Amniotic fluid alkaline phosphatase as a biomarker of fetal growth and development

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February, 2007

A thesis is submitted to McGill University in partial fulfillment of the requirements of the degree of Masters of Science.

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ABSTRACT

The objectives of this study were to assess the concentration of alkaline phosphatase (ALP) and its isoenzymes (intestinal, liver/bone/kidney, placental) in 2nd trimester amniotic fluid (AF) and to establish whether total ALP or placental ALP were related to infant birth weight (BW) or gestational age (GA). 518 biobanked AF samples were analyzed using fluorometry to measure ALP concentrations. Analysis of variance showed that mothers giving birth to small-for-gestational-age infants had higher placental AF ALP concentration compared to those giving birth to appropriate-for-gestational-age infants, when controlling for established predictors of birth weight. Placental ALP was also elevated in the AF of mothers giving birth to females compared to males and in smokers compared to non-smokers. Multiple regression analysis demonstrated that 2nd trimester AF placental ALP negatively predicted BW (in grams and as percentiles) in term infants. AF ALP may emerge as an early indicator of placental tissue status or immunity which could subsequently affect fetal growth.
SOMMAIRE

Les objectifs de cette étude étaient d’évaluer la concentration de phosphatase alcaline (ALP) et de ses iso-enzymes (intestinaux, hépatiques, rénales, placentaires) dans le liquides amniotique (AF) du 2e trimestre, et d’établir si l’ALP totale ou l’ALP placentaire sont associées au poids à la naissance ou à l’âge gestationnel. Un total de 518 échantillons rassemblés biologiquement ont été analysés en utilisant la fluorométrie pour mesurer l’ALP concentrations. L’analyse de variance a montré que les mères donnant naissance à des enfants petits pour leur âge gestationnel avaient la concentration de l’ALP placentaire du AF plus élevée que celle des mères donnant naissance à des enfants appropriés pour leur âge gestationnel, en contrôlant les prédicateurs du poids à la naissance. L’ALP placentaire était aussi élevé dans l’AF des mères donnant naissance à des filles comparativement à des garçons et chez les fumeuses comparativement aux non-fumeuses. L’analyse de régression multiple a démontré que l’ALP dans l’AF placentaire du 2e trimestre a prédit inversement le poids à la naissance (en grammes et percentiles) chez les enfants à terme. L’ALP du AF pourra émerger comme un indicateur précoce des tissus placentaires deviendra peut-être un prédicateur du statut des tissus placentaires et immunité qui peut affecter subséquemment la croissance fœtale.
ACKNOWLEDGEMENTS

First, I would like to express my sincere appreciation to my supervisor Dr. K. Koski. Her constant guidance, patience, expertise and dedication have provided me with valuable knowledge not only about the field but she has helped me in identifying my personal strengths and improving my weaknesses as a graduate student. Thank you to my committee members: Dr. Kubow and Dr. C Skinner for their time and valuable input into my research.

I would also like to give thanks to Lise Grant for her resourcefulness and guidance throughout my Masters. I am grateful to my colleagues for their support both inside and outside of the lab: Kebba Sabally, Dan Tisi, Mike Massie and my roomie and friend Dima El-Halabi.

Thank you my friends in Montreal who have provided me encouragement and most of all, the many laughs throughout my Masters. Thank you to my dear friend Swami who provided me emotional support through frequent long distance calls.

Finally, I would like to express my most heartfelt gratitude to my family who has always supported and encouraged me with every decision I’ve made. To my parents thank you for giving me the world and helping me to achieve my dreams; to my brother for being the big brother I look up to, for keeping me grounded and for never allowing me to give up. Without the love of my family, I would not be where I am today.
CONTRIBUTION OF AUTHORS

The research presented in this project was developed in collaboration with Dr. Kristine Koski. The author adapted established assay methods in literature for use in this study with the support of Kebba Sabally. The author completed analysis of alkaline phosphatase in amniotic fluid samples by fluorometry with the assistance of another student. Michael Massie, a summer NSERC student, assisted in the analysis of alkaline phosphatase in amniotic fluid samples under the instruction of the author. The author participated in the recruitment of participants, medical chart review and the cataloguing and management of amniotic fluid samples. Statistical analysis, writing of the thesis and paper were completed by the author.
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LIST OF ABBREVIATIONS

AF: Amniotic fluid
AGA: Appropriate-for-gestational-age
A.A.: Amino Acid
ANOVA: Analysis of variance
ANCOVA: Analysis of covariance
BBt: 2’-[2-benzothiazoyl]-6’-hydroxybenzothiazole phosphate
BMI: Body Mass Index
BW: Birth weight
GA: Gestational age
GDM: Gestational Diabetes Mellitus
Ht: Height
IGF-1 and IGF-II: Insulin-like growth factors (IGF-1 and IGF-II)
IGF-BP: Insulin-growth-factor binding protein
IgG: Immunoglobulin G
IUGR: Intrauterine growth retardation
LBK: Liver/Bone Kidney
LBW: Low birth weight
LGA: Large-for-gestational-age
LMP: last menstrual period
PPWkg: Pre-Pregnancy Weight
PPWR: Post partum weight retention
SGA Small-for-gestational-age
TNS ALP: Tissue Non-Specific Alkaline Phosphatase
TS ALP: Tissue Specific Alkaline Phosphatase
VLBW: Very Low Birth Weight
I. OVERVIEW

Amniotic fluid (AF) is important for fetal growth and development (Cellini C et al., 2006). Not only does AF physically protect the fetus from mechanical disturbances and bacterial substances, it functions to regulate pH, temperature and provides nutrients and growth factors (Cellini C et al., 2006; Underwood MA et al., 2005). Daily, a fetus may swallow up to 100-300 ml of AF per kg of their body weight (Ross MG et al., 2001). Strong evidence shows that fetal ingestion of AF has an impact on fetal growth and development (Cellini C et al., 2006; Ross MG et al., 2001; Underwood MA et al., 2005; Buchmiller TL et al., 1994; Pitkin RM et al., 1975). In animal studies, impairment of AF swallowing has lead to delayed gastrointestinal development (Ross MG et al., 2001). More seriously, impaired AF swallowing may cause a reduction in fetal weight (Cellini C et al., 2006; Ross MG et al., 2001).

Researchers have attempted to examine the numerous biological components (Gurekian N et al., 2005) present in AF including enzymes (Tisi DK et al., 2004; Burc L et al., 2001; Benzie RJ et al., 1974). In AF, enzymes may originate from maternal plasma, maternal uterogenital tract, the placenta, the umbilical cord, AF cells, fetal urine, meconium, and various other secretions from the fetus (McCarthy T et al., 1978; Hahnemann N et al., 1974). One enzyme, which is of growing interest, is non-hormonal alkaline phosphatase (ALP). Research, which has mostly involved the analysis of maternal plasma, has shown an increased risk of preterm pregnancies in pregnant women with elevated total ALP activity (Moawad AH et al., 2002; Goldenberg RL et al., 1997). Concurrent studies have also examined the role of placental ALP in relation to low birth weight (LBW) (Best RG et al., 1991, Brock DJH et al., 1988) and prematurity (Meyer RE et al., 1995) During pregnancy, evidence suggests placental ALP transports Immunoglobulin G (IgG) from the mother to the fetus (Stefaner B et al., 1997; Makiya R et al., 1992) and stimulates cell proliferation in fetal cells (She QB et al., 2000a; She QB et al., 2000b). These studies imply a potential relationship between the placenta, fetal growth and ALP.

Abnormal ALP values in both maternal serum and AF can indicate Down’s syndrome (Ben-Ami M et al., 2002; Ind TE et al., 1993; Vergnes H et al., 2000). In addition, ALP in AF is a strong predictor of cystic fibrosis (Sembaj A et al., 1995; Moss...
Clinically, ALP throughout gestation can be a valuable tool for understanding fetal development. However, there is a lack of data analyzing ALP in AF.

This study investigates whether human AF total ALP and/or placental ALP are predictors of gestational age (GA) and/or birth weight (BW). Participants for this study were recruited from a large population of women undergoing routine amniocentesis for age and related genetic testing. Obtained second trimester AF samples were biobanked and frozen for the purposes of this study. The concentration of AF ALP and its family of isoenzymes - intestinal, liver, bone, kidney and placental - were assessed and their association to GA and fetal BW were analyzed. The aim of this study is to provide more information regarding the early recognition of aberrant birth weights in hopes of improving prenatal care.

This thesis has been prepared as a manuscript based thesis. The sections of this thesis include a comprehensive literature review, a manuscript for journal submission, a general discussion and a list of cited references.
II. LITERATURE REVIEW

A. Fetal Growth

Fetal birth weight is a determining factor of perinatal morbidity and mortality (McCormick MC, 1985). More recently, a strong association between size at birth and adult onset of adverse health diseases (Ong KK et al., 2004; Baschat AA et al., 2004; Aplin J, 2000; Krishnaswamy K et al., 2002) has been observed. It is now understood that the growth potential of a fetus in utero is determined by a complex interrelationship between three influential factors: the fetus, the mother and the placenta (Baschat AA et al., 2004; Valero De Bernabé J et al., 2004; Kramer MS, 1987a). The following sections describe these factors in more detail.

1. Fetal Factors

Glucose, metabolized by the fetus, directly influences fetal growth (Ong KK et al., 2004). In instances when the fetal environment fails to provide adequate nutrition, the fetus responds and adapts. Fetal hypoinsulinemia, caused by impaired trans-placental glucose transfer, limits oxygen and subsequently tissue growth (Baschat AA et al., 2004; Scholl TO et al., 2001). This condition is commonly seen in intrauterine growth retardation (IUGR) infants. Conversely, fetal hyperinsulinemia has been associated with macrosomia (Jansson T et al, 2006). Initially, such fetal adaptations enable the fetus to survive in its dynamic environment. However, research has shown that these adaptations can lead to long term morbidities including hypertension and obesity (Godfrey KM et al., 2000).

Genetic mutations in the fetus causing an over-expression or under-expression of insulin-like growth factors, IGF-1 and IGF-II, have been linked to both excess and restricted fetal growth, respectively (Ong KK et al., 2004). Unlike the above mentioned fetal environmental factors, medical intervention for fetal chromosomal or anatomical abnormalities such as trisomy 21 will not alter fetal growth outcome (Valero De Bernabé J et al., 2004).
2. Maternal Factors

Fetal size at birth is a composite of numerous maternal factors that come into play prior to conception and continue up until delivery (Kramer MS, 1987a). Maternal factors which have been the most widely studied as they appear to have the most influence on fetal growth will be described in the following sections.

Gestational Age

Length of gestation directly determines fetal birth weight (Oken E et al., 2003; Hindmarsh PC et al., 2002; Muslimatun S et al., 2002; Kramer MS, 1987b). It has been reported that birth weight increases with gestation length non-linearly (Oken E et al., 2003). The most rapid weight gain occurs in the 3rd trimester and tapers off at 40 weeks (Oken E et al., 2003). In addition, neonatal length and head circumference develop with progressing gestational age (Hindmarsh PC et al., 2002). Therefore, shortened or prolonged gestation length directly impacts neonatal weight. More specifically, for every week of gestation, birth weight has been demonstrated to increase by 124 g (Hindmarsh PC et al., 2002).

Preterm delivery has been defined as less than 37 weeks gestation (Wen SW et al., 2004). However, other definitions of preterm delivery have been suggested. In addition, GA is typically incorrectly estimated using a mother’s last menstrual period (LMP). Research has proposed that 38 weeks gestation more accurately reflects a term infant since it is associated with the lowest risk of perinatal death (Smith GC, 2001). Premature infants are more often than not of low birth weight and incur health risks including neurological disorders, cerebral palsy and birth defects (Lancet, 2006). Despite advances in prenatal care, in Canada, preterm births have increased from 6.6% in 1991 to 7.6% in 2000 (Canadian Perinatal Health Report, 2003).

At the other end of the spectrum, a gestation length of > 42 weeks, post term delivery, is often linked to macrosomia (>4500 g) (Heiskanen N et al., 2006; Stotland NE et al., 2004). Women with prolonged gestation lengths have a three to four fold risk of delivering macrosomic infants (Heiskanen N et al., 2006; Stotland NE et al., 2004). Canadian rates of post term deliveries have declined from 4.4% to 1.2% from 1991 to 2000 (Canadian Perinatal Report, 2003). Recent literature has demonstrated that this has
resulted from an overall increase in infant birth weight (Kramer MS et al., 2002), strongly suggesting that other factors such as a reduction in cigarette smoking and an increase in maternal stature contribute to macrosomia.

**Infant Gender**

Existing literature regarding the effect of gender on fetal size at birth concludes that males have a lower risk of IUGR and are hence born with a higher birth weight than females (Valero De Bernabé J et al., 2004; Yunis KA et al., 2004; Oken E et al., 2003, Hindmarsh PC et al., 2002, Muslimatun S et al., 2002, Kramer MS, 1987a). Besides being heavier, male neonates are generally longer in length and have larger head circumferences (Muslimatun S et al., 2002). In developed countries, males are heavier by a magnitude of 126.4 g (Kramer MS, 1987a) to 130 g (Hindmarsh PC et al., 2002). Meanwhile, in developing countries, the birth weight discrepancy between genders is less at approximately 100 g (Muslimatun S et al., 2002; Kramer MS, 1987a). Male infants have two times the likelihood to be macrosomic (Heiskanen N et al., 2006; Stotland NE et al., 2004).

In contrast, female infants have a relative risk of 1.94 for small-for-gestational-age (SGA) (Yunis KA et al., 2004). This sexual dimorphism exists *in utero*. In early gestation, females have smaller head and abdominal circumferences than males (Hindmarsh PC et al., 2002).

**Maternal Height**

Maternal height has been demonstrated to have a positive effect on fetal birth weight (Muslimatun S et al., 2002, Kramer MS, 1987a). A woman’s height can determine whether she can provide an optimal growing environment for the fetus and thus, genetically determines a developing fetus’ growth potential (Kramer MS, 1987a). Based on a review of eight studies with an accumulated sample size of 52 371, it was demonstrated that the exact effect of maternal height on birth weight is 7.8 g for every 1 cm increment of height (Kramer MS, 1987a). Meanwhile a more recent study conducted in the United Kingdom (n=1129) showed an increase of 16 g for every 1 cm of maternal
height (Hindmarsh PC et al., 2002). Short stature women with a height of <155 cm have almost a three fold risk of delivering a SGA infants (Yunis KA et al., 2004).

**Pre-pregnancy Weight**

Similar to maternal height, a woman’s weight prior to pregnancy contributes to fetal birth weight (Kramer MS, 1987a). It has been shown that an increase of 9.5 g in fetal birth weight can occur for every additional 1 kg of pre-pregnancy weight (Kramer MS, 1987a).

Lean women with a pre-pregnancy weight of < 50 kg are at a two fold risk of delivering an SGA infant (Yunis KA et al., 2004). Other research has shown that lean women of less than 100 lbs are at an increased risk for delivering very low birth weight (VLBW) (1500 g) babies compared to women weighing 45-68 kg (Murakami M et al., 2005, Rosenberg TJ et al., 2003). Murakami et al. (2005) showed that women with a BMI <18.5 have a three fold risk over women with a BMI of 18.5-25 of delivering a LBW baby. Compared to women with a normal BMI of 19.8-26, women who are morbidly obese or have higher body mass indices are significantly more at risk of delivering macrosomic infants (Heiskanen N et al., 2006) and for Gestational Diabetes Mellitus (GDM) (Cedergren MI 2004; Rosenberg TJ et al., 2003).

**Maternal Weight Gain**

Maternal weight gain during pregnancy has a direct impact on fetal birth weight (Kramer MS, 1987a). It is evident that pregnancy requires an increased demand of nutrients to ensure adequate growth of the fetus, placenta and volume of AF. Thus, it has been advised that during pregnancy women should gain at least 12-16 kg (Lasker JN et al., 2005, Valero De Bernabé J et al., 2004). The American College of Obstetricians and Gynecologists has suggested the following recommendations for maternal weight gain: BMI <18.9 gain 28-40 lbs (12-18 kg), BMI 19-24.9 gain 25-35 lbs (11-15 kg), BMI > 25 gain 15-25 lbs (6-11 kg) (Shapiro C et al., 2000). Literature shows an increase of 20.3 g in birth weight for every 1 kg increase in maternal weight gain (Kramer MS, 1987a).

A collection of literature has shown that inadequate weight gain during pregnancy of less than 10 kg puts a woman at two times the risk for IUGR and SGA:
• < 7 kg: 1.98 for IUGR (Kramer MS, 1987a)
• < 8.5 kg: 1.26 for LBW (Murakami M et al., 2005)
• < 10 kg: 1.75 risk for SGA (Yunis KA et al., 2004)

Moreover, a weekly weight gain of less than 0.2 kg is associated with an increased risk for SGA (Cheng CJ et al., 2004). It has been shown that inadequate maternal weight gain leads to significantly smaller placental volumes. This can subsequently lead to fetal growth retardation (Thame M et al., 2004).

Mothers that have gained >14 kg during the course of pregnancy decrease the risk of LBW by half (Lasker JN et al., 2005). However, a maternal weight gain of over 12 kg during pregnancy may lead to post partum weight retention (PPWR) (Muscati SK et al., 1996). This trend was strongest for weight gained during the first and second trimester of pregnancy. This suggests that it is advantageous for maternal weight gain to occur in late pregnancy to optimize fetal growth and limit the risk of PPWR (Muscati SK et al., 1996).

Excessive weight gain of more than 20 kg during pregnancy remains one of the contributing factors for macrosomia (Bérard J et al., 1998). It has been suggested that heavier women have larger volumes of plasma and hence, have increased placental perfusion providing an enhanced amount of nutrients to the fetus (Goldenberg RL et al., 1996).

Maternal Age

Research has concluded that maternal age does not directly correlate with birth weight. Instead, factors associated with maternal age influence birth weight (Kramer MS, 1987a). Results from various studies demonstrate that women from the two ends of the age spectrum are more prone to delivering low birth weight babies (Lasker JN et al., 2005; Cheng CJ et al., 2004; Fraser AM et al., 1995; Reichman NE et al., 1997). The exact age specifications are, however, unclear.

Some research suggests that adolescents less than 15 years old (Reichman NE et al., 1997) are at an increased risk while others suggest that women < 20 years are (Lasker JN et al., 2005; Cheng CJ et al., 2004). Adolescent mothers, who are still in the process of growing themselves, compete with the fetus for nutrients (Lasker JN et al., 2005; Fraser AM et al., 1995). This reasoning provides an explanation for the increased
propensity for LBW deliveries in this age group. Furthermore, women in this age bracket are more inclined to exhibit other unfavourable factors such as lower pre-pregnancy weights, inadequate weight gain during pregnancy (Lasker JN et al., 2005; Fraser AM et al., 1995), smoking, and alcohol consumption. Moreover, these pregnancies tend to be unplanned and unwanted (Kramer MS, 1987a).

Women of advanced maternal age have a significantly higher risk of delivering a LBW neonate (Lasker JN et al., 2005; Reichman NE et al., 1997). Older women > 35 years (Lasker JN et al., 2005) as well as women 30-40 years of age (Reichman NE et al., 1997) have been identified to be at significant risk for LBW. It has been demonstrated that women >40 years of age have an almost three fold risk of LBW compared to women of age 20-29 years (Lasker JN et al., 2005). Research has shown that mothers in the advanced age range have an increased chance of medical diseases like diabetes and hypertension that have a negative impact on fetal growth (Cnattingius S et al., 1992). Recent studies have also demonstrated that of all age groups, women with a maternal age of 30-40 were more likely to deliver more macrosomic infants than infants of normal birth weight (Heiskanen N et al., 2006, Stotland NE et al., 2004). In contrast to the majority of literature, research has also suggested that maternal age bears no relevance to birth weight (Muslimatun S et al., 2002).

**Ethnicity**

Natality data from different regions and countries across the world have shown a disparity of reported birth weights suggesting that ethnicity has some effect on birth weight (Kramer MS, 1987a).

- **White**

  Compared to other ethnicities, North American Whites and European Caucasians have the lowest rate of LBW (Lasker JN et al., 2005). While they are at lower risk for LBW, studies have also found that this ethnic group has the highest rate of macrosomic infants (Stotland NE et al., 2004). Larger-sized infants are also more apparent in Indian and Jewish communities (Kramer MS, 1987a). It is also evident from literature that predictors of LBW are non-white ethnic groups (Shiao SY et al., 2005).
• African Americans

It has long been established that African Americans have the highest rate of LBW (Lasker JN et al., 2005; Shiao SY et al., 2005; Shi L et al., 2004; Steward DK et al., 2004; Valero De Bernabé J et al., 2004). The incidence of LBW in this population is twice that of the European Caucasian population (Lasker JN et al., 2005). Studies have consistently found that African Americans have 2.64 times the risk for SGA (Valero De Bernabé J et al., 2004; Alexander GR et al., 1999). The high incidence of LBW is also accompanied by the highest infant mortality rate (Shiao SY et al., 2005; Alexander GR et al., 1999). It has been postulated that this significant birth weight discrepancy between these populations may be attributed to a higher proportion of mothers smoking during pregnancy (Lasker JN et al., 2005) and an increased incidence of infection and hypertension (Shiao SY et al., 2005). Other research has found that African Americans are significantly more likely to have children at a younger age than North American Whites (Chang SC et al., 2003), which increases the likelihood of SGA (Reichman NE et al., 1997; Lasker JN et al., 2005). On the other hand, North American Whites are more likely to have pregnancies after the age of 30 (Reichman NE et al., 1997). Literature also shows that African Americans have the highest incidence of preterm deliveries (Patel RR et al., 2004) putting this ethnic group at extremely high risk of adverse perinatal outcomes, including SGA.

• Asians

The research regarding Asians and birth weight trends remains limited (Qin C et al., 2006). Asians reportedly have a high propensity of delivering infants born IUGR (Shiao SY et al., 2005; Steward DK et al., 2004) which may be a result of their smaller maternal stature (Steward DK et al., 2004). The average BMI of some oriental Asians during pregnancy has been reported at 20.9 with an average weight gain of 10.5 kg (Murakami M et al., 2005). After African Americans, Asians have the 2nd highest rate of preterm deliveries (Patel RR et al, 2004). However, in the available literature, the definition of Asian has been unclear. Research has also identified Indians and Pakistanis...
(Kramer MS, 1987a) as having an increased likelihood for SGA or LBW compared to Whites.

Emerging literature by Patel RR et al. suggests that gestation length varies with ethnicity demonstrating that Asians (39 weeks) and African Americans (39 weeks) have shorter gestation lengths compared to North American White women (40 weeks). As mentioned in previous sections, gestation length has a bearing on fetal size at birth (Patel et al., 2004). Recent research suggests that acculturation, immigrant status (Qin C et al., 2006) and socioeconomic status (Lasker JN et al., 2005) may explain the various ethnic disparities of birth weight.

**Parity**

Many studies have found that the first born child tends to be lighter and shorter at birth (Muslimatun S et al., 2002; Hindmarsh PC et al., 2002). The magnitude of disparity in birth weight has been approximated at 186 g (Hindmarsh PC et al., 2002). Hence, nullparous women expecting their first child have a slightly increased risk of delivering SGA infants (Yunis KA et al., 2004).

- **Multiparity**

   After the first child, the birth weight of each following child is reported to increase (Valero De Bernabé J et al., 2004; Oken E et al., 2003). Literature has suggested that birth weight increases with additional births because in each birth, a more optimal environment is provided by the placenta (Roth J et al., 1998). However, literature regarding the effect of multi-parity on fetal outcome has been somewhat inconsistent (Aliyu MH et al., 2005). This may be attributed to numerous confounding factors of parity including: maternal age, maternal height, pre-pregnancy weight, and socioeconomic status (Aliyu MH et al., 2005; Kramer MS, 1987a). A strong inverse relationship between parity ≥ 5 and BW as well as GA has been demonstrated, despite controlling for confounding factors (Aliyu MH et al., 2005; Silva AA et al., 1998). Increased incidence of placental previa and placental abruption, conditions commonly seen in preterm deliveries, increase with parity (Aliyu MH et al., 2005).
However, increasing parity - multiparas of 5 or more live births – may also be an independent risk factor for macrosomia (Aliyu MH et al., 2005; Toohey JS et al., 1995). Other research suggests a parity of > 7 (Heiskanen N et al., 2006) or 10-14 (Stotland NE et al., 2004) increases a woman’s likelihood of delivering a macrosomic infant. Emerging literature has reported that women with a previous delivery of a macrosomic (Heiskanen N et al., 2006) or LBW infant are highly more likely to have a recurrence of the same perinatal outcome (Adams MM et al., 2006).

Cigarette Smoking

Maternal smoking during pregnancy has been strongly established as a risk factor for LBW and IUGR (Jauniaux E et al., 2001; Horta BL et al., 1997; Kramer MS, 1987a). Accumulated studies show that smokers have twice the risk of having an IUGR or LBW baby (Dejmek J et al., 2002; Horta BL et al., 1997; Kramer MS, 1987a). Research suggests that 18% of all IUGR cases are attributed to this modifiable habit (Thompson JM et al., 2001).

A disparity of 142 g has been reported between babies delivered by mothers who smoked during their pregnancy compared to babies of non-smoking mothers (Horta BL et al., 1997). A dose-dependent relationship exists between the number of cigarettes smoked and a reduction in fetal birth weight (Dejmek J et al., 2002; Horta BL et al., 1997; Abel EL, 1980). An average decrease of a 150g of fetal birth weight has been observed in mothers who smoked 1-10 cigarettes/day (Dejmek J et al., 2002). Meanwhile, a reduction of 260 g was reported in those who smoked an excess of 10 cigarettes/day. Mothers who smoke late into the 2nd and 3rd trimester of pregnancy are at a higher predisposition for delivering a LBW baby (Dejmek J et al., 2002; Horta BL et al., 1997). The risk of delivering IUGR infants was comparable for those who stopped smoking early in pregnancy to that of non-smoking mothers (Dejmek J et al., 2002; Horta BL et al., 1997).

The components of cigarette smoking are known to affect birth weight through three means: fetal hypoxia, nicotine and cyanide (Horta BL et al., 1997). Increased carbon monoxide increases carboxyhaemoglobin levels can to lead fetal hypoxia. This then causes a decreased oxygen supply to the fetus and inadequate blood supply to the placenta (Abel EL, 1980). Evidence demonstrates altered placental pathophysiology in
smokers (Rath G et al., 2001). Cord blood of chronic smokers demonstrates that fetoplacental transfer of amino acids and enzymes are impaired possibly attributing to growth retardation (Jauniaux E et al., 2001). Toxic compounds in cigarettes such as nicotine, an appetite suppressant, have been suggested to cause uterine vasoconstriction (Quigley M et al., 1979) while cyanide compounds may play a role in disturbing fetal oxidative metabolism (Andrews J, 1973). Other researchers have also proposed a mechanism whereby a decrease in plasma volume, seen in smokers, results in decreased placental perfusion (Valero De Bernabé J et al., 2004).

Non-smoking mothers, compared to smokers, are slightly predisposed for delivering macrosomic infants (Heiskanen N et al., 2006). Moreover, the effect of smoking on birth weight has not only been limited to maternal smoking. Exposure to environmental tobacco smoke or second hand smoke puts a woman at further risk for LBW or IUGR (Dejmek J et al., 2002; Horta BL et al., 1997).

3. Placental Factors

The placenta is a key determinant of fetal growth and development (Salafia CM et al., 2006; Regnault TR et al., 2005; Baschat AA et al., 2004; Redmer DA et al., 2004). Given that the exchange of nutrients between mother and fetus takes place at the placenta, optimal intrauterine growth heavily relies on optimal placental function (Salafia CM et al., 2006; Redmer DA et al., 2004; Aplin J, 2000). The placenta is responsible for transporting essential amino acids, glucose and oxygen which are essential for fetal growth (Regnault TR et al., 2005; Baschat AA et al., 2004). The simple pathway summarized by Salafia CM et al. regarding the inter-relationship between placental growth and fetal growth will be discussed in the following sections:

Uteroplacental Vascular Pathology → Placental Growth → Fetal Growth

a. Placental Growth

Placental growth occurs during the first and second trimester of pregnancy to prepare for the nutritional requirements of the growing fetus (Redmer DA et al., 2004). Typical measures of placental growth as reported by Salafia CM et al. (2006) include placental disk shape, placental disk diameter and thickness, location of umbilical cord
insertion and placental weight. The gross growth of the placenta allows for the aborization and development of the placental vascular system required for nutrient transport (Salafia CM et al., 2006).

Throughout progressing gestation, the volume and weight of the placenta is expected to increase (Thame M et al., 2001). Placental volumes have been reported to drastically increase from 116 ml to 359 ml from early 2\textsuperscript{nd} trimester to late 2\textsuperscript{nd} trimester (Thame M et al., 2004), signifying rapid placental growth in the 2\textsuperscript{nd} trimester (Redmer DA et al., 2004). Placental weights at birth have been reported to vary widely between 142 g to 1200 g (Thame M et al., 2004). It has been reported that 1kg/m\textsuperscript{2} of BMI is associated with 0.08 unit increase in the square root of placental volume (Thame M et al., 2004) demonstrating that maternal conditions can affect placental growth.

Evidently, placental growth is related to placental function (Salafia CM et al., 2006). Transport of nutrients to meet the requirements of the growing fetus is facilitated through proper placental vascular development (Smith, GC 2004; Regnault TR et al., 2005). During pregnancy, total placental surface area dramatically increases to enhance the nutrient transfer capacity of the placenta (Regnault TR et al., 2005). Thus, it has been established that placental growth characterized by (weight, volume, thickness surface area) determines fetal growth (Salafia CM et al., 2006; Regnault TR et al., 2005; Redmer DA et al., 2004).

b. Placenta and Fetal Growth

Several studies have investigated 2\textsuperscript{nd} trimester placental size and its relationship with infant size at birth (Thame M et al., 2001; Thame M et al., 2004; Clapp JF et al., 1995; Wolf H et al., 1989). Placental volume and placental rate of growth in the 2\textsuperscript{nd} trimester are both positively related to biparietal diameter, head circumference, abdominal circumference and femoral length (Thame M et al., 2004).

Research suggests that early second trimester placental volume strongly predicts birth weight (Thame M et al., 2001; Clapp JF et al., 1995; Wolf H et al., 1989). It has been demonstrated that for every increase of 0.117-0.137 ml in placental volume, there is a 1 kg increase in birth weight (Thame M et al., 2001). Moreover, this study showed that for every standard deviation decrease in placental volume in early 2\textsuperscript{nd} trimester, the odds ratio
for LBW increases by 1.68. This suggests that prenatal outcomes of low birth weight are preceded by small placental volumes in the 2nd trimester. Suboptimal placental growth and development can hamper placental function leading to inhibited fetal growth (Aplin, J, 2000; Wallace JM et al., 2004).

c. Placental and Vascular Pathology

IUGR has been correlated with aberrations in placental mass, placental surface area and placental thickness (Wallace JM et al., 2004, Redmer DA et al., 2004). Inadequate placental development has been reported to cause alterations in placental vascular development (Redmer DA et al., 2004). Other placental adverse conditions: 1) placental infarctions, 2) placental abruption where the placenta is prematurely displaced and 3) placenta previa where the placental decidua ruptures have also been correlated with IUGR (Valero De Bernabé J et al., 2004).

A reduction in placental mass can cause fetal hypoxia and alterations in placental vascular development (Redmer DA et al., 2004; Regnault TR et al., 2005). “Fetal birth weight is reduced in a significantly smaller placenta” (Salafia CM et al., 2006). In addition, adolescent mothers are normally characterized by small sized placentas as they are still in the process of growing (Lasker JN et al., 2005; Fraser AM et al., 1995). Small sized placentas resulting in suboptimal nutrient transfer capacity may explain the high propensity of growth retarded infants in this adolescent population (Wallace JM et al., 2004). Low maternal weight gain in the first trimester is associated with smaller placenta and smaller fetal abdominal circumference at 35 weeks (Thame M et al., 2004).

Research has also discovered evidence to suggest that an abnormally thick placenta is correlated with numerous perinatal outcomes including IUGR and SGA (Raio L et al., 2004). Because of the increased thickness of the placenta, placental resistance and fetal metabolic demands are increased (Raio L et al., 2004). Moreover, it has been suggested that the thickness of the placenta is a compensatory mechanism for the inadequate development of the placental vascular system earlier in pregnancy. IUGR is associated with placentas characterized by reduced volumes of intervillous space and villi that results in smaller exchange surface (Salafia CM et al., 2006).
Placental insufficiency can result in IUGR (Salafia CM et al., 2006; Regnault TR et al., 2005). Manifestations of placental insufficiency include impaired placental angiogenesis and reduction in cytotrophoblast proliferation (Ong CYT et al., 2001). Altered placental pathophysiology (Rath G et al., 2001) and impaired nutrient transfer capacity of the placenta is commonly seen in smokers (Jauniaux E et al., 2001; Abel EL, 1980). This provides evidence to explain the strong correlation between smoking and LBW (Jauniaux E et al., 2001; Horta BL et al., 1997; Kramer MS, 1987a).

The aforementioned placental conditions can cause “intra-uterine malnutrition” (Valero De Bernabé J et al., 2004) due to inadequate placental perfusion. This subsequently provides inadequate transport of oxygen and nutrients from the placenta to the fetus (Regnault TR et al., 2005), resulting in growth retardation.

Optimal conditions of the aforementioned variables would normally result in a healthy baby include

- a 38-41 week gestation length
- maternal stature of BMI 18.5-25
- maternal weight gain of 12-16 kg
- maternal age between 20-30 years of age
- non-smoking mother in a smoke-free environment
- optimal placental growth of average placental weight, volume and subsequently proper vascular development

Unfavourable conditions may result in aberrations of birth weight resulting in IUGR or macrosomia (Baschat AA et al., 2004).

**B. Birth Weight Aberrations**

The two extremes of fetal growth, namely, intrauterine growth retardation (IUGR) and macrosomia are strong causes of increased neonatal morbidity and mortality (Baschat AA et al., 2004). The following sections discuss these two extremes of fetal growth in detail (Baschat AA et al., 2004).
1. Low birth weight (LBW)

It has been long established by the World Health Organization (WHO) that all neonates born with a birth weight of less than 2500 g are considered (LBW) infants (Cheng CJ et al., 2004; Valero De Bernabé J et al., 2004). LBW can arise from two factors: 1) prematurity, typically defined as <37 weeks gestation (Wen SW et al., 2004) or 2) intrauterine growth retardation (Kramer MS, 1987; Horta BL et al. 1997). However, it has been suggested that <38 weeks more correctly reflects prematurity. The rationale is that 38 weeks gestation is considered term since it is associated with the lowest risk of perinatal death (Smith GC, 2001). Small-for-gestational-age (SGA) is a term that is used to categorize infants with a birth weight of less than the 10th percentile for their gestational age (Kramer MS et al., 2001). In Canada, the incidence of SGA newborns have decreased from 10.9 to 7.9% from 1991 to 2000 (Canadian Perinatal Health Report, 2002).

IUGR can be divided into two distinct subgroups: proportional and disproportional (Kramer MS, 1987a). Newborns categorized with disproportional IUGR characteristically have a low weight for length but normal length and head circumference (Kramer MS, 1987a). Conversely, weight, length and head circumference are all equally reduced in newborns with proportional IUGR (Kramer MS, 1987a). Infants in this category exhibit less catch-up growth potential and are more prone to have neurological abnormalities compared to those with disproportional IUGR (Ong KK et al., 2004; Valero De Bernabé J et al., 2004).

Infants born LBW, either IUGR or premature, are associated with numerous morbidities following birth and into adulthood (Valero De Bernabé J et al., 2004). It has been shown that LBW infants lacking adequate nutrition while in utero tend to become overweight later in life. Excess adaptive insulin resistance and excess central fat deposits developed in utero may explain this trend (Ong KK et al., 2004; Hales CN et al., 2001). Of more concern, LBW infants have been strongly linked to infant mortality (Kramer MS et al., 2005; Valero De Bernabé J et al., 2004; Kramer MS et al., 2001).
2. Macrosomia

A lack of consensus regarding the definition of macrosomia has produced contrasting indices (Heiskanen N et al., 2006). Definitions have ranged from greater than 4000 g to greater than 5000 g (Heiskanen N et al., 2006). However, the American College of Obstetricians and Gynecologists state that the definition of macrosomia is a birth weight greater than 4500 g (ACOG, 2000). This definition has been most commonly used. Large-for-gestational-age (LGA) is as a neonate with a birth weight greater than the 90th percentile (Kramer MS et al., 2001). This classification accounts for the effect of gestational age on birth weight (Kramer MS et al., 2001).

In developed countries where reported indices of macrosomia range from 0.8-1.7%, there has been a significant increase in average birth weight (Arbuckle TE et al., 1989). A recent Montreal based hospital study of 61,437 births discovered an increase of average birth weight of 3419 g to 3476 g between 1978 to 1996 in term infants (Kramer MS et al., 2002). In addition, the percentage of LGA infants increased from 8 to 11% supporting that babies, at least in Quebec, are born bigger. Researchers of this study attributed this bigger baby trend to a reduction in cigarette smoking from 12.7% in 1978 to 5.6% in 1996 as well an increase in maternal stature (BMI and weight gain during pregnancy) and gestational diabetes (Kramer MS et al., 2002). In Canada, the rate of LGA infants has significantly increased from 9.5% to 12% (Canadian Perinatal Health Report, 2002).

Most often, growth restricted pregnancies have received more attention due to associated complications. But, neonates born with a birth weight of >4500 g are at an increased risk of lowered APGAR scores and fetal distress (Cedergren MI, 2004). They are also at risk of birth asphyxia, and delivery-related traumas including shoulder dystocia (Heiskanen N et al., 2006; Cedergren MI, 2004; Bérard J et al., 1998). Labour associated with macrosomic infants delivered vaginally is typically long and difficult for the mother with an increased frequency of cervical and perineal tears and postpartum infection and hemorrhaging (Stotland NE et al., 2004, Lim JH et al., 2002).

The risk of macrosomia remains a challenge for obstetricians to diagnose. However, studies have recognized several factors associated with a macrosomic fetus: excess maternal weight gain, increased maternal age, multiparity, reduced cigarette
smoking, previous macrosomic infant and an increased likelihood of caesarean section (Heiskanen N et al., 2006; Kramer MS et al., 2002). Male infants have a higher predisposition of being macrosomic (Bérard J et al., 1998).

Researchers have observed a 5-14% increased risk of macrosomia in women with a triad of the leading risk factors: diabetes, obesity and postmaturity (Spellacy WN et al., 1985). Excess maternal weight gain remains one of the highest risk factors predisposing fetuses to macrosomia (Bérard J et al., 1998). In early gestation a BMI > 40 had an almost four fold risk for LGA (Cedergren MI, 2004).

A possible explanation of the link between obesity and macrosomia has been that larger women tend to have an increased volume of plasma thus increasing placental perfusion (Goldenberg RL et al., 1996). Moreover, obesity places a woman at high risk for developing diabetes, the other component of the triad (Stotland NE et al., 2004). Increased maternal glycemia provides the fetus with an over abundance of substrates, including glucose, amino acids and lipids (Jansson T et al, 2006). Pedersen’s hypothesis, which has long been reported, states that the fetus adapts to this increased influx of glucose by over producing insulin which leads to increased fat deposition and subsequently increased weight gain (Scholl TO et al., 2001). A study has demonstrated a 50 g increase in birth weight in women with glucose concentrations of 90-130 mg/dl (Scholl TO et al., 2001). In this same study, maternal glycemia levels of >130 mg/dl was correlated with a 160-200 g increase and subsequently LGA. The development of gestational diabetes secondary to obesity or a family history of diabetes also accounts for the prevalence of macrosomia (Bérard J et al., 1998). Not only does excess weight gain during pregnancy affect glucose metabolism, studies have demonstrated that it is associated with postmaturity, with the incidence of macrosomia increasing from 12% to 21% from 40 to 42 weeks, respectively (Boyd, 1983).

C. Role of Alkaline Phosphatase in Pregnancy

1. Introduction to Alkaline Phosphatase

In addition to the aforementioned maternal, fetal and placental characteristics, biomarkers such as enzymes in biological fluids may also provide insight into fetal
growth and development. Alkaline phosphatase (ALP) (EC 3.1.3.1) is a dimeric metalloglyco-enzyme that hydrolyzes monophosphate esters into inorganic phosphates and alcohols (Seleen JC, 1978; Nozawa S et al., 1982). The molecular weight of this enzyme and its isoenzymes ranges from 116 000 to 170 000 Daltons (Herz F, 1985; Nozawa S et al., 1982). Optimal enzyme activity requires alkaline conditions and the binding of 2 zinc and 1 magnesium cofactors to each monomer (Price CP, 1993, Herz F, 1985). Total alkaline phosphatase is a composite of various isoenzymes that have been identified and are produced by various body tissues, which include the intestine, bone, liver, kidney and placenta (Wojcicka-Bentyn J et al., 2004). These five isoenzymes, although similar in function, differ structurally in amino acid (a.a) sequence and can be differentiated based on their individual chemical and physical properties (Price CP, 1993; Nozawa S et al., 1982; Herz F, 1985; Mulivor RA et al., 1985; Mulivor RA et al., 1979) (Table 1). The isoenzymes of ALP can be categorized into three categories based on the three different structural gene loci that determine their protein structure: 1) Tissue Non-Specific (TNS) ALP – Liver/Bone/Kidney (LBK) 2) Tissue Specific (TS) Intestinal and 3) (TS) Placental (She QB et al., 2000a; Herz F, 1985).

The physiological role of ALP is not completely understood. Literature has shown evidence that ALP participates in the active transport of phosphates (Corathers SD, 2006) in the intestinal mucosa, renal tubuli, bone and placenta (Kaldor G, 1983; Seleen JC, 1978) or across membranes (Vongthavaravat V et al., 2000).

2. ALP - Physiological and Disease Marker

ALP, in serum, has been widely used as a biomarker in clinical practice. Its activity is reported in IU/L. An enzyme unit (U) is defined as the amount of enzyme required to convert 1.0 µmole of substrate per minute. A high degree of inter-individual variability but minimal intra-individual variability is exhibited by this enzyme (Price CP, 1993). Adult serum reference ranges of ALP extend from 40-140 IU/L (Vongthavaravat V et al., 2000). Serum levels of ALP isoenzymes vary greatly due to differences in clearance rates and half lives stemming from distinct chemical structures. Liver and bone isoenzymes make up 95% of measured adult serum ALP values (Millan JL, 2005). Serum levels of
<table>
<thead>
<tr>
<th>Isoenzyme</th>
<th>Category</th>
<th>Source</th>
<th>Molecular Weight (Daltons)</th>
<th>Amino Acid (a.a) Composition</th>
<th>Inhibited by</th>
<th>Heat stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>TNS</td>
<td>Sinusoidal membrane, microvilli, endothelial cells, hepatocytes</td>
<td>136 000 - 170 000</td>
<td>507 a.a.</td>
<td>L-Homoarginine</td>
<td>Heat Labile</td>
</tr>
<tr>
<td>Bone</td>
<td>TNS</td>
<td>Cell surfaces of osteoblasts and chondrocytes</td>
<td>136 000 - 170 000</td>
<td>507 a.a.</td>
<td>L-Homoarginine</td>
<td>Heat Labile</td>
</tr>
<tr>
<td>Kidney</td>
<td>TNS</td>
<td>Proximal convoluted tubule, kidney brush border</td>
<td>136 000 - 170 000</td>
<td>507 a.a.</td>
<td>L-Homoarginine</td>
<td>Heat Labile</td>
</tr>
<tr>
<td>Intestinal</td>
<td>TS</td>
<td>Intestinal mucosal cells, brush border of intestinal epithelium</td>
<td>140 000 - 170 000</td>
<td>509 a.a</td>
<td>L-Phenylalanine</td>
<td>Heat Labile</td>
</tr>
<tr>
<td>Placental</td>
<td>TS</td>
<td>12th week of gestation onward from the placenta</td>
<td>116 000 - 125 000</td>
<td>513 a.a.</td>
<td>L-Phenylalanine</td>
<td>Stable exceeding 56-65 ° C</td>
</tr>
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(Table compiled using references: Price CP, 1993; Herz F, 1985; Mulivor RA et al., 1985; Mulivor RA et al., 1979)
this enzyme can indicate both physiological and disease processes within the body which will be discussed in the coming sections.

Elevations in serum enzyme concentrations can indicate both altered tissue function (dead or damaged tissues) as well as proliferating or stimulated cell growth (Corathers SD, 2006; Moss D, 1987). With regards to normal development, this enzyme also increases with age (Tobiume H et al., 1997). During reproduction, a significant but normal increase in total ALP in maternal serum has also been reported during pregnancy (Okesina AB et al., 1995). Elevations in ALP can also indicate carcinomas or lesions in its various originating tissues (Vongthavaravat V et al., 2000; Herz F, 1985). Decreased clearance of enzymes from serum can also elicit unexpected elevations (Moss D, 1987).

a. Bone ALP – Physiological and Disease Processes

Bone ALP is the most understood of all ALP isoenzymes. ALP originating from the bone is found on the cell membranes of osteoblasts, chondrocytes, and matrix vesicles (Faerk J et al., 2005; Anh DJ et al., 2001). Evidently, it is recognized as an index of osteoblastic activity. It functions by hydrolyzing inorganic phosphates that subsequently allows for bone mineralization/crystallization to occur (Faerk J et al., 2005; McComb RB et al., 1979). Animal studies found cases of reduced extra vesicular crystal propagation in mice that were deficient of bone ALP (Anderson HC et al., 2004; Anderson HC et al., 1997). Once adequate crystallization occurs, calcification ceases and bone ALP enters circulation where vesicles rupture due to osteoblastic apoptosis (Faerk J et al., 2005; Farley JR et al., 2001). Thus, serum concentrations of bone ALP reflect osteoblastic activity.

Bone formation and turnover elicits elevations in bone ALP (Corathers SD 2006; Faerk J et al., 2005, Tobiume H et al., 1997). Consequently, growing infants and children have the most notable elevations in ALP secondary to bone growth (Corathers SD, 2006, Tobiume H et al., 1997). Studies have reported instances of ALP three times the normal adult upper limit in growing infants and in individuals with healing fractures (Corathers SD, 2006, Crofton PM et al., 1987, Moss D, 1987, Haworth JC et al., 1986).
In adolescents, following puberty, ALP steadily declines as values fall back to normal reference ranges established for adults. This trend is most attributed to bone growth occurring during these developmental stages (Tobiume H et al., 1997).

In addition, serum concentrations of ALP vary with age from birth through childhood. Fetal serum ALP is shown to be correlated with the week of gestation (Jauniaux E et al., 2000; Nava S et al., 1996). Serum taken from fetus’ between the 11-17th week of gestation were higher (301 IU/L) compared to serum taken between 20-26 weeks of gestation (Jauniaux E et al., 2000). Conversely, higher values at 8 weeks have also been reported (Nava S et al., 1996). Two weeks postpartum, only bone ALP was detected in the serum of neonates born term (>36 weeks) and was devoid of all other isoenzymes (Crofton PM, 1987). Meanwhile, both bone ALP and intestinal ALP were identified in the serum of preterm infants (<35 weeks) (Crofton PM, 1987).

Clinically, ALP has been established as a marker for bone related diseases (Vongthavaravat V et al., 2000). Paget’s, rickets, and osteoporosis can cause elevated serum ALP (Corathers SD, 2006). The mechanism behind serum elevations is due to osteoblastic proliferation which causes an increased release of ALP into the blood stream. Elevated serum ALP can also represent bone malignancy and blastic lesions (Corathers SD, 2006). It has been noted that the more progressive the skeletal condition, the more severe the rise in serum ALP. Hypophosphatasia, an autosomal recessive disease characterized by defective skeletal mineralization, can produce lower than expected serum ALP activity (Mulivor RA et al., 1978).

b. Liver ALP – Physiological and Disease Processes

The liver isoenzyme cannot be detected in neonates (Crofton PM, 1987). However, at 6 months of age, as synthesis begins, a rise in liver ALP is noted. The hepatic form of ALP originates from several regions of the liver: sinusoidal membrane, microvilli and endothelial cells (Wolf PL, 1978). It has been suggested that liver ALP plays a role in intrahepatic biliary epithelium secretory activities (Alvaro D et al., 2000).

Clinically, liver function has been readily assessed using ALP. It is most notable for its association with cholestasis (Corathers SD, 2006). Increased ALP levels in serum are commonly seen in cholestasis, extra hepatic and intra hepatic obstruction or cirrhosis
(Corathers SD, 2006; Vongthavaravat V et al., 2000; Wolf PL, 1978). An obstruction of the bile duct or biliary passages can lead to a three to ten fold increase of normal serum values. Mechanisms to describe these measured elevations include stimulation of increased ALP synthesis by hepatocytes secondary to obstruction (Price CP, 1993). At the same time, the obstruction causes bile constituents to overflow into the circulatory system. Injury to hepatocytes can also elicit elevations in ALP (Price CP, 1993).

c. Kidney ALP – Physiological and Disease Processes
A limited understanding of kidney ALP exists. It has been suggested that kidney tissue ALP may play a role in phosphate resorption through the brush border of the proximal convoluted tubules (McComb RB et al., 1979). ALP originating from the proximal convoluted tubule may be indicative of infarcts in the kidney (Wolf PL, 1978).

d. Intestinal ALP – Physiological and Disease Processes
Intestinal ALP is found in the apical brush border membrane of the enterocyte or brush border of the intestinal epithelium. Intestinal ALP originates from mucosal cells of the small intestine (Wolf PL, 1978). Postprandial increases in serum intestinal ALP activity occur following a fatty meal (Corathers SD, 2006; Alpers DH et al., 1990; McComb RB et al., 1979). Animal studies of intestinal ALP reveal its ability to bind to and decrease the toxicity of endotoxins (Poelstra K et al., 1997) suggesting that ALP could have a protective role. Lesions in the gastrointestinal tract can trigger abnormal elevations in ALP (Wolf PL, 1978).

e. Placental Alkaline ALP – Physiological Processes
Placental ALP is associated with growth. It may play a role in the regulation of cell division and DNA synthesis (She QB et al., 2000a; She QB et al., 2000b). Pregnancy elicits the production of placental ALP by the microvilli of the syncytiotrophoblast in the placenta (Gol M et al., 2005; Fishman WH et al., 1979). As it is of fetal origin, the phenotype of placental ALP is determined by the genotype of fetal ALP (Magrini A et al., 2003; Beckman G et al., 1995). Elevated serum levels of total ALP have been observed in pregnant women with the most significant increase in the 3rd trimester. The increased
production or release of placental ALP into circulation is responsible for the rise in total ALP (Goldenberg RL et al., 1997; Meyer RE et al., 1995; Okesina AB et al., 1995).

Several important functions of placental ALP during pregnancy have been reported in literature. Evidence suggests that placental ALP in maternal serum indicates the development of syncytiotrophoblast microvilli and overall placental function (Mezzano L et al., 2005; Kaneda T et al. 1997; Okpere E et al., 1986; Messer HH et al., 1975). Research investigating maternal serum has proposed that placental ALP may indicate or reflect maternofetal transfusion (Kaneda T et al. 1997) or feto-maternal metabolism (Okamoto T et al., 1990).

Second and 3rd trimester serum levels have shown higher total and placental ALP levels in mothers pregnant with female fetuses. (Gol M et al., 2005). This suggests a possible gender difference in placental growth and subsequently, serum placental ALP values (Vergnes CJ et al., 1998). Researchers have proposed that an increase in placental ALP found in neutrophils may be a result of accelerated placental growth, more common in female than male infants (Vergnes CJ et al., 1998).

**f. Placental ALP – Disease Processes**

Researchers have proposed that placental ALP may indicate disturbances of placental function. Abnormal increases in total and/or placental ALP may be secondary to placental lesion, infarction or toxemia (Wolf PL, 1978; Fishman WH et al., 1972) – but a more recent paper (Vongthavaravat V et al., 2000) denies any relationship. Even more recent literature has demonstrated an association between maternal smoking, ALP and the possible effect of smoking on the placenta (Jauniaux E et al., 2001). In cases where mothers smoked during pregnancy, results showed increased serum ALP in early gestation, originating from the placenta. Conversely, the same study showed that chronic smoking by the mother resulted in a decrease of total ALP due to reduced microvilli from the syncytiotrophoblast (Jauniaux E et al., 2001).
D. ALP and Pregnancy

1. Developmental Profile during Pregnancy

During pregnancy, total ALP found in maternal serum increases with progressing gestation (Goldenberg RL et al., 1997). Isoenzymes that make up total maternal serum ALP include hepatic, renal, osteal and intestinal ALP. During pregnancy, the developmental profile of maternal ALP is affected by stage of pregnancy.

Placental ALP is responsible for the substantial rise in total ALP as pregnancy progresses (She QB et al., 2000a, b; Okesina AB et al., 1995). It has been suggested that placental ALP is produced by the syncoiotrophoblasts (Okesina AB et al., 1995) as early as 7-8 weeks of gestation (Okamoto T et al., 1990) with the most marked increase in the villi. This marked increase of placental ALP in the placental villi consequently coincides with the rapid growth of the placental during the 2nd trimester. Provided that placental ALP is distinct only to pregnancy in healthy adults, ALP levels return to normal reference range values 20-24 weeks postpartum (Wojcicka-Bentyn J et al., 2004).

Late in the first trimester (8-12 weeks gestation) serum ALP of healthy pregnant women consists predominantly of bone ALP closely followed by non-bone ALP (Okesina AB et al., 1995). By the third trimester at >38 weeks, placental ALP becomes the most predominant ALP isoenzymes in maternal circulation (She QB et al., 2000a, b; Okesina AB et al., 1995). Bone ALP is found secondary to placental ALP with the remaining amount credited to non-bone ALP. Investigations of bone ALP in the serum of healthy pregnant women reported a 38% increase of 3rd trimester bone ALP compared to non-pregnant women (Okesina AB et al., 1995). This suggests that bone ALP, a well established indicator of bone turnover, may indicate maternal bone turnover in the 3rd trimester. This occurs in order to meet the high fetal demands of calcium for fetal bone growth (Bezerra FF et al., 2002). Both bone and placental ALP are responsible for the significant rise in 3rd trimester ALP (Okesina AB et al., 1995).

2. Quantity

The serum ALP of healthy pregnant women is expected to reach values two times that of the normal upper limit of 40-140 IU/L (Wojcicka-Bentyn J et al., 2004;
Vongthavaravat V et al., 2000; Okesina AB et al., 1995). With progressing gestation, maternal serum ALP rises steadily from 73.4 IU/L to 267.9 IU/L at >38 weeks gestation (Okesina AB et al., 1995). However, other studies have reported maternal serum ALP values within normal adult reference ranges:

- 38 IU/L at 19 weeks gestation to 130.2 IU/L at delivery (Goldenberg RL et al., 1997)
- 45 IU/L at 24 weeks (Moawad AH et al., 2002)

This suggests that ALP during pregnancy can have a high degree of variability.

3. Extreme Maternal Serum ALP

Recent cases of severe elevated maternal serum ALP have been associated with “unexplained” isolated incidences of uncomplicated pregnancy (Vongthavaravat V et al., 2000) and with gestational diabetes (Wojcicka-Bentyn J et al., 2004).

- Case #1

  In an uncomplicated pregnancy, 2nd trimester maternal serum ALP was reported at 10 times the upper limit at 1191-1342 IU/L. Analysis revealed evidence of placental infarction and the increase in ALP was attributed to placental ALP. However, researchers could not explain the marked elevation of serum ALP nor explain the relevance of the placental infarction to serum ALP (Vongthavaravat V et al., 2000).

- Case #2

  In the 3rd trimester of pregnancy, a woman presented with serum ALP of 2037 IU/L and elevated placental ALP. Maternal conditions included GDM. Clinicians were not able to determine the cause of such elevation but speculated a genetic anomaly (Wojcicka-Bentyn J et al., 2004).

E. ALP and Fetal Growth

Numerous studies have investigated the relationship between maternal serum ALP and fetal growth. ALP has been suggested to provide insight into predicting both gestational age (Moawad AH et al., 2002; Meyer RE et al., 1995; Brock DJH et al., 1988) and birth weight (Best RG et al., 1991; Brock DJH et al., 1988; Holmgren PA et al., 1979; Spellacy WN et al., 1977; Kunz J et al., 1976). In addition, maternal serum ALP
values may indicate genetic fetal conditions such as Down syndrome (Gol M et al., 2005). It must be noted that the majority of studies consistently report the association between ALP and abnormal birth outcomes but rarely in relation to normal fetal growth. Unlike other ALP isoenzymes that predominantly reflect disease states, placental ALP has demonstrated important roles in fetal growth in utero. The following sections will discuss the relationship between ALP and complicated prenatal outcomes as well as address the role of placental ALP in normal fetal growth.

1. ALP and Gestational Age

Several researchers have suggested the potential of serum ALP as a predictor of preterm birth (Moawad AH et al., 2002; Goldenberg RL et al., 1997; Meyer RE et al., 1995). In the Preterm Prediction Study (n=254), total ALP measured in the 3rd trimester serum of women who had a subsequent preterm birth was significantly higher than those who gave birth to a term infant. This finding was even more marked in women giving birth to infants < 32 weeks of gestation (OR = 6.8) than those who gave birth to infants < 35 weeks births of gestation (OR = 5.8) (Moawad AH et al., 2002). It is important to note that despite the elevated serum ALP within this population; all values were still within normal adult reference ranges.

Added research has attempted to substantiate this relationship between the increased risk of preterm births in African American women (n=580) with elevated serum total ALP (Goldenberg RL et al., 1997). Women in the highest quartile of 3rd trimester serum total ALP were more likely to give birth prematurely (<37 weeks). In this study, researchers concluded that women with high plasma serum total ALP were three times more likely to give birth prematurely. Moreover, these women were more likely to deliver significantly lighter babies than women with lower serum total ALP. These results could not be duplicated in the analysis of 2nd trimester serum. One of the strengths of this study was that it accounted for several cofounders including: maternal BMI, smoking status, zinc supplementation, plasma folate, albumin and copper.

A large scale study (n=1868) discovered that the greater the elevation of mid-trimester maternal serum placental ALP, the greater the risk of preterm delivery (<37 weeks) (Meyer RE et al., 1995). In addition to its large sample size, this study corrected
for risk factors associated with birth weight including: maternal weight at mid trimester, infant gender, ethnicity and amniocentesis week. Most recently, a woman with serum ALP of 1194 IU/L gave birth to a premature infant at 36 weeks. Researchers demonstrated that placental ALP was responsible for the marked increase in measured serum ALP. This suggests that serum ALP may provide potential in indicating preterm birth (Bashiri A et al., 2006).

In fetal serum, studies have shown that bone mineral content is lower in preterm infants than in term infants (Crofton and Hume, 1987). In a study of 12 VLBW and premature infants, serum bone ALP increased steadily postpartum, signifying compensatory bone formation (Litmanovitz I et al., 2004). Levels of fetal intestinal ALP have also been reported to be correlated with GA (Crofton PM et al., 1987).

2. ALP and Birth Weight

The most strongly implicated ALP isoenzyme relevant to fetal growth is placental ALP (Best RG et al., 1991, Brock DJH et al., 1988, Meyer RE et al., 1995, She QB et al., 2000a, She QB et al., 2000b). However, the relationship between placental ALP and birth weight remains somewhat controversial. Both elevated (Best RG et al., 1991; Brock DJH et al., 1988) and low (Holmgren PA et al., 1979, Spellacy WN et al., 1977, Kunz J et al., 1976) levels of maternal serum placental ALP have been correlated with infant birth weight.

Brock and Barron (1988) conducted several immunoassay studies investigating the use of placental ALP as a predictor of low birth weight. These researchers found that women with placental ALP twice the normal median were four times more likely to give birth to a LBW infant. This correlation was demonstrated in both serum collected in the 2nd trimester and at term. Results were produced using frozen biobanked serum samples from two previous studies (Brock DJH et al., 1982; Brock DJH et al., 1980) initially used to analyze alpha-fetoprotein. Although not specified, samples may have been stored for up to 8 years. Similar findings were replicated in another study that controlled for ethnic origin, age and mid trimester maternal weight (Best RG et al., 1991). The use of serum alpha-fetoprotein in conjunction with serum placental ALP has been suggested to enhance the predictive power in determining low birth weight (Brock DJH et al., 1988). These
studies also suggested the clinical potential of 2nd trimester serum placental ALP to predict LBW.

Studies have also provided evidence to believe IUGR is associated with low serum values of placental ALP (Holmgren PA et al., 1979; Spellacy WN et al., 1977; Kunz J et al., 1976). Meanwhile, other studies using similar immunoassays found placental ALP to provide no predictive value of LBW (Contractor SF et al., 1985; McLaughlin PJ et al., 1983).

Interestingly, almost all of the aforementioned studies, predicting birth weight and gestational age, have implicated placental ALP. This could provide insight that the etiology of these incidences may be related to placental function.

3. Relationship between Placental ALP and Fetal Growth

In previous sections of this literature review, placental ALP demonstrated its potential as an indicator of placental function and possible dysfunction. The placenta which may elicit either above normal or below normal placental ALP may provide explanation as to why the aforementioned trends are being seen in LBW and preterm infants. In an attempt to explain such results, researchers have theorized that uncharacteristic release of placental ALP may be a result of damage or injury to the placenta or placental tissues. This could be a result of placental infarction, abruption, toxemia or chorionamnion infection (Goldenberg RL et al., 1997; Meyer RE et al., 1995; Wolf PL, 1978; Oesterling MJ et al., 1977).

Goldenberg RL et al. (1997) attempted to explain the relationship between gestational age and ALP by suggesting increased production of placental ALP is secondary to chorionamnion infection, commonly seen in preterm births. Meanwhile, researchers of a recent isolated case of extreme placental ALP and preterm concluded no connection between indication of placental chorionic plate inflammation and placental ALP (Bashiri A et al., 2006). Depressed serum values of ALP in women who subsequently gave birth to IUGR infants found total ALP to be correlated with feto-placental weight (Holmgren PA et al., 1979). Commonly associated to LBW and IUGR, smoking during pregnancy has been reported to cause elevated serum ALP while chronic smoking results in a decrease of serum ALP. It has been suggested that chronic smoking
may cause a decrease in syncytiotrophoblastic microvilli from where placental ALP originates (Jauniaux E et al., 2001). The accumulated studies suggest that fluctuations in ALP may be attributed to placental health and subsequently on fetal growth.

Placental ALP may not only indicate placental health but support fetal growth and development. The isoenzyme acts as a receptor for IgG, aiding in the transplacental transport of IgG from maternal blood to the fetus (Stefaner B et al., 1997; Makiya R et al., 1992). Therefore, it appears that placental ALP helps to provide passive immunity to the fetus during gestation (Makiya R et al., 1992). However, the extent of transplacental transport of IgG is limited by the genotype of fetal placental ALP (Magrini A et al., Beckman G et al., 1995; Grozdea J et al., 1995). Interestingly, one study suggests that one fetal genotype of placental ALP places a fetus a higher risk of LBW than other fetal genotypes (Amante A et al., 1996). In addition, human fetal fibroblasts incubated with placental ALP have demonstrated DNA synthesis and cell proliferation. This suggests that placental ALP may play a vital role in the fetal growth of tissues during the 2nd and 3rd trimester and more importantly, fetal development (She QB et al., 2000a; She QB et al., 2000b).

F. Summary

It has been determined that the fetus, the mother and the placenta play vital roles in providing the fetus with an optimal environment for fetal growth. Evidence has been conclusive regarding the direct impact of maternal anthropometrics, sociodemographic and lifestyle factors on birth weight. Maternal characteristics including: weight gain, pre-pregnancy weight, maternal height, parity as well as infant gender and gestational age are known established determinants of fetal birth weight.

Serum ALP has been widely researched and used as a biomarker in clinical practice years. In clinical settings, serum ALP has been routinely used as an indicator of liver and bone disease as well as growth. Most recent ALP literature has focused on pregnancy where ALP activities in both maternal and fetal serum have been measured. Researchers have mapped the developmental profile of ALP during healthy pregnancies attributing elevations to a normal increased production from the placenta. Investigations
of placental ALP report several possible functions in relation to fetal growth: promotion of fetal cell synthesis and participation in immune defenses. Contrastingly, literature has also provided evidence to suggest an association between elevated maternal serum ALP levels and the increased likelihood of LBW and premature infants. Despite the accumulated knowledge on ALP, this enzyme remains poorly understood. With its numerous isoenzymes, researchers have had difficulty determining which isoenzyme is responsible for above normal serum ALP values. However, some existing literature has suggested that the elevations in serum ALP are secondary to adverse placental conditions which could subsequently affect fetal growth. Furthermore, current literature has examined possible covariates such as fetal gender affecting serum ALP activity in maternal serum during pregnancy to increase the validity of serum ALP as a prenatal indicator. Several connections between both maternal and fetal ALP and fetal growth and development have been demonstrated to be a status indicator during pregnancy.
III. STATEMENT OF PURPOSE

1. Rationale

ALP is found not only in biological mediums such as maternal and fetal serum but also in amniotic fluid, the medium that bathes the fetus during gestation. Vital to the development of the fetus, the fluid provides physical and bacterial protection and regulates pH and temperature to facilitate growth (Cellini C et al., 2006; Underwood MA et al., 2005). The pool of AF is constantly recycled by the continual fetal swallowing and excretion of AF that contains nutrients, digestive enzymes, growth factors and waste products (Ross MG et al., 2001). Maternal and fetal contributions originating from plasma, the placenta, umbilical cord, fetal skin, gastrointestinal tract and kidneys also make their way into AF (McCarthy T et al., 1978). Proteins such as ALP enter AF through two routes: 1) directly from the fetus and 2) from the mother through transport across the placenta into AF (Surana R et al., 1994). Therefore, AF and its constituents provide an interesting window into fetal growth since it is a composite of maternal, fetal and placental components.

Recently, numerous studies have demonstrated the association between several AF constituents and fetal outcomes. IGF-BP3 has been shown to positively predict fetal birth weight whereas total protein (Tisi DK et al., 2004), insulin growth factor binding protein 1 (IGF-BP1) and IGF-2 (Tisi DK et al., 2005) have been demonstrated to have a negative predictive value. This evidence provides grounds to assume that other proteins present in AF, such as ALP, may possess the power to predict fetal development.

Total ALP in AF exhibits a high degree of variability during normal pregnancies. Its activity increases in early gestation (20 IU/L) with levels peaking in early 2nd trimester (31 IU/L), decreasing thereafter (7 IU/l), and once again increasing at 24-28 weeks (15 IU/L) until the end of term (Burc L et al., 2001; Gulbis B et al., 1998; Salgo L et al., 1989; Brocklehurst D et al., 1980; Benzie RJ et al., 1974; Hahnemann N et al., 1974).

The exact origin of each ALP isoenzyme (intestinal, LBK and placental) in AF remains poorly understood (Mulivor RA et al., 1979). Similar to serum, the following isoenzymes are also found in 2nd and 3rd trimester AF: intestinal, liver/bone/kidney and placental ALP. In the 2nd trimester, ALP is primarily of the fetal intestinal form (80%) with minor contributions from LBK (15%) and trace amounts from placental ALP (4%).
(Burc L et al., 2001; Brock DJH, 1983; Mulivor RA et al., 1979). At this point in pregnancy, the liver/bone/kidney forms of ALP are derived from maternal serum that cross the placenta while placental ALP is released directly from the placenta into AF. In contrast, 3rd trimester ALP is predominantly of the liver/bone/kidney form (69%) followed by placental (27%) and intestinal (5%) ALP (Burc L et al., 2001; Mulivor RA et al., 1979). The majority of fetal intestinal ALP is believed to stem from desquamated intestinal mucosal cells while the liver/bone/kidney form in late gestation is largely of fetal origin as organs develop.

Largely, ALP’s physiological role remains poorly understood. However, in fluctuations of ALP isoenzymes in AF during gestation appear to signify normal developmental stages of the fetus.

- LBK – increasing levels indicate fetal organ maturation occurring in the 3rd trimester
- Intestinal – decreasing levels of this isoenzyme indicates the development of the anal sphincter similarly, low levels can also indicate gastrointestinal obstructions which could affect the ingestion and absorption of AF
- Placental – enters AF from both fetal and maternal sources during gestation

ALP and its association with fetal growth have been limited to serum analyses (Moawad AH et al., 2002; Meyer RE et al., 1995; Best RG et al., 1991; Brock DJH et al., 1988; Holmgren PA et al., 1979; Spellacy WN et al., 1977; Kunz J et al., 1976). Moreover, maternal serum placental ALP has been implicated in fetal cell synthesis (She QB et al., 2000a) and immunoreactivity (Makiya et al., 1994). Although ALP in AF has been measured (Mulivor RA et al., 1985, Mulivor RA et al., 1979), to our knowledge, no study has examined the link between ALP in AF and fetal birth weight.

Although ALP in AF has been quantified during pregnancy, the majority of these studies have been limited by their methodology. All studies were limited by a smaller sample size, did not control for confounders of BW, GA or ALP concentrations and were limited to the use of the substrate p-nitrophenthylphosphate. This study will implement the use a novel substrate to increase sensitivity: 2’-[2-benzothiazoyl]-6’-hydroxybenzothiazolen phosphate (BBt), control for confounders and utilize a larger sample size. Consequently, this study aims to evaluate the potential of ALP in AF to
predict fetal growth and development and to analyze the clinical potential of 2nd trimester AF total and/or placental ALP to predict LBW and/or gestational age.

2. Hypothesis

The hypothesis of this study is 2nd trimester amniotic fluid alkaline phosphatase will be associated with fetal growth.

3. Objectives

The specific objectives of this study were two-fold: 1) To quantify total ALP and its isoenzymes (intestinal, LBK, placental) in 2nd trimester human AF and 2) to determine whether total AF and placental ALP were associated with or predictive of infant birth weight and/or gestational age.
IV. MANUSCRIPT

Placental Alkaline Phosphatase in Human Amniotic Fluid is Associated with Fetal Growth 1

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Number of Tables: 2

Number of Figures: 1

4492 words from title through references

1Supported by the National Sciences and Engineering Research Council (NSERC) Canada
2Abbreviations used: AF - amniotic fluid; AGA - appropriate-for-gestational-age; ALP - alkaline
phosphatase; ANCOVA - Analysis of Covariance; ANOVA - Analysis of Variance; BBt - 2'-(2-
benzothiazoyl)-6'-hydroxybenzothiazole; BW - birth weight; GA - gestational age; IUGR - Intrauterine
Growth Retardation; LBK - Liver/Bone Kidney; LGA - large-for-gestational-age; SGA - small-for-
gestational-age; weeks - wks
* To whom correspondence should be addressed. Email: Kris.Koski@mcgill.ca
Abstract

Background
Elevations in maternal serum alkaline phosphatase (ALP) are associated with an increased risk of preterm births and low birth weight. Few studies have examined ALP and/or its isoenzymes in amniotic fluid (AF) in association with fetal outcomes.

Aims
We determined whether 2nd trimester AF total ALP and isoenzymes (intestinal, liver/bone/kidney (LBK) and placental) were associated with infant birth weight (BW) or gestational age (GA) at birth.

Study Design and Outcome Measures
AF samples (n=518) were analyzed for ALP using fluorometry with heat denaturation and amino acid inhibition; maternal and fetal characteristics were obtained through questionnaires and medical chart review. We compared AF ALP concentrations among small-for-gestational-age (SGA), appropriate-for-gestational-age (AGA), large-for-gestational-age (LGA) and across preterm, term, post-term infants. Using multiple regression analyses, we also investigated whether AF ALP concentrations were associated with BW or GA.

Results
Placental ALP was elevated in the AF of mothers giving birth to females compared to males and in smokers compared to non-smokers. Mothers giving birth to SGA infants had higher placental AF ALP concentrations compared to those giving birth to AGA infants. AF placental ALP also entered as a significant independent predictor of birth weight (in grams and percentiles) in healthy term infants (38-41 weeks). No other 2nd trimester AF ALP isoenzymes were associated with BW or GA.

Conclusions
Placental ALP when measured in 2nd trimester AF may emerge as an early indicator of fetal growth in utero.

Keywords: birth weight, alkaline phosphatase, fetal growth, amniotic fluid
1. Introduction

Amniotic fluid (AF) serves numerous functions throughout gestation. It functions to physically protect the fetus from mechanical disturbances and bacterial substances as well as regulate pH and temperature. Fetal swallowing of AF also provides nutrients and growth factors to the fetus (Cellini C et al., 2006; Underwood MA et al., 2005). Evidence shows that fetal ingestion of AF has an impact on fetal growth (Cellini C et al., 2006; Ross MG et al., 2001; Underwood MA et al., 2005; Buchmiller TL et al., 1994; Suranna R et al., 1994; Pitkin RM et al., 1975) while the impairment of AF swallowing has lead to delayed gastrointestinal development (Ross MG et al., 2001) and a reduction in fetal weight (Cellini C et al., 2006; Ross MG et al., 2001; Surana R et al., 1994).

Various enzymes have been identified in amniotic fluid (AF) (Burc L et al., 2001; Benzie RK et al., 1974), but one which is of growing interest is non-hormonal alkaline phosphatase (ALP) (EC 3.1.3.1). Literature has shown that ALP functions optimally in alkaline conditions and participates in the active transport of sugars and phosphates in intestinal mucosa, renal tubuli, placenta, and bone, (Kaldor G, 1983) but, researchers remain unclear on the exact physiological role of ALP and its isoenzymes. Recently, it has been suggested that placental ALP may play a role in DNA synthesis and cell proliferation during fetal growth (She QB et al., 2000a; She QB et al., 2000b) and transplacental transport of Immunoglobulin G (IgG) to the fetus (Makiya R et al., 1992).

Researchers have isolated and quantified intestinal, liver/bone/kidney (LBK) and placental isoenzymes of ALP in AF (Vergnes H et al., 2000; Mulivor RA et al., 1979). AF ALP activity peaks at 17-19 weeks of gestation, decreasing thereafter but once again increases at 24-28 weeks till term (Burc L et al., 2001; Gulbis B et al., 1998; Salgo L et al., 1989; Brocklehurst D et al., 1980; Benzie RK et al., 1974; Hahnemann N et al., 1974). In early pregnancy, the majority of AF total ALP is intestinal from desquamated fetal intestinal cells; placental ALP directly enters AF from the placenta (Mulivor RA et al., 1979). Fluctuations in ALP isoenzymes throughout gestation appear to signify the development of the fetus and its organs: the degree of and subsequent absence of intestinal ALP present in AF between the 2nd and 3rd trimester depends on the proper development of the anal sphincter while the dramatic increase of LBK ALP in 3rd
trimester AF reflects the development of fetal organs in late gestation (Gulbis B et al., 1998).

At mid-gestation, lower than expected total AF ALP has been associated with Down’s syndrome (Ben-Ami M et al., 2002; Vergnes H et al., 2000; Ind et al., 1993), cystic fibrosis (Sembaj et al., 1995; Brock DJH, 1983) and intestinal distension (Stefos T et al., 1993). Moreover, lower than expected fetal intestinal ALP has been associated with inadequate development of the fetal anal membrane in the 1st trimester (Mulivor RA et al., 1979) and the presence of a duodenal atresia (Vergnes H et al., 2000), both of which could affect the fetal swallowing and absorption of nutrients in AF. Past research has demonstrated relationships between 2nd trimester maternal serum ALP levels with preterm birth and low birth weight (Moawad AH et al., 2002; Goldenberg RL et al., 1997; Best RG et al., 1991; Meyer RE et al., 1995; Brock DJH et al., 1988); however, little research has explored AF ALP as a possible biomarker of fetal growth, development or gestational age during normal development.

Thus, we explored whether AF ALP concentrations, in early pregnancy could be a biomarker of fetal growth. Our specific objectives were: 1) to quantify total ALP and its isoenzymes (intestinal, LBK, placental) in 2nd trimester human AF and 2) to determine whether AF ALP (total and/or placental) was associated with infant BW and/or GA.

2. Materials and Methods

Study Design and Recruitment. Between 2000 to 2004, pregnant women undergoing routine amniocentesis for genetic testing at St. Mary’s Hospital Centre in Montreal, Canada were approached to participate in the study. Ethics were approved by the Institutional Review Board of McGill University and St. Mary’s Hospital Centre. Informed consent permitted researchers to obtain AF samples from the Montreal Children’s Hospital following age-related genetic testing and to access medical charts. At the time of recruitment, subjects, assisted by a researcher, completed questionnaires detailing ethnicity, height, pre-pregnancy weight, age, smoking status, amniocentesis wk and parity. Confirmation of this information as well as infant gender, infant BW and GA were obtained through medical chart review following parturition. GA was determined by the physician based on the date of the mother’s last menstrual period. The exclusion
criteria for this study consisted of non-singleton pregnancies and mothers with genetic anomalies, resulting in 518 mother-infant pairs.

**Biochemical Analysis** AF samples, stored at -80°C, were analyzed for ALP and its isoenzymes. Manufacturer’s protocol for Promega AttoPhos® AP Fluorescent Substrate System (Promega; Madison, Wisconsin) was followed and performed in duplicates using 96 well plates. The kit was chosen for its novel substrate of 2’-[2-benzothiazoyl]-6’-hydroxybenzothiazole (BBt) phosphate, a substrate that is more sensitive than the traditional p-nitrophenylphosphate (Parandoosh et al., 1998). Methodology for the quantification of ALP isoenzymes was based on previously described chemical and physical properties (Mulivor RA et al. 1985). Unheated samples of AF were assayed in the presence of 2.5 mmol/L of L-phenylalanine and 10.0 mmol/L of L-homoarginine to determine LBK and intestinal ALP concentrations, respectively (Mulivor RA et al., 1985). AF samples were also heated at 65°C in a water bath for 90 minutes to destroy heat-labile isoenzymes (LBK and intestinal ALP) and then assayed to determine placental ALP concentration. All reaction mixtures were incubated at 37°C to mimic physiological conditions prior to assaying. Absorbance readings were taken using the Wallac fluorometer (Turku, Finland), set at 430-440 nm for the excitation filter and 550-560 nm for the emission filter.

**Statistical Analyses.** All data were analyzed using SAS (Version 8.0, SAS Institute, Cary, N.C.). All enzymes were normalized using square root transformation (total ALP, intestinal, LBK and placental ALP). BW was categorized into percentile groups corrected for gender and gestational age: 1) small-for-gestational-age (SGA) <10%, 2) appropriate-for-gestational-age (AGA) 10-90% and 3) large-for-gestational-age (LGA) >90% (Kramer MS et al., 2001). National data compiled to develop the population based Canadian reference for birth weight for gestational age was used in this study to determine percentile ranking (Kramer MS et al., 2001). A percentile ranking was assigned to each infant following a comparison between infant birth weight and expected infant birth weight for gestational age and gender. Gestational age (GA) was divided into: GA < 38 wks, GA 38-41 wks and GA >41 wks based on literature that mother’s last
menstrual period is often miscalculated and 38 wks gestation is associated with the lowest risk of perinatal death (Smith GC, 2001). Analysis of variance (ANOVA) and covariance (ANCOVA) were performed based on these subgroups. Covariates for birth weight included maternal height, pre pregnancy weight, parity, maternal smoking status, infant gender and GA based on existing literature demonstrating their associations with infant birth weight (Kramer MS, 1987). Storage length, based on the difference between day of amniocentesis and the day the sample was assayed, was included as a covariate based on its possible effect on ALP concentration (Wolf PL, 1978). Multiple linear regression analysis of birth weight as a continuous dependent variable (in grams and percentiles) was performed for the entire population and within subgroups: gestational age and percentile groups corrected for gender and gestational age. Significance was set at $p \leq 0.05$.

3. Results

The study population consisted of 518 multi-ethnic mother-infant pairs (59% Caucasian, 19%, Asian and 22% Blacks, Hispanics or Middle Eastern); the majority were non-smokers (87%). Mean maternal age was 37.5 ± 2.8 years with a parity of 1.2. Forty nine percent of women had spontaneous vaginal births. Mean birth weight was 3418 ± 503 grams with female infants making up 48% of the population where 8, 81 and 11% were born SGA, AGA and LGA, respectively. Average gestational age was 39.5 wks with 87% being born at term (38-41 wks).

AF samples taken between the 12th and 24th week of gestation showed a wide range of concentrations for total ALP (0.081 ± 0.002 M/L) and each ALP isoenzyme: intestinal (0.064 ± 0.0019 M/L), LBK (0.061± 0.0017 M/L), placental (0.0063 ± 0.0002 M/L). In our population of healthy mothers, AF placental ALP was correlated with maternal height ($r = 0.10822, p<0.0137$) and with smoking status ($r = 0.113, p<0.0102$). Higher AF placental ALP was found for female infants ($r = -0.103, p<0.0184$). All enzymes were positively correlated with each other.

Comparison of ALP and its isoenzymes across maternal height, pre pregnancy weight, age, parity, birthing method and ethnicity did not differ with the exception of smoking. Higher concentrations of placental ALP were seen in smokers versus non-
smokers (0.0078 ± 0.0007 M/L, 0.0062 ± 0.00002 M/L) (Figure IV-1A) and in females versus males (0.0069 ± 0.0003 M/L, 0.0059 ± 0.0003 M/L) (Figure IV-1B).

Comparisons of enzymes across birth-weight-corrected-for-gender-and-gestational-age showed that placental ALP differed across groups with the inclusion of the following covariates maternal height, pre-pregnancy weight, parity, smoking status and storage time. Placental ALP was higher in SGA (3.30 ± 0.34 mg/ml) than in AGA infants (2.83 ± 0.1 mg/ml) (Table IV-1). Further analysis showed a statistically significant difference between term SGA (3.36 ± 0.39 mg/ml) and term AGA infants (2.87 ± 0.12 mg/ml) (Table IV-1). None of the other enzymes differed across the birth weight categories.

Finally, multiple regression analysis identified placental ALP as a negative predictor of infant birth weights at term measured either in grams or in birth-weight-corrected-for-gender-and-gestational-age (Table IV-2). No AF ALP measurement predicted gestational age.
**Figure IV-1** - Differences in ALP and ALP isoenzyme concentrations between (A) maternal smoking status and (B) infant gender. Superscripts indicate significant difference between groups, $P \leq 0.05$. 
Table IV-1 - Infant, maternal and amniotic fluid characteristics stratified by birth-weight- for-gestational-age for entire population\(^1\) and for term infants\(^2\)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>SGA</th>
<th>AGA</th>
<th>LGA</th>
<th>P</th>
<th>SGA</th>
<th>AGA</th>
<th>LGA</th>
<th>P</th>
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<tbody>
<tr>
<td><strong>Infant</strong></td>
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<tr>
<td>Birth Weight, g</td>
<td>2761 ± 58(^a)</td>
<td>3377 ± 19(^b)</td>
<td>4239 ± 51(^c)</td>
<td>&lt;0.0001</td>
<td>2754 ± 30(^a)</td>
<td>3425 ± 18(^b)</td>
<td>4242 ± 45(^c)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Gender, % female</td>
<td>49</td>
<td>48</td>
<td>45</td>
<td>NS</td>
<td>50</td>
<td>46</td>
<td>46</td>
<td>NS</td>
</tr>
<tr>
<td>Gestational Age, wks</td>
<td>40.0 ± 0.52(^a)</td>
<td>39.4 ± 0.66(^b)</td>
<td>39.5 ± 1.02(^ab)</td>
<td>0.0359</td>
<td>40.0 ± 0.13(^a)</td>
<td>39.6 ± 0.04(^b)</td>
<td>39.3 ± 0.12(^ab)</td>
<td>0.002</td>
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<td><strong>Maternal</strong></td>
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<tr>
<td>Height, m</td>
<td>1.6 ± 0.01(^a)</td>
<td>1.62 ± 0.003(^a)</td>
<td>1.65 ± 0.009(^b)</td>
<td>&lt;0.0001</td>
<td>1.6 ± 0.01(^a)</td>
<td>1.62 ± 0.004(^a)</td>
<td>1.66 ± 0.009(^b)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Pre pregnancy Weight, kg</td>
<td>58 ± 1(^a)</td>
<td>62 ± 1(^a)</td>
<td>71 ± 2(^b)</td>
<td>&lt;0.0001</td>
<td>57 ± 2(^a)</td>
<td>63 ± 1(^a)</td>
<td>74 ± 3(^b)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Nonsmoking, %</td>
<td>86</td>
<td>85</td>
<td>92</td>
<td>NS</td>
<td>86</td>
<td>85</td>
<td>89</td>
<td>NS</td>
</tr>
<tr>
<td>Parity</td>
<td>0.8 ± 0.17(^a)</td>
<td>1.1 ± 0.056(^a)</td>
<td>1.2 ± 0.13(^ab)</td>
<td>0.0382</td>
<td>0.9 ± 0.2(^a)</td>
<td>1.2 ± 0.067(^a)</td>
<td>1.2 ± 0.13(^ab)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Amniotic Fluid</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Amniocentesis week, wk</td>
<td>15.4 ± 0.2</td>
<td>15.4 ± 0.1</td>
<td>15.5 ± 0.1</td>
<td>NS</td>
<td>15.4 ± 0.2</td>
<td>15.4 ± 0.1</td>
<td>15.5 ± 0.1</td>
<td>NS</td>
</tr>
<tr>
<td>Total ALP, M/L(^3)</td>
<td>0.073 ± 0.0054</td>
<td>0.082 ± 0.0025</td>
<td>0.080 ± 0.0085</td>
<td>NS</td>
<td>0.070 ± 0.0057</td>
<td>0.081 ± 0.0027</td>
<td>0.086 ± 0.0113</td>
<td>NS</td>
</tr>
<tr>
<td>Intestinal(^3)</td>
<td>0.055 ± 0.0048</td>
<td>0.065 ± 0.0022</td>
<td>0.063 ± 0.0076</td>
<td>NS</td>
<td>0.052 ± 0.0051</td>
<td>0.064 ± 0.0025</td>
<td>0.069 ± 0.0102</td>
<td>NS</td>
</tr>
<tr>
<td>LBK(^3)</td>
<td>0.055 ± 0.0041</td>
<td>0.061 ± 0.0018</td>
<td>0.062 ± 0.0082</td>
<td>NS</td>
<td>0.053 ± 0.0044</td>
<td>0.061 ± 0.0021</td>
<td>0.068 ± 0.0113</td>
<td>NS</td>
</tr>
<tr>
<td>Placental(^3)</td>
<td>0.007 ±</td>
<td>0.006 ±</td>
<td>0.006 ±</td>
<td>0.0364</td>
<td>0.007 ±</td>
<td>0.006 ±</td>
<td>0.006 ±</td>
<td>0.0432</td>
</tr>
<tr>
<td></td>
<td>0.0008(^a)</td>
<td>0.0002(^b)</td>
<td>0.0007(^ab)</td>
<td></td>
<td>0.0009(^a)</td>
<td>0.0003(^b)</td>
<td>0.0008(^ab)</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) ANOVA of all infants born between 33-46 weeks of gestational age. Values are means ± SEM or %, n= 506-518. Percentile subcategories include SGA (<10%) n= 42-43, AGA (10-90%) n= 410-419 and LGA (>90%) n= 53-56. Means in a row with different superscripts differ, P ≤ 0.05.

\(^2\) ANOVA of only term infants born between 38-41 weeks of gestational age. Values are means ± SEM or %, n=391-399. Percentile subcategories include SGA (<10%) n=35-36, AGA (10-90%) n=318-324 and LGA (>90%) n=38-39. Means in a row with different superscripts differ, P ≤ 0.05.

\(^3\) Enzymes were corrected for the following covariates: maternal height, pre-pregnancy weight, parity, smoking status and storage length.
Table IV-2 - Regression analysis for birth weight in all term infants (38-41 weeks gestation)

<table>
<thead>
<tr>
<th>Variable</th>
<th>MODEL 1-TERM INFANTS, %&lt;sup&gt;1&lt;/sup&gt;</th>
<th>P</th>
<th>MODEL 2-TERM INFANTS, g&lt;sup&gt;2&lt;/sup&gt;</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placental, M/L</td>
<td>-1.65 ± 0.7</td>
<td>0.0216</td>
<td>-21.70 ± 10.85</td>
<td>0.0463</td>
</tr>
<tr>
<td>Height, m</td>
<td>59.3 ± 23.5</td>
<td>0.0122</td>
<td>926.50 ± 360.65</td>
<td>0.0107</td>
</tr>
<tr>
<td>Pre-Pregnancy weight, kg</td>
<td>0.49 ± 0.13</td>
<td>0.0002</td>
<td>7.38 ± 1.99</td>
<td>0.0002</td>
</tr>
<tr>
<td>Parity</td>
<td>2.03 ± 1.37</td>
<td>NS&lt;sup&gt;3&lt;/sup&gt;</td>
<td>14.56 ± 20.90</td>
<td>NS</td>
</tr>
<tr>
<td>Smoking Status, 0=non-smoker, 1=smoker</td>
<td>-4.33 ± 4.46</td>
<td>NS</td>
<td>-87.84 ± 67.32</td>
<td>NS</td>
</tr>
<tr>
<td>Gender, 0=f, 1=m</td>
<td>NE</td>
<td>NE</td>
<td>115.43 ± 46.09</td>
<td>0.0128</td>
</tr>
<tr>
<td>Gestational Age, wks</td>
<td>NE</td>
<td>NE</td>
<td>93.21 ± 29.32</td>
<td>0.0016</td>
</tr>
<tr>
<td>Storage Length, years</td>
<td>2.62 ± 0.87</td>
<td>0.0028</td>
<td>33.95 ± 13.10</td>
<td>0.01</td>
</tr>
<tr>
<td>Variability captured (R&lt;sup&gt;2&lt;/sup&gt;*100)</td>
<td>13.23</td>
<td></td>
<td>16.08</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>MODEL 1: Regression analysis for birth-weight- corrected-for-gestational-age-and-gender in all term infants (38-41 weeks) (n=317). NE, not entered into analysis as independent variables.

<sup>2</sup>MODEL 2: Regression analysis for birth weight represented by grams in all term infants (38-41 weeks) (n=320)

<sup>3</sup> NS, not significant, P ≤ 0.05.
4. Discussion

Serum ALP has been extensively studied as a biomarker, where a negative association between maternal serum ALP and prematurity or low birth weight has been reported (Moawad AH et al., 2002; Goldenberg RL et al., 1997; Meyer RE et al., 1995; Best RG et al., 1991; Brock DJH et al., 1988). Higher 2nd and 3rd trimester maternal serum ALP activity has been associated with spontaneous preterm birth (Moawad AH et al., 2002; Meyer RE et al., 1995) and low birth weight (Goldenberg RL et al., 1997) and two studies have shown that elevated placental ALP in 2nd trimester maternal serum increased the risk of delivering a low birth weight infant (Best RG et al., 1991; Brock DJH et al., 1988). We report for the first time a similar negative association using 2nd trimester AF placental ALP with birth weight.

Previous researchers have attempted to explain this relationship between elevated placental ALP and low birth weight (Best RG et al., 1991; Brock DJH et al., 1988) and/or prematurity (Moawad AH et al., 2002; Goldenberg RL et al., 1997; Meyer RE et al., 1995) in maternal with the release of cellular following placental tissue disruption (Wolf PL 1978). In AF, placental ALP originates directly from the trophoblast cells of the placenta (Gol M et al., 2005; Goldenberg RL et al., 1997) and can indicate both active placental growth and/or placental damage during gestation (Best RG et al., 1991) as well as transplacental transport of IgG (Makiya R et al., 1992). Although placental ALP is found in relatively small concentrations in 2nd trimester AF, it is not surprising that this isoenzyme displayed an association with fetal growth. Placental ALP is of fetal origin (Magrini A et al., 2003; Beckman G et al., 1995). The fetal genotype of this isoenzyme has been linked to LBW (Amante A et al., 1996) as well as transplacental transport of IgG (Makiya R et al., 1992). In addition, our 2nd trimester AF samples were obtained at a point in gestation when maximum placental growth was occurring (Redmer RA et al., 2004) but considering that only birth weight was measured, we cannot comment on the effect of placental volume, placental histopathology or IgG in our study population.

Several explanations for this inverse relationship have been proposed. (1) One theory is that the higher concentrations of AF protein are a result of limited AF volume expansion (Sherer DM, 2002). Uteroplacental insufficiency or poor placental perfusion, which is associated with limited AF volume expansion, is common in fetal growth restriction such
as SGA (Ong CY et al., 2001). (2) Another proposed theory for the inverse relationship suggests that a larger fetus swallows and digests more AF and thus AF proteins for its growth (Ross MG et al., 2001) leaving a smaller amount of protein remaining in AF. (3) Recent literature has also suggested that placental ALP may play an important role in the growth of fetal tissues during the 2nd and 3rd trimester (She QB et al., 2000a; She QB et al., 2000b). These researchers provided evidence that placental ALP stimulates DNA synthesis and cell proliferation when incubated with human fetal fibroblasts (She QB et al., 2000a; She QB et al., 2000b). 4) The two sources of ALP in AF during the 2nd trimester of pregnancy originate from maternal plasma (Mulivor R et al., 1979; Johnson AM et al., 1974) with minor contributions from the placenta which is of fetal origin (Mulivor R et al., 1979; Sutcliffe RG et al., 1973). Elevated levels of placental ALP in maternal serum can arise from increased synciotrophoblast synthesis or increased dissociation from disrupted synciotrophoblasts (Ind Te et al., 1993). Consequently, elevations of placental ALP, observed in our study, may be due to elevated maternal serum placental ALP traveling into AF. 5) Placental ALP demonstrates immunoreactivity during pregnancy as it transports IgG across the placenta from maternal serum to the fetus as well as AF (Ind TE et al., 1993; Makiya R et al., 1992). Commonly seen in Down’s syndrome, disturbed trophoblast function elicits increases in maternal serum placental ALP and decreased levels of AF placental ALP. This thereby limits the transfer of IgG to the fetus (Ind TE et al., 1993). In our study, it is uncertain whether the growth restricted fetus requires an enhanced production of placental ALP in AF in order to transport IgG more readily. Comparatively, it is possible that lower levels of AF placental ALP observed in larger babies do not require the protective role of IgG to the same extent of SGA infants. In fact, a combination of one or more of these proposed mechanisms may underscore the inverse relationship that occurs in 2nd trimester AF ALP.

Our study also revealed that mothers who smoked demonstrated higher AF placental ALP concentration compared to non-smokers. Smoking leads to decreased placental blood perfusion causing impaired utero-placental transfer of nutrients and oxygen to the fetus which can lead to IUGR (Baschat AA et al., 2004; Burton GC et al., 1989). Moreover, documented morphological changes of the placenta exposed to cigarette smoke include a higher number of syncytial buds, apoptosis, increased thickness of the
villous membrane and synciotrophoblastic necrosis (Zdravkovic T et al., 2005; Rath G et al., 2001; Jauniaux E et al., 1992). Our findings confirm previous literature reporting that the effect of smoking on infant birth weight impacts placental ALP (Magrini A et al., 2003). It has been demonstrated maternal smoking during pregnancy increases ALP activity in fetal plasma (Jauniaux E et al., 2001) suggesting altered cellular function of placental tissues and enzyme activity as a result of smoking. Our finding suggests that smoking influences placental ALP concentration and must be considered as a confounder of placental ALP concentration.

In addition, this study demonstrated that mothers who gave birth to female infants had higher AF placental ALP concentration than those who gave birth to male infants. Pregnant mothers carrying female fetuses had higher 2nd and 3rd trimester serum ALP activities for total and placental ALP (Gol M et al., 2005). Vergnes et al. (1998) proposed that the more mature female placenta in the 2nd trimester causes increased maternal hormonal secretions that may trigger increased maternal ALP synthesis in those carrying female fetuses (Vergnes et al., 1998) while others have proposed that there is a link between the production of human chorionic gonadotropin and maternal ALP activity during pregnancy (Gol et al., 2005; Steier J et al., 2002). Therefore the results of our study and the study by Gol et al. (2005) suggest gender-related effects on ALP concentration must be considered and controlled for in any statistical analyses when investigating total and/or placental ALP as a biomarker of birth outcomes.

In summary, the results of our study show that 2nd trimester placental ALP in AF is negatively associated with birth weight in women giving birth to healthy term infants. This suggests placental function may be perturbed or an immune response may be triggered but placental weight; histopathology, function and the transport of IgG must be further explored to understand these relationships.
5. Literature Cited


Moawad AH, Goldenberg RL, Mercer B, Meis PJ, Iams JD, Das A, Caritis SN,


V. GENERAL DISCUSSION

A. Study Population

The results of this study were based on 518 multi-ethnic women (Table V-1) and were comparable to that of the general Canadian population. Although our population of mothers was older than the reported Canadian average of 30 years old, they were healthy with an average BMI of 23 and predominantly primagravida. Non-smokers represented 87% of the population similar to published Canadian statistics of 81% (Canadian Perinatal Report, 2003). Our newborns were born at an average gestational age of 39 weeks with a BW of 3421 g. Canadian newborns weigh in at 3420 g. Thus, even though our population consisted of older mothers with an average age of 37, we can conclude that our study population was representative of the Canadian population at large.

B. Major Findings

The findings of this study, similar to serum ALP studies (Moawad AH et al., 2002, Brock DJH et al., 1988, Meyer RE et al., 1995, Brock DJH et al., 1988, Best RG et al., 1991, Holmgren PA et al., 1979, Spellacy WN et al., 1977, Kunz J et al., 1976), suggest that placental ALP is indeed related to fetal growth and may have important implications for placental health. An inverse relationship was observed between AF placental ALP and birth weight in both healthy term (38-41 weeks). Moreover, compared to AGA infants, SGA infants had higher AF placental ALP concentrations. This was demonstrated in both the entire population as well as in term infants. Provided that placental growth is limited to the first and second trimester of pregnancy (Redmer DA et al., 2004) the AF samples collected for this study would reflect a time period of placental growth. Subsequently, the AF placental ALP measured in this study may reflect early placental tissue health or immunoreactivity and via this relationship explain its association with fetal growth or infant birth weight.

Although placental ALP only contributes small amounts to total ALP in AF, it is not surprising that only this isoenzyme demonstrated relationships to fetal growth. Placental ALP is of fetal genotype (Magrini A et al., 2003; Beckman G et al., 1995) and has been suggested to play important roles in fetal cell synthesis (She QB et al., 2000a; She QB et al., 2000b) and immunoreactivity (Makiya R et al., 1992). Moreover, the
extent of transplacental transport of IgG is determined by the fetal genotype of placental ALP (Beckman G et al., 1995). LBK and intestinal ALP did not reveal any relationships with fetal growth nor did we expect them to considering that uncharacteristic levels of both are used to identify abnormal conditions such as intestinal obstruction (Stefos T et al., 1993), cystic fibrosis and Down’s syndrome (Ben-Ami M et al., 2002, Ind TE et al., 1993, Vergnes H et al., 2000, Stinson RA et al., 1987, Moss D, 1987, Sembaj A et al., 1995). In addition, LBK in samples obtained in the 2\textsuperscript{nd} trimester would not reflect fetal maturation but rather the transport of maternal serum proteins across the placenta (Mulivor R et al., 1979). Moreover, mother-infants pairs only in this study only included healthy pregnancy outcomes.

C. Sample Size

Routine amniocentesis for genetic testing allowed for the ongoing collection of amniotic fluid samples for the purposes of this study. A total of 518 biobanked samples were analyzed for alkaline phosphatase and its family of isoenzymes. Nevertheless, our study did not produce any significant findings between AF ALP and preterm births. In attempt to explain why our study did not find similar results between ALP and prematurity (Moawad AH et al., 2002; Goldernberg RL et al., 1997; Meyer RE et al., 1995), we eliminated the inclusion of confounders but were still unable to find any correlation. We can speculate that sample size may have contributed to this discrepancy. Serum studies involving ALP and fetal growth had substantially larger sample sizes: n=2929 (Moawad AH et al., 2002); n=1868 (Meyer RE et al., 1995); n=580 (Goldernberg RL et al., 1997). However, in the study by Moawad AH et al., findings were significant with lower prematurity cut offs that were set at < 32 week and <35 weeks whereas our 56 preterm infants were categorized at <38 weeks which may have provided a more accurate categorization of premature and term infants (Smith GC, 2001). Given that sample size may have been a limitation to our study, Smith’s recent definition of prematurity (<38 weeks) was utilized to increase statistical power.

The discrepancy between samples sizes must consider that serum samples are more readily collected and less invasive than amniocentesis. The only study with a population comparable to that of ours is the study by Goldernberg RL et al. with 45
infants born <37 weeks of gestation. However, the results of the study, which found high serum ALP in mothers who gave birth to preterm infants, may not be generalizable as the study population consisted of only African American mothers. We must recognize that ALP may behave differently in serum than in AF. However, a lack of literature exists regarding ALP AF and fetal growth. This study may help to provide further information into understanding AF ALP and fetal growth.

D. Development of Covariates

This study is novel in that it attempts to control for confounding factors by taking into consideration other variables that could possibly influence birth weight, gestational age and ALP concentration. Earlier studies investigating serum ALP did include covariates in their analysis but did not account for all factors that may affect BW, GA and ALP. When investigating ALP with GA: Meyer RE et al (1995) included mid-trimester maternal weight, ethnicity, infant gender, and amniocentesis week while Moawad AH et al. (2002) controlled for ethnicity and parity. Goldenberg RL et al. (1997) considered micronutrients and corrected for maternal BMI, smoking status, zinc supplementation, plasma folate, albumin and copper. Best RG et al. (1991) controlled for ethnicity, maternal age, mid trimester maternal weight when determining whether ALP was associated with BW. Meanwhile, Brock DJH et al. (1988) provided no evidence of including covariates. In our study, established covariates for birth weight included: maternal smoking status, maternal height, pre-pregnancy weight, ethnicity, parity and infant gender and were used in statistical analysis to test for birth weight outcomes (Kramer MS, 1987a). In addition, covariates for gestational age included: ethnicity, maternal height, parity, smoking status and gender.

Acknowledging the complexity of amniotic fluid as well as the enzymology of ALP, our study included an extensive assessment of covariates that could affect ALP concentrations (Table V-2). Although not significant, total, intestinal and LBK AF ALP increased with amniocentesis week supporting the development profile reported by previous literature (Benzie RJ et al., 1974) (Figure V-1A). As expected, placental AF ALP stayed relatively constant across amniocentesis weeks since its most marked increased is in the 3rd trimester (Mulivor RA et al., 1979).
Placental ALP concentrations showed a clear distinction between genders and maternal smoking status (Table V-2). Literature has suggested that gender differences affect placental growth (Vergnes CJ et al., 1998) while maternal smoking during pregnancy has been strongly linked to altered placental pathophysiology and placental insufficiency (Horta BL et al., 1997). Literature has reported that ALP varies with age throughout adulthood (Corathers SD, 2006). However, maternal age (25-47 years) did not have an effect on ALP concentration in our study population. Initial statistical analysis revealed that storage length had a negative linear effect on placental ALP concentrations and was included as a covariate. No knowledge exists on the behaviour of ALP in AF. Further studies are required to fully understand storage length’s impact on ALP in AF.

Covariates in our ANCOVA across birth-weight-corrected-for-gestational-age-and-gender categories included established maternal factors known to affect birth weight: maternal height, PPWkg, parity and smoking status. Provided that the majority of mothers were primagravida, inter-birth time interval was not considered to be a covariate. Storage length was included as a covariate of ALP concentration. Despite controlling for maternal factors, the results demonstrate that there is a fetal residual effect on placental ALP suggesting that the placental ALP may originate and be influenced by the fetal environment.

E. Relevance to the Field of Research

Researchers have two proposed mechanisms to explain for the inverse relationship between birth weight and AF proteins (Ross MG et al., 2001; Sherer DM, 2002). 1) A potential mechanism is that compared to smaller fetuses; larger fetuses swallow and absorb a greater amount of amniotic fluid in utero (Ross MG et al., 2001). Hence, smaller infants swallow smaller quantities of protein components leaving behind more to be contributed to the total AF concentration. 2) Higher concentrations of AF proteins may also be a result of limited AF volume expansion, which can suggest uteroplacental insufficiency or poor placental perfusion (Sherer DM, 2002). Consequently, fetal growth restriction such as SGA is common in cases of placental insufficiency (Baschat AA et al., 2004). In relation to our findings, it could be that perturbations in placental tissues that are known to restrict fetal growth may also elicit elevations in placental ALP.
Interestingly, the same inverse relationship between placental ALP and birth weight was also demonstrated in term infants. In support of Sherer (2001), the decreased placental AF ALP in our heavier term infants could be a result of increased AF expansion, which could indicate adequate placental perfusion. Moreover, these heavier healthy term infants could potentially be swallowing more placental ALP leaving less remaining placental AF ALP. Subsequently, adequate placental perfusion would provide an optimal environment for growing fetus. In addition to these proposed mechanisms, placental ALP has also been known to promote fetal cell synthesis and to demonstrate immunoreactivity.

F. Alkaline Phosphatase Assay Techniques

Compared to existing literature, our study is novel in that it measured the concentration of AF ALP and its isoenzymes using a new kit, a more sensitive substrate and we corrected for confounders during statistical analysis. A comparison study of biochemical and immunological assays to quantify ALP reported similar activities of total and ALP isoenzymes regardless of the method (Mulivor RA et al., 1985). Using spectrophotometry with p-nitrophenylphosphate as the substrate, Mulivor et al., found 2nd trimester AF ALP activity levels ranging from 0.19-118 mIU/ml in a sample of 42 mothers (Mulivor RA et al., 1979). In a recent study conducted on a sample of 59 2nd trimester AF samples, a range of activities were obtained between 2.8-112 IU/l (Vergnes H et al., 2000).

The Promega AttoPhos kit used in this study was developed to detect ALP in solution. Compared to previous studies, our methods were comparable and varied only with the choice of substrate. Unlike prior studies which utilized the substrate: p-nitrophenylphosphate, this study used 2’-[2-benzothiazoyl]-6’- hydroxybenzothiazole phosphate (BBt). To our knowledge; this is the first study to use this kit and substrate to measure ALP in amniotic fluid. Manufacturers of the kit: AttoPhos AP Fluorescent Substrate System (Madison, WI) claims that BBt phosphate is a highly sensitive substrate (Parandoosh et al., 1998) compared to other substrates available for ALP. The kit also claims an increased detection sensitivity and lower background fluorescence for better results.
The method used in this study did not allow for the separation of the tissue non-specific isoenzymes: liver/bone/kidney and is limited compared to the immunological method (e.g. ELISA). However, this was not a concern for us as amniotic fluid samples were collected during the 2nd trimester where LBK ALP is of maternal origin and thus indicates maternal health status. The fetal organs in the 2nd trimester are still maturing and do not release tissue non-specific ALP until the 3rd trimester when they are developed. The focus of this study was to determine AF total ALP and AF placental ALP.

G. Limitations

1. Alkaline Phosphatase Concentrations

Considering that our study measured ALP AF concentration, controlled for covariates, and utilized a different substrate, it is difficult to compare our AF ALP concentrations: (Total ALP = 0.081 ± 0.002 M/L, intestinal = 0.064 ± 0.0019 M/L, LBK 0.061± 0.0017 M/L, placental = 0.0063 ± 0.0002 M/L) to previous studies that quantified AF ALP activity. However, the proportions of intestinal and placental ALP in comparison to total ALP were comparable to literature. Early gestation activity is expected to consist of intestinal, LBK, and trace amounts of placental (81%, 15%, 4%), respectively (Mulivor RA et al., 1979). When using measured total AF ALP our samples of 2nd trimester AF, ALP was composed of the following proportions of ALP isoenzymes: intestinal ALP (76%), LBK ALP (75%) with minor amounts of placental ALP (10%). Conversely, while calculating isoenzymes proportions based on the sum of intestinal, LBK and placental ALP the following proportions were as follows: intestinal (47%), LBK (46%) and placental (6%). This suggests that either intestinal and/or LBK concentrations may be overestimated. Early research suggests that meconium-stained AF may contain excessive quantities of the heat-labile isoenzymes (Sutcliffe RG et al., 1972b). A limitation to our study was that our AF samples did not exclude meconium-stained samples. Thus, this may explain for the observed overestimated concentrations of intestinal and/or LBK isoenzymes in our study. In order to confirm the ALP concentrations obtained, verification by way of another assay is warranted with the exclusion of meconium stained AF samples.
2. Impact of Storage Length and pH

The quantities of ALP measured in this study may indicate that there were interfering substances that may have altered the concentration of ALP such as storage length. In serum, earlier studies did not specify storage length of samples and did not correct for the storage length when literature suggests that an increase of ALP activity can occur in the prolonged storage of sera (Wolf PL, 1978). An increase in pH results from stored serum as a result of carbon dioxide loss and an increase in ammonia thereby producing an optimal pH for ALP activity (Wolf PL, 1978). To our knowledge, no literature exists on whether ALP in AF is affected by storage length or pH but because of initial statistical analysis, storage length was included a covariate in our analyses. The average pH of AF samples was measured at 37° C. Statistical analysis revealed that was ALP concentrations did not differ across pH and was therefore not included as a covariate (Figure V-1B). However, recorded pH readings were not measured at the time of the assay and thus, may not provide an accurate representation of the pH at the time the AF samples were assayed for concentration. Future work may include the measurement of pH at the time of the assay to understand whether and how storage length, pH and ALP concentration are related.

3. Collection of Placental Information

Data collected during this study was limited regarding the pathophysiology of placentas. Physicians do not routinely record information such as placental weight or placental pathophysiology. Moreover, the examination of the placenta is only ordered following complicated pregnancies. Therefore, in this study, a large enough sample size was not available to determine the relationship between placental ALP, the placenta and subsequently fetal growth. In order to understand the underlying mechanism of elevated placental ALP and birth weight, future work is required to collect information on placental conditions during gestation and post-partum. This may include: placental weight, placental infarctions and any other placental pathophysiology available.
H. Future Work

Provided that placental ALP transports IgG from maternal serum to the fetus and AF, the amount of IgG present in AF and maternal serum in relation to placental ALP should be examined to test the immunogenic capacity of this isoenzyme in AF and its impact on fetal growth. Maternal nutritional status may provide valuable information into fetal birth weight and the composition of AF. While literature has not provided findings linking protein supplementation with birth weight, caloric intake during pregnancy may have a bearing on infant birth weight (Kramer MS, 1987a). Data on maternal diet for this population included intake of vitamins, supplements, coffee, tea and alcohol consumption but did not include average intake of macronutrients. A more comprehensive questionnaire regarding maternal diet and perhaps a 24-hour recall could be included in further analysis. Although most women took supplements, details regarding dosage of vitamins and supplements were limited. Considering ALP contains zinc and magnesium cofactors, it may be worthwhile to look at varying levels of zinc and magnesium or supplementation in the diet and their effect on ALP concentration in AF. Literature has also suggested that nutritional deficiencies including Vitamin C may elicit lower than expected ALP concentrations (Corathers SD, 2006).

Proteins found in amniotic fluid during the 2nd trimester of pregnancy originate from maternal plasma (Johnson AM et al., 1974) with minor contributions from the fetus (Sutcliffe RG et al., 1973). Considering the gestational point of our amniocentesis collection ranged between 12-22 weeks, the large proportion of ALP in AF originated from the mother. For the purposes of this study, the other ALP isoenzymes were quantified in order to quantify placental ALP by difference. Albeit in small concentrations compared to other isoenzymes, 2nd trimester placental ALP originates from the fetus and may provide the insight into fetal growth. However, for future work, medical chart review regarding maternal conditions/diseases where ALP is affected should be noted to determine whether it has an effect on AF ALP.

It has been suggested in our study that ALP concentrations in our frozen AF samples may be affected by storage length and through this possibly pH. In regards to study design, future studies utilizing fresh AF samples and measuring pH at the time of assay is warranted in order to totally understand the behaviour of AF ALP and the effect
of storage length and pH on its concentrations. Moreover, provided that the findings of our study centered on healthy term infants, it may be interesting to use a larger study population to determine whether AF ALP is related to prematurity as seen in previous serum studies.

Ultrasounds providing anthropometric measurements are important in indicating proper fetal growth and development but our study is novel in that it considers the effect of the fetal metabolic environment on fetal growth. To our knowledge, this study is novel in its attempt to examine whether in early gestation AF ALP and/or its isoenzymes could predict fetal growth and development. This study not only provided data linking fetal birth weight with fetal metabolic environment, it suggests that AF may reflect the biological state of the developing placenta. Our findings show that AF of SGA infants had higher placental ALP concentrations than AGA infants in both the entire population and in only term infants (38-41 weeks). What is even more interesting, placental ALP also negatively predicted birth weight in term infants, which by all accounts should not demonstrate metabolic aberrations. Interestingly, the results of this study demonstrate the complex nature of enzymes. Both damaged as well as normal tissue growth can elicit elevations in enzymes.

Currently, ultrasounds and pelvic exams allow clinicians to visualize placental implantation. These techniques help to alert conditions such as placental abruptions or placental previa. However, little is available to warn clinicians of early alterations in placental morphology or pathophysiology that could impact pregnancy outcomes. Post-partum examinations on the histology or pathology (blood clots, infections, tissue damage) of the placenta are only completed after the event of a complicated pregnancy. Thus, this study strongly demonstrates that 2nd trimester placental ALP in AF is negatively associated with birth weight and that elevated 2nd trimester concentrations of this isoenzyme may help identify lower birth weight outcomes. Our findings propose that AF placental ALP may emerge as an early biomarker of growing or disrupted placental tissue that could subsequently have affect fetal growth and development. AF placental ALP may be able to signify to clinicians early signs of placental tissue status or the transport of IgG to the fetus and allow for earlier intervention. Findings of this study
suggest that placental ALP in AF may shed further light on the link between placental health and fetal growth.
Table V-1 Characteristics of the sample population, n = 518

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MATERNAL</strong></td>
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</tr>
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<td><strong>Age, years</strong></td>
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</tr>
<tr>
<td><strong>Height, m</strong></td>
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</tr>
<tr>
<td><strong>Pre Pregnancy Weight, kg</strong></td>
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<td><strong>BMI</strong></td>
<td>23.9 ± 4.1</td>
</tr>
<tr>
<td><strong>Parity</strong></td>
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<td><strong>Non Smokers, %</strong></td>
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<td><strong>FETAL</strong></td>
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<td><strong>Infant Gender, % female</strong></td>
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<tr>
<td><strong>Birth weight, grams</strong></td>
<td>3418 ± 503</td>
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<td><strong>Gestational Age, wks</strong></td>
<td>39.47 ± 1.49</td>
</tr>
<tr>
<td><strong>Birth outcome, %</strong></td>
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</tr>
<tr>
<td>IUGR (&lt;2500 g)</td>
<td>3</td>
</tr>
<tr>
<td>Normal (2500-3999 g)</td>
<td>84</td>
</tr>
<tr>
<td>Macrosomia (≥ 4000 g)</td>
<td>13</td>
</tr>
<tr>
<td>SGA (&lt;10%)</td>
<td>8</td>
</tr>
<tr>
<td>AGA (10-90%)</td>
<td>81</td>
</tr>
<tr>
<td>LGA (≥ 90%)</td>
<td>11</td>
</tr>
<tr>
<td>Preterm (&lt;38 weeks)</td>
<td>11</td>
</tr>
<tr>
<td>Term (38-41 weeks)</td>
<td>77</td>
</tr>
<tr>
<td>Post-term (&gt;41 weeks)</td>
<td>12</td>
</tr>
<tr>
<td><strong>AMNIOTIC FLUID CHARACTERISTICS</strong></td>
<td></td>
</tr>
<tr>
<td>Amniocentesis Week, wk</td>
<td>15.4 ± 1.2</td>
</tr>
<tr>
<td>pH</td>
<td>(348) 9.51 ± 0.017</td>
</tr>
<tr>
<td>Alkaline Phosphatase, M/L</td>
<td>0.081 ± 0.002</td>
</tr>
<tr>
<td><strong>Intestinal</strong></td>
<td>0.064 ± 0.0019</td>
</tr>
<tr>
<td><strong>LBK</strong></td>
<td>0.061 ± 0.0017</td>
</tr>
<tr>
<td><strong>Placental</strong></td>
<td>0.0063 ± 0.0002</td>
</tr>
</tbody>
</table>
Table V-2 – Alkaline Phosphatase variation by Maternal, Fetal and Environmental Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>N</th>
<th>Total ALP M/L</th>
<th>Intestinal M/L</th>
<th>Liver/Bone/Kidney M/L</th>
<th>Placental M/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>P=0.4089</td>
<td>P=0.6931</td>
<td>P=0.4685</td>
<td>P=0.0194</td>
</tr>
<tr>
<td><strong>Infant Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>247</td>
<td>0.0835 ± 0.0036</td>
<td>0.0655 ± 0.0031</td>
<td>0.0628 ± 0.0029</td>
<td>0.0069 ± 0.0003⁻¹</td>
</tr>
<tr>
<td>Male</td>
<td>271</td>
<td>0.0798 ± 0.0027</td>
<td>0.0640 ± 0.0025</td>
<td>0.0602 ± 0.0021</td>
<td>0.0059 ± 0.0003⁻²</td>
</tr>
<tr>
<td><strong>Maternal Height (m)</strong></td>
<td></td>
<td>P=0.3170</td>
<td>P=0.5172</td>
<td>P=0.3922</td>
<td>P=0.05</td>
</tr>
<tr>
<td>&lt;1.5748</td>
<td>119</td>
<td>0.0789 ± 0.0049</td>
<td>0.0633 ± 0.0044</td>
<td>0.0582 ± 0.0038</td>
<td>0.0055 ± 0.0003</td>
</tr>
<tr>
<td>≥1.5748 and &lt; 1.6256</td>
<td>128</td>
<td>0.0838 ± 0.0046</td>
<td>0.0660 ± 0.0042</td>
<td>0.0637 ± 0.0039</td>
<td>0.0069 ± 0.0004</td>
</tr>
<tr>
<td>≥1.6256 and &lt; 1.67</td>
<td>138</td>
<td>0.0771 ± 0.0040</td>
<td>0.0609 ± 0.0033</td>
<td>0.0595 ± 0.0031</td>
<td>0.0061 ± 0.0004</td>
</tr>
<tr>
<td>≥1.67</td>
<td>133</td>
<td>0.0863 ± 0.0045</td>
<td>0.0687 ± 0.0041</td>
<td>0.0641 ± 0.0033</td>
<td>0.0070 ± 0.0005</td>
</tr>
<tr>
<td><strong>Parity</strong></td>
<td></td>
<td>P=0.5317</td>
<td>P=0.5279</td>
<td>P=0.6591</td>
<td>P=0.2148</td>
</tr>
<tr>
<td>0</td>
<td>152</td>
<td>0.0877 ± 0.0046</td>
<td>0.0701 ± 0.0042</td>
<td>0.0655 ± 0.0035</td>
<td>0.0069 ± 0.0004</td>
</tr>
<tr>
<td>1</td>
<td>212</td>
<td>0.0803 ± 0.0035</td>
<td>0.0634 ± 0.0030</td>
<td>0.0607 ± 0.0028</td>
<td>0.0061 ± 0.0003</td>
</tr>
<tr>
<td>2</td>
<td>102</td>
<td>0.0775 ± 0.0043</td>
<td>0.0618 ± 0.0042</td>
<td>0.0590 ± 0.0035</td>
<td>0.0065 ± 0.0005</td>
</tr>
<tr>
<td>3</td>
<td>26</td>
<td>0.0774 ± 0.0075</td>
<td>0.0602 ± 0.0063</td>
<td>0.0581 ± 0.0057</td>
<td>0.0051 ± 0.0008</td>
</tr>
<tr>
<td>≥4</td>
<td>22</td>
<td>0.0767 ± 0.0109</td>
<td>0.0612 ± 0.0097</td>
<td>0.0575 ± 0.0084</td>
<td>0.0063 ± 0.0010</td>
</tr>
<tr>
<td><strong>Pre-Pregnancy Weight (kg)</strong></td>
<td></td>
<td>P=0.2942</td>
<td>P=0.4056</td>
<td>P=0.2627</td>
<td>P=0.1587</td>
</tr>
<tr>
<td>&lt;55</td>
<td>132</td>
<td>0.0789 ± 0.0040</td>
<td>0.0631 ± 0.0036</td>
<td>0.0595 ± 0.0031</td>
<td>0.0057 ± 0.0004</td>
</tr>
<tr>
<td>≥55 and &lt;60.7824</td>
<td>131</td>
<td>0.0903 ± 0.0059</td>
<td>0.0719 ± 0.0051</td>
<td>0.0685 ± 0.0048</td>
<td>0.0067 ± 0.0004</td>
</tr>
<tr>
<td>≥60.7824 and ≤ 68.04</td>
<td>128</td>
<td>0.0786 ± 0.0037</td>
<td>0.0615 ± 0.0034</td>
<td>0.0585 ± 0.0027</td>
<td>0.0066 ± 0.0004</td>
</tr>
<tr>
<td>≥70</td>
<td>127</td>
<td>0.0781 ± 0.0038</td>
<td>0.0623 ± 0.0035</td>
<td>0.0589 ± 0.0030</td>
<td>0.0065 ± 0.0005</td>
</tr>
<tr>
<td><strong>Smoking Status</strong></td>
<td></td>
<td>P=0.6069</td>
<td>P=0.7432</td>
<td>P=0.4393</td>
<td>P=0.0349</td>
</tr>
<tr>
<td>Non-Smoker</td>
<td>441</td>
<td>0.0812 ± 0.0025</td>
<td>0.0645 ± 0.0022</td>
<td>0.0609 ± 0.0019</td>
<td>0.0062 ± 0.0000²⁻¹</td>
</tr>
<tr>
<td>Smoker</td>
<td>68</td>
<td>0.0835 ± 0.0055</td>
<td>0.0664 ± 0.0048</td>
<td>0.0650 ± 0.0043</td>
<td>0.0078 ± 0.0007²⁻²</td>
</tr>
<tr>
<td><strong>Amniocentesis Week (Weeks)</strong></td>
<td></td>
<td>P=0.1116</td>
<td>P=0.1543</td>
<td>P=0.2568</td>
<td>P=0.6533</td>
</tr>
<tr>
<td>≤14.5</td>
<td>96</td>
<td>0.0750 ± 0.0046</td>
<td>0.0602 ± 0.0042</td>
<td>0.0577 ± 0.0037</td>
<td>0.0063 ± 0.0004</td>
</tr>
<tr>
<td>&gt;14.5 and ≤ 15.5</td>
<td>214</td>
<td>0.0786 ± 0.0033</td>
<td>0.0618 ± 0.0031</td>
<td>0.0598 ± 0.0028</td>
<td>0.0065 ± 0.0003</td>
</tr>
<tr>
<td>&gt;15.5 and ≤16.5</td>
<td>158</td>
<td>0.0858 ± 0.0034</td>
<td>0.0683 ± 0.0031</td>
<td>0.0644 ± 0.0025</td>
<td>0.0066 ± 0.0004</td>
</tr>
<tr>
<td>&gt;16.5</td>
<td>50</td>
<td>0.0931 ± 0.0117</td>
<td>0.0744 ± 0.0095</td>
<td>0.0659 ± 0.0083</td>
<td>0.0055 ± 0.0006</td>
</tr>
</tbody>
</table>

¹ Values are mean ± SEM. Means in a row with different superscripts differ, P ≤ 0.05
Figure V-1 Differences in ALP and its isoenzymes across amniocentesis weeks and pH
(A) ALP concentrations by amniocentesis week (n=518), (B) ALP concentrations by pH
(n=348). Means in a row with different superscripts differ, P <0.05.
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APPENDIX A

McGill University Ethics Approval
12 September 2006.

Dr. Kristine G. Koski  
School of Dietetics and Human Nutrition  
MacDonald Campus  
21,111 Lakeshore Road  
Ste-Anne-de-Bellevue QC H9X 3V9

RE: IRB Study Number A03-M03-98

Dear Dr. Koski,

Thank you for submitting an application for continuing review for the above-referenced study entitled, *The Role of Amniotic Fluid Constituents in Predicting Human Fetal Growth.*

The study progress report underwent review and full Board re-approval for this study was provided on September 11, 2006. The ethics renewal certificate (enclosed) is dated 2007.

It is the investigator's responsibility for ensuring that all documents approved by this Board are reported to, and meet the requirements in force at the institution/s where subject recruitment occurs and/or where study data is collected. Please contact the individual Research Ethics Offices for instruction on fulfilling this obligation.

If any study modifications or unanticipated study developments occur prior to the next annual review, including study terminations, please notify the IRB promptly. Regulation does not permit the implementation of study modifications prior to IRB review and approval.

Sincerely,

J. Lawrence Hutchison, MD  
Co-Chair  
Institutional Review Board

Cc: Ms. E. Flomp - SMH  
A03-M03-98
APPENDIX B

St. Mary’s Hospital Center Ethics Approval
In response to your request dated June 1, 2005 for an interim review of the above-mentioned protocol, please be advised that the interim report received re-approval by the Research Ethics Committee (REC) at its meeting held on June 15, 2005. Therefore, the REC has recommended an approval period of one year from June 15, 2005 to June 14, 2006.

Please note that the name of the patient representative on the consent form should be changed to Caroline Roy. Please make this change and send me the revised English and French consent forms so that they can be stamped to reflect this approval. Note that only stamped copies of these consent forms should be used in your study.

If you have any questions or require additional information, please do not hesitate to contact me at 345-3511 extension 3698.

Elizabeth Plomp
Research Administrative Secretary
APPENDIX C

Study Consent Form-English Version
St. Mary’s Hospital
Department of Obstetrics & Gynecology

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
Research Assistant: April D. Johansen, Ph.D. Candidate
St. Mary’s Hospital On-Site Contact: Dr. Gary Lusky, M.D. tel: 731-1253
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 min. 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 it may lead to better prenatal care in the future.
• Your identity and that of your baby will not be known to anyone except the researcher involved. All samples and results will be coded. Group results may be presented to scientific or professional audiences, but there will be no means of identifying you or your baby.

I, ______________________________, the undersigned, hereby consent to participate as a subject in the above named research on The Role of Amniotic Fluid in Predicting Human Growth, a project conducted by the School of Dietetics and Human Nutrition at McGill University. I have read the above information and agree to provide permission to the investigators

1) to receive 15 cc of my amniotic fluid sample that I have just delivered to the Montreal Children’s Hospital, and

2) to obtain birth weight, length, gender, last maternal weight before delivery from my hospital chart following my delivery.

If you have any questions regarding your rights, please call Ms. Monique Robitaille, at (514) 734-2618, or write to her at the following address:

   Ms. Monique Robitaille
   Patient Representative
   St. Mary’s Hospital
   3830 Lacombe Avenue
   Montréal, Qc
   H3T 1M5

I understand that my research participation is completely voluntary, that my confidentiality will be maintained at all times, and I am free to discontinue my participation at any time.

Subject’s Signature ___________________________ Date ________________

Witness ___________________________ Date ________________

Please proceed to filling in attached questionnaire.
APPENDIX D

Maternal Questionnaire
**Questionnaire**

*Please answer this brief questionnaire. You are reminded that all the information provided will be kept strictly confidential.*

Name: ______________________________ Telephone number: ______________________________

Date of birth: ________________________ Height: ______ feet ______ inches or ______ meters

Ethnic background: North American ______ South American ______

European ______ African ______

Middle Eastern ______ Asian ______

Other ______

Number of children I have already given birth to: ______

_The following information pertains to this pregnancy only:_

Weight prior to pregnancy: ______ pounds or ______ kg

I am in my ______ * week of pregnancy Due date: __________________________

Hospital where I will deliver: Royal Victoria ______

Lakeshore General ______

Jewish General ______

St. Mary's ______

Other ______

Name of Obstetrician/Gynecologist: __________________________

I am a smoker: Yes ______

If Yes, while pregnant I smoke ______ cigarettes / day

Yes, but stopped while pregnant ______

No ______

While pregnant, I consume an average of: 0-1 alcoholic drinks / week ______

2-5 alcoholic drinks / week ______

6-10 alcoholic drinks / week ______

11-15 alcoholic drinks / week ______

0-1 cups of coffee/tea / week ______

2-5 cups of coffee/tea / week ______

6-10 cups of coffee/tea / week ______

11-15 cups of coffee/tea / week ______

I am currently taking medication (prescribed by my doctor or over-the-counter): Yes ______ No ______

If you checked yes, please specify __________________________

THANK-YOU
APPENDIX E

Copyright Release by Coauthors of Manuscript
February 17, 2007

To whom it may concern:

This letter is to confirm that the co-authors (Kebba Sabally, Michael Massie, Kristine Koski) agree that MSc. candidate (Joanna Cheung) include the manuscript entitled *Placental ALP in Human Amniotic Fluid is Associated with Fetal Growth* in her thesis submission.

The author adapted established assay methods in literature for use in this study with the support of Kebba Sabally. Analysis of alkaline phosphatase in amniotic fluid samples by fluorometry and the management of data were completed by the candidate with the assistance of a summer research assistant. Michael Massie, the summer NSERC student, assisted in the analysis of alkaline phosphatase in amniotic fluid samples and management of data under the instruction of the candidate. The author participated in the recruitment of participants, medical chart review and the cataloguing and management of amniotic fluid samples. Statistical analysis and the writing of the thesis and paper were completed by the candidate. Modifications were made to the manuscript based on comments from co-authors: Kristine Koski and Kebba Sabally.

Joanna Cheung

I, the co-author, agree that the candidate, Joanna Cheung, include the manuscript entitled *Placental ALP in Human Amniotic Fluid is Associated with Fetal Growth* in her thesis.

Kebba Sabally

Michael Massie

Kristine Koski