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RESEARCH ARTICLE

BIOMARKERS IN HUMAN AMNIOTIC FLUID: VARIOUS PROTEOMICS APPROACH

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ABSTRACT

Pregnancy is a special time during a woman's reproductive life as a result of the unique physiology and the presence of a developing fetus. Despite an impressive amount of effort and extensive research over the past century, our knowledge of the development, physiology and pathophysiology of the fetus and its environment remains limited. Thus, study of amniotic fluid (AF) provides unique as well as vital information about the understanding of the biochemistry and physiology of AF and to foresee ways in which the fluid might be used in other diagnostic problems. AF is a complex and dynamic biological fluid that surrounds the fetus in the amniotic cavity which protects the fetus from mechanical as well as thermal shock and also contains nutrients and growth factors that facilitate fetal growth. AF is known to contain large amounts of proteins whose expression profile reflects the genotypic constitution of the fetus and regulates feto-maternal physiological interaction. An intricate balance of proteins is required throughout pregnancy and in cases of pregnancy complications or fetal genetic abnormalities, the balance may be disturbed. So, identification of these changes therefore, may be used for the detection of a particular type of pathology. Proteomics have additional relevance in understanding pathophysiology and the development of molecularly targeted therapeutics. Comparison of normal human AF proteome with that coming from pregnancies carrying fetuses with chromosomal abnormalities facilitated the detection of panels of potential biomarkers for prenatal detection of fetal aneuploidies. The discovery of novel protein biomarkers in either drug development or the study of disease in AF is often hindered by certain particular proteins present at relatively high concentrations. The ability to deplete these proteins specifically, reproducibly and high selectivity is increasingly important in proteomic studies and success in this procedure is leading to an ever-increasing list of low abundant proteins being identified in AF. AF proved to be a promising target for biomarker discovery of pre-mature rupture of amnion, intra-amniotic infection, diseases like DS (Down syndrome) and to distinguish pre-eclampsia from chronic hypertension and normotensic controls.

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INTRODUCTION

Amniotic fluid (AF) surrounds the fetus in the amniotic cavity and protects it from mechanical and thermal shocks, contains nutrients and growth factors that facilitate fetal growth and antimicrobial factors that protect the fetus and allows assessment of fetus maturity and diseases (Schmidt, 1992; Stewart *et al*., 2001). AF is derived from both maternal perfusion activity of chorioamnion and fetal urine. During embryogenesis volume of AF increases faster than the embryonic size.

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The water in AF comes from maternal plasma that passes through the fetal membranes based on hydrostatic and osmotic forces. When the placenta and fetal vessels develop, the water and solutes from the maternal plasma pass across the placenta to the fetus and then to the AF. In second and third trimester during maturation, the composition of AF is altered by fetal urine (300 ml/kg fetal weight/day or 600 to 1200 ml/day near term) as well as the secretion of oral, nasal, tracheal and pulmonary fluids (60 to 100 ml/kg fetal weight/day)(Gilbert and Brace, 1993). In the first half of pregnancy the AF volume appears to increase in association with growth of the fetus. During 20 and 30 weeks of gestation, fetal swallowing provides a means of removing water and proteins equally from the amniotic cavity (being a form of "bulk flow" rather than diffusion) whilst water is replaced by fetal urine. During the first half of the pregnancy, AF appears to maintain the

extracellular fluid chemistry of the fetus and in the second half AF reflects the development of renal function and by virtue of the cells fetus sheds by the morphological development of the skin and the mucous membranes. Keratinization of fetal skin begins at 19 to 20 weeks of gestation and is usually completed at 25 weeks after conception. After the keratinization is completed the AF volume changes but not linearly with the fetus size. When the fetal skin is fully keratinized, the volume of AF is determined by different mechanisms which comprise the AF circulation. Five pathways (Figure 1) of exchange have been identified between the AF space and the surrounding tissue (Underwood *et al*., 2005).

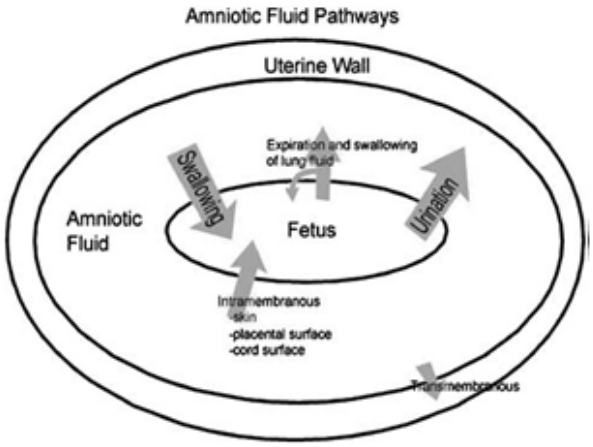

Figure 1. Amniotic fluid path ways

During the second half of the pregnancy, concentration level of sodium and chloride decreases, urea and creatinine concentration increases and overall decrease in AF osmolarity has been reported (Wintour and Shandley, 1993). The fetus largely depends on placenta for the transport of the nutrients and is also protected from marked fluctuations in the maternal metabolism. Gulbis *et al*. (1996) suggested that increase in creatinine, γ-glutamyltrasferase and β-microglobulin concentration in AF after 10 weeks of gestation confirms the maturation of fetal glomerular function and reflects the fetal kidney development from mesonephros to the metanephros. AF has low specific gravity (1.008) and a pH of 7.2. During pregnancy, the protein concentration increases in the AF from the mean of 3.5 mg/ml at 12 weeks of gestation to a maximum of 6.6 mg/ml at about 25 weeks. But after 25 weeks protein concentration gradually falls to about 3 mg/ml upto 35 weeks. During 35-39 weeks of gestation there are little changes in the concentration of proteins. In case of mild and moderate Rh-isoimmunization no consistent change in the protein concentration has been reported, but in very serve cases more random patterns are observed (Queenan *et al*., 1970).

The concentration of albumin, alpha-antitrypsin, group specific component and transferrin has also been analyzed in AF during gestation and has been shown to be at highest level between 20 to 30 weeks gestation (Sutcliffe and Brock, 1973). The reason for changes in protein concentration during pregnancy in different gestation is not clear because little is known of the rate of flux of water and proteins across the various structures of the fetus and amniotic cavity. However, the process of fetal swallowing and micturation could be advanced as possible reason for decline in the concentration of proteins in the AF towards the terms. The most of the proteins are stable at that gestation period so that decline in concentration of fluid does not cause any protein degradation (Sutcliffe and Brock, 1973). Thus, it is possible to emphasize that in the absence of gross changes in total volume of AF, the bulk flow of AF in the amniotic cavity is an important factor in the regulation of the protein concentration, especially towards the end of the pregnancy.

Amniocentesis

The removal of AF through invasive technique for analysis had been performed as early as 1881 (Lambi, 1881). First definite investigation procedure in AF spectrophotometry has been reported by Liley (Liley, 1961). This method is widely used for predicting the risk of fetal anemia. Therefore for this remarkable work many regarded Liley as father of fetal medicine. Liley has devised in zones that represent fetal risk categories based on the concentration of bilirubin in the AF. The zones are upper, mid and lower. In AF the break down product from the haemolysed fetal red cells appears as bilirubin. Bilirubin concentration creates a serious problem for the fetus. Bilirubin is quantified usually by spectroscopic analysis, the maximum absorption for bilirubin being at a wavelength of 450 nm. The optical density difference is plotted on the Liley's chart for predicting severity of hemolytic diseases such as rhesus disease. Amniocentesis is most commonly performed during second and third trimesters with little risk to mother and fetus. Nowadays amniocentesis is an established standard tool for the assessment of pregnancy that is at risk for variety of chromosomal disorder, single gene defects, biochemical analysis and fetal infections. Thus, it has become important to gain a possible understanding of the biochemistry and physiology of AF and to foresee ways in which this fluid might be used in other diagnostic problems.
Common indications of amniocentesis:

In the second trimester

- Advanced maternal age (=35 years) and older.
- Previous child or pregnancy with birth defects.
- Blood test or ultra sound suggesting birth defect such as DS or neural tube defects.
- Family history of genetic disorder such as cystic fibrosis.

In the third trimester

- To determine maturity of baby's lung (early delivery is necessary).
- To diagnose a uterine infection.
- To check for anemia in baby with Rh incompatibility.

Origin of protein in amniotic fluid

A wide range of proteins have been identified in human AF (Queenan, 1978; Burdett *et al*., 1982; Drohse *et al*., 1998). These proteins can enter the AF from the maternal uterine tissues, umbilical cord, AF cells, meconium, fetal urine, and other fetal secretions which include transduction through fetal skin (Queenan, 1978; Sutcliffe, 1981; Jauniaux *et al*., 1998).

AF proteins are principally of maternal origin have concentrations lower than maternal serum and reportedly vary between 0.2 to 7 g/l (Jauniaux *et al*., 1991; Campbell *et al*., 1992; Jauniaux *et al*., 1994a; Jauniaux *et al*., 1998). For the dynamic temporal pattern with AF total protein concentrations rising from 7 to 20 weeks of gestation and declining there after (Benzie *et al*., 1974; Queenan, 1978; Jauniaux *et al*., 1994b). Studies on the constituent proteins in AF, provides a volume of evidence which suggest that AF is derived largely from maternal sources (Sutcliffe, 1975). The proteins in AF exist in a number of different forms. It may be part of the intact cells or may be cellular organelle or soluble proteins. Throughout the pregnancy, majority of the soluble proteins in AF are derived from the mother rather than the fetus are mainly serum proteins. Many maternal serum proteins have gain access to the AF, there by complicating the use of this fluid for prenatal diagnosis.

There are a number of possible sources which might contribute proteins of the fetal origin to the AF. Proteins of the fetal origin are probably derived from skin, amnion, chorionic, umbilical cord, urine, bronchial, buccal and gastrointestinal secretions. Phenotyping (Serum proteins polymorphism) studies have suggested that α_2-group-specific component (Gc) present in AF in early stage of gestation is of maternal origin. It seems that this protein enters through the placenta or the fetal membranes (Sutcliffe and Brock, 1972). In AF, β_2 - microglobulin is fetal origin. Its concentration in AF changed during pregnancy in a way similar to that observed for the total protein concentrations. Further, it was always in excess of the β_2 - microglobulin concentration in maternal serum. At term its concentration in AF is similar to that in cord serum but twice the concentration found in maternal serum. Relatively low level of β_2-microglobulin in maternal serum suggests that this protein in AF is of fetal origin and it is excreted in AF by the fetal kidney (Jonasson *et al*., 1974). The concentration of low molecular weight protein (LMWP) i.e. α_1- microglobulin and β_2 - microglobulin increases in AF until the end of second trimester and as the pregnancy advances a progressive decrease of these occur parallel to the fetal renal maturation. After 26 weeks gestation strong correlations have been identified between LMWP levels and α_1- microglobulin and between LMWP and β_2 - microglobuline. Further no significant differences have been detected between LMWP levels in the first urine of the neonates and in the AF.

This suggested that microproteins in fetal urine are of fetal origin. The fetal renal maturation can be evaluated by measuring miroproteins in AF. Fetal renal maturation is best reflected by α_1-microglobulin (Cagdas *et al*., 2000). To investigate the origin of insulin in AF, amniocenteses have been carried out by Greco *et al*. (1980) in pregnancies with live, dead and anencephalic fetuses. They found that AF insulin of pregnant women bearing live fetuses was 9.0±2.1 microU/ml but AF insulin was not detected in six women with dead fetuses. A significant positive correlation has been observed between gestational age and AF concentration of insulin. In the AF of four women bearing anencephalic fetuses, the amount of hormone is within normal limits (10.0 ± 1.4 microU/ml). Intravenous glucose administration (0.33 g/kg body weight) to the mother doesn't influence levels of insulin in AF, changes in AF glucose concentration occurs. These findings supported the conclusion that human AF insulin is of fetal rather than maternal origin (Greco *et al*., 1980). Insulin like growth factor binding protein-1 (IGFBP-1) is probably the predominant member of IGFBPs (Drop *et al*., 1984). It is the main secretory product of decidualized endometrial cells (Koistinen *et al*., 1986; Bell *et al*., 1988). In AF, IGFBP-1 concentrations are three times greater than those measured in maternal serum. Since IGFBP-1 is either none or less phosphorylated, several studies have suggested that highly phosphorylated IGFBP-1 is not found in AF (Jones *et al*., 1991; Westwood *et al*., 1994). Many studies have indicated that maternal decidua is the likely source of this IGFBP-1. Decidualized endometrium secretes IGFBP-1 in the non- and less phosphorylated isoforms (Frost and Tseng, 1991; Martina *et al*., 1997; Westwood *et al*., 1998) in addition to the highly phosphorylated species. In the beginning of the second trimester concentration levels of IGFBP-1 in the AF are low until the amnion fuses with the chorionic-deciduas (Wathen *et al*., 1993).

It indicated that the additional IGFBP-1 isoforms observed in the maternal circulation during pregnancy may originate at the fetal maternal interface. This hypothesis is supported by the above study also that, in response to oral oestrogen administration non-pregnant women whose circulating IGFBP-1 concentrations are increased, non- and less phosphorylated isoforms cannot be detected instead only the highly phosphorylated isoforms are present (Westwood *et al*., 1999). Adiponectin is an adipose tissue derived protein with important metabolic effects and a strong correlation with insulin sensitivity. In pregnancy there is progressive increase in insulin resistance, where as in plasma adiponectin concentrations decrease in the second half of gestation (Fuglsang *et al*., 2006). It is suggested that amniotic adiponectin might be of fetal origin and maternal origin seems unlikely because there are no correlations between plasma and amniotic adiponectin or between amniotic adiponectin and maternal age or BMI (Body Mass Index). In addition, Corbetta *et al*. (2005) found no adiponectin in placental tissue. Thus, the fetal origin of amniotic adiponectin appears likely. Insulin production of the human fetus from 11 weeks of gestation age has been demonstrated in an experimental study (Reiher *et al*., 1983) and a strong correlation between amniotic adiponectin and amniotic insulin has been demonstrated (Scioscia *et al*., 2006). Further, the statistically significant differences between female and male fetuses support the theory that the amniotic adiponectin is of fetal origin.

Clinical and experimental studies demonstrated that the sex dimorphism of adiponectin concentrations might be caused by testosterone induced inhibition of its secretion from adipocytes (Xu *et al*., 2005). Recent studies based on 50 normal pregnancies at the time of mid trimester amniocentesis have suggested that adiponectin found in the AF is of fetal origin (Scioscia *et al*., 2006). Fetal urine is the major source of Alpha fetoprotein (AFP) in AF of normal pregnancy (Weiss *et al*., 1976). The concentration in maternal serum is valuable in prenatal diagnosis of neural tube defects and other fetal malformations. Fetal serum contains AFP in a concentration 150 times that of maternal serum. Several fetal abnormalities other than neural tube defects have now been reported where

the level of AFP have been increased in the AF. These include congenital nephrosis (Kjessler *et al*., 1975; Thom *et al*., 1977), oesophageal atresia (Seppala *et al*., 1974), duodenal atresia (Weinberg *et al*., 1975), omphalocele (Nevin and Armstrong, 1975) and Meckel's syndrome (Chemke *et al*., 1977). Raised AF and maternal serum AFP levels have been associated with multiple fetal abnormalities including gross distension of the bladder caused by the absence of urethra, intestinal atresia, transposition of the aorta and an interventricular septal defect (Nevin *et al*., 1978).

High abundance proteins in amniotic fluid

Many Proteomic analysis efforts have been considered, high abundance proteins to be a hindrance in the search for more interesting low molecular proteins in AF. The high abundance proteins in AF represent many physiologically important molecules such as apolipoproteins, immunoglobulins, coagulation factors, protease inhibitors, complement factors and carrier molecules (Putnam, 1975; Peters, 1983; Anderson and Anderson, 2002; Johnson, 2006). The high abundance proteins predominantly represent major secretary proteins released from abundant tissue such as liver, lymphoid, hematopoietic tissues, and intestine. Quantitative analysis of many of these high abundance components is diagnostically useful for assessing nutrition, immune status and disorders of coagulation or fibrinolysis. The disorders of lipoproteins are acute phase responses to injury or disease. Therefore, measurements of these molecules are an important part of clinical chemistry practice. However, presence of high abundance proteins in AF produces hindrances in the proteomic analysis of this fluid in search of interestingly low molecular weight Proteins. Chao *et al*. (2007) have reported 15 proteins with largest number of unique peptides in AF along with the top 15 proteins from the human plasma proteome (Tirumalai *et al*., 2003) for comparison (Table 1).

Proteins in AF have been ranked by the number of unique peptides identified from SAX (strong anion exchanger) and SCX (strong cation exchanger) fractionation methods. They have compared top 15 proteins from plasma proteome and AF proteome and found some significant differences. Transforming growth factor β- induced protein ig-h3 precursor, α-fetoprotein and periostin (among 15 abundant proteins in AF) are found in relatively low concentration in plasma and not placed in top 15 plasma protein. Haptoglobulin which is one of the most abundant proteins in plasma is found at low concentration in AF. Apolipoprotein B also seems to be low abundance protein in AF because only one group has been identified in AF (Michel *et al*., 2006).

Depletion of high abundance proteins

AF protein analysis has been largely hampered by the predominance of several high abundance proteins including albumin (HSA), Immunoglobulins, Alpha-1-antitrypsin (A1AT), fibrinogen and haptoglobilin (HG) and their isoforms and fragments. There are 22 different proteins comprising roughly 98% of the total protein content of the serum (Anderson and Anderson, 2002). The remaining 1-3% of the proteins are in low abundance, which has been focused into new biomarkers, since it has been proposed that subtle change in pathophysiology of various conditions may be reflected in the small pool of proteins. Albumin, the single most abundance protein in AF is present in mg/ml range compared to pg/ml for cytokines (Bishop *et al*., 2000). Chao *et al*. (2007) have studied the total proteins and albumin analysis of AF. They have reported that albumin comprises nearly 70% of the total protein content of AF with the immunoglobulins being the second most abundance protein. These two major proteins in AF complicate proteomic profiling. Depletion of these proteins certainly improves identification of protein range in AF. This is mostly attributed to the so called "Sponge effect" of these major proteins (especially albumin); to which small proteins and peptides may bind to large proteins that normally serving as their carriers (Moritz *et al*., 2004; Moritz *et al*., 2005). Depletion of these highly abundance proteins are essential prior to proteome analysis in the search of new biomarkers of diseases.

Table 1. Top 15 high abundance proteins in AF, Proteins in bold are indicate high abundance found in either AF or plasma

Amniotic fluid Proteome			Plasma Proteome	
Gene	Protein	Rank	Gene	Protein
ALB	Albumin	1	ALB	Albumin
	Immunoglobulin's	2		Immunoglobulin's
FN1	Fibronectin	3	TF	Serotransferrin
TF	Serotransferrin	4	FG	Fibrinogen
C3	Complement C3	5	AMBP	α_1- microglobulin
SERPINA1	α_1- antitrypsin	6	SERPINA1	α -1-antitrypsin
CP	Ceruloplasmin	7	C3	Complement C3
AFP	α -fetoprotein	8	HP	Haptoglobin
GC	Vitamin-D-binding protein	9	APOA1	Apolipoprotein A-I
POSTN	Periostin	10	APOB	Apolipoprotein B
APOA1	Apolipoprotein A-I	11	ORM1	α_1-Acid glycoprotein
SERPINC1	Antithrombin III	11/ 12		Lipoprotein
TGFBI	Transforming growth factor-beta-induced protein-ig-h3 precursor	13	CFH	Factor H
AMBP	α_1-microglobulin	14	CP	Ceruloplasmin
PLG	Plasminogen	15	C4	Complement C4

The classical depletion of albumins involves the use of hydrophobic dye Cibacron blue (CB) and a chlorotriazine dye which has higher affinity for albumin (Travis and Pannell, 1973; Travis *et al*., 1976; Leather barrow and Dean, 1980; Gianazza and Arnaud, 1982a; Gianazza and Arnaud, 1982b). Because of its relatively low cost this method is still

sometimes used for the depletion of albumin (Kubo *et al*., 2000; Ahmed *et al*., 2003; Shaw and Riedere, 2003; Hammack *et al*., 2003; Ahmed *et al*., 2004; Li and Lee, 2004). However, it has been reported that the binding of albumin with CB dye is non-specific; sensitivity and specificity are not as effective as mAb-based immunoaffinity resins or columns (Ahmed *et al*., 2003; Steel *et al*., 2003; Bjorhall *et al*., 2005; Zolotarjova *et al*., 2005). Chemical methods such as protein precipitation with TCA/Acetone or NaCl/Ethanol also appear to be useful for depletion of albumins (Chen *et al*., 2005; Huang *et al*., 2005). The important draw back of CB dye is its indeterminate specificity and poor robustness. CB dye has been shown to bind albumin but has also been used for the binding of NAD (nicotinamide adenine dinucleotide), FAD (flavin adenine dinucleotide) and ATP binding sites of the proteins (Thresher and Swaisgood, 1990; Prestera *et al*., 1992). This multifunctionality might result in the removal of proteins of interest through a rather broad and non-specific interaction, which can only be partly mitigated by judicious selection of mobile phase condition.

Protein A/G affinity resin or column (Bjorck and Kronvall, 1981; Guss *et al*., 1986) can be used for the removal of Immunoglobulins (Wang *et al*., 2003; Greenough *et al*., 2004). Immunoglobulins represent the second most abundance protein in the AF. A low cost depletion kit for simultaneous depletion of albumin and immunoglobulins (Cat.No.PROTIBA) is also available which includes both types of the above mentioned resins. Comparative studies indicated that immunoaffinity depletion using multiple affinity removal column (MARC) for HSA and IgG give effective results as compared to tradional CB dye/ protein A or G depletion method (Bjorhall *et al*., 2005; Zolotarjova *et al*., 2005; Echan *et al*., 2005) because, MARC can simultaneously remove multiple abundant proteins with minimal carryover, high longitivity and minimal non-specific binding (Pieper *et al*., 2003; Bjorhall *et al*., 2005; Zolotarjova *et al*., 2005; Echan *et al*., 2005). Because of its high specificity, the trends are now towards the use of immunoaffinity column for most protein analysis. We have also reported two different methods for depletion of albumin from AF and the results demonstrated that specific depletion of albumin using affinity ligand based resin is more efficient as compared to conventional CB dye method (Alam *et al*., 2010). Affinity column are made up of matrices with covalently attached antibodies to the specific proteins (Wang *et al*., 2003; Govorukhina *et al*., 2003; Pieper *et al*., 2003; Anderson *et al*., 2004). As immunoaffinity depletion methods is more specific in removing good proportion of Albumin/IgG proteins, but not all the highly abundance proteins, these column may also remove other proteins (e.g., Cytokines) by non-specific binding (Yocum *et al*., 2005; Granger *et al*., 2005).

Therefore, the possible approach for dealing this problem is to disrupt the binding of low molecular weight binding proteins to the carrier proteins albumin/IgG. By adding 5 or 20% ACN as denaturating agents that may disrupts the binding between low molecular proteins and albumin/IgG resulting in increased number of proteins detected under denaturating condition as compared to native conditions. The presence of 5% ACN in the serum provides better enrichment of low molecular proteins as compared to 20% ACN condition (Huang *et al*., 2005).

Proteomics of amniotic fluid

Proteomics is widely used in biomedical research for protein screening in tissues or fluid in healthy and diseased states. The clinical advantage of this method is that very low amount of biological fluid is used for generating protein profile by two dimensional (2D) gel electrophoresis followed by mass spectroscopy that may be distinctive to the pathology under consideration. AF contains the secretary products from the amnion cells in the amniotic cavity. Amnion is composed of fetal cells which play active role in pregnancy. The protein profile of amnion and AF would reflect the pathophysiological changes occurring in uterus, which affects the fetus and the mother. Therefore, the proteomic analysis of amnion and AF has been used for identifying putative biological markers of high risk pregnancy. Recently proteomics have been widely used to investigate AF in order to better understand its biochemical complexities, and to discover disease specific biomarkers for fetal aneuploidies, pregnancy related complications, intra amniotic infection and in prenatal diagnosis. Initially, 2DE, LC-MS/MS has been proven its usefulness for the identification of potential biomarkers in clinical samples. But, now LC coupled to triple-quad instruments (multiple reaction monitoring/selective reaction monitoring [MRM/ SRM]) is used for validation of protein-based biomarkers in a clinical proteomics. The CE-MS (Capillary electrophoresis-Mass spectrometry) can be directly applied in clinical proteomics, while, 2DE-MS and LC-MS are not easily transferable to routine use in the clinic samples. However, MRM/SRM strategies, variants of LC-MS, are already being used in proteomics for the targeted and quantification of multiple low-molecular weight molecules such as drugs and metabolites (Lehotay *et al*., 2011; Seger *et al*., 2009; Van Den Ouweland and Kema, 2012). Proteomics have been applied in the prenatal diagnosis of DS by analysing protein levels in the tissues of fetus with comparison with normal fetuses to discover possible diagnostic tools (Larque *et al*., 2002; Fang *et al*., 2004). Several studies have reported for proteomic profile of AF using different techniques; Liberatori *et al*. (1997) identified 31 proteins in AF supernatant by immune-blot analysis and have also reported a 2D map of human AF, obtained during genetic amniocentesis at 17 weeks gestation. Nilsson *et al*. (2004) examined AF protein composition using LC-Fourier transform-ion cyclotron resonance MS to identify 58 proteins from AF in the 15^{th}-18^{th} weeks of gestation.

They are the first to use MS for profiling AF proteome and they also for the first time depleted albumin from AF to identify more proteins. Gravett *et al*. (2004) used SELDI/MS and LC-MS/MS to observe over-expression of several proteins from AF of infected patients undergoing preterm labor. They have also carried out extensive proteome analysis in human amnion and AF at term by 2-DE and MALDI-TOF/MS. Several proteins are showed differential expression in AF at term between patients with and without *Ureaplasma urealyticum*. By enzyme-linked immunosorbent assay (ELISA) and Western blot analysis, Kim *et al*. (2013), suggested the

elevated levels of interleukin-6 (IL-6) and matrix metalloproteinases-8 (MMP-8) in AF at mid-trimester are predictive of preterm delivery, and that vascular endothelial growth factor (VEGF) which is representative of angiogenesis can be a new and useful predictor of preterm delivery. Proteomics has been extensively used in the recent past to search for biologically relevant biomarkers to generate protein profiles characteristic of intra-amniotic inflammation (Gravett *et al*., 2004; Buhimschi *et al*., 2005a; Buhimschi *et al*., 2005b; Buhimschi *et al*., 2005c; Buhimschi *et al*., 2005d; Ruetschi *et al*., 2005; Weiner *et al*., 2005; Buhimschi *et al*., 2007). Park *et al*. (2006) have identified a total of 111 and 35 proteins from human amnion and AF respectively at term by 2-DE and MALDI-TOF/MS analysis.

They have identified Calgranulin A and B by MALDI-TOF-TOF/MS which are considered to be putative markers of pregnancies complicated by intra amniotic microbial infection. Thus, AF proteome reflects closely the fetal inflammatory response to intra-amniotic infection. These findings were sparked by the clinical observation that although inflammatory intrauterine processes adversely affect the fetus prior to birth (Leviton, 1993; Gonzalez *et al*., 2003; Keogh and Badawi, 2006; Babnik *et al*., 2006; Costantine *et al*., 2007), neither the biophysical profile nor evaluation of the fetal heart rate reliably provides early identification of the sick fetuses and need for immediate delivery (Ghidini *et al*., 2000; Aina-Mumuney *et al*., 2007; Buhimschi *et al*., 2008). Chao *et al*. (2007) have reported on the most extensive protein profile by analyzing the 16 AF samples between gestation ages of 16^{th}-18^{th} weeks from women carrying chromosomally normal fetuses. All identified proteins have been combined to generate the AF proteome that comprise 1,026 unique gene matches or 842 non-redundant proteins. Tsangaris *et al*. (2005) have constructed a 2D reference map for the normal human amniocytes that consisted of 432 different gene products. Later on in 2006 they reported the 2D protein database of the normal human AF supernatant (AFS) of ten AFS samples from women carrying normal fetuses. A mean of 412 spots per gel have been analyzed and protein identification have been carried out by MALDI-MS and MALDI-MS/MS.

The 2D protein map comprised of 136 different gene products, and the majority of the identified proteins are regulatory proteins, secreted proteins, carriers, immunoglobulins, and enzymes, (Tsangaris *et al*., 2006b). Queloz *et al*. (2007) compared the proteomic profile of normal AF, obtained either at 17 or 40 weeks of pregnancy with those of fetus presenting with congenital diaphragmatic hernia (CDH). They have used 2D and silver staining as well as 2D fluorescence difference gel electrophoresis techniques for proteomics purposes and have identified proteins more abundant in early pregnancy. Alternations in protein expression have been further confirmed by Michel *et al*. (2006) using immunodepletion methods to remove high abundance proteins and compared maternal plasma to human AF by Isoelectric focusing and LC-MS/MS to find potential markers for premature rupture of membranes (PROM). They also identified 69 proteins from depleted AF. In another study they have reported the comparison of the first, second and third trimester AF samples by fluorescence 2D-DIGE (two-dimensional differential in gel electrophoresis) that revealed significant differences in the relative abundance of AF proteins between the first and second trimesters (Michaels *et al*., 2007). The Preterm premature rupture of membranes (PPROM) is also major cause of preterm birth and neonatal disease. In this disease, PPROM results in placental and fetal membrane inflammation, being responsible for serious neonatal complications including chronic pulmonary disease and neurodevelopmental disease. Thus, identification of the infectious phenotype of PPROM is crucial for improving the outcome of the disease (Edmondson *et al*., 2009). The iTRAQ (isobaric tags for relative and absolute quantification) LC–MS/MS proteome analysis was performed using, pooled AF samples from women with PPROM (n = 19 per group) with and without associated bacterial infection and chorioamnionitis (Tambor *et al*., 2012). The top five proteins found deregulated between the two groups were three distinct histone proteins, cathelicidin and myeloperoxidase. An ELISA analysis confirmed increased abundance of cathelicidin in AF of new PPROM patients with (n = 38) and without bacterial infection and chorioamnionitis (n = 38) with high specificity (90%) but low sensitivity (48%).

Biomarkers Identified in amniotic fluid

The progress of pregnancy and delivery of fetus depend on a complex interaction of intracellular and extracellular signals; which include hormones, proteins, adhesion molecules, growth factors and immunomodulators (Pellicer *et al*., 1999). An intricate balance of these factors is required throughout pregnancy and in cases of fetal genetic abnormalities or gestational disease this balance may be disturbed. The identification of such changes in the balance of signals may be used to detect a specific type of pathology or to ascertain its severity (Wunder *et al*., 2005). Identification of proteins specific to pregnancy is likely to contribute to the comprehension of the underlying pathophysiology and to the discovery of biomarkers relevant to fetal genetic diseases or pregnancy complications (Brewis, 1999; Page *et al*., 2002; Jauniaux *et al*., 2003). The main challenge is to identify biomarkers that are fetal specific and differ in an aneuploid or disease affected pregnancy from a normal one. Many of the principles described for discovering biologically relevant fluid biomarkers in AF using SELDI-TOF (Buhimschi, 2012). If biomarkers are detected in early pregnancy or before the development of clinical symptoms, than they can be used as suitable disease markers, and whereas those detected at later stages are likely to be more specific and may be closely related to the phenotype of the disease.

AF is known to contain large amounts of proteins, whose expression profile changes throughout pregnancy and its protein profile reflects both physiological and pathological changes, affecting the fetus and the mother (Orczyk-Pawilowicz *et al*., 2005). Proteomic profiling of AF samples obtained from cases known to carry chromosomally abnormal fetuses revealed many candidate biomarkers for fetal aneuploidies. Proteomic changes in AF samples detected in pregnant women that carried DS fetuses exhibit significant derangement in the carbohydrate, amino acid levels, purine, intermediary metabolism, and miscellaneous metabolic pathways (Oh *et al*., 2004). The proteomics, based on MS/MS

have been used to identify biomarkers in AF samples from pregnancies with DS fetuses and chromosomally normal ones. This comparison revealed that seven proteins were differentially expressed in samples obtained from pregnancies with DS fetuses as compared to the control group. In pregnancies with DS fetuses expression increased for α-1-microglobulin, collagen α- 1(I) chain, collagen α- 1(III) chain, collagen α- 1(V) chain d and basement membrane-specific heparin sulfate proteoglycan core protein, whereas in chromosomally normal fetuses insulin-like growth factor binding protein is decreased by 40%. In the same study, splicing factor arginine/ serine-rich protein have been identified as present only in AF samples from cases with DS fetuses and was completely absent in the chromosomally normal ones (Tsangaris *et al*., 2006a).

hypertensive disorder characterized by a sudden onset of hypertension and the appearance of proteinuria and edema after 20 weeks of gestation (ACOG, 2002). The discovery of relevant biomarkers to aid with the early prediction, rapid confirmation of the diagnosis and treatment of PE has been crucial. Therefore, proteomics seem to be providing the needed breakthrough in understanding the pathology of PE through the discovery of biomarkers. Michel *et al*. (2006) compared the maternal plasma to AF by off-gel Isoelectric focusing technique followed by tryptic digestion of the proteins and by LC-MS/MS. The comparison revealed that nineteen proteins were specifically present in the AF and absent in maternal plasma.

Table 2. Markers for pathological conditions that were identified in AF

Pathology	Bio-Markers Identified
Down Syndrome (DS)	hCG β chain, AFP, Inhibin A (β-A chain), glyceraldehydes-3-phosphate dehydrogenase (Lubec *et al*., 1999), AMBP, collagen α 1 (I), collagen α 1 (III), collagen α 1 (V), basement membrane-specific heparin sulfate proteoglycan core protein, IBP-1 (Tsangaris *et al*., 2006a), activin A (β-A chain) (Wallace *et al*., 1999).
Trisomy 13	AFP, hCG β chain
Trisomy 18	AFP, hCG β chain
Preterm Delivery	CD163 (Vogel *et al*., 2005)
Feto-placental Hypoxemia	activin A (β-A chain) (Jenkin *et al*., 2001)
Intra-amniotic Infection	Calgranulin A (Ruetschi *et al*., 2005), Calgranulin B (Gravett *et al*., 2004), Calgranulin C (Buhimschi *et al*., 2005a), Vitamin D binding protein, IGFBP-1(Gravett *et al*., 2004), neutrophil defensin-1 (Buhimschi *et al*., 2005a).
Pre-eclampsia	Fibronectin (Stubbs *et al*., 1984), intercellular adhesion molecule-1 (Krauss *et al*., 1997), plasminogen activator inhibitor (Gao *et al*., 1996).
Rupture of the membrane	Prolactin, hCG β chain, fibronectin (Michel *et al*., 2006), AFP, IGFBP-1(Rutanen *et al*., 1996), agrin, plasma retinol-binding protein precursor, apolipoprotein A-I, B-factor (Vuadens *et al*., 2003).
Ureaplasma Infection	intercellular adhesion molecule-1 (Hadar *et al*., 2006)

An another study the same group used MS based proteomics in AF samples obtained from pregnancy with Turner's syndrome fetuses, has demonstrated seven biomarkers for the syndrome. Serotransferrin, lumican, plasma retinol-binding protein and apolipoprotein A-I expression levels are increased in Turner syndrome, whereas kininogen, prothrombin and apolipoprotein A-IV are decreased (Mavrou *et al*., 2008). Wang *et al*. (2009) have reported 2DE followed by MS to identify proteins that were differentially expressed in AF of trisomy 18 and DS fetuses. The proteins which exhibit significant differential expression in DS are APOA1, SERPINA3, prealbumin (transthyretin, TTR) and transferrin (TF). The expression levels of apolipoprotein A1, AP-3mu and antitrypsin are significantly decreased in trisomy-18 AFS; whereas placental protein-14 (PP-14) was increased. On the other hand, apolipoprotein A1 is decreased in pregnancy with trisomy-21 AF, whereas antitrypsin, prealbumin and transferrin are increased in trisomy 21. From the overall findings mentioned above it is revealed that the proteins of the trisomy18 AF are involved in immune processes, platelet disorders and dysfunction of skin pigmentation; whereas those of trisomy 21 have been associated with dysfunctional lipid and cholesterol metabolism processes, metal ion transport, ATP metabolism and energy coupled protein transport. The Pregnancy-related disorders such as preeclampsia (PE), intrauterine growth restriction (IUGR), preterm labor (PTL) and intra amniotic infection (IAI) have been found to contribute significantly to maternal and fetal mortality. Each of these disorders, considered to have multifactorial etiology, has a prevalence of 5-10 (WHO, 1987; Lumley, 2003). PE is a pregnancy-specific

Of these ten proteins have been previously described as pregnancy or placenta specific and therefore, could be further characterized as potential PROM biomarkers. PTL can spontaneously occur either as PTL or preterm prelabor rupture of the membranes (PPROM). IAI is often associated with preterm birth and adverse neonatal sequel. Early diagnosis of IAI is problematic because the clinical symptoms are late manifestations of this condition. Through a number of investigation on the AF proteomic profiling using different techniques, a few putative markers including human neutrophil protein (HNP) 1-3, Calgranulin A, B, C and IGFBP-1 have been identified as potentially useful for diagnosis of the IAI (Gravett *et al*., 2004; Buhimschi *et al*., 2005a; Buhimschi *et al*., 2005b; Ruetschi *et al*., 2005; Buhimschi *et al*., 2006). IGFBP-1 may also be used in the diagnosis of premature rupture of membranes, a condition associated with increased maternal and fetal morbidity and mortality unless detected and treated quickly (Bujold *et al*., 2008).

Park *et al*. (2006) found altered expression of Calgranulin A and B in human amnion and AF samples obtained from pregnant women infected with *Ureaplasma urealyticum*, but not found in any of the patients without infection. Ruetschi *et al*. (2005) have analyzed AF and CVF (Cervical-vaginal *fluid*) samples from pregnant women with clinical signs of PLT with or without IAI using SELDI-TOF. They have identified 17 proteins significantly over expressed in the AF samples in pregnancies complicated with inflammation. Presently, a vast amount of biomedical literature has amassed concerning the use of Human alpha-fetoprotein (HAFP) during

pregnancy as a biomarker in human maternal serum and AF. 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 multiple congenital malformations of the embryo and fetus. The first developmental abnormalities to be associated with abnormal AFP levels were neural tube defects and brain/spinal cord malformations (Drugan *et al*., 2001; Muller, 2003). Later, other types of birth defects were also found to reflect discordant AFP levels, including chromosomal abnormalities (anaploidies) and various anatomic congenital disorders (Duric *et al*., 2003; Benn and Ying, 2004). AFP serum levels during pregnancy also have been used as an ancillary aid in the diagnosis of pregnancy-related hematologic disorders (anemias), placental abnormalities, fetal death, growth restriction/retardation and preterm labor (Bartha *et al*., 1999). Recently, Tambor *et al*. (2012) suggested that cathelicidin as a candidate marker that should be considered for a panel of AF proteins permitting identification of PPROM women with microbial invasion of the amniotic cavity (MIAC) leading to histological chorioamnionitis (HCA). Cho *et al*. (2007) reported on the most extensive protein profile of the second trimester normal human AF, which comprised of 1026 unique gene products from 842 different genes. This list includes most of the currently used biomarkers for pregnancy associated pathologic conditions such as intra amniotic infection, preterm delivery, and chromosomal anomalies of the fetus (Table 2).

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