of Control plasma samples

Control plasma samples were analysed to determine whether ITIH4 is constitutively present in plasma (Figure 3). Samples underwent dilution as described in the materials and methods section. 10µl of each sample was added to the corresponding well, protein concentration was not determined. Both Gels A & B (Figure 3) show the presence of ITIH4 at 125kDa in all the plasma samples, as well as a smaller fragment, cleaved by kallikrein, demonstrated in some samples at around 35kDa.

Western blot analysis of Maternal Plasma samples from known DS pregnancies

After Bradford Protein assay carried out to determine concentration of protein present in each sample, 18.5µg of protein added to each well in Gel A (Figure 4). Gel B (Figure 4) samples were diluted as previously carried out on Gel B (Figure 2). Results for both Gel A and B (Figure 4) demonstrate the presence of ITIH4 in all samples. However, KC05 and KC06 seemed to have little to none of the complete 125kDa form of ITIH4. These gels also suggest that the remaining samples only have the full length ITIH4 with no 35kDa fragments present. The maternal/gestational age do not seem to correspond with the fragmentation pattern seen (Figure 4). A repeat experiment using the same methods, but in a different order to ensure that this had no effect on the results seen and to ensure no errors had been made with sample labelling, was carried out (Figure 5). Whilst more background noise was demonstrated than the previous results (due to different times between colour development and image acquisition), a much stronger result occurred. The presence of the 35kDa fragment was demonstrated to be present alongside the full ITIH4 in both samples KC06B and KC09, this was also found in Figure 6, using the ECL colour development procedure with a maximum exposure of 25 seconds. All other results were in accordance with the results seen in Figure 4.

Control Western Blot

This blot was carried out using only Rabbit anti-goat secondary antibody to ensure that the results being seen are only due to binding of the primary antibody. Method carried out as previously described, without the primary antibody steps. Figure 6 demonstrates some inappropriate binding of the secondary antibody. However, no binding has taken place within the areas of interest, 125kDa or 35kDa, and therefore the secondary antibody is not affecting the results that are being discussed.

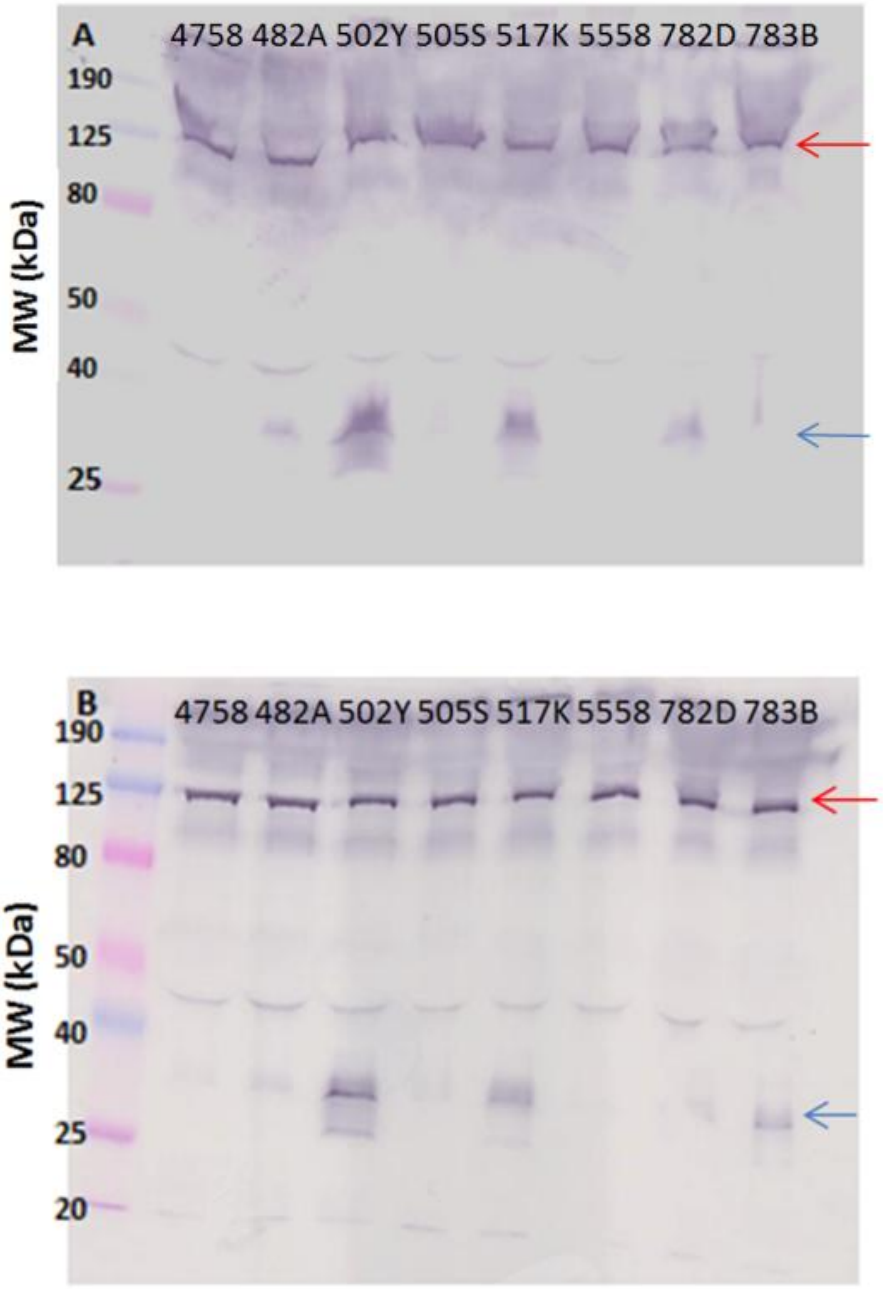

Figure 3. Western Blot analysis of non-pregnant plasma samples. Aims to identify the presence of ITIH4 using goat primary antibody and rabbit anti-goat secondary antibody and a HRP colour development procedure, using 10% SDS-polyacrylamide gel. Samples added to Gel B were diluted. In both gels, 10µl of maternal plasma sample was added to each well. ITIH4 presence demonstrated at around 125kDa (red arrow) and a 35kDa (blue arrow) fragment

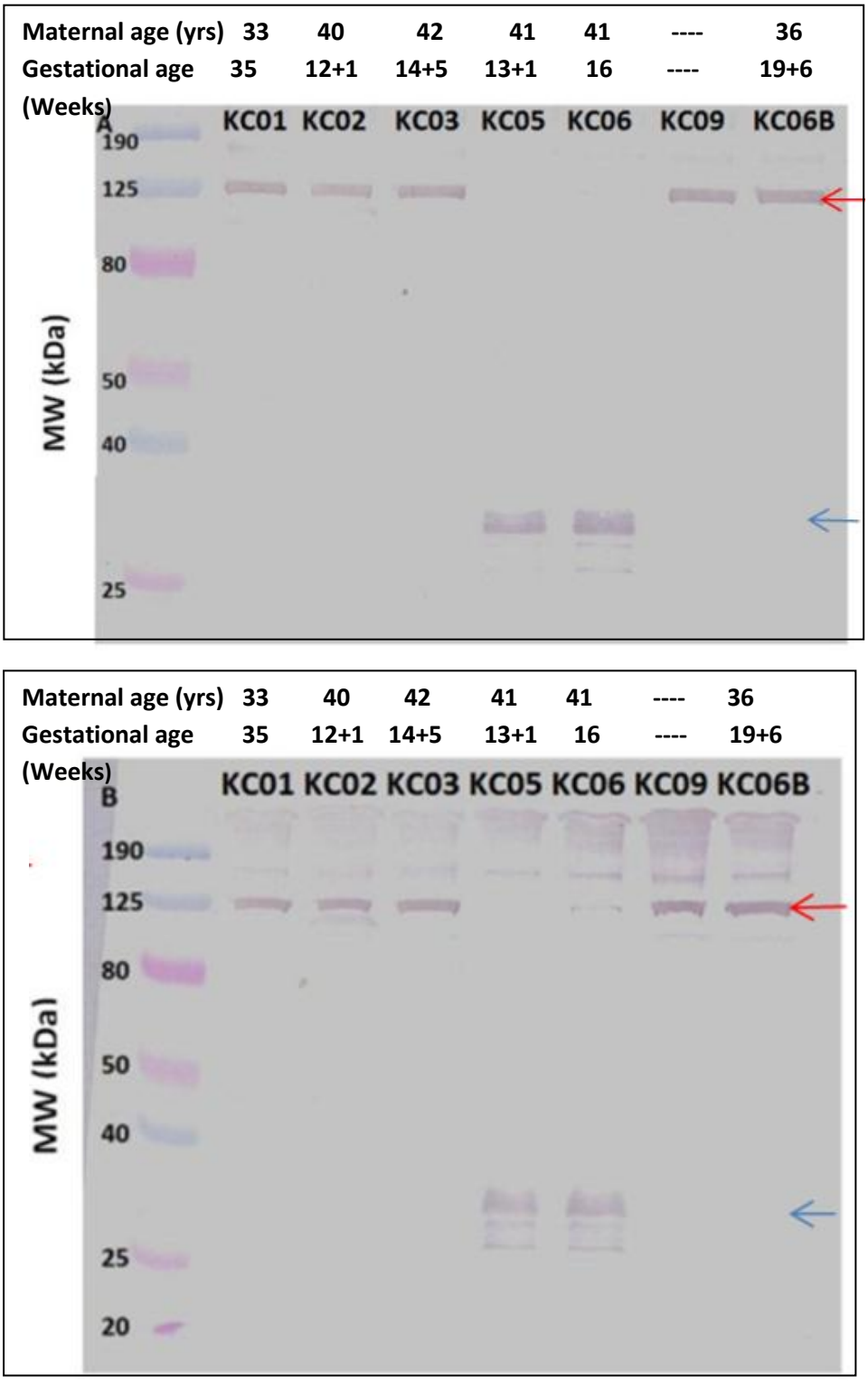

Figure 4. Western blot analysis of maternal plasma from known DS pregnancies to identify the presence of ITIH4 using goat primary antibody and rabbit anti-goat secondary antibody and a HRP colour development procedure. 18.5µg of protein added to each well in Gel A. Samples added to Gel B were diluted. Presence of ITIH4 demonstrated in all samples, with only the 35kDa fragment (blue arrow) present in samples KC05 and KC06. Red arrow highlights 125kDa region of full length ITIH4. Maternal and gestational age details given above sample numbers, does not suggest an association.

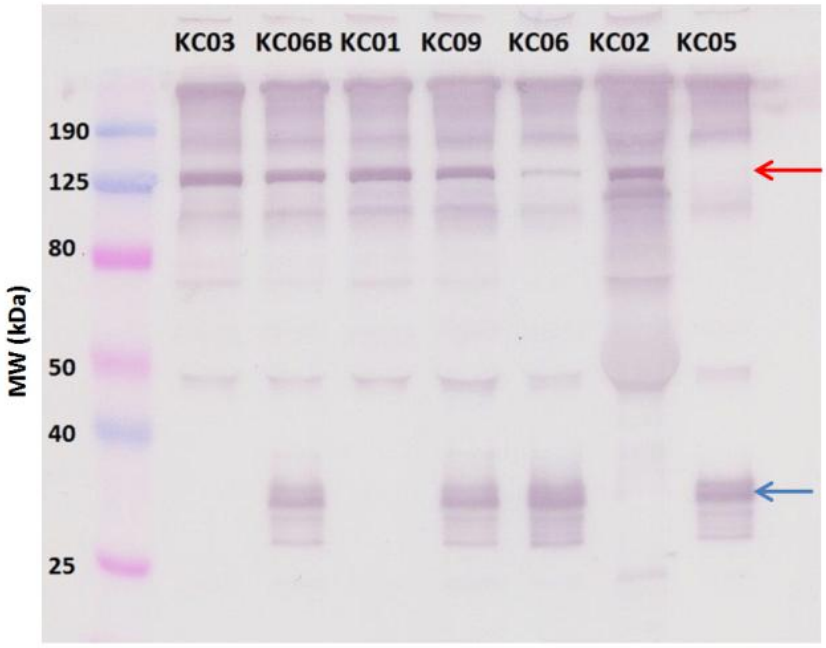

Figure 5. Repeat analysis of the DS pregnancy samples using Western Blot analysis and the HRP colour development procedure. 18.5µg of protein added to each well. 35kDa ITIH4 fragment (blue arrow) present in KC06B, KC09, KC06 and KC05. Presence of ITIH4 demonstrated in all samples. Red arrow highlights region of full length 125kDa ITIH4 fragment

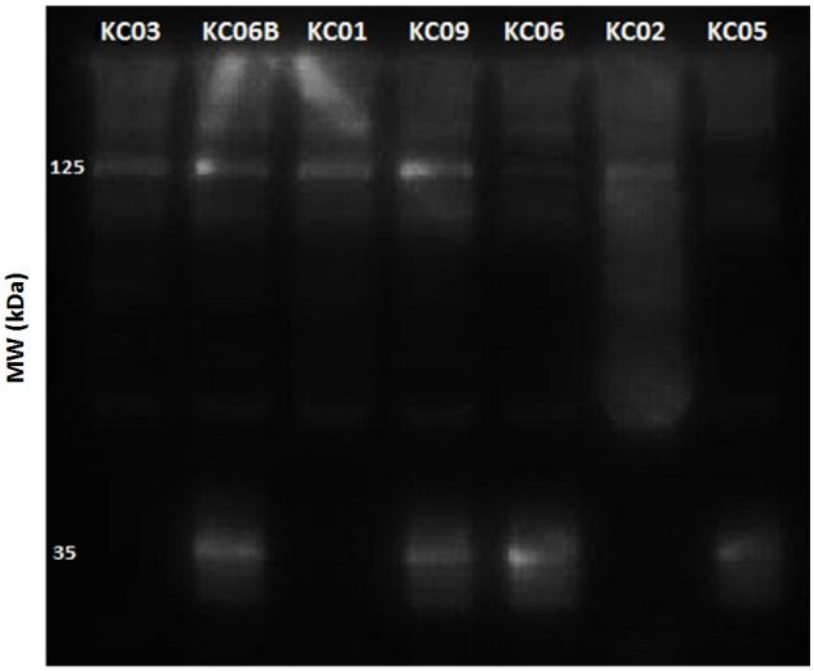

Figure 6. Repeat analysis of the DS pregnancy samples using Western Blot analysis and the ECL colour development procedure. 18.5µg of protein added to each well. 25 second exposure. Demonstrates presence of ITIH4 in all samples

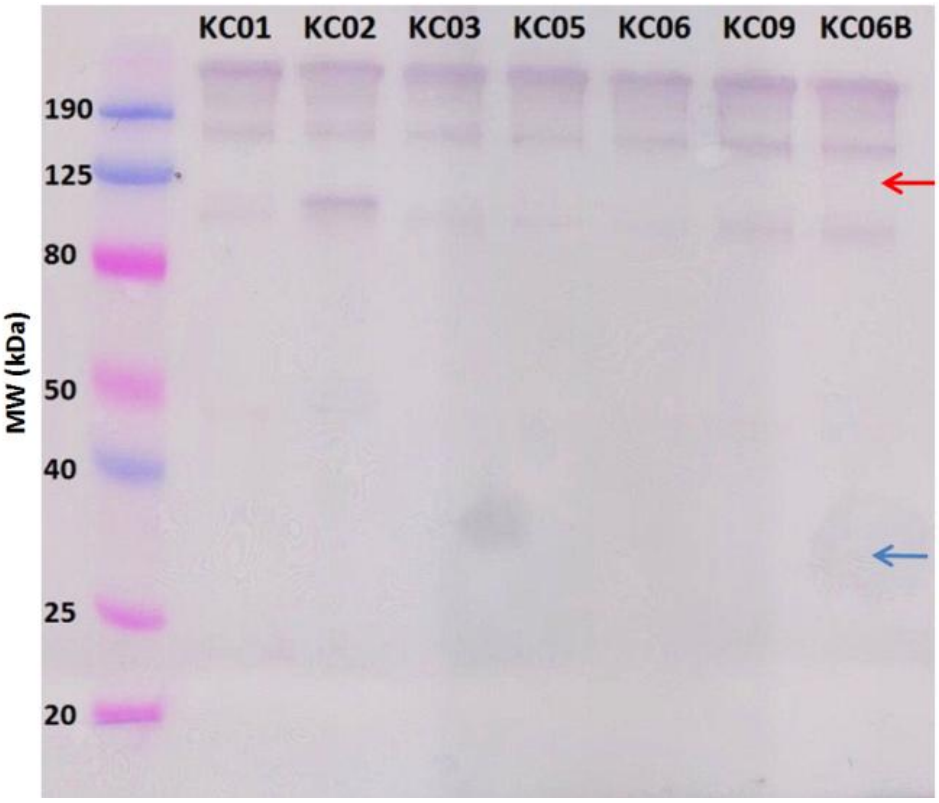

Figure 7. Control Western Blot on DS pregnancy samples using only ITIH4 Rabbit anti-goat secondary antibodies. No binding has occurred in ITIH4 regions, 125kDa and 35kDa, red and blue arrows, respectively. Demonstrates the secondary antibody may be responsible for the background noise within our results

Fetal Sex-determination using Real-time PCR

Real-time PCR was carried out to determine whether the differences seen between the maternal plasma samples and the presence of the 35kDa fragment were due to the sex of the fetus. DNA was extracted from each sample, excluding KC01 due to insufficient volume. KC02 and KC06B samples had protease inhibitor added at the time of sample preparation. Table 5 gives the sex of the fetus for these samples, determined using both *SRY* and *DYS14*, with all results corresponding to both genes. As real-time PCR suggests KC05 was female (Figure 8) and KC06 male (Figure 9), and the 1D Western blot for these samples only presented the 35kDa fragment, it seems unlikely that the sex of the fetus is influencing ITIH4 fragmentation.

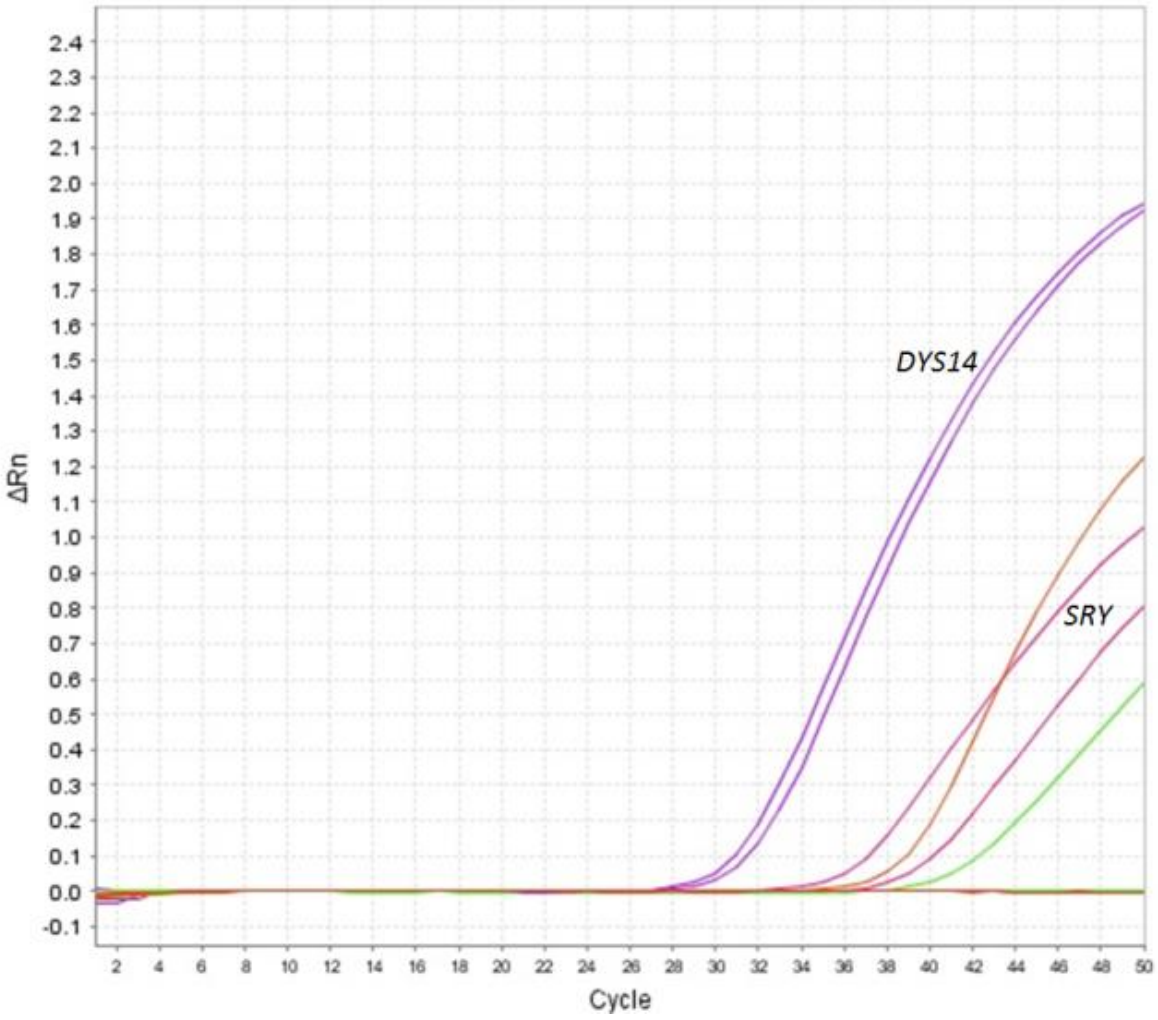

Figure 8. Real-time PCR amplification plot for KC05 using *DYS14* and *SRY* as Y chromosome markers (Purple and pink lines, respectively). Despite some contamination (red and green line), the plot suggests KC05 is female

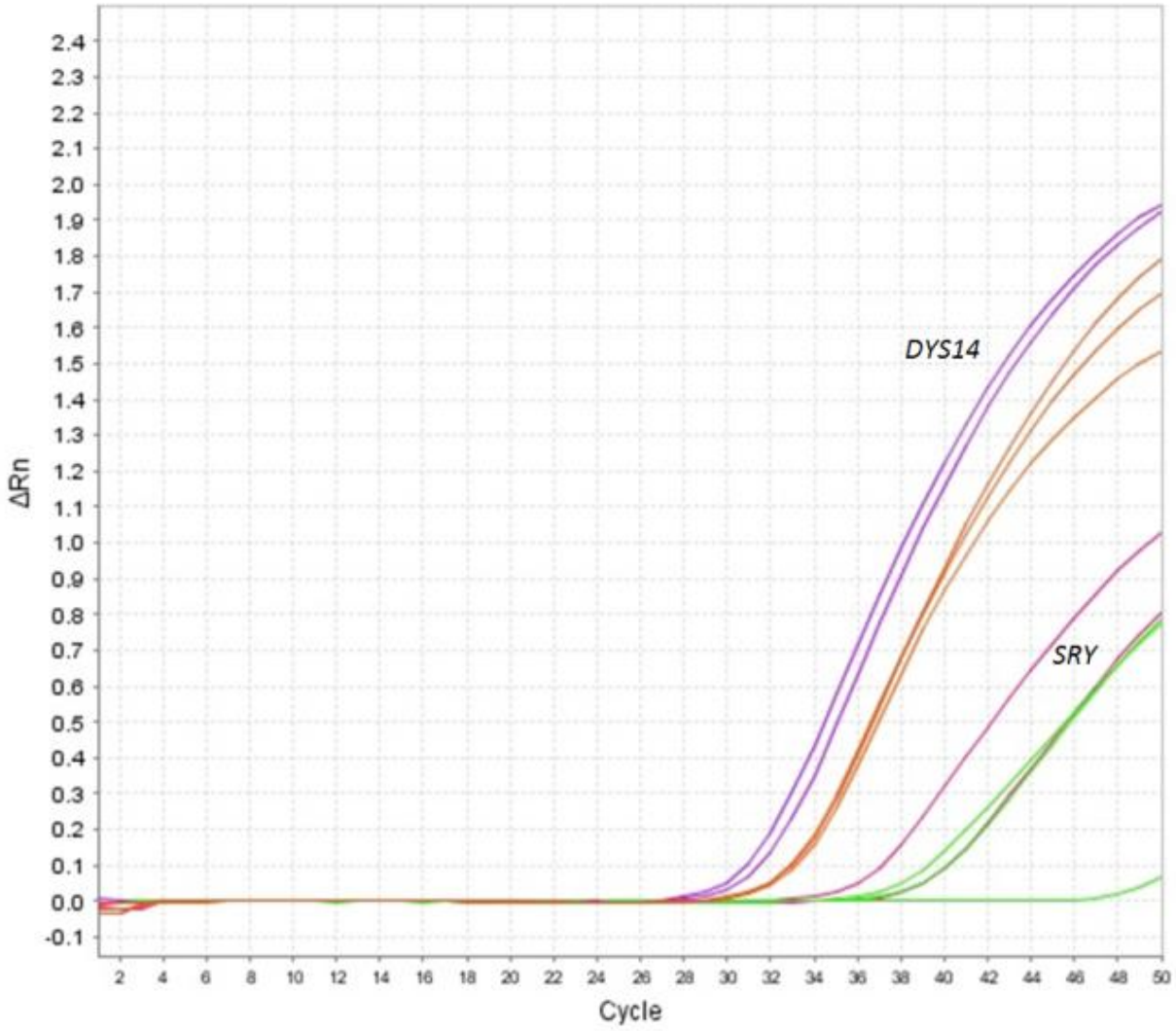

Figure 9. Real-time PCR amplification plot for KC06 using *DYS14* and *SRY* as Y chromosome markers (Purple and pink lines, respectively). Strong results for both *DYS14* and *SRY* demonstrated by the orange and green line, respectively, suggest that the fetus from sample KC06 is male

Table 5. Determination of fetal sex in each sample using Real-Time PCR and *DYS14* and *SRY* as Y Chromosome specific markers

Sample	*DYS14*	*SRY*
KC01	-	-
KC02	M	M
KC03	F	F
KC05	F	F
KC06	M	M
KC06B	M	M
KC09	F	F

2-DE analysis of maternal plasma samples KC01 and KC05

2-DE was chosen as the next step in analysing some specified samples as it is a more sensitive method and has been used in many studies to identify biomarkers [24][27][28](Heywood *et al*, Unpublished data). Therefore, it was thought that this would give more detailed information on the differences being seen between two different DS pregnancy samples. KC01 and KC05 were chosen as Western Blot analysis had demonstrated that KC01 had only the full 125kDa ITIH4 present and KC05 had only the 35kDa fragment present. After albumin and IgG depletion, to remove abundant proteins that may mask the presence of the lower abundance ITIH4, 40µl of sample was added to hydrated IPG (pH5-8) strips. Four IPG strips underwent isoelectric focusing for 35-40k volt hours. Two strips underwent Western Blotting. The IPG strip was placed in pre-cast gels (4-12%) and the Western Blot was carried out as described previously, including HRP colour development (Figure 10). The KC01 image in figure 10 demonstrates the presence of the 35kDa fragment as well as the full 125kDa ITIH4, which was unseen in the previous Western Blots. Supporting Figures 4 and 5, the results for KC05 using 2-DE (Figure 10) demonstrate the presence of the 35kDa fragment only. The remaining two gels were fixed and stained (see materials and methods).

The coomassie stained gel for KC05 (Figure 11), suggests no 35kDa was present, in contrast to previous results (Figure 4 and 5), possibly due to low abundance of the ITIH4 fragment. Results for KC01 suggest presence of a protein around 125kDa, which may be ITIH4. In contrast to the 2D Western Blot carried out (Figure 10), but supporting the Western Blots depicted in Figures 4 and 5 no 35kDa fragment was found in the results for KC01. Figure 12 depicts superimposed images of KC01 from Figure 10 and KC01 from Figure 11, with regions highlighting the possible presence of ITIH4 on the Coomassie stained gel. However, slight differences demonstrated in the pI suggesting that it is not the presence of the full 125kDa ITIH4, as ITIH4 pI is 6.51 (ExPASy http://expasy.org/cgi-bin/pi_tool) and the coomassie gel suggests the pI of the region suspected to be ITIH4 is greater than this.

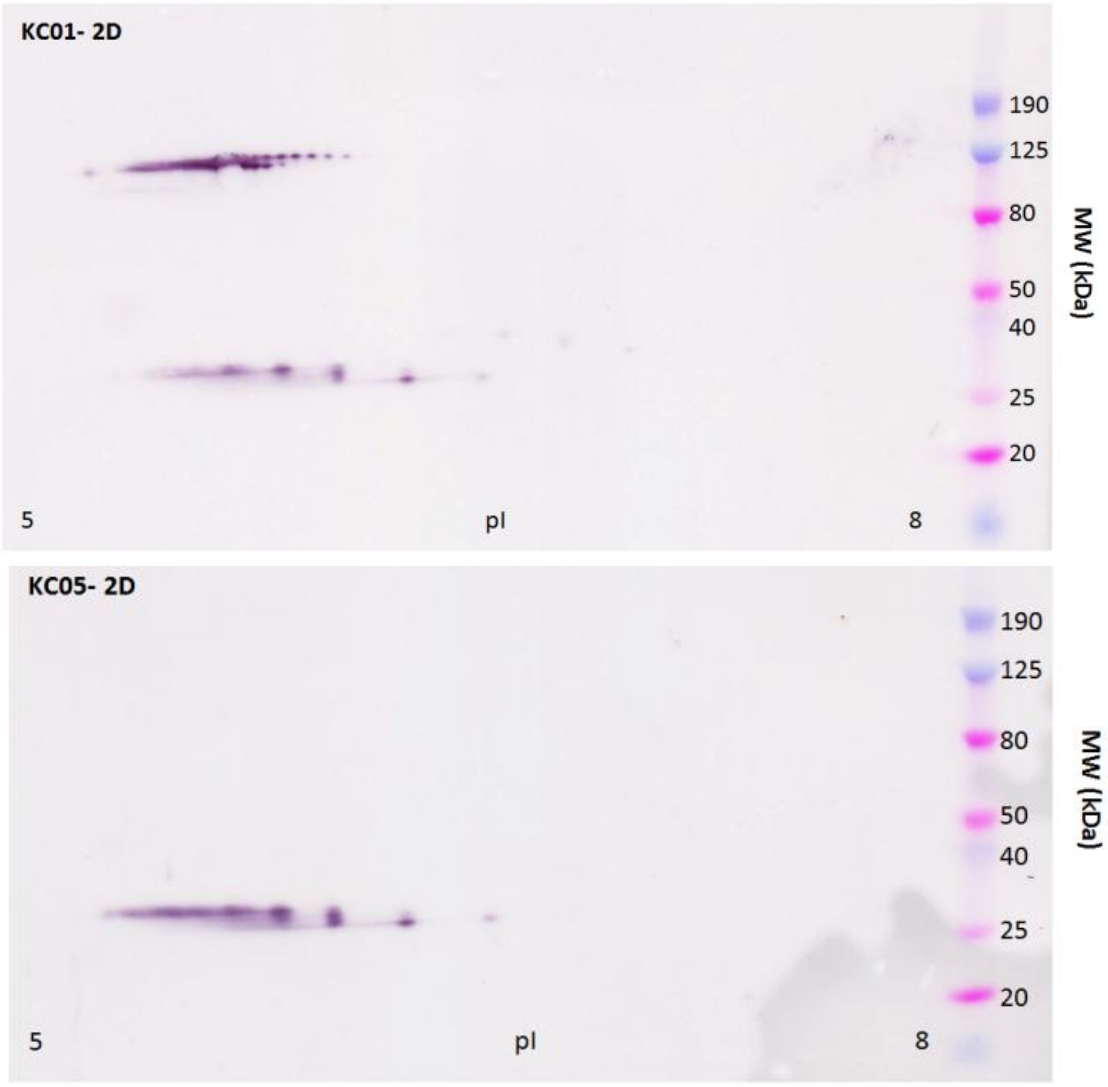

Figure 10. 2-DE of KC01 and KC05. 40µl of sample added to hydrated IPG strip (pI 5-8). Isoelectric focusing undertaken over 35-30k volt hours. Strips added to precast gels (4-12%) before Western Blot and HRP colour development, demonstrates depletion has worked well. ITIH4 125kDa protein and 35kDa fragment identified in KC01. 35kDa fragment identified in KC05

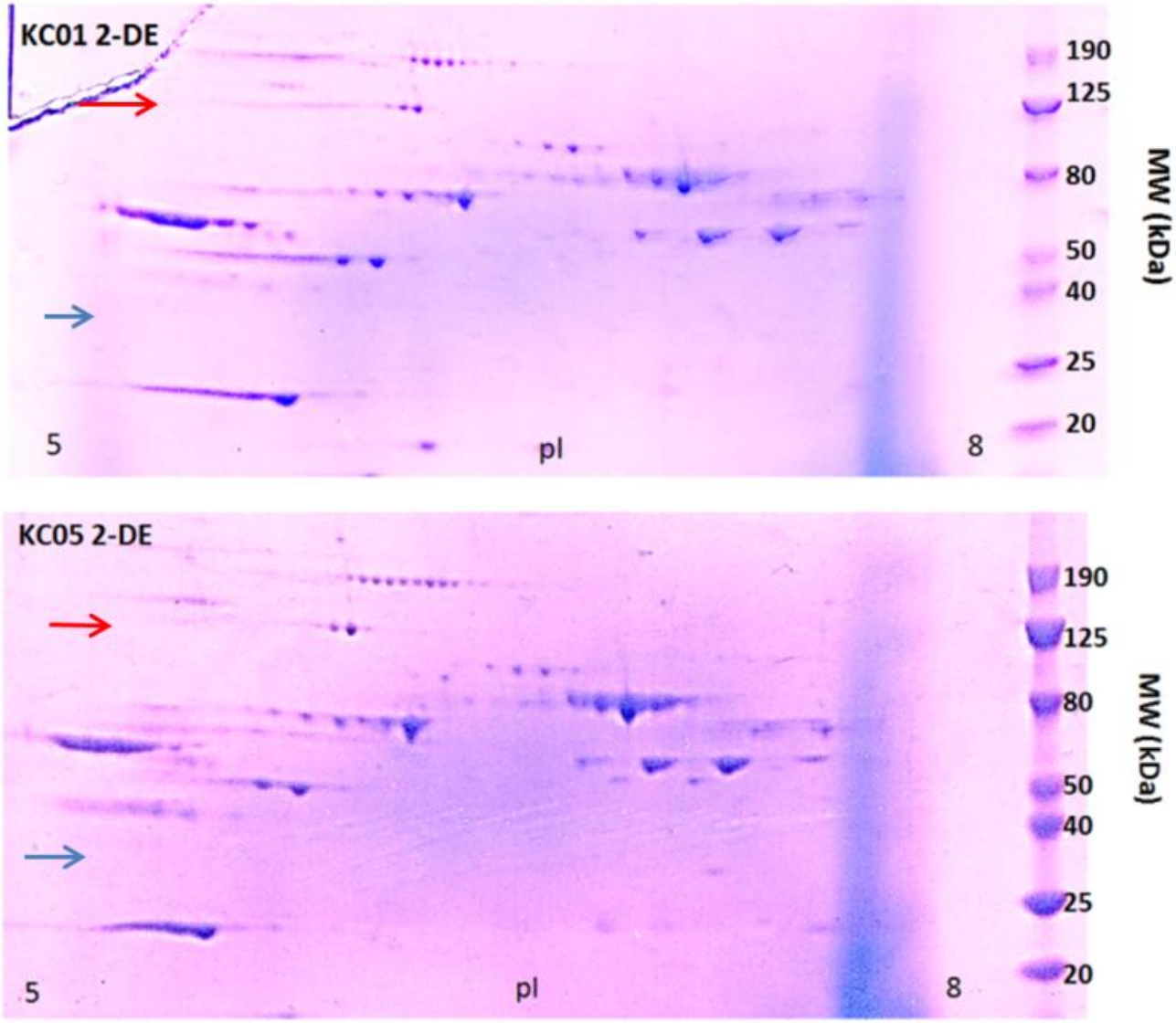

Figure 11. 2-DE of KC01 and KC05 using Coomassie staining to highlight the most abundant proteins. Regions of interest depicted by arrows at 125kDa (red) and 35kDa (blue). No result depicted around 35kDa on the KC01 or KC05 coomassie gel, possibly due to high abundance proteins masking its presence. kC01 gel depicts spots and trail of protein around region of where the full 125kDa ITIH4 fragment would be expected

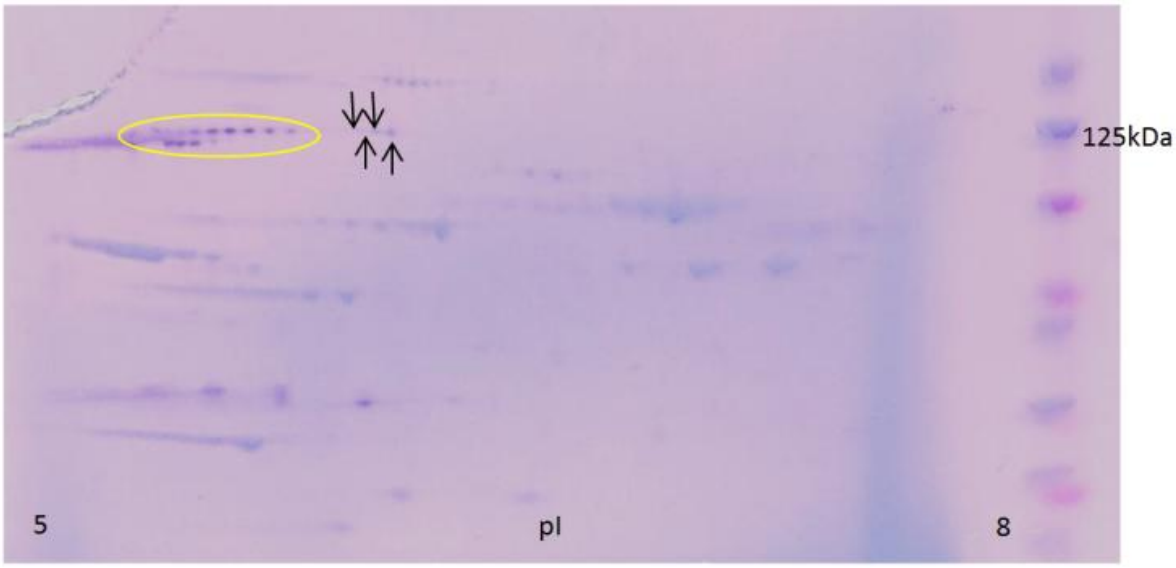

Figure 12. 2-DE of KC01 using 2D Western Blot image superimposed onto Coomassie stain image to determine region of ITIH4 on the Coomassie gel. Circle highlights ITIH4 identified through Western Blot analysis at 125kDa. Arrows highlighting coomassie stained spots that underwent MS analysis, as trail seemed to correspond with 2D Western gel image

MS Profiling to Determine identity of Spots of interest from KC01 Coomassie stained gel

This method is a high-throughput technique that only requires small amounts of protein (~0.1µg per MALDI spot) [24]. Therefore, this method was used to determine whether the spots within the coomassie stained KC01 gel, that seemed to correspond with the ITIH4 fragment, (Figure 12), was ITIH4. However, results found Complement component C3 was present in the analysed spots, possibly due to ITIH4 only being present in low abundance and therefore, not detected by the coomassie stain. Table 6 indicates the top four results gained from the MS analysis. The data indicates chain B was present however, when investigating, the known molecular weight (MW) for Complement component C3 Chain B (beta) was 71.3kDa with a theoretical pI of 6.82 (Expasy). This data does not correspond with the data outlined in Table 6, or from the results of the coomassie stain, which suggested the MW was similar to that of ITIH4, at around 125kDa. This suggested that the chain identified was Complement component C3 alpha chain, as the known MW is 113.0kDa with a theoretical pI of 5.55 (Expasy) which is strongly supportive of the rank 1 result described in table 6.

Table 6. Proteins identified using MS and the suggested MW and pI. Protein scores >66 indicate protein identity as significant (p<0.05)

Rank	Protein Name	Protein MW (kDa)	Protein pI	Protein Score	Accession Number	No' of Peptide Hits
1	Chain B, Human Complement Component C3	114.2	5.55	714	78101268	46
2	Chain B, Human Complement Component C3	114.2	5.55	714	78101268	48
3	Chain B, Human Complement Component C3	114.2	5.55	656	78101268	47
4	Chain B, Human Complement Component C3	114.2	5.55	415	78101268	40

Discussion

Through the use and addition of new maternal serum biomarkers, including PAPP-A and AFP, prenatal DS screening has rapidly evolved reducing the need for invasive procedures [24] including CVS that carry a small but significant ~1% risk of miscarriage [9]. With the occurrence of DS as much as 3 in 1000 births [1] increasing the detection rate from its current levels of 80-87% [7] is of significant importance to prenatal medicine and in particular for better non-invasive prenatal screening methods.

Presence of ITIH4

ITIH4 is a plasma glycoprotein secreted from the liver [38] and known to be present in plasma at concentrations of 80µg/ml [30]. Through the use of Western Blotting it has been possible to identify the presence of ITIH4 in all control and pregnant samples known to be carrying a DS fetus, as expected. However, the study would have been strengthened by an investigation into the concentration of ITIH4 present, to allow comparisons to be made between the concentration of ITIH4 in the pregnant and control samples and to identify any changes over the pregnancy. Nagalla *et al*., [24] used 2D-DIGE and 2D-CF to identify a 2.67 fold increase in ITIH4 in DS vs control samples in the first trimester and a 1.69 fold increase in the second trimester. These advances in proteomic separation technology allow much easier detection of differentially expressed proteins between samples and highlight the significant increase in information that can be obtained. However, this study was carried out on maternal serum, in contrast to our study, and therefore it may not be possible to use the study to suggest that this increase may have occurred in our samples. On the other hand, Kolla *et al*., [29], carried out a study on first trimester maternal plasma samples using iTRAQ and also found ITIH4 to be significantly upregulated ($p < 0.001$) in DS pregnancies compared to controls, suggesting ITIH4 is a potentially significant plasma and serum biomarker of DS. In contrast, Heywood *et al.,* (Unpublished data) only found ITIH4 to be upregulated in maternal plasma samples carrying a DS fetus, in the second trimester, using 2-DE.

ITIH4 as an Acute Phase Protein

Although again based on serum biomarker proteins Nagalla *et al*., [24] suggested candidate biomarkers fall into three major functional groups; serum carrier proteins, protease inhibitors and acute-phase proteins. ITIH4 is related to a group of protease inhibitors, however, ITIH4 itself lacks the bikunin chain which contains the protease inhibitor domains [31]. Nonetheless, ITIH4 has been identified as a major acute phase protein (APP) in pigs [39] and cattle [40] and has been further characterised as a type II APP in humans [31]. Pineiro *et al.,* [31] observed a dose dependent upregulation of ITIH4 mRNA expression after exposure to interleukin-6 (IL-6) in hepatocarcinoma HepG2 cells, alongside an increase in secretion of the mature protein within the cellular media. This is significant as Licastro *et al.,* [41] has demonstrated a significant increase in IL-6 levels in plasma of DS children, this is supported by Corsi *et al.,* [42]. Whilst studies would have to be carried out to determine whether elevated levels of IL-6 occur prenatally this evidence suggests that the increase in ITIH4 in maternal serum and plasma in Down syndrome pregnancies may be caused by an increase in plasma IL-6 levels within the fetus. Further research would be needed to determine whether the increased levels of IL-6 amplifies expression of ITIH4 in the fetus which passes to the mothers blood resulting in the observations of Nagalla *et al*., [24] and Kolla *et al.,* [29], or whether increased levels of IL-6 increase maternal expression of ITIH4.

Limitations

An important limitation of our study is the lack of maternal plasma samples carrying known euploid fetuses, this places significant limitations on the conclusions that can be drawn from these results on the use of ITIH4 as a prenatal biomarker of DS. The use of maternal plasma samples from a known euploid fetus would be a benefit for comparisons to be made in a future study. The method could also be improved by including matched controls for factors including age, race and gestational age. As well as matching non-pregnant controls, which would attempt to remove any confounding factors. A further limitation, is the lack of information on the sex of the sample from KC01. Information on the sex of this sample would have been useful to see if it influenced the 2-DE results (Figure 10). Nonetheless, the results obtained from the Western Blots (Figure 4 and 5), and real-time PCR (Table 5) suggest that the sex of the fetus had no effect on the differences seen between the samples.

Kallikrein and ITIH4

Kallikrein cleaves ITIH4 into a 100 and 35kDa fragment, with the 100kDa fragment subsequently cleaved to yield a 70kDa fragment [30]. The Western Blot analysis identified the 35kDa fragment and the full 125kDa protein. However, the full MW is in contrast to a number of other studies including [30], and Pineiro *et al.,* [40] who found ITIH4 to have a MW of 120kDa. This may have been due to differences in the marker used. Nonetheless, the results for KC05 and KC06 suggested the full ITIH4 molecule was not present. This is in contrast to the other results, from both control and pregnant samples, which found the full fragment to be present and in some cases, the 35kDa fragment aswel. No correlation was found between this result and the gestational or maternal age(Figure 4 and 5), or the sex of the fetus.

A potential explanation for the fragmentation differences seen could be due to variations in kallikrein activity. Recently, Almeidaa, et al., [43] demonstrated that polymorphisms in the Angiotensin I-converting enzyme (ACE), II, ID and DD, affected plasma kallikrein activity. With the DD polymorphism associated with a 60%

and 30% increase in plasma kallikrein activity when compared with the II and ID polymorphisms, respectively. This may mean that an individual with a DD polymorphism has increased kallikrein activity which may result in more fragmentation of ITIH4, potentially resulting in the lack of full length 125kDa ITIH4 seen in KC05 and KC06. However, this study has not been followed up by any other investigations and therefore it is unclear as to whether these results could be replicated.It is also possible the differences between the fragmentation patterns of the samples may be due to the type of samples and how they were collected. For example, if serum was used instead of plasma, proteases, activated by the clotting process such as Kallikrein, may reveal different fragmentation patterns [38]. However, Song *et al.,* [38] identified no alteration in fragmentation samples when using either serum or plasma from the same patient.

Mass Spectrometry Analysis

The MS analysis of the KC01 coomassie stained Gel (Figure 11) did not find ITIH4 to be present but complement component C3. This is likely to be due to the low protein concentration that was run on the gel which despite the protein depletion procedure meant there were more abundant proteins that masked the presence of ITIH4. If this study were to be carried out again then it would be useful to confirm the presence of ITIH4 and the methods could have been improved to reflect this, including running bigger IPG strips that would have allowed more protein to be added to the gel which would have been likely to yield the presence of the low abundance protein, ITIH4.

The use of Proteomics

Although proteomic separation technology has advanced, one of the major issues that hinders proteomic studies is the complex nature of the serum/plasma proteome, where many low abundance proteins are masked by the presence of a few highly abundant proteins [29], which has been highlighted in our study when it was not possible to identify the presence of ITIH4 after 2-DE, despite undergoing Albumin/IgG depletion. It is likely that this causes the large variation in results from different studies, which is further compounded by the variation in methods between studies [24]. ITIH4 also highlights the complication of post-translational modification including cleavage, which results in fragments that are present at an even lower concentration that the full protein, making detection increasingly difficult.

Future directions

Future work could focus on the ACE polymorphisms and plasma kallikrein activity, as to why we see the different fragmentation patterns, which may be completely independent of DS. It would be interesting to use another antibody to identify the presence the 70kDa fragment, to gain more information on how ITIH4 fragments. It would also be useful to investigate maternal plasma samples carrying known euploid foetuses, this would allow direct comparisons to be made on the cleavage patterns and to identify whether the cleavage patterns seen in DS samples KC05 and KC06 only occur in DS samples. Need to carry out large-scale studies using similar methods to efficiently determine effective biomarkers.

Conclusions

This study has been unable to determine whether ITIH4 would be suitable as a biomarker in DS pregnancies. However, the presence of ITIH4 was determined in all control samples and maternal plasma samples known to be carrying a DS fetus, as expected. Variations were found in the fragment pattern of ITIH4 between DS

samples, which was not seen in the control samples. It is unclear as to what causes this variation, which was not associated with gestational or maternal age, or the fetal sex.

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