THE ASSOCIATION BETWEEN AMNIOTIC FLUID ALBUMIN, PREALBUMIN OR TRANSFERRIN AND THE FETAL GROWTH

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ABSTRACT

The study objectives were to measure the concentrations of albumin, prealbumin and transferrin in amniotic fluid (AF), and to establish if these concentrations were associated with infant birth weight (BW). At St Mary's Hospital (Montreal, Quebec), 294 AF samples were collected from mothers undergoing routine amniocentesis (12-19 weeks gestation). Exclusion criteria included subjects having gestational diabetes, multiple births or fetal genetic abnormalities. AF samples were analyzed by capillary electrophoresis (CE) at 190 nm. Analysis of variance and multiple linear regressions were performed. AF prealbumin could not be detected by CE. However, ANCOVA showed that transferrin was different among BW categories. Multiple regressions showed the parameter estimates for transferrin and albumin were negative, but neither was associated with BW in our study population. In contrast, transferrin was negatively associated with BW in our LBW infants. Our study shows that 2nd trimester AF transferrin may emerge as a biomarker for poor in-utero growth.

SOMMAIRE

Nos objectifs étaient de mesurer les concentrations de l'albumine, préalbumine et transferrine dans le liquide amniotique (LA), et d'établir si ces concentrations sont associées au poids de naissance (PN). À l'hôpital St Mary (Montréal, Québec), 294 échantillons de (LA) furent rassemblés des mères subissant l'amniocentèse (12-19 semaines de grossesse). Les sujets avec complications de grossesse et foetales et plus qu'un bébé furent exclus. L'analyse fut par électrophorèse capillaire à 190 nm. ANOVA, ANCOVA et régressions linéaires multiples furent exécutés. La préalbumine ne fut pas détectée. ANCOVA prouva que la transferrine était différente entre les catégories de PN. Les régressions multiples montrèrent que les paramètres d'évaluation de la transferrine et de l'albumine étaient négatives sans être associés au PN. La transferrine fut négativement associée au PN pour les enfants au poids léger. Donc, la transferrine du 2ème trimestre est différente entre les catégories de PN, et négativement associée au PN au poids léger.

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Statement of Originality

In conjunction with Dr. K. Koski, the author developed the present research project. The analysis the amniotic fluid samples using the capillary electrophoresis was performed by the author and two students, to coordinate the time on the machine. The author contributed to 61% of the samples analyzed, and the rest was divided between the two other students: Tao Gao, and Celine La Croix. Celine LaCroix tested the amniotic fluid samples stability to room temperature as well as the stability to freezing and thawing. Dr. Skinner provided training on the CE machine located at Concordia University. Dr. Burns provided the statistical support for the conversion of the electropherograms' protein peak areas into quantities. The author recruited subjects and reviewed the medical charts. The author was responsible of the statistical analysis, data management, and writing of the thesis and paper.

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LIST OF ABBREVIATIONS

AC	Abdominal circumference
AF	Amniotic fluid
AGA	Appropriate-for-gestational-age
ANOVA	Analysis of variance
ANCOVA	Analysis of covariance
BMI	Body mass index
BPD	Biparietal diameter
BW	Birth weight
CE	Capillary electrophoresis
CZE	Capillary zone electrophoresis
EDTA	Ethylenediamine tetra-acetic acid
EOF	Electro-osmotic flow
FL	Femur length
GA	Gestational age
Ht	Height
IGF-BP	Insulin-growth-factor binding protein
IUGR	Intrauterine growth retardation
LBW	Low birth weight
LGA	Large-for-gestational-age
MCH	Montréal Children's Hospital
MDQ	Method development and quantitation
NaOH	Sodium hydroxide
PDA	Photodiode array
PPw	Prepregnancy weight
SDS	sodium dodecyl sulfate
SGA	Small-for-gestational-age
UV	Ultra-violet
VLBW	Very low birth weight

I. Overview

In both developed and developing countries birth weight (BW) influences neonatal mortality and infant and childhood morbidity (Mc Cormick, MC 1985). While various genetic and environmental factors affect the birth weight of the newborn infant (Kirchengast S *et al.,* 1998 b) intrauterine growth depends mainly on three factors: fetal, placental and maternal factors (Grassi A E, Giuliano M A, 2000). The two extremes of irregular fetal growth are fetal macrosomia and fetal growth restriction (Langer O, 2000). These can lead to short-term complications like caesarian deliveries, birth injuries, and shoulder distocia as well as long-term complications like diabetes, obesity, mental health problems, impaired cognitive and behavioral development (Chescheir NC *et al.,* 2004; Boulet SL *et al.,* 2003; Langer O, 2000; Grassi AE *et al.,* 2000; Zubrick SR *et al.,* 2000; Hack M, 1998) that are of considerable concern to health professionals. Early diagnosis of these conditions would be of considerable benefit.

Amniotic fluid (AF) originates from the mother, the placenta, the cord, as well as directly from the fetus; its origin varies with different gestational ages (McCarthy T *et.al,* 1978). Amniotic fluid consists of 98% water and has analogous characteristics to maternal serum in early pregnancy (Finegan JA, 1984). AF protects the infant, prevents mechanical and thermal shock, contains nutritional factors, assists in acid/base balance, and possesses antimicrobial activity (Finegan JA, 1984). It enables normal development of the respiratory, gastrointestinal, urinary tracts of the fetus and its musculoskeletal system (Sherer DM, 2002). The fetus contributes to the composition of the AF through the lungs, the trachea, swallowing, and producing urine (Stewart CJ *et al.,* 2001).

Proteins have been associated with many outcomes related to the fetal development (Jauniaux E *et al.,* 2000, Jauniaux E *et al.,* 1994, Raubenstine D A et al, 1990). Most importantly, total AF proteins are negatively associated with BW at term (Tisi DK *et al.,* 2004) and some specific proteins such as insulin growth factor binding protein 1 (IGF-BP1) are negatively associated with BW, while others like IGF-2 and IGF-BP3 were positive predictors of BW (Tisi DK *et al.,* 2005). Prealbumin, albumin and transferrin have also been linked to some fetal outcomes: Newborn BW correlates with

maternal and fetal plasma prealbumin concentrations (Jain SK *et al.,* 1995) as well as with fetal serum prealbumin (Raubenstine DA *et al.,* 1990). Serum prealbumin may indicate poor protein nutritional status in very low birth weight (VLBW) infants (Polberger SK *et al.,* 1990). AF prealbumin is also considered as a potential biochemical marker for certain complications of pregnancy and fetal defects (Lolis D *et al.,* 1995). Newborn serum albumin correlates with BW (Raubenstine DA *et al.,* 1990). Intrauterine growth restricted (IUGR) fetus are hypoalbuminemic (Nieto-Diaz A *et al.,* 1996). Fetal intestinal tract abnormalities also can be diagnosed early by electrophoretic detection of abnormal AF albumin bands (Stefos T *et al.,* 1993). As a final point, serum transferrin of newborns correlates with BW (Raubenstine DA *et al.,* 1990). AF transferrin is known to increase in pathological conditions (Bardos P *et al.,* 1976). Plasma transferrin may be related to protein nutritional status in VLBW infants where it strongly correlates with the weight and growth of VLBW fetuses (Polberger SK *et al.,* 1990).

To date, no association between the amniotic fluid content of albumin, transferrin and prealbumin and fetal growth has been studied. The objectives of our study were 2-fold: 1) to measure the concentrations of albumin, prealbumin and transferrin in the amniotic fluid, and 2) to establish if albumin, prealbumin, transferrin concentrations in the amniotic fluid are associated with infant birth weight. We used the newly developed birth-weight percentiles that define small-for-gestational age (SGA) as <10% and large-for-gestational age (LGA) as >90% based on gestational age and gender for our classification system (Kramer MS, 2001).

The weight of the fetus can be approximated using ultrasound measurements around the 24th week of gestation. Since this study deals with measurements at earlier times, its originality lies in the possibility of predicting the weight of the infant at an earlier gestational age using amniotic fluid constituents. Ultrasound infant birth measurements are based on anthropometric measurements only. Subsequently, our research has another advantage in that it takes into account the composition of the metabolic environment of the fetus, which is absent using ultrasound measurements. Therefore, this study postulates broadly that the composition of the AF in the early gestational period might provide a new window on fetal growth and development.

This thesis is a manuscript-based thesis. It includes a comprehensive review of the literature, a materials and methods section, followed by a paper to be submitted to the Journal of Nutrition, then a general discussion, and a thorough bibliography. The appendix will include ethics certificate and the consent forms used in the study.

II. Literature Review

Fetal Growth

Fetal size is a major determinant of the infants' survival at the beginning of their lives (Kirchengast S *et al.,* 1998). In fact, birth weight (BW) is probably the most important factor that influences neonatal mortality, and infant and childhood morbidity. This can be seen in both developed and developing countries (McCormick MC, 1985). Under normal circumstances, the infant has a built-in growth potential that results in a normal height and weight (Vandenbosche RC *et al.,* 1998). It is recognized that the linear growth of the fetus is continuous while the speed of growth varies during gestation; the rate of growth in fetal weight slows during the second and third trimesters and near term (Kliegman RM *et al.,* 2001; Creasy RK *et al.,* 1999; Luke B, 1994). Various standard curves for fetal growth are used, as reviewed by Resnik (Resnik R, 2002). BW is controlled mainly by the intrauterine growth rate and the duration of gestation (Kramer MS, 2001; Kramer MS, 1987). Two fetal growth classification systems are usually used; the first one is: small for gestational age (SGA) < 10^{th} percentile, appropriate for gestational age (AGA), between 10^{th} and 90^{th} percentile, and large for gestational age (LGA)> 90^{th} percentile (Kramer MS, 2001). The second one is: low birth weight (LBW) < 2500 g, and macrosomic > 4000 g, with healthy birth weights lying in between these two extremes.

It is also possible that various genetic as well as environmental factors affect the size of the newborn infant (Kirchengast S *et al.,* 1998 b). In utero, intrauterine growth is influenced mainly by three compartments: fetal, placental and maternal factors. The fetal factors are mainly hormones and growth factors such as insulin-like growth factors, thyroid hormones and insulin. Hyperinsulinemic fetuses will have macrosomia, while hypoinsulinemic fetuses usually end up having IUGR (Grassi AE *et al.,* 2000). The development of the placenta affects the fetal growth (Robinson J *et al.,* 1997). Placental factors include mostly single umbilical arteries (Bjoro K Jr, 1983) and placenta previa

(McFarlin BL 1994; Naeye RL 1978). Maternal factors will be discussed in the following paragraphs. The two extremes of irregular fetal growth are fetal growth restriction and fetal macrosomia (Langer O, 2000).

Macrosomia

There is no uniform standard for macrosomia; in fact, there are several definitions for macrosomia which are either >90th percentile for gestational age or > 4,000g or > 4,500g of BW (Grassi AE *et al.,* 2000). The American College of Obstetricians and Gynecologists in 1991 defined macrosomia as an infant having more than 4,500g of BW (ACOG, 2000). Macrosomic infants are sometimes called large-for-gestational-age (LGA) infants (Haram K et al, 2002). World-wide, 10% of neonates are LGA, and the majority of these are macrosomic (Berkus MD *et al.,* 1999). In the United States, more than 100,000 LGA infants are born each year (Langer O, 2000). In Canada, the prevalence of LGA infants is 12 % (Canadian Perinatal Health Report 2003). A rise in the incidence of macrosomia has been noticed lately (Surkan PJ *et al.,* 2004; Orskou J *et al.,* 2001; Grassi AE *et al.,* 2000). The increased BW was attributed to the increase in fetal growth (Kramer MS *et al.,* 2002) and an increase in gestational diabetes (Grassi AE *et al.,* 2000). In addition, in a large prospective cohort study, it was found that changes in some factors over time explained the increasing proportion of high BW infants found in the 1990's (1990-1999). The factors were: high maternal prepregnancy weight and height, being a nonsmoker, a low level of caffeine intake, high parity, gestational age, and male infant gender increased the risk of delivering an infant with a BW above 4000 g (Orskou J *et al.,* 2003). On the other hand, simultaneous increases in maternal body mass index and decreases in maternal smoking explain the increasing proportions of LGA births over time (Surkan PJ *et al.,* 2004).

Fetal macrosomia has been shown to affect the health of the fetus. Indeed, an association between the large infants and high maternal and perinatal morbidity and mortality ratios has been found (Langer O, 2000). Furthermore, fetal macrosomia is one of the major obstetric determinants that induce the infant to face presentation, which is a relatively uncommon labor presentation, when the infant is head down but has its neck

extended (Duff P, 1981). There are additional complications due to macrosomia like shoulder dystoxia, perinatal asphyxia, protracted labor (Grassi AE *et al.,* 2000), increased risk of cesarean delivery (Boulet SL *et al.,* 2003; Grassi AE *et al.,* 2000), birth injuries and cephalopelvic disproportion (Boulet SL *et al.,* 2003). Besides, BW has an inverse impact on the blood pressure by the age of 7 (Yiu V *et al.,* 1999).

Concerning the long-term complications of macrosomia, there is a positive association with Wilms tumor, neuroblastoma, and acute lymphocytic leukemia (Yeazel MW *et al.,* 1997). While the frequency of adverse birth outcomes increases slightly with increasing BWs, there is no report of adverse outcome or death in most of the macrosomic infants, but the increased risk of death is only evident in the highest BW group of more than 5000g (Boulet SL *et al.,* 2003).

There is no early known diagnosis for most macrosomia (Berkus MD *et al.,* 1999). Many factors are known to cause the occurrence of macrosomia. It has been shown that the strongest two factors are a previous history of macrosomia (Boulet SL *et al.,* 2003) and maternal diabetes mellitus (Boulet SL *et al.,* 2003; Brunskill *et al.,* 1991). Diabetic women entering the pregnancy are at an increased risk of having macrosomic infants, followed by women having gestational diabetes with a fasting glucose of $\geq$ 6 mmol/l, followed lastly by women having blood glucose < 6 mmol/l (Caulfield LE *et al.,* 1998). Obviously, a major cause of macrosomia is glucose (Langer O, 1991). In fact, the mother with elevated glucose tolerance, not necessarily diabetes, may transfer more glucose to her fetus. This may result in a symmetrically large infant (Langhoff-Ross J, 1987). On the other hand, hyperinsulinemia is another major cause of macrosomia, as well as irregularities in insulin growth factor (IGF) secretion (Grassi AE *et al.,* 2000). The diabetic macrosomic infants show hypocalcaemia, cardiac failure, hypoglycemia, hyperbilirubinemia, polycythemia, cerebral edema from asphyxia or trauma. In nondiabetic macrosomic neonates, the incidence of hypoglycemia is not clear (Berkus MD *et al.,* 1999). Additional factors have been associated with macrosomia like increased maternal weight and height, excessive maternal weight gain during pregnancy, postdate, prolonged or difficult labor (Udall JN *et al.,*1978; Stallone LA *et al.,* 1974), ethnic origin (Grassi AE *et al.,* 2000; Udall JN *et al.,*1978; Stallone LA *et al.,* 1974), maternal obesity (Dar *et al.,* 2000), multiparity (Brunskill *et al.,*1991; Udall JN *et*

al.,1978; Stallone LA *et al.*, 1974), various congenital disorders (Grassi AE *et al.*, 2000) and hypertension (Boulet SL *et al.*, 2003). Macrosomia deliveries are common for obese women (Kliegman RM *et al.*, 2001). On the other hand, it has been shown that macrosomic infants tend to be male and are usually born after 33-38 weeks (Brunskill *et al.*, 1991). Some studies have shown that advanced maternal age (Mondalou HD *et al.*, 1980; Sack RA 1969) is associated with the incidence of macrosomia while others did not find any relation between the two (Brunskill *et al.*, 1991). Without a doubt, being less than 18 years old is less likely for a woman to give birth to a macrosomic infant. On the other hand, it has been shown that a previous pregnancy loss is associated with the incidence of macrosomia (Boulet SL *et al.*, 2003). Lastly, the risk of macrosomia is lowered in the presence of maternal smoking (Boulet SL *et al.*, 2003; Brunskill *et al.*, 1991). Figure 1 shows the rate of LGA infants in Canada from 1991-2000.

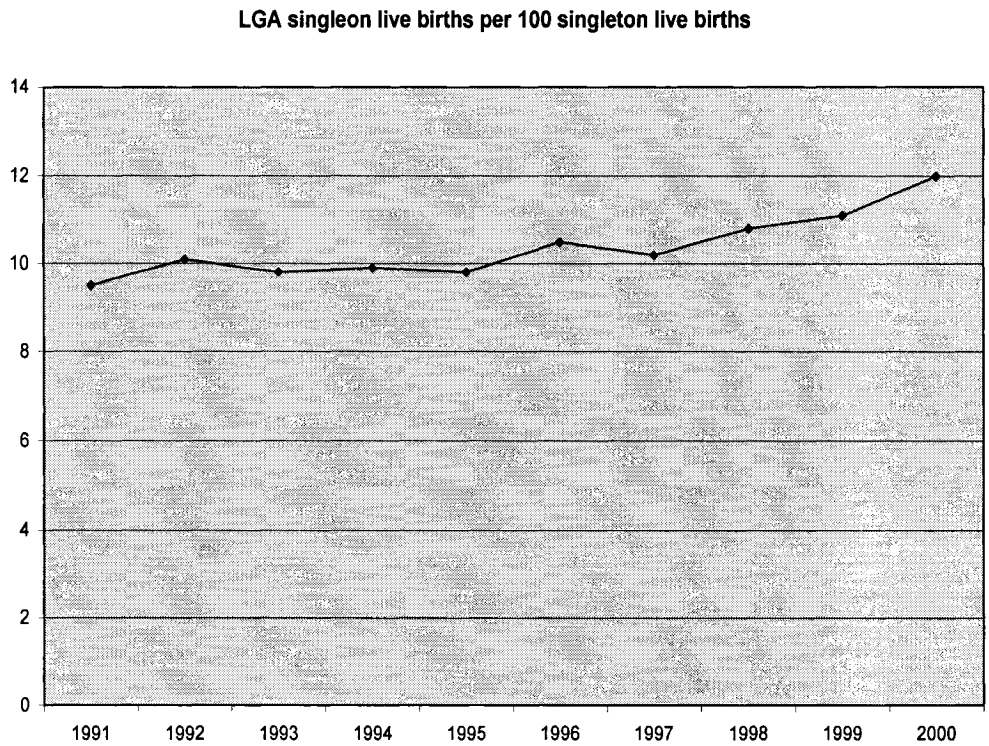

Figure 1. Rate of LGA, Canada, excluding Ontario, 1991-2000; Statistics Canada, Canadian Vital Statistics System, 1991-2000

Low birth weight

All newborns weighing less than 2500 g are classified as having low birth weight (LBW) according to the World Health Organization (WHO) (Creasy RK *et al.,* 1999; Vandenbosche RC *et al.,* 1998). LBW is defined as corresponding to one of the following categories:

1) Preterm and born before 37 completed weeks of gestation and of appropriate- weight-for-gestational age (AGA)

2) Preterm or growth retarded or born before 37 completed weeks of gestation and are small-for-gestational age (SGA)

3) Term and growth retarded or born after 37 completed weeks of gestation and are small-for-gestational age (SGA) (Kliegman RM *et al.,* 2001; Creasy RK *et al.,* 1999).

LBW is most commonly caused by short gestation, intra-uterine growth retardation (IUGR), or both (Save the Children, 2001). World wide, in 1979, 17% of all the births were LBW infants (WHO, 1980). In 1982, this rate dropped to 16% (WHO, 1984). Recently, around 16 million LBW infants are born each year (Save the Children, 2001). According to the WHO, 17 % of infants are born LBW in developing countries compared to 6 % in developed countries (Save the Children 2001). In 1998, Kramer MS concluded that there had been a modest decline in the incidence of LBW in Canada and the United States in the last several decades (Kramer MS, 1998); the lower incidence of intra uterine growth retarded (IUGR) rather than preterm birth caused this downtrend; in addition, the size of term infants had been increasing (Division of Nutrition, National center for Chronic Disease Prevention and Health Promotion, 1994). In Canada, 5.8% of deliveries in 1995 were LBW infants (Perinatal Education Program of Eastern Ontario, 1998); In fact, 85% of the stillbirths and 65% of the deaths in the first week of life were caused by perinatal complications associated with LBW (Nault, F, 1997). At all ages of mothers, the rate of LBW was 5.6% in 2000, 5.5% in 2001, and 5.7% in 2002; and specifically, for mothers aged between 35 and 49 years, the rate of LBW was: 6.6% in 2000, 6.6% in 2001, 6.8% in 2002 (Statistics Canada, 2002). While the rate of SGA infants is 8% (Canadian Perinatal Health Report, 2003).

LBW is linked with poor ensuing growth in infancy and childhood (Albertsson-Wikland K *et al.,* 1997), and with impaired behavioral and cognitive development (Hack

M, 1998). In addition, preterm LBW infants have increased rate of health problems in the postnatal period as well as in childhood. Twelve to 18 % of those preterm infants will have impediments like learning difficulties and cerebral palsies (Moutquin J.M *et al.,* 1996) Infants born LBW (Kogan MD, 1995) and SGA (Barker D, 1994) have higher risks of developing diseases and health problems in adulthood. Three to 10% of pregnancies result in IUGR infants (Palo P, 1992). IUGR is a deflection from an anticipated sequence of fetal growth and is the major cause of LBW in developing countries (Kliegman RM *et al.,* 2001). IUGR is commonly defined as BW less than 10th percentile for gestational age, as BW less than 2500 g with gestation greater or equal to 37 weeks, less than 2 standard deviations below the mean value for gestational age (Kramer MS, 1987) and whose abdominal circumference is less than the 2.5th percentile. IUGR is described as the "pathologic counterpart" of SGA (Vandenbosche RC *et al.,* 1998). Actually, the causes of IUGR are not the same as those of SGA; many infants who are IUGR are not SGA and not all SGA infants are IUGR (Chescheir NC *et al.,* 2004). In fact, not all infants with IUGR are SGA (Kliegman RM *et al.,* 2001). Some infants are genetically small, but develop normally and are well-proportioned, while the growth-restricted infants are not (Vandenbosche RC *et al.,* 1998) and have not completed their growth potential (Resnik R, 2002). More to the point, if the fetus is SGA before 33 weeks of gestation, this is associated with IUGR (Thiebaugeorges O *et al.,* 2000). There are three different types of IUGR: the symmetric type, the asymmetric and the combined type. The symmetric type is distinguished by stunting of the linear skeletal growth (Miller HC, 1985) and by a proportional decrease of the fetal abdomen and head (McFarlin BL, 1994; Pollack RN *et al.,* 1992). It happens early in gestation (Kliegman RM *et al.,* 2001). The asymmetric type is characterized by a disproportionate decrease of the abdomen of the fetus with respect to the fetal head (McFarlin BL, 1994; Pollack RN *et al.,* 1992); it is called the brain-sparing effect (Chescheir NC *et al.,* 2004) or head-sparing effect (Pollack RN *et al.,* 1992) and is the less serious and the most frequently occurring type. The third type is not as frequent as the first two but is the most severe form. It is a combination of the two (Miller HC, 1985). The eventual growth potential for IUGR infants seems adequate (Bernstein I *et al.,* 2002) with only a 0.2 to 1 % of mortality rate (Bernstein I *et al.,* 1996). The majority of the catch-up growth occurs in the first year of life. On the

other hand, a portion of IUGR infants have continuing growth failure (Karlberg JP *et al.*, 1997).

IUGR is a combination of maternal and fetal disorders and is not a specific disease. It is strongly associated with congenital malformations and chromosomal disorders (Resnik R, 2002). Among infants born IUGR, the risk of developing type II diabetes is increased in adulthood (Chescheir NC *et al.*, 2004). In addition, mental health problems might result from having IUGR (Zubrick SR *et al.*, 2000). Recently, the definition of IUGR has changed from intrauterine growth retardation to intrauterine growth restriction (Chescheir NC *et al.*, 2004). Figure 2 shows the rate of SGA infants in Canada from 1991-2000.

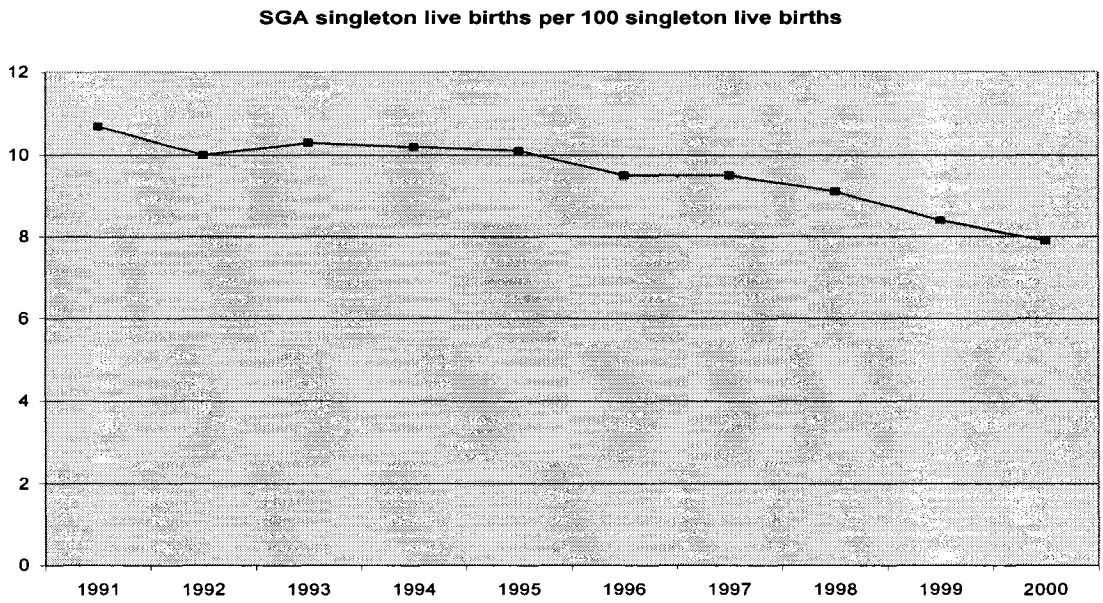

Figure 2. Rate of SGA, Canada excluding Ontario, 1991-2000, Statistics Canada, Canadian Vital Statistics System, 1991-2000

Determinants of birth weight

"The causality of LBW is multifactorial" (Kramer MS, 1987). Many factors are known to affect the BW. Listed below are the main determinants.

Gestational age

Eighty per cent of pregnancies last for 38-42 weeks, 10% are preterm and the rest are post-term (Berkus MD *et al.,* 1999). Preterm delivery is defined as less than 37 completed weeks of gestation (Cheng CJ *et al.,* 2004). It has been shown that the utmost determinant of BW is gestational age (Muslimatun S *et al.,* 2002, Catalano PM *et al.,* 2001). Furthermore, fetal BW and gestational age have been found to be strongly associated (Langer O, 2000). Undeniably, the growth of the fetus in weight and height is primarily established by the length of gestation (Luke B, 1994). On the other hand, preterm delivery affects SGA infants by producing an odds ratio of 1.7 for SGA (Cheng CJ *et al.,* 2004). Interestingly enough, it has been determined that gestational age is affected by race and ethnicity. In fact, white women (Luke B, 1994), especially European (Patel RR *et al.,* 2004), tend to have naturally longer pregnancies than black and Asian women (Patel RR *et al.,* 2004).

Cigarette smoking

In developed countries, cigarette smoking is the most important factor affecting intrauterine growth (Gross TL, 1989; Kramer MS, 1987 b) where the rate of smoking is higher in women from lower socio-economic settings (Kogan MD, 1995; Hagland B *et al.,* 1993; Subcommittee on nutritional status and weight gain during pregnancy, 1990). Although maternal smoking is rare in developing countries, many habits pertaining to their lifestyles result increased levels of carbon monoxide that leads to the same impact as smoking (Dary O *et al.,* 1981). In Canada the percentage of women who smoke during pregnancy is 20.6 % (Statistics Canada, 1998, as referred by Perinatal Education Program of Eastern Ontario 1998). Thirty per cent of mothers smoke in developed countries. In those countries, the etiologic factor associated with cigarette smoking is quite high (Kramer MS, 1998). Smokers are 2.42 times more likely to have LBW and IUGR infants than non-smokers (Kramer MS, 1987). Cigarette smoking is well established as a risk factor for LBW (Floyd RL *et al.,* 1993; Kramer MS, 1987; Institute of Medicine, 1985) and IUGR (Steward DK *et al.,* 2004, Floyd RL *et al.,* 1993; Kramer MS, 1987; Institute of Medicine, 1985) which are strongly affected by the direct impact of smoking (Kramer MS, 1987). According to the Institute of Medicine, cigarette smoking is the absolute risk

factor for LBW (Institute of Medicine, 1985) which is caused by an increase in the incidence of SGA and preterm birth (Kramer MS, 1987). This direct effect is not changed by altered maternal nutritional intake (Scott A *et al.,* 1981). Studies show that IUGR rather than prematurity appears to cause the increased incidence of LBW among smokers (Abel EL, 1980). Furthermore, a symmetrically smaller infant is the result of smoking (Bernstein I *et al.,* 2002; Cliver SP *et al.,* 1995). Nicotine is usually the major entity that causes the pharmacological effects of smoking on BW (Abel EL, 1980). In addition to the carbon monoxide, they constitute the major contributors to the intrauterine growth retardation (Perinatal Education Program of Eastern Ontario, 1998; Bernstein PS *et al.,* 1997; Kramer MS, 1987). The cyanide compounds contained in the tobacco smoke also affect the intrauterine growth (Andrews J, 1973). The main impact of smoking on the fetus is intrauterine hypoxia, increased carboxyhemoglobin (Abel EL, 1980, Longo LD, 1977), reduced blood flow and changes in the placenta (Abel EL, 1980). There is a significant reduction in BWs of the infants of smoking mothers. This reduction augments with increasing number of cigarettes smoked showing a dose-response relationship (Cheng CJ *et al.,* 2004; Bernstein PS *et al.,* 1997; Muscati SK *et al.,* 1996; Kiely JL *et al.,* 1994; Kramer MS, 1987; Dougherty CR *et al.,* 1982; Abel EL, 1980). The effect of smoking in the last trimester is mostly observed, so the period of gestation when the mother smokes affects the cigarette smoking impact (Fried PA *et al.,* 1987; Kramer MS, 1987). There is reduced impact on BW if smoking is stopped before the last trimester (Cliver SP *et al.,* 1995). Studies show that after intrauterine exposure to cigarette smoking, infants usually stay below the growth average in height and weight on a long-term basis (Abel EL, 1980). The average of BW reduction related to maternal smoking was determined as 160g (Grjibovski A *et al.,* 2004) while others found it to be 200 g (Catalano PM *et al.,* 2001; Creasy RK *et al.,* 1999; Abel EL, 1980), ranging from 40 to 430 g (Abel EL, 1980). Height is also affected were newborns of smoking mothers are about 1.4 cm shorter (Meredith HV, 1975). The relative risk of BW for smokers is -149.9 g reduction in BW, while a reduction of -11.1g of BW/cigarette/day is observed (Kramer MS, 1987), whereas newer studies showed that it was a reduction of 27g of BW/ cigarette/ day (Grjibovski A *et al.,* 2004). With advanced maternal age, the impact of smoking increases (Kliegman RM *et al.,* 2001; Backe E, 1993; Kim I *et al.,* 1992) and

this can be seen in younger maternal age too (Cornelius MD *et al.*, 1995). Moreover, underweight smoker women older than 30 years of age who entered pregnancy are at a greater hazard of having LBW siblings (Luke B, 1994). The growth-retarding impact of smoking on BW could be reduced by increasing maternal pregravid weight and gestational weight gain (Luke B, 1994). The studies reviewed by Kramer MS showed that the risk of prematurity and reduction in gestational age appears to increase with maternal cigarette smoking (Kramer MS, 1987). Some studies showed that the impact of cigarettes can also be seen in passive smoking (Windham GC *et al.*, 2000; Ellard GA *et al.*, 1996; Rubin DH *et al.*, 1986). This impact is high in the low socioeconomic classes (Rubin DH *et al.*, 1986).

Maternal weight gain

Maternal weight gain during gestation is associated (Bernstein PS *et al.*, 1997) and correlates positively with BW (Catalano PM *et al.*, 2001). Prepregnancy weight gain (Luke B, 1994; McFarlin BL, 1994) and weight are the primordial factors determining the fetal weight, and although these two factors are independent, their effect is additive (Luke B, 1994). It has been shown that a poor weight gain during pregnancy has a greater risk of delivering an infant weighing less than 2500 g (Abrams BF *et al.*, 1986). In addition, weight gain of the pregnant woman during gestation has a direct impact on intrauterine growth (Kramer MS, 1987 b); indeed, maternal weight gain and IUGR are significantly associated (Steward DK *et al.*, 2004). More precisely, a maternal weight gain of less than 10 kg by 40 weeks of gestation is a risk factor for IUGR (Creasy RK *et al.*, 1994). Interestingly enough, maternal weight gain between subsequent gestations decreases the risk of delivering a SGA infant (Cheng CJ *et al.*, 2004) where women with higher weight gains gave birth to LGA infants (Ehrenberg HM *et al.*, 2004).

Prepregnancy weight

"The potential growth of a fetus seems to be limited by maternal size" (Osrin D *et al.*, 2000). In fact, environmental and genetic determinants affect maternal prepregnancy weight (Kramer MS, 1987) and consequently, prepregnancy maternal weight affects the BW (Muslimatun S *et al.*, 2002; Luke B, 1998; Kirchengast SI *et al.*, 1998 a; Bernstein

PS *et al.,* 1997; Luke B, 1994; Arbuckle TE et al, 1989; Abrams BF *et al.,* 1986; Kleinman JC *et al.,* 1985) in a direct (Kramer MS, 1987 b), positive association (Kirchengast SI *et al.,* 1998 a; Kramer MS, 1987; Ounsted M et al, 1981; Stein Z *et al.,* 1975). The impact of every kg of maternal prepregnancy weight on BW is 9.5 g (Kramer MS, 1987). Some studies mention that the association between prepregnancy weight and BW is not that strong (as reviewed by Osrin D *et al.,* 2000) others contend it is a strong predictor of BW (Catalano PM *et al.,* 2001; Kirchengast S *et al.,* 1998 a; Nandi C *et al.,* 1992). Undeniably, pregnant women who have lower prepregnany weight usually give birth to lower weight infants (Kliegman RM *et al.,* 2001; Osrin D *et al.,* 2000; McFarlin BL, 1994; Nandi C *et al.,* 1992) and particularly underweight women (Cheng CJ *et al.,* 2004) are at more risk to deliver SGA (Cheng CJ *et al.,* 2004; Stewart PJ *et al.,* 1994) and LBW infant (McFarlin B L, 1994). On the other hand, women with significant heavier prepregnancy weights give birth to LGA infants (Ehrenberg HM *et al.,* 2004). Similarly, an increase in prepregnancy weight results in a heavier BW infant (Kirchengast S *et al.,* 1998 b; McFarlin BL, 1994). In fact, overweight women entering gestation rarely deliver LBW infants (Nandi C *et al.,* 1992) and obese women rarely give birth to SGA infants (Kliegman RM *et al.,* 2001); so, pregnant women deliver infants proportional to their sizes (Thomson AM, 1959). In addition, pregravid weight is associated with IUGR (Steward DK *et al.,* 2004). The relative risk of IUGR for a less than 49.5 kg of prepregnancy weight is 1.84 (Kramer MS, 1987).

Parity

Primiparity is more prevalent in developed countries than in developing countries (Kramer MS, 1987). It is well established that parity impacts the mean BW (Gardosi J *et al.,* 1992; Altman DG *et al.,* 1980) and the intrauterine growth (Miller HC, 1979) directly (Kramer MS, 1987 b). Moreover, the birth weights of subsequent pregnancies influence each other (Luke B, 1994). In fact, BW is significantly increased with increasing parity (Kramer MS, 1987) where later-born infants are heavier than first-born infants (Kliegman RM *et al.,* 2001; Miller HC, 1985). The sample-size weighted effect was determined as 43.4 g per birth (Kramer MS, 1987); on the other hand, a mean increase of 100 to 150 g in succeeding BW was found to be associated with parity (Thomson AM *et al.,* 1968).

This might be due to mothers subsequently having increased prepregnancy weights. There was a 23% additional risk for primiparous women to have IUGR compared to multiparous women according to data from 4 studies with a large sample size (Kramer MS, 1987). It has been shown that a poor obstetric history is also associated with the succeeding IUGR deliveries (Bernstein I *et al.,* 2002; Bernstein PS *et al.,* 1997). In fact, women with no history of LBW deliveries have a 2.75 less risk of delivering an infant with IUGR than women with previous LBW infants (Kramer MS, 1987). Similarly, it has been recently shown that a previous SGA birth has the utmost unadjusted risk for a successive SGA birth (Cheng CJ *et al.,* 2004). More precisely, a woman who previously had a SGA infant is eight times more at risk of having a SGA delivery than a woman who had a previous AGA infant (Scott A *et al.,* 1981). There is an ambiguous proof as for high parity having a meaningful independent detrimental impact on BW (Kramer MS, 1987); in fact, it has been shown that the impact of parity on BW is reduced with high parity (Catalano PM *et al.,* 2001). On the other hand, parity has been recently considered a risk factor for LGA infants (Ehrenberg HM *et al.,* 2004).

Infant gender

Fetal gender has been shown to be a strong predictor of BW (Muslimatun S *et al.,* 2002; Catalano PM *et al.,* 2001; Gardosi J *et al.,* 1992; Altman DG *et al.,* 1980) and IUGR (Miller HC *et al.,* 1979). It is a factor with a direct impact on fetal growth (Kramer MS, 1987 b). Compared to males, female infants are smaller (Miller HC, 1985), have lower birth weights (Catalano PM *et al.,* 2001; Kliegman RM *et al.,* 2001; Kramer MS, 1987; Miller HC, 1985) and a higher risk of being born IUGR (Kramer MS, 1987). It has been shown that female fetuses have a slower rate of growth than male fetuses (Luke B, 1994). After 28 weeks of gestation, the difference of weight becomes dominant (Kliegman RM *et al.,* 2001). The sex difference between males and females for the BW is 126.4g in industrialized countries, while it is 93.1g in developing countries. The relative risk for females is 1.19 (Kramer MS, 1987). On the other hand, male gender is considered a risk factor for LGA (Ehrenberg HM *et al.,* 2004). Recently conflicting results were found showing that male fetuses have a higher risk of being born IUGR (Steward DK *et al.,* 2004).

Maternal height

Maternal height (Gardosi J *et al.,* 1992; Altman DG *et al.,* 1980) and height adjusted for weight (Arbuckle TE et al; 1989 Kleinman JC *et al.,* 1985; Love EJ *et al.,* 1965) have been shown to affect the BW, in a direct (Kramer MS (b), 1987) and positive association (Kirchengast S *et al.,* 1998 a; Kramer Ms, 1987; Ounsted M et al, 1981). The impact of every centimeter of maternal height on BW is 7.8g (Kramer MS, 1987). In addition, maternal height might affect intrauterine growth (Bernstein PS *et al.,* 1997; Miller HC *et al.,* 1979) by either environmental or genetic processes (Kramer MS, 1987). They are negatively associated with a relative risk of 1.27 for less than 157.5 cm of height. If the prevalence of short stature is high in a population, this factor contributes to a high proportion of IUGR deliveries (Kramer MS, 1987).

Ethnicity

BW and fetal growth differ among various regions and populations (Frisbie WP *et al.,* 1997; Kramer Ms, 1987) where it has been shown that ethnicity has an impact on BW (Luke B, 1994). The weight variations between different ethnic groups are most likely due to genetic factors rather than social determinants (Brooke OG *et al.,* 1981). Interestingly, the ratios of LBW vary in the same racial group in various geographical regions (Kogan MD, 1995). Among all ethnic groups, blacks have the highest rate of LBW deliveries (Luke B, 1994). This might be in part dependent of socioeconomic factors (Kliegman RM *et al.,* 2001). It seems that North American whites and Europeans have higher birth weights than Blacks, Pakistanis and Indians; whilst North African Jews and North American Indians give birth usually to larger infants (Kramer MS, 1987). Specifically, white mothers have a 5.6% rate of giving birth to LBW infants, American Indians 6.1%, Asian 6.2% and Hispanic 6.2% compared to blacks having an incidence of 13% of LBW infants (Luke B, 1994). Those dissimilarities in growth and weight gain are shown in utero by ultrasound measurements (Gardosi J, 1995). A significant difference in mean birth weights exists between white and black infants (Kramer MS, 1987) where white infants are larger (Miller HC, 1985) and weigh heavier than black (Kramer MS, 1987; Miller HC, 1985) by 108g (Kramer MS, 1987). Kramer MS concluded that the relative risk for black infants is 1.39 (Kramer MS, 1987) while other sources mention that

the risk is about twice (Kliegman RM *et al.,* 2001; NCHS 1993; Kallan JE, 1993; Kramer MS, 1987; Institute of Medicine, 1985; Thomson AM, 1959) or 3 times that for blacks to deliver LBW infants than for whites. Among all races, the infant mortality was reduced during the past 2 decades, but the black-white disparity for infant mortality has enlarged (MMWR, 2002). In fact, the rate of LBW was reduced among white more than among black between 1973 and 1983 (Kleinman JC *et al.,* 1987). The black-white difference in BW is caused by unidentified maternal factors tightly linked to maternal ethnicity (Collins JW Jr *et al.,* 1993). Among the combination of the parental racial groups, it was shown that the mean BW progressively increases from both parents being black, to black mother and white father, to white mother and black father, to both parents white, and LBW decreases in the same order (Migone A *et al.,* 1991). The paternal and infant ethnicity do not impact the BW sequence of the infants whose mothers are white and fathers are black (Collins JW Jr *et al.,* 1993). In addition to the BW, the ratios of IUGR deliveries vary in a large extent by race (Horon IL *et al.,* 1983). Ethnicity impacts intrauterine growth (Bernstein PS *et al.,* 1997; Miller HC *et al.,* 1979) directly (Kramer MS, 1987 b). In fact, black fetuses have a slower rate of growth than white fetuses (Luke B, 1994). A higher proportion of IUGR is seen among the black community of the United States, among the indigenous people of Australia, and the Asians in the United Kingdom (Kramer MS, 1998), and among Asian American or African American (Steward DK *et al.,* 2004). The cause might be related to socioeconomic factors as well as to the biologic versus genetic differences (Kramer MS, 1998), behavior and health awareness (CDC, 1993). On the other hand, a low risk of LGA exists among the black race (Ehrenberg HM *et al.,* 2004).

Alcohol consumption

In developed countries the rate of alcohol consumption was higher than in developing countries despite its increase in the latter (Walsh B *et al.,* 1985). Eleven per cent of pregnant women consume alcohol (McFarlin BL, 1994; Pollack RN *et al.,* 1992). In North America, most studies show that 2-3% of pregnant women consume more than 2 drinks per day (Kramer MS, 1987). In Canada specifically, the percentage of women who consume alcohol during pregnancy is 17 % (Statistics Canada, 1998, as referred by

Perinatal Education Program of Eastern Ontario, 1998). Alcohol traverses the placenta freely (Driscoll C *et al.,* 1990). The growth inhibition is caused by reduced integration of amino acids into proteins (Abel EL 1982) or by fetal hypoxia (Pollack RN *et al.,* 1992; Mukherjee AB *et al.,* 1982) and acidosis (Pollack RN *et al.,* 1992). LBW has been shown to be significantly associated with maternal alcohol consumption, specifically at more than two drinks per day. A decrease of 155 g per weight was noticed for a consumption of more than 2 drinks of alcohol per day (Kramer MS, 1987). So a significant dose–response effect was established according to many studies reviewed by Kramer MS (Kramer MS, 1987). At less than this amount, some studies showed an impact on BW while others couldn't show any effect (Kramer MS, 1987). The impact of alcohol on BW can still be seen at 8 months of age, but is absent thereafter (Sampson PD *et al.,* 1994). On the other hand, a recent study has shown that maternal alcohol consumption is positively associated with BW (Grjibovski A *et al.,* 2004). On the other hand, it has been shown that women who binged anytime during gestation were not significantly more likely to deliver a SGA infant. However, due to small sample size, these estimates were not very precise (Whitehead N *et al.,* 2003). Heavy drinking, on the contrary was associated with increased risk of SGA (Whitehead N *et al.,* 2003). On the other hand, alcohol consumption has a direct impact on intrauterine growth (Kramer MS, 1987; Kramer MS, 1987 b) that might be negatively affected (Bernstein I *et al.,* 2002; Kramer MS, 1998; Manning FA, 1995; Luke B, 1994; Kramer MS, 1987), even if fetal alcohol syndrome is not fully present (Kiely JL *et al.,* 1994; Kramer MS, 1987). A dose-response relationship was also established as in the case of LBW risk. The existence of a safe "threshold" of alcohol consumption might not stand (Kramer MS, 1987). On the other hand, the 'J-shaped function' was suggested between alcohol intake and BW that shows a significant increase in BW with a small quantity of alcohol (Abel EL *et al.,* 1995). Indeed, newer studies show that mild drinking had a significant protective impact on IUGR in the first month of pregnancy (Lundsberg LS *et al.,* 1997). Moreover, moderate consumption of alcohol during pregnancy didn't reduce the BW, nor the weight at 5 years of age (O'Callaghan FV *et al.,* 2003). The Relative Risk of IUGR for more than 2 drinks per day is 1.78 (Kramer MS, 1987). The timing of alcohol intake during gestation is important; in fact, at a later stage of pregnancy the effect of alcohol might be more

considerable (Kramer M S, 1 987). Indeed, IUGR h as b een n oticed i n s econd a nd t hird trimester consumption of alcohol (Pollack RN *et al.,* 1992). Because of the low prevalence of exposure to alcohol, its etiologic fraction is not too high (Kramer MS, 1998). On the other hand, maternal alcohol effect on gestational age has no proven evidence (Kramer MS, 1987).

Caffeine consumption

During pregnancy, around 90% of women report consumption of caffeine (Fried PA *et al.,* 1987). During the second and third trimesters of pregnancy the rate of caffeine clearance is reduced (Aldridge A *et al.,* 1981). The impact of high caffeine intake during pregnancy is polemical (Kramer MS, 1998); some studies have shown that intake of caffeine during gestation is associated with LBW (McFarlin BL, 1994) whereas others have shown that coffee or caffeine consumption during pregnancy has no significant impact on intrauterine growth (Clausson B *et al.,* 2002; Mills JL *et al.,* 1993; Kramer MS, 1987; Fried PA *et al.,* 1987) or BW (Clausson B *et al.,* 2002; Perinatal Education Program of Eastern Ontario, 1998; Kramer MS, 1987). Furthermore, a study done by Mills JL *et al.,* in 1993, has shown that moderate consumption of caffeine might be safe (Mills JL *et al.,* 1993). Its relative risk is only weakly, if at all elevated (Fortier I *et al.,* 1993; Dlugosz L *et al.,* 1992; Martin TR *et al.,* 1987).

Maternal age

It has been determined that maternal age has an indirect impact on intrauterine growth (Kramer MS, 1987 b). Considering age an independent determinant of intrauterine growth has been controversial (Berendes HW *et al.,* 1991; Kramer MS, 1987). In reality, very few studies found an independent effect of maternal age on BW (Kirchengast S *et al.,* 1998 b; Nandi C *et al.,* 1992), intrauterine growth (Miller HC *et al.,* 1979) while others did not detect an age impact on either BW or intrauterine growth at all (Muslimatun S *et al.,* 2002; Catalano PM *et al.,* 2001; Kramer MS, 1987). Some studies agreed on the impact of age extremes (Johnstone F *et al.,* 1974) without setting clear age cut-offs (Luke B, 1994). Others have found that at less than 17 years and more than 34 years of age, there is an increased risk of having a LBW infant (Institute of Medicine

1985; Berendes HW *et al.,* 1991). Recently, a large study showed that mothers younger than 20 years old are at a higher risk of having a SGA infant (Cheng CJ *et al.,* 2004). Adolescent mothers were found to have increased risk for LBW (Slap GB *et al.,* 1989) and IUGR (Steward DK *et al.,* 2004; Luke B, 1994) because they are still in the process of growing up, while older women were at increased risk because of the high incidence of diseases at their age (Luke B, 1994).

Medication/multivitamin intake

Fetal growth is importantly determined by maternal nutrition (Catalano PM *et al.,* 2001) where poor nutrition is one of the causes of LBW (Ramakrishnan U *et al.,* 2003). Micronutrient deficiencies contribute to impaired growth, health, and development (Ladipo OA, 2000). It has been shown that upon supplementation with micronutrients, the birth weights of the infants improved increasing by 10% (Hininger I *et al.,* 2004). Furthermore, in a study done in the United States, it was suggested that the use of multiple vitamin and mineral supplements reduces the risk of preterm delivery and LBW (Scholl TO *et al.,* 1997). Moreover, a study done in Tanzania concluded that multivitamin supplementation dramatically reduced preterm delivery by 39% and LBW by 44% while the mean BW increased by 120 g (Fawzi WW *et al.,* 1998). On the other hand, a recent review done by Shah D *et al.* has shown that the approach of multiple micronutrient supplements should be more adequately tested to lead to more reliable results (Shah D *et al.,* 2004). The impact of many individual minerals and vitamins on fetal growth has been studied. Through increasing length of gestation, some micronutrients affect fetal growth like: folate, magnesium, calcium, vitamin A, long-chain n-3 fatty acids and iron (Luke B, 1994). However it has been concluded that there is no difference between multiple micronutrient supplementation and iron supplementation in infant birth size (Ramakrishnan U *et al.,* 2003). Particularly, there is no significant impact of iron on intrauterine growth (Kramer MS, 1987) and no additional BW (Muslimatun S *et al.,* 2002; Rasmussen K, 2001). It was hypothesized with no proof that iron deficiency causes LBW (Rasmussen KM *et al.,* 2003). No rigorous study was done in developing countries, where the prevalence of iron-deficiency anemia is high, to rule out the effect of iron supplementation (Kramer MS, 1987). On the other hand, in

well-nourished populations, there is no proof for the benefit of supplementing routine versus elective iron in pregnant women (De Onis M *et al.,* 1998). Hence, antenatal supplementation with iron has uncertain effects on fetal growth (Mahomed K, 1997 a, Mahomed K 1997 b). In addition to iron, antenatal supplementation with folate has uncertain effects on fetal growth (Mahomed K, 1997 a, Mahomed K 1997 b). Folic acid might play a role in intrauterine growth given that his micronutrient is important for DNA synthesis (Kramer MS, 1987). On the other hand, although there are many limitations to the studies done on folate supplementations, the results show a possible benefit in reducing the risk of LBW (De Onis M *et al.,* 1998). But still, the evidence of the major role of folic acid on intrauterine growth is weak (Kramer MS, 1987).

Amniotic Fluid

Amniotic fluid (AF) is important to the fetal health and development as it protects the infant and prevents mechanical and thermal shock (Finegan JA, 1984). Throughout gestation, the fluid enables normal development of the respiratory, gastrointestinal, urinary tracts of the fetus and its musculoskeletal system (Sherer DM, 2002). AF consists of 98% water and has analogous characteristics to the maternal serum in early pregnancy. It contains nutritional factors, assists in acid/base balance, and possesses antimicrobial activity (Finegan JA, 1984). The AF composition changes with different gestation periods (Gilbert WM *et al.,* 1993). This fluid originates from secretion or ultrafiltrate from the mother, the placenta and cord, and the fetus, and its origin varies with different gestational ages (McCarthy T *et al.,* 1978). The fetal trachea and lungs add to the AF composition. At the 11^{th} week, the fetus starts swallowing and producing urine. As the skin is not keratinized until the 26^{th} week, it allows the fluid to cross through the amniotic cavity. The turnover of the amniotic fluid is every 3 hours (Stewart CJ *et al.,* 2001).

Total Proteins

Quantity

In amniotic fluid of humans, the concentration of total protein appears to decrease with advancing gestation (Guibaud S *et al.,* 1973); it is at its maximum of 4.7 g/l around the 25th week of gestation and then decreases until 2.4g/l at delivery (Legras B *et al.,* 1978). From 16 to 41 weeks of gestation, the main factors influencing the concentration of AF proteins seem to be fetal urination and swallowing (Lolis D *et al.,* 1995). Fetal swallowing might be accompanied by fetal digestion of the protein (Gitlin D *et al.,* 1972).

Pattern

In normal pregnancies, the AF total protein pattern varies to a great extent in intensity, number, width and mobility of gel electrophoresis bands. The reason behind it might be their stage of development and inherent genetic make up. Different stages of gestation exhibit different protein patterns; specifically in late gestation where 14 to 17 bands of amniotic fluid proteins are usually seen. However, the pattern of total AF proteins has a simpler and lower number of bands than a normal adult (Roychoudhury A *et al.,* 1978).

Source

The source of the amniotic fluid proteins varies throughout the pregnancy. It has been shown that during the third trimester, the AF proteins are mainly of maternal origin (Sutcliffe RG *et al.,* 1973). Indeed, as early as 15 weeks of gestation, it has been proven that the proteins in the amniotic fluid are originally a simple filtrate of the maternal plasma protein (Johnson AM *et al.,* 1974). In early gestation, fetal serum proteins do not contribute largely to the amniotic fluid (Sutcliffe RG *et al.,* 1973), whereas it has been shown later on that during the first half of pregnancy, the main source of most of the proteins present in the amniotic fluid are the embryo and its yolk sac and subsequently the fetus (Jauniaux E *et al.,* 1994). Sutcliffe RG *et al.* concluded that at various gestational periods, the protein concentration in fetal and maternal sera is higher than in

amniotic fluid; as well as the gradient between the fetal serum and the amniotic fluid is lower than between the maternal serum and the fluid. In addition, this study established that the protein concentration in amniotic fluid and maternal serum are correlated (Sutcliffe RG *et al.,* 1973).

Clearance

Concerning the rate of clearance of the AF proteins, a study done on 19 women, at 34 to 40 weeks of gestation, concluded that labeled proteins are cleared from the amniotic fluid at similar rates, even though these proteins differ markedly in the molecular weights and metabolic functions. The protein turnover was 63% ± 5 (mean ± SEM) per day. Each protein that leaves the amniotic fluid is replaced by an identical amount of that protein entering that fluid, and, in the presence of a dead fetus, the AF protein is cleared at a very low rate (Gitlin D *et al.,* 1972).

Relative concentrations

Additional compartments in the maternal–fetal system contribute to the protein movement in pregnancy. Some conclusions have been obtained from various studies.
Total proteins have been found in higher concentrations in the maternal serum than in the exocoelomic and amniotic fluids (Gulbis B *et al.,* 1992). In a study done on 35 fetuses between 11 and 17 weeks of gestation, it was determined that the total protein concentration was higher in maternal as compared to fetal serum, and that these 2 concentrations were not correlated. Moreover, as pregnancy advances, total fetal serum protein concentration has been found to increase (Jauniaux E *et al.,* 2000). On the other hand, a transudate of maternal serum through the placental chorion forms the exocoelomic fluid with the exception of alpha-fetoprotein (Gulbis B *et al.,* 1992). With advancing gestation, the total protein concentration decreases in the maternal serum, while it increases in the exocoelomic cavity (Jauniaux E *et al.,* 1994). A non-permeable membrane separates the exocoelomic and amniotic fluid, and the protein patterns of the latter are not the same as those found in the amniotic fluid, in the same period of gestation (Gulbis B *et al.,* 1992). Another assertion is that during the first trimester,

changes in maternal serum protein concentration have no impact on the total protein concentration in the coelomic fluid as the total protein concentration in this compartment increases. Whereas the total proteins in both matched cavities of coelomic fluid and amniotic fluid are not correlated, their concentration is 54 times lower in the amniotic than in the coelomic cavity. In this same study, a significant decrease was noticed from maternal serum to the coelomic cavity and from the latter to the amniotic cavity in total protein concentration (in matched samples of coelomic and amniotic fluid) (Jauniaux E *et al.,* 1994). It has been concluded that the secondary yolk sac dispenses proteins into the exocoelomic fluid in early gestation (Gulbis B *et al.,* 1992). Besides, from 8 to 12 weeks of gestation, it has been shown that the yolk sac is an important channel for the high molecular weight proteins to reach the embryonic circulation. The yolk sac fluid contains higher total protein levels as compared to AF samples (Gulbis B *et al.,* 1998). A significant correlation between the cord blood and newborn blood protein concentrations has been established (Raubenstine DA *et al.,* 1990).

Proteins and fetal growth

Proteins have been proven to be associated with many outcomes related to the fetal development; most importantly, total AF protein from 12-20 weeks of gestation is negatively associated with BW at term (Tisi DK *et al.,* 2004). Moreover, IGF-BP 1 and IGF-2 are negatively associated with BW while IGF-BP3 is a positive predictor of BW (Tisi DK *et al.,* 2005). Additional conclusions have been drawn from other studies. In samples of amniotic fluid collected before 11 weeks of gestation, no evident trend was found between the concentration of total proteins and gestational age (Jauniaux E *et al.,* 1994), whereas, a difference in protein quantities was found between early and late gestation AF protein samples. Gestational age and the total protein concentration of maternal serum were negatively linearly correlated, while gestational age and the concentration of total proteins in the coelomic fluid were positively linearly correlated (Jauniaux E *et al.,* 1994). Additionally, gestational age and the concentration of fetal serum total protein were significantly positively linearly associated between 11 and 17 weeks of gestation (Jauniaux E *et al.,* 2000). In a study done on 21 newborns, it had been shown that the weight of newborns was correlated with cord and newborn protein mass

(Raubenstine DA *et al.,* 1990). From 20 blood measurements of newborns and pregnant women at delivery, it has been shown that maternal protein nutrition may have an effect on the fetus BW (Jain SK *et al.,* 1995); It is possible that, in this study, the acute protein nutrition status examined is a reflection of the chronic protein nutrition status, and that the association between acute protein nutrition and BW reported is due to the lower socio-economic status of these participants who were at the margin of protein deficiency (Jain SK *et al.,* 1995). Concerning the protein clearance implications, it has been determined that the BW of the infant has no apparent correlation with the protein clearance (Gitlin D *et al.,* 1972). It has been shown that fetal swallowing might be followed by fetal digestion of the protein (Gitlin D *et al.,* 1972). No relationship has been observed between fetal weight and absorption of proteins in clinical setting (Cheng W *et al.,* 1996), but recent literature has shown that the absence of fetal swallowing is associated with fetal growth retardation (Ross MG *et al.,* 2001). Additional information could be drawn from protein measurements: at delivery, no statistically significant difference was found between mothers of normal infants and mothers of IUGR in protein values (Nieto-Diaz A *et al.,* 1996).

Animal studies

Proteins and fetal growth have been studied in animals as well, leading to the following conclusions. In rats, the concentration of the total protein increases noticeably near term. Indeed, the concentrations of proteins increase in fetal plasma during gestation and slightly in extraembryonic fluid; the latter mirrors the changes of the protein pattern of the fetal plasma (Tam PP *et al.,* 1977), whereas in intestinal atresia chicks, the level of total serum proteins increases significantly, and the protein fractions change (Lopez de Torre B *et al.,* 1992). In fetal pigs, the concentration of the total proteins in the amniotic fluid is at its lowest at day 26 and increases to a maximum at day 58 before declining (McKenna P, 1984). In mice, with the exception of albumin, AF proteins are of fetal origin, and the yolk sac may be important for the synthesis of some proteins and their transfer (Renfree MB *et al.,* 1974).

Prealbumin

Chemistry and biology

Prealbumin, or human transthyretin, is a tetrameric protein of a molecular size of 50,000-70,000 Daltons. This protein is synthesized in the liver, and is rich in tryptophan. In the body, prealbumin transports thyroid hormones and retinol (vitamin A). The level of this protein decreases in hepatic damage, tissue necrosis, and acute inflammatory response. Prealbumin indicates the protein nutritional status and has a short half-life of 2 days (Lindsey BJ, 2005).

Quantity

Prealbumin concentration in the amniotic fluid decreases when gestational age advances (Lolis D *et al.,* 1995).

Relative concentrations

The levels of prealbumin have been measured and compared in various maternal and fetal compartments. In fetal blood, it has been shown that prealbumin levels decrease significantly as gestation progresses (Fryer AA *et al.,* 1993). From 25 to 42 weeks of gestation, prealbumin levels in cord blood increase with increasing gestational age (Sasanow SR *et al.,* 1986). In the maternal serum, the mean prealbumin concentration is 1.14 +- 0.2 g/l (Jauniaux E *et al.,* 1994). As a matter of fact, maternal plasma concentrations are significantly higher compared with respective plasma transthyretin concentrations from the cord blood of the newborns; however the two are significantly correlated (Jain SK *et al.,* 1995). Specifically, maternal values are almost half serum prealbumin concentrations in cord blood (Sasanow SR *et al.,* 1986). On the other hand, during the first trimester, prealbumin levels increase in the coleomic fluid (Jauniaux E *et al.,* 1994). Besides, prealbumin levels in the coelomic fluid and yolk sac volume are correlated (Jauniaux E *et al.,* 1994). Between 7 and 14 weeks gestation, no correlation was found between prealbumin values in maternal serum and those in coelomic fluid; this shows that prealbumin rate of transfer might occur independently of its level at each side

of the placenta (Jauniaux E *et al.,* 1994). From 20 blood measurements of newborns and pregnant women at delivery, it was shown that prealbumin is transferred through the placenta (Jain SK *et al.,* 1995) and between 26 and 42 weeks of gestation, prealbumin transfer does not occur actively (Sasanow SR *et al.,* 1986). On the other hand, from blood measurements of 28 very low birth weight infants (VLBW) aged 19 days, it has been shown that, at different nutritional regimens, it is the protein and not the total energy intake that mainly contributes to the changes in the levels of plasma transthyretin (Polberger SK *et al.,* 1990).

Prealbumin and fetal growth

Prealbumin values in different maternal and fetal compartments have been linked to many gestation outcomes. Among them we can conclude most importantly that the newborn BW correlates with the maternal and fetal plasma transthyretin concentrations (Jain SK *et al.,* 1995). As well, the fetal plasma levels of this protein and the weight growth of the AGA VLBW infants are strongly correlated (Polberger SK *et al.,* 1990). Whereas, in premature infants, the serum prealbumin correlates poorly with growth, measured as changes in weight, length, and head circumference (Moskowitz SR *et al.,* 1983).

Between subgroups of mothers presenting with various serum prealbumin values, there was no significant difference for the protein concentration, crown-rump length, and yolk sac volume in the coelomic fluid. The gestational age and the maternal serum concentration of prealbumin were negatively linearly correlated (Jauniaux E *et al.,* 1994). As well, from blood measurements of 21 newborns, it has been found that the newborn serum prealbumin is correlated with the gestational age, weight, length of the infant (Raubenstine DA *et al.,* 1990). Besides, Lolis et al. stated that prealbumin values during pregnancy are inversely correlated with gestational age (Lolis D *et al.,* 1995), while others concluded that gestational age and prealbumin concentration in the coelomic fluid are positively linearly correlated (Jauniaux E *et al.,* 1994). Measurements on the cord prealbumin level concluded that this protein level is significantly different between the pre and term groups (Warner A *et al.,* 1990). More specifically, this level is significantly greater in the term and postterm AGA infant than in the more immature infants (Sasanow

SR *et al.,* 1986; Pittard WB 3rd *et al.,* 1985). Moreover, cord blood prealbumin level is significantly lower in SGA than in LGA and AGA neonates. In addition, this study proved that cord prealbumin could be a useful marker for differentiating newborn infants who are small or large for dates on a constitutional basis versus those whose LGA or SGA status is related to nutrition (Pittard WB 3rd *et al.,* 1985). Besides, in premature AGA infants with a BW of 1000 g or less, serum prealbumin level is the most sensitive indicator of nutritional intake, and in detecting protein-calorie deficits in premature AGA infants (Moskowitz SR *et al.,* 1983). As a matter of fact, serum prealbumin and protein intake are strongly positively correlated in VLBW (Polberger SK *et al.,* 1990; Moskowitz SR *et al.,* 1983) so this protein could indicate protein nutritional status in VLBW infants (Polberger SK *et al.,* 1990). But interestingly enough, prealbumin is not considered as a potential maturity marker (Warner A *et al.,* 1990). In specific cases, like maternal hypertension, it has been determined that there is a significantly lower prealbumin level in the cord blood of their fetuses than in the cord blood of the AGA neonates whose mothers are normotensive. However, the cord blood prealbumin level is not affected by maternal hypertension enough to distinguish SGA infants of hypertensive mothers from normotensive mothers at delivery (Pittard WB 3rd *et al.,* 1985). Interestingly enough, from amniotic fluid measurements at 16-41 weeks of gestation, it has been found that AF prealbumin, when expressed as a ratio of the total protein content, is considered as a potential biochemical marker for certain complications of pregnancy and fetal defects, but it is not specific for a particular disorder (Lolis D *et al.,* 1995). Actually, higher values of AF prealbumin have been observed during fetal abnormalities. Moreover, in SGA fetuses, in the second trimester, high amniotic fluid prealbumin levels are found (Lolis D *et al.,* 1995). In premature infants, the degree of prealbumin synthesis might be a restricting factor in the transport of vitamin A (Sasanow SR *et al.,* 1986).

Albumin

Chemistry and biology

Albumin is a globular protein of a molecular size of 69,000 Daltons, spherical and highly soluble, consisting of a single polypeptide chain of 585 amino acids. Albumin is

very flexible and changes shape readily with binding of ligands and with variations in environmental conditions. In the human body, albumin has several functions. One of its major functions is maintaining the colloid oncotic pressure (COP), and the regulation of the tissue fluid distribution. Another albumin function is binding and transport of compounds, like drugs and endogenous compounds; bilirubin, haematin, ascorbate, tryptophan, bile acids, copper, zinc, folate, and eicosanoids. Additional functions of albumin are its anticoagulant effects, acting as a plasma buffer, having antioxidant properties. Furthermore, albumin is considered as a negative acute-phase protein, as it decreases by 25% during physiologic stress (Fuhrman et al, 2004). Albumin synthesis in humans takes place only in the liver, and it is released into the portal circulation as soon as it is manufactured. The rate of synthesis varies with nutritional and disease status. Synthesis responds to COP variations (Fuhrman et al, 2004). The degradation of the albumin is 5 % of the daily whole protein turnover, which adds to the amino acid pool in the cells and plasma (Sutcliffe RG *et al.,* 1973).

Quantity

In the amniotic fluid a large proportion of the protein concentration in the amniotic fluid is albumin (Sutcliffe RG *et al.,* 1973). Indeed, at 34 to 40 weeks of gestation, albumin is half of the soluble protein content in the amniotic fluid, and, consequently, is half of the total AF soluble protein turnover (Gitlin D *et al.,* 1972). From 10 to 20 weeks of gestation, the AF albumin level increases linearly, then stabilizes, subsequently decreases rapidly between 30 weeks and term (Sutcliffe RG et al., 1973). More precisely, it reaches maximum around the 25^{th} week, with a concentration of 2.8 g/l and then decreases until delivery to 1.24g/l along with the total proteins (Legras B et. al, 1978). Benzie et al. reported the amniotic fluid albumin to be 3±2 g/l (Benzie RJ *et al.,* 1974). Newer sources have established by spectrophotometry the albumin concentration in the amniotic fluid to be 3.9 g/l from 16 to 22 weeks of gestation (Adama van Scheltema *et al.,* 2005).

Pattern

Albumin is among the proteins that exhibit maximum variability in human AF of normal pregnancies. Albumin pattern increases in mobility as gestation advances (Roychoudhury A *et al.,* 1978). Besides, the albumin band decreases while all electrophoretic bands increase with advancing gestation (Gulbis B *et al.,* 1992).

Source

At different stages of gestation, amniotic fluid albumin is mainly of maternal origin (Sutcliffe RG *et al.,* 1973) but the fetus contributes to some of the AF albumin. As a matter of fact, in a study done on amniotic fluid of 19 women, at 34 to 40 weeks of gestation, it has been determined that less than 5% of the albumin present in the amniotic fluid is supplemented by fetal urine. Moreover, albumin is almost half of the AF soluble protein swallowed and digested by the fetus (Gitlin D *et al.,* 1972).

Relative concentrations

Many studies have looked into additional compartments in the maternal–fetal system that contribute to the albumin values in pregnancy. Some conclusions can be made from plasma measurements of 22 human fetuses between 13 and 22 weeks of gestation and 66 infants born between 24 and 41 weeks of gestation, it was shown that albumin is the major plasma protein during pregnancy. Besides, as gestation progresses, the ratio of albumin to total protein in the plasma has been determined to increase significantly (Fryer AA *et al.,* 1993). From maternal blood and coelomic fluid measurements between 7 and 14 weeks gestation, no correlation was found between albumin values in maternal serum and those in the coelomic fluid; this shows that the albumin rate of transfer might occur independently of its level at each side of the placenta (Jauniaux E *et al.,* 1994). In a study of 11 pregnant women, it was concluded that albumin is present in the yolk sac from 8-12 weeks of gestation (Gulbis B *et al.,* 1998). During the first trimester, there was not much variation in the albumin concentrations in coelomic fluid (Jauniaux E *et al.,* 1994).

Albumin and pregnancy outcomes

Albumin has been shown to be associated with many outcomes related to fetal development. Although the gestational age and the albumin level in the coelomic fluid and the total protein in samples of amniotic fluid collected before 11 weeks of gestation show no noticeable trend between them, it has been determined that the gestational age and the maternal serum concentration of albumin are negatively linearly correlated (Jauniaux E *et al.,* 1994). A correlation was found between albumin newborn serum protein masses and the gestational age, the weight and length of the infants (Raubenstine DA *et al.,* 1990). A research done on 45 IUGR infants concluded that the cord blood albumin and the birth length of IUGR infants are positively associated. This same study proved that the IUGR fetus would be hypoalbuminemic (Nieto-Diaz A *et al.,* 1996). In one case-study, it has been proven from measurements of amniotic fluid at 34 weeks of gestation that the fetal intestinal tract abnormalities can be diagnosed early by protein electrophoresis of abnormal albumin bands in the amniotic fluid (Stefos T *et al.,* 1993).

Animal studies

In addition to human samples, albumin and fetal growth were studied in animal models. In pigs, in the first 2/3 of gestation (day 26 to 74, term is 114 days), it has been concluded that albumin is one of the major proteins of the amniotic fluid but not of the allantoic fluid. This protein was detected at day 38 and rose gradually through gestation to reach a peak at the end of the study (McKenna P, 1984). In mice, the amniotic fluid proteins, except albumin, are of fetal origin (Renfree MB *et al.,* 1974). Studies in the chick embryo showed that the majority of amniotic fluid protein in the intestinal atresia chicks is egg-albumin, and it decreases significantly in those chicks (Lopez de Torre B *et al.,* 1992).

Transferrin

Chemistry and biology

Transferrin is a glycoprotein synthesized in the liver. It has a molecular size of 76,000 Daltons (Rand ML *et al.,* 2000). It consists of a single polypeptide chain of 679 amnio acid residues. This molecule can be divided into two globular domains: the N-terminal and the C-terminal (De Jong G *et al.,* 1990). This protein is essentially synthesized in the liver and secreted into the plasma, but other cells have been shown to synthesize transferrin (Cassia *et al.,* 1997). The concentration of transferrin is increased during iron-deficiency anemia and pregnancy and decreases in hemocromatosis (De Jong G *et al.,* 1990). The major function of transferrin is transport and delivery of iron to target cells. Besides, this protein has a role in bacteriostasis, as well as in the growth and differentiation of cells (De Jong G *et al.,* 1990). In fact, from blood measurements of 113 pregnant women, 35 normal parturient women and umbilical blood, and in 30 samples of placental villous tissue, it has been shown that the iron bound to transferrin in the maternal blood is the main source of iron to the fetus (Okuyama T *et al.,* 1985). On the other hand, from blood measurements of 36 women at 7 to 13 weeks of gestation, 26 samples of coelomic fluid, 22 amniotic fluid samples, it has been concluded that iron and iron-binding protein levels between the coelomic fluid and maternal serum are non-significantly correlated (Gulbis B *et al.,* 1994).

Source

The trophoblast is the source and supplier of the transferrin in the human amniotic fluid (Jeschke U *et al.,* 2003). Mainly, amniotic fluid transferrin is of maternal origin (Sutcliffe RG *et al.,* 1973). From 34 to 40 weeks of gestation, it has been shown that less than 2% of the transferrin present in the amniotic fluid is supplemented by fetal urine (Gitlin D *et al.,* 1972).

Quantity

This protein is the major beta-globulin in amniotic fluid. It is constantly present at 6 months of gestation (Sutcliffe RG *et al.*, 1973). The transferrin concentration in the amniotic fluid reaches its maximum around the 25th week of gestation and then decreases until delivery with total proteins (Legras B *et al.*, 1978); specifically, from 7 to 13 weeks of gestation, transferrin concentration is less than 0.08g/l, and so, undetectable in amniotic fluid samples (Gulbis B *et al.*, 1994). Upon testing 40 samples from 18 to 40 weeks of gestation, using Mancini's simple radial diffusion method, polyacrylamide gel electrophoresis, and Ouchterlony's method of double gel diffusion, a noticeable variation was found between AF transferrin values from one sample to the other (0.015g/l to 0.604g/l) (Guibaud S *et al.*, 1973). Another study found that in the 3rd trimester, the concentration of AF transferrin is 0.151g/l and this value ranges between: 0.012 and 0.325 g/l (Bardos P *et al.*, 1976). Dissimilar values were found in a different study that used the single radial diffusion. It was found that from 16 to 42 weeks of gestation the range of AF transferrin is from 0.14 to 0.445 g/l and its mean value is 0.299g/l. Transferrin value didn't increase through the gestation period tested. This study was done on 52 samples, and the variations in AF transferrin level were not perceptible in the individual patient (Larsen B *et al.*, 1973). On the other hand, from 10 to 20 weeks, using antibody-antigen crossed electrophoresis, the AF transferrin level was shown to rise linearly, stabilize, then fall rapidly between 30 weeks and term (Sutcliffe RG *et al.*, 1973). The different techniques used in literature gave several AF transferrin values, with wide concentration ranges. Using capillary electrophoresis to measure AF transferrin will provide more sensitive readings and a more precise transferrin concentration.

Pattern

Transferrin doesn't vary significantly in the amniotic fluid in the third trimester (Bardos P *et al.*, 1976; Larsen B *et al.*, 1973) until term (Larsen B *et al.*, 1973). On the other hand, a study done on 66 samples concluded that transferrin is among the proteins that exhibit maximum variability in human AF of normal pregnancies while transferrin phenotypes didn't vary (Roychoudhury A *et al.*, 1978).

Relative concentrations

Transferrin is present in various compartments of the maternal-fetal system. Concerning their related levels, coelomic fluid has significantly lower transferrin levels as compared to maternal serum (Gulbis B *et al.,* 1994). Fetal plasma transferrin can be affected by some factors. Indeed, from blood measurements of 28 VLBW infants aged 19 days, it has been shown that, at different nutritional regimens, it is the protein and not the total energy intake that mainly contributes to the changes in the levels of plasma transferrin (Polberger SK *et al.,* 1990).

Transferrin and fetal growth

Certain fetal outcomes can be concluded from transferrin levels in various maternal-fetal departments: A correlation has been determined between transferrin newborn serum protein masses and the gestational age, weight, length of the infant (Raubenstine DA *et al.,* 1990). On the other hand, during the 2^{nd} and 3^{rd} trimester, the gestational age and the maternal serum concentrations of transferrin were not correlated (Gulbis B *et al.,* 1994). Besides, between 7 and 11 weeks gestation no correlation has been found between the level of transferrin in coelomic fluid and the gestational age (Gulbis B *et al.,* 1994). On the other hand, it has been shown that transferrin and the protein intake are positively correlated in VLBW infants (Polberger SK *et al.,* 1990). Interestingly enough, transferrin measurements can be suggestive of some specific disorders: from measurements of 69 AF samples, it has been found that the difference between transferrin values in the pathological population is significantly different from normal population. In addition, as transferrin increases to more than 18mg/100ml, the probability of iso-immunisation is high at 65% (Bardos P *et al.,* 1976). On the other hand, plasma transferrin and protein intake were determined to be strongly positively correlated therefore this protein can be an indicator of protein nutritional status in VLBW infants. Moreover, it has been concluded that the levels of transferrin and the weight growth of the VLBW fetuses were strongly correlated (Polberger SK *et al.,* 1990).

Animal studies

Some conclusions about transferrin and fetal growth can be drawn from animal studies. In amniotic and allantoic fluids measurements in the first two thirds of gestation of pigs (day 26 to 74, term is 114 days), it has been shown that transferrin is one of the major proteins in amniotic but not allantoic fluid, more precisely during the second trimester of gestation. This protein was detected in AF at day 26, at a concentration of 4.0 +-0.1mg/dl. This level increased to a maximum in mid-gestation and decreased in the third quarter of gestation (McKenna P, 1984). From a study on intestinal atresia chicks, it has been concluded that transferrin was among the major amniotic fluid proteins (Lopez de Torre B *et al.,* 1992).

Summary

As we can notice from this overall review, birth weight, be it LBW or macrosomia, is an important factor to take into consideration in clinical practice and in analyses of research data where one is trying to make a causal association. It might impact the health of the growing fetus, and affect the overall status of the infant at short or long term basis. Many maternal, fetal and placental factors have been determined as direct or indirect determinants of the BW. Gestational age, cigarette smoking, maternal height and prepregnancy weight, infant gender, parity, maternal weight gain, and ethnicity are proven to directly impact the BW. Other factors like alcohol, caffeine and vitamin intake are more controversial, where some studies conclude a direct effect and others do not. Amniotic fluid (AF) is a key component for the growth of the fetus, and its analytes might be associated with the fetal development. The growth of the fetus during pregnancy was studied in relation to the protein content of the maternal blood, the fetal, or the cord blood, in human and animal studies, whereas no association has been studied between the amniotic fluid content of albumin, transferrin and prealbumin and fetal growth. However, since as already mentioned, AF is an exudate of maternal plasma, whose protein levels are modulated by the growing fetus. Therefore there is reason to postulate that these same constituents (albumin, transferrin and prealbumin) can be postulated as fetal growth determinants when measured in AF in early gestation.

III. Statement of purpose

Rationale

Birth weight extremes engender risks for the growing infant (Chescheir NC *et al.,* 2004; Boulet SL *et al.,* 2003; Langer O, 2000; Grassi AE *et al.,* 2000; Zubrick SR *et al.,* 2000; Hack M, 1998). Amniotic fluid is a crucial element and vital medium for fetal growth (Finegan JA, 1984). It originates from the mother, the placenta and cord, or the fetus (McCarthy T *et al.,* 1978). Proteins have been associated with many outcomes related to the fetal development; most importantly, total AF proteins (Tisi DK *et al.,* 2004) and insulin growth factor binding protein 1 (IGF-BP1) and IGF-2 (Tisi DK *et al.,* 2005) were found to be negatively associated with BW, while IGF-BP3 were recognized as positive predictors of BW (Tisi DK *et al.,* 2005). This had lead to the assumption that other individual AF proteins might be associated with BW. Prealbumin, albumin and transferrin have been linked to fetal growth in studies mainly dealing with blood measurements (Nieto-Diaz A *et al.,* 1996; Jain SK *et al.,* 1995; Polberger SK *et al.,* 1990; Raubenstine DA *et al.,* 1990) but no study has dealt so far with the AF content of these proteins with regards to the BW. As a result, this study widely assumes that the AF makeup in the early gestational period might provide a new horizon for assessment of fetal development.

Hypothesis

The hypothesis of the study is that there will be an association between amniotic fluid albumin, prealbumin or transferrin in early gestation and the fetal growth.

Research Questions

1. Do albumin, transferrin or prealbumin concentrations in the amniotic fluid change by different birth weight categories?

2. Can any or all of these amniotic fluid proteins be associated with and predict the birth weight?

Objectives

The specific objectives of this study were two-fold:

1. To measure the albumin, transferrin and prealbumin concentrations in the amniotic fluid (AF) using capillary electrophoresis.
2. To establish if albumin, prealbumin, transferrin concentrations collected at the time of routine amniocentesis for genetic testing (12-19 weeks of gestation) are associated with infant birth weight, when categorized by percentile or gram weight classifications.

IV. Materials and Methods

Overview of Design

This prospective study aimed at defining the relationship between the amniotic fluid content of albumin, prealbumin or transferrin and fetal growth. Three hundred subjects were recruited from St Mary's Hospital center, Montreal, between 2000 and 2003, and were asked, via second signed consent, to give away the leftover, or discarded portion of their amniotic fluid, and to fill out a questionnaire. Amniotic fluid samples were analyzed using Capillary Electrophoresis. Two fetal growth categories to classify infant birth weight were used in this study: 1) the percentiles: small-for-gestational-age (SGA) < 10^{th} percentile, appropriate-for-gestational-age (AGA), between 10^{th} and 90^{th} percentile, and large-for-gestational-age (LGA)> 90^{th} percentile; 2) the gram weights: Low birth weight (LBW) < 2500 g, healthy between 2500 g and 4000 g, and macrosomic > 4000 g. Statistical analysis was done using SAS program Version 8 (Cary, NC), analysis of variance (ANOVA), analysis of covariance (ANCOVA) and multiple regressions. The maternal and fetal covariates needed for the multiple regression analysis were provided by the questionnaires and the medical chart reviews.

Ethical Considerations

The study was approved by the McGill Institutional Reviews Board though the Faculty of Medicine, and by St. Mary's Hospital center (Appendix A, B).

Subject confidentiality

Confidentiality was maintained by numerically coding the samples. Numbers were assigned to the subjects and they were used in place of names on all assessment materials. The list linking numbers with names and data was stored in locked and secure files in McGill University, Macdonald campus. The information obtained was accessible only to the research staff, and no subject was identified in any report of the project. The

staff contributing to the study was aware of the ethical issues associated with this research, with specific attention to confidentiality.

Potential risks to subjects

There are no known risks associated with participation in this study, since the physician doesn't extract additional fluid for the study purpose. The amount of the amniotic fluid used in our study was normally discarded after genetic testing. Contributing to better prenatal care eventually is a benefit for participating in the study.

Consent forms

Women undergoing amniocentesis had to sign two consent forms. The first one agreeing to undergo the amniocentesis with no third party involved. The second form was to give us - the third party - their authorization to collect the discarded portion of the amniotic fluid having no genetic abnormalities. By signing the consent form, permission was given to retrieve the normally discarded portion of the fluid from Montreal Children's Hospital. Consent Forms were filed together and kept in locked file cabinets (Appendix C, D)

Study Subjects

Subject recruitment

From 2000-2003, pregnant women undergoing routine amniocentesis at St Mary's Hospital Center in Montreal, Canada, were asked to be part of the study. Pregnant women were already scheduled to come on Thursday mornings to the hospital to undergo amniocentesis for genetic testing. Accordingly, every Thursday, approximately 10 pregnant women were approached, and usually most of them were successfully recruited. The author explained the study objectives to the participants face to face, replied to their questions, and clarified their concerns in four different languages (English, French, Arabic and Spanish). If the woman agreed to participate, she had to fill out a questionnaire and sign a consent form. The questionnaires were written in both English

and French. When necessary, translation was provided by the author to Spanish or Arabic. Additional language translations were provided by the accompanier of the pregnant woman. The questionnaires were filled out either by the woman, by the author or by her accompanier if she had problems with the language. But the woman herself signed the consent form. The AF samples to be tested were taken by the mothers to the Montreal Children's Hospital. After completion of genetic testing, we were allowed to get the leftover portion of the normal AF of each mother who would have signed the consent forms.

Inclusion and exclusion criteria

The participant had to have a singleton pregnancy, a normal gestation, and no fetal complications. Pregnant women having pregnancy complications, like gestational diabetes, or fetal complications, like abnormal fetuses with chromosome abnormalities, or multiple pregnancies were excluded from the study on the basis that these factors may interfere with the results of the study. Post-hoc exclusions were made based on the following: a birth weight of less than 1000g, genetic abnormalities revealed by amniocentesis, a maternal disease, discovery of a multiple pregnancy, miscarriages, abortion, samples mishandled, or lost or in very low quantity for biochemical analysis, samples whose charts are missing or incomplete were excluded from the study.

Sample size estimate

To estimate the sample size, the hypothesis had to be specified. The null hypothesis of our study was "There is no association between amniotic fluid albumin, prealbumin, transferrin and fetal growth." The study would be analyzed using ANOVA followed by Scheffe's multiple comparisons test. To determine the effect size, which is the strength or magnitude of the difference between two sets of data and the standard deviation, results of prior related studies were consulted (Tisi DK *et al.*, 2004). The effect size was equal to 4.0-3.8 = 0.2. The standard deviation of protein content in amniotic fluid was 0.2. Given that the standardized effect size is equal to effect size / standard deviation, the calculated standardized effect was equal to 1. Levels of α and β needed to

be set up for estimation of the sample size. Given that it is possible for the amniotic fluid samples to have a level of individual proteins either higher or lower than the average, we chose to use a two-sided α of 0.05 and β of 0.2, a power of 80%. In order to have a standardized effect size of 0.2 and α (two-sided) = 0.05; β = 0.2, 17 subjects were required for this subgroup of SGA (Warren SB *et al.*, 2001). Since SGA represents 8% of the Canadian population (Canadian Perinatal Health Report, 2003), so to find 17 subjects in this subgroup we needed to collect 212 samples.

We collected 294 amniotic fluid (AF) samples to obtain the percentage desired. Due to specific instrumental errors such as blockage of the capillary or current failure, some samples were lost. Electropherograms were collected for 279 AF samples. Of these we kept only the samples whose variation in duplicates was < ±2sd. This resulted in 224 AF samples to be used in the statistical analyses. Due to some missing medical information regarding maternal or infant characteristics in the medical charts, only 196 AF samples with complete information were analyzed statistically.

Measured Variables

Main predictor variable

The main biochemical predictor variables were amniotic fluid transferrin, albumin, and prealbumin.

Outcome variable

The birth weight of the infants was the outcome variable we measured. Two fetal growth classification systems were used; the first one is small-for-gestational-age (SGA) < 10^{th} percentile, appropriate-for-gestational-age (AGA), between 10^{th} and 90^{th} percentile, and large-for-gestational-age (LGA)> 90^{th} percentile. The second one is low birth weight (LBW) < 2500 g, and macrosomic > 4000 g, with healthy birth weights lying in between these two extremes.

Potential confounding variables of infant birth weight

The factors we controlled for were: maternal height, pre-pregnancy weight, maternal weight gain, smoking status, gestational age, infant gender, parity, and ethnic background. Since all of those determinants are known to affect the birth weight based on the results of previous research (Kramer MS, 1987), they were included in every statistical model. Amniocentesis week was also included in our models since through our analysis, this factor turned out to modify our biochemicals studied.

This data was obtained from one-page questionnaires that were given to the mothers undergoing the amniocentesis to get the maternal characteristics. The questionnaires included maternal characteristics: maternal pre-pregnancy weight, height, age, smoking status, amniocentesis week, parity, multivitamin and/or medication use, alcohol and coffee/tea intake; mother's and father's ethnicity was asked too. The alcohol and coffee/tea consumption were recorded by drinks per week using four categories (0-1, 2-5, 6-10, or 11-15 drinks). The smoking status was assessed in three categories as: currently smoking, and how much, quit during pregnancy or never smoked. The ethnic background was divided into: North American, South American, European, African, Middle Eastern, Asian and other. The body mass index (BMI) was organized into: Underweight = <18.5, Normal weight = 18.5-24.9, Overweight = 25-29.9, Obesity = BMI of 30 or greater (Health Canada, 2003). Infant characteristics like infant gender, birth weight, gestational age and some maternal characteristics like the maternal age, pre-pregnancy weight, height, parity, were self-reported and verified from medical chart review. The birth weight was measured in two ways: in percentiles (new methodology) (Kramer MS *et al.,* 2001), and in g/wt (traditional methodology) (Vandenbosche RC *et al.,* 1998, Grassi AE *et al.,* 2000). The percentile method corrects for gender and gestational age. Percentile subcategories were divided in three categories: small-for-gestational-age, SGA <10%, average-for-gestational-age, AGA (10-90%), and large-for-gestational-age, LGA >90% (Kramer MS *et al.,* 2001). Percentile rankings are more precise since they correct for gender and gestational age.

Ultrasound and Amniocentesis Measurements

Ultrasound

Gestational age was based on ultrasound measurements. Ultasound examinations are the most common method for the evaluation of fetal growth and growth disturbances (Little D *et al.,* 1982; Campbell S, 1974). Estimation of fetal weight by ultrasound consists of different fetal measurements. The simplest methods that give reasonably accurate results are based on two measurements:1) abdominal circumference (AC) and biparietal diameter (BPD) (Warsof SL *et al.,* 1982; Shepard MJ *et al.,* 1982) or 2) AC and femur length (FL) (Campbell WA *et al.,* 1985; Hadlock FP *et al.,* 1984). The fetal size is estimated by combining these parameters into formulae. Although many formulaes have been published, the ideal one has not been found yet (Schwartz J *et al.,* 2003). However, the best single ultrasound measurement of fetal size is the AC (Gallivan S.*et al.,* 1993). At St Mary's Hospital Center, the measurements used were the standard ones used worldwide: the BPD, FL, head circumference and AC measurements. Serial measurements of fetal parameters should be made to diagnose an IUGR infant, instead of single measurements (Leung KY *et al.,* 1998). In addition, detecting IUGR accurately depends on precise estimation of gestational age. If the menstrual cycles are irregular or the last menstrual date is imprecise, ultrasound examinations are obligatory (Leung KY *et al.,* 1998). Interestingly, 85% of growth-retarded fetuses were detected by AC ultrasound measurement around the 34^{th} week of gestation (Pearce JM *et al.,* 1983). Knowing that antenatal detection of growth-restricted fetuses by routine ultrasonography shows varying rates of detection and false-positive results (Chang *et al.,* 1992; Larsen *et al.,* 1992). De Jong et al. have determined that the 10^{th} fetal weight percentile is a good cut-off limit for predicting perinatal problems caused by fetal growth restriction (De Jong CL *et al.,* 2000). At St Mary's Hospital center, this same cut-off is used to detect SGA or IUGR, and the 90^{th} fetal weight percentile is used to detect the LGA or macrosomic infant.

Amniocentesis

Amniocentesis is a diagnostic procedure which, using ultrasound as a guide, is performed by inserting a hollow needle through the abdominal wall into the uterus and withdrawing a small amount of fluid from the sac surrounding the fetus. This procedure is usually used to detect genetic disorders. Amniocentesis has been associated with some drawbacks. Indeed, women having second trimester genetic amniocentesis were prone to an increased risk of delivering preterm infants (Medda E *et al.,* 2003). Genetic amniocentesis results in mild pain and discomfort (Harris A *et al.,* 2004). Some factors like a previous amniocentesis, insertion of the needle in the lower uterus, a history of menstrual cramps, and increased maternal anxiety were shown to increase the pain of the procedure (Harris A *et al.,* 2004). On the other hand, "mid-trimester amniocentesis remains the safest invasive procedure" (Jauniaux E *et al.,* 2000). Furthermore, second-trimester procedure is considered a safe procedure, with a risk of pregnancy loss of 1.3% (Marthin T *et al.,* 1997). In addition, in a study done on 562 amniocentesis procedures in the third-trimester, the complication rate was even lower, at 0.7%; the complications mainly consisted of placental abruption, spontaneous labor in a preterm gestation, fetal-maternal hemorrhage, and premature rupture of the membranes. The overall conclusion was that this third-trimester procedure has a "high success rate and low risk of complications" (Gordon MC *et al.,* 2002).

Collection of Amniotic Fluid Samples

After undergoing the routine amniocentesis at St. Mary's Hospital Center, the pregnant women took their amniotic fluid samples to the Montreal Children Hospital Center (MCH) for genetic testing. Collection of amniotic fluid samples for which consent was obtained, was completed every month from the MCH. The length of time until the samples reached the hospital and the temperature in which they were transported were not easy to control. After being transported in a cooler full of dry ice, the AF samples were directly put in the -80 ° C freezer in the laboratories. The volume of individual samples ranged between 5 and 20 ml, depending on the volume taken at amniocentesis.

Those samples were arranged by name of subject and amniocentesis date. The stability of the AF samples was tested in the lab as will be shown in the upcoming sections.

Biochemical Assessment

The biochemical assessment of the amniotic fluid samples was done at Concordia University - Loyola Campus in Montreal, Canada. The machine used was a MDQ Capillary Electrophoresis (Beckman-Coulter, Mississanga, Ontario).

Capillary electrophoresis (CE)

Electrophoresis is an important analytical separation technique for biological compounds such as proteins (Janson JC *et al.,* 1998). Electrophoresis stands for the process in which a molecule with a net charge moves in presence of an electric field (Stryer *et al.,* 1995). Electrophoresis consists of five elements: the driving force, or electric field, the support medium, the buffer, the sample, and the detecting system (Wu AH, 2005). Capillary electrophoresis (CE) includes several analysis methods based on separation executed in capillaries. Capillary zone electrophoresis (CZE) is a mode of CE where ionic compounds separate into discrete bands where each analyte mobility is different from the others (Weinberger R, 2000 a). In CZE, the capillary is filled with an aqueous buffer (Wu AH, 2005). Electrophoretic mobility is the essential factor governing CZE, and the buffer pH is the main experimental variable (Weinberger R, 2000 a). Electro-osmotic flow (EOF) is the essential CE concept. This is the bulk flow of liquid in the capillary toward the cathode upon application of an electric field (Wu AH, 2005). Usually all ions will migrate with the EOF to the detector end yet with different mobility depending on charges and sizes (Wu AH, 2005). CE is applied in areas were the separation of biologically and biochemically important samples necessitate a microseparation technique that guarantees high selectivity and high efficiency. CE is usually used for ions, small molecules and biomacromolecules (Shen Z *et al.,* 2002). It is a powerful seperation technique (Bergman T, 1993; Bergman T *et al.,* 1991) which provides low buffer consumption, very small sample volume consumption (Bergman T, 1993; Bergman T *et al.,* 1991), good resolution, and short analysis time (Ummadi M *et*

al., 2002; Stewart CJ *et al.,* 2001; Bergman T, 1993; Bergman T *et al.,* 1991). Amniotic fluid analysis is usually difficult to achieve in ordinary techniques since the volume of fluid required is very large. CE offers the advantage of requiring minute amounts of sample. Detection of proteins in amniotic fluid using CE was done for the first time in 2001 (Stewart CJ *et al.,* 2001).

This method has some limitations when used for protein separation: proteins are large amphoteric molecules (Wu AH, 2005; Janson JC *et al.,* 1998) that stick to the capillary surface, which might lead to tailing, irreproducibility of separations (Weinberger R, 2000 a), and band broadening (Weinberger R, 2000 a; Janson JC *et al.,* 1998), even, at times, to loss of the polypeptides due to adsorption (Janson JC *et al.,* 1998). Capillaries we used were made of fused silica that permits high separation voltages, convenient detection in the ultraviolet and effective heat removal (Janson JC *et al.,* 1998). On the other hand, the capillary wall has a negative charge due to silanol ionization at most pH values (Weinberger R, 2000 a). When separation of a protein occurs below the pI, this phenomenon leads to a cationic solute that ion-pairs to the capillary surface (Weinberger R, 2000 a) resulting in protein adsorption and poor separation. To circumvent this problem, most CZE separations are carried-out at elevated pH's. The more the pH differs from the pI, the faster the protein will move in the electric field (Wu AH, 2005). Throughout this study, we used procedures and reagents to minimize protein adsorption and provide a high resolution separation (Table 1).

Amniotic fluid CE analysis procedure

Every morning, the instrument was prepared for data collection by first warming-up the instrument, cleaning the capillary and running a standard pooled sample. The instrument was warmed-up by igniting the lamp prior to running the cleaning method to ensure stable baselines by the time the first sample was run. The platinum electrodes were cleaned with ethanol. Subsequently, a cleaning method (Table 2) was run to wash the capillary of any adsorbed residues. Sodium dodecyl sulfate (SDS) is a known efficient decontaminating agent for capillaries used in bioanalytical separations. Indeed, SDS cleans the walls of the capillary and dissolves absorbed proteins (Lloyd *et al.,* 1995).

NaOH (0.1M) is also frequently used to rinse the capillary between runs (Mandrup G, 1992) as a reducing agent to expose the SiO^- groups and ensure a consistent EOF.

The separation buffer was a 75 mM borate buffer. This buffer has a low conductivity, and gives the best resolution with regards to peak shape (Stewart CJ *et al.,* 2001). The pH was adjusted to 9.27 (around 9.3 mentioned by Weinberger R, 2000 a; and 9 by Stewart CJ *et al.,* 2001), and 0.8 mM EDTA- ethylenediamine tetra-acetic acid-was added to it. EDTA was added to inactivate enzymatic action.

The internal standard used was thiamin, or vitamin B_1, at a concentration of 0.5mg/ml in distilled water. According to literature, dilution of AF in water gives the best resolution (Stewart CJ *et al.,* 2001), accordingly, a 1:1 dilution of the samples in thiamin solution was done to reduce the salt content of the samples while simultaneously introducing the internal standard. In order to minimize protein denaturation, AF samples were thawed on wet ice before introduction of the internal standard and the solutions were mixed gently with the pipettor. The AF separations were carried out at 28 C using a voltage of 20 kV with a fault current limit of 300 μA. The capillary was 60 cm long (effective length of 50 cm), an internal diameter of 75 μm and an external diameter of 365 μm. The reagents used were: SDS (sodium dodecyl sulfate), borate (sodium tetraborate decahydrate), EDTA (Ethylenediamine tetracetic acid), thiamin (vitamin B_1), NaOH (sodium hydroxide), described in Table 3. All the solutions were filtered through a 0.45 μm filter and kept in a 4C fridge.

Detection wavelengths

Concerning the detection mode, we used UV detection with a photodiode array (PDA) detector that permits the acquisition of full spectra data from the UV into the visible. Peak integration was done at 190, 254 and 275 nm with the PDA detector data. Detection of proteins is best at 200 nm (Weinberger R, 2000 a), and 195 nm is a sensitive wavelength for protein determination in a biofluid (Stewart CJ *et al.,* 2001). The larger wavelengths are usually used to detect polynucleotides and the aromatic amino acids in the proteins (Weinberger R, 2000 a).

Modifications to methodology procedures

Throughout the analysis, we had to do some modifications to the methodology. During routine AF sample analyses, we encountered the problem of persistent current failure. This was interrupting the on-going analysis and the sample in question had to be repeated. One possible cause of the problem was that the current failure might be due to the blockage of capillary by particles. Filtering the solutions, SDS, NaOH, thiamin and buffer, using a 0.45 μm syringe filter (Stewart CJ *et al.,* 2001), was first tried. But this attempt didn't provide any positive results; therefore, the second try was to degas the buffer. The justification to this is that the temperature of the buffer increases when the current is applied in the capillary. If there is gas disolved in the buffer the increased temperature leads to the formation of gas bubbles. This might explain the repetitive current failure. This procedure was done by warming up the buffer and degassing by bubbling inert nitrogen gas into it. This degassing step was added to the analysis preparation procedure. This procedure gave satisfactory results, since very few current failures were observed after implementing it.

Table 1

***Capillary electrophoresis steps*[1]**

EVENT	TIME	DESCRIPTION	
Pressure rinse	1 min	SDS rinse 5 mM	1379 mBar
Wait	0.5 min	SDS soak	
Pressure rinse	1 min	NaOH rinse 100 mM	1379 mBar
Wait	0.5 min	NaOH soak	
Pressure rinse	2 min	Buffer rinse	1379 mBar
Pre-separation	1 min	Equilibration	25 Kv
Pressure injection	10 sec	Sample injection	34.5 mBar
Wait	0.01 min	Buffer electrode rinse	
Separation[2]	20 min	Separation	20 kV

[1] SDS and NaOH were used to clean and rinse the capillary walls

[2]The separation of each AF sample took 20 min

Table 2

Washing method for capillary conditioning[1]

EVENT	TIME	DESCRIPTION	
Rinse Pressure	3 min	5mM SDS	20 PSI
	2 min	5mM SDS	
Rinse Pressure	3 min	100 mM NaOH	20 PSI
	2 min	100 mM NaOH	
Rinse Pressure	2 min	Borate (75mM) Buffer, 0.8mM sodium EDTA pH~9.25	20 PSI
Equilibration - Voltage	2 min	Borate (75mM) Buffer, 0.8mM sodium EDTA pH~9.25	

[1] SDS and NaOH were used to clean and rinse the capillary walls

Table 3

List of reagents

Reagent		Provider
SDS	Sodium dodecyl sulfate	Ultra pure from Schwarz/Mann biotech, Cleveland OH, USA
Borate	Sodium tetraborate decahydrate	Sigma Ultra, St. Louis MO, USA
EDTA	Ethylenediamine tetracetic acid	Disodium salt dihydrate, Sigma Ultra, approx 99% (Titration)
Thiamin	Vitamin B_1	Hydrochloride, Sigma , St. Louis MO, USA
NaOH	Sodium hydroxide	ACS Reagent Grade, ICN Biochemicals, Aurora OH, USA

Monitoring the machine variation

Pooled samples

A duplicate of AF pooled samples was run to check the machine variability. Pooled samples were prepared by mixing 1.0 ml of amniotic fluid samples taken from the MCH where genetic testing of amniotic fluid was done, from 11 mothers not included in the study. They were aliquotted into 10 µl volumes, and were mixed well to avoid settlement of layers in the vials. Pooled samples were analyzed the same way as regular AF samples. An equal volume of 0.5g/l thiamin solution was added to each sample before running it. Samples were run in duplicates. These were used as a reference and for monitoring machine problems. A pooled sample was injected at the beginning, the end of the day, and every 10 injections. This sample compared to previous injections in order to detect the occurrence of problems with the procedure or the equipment.

AF pooled sample transferrin had a mean of 0.32± 0.05 g/l (mean ± SD), while albumin had a mean of 3.24±1.13 g/l (mean ± SD). As noted from the scattered plots in Figure 3, where the squares represent the first runs of the day, and the triangles the runs during and at the end of the day, there was no remarkable difference between the runs done at the beginning of the days, and the rest of the day. Therefore, the CE was running efficiently, and the large variation noticed in AF albumin pooled samples is not due to machine variability.

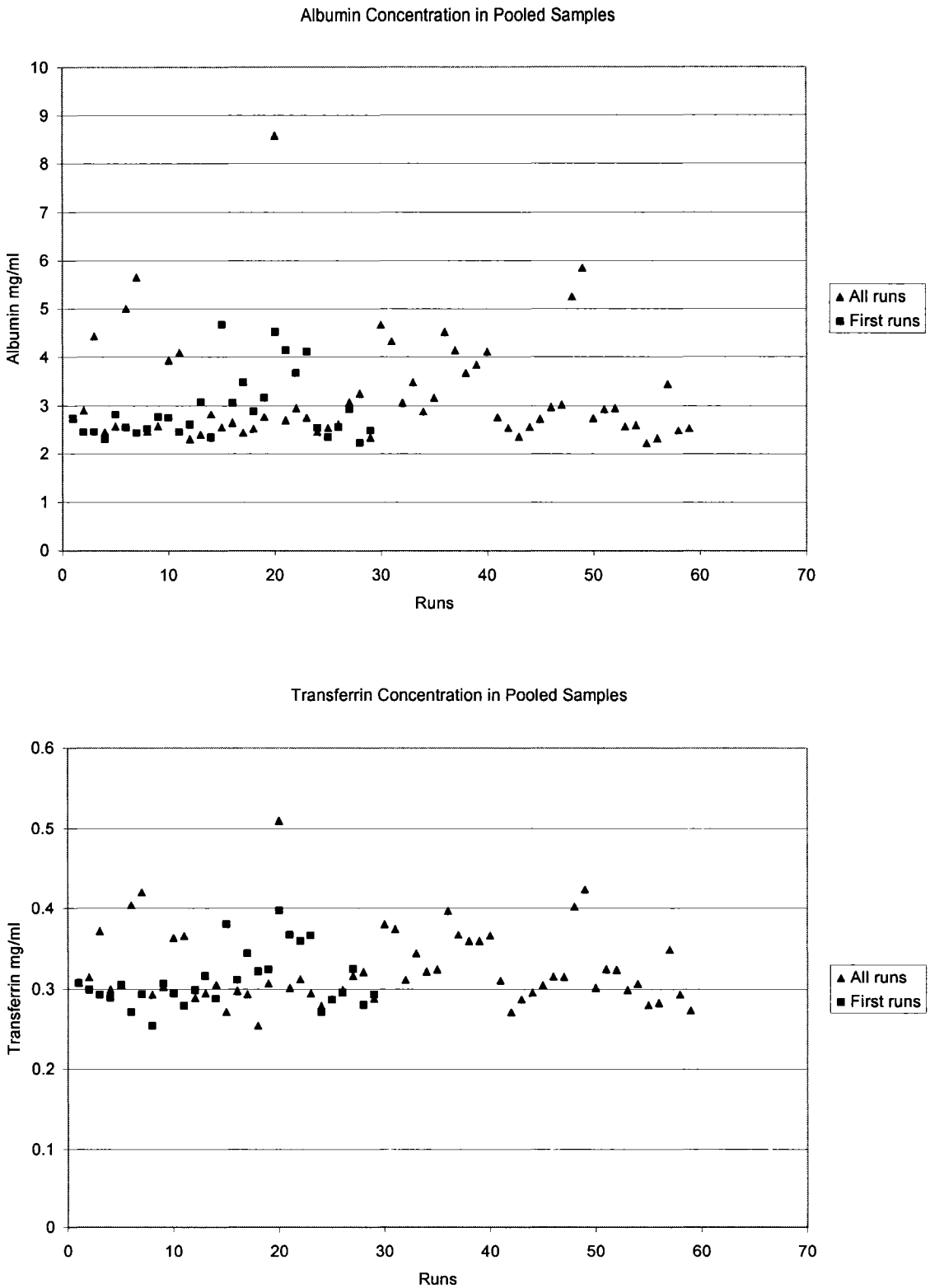

Figure 3. Pooled samples scattered plots. The X axis represents the runs of the days, the Y axis the AF albumin and transferrin concentrations. No visible difference was noticed between the first runs and the last runs of the days. This shows that the CE was running efficiently.

Block effect

Three persons in the lab, using the same equipment, methodology and step-by-step procedures, carried out the analysis of the amniotic fluid samples. Around two thirds of the samples were run by one person, around one quarter by the other, and the rest by the third person. There was no significant difference between the runs of the distinct individuals for transferrin and albumin values of the amniotic fluid samples, as well as birth weight categories and percentiles: transferrin ($p=0.7909$) albumin ($p=0.8829$).

Monitoring the capillary status

The electro-osmotic flow (EOF) causes a pumping effect in the capillary. As we mentioned before, the flow in the capillary is related through the EOF to the voltage applied on the capillary. Hence, using the computer for data acquisition and by monitoring the current, the course of the analysis was measured. The current signal mainly relied on: the voltage applied, the flow in the capillary and the resistance of the buffer. This signal can be used to monitor the success of the separation, the state of the capillary and the lifetime of the capillary. Indeed, given that air is an insulator, any air bubbles would interrupt current and interfere with the separation. On the other hand, as the silica is dissolved off the capillary walls, the cross sectional area of the capillary increases and the resistance is lower resulting in a general increase in current from day to day. Consequently, as the capillary gets damaged, the current intensity increases (Figure 4).

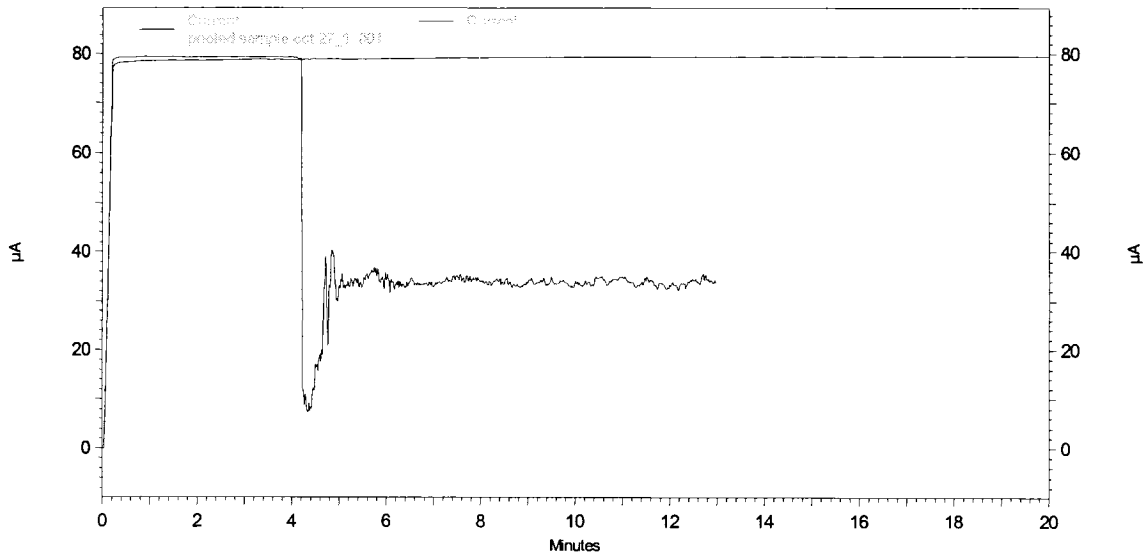

Figure 4. Example of current failure signal and proper current signal. The X axis represents the minutes, the Y axis represents the μA. The above steady line is an appropriate signal, while the bottom non-steady line represents a current failure.

Testing sample stability

Since AF samples were brought from the MCH to the McGill labs where they were stored frozen until transferred and analysed at Concordia University where they were thawed, repartitioned in smaller quantities for CE analysis, and then frozen again; thus, stability to freezing and thawing had to be tested. This procedure was done by testing 10 pooled samples. These samples were taken out of the freezer, thawed, and the first portion was analyzed immediately. The 9 remaining samples were put back in the -80°C freezer for the analysis time. These 9 samples were then all taken out of the freezer, thawed, and the following sample was analyzed. The 8 remaining samples were placed back in the freezer, and so on, until all the samples were analyzed. Data acquisition was done for 60 minutes rather than the usual 20 min in order to see if due to degradation any constituents would elute at later times. Furthermore, in order to be able to run AF samples in sequence, we had to test the stability of samples at room temperature. Since the instrument used was not provided with a cooling system for the samples, the stability of the analytes at room temperature had to be verified. It was necessary to verify whether

or not the samples composition alters over time when exposed at room temperature. The main concern was the occurrence of proteolytic activity or protein denaturation and aggregation. The method reproducibility was tested by using several successive runs of pooled samples and comparing them. The test was done over 10 hours. Every hour a sample was injected and analyzed. To be able to detect degradation compounds, we acquired the data over 60 minutes. From the electropherograms shown in Figures 5 and 6, most importantly, the sample pattern appears to be conserved in a way that none of the peaks diminishes in area and no new peaks appear. The small changes in time are probably related to the separation buffer beginning to be electrodepleted causing a small change in pH and ionic strength. This is fairly common in CE. Dividing peak areas by retention time corrects for these small changes in migration time. In addition, the peaks of interest (transferrin and albumin) elute in the window where the peaks are visibly reproducible in area and shape. Therefore, amniotic fluid samples are more or less stable over successive thawing and freezing steps, and at room temperature.

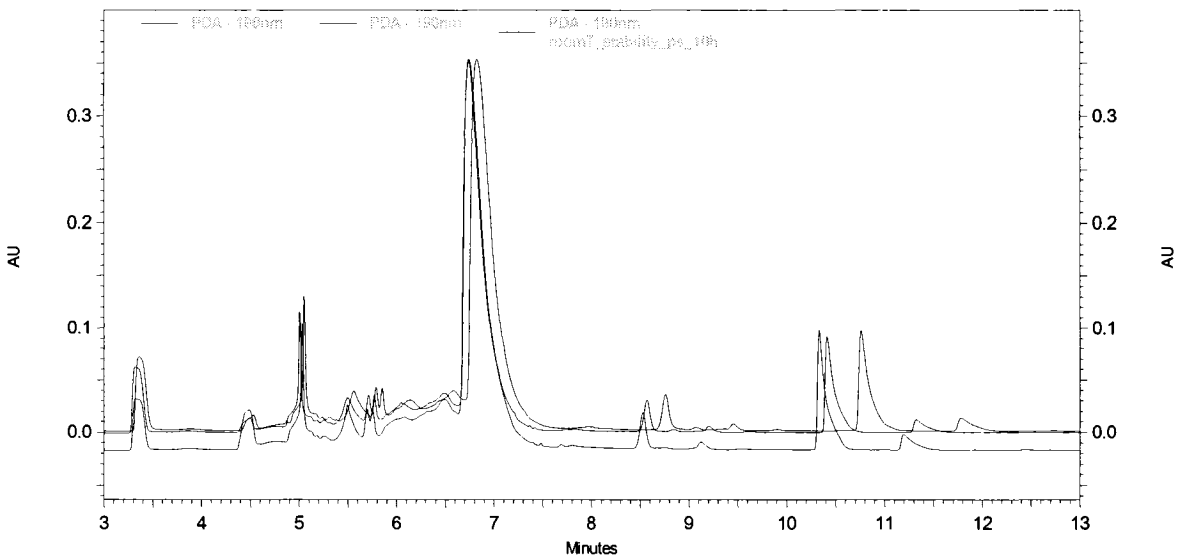

Figure 5. Comparison of elecropherograms from the room temperature test, the t=0, t=1, t=10 samples, runtime of 60 min, zoom, detection at 190 nm. The first line represents the t=0, the second line represents t=1, the third line represents t=10. Albumin peak shows at around 7 min, transferrin peak shows at around 5.5 min. The sample pattern appears to be conserved in a way that none of the peaks diminishes in area and no new peak appears. The small changes in time were corrected. In addition, transferrin and albumin peaks elute in the window where the peaks are visibly reproducible in area and shape.

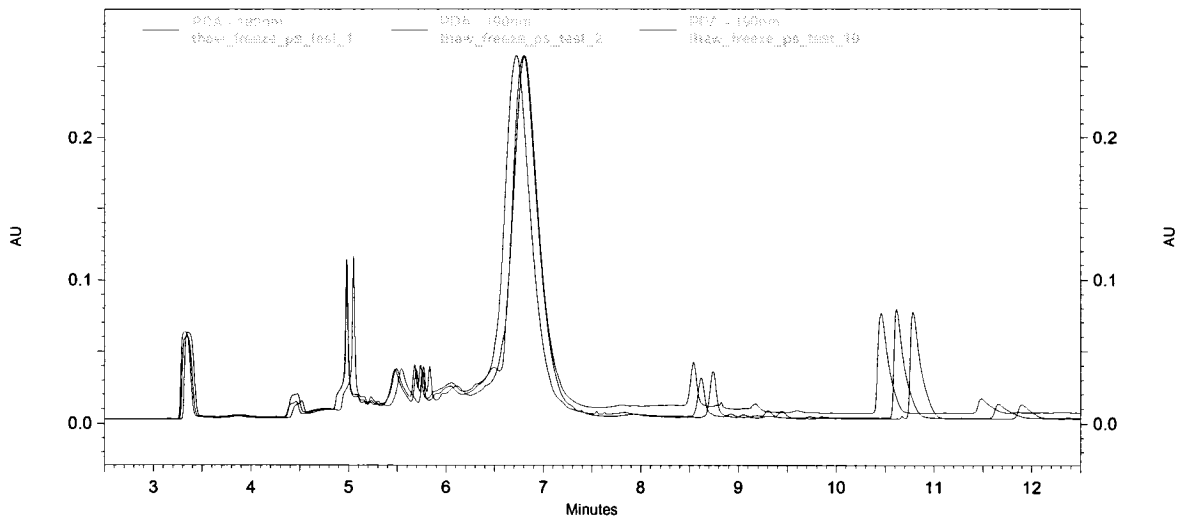

Figure 6. Comparisons of electropherograms from freezing and thawing experiment- the 1st, 2nd and 10th samples, zoom, detection at 190 nm. The first line represents the 1st sample, the second line represents the 2nd sample, the third line represents the 10th sample. Albumin peak shows at around 7 min, transferrin peak shows at around 5.5 min. The sample pattern appears to be conserved in a way that none of the peaks diminishes in area and no new peak appears. The small changes in time were corrected. In addition, transferrin and albumin peaks elute in the window where the peaks are visibly reproducible in area and shape.

Analysis of biochemical data

In order for us to unambiguously identify the peaks of the 3 proteins we were interested in, albumin, transferrin and prealbumin, we used two methods: 1) we ran pure serum albumin, transferrin and prealbumin standards in addition to the internal AF standard to see when exactly the individual protein peaks elute; 2) furthermore, we added those pure proteins one by one to pooled AF sample, in a bigger quantity than expected. In this case, the protein peak of interest increased in size. After running the samples, the migration times of the peaks, in addition to their relative areas were determined. To calibrate the method and quantitate the protein levels, we used peak areas rather than peak heights, since they are best used in quantitative analysis (Weinberger R, 2000 b).

In order to calculate the final area under the curve for each peak, each peak area reported was divided by the peak migration time to correct for variations in migration velocity (Huang X *et al.,* 1988). The peak area is the product of the peak height and the peak width. Variations in the migration velocity will cause the peak width to change

proportionally with the migration velocity thus dividing the peak area by the migration time removes this source of variation. The second normalization step was to divide the corrected analyte peak areas by the internal standard thiamin area. This correction compensates for variations in injected volume that are the result of differences in viscosity or instrumental errors during injection.

Since our objective was to determine the concentration of the proteins in the amniotic fluid, we ran 5 different quantities of the pure proteins by serial dilution, and matched the corresponding peak areas. The quantities used for albumin were: 9.1 g/l, 4.55 g/l, 2.27 g/l, 1.14 g/l, 0.57 g/l. The concentrations of transferrin were: 3.4 g/l, 1.7 g/l, 0.85 g/l, 0.42 g/l, and 0.21 g/l. By the resultant calibration curves, we calculated the AF protein concentrations of the specific proteins. The areas under the curves were analysed using an external calibration. A calibration of the protein was obtained using a least squares fit of a second order polynomial to the peak areas. The least squares fit was calculated using Matlab. The equations were:

1) Albumin (g/l) = $(4.38 \times 10^{-1}) + (2.03 \times 10^{-1}) \times \text{area} + (1.95 \times 10^{-2}) \times \text{area}^2$.

2) Transferrin (g/l) = $(1.65 \times 10^{-1}) + (2.74 \times 10^{-1}) \times \text{area} + (2.69 \times 10^{-2}) \times \text{area}^2$.

Amniotic fluid electropherograms

Figure 7 shows a typical electropherogram of pooled amniotic fluid proteins elution at 190 nm. Although every sample separation took 20 minutes, all the proteins of interest eluted before 10 minutes. On average, after adding the internal AF standard (thiamin), we found the corresponding peak retention times: the thiamin peak was the first peak to elute at a retention time of roughly 2.6 min. Albumin's retention time was around 5 min, while transferrin's retention time was in the region of 4.2 min.

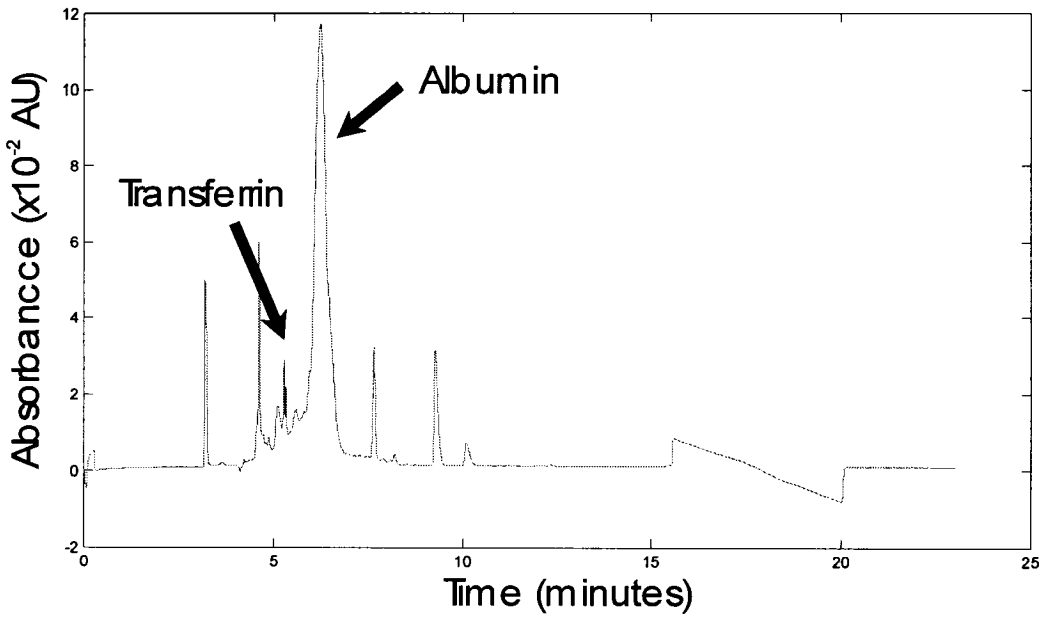

Figure 7. Electropherogram of pooled AF from 190-195 nm, Y axis in 10^{-2} AU, X axis in minutes. Albumin and transferrin are shown by arrows.

Prealbumin

As mentioned in the methodology section, each pure protein was measured separately and spiked into the pooled AF to see exactly where they appeared on the electropherogram. Figure 8 shows that prealbumin added to the amniotic sample had a migration time of 6.3 min. The low concentration of prealbumin in AF precluded detection of prealbumin in any of the normal amniotic fluid electropherograms.

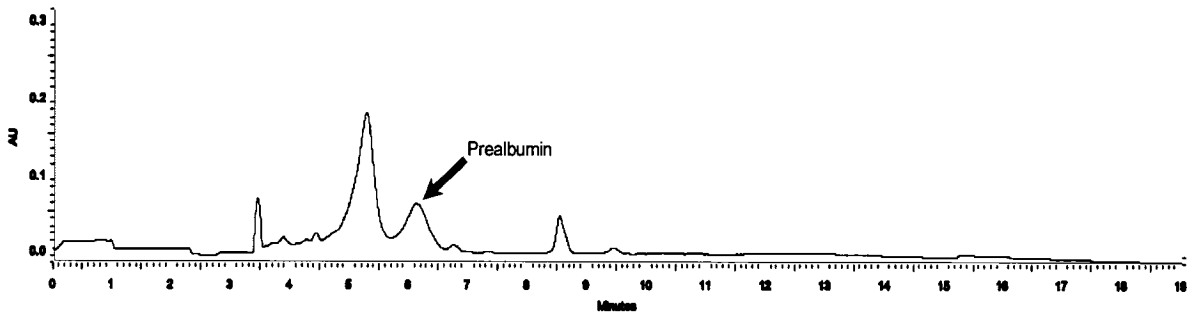

Figure 8. Prealbumin spiking in amniotic fluid samples (showed by the arrow), appearing at 6.3 min after the albumin peak. X axis in min, Y axis in AU. Detection at 190 nm.

Statistical analyses

SAS program version 8 (SAS Institute Inc., Cary, NC) was used to analyze the data. The level of significance was set to $P< 0.05$. We divided the birth weights into percentiles which corrected for gender and gestational age (Kramer MS, 2001); non-normally distributed data was transformed using log based 10 which was based on individual parameter's skewness and kurtosis. We reported the mean concentrations of the amniotic fluid albumin and transferrin at 190 nm. ANOVA and Scheffé's multiple comparison tests were performed to see if albumin and transferrin concentrations changed by birth weight categories including several maternal characteristics: maternal height, maternal pregravid weight, maternal weight gain, ethnicity, amniocentesis week and parity as covariates. We chose to use ANCOVA since it is usually used to test a hypothesis concerning the means of three or more populations. We used Scheffe's test because it is used to find where the differences between means lay when the ANCOVA indicated the means were not all equal. It allowed testing for unplanned comparisons among means without inflating the Type I error rate. Pearson and Spearman correlations were run between the individual proteins and the birth weights. Pearson's correlation reflects the degree of linear relationship between two rank-ordered variables, while Spearman is a nonparametric (distribution-free) rank statistic proposed as a measure of the strength of the associations between two variables. Multiple linear regressions with birth weight as the dependant variable for the entire population and for each individual birth weight category as a dependent variable were run with the results for each individual AF protein assay entered individually and run in separate regression models. In each case the covariates affecting birth weight were included in all models (gestational age, infant gender, amniocentesis week, pregravid weight, height, parity, ethnicity, smoking behavior). Multiple linear regressions with gestational age as the dependant variable for the entire population and for the preterm, term and postterm categories were run with the results for each individual AF protein assay entered individually and run in separate regression models. In each case birth weight, infant gender, amniocentesis week, pregravid weight, height, parity, ethnicity, and smoking behavior were included in the models. Stepwise, forward and backward regressions were also performed.

V. Section Introduction

The following section of the thesis includes the paper which is to be submitted to the Journal of Nutrition. The candidate chose to prepare a paper and to include this document in the thesis in lieu of writing a traditional thesis.

The amniotic fluid sample data were initially analysed at 3 wavelenghts: 190, 254 and 275 nm but only 190 nm is reported in the following paper, since detection of proteins is best at 200 nm (Weinberger, 2000), and 195 nm is a sensitive wavelength for protein determination in a biofluid (Stewart *et al.,* 2001).

Results where significance level of 0.05 was obtained were reported as dictated by the instructions to authors for Journal of Nutrition. The traditional classification using gram weights as well as the percentile classification of birth weight were both included in the manuscript despite the fact that this later classification is the preferred method by the editorial board of the Journal of Nutrition. The student author wanted readers to know that the findings using the gram weight classification system were similar in large part to those results using the newer percentile classification system. Results that did not attain a 0.05 level of significance but that were relevant to the overall understanding to the role of albumin and transferrin in fetal growth are also discussed in the the manuscript.

VI. Paper I

Association between amniotic fluid albumin, prealbumin or transferrin and the fetal growth

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Number of Tables: 9
Number of Figures: 3

6,895 words from title through references

In preparation for submission to Journal of Nutrition

Paper I

ABSTRACT. The objectives of our study were 2-fold: 1) to measure the concentrations of albumin, prealbumin and transferrin in amniotic fluid, and 2) to establish if albumin, prealbumin, and/or transferrin concentrations in amniotic fluid were associated with infant birth weight. From 2000-2003, 294 samples of AF were collected from mothers undergoing routine amniocentesis (12-19 weeks gestation) at St Mary's Hospital (Montreal, Quebec). Exclusion criteria included subjects having gestational diabetes, multiple pregnancies and fetal genetic abnormalities. Samples were analyzed using capillary electrophoresis at 190 nm. Statistical analysis was done using SAS V8. Analysis of variance as well as multiple linear regressions were performed. ANCOVA showed that transferrin differed across BW categories. Multiple regressions showed the parameter estimates of transferrin and albumin were negative, but neither was associated with birth weight for our study population. However, multiple regressions run by individual birth weight categories showed that transferrin was negatively associated with BW in low birth weight (LBW) infants and positively with postmature infants. In conclusion, our study shows that 2nd trimester AF transferrin may emerge as a biomarker for poor in-utero growth and/or postmaturity.

KEY WORDS: *amniotic fluid. albumin. transferrin. birth weight. capillary electrophoresis*

INTRODUCTION

Birth weight (BW) is an important factor that influences neonatal mortality and infant and childhood morbidity (Mc Cormick MC, 1985). Various genetic and environmental factors affect the size of the newborn infant (Kirchengast S *et al.,* 1998). However, intrauterine growth generally depends on three factors: maternal, fetal and placental factors (Grassi AE *et al.,* 2000). The two extremes of irregular fetal growth are fetal macrosomia and fetal growth restriction (Langer O, 2000) both of which lead to short-term and long-term complications (Chescheir NC *et al.,* 2004; Boulet SL *et al.,*

2003; Grassi AE *et al.,* 2000; Langer O, 2000; Zubrick SR *et al.,* 2000; Yiu V *et al.,* 1999; Hack M, 1998; Albertsson-Wikland K *et al.,* 1997; Yeazel MW *et al.,* 1997; Kogan MD, 1995).

The developing fetus is surrounded by amniotic fluid (AF). This fluid originates from the mother, the placenta and cord, or the fetus and varies with different gestational ages (GA) (McCarthy T *et al.,* 1978). AF consists of 98% water and has analogous characteristics to the maternal serum in early pregnancy (Finegan JA, 1984). It protects the infant, contains nutritional factors, and possesses antimicrobial activity (Finegan JA, 1984). It enables normal development of fetal internal systems (Sherer DM, 2002). The fetus contributes to the composition of the AF through the lungs, the trachea, swallowing, and producing urine (Stewart CJ *et al.,* 2001).

Proteins have been associated with many outcomes related to fetal development (Tisi DK *et al.,* 2005; Tisi DK *et al.,* 2004; Ross MG *et al.,* 2001; Jauniaux E *et al.,* 2000; Jain SK *et al.,* 1995; Jauniaux E *et al.,* 1994; Raubenstine DA *et al.,* 1990; Gitlin D *et al.,* 1972); Specifically plasma and serum prealbumin, albumin and transferrin measurements have been linked to some fetal outcomes. Newborn BW has been correlated with maternal and fetal plasma prealbumin concentrations (Jain SK *et al.,* 1995) as well as with fetal serum prealbumin (Raubenstine DA *et al.,* 1990). Serum prealbumin has been associated with protein nutritional status in very low birth weight (VLBW) infants (Polberger SK *et al.,* 1990) and is considered as a potential biochemical marker for certain complications of pregnancy and fetal defects (Lolis D *et al.,* 1995). Newborn serum albumin concentrations reportedly correlates with BW (Raubenstine DA *et al.,* 1990), and intrauterine growth restricted (IUGR) fetuses have been described as hypoalbuminemic (Nieto-Diaz A *et al.,* 1996). A newborn's serum transferrin concentrations are also known to correlate with BW (Raubenstine DA *et al.,* 1990), As well, plasma transferrin indicates poor protein nutritional status in VLBW infants, where transferrin is positively correlated with protein intake. Moreover, it strongly correlates with the weight of VLBW fetuses (Polberger SK *et al.,* 1990). With regards to AF measurements, more recent observations have shown that total AF protein has been negatively associated with BW at term (Tisi DK *et al.,* 2004) and insulin growth factor binding protein 1 (IGF-BP1) has been negatively associated with BW (Tisi DK *et al.,*

2005). Fetal intestinal tract abnormalities can be diagnosed early by protein electrophoresis of abnormal albumin bands in amniotic fluid (Stefos T *et al.,* 1993). AF transferrin increases in pathological populations (Bardos P *et al.,* 1976). From 16 to 41 weeks of gestation, the main factors influencing the concentration of AF proteins seem to be fetal urination and swallowing (Lolis D *et al.,* 1995). Fetal swallowing might be accompanied by fetal digestion of the protein (Gitlin D *et al.,* 1972). The absence of fetal swallowing of AF is associated with fetal growth retardation (Ross MG *et al.,* 2001).

The association between amniotic fluid albumin, transferrin and prealbumin and fetal growth has not been studied. But given that concentrations of all 3 constituents are found in amniotic fluid early in pregnancy (Adama van Scheltema *et al.,* 2005; Lolis D *et al.,* 1995; Legras B *et al.,* 1978; Sutcliffe RG *et al.,* 1973), we chose to explore the possibility that each might be related to fetal growth. The specific objectives of our study were 2-fold: 1) to measure the concentrations of albumin, prealbumin and transferrin in 2^{nd} trimester amniotic fluid, and 2) to establish if the protein concentrations were associated with infant birth weight.

METHODS

From 2000-2003, pregnant women undergoing routine amniocentesis for testing of age related genetic abnormalities at St Mary's Hospital Center in Montreal, Canada, were approached to be part of the study. They were asked to fill out a questionnaire and sign a consent form in order for the researchers to analyze the remaining AF following completion of genetic testing. After going through the medical charts, subjects having gestational diabetes, multiple pregnancies and fetal genetic abnormalities were excluded from the study. Two hundred ninety four women were recruited but due to technical problems and missing information, we ended up with 196 samples in the study set.

Biochemical assessment was done by Capillary Electrophoresis (CE) using a MDQ from Beckman-Coultier (Mississanga, Ontario). Capillary electrophoresis is a powerful separation technique which features very small sample consumption (Bergman T *et al.,* 1993; Bergman T *et al.,* 1991) low buffer consumption, good resolution, and short analysis time (Ummadi M *et al.,* 2001; Stewart CJ *et al.,* 2001; Bergman T *et al.,*

1993; Bergman T *et al.,* 1991). Before analysis, the instruments was prepared by igniting the deuterium lamp, cleaning the electrodes with ethanol and conditioning the capillary with SDS, NaOH and buffer rinses. All AF samples were injected hydrodynamically but changes in sample viscosity necessitated the use of an internal standard to correct for injection volume variations. The internal standard was thiamin, at a concentration of 0.5mg/ml in distilled water. A 1:1 dilution of the AF samples with the thiamin standard was done prior to analysis. To monitor the instrumental quality, pooled AF samples were separated at the beginning, the end of the day, and every 10 sample injections.

The separation buffer was 75 mM borate with 0.8 mM EDTA, adjusted to a pH of 9.27. The separation was carried out at a voltage of 20 kV, and temperature at 28°C in a 60 cm long capillary (50 cm effective length). Detection with a photodiode array (PDA) detector permitted detection from 190-350 nm with only peak integration only at 190 nm reported. Prior to assaying the study set samples, the stability of AF against freezing and thawing as well as the stability at room temperature was tested. Stability was verified by observing no apparent changes in the CE protein profile of AF samples subjected to 10 freeze/thaw cycles and 10 hours storage at room temperature. The buffer was degassed to reduce current failures encountered in the early stages of the analysis.

The proteins peak areas were converted into g/l. The areas under the curves were analysed using an external calibration. A calibration of the protein was obtained using a least squares fit of a second order polynomial to the peak areas. The least squares fit was calculated using Matlab. The equations were: 1) Albumin (g/l) = $(4.38 \times 10^{-1}) + (2.03 \times 10^{-1}) \times \text{area} + (1.95 \times 10^{-2}) \times \text{area}^2$. 2) Transferrin (g/l) = $(1.65 \times 10^{-1}) + (2.74 \times 10^{-1}) \times \text{area} + (2.69 \times 10^{-2}) \times \text{area}^2$.

The SAS program (Version 8, Cary, NC) was used to analyze the data; two fetal growth classification systems were used. The first one employed was gender-corrected-percentiles that included small-for-gestational-age (SGA) < 10^{th} percentile, appropriate-for-gestational-age (AGA), between 10^{th} and 90^{th} percentile, and large-for-gestational-age (LGA)> 90^{th} percentile (Kramer MS, 2001). The second is the more traditional classification: low birth weight (LBW) < 2500 g, and macrosomic > 4000 g, with healthy birth weights lying in between these two extremes. Non-normal data (gestational age (GA), maternal age, smoking, ethnicity, transferrin and albumin) were transformed using

log_{10}. ANOVA, followed by Scheffe's test and ANCOVA were run to test if protein concentrations changed by birth weight (BW) categories. Multiple regressions for BW categories were made to determine those proteins predictive of BW. Covariates known to affect BW like gender, GA, maternal height and prepregnancy weight, parity, ethnicity and smoking behaviour were included in the analysis (Kramer MS, 1987). These variables were collected from the questionnaires and the medical chart reviews. Significance was set at $P<0.05$.

RESULTS

Maternal and infant characteristics are summarized in Table 4. Albumin and transferrin concentrations were moderately positively skewed, with means of 4.35g/l and 0.36 g/l respectively (Figure 9).

The histograms in Figure 9 describe the population distributions for amniotic fluid albumin and transferrin. The prealbumin peak was resolved from the other proteins but could not be detected by CE due to its low concentration. The display of both AF transferring and albumin in Figure 9 showed that their distribution was moderately positively skewed, and therefore were logarithmically transformed in our statistical analyses.

Concentrations of AF albumin and transferrin exhibited an increasing trend with increasing gestation (12-19 weeks); (albumin $p = 0.18$; transferrin $p = 0.09$) (Figure 10). Even though this trend didn't reach significance, we included amniocentesis week as a covariate in our statistical models.

Amniotic fluid albumin and transferrin concentration were compared among the different categories of ethnicity, gestational age, BMI categories, parity, gender, and smoking behaviors. As can be noted from Table 5 albumin and transferrin concentrations did not differ across these different categories of known birth weight determinants.

A significant Spearman's correlation between AF albumin and birth weight was observed ($r = -0.1527$, $p = 0.0326$), AF transferrin was borderline ($r = -0.1259$, $p = 0.08$). Neither negative relationship was significant using a Pearson's correlation.

Using the two classification systems, ANOVA results showed that at 190 nm, amniotic fluid albumin and transferrin had a decreasing trend from SGA to AGA to LGA, and from LBW to normal to macrosomic infants (Table 6, Table 7). This trend can be seen clearly in Figure 11, where the bar graphs depict the decreasing concentration of the specific proteins with augmenting birth weight in both classifications. This decline did not reach significance in the percentile classification for amniotic fluid albumin ($p= 0.28$) and transferrin ($p= 0.34$) nor using gram/weight classifications {albumin ($p= 0.11$), transferrin ($p= 0.13$)}. By percentile classification (Table 6), ANCOVA showed that amniotic fluid transferrin was different among SGA, AGA and LGA groups ($p=0.02$) when maternal height, prepregnancy weight, amniocentesis week, and parity were controlled for. Amniotic fluid albumin was borderline ($p=0.06$). Similar results were found for the gram weight classification (Table 7).

Multiple linear regressions for birth weight for the entire study population were run, and GA, smoking, infant gender, ethnicity, parity, maternal height and prepregnancy weight were controlled for. Amniotic fluid albumin and transferrin shared a negative association with birth weight according to the negative parameter estimate, but were not significant predictors of BW. Only the previously established birth weight determinants (Kramer MS, 1987): GA, pregravid weight, height, amniocentesis week, and parity of the pregnant women were entered as determinants of birth weight by percentile classification (Table 8). However, when linear regressions were run by the three gram weight classes LBW, normal and macrosomic individually, transferrin was negatively associated with BW in the LBW category ($p= 0.0158$) (Table 9).

Table 4

Characteristics of the sample population[1]

Maternal characteristics	
Height, *m*	(191) 1.62 ± 0.01
Prepregnancy weight, *kg*	(191) 62.9 ± 0.9
BMI *kg/m*^{2}	(187) 23.83±0.30
BMI, *%*	
<18.5 *kg/m*^{2}	7
18.5–24.9 *kg/m*^{2}	64
25–29.9 *kg/m*^{2}	21
≥30 *kg/m*^{2}	8
Parity	(194) 1.1 ± 0.1
Ethnicity, *%*	
Caucasian	64
Asian	21
Other[2]	15
Nonsmoking, *%*	87
Infant characteristics	
Gestational age, *wk*	(196) 39.3± 0.1
Gender, *% female*	47
Birth weight, *g*	(196) 3431± 48
Birth outcome, %	
LBW (<2500 g)	7
Macrosomia (≥4000 g)	21
SGA (<10%)	9
LGA (≥90%)	16
Amniotic fluid characteristics	
Amniocentesis, *wk of gestation*	(194) 15.2 ± 0.1
Transferrin *g/l*	(196) 0.36 ± 0.01
Albumin *g/l*	(196) 4.35 ± 0.24

[1] Values are mean ± SEM or %, values in parentheses are n
[2] Other includes Black, Middle-Eastern and Hispanic

Table 5

Albumin and transferrin variation by birth weight determinants[1]

				P values	
Determinant	**Categories**	**Transferrin**	**Albumin**	**Transferrin**	**Albumin**
Ethnicity				0.11	0.07
(125)	Caucasian	0.36±0.01	4.57±0.33		
(41)	Asian	0.38±0.02	4.49±0.40		
(29)	Other[2]	0.32±0.02	3.20±0.45		
GA				0.15	0.14
(17)	<37 wks	0.34±0.02	4.32±0.57		
(53)	≥37&<39 wks	0.37±0.02	4.63±0.57		
(52)	≥39&≤ 40 wks	0.38±0.02	4.93±0.46		
(74)	>40 wks	0.33±0.01	3.75±0.33		
BMI				0.30	0.85
(14)	< 18.5	0.39±0.04	4.67±0.88		
(122)	≥18.5&≤ 24.9	0.35±0.01	4.30±0.31		
(40)	>25&<29	0.31±0.02	4.47±0.53		
(16)	≥ 30	0.40±0.03	4.79±0.74		
Parity				0.90	0.62
(54)	0	0.37±0.02	4.95±0.55		
(87)	1	0.35±0.01	4.00±0.30		
(44)	2	0.36±0.02	4.34±0.57		
(6)	3	0.37±0.04	4.64±0.96		
(3)	4	0.34±0.06	3.69±1.17		
Gender				0.90	0.56
(92)	Females	0.36±0.01	4.46±0.35		
(104)	Males	0.36±0.01	4.26±0.33		
Smoking				0.59	0.40
(169)	Non-Smoker	0.36±0.01	4.31±0.26		
(26)	Smoker	0.37±0.02	4.67±0.58		

[1] Values are mean ± SEM, values in parentheses are n
[2] Other includes Black, Middle-Eastern and Hispanic combined

Upon division of GA into three categories of preterm, term and post-term, no significant difference for either protein was found when BW, smoke, infant gender, prepregnancy weight, height, amniocentesis week, parity and ethnicity were controlled for. No surprisingly, only birth weight was associated with GA ($p<0.01$) (Table 10).

Further analysis using a stepwise regression with birth weight, smoking, infant gender, pregravid weight, height, amniocentesis week, parity, ethnicity and transferrin or albumin in the models, resulted in a significant model at a 0.15 level (R^2=0.518). A forward regression gave a model that included birth weight, infant gender, parity, amniocentesis week and ethnicity ($p<0.5$) (R^2=0.5180), whereas the backward regression resulted in only birth weight and infant gender in the model ($p<0.01$) (R^2=0.499) (Table 11). The stepwise regression was performed in 1 step, the backward regression in 7 steps, while the forward regression was performed in 5 steps.

In sharp contrast to regression for the entire study population, regressions run for separate subcategories of preterm, term and post-term infants individually showed that transferrin was positively associated with post-term infants (> 40 weeks), ($p = 0.0286$). Birth weight was positively associated with post-term infants while infant gender was negatively associated with post-term infants, with respective $p=0.0168$ and $p=0.0194$ (Table 12). On the other hand, albumin shared a positive parameter estimate with post-term infants, but the association did not reach significance ($p=0.0909$).

DISCUSSION

The participants in our study are representative of the Canadian population at large, and the findings in our study are comparable to what was previously stated in the literature. Our multi-ethnic population was generally healthy. Most (87%) were non-smokers compared to the Canadian population where 81% are non-smokers (Canadian Perinatal Health Report, 2003). The age of the mothers participating in the study fell between 26 and 45 years old, with a mean of 38±0.2 (mean± SEM). Seventy eight percent of the Canadian pregnant women have a similar age range (Statistics Canada, 2002 a). Our mothers had an average height of 1.62 m similar to the Canadian average of 1.63 m (Gilmore J, 1999), average pregravid weight was 62.9 kg, and the Canadian average is 65.8 kg (Gilmore J, 1999). Seven percent of our women were underweight, the women with normal BMI were 64%, and overweight and obese women were 21%, and 8% of our study population. For the Canadian population at large, the distribution was 3%, 57%, 23% and 13% respectively (Canadian Community Health Survey, 2003). The mean BW of all Canadian newborns is 3,403 g (Statistics Canada, 2002 b), whereas the

mean BW of our sample was 3,431 g, with a mean gestational age of 39 weeks (Statistics Canada, 2002 c). Seven percent of our infants were LBW compared to a rate of 5.5% in Canada (Statistics Canada, 2001). Nine per cent were SGA, and around 16% were LGA compared to 8 % and 12% respectively for the Canadian population at large (Canadian Perinatal Health Report, 2003). We can conclude that the sample of mothers and corresponding infants included in our study are comparable to the Canadian population as a whole. Therefore, despite being older moms, our mothers typified the Canadian population.

The parameter estimates of the birth weight determinants measured in our sample for the whole population - gestational age, infant gender, pregravid weight, height, amniocentesis week, parity, and ethnicity - were also comparable to previous studies (Tisi DK *et al.,* 2005; Tisi DK *et al.,* 2004; Kramer MS, 1987). Literature states that infant gender affects the birth weight with an increase of 126.4 g for males in developed countries; the ethnicity of the mother, specifically blacks, also is known to influence the birth weight by a decrease of 108 g (Kramer MS, 1987). The effect of other ethnic origins has not been described. Every centimeter increase in maternal height increased the birth weight by 7.8 g, while every kg increase in pregravid maternal weight increased the birth weight by 9.5 g (Kramer MS, 1987). Parity on the other hand increased the birth weight by 43.3 g, and the difference between multiparae and primiparae was 82.7g by birth weight (Kramer MS, 1987). Smoking decreased the birth weight of the infant by 149.4g, and every cigarette per day decreased the birth weight by 11.1 g of the newborn weight at delivery (Kramer MS, 1987). Therefore, the BW determinants measured in our study had a similar impact on BW as the previously mentioned BW determinants in literature.

AF transferrin concentrations reportedly range from 0.015g/l to 0.604g/l using Mancini's simple radial diffusion method, polyacrylamide gel electrophoresis, and Ouchterlony's method of double gel diffusion (Guibaud S *et al.,* 1973). Larsen *et al.,* using single radial diffusion, found that from 16 to 42 weeks of gestation, AF transferrin ranged from 0.14 to 0.44g/l with a mean value of 0.299g/l (Larsen B *et al.,* 1973). In our study, AF transferrin concentration between 12 and 19 weeks of gestation averaged 0.36±0.01 g/l (mean ± SEM), and ranged between 0.18 g/l and 0.83 g/l. Thus our AF transferrin concentration range was comparable to previous findings. As for albumin, the

reported the AF albumin concentration between 15 and 22 weeks of gestation was 3±2 g/l using a dye-binding method (Bromocresol Green) on an automated technicon autoanalyzer (Benzie RJ *et al.,* 1974). Newer spectrophotometry methodologies have established AF albumin concentration at 3.9±1.1 g/l from 16 to 22 weeks of gestation (Adama van Scheltema PN *et al.,* 2005) which was of a similar magnitude to concentrations measured in our study (4.35 g/l ± 0.24, mean ± SEM).

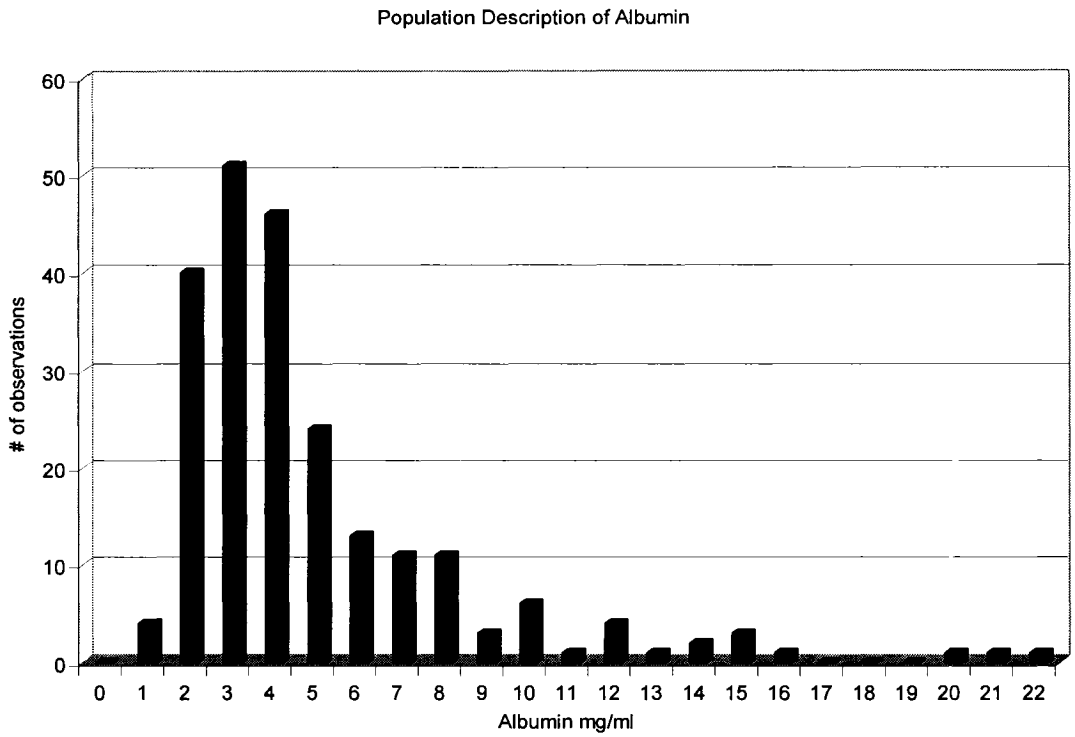
Previous work had demonstrated that from 12 to 20 weeks of gestation, amniotic fluid proteins were negatively associated with the birth weight (Tisi DK *et al.,* 2005; Tisi DK *et al.,* 2004; Ostlund E *et al.,* 2000; Buescher *U et al.,* 1998; Chevallier B *et al.,* 1998; Heyborne KD *et al.,* 1994). There are a series of possible explanations for our negative associations which are the following: 1) Low-birth-weight infants are often associated with oligohydramnios (Chamberlain PF *et al.,* 1984) which 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 DM, 2002). 2) Another explanation might be that there is increased amniotic fluid swallowing and absorption in larger fetuses compared to smaller infants (Ross MG *et al.,* 2001). Indeed, in late gestation, the fetus swallows and digests on average 0.19 g/kg body weight of soluble amniotic fluid protein per day (Gitlin D *et al.,* 1972). So, if this also pertains to the early gestational period, the larger the infant, the more protein he swallows, the less the AF protein concentration. 3) Increased transferrin occurred in the LBW infants and might be related to maternal iron deficiency and the increased maternal transferrin transfer since early in gestation the principal source of AF proteins are maternal serum (Johnson AM *et al.,* 1974; Smith NJ *et al.,* 1974). However it is entirely possible that fetal hypoxia, which would be associated with LBW, could exist (Shepherd RW *et al.,* 1992). The fetus produces some transferrin during the first half of gestation (Jauniaux E *et al.,* 1994) and therefore could be producing its own as a result of maternal iron deficiency because it is experiencing hypoxia. 4) If the rise of AF transferrin concentration of those small infants is supplied by the modest fetal source (Jauniaux E *et al.,* 1994), and since it is known that the major function of transferrin is transport and delivery of iron to target cells (De Jong G *et al.,* 1990), this might lead to the assumption that those small infants could be anemic or iron-starved, and consequently transferrin is upregulated to provide more iron to the

fetus. 5) An additional speculation might be related to the progesterone in the amniotic fluid. Studies have shown that AF transferrin is upregulated by progesterone secretion in the amniotic fluid (Jeschke U *et al.,* 2004).

Of significant interest in our study was the observation that higher concentrations of AF transferrin when measured early in gestation were associated with an infant subsequently being born post term. Depletion of maternal iron stores at term can not be prevented (Milman N *et al.,* 1994), which leads to an upregulation of maternal serum transferrin at term. Therefore, assuming that advancing gestation adds to the maternal iron depletion, we can assume that a post term infant may already be faced with a depleted maternal iron stores, with increased maternal serum transferrin and consequently AF transferrin. On the other hand it is entirely possible that the elevation in the concentration of transferrin is a mere reflection of oligohydramnios and it is this factor that is associated with postmaturity (Rayburn WF *et al.,* 1982). In animal models, postmaturity is maintained through progesterone injections (Dupouy JP *et al.,* 1986) and indeed if progesterone raises transferrin then there could be based on our observations a change in the fetal metabolic environment with early elevations in progesterone leading to increased transferrin and postmaturity. However, more investigation is needed concerning the association between gestational age and transferrin and/or albumin.

With regards to albumin, given that it is the major amniotic fluid protein (Sutcliffe RG *et al.,* 1973), it would decrease in the amniotic fluid of smaller infants compared to larger infants, as described previously. In our study, albumin exhibited a negative trend with increasing birth weight, but significance could not be reached probably because of increased variability and reduced power in our study population or due to instrumental effects. However, more investigation should be done with regards to AF albumin and fetal growth.

To summarize, our study shows that 2nd trimester AF transferrin concentration is different among the birth weight categories and that AF transferrin is negatively associated with birth weight at the LBW category. These findings are supported by the literature that also reports negative relationships between amniotic fluid proteins and fetal growth. Further investigations might help to clarify and improve our understanding of the association of AF albumin with the fetal growth.



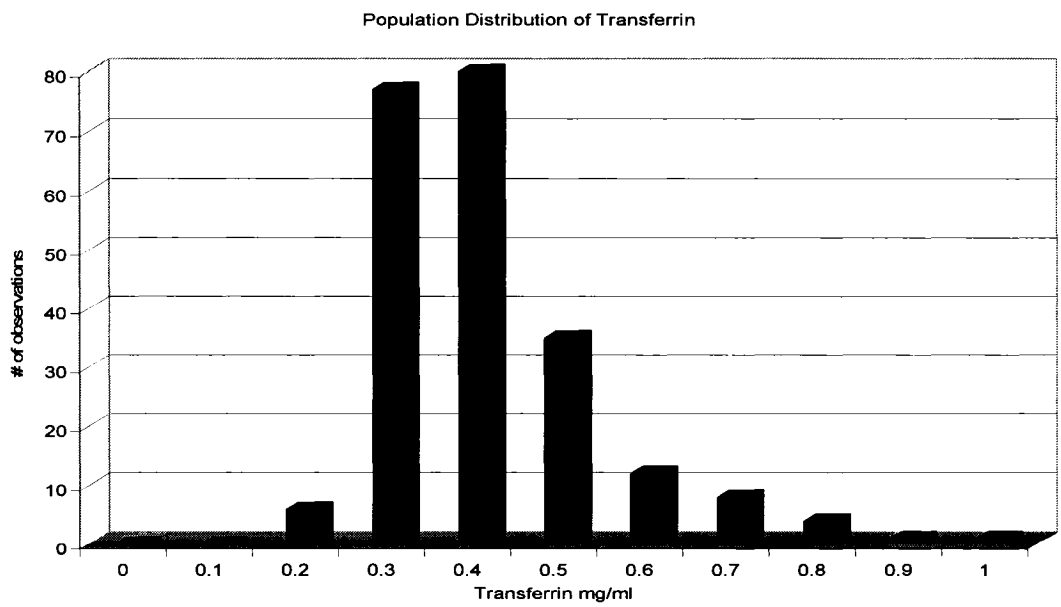


Figure 9. Population distribution (n=224) of amniotic fluid albumin and transferrin concentration obtained via routine amniocentesis in pregnant women. Albumin and transferrin was moderately positively skewed. Transformation using $\log_{10}$ was required for them to be normally distributed.

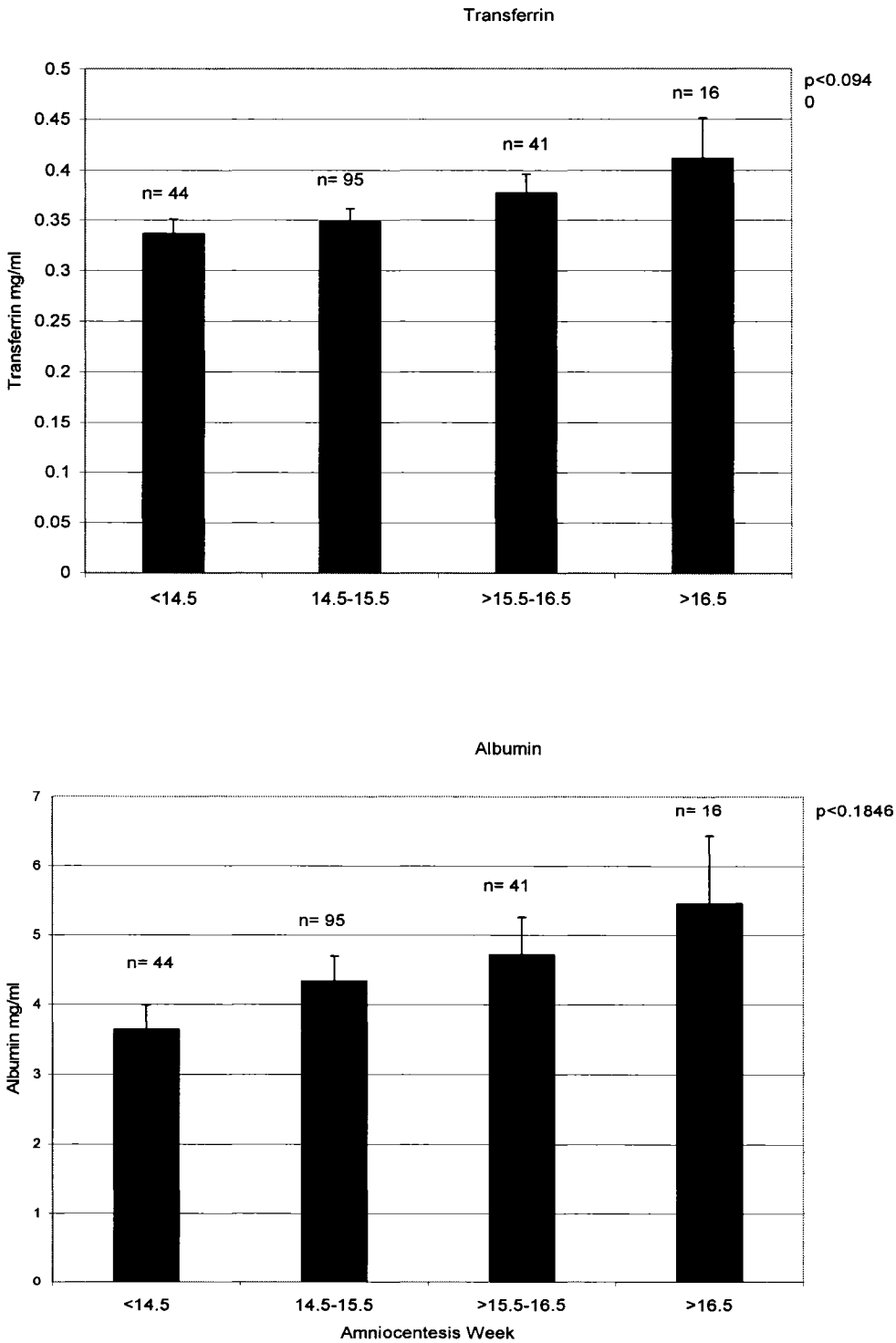


Figure 10. AF transferrin and albumin variation according to week of gestation when amniocentesis was performed. An increasing trend can be seen with increasing gestation.

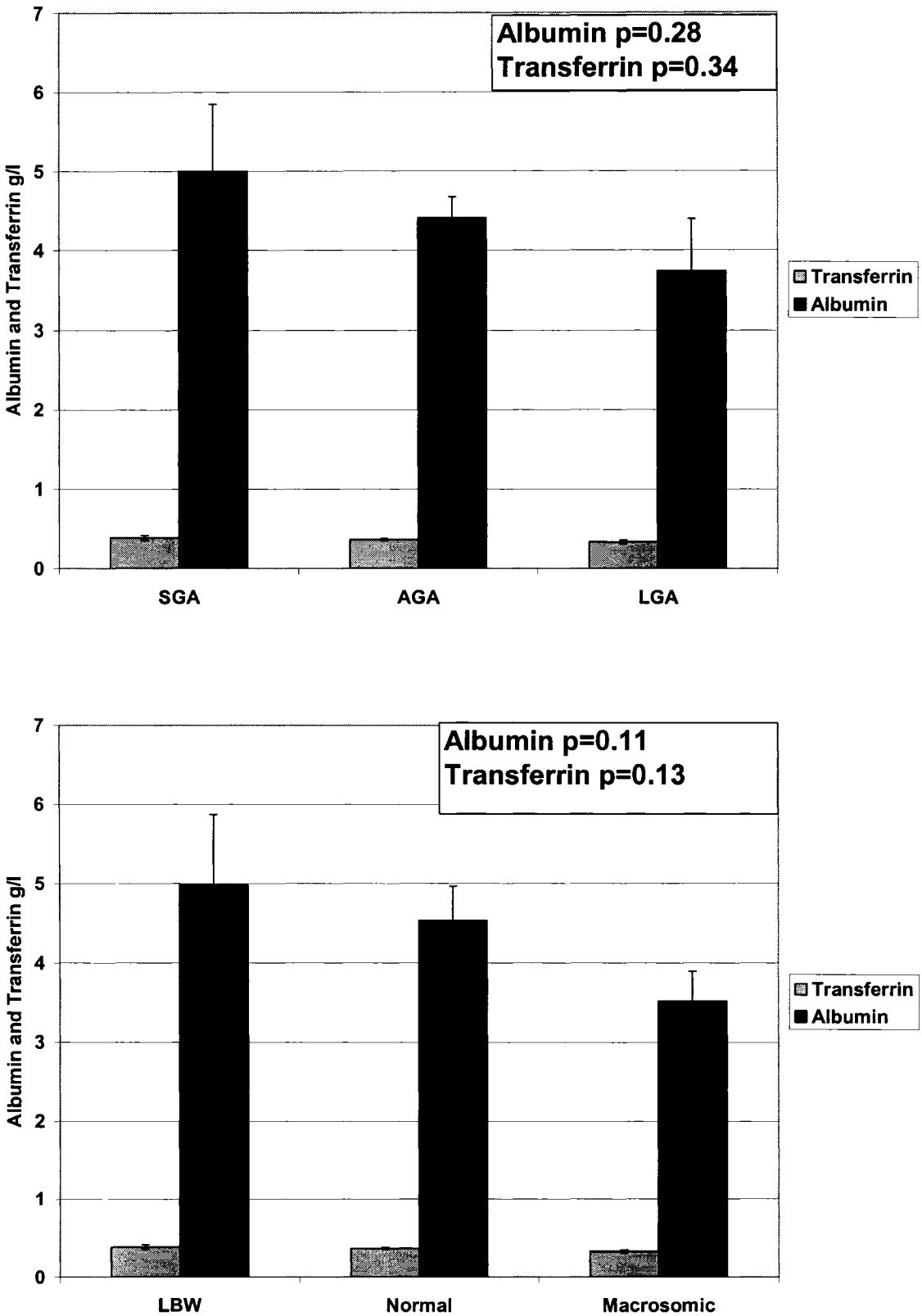


Figure 11. Transferrin and albumin variation by birth weight categories. A visible decreasing trend can be noticed with increasing birth weight.

Table 6

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

CHARACTERISTIC	SGA	AGA	LGA	F	P
Infant					
Birth weight, (*n*) *g*	(18) 2415 ± 140	(147) 3367± 41	(31) 4328± 61	95.32	<0.01
Gender, *% female*	44	46	52	0.17	0.84
Gestational age, (*n*)	(18) 38.2 ± 0.6[a]	(147) 39.2± 0.1[ab]	(31) 40.0± 0.2[b]	5.87	<0.01
Maternal					
Height, (*n*) *m*	(18) 1.58 ± 0.01[a]	(142) 1.62 ± 0.01[ab]	(31) 1.66 ± 0.01[c]	7.42	<0.01
Age,*yrs*	(18) 39 ± 0.6	(139) 38 ± 0.2	(29) 38 ± 0.4	0.97	0.38
Pregravid wt, (*n*) *kg*	(18) 59.0 ± 2.9[a]	(143) 61.9 ± 1.0[ab]	(30) 70.0 ± 2.4[c]	6.54	<0.01
BMI, (*n*) *kg/m2*	(18) 23.8 ± 1.2	(139) 23.5 ± 0.3	(30) 25.3 ± 0.7	2.44	0.09
Ethnicity, *%*				*0.23*	0.79
Caucasian	56	62	77		
Asian	39	22	6		
Other[2]	5	16	16		
Nonsmoking, *%*	83	85	97	1.56	0.21
Parity (*n*)	(17) 0.6± 0.2[a]	(146) 1.0± 0.1[ab]	(31) 1.3± 0.2[b]	3.37	0.04
Amniotic fluid					
Amniocentesis, (*n*) *wk*	(18) 15.3 ± 0.2	(145) 15.1 ± 0.1	(31) 15.3 ± 0.2	0.66	0.52
Transferrin	(18) 0.38 ± 0.03	(147) 0.36 ± 0.01	(31) 0.33 ± 0.02	1.09	0.34
ANCOVA[3]				3.02	0.02
Albumin	(18) 5.00 ± 0.85	(147) 4.40 ± 0.27	(31) 3.74 ± 0.65	1.28	0.28
ANCOVA[3]				2.25	0.06

[1] Values are mean ± SEM or %, values in parentheses are n

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

[3] Covariates included height, pregravid weight, amniocentesis week, and parity

Table 7

Baseline characteristics by gram weight classification[1]

CHARACTERISTIC	LBW	Normal	Macrosomic	F	P
Infant					
Birth weight, (*n*) *g*	(14) 1906 ± 122	(141) 3335± 31	(41) 4284± 46	244.38	<0.01
Gender, *% female*	50	50	37	1.11	0.33
Gestational age, (*n*)	(14) 35.2 ± 0.7[a]	(141) 39.3± 0.1[b]	(41) 40.5± 0.1[c]	78.16	<0.01
Maternal					
Height, (*n*) *m*	(14) 1.58 ± 0.01[a]	(137) 1.61 ± 0.01[ab]	(40) 1.65 ± 0.01[c]	6.04	<0.01
Age,*yrs*	(14) 38 ± 0.5	(133) 38 ± 0.2	(39) 37 ± 0.3	1.26	0.29
Pregravid wt, (*n*) *kg*	(13) 57.8 ± 3.4[a]	(138) 61.2 ± 1.0[ab]	(40) 70.6 ± 2.0[c]	11.1	<0.01
BMI, (*n*) *kg/m2*	(13) 22.9 ± 1.3[ab]	(135) 23.4 ± 0.3[a]	(39) 25.6 ± 0.6[b]	4.92	0.01
Ethnicity, *%*				1.31	0.27
Caucasian	36	65	71		
Asian	50	22	7		
Other[2]	14	13	22		
Nonsmoking, *%*	86	84	95	1.52	0.22
Parity (*n*)	(13) 0.9± 0.2	(140) 1.0± 0.1	(41) 1.2± 0.2	1.25	0.29
Amniotic fluid					
Amniocentesis, (*n*) *wk*	(14) 15.3 ± 0.2	(139) 15.1 ± 0.1	(41) 15.3 ± 0.1	0.80	0.45
Transferrin *g/l*	(14) 0.38 ± 0.03	(141) 0.36 ± 0.01	(41) 0.33 ± 0.02	2.06	0.13
ANCOVA				3.52	0.02
Albumin *g/l*	(14) 4.98 ± 0.89	(141) 4.53 ± 0.43	(41) 3.52 ± 0.38	2.23	0.11
ANCOVA				2.44	0.07

[1] Values are mean ± SEM or %, values in parentheses are n

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

[3] Covariates included height, pregravid weight and amniocentesis week

Table 8

Multiple regressions of birth outcomes by percentile and gram weight for the entire population[1]

Variable	Birth Weight							
	Albumin *g/l*				Transferrin *g/l*			
	Percentile		Gram weight		Percentile		Gram Weight	
	β±SEM	P	β±SEM	P	β±SEM	P	β±SEM	P
AF biochemical Albumin *g/l*	-0.21±0.63	0.73	-4.35±10.35	0.67				
AF biochemical Transferrin *g/l*					-7.68±18.23	0.67	-165.07±298.62	0.58
Gestational age, *wk*	2.84±1.17	0.02	235.28±19.18	<0.01	2.83±1.17	0.02	235.05±19.17	<0.01
Smoking behavior, *0=smoker, 1=non*	-8.69±6.08	0.15	-128.54±99.60	0.20	-8.68±6.08	0.15	-128.35±99.57	0.20
Gender, *0=female, 1=male*	0.50±4.20	0.91	153.84±68.95	0.03	0.53±4.20	0.90	154.41±68.92	0.03
Pregravid weight, *Kg*	0.38±0.19	0.05	5.64±3.19	0.08	0.39±0.20	0.05	5.77±3.20	0.07
Height, *m*	69.28±32.70	0.04	981.21±535.53	0.07	68.17±32.94	0.04	956.15±539.40	0.08
Amniocentesis week, *wk*	4.70±2.27	0.04	70.82±37.24	0.06	4.75±2.28	0.04	72.03±37.37	0.06
Parity, *1=1 child*	5.58±2.48	0.03	91.32±40.67	0.03	5.56±2.48	0.03	90.93±40.65	0.03
Ethnicity,*1=Caucasian, 2=Asian 3=Others*[2]	1.32±2.17	0.54	16.94±35.57	0.67	1.32±2.16	0.54	16.86±35.42	0.63
Variability captured (R^2 * 100), *%*	20.81		56.88		20.84		56.91	

[1]Birth weight is the dependant variable. n=181. Transferrin and albumin showed no significant association with BW by percentile and gram weight classifications. Gestational age, prepregnancy weight, height, amniocentesis week, and parity, were significantly associated with BW by percentile classification. Gestational age, infant gender, and parity, were significantly associated with BW in the gram weight classification. By percentile and gram weight classification respectively, model with albumin $R^2 = 0.2081$, and $R^2 = 0.5688$ respectively, model with transferrin $R^2 = 0.2084$, and $R^2 = 0.5691$ respectively

[2]Others include Middle-eastern, Black and South American

Table 9

Multiple linear regressions of birth outcomes by gram weight in LBW category[1]

	LBW (n=11)	
Variable	**β coefficient**	**P**
Gestational age, *wk*	72.36	0.02
Gender, ***0=female, 1=male***	1022.81	0.01
Pregravid weight, *kg*	-26.67	0.03
Height, *m*	7742.60	0.01
Amniocentesis week, *wk*	-435.27	0.02
Ethnicity*1=Caucasian, 2=Asian, 3=Others*[3]	-196.58	0.01
Transferrin *g/l*[2]	-3851.41	0.02
Variability captured (R^2 * 100), *%*	99.79	

[1] The non-significant covariates, smoking and parity, are not shown in the table
[2]Transferrin showed a significant association with BW at the low-birth-weight category. R^2= 0.9979 Gestational age, infant gender, prepregnancy weight, height, amniocentesis week and ethnicity were significantly associated with BW in LBW category
[3]Others include Middle Eastern, Black and South American

Table 10

Multiple linear regressions by gestational age for the entire population[1]

Variable	**Gestational age (n=181)**			
	Multiple linear regression			
	Albumin g/l		**Transferrin g/l**	
	β±SEM	P	β±SEM	P
AF biochemical: Albumin *g/l*	-0.41±0.87	0.57		
AF biochemical:Transferrin *g/l*			-0.41±0.87	0.64
Birth weight, *g*	0.002±0.00	<0.01	0.002±0.00	<0.01
Smoking behavior, *0=smoker, 1=non*	0.01±0.29	0.96	0.01±0.29	0.96
Gender, *0=female, 1=male*	-0.32±0.2	0.11	-0.32±0.20	0.11
Pregravid weight, *Kg*	0.003±0.01	0.80	0.003±0.01	0.78
Height, *m*	0.03±1.57	0.98	0.001±1.58	1.00
Amniocentesis week, *wk*	-0.17±0.11	0.11	-0.17±0.11	0.11
Parity, *1=1 child*	-0.19±0.12	0.11	-0.19±0.12	0.12
Ethnicity,*1=Caucasian,2=Asian 3=Others*[2]	-0.13±0.10	0.21	-0.13±0.10	0.22
Variability captured (R^2 * 100), *%*	51.91		51.89	

[1] Neither transferrin nor albumin were associated with GA. Only birth weight was associated with gestational age. Model including albumin R^2 = 0.5191, model including transferrin R^2 = 0.5189
[2]Others include Middle Eastern, Black and South American.

Table 11

Stepwise, forward and backwards regressions for gestational age for the entire population (n=181)[1]

Variable	Stepwise regression				Forward regression				Backward regression			
	Albumin g/l		Transferrin g/l		Albumin g/l		Transferrin g/l		Albumin g/l		Transferrin g/l	
	Partial R^2	P	Partial R^2	P	Partial R^2	P	Partial R^2	P	Partial R^2	P	Partial R^2	P
Albumin *g/l*	0.0009	<0.15			NI	NS			0.0009	NS		
Transferrin *g/l*			0.0006	<0.15			NI	NS			0.0006	NS
Birth weight, *g*	0.4208	<0.15	0.4206	<0.15	0.4874	<0.5	0.4874	<0.5	β±SEM: 0.002±0.00	<0.01	β±SEM: 0.002±0.00	<0.01
Smoking behavior, *0=smoker, 1=non*	0.0000	<0.15	0.0000	<0.15	NI	NS	NI	NS	0.0000	NS	0.0000	NS
Gender *0=female, 1=male*	0.0073	<0.15	0.0072	<0.15	0.0120	<0.5	0.0120	<0.5	β±SEM: -0.41±0.20	<0.01	β±SEM: -0.41±0.20	<0.01
Pregravid weight, *Kg*	0.0002	<0.15	0.0002	<0.15	NI	NS	NI	NS	0.0003	NS	0.0003	NS
Height,*m*	0.0000	<0.15	0.0000	<0.15	NI	NS	NI	NS	0.0000	NS	0.0000	NS
Amnio week, *wk*	0.0071	<0.15	0.0071	<0.15	0.0068	<0.5	0.0068	<0.5	0.0068	NS	0.0068	NS
Parity, *1=1 child*	0.0070	<0.15	0.0070	<0.15	0.0076	<0.5	0.0076	<0.5	0.0076	NS	0.0076	NS
Ethnicity, *1=Caucasian, 2=Asian 3=Others*[2]	0.0044	<0.15	0.0042	<0.15	0.0042	<0.5	0.0042	<0.5	0.0042	NS	0.0042	NS

[1]n= 181. NS= Non-significant. NI= Not included. All factors were included in the model in the stepwise regression at $p<0.15$ (R^2=0.5183, variability captured = 51.83%). In the forward regression, only birth weight, gender, amniocentesis week, parity and ethnicity were included at $p<0.5$ (R^2=0.5180, variability captured = 51.8%). In the backward regression, only birth weight and gender were included in the model at $p<0.01$ (R^2=0.4994, variability captured = 49.94%). The stepwise regression was performed in 1 step, the backward regression in 7 steps, while the forward regression was performed in 5 steps.

Table 12

Multiple linear regressions run by post-term category[1]

	Post-term (n=68)	
Variable	**β coefficient**	**Pr> |t|**
Birth weight, *g*	0.000	0.02
Gender, ***0=female, 1=male***	-0.320	0.02
Transferrin *g/l*	1.503	0.03
Variability captured (R^2 * 100), *%*	22.44	

[1] Gestational age is the dependant variable. Transferrin as significantly associated with GA in the post-term. The non-significant covariates, smoke, prepregnancy weight, height, amniocentesis week, parity, and ethnicity were not included in the table. $R^2 = 0.2244$

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VII. General Conclusions

Our multi-ethnic population was generally healthy. Most (87%) were non-smokers; the Canadian population consists of 81% non-smokers (Canadian Perinatal Health Report 2003). The mean BW of all Canadian newborns is 3,403 g (Statistics Canada, 2002 b), whereas the mean BW of our sample was 3,431 g, with a mean gestational age of 39 weeks (Statistics Canada, 2002 c). In general we concluded that our population of older mothers was similar in most parameters except age with the Canadian population at large which is now giving birth on average at the age of 30 years (Statistics Canada, 2005).

Sample size

For the purpose of this study, 224 amniotic fluid specimens were collected. Since 212 samples were needed at the beginning of the study to have a power of 80%, having 224 AF samples to analyze at the end was an adequate number. The missing information of the maternal and infant characteristics from the medical charts resulted in a smaller sample size, around 196 samples, to analyze in SAS. So, this number was below the value we projected to get a power of 80 % in our study. Since albumin had the largest variation, and now that the effect size and standard deviation of albumin were found, estimation of the necessary sample size could be recalculated to detect a difference between SGA and LGA. Calculation showed that 132 subjects were required for each of SGA and LGA groups to determine differences in albumin across our birth weight categories. Estimating again the sample size needed to detect a difference in AF transferrin between SGA and LGA groups based on our study population variability, we found that 64 samples were required for each group. However, even though we had a smaller sample size in SGA and LGA (18 and 31 respectively), we were able to detect difference which shows that the transferrin differences across birth weight categories is much easier to detect. This cannot be stated with regards to AF albumin, so it appears that AF transferrin is more sensitive to pick up difference that AF albumin. Increasing the sample size would be required to identify differences in AF albumin across the birth

weight categories. This is underway as a cheaper method using ELISA is being conducted on n=600 AF in order to ascertain this.

Comparing with literature

AF transferrin was found to be statistically different between the three different birth weight categories by the percentile and gram weight classifications. This finding is comparable to previous amniotic fluid protein findings (Tisi DK *et al.,* 2005; Tisi DK *e al.,* 2004; Chevallier B *et al.,* 1998). Albumin significance was at borderline; significance could not be reached due to non-biological significance because of increased variability and reduced power in our study population and was not most probably due to technical problems, since both albumin and transferrin have the same source in the amniotic fluid-mostly maternal- and peak at the same period in gestation (Legras B et.al, 1978, Sutcliffe RG et.al, 1973).

Previous work had demonstrated that from 12 to 20 weeks of gestation, amniotic fluid proteins were negatively associated with the birth weight (Tisi DK *et al.,* 2004). As well, erythropoietin (EPO) has been negatively associated with birth weight as fetal hypoxia is produced by a high concentration of EPO (Ostlund E *et al.,* 2000; Buescher U et.al, 1998). In addition, low IGF-BP1 levels are associated with higher birth weight infants while high levels of this protein are associated with SGA infants (Tisi DK *et al.,* 2005, Chevallier B et.al, 1998). Moreover, high AF interleukin-10 concentrations are found in the amniotic fluid protein of SGA infants (Heyborne KD *et al.,* 1994). As can be noticed, the AF transferrin and albumin declining trend in concentration from SGA (or LBW) to AGA (or normal) to LGA (or macrosomic) is comparable to the trend of other amniotic fluid proteins in the literature. There are a series of possible explanations for these negative associations. First, low-birth-weight infants have been associated with oligohydramnios (Chamberlain PF *et al.,* 1984). These infants are consequently associated with placental perfusion which might be explained by this limited amniotic fluid expansion. This could lead to an increased concentration of the proteins in the amniotic fluid (Sherer DM, 2002). Another explanation might be because of the increased amniotic fluid swallowing and absorption in larger fetuses compared to smaller infants (Ross MG *et al.,* 2001). Indeed, the fetus swallows and digests on average 0.19 g/kg body

weight of soluble amniotic fluid protein (Gitlin MD *et al.,* 1972). So, the larger the infant, the more protein he swallows, the less the AF protein concentration.

As early as 15 weeks of gestation, amniotic fluid proteins are originally a simple filtrate of the maternal plasma protein (Johnson AM *et al.,* 1974). In early gestation, fetal serum proteins do not contribute largely to the amniotic fluid (Sutcliffe RG *et al.,* 1973). Since our study dealt with a gestation period from 12 to 19 weeks, most of the proteins may likely reflect the maternal plasma proteins. The concentration of prealbumin in plasma protein was 0.1-0.4 g/l, while the transferrin and the albumin concentration were respectively 2.04-3.60 g/l and 35-55 g/l (Lindsey BJ *et al.,* 2005). As can be noticed, the prealbumin level is very low compared to that of albumin, which might clarify the reason why the amniotic fluid prealbumin could not be detected by the capillary electophoresis due to the very low concentration of this protein. To our knowledge, no study has previously reported the concentration of prealbumin in the amniotic fluid.

Instrumental effects

Amniotic fluid samples were integrated at 3 wavelengths: 190, 254 and 275 nm. At the latter 2 wavelengths, the majority of the albumin peaks eluted as two different visible peaks. A possible explanation for the numerous peaks seen in the 254 and 275 nm wavelengths, is one of the functions of albumin in the body, that is binding and transport of compounds, like drugs and endogenous compounds; bilirubin, haematin, ascorbate, tryptophan, bile acids, copper, zinc, folate, and eicosanoids (Fuhrman et al, 2004). Therefore, the various peaks visible at the 254 and 275 nm wavelengths might be various compounds eluted with albumin. Although this might be a probable explanation, a more thorough study of those peaks lead to the conclusion that albumin was co eluting with the internal AF standard we used, thiamin. This internal standard combined with albumin and formed a peak before pure albumin. The impact of this co-elution problem was largely mitigated in the 190 nm wavelength electropherograms since the albumin absorption signal is strongest at 190 nm.

Protein peak areas were divided by their migration time. Consequently, these are divided by the internal standard (thiamin) peak area; this final step is usually done to control for the changes in volumes injected. But due to the co-elution with albumin,

which might be the ones discussed previously, the internal standard did not perfectly control for the variation in injection volume. Thiamin solution is heat and light-sensitive but was always kept in the fridge at 4° C, and wrapped up in aluminum paper to lessen its exposure to light. The deterioration of this vitamin by consequent exposure to offending environments could have affected its composition. This might have lead to different peak areas proportions while combining with albumin. These possibilities might explain the slight difference between the amniotic fluid albumin level reported in the literature and the albumin concentration in our study, where we have a slightly overestimated albumin concentration, and the fact that we didn't find any significance with regards to albumin.

As mentioned before, a large variation in albumin concentration was found among the amniotic fluid pooled samples. The co-elution of thiamin and albumin might explain the large variation in the albumin concentration in the pooled samples (34%) even though the concentration of albumin found in the pooled samples 3.24 ± 1.13g/l (mean ± SD) was comparable to literature. Since there was no visible difference between the runs of the pooled samples in the beginning of the day and the end of the day, we can assure that the capillary electrophoresis machine was running efficiently (Figure 3). The large variation in AF albumin concentration might have affected our results and lead to the non-significant negative association between albumin and birth weight. In addition, non-significant results might be the results of the low sample size. It can be estimated again.

Future work

The consent forms used were well-organized and very comprehensive. The questions covered all the topics of interest, and women had no problem in answering them. However, it is possible that there may be other disease conditions, which we did not control for that could be confounding our results. While we excluded for diabetes, other conditions may be related to AF transferrin, like infections. However, the consent forms need some improvement in certain areas. It was demonstrated that amniotic fluid can be modified by maternal diet (Koski KG *et al.,* 1992). In addition, caloric intake of a pregnant woman affects the birth weight of her infant (Kramer MS, 1987), while protein supplementation has not shown any significant effect on birth weight (Kramer MS, 1987). Our consent forms did not cover the maternal dietary intake, mainly her energy or

protein intake, and it would be worth looking at this issue. The only information we could gather from that topic was the pregnant woman's intake of vitamins, coffee/tea, and alcohol. Since other food might contain caffeine, the caffeine intake of the mother was not fully reported. Alcohol intake could have a different categorization in the consent form. Instead of having a choice of 0-1 alcoholic drinks/week, those two quantities should be categorized separately, since as mentioned in the literature, very low intake of alcohol versus no intake at all might affect the infant (Kramer MS, 1987). Updating the consent forms will help include better controlled covariates in the analyses.

For future work, the limitations encountered during our study can be overcome by using a methodology with better strategy in certain areas. The technical problems encountered in our method could be handled to give more reliable results in some areas of the study. Three directions should be investigated in the future:

1) Amniotic fluid samples should be analyzed with a different internal standard not co-eluting with any amniotic fluid constituent, or at least the analyte of interest. Further analysis should be done specifically concerning the albumin to give more reliable results. Indeed, AF pooled sample albumin had a mean of 3.24±1.13 g/l (mean ± SD), with a variation of 35%. So, this wide variation would be narrowed with the use of another internal standard.

2) Furthermore, another possible way to calculate the albumin and transferrin peak areas might be using the relative areas of these proteins compared to the range of proteins in the amniotic fluid. This gives the distribution of proteins in the amniotic fluid rather than the absolute values. This could provide a better control over the peak variations since all the peak areas in a specific sample will behave in the same manner, either decreasing or increasing in the same fashion. As can be noticed in Figure 12, plotting the two proteins has shown that they appear fairly well, nonlinearly correlated, and that there might be biochemical information in the distribution, not in the absolute concentrations. Using the relative concentrations is a method of evaluating the distributions.

3) In addition, there are many protein peaks eluting on the electropherograms. Inclusion of other protein peak data, and studying their relation to fetal growth might be

an interesting subject to explore. We only used 2 peaks out of multiple peaks due to time limitations but the data is there ready to use for the future.

Verification of the amniotic fluid constituents by another method would also be useful. Now that we have calculated the concentration of these two proteins by capillary electrophoresis, another calculation of the amniotic fluid content of these two proteins is underway by enzyme-linked immunosorbent assay (ELISA). This test has many advantages in that it is cheaper, and generally highly sensitive and specific. ELISA might catch the large variability found in the CE. This test does not involve thiamin, so the comparison between the two outcomes will give us a clear view of the amniotic fluid content of these two proteins.

Currently, the fetal weight early in gestation cannot be predicted using ultrasound measurements. Since our study dealt with measurements at early times, therefore, its originality lies in the hypothesis that evaluates the possibility of predicting the infant birth weight at an earlier gestational age by measuring transferrin, albumin and prealbumin in the amniotic fluid, at the time of routine amniocentesis and associating these measures with birth outcomes. Moreover, our research had another advantage in that it took into account the composition of the metabolic environment of the fetus, which is absent using ultrasound measurements, since the latter are based on anthropometric measurements only. Our study showed that 2nd trimester amniotic fluid transferrin is significantly different between SGA, AGA and LGA infants, and transferrin was found to be negatively associated with the birth weight at the low birth weight category.

Although human studies are needed, animal studies have demonstrated that maternal diet affects the amniotic fluid composition (Koski KG et al, 1992). Consequently, an intervention or manipulation of the amniotic fluid components could be made to prevent the occurrence of the birth weight extremes. Therefore, this study suggests that the composition of the AF in the early gestational period provides a new window on fetal growth and development, that transferrin is different between SGA, AGA and LGA infants, and is associated with low birth weight infants. These constituents can help prevent or protect against the incidence of the birth weight extremes.

In conclusion, our study shows that 2nd trimester AF transferrin concentration is different among the birth weight categories, and that AF transferrin is negatively associated with birth weight at the LBW category.

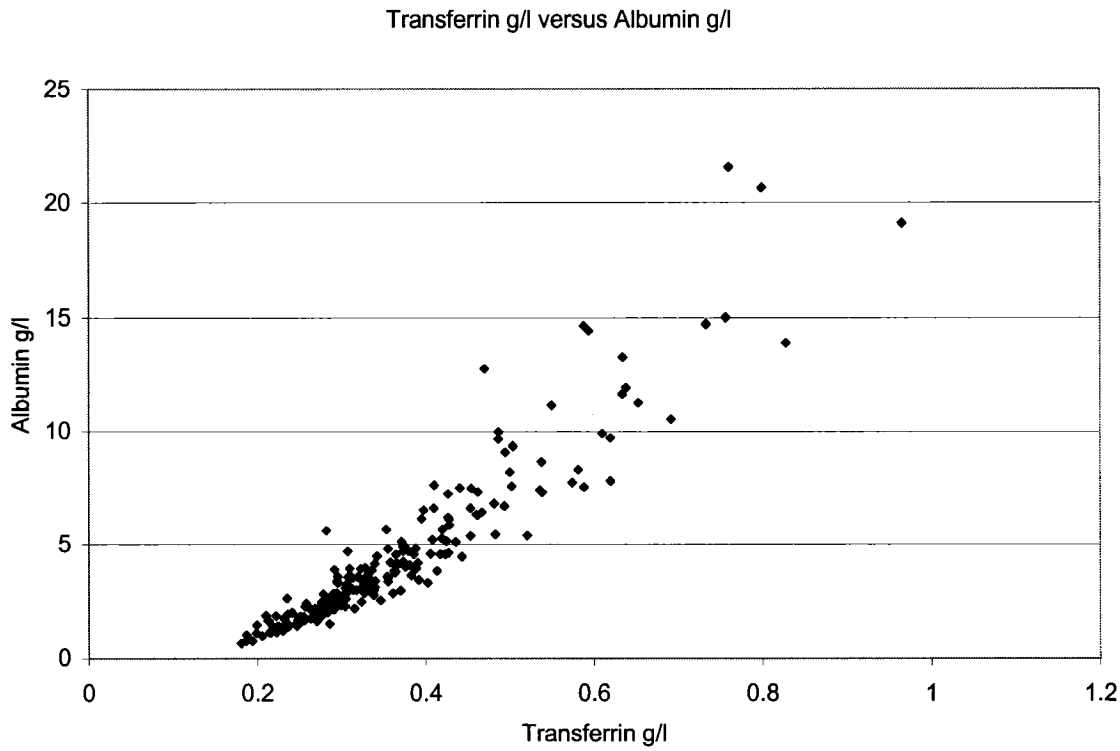

Figure 12. Plot of AF transferrin versus AF albumin concentrations.

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APPENDICES

McGill

School of Dietetics and Human Nutrition
École de diététique et nutrition humaine
Tel.: (514) 398-7842
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Faculty of Agricultural and Environmental Sciences
Faculté des sciences de l'agriculture et de l'environnement
21,111 Lakeshore
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Université McGill
Campus Macdonald

SUBJECT CONSENT FOR RESEARCH PROTOCOL

Protocol #_____ Patient Name_____ Telephone #_____

The Role of Amniotic Fluid in Predicting Human Fetal Growth

School of Dietetics and Human Nutrition
MacDonald Campus of McGill University

Principal Investigator: Dr. Kristine G Koski, Ph.D.-R.D. tel: 398-7845
St. Mary's Hospital On-Site Contact: Dr. Gary Lusky, M.D. tel: 731-5566
Montreal Children's Hospital On-Site Contact: Dr. Louis Beaumier, M.D.

Research Protocol:

The aim of this study is to examine the possibility that certain substances that help the baby grow, called nutrients, as well as natural hormones, which are normally found in amniotic fluid, might be used to assess fetal growth and development.

Participation in the study in no way implies that you or your baby's health is in jeopardy or at risk.

We are requesting that you fill out our study consent form. By signing our consent form, you give your permission to the Montreal Children's Hospital to give the normally discarded portion of your current sample to the above named researchers for their investigation. Without your signature on this form, the Montreal Children's Hospital is not permitted to give your amniotic fluid sample to a third party.

If you agree to participate in the study, we will collect, following the completion of your genetic testing, a small portion (15cc) of your leftover amniotic fluid, which will be used to measure the natural levels of substances (growth hormones and nutrients) that help the baby grow. The same amniotic fluid would otherwise be thrown away after genetic testing. No additional amniotic fluid will be required now, or in the future. Your medical record and your baby's medical record at birth will be reviewed for information about the pregnancy and the birth. You will be asked to complete a short questionnaire related to your health and your pregnancy. It should take about 5 minutes to complete.

- Please be assured that your decision will in no way interfere with the Montreal Children's Hospital analysis or the reporting of the results to your physician. You will receive the usual care.
- There are no known risks associated with participation in this study.
- There are no known benefits to you or your family from participation in this study, although lead to better prenatal care in the future.

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Le présent projet de recherche est dirigé par l'Université de McGill avec la collaboration des hôpitaux affiliés de McGill. Toutes les femmes enceintes subissant une routine amniocentèse sont appelées à participer. La cible à long terme du projet est d'améliorer les soins prénatals et d'avancer les connaissances dans ce domaine.

Nous encourageons votre participation dans cette étude et dans ce but, nous vous fournissons un numéro de téléphone pour toutes questions au sujet du formulaire de consentement ou de l'étude. Dans ce cas, n'hésitez pas à contacter, Dr. Kristine G. Koski, coordinatrice de l'étude, au (514) 398-7845 entre 9h00 et 16h00, du lundi au vendredi.

Si vous avez besoin d'informations supplémentaires à propos de l'étude avant de participer, le formulaire de consentement et le questionnaire pourront être postés à l'adresse suivante :

Kristine G. Koski
School of Dietetics and Human Nutrition
Faculty of Agricultural and Environmental Sciences
McGill University – Macdonald Campus
21,111 Lakeshore Road
Ste-Anne-de-Bellevue, Québec
H9X 3V9

Veuillez agréer, madame, mes meilleures salutations.

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FORMULAIRE DE CONSENTEMENT POUR UN PROTOCOLE DE RECHERCHE

Protocole n° _____ Nom du patient _____ N° de téléphone _____

Rôle du liquide amniotique dans l'évaluation de la croissance du foetus humain

École de diététique et de nutrition humaine
Campus Macdonald de l' Université McGill

Chercheur principal : Dr Kristine G. Koski, Ph.D.-R.D. tél : 398-7845
Personne-ressource sur place du Centre Hospitalier de St. Mary : Dr. Lusky, médecin tél : 731-5566
Personne-ressource sur place de l'Hôpital de Montréal pour Enfants: Dr Louis Beaumier, médecin

Protocole de recherche :

L'objectif de cette étude est d'étudier la possibilité que certaines substances qui contribuent à la croissance du foetus, appelées nutriments, ainsi que les hormones naturelles que l'on trouve normalement dans le liquide amniotique, puissent servir à évaluer la croissance et le développement du foetus.

Votre participation à cette étude n'implique nullement que votre santé ou celle de votre bébé est en danger ou court des risques.

Nous vous demandons de bien vouloir remplir le formulaire de consentement. En apposant votre signature, vous autorisez l'Hôpital de Montréal pour Enfants à remettre la partie normalement éliminée de votre liquide amniotique aux chercheurs, dont les noms figurent ci-dessus pour leur recherche. À défaut de signer ce formulaire, l'Hôpital de Montréal pour Enfants n'est pas autorisé à remettre l'échantillon de votre liquide amniotique à un tiers.

Si vous acceptez de participer à cette étude, nous prélèverons, après avoir terminé votre test génétique, une petite partie (15 cc) du restant de votre liquide amniotique, qui servira à mesurer les niveaux naturels des substances (hormones de croissance et nutriments) qui contribuent à la croissance du foetus. Ce même liquide amniotique serait autrement éliminé après le test génétique. Aucun prélèvement de liquide amniotique supplémentaire n'est prélevé maintenant ou en tout autre moment. Votre dossier médical sera analysé pour en extraire des informations sur la grossesse et la naissance. Nous vous demandons de remplir un court questionnaire au sujet de votre santé et de votre grossesse. Cela devrait vous prendre environ 5 minutes.

Vous pouvez être assurée que votre décision n'aura aucune incidence sur l'analyse de l'Hôpital de Montréal pour Enfants ou sur la communication des résultats à votre médecin. Vous bénéficierez des soins habituels.

- **Aucun risque connu ne se rattache à la participation à cette étude.**

- **Votre participation à cette étude ne présente aucun avantage connu pour vous ou pour votre famille, si ce n'est qu'elle peut se traduire par une amélioration des soins prénatals à l'avenir.**

- **Votre identité et celle de votre bébé ne seront divulguées à personne, en dehors des chercheurs participant à ce protocole. Tous les échantillons et les résultats seront codés. Les résultats collectifs pourront être présentés dans le cadre de tribunes scientifiques ou professionnelles, sans toutefois qu'il soit possible de vous identifier, vous ou votre bébé.**

Je soussignée _____ **consens par la présente à participer comme sujet au protocole de recherche mentionné ci-dessus** ***intitulé Le rôle du liquide amniotique dans l'évaluation de la croissance du foetus humain***, **projet réalisé par l'École de diététique et de nutrition humaine de l'Université McGill. J'ai pris connaissance des données ci-dessus et j'autorise les chercheurs**

1) **à prélever 15 cc de liquide amniotique sur l'échantillon qui vient d'être prélevé à l'Hôpital de Montréal pour Enfants; et**

2) **à obtenir le poids de naissance, la taille, le sexe, le dernier poids de la mère avant l'accouchement sur le dossier hospitalier après l'accouchement.**

Si vous avez des questions concernant vos droits comme patiente, veuillez contacter Mme Monique Robitaille au (514) 734-2618 ou écrivez à l'adresse suivante :

Mme Monique Robitaille
Centre Hospitalier de St. Mary
3830 Lacombe
Monréal, Qc
H3T 1M5

Il est entendu que ma participation à ce protocole est entièrement volontaire, que les données me concernant resteront confidentielles et que je suis libre de me désister de cette étude à tout moment.

Signature du sujet _____ **Date** _____

Témoin _____ **Date** _____

Veuillez remplir le questionnaire ci-joint.

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Québec, Canada H9X 3V9

Questionnaire

Veuillez répondre à ce bref questionnaire. Nous vous rappelons que toutes les données fournies seront rigoureusement confidentielles.

Nom : _____ Numéro de téléphone : _____

Date de naissance : _____ Taille : ____ pieds ____ pouces ou ____ mètres

Origine ethnique :
Nord Américaine ____ Sud Américaine ____
Européenne ____ Africaine ____
Moyen-Orientale ____ Asiatique ____
Autre _____

Nombre d'enfants à qui j'ai déjà donné naissance : ____

Les renseignements suivants n'ont trait qu'à cette grossesse :

Poids avant la grossesse : ____ livres ou ____ kilos

J'en suis à ma ____ e semaine de grossesse. Date d'accouchement : _____

Hôpital où j'accoucherai :
Royal Victoria _____
Général Juif _____
St. Mary's _____
Autre _____

Nom de l'obstétricien/gynécologue : _____

Je suis fumeuse : Oui ___
Dans l'affirmative, pendant que j'étais enceinte, j'ai fumé ___ cigarettes par jour
Oui, mais j'ai arrêté pendant ma grossesse ____
Non ____

Pendant ma grossesse, j'ai consommé en moyenne :
0-1 boisson alcoolique par semaine ____
2-5 boissons alcooliques par semaine ____
6-10 boissons alcooliques par semaine ____
11-15 boissons alcooliques par semaine ____

0-1 tasse de café/thé par semaine ____
2-5 tasses de café/thé par semaine ____
6-10 tasses de café/thé par semaine ____
11-15 tasses de café/thé par semaine ____

Je prends actuellement des médicaments et/ou vitamines (prescrits par mon médecin ou en vente libre) : oui ___ non ___

Si vous avez coché oui, veuillez préciser lesquels _____
_____ MERCI