Measurements Using Capillary Zone Electrophoresis of Amniotic Fluid Proteins and Uric Acid

By

Tao Gao

School of Dietetics and Human Nutrition
McGill University
Montreal, Québec

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I. ABSTRACT

The objectives of the study were to measure the concentrations of albumin, transferrin, IgG and uric acid in 2nd trimester amniotic fluid (AF) and to establish if these concentrations were associated with infant birth outcomes.

Amniotic fluid samples (n=230) were collected from mothers undergoing routine amniocentesis (12-20 wk). Maternal characteristics like height, pre- pregnancy weight, age, smoking status, parity and infant gender, birth weight and gestational age were collected from questionnaires and obstetrical medical chart review. AF samples were analyzed by capillary zone electrophoresis (CZE).

The results showed that the 2nd trimester AF uric acid was a significant predictor of infant birth weight (grams) and transferrin was negatively associated with gestational age in term infants.

II. RÉSUMÉ

L'Objectif de cette étude est de mesurer la concentration en albumine, transferrine, IgG et acide urique dans le fluide amniotique (FA) du 2eme trimestre et de déterminer si ces concentrations sont associées aux conditions de naissance de l'enfant.

Des échantillons de fluide amniotique (n=230) ont été collectés de mères suivant une routine d'amniocentèses (12-20 semaines de gestation). Les caractéristiques de la mère, soit la grandeur, le poids lors de la grossesse, l'âge, le statu de fumeur, le nombre de grossesse antérieur et les caractéristiques du nouveau né, soit le genre, le poids, la durée de la gestation ont été collectées à partir de questionnaires et de tableaux médicaux obstétriques. Les échantillons de FA ont été analysés par électrophorèses de zone capillaire.

Les résultats démontrent que le FA urique acide du 2eme trimestre est un indice du poids de l'enfant à la naissance (grammes) et la transferrine est associée de manière négative avec la durée de la gestation du nouveau né.

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AF	Amniotic fluid
AGA	Appropriate-for-gestational-age
ANOVA	Analysis of variance
BMI	Body mass index
BW	Birth weight
CE	Capillary electrophoresis
CZE	Capillary zone electrophoresis
Fab	Fragment antigen binding
Fc	Fraction crystallizable
GA	Gestational age
Ig	Immunoglobulin
IGFBP	Insulin-like growth factor binding protein
IgG	Immunoglobulin G
IUGR	Intrauterine growth retardation
LBW	Low birth weight
LGA	Large-for-gestational-age
NBW	Normal birth weight
PDA	Photo diode array
SGA	Small-for-gestational-age
UV	Ultra-violet

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IX. STATEMENT OF ORIGINALITY

The author has worked with Dr Koski to develop the research project. Together with two other students Nadine Zablith and Celine La Croix, the author performed separations of the amniotic fluid samples by capillary zone electrophoresis. The author did all of the electropherogram integration, amniotic fluid data collection and statistical analysis. Dr Koski provided the direction of the whole project and regulated the amniotic fluid sample collection and datasheet organization. Dr Burns offered help with data calibration and statistical analysis. Dr Skinner offered biomedical method development of CE separation of AF proteins and helped with the experimental analysis at his lab in Concordia University and had significant input into the first paper.

Chapter I

Literature Review

1.1 AMNIOTIC FLUID: SIGNIFICANCE, SOURCE AND CIRCULATION

Amniotic fluid (AF), the serous fluid within the amnion that surrounds the fetus, plays a very important role in fetal growth and development. Amniotic fluid serves numerous functions in the growing fetus as follows: the amniotic fluid permits symmetrical external growth of the embryo and fetus, provides a physical barrier to protect the developing fetus against trauma and it may also serve as an immunologic barrier between the fetus and the maternal and external environments, thereby protecting against intrauterine infections. AF is also involved in maintaining the fetal homeostasis of fluid and electrolytes (Moore *et al.*, 2003).

The amniotic cavity develops during the third week of pregnancy from the inner mass cell of the implanted blastocyst and grows inside the extraembryonic coelom fusing with the placental chorionic plate at the end of the first trimester (Moore, 1982). The volume of amniotic fluid normally increases slowly, reaching about 30 ml at 10 weeks, 350 ml at 20 weeks, and 700 to 1000 ml by 37 weeks (Moore *et al.*, 2003).

The source and circulation of amniotic fluid varies with gestational age. Before the fetal skin becomes keratinized during the second trimester, it is a major pathway for passage of water and solutes from fetal tissue fluid to the amniotic cavity (Moore *et al.*, 2003). The rupture of the oropharyngeal and cloacal membranes around the end of the 5th and 7th week of gestation (Moore, 1982), respectively, allows a free circulation between fetal digestive and respiratory tracts and the amniotic cavity and influences the amniotic fluid composition after 8 weeks of gestation (Gulbis *et al.*,

1998). Amniotic fluid is secreted by the fetal respiratory tract and enters the amniotic cavity. The daily rate of contribution of fluid to amniotic cavity from the respiratory tract is 300 to 400 ml (Moore *et al.*, 2003).

The fetal kidneys start to develop during the 4th and 5th weeks of gestation and begin to excrete urine into the amniotic fluid at the 8th to 11th week (Brenner, 1990; Nigam *et al.*, 1996; O'Rahilly *et al.*, 1992). From that stage fetal urine becomes the main source of amniotic fluid. By late pregnancy about a half-liter of urine is added daily (Moore *et al.*, 2003). When the definitive placenta has formed, movements of water and electrolytes also take place across the free placental membranes (Lind, 1978). Amniotic fluid is swallowed by the fetus and absorbed by the fetus's respiratory and digestive tracts. It has been estimated that during the final stages of pregnancy, the fetus swallows up to 400 ml of amniotic fluid per day (Moore *et al.*, 2003).

1.2 FUNCTION AND STRUCTURE OF MAJOR AF PROTEINS

Although amniotic fluid originates from a great many sources, most of the amniotic fluid proteins originate from the pregnant mother and the protein composition approximates that of maternal serum (Anderson *et al.*, 2002; Galdhar *et al.*, 1987). Albumin, transferrin, and immunoglobulin are major proteins in the second trimester amniotic fluid (Haddow *et al.*, 1978; Liberatori *et al.*, 1997).

ALBUMIN

Albumin has a molecular weight of 67-kDa and is the most abundant protein in the plasma (Sutcliffe *et al.,* 1973). The main biological function of albumin is to regulate the colloidal osmotic pressure of blood. It is also the main component of blood transport system and binds calcium, fatty acids, bilirubin, steroid hormones and other physiologically important compounds (Scott *et al.,* 1988).

TRANSFERRIN

Transferrin is an 80-kDa glycoprotein with homologous N-terminal and C-terminal iron-binding domains (Huebers *et al.,* 1987). The N-terminal and C-terminal domains of these proteins are globular moieties of about 330 amino acids. Each of these domains is divided into two subdomains, with the iron- and anion-binding sites found within the intersubdomain cleft (Aisen *et al.,* 1980). The liver synthesizes transferrin and secretes it into the plasma. The function of transferrin is to transport iron through the blood to the liver, spleen and bone marrow. Under normal circumstances, approximately one-third of transferrin iron-binding pockets are filled. The average transferrin molecule, with a half-life of eight days, may be used up to one hundred times for iron delivery (Harford *et al.,* 1994).

IMMUNOGLOBULIN

There are five major classes of immunoglobulins (Ig): IgG, IgM, IgA, IgE and IgD. The basic structure of all immunoglobulin molecules consists of two identical L chains and two identical H chains. The antibody molecule consists of three major

domains connected by a hinge region. Two domains are identical and are called fragment antigen binding (Fab), and the third domain is called fraction crystallizable (Fc). Fab interacts with the antigen, and Fc binds to Fc-receptors on different cells.

IgG is the dominant immunoglobulin in extracellular fluids. There are four subclasses of IgG. IgM is found principally in blood, but also occurs in external secretions. It binds most efficiently the C1q subunit of the first component of complement. There are two principal molecular forms of IgA, monomers and dimers. Monomeric IgA, the second most common immunoglobulin in adult serum, is primarily produced by plasma cells in the bone marrow, whereas dimeric IgA, the dominant immunoglobulin in external secretions, is produced by plasma cells at mucosal sites. IgE binds avidly to circulating blood basophils and mast cells in the submucosal sites and the skin. Cell-bound IgE antibodies defend against tissue parasites and initiate the pathogenesis of immediate hypersensitivity by triggering the release of low-molecular weight vasoactive compounds. Only small amounts of IgD are found in extracellular fluids (Baron *et al.*, 2006).

During pregnancy, the maternal serum proteins get into the amniotic cavity by placental transfer and all of these major proteins in the serum are also found in the amniotic fluid and have similar properties and biological functions.

1.3 SOURCES OF FETAL SERUM PROTEINS AND THEIR ASSOCIATION WITH BIRTH OUTCOMES

Many studies have examined the relationships between fetal serum protein and birth outcomes including birth weight and gestational age. Fetal serum proteins come from many various sources and in general they are from two major routes: the placental transfer of maternal serum proteins and fetuses' own protein synthesis.

1.3.1 PLACENTAL TRANSFER OF MATERNAL SERUM PROTEINS

The placenta is a fetomaternal organ that has two components: a fetal part that develops from the chorionic sac and a maternal part that is derived from the endometrium (Moore *et al.,* 2003). During fetal development the placenta is the primary site of nutrient and gas exchange between the mother and fetus. It provides oxygen and nutrients to the fetus, disposes of fetal waste products, synthesizes and secretes hormones, growth factors, cytokines and other bioactive molecules, protects the fetus against pathogens and forms an immunologic barrier between mother and fetus (Schneider, 1991).

The placenta also plays a central role in regulating transfer of proteins. *In vitro* experiments have shown that the transport of intact proteins through the trophoblastic layer involves pinocytosis and endocytosis and probably exocytosis to allow expulsion of the proteins into the fetal circulation (Sibley *et al.,* 1992). Fetal serum albumin comes mostly from maternal origin through placental transfer (Sutcliffe *et al.,* 1973). Transferrin crosses the placental membrane and carries iron to the embryo or fetus. The placental surface contains special receptors for this protein (Moore *et al.,*

2003). The transport of iron across the placental membrane is an active process, which is mediated via binding of maternal transferrin bound iron to transferrin receptors in placenta and subsequent transfer of iron into the fetal circulation (Bergamaschi *et al.,* 1990). The efficiency of this transport system implies that iron deficiency in the newborn is encountered only at extreme iron deficiency in the mother (Fletcher *et al.,* 1969; Bergamaschi *et al.,* 1990). While maternal iron deficiency during pregnancy induces anemia in the developing fetus, the severity tends to be less than in the mother. Expression of placental proteins of iron transport is up-regulated in maternal iron deficiency, resulting in an increased efficiency of iron flux and a consequent minimization of the severity of fetal anemia (Gambling *et al.,* 2001).

The human fetus and neonate has an immature immune system and acquires maternal IgG antibodies exclusively via the placenta. By using specific genetic typing systems (Gm factors), it is proven that the majority of IgG found in the AF is of maternal origin (Cederqvist *et al.,* 1972). A statistically significant correlation between the values of IgG concentration in the maternal serum and the umbilical blood of the neonates has been reported (Grybos, 1984). One study measured both the maternal and infant serum immunoglobulin levels. The mean maternal IgG, and IgM, and IgA were 2112 mg/dl, 118 mg/dl and 251 mg/dl, respectively, while in corresponding infants, the mean levels were 2194 mg/dl, 36 mg/dl and 19 mg/dl. At term, fetal IgG levels reportedly exceed those in the maternal circulation, indicating active transport

against a concentration gradient and thus receptor-mediated IgG transportation (Prabhakar *et al.*, 1985).

Experiments using radioactive plasma proteins have demonstrated that the transfer of IgG is more efficient than the transfer of albumin suggesting a selective transfer not related to molecular weight (Dancis *et al.*, 1961; Gitlin *et al.*, 1964; Jauniaux *et al.*, 1995). It has been demonstrated that the active transfer of IgG across the placenta is mediated by specific receptors for the Fc fragment of IgG, which are attached to the syncytiotrophoblast surface (Saji *et al.*, 1994). Transfer of all four subclasses of IgG preparation across the human placenta is demonstrated by Malek *et al* using the dual in vitro perfusion system. In the experiment the ratio of IgG1 to IgG2 in the fetal perfusate is significantly higher than in the maternal perfusate, suggesting preferential transfer of IgG1 (Malek *et al.*, 1995).

In vitro studies have indicated that IgA could cross the placental barrier in small quantities (Gitlin *et al.*, 1969). The fact that the coelomic liquid of 6-12-week-old human embryos and blood of those mothers have similar levels of specific IgG and IgA antibodies to rubella, toxoplasma, and cytomegalovirus proves maternal origin of embryonic immunoglobulins and suggests that the placental transfer of IgG and IgA has started very early in pregnancy, probably from the first placental villus is formed (Jauniaux *et al.*, 1995). Trace amounts of IgG and IgM have been found in cultures of human fetal liver and spleen only from the end of the third trimester (Gitlin *et al.*, 1969) and IgA synthesis is not demonstrated *in vitro* until after 30 weeks of gestation

(Gitlin, 1984). Thus the fetal immunoglobulins present at early gestation most likely originate from the mother.

1.3.2 FETAL PROTEIN SYNTHESIS AND HEMATOPOIESIS

The embryo yolk sac begins to develop at the third week and disappears at 10 weeks. The secondary yolk sac is probably the main source of protein synthesis inside the gestational sac in early pregnancy (Gulbis *et al.,* 1992). The liver begins development early in the fourth week and grows rapidly. From the fifth to tenth weeks, it fills a large part of the upper abdominal cavity. Hematopoiesis begins during the sixth week, giving the liver a bright reddish appearance. By the ninth week, the liver accounts for about 10% of the total weight of the fetus (Moore *et al.,* 2003).

Studies have shown that the secondary yolk sac is capable of various protein syntheses including principally prealbumin, albumin, AFP, a1-protease inhibitor and transferrin (Gitlin *et al.,* 1969; Gitlin *et al.,* 1970; Shi *et al.,* 1985) until the embryonic liver matures sufficiently to carry out these functions. In the human, the synthesis of transferrin has been demonstrated as early as 30 days' gestation and high levels of transferrin have been measured in human fetal plasma as early as 13-14 weeks of gestation (Gitlin *et al.,* 1969; Fryer *et al.,* 1993).

Fetal immunoglobulins come from hematopoietic organs. Hematopoiesis in the fetus originates in the blood islands of the secondary yolk sac from the third week and at approximately 6 weeks' gestation there is migration to the fetal liver, which then acts as the major hematopoietic organ during gestation. The spleen, a vascular lymphatic organ, begins to develop during the fifth week. The spleen functions as a hematopoietic centre until late in fetal life. At approximately 16 weeks' gestation, migratory hematopoietic cells can be observed in the bone marrow, which then acts as the major hematopoietic organ during postnatal life (Keleman *et al.,* 1979; Medvinsky *et al.,* 1996; Pahal *et al.,* 2000; Moore *et al.,* 2003).

As the hematological system develops, the corresponding lymphocytes and their products can be detected in the fetus successively. Histological studies of fetal tissue have reported the presence of lymphocyte like cells in the fetal liver, thymus and spleen, and lymph nodes from 8, 10, and 13 weeks' gestation, respectively (Keleman *et al.,* 1979). From 14 weeks B-lymphocytes could be seen in the lungs and gut and by 19-20 weeks circulating B cells have detectable surface IgM (Hayward, 1981). T-lymphocytes could also be detected from as early as 12 weeks' gestation (Pahal *et al.,* 2000; Warner *et al.,* 2000). IgE-bearing lymphocytes have been recognized in the liver and lungs of 11-week-old fetuses, and in the spleen of 21-week-old fetuses (Miller *et al.,* 1973).

Many studies have shown that except for IgG, other subclasses of immunoglobulin are synthesized by the fetus. By determining Am2 (a genetic marker for human

alpha2 heavy chains), it is concluded that the fetus synthesizes IgA2 in uterus and secretes it into the AF (Cederqvist *et al.,* 1974). Specific IgA antibodies to Candida albicans in AF with a higher ratio between IgA and IgG titers in AF than in cord serum also indicate a fetal IgA origin (Auger *et al.,* 1980). The finding of secretory IgA and IgM antibodies to E. coli and poliovirus type I antigen in early saliva and meconium samples from infants during the first day of life, especially from infants of low immunoglobulin mothers, also supports a fetal origin for at least part of the secretory antibodies (Mellander *et al.,* 1986). And the fact that there is practically no IgE concentration difference between the arterial and venous cord blood indicates that cord blood IgE is essentially a product of fetal lymphocytes (Cederqvist *et al.,* 1984; Schreyer *et al.,* 1989).

1.3.3 FETAL SERUM PROTEINS AND BIRTH OUTCOMES

ALBUMIN

Studies have shown that the fetal serum albumin, transferrin and total protein increase throughout gestation (Moniz *et al.,* 1985; Fryer *et al.,* 1993). Serum albumin concentrations in the fetus reach lateau by 22 to 24 weeks' gestation and remain at these levels through the neonatal period; the average concentration of albumin in the neonatal period is slightly higher than that in the mothers (Gitlin *et al.,* 1964; Bala *et al.,* 1987). IUGR infants have lower cord blood albumin than normal birth weight infants (Nieto-Diaz *et al.,* 1996).

TRANSFERRIN

Cord blood transferrin levels in term infants are significantly below normal adult levels. Transferrin levels showed a positive correlation with birth length, weight, gestational age and albumin levels in the newborn infants (Misaki *et al.,* 1987). A predictable rise in various serum protein concentrations, including transferrin, during the last trimester has been attributed to increased transplacental amino acid transfer and fetal hepatic protein synthetic ability (Chockalingam *et al.,* 1987; Thom *et al.,* 1967).

While transferrin can usually reflect protein-energy status, in infants with uteroplacental vascular insufficiency or chronic hypoxia, there is a significant inverse correlation between cord transferrin and ferritin levels, which means that higher cord transferrin is associated with impaired iron stores (Chockalingam *et al.,* 1987). When nutrient transfer (for example iron transfer) is limited by uteroplacental vascular insufficiency, fetal growth retardation may result (Chockalingam *et al.,* 1987).

IMMUNOGLOBULIN

The significant change of the levels of fetal immunoglobulin during pregnancy reflects the change of source and metabolism. In the first trimester, IgG and IgA concentrations are respectively 28 and 128 times lower in coelomic fluid (which probably reflects fetal serum concentrations) than in maternal serum (Jauniaux *et al.,* 1995). Cord blood IgG and IgM both increase with gestational age until term, while

IgA and IgD are unaffected by gestational age (Cederqvist *et al.,* 1978). By the end of pregnancy, the cord blood contains approximately adult levels of IgG, 10% of the normal adult levels of IgM, but still very little IgA (South *et al.,* 1980).

Studies have shown that the cord blood IgG is positively correlated with gestational age and birth weight (Mahulja *et al.,* 1993; Sharma *et al.* 1986). Neonates with severe IUGR and preterm babies have significantly lower concentrations of IgG (Singh *et al.,* 1978; Ladipo *et al.,* 1978). It has been explained by the immature liver functions and inadequate transfer of IgG across the placenta, which mainly occurs during the last trimester of pregnancy. Impaired transfer of IgG in pregnancies where intrauterine growth retardation occurs may also result from placental dysfunction (Sharma *et al.,* 1986). However another study shows no relation as variation in the gestational age, birth weight, duration of labor and maternal preeclamptic toxemia do not significantly affect the IgG levels (Prabhakar *et al.,* 1985).

IgG concentrations are lower in neonates born to preeclamptic toxemia mothers when compared to neonates of normal mothers. Low birth weight (LBW) babies show lower IgG values than normal birth weight (NBW) babies. The lower level of IgG in LBW neonates has been attributed to blockage of the IgG-specific Fc receptor site in the placenta due to acute atherosis and reduced uteroplacental perfusion (Das *et al.,* 1998).

Cord blood IgM has been shown to be higher in fetuses weighing 2,000 grams or more, while IgA is not influenced by birth weight (Cederqvist *et al.,* 1978).

1.4 CONCENTRATION AND SOURCES OF AMNIOTIC FLUID PROTEINS

The second trimester amniotic fluid proteins come from various sources. Part of the proteins can come from the mother by transudation of maternal plasma through the amniotic membrane and most of the proteins come from the fetus by a variety of sources like fetal skin, fetal urine, meconium, and the nasopharyngeal, oral and lachrymal secretions of the fetus (Moore *et al.,* 2003; Queenan *et al.,* 1978). Because of the particular characteristics of different fetal proteins, they can reach the amniotic fluid in different ways.

1.4.1 CONCENTRATION OF MAJOR AMNIOTIC FLUID PROTEINS

ALBUMIN

Through the pregnancy, albumin levels increase until 30 weeks, and subsequently decrease to the term. The mean value of the amniotic fluid albumin in the second trimester is 3 ± 2 g/l (Benzie *et al.,* 1974). Another study has established the albumin concentration in the AF by laser nephelometry to be 3.6 g/l (Tatra *et al.,* 1987).

TRANSFERRIN

From 7 to 13 weeks of gestation, transferrin concentration is less than 0.08 g/l in the amniotic fluid samples (Gulbis *et al.,* 1994). From 16 to 42 weeks of gestation, the range of AF transferrin is between 0.14 and 0.445 g/l with the mean value being 0.299 g/l and its concentration does not increase throughout this tested gestation period (Larsen *et al.,* 1973).

IMMUNOGLOBULIN FAMILY

IgG: IgG is the major subclass of immunoglobulin in amniotic fluid (Quan *et al.,* 1999). IgG concentrations can be detected in AF samples between 6 and 12 weeks of gestation (Jauniaux *et al.,*1995); then IgG levels increase until the 20-24th weeks of gestation and decline significantly at term. The mean IgG level of second trimester and term AF is 34 ± 14 mg/dl and 17.9 ± 11.1 mg/dl respectively (Davis *et al.*, 1983; Blanco *et al.*, 1983; Tatra *et al.,* 1987).

IgA: IgA is much less abundant than IgG in AF and its concentration increases during normal pregnancy (Donat, 1979; Tatra *et al.,* 1987). As to the main forms of IgA existing in AF, one study shows that IgA is monomeric, with a low level of secretory IgA (S-IgA) and with various amounts of free secretory component (SC) while another study shows that the major forms of IgA contain SC, but range in size between 170 and 200-kDa, unlike the 380-kDa size of the typical S-IgA (Quan *et al.,* 1999; Cleveland *et al.*, 1991).

IgM: The concentration of IgM is relatively low in the amniotic fluid (Jauniaux *et al.*, 1995; Quan *et al.*, 1999) and increases with gestation to term (Cederqvist *et al.*, 1978; Tatra *et al.*, 1987).

1.4.2 OTHER HIGH ABUNDENT PROTEINS IN AMNIOTIC FLUID

A1-antitrypsin

A1-antitrypsin is a member of the serine proteinase inhibitor (serpin) superfamily and a potent inhibitor of neutrophil elastase. It is a highly polymorphic 52-kDa acute phase glycoprotein (Parfrey *et al.*, 2003). Haddow et al had shown that the level of α_1-antitrypsin in the second trimester amniotic fluid was 18.0 ± 8.4 mg/dl (Mean ± SD) (Haddow *et al.*, 1978).

IGFBP1

Insulin-like growth factor binding protein 1 (IGFBP1) is a 25.3-kDa phosphoprotein synthesized primarily in the liver and largely distributed in intravascular and extravascular spaces. It is a member of the family of high-affinity binding proteins that have been proposed to coordinate and regulate the biological activity of the insulin-like growth factors (Jones *et al.*, 1995). In the amniotic fluid, IGFBP1 is the most abundant one. The levels of IGFBP1 increased by four orders of magnitude after 10 weeks and reached a peak (median 145.2 mg/l) at 16 weeks (Wathen *et al.*, 1993) while another study showed that the mean IGFBP1 concentration in amniotic

fluid from 13 to 19 gestational weeks was 68.2 ± 49.7 mg/l (Mean ± SD) (Chevallier *et al.,* 1998).

DBP

Vitamin D-binding protein (DBP), also known as group-specific component (Gc-globulin), is a 58-kDa α_2-globulin. Its role in the serum is the transportation of vitamin D and its metabolites in the circulation as well as modulating certain immune and inflammatory responses. Mean serum DBP level is 256.4 ± 24.3 mg/l (Hamashima *et al.,* 2002).

Ceruloplasmin

Ceruloplasmin (Cp) is a 135-kDa blue copper oxidase that is synthesized predominantly in the liver. Originally identified as an acute-phase protein, ceruloplasmin has diverse functions. It is essential for iron homeostasis and is involved in angiogenesis and cellular prooxidant and antioxidant processes, as well as antibacterial host defense. The concentration of Cp in the human serum is 306.1 mg/l, and the oxidase activity is 127.2 U/l (Louro *et al.,* 1989). Cp Oxidase activity in the amniotic fluid is 2.68 U/l at 20-30 weeks gestational age (Chan *et al.,* 1980).

1.5 SUMMARY

Albumin, transferrin and IgG are major proteins in the amniotic fluid. Many studies have found that fetal serum proteins were associated with fetal growth and

development though quite a few studies have been done about major amniotic fluid proteins. The majority of the fetal serum proteins are originated from maternal serum through active transfer of placenta. So the function of the placenta is essential for fetal growth. With the development of the fetal liver and hematopoietic organs, the second trimester fetus can also synthesize some of its own proteins. Since amniotic fluid continuously circulates between the fetus and the amniotic cavity, some protein levels in AF could reflect the levels in fetus. Analysis of AF proteins could offer us a valuable opportunity to investigate fetal growth and development in pregnancy as second trimester AF provides an important window into fetal metabolism.

Chapter II

Hypothesis and Objectives

Amniocentesis is a common invasive prenatal diagnostic procedure. During amniocentesis, amniotic fluid is sampled by inserting a hollow needle through the mother's anterior abdominal and uterine walls into the amniotic cavity by piercing the chorion and amnion (Moore *et al.,* 2003).

Since routine amniocentesis for genetic testing is usually performed in early gestation, analysis of amniotic fluid components after genetic testing has been done might offer us a valuable window to investigate fetal growth and development as early as the second trimester.

Capillary electrophoresis (CE) is a family of related separation techniques that use narrow-bore fused silica capillaries to separate a complex array of large and small molecules. High voltages are used to separate molecules based on differences in charge and size. Capillary zone electrophoresis (CZE) is the simplest and most universal of the techniques, having been shown to be useful for the separation of both low molecular weight and macromolecular analytes including proteins.

Despite CE having been proven useful in the analysis of proteins in other biofluids including serum/plasma, cerebrospinal fluid and so on (Oda *et al.,* 1997; Cowdrey *et al.,* 1995) there are not many studies about analyzing amniotic fluid with CE. Stewart *et al* have tried to use CZE to separate human amniotic fluid samples and identified a few peaks as proteins in the electropherogram, but the capability of using

CZE to analyze large numbers of samples have not been demonstrated (Stewart *et al.*, 2001).

So the objective of this study is in two fold. The first is to determine whether capillary zone electrophoresis could be used to measure AF proteins and if possible, develop and characterize a capillary electrophoresis method for the analysis of 2nd trimester human amniotic fluid with the potential for use in biomarker discovery. The second is to assess the concentrations of major components in the human amniotic fluid in early gestation and identify those AF components (proteins plus uric acid) that might be associated with infant birth weight or gestational age.

In the following two chapters we will talk about the research method and results of our study for these two objectives. In chapter 3 (Paper 1), we will focus on the application of the method of capillary zone electrophoresis to analyze the second trimester amniotic fluid samples. Paper 1 will include method development, examination of sample stability at room temperature and freeze/thaw cycle, selection of internal standard, identification of major AF peaks and analysis of a pooled AF sample set to investigate method reproducibility.

In chapter 4 (Paper 2) the data from our study population were used to investigate the relationship between the amniotic fluid proteins and the birth outcomes including infant birth weight and gestational age. In the study, the maternal and infant characteristics were obtained from questionnaires and obstetrical chart review.

Statistical analyses including analysis of variance and multiple regressions were performed to detect the association between the major amniotic fluid components and birth outcomes.

Chapter III

Identification and Characterization of Human Amniotic Fluid Components Using Capillary Zone Electrophoresis

Tao Gao,[1] David H. Burns,[2] Kristine G. Koski,[1] Cameron D. Skinner[3]

[1] School of Dietetics and Human Nutrition, McGill University (Macdonald Campus), Montreal, Canada H9X 3V9

[2] Department of Chemistry, McGill University, Montreal, Canada H3A 2K6

[3] Department of Chemistry and Biochemistry, Concordia University Montreal, Quebec, Canada H3G 1M8. Email: CSkinner@Alcor.concordia.ca, Tel: 1 (514) 848-2424 x 3341, Fax: 1 (514) 848-2868

3.1 ABSTRACT

Human amniotic fluid was separated by capillary electrophoresis. Albumin, IgG, transferrin and uric acid were quantitated in 230 amniotic fluid samples collected from 12-20 weeks gestation. All major components were detected within 10 minutes. Species were quantitated by external calibration with the aid of thiamine as internal standard. The resolved major species were quantitated while other proteins like α_1-antitrypsin, IGFBP1, Gc globulin, α_1 acid-glycoprotein, and ceruloplasmin could not be fully resolved. The method's reproducibility was assessed from 81 repeated measurements of a pooled amniotic fluid sample interspersed in the study. Migration time reproducibility was 3.0 and normalized peak areas were 12 %RSD or better at 190 nm. The separation profile was not affected by 10 freeze/thaw cycles or 10 hours of storage at room temperature.

3.2 INTRODUCTION

Amniotic fluid (AF), the serous fluid within the amnion that surrounds the fetus, plays important roles in fetal growth and development. AF protects the infant by preventing mechanical and thermal shock, assists in acid/base balance, possesses antimicrobial activity, and contains nutritional factors. Amniotic fluid originates from both maternal and fetal sources, contains 98% water and metabolic species that are necessary for, and by-products of, biological processes *e.g.* proteins, salts, glucose, uric acid etc. Its protein composition approximates that of maternal serum early in pregnancy (Finegan, 1984; Anderson *et al.,* 2002), albeit at a significantly lower concentration and lower complexity (Anderson *et al.,* 2002; Galdhar *et al.,* 1987; Johnson *et al.,* 1974).

In human AF, the concentration of total protein appears to increase with advancing gestation (Guibaud *et al.,* 1973); it reaches its maximum of 4.7 g/l by the 25^{th} week of gestation and then decreases to 2.4 g/l at delivery (Legras *et al.,* 1978). As with serum, there are a handful of high abundance proteins and several hundred lower abundance proteins (Haddow *et al.,* 1978; Liberatori *et al.,* 1997). In normal pregnancies, the AF protein pattern varies to a great extent in intensity, number, width and mobility of gel electrophoresis bands (Galdhar *et al.,* 1987). Variations in the protein profile may be due to developmental stage, consumption and inherent genetic make up of both mother and fetus (Galdhar *et al.,* 1978; Haddow *et al.,* 1978).

Albumin, IgG, α_1-antitrypsin and transferrin are the major proteins in the AF during the second trimester (Galdhar *et al.,* 1978; Haddow *et al.,* 1978). There are also other significant proteins such as insulin-like growth factor binding protein 1 (IGFBP1), Gc globulin (Vitamin D binding protein), α_1 acid-glycoprotein and ceruloplasmin in the AF (Tisi *et al.,* 2005; Johnson *et al.,* 1974). The relatively fast clearance time of 1.2 days means that the proteins observed in AF provide a unique window onto the biological process occurring within the fetus (Gitlin *et al.,* 1972). Also, AF proteins may act as biomarkers of fetal development but have yet to receive sufficient attention to validate this approach (Tisi *et al.,* 2005; Tisi *et al.,* 2004; Sharma *et al.,* 1986; Mahulja *et al.,* 1993). The work that is presented here demonstrates the development and characterization of a capillary electrophoresis method for the analysis of 2nd trimester human amniotic fluid with the potential for use in biomarker discovery.

3.3 EXPERIMENTAL METHODS

3.3.1 SAMPLES AND REAGENTS

Pregnant women (N = 230) undergoing routine amniocentesis (12-20 wks) for age-related genetic testing at St Mary's Hospital Center (Montreal, Canada) were invited to participate in this study. Ethical approval was obtained from both McGill and Concordia Universities and from St Mary's Hospital Center. A signed consent allowed collection of $\approx$ 1.5 ml AF from each participant, once genetic testing was completed, that was stored at –85°C until analyzed. A pooled amniotic fluid sample

was prepared by mixing ten AF samples together followed by aliquotting and freezing at -85°C. Samples (including the pooled) were not filtered due to the small volume available and the potential for analyte loss. A consequence was that occasionally particles would cause the separation to fail thus samples were separated in duplicate to insure that at least one separation was available from each sample.

Human IgG and IgA were purchased from Bethyl (Montgomery, TX, US). Albumin, transferrin, α_1-antitrypsin, Gc globulin and α_1 acid-glycoprotein were purchased from Sigma-Aldrich (Oakville, ON, CA). IGFBP1 was purchased from R&D Systems (Minneapolis, MN, US) and ceruloplasmin was purchased from the Biogenesis (Kingston, NH, US). Sodium tetraborate (electrophoresis grade), ethylenediamine tetraacetic acid (disodium salt) (EDTA) and thiamine (Vitamin B_1) were purchased from Sigma (St. Louis, MO, US). Sodium dodecyl sulfate (SDS) was purchased from Schwarz/Mann biotech (Cleveland, OH, US). Sodium hydroxide (NaOH) was purchased from ICN Biochemical (Aurora, OH, US).

3.3.2 CAPILLARY ELECTROPHORESIS

All CE separations were performed on a Beckman Coulter P/ACE Series MDQ capillary electrophoresis system (Beckman, Fullerton, CA) using Beckman 32 Karat Software Version 5.0 for instrument control, data acquisition and analysis. Untreated fused silica capillary (75µm ID, 360µm OD) purchased from Polymicro Technologies (Phoenix, AZ, US) was cut to 60 cm in length. A window was made 50 cm from the

inlet by removing the polyimide with a nickel-chrome wire electrically heated to a dull yellow incandescence.

The capillary was conditioned in-between runs by sequentially flushing for 3 minutes at 1.4 bar (20 PSI) followed by a 1 minute wait at 0 bar with 5 mM SDS then 100 mM NaOH. The capillary was then rinsed and filled with the separation buffer (2 minutes at 1.4 bar) and conditioned under 25 kV for 1minute (0 bar). At the beginning of each day a similar conditioning step was performed except the pressure rinses were 5 minutes and the waits were 2 minutes. All prepared solutions were filtered through 0.45 μm syringe filters and degassed before use. Prior to separation, frozen (-85°C) AF samples were thawed in an ice-water bath and diluted 1:1 (v/v) with 0.5 mg/ml thiamine in water. Samples were injected hydrodynamically (34.5 mbar, 0.5 PSI) for 10 s and separated using 75mM borate, 0.8 mM EDTA pH 9.27 buffer at 25 kV for 20 minutes with the temperature set at 28 °C.

Detection was with a photo diode array (PDA) detector at 4 Hz covering the 190-350 nm spectral range, peak integration was done at 190, 254 and 275 nm (5 nm bandwidth) using the Beckman 32 Karat Software.

3.4 RESULTS AND DISCUSSION

We have had some success identifying proteins in amniotic fluid using single analyte techniques such as immunoassays, where we have been able to characterize total

protein, IGF2, IGFBP1 and IGFBP3 (Tisi *et al.*, 2005; Tisi *et al.*, 2004). However, this approach fails to measure multiple analytes in a single amniotic fluid sample. A few groups have demonstrated gel electrophoresis (Galdhar *et al.*, 1987) and 2D-PAGE (Jones *et al.*, 1981; Burdett *et al.*, 1982; Kronquist *et al.*, 1984) separations but their low throughput makes them unappealing for the analysis of large numbers of samples. Stewart *et al* developed an efficient CZE method in 2001 that we used as the basis for the method presented here (Stewart *et al.*, 2001).

We opted to extend the length of the capillary from 36 cm to 50 cm and the separation time compared to Stewart *et al* to improve the resolution and to detect the triangular peak that begins at 15 minutes and carries through to 19 minutes spanning the entire wavelength region (190-350 nm). This peak showed strong electrodispersion indicating the detection of ionic species, probably the inorganic ions (data not shown). We felt that this peak might contain some useful biochemical information about the AF and should not be discarded until demonstrated that it was not relevant. However, the majority of species migrated as "well-behaved" peaks, as shown in Figure 1, in 10 minutes compared to the 6.7-7.0 minutes previously reported by Stewart *et al* (2001).

3.4.1 SAMPLE STABILITY AT ROOM TEMPERATURE

The CE instrument did not have a cooled sample storage compartment. The amount of time that the samples spent in the instrument, at room temperature, depended on their position in the analysis queue and ranged from half an hour to eight hours for the

first sample and the last sample respectively. To address the potential for sample degradation the stability of pooled AF samples stored at room temperature was examined. Several successive separations of pooled samples, diluted with the thiamine internal standard (IS), maintained at room temperature over 10 hours, were performed. Comparison of the elution profiles measured at 190 nm, where the sensitivity is the highest, showed that the profile did not significantly change (data not shown) with the exception of two new peaks at 254 and 275 nm (discussed below). The consistency of the elution profile indicates that the major components of AF were stable and allowed the analysis, even after extended periods at room temperature.

3.4.2 SAMPLE STABILITY TO SUCCESSIVE FREEZING AND THAWING

Collection, aliquotting and analysis require the samples to be frozen and thawed repeatedly. Therefore, stability to freezing and thawing was assessed by subjecting aliquots of the pooled sample to increasing numbers of freeze/thaw cycles. Ten samples were taken out of the freezer, thawed on wet ice and one sample was analyzed immediately. The remaining samples were refrozen (-85°C). This cycle was repeated until the last sample had been frozen/thawed ten times. Comparison showed no apparent changes in the 190, 254 or 275 nm electropherograms (data not shown) indicating that the concentrations of the major AF components are stable to successive freeze thaw cycles. The effect of multiple freeze thaw cycles on their biological activity was not measured here but could be expected to be significantly reduced (Chang *et al.,* 1996; Cao *et al.,* 2003).

3.4.3 SELECTION OF INTERNAL STANDARD

Thiamine, or vitamin B_1, was used as the internal standard to act as a point of reference for peak alignment, data processing and allow normalization of data by correcting for variations in injected volume due to sample viscosity and electroosmotic flow variations. Before separation, the AF samples were diluted 1:1 with the thiamine solution thereby reducing the salt content of the samples while simultaneously introducing the internal standard. Thiamine was chosen because it has strong absorbance across the 190-300 nm band and is positively charged at pH 9.2. The cationic nature insures that it has the highest electrophoretic velocity and is the first to reach the detector distinguishing it from all other amniotic fluid components. This was confirmed by injection of the pooled sample without the internal standard.

Even though thiamine has many desirable properties what was not immediately apparent from our initial experiments was that thiamine also interacts with components in the AF and produced thiamine-AF complex peaks. One peak has been positively identified as a complex of albumin by the methods of spectral subtraction and separation of mixtures of thiamine and albumin. This albumin complex migrates with a slight positive shift in electrophoretic mobility as expected , compared to albumin, and can be seen as a partially resolved peak in the 254 and 275 nm wavelength data where the thiamine strongly absorbs but is not obvious at 190 nm due to the much stronger protein absorption as shown in Figure 2. One major biological function of albumin is to act as a carrier within blood and therefore it is

likely to bind many species of proteins, micronutrients *etc.* and apparently this includes thiamine.

In the case of the peak numbered 2* in Figure 2 we were not able to determine what species had combined with thiamine but subtraction of the thiamine spectrum yielded a residual spectrum with an intensity less than 5% of the complex at 275 nm indicating a small contribution to the signal from the protein.

3.4.4 THIAMINE AS AN INTERNAL STANDARD

The use of thiamine as an internal standard was complicated by the fact that the thiamine interacted with components in AF and that the extent of combination was time dependant. From spectral subtraction it was established that the majority of the peak area of the two complex peaks (3.45 and 5.0 minutes in Figure 2) was from thiamine. Corroborating this was the plot of the three thiamine containing peaks plotted as a function of time shown in the inset of Figure 2. This data showed that the pure thiamine peak decreased while those of the complexes increased. However, even after 10 hours the total area of the three peaks is only 105 % of the initial area indicating that the contribution of the protein at 275 nm to the peak area is minimal. If there were significant contribution we would have expected the total area to rise significantly as the concentration of the complexes increased. Thus to calculate the total area of the internal standard peak at 275 nm we took the sum of the three thiamine containing peaks at 275 nm. To calculate the total area of the internal standard at the other wavelengths we assumed that the same distribution of peak areas

observed at 275 nm also applied and using the easily integrated pure thiamine peak allowed calculation of the total area expected for thiamine.

3.4.5 IDENTIFICATION OF THE MAJOR AF PEAKS

There are eight distinct peaks in the 190nm electropherogram of the pooled AF shown in Figure 1. Stewart *et al* identified several of the proteins but many of the proteins expected in AF were not determined (Stewart *et al.,* 2001). Purified standards were run separately and spiked into pooled AF to identify the migration time and spectra of the species. Standards of IgG, transferrin, α_1-antitrypsin, IGFBP1, Gc globulin, α_1 acid-glycoprotein, albumin, uric acid, xanthine, and hypoxanthine were measured based on reports of the expected high abundance species in AF (Stewart *et al.,* 2001; Johnson *et al.,* 1974; Tisi *et al.,* 2005). Figure 1 summarizes our findings, several analytes migrate as pure species however the majority of proteins cannot be fully resolved with CZE. Analysis of the pure standard of α_1-antitrypsin in bare fused silica capillary revealed two resolved peaks (data not shown) in contrast to the six isoforms that were resolved by Chang *et al* using dynamically coated capillaries in a phosphate buffer at pH 6 (Chang *et al.*, 2005). When spiked into pooled sample, the peak numbered 7 in Figure 1 increased and was identified as α_1-antitrypsin.

The peaks in the range of numbers 2-8 are a complex of unresolved species and contain the majority of the protein species. Similar results were observed by Stewart *et al* (2001) as well as others using gel electrophoresis of AF and serum (Galdhar *et*

al., 1987; Gay-Bellile *et al.,* 2003). This significant number of overlapping species makes quantitation of individual species difficult even though the current method employs a longer capillary with a higher resolution than Stewart *et al* (2001).

Peak 9 in Figure 1 is identified as uric acid, however in the study of Stewart *et al* (2001) it was identified as xanthine. Stewart *et al* (2001) developed a panel of standards (including xanthine, hypoxanthine but not uric acid) based on the expected composition of AF and spiked these standards into AF to verify the identity of the peaks. When their experiments were reproduced we found that xanthine showed a slight difference in migration time (data not shown) and a large spectral difference. Slightly better resolution, and ability to detect small differences in migration time, of all components was possible with the longer 50 cm vs. the 36 cm capillary (to detector) used by Stewart *et al* (2001). More obviously, there was a marked difference in absorbance spectrum between xanthine and the 9^{th} peak. Believing that there had been a typographic error, we also measured hypoxanthine but again found a large discrepancy in the spectrum. After examining the literature for non-proteinaceous species in AF and urine, uric acid was determined to be a likely candidate. Figure 4 demonstrates a perfect spectral overlap and spiked studies showed co-migration. Uric acid is a metabolic by-product and is released into the AF by the developing fetus with a typical concentration of 3-4 mg/dl (Oliveira *et al.,* 2002; Benzie *et al.,* 1974).

3.4.6 ANALYSIS OF THE POOLED AF SAMPLE SET

To analyse the study set of 230 AF samples required developing a reproducible method that showed no operator bias and that could be performed unattended on an automated instrument. To monitor equipment operation, the pooled reference sample was run at the beginning and end of each set of 5-8 AF samples. Over the course of method development and routine experiments we successfully separated the pooled sample a total of 81 times.

If only the first peak of thiamine (Peak 1, Figure 1) was used for normalization of the analyte peak areas the relative error for the albumin peak was 31.5 %RSD. When the total thiamine peak area was used the relative error was reduced to 9.4 %RSD. Similar increases in precision were observed for the other peaks justifying the use of the corrected thiamine peak area as reported in Table 1.

In addition to using the thiamine internal standard we also calculated the error on the ratio of peak areas of select peaks to those of other peaks in the same electropherogram *e.g.* albumin/uric acid = 7.78 %RSD, transferrin/uric acid = 10.5 %RSD. This ratio should be largely independent of dilution and injection volume effects for resolved peaks since all peak areas should scale with the mass of sample injected. The range was 7-11 %RSD indicating that the separation has a fundamental variability of approximately 7-11 % and that the 10.5 % error calculated with the total thiamine was close to the maximum achievable with this analytical method.

In this study, 230 sample and 81 pooled sample separations were successfully completed by three operators over a period of four months during which the capillary was replaced several times due to breakage and plugging. The pooled sample data set was examined using ANOVA for operator, temporal and capillary bias using the 190 nm normalized (to total thiamine) albumin, IgG and uric acid peak areas. No trends were visible in the data nor were any significant differences found at the 95% confidence level.

3.4.7 CONCENTRATIONS OF ANALYTES IN AMNIOTIC FLUID

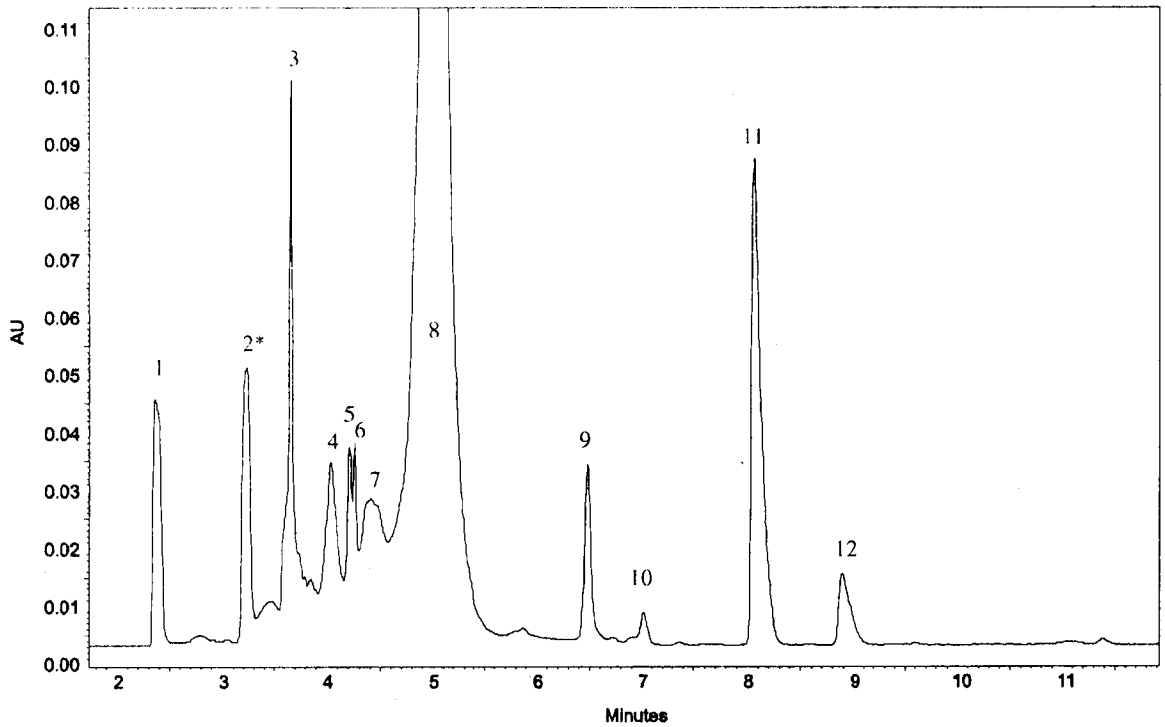
Analyte concentrations were determined in the AF via external standardization with the limits of peak integration determined by spiking standards into the pooled sample. Concentration results in **Table 3.1.** are from the pooled sample, **Table 3.2.** are the average from the 230 AF samples.

3.5 CONCLUSIONS

A robust capillary electrophoresis separation method has been developed for the analysis of high abundance bio-analytes in AF. The concentrations of major components in AF were surprisingly stable to both room temperature storage and freeze/thaw cycles however their biological activity was not assessed here. A benefit of this stability is that a greater number of samples can be loaded into instruments that do not have sample cooling systems for unattended analysis. Using an internal

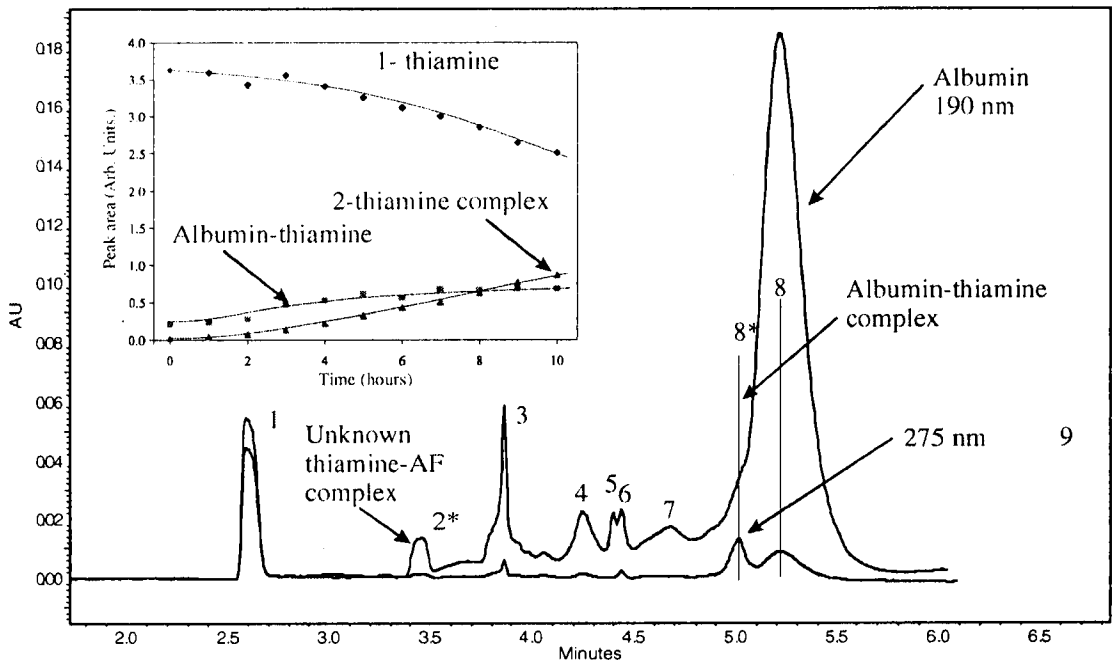
standard improves the precision of the method, however thiamine has definite limitations as it complexes with components in AF resulting in additional peaks in the electropherogram. An alternate cationic internal standard with a broad absorption spectrum would be preferred since it migrates outside of the normal zone occupied by neutral and anionic species. The complexity of AF, with a few high-abundance species and a plethora of lower abundance species results in a relatively low-resolution separation on a broad baseline with multiple species demonstrating overlapping bands. This places severe limits on the number of species that can be reliably quantitated using traditional peak integration, even when multispectral information is used. However, this same data offers an exciting opportunity for more sophisticated analysis techniques when looking for species associated with fetal outcome. The albumin, IgG, transferrin and uric acid results from the 230 AF samples are comparable to those reported in the literature. A separation method with greater resolving power could provide a distinct advantage in quantitating additional species.

Figure 1: Identification of prominent peaks in amniotic fluid at 190 nm



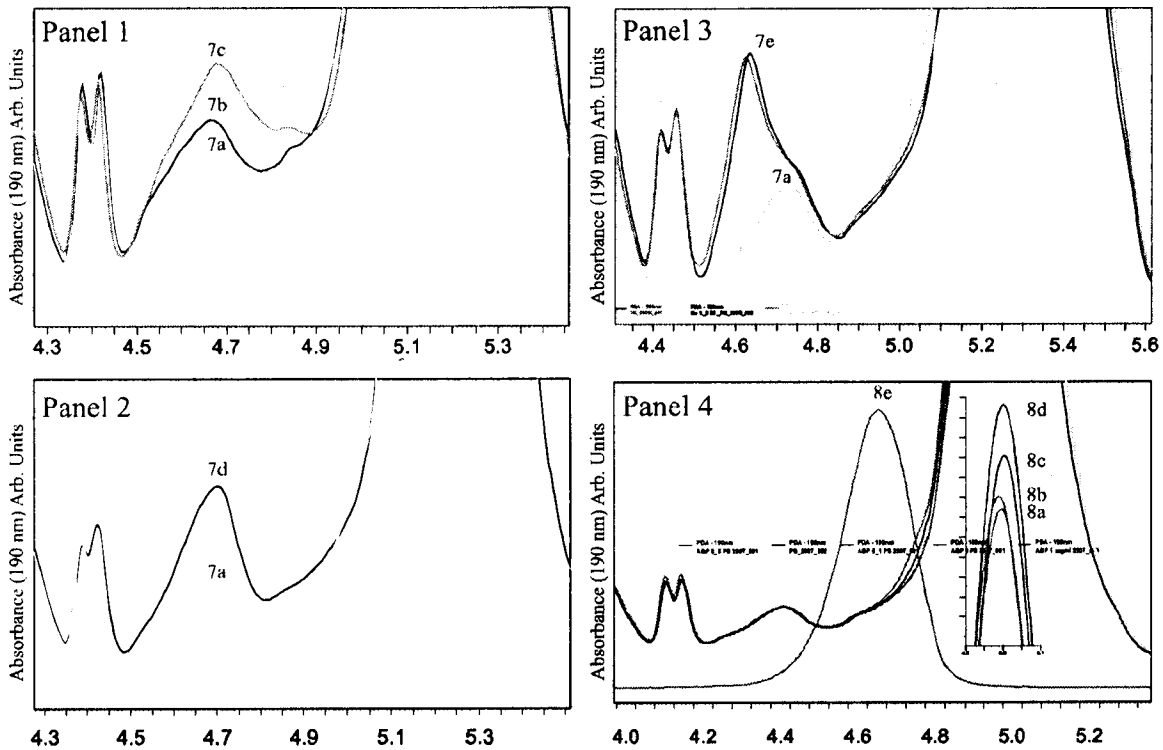
Electropherogram of pooled AF shows the prominent peaks: thiamine (1), thiamine complex (2*), IgG (3), transferrin (4), unknown species (5-6), partially resolved α_1-antitrypsin, IGFBP1 and Gc globulin (7), albumin, α_1 acid-glycoprotein (8), uric acid (9), unknown species(10, 11, 12), identified as proteins in study of Stewart *et al* (2001).

Figure 2: Thiamine interaction with AF components and production of complex peaks at 190nm and 275nm



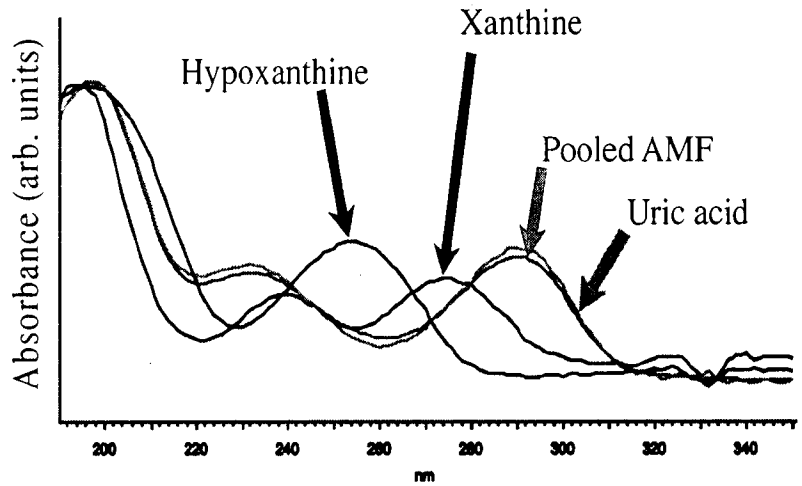
Peaks are numbered as in Figure 1. Thiamine is detected at 2.6 minutes but thiamine complexes (noted with the * on the peak number) are observed at 3.5 minutes and 5.0 minutes. The albumin complex (8*) is particularly evident at 254 (not shown) and 275 nm due to the strong absorption of the thiamine and the significantly reduced absorption of albumin. Inset shows time course of the combination at 275 nm and reduction in thiamine. Lines are drawn only to guide the eye.

Figure 3: Peaks identification by adding different pure protein standards into AF at 190nm



Peak numbering is the same as in Figure 1. Panel 1, pooled sample (7a), pooled sample + 0.3 (7b), + 0.6 mg/ml (7c) α1-antitrypsin. Panel 2, pooled sample (7a), pooled sample + 0.25 mg/ml IGFBP1 (7d). Panel 3, pooled sample (7a), pooled sample + 0.2 mg/ml Gc globulin (7e). Panel 4, pooled sample (8a), pooled sample + 0.1 (8b), +0.5 (8c), +1.0 (8d) mg/ml α_1 acid-glycoprotein and 1.0 mg/ml α_1 acid-glycoprotein standard alone (8e)

Figure 4: Spectral comparison of xanthine, hypoxanthine, uric acid and pooled AF



Spectra have been scaled to the same maximum absorbance. The spectrum of the peak in the pooled AF compared with the pure chemicals is the 9th peak in Figure 1.

Table 3.1: Data calculated from 81 measurements of the pooled AF sample.

Peak number (Figure 1) N=81	Species	%RSD migration time	% RSD peak area	% RSD peak area using thiamine peak 1	% RSD peak area using total thiamine	Concentration (mg/dl)
Internal Standard						
1	Thiamine	1.3	20	N/A	N/A	N/A
1+2*+8*	Total thiamine	N/A	9.9	N/A	N/A	N/A
Analytes						
3	Transferrin 190nm	2.1	11	29	9.0	32
4	IgG 190nm	2.0	11	31	11	26
8	Albumin 190nm	2.3	11	31	9.0	390
9	Uric acid 190nm	3.0	16	32	12	3.6
9	Uric acid 254nm	3.0	30	40	24	3.2
9	Uric acid 275nm	3.0	19	35	14	3.0

Table 3.2: Data calculated from 230 measurements of AF samples

Peak number (Figure 1) N=230	Species measured at 190 nm	% RSD migration time	% RSD on peak area using total thiamine	Concentration (mg/dl)
Analytes				
3	Transferrin	2.97	43.4	33
4	IgG	2.63	41.9	28
8	Albumin	3.70	39.8	441
9	Uric acid 190nm	5.40	37.6	3.95
9	Uric acid 254nm	5.42	48.9	3.25
9	Uric acid 275nm	5.40	54.9	3.17

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CONNECTING PARAGRAPH

In the last chapter (Paper 1) we have established a robust method for the analysis of amniotic fluid samples. The method of capillary zone electrophoresis has been proven to be precise and with high reproducibility. Major proteins like albumin, transferrin, IgG and uric acid in the second trimester amniotic fluid were identified. The amniotic fluid samples (n= 230) of our study population were analyzed and the concentration of major components were measured. In the next chapter (Paper 2) we used the data about the maternal and infant characteristics of our study population and associated them with the amniotic fluid protein levels to investigate if there was any relationship between the concentrations of these amniotic fluid constituents and infant birth outcomes including the gestational age and the birth weight.

Chapter IV

Second Trimester Amniotic Fluid Transferrin and Uric Acid are Associated with Adverse Birth Outcomes

Tao Gao[1] David H. Burns,[2] Cameron D. Skinner,[3] Kristine G. Koski [1]

[1] School of Dietetics and Human Nutrition, McGill University (Macdonald Campus), Montreal, Quebec, Canada H9X-3V9 Email: kris.koski@mcgill.ca Tel: 1(514)398-7845 Fax: 1(514)398-7739

[2] Department of Chemistry, McGill University, Montreal, Canada H3A-2K6

[3] Department of Chemistry and Biochemistry, Concordia University, Montreal, Canada H3G-1M8

4.1 ABSTRACT

Objective: The objective of our study was to establish whether early amniotic fluid (AF) protein and uric acid concentrations were associated with and predictive of infant birth weight or gestational age.

Methods: Amniotic fluid samples (n=230) were obtained from pregnant women undergoing routine amniocentesis for genetic testing in the second trimester. The AF sample separations were performed by capillary zone electrophoresis (CZE) and albumin, IgG, transferrin and uric acid were quantified. From questionnaires and maternal obstetrical chart review, maternal and infant characteristics were obtained.

Results: Preterm infants had higher concentrations of AF albumin (p=0.0266) and transferrin (p=0.0215). Multiple regression analyses revealed that uric acid was a significant predictor of birth weight (grams) while transferrin was negatively associated with the gestational age in term infants.

Conclusion: Second trimester amniotic fluid albumin, transferrin and uric acid concentrations are related to subsequent birth outcomes including the infant's gestational age and birth weight. Analysis of AF proteins and uric acid may provide an important window into early fetal development.

Key words amniotic fluid, albumin, transferrin, uric acid, gestational age, birth weight.

4.2 INTRODUCTION

Amniotic fluid (AF), the serous fluid within the amnion that surrounds the fetus, plays an important role in fetal growth and development. Second trimester amniotic fluid originates from a great many sources including transudation of maternal plasma through the amnion or from the fetus via unkeratinized fetal skin, urine excretion from the developing fetal kidney, and from fetal nasopharyngeal, oral and lachrymal secretions (Moore *et al.,* 2003; Queenan, 1978). Its protein composition approximates that of maternal serum (Anderson *et al.,* 2002; Galdhar *et al.,* 1987) and albumin, transferrin, and IgG are major proteins in the second trimester amniotic fluid (Haddow *et al.,* 1978; Liberatori *et al.,* 1997).

Several studies have examined the relationships between maternal serum or cord blood proteins and birth outcomes, including birth weight and gestational age. A significant inverse correlation between third trimester maternal serum albumin and birth weight was found (Forest *et al.,* 1996). Studies have shown a relationship between maternal anemia during pregnancy and low infant birth weight and/or premature birth (Scholl *et al.,* 1992; Klebanoff *et al.,* 1991; Lieberman *et al.,* 1988). Newborn infants who were at risk of impaired uteroplacental blood flow or chronic fetal hypoxia had elevated serum transferrin levels and depressed calculated iron stores at birth (Chockalingam *et al.,* 1987). Studies have also shown that the cord blood IgG was positively correlated to the gestational age and birth weight (Mahulja *et al.,* 1993; Sharma *et al.,* 1986). The neonates with severe IUGR and

preterm babies had significantly lower IgG levels (Singh *et al.,* 1978; Ladipo *et al.,* 1978).

Since amniotic fluid continuously exchanges with the fetus via swallowing and through unkeratinized skin, the protein levels in the AF can reflect the levels in fetus. Using capillary zone electrophoresis albumin, transferrin, IgG and uric acid were found in the amniotic fluid and AF provided an important window into fetal metabolism (Gao *et al.,* submitted). Thus analysis of second trimester AF proteins offers us a valuable opportunity to investigate fetal growth and development in pregnancy. The objective of the present study was to assess the concentrations of major protein components (albumin, transferrin, IgG) in human amniotic fluid early in gestation and to determine if they were associated with, and predictive of, infant birth outcomes, especially infant gestational age and/or birth weight.

4.3 MATERIALS AND METHODS

4.3.1 RECRUITMENT

From the year of 1999 to 2003, pregnant women undergoing routine amniocentesis between 12-20 gestational weeks at St Mary's Hospital Center in Montreal, Canada were invited to participate in this study. Ethical approval was obtained and signed consents allowed researchers to obtain amniotic fluid from Montreal Children's Hospital once genetic testing was completed. Application of inclusion criteria

(singleton pregnancy) and exclusion criteria (multiple births, genetic anomalies) resulted in 230 mother-infant pairs for whom amniotic fluid samples were analyzed and birth outcomes were obtained from the maternal obstetrics chart.

4.3.2 MATERNAL AND FETAL CHARACTERISTICS

From questionnaires and maternal obstetrical chart review maternal characteristics including maternal age, height, prepregnancy weight, smoking status, parity, amniocentesis week and infant characteristics like gender, birth weight and gestational age were obtained. Gestational age was uniformly calculated on the basis of physicians' estimates using last menstrual period. Ethnicity was classified and the following three groups were created: Caucasian (North American and European), Asian, and other that included Caribbean and African Blacks, Hispanics of Mexican, Central and South American descent and those of Middle-Eastern decent. Body mass index (BMI) was categorized into 3 groups using NIH criteria (NIH, 2000). Infants were divided into three groups according to their gestational age at birth: those born before 38 weeks were preterm infants, after 41 weeks were post-term infants, and those between 38 and 41 weeks were term ones. Birth weight was determined using two categories. One was the recently developed birth-weight-for-gestational-age category where infants < 10% fetal growth percentiles were classified as small-for-gestational-age (SGA), those > 90% as large-for-gestational-age (LGA) and the infants between them as appropriate-for-gestational-age (AGA) (Kramer *et al.*, 2001). The other was birth weight by grams category in

which birth weight <2500 grams were classified as low birth weight (LBW), > 4000 grams as macrosomia, and those between these two values as normal (Grassi *et al.*, 2000; Creasy *et al.*, 1999).

4.3.3 BIOCHEMICAL ANALYSES

For the external standards, human serum IgG was purchased from Bethyl (Montgomery, TX, US) and albumin, transferrin and uric acid were purchased from Sigma-Aldrich (Oakville, ON, CA).

Detection of amniotic fluid components was performed on a Beckman Coulter P/ACE Series MDQ capillary electrophoresis system (Fullerton, CA, US) using Beckman 32 Karat Software Version 5.0 for instrument control, data acquisition and analysis (Gao *et al.*, submitted). Prior to separation, frozen (-85 °C) AF samples were thawed in an ice water bath and diluted 1:1 (v/v) with 0.5 mg/ml thiamine in water, which was used as the internal standard as described previously. Samples were injected hydrodynamically (0.5 PSI) for 10 s into the untreated fused-silica capillary (75µm ID, 360µm OD) and separated at 25 kV for 20 minutes using 75mM borate, 0.8mM EDTA, pH 9.27 buffer with the temperature set at 28 °C. Detection was with a photo diode array (PDA) detector and peak integration was done at 190, 254 and 275 nm.

4.3.4 STATISTICAL ANALYSES

All data were analyzed using SAS (Version 9.1, SAS Institute). Pearson and Spearman correlations were performed among the maternal, infant and amniotic fluid characteristics. ANOVA followed by Scheffé multiple comparison tests were performed to determine whether the concentrations of amniotic fluid components and maternal characteristics (height, prepregnency weight, BMI, amniocentesis week) differed among the birth outcomes including birth weight (in grams or percentiles) and gestational age. Multiple regressions for birth weight and gestational age were examined with the results for each AF component entered individually and analyzed in separate regression models; in the cases of birth weight, the several well-documented birth weight determinants like maternal height, prepregnancy weight, smoking status, gestational age, and infant gender were included (Kramer, 1987). Significance was established as $P < 0.05$.

4.4 RESULTS

Maternal, fetal and amniotic fluid characteristics of the study population (n =230) are summarized in **Table 4.1**. Though the mothers in our study were older (37.8 ± 0.2 years) than the average Canadian mothers, which were the major reason for their amniocentesis, the majority were within normal BMI range (23.97 ± 0.27) and 84% were non-smokers. The average infant birth weight was 3478 ± 43 grams.

Concentrations of AF constituents were correlated with maternal and infant characteristics. By Pearson correlations, AF components were positively correlated with amniocentesis week: for albumin ($r = 0.1426$, $p = 0.0314$), transferrin ($r = 0.1450$, $p = 0.0283$) and uric acid ($r = 0.1537$, $p = 0.0203$). Two proteins in AF were also positively correlated with parity: transferrin ($r = 0.1859$, $p = 0.0048$) and IgG ($r = 0.2057$, $p = 0.0018$).

The maternal, infant and amniotic fluid characteristics were compared among two birth weight categories: birth weight in grams (**Table 4.2**) and in percentiles (**Table 4.3**) and one gestational age category (**Table 4.4**). The results of analysis of variance (ANOVA) showed that the AF protein concentrations did not differ between by either birth weight classifications. In contrast, preterm infants had significantly higher concentrations of second trimester AF albumin compared to post-term infants ($p = 0.0266$). Also AF transferrin concentrations of preterm infants were significantly higher than term infants ($p = 0.0215$).

Multiple regression analyses were performed with all established birth weight predictors controlled for in each of the two separate birth weight models (**Table 4.5**). Neither albumin, transferrin nor IgG entered as predictors of birth weight measured in grams or percentiles. In contrast uric acid was significantly associated with birth weight when measured in grams. Interestingly the relationship of AF uric acid with birth weight was polynomial. Infants had the lowest birth weight when the concentration of AF uric acid was about 0.05 g/l. As uric acid

concentrations of most AF samples in our study were less than 0.05 g/l, infant birth weights in our study were negatively associated with second trimester AF uric acid concentrations.

Multiple linear regression analyses for gestational age were performed for the whole population and for each gestational age group. Transferrin was an independent negative predictor for the gestational age of term infants only (**Table 4.6**): the higher the transferrin concentration, the shorter the infant's gestational age.

Table 4.1 Characteristics of the sample population [1]

Maternal characteristics	
Height, *m*	1.62 ± 0.01
Prepregnancy weight, *kg*	63.18 ± 0.81
BMI, kg/m^2	23.97 ± 0.27
BMI, %	
<18.5 kg/m^2	6.1
18.5–24.9 kg/m^2	60.9
≥25 kg/m^2	33.0
Parity	1.15 ± 0.07
Ethnicity, %	
Caucasian	62.9
Asian	21.5
Other [2]	15.6
Non-smoking, %	84
Infant characteristics	
Gestational age, *wk*	39.2 ± 0.1
Gender, *% female*	44.9
Birth weight, *g*	3478 ± 43
Amniotic fluid characteristics	
Amniocentesis wk	15.15 ± 0.06
Albumin, *g/L*	4.41 ± 0.17
Transferrin, *g/L*	0.33 ± 0.01
IgG, g/L	0.28 ± 0.01
Uric acid, mg/dL	3.95 ± 0.09

[1] Values are means ± SEM or %, n=230.

[2] Other includes Black, Middle-Eastern and Hispanic combined

Table 4.2 Infant, maternal and amniotic fluid characteristics stratified by gram birth weights [1,2]

Characteristic	LBW (n=15)	Normal (n=161)	Macrosomia (n =54)	F	P
Infant					
Birth weight, *g*	1821± 93 [a]	3332± 27 [b]	4334± 46 [c]	344.48	<0.0001
Gender, *% female*	47	48	35	1.57	0.21
Gestational age, *wk*	34.4± 0.4 [a]	39.4± 0.1 [b]	40.1± 0.2 [c]	89.26	<0.0001
Maternal					
Age, *yrs*	38.5± 0.6	37.9 ± 0.2	37.4 ± 0.3	1.46	0.24
Height, *m*	1.60 ± 0.02 [ab]	1.61 ± 0.01 [a]	1.65± 0.01 [b]	4.80	0.009
Prepreg wt, *kg*	58.9 ± 3.2 [a]	61.1± 0.9 [a]	70.7 ± 1.6 [b]	14.70	<0.0001
BMI, *kg/m2*	23.0 ± 1.1 [ab]	23.4 ± 0.3 [a]	26.0 ± 0.5 [b]	9.12	0.0002
Non-smoking, *%*	87	81	93	2.51	0.08
Amniotic fluid					
Amniocentesis wk	15.40± 0.23	15.08 ± 0.07	15.28 ± 0.12	1.69	0.19
Albumin, *g/L*	5.11± 0.66	4.50 ± 0.20	3.96 ± 0.35	1.48	0.23
Transferrin, *g/L*	0.365 ± 0.026	0.334 ± 0.008	0.324 ± 0.013	1.01	0.36
IgG, *g/L*	0.290 ± 0.036	0.284 ± 0.010	0.248 ± 0.018	1.58	0.21
Uric acid, *mg/dL*	3.54 ± 0.35	4.03 ± 0.10	3.80 ± 0.18	1.35	0.26

1. Values are means ± SEM or %. Means in a row with superscripts without a common letter differ, P< 0.05.

2. Infants are divided into three groups: LBW (BW< 2500g), Normal (2500g ≤ BW≤ 4000g) and Macrosomia (BW>4000g).

Table 4.3 Infant, maternal and amniotic fluid characteristics stratified by birth-weight-for-gestational-age [1,2]

Characteristic	SGA (n=16)	AGA (n=166)	LGA (n=48)	F	P
Infant					
Birth weight, *g*	2383 ± 116 [a]	3337 ± 35 [b]	4365 ± 67 [c]	141.5	<0.0001
Gender, *% female*	41	46	43	0.11	0.90
Gestational age, *wk*	38.2 ± 0.5 [a]	39.2 ± 0.1 [ab]	39.8 ± 0.3 [b]	5.1	0.0067
Maternal					
Age, *yrs*	38.2 ± 0.6	37.8 ± 0.2	37.8 ± 0.4	0.29	0.75
Height, *m*	1.59 ± 0.02 [a]	1.61 ± 0.01 [a]	1.66 ± 0.01 [b]	8.27	0.0003
Prepreg wt, *kg*	59.8 ± 2.9 [a]	61.3 ± 0.9 [a]	71.6 ± 1.7 [b]	14.68	<0.0001
BMI, kg/m^2	23.6 ± 1.0 [ab]	23.5 ± 0.3 [a]	26.0 ± 0.6 [b]	7.23	0.0009
Non-smoking, %	82	82	92	1.41	0.25
Amniotic fluid					
Amniocentesis wk	15.12 ± 0.22	15.09 ± 0.07	15.37 ± 0.13	1.93	0.15
Albumin, *g/L*	4.75 ± 0.64	4.46 ± 0.20	4.15 ± 0.37	0.41	0.66
Transferrin, *g/L*	0.341 ± 0.025	0.334 ± 0.008	0.330 ± 0.014	0.08	0.92
IgG, *g/L*	0.277 ± 0.033	0.281 ± 0.010	0.259 ± 0.019	0.53	0.59
Uric acid, *mg/dL*	4.00 ± 0.34	4.04 ± 0.10	3.61 ± 0.19	1.95	0.14

1. Values are means ± SEM or %. Means in a row with superscripts without a common letter differ, $P< 0.05$.

2. Infants are divided into three groups: SGA (< 10%), AGA (10% ≤ percentile≤ 90%) and LGA (>90%).

Table 4.4 Infant, maternal and amniotic fluid characteristics stratified by gestational age [1,2]

Characteristic	Preterm (n=39)	Term (n=161)	Post-term (n=30)	F	P
Infant					
Birth weight, *g*	2561 ± 86^{a}	3595 ± 42^{b}	3924 ± 90^{c}	72.77	<0.0001
Gestational age,*wk*	36.0 ± 0.18^{a}	39.5 ± 0.09^{b}	41.5 ± 0.19^{c}	226.51	<0.0001
Maternal					
Height, *m*	1.59 ± 0.01^{a}	1.62 ± 0.01^{ab}	1.64 ± 0.01^{b}	4.19	0.016
Prepreg wt, *kg*	60.3 ± 2.0	63.3 ± 1.0	65.4 ± 2.1	1.55	0.21
BMI, kg/m^2	23.7 ± 0.68	24.0 ± 0.32	24.2 ± 0.71	0.16	0.85
Amniotic fluid					
Amniocentesis wk	15.15 ± 0.14	15.15 ± 0.07	15.13 ± 0.15	0.01	0.99
Albumin, *g/L*	5.18 ± 0.41^{a}	4.40 ± 0.20^{ab}	3.50 ± 0.46^{b}	3.69	0.0266
Transferrin, *g/L*	0.372 ± 0.015^{a}	0.327 ± 0.008^{b}	0.319 ± 0.017^{ab}	3.91	0.0215
IgG, *g/L*	0.308 ± 0.021	0.272 ± 0.011	0.259 ± 0.024	1.45	0.2367
Uric acid, *mg/dL*	3.88 ± 0.22	3.94 ± 0.10	4.04 ± 0.22	0.14	0.87

1. Values are means ± SEM. Means in a row with superscripts without a common letter differ, $P < 0.05$.

2. Infants are divided into three groups: Preterm (GA<38w), Term ($38w \leq GA \leq 41w$) and Post-term (GA>41w).

Table 4.5 Multiple regressions of infant birth weight by percentile and grams

Indicator	Birth weight (n=220)			
	Percentile		Gram	
	β±SEM	P	β±SEM	P
Maternal height, *m*	59.75 ± 29.8	0.046	411.9 ± 534.2	0.441
Maternal prepreg weight, *kg*	0.66 ± 0.17	<0.001	12.7 ± 2.9	<0.001
Smoking behavior, *0=non, 1=smoker*	-7.73 ± 5.28	0.144	-144.4 ± 93.5	0.123
Parity	3.17 ± 1.77	0.075	62.3 ± 31.4	0.049
Ethnicity [1]	-0.15 ± 1.87	0.936	-12.8 ± 33.3	0.701
Gestational age, *wk*	*N/A*		215.8 ± 17.1	<0.001
Infant gender, *0=female, 1=male*	*N/A*		219.6 ± 67.0	<0.001
Amniocentesis wk	4.93 ± 2.02	0.016	74.4 ± 36.1	0.041
Uric acid, *mg/dL*	-13.79 ± 8.22	0.094	-321.5 ± 145.9	0.029
(Uric acid) [2]	1.32 ± 0.84	0.121	30.1± 15.0	0.046
R-square	0.20		0.52	

[1] For ethnicity, 1=Caucasian, 2=Asian, 3= other, includes Black, Middle-Eastern and Hispanic

Table 4.6 Multiple linear regression of gestational age in the term population only [1]

Indicator	GA in Term Infants	
	β±SEM	P
Maternal prepreg weight, *kg*	0.01 ± 0.004	0.78
Birthing code [2]	0.228 ± 0.07	<0.01
Amniocentesis wk	-0.08 ± 0.06	0.23
Transferrin, *g/L*	-1.52 ± 0.70	0.03

[1.] The term infants' population includes the infants born between 38-41 gestational weeks. (n=155)

[2.] For birthing method code, 0 = Cesarean section, 1 = Induction, 2 = Spontaneous vaginal delivery.

4.5 DISCUSSION

Earlier reports in the literature had shown that the concentration of total protein in the second trimester amniotic fluid was negatively associated with infant birth weight (Tisi *et al.,* 2004). In the present study, we did not find the same negative association existed between amniotic fluid albumin, transferrin or IgG and infant birth weight. The reason may be that each single protein in the amniotic fluid has its own physiology and metabolism, which makes them differ from total protein as to the relationship with birth weight.

Preterm labor is the leading cause of perinatal mortality and morbidity (Knoches *et al.,* 1993). In our study, we did show that concentrations of second trimester amniotic fluid albumin and transferrin differed across infant gestational ages, but when included in a multiple regression model, only transferrin predicted gestational age. Several mechanisms may be responsible for this result. First, it could be attributed to the change of amniotic fluid volume. It is known that mothers with oligohydramnios tend to have a premature delivery (Varma *et al.,* 1988; Garmel *et al.,* 1997). Oligohydramnios may result from poor placental perfusion and limited amniotic fluid expansion and lead to an increased concentration of the proteins in the amniotic fluid (Sherer, 2002; Roberts *et al.,* 1998).

Maternal iron deficiency anemia can result in premature birth of the infant (Scholl *et al.,* 1992; Klebanoff *et al.,* 1991). The causes are multi-factorial. First, anemia

may decrease the maternal oxygen delivery to the placenta and the fetus (Bondevik *et al.,* 2001). Second, the iron supply to the fetus may also be reduced and result in fetal anemia (Gambling *et al.,* 2001). As amniotic fluid transferrin comes mostly from fetus, high fetal serum transferrin could result in high AF transferrin (Queenan, 1978). It might explain why in our study high amniotic fluid transferrin was related to earlier delivery in our term infants.

Studies show that pregnant women with low BMI have a tendency for preterm delivery, because women with low BMI have less capacity for fluid expansion during pregnancy, which can reduce placental circulation (Schieve *et al.,* 2000; Kramer *et al.,* 1995). In support of this Maher *et al* showed that maternal serum albumin was negatively associated with BMI (Maher *et al.,* 1993). As most of the albumin in the AF is ultimately of maternal origin (Johnson *et al.,* 1974), the lower AF albumin concentrations in women with larger BMI agree with the similar observations with maternal serum albumin and BMI.

Amniotic fluid uric acid predicted infant birth weight. Most of our AF uric acid concentrations were less than 0.05 g/l, and in this range the infant birth weight was negatively associated with uric acid. Others have shown that mothers with high serum uric acid were more likely to have SGA infants (Roberts *et al.,* 2005; Chang *et al.,* 1987). Usually high uric acid in the maternal serum occurs in a setting of maternal hypoxia, local acidosis, increased tissue breakdown or reduced renal function and all of the above factors could negatively affect fetal growth (Roberts *et*

al., 2005). Since uric acid is a small molecule and can freely cross the placenta, high fetal uric acid may simply be a result of high maternal serum uric acid and reflect compromised nutritional or health status (Chang *et al.,* 1987). On the other side, increased fetal uric acid can also inhibit renal endothelial cell proliferation which is a necessary step of nephron development and can reduce the final nephron number at birth (Feig *et al.,* 2004). Impaired renal development and reduced nephron number at birth are correlated with both intrauterine growth retardation (IUGR) and low birth weight (LBW) (Konje *et al.,* 1996). All offer explanation why infants with higher AF uric acid concentrations would cause lower birth weights.

In conclusion, in our study high concentrations of second trimester amniotic fluid transferrin was associated with subsequent earlier delivery in term infants. Also, for the first time, our study showed that the concentration of second trimester AF uric acid was negatively associated with infant birth weight. Since the routine amniocentesis for high risk pregnancy are usually performed about 15 weeks of gestation, analysis of major amniotic fluid components might offer us a valuable opportunity to investigate fetal growth and development as early as the second trimester.

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Chapter V

Summary and Conclusions

In this study we measured the second trimester major proteins in the amniotic fluid with CZE and discovered important relationships between second trimester amniotic fluid proteins and the birth outcomes, including birth weight and gestational age. The results supported our hypothesis that showed that capillary electrophoresis is a robust method to analyze major components in the amniotic fluid, that the 2nd trimester AF uric acid is a significant predictor of infant birth weight (grams) and that transferrin is negatively associated with gestational age in term infants.

5.1 CAPILLARY ZONE ELECTROPHORESIS

There are a number of characteristics that make CZE ideal for clinical protein analysis, for example, automation, on-line detection, multi-mode capabilities, low volume sample and reagent consumption and so on (Oda *et al.,* 1997). Most important of all, it simultaneously detects multiple proteins using one sample. During our separation, all major AF peaks eluted within ten minutes. The concentrations of major proteins in AF were stable to both room temperature storage and to multiple freeze/thaw cycles. We discovered that there were eight distinct peaks in the 190nm electropherogram of the pooled AF and four of them were identified and utilized: IgG, transferrin, albumin and uric acid.

5.2 AF PROTEINS WITH BIRTH WEIGHT AND GESTATIONAL AGE

Infant birth weight is an important measurement for the fetal intrauterine growth. Many factors affect infant birth weight, for example, maternal height, prepregnancy weight, cigarette smoking, parity, ethnicity and infant characteristics like gender, gestational age and so on. In our study, above well known maternal and infant factors were measured and each of the amniotic fluid component was included in the regression model. For birth weight, we used both the birth weight in grams category and the birth weight for gestational age category.

Tisi et al have shown that the concentration of total protein in the second trimester amniotic fluid was negatively associated with infant birth weight (Tisi *et al.*, 2004). In our study, we could not demonstrate the same association between each major amniotic fluid protein (albumin, transferrin, IgG) and infant birth weight. One study has found that maternal albumin is negatively associated with infant birth weight because albumin is negatively associated with maternal BMI (Maher *et al.*, 1993). That may explain why in our study albumin is not correlated with the infant birth weight when both albumin and BMI (or maternal prepregnancy weight and height) are included in the regression model.

Preterm labor is the leading cause of perinatal mortality and morbidity (Knoches *et al.*, 1993). In our study preterm infants had significantly higher concentrations of second trimester AF albumin and transferrin, but only transferrin was an independent negative predictor for the gestational age and in term infants only. It could be attributed to the change of amniotic fluid volume. It is known that

mothers with oligohydramnios tend to have premature delivery (Varma *et al.*, 1988; Garmel *et al.*, 1997). Oligohydramnios may result from poor placental perfusion and limited amniotic fluid expansion and lead to an increased concentration of the proteins in the amniotic fluid (Sherer, 2002; Roberts *et al.*, 1998).

5.3 AMNIOTIC FLUID URIC ACID AND INFANT BIRTH WEIGHT

Because capillary zone electrophoresis (CZE) separation of molecules based on differences in charge and size, simultaneous separation of both low molecular weight and macromolecular analytes including proteins were achieved in our study. Although the purpose of our study was to use CZE to measure major proteins in the amniotic fluid, uric acid showed up as a well resolved peak with strong absorbance at all the three (190, 254, 275nm) wavelengths and more importantly, it was associated with infant birth weight.

In our birth weight in gram model multiple regression analysis with all established birth weight predictors controlled for showed that the relationship of second trimester amniotic fluid uric acid and infant birth weight was polynomial. Uric acid is the end product of purine metabolism (purines are building blocks of RNA and DNA). The polynomial relationship between AF uric acid and birth weight might be the outcome of many factors working together.

First, high AF uric acid can result from increased maternal plasma uric acid. An overproduction of uric acid occurs when there is excessive breakdown of cells (which contain purines) or an inability of the kidneys to excrete uric acid. Uric acid is a small molecule and can pass through the placenta membrane by simple diffusion (Moore *et al.*, 2003). Free transfer of uric acid via placenta in both directions can also be approved by the high correlation and minimal concentration difference between maternal and neonatal uric acid (Anderer *et al.*, 1975; Chang *et al.*, 1987). Therefore high maternal serum uric acid could lead to high fetal uric acid and finally reach the amniotic cavity through the amniotic fluid circulation.

Both maternal uric acid and neonatal serum uric acid have negative correlations with birth weight and the one and five-minute Apgar scores (Chang *et al.*, 1987). Preeclampsia is a typical example. There is a marked rise in serum uric acid in the mothers with preeclampsia during the third trimester along with elevated levels of uric acid in the fetus and a high frequency of IUGR and low birth weights (Chang *et al.*, 1987). Animal studies have also shown that amniotic fluid uric acid is negatively associated with fetal weight, plasma glucose, maternal and fetal liver glycogen (Koski *et al.*, 1992). Decreased maternal dietary carbohydrate intake during pregnancy, which is an essential nutrient for fetal growth and development, can lead to a higher metabolic requirement for gluconeogenesis, hence the rise in plasma and amniotic fluid uric acid levels (Koski *et al.*, 1992).

On the other hand, the level of AF uric acid can reflect fetal kidney development which can affect the overall fetal growth. It is known that uric acid enters the amniotic fluid through fetal production of urine in two ways. In fetal kidney, uric acid is filtered at the glomerulus and renal tubule secretes uric acid to the tubular lumen, then the urine passes into the amniotic cavity (Fathallah-Shaykh *et al.,* 2006). Amniotic fluid uric acid significantly increases throughout gestation and its level in the amniotic fluid is significantly higher than the levels in maternal vein serum or umbilical cord serum (Oliveira *et al.,* 2002). The concentration of AF uric acid is significantly correlated with the concentration of AF creatinine. So AF uric acid can be seen as indicators of fetal kidney maturation and renal function (Oliveira *et al.,* 2002). It is known that impaired renal development is correlated with intrauterine growth retardation (IUGR) and low birth weight (LBW) (Konje *et al.,* 1996).

Uric acid can also function as biological antioxidant. Uric acid is shown to be a powerful antioxidant and a scavenger of singlet oxygen and peroxyl radicals (Ames *et al.,* 1981;Wayner *et al.,* 1987). Urate can also act as a plasma antioxidant by binding transition metal ions (Becker, 1993). It has been suggested that neonates, in particular preterm and small-for-gestational-age babies, have an increased risk of oxidant stress due to their reduced ability to bind transition metal ions (Sullivan & Newton, 1988; Lindeman *et al.,* 1992). Although total plasma lipids are lower in neonates than in adults, there is a higher proportion of polyunsaturated fatty acids (PUFA) in cord plasma compared with adult plasma (Oostenbrug *et al.,* 1998). As

PUFA are susceptible to oxidation, this may increase the risk of prooxidant activity in cord plasma. Therefore increased levels of fetal uric acid can protect the fetus from oxidant stress and promote ideal fetal growth.

5.4 LIMITATIONS AND FUTURE STUDIES

Because of the various sources of amniotic fluid, the composition of second trimester AF is quite complicated. Besides major proteins like albumin, transferrin and IgG, there are a lot of other less abundant proteins in the amniotic fluid, for example α_1-antitrypsin, IGFBP1, Gc globulin, α_1 acid-glycoprotein (Chevallier *et al.,* 1998; Hamashima *et al.,* 2002; Johnson *et al.,* 1974). As the result, the electropherogram shows a relatively low-resolution separation on a broad baseline with multiple species demonstrating overlapping bands. This places severe limits on the number of species that can be reliably quantitated using traditional peak integration and also affects the accuracy of integrated peaks. By changing the conditions of the separation, improved resolution of the electrophoresis may be achieved.

Another limitation of the study is using thiamine as the internal standard. Even though thiamine has many desirable properties, thiamine has definite limitation as an internal standard because of the fact that it complexes with components in AF resulting in additional peaks in the electropherogram and interfering with the calibration.

For birth outcomes we only used infant birth weight and gestational age. In future studies other outcome measurements could be explored like infant height, biparietal diameter, femur length, abdominal circumference and so on. On the other hand, not only serum proteins can represent nutrition status of the mother and the fetus, each specific protein is also associated with specific functions in the body. We can try to detect the association of protein levels with certain conditions like intrauterine infection, hypertension, diabetes and so on.

Although the aforementioned limitations were present, our study was novel and presented strengths. Our study is the first to use the new and efficient method of capillary zone electrophoresis to measure mass amniotic fluid samples. For the first time we found that second trimester amniotic fluid uric acid is significantly associated with birth weight and AF transferrin is a negative predictor of gestational age for term infants. These major AF components can be used as biomarkers of fetal growth and development in early pregnancy and as pre-diagnostic tool they may aid in promoting optimal fetal growth and prevention of unfavourable birth outcomes.

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APPENDIX I

Method of Quantitative Analysis of Electrophoregram

In the Beckman P/ACE MDQ system that we have used to analyze amniotic fluid, AF sample is injected into a fused-silica capillary and then sample components migrate differentially through the capillary under the influence of high voltage. As these components pass the detection window near the end the capillary, a multiwavelength photodiode array detector (PDA) measures absorbance and then converts the signal into electropherogram. In the electropherogram, the area under a certain peak is directly related to the amount of the analyte present in the sample. The process of determining this area is known as integration. In practice, integration includes steps that determine the points at which peak start and stop, and the shape and position of the baseline. After we acquire the area of each peak in the electropherogram, peak area should be converted to concentration for further analysis.

To quantitative analysis of amniotic fluid components, we had run a series of runs with known concentrations of each AF component as external standard calibration. Solutions at different concentration were obtained by serial dilution for albumin at 0.56875, 1.1375, 2.275, 4.55, 9.1 g/l, transferrin at 0.2125, 0.425, 0.85, 1.7, 3.4 g/l, IgG at 0.09375, 0.1875, 0.375, 0.75 g/l and uric acid at 0.675, 1.35, 2.7, 5.4 mg/dl.

Then the corresponding peak areas in the electropherogram for each amniotic fluid component of known concentrations were measured and normalized by thiamine which acted as the internal standard. Using second order polynomial least squares regression a standard curve for peak area versus concentration was generated for each amniotic fluid component as shown in the following: concentration of AF uric acid 190nm = (-2.46E-

02)+(29.000) *area+(-15.472)*(area *area); concentration of AF albumin = (0.483)+ (0.213)*area + (5.841E-02)*(area*area); concentration of AF transferrin = (0.123)+ (0.410)*area + (8.178E-02) * (area*area); concentration of AF IgG = (-3.4E-03)+ (0.317)*area + (0.078)*(area* area). Then using the standard curve, the concentrations of the amniotic fluid proteins and uric acid were obtained.

APPENDIX II

Ethics Approval - Human Study

APPENDIX III

Co - author Approval