Amniotic Fluid Amino Acids as Biological Indicators of Fetal Growth in Human and Rat Models

By

Christine N. Gurekian

School of Dietetics and Human Nutrition

McGill University

Macdonald Campus

Ste. Anne-de-Bellevue, Québec

H9X 3V9

MSc. Thesis Copy 1

A thesis submitted to the Faculty of Graduate Studies and Research in the partial fulfillment of the requirements for the degree of Master of Science

August 2005

Your file Votre référence
ISBN: 978-0-494-24687-0
Our file Notre référence
ISBN: 978-0-494-24687-0

I. ABSTRACT

Amniotic fluid (AF) is a protective pool and a resource of amino acids for the growing fetus. In study 1, we investigated if any of these AF amino acids at mid gestation were associated with fetal development in humans. Nineteen amino acids differed across birth weight percentiles. Arginine, 3-methyl histidine and tryptophan were positive predictors of birth weight, while ornithine was a negative predictor. In study 2, we used a diet induced model of IUGR to see if specific AF amino acids were predictive of fetal weight near term. Methionine and phenylalanine were modified by diet, and 12 amino acids were independently modified by gestational age, respectively. Cysteine, lysine, methionine and tyrosine were predictors of fetal weight. Thus, the AF amino acid pool is associated in animals and humans with fetal growth.

II. RÉSUMÉ

Le liquide amniotique (LA) est une mare protectrice ainsi que d'une source des acides aminés au foetus croissant. Dans l'étude 1, nous avons examiné l'association de ces acides aminés du LA à la mi-gestation et du développement foetal. Dix-neuf acides aminés ont différé à travers les pourcentile de poids de naissance. Arginine, 3-methyl histidine et tryptophane étaient des indicateurs positifs de poids de naissance, pendant que l'ornithine était un indicateur négatif. Dans l'étude 2, un régime alimentaire porté à un modèle de IUGR est utilisé afin de voir si des acides aminés specifiques du LA qui pouvaient prédire le poids foetal près du terme. Méthionine et phenylalanine étaient modifiés par la régime alimentaire et 12 acides aminés du LA ont été modifiés d'une manière indépendante par l'âge de grossesse. Cystéine, lysine, méthionine et tyrosine étaient des indicateurs de poids foetal. Ainsi, la mare d'acide aminé du LA dans les animaux et humains est associée au développement fœtal.

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V. LIST OF ABBREVIATIONS

AA, amino acid

AGA, appropriate for gestational age

ALA, alanine

ARG, arginine

ASN, asparagine

ASP, aspartic acid

BCAA, branched chain amino acids

CYS, cysteine

FMOC, 9-fluorenylmethyl oxychloroformate

GLN, glutamine

GLU, glutamic acid,

GLY, glycine

HIS, histidine

HPLC, high performance liquid chromatography

ILE, isoleucine

IUGR, intrauterine growth retardation

LEU, leucine

LGA, large for gestational age

LYS, lysine

MET, methionine

3METHIS, 3-methyl histidine

ORN, ornithine

PHE, phenylalanine

PITC, phenylisothiocyanate

PRO, proline

RP, reverse phase

SER, serine

SGA, small for gestational age

TAU, taurine

THR, threonine

TRP, tryptophan

TYR, tyrosine

VAL, valine.

VI. ACKNOWLEDGMENTS

I would like to thank the following people for their help and support during the course of my graduate studies:

My supervisor, **Dr. Kristine Koski**, for your wisdom and knowledge, and for always keeping my best interests in mind concerning both my studies and future career. Thank you for guiding me throughout the past few years, even prior to my graduate studies and giving me the confidence to accomplish projects and tasks that I never thought I could prior. It has been an honour to have worked under your supervision.

Dr. Linda Wykes, for your encouragement, and for your extended knowledge of both amino acids and the HPLC. I also thank you for giving me the opportunity to expand my knowledge with other projects under your supervision.

Dr. Gordon Zello, for your background knowledge on this project, the HPLC and for your prompt feedback concerning this project, even from afar.

Zovig Kevorkian, for getting this project started and putting so much time into the beginning stages of this project.

Kebba Sabbally, for training me on the HPLC and for his continual patience and availability for my questions.

This project wouldn't have been possible without the agreement from the **mothers** at St. Mary's Hospital, who I thank for allowing us to use the amniotic fluid from their pregnancies to further understand the fetal growth process.

Great thanks to Dr. Koski's research team: **Daniel Tisi, Erin Enros, Joanna Cheung, Nadine Zablith, Maria Rubino, Dima El Halabi, Michael Massie, Natacha Tardio, and Vanessa Tardio** for your support along the way, and making my time in the lab an enjoyable one.

I thank everyone in the School of Dietetics and Human Nutrition who work so hard to keep things running smoothly, particularly: **Francine Tardif, Lise Grant, Jocelyne Bégin and Judy Campbell-Gordon.** I also thank my friends within the School for bringing a sense of humour to daily research life, but most importantly, for your valuable friendships; **Keely Fraser, Scott Harding, Mélanie Journoud, Guylaine Charbonneau, Lindsay MacKinnon, Makiko Yoshida, and Naoyuki Ebine.**

Endless thanks to **Chris** and my **family** for filling my life with so much love and encouragement. I thank you for always selflessly supporting my goals.

VII. CONTRIBUTION OF AUTHORS

STUDY 1: ***Amniotic fluid amino acids in early pregnancy as biological indicators of fetal growth and infant birth weight***

I, **Christine Gurekian**, as first author, was responsible for writing the draft and incorporating the input of the other authors for the manuscript that appears in this thesis. I was also responsible for completing laboratory analysis, assembling the data from all time points, completing required calculations and conducting the statistical analysis.

Mrs. Zovig Kevorkian was involved in participant recruitment, and method development for measurement of amino acids in half of the amniotic fluid samples, which was performed in **Dr. Gordon Zello's** laboratory in the initial stages of this project.

Dr. Linda Wykes provided direction for problem solving concerning HPLC analysis, chromatogram interpretation and knowledge concerning amino acid metabolism, for the second half of the samples done in Dr. Kristine Koski's laboratory.

Dr. Kristine Koski provided her laboratory and the direction for the project for amniotic fluid amino acid determination, and originated the original concept to measure amniotic fluid amino acids as biomarkers of fetal growth and development.

This manuscript will be submitted for publication to the American Journal of Clinical Nutrition in September, 2005.

STUDY 2: Amniotic fluid amino acid concentrations are modified by maternal dietary glucose, gestational age, and fetal growth in rats.

I, **Christine Gurekian**, as first author, was responsible for writing the draft and incorporating the input of Dr. Koski for this manuscript which was recently published in the September 2005 issue of the Journal of Nutriton (pages 2219-2224). Initially, laboratory work was previously completed by a technician of Dr. Kristine Koski's laboratory. I completed all further analysis, including data management, where I assembled the chromatogram results, identified each amino acid, and quantified the amino acid concentrations based on previous standards run during analysis. Furthermore, I completed all statistical analysis, assembled information for the manuscript, and completed the writing of the manuscript.

Ms. Michelle Mackenzie was highly involved in carrying out the rat study, and obtaining amniotic fluid samples from this animal model.

Dr. Kristine Koski provided her laboratory and the direction for this project during the feeding trial, and during amniotic fluid amino acid determination. She had significant input into this manuscript.

CHAPTER 1:

Literature Review

Amniotic Fluid Amino Acids as Biological Indicators of Fetal Growth in Human and Rat Models

1.1 THE IMPACT OF MATERNAL DIET ON FETAL AMINO ACIDS

As a precursor for all non-essential amino acids except tyrosine, glucose (Cetin et al. 1988) is considered an important regulatory fuel during fetal development (Koski & Fergusson 1992; Koski & Hill 1990). It acts to sustain numerous energy-dependent systems, including placental amino acid uptake (Yudilevich & Sweiry 1985), and to promote and maintain fetal growth (Koski & Fergusson 1992). Several studies have investigated the impact of starvation or carbohydrate restriction on amino acid metabolism (Swendseid et al. 1967; Adibi & Drash 1970; Felig et al. 1970; Vazquez et al. 1985; Kaloyianni & Freedland 1990), but few have done so during pregnancy (Felig et al. 1972; Girard et al. 1977; Lemons & Schreiner 1983; Bell et al. 1999), and far fewer have measured their effect on AF composition (Gurekian & Koski 2005; Felig et al. 1972; Bernstein et al. 1992). Previous research in our laboratory showed that if maternal dietary glucose was limiting, there was high perinatal mortality, morbidity, and intrauterine growth retardation (IUGR), proportional to the degree of maternal dietary glucose restriction (Koski & Hill 1986; Koski & Hill 1990; Lanoue et al. 1992). Increasing amounts of maternal dietary glucose are associated not only with fetal growth in a dose-dependent manner but also with increasing tissue glycogen reserves and the emergence of important regulatory enzymes associated with perinatal glycogenolysis and gluconeogenesis (Liu & Koski 1997; Koski et al. 1993; Lanoue et al. 1999). Restriction of maternal dietary glucose can produce intra-uterine growth retardation (IUGR) (Koski & Fergusson 1992; Koski & Hill 1986, Lanoue et al. 1992; Koski et al. 1990), where both short and long-term glucose deprivation (DiGiacomo & Hay 1989) result in oxidation of gluconeogenic substrates including lactate and several amino acids (BCAAs, arginine, methionine, phenylalanine) to meet fetal needs (Arola et al. 1982; Godfrey et al. 1996; Hay 1999). Starvation markedly increased plasma BCAA concentrations (Felig et al. 1972), whereas hyperglycemia decreases all maternal and fetal plasma amino acids by >50%, especially fetal BCAAs, and essential amino acids (Thureen et al. 2000). Jozwik et al (2001) reported that fetal plasma glucose levels were not changed after an infusion of BCAAs, suggesting that the fetal utilization of BCAAs is independent of changes in maternal plasma glucose concentrations (Jozwik et al. 2001).

In AF, food deprivation in late gestation lowered the concentrations of BCAAs, alanine, and glycine (Bernstein et al. 1992) whereas energy restriction produced higher concentrations in AF taurine and BCAAs (Felig et al. 1972). In a rat model, methionine and phenylalanine were lower in the AF of dams fed a diet with =12% glucose (Gurekian & Koski 2005). During starvation, in addition to increased gluconeogenesis, the placenta decreased its high consumption of glucose (Hay 1991) without changing its size (Lanoue et al. 1992) to decrease its demand on maternal glucose and to promote diversion of some maternal glucose from the placenta to the fetus (Hay 1991). This occured without alterations in the expression and activity of placental glucose transporters in IUGR fetuses (Cetin et al. 2004). However, this inhibition of glycolysis and aerobic metabolism was shown to suppress amino acid transport across the placenta (Illsley et al. 1984; Milley 1988; Smith 1981), particularly essential amino acids in growth retarded infants (Cetin et al. 1988) via a reduced activity/expression of amino acid placental transport systems (Cetin et al. 2004).

1.2 CHANGES OF AF AMINO ACIDS WITH GESTATIONAL AGE

As gestation progresses, AF changes in volume and in nutrient profile (Queenan 1978). Most amino acids found in AF during the second and third trimesters are of fetal origin, as they enter the AF through the fetal skin, kidneys, lungs and digestive tract secretions (Queenan 1978; Mesavage et al. 1985).

Ample research has reported decreases in human AF amino acids across gestation (Dallaire et al. 1974, Mesavage et al. 1985, Rabier et al. 1996, Dawson et al. 1999), reporting a marked decrease of most amino acid concentrations in the second trimester of pregnancy (Queenan 1978). Dawson et al (1999) reported a steady decrease in amino acids from 16 to 20 wk gestation with the exception of serine, whereas Rabier et al. (1996) reported that the concentration of the majority of amino acids analyzed between 10 to >27 wk gestation progressively decreased, with the exception of aspartic acid, serine, glutamine and glycine concentrations, which remained constant. In an earlier study, Mesavage et al. (1985) reported a decrease in the concentrations of amino acids with gestational age by 24 to 61% between 13 and 20 wk gestation, with the exception of a glutamine increase. Evidently, the majority of amino acids are reported to decrease in

concentration as gestational age progresses in humans (Mesavage et al. 1985, Rabier et al. 1996, Dawson et al. 1999).

In rats, the opposite trend is seen. In rat AF, McEvoy-Bowe et al. (1987) reported a doubling in concentration of amino acids, and in agreement, Gurekian & Koski (2005) reported an increase in all amino acids across the last 4 days of gestation with the exception of taurine, which decreased. The differences observed between amino acid trends for human and rat AF can possibly be explained by the level of infant vs. pup development at term, the length of gestation, and the number of infants vs pups for each delivery.

Changes in amino acid concentrations in AF across gestational age can be explained by fetal requirements of amino acids associated with rates of tissue growth, protein deposition and fetal energy demands which change with gestational age (Bell et al. 1999). Moreover, biological events occurring in the fetus such as the keratinization of skin at wk 20 (Jauniaux et al. 1999) or the maturation of renal functions (Domenech et al. 1986) are thought to have a direct effect on the levels of free amino acids in AF. As gestation progresses, decreases in AF amino acid concentrations in humans may be explained by the fetal use of amino acids as a source of energy, and as precursors for the synthesis of DNA, RNA, creatinine, and neurotransmitters (Duggleby & Jackson 2002). Other explanations may be the relative rates of umbilical uptake of amino acids which accompany the decline seen in the fractional rates of fetal protein synthesis and oxygen consumption (Bell et al. 1999).

During the beginning of the second trimester (12-20 wk), which is the time period of interest for this study, numerous biological events occur which involve the utilization and/or production of many amino acids in both human and animal models. The following are some key events that occur during this time period, and the amino acids related to their function (National Library of Medicine 2004).

1) **Lanugo** starts to grow on the infant's head, involving the utilization of cysteine, present in alpha-keratin, the key protein in hair (Balch 1997).

2) The **skin** becomes transparent, and once again, cystine's role in alpha-keratin is important, as it is also the key protein in the skin (Shoveller et al. 2005). Additionally,

glycine and proline play a role in collagen production (Rees et al. 1999), and proline is also important for skin texture (Balch 1997).

3) The fetal **muscles** start to develop, and the fetus starts to make active movements. During this time, alanine, highly concentrated in the muscle plays a role by being released to provide energy (Felig et al. 1970). Of great importance are the BCAAs (valine, isoleucine, leucine), unique due to the fact that they are metabolized in the muscle and key amino acids for growth maintenance (Platell et al. 2000). Threonine is also present in high concentrations in skeletal muscle (Lima & Jaffe 1998, Young et al. 1972), therefore incorporation of threonine would presumably be increased.

4) Fetal **bones** begin to develop and become harder. Proline and glycine are once again key amino acids in the formation of collagen and elastin (Rees et al. 1999) and are involved in the maturation of fetal bones.

5) The fetal **gastrointestinal tract** starts to produce meconium, and numerous amino acids may play a role during this time. Glutamine is known to be a key oxidative fuel for the gut, and additionally, proline and cysteine, which are precursors to mucins, act to protect the intestine (Rees et al. 1999). In adults, arginine, ornithine, and proline are important for gut function and metabolism (Burrin & Stoll 2002), and threonine is important for intestinal growth (Burrin & Stoll 2002).

6) The **liver** and **pancreas** start to produce appropriate fluid secretions, and for these functions, many amino acids are involved with these 2 organs. Alanine, threonine, valine, histidine and proline are known to be taken up by the fetal liver, whereas ornithine is released from it (Regnault et al. 2002). The movement of these amino acids to and from the fetal liver is indicative that the liver is utilizing these amino acids for growth and for enzyme production. BCAAs, especially leucine, have been reported to play a critical role in the pancreatic development and insulin release of the fetus (Domenech et al .1986), whereas taurine is synthesized in the fetal liver and is also associated with pancreatic islets and their ability to release insulin (Rees et al. 1999).

7) The **kidney** begins to develop in the later part of this stage, and the fetal kidney has been reported to uptake glutamine, proline, and citrulline, and to release serine, cystine, arginine, tyrosine, histidine, threonine, lysine and leucine (Garibotto et al. 1999). The movement of these amino acids is important, as the nitrogen balance across the

kidney contributed by amino acids and ammonia is a good indicator of protein degradation and synthesis (Garibotto et al. 1999).

1.3 AMINO ACIDS AND THEIR ASSOCIATION WITH FETAL WEIGHT

Infant birth weight, particularly in relation to gestational age (Kramer et al. 2001) is an excellent indicator of the infant's immediate and future health (Arbuckle & Sherman 1989). A number of studies have investigated the relationship between birth weight and health outcomes during childhood and adulthood (Barker 1997, Whitaker 1998, Shiell et al. 2000, McLellan 2001), although none have investigated fetal health during mid pregnancy and its association with term birth weight. Investigations at mid pregnancy would demonstrate the ideal surroundings for the fetus to produce an infant born at a healthy weight for gestational age.

Fetal growth has been associated with certain amino acids; Regnault et al. (2002) stated that regardless of a human or sheep model, the transplacental flux of leucine, threonine and phenylalanine was significantly reduced with fetal growth restriction. Cetin et al. (1990) reported that small for gestational age fetuses had lower plasma levels of valine, leucine, isoleucine, lysine and serine, while McClain et al. 1978 reported that fetal plasma levels of alanine and tyrosine were positively associated with birth weight.

While great interest has been placed on the health risks for small for gestational age infants, the rise of obesity in North America presents us with many health concerns that accompany infants born large for gestational age. The most common cause of large for gestational age infants has been documented to be maternal hyperglycemia, occurring from gestational diabetes (Novak 2002), and leading to fetal adiposity and macrosomia (Metzger 1990). These infants born large for gestational age are themselves at a higher risk for obesity in adolescence and adulthood, and in turn, increased morbidity (Novak 2002). It is evident that appropriately sized infants, rather than either small or large for gestational age infants are associated with the best health outcomes for the infant (Novak 2002). Concentrations of mid gestation AF amino acids for appropriate for gestational age infants would indicate the ideal nutrient surroundings for the fetus in relation to sufficient protein metabolism and growth.

1.4 AMINO ACID METABOLISM AND FETAL GROWTH

Despite the fact that free amino acids only represent 5% of the entire amino acid content in the human body (Lima 1998), they play a significant role as indicators for protein synthesis and degradation (Pastor-Anglada 1986). Their involvement in protein metabolism is related to many biological events in the fetus, such as muscle formation, and the beginning of organ functions, and while higher fetal amino acid levels have been suggested to indicate protein breakdown, lower maternal amino acid levels are suggested to represent overutilization by the mother (Hay 1999).

Various studies have investigated free amino acid concentrations during pregnancy, although these were primarily done using maternal or fetal plasma (Moghissi et al. 1975, McClain et al. 1978, Lemons 1979, Cetin et al. 1992, Braida et al. 2001), and seldom in AF (Jauniaux et al. 1999; Mesavage et al. 1985). Moreover, none of the studies that have investigated AF amino acids has associated amino acid concentrations with fetal growth and specifically infant birth weight. This is predominantly explained by the fact that most research has obtained AF samples during the time of therapeutic abortion (Weissman et al. 2003, Jauniaux et al. 1999, Jauniaux 1998, A'Zary et al. 1973, Scott et al. 1972, Cockburn et al. 1970), ruling out the possibility of reporting any full term outcomes of the infant including birth weight.

Regardless of sample size, time of collection, method of analysis, and even human versus animal models, those amino acids present in the lowest and highest concentrations in AF remain constant. The highest concentrations of amino acids in AF are repeatedly alanine, glutamine, glutamic acid, lysine, and valine (Jauniaux et al. 1999, Rabier et al. 1996, Mesavage et al. 1985), and the lowest concentrations are frequently reported to be aspartic acid, citrulline, cystine, serine, and tryptophan (Jauniaux et al. 1999, Rabier et al. 1996, Mesavage et al. 1985).

Amino acids can be grouped based on the human body's ability to produce them, where those amino acids which can be synthesized by the human body are labeled as non-essential (alanine, aspartic acid, asparagine, glutamic acid, serine), and those which cannot be synthesized are labeled as essential (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine). The amino acids that are conditionally essential (arginine, cysteine, glutamine, glycine, proline, tyrosine) can be

synthesized from other amino acids and are often limited under special pathophysiological conditions (Dietary Reference Intakes 2002).

Another method of grouping is based on the unique structure of the amino acid side chain. Seven groups exist; (1) aliphatic (glycine, alanine, valine, leucine, isoleucine), (2) aromatic (phenylalanine, tyrosine, tryptophan), (3) hydroxyl (serine, threonine), (4) sulfur (cysteine, methionine), (5) acidic (glutamic acid, glutamine, aspartic acid, asparagine), (6) basic (arginine, lysine, histidine), and (7) imino (proline). Four other amino acids of interest to us are citrulline, 3-methyl histidine, ornithine, and taurine. Although still precursors for many biological functions, these 4 amino acids do not fit into any of the above categories as they are not components of any major proteins or enzymes. Many amino acids have been studied during pregnancy, a great amount of literature focuses on amino acid concentrations in the maternal and fetal plasma and the relationship between the two. Amino acids have also been detected and quantified in AF, although there is very scarce data linking these AF amino acids with fetal development. The following details each amino acid, and brings together literature on amino acids involved in growth and fetal development, and when possible, in AF.

1.4.1 ALIPHATIC AMINO ACIDS

Alanine functions as a major energy source, and is the key amino acid released by the muscle (Felig et al. 1970). Fluctuations between the maternal and fetal compartments during starvation suggest compensation reactions by the mother in order to provide sufficient energy to the fetus during growth. In healthy pregnancies, the fetus takes up alanine in an excessive amount, exceeding its contribution to body protein (McEvoy-Bowe et al. 1987), which explains the consistently high levels of free AF alanine (Jauniaux et al. 1999, Rabier et al. 1996, Mesavage et al. 1985). Fetal serum levels of alanine are reportedly higher in small for gestational age fetuses than in appropriate for gestational age fetuses (Economides et al. 1989). In a starved pregnant state, alanine plays a significant role in gluconeogenic conversions (Felig et al. 1970, Balch 1997), and the mother reportedly increases endogenous plasma alanine, as the alanine levels in the AF decrease (Felig et al. 1972). More recent studies have reported that starvation during pregnancy causes for an increase in fetal plasma alanine levels (MacMahon et al. 1990).

The lack of a side chain for **glycine** makes it an important amino acid as a structural unit for proteins, such as collagen, which contains 30% glycine and makes up 20-25% of the body's protein (Neuberger 1981). The fetus requires large amounts of glycine to meet both its structural and metabolic demands (Lewis et al. 2005), but the many products of glycine metabolism, such as nucleic acids, heme and creatine represent the end point for glycine, and do not recycle glycine back into the amino acid pool (Lewis et al. 2005). A large fraction of glycine is also taken up by the fetal liver and used for oxidation and for fetal hepatic serine production (Cetin et al. 1991, Battaglia 1992) and little glycine is taken up by the placenta from the fetal circulation (Battaglia 1992). An overall low fetal plasma glycine concentration from inadequate endogenous production provides evidence that the demand for glycine exceeds the available supply for the fetus (Jackson 1991). It is for this reason that glycine is considered conditionally indispensable essential during the fetal life (Battaglia 1992, Lewis et al. 2005). More recently, glycine reportedly presents a benefit after birth, where a low protein maternal diet supplemented with glycine prevented the development of elevated blood pressure and vascular dysfunction in the offspring (Brawley et al. 2004).

Branched-chain amino acids (BCAA); valine, leucine, isoleucine are unique because they are primarily metabolized extrahepatically in the skeletal muscle (Platell et al. 2000). They are involved in stress, energy and muscle metabolism (Platell et al. 2000), and are highly associated with protein synthesis, growth and tissue repair (Harris et al. 1994). BCAA's are also known to promote insulin release from the developing pancreas (Domenech et al. 1986) and even aid in the improvement of respiratory function in premature infants (Imura & Okada 1998). BCAAs play a critical role in the synthesis of other amino acids. For example, the carbon in alanine is derived in large part by valine, and exported to the liver to form glucose (Platell et al. 2000). During fasting, endogenous BCAAs are released by protein breakdown and are the principal precursors for glutamine de novo synthesis, which is then metabolized in the gut as an energy substrate (Platell et al. 2000). Furthermore, BCAAs are known to modify central nervous system uptake of tryptophan, phenylalanine and tyrosine for neurotransmitter synthesis (Bender 1983). During pregnancy, research has demonstrated that a shortage of BCAAs restrict protein synthesis and in turn, impair growth and tissue repair, and that BCAA

plasma concentrations are reduced in the circulation of IUGR fetuses (Battaglia 1992, Cetin et al. 1992). The sum of the BCAA concentrations in the umbilical vein is reported to be directly related to maternal arterial values in both appropriate and small for gestational age fetuses, although maternal arterial concentrations were slightly lower in small for gestational age fetuses (Heird 1998). The ability of BCAAs to stimulate protein synthesis, inhibit degradation of isolated muscle and increase the reutilization of amino acids in many organs (Heird 1998), suggests that during muscle and organ development in the fetal period (13-20 wk) (National Library of Medicine 2004), levels of free AF BCAA's may fluctuate. This presents the possibilities that larger infants 1) receive an excess of BCAA's from maternal sources (placenta, umbilical cord) and the unused free BCAA's remain in the AF, or 2) a higher level of BCAA precursors are present in the AF which in turn produces large free BCAA pools.

1.4.2 AROMATIC AMINO ACIDS

Phenylalanine is an indispensable essential amino acid that has been reported to be highly retained by fetal and maternal tissues (Velazquez et al. 1976). After both a 1 and 12 hour maternal amino acid infusion by MacMahon et al. (1990) and Jozwik et al. (1999), respectively, increases in fetal plasma phenylalanine concentrations were reported, demonstrating that the fetal phenylalanine supply primarily comes from maternal sources. Phenylalanine is highly hydroxylated by a non-reversible reaction to **tyrosine**, making this next aromatic amino acid conditionally indispensable essential (Balch 1997). Tyrosine and **tryptophan** are both precursors to neurotransmitters (Fernstrom 2000), and while tyrosine plays a role in the mobilization of stored carbohydrate and fat to provide immediate available energy, tryptophan plays a role in the secretion of growth hormone and prolactin (Balch 1997). There is a lack of literature relating any of these aromatic amino acids with fetal growth or metabolism, although their association with brain neurotransmitters should be noted (Fernstrom 2000).

1.4.3 HYDROXYL AMINO ACIDS

With the use of tracer methodology, it was reported that **serine** was not transported from the maternal to the fetal circulation (Battaglia et al. 1994). A net loss of

serine is seen from the fetal circulation into the placenta, a loss which is greater at midgestation compared to term (Battaglia et al. 1994). This indicates low levels of serine in fetal circulation and AF, regardless of sufficient amounts in the maternal diet. **Threonine** is significantly retained by fetal tissues, and is abundant in human plasma, particularly in newborns (Velazquez et al. 1976). It plays a role in the formation of collagen and elastin and works with aspartic acid and methionine to aid in liver and lipotropic functions by preventing fatty acid buildup (Balch 1997). Threonine is present in the heart, central nervous system, and skeletal muscle of the human adult (Balch 1997), although levels in fetal organs have not yet been reported. In piglets, threonine has been reported to be especially important for intestinal growth, where formula fed piglets used up to 60% of their dietary threonine in the gut (Burrin & Stoll 2002), and the high intestinal requirement for threonine is suggested to be for the synthesis of threonine rich mucins by the goblet cells (Burrin & Stoll 2002). Unlike serine, fetal threonine levels were reported to be affected by the maternal diet, where a 90 hour fast resulted in a decrease in AF threonine concentrations (Felig et al. 1972).

1.4.4 SULFUR AMINO ACIDS

Both sulfur amino acids are highly interrelated, as **methionine** plays a vital role in the production of **cysteine** (Balch 1997) as well as in DNA and tRNA methylation (Lemons 1979; Steegers-Theunissen et al. 1997). The liver is generally considered to be the primary site of dietary methionine and cysteine metabolism in the body (Shoveller et al. 2005), although many tissues in the body are capable of converting methionine to cysteine via the enzymatic processes of transmethylation and transsulfuration (Finkelstein & Martin 2000). Some studies have shown that providing cysteine in the diet can decrease the need for methionine by 50-80% in mammals (Shoveller et al. 2005), although due to the lack of cystathionase in the fetal human liver and brain, both cysteine and methionine are essential to the fetus and conserved for protein synthesis during rapid growth (Lemons 1979). This conservation is demonstrated in human pregnancies at 8-12 weeks gestation; at that time point, methionine was reported to be 4 times higher in the extra-embryonic coelomic fluid and 2 times higher in the AF versus maternal serum (Steegers-Theunissen et al. 1997). Varying levels of methionine between fetal

compartments indicates active transport from maternal serum to the coelomic cavity where it is stored prior to active transport or diffusion to AF for utilization by the growing fetus, which has reported to significantly retain methionine (Velazquez et al. 1976). In rats, a shortage of methionine during pregnancy has caused disturbed morphogenesis, especially in the development of neural tube defects (Steegers-Theunissen et al. 1997).

Cysteine, is important for energy metabolism, and required for protein synthesis and hence, growth and nitrogen balance (Stipanuk 1999). Preterm infants (=32 wks gestation) were reported to have low plasma cysteine concentrations (Stipanuk 1999), and intravenous feeding with low amounts of cysteine supplementation resulted in low levels of circulating cysteine in human infants (Zlotkin et al. 1981). This indicates that infant immaturity may be associated with a cysteine requirement. Moreover, cysteine is a precursor for glutathione in the liver, which is essential for normal intestinal function (Martensson et al. 1990), and plays a crucial role in the detoxification of the body's endogenous peroxides and exogenous chemical compounds (Heird 1998). In relation to skin development, the suggestion has even been made that cysteine availability and local glutathione concentration have a direct influence on epithelial cell proliferation and survival, primarily due to the high sulfur content in skin, hair and nails (Shoveller et al. 2005).

1.4.5 ACIDIC AMINO ACIDS

Glutamic acid, glutamine, aspartic acid and **asparagine** are seldom reported in the literature to be affected by gestational age (Mesavage et al. 1985, Rabier et al. 1996), and there is little proof of their importance for fetal growth other then reports related to glutamine and glutamic acid. Glutamine is important for the fetus, as it is delivered into the fetal circulation at a rate that is the highest of all amino acids (Battaglia 2000), whereas 90% of glutamate is removed from the fetal circulation by the placenta (Battaglia 2000). Glutamine is associated with being an important energy substrate for rapidly proliferating cells, such as enterocytes and lymphocytes, and also acts as a precursor of nucleic acids, nucleotides, amino sugars, amino acids and glutathione (Burrin & Stoll 2002). It has been discovered to be a key oxidative fuel for the gut in rats

(Windmueller & Spaeth 1974), and in cultured enterocytes, glutamine was shown to stimulate cell proliferation, protein synthesis, and the synthesis of early genes and polyamines (Higashiguchi et al. 1993, Kandil et al. 1995). It plays a critical role as an important fuel source for stressed individuals or those with compromised gastrointestinal function (Heird 1998). In the liver, glutamine is used for glutamate production, and the rate of fetal hepatic glutamate production is sufficient to account for all of the fetal glutamate requirements (Moores et al. 1994). After production in the fetal liver, glutamate is exported back to the placenta (Novak 2002), where it is almost completely oxidized (Moores et al. 1994), and has even been suggested to be the primary carrier of excess nitrogen, as the immature fetus may not possess an efficient urea cycle at this time (Lemons 1979).

1.4.6 BASIC AMINO ACIDS

Arginine, is conditionally indispensable essential (Imura & Okada 1998) and multifunctional in the human body. It has been involved with 1) cell proliferation (Witte & Barbul 2003), 2) tissue repair and healing (Witte & Barbul 2003), 3) nitric oxide synthesis relating to blood vessels and improving fetal-maternal blood flow (Appleton 2002; Xia & Li 2005), 4) the maintenance of mucosal blood flow and immune function (Burrin & Stoll 2002, Cynober 1994), 5) gut function (Burrin & Stoll 2002), 6) the release of the pituitary growth hormone, prolactin (Appleton 2002, Heys & Gardner 1999, Cynober 1994) and glucagon (Visek 1986), 7) the synthesis of polyamines important for growth and differentiation of intestinal mucosal cells (Cynober 1994), and 8) renal synthesis of creatine (Heys & Gardner 1999), an important constituent of skeletal muscle (Wu & Morris 1998). Xia & Li (2005) reported that an arginine infusion given to women with asymmetric fetal growth restriction significantly increased infant birth weight. Although complex, the multifunctional role of arginine biologically can easily indicate that higher concentrations of this amino acid would promote a healthier fetal growth and possibly, an increased birth weight.

Histidine, which is present in large quantities in skeletal muscle, is reported to be a key component of carnosine (Kriengsinyos et al. 2002). During the consumption of a histidine deficient diet, the amount of carnosine in muscle tissue is sufficient to provide

enough histidine to maintain nitrogen balance in older children and adults for several weeks, but not in the case of infants due to the limited amount of the enzyme carnosinase (Lenney et al. 1982). A histidine free diet also indicates an overall deceleration of protein turnover, and a decrease in hemoglobin synthesis (Kriengsinyos et al. 2002). Thus, the need for histidine to be supplied to the fetus in sufficient amounts from maternal sources is necessary due to the lack of carnosinase and the increased need of this amino acid to develop proper muscle tissue.

Similar to histidine, **lysine** cannot be synthesized by the human body. Lysine's role in fetal growth is not well documented as of yet, although consistently high concentrations of this amino acid in AF may be explained by the fact that levels of lysine are taken up by the fetus in amounts that exceed its contribution to body protein (McEvoy-Bowe et al. 1987).

1.4.7 IMINO AMINO ACID

Proline plays an important role in skin texture, collagen production, connective tissue and the strengthening of joints, tendons and the heart muscle (Balch 1997). Proline is additionally important for gut function and metabolism and serves as a key precursor for both arginine and ornithine synthesis (Burrin & Stoll 2002). Despite the fact that this amino acid has not been directly associated with fetal growth, it is expected that proline plays a significant role in fetal development during gut development, skin keratinization, and the beginning of muscle movements.

1.4.8 OTHER AMINO ACIDS

Ornithine plays a large role in the urea cycle, due to the fact that it is the amino acid at the start and end of this nitrogen excreting cycle (Cynober 1994). Although ornithine is not a constituent of protein, it is synthesized by glutamic acid, acts as a precursor to citrulline and arginine (Blachier et al. 1995; Wu & Morris 1998; van de Poll et al 2004), and has been reported to be important for gut function and metabolism (Cynober 1994; Gardiner et al. 1995, Burrin & Stoll 2002). Its role in the urea cycle may suggest that larger infants with more functional livers take in larger amounts of ornithine from the AF. At mid-gestation, an excess of amino acids are available for oxidation,

producing a high production rate of urea (Battaglia 1992), and indicating an increased need for amino acids involved in the urea cycle.

Taurine is an interesting amino acid, as it is a predominant intra cellular free amino acid (Chesney et al. 1998, Aerts & Van Assche 2002), it's synthesized from cysteine in the liver and is the second highest amino acid present in the liver (Lima 1998). It is reported to play a role in children's retinal patterns and in rat glial cell function (Lima 1998). A potent antioxidant (Lima 1998), taurine also serves as an important organic osmolyte in the brain and kidney, contributing to cell volume regulation, where its pool size is uniquely regulated by the kidney (Aerts & Van Assche 2002). During pregnancy, a maternal taurine deficiency has been associated with growth restriction, and impaired perinatal development of the central nervous system and pancreas (Aerts & Van Assche 2002). Velazquez et al. (1976) reported high levels of taurine in the AF of infants born AGA at full term. This amino acid is also of great interest due to its unique trends in comparison with other amino acids. AF taurine levels have been reported to have contradicting trends to other amino acids found in AF in relation to gestational age, where the majority of amino acids increase with gestational age and taurine decreases, both in rats (Gurekian & Koski 2005), and humans (O'Neill et al. 1971). In preterm infants with a gestational age of <33 wks, taurine supplementation showed a decrease in cholesterol synthesis and an increase in bile acid excretion and fatty acid absorption, although this was not seen in older or term infants (Chesney et al. 1998). These are not the only effects seen in preterm infants concerning taurine, as renal immaturity has been reported to prevent proper resorption of taurine by the kidneys, and therefore the large loss of taurine from the kidneys poses detrimental effects on the brain and retina (Lima 1998). In children, malnourishment reported high plasma taurine concentrations, which may be related to the internal movement of taurine from tissues (liver, muscle) to other compartments such as the CNS (Lima 1998).

Three-methylhistidine is produced from histidine by posttranslational methylation and is found in the skeletal muscle, gastrointestinal tract and skin (Young et al. 1972). This amino acid , but cannot be used for muscle protein synthesis (Chinkes 2005), therefore any increase in concentration of three-methylhistidine could indicate

muscle degradation,changes in concentration seen in AF of this amino acid or may be related to gastrointestinal or skin development in the fetus (Chinkes 2005).

As represented above, all amino acids have unique functions, many of these fundamental to fetal growth. Although all amino acids have been measured in AF (Jauniaux et al. 1999; Mesavage et al. 1985), only one study (Mesavage et al. 1985) has looked at mid gestation concentrations and related them to fetal outcome of gestational age. Evidently from the literature cited below, it will be clear that no studies have yet investigated mid gestation concentrations with final infant birth weight.

The complexity of amino acid metabolism makes it very challenging to pinpoint specific amino acids, or even amino acids as a whole to have sole responsibility for precise aspects of fetal growth. Not only are amino acids being transferred into AF from several external sources, but differences in amino acid concentrations are seen between the maternal plasma, fetal plasma, coelomic fluid, and the AF (Jauniaux et al. 1999; Felig et al. 1970). Consequently, amino acid concentrations in these compartments can give us indications of fetal amino acid use, although more research needs to be done to understand the true relationship between them all.

1.5 AMNIOTIC FLUID AND FETAL HEALTH

Amniotic fluid (AF) is an essential contributor to fetal health, acting as not only a protective pool, but also as an abundant and readily accessible resource of nutrients (Queenan 1978). Contributions to AF originate from the mother across the placenta and umbilical cord, and from the fetus itself through the skin, gastrointestinal tract, tracheobronchial tree or kidneys (McCarthy & Saunders 1978). A constant circulation of AF is seen as the fetus swallows up to 750 ml each day (Pritchard 1966), and water turnover increases from >350 ml/hr at 26 wks to >500 ml/hr at term (O'Neill et al. 1971). The numerous biological events during pregnancy, such as the keratinization of fetal skin (Jauniaux et al. 1999), the start of fetal tracheal secretions (O'Neill et al. 1971), fetal plasma filtrate through skin or umbilical cord (O'Neill et al. 1971) and the development of fetal organs (Regnault et al. 2002) affect the flow and utilization of nutrients for the fetus, and alters the composition and volume of the AF. Prior to the keratinization of the fetal skin at approximately 20 wk gestation (Jauniaux et al. 1999), AF is of fetal origin

and is a dialyzate of fetal plasma through the semipermeable fetal skin, and concentrations of AF constituents are therefore closer to fetal plasma concentrations than to maternal plasma concentrations (LeMoyec et al. 1994). During this time, it is expected that AF is a very important indicator of fetal health, as many nutrients; including amino acids are easily diffused between the fetal circulation and the AF (Jauniaux et al. 1999), and the fetus is dependent on AF for 10-14% of its normal caloric intake (Mulvihill et al. 1985). As gestation progresses, the fetus uses amino acids as precursors for the synthesis of protein, DNA, RNA, creatinine, and neurotransmitters, as well as for a source of energy (Duggleby & Jackson 2002), which are all important to consider when investigating AF amino acid concentrations. Due to the present use of ultrasound and the increasing number of older mothers being tested for genetic abnormalities, amniocentesis has become a much more common practice, and has allowed researchers to investigate AF and its nutrients at various time points during gestation to better understand fetal development. This exciting step forward in the examination of pregnancy gives us the opportunity to further investigate AF amino acids and their importance in fetal development.

Several studies have looked at amino acids in human AF of healthy pregnancies at mid gestation (Dawson et al. 1999; Rabier et al. 1996; Mesavage et al. 1985; Pettit & Allen 1980; Schulman et al. 1972; Reid et al. 1972), and some at the time of therapeutic abortion (Jauniaux et al. 1999; A'Zary et al. 1973; Scott et al. 1972; O'Neill et al. 1971; Cockburn et al. 1970). While only one study has associated mid gestation amino acid concentrations with gestational age (Mesavage et al. 1985), no study has yet associated them with infant birth weight.

Please note that all references are combined beginning on page 62.

OVERVIEW AND STATEMENT OF PURPOSE

For the developing fetus, AF is multifunctional as a protective pool and a readily accessible resource of nutrients (Sandler 1981). Both its volume and composition play an important role in fetal growth and development. AF originates from the mother via the placenta and umbilical cord, and from the fetus itself through the skin, gastrointestinal tract, tracheobronchial tree and kidneys (McCarthy & Saunders 1978). The importance of AF in fetal growth was made evident by Mulvihill et al. (1985) who reported that esophageal ligation in rabbits decreased birth weight, rump length, and gastric and intestinal tissue weight. This indicates that fetal AF swallowing is essential in gastrointestinal development and for optimal birth weight. Before 20 wk gestation, the unkeratinized fetal skin in the human allows for a free flow of nutrients and amino acids to transfer between the fetus and the AF (Jauniaux 1998).

In AF, free amino acids have been suggested to be indicators of fetal protein metabolism (Jones & Rolph 1985), and while several studies have identified a relationship between AF amino acids and fetal metabolic maturity near birth (Jauniaux et al. 1999; Jones & Rolph 1985), no study has yet taken a step back to investigate these concentrations at mid gestation as indicators of later fetal growth and development. A greater understanding of mid gestation AF amino acids and final birth outcomes would indicate an optimal environment for the fetus to grow in.

The objectives of our first study were 1) to determine the overall free amino acid profile of AF at mid-gestation from healthy pregnancies, 2) to compare concentrations of AF amino acids across percentile categories of infants born small for gestational age (SGA), appropriate for gestational age (AGA), and large for gestational age (LGA), and 3) to investigate if any AF amino acids at mid-gestation are predictive of birth weight and/or gestational age. This study looks at the concentrations of AF amino acids, but does not use a dietary intervention method since this is unethical in a model of human pregnancy.

Some studies have investigated the impact of diet by starvation or carbohydrate restriction during pregnancy on amino acid metabolism (Bell et al. 1999; Lemons & Schreiner 1983; Girard et al. 1977; Felig et al. 1972), but few have measured their effect on AF composition (Felig et al. 1972; Bernstein et al. 1992). Of those studies that did,

AF BCAAs, alanine and glycine were reported to decrease with food deprivation in late gestation (Bernstein et al. 1992), whereas during prolonged energy restriction in pregnant women, AF concentrations of alanine decreased while AF taurine and BCAAs increased (Felig et al. 1972).

More recently in our laboratory, a diet limiting in maternal dietary glucose caused high perinatal mortality, morbidity, and intrauterine growth retardation (IUGR) in rats, proportional to the degree of maternal dietary glucose restriction (Koski & Hill 1986, Koski & Hill 1990; Lanoue et al. 1992). Glucose is a precursor to all non-essential dispensable amino acids except tyrosine (Cetin et al. 1988), and also plays a role in fetal development, as it is considered to be the principal metabolic fuel for the fetus in utero (Koski & Fergusson 1992; Koski & Hill 1990), acting to sustain numerous energy-dependent systems (Yudilevich & Sweiry 1985), and to promote and maintain fetal growth (Koski & Fergusson 1992). The inhibition of glycolysis and aerobic metabolism was shown to suppress amino acid transport across the placenta (Illsley et al. 1984; Milley 1988; Smith 1981), particularly essential amino acids in growth retarded infants (Cetin et al. 1988) via a reduced activity/expression of amino acid placental transport systems (Cetin et al. 2004). Thus, the possibility existed that the diet-induced model from our laboratory, employing restriction of maternal dietary glucose to produce intrauterine growth retardation could be used to determine those AF amino acids specifically related to optimal fetal growth. Thus, our objectives were to use a pregnant rat model to: 1) determine whether a restriction of maternal dietary glucose modulates the free AF amino acid pool, and 2) establish whether any diet-induced changes were predictive of fetal weight near term (d 21.5).

The overall aim of this research was to explore the possibility that the amino acid profile of AF may be related to fetal growth, and to explore which pregnancy factors may have an impact on the AF amino acid pool.

CHAPTER 2:

Amniotic Fluid Amino Acid Profile in

Early Pregnancy as a Biological Indicator of Fetal Growth

Christine N. Gurekian[1], Linda J. Wykes[1], Gordon A. Zello[2]
and Kristine G. Koski[1]

[1]School of Dietetics and Human Nutrition, McGill University (Macdonald Campus),
Ste. Anne-de-Bellevue, QC, Canada, H9X 3V9

[2]College of Pharmacy and Nutrition, University of Saskatchewan,
Saskatoon, Saskatchewan, Canada, S7N 5C9

2.1 ABSTRACT

Amniotic fluid (AF) is important for fetal health, acting to protect and to supply nutrients to the developing fetus. However, few research studies have investigated the free amino acid profile of AF and none has associated AF amino acids at mid-gestation with final pregnancy outcomes. Our objectives were 1) to characterize the free amino acid profile of AF at mid-gestation, 2) to compare these concentrations across percentile categories of infants born small- for-gestational-age (SGA), appropriate-for-gestational-age (AGA), and large-for-gestational-age (LGA), and 3) to investigate if any AF amino acids at mid-gestation are predictive of birth weight and gestational age. AF samples (n=361) were collected between 12 and 20 weeks gestation from mothers undergoing amniocentesis for genetic testing. Samples were precolumn derivatized with phenylisothiocyanate (PITC) and analyzed by reverse phase HPLC; 23 amino acids were detected by UV absorbance. Analysis of variance showed that total and 12 individual amino acids differed by amniocentesis sampling week, with 8 having a nadir at ≥ 16 wks. By percentile categories for birth weight, total and 13 individual amino acids had their highest concentrations for infants born AGA, while 5 amino acids peaked in SGA or LGA infants. Arginine, 3-methyl histidine and tryptophan were positive predictors of birth weight, while ornithine was a negative predictor. The characterization of the AF amino acid profile at mid gestation has shown that AF amino acids are related to fetal growth and some predict infant birth weight.

2.2 INTRODUCTION

Amniotic fluid (AF) is an essential component for fetal health because it acts as a protective environment as well as an abundant and readily accessible source of nutrients (Sandler 1981). Contributions to AF originate from the mother via the placenta and umbilical cord, and from the fetus itself through the skin, gastrointestinal tract, tracheobronchial tree or kidneys (McCarthy & Saunders 1978). Early in gestation, AF is of fetal origin (LeMoyec et al. 1994), and the unkeratinized fetal skin at this time allows for a free flow of nutrients and amino acids to transfer between the fetus and the AF (Jauniaux 1998). Even though free amino acids only represent a small proportion of the body's total amino acid pool, this early dynamic exchange makes the amino acid profile of AF a potentially important biological indicator of fetal health. Free AF amino acid concentrations early in gestation may be indicators of fetal protein synthesis and breakdown at this critical time when muscle development and increased organ function is occurring (Jones & Rolph 1985).

Over 30 free amino acids have been detected in AF (Jauniaux 1998; Mesavage et al. 1985; A'Zary et al. 1973; Scott 1972), and several studies have identified a trend of decreasing AF amino acid concentrations as gestation progresses (Dawson et al. 1999; Rabier et al. 1996, Mesavage et al. 1985; Schulman et al. 1972). Some studies have examined the relationship of amino acids with fetal metabolic maturity (Jauniaux et al. 1999; Jones & Rolph 1985), although no study has yet investigated AF amino acids in early gestation as indicators of subsequent fetal growth and development.

The objectives of this study were therefore 1) to determine the overall free amino acid profile of AF at mid-gestation from healthy pregnancies, 2) to compare concentrations of AF amino acids across percentile categories of infants born small for gestational age (SGA), appropriate for gestational age (AGA), and large for gestational age (LGA), and 3) to investigate if any AF amino acids at mid-gestation are predictive of birth weight and/or gestational age at birth.

2.3 MATERIALS AND METHODS

2.3.1 Recruitment

AF samples (n=361) were collected from mothers undergoing routine amniocentesis for genetic testing at St. Mary's Hospital (Montréal, Québec) during the years 2000 1998 through 2003. Candidates were excluded for non-singleton pregnancies, and any genetic abnormalities. Fifteen ml of AF were collected from each mother between 12 and 20 wk gestation, and immediately stored at -80 °C until analyzed. Ethics approval for the study was obtained from Institutional Review Boards of McGill University, the Montreal Children's Hospital, and St. Mary's Hospital Center in Montreal, Canada. At the time of consent, women filled out a brief questionnaire reporting age (yr), height (m), pre pregnancy weight (kg), parity, ethnicity, smoking habits, and wk of amniocentesis. Within 6 months after birth, maternal medical charts were reviewed and data pertaining to the delivery, gestational age and infant birth weight were recorded.

Gestational age was calculated on the basis of the physicians' estimates using last menstrual period. Infants were categorized by birth weight percentiles (Kramer et al. 2001) and compared across birth weight categories while controlling for infant birth weight and gender, as well as the gestation length of the pregnancy (Kramer 1987). SGA infants represented those infants that had birth weights less than the 10^{th} percentile, AGA infants represent those between the 10^{th} and 90^{th} percentiles, and LGA infants represent those greater than the 90^{th} percentile (Kramer et al. 2001). Samples were selected from our large pool of AF samples to represent the Canadian population where infants were born SGA (8%), AGA (80%) and LGA (12%) (Canadian Perinatal Health Report 2003).

2.3.2 Laboratory Analysis

Measurements of amino acids in AF were performed by RP-HPLC using the Pico Tag method (Waters HPLC System, Waters Chromatography Division, Millipore Corp.). The HPLC system consisted of a Varian 9012 pump, a Varian 9050 UV detector, and a Varian Prostar 410 Autosampler. A Varian C18 Hypersil 25 x 4.6 cm column was used and kept at a constant temperature of 46 °C (Buzzigolli et al. 1990) during analysis. One hundred µL of AF was added to the internal standard, norleucine (200 mmol/L) in a 1:1

ratio (Davey & Ersser 1990), and deproteinized by ultracentrifugation with 10,000 M Millipore cut off membranes (1500 g for 30 min). Twenty-five μL of sample was transferred into glass tubes and vacuum dried, followed by the addition of 10 μL of drying solution (methanol: 1M sodium acetate: triethylamine; 2:2:1 ratio) to each sample and once again vacuum dried. Twenty μL of freshly prepared derivatizing agent (triethylamine: doubly distilled water: PITC: methanol; 1:1:1:7 ratio) was then added to each sample and left to sit at ambient temperature for 20 minutes. Samples were then redried under vacuum and reconstituted with 100 μL of Waters Pico Tag diluent. Three mobile phases were used during analysis, 1) HPLC grade acetonitrile, 2) doubly distilled water (vacuum filtered), and 3) eluent consisting of 140 mmol/L sodium acetate trihydrate, 3.6 mmol/L triethylamine, and 0.2 mL/L of stock EDTA solution, titrated to a pH of 6.4. Twenty μL of reconstituted sample was eluted at a flow rate of 1-1.5 ml/minute (Buzzigolli et al. 1990, Campanella et al. 1998) and detected by UV absorbance at 245 nm.

RP-HPLC was chosen because of its higher sensitivity and accuracy as compared to previous studies which employed other/older analytical techniques such as ion-exchange chromatography (Pettit & Allen 1980, Mesavage et al. 1985, Rabier et al. 1996), cation-exchange chromatography (A'Zary et al. 1973), gas chromatography (Schulman et al. 1972, Kwokei et al. 1982), proton magnetic resonance spectroscopy (Le Moyec et al. 1994, Bock 1994, Sims et al. 1993), mass spectrometry (Braida et al. 2001), and amino acid analyzers (Dawson et al. 1999, Jauniaux et al. 1999, Jauniaux 1998, Rabier et al. 1996). More particularly, the use of pre column derivatization of amino acids with PITC was used due to the fact that this method has the ability to detect primary and secondary amino acids in a concentration < 1 pmol (Cohen & Strydom 1998), with good sensitivity and specificity (Cohen & Strydom 1986, Buzzigolli et al. 1990) in biological fluids (Buzzigoli et al. 1990, Campanella et al. 1999).

2.4 STATISTICAL ANALYSIS

All statistical analyses were done using SAS version 8.0 (Cary, North Carolina). Amino acid values were tested for normality and normalized by log10 where required (arginine, citrulline, lysine, methionine, serine, taurine, tryptophan), and amino acids

were also grouped into essential, non essential and conditionally essential groups. Amino acid concentrations were calculated with the use of the known concentration of internal standard, norleucine. In order to identify whether amino acid levels were affected at the time of AF sampling, concentrations were tested by ANOVA across amniocentesis sampling wk. Additionally, in order to determine if any differences in amino acid concentrations existed across birth weight percentiles (Kramer et al. 2001), an ANCOVA was performed across categories of infants born SGA, AGA and LGA, controlling for wk of amniocentesis sampling, maternal smoking, and maternal pre-pregnancy BMI (Kramer 1987). We included amniocentesis wk as an additional covariate due to changes seen in AF amino acids across gestation in our study; maternal smoking and pre-pregnancy BMI were used as covariates because these two factors have been reported to be significant determinants of birth weight (Kramer 1987). Due to the fact that analysis was done in two different laboratories, a statistical control was also placed on this model. By using percentiles as a categorical value, this controlled for the gestation length of the pregnancy as well as the infant's birth weight and gender, so these were not entered as covariates. Multiple regressions with gestational age, maternal BMI, maternal smoking, and infant gender as independent variables were examined to identify if any amino acids were predictors of birth weight. Amino acids were individually added into the regression model due to their co linearity (Mesavage et al. 1985). Significance was reported at $P < 0.05$.

2.5 RESULTS

Mothers in our study population (n=361) had a mean pre-pregnancy weight of 63 ± 0.7 kg and a height of 1.6 ± 0.5 m. More than half of the population (52%) had a pre-pregnancy BMI of 20 to 25 kg/m^2, and the great majority (88%) were non-smokers. Most (66%) mothers were of Caucasian descent, and had given birth to a mean of 1 ± 0.4 infants prior to this pregnancy (Table 2.1).

Twenty-three AF amino acids were measured in 361 AF samples (Table 2.2). Cystine could not be measured in these samples; PITC derivatization has been chronicled as unreliable in the quantification of cystine in biological samples (Furst et al. 1990). The 5 amino acids with the highest concentrations were alanine, glutamic acid +

glutamine, lysine, proline and valine together totaling over half (54%) of the total amino acid concentration. Those amino acids with the lowest concentrations were tryptophan, citrulline, ornithine, serine and 3-methyl histidine, totaling no more than 4% of the total amino acid concentration. Taken together these data demonstrate the great range in concentration in amino acids found in human AF. Despite the differences in concentrations, correlation coefficients between all these amino acids were as high as 0.87.

Between 12 and 20 wk gestation, the concentrations of total, essential, non-essential and 8 individual amino acids consistently declined across wk of amniocentesis sampling (Table 3). All amino acids reported a nadir at = 16 weeks, with the exception of glutamic acid + glutamine (lowest at 14-15 wks). While arginine, citrulline and tryptophan reported significant differences across wk of amniocentesis, they did not show significance when testing with Student Neuman Keuls post hocs.

Eighteen individual amino acids differed in concentration in the AF of infants subsequently born SGA, AGA or LGA. We observed that 13 AF amino acids were highest for those infants born AGA (alanine, aspartic acid+asparagine, glutamic acid+glutamine, glycine, histidine, isoleucine, lysine, ornithine, phenylalanine, proline, threonine, tyrosine, valine), although some were highest for LGA (arginine, citrulline, 3-methyl histidine, tryptophan) and SGA (methionine). Differences for the total, essential, non-essential, and conditionally essential groups were also observed, all reporting peak concentrations for infants born AGA. Controlling factors in the model were amniocentesis wk, maternal smoking and maternal pre-pregnancy BMI.

A series of multiple regressions, with birth weight as a dependent variable in a model that included gestational age, infant gender, maternal pre-pregnancy BMI, smoking, and parity as independent variables, was examined. Each amino acid was added individually into each model. Arginine, 3-methyl histidine and tryptophan emerged as positive predictors of birth weight; in contrast, ornithine was negatively associated with infant birth weight. When looking further into the separate percentile groups, no amino acids emerged as predictors individually in either of the SGA, AGA or LGA groups. The greatest predictor of infant birth weight was maternal smoking, where non smoking mothers gave birth to infants 180 g larger than infants of smoking mothers.

Male infants were born 141 g larger than the female infants, while other significant predictors were gestational days (β-coefficient of 27 g), and maternal prepregnancy BMI (β-coefficient of 19 g).

2.6 DISCUSSION

Our results showed that our AF samples were taken from a healthy maternal population, where the majority were nonsmokers and had a healthy prepregnancy BMI. Precolumn derivatization with PITC allowed for the quantification of 23 amino acids, greatly ranging in concentrations. Interestingly, we also determined that 1) AF amino acid concentrations measured early in gestation were affected by the timing of amniocentesis collection, 2) the majority of AF amino acids measured differed by percentiles of fetal growth when corrected for wk of amniocentesis, maternal pre-pregnancy BMI, parity and smoking habits, as well as the infant's birth weight and gender which were corrected for by using percentiles of fetal growth and 3) as expected, several maternal characteristics (gestational age, pre pregnancy BMI, infant gender, maternal smoking) were associated with infant birth weight, and 4) we were able to establish that 4 2^{nd} trimester amino acids (arginine, 3 methyl histidine, ornithine, tryptophan) were significant predictors of infant birth weight.

We measured free AF amino acid concentrations between 12 and 20 wk gestation in healthy pregnancies for over 350 mother-infant pairs. This study is unique in comparison with previous research on AF amino acids, because it characterized the AF amino acid profile early in the 2^{nd} trimester using AF samples collected at the time of amniocentesis. We were able to associate certain AF amino acids with fetal growth in women having healthy pregnancy outcomes. We discovered not only that ornithine, arginine, 3-methyl histidine and tryptophan were predictors of birth weight, but that 18 AF amino acid concentrations differed at mid gestation for infants born SGA, AGA, and LGA. Previous research investigating AF amino acid concentrations early in gestation had repeatedly collected AF from therapeutic abortions (Weissman et al. 2003, Jauniaux et al. 1999, Jauniaux 1998, Jauniaux et al. 1994, A'Zary et al. 1973, Scott 1972, Cockburn et al. 1970), making it impossible to associate AF concentrations with full term healthy fetal outcomes, which we report for the first time in this study.

Fetal weight increases the most rapidly close to 30 wk gestation (Duggleby & Jackson 2002), thus the wks just prior are intended to supply the fetus with enough nutrients and amino acids for tissue growth and development. In our study, a multiple regression reported that 2nd trimester AF ornithine was a negative predictor of birth weight with a ß-coefficients of –3.6 g. Although not a constituent of protein, ornithine is an important amino acid in the urea cycle and is used both as a precursor for the synthesis of arginine as well as a product of arginine metabolism (Blachier et al. 1995; Wu & Morris 1998; Van de Poll et al. 2004). Its role in the urea cycle may explain its importance as a negative birth weight predictor, and may suggest that larger infants with more functional livers in the early 2nd trimester incorporate larger amounts of ornithine, and leave smaller concentrations of free amino acids in the AF. At mid-gestation, an excess of amino acids requiring oxidation can produce high concentrations of urea (Battaglia 1992), which indicates the increased need for amino acids important for the urea cycle. Ornithine's importance for gut function and metabolism should also be noted (Cynober 1994; Gardiner et al. 1995).

Multiple regressions also reported that 2nd trimester AF tryptophan, 3-methyl histidine, and arginine and tryptophan were positive predictors of birth weight with ß-coefficients of, 6.0, 2.14, and 0.56 g, respectively. Arginine's positive association with birth weight may be related to its numerous functional roles in a) cell proliferation (Witte & Barbul 2003), b) the synthesis of polyamines, important for growth and differentiation of intestinal mucosal cells (Cynober 1994), c) renal synthesis of creatine (Heys & Gardner 1999), an important constituent of skeletal muscle (Wu & Morris 1998), d) the maintenance of mucosal blood flow and immune function (Cynober 1994; Wallace & Tom 2000), e) the normalization and improvement of tissue repair and healing (Witte & Barbul 2003), f) the release of pituitary growth hormone, prolactin (Heys & Gardner 1999, Cynober 1994) and glucagon (Visek 1986) and g) as a nitrogen donor for nitric oxide synthesis in the neonatal and therefore possibly fetal gut (Appleton 2002). Although complex, the multifunctional biological role of arginine may indicate that higher concentrations of this amino acid would be a sign of healthier fetal growth and an increased birth weight. The majority of 3-methyl histidine is found in the skeletal muscle, gastrointestinal tissues and in the connective tissues of the skin (Young et al.

1972), and due to the fact that 3-methyl histidine cannot be reutilized for muscle protein synthesis (Chinket 2005), any concentration changes for this amino acid in AF may be related with gastrointestinal or skin development. The positive association between early AF tryptophan concentrations and final infant birth weight has not been previously explored, although tryptophan's crucial role as a precursor to brain serotonin (Hernandez-Rodriguez & Manjarrez-Gutiérrez 2001; Fernstrom 2000) suggests a relationship with brain development, which has been indirectly associated with birth weight. Considering the importance of BCAAs for brain development (Fernstrom 2005), and especially leucine in the stimulation of muscle protein synthesis (Garlick 2005; Buse & Reid 1975), it was unexpected that no BCAAs emerged as predictors of birth weight, especially surprising since lower plasma concentrations of BCAAs have been reported in intrauterine growth retarded fetuses (Cetin et al. 1988), and a shortage of BCAAs has been shown to restrict protein synthesis and in turn, impair growth and tissue repair (Harris et al. 1994).

Since the amino acids are freely diffusible between the AF and fetal plasma across the unkeratinized skin (Queenan 1978) from 12 to 20 wk gestation, and are also swallowed by the developing fetus beginning at approximately 11 wk gestation (Queenan 1978), higher concentrations in AF could provide a larger precursor pool that could enhance fetal growth and organ development, as well as lead to an infant with a birth weight that is appropriate or even large for gestational age. While many amino acids did not emerge as predictors of birth weight in the multiple regression models, analysis of variance across percentile categories, while controlling for wk of amniocentesis sampling, maternal pre-pregnancy BMI, smoking, infant gender and gestational age, showed that total amino acids and 18 individual amino acids differed across percentile categories. Thirteen of these amino acids were highest for infants born AGA (alanine, aspartic acid+asparagine, glutamic acid+glutamine, glycine, histidine, isoleucine, lysine, ornithine, phenylalanine, proline, threonine, tyrosine, valine), but more intriguing were those AF amino acids that were higher in SGA (methionine) and LGA (arginine, citrulline, 3-methyl histidine, tryptophan) infants. Interestingly, arginine, 3-methyl histidine and tryptophan also emerged as predictors of birth weight in this study, where previously only methionine emerged as a predictor of pup weight using a rat model and

measuring near term in a diet induced model of IUGR (Gurekian & Koski 2005). The reason that methionine did not emerge as a predictor for birth weight in humans is interesting, although it might be explained by the small sample size for the small-for-gestational-age infants.

Statistically, we included controls for previously established determinants of birth weight; maternal height, pre-pregnancy weight, and smoking habits, gestational age, and infant gender (Kramer 1987) and additionally entered wk of amniocentesis sampling as a covariate due to differences in AF amino acid concentrations seen across gestation in our study as well as in past research (Dawson et al. 1999; Rabier et al. 1996, Mesavage et al. 1985; Schulman et al. 1972). The strongest predictor of infant birth weight was maternal smoking where nonsmoking mothers gave birth to infants 179 g larger than smoking mothers. In agreement, Adriaanse et al. (1996) reported that for women who still smoked after 7 months of pregnancy, the birth weight of their infant decreased by 24 g for every cigarette smoked per day (Adriaanse et al. 1996). Logically, other positive predictors were pre-pregnancy BMI, gestational age (days), and infant gender (female to male), with ß-coefficients of 18.8 g, 26.9 g, and 141.4 g, respectively. As maternal pre-pregnancy weight was one of our controlling factors, it was of great interest to note that during gestation, it has been previously shown to modulate the amino acid concentrations in the maternal plasma; obese women reported lower overall amino acid concentrations, and more specifically, lower alanine, histidine, lysine, proline, serine, taurine and threonine levels than lean control subjects (Kalkhoff et al. 1988). Postpartum, these plasma levels returned to high concentrations in the obese women, and levels of tyrosine, phenylalanine and BCAAs even exceeded those of lean mothers (Kalkhoff et al. 1988). This finding had prompted the suggestion that despite these high levels of amino acids in the plasma of obese mothers before pregnancy, the fetus' of obese mothers may utilize greater amounts of amino acids in-utero, consequently lowering maternal plasma levels during pregnancy. We, however, showed that maternal pre-pregnancy BMI was significantly higher in mothers of infants born LGA, although no amino acids differed across maternal pre-pregnancy BMIs as would have been expected, indicating that AF amino acids are not simply driven by high infant or maternal weights as had been reported for maternal amino acid profiles.

Analytically, our choice of RP-HPLC provided high sensitivity and accuracy for detecting both primary and secondary amino acids in AF. This was the case for citrulline, glutamine, 3-methyl histidine and tryptophan, which had not been reported for a similar gestational age in earlier literature (Jauniaux 1998; Rabier et al. 1996; Jauniaux et al. 1994; Mesavage et al. 1985; Pettit & Allen 1980; A'Zary et al. 1973; Scott 1972, Schulman et al. 1972; Reid 1971, Cockburn et al. 1970, Levy & Montag 1969). Cystine was undetectable in our study, although the inability to detect cystine using PITC derivatization is well documented (Furst et al. 1990), and was also prominent in other methods investigating AF amino acids (Rabier et al. 1996; Jauniaux et al. 1994; Scott 1972, Schulman et al. 1972, Reid 1971). Our results showed a large variation between the 23 detected AF amino acids, with concentrations ranging from 13 to 424 μmol/L. The examination of these concentrations demonstrated that our highest 5 amino acids (alanine, glutamine + glutamic acid, lysine, proline, valine) and lowest 5 amino acids (tryptophan, citrulline, ornithine, serine, 3-methyl histidine) corresponded, even in our narrow developmental range, with similar rank orders of previous researchers who measured AF amino acids throughout fetal development (Braida et al. 2001; Jauniaux 1998; Rabier et al. 1996; Jauniaux et al. 1994; Sims et al. 1993; Mesavage et al. 1985; A'Zary et al. 1973; Scott 1972; Schulman et al. 1972; Reid 1971; Cockburn et al. 1970).

An important strength of our study was our large sample size (n=361 mother-infant pairs) which exceeded previous studies on AF amino acids with sample sizes of n<50 (Dawson et al. 1999; Jauniaux 1998; Rabier et al. 1996; Sims et al. 1993; Pettit & Allen 1980; A'Zary et al. 1973; Scott 1972; Schulman et al. 1972; Reid 1971; Cockburn et al. 1970; Levy & Montag 1969) and even larger studies with n<200 AF samples (Braida et al. 2001; Bock 1994; Le Moyec et al. 1994; Mesavage et al 1985). Moreover, our limited time frame (95% of samples taken between 14-17 wk gestation) gave us a narrow window to investigate the possibility of AF amino acids as biomarkers of in-utero growth given our knowledge that AF is a dynamically changing throughout development. We studied early 2nd trimester AF where passive diffusion through the large surface of unkeratinized fetal skin is most likely the primary mechanism for the movement of amino acids between the fetal circulation and the amniotic cavity (Jauniaux et al. 1999) although swallowing of AF is occurring (Ross & Brace 2001). We reported an overall decrease in

concentration for total and 13 individual AF amino acids from 12 to 20 wk gestation, in agreement with previous literature observing steady decreases from 10 to >27 wk gestation (Dawson et al. 1999; Rabier et al. 1996, Mesavage et al. 1985; Schulman et al. 1972). It is believed that this decline in AF amino acids is used by the developing fetus for the formation of peptides and proteins for tissue deposition (Duggleby & Jackson 2001; Liechty & Denne 1998) and as precursors for the synthesis of protein, DNA, RNA, creatinine, and neurotransmitters, and energy (Duggleby & Jackson 2002).

In summary, as a result of our large sample size and improved analytical and statistical approaches, our findings described for the first time several novel outcomes: 1) first and most importantly, we showed that AF arginine, 3-methyl histidine, ornithine and tryptophan measured early in the second trimester (15 ± 0.1wks) were independent predictors of birth weight in healthy mothers carrying pregnancy to term, 2) we found that several AF amino acid concentrations were uniformly higher in the AF of AGA infants (alanine, aspartic acid+asparagine, glutamic acid+glutamine, glycine, histidine, isoleucine, lysine, ornithine, phenylalanine, proline, threonine, tyrosine, valine), while a few were also highest for SGA (methionine) and LGA (arginine, citrulline, 3-methyl histidine, tryptophan), and 3) in agreement with previous literature (Rabier et al. 1996, Mesavage et al. 1985, Scott 1972), we showed a steady decline in concentration for the majority of AF amino acids between 12 and 20 wk gestation, supporting our claim that amniocentesis wk should be statistically controlled for when evaluating the impact of AF amino acids on fetal outcomes. Our overall conclusion is that several amino acids should be further explored as biomarkers of in-utero fetal growth at mid gestation.

Please note that all references are combined beginning on page 62.

TABLE 2.1

Characteristics of the sample population[1]

Pregnancy characteristics	
Amniocentesis sampling, *wk*	15.1 ± 0.1
Gestational age, *wk*	39.5 ± 0.1
x = 38 wk	15%
38 - 40 wk	45%
x > 40 wk	40%
Maternal characteristics	
Age, *yr*	37.9 ± 0.1
Pre-pregnancy weight, *kg*	62.9 ± 0.7
Height, *m*	1.6 ± 0.004
Pre-pregnancy BMI, kg/m^2	23.8 ± 0.3
= 20 kg/m^2	16%
20-25 kg/m^2	52%
25-30 kg/m^2	24%
=30 kg/m^2	8%
Ethnicity	
Caucasian	66%
Asian	18%
Other[2]	16%
Non-smoker	88%
Parity	1.1 ± 0.4
Fetal characteristics	
Infant gender	
Males	57%
Females	43%
Birth weight, *g*	3509 ± 31
[3]SGA (<10%)	2661 ± 69
[3]AGA (10-90%)	3382 ± 26
[3]LGA (>90%)	4255 ± 34

Footnotes for Table 2.1

[1]Values are means ± SEM, or % with n=361 mother-infant pairs. [2]Other consists of Middle Eastern, African and South American combined. [3]SGA: small for gestational age (n=32), AGA: appropriate for gestational age (n=250), LGA: large for gestational age (n=79).

TABLE 2.2

Amino acid concentrations in human amniotic fluid during early gestation

Amino Acids	Mean ± SEM	Median
Alanine	424 ± 9	424
Arginine	186 ± 6	165
Aspartic Acid + Asparagine	47 ± 2	28
Citrulline	16 ± 1	11
Glutamic Acid + Glutamine	342 ± 8	144
Glycine	141 ± 4	140
Histidine	100 ± 2	102
Isoleucine	175 ± 6	155
Leucine	132 ± 4	122
Lysine	261 ± 9	259
Methionine	49 ± 3	32
3-Methyl Histidine	41 ± 2	35
Ornithine	25 ± 1	26
Phenylalanine	92 ± 2	94
Proline	215 ± 7	240
Serine	25 ± 1	22.7
Taurine	57 ± 3	55
Threonine	58 ± 2	53
Tryptophan	13 ± 1	10
Tyrosine	108 ± 3	106
Valine	194 ± 5	208
TOTAL	**2679 ± 50**	**2739**

[1]Values in μmol/L where n=361.

TABLE 2.3

Amino acid concentrations across week of amniocentesis[1]

Amino Acids	Amniocentesis week =14	14-15	15-16	=16	p-value
Individual					
Alanine	473 ± 15^{b}	413 ± 14^{ab}	417 ± 19^{ab}	368 ± 34^{a}	0.0102
Arginine	201 ± 10	174 ± 7	203 ± 19	178 ± 23	0.0477
Citrulline	17 ± 2	15 ± 2	13 ± 2	19 ± 6	0.0152
Glutamic Acid + Glutamine	365 ± 13^{b}	316 ± 10^{a}	369 ± 20^{b}	372 ± 34^{ab}	0.0130
Histidine	113 ± 4^{b}	96 ± 4^{a}	97 ± 5^{a}	94 ± 10^{ab}	0.0247
Lysine	302 ± 16^{b}	258 ± 13^{ab}	246 ± 21^{ab}	206 ± 44^{a}	0.0118
Ornithine	30 ± 2^{b}	24 ± 1^{ab}	24 ± 2^{ab}	18 ± 3^{a}	0.0046
Phenylalanine	102 ± 4^{b}	91 ± 4^{ab}	88 ± 5^{ab}	74 ± 7^{a}	0.0310
Proline	243 ± 13^{b}	206 ± 10^{ab}	216 ± 14^{ab}	168 ± 20^{a}	0.0341
Threonine	67 ± 4^{b}	56 ± 3^{ab}	57 ± 4^{ab}	45 ± 5^{a}	0.0193
Tryptophan	13 ± 1	15 ± 2	11 ± 1	12 ± 3	0.0488
Valine	218 ± 10^{b}	188 ± 8^{b}	196 ± 11^{b}	144 ± 16^{a}	0.0085
Grouped					
Non-Essential[2]	1000 ± 29^{b}	871 ± 28^{a}	912 ± 39^{ab}	847 ± 68^{a}	0.0280
Conditionally Essential[3]	736 ± 23^{b}	636 ± 19^{a}	712 ± 37^{b}	635 ± 57^{ab}	0.0190
TOTAL	2935 ± 84^{b}	2582 ± 76^{ab}	2725 ± 118^{ab}	2376 ± 184^{a}	0.0123

[1]Values as means ± SEM in μmol/L where n=93 (=14 wks), n=164 (14-15 wks), n=72 (15-16 wks), and n=26 (=16 wks); values with different superscripts represented by the letters a, b, c present differences across groups from post hoc Student Newman Kneuls tests; post hoc tests did not emerge for arginine, citrulline, and tryptophan. [2]Non-Essential amino acids (alanine, aspartic acid, asparagine, glutamic acid, serine) [3]Conditionally Essential amino acids (arginine, cysteine, glutamine, glycine, proline, tyrosine)

TABLE 2.4

Amniotic fluid amino acid concentrations across percentile categories[1]

	Percentiles			
	SGA <10%	AGA 10-90%	LGA >90%	p-value[2]
Maternal pre-pregnancy BMI (kg/m^2)	24 ± 1^a	23 ± 0.5^a	26 ± 1^b	<0.0001
Amino Acids				
Individual				
Alanine	330 ± 37^a	452 ± 9^b	373 ± 23^a	<0.0001
Arginine	193 ± 23^{ab}	174 ± 5^a	222 ± 20^b	0.0212
Aspartic Acid + Asparagine	24 ± 6^a	55 ± 1^c	32 ± 3^b	<0.0001
Citrulline	14 ± 4	15 ± 1	18 ± 3	<0.0001
Glutamic Acid + Glutamine	267 ± 21^a	367 ± 8^b	294 ± 20^a	<0.0001
Glycine	95 ± 13^a	155 ± 5^b	112 ± 9^a	<0.0001
Histidine	73 ± 11^a	108 ± 2^b	86 ± 6^a	<0.0001
Isoleucine	123 ± 17^a	184 ± 7^b	167 ± 13^b	<0.0001
Lysine	185 ± 35^a	280 ± 9^c	234 ± 26^b	<0.0001
Methionine	78 ± 14^b	38 ± 2^a	71 ± 8^b	<0.0001
3-Methyl Histidine	30 ± 5^a	41 ± 2^b	47 ± 4^b	0.0222
Ornithine	16 ± 3^a	29 ± 1^b	16 ± 2^a	<0.0001
Phenylalanine	75 ± 12^a	99 ± 2^b	77 ± 6^a	<0.0001
Proline	112 ± 21^a	251 ± 6^c	140 ± 15^b	<0.0001
Serine	25 ± 2	25 ± 1	26 ± 2	0.0002
Threonine	44 ± 6^a	63 ± 2^b	50 ± 4^a	<0.0001
Tryptophan	13 ± 4^a	11 ± 0.5^a	21 ± 4^b	<0.0001
Tyrosine	95 ± 15	111 ± 3	101 ± 9	0.0003
Valine	123 ± 19^a	216 ± 5^c	151 ± 13^b	<0.0001
Grouped				
Essential	824 ± 87^a	1108 ± 21^c	983 ± 52^b	<0.0001
Non-Essential	692 ± 69^a	974 ± 18^b	788 ± 45^a	<0.0001
Conditionally Essential	531 ± 49^a	715 ± 14^b	605 ± 38^a	<0.0001
TOTAL	2076 ± 199^a	2837 ± 51^c	2423 ± 127^b	<0.0001

[1]Values as means ± SEM in µmol/L where n=41 (SGA), n=250 (AGA), and n=75 (LGA); covariates used: week of amniocentesis sampling, maternal smoking and prepregnancy BMI; values with different superscripts represented by the letters a, b, c present differences across groups from post hoc Student Newman Kneuls tests; post hoc tests did not emerge for citrulline and tyrosine. [2]Essential amino acids (histidine, isoleucine, leucine, lysine, methioinie, phenylalanine, threonine, tryptophan, valine). [3]Non-Essential amino acids (alanine, aspartic acid, asparagine, glutamic acid, serine). [4]Conditionally Essential amino acids (arginine, cysteine, glutamine, glycine, proline, tyrosine).

TABLE 2.5 Amniotic fluid amino acids as predictors of birth weight (g)[1]

	ß–coefficient, *g*	p-value	Variability Captured, %
Gestational age, *d*	26.90	<0.0001	27.35
Prepregnancy BMI, *kg/m²*	18.79	0.0008	
Infant Gender	141.40	0.0087	
Smoking	178.90	0.0283	
Amino acids[2]			
Arginine	0.56	0.0264	28.17
3-Methyl Histidine	2.14	0.0180	28.46
Ornithine	-3.59	0.0269	28.12
Tryptophan	5.95	0.0001	30.41

[1] Birth weight (g) as the dependent variable with n=361. [2]Amniocentesis wk was not included in the model because it was not a significant predictor of birth weight.
[3] Each amino acid was added individually into the multiple regression model, and only those with a significance of $P < 0.05$ are reported.

CONNECTING PARAGRAPH

Results from the first study indicate that AF amino acids can be associated with fetal growth; some have even demonstrated to be predictive of infant birth weight. In a rat model, our laboratory has previously shown that restriction of maternal dietary glucose will produce IUGR in rats (Koski & Fergusson 1992; Koski & Hill 1986; Lanoue et al. 1992; Koski et al. 1990). Moreover, other studies that studied both short- (DiGiacomo & Hay 1989) and long-term (DiGiacomo & Hay 1989) glucose deprivation showed that the oxidation of several amino acids (BCAAs, arginine, methionine, phenylalanine) occurred in order to meet fetal needs (Arola et al. 1982; Godfrey et al. 1996; Hay 1999). Also, food deprivation in pregnant women even reported changes in AF amino acids (Bernstein et al. 1992; Felig et al. 1972). The following study was conducted in order to further investigate whether any AF amino acids as predictors of fetal growth and development might be similar in humans versus animals in both early and late gestation. We also added a new variable of maternal dietary restriction into the following study and we chose a rat model for this study since it is unethical to impose dietary restriction in human pregnancy.

CHAPTER 3:

Amniotic fluid amino acid concentrations are modified by maternal dietary glucose, gestational age, and fetal growth in rats

Christine N Gurekian[1] and Kristine G Koski[1]

[1]School of Dietetics and Human Nutrition, McGill University (Macdonald Campus), Ste. Anne-de-Bellevue, QC, Canada, H9X 3V9

This manuscript has been accepted for publication in thepublished

Journal of Nutrition Sept 135(9): 2219-2224 (2005)

3.1 ABSTRACT

Amniotic fluid (AF) contains free amino acids that enter via transplacental and transmembraneous routes from maternal sources; subsequently the developing fetus 'ingests' these amino acids early in gestation through unkeratinized skin and later through continuous AF swallowing. Our objectives were as follows: *1)* to determine whether a restriction of maternal dietary glucose modulates the free AF amino acid pool, and *2)* to establish whether any diet-induced changes were predictive of fetal weight near term (d 21.5). To produce varying in-utero growth rates, pregnant rat dams were fed varying levels of glucose (0, 12, 24, 60%) throughout pregnancy. AF samples, collected on gestational days 18-21, were precolumn derivatized by 9-fluorenylmethyloxy chloroformate (FMOC) to produce stable primary and secondary amino acid derivatives required for HPLC detection at low amino acid concentrations. Eighteen amino acids were identified. A 2-way ANOVA with main effects of diet (=12% and =24% glucose), and gestational age (days 18/19 and 20/21) showed that 2 AF amino acids, methionine and phenylalanine, and 12 AF amino acids were independently modified by diet and gestational age, respectively. Of note were the 364% increase in AF methionine and the constant decline in AF taurine as both gestational age lengthened and fetal weight increased. Multiple regression demonstrated in addition to methionine, 3 specific AF amino acids- cysteine, lysine, and tyrosine, predicted fetal weight. These results demonstrate that the AF amino acid pool can be modified by the glucose content of the maternal diet and that specific AF amino acids are associated with gestational age and fetal growth.

3.2 INTRODUCTION

Amniotic fluid (AF)[3] is swallowed by the developing fetus (Queenan 1978), and both its volume and composition play an important role in fetal growth and development. AF, which is a dynamically changing nutrient reservoir, is a composite of secretions from maternal plasma, from the placenta and from the developing fetal urinary, respiratory and alimentary tracts (Mandelbaum & Evans 1969). Within this complex nutrient matrix, AF glucose, which is considered an important regulatory fuel during fetal development, acts to sustain numerous energy-dependent systems, including placental amino acid uptake (Yudilevich & Sweiry 1985), and promotes and maintains fetal growth (Koski & Fergusson 1992).

Several studies have investigated the impact of starvation or carbohydrate restriction on amino acid metabolism (Swendseid et al. 1967; Adibi & Drash 1970; Felig et al. 1970; Vazquez et al. 1985; Kaloyianni & Freedland 1990), but few have done so during pregnancy (Felig et al. 1972; Girard et al. 1977; Lemons & Schreiner 1983; Bell et al. 1999), and far fewer have measured their effect on AF composition (Felig et al. 1972; Bernstein et al. 1992). Food deprivation in late gestation reportedly decreases AF BCAAs, as well as the gluconeogenic amino acids, alanine and glycine (Bernstein et al. 1992). In pregnant women, energy restriction lowered AF concentrations of alanine and results in higher concentrations of AF taurine and BCAAs (Felig et al. 1972). Evidence relating to other dietary-induced changes in amino acid concentrations in AF is lacking.

Our aim was to explore the possibility that the amino acid profile of AF might be related to fetal growth. Previous research in our laboratory showed that if maternal dietary glucose was limiting, there was a high perinatal mortality, morbidity, and intrauterine growth retardation (IUGR), proportional to the degree of maternal dietary glucose restriction (Koski & Hill 1986, Koski & Hill 1990; Lanoue et al. 1992). Glucose is considered to be the principal metabolic fuel for the developing fetus in utero (Koski & Fergusson 1992; Koski & Hill 1990). Increasing amounts of maternal dietary glucose are associated not only with fetal growth in a dose-dependent manner but also with increasing tissue glycogen reserves and the emergence of important regulatory enzymes associated with perinatal glycogenolysis and gluconeogenesis (Liu & Koski 1997; Koski et al. 1993; Lanoue et al. 1999). Thus, the possibility existed that this diet-induced model

employing restriction of maternal dietary glucose to produce fetal growth retardation could be used to ascertain those AF amino acids specifically related to optimal fetal growth and gestational age and those specific AF amino acids that might be associated with poor maternal dietary glucose intake and predictive of intrauterine growth retardation.

3.3 MATERIALS AND METHODS

3.3.1 Experimental Design

Using a 4 x 4 factorial design, we investigated how 4 levels of maternal dietary carbohydrate (0, 12, 24, 60 % glucose) fed throughout pregnancy would affect the AF amino acid profile in rats during the last 4 gestational days (Liu & Koski 1997; Koski et al 1993; Lanoue et al. 1999; Lanoue & Koski 1994). Time-bred female Sprague-Dawley rats (180-200 g, Charles River) were housed in individual suspended wire screen cages maintained in a temperature-controlled room (20 °C) with fluorescent lighting (12 h daily from 0700-1900 h.) and were randomly assigned on gestational d 0/1 to one of the 4 previously published experimental diets (Lanoue & Koski 1994). Individual body weights and food intake were measured every other day. Water and food were freely provided.

The rationale for the use of these 3 low-carbohydrate diets was based on our earlier observations reporting a change in AF glucose as maternal dietary glucose varied that was proportional to birthweight (Koski & Fergusson 1992). Additionally, maternal glucose restriction resulted in an increase in AF uric acid that was negatively associated with maternal dietary glucose, which was positively correlated with maternal and fetal liver glycogen and in utero growth (Koski & Fergusson 1992). Thus, these results strongly suggested that this maternal dietary glucose restriction could also modity AF amino acid concentrations; use of this dietary model could provide insight into the contribution of individual AF amino acids to fetal growth.

3.3.2 Amniotic fluid collection and biochemical analysis

Fetuses were delivered from dams by caesarean section on d 18, 19, 20 or 21 of gestation between 0730 and 1230 h. All dams were killed in the postabsorptive (fed)

state under anesthesia with Ketamine-HCl (30 mg/kg, Rogarsetic, Rogar/STB). Immediately after cardiac puncture, intact uteri were removed and an aliquot of AF was collected from each individual sac and pooled by litter. Fetuses were weighed individually and then killed by exsanguination. All procedures were conducted in conformance with the guidelines for experimental procedures set forth by the local animal care committee of McGill University and by the Canadian Council on Animal Care (Canada Council on Animal Care 1984). The pooled AF was stored at -80°C until the day before analysis. Immediately after thawing, samples were deproteinized by ultracentrifugation (1500 g for 30 mins) using 10,000 mol/L Millipore cut off membranes. The concentrations of 18 amino acids in AF (alanine, arginine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, taurine, threonine, tyrosine, and valine) were measured by HPLC Aminotag, using norleucine as an internal standard. Samples were precolumn derivatized by 9-fluorenylmethyloxychloroformate (FMOC), which was selected for its ability to derivatize low picomolar amounts of amino acids under fluorescence and to produce stable derivatives of both primary and secondary amino acids (Malmer & Schroeder 1990; Miller et al. 1990; Schuster 1988). FMOC also surpasses other derivatization methods that have a lengthy analysis time (ninhydrin), produce unstable derivatives (dansyl chlorides), are not able to detect secondary amino acids (o-phthalaldehyde), or whose samples have to be evaporated before analysis (phenylisothiocyanate) (Malmer & Schroeder 1990). Moreover, FMOC derivatization is less sensitive to interference from extraneous sample components in comparison with other methods, and can derivatize amino acids in <1 min (Malmer & Schroeder 1990). Our samples were derivatized immediately before injection into the HPLC to avoid having to extract excess FMOC with amantadine (Malmer & Schroeder 1990).

3.4 STATISTICAL ANALYSIS

All statistical analyses were performed on SAS, version 8.0 (SAS Institute Inc. 2000). Amino acid concentrations were analyzed for AF pooled by litter. In addition to individual amino acids, amino acid concentrations were also categorized into essential, non essential and conditionally essential groups based on guidelines set by Domenech et

al. (1986) in which all essential amino acids were considered the same for growing rats as for humans, with the addition of arginine, asparagine, glutamic acid, proline and tyrosine for rats. The concentration of total free amino acids was also calculated for analysis. The amino acids of glutamic acid and glutamine were combined because inter-conversion between the 2 is common (Arola et al. 1982). Concentrations of individual and grouped amino acids were categorized for diets as =12% glucose and =24% glucose, as well as for gestational age as d 18/19 and d 20/21. AF amino acids were tested by a two by two interaction anova (diet x gestational age). AF amino acids were tested using a 2 x 2 interaction ANOVA (diet x gestational age). AF amino acid values were also compared using a 1-way ANOVA across quartiles of fetal weight that included gestational age as a covariate. To determine whether any AF amino acids acted as predictors for fetal weight, a multiple regression was performed with mean fetal weights for each dam as a dependent variable, gestational age as an independent variable, and each amino acid or amino acid group individually introduced into the model. Introduction of individual amino acids or amino acid groups into the model was done because there were noteworthy correlations among many amino acids (Mesavage et al. 1985). A $P < 0.05$ was chosen for significance for all statistical analyses including the Student's *t* test and Student-Newman-Keuls post-hoc tests.

3.5 RESULTS

Eighteen AF amino acids were measured and quantified (n = 73 litters): alanine, arginine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, taurine, threonine, tyrosine, and valine. The 3 amino acids with the highest concentrations were glutamine, lysine and alanine, together totaling 47.3% of the total AF amino acid pool, whereas the 3 lowest were cysteine, glutamic acid and taurine, totaling 1.3 % of the total AF amino acid pool.

There were no significant interactions between diet and gestation age (2-way interaction ANOVA). Diet was the main effect for only 2 amino acids, methionine and phenylalalnine, and concentrations of these amino acids were higher in dams fed = 24% glucose (n = 32) than in those fed = 12% glucose (n = 41) (Table 1). These values were also associated with a higher fetal weight (3.14 ± 0.17 g vs 4.07 ± 0.23 g for =12 vs =24

%, respectively). Gestational age was a significant main effect for several individual amino acids: alanine, glutamic acid + glutamine, glycine, isoleucine, leucine, methioinine, phenylalanine, proline, serine, taurine, threonine and valine, as well as for grouped amino acids: essential, nonessential, conditionally essential and total amino acids (Table 1). With the exception of taurine, all AF amino acids increased with gestational age. It may be expected that because AF volume decreases with increasing gestational age, a dilution effect may be contributing to the reported increase in concentration of amino acids as pregnancy progressed; however, there was no constant percentage increase across all amino acids.

With the mean fetal weight / litter divided into quartiles of <2.65, 2.65-3.30, 3.31-4.21, and >4.21 g, (n = 18/group) and with gestational age included as a covariate, AF alanine, cysteine, glutamic acid + glutamine, glycine, leucine, lysine, methionine, phenylalanine, proline, taurine, threonine, and tyrosine differed across these 4 birth weight classifications. Moreover, differences with the essential, nonessential, conditionally essential and total amino acid groups also occurred. Concentrations of all of these individual and grouped amino acids, with the exception of threonine and taurine, were lowest when birthweight was in the 2 lowest quartiles (=3.30 g) and highest when birthweight was >4.21 g. Taurine declined steadily as term fetal weight increased, with a peak concentration at the lower quartile (<2.65g) and a nadir with the highest quartile weights (>4.21g), whereas threonine had a peak in concentration between 3.3 to 4.21 g (Table 2).

A multiple regression, with the mean fetal weights for each litter (n = 73) as a dependent variable, gestational age as an independent variable, and each amino acid individually introduced into the model, yielded 4 amino acids (cysteine, lysine, methionine, tyrosine) that were positively associated with fetal weight near term (Table 3). As expected, gestational age was the major predictor ($P < 0.0001$) of fetal weight, capturing 76.6% of its variability and accounting for a 1.2-g daily increase in birthweight over the last 4 d of gestation. Based on the magnitude of the β-coefficients, the biological effect of a 1 μmol/L increase was to increase fetal weight by 0.3 mg for lysine, 2.4 mg for methionine, 6.7 mg for tyrosine, and 62.4 mg for cysteine.

These results showed that the AF amino acid pool was altered by the following: *1*) glucose level in the maternal diet (Table 1); *2*) gestation length (Table 1); and *3*) mean fetal weight for each litter (Table 2). In addition, 4 amino acids acted as significant predictors of pup birthweight (Table 3).

3.6 DISCUSSION

Restriction of maternal dietary glucose can produce intra-uterine growth retardation (IUGR) (Koski & Fergusson 1992, Koski & Hill 1986; Lanoue et al. 1992; Koski et al. 1990), where both short- (DiGiacomo & Hay 1989) and long-term glucose deprivation (DiGiacomo & Hay 1989) result in oxidation of gluconeogenic substrates including lactate and several amino acids (BCAAs, arginine, methionine, phenylalanine) to meet fetal needs (Arola et al. 1982; Godfrey et al. 1996; Hay 1999). Our study reports novel findings that demonstrate that the level of glucose in the maternal diet can modify the AF amino acid precursor pool. We showed a reduction in AF methionine (90%) and phenylalanine (30%) when maternal dietary glucose was restricted during pregnancy, an observation that both agrees with and differs from earlier reports (Felig et al. 1972; Bernstein et al. 1992) that employed food deprivation to restrict glucose delivery to the developing fetus. In these earlier studies, food deprivation in late gestation lowered the concentrations in AF BCAAs, and the gluconeogenic amino acids (alanine, glycine) (Bernstein et al. 1992) whereas energy restriction produced higher concentrations in AF taurine and BCAAs (Felig et al. 1972). In our study, methionine and phenylalanine were modulated independently by the maternal diet. Our findings confirm earlier observations (Jozwik et al. 1999; Thureen et al. 2000) that maternal dietary glucose restriction lowered AF phenylalanine concentrations. AF amino acids, including alanine, cysteine, glutamic acid + glutamine, glycine, leucine, lysine, methionine, proline, threonine, and tyrosine differed across the 4 quartiles of fetal weight, if gestational age was included as a covariate. All were found in lower concentrations in pups with IUGR, with the exception of taurine. Taurine, as reported earlier (Felig et al. 1972), was the only AF amino acid to decline as gestation progressed and fetal weight increased. In our study, the BCAAs were related to gestational age and not to fetal weight as previously described (Felig et al. 1972; Bernstein et al. 1992). In our study, only 4 essential AF amino acids, cysteine,

lysine, methionine, and tyrosine, were independent predictors of fetal weight in a multiple regression model that controlled for gestational age; among these, only methionine has been previously reported (Felig et al. 1972).

During starvation, in addition to increased gluconeogenesis, the placenta decreased its high consumption of glucose (Hay 1991) without changing its size (Lanoue et al. 1992) to decrease its demand on maternal glucose and to promote diversion of some maternal glucose from the placenta to the fetus (Hay 1991). This occurs without alterations in the expression and activity of placental glucose transporters in IUGR fetuses (Cetin et al. 2004). However, this inhibition of glycolysis and aerobic metabolism was shown to suppress amino acid transport across the placenta (Illsley et al. 1984; Milley 1988; Smith 1981), particularly essential amino acids in growth retarded infants (Cetin et al. 1988) via a reduced activity/expression of amino acid placental transport systems (Cetin et al. 2004). Our data supported this observation for two amino acids because AF concentrations of methionine and phenylalanine were lower in glucose-deprived rat dams. Our data did not support the suggestion (Philipps et al. 1978) that the placenta, in times of nutritional deprivation, would supply amino acids to meet fetal needs and regulate fetal growth (Cetin et al. 2004).

Methionine's importance in growth is demonstrated by its vital role in the production of cysteine and in facilitating DNA and tRNA methylation (Lemons 1979; Steegers-Theunissen et al. 1997). Due to the lack of cystathionase in the fetal human liver and brain, both cysteine and methionine are essential to the fetus and conserved for protein synthesis during rapid growth (Lemons 1979). This conservation is demonstrated in human pregnancies at 8-12 weeks gestation; at that time point, methionine was reported to be 4 times higher in the extra-embryonic coelomic fluid and 2 times higher in the AF versus maternal serum (Steegers-Theunissen et al. 1997). In our study, AF methionine concentrations had the most notable increase, rising 87% when maternal glucose intake increased from ≤12% to =24%. On the other hand, the concentration of AF methionine increased 364%, from 45 ± 16 μmol/L at a fetal weight of <2.65 g to 209 μmol/L with mean fetal weights >4.21 g. Additionally, methionine as well as cysteine had high β-coefficients when associated with fetal weight in a multiple linear regression model. Thus, methionine's decidedly lower concentration in AF in our growth-retarded

fetuses supports the possibility that its shortage during pregnancy may be detrimental to fetal growth and development; previous reports in rats described disturbed morphogenesis and the development of neural tube defects (Steegers-Theunissen et al. 1997). For cysteine, a high β-coefficient was associated with a maximum change of 2 μmol / L from d 18 – 21, which would produce no more than a 124 mg increase in fetal weight. The existence of these low concentrations of AF cysteine is likely due to the absence cystathionase in the fetal brain and liver or to slow placental transfer of cysteine to protect the fetus from toxic cysteine metabolites (Lemons 1979).

Starvation markedly increased plasma BCAA concentrations (Felig et al. 1972), whereas hyperglycemia decreases all maternal and fetal plasma amino acids by >50%, especially fetal BCAAs, and essential amino acids (Thureen et al. 2000). BCAAs have regularly been associated with protein synthesis, growth and tissue repair (Harris et al. 1994) and pancreatic development (Milner et al. 1972). BCAAs are of particular interest in fetal growth as they may be transported to the fetus preferentially (Jozwik et al. 2001), rapidly crossing the placenta (Chung et al. 1998). Others have suggested the existence of non-maternal sources such as the yolk sac as an important precursor pool (Beckman et al. 1997). Our study reported that concentrations of AF BCAAs were unaltered by maternal glucose intake if gestational age was controlled for in the model and strongly suggested that limiting only maternal dietary glucose was not sufficient to perturb AF BCAA concentrations. BCAAs in our study were associated with gestational age. Because of these results, it is likely that these amino acids were not the regulators of sustained fetal gluconeogenesis when glucose was limited, as was suggested (Hay 1991). In agreement with our results, Jozwik et al (Jozwik et al. 2001) reported that fetal plasma glucose levels were not changed after an infusion of BCAAs. This suggests that the fetal utilization of BCAAs is independent of changes in maternal plasma glucose concentrations (Jozwik et al. 2001).

In humans, concentrations of several AF amino acids, including BCAAs, alanine, and lysine reportedly decrease towards the end of pregnancy (Mesavage et al. 1985; Dallaire et al. 1974; O'Neill et al. 1971); however, small increases in AF methionine, phenylalanine, taurine and tyrosine were also reported (Felig et al. 1972; McEvoy-Bowe et al. 1987) in rat AF, with the exception of a decreasing trend for taurine (McEvoy-

Bowe et al. 1987), which was also observed in the present study. Changes in AF amino acid concentrations as gestational age progressed were most likely regulated by tissue growth, protein deposition, and fetal energy demands, which are developmentally regulated (Bell et al. 1999). Other variations in AF concentrations during development may also be attributed to factors such as fetal skin keratinization, maturation of renal functions (Domenech et al. 1986), and fetal swallowing (Queenan 1978). It is believed that mothers retain essential and nonessential amino acids equally, whereas the fetus retains more essential amino acids (Arola et al. 1982; Velazquez et al. 1976). Amino acids that are generally retained by the fetal tissues are aspartic acid, cysteine, methionine, lysine, phenylalanine, serine, and threonine (Velazquez et al. 1976); therefore, it was not surprising to find that these AF concentrations changed as development progressed in our study. Those that were released by fetal tissues are arginine, glutamic acid, glutamine, and proline (Velazquez et al. 1976), and in our study, all of these were correlated with changes in gestational age.

AF taurine differed notably from all other amino acids as it was negatively associated with birth weight. As a multifunctional amino acid, it was not surprising that our study reported levels of AF taurine that varied with fetal weight, although the tendency to repeatedly from other amino acid trends was intriguing. As a predominant intracellular free amino acid (Chesney et al. 1998) and a potent antioxidant (Lima & Jaffe 1998), taurine reportedly played a role in retinal development and rat glial cell function (Moran et al. 1996). It also serves as an important organic osmolyte in the brain and kidney, contributing to cell volume regulation (Aerts & Van Assche 2002). It may also play this role for AF volume near term as both declined. Throughout gestation, taurine is present in the human fetal brain, liver, retina, plasma and placenta in higher amounts than in the respective adult tissues (Lemons 1979), and it is found in high concentrations (68 μmol/L) in the AF of infants born appropriate for gestational age (Velazquez et al. 1976). However, its specific role in fetal growth is open to interpretation and speculation. We suggest that the higher concentrations of AF taurine that were associated with the lower fetal weights in our study could point to its lower utilization by the relatively immature pups given its involvement in several growth processes.

Please note that all references are combined beginning on page 62.

TABLE 3.1

Dietary and developmentally induced changes in AF amino acid concentrations during the last 4 d of gestation in rats whose dams were fed varying levels of carbohydrate

	Dietary glucose,[1] %		
	=12%	=24%	Main effect[2]
	μmol/L		
Individual amino acids			
Methionine	72 ± 12[a]	135 ± 20[b]	0.0281
Phenylalanine	436 ± 24[a]	565 ± 40[b]	0.0297
	Gestational Age,[3] %		
	18/19	20/ 21	Main effect[4]
Individual amino acids			
Alanine	833 ± 50[a]	1239 ± 96[b]	0.0005
Glutamic Acid + Glutamine	1409± 61[a]	1910 ± 141[b]	0.0012
Glycine	298 ± 14[a]	438 ± 32[b]	0.0001
Isoleucine[5]	205 ± 14[a]	254 ± 12[b]	0.0079
Leucine	357 ± 32[a]	520 ± 36[b]	0.0011
Methionine	39 ± 5[a]	165 ± 18[b]	<0.0001
Phenylalanine	396 ± 26[a]	587 ± 32[b]	<0.0001
Proline	819 ± 24[a]	973 ± 43[b]	0.0029
Serine	410 ± 28[a]	575 ± 39[b]	0.0017
Taurine	67 ± 7[b]	34 ± 5[a]	<0.0001
Threonine	245 ± 23[a]	428 ± 31[b]	<0.0001
Valine	435 ± 27[a]	587 ± 32[b]	0.0012
Grouped Amino Acids			
Essential[6]	4257 ± 157[a]	5618 ± 267[b]	<0.0001
Non Essential[7]	1777 ± 93[a]	2470 ± 167[b]	0.0003
Conditionally Essential[8]	1358 ± 60[a]	1840 ± 133[b]	0.0011
Total amino acids	7223 ± 276[a]	9744 ± 525[b]	<0.0001

[1]Values are means ± SEM, n = 41 (=12% glucose) and n = 32 (=24% glucose). [2]Main effect of diet. [3]Means ± SEM; gestational age is divided into 2 subgroups: 18/19 (n = 38 litters) and 20/21 (n = 35 litters). [4]Main effect of gestational age. [5]Isoleucine; gestational age d 18/19, n = 27 litters; gestational age d 20/21, n = 31 litters. [6]Essential amino acids (arginine, asparagine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tryptophan, tyrosine, valine). [7]Non-Essential amino acids (alanine, aspartic acid, serine) [8]Conditionally Essential amino acids (cysteine, glutamine, glycine)

TABLE 3.2

AF amino acid concentrations across quartiles of fetal weights in rats whose dams were fed varying levels of carbohydrate[1]

	Fetal weight, g				
	<2.65	2.65 - 3.30	3.31 - 4.21	>4.21	p-value
Mean fetal weight (g)	2.16 ± 0.08	2.93 ± 0.05	3.75 ± 0.07	5.31 ± 0.15	p<0.0001
			µmol/L		
Individual Amino Acids					
Alanine	853 ± 93[a]	839 ± 74[a]	1075 ± 116[ab]	1354 ± 134[b]	0.0064
Cysteine	7 ± 1[a]	6 ± 0.5[a]	6 ± 0.5[a]	9 ± 1[b]	0.0048
Glutamic Acid + Glutamine	1410 ± 107[a]	1416 ± 121[a]	1724 ± 128[ab]	2082 ± 225[b]	0.0151
Glycine	296 ± 27[a]	320 ± 25[a]	390 ± 45[ab]	458 ± 39[b]	0.0016
Leucine	358 ± 51[a]	378 ± 46[a]	458 ± 51[ab]	551 ± 49[b]	0.0431
Lysine	1324 ± 92[a]	1193 ± 42[a]	1318 ± 84[a]	1769 ± 208[b]	0.0322
Methionine	45 ± 16[a]	42 ± 11[a]	106 ± 13[b]	209 ± 24[c]	<0.0001
Phenylalanine	367 ± 38[a]	389 ± 27[a]	573 ± 39[b]	649 ± 47[b]	<0.0001
Proline	867 ± 36[a]	786 ± 26[a]	840 ± 44[a]	1082 ± 66[b]	0.0005
Taurine	67 ± 6[a]	62 ± 15[a]	46 ± 7[a]	29 ± 7[b]	<0.0001
Threonine	186 ± 31[a]	305 ± 41[b]	429 ± 44[c]	419 ± 34[c]	<0.0001
Tyrosine	66 ± 4[a]	52 ± 3[a]	54 ± 4[a]	93 ± 13[b]	0.0009
Grouped Amino acids					
Essential[2]	4280 ± 252[a]	4233 ± 233[a]	5002 ± 252[a]	6157 ± 410[b]	<0.0001
Non Essential[3]	1820 ± 177[a]	1808 ± 137[a]	2198 ± 207[ab]	2626 ± 233[b]	0.0084
Conditionally Essential[4]	1348 ± 103[a]	1359 ± 117[a]	1703 ± 130[ab]	1960 ± 202[b]	0.0156
Total amino acids	7277 ± 475[a]	7230 ± 439[a]	8748 ± 546[a]	10535 ± 788[b]	0.0002

[1] Values are means ± SEM, n = 18/quartile with gestational age (d 18-21) included as a covariate. Means in a row with superscripts without a common letter differ, $P < 0.05$. [2]Essential amino acids (arginine, asparagine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tryptophan, tyrosine, valine). [3]Non-Essential amino acids (alanine, aspartic acid, serine). [4]Conditionally Essential amino acids (cystine, glutamine, glycine).

TABLE 3.3

Amino acid concentrations in rat AF as a predictor of fetal weight using multiple regression[1,2]

	ß-coefficient	P-value	Variability captured
	mg		*%*
Gestational age	1167.67	<0.0001	76.6
Amino acids			
Cysteine	62.410	0.0046	79.8
Lysine	0.302	0.0306	79.3
Methionine	2.410	0.0252	79.4
Tyrosine	6.680	0.0034	80.7

[1] Mean fetal weight (mg) per litter as dependent variable with n = 73
[2] Each amino acid was added individually to the multiple regression model, and only those that were significant are reported.

SUMMARY AND CONCLUSIONS

The present project was the first to investigate the role of AF amino acids as predictors of infant birth weight at mid gestation, and the first to produce an intrauterine growth retarded model by using graded levels of carbohydrate restriction to investigate its effect on AF amino acids.

Several amino acids emerged as birth weight predictors for both studies, although no amino acids were similar for both. When investigating AF amino acids at mid gestation in humans; arginine, 3 methyl histidine, and tryptophan were positive predictors of final birth weight, while ornithine was a negative predictor. When looking at different levels of carbohydrate restriction in rats; cysteine, lysine, methionine and tyrosine in late gestation were all positive predictors of pup weight. Although there are discrepancies seen between the amino acids that emerged as predictors for the human study versus those for the rat study, the variations between the two studies would easily explain these differences. Biologically, the rat versus human model differ by length of gestation, AF volume, and the fact that human pregnancies in this study only had a single birth, while the rat models gave birth to large litters, putting more strain on the rat dam during a shorter gestation time. Other factors which were imposed by us as investigators, were the dietary restrictions on the rat model and not in the human model, and the time of AF sampling, as AF was sampled in late gestation for the rat pregnancies and at mid gestation for the human pregnancies.

In our human study, several maternal characteristics were reported to be significant predictors of infant birth weight, such as gestational age, infant gender, maternal smoking and pre pregnancy BMI. The use of percentiles in this study to rate the level of fetal growth is important and novel as this type of classification controls for the infant's birth weight, gestational age and gender (Kramer et al. 2001). Additional covariates of maternal smoking and prepregnancy BMI were included in our analysis of variance model, as they are reported to be significant predictors of infant birth weight by Kramer (1987). Previous research has looked at the effect of maternal body composition on infant birth weight, where Aguirre et al. (2003) reported that fat-free mass and total body water explained a major proportion of the variability of birth weight in comparison with the mother's weight gain during the pregnancy period. In maternal plasma, amino

acid concentrations were reported to be affected by maternal weight, where during pregnancy, obese women reported lower overall amino acid concentrations, and lower alanine, histidine, lysine, proline, serine, taurine and threonine levels than lean control subjects (Kalkhoff et al. 1988). Postpartum, these plasma levels returned to high concentrations in the obese women, and levels of tyrosine, phenylalanine and BCAAs even exceeded those of lean mothers (Kalkhoff et al. 1988). This finding had prompted the suggestion that despite these high levels of amino acids in the plasma of obese mothers before pregnancy, the fetus' of obese mothers may utilize greater amounts of amino acids in-utero, consequently lowering maternal plasma levels during pregnancy. Maternal smoking, another strong controlling factor has been reported to have an independent influence on birth weight loss, where an average of 19 g of birth weight is lost for each cigarette smoked per day during pregnancy (Adriaanse et al. 1996).

When investigating gestational age, several studies have identified a decreasing trend in AF amino acid concentrations throughout gestation (Dawson et al. 1999; Rabier et al. 1996, Mesavage et al. 1985; Schulman et al. 1972). Some have examined the relationship of amino acids with fetal metabolic maturity (Jauniaux et al. 1999; Jones & Rolph 1985), although only one has associated AF amino acids at mid gestation with subsequent gestational age of the infant (Mesavage et al. 1985). Numerous amino acids differed across amniocentesis wk for the human trial and across gestation days for the rat trial, although it was interesting that none emerged as predictors of gestational age for either study.

RP-HPLC analysis was used for both human and rat studies, which was chosen because of its higher sensitivity and accuracy as compared to previous studies which employed other analytical techniques such as ion-exchange chromatography (Pettit & Allen 1980, Mesavage et al. 1985, Rabier et al. 1996), gas chromatography (Schulman et al. 1972, Kwokei et al. 1982), and amino acid analyzers (Dawson et al. 1999, Jauniaux et al. 1999, Jauniaux 1998, Rabier et al. 1996). Using the PICOTAG method, pre column derivatization of amino acids with PITC was able to detect 23 primary and secondary amino acids in concentrations of < 1 pmol (Cohen & Strydom 1998), with good sensitivity and specificity (Cohen & Strydom 1986, Buzzigolli et al. 1990), although cysteine was undetectable. FMOC derivatization was selected for its ability to derivatize

low picomolar amounts of stable amino acids under fluorescence in a short amount of time (Malmer & Schroeder 1990; Miller et al. 1990; Schuster 1988), and detected 18 amino acids including cysteine. The two methods differed where the PICOTAG analysis was longer to carry out as the samples needed to be vacuum dried after each step, and derivatization was done in the laboratory for 20 minutes before samples were injected into the HPLC. FMOC analysis did not require any vacuum drying, and samples were derivatized immediately prior to injection into the HPLC system by the autoanalyzer, greatly decreasing analysis time. Over 30 amino acids have been detected in AF over the past 30 years by various laboratory techniques (Jauniaux 1998; Mesavage et al. 1985; A'Zary et al. 1973; Scott 1972). We used HPLC analysis for this study, and regardless of technique, the amino acid concentrations in human AF were in agreement with previous research (Jauniaux et al. 1999, Rabier et al. 1996, Mesavage et al. 1985), where we found the highest amino acid concentrations in human AF to be alanine, glutamic acid + glutamine, lysine, proline and valine, and the lowest to be tryptophan, citrulline, ornithine, serine and 3-methylhistidine. Although these studies were limited to studying a maximum of 23 amino acids, we recognize that other related products have been detected in amniotic fluid, and that these may be detected in future studies.

Some limitations were evident for these two studies. When investigating the amino acid concentrations in human AF, several factors would have been interesting to incorporate into our analysis, such as trends in the maternal diet due to research showing evidence in humans (Felig et al. 1972; Adibi & Drash 1970) and animals (Pastor-Anglada et al. 1986; Arola et al. 1982) that the maternal diet can affect the amino acid content of the fetal compartment and even AF. Other factors may be maternal vitamin intake and exercise frequency, as well as trends in paternal age, race, diet, smoking and exercise. Another limitation was that of sample size. While the overall sample was very large in comparison with past research (Dawson et al. 1999, Jauniaux 1998, Rabier et al. 1996, Sims et al. 1993, Mesavage et al. 1985), and represented the birth weights of the Canadian population, the high rate of health conscious older mothers provided us with a very small sample size of infants born small for gestational age. Sample size was also an issue for our rat trial, where small sample sizes within each group forced us to collapse our 4x4 factorial analysis into a 2x2 factorial analysis. This did not allow us to

investigate the differences between AF amino acids in pups of mothers fed 0% vs 12% carbohydrate, 24% vs 60% carbohydrate, or across gestational days 18 vs 19, or gestational days 20 vs 21.

Although the aforementioned limitations were present, both studies were novel and presented strengths. The human study was the first to associate mid gestation AF amino acids with fetal growth in such a large overall sample size of healthy, full term pregnancies. Our second study, using a rat model, was the first to create an intrauterine growth retarded model and associate graded levels of carbohydrate with AF amino acids. Overall, our results suggest that several AF amino acids are associated with and predictive of infant or pup weight at birth. This research gives us great insight into the role that AF amino acids play as a nutrient source to the developing fetus, and as early biomarkers of in-utero fetal growth.

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APPENDIX I

PICOTAG METHOD

PICOTAG Analysis Protocol

A. PREPARATION OF STOCK SOLUTIONS / REAGENTS

1. Internal Standard Preparation

   Norleucine solution (200 µM)

   In a volumetric flask, mix

   - 0.0026 g norleucine (MW 131.18 g)
   - 100 ml DDW

   Store in the refrigerator

2. Eluent Preparation

   Eluent A

   a. In a volumetric flask, prepare the stock EDTA solution
      - 100mg EDTA with 100ml DDW, label and refrigerate
   b. In a 2L Erlenmeyer flask, prepare the Sodium acetate trihydrate
      - 38 g sodium acetate trihydrate
      - 2 L DDW
      - Add 1 ml triethylamine (TEA) under stirring
      - Add 0.4 ml stock EDTA solution
      - Titrate to pH 6.2 to 6.44 with glacial acetic acid
      - Filter and degas by sonicating under vacuum

   Eluent B – Acetonitrile – HPLC grade

   Eluent C – DDW

3. Reagent Preparation

   Drying solution (make 500 µL):

   - Prepare 1 M sodium acetate (1.36 g in 10 ml)
   - Mix methanol: sodium acetate (1M) : triethylamine in a 2:2:1 ratio

Derivatization Reagent (make 10 ml):

- Mix methanol: triethylamine: DDW: phenylisothiocyanate (PITC) in a 7:1:1:1 ratio
- Close vial under nitrogen
- Do not make in advance, since PITC is very sensitive to atmospheric oxygen. When you open the 10 X 1 ml vial, you must store the remainder under nitrogen in an epindorff tube labeled PITC - in the –80 freezer until you are ready to make the next batch.

B. SAMPLE PREPARATION

1. Obtain samples from –80 freezer and thaw in refrigerator overnight.
2. Vortex thawed sample for 5-10 seconds
3. In an ultracentrifuge device, place a 1:1 ratio of sample: internal standard (Norleucine). (100 µL : 100 µL). Vortex to mix.
4. Label ultracentrifuge device with sample number followed by – dp. (e.g. A163 – dp). This indicates that the sample has internal standard added to it and has been deproteinated.
5. Centrifuge at 1500g for 30 minutes to deproteinate.
6. Discard top portion of ultracentrifuge device.
7. Sample storage: Store deproteinated samples in zip lock bags in –80 Freezer
   On a large zip lock bag indicate that samples that have been deproteinized
   E.g. KE# Amniotic Fluid
   Sample + Norleucine (200µM) (1:1 – 100 µL : 100 µL)
   Deproteinated Samples
8. When ready for analysis, remove samples from freezer and thaw overnight
9. Pipette 25 µL of filtered sample into 6 x 50 mm sample tube and vacuum dry. Freeze remainder of sample + norleucine at -20 C
10. Add 10 µL of drying solution, vortex to mix and vacuum dry
11. Add 20 µL derivatization reagent, vortex to mix and let stand for 20 minutes at room temperature. Vacuum to dry

12. Reconstitute dried sample with 100 µL Pico.Tag Diluent (Waters). Vortex to dissolve
13. Transfer sample into HPLC sample tubes for analysis
14. Samples can be stored in refrigerator for 48 hours or freezer at -20 C for 96 hours

C. STANDARD PREPARATION

1. Using the standard amino acid solutions (10 X 1ml vials) – these solutions have 18 amino acids in them, all at 2.5mM except cystine at 1.25mM. Any amino acids not included in these 18 must be processed separately
   a. Label epindorff tubes S-01 to S-10. Make the dilutions shown in the table (IS = Internal Standard = Norleucine (2.5mM))

	S-1	S-2	S-3	S-4	S-5	S-6	S-7	S-8	S-9	S10
AA std	100	90	80	70	60	50	40	30	20	10
DDW	0	10	20	30	40	50	60	70	80	90
IS	100	100	100	100	100	100	100	100	100	100
[aa] (mM)	2.5	2.25	2	1.75	1.5	1.25	1	0.75	0.5	0.25
[cystine]	1.25	1.13	1	0.88	0.75	0.63	0.5	0.38	0.25	0.13

2. Store all standards at –80 immediately
3. Label standards as 'standards + (2.5mM) (1:1), date made, PICOTAG METHOD.

D. PEAK IDENTIFICATION

Prepare 2.5mM solutions of each amino acid and dilute to make 0.25mM solutions (1ml 2.5mM : 9ml DDW) of each. Run individually.

NOTE:

- You will need to run standards each time you change the column. A PICOTAG column is good for about 150-200 runs. Please read the instructions regarding column care and maintenance – to increase the life of the column – especially if you are not planning to conduct any runs for a period of time.
- Allow PITC to come to room temperature before opening to prevent condensation
- Be sure PITC is completely dissolved yielding a clear solution with no oily droplets at bottom of vial
- Vortex reagent and allow to stand 5 mins before using. Use solution within 2 hr
- Blanket TEA with N gas and store in fridge (maximum 3 months)
- Transfer PITC to small vial with Teflon cap for storage. Blanket with N gas, cap tightly and store in freezer for up to 3 weeks from date of opening ampule.

E. ELUTION ORDER

Time	Flow	%Eluent	%H2O	%ACN
	1	94	0	6
1	1	94	0	6
21	1	56	16	28
21.5	1	0	40	60
22	1.5	0	40	60
26	1.5	0	40	60
26.5	1.5	94	0	6
39	1.5	94	0	6
39.5	1	94	0	6

Column: 250 x 4.6 mm reverse phase

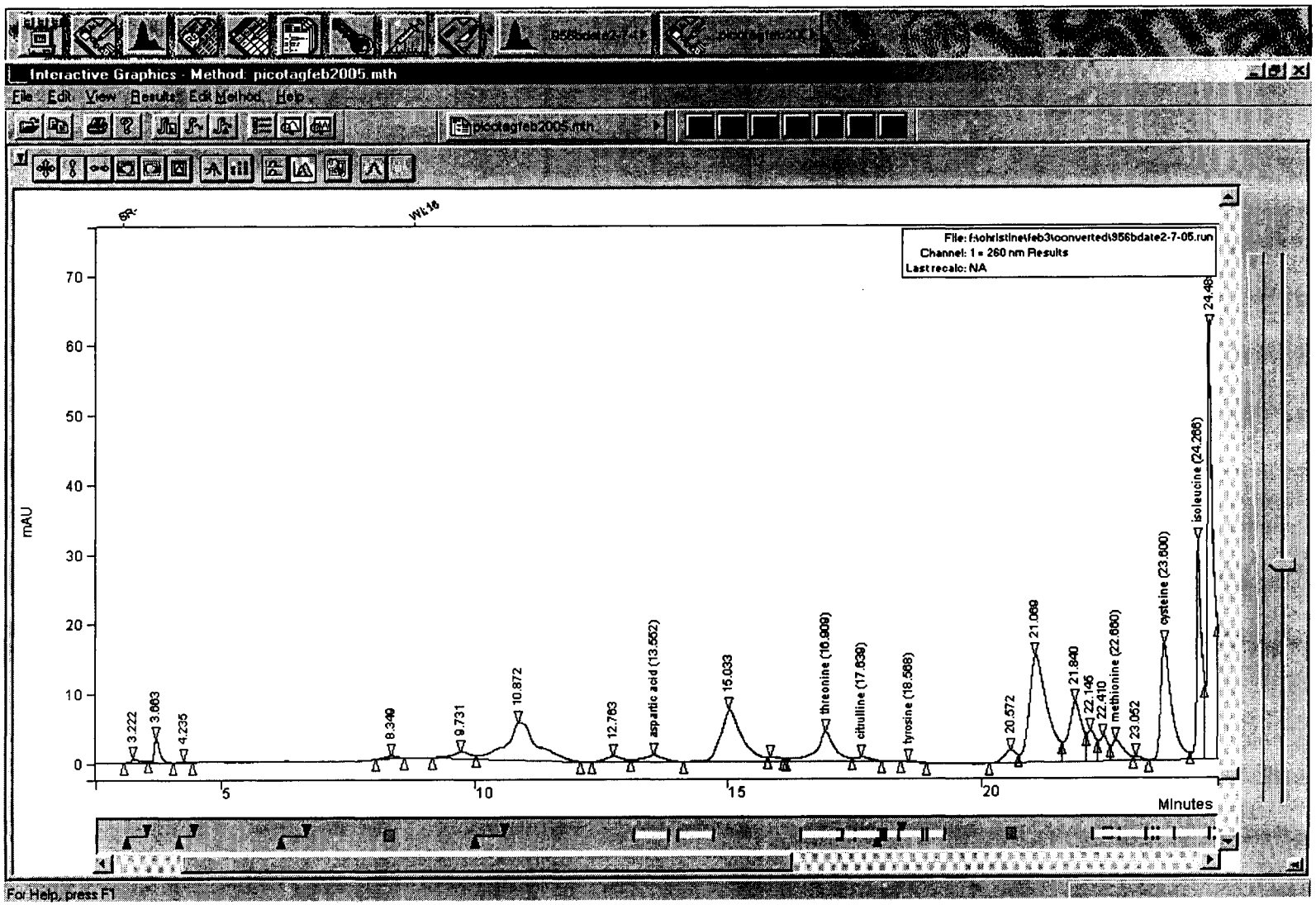
Temperature: 46 degrees Celsius

Inject volume: 20 uL

UV detector: 245 nm (maxima for PTC derived amino acids)

APPENDIX II

Sample chromatogram from PICOTAG Analysis


Interactive Graphics - Method: picotagfeb2005.mth
File: f:\ohristine\feb3\converted\956bdate2-7-05.run
Channel: 1 = 260 nm Results
Last recalc: NA
mAU
Minutes
3.222
3.663
4.235
8.349
9.731
10.872
12.783
aspartic acid (13.552)
15.033
threonine (16.909)
citrulline (17.639)
tyrosine (18.568)
20.572
21.069
21.840
22.145
22.410
methionine (22.660)
23.052
cysteine (23.800)
isoleucine (24.266)
24.48
For Help, press F1

APPENDIX III

Comparison between PICOTAG and FMOC methods

	PITC	FMOC
Derivatization Method	Precolumn[1]	Precolumn[1]
Derivatization Time	20 mins[2]	5 mins[3]
Analysis Time	60 mins[4]	23 mins[5]
Lifetime of Column	Shorter than FMOC[5]	Long[5]
Detection Method	UV 254 nm[4]	UV or fluorescence[5]
Sensitivity	50 x lower than FMOC[5]	High[5]
Primary & Secondary AAs	YES[5]	YES[5]
Problematic Amino Acids	Ornithine, Histidine, Tryptophan, Cysteine[5]	Histidine, Tryptophan, Cysteine[5]

[1]Bidlingmeyer et al (1984), [2]Fierabracci et al (1991), [3]Sarwar et al (1993), [4]Cohen & Strydom (1988), [5]Furst et al (1990)

APPENDIX IV

Composition of rat diet

Dietary Composition of rat diets

	Glucose Level			
Ingredient, g	**0%**	**12%**	**24%**	**60%**
Glucose	0	12	24	60
Soybean oil	39.64	34.91	30.18	16.00
Cellulose	41.36	34.09	26.82	5.00
Casein	11	11	11	11
Vitamin mix	1.2	1.2	1.2	1.2
Mineral mix	5.5	5.5	5.5	5.5
DL-Methionine	0.34	0.34	0.34	0.34
Sodium bicarbonate	1.00	1.00	1.00	1.00
Weight, g	100.04	100.04	100.04	100.04
Metabolizable energy kJ/g	17.4	17.4	17.4	17.4

- **Glucose** – Dextrose (anhydrous), ICN Biochemicals Canada, Mtl, Qc
- **Soybean oil** – Degummed soybean oil, Canadas Packers, Mtl, Qc
- **Cellulose** – Alphacel, ICN Biochemicals Canada
- **Casein** – High nitrogen casein ((CN Biochemicals Canada), containing 86.5% ptn (N*6.25) or 89.1% ptn (N*6.71)
- **Vitamin mix** (mg/kg diet): niacin 100; calcium pantothenate, 32; riboflavin, 12; pyridoxine hydrochloride, 24; thiamin hydrochloride, 16; folacin, 4; biotin, 1; cyanocobalamine, 0.2; all-rac-alpha-tocopheryl acetate, 60; menaquinone, 0.6; choline chloride, 4000; cholecalciferol 0.039; retinyl palmitate, 2.75.
- **Mineral mix** (g/kg diet): CaHPO4, 26.44; KHCO3, 20.35; NaCl, 2.82; MgSO4, 4.38; CrK(SO4)2-12H2O, 0.0064; CuCO3-Cu(OH)2-H2O, 0.0198; KIO3, 0.0006; FeSO4-7H2O, 0.3931; MnCO3, 0.1046; ZnCO3, 0.1726; Na2SeO3, 0.0005; NaMoO4-2H2O, 0.0177; KF-2H2O, 0.0111.

APPENDIX V

Ethics Approval – Human Study

McGill

School of Dietetics and Human Nutrition

Faculty of Agricultural and Environmental Sciences

McGill University
Macdonald Campus

École de diététique et nutrition humaine

Faculté des sciences de l'agriculture et de l'environnement

Université McGill
Campus Macdonald

Tel.: (514) 398-7842
Fax: (514) 398-7739

21,111 Lakeshore
Ste-Anne-de-Bellevue
Québec, Canada H9X 3V9

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

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

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

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

Veuillez agréer, madame, mes meilleures salutations.

McGill

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FORMULAIRE DE CONSENTEMENT POUR UN PROTOCOLE DE RECHERCHE

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

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

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

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

Protocole de recherche :

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Signature du sujet _____ Date _____

Témoin _____ Date _____

Veuillez remplir le questionnaire ci-joint.

McGill

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Questionnaire

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

Nom : _____ Numéro de téléphone : _____

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

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

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

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

Poids avant la grossesse : ____ livres ou ____ kilos

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

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

Nom de l'obstétricien/gynécologue : _____

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

Pendant ma grossesse, j'ai consommé en moyenne :

0-1 boisson alcoolique par semaine ____
2-5 boissons alcooliques par semaine ____
6-10 boissons alcooliques par semaine ____
11-15 boissons alcooliques par semaine ____

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

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

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

APPENDIX VI

Ethics Approval – Animal Study

APPENDIX VII

Co-author Approval

APPENDIX VIII

Copyright Waiver Form