Determination of protein requirements of healthy pregnant women during early and late gestation using the indicator amino acid oxidation technique

by

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Abstract

Adequate maternal dietary protein (PRO) intake is necessary to support rapid tissue accretion during a healthy pregnancy. Both insufficient and excessive maternal PRO intake during pregnancy is associated with intrauterine growth restriction (IUGR) of the fetus. IUGR increases the risk of neonatal morbidity and mortality, and is associated with an increased risk of future health problems, including cardiovascular disease, kidney disease, obstructive airway disease, and obesity. However, current PRO intake recommendations for healthy pregnant women are based on factorial calculations of nitrogen balance data derived from non-pregnant adults. Thus, an estimate of PRO requirements based on pregnancy-specific data is needed. PRO requirements of healthy pregnant women at 11-20 (early) and 31-38 (late) weeks gestation were determined using the indicator amino acid oxidation (IAAO) method. Twenty-nine healthy women (age 24-37) each randomly received a different test PRO intake (range = 0.22-2.56 g/kg/day) during each study day in early and late gestation (n_{early} = 37, n_{late} = 44). The diets were isocaloric and provided energy at 1.7 X resting energy expenditure (REE). PRO was given as a crystalline amino acid mixture based on egg PRO composition, except phenylalanine and tyrosine, which were maintained constant across intakes. PRO requirements were determined by measuring the oxidation of L-[1-^{13}C]phenylalanine to $^{13}CO_2$ ($F^{13}CO_2$). Breath and urine samples were collected at baseline and isotopic steady state. Linear regression crossover analysis identified a breakpoint (requirement) at minimal $F^{13}CO_2$ in response to different PRO intakes.

The estimated average requirement (EAR) for PRO in early and late gestation was determined to be 1.22 and 1.52 g/kg/d, respectively. Both of these estimates are significantly greater than the EAR of 0.88 g/kg/d currently recommended by the Dietary Reference Intakes (DRI 2005). Our results indicate increased demand for PRO before 20 weeks gestation (on a gram per kilogram body weight basis), a consideration that has not been addressed by current DRI recommendations. This study is the first to directly estimate gestational PRO requirements in a population composed solely of healthy pregnant women, and suggests that current recommendations based on the nitrogen balance method and factorial calculations underestimate PRO requirements.

Preface

This project was approved by the University of British Columbia and the British Columbia Children's and Women's Hospital research ethics board in accordance with the Declaration of Helsinki principles. Human ethics certificate number: H11-00070. Clinical Trials.gov identifier: NCT01784198.

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BIA – bioelectric impedance analysis

DRI – Dietary Reference Intakes

EAR – estimated average requirement

EG – early gestation

$F^{13}CO_2$ – rate of appearance of heavy carbon (^{13}C) labeled carbon dioxide in breath

FAO – Food and Agriculture Organization

FFM/LBM – fat free mass/lean body mass

hCG – human chorionic gonadotropin

IAAO – indicator amino acid oxidation

IQR – interquartile range

IUGR – intrauterine growth restriction

LBW – low birth weight

LG – late gestation

RDA – recommended dietary allowance

REE – resting energy expenditure

UN – United Nations

V_{CO2} – volume of carbon dioxide production per minute

V_{O2} – volume of oxygen production per minute

WHO – World Health Organization

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Dedication

For Mom, Dad, and Jeff

Chapter 1. Introduction

Arguably the most exquisite event in the human lifecycle is the series of highly orchestrated biological pathways that transform two haploid cells into an autonomous being during a 40-week gestation period. Strong genetic selection has molded human reproduction into a relatively predictable process, but many environmental influences represent modifiable determinants of pregnancy outcomes. Increasingly it has become apparent that sub-optimal maternal nutrition adversely affects maternal and fetal health. For example, insufficient folic acid intake before and during pregnancy is associated with neural tube, cardiovascular, and urinary tract defects, and cleft palate (Hernández-Díaz *et al.*, 2000); fetal brain development and early childhood visual acuity is linked to docosahexaenoic acid (DHA) intake during pregnancy (Innis, 2007; Innis and Freisen, 2008); and maternal fat and carbohydrate intake is associated with changes in hormone balance that affect fetal growth (King, 2006; Jansson *et al.,* 2008). Globally, the most pervasive affliction caused by sub-optimal maternal nutrition is intrauterine growth restriction (IUGR), resulting in low birth weight infants (LBW; WHO, 2002). According to the WHO, 24% of children born annually are classified as LBW (WHO, 2002). LBW infants are at an increased risk of neonatal morbidity and mortality, with an associated increase in disability-adjusted lifestyle years and disease susceptibility in later life (WHO, 2002). Central to this issue is the availability of high-quality or 'complete' protein in the diet, because it has been suggested that dietary protein is the macronutrient with the single greatest influence on birth

weight (Cuco *et al*., 2006). Epidemiological studies have linked both insufficient and excessive dietary protein intake to an increased risk of IUGR, although the mechanism for the latter remains unclear (Godfrey *et al*., 1996; Sloan *et al*., 2001; Kramer and Kakuma, 2003; Moore *et al*., 2004; Imdad and Bhutta, 2011). Dietary protein and IUGR are related in developing and developed countries for opposing reasons. In developing countries, access to complete dietary protein is limited by socio-economic status, and women are more likely to suffer from insufficient protein intake (Gebre-Medhin and Gobezie, 1975; Müller and Krawinkel, 2005). In developed countries, the popularity of high-protein diets may be cause for concern if the percentage of energy from protein exceeds a healthy intake (Kramer and Kakuma, 2003). However, current protein intake recommendations for healthy pregnant women are based on factorial calculations of nitrogen balance data derived from non-pregnant adults. Thus, it is necessary to define protein requirement directly during healthy pregnancy, and thereby encourage maternal nutritional status that optimizes pregnancy outcomes. The objective of this study was to define optimal protein requirement during healthy pregnancy using a modern, state-of-the-art stable isotope-based method – the indicator amino acid (IAAO) method.

Chapter 2. Background

2.1. Metabolic adaptations to pregnancy

Pregnancy is an exceptional stage of life defined by rapid growth and development, and enormous maternal physiological changes from the time of conception to parturition. In addition to fetal development, there is rapid growth of maternal tissues like the heart, blood, breasts and uterus, and fetal-support tissues like the placenta and extra-embryonic membranes (Boron and Boulpaep, 2003). To accommodate this increased demand, maternal metabolic adaptations to pregnancy arise. The overall effect of these adaptations results in two discrete metabolic phases: anabolic and catabolic. The first two-thirds of gestation are defined by anabolic adaptations that prepare the pregnant body for the impending energetic demands of the fetus by building fat stores (Knopp *et al.,* 1981; Herrera, 2000). Fat accumulation is facilitated by increased insulin release, glucose utilization, and lipogenesis, normal glucose tolerance, and decreased lipolysis (Knopp *et al.*, 1981; Butte, 2000). During this stage, the demand for energy is high, with ~36,000 kcal of energy required to build fat stores (King, 2006). During the last third of gestation, nutritional demands of the fetus increase markedly. In response, maternal lipid utilization increases, and glucose and amino acids are spared for the fetus. This phase is defined by catabolic adaptations that include decreased protein catabolism, increased maternal insulin resistance, hepatic glucose production, lipolysis, and fat mobilization, and decreased glucose tolerance and lipogenesis (Knopp *et al.*, 1981; Butte, 2000; King, 2006). Despite this elegant metabolic response, mothers must

consume more nutrients in order to provide the additional biochemical substrates needed to support a healthy pregnancy.

2.2. Maternal nutrition

2.2.1. Impact of maternal nutrition on infant health

To support gestational demands, healthy pregnant women have different nutritional requirements than non-pregnant adults. The importance of maternal nutrition is illustrated in a number of epidemiological studies, perhaps most notably by the exhaustive follow-up of Dutch famine survivors. During the winter of 1944-1945, German troops restricted food supply to many areas in the Netherlands, resulting in adult energy intakes of less than 600 kCal per day. Prenatal exposure to these conditions resulted in lower birth weight, lower placental weight, reduced length at birth and reduced head circumference in the offspring (Stein and Susser, 1975). Long-term cohort studies have demonstrated an increased prevalence of glucose intolerance, coronary heart disease, disturbed blood coagulation, obesity, and obstructive airway disease among survivors (Roseboom *et al.*, 2006). As evidenced by the Dutch famine studies, inadequate maternal nutrition is associated with a variety of adverse pregnancy outcomes, including both short- and long-term infant morbidities and mortality. In the short-term, malnourished mothers are more likely to produce IUGR infants. IUGR infants are at an increased risk of hypoxia/asphyxiation, meconium aspiration, and hypoglycemia, which may result in death without adequate medical intervention and perinatal care (Kendig, 2007). In

the long-term, inadequate prenatal nutrition is associated with an increased risk of hypertension, coronary heart disease, respiratory disease, kidney disease and obesity (Harding, 2001). However, the hazards of poor prenatal nutrition are not limited to *insufficient* dietary intake. Unprecedented food security in recent generations has revealed that *excessive* maternal nutrition can also be detrimental (Godfrey *et al.*, 1996, Kramer and Kakuma, 2003, Moore *et al.*, 2004; King, 2006). Prospective observational studies of maternal dietary intake patterns have revealed that superfluous dietary protein or carbohydrate intake is also associated with IUGR, and excessive energy intake with infant macrosomia (i.e. birth weight >4.5 kg; Godfrey *et al.*, 1996, Sloan *et al*., 2001; Kramer and Kakuma, 2003, Moore *et al.*, 2004; King, 2006; Imdad and Bhutta, 2011). Macrosomia is associated with complicated delivery and perinatal hypoglycemia, and an increased risk of metabolic syndrome in later life (Kendig, 2007). Thus, the classic conception of 'eating for two' during pregnancy has been debunked, and the importance of 'optimal and adequate' nutrition must be emphasized.

2.2.2. Protein and amino acid nutrition during pregnancy

Dietary protein is of particular importance to maternal nutrition. Dietary protein supports fetal and placental tissue accretion, permits uterine expansion and mammary growth, and allows for an increase in blood volume via hematocrit and plasma genesis (Boron and Boulpaep, 2003). Proteins serve a variety of structural and functional purposes in the body, acting as enzymes, transporters, signaling molecules, contractile units, connective tissue, and many other roles. The vast

diversity of protein types and functions is possible because of their composition. 20 amino acids act as the building blocks from which mammalian proteins are synthesized, creating the potential for millions of unique peptide sequences (DRI, 2005; Otten *et al.*, 2006; **Table 1**). From a nutritional perspective, 9 of these amino acids are recognized as indispensable in the diet, owing to the fact that they cannot be endogenously synthesized "out of materials ordinarily available to the cell at a speed commensurate with normal growth" (Reeds, 2000; Borman *et al.*, 1946). The remaining 11 amino acids can potentially be produced in nutritionally sufficient quantities via de novo synthesis or precursor molecule modification, so long as a sufficient nitrogen source is provided (Reeds, 2000). Under specific conditions the human body may require arginine, cysteine, glutamine, glycine, proline and tyrosine – the conditionally indispensable amino acids – from dietary sources (Reeds, 2000).

However, the dispensable/indispensable paradigm is misleading because it implies that a healthy diet need only contain sufficient quantities of isoleucine, leucine, histidine, lysine, tryptophan, phenylalanine, valine, methionine, and threonine, in order to meet the needs of protein metabolism. Protein metabolism in humans is a highly integrated process dependent upon the biological availability of all 20 amino acids (WHO, 2007). All 'nonessential' amino acids require an amino acid source of nitrogen for biosynthesis (Reeds, 2000). In this regard, all 20 amino acids required for protein metabolism in humans may be adversely affected by limited protein intake. Extremes in amino acid availability, resulting from insufficient or excessive protein intake, have been identified as underlying mechanisms for IUGR. Prolonged exposure to insufficient protein intake causes

Table 1. Classification of amino acids as dispensable, conditionally indispensable, and indispensable in the diet

Indispensable	**Dispensable**	**Conditionally Indispensable**
Histidine	Alanine	Arginine
Isoleucine	Aspartic Acid	Cysteine
Leucine	Asparagine	Glutamine
Lysine	Glutamic Acid	Glycine
Methionine	Serine	Proline
Phenylalanine		Tyrosine
Threonine		
Tryptophan		
Valine		

depletion of the body's amino acid pool, thereby reducing protein synthesis and tissue accretion. Specifically, the biochemical contributions made by individual amino acids can hinder intrauterine growth. For example, arginine is integral to the synthesis of nitric oxide and polyamines, which contribute to placental angiogenesis (Wu *et al*., 2000). While healthy pregnant women are capable of synthesizing arginine, the fetus cannot and must procure arginine via the placenta (Wu *et al.,* 2000). If fetal uptake diminishes maternal arginine supplies more quickly than they can be replenished via endogenous synthesis, deficiency will ensue unless dietary protein intake can compensate for the increased demand. Alternatively, excessive amino acid availability may cause IUGR due to competition between amino acids for placental transport systems (Ronzoni *et al*., 2002; Jozwik *et al.*, 2004). Evidence from human and animal studies suggests that when placental transport systems are saturated with amino acids, competition arises for these carriers resulting in reduced transport of certain essential amino acids (Ronzoni *et al*., 2002; Jozwik *et*

al., 2004). This can then lead to deficiencies and reduced protein synthesis. Therefore, finding the 'optimal' balance among all amino acids during pregnancy is critical to ensure a healthy pregnancy.

2.3. Fetal and placental amino acid metabolism

2.3.1. Current understanding of fetal amino acid metabolism

Fetal plasma amino acid concentrations are significantly higher than maternal plasma concentrations, and this phenomenon persists throughout pregnancy (Cetin *et al.,* 1996). Although ethical and technical constraints have hindered *in vivo* studies of fetal amino acid metabolism in humans, the development of stable isotope-based methods has revealed new information (Chein *et al.,* 1993; Marconi *et al.,* 1999; Galan *et al.,* 2009; van den Akker *et al.,* 2009 and 2011). Using ^{13}C leucine and ^{15}N phenylalanine infusion into the umbilical cord of 6 pregnant women at term, protein synthesis in the fetus was calculated as ~13 g/kg/d, with net protein accretion of 2-4 g/kg/d (Chein *et al.,* 1993). This study demonstrated that protein turnover is increased in the fetus, and similar to turnover rates observed in neonates. A more recent study came to a very different conclusion, estimating net protein accretion as 1.7 g/kg/d in human fetuses at term (van den Akker *et al.,* 2009). Amino acid kinetics for leucine, phenylalanine, tyrosine, methionine, and valine have also been examined in human fetuses at term using [1-^{13}C]phenylalanine, [ring-D_4]tyrosine, [1-^{13}C, ^{15}N]leucine, [U-$^{13}C_5$]valine, and [1-^{13}C]methionine (van den Akker *et al.,* 2009 and 2011). Net accretion of 40, 28, 9.2,

and 17.5 μmol/kg/h was calculated for leucine, valine, methionine, and phenylalanine, respectively. The metabolized fraction of leucine, valine, methionine, phenylalanine and tyrosine was considerably less than what was available, suggesting a large amino acid reserve capacity in the fetus (van den Akker *et al.,* 2009 and 2011).

Although each of these studies provides new insight into how the fetus uses protein and amino acids, current data is limited to the fetus at term, and offers conflicting results. It is clear that much remains to be determined regarding fetal protein and amino acid metabolism.

2.3.2. Placental transport of amino acids

The placenta is a highly metabolically active organ that participates in protein synthesis, oxidation, transamination, and nonessential amino acid production (Hay, 1991; Battaglia and Regnault, 2001; Cetin 2001; Lewis *et al.,* 2012). Amino acids are more concentrated in the placenta than either the fetal or maternal compartments, and transfer of amino acids is asymmetric but preferentially directed towards the fetus (Schneider *et al.,* 1987). Evidence suggests that while some amino acids move rapidly from mother to fetus (e.g. lysine), others exhibit no net transfer across the placenta (i.e. glutamate, aspartate) and others still appear to flow from the mother and fetus into the placenta (i.e. serine) for the purpose of placental amino acid production (i.e. glycine; Cetin, 2001; Jannson, 2001; Avagliano *et al.,* 2012; Lewis *et al.,* 2012).

Transplacental amino acid transport occurs at strikingly different rates depending on the amino acid (Galen *et al.,* 2009). Although not fully understood,

several characteristics of the placenta contribute to this variation. The placenta uses multiple transport systems to move amino acids from the maternal side (across the microvillus plasma membrane) to the fetal side (across the basal plasma membrane; Jansson, 2001; Avagliano *et al.,* 2012; Lewis *et al.,* 2012). For example, the System A family accumulative transporters move small, neutral amino acids, while the System X^-_{AG} family exchange transporters move aspartic acid, glutamic acid, and cysteine (Lewis *et al.,* 2012). Each of these transport systems exhibits high substrate specificity, and uses a different mode of action. Common amongst these transporters is their sensitivity to maternal-fetal concentration gradients. Accumulative transporters are driven by electrochemical differences across the microvillus and basal plasma membranes; exchange transporters qualitatively change the amino acid profile across the membrane without altering osmolality; and facilitated transporters move amino acids down their concentration gradient (Lewis *et al.,* 2012). The unique contribution made by each transport system to the overall establishment of concentration gradients means that placental amino acid transporter activity is very interdependent. For example, increased transport of lysine into the fetal compartment will alter the osmolality and electrochemical gradients across the basal plasma membrane, causing a shift in the rate at which other amino acids are transferred. Thus, each of the individual placental transport systems influences amino acid transfer rates and variability.

As the elaborate relationships governing placental transfer of amino acids are unraveled, it becomes clear that the placenta is not a passive conduit but an integral organ in the highly orchestrated process of gestational protein metabolism.

2.4. Protein metabolism changes during different stages of pregnancy

Maternal glucose and fatty-acid metabolism becomes increasingly adapted as gestation progresses so that the availability of these energy-yielding molecules is commensurate with the increasing demands of pregnancy (Kalhan, 2000). In contrast, adaptations to maternal protein metabolism begin early in gestation, before there is a significant increase in conceptus demand (Kalhan, 2000). Changes to maternal protein metabolism, as compared to non-pregnant women, are evidenced by differences in plasma amino acid concentration as early as 5 weeks gestation (Shoengold *et al.*, 1978). With the exception of lysine, histidine and threonine, maternal plasma amino acid concentrations are significantly lower than those of non-pregnant women (Shoengold *et al.,* 1978; Cetin *et al.*, 1996). A comparison of plasma amino acid profiles in early versus late pregnancy has also indicated significant differences in amino acid concentration – namely a significant increase in threonine, methionine, serine, and glycine in late gestation (Cetin *et al.*, 1996). Similarly, gestational differences in protein metabolism are evident in the amino acid profile of amniotic fluid (Reid *et al.,* 1971; Rabier *et al.*, 1996). With the exception of aspartic acid, serine, glutamine and glycine, all other amino acids significantly decrease in concentration within the amniotic fluid as gestation progresses (Reid *et al.,* 1971; Rabier *et al.*, 1996).

Nitrogen metabolism studies conducted using ^{15}N-leucine also show gestational differences: significantly higher nitrogen retention, and significantly lower urea synthesis and branched-chain amino acid oxidation in late versus early

pregnancy (Kalhan, 2000; King, 2000). These findings suggest that adaptations to protein metabolism in the late stages of pregnancy favor nitrogen conservation.

Recently, stable isotope techniques have been used to determine threonine and lysine requirement in pregnant sows (Levesque *et al.*, 2009; Levesque *et al.*, 2010; Samuel *et al*., 2012). These studies identified a 200% and 85% increase in threonine and lysine requirement, respectively, during late as compared to early gestation (Levesque *et al.*, 2009; Levesque *et al.*, 2010; Samuel *et al*., 2012). Similar experiments have not been published in human pregnancy, but it is clear that in addition to changes in maternal protein metabolism there exists changing demand for dietary protein and amino acids. Therefore, there is a need to determine protein and amino acid requirements during early versus late gestation.

2.5. Protein requirements during pregnancy

2.5.1. Current protein recommendations during pregnancy

The United Nations' (UN) World Health Organization (WHO) is the leading international public health authority, responsible for determining human nutrition requirements and providing dietary intake recommendations. In conjunction with the UN Food and Agricultural Organization (FAO), the WHO/FAO/UNU expert council has set international protein intake recommendations during healthy pregnancy as an additional 0.7, 9.6, and 31.2 grams of high quality protein per day (g/d) above the healthy non-pregnant adult recommended protein intake of 0.66 grams per kilogram per day (g/kg/d) in the 1st, 2nd, and 3rd trimesters of pregnancy,

respectively (WHO, 2007; **Table 2**). This estimate was derived by a factorial approach using data from nitrogen balance studies of non-pregnant adults, and mean protein deposition during pregnancy obtained from studies of total body potassium (WHO, 2007).

In North America, nutrient intake recommendations are set by the Dietary Reference Intakes for macronutrients (DRI), which is published by the Institute of Medicines' Food and Nutrition Board (DRI, 2005). The current DRI recommends that healthy pregnant women consume at minimum 0.88 g/kg/d - the equivalent of an additional 21 g/d - of high quality protein beyond 20 weeks gestation (DRI, 2005). This value represents the estimated average requirement (EAR), or the protein intake that will ensure 50% of the populations' dietary protein needs are met. To ensure that the dietary protein needs of 95% of the population are met, the DRI recommends consuming an intake that is 2 standard deviations beyond the EAR, a value referred to as the recommended daily allowance (RDA). The current DRI RDA for protein intake during healthy pregnancy is 1.1 g/kg/d beyond 20 weeks gestation (DRI, 2005). The DRI protein requirement estimate for healthy pregnant women is also derived by a factorial approach using data from nitrogen balance studies of non-pregnant adults, and mean protein deposition during pregnancy obtained from studies of total body potassium (DRI, 2005; Appendix A).

The study described in this thesis was conducted in a North American context, so the DRI protein intake recommendations will serve as the primary point of comparison for our estimation of protein requirement of healthy pregnant women.

Table 2. Comparison of dietary protein intake recommendations by the DRI (2005), WHO (2007), and IAAO method, and dietary intake patterns of pregnant women in BC, Canada.

Dietary Protein g/kg/d	Adult (19-50 y) DRI 2005		Pregnancy (all age groups) DRI 2005		Pregnancy (all age groups by trimester) WHO 2007			Adult (19-50 y) Elango *et al.,* 2009		Dietary protein intake of pregnant women in BC, Canada Stephens *et al.* (unpublished)	
	EAR	RDA	EAR	RDA	1st	2nd	3rd	EAR	RDA	Intake 16wk	Intake 36wk
	0.66	0.80	0.88	1.1	0.7[1]	9.6[1]	31.2[1]	0.93	1.2	1.5	1.3

[1]Additional grams of protein per day (g/d) above adult recommended intake of 0.66 g/kg/d

2.5.2. Evaluation of DRI during pregnancy

As previously stated, the current DRI recommends that healthy pregnant women consume 1.1 grams of dietary protein per kilogram of body weight per day (**Table 2**; Appendix A). This estimate is largely based on research conducted by J.C. King, D. Calloway and S. Margen in pregnant adolescent girls (King *et al.,* 1973). King *et al.* (1973) examined the balance of nitrogen and total body potassium in order to determine nitrogen retention in 10 pregnant girls aged 15-19 years. The accuracy of nitrogen balance studies has been critically examined in a number of scientific publications (Hegsted, 1976; Young, 1986; Waterlow, 1999). In general, nitrogen balance estimates are cumbersome due to: the difficulty of accounting for miscellaneous losses (e.g. hair, nails, sweat) leading to underestimates of nitrogen loss; the tendency to overestimate nitrogen intake; the lengthy period (~7 days) required to adapt to an experimental diet before nitrogen excretion data can be

collected; the necessity for subjects to strictly adhere to the experimental diet; and the decrease in protein utilization efficiency that is associated with dietary test protein intakes approaching zero nitrogen balance (Hegsted, 1976; Young, 1986; Waterlow, 1999). Overall, these variables tend to confound nitrogen retention estimates, resulting in an underestimate of protein requirements. The King *et al.* (1973) study rigorously diminished environmental variability by confining subjects to an experimental site, allowing for complete measurement of all nitrogen intakes and losses. However, according to the United Nations University Food and Nutrition department's criteria for upholding the validity of nitrogen balance assessments of protein requirements, the King *et al.* (1973) study violated at least two criteria (Scrimshaw, 1996). First, the researchers allowed subjects to freely consume snacks devoid of protein (e.g. juice, taffy, soda). This violates the criterion that energy intake must match energy requirements. Second, because subjects were free to consume beverages *ad libitum*, urinary nitrogen retention estimates may have been artificially increased due to uncontrolled fluid intake.

The DRI recommendations also take into account six studies of total body potassium (TBK) content during pregnancy, as an indirect estimate of protein accretion. In each of these studies, TBK was measured using the 4π liquid scintillation counter method, which is well validated (Barnaby and Jasani, 1968; Smith *et al.*, 1979). However, the potassium/nitrogen ratio used in the DRI's calculation of average protein deposition (2.15 mmol K/g N) is based on infant carcass analysis (Hamilton and Moriarty, 1929; Iob and Swanson, 1934; Fee and

Weil, 1963; Widdowson and Dickerson, 1964). The problem with using this value when investigating pregnant women is that it differs from the value obtained using adult tissue (2.7 mmol K/ g N), and thus may not accurately represent adult protein deposition (Reifenstien *et al.*, 1945).

Additionally, the DRI derivation for protein requirements during pregnancy assumes a protein utilization efficiency value of 0.43, which is less than the efficiency value used in calculating non-pregnant adult protein requirement (0.47; DRI, 2005; Otten *et al.*, 2006). It is contradictory that during a period of increased protein requirement (gestation), the pregnant body would adapt by decreasing protein utilization efficiency (King, 2000). Given that the 0.43 efficiency value is based on the slope of a regression line for protein intake versus nitrogen balance from the King *et al.* (1973) data, it is possible that this value was distorted by the aforementioned violations of criteria necessary for upholding the validity of nitrogen balance assessments of protein requirements. Alternatively, the biologically implausible efficiency value may be due to the fact that King *et al.* (1973) used a linear regression analysis to determine protein utilization efficiency. Because there is a decline in protein utilization efficiency from test protein intakes as zero balance is approached, linear regression analysis is not a valid statistical tool for assessing the relationship between protein intake and nitrogen balance (Rand and Young, 1999).

2.5.3. Fundamental criticisms of nitrogen balance studies

It is important to address three fundamental criticisms of methodology used hitherto when determining human protein requirements. First, nitrogen balance

studies are highly invasive. Subjects must strictly adhere to experimental diets over relatively long periods of time, must limit their physical activity, and are often confined to experimental units in order to reduce environmental sources of variability in the data. Second, in order to determine protein requirements, subjects must adapt to diets designed to include test protein intakes both above and below the expected protein requirement. For vulnerable populations such as pregnant women, a prolonged, induced state of protein deficiency or excess could pose serious risk, and as such is unethical (Bruton *et al.,* 1998). Third, nitrogen balance and total body potassium assessments have fundamental experimental flaws that cannot be easy corrected. Therefore, it is essential to the study of nutrition, to public health initiatives, and to the clinical application of dietetics, that novel techniques be employed in reassessing protein requirements. In this pursuit, the indicator amino acid oxidation (IAAO) method - with the use of stable isotopes - has emerged as a compelling technique due to its minimally invasive procedure.

2.6. Stable isotopes and their use in pregnancy studies

2.6.1. Stable isotopes as tracers

Stable isotopes are naturally occurring compounds present in the foods we eat and the environment we live in. For the 4 most common atoms that make up living organisms (carbon, hydrogen, oxygen and nitrogen) there are stable isotopes. For example, carbon 13 (^{13}C) is the stable isotope of the commonly occurring carbon 12 (^{12}C), and ^{15}N is the stable isotope to the more common ^{14}N. Stable isotopes are present in living things at very low enrichments - expressed as atomic percent

(atom%). For example the natural abundance of ^{13}C is 1.11 atom%, and ^{15}N is 0.37 atom%. These isotopes differ from their common atomic counterparts in that they posses an extra neutron, and therefore extra mass. The atomic mass difference can be detected, and overall isotope enrichment quantified, from biological samples using a mass spectrometer. Thus, stable isotopes can be used as labels to follow substrate metabolism, making them ideal experimental tracers. Stable isotope tracers have become an alternative to radioactive tracers, which decay over time and emit radiation, making them carcinogenic.

2.6.2. Protein metabolism during pregnancy studied using stable isotopes

In addition to the aforementioned studies of fetal amino acid metabolism using stable isotopes (Section 2.3.1.), several studies have examined whole-body protein metabolism during pregnancy. Using stable isotope-labeled amino acids, oxidative disposal (e.g. catabolism of amino acids to urea and CO_2) and non-oxidative disposal (e.g. anabolic uptake of amino acids for protein synthesis) are measured, revealing changes to amino acid usage with test protein intakes. Maternal whole-body protein metabolism was studied at 13, 24 and 35 weeks gestation in six healthy women using a continuous infusion of [1-^{13}C]leucine (Thompson and Halliday 1992; Appendix H). Mean protein synthesis increased from 5.3 (±0.6 SD) grams per kilogram fat free mass per 24 hours (g/(kg FFM*24h)) at 13 weeks to 5.9±0.5 g/(kg FFM*24h) ($p<0.1$) at 24 weeks and 6.1±0.6 g/(kg FFM*24h) at 35 weeks. Protein synthesis at 24 and 35 weeks gestation was significantly greater when compared to a group of 17 healthy, non-pregnant women (4.9±0.6 g/(kg FFM*24h); $p<0.001$). These data indicate that there are substantial increases in

protein turnover during pregnancy. However, some recent studies have found no significant increase in protein turnover in the 3rd trimester, suggesting that protein conservation occurs in the final stage of gestation (Whittaker *et al.,* 2000; Appendix H). Whittaker and colleagues infused 6 pregnant women with [1-^{13}C]leucine between 34-38 weeks gestation. They studied the same women post-partum and concluded that protein turnover was not significantly different between the 3rd trimester and non-pregnant state. This study also suggested that the sum of leucine kinetic differences (oxidative disposal versus non-oxidative disposal) resulted in a net increase in protein conservation. Similar results have been demonstrated by Jolly *et al.* (2004) using [1-^{13}C]leucine and Whittaker *et al.* (1999) using [ring-$^{2}H_5$]phenylalanine. To investigate the apparent decrease in protein turnover in the 3rd trimester of pregnancy, Kalhan and Parimi (2006) studied protein and amino acid metabolism using 1-[^{13}C]leucine, 1-[^{13}C]phenylalanine and [^{15}N]glycine. Kalhan and Parimi's data suggests that during pregnancy major physiological adaptations result in changes to maternal protein metabolism that conserve protein for protein synthesis in the maternal and fetal compartments.

The combined results show that stable isotopes can be used to examine protein and amino acid metabolism in pregnant populations reliably. However, no study has yet applied stable isotope techniques to determine protein or amino acid *requirements* during pregnancy. Two possible reasons for why stable isotope techniques have not been applied more broadly to consider requirements during pregnancy are that 1) most of the studies already conducted involve intravenous infusion of isotopes and 2) studies to date have been conducted in the fasted state

when the rate of protein synthesis decreases. A relatively non-invasive, fed-state method is necessary to conduct nutrient requirement studies in pregnant women, and the IAAO technique is well suited for this purpose. It has been applied previously to study protein requirements of healthy adult men and healthy school-age children using oral stable isotope (1-[^{13}C]phenylalanine) infusion, and collection of breath and urine to measure protein synthesis (Humayun *et al.*, 2007; Elango *et al.,* 2011).

2.7. The IAAO method: a robust model for determining protein requirements of vulnerable populations

2.7.1. IAAO method: principles and protocol

The IAAO method is based on the principle that if a single indispensable amino acid is deficient for protein synthesis, then all other amino acids will be oxidized (Ball and Bayley, 1984; Elango *et al.,* 2008). This is because excess amino acids are not effectively stored in the body and are therefore disposed. In practice, an isotopically labeled amino acid (usually L-[1-^{13}C]phenylalanine) is taken orally, along with an amino acid mixture representative of a complete protein (complete protein defined here as the amino acid combination present in egg protein; Elango *et al.,* 2008). The amount of protein administered is graded to give intakes both above and below the predicted requirement. Subsequent changes in protein dynamics are measured as the amount of heavy carbon (^{13}C) produced from the indicator amino acid in breath ($^{13}CO_2$) and urine (^{13}C-amino acid) samples (Elango *et al.,* 2008). Exhaled $^{13}CO_2$ is plotted against the range of administered test protein intakes (**Figure 1**). When these data points are analyzed using a two-phase linear regression crossover model,

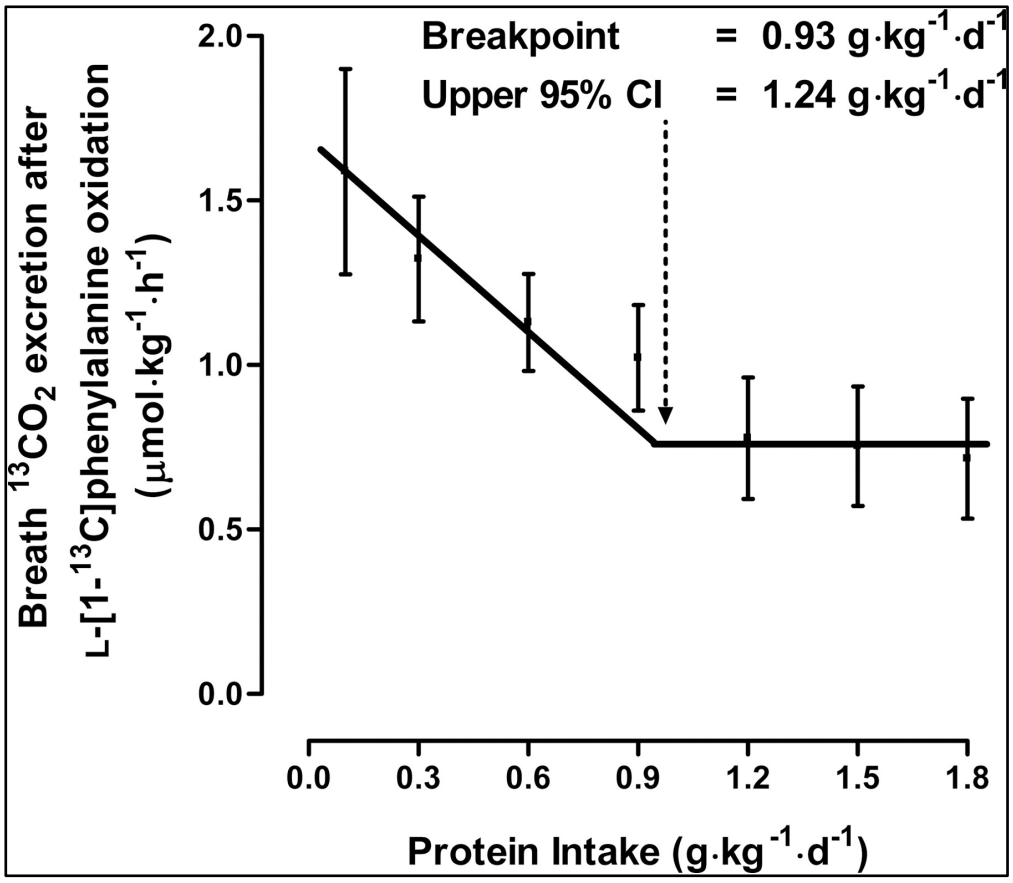


Figure 1. Estimated average protein requirement analysis using IAAO method (from Humayun *et al*., 2007)

a breakpoint is produced where the rate of IAAO plateaus. Because IAAO is inversely proportional to protein synthesis, an increase in the amount of protein intake – when deficient - is associated with a corresponding decrease in the rate of IAAO. Where the rate of IAAO plateaus, it can be inferred that the test intake is sufficient for protein synthesis to occur, and thus represents the estimated average protein requirement (Brunton *et al*., 1998; Elango *et al*., 2008).

2.7.2. Concerns with the IAAO method

Criticism of the IAAO method has focused heavily on the minimal adaptation period to the test protein/amino acid intake (Elango *et al*., 2010). In response to

these concerns, Elango *et al*. (2009) compared the results of IAAO in healthy young men following 8 hours (current minimal invasive IAAO model), 3 days and 7 days, adaptation. The results yielded no significant difference in $F^{13}CO_2$ (L-[1-^{13}C]phenylalanine indicator used), indicating that breakpoint determination was not affected by adaptation period.

Additionally, the validity and sensitivity of measuring breath $^{13}CO_2$ flux as an indication of protein dynamics has been contested. Recent evidence has helped to dispel these concerns (Elango *et al*., 2009; Rafii *et al.*, 2008). When the breakpoint for IAAO measured using $F^{13}CO_2$ flux from breath samples was compared with the breakpoint for phenylalanine hydroxylation measured using apolipoprotein B-100 – a protein synthesized from intrahepatocyte amino acids – the two values were found to be very similar (Elango *et al*., 2009). This indicates that $F^{13}CO_2$ measured from breath samples reflects changes in protein synthesis similar to an intracellular index.

2.7.3. Advantages of the IAAO method

The benefits of utilizing the minimally invasive IAAO model are substantial (Zello *et* al., 1995; Brunton *et al*., 1998; Elango *et al.*, 2008). Without the need for a prolonged adaptation period, subjects are only required to fast overnight then adhere to an experimental diet for 8 hours the following day. By reducing the amount of time researchers are required to disrupt the subject's diet, protein requirements can be directly examined in vulnerable populations. During the study day, the indicator amino acid is administered orally, and does not involve the more invasive approach - intravenous infusion. Samples are collected in the form of

breath and urine, and all samples are collected during the 8-hour study day, reducing subject discomfort and lifestyle disruption. From a methodological perspective, the IAAO model is the first direct measure of protein metabolism that has been accepted as an appropriate application for determining amino acid requirements (Institute of Medicine Food and Nutrition Board, 2002; Note: the IAAO approach to determining protein requirements is still the subject of debate). Furthermore, by measuring an end product of protein catabolism (CO_2) we arrive at a single estimate of protein requirements that represents the pregnant body as a whole, thereby eliminating complications that may arise due to differential protein metabolism in the maternal, placental or fetal compartments.

Cumulatively, the minimally invasive qualities that characterize the IAAO method have made it an attractive new approach to determining protein requirements, particularly in vulnerable populations such as pregnant women.

2.8. Summary of background

The critical importance of maternal nutrition in assuring healthy pregnancy outcomes is undeniable. Adequate and optimal protein intake is paramount in this pursuit. Currently, there is a paucity of evidence supporting protein requirements during pregnancy, and no studies identifying protein requirements during different stages of pregnancy. This is a direct consequence of a dependency on the nitrogen balance technique, which has many assumptions and flaws, and is unsuitable for vulnerable populations. The minimally invasive IAAO model is a sensitive and robust technique, which presents an opportunity to better identify protein requirement during pregnancy.

Chapter 3. Objective and hypothesis

The objective of this study was to determine protein requirements of healthy women aged 19-35y during early (11-20 weeks) and late (31-38 weeks) gestation by use of the minimally invasive IAAO model, where oxidation of L-[^{13}C]phenylalanine to $^{13}CO_2$ (collected from expired breath) in response to a graded intake of crystalline L-amino acid based on the composition of egg protein is measured.

IAAO research from Humayun *et al.* (2007) indicates that the current DRI underestimates protein requirements, based on the finding of a 41% discrepancy between the DRI EAR and IAAO-derived protein requirement estimated from healthy adults. Elango *et al.* (2011) achieved similar results that suggest a 78% discrepancy between the DRI estimated average protein requirement and IAAO-derived protein requirement of healthy children. Based on these findings, we hypothesize that the current recommendations for protein intake during pregnancy are underestimated (**Table 2**).

Nitrogen retention and protein deposition studies indicate significantly greater protein accretion during the late stages of pregnancy (Pitkin, 1977; Marino, 1983; Denne *et al.*, 1991). Based on these findings, we hypothesize that protein requirements during late gestation will be greater than early gestation.

Chapter 4. Methods

4.1 Subjects

4.1.1. Age demographic

This study examined healthy, pregnant women aged 19 to 35 years (**Figure 2**). The upper and lower limit of this demographic was selected to reduce the risk of pregnancy complications that might skew our results. Pregnancy during adolescence is associated with an increased risk of pre-term and very pre-term birth, low and very LBW, small for gestational age neonates, low and very low Apgar score, and fetal and neonatal mortality (Otterbald Olausson *et al.*, 1999; Chen *et al.*, 2007). Adolescents are more likely to partake in social behaviors such as cigarette use, illicit drug use, frequent alcohol consumption, and sexual promiscuity, all of which adversely affect pregnancy (McAnarney, 1987). By 19 years of age, the adverse affects associated with adolescent pregnancy are diminished (Chen *et al.*, 2007). For this reason, a lower limit of 19 years was selected for our study demographic.

In recent decades there has been a significant increase in the proportion of women in Western nations who delay childbearing (Johnson and Tough, 2012). As a result, there has been increased interest in the implications of reproduction in the latter part of childbearing years (Seoud *et al.,* 2002, Johnson and Tough, 2012). Evidence suggests that from 35 years of age onwards, maternal age is associated with a statistically significant increase in pregnancy complications (Hansen, 1986;

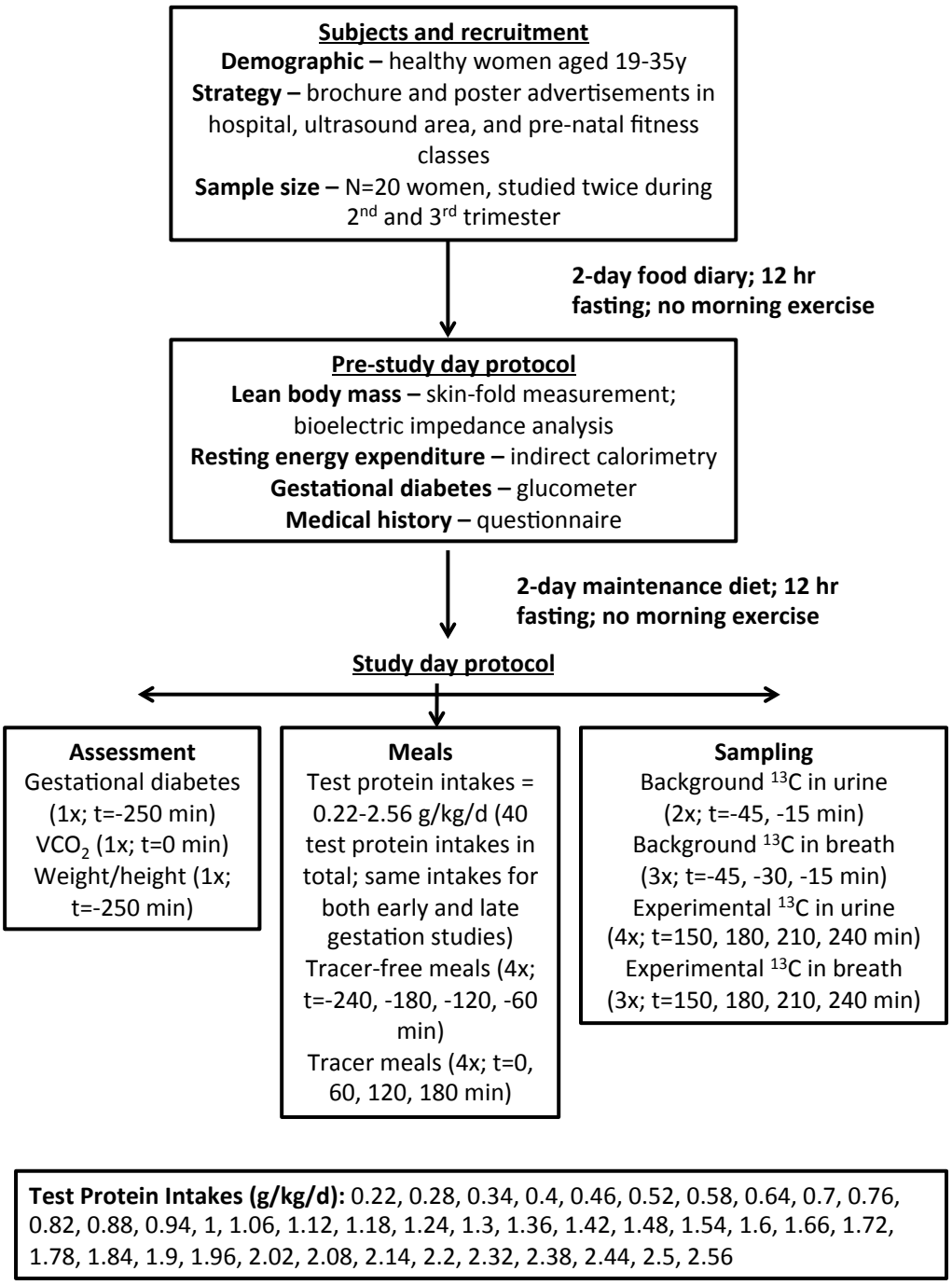


Figure 2. Flow diagram of our experimental design

Seoud *et al.,* 2002; Luke and Brown, 2007, Johnson and Tough, 2012). Complications include chronic hypertension, gestational diabetes, pregnancy-associated hypertension, low and very LBW, preterm birth, preeclampsia, and spontaneous abortion (Nybo Andersen *et al*., 2000; Seoud *et al*., 2002; Luke and Brown, 2007). Therefore, the upper limit of our study demographic was set at 35 years of age.

4.1.2. Pregnancy considerations

This study included women pregnant with a single fetus who had not recently given birth (<18 months). Minimal inter-pregnancy interval is associated with maternal nutrient depletion resulting in preterm birth and/or fetal growth restriction (King, 2003). Multiple pregnancy augments maternal nutritional needs, and is associated with an increased risk of complications such as preeclampsia, spontaneous abortion and IUGR (Kahn *et al*., 2003; DRI, 2005; Duckitt and Harrington, 2005). Similarly, use of assisted reproductive technologies (ARTs) for conception increases the risk of pregnancy complications, so preference was given to naturally conceived pregnancies (Reddy *et al.,* 2007).

4.1.3. Gestational stage considerations

Pregnancy can be divided into several distinct stages. The most common is by trimester, defined as 1-12 weeks (1^{st}), 13-28 weeks (2^{nd}), and 29-40 weeks (3^{rd}). Others describe the quarters of pregnancy (1-10^{th}, 11-20^{th}, 21-30^{th}, 31-40^{th} weeks gestation), or simply the halves (1-20^{th} and 21-40^{th} weeks gestation; King, 2000). Here, we refer to the 2^{nd} and 4^{th} quarters of pregnancy as early and late gestation.

We studied women from 11-20 weeks and 31-38 weeks gestation, respectively (i.e. the 2nd and 4th quarters of pregnancy). The purpose of this design was to account for protein requirements during different stages of pregnancy, allowing for a temporal gap between the stages studied so as to detect significant changes in protein metabolism, while avoiding most of the first trimester - when many women experience nausea and vomiting (Gadsby *et al.*, 1993). The minimally invasive IAAO model requires that subjects adhere to specific dietary consumption patterns before and during the study day. To reduce subject discomfort, and to increase the accuracy of our results, we did not study women experiencing severe nausea, food avoidance and/or vomiting, or women <11 weeks gestation. In addition, physiological changes associated with the first 10 weeks of pregnancy are predominantly characterized by up-regulation of hormone production and secretion, and are not believed to substantially affect nutritional needs (Blackburn and Loper, 1992; King, 2000; Picciano, 2003).

4.1.4. Participant health

Health status, medication use, and social behaviors were considered during subject selection (Appendix B). Women studied were in apparent good health, with preexisting health conditions assessed on an individual basis. Subjects with preexisting health conditions directly linked to metabolic dysfunction were excluded (e.g. diabetes). Medication use was also considered on a case-by-case basis. The most commonly used medication that delayed participation was Diclectin® (doxylamine succinate/pyridoxine hydrochloride), which is prescribed for controlling nausea. Potential participants taking Diclectin® were asked to

discontinue use (if possible) for 1 week prior to the study. Potential participants were asked if allergic to eggs or egg protein due to the fact that the amino acid composition of the experimental diets followed egg protein composition. Participant body mass index (BMI; kg/m^2) was also taken into consideration. Low maternal BMI is associated with poor pregnancy outcomes, including preterm labor, IUGR and LBW (Erhenberg *et al.*, 2003). Maternal obesity disrupts metabolic adaptations to pregnancy, disrupts fetal growth, and increases the risk of preeclampsia (King, 2006; Nelson *et al.*, 2010). Therefore, the lower and upper pregravid BMI limits were set at 18.5 kg/m^2 and 25 kg/m^2, respectively.

4.1.5. Participant recruitment

Recruitment of participants was conducted by posting advertisements and brochures within the British Columbia Children's Hospital, and community centers such as prenatal fitness classes and coffee shops (Appendix C). Subjects were invited to participate in 2 study days per gestational period, for a total of 4 studies per individual. Subjects were compensated for transportation costs (public transit passes or parking passes), and offered an honorarium ($100/ study day) for their participation.

4.1.6. Inclusion and exclusion criteria

We included women who were 19 to 35 years of age, pregnant with a single fetus, 11-20 or 31-38 weeks gestation, in apparent good health, belonging to any ethnicity. Potential participants were excluded based on the following criteria: women who were not pregnant or pregnant with more than one fetus; women who

were not 19-35 years of age; women with a metabolic, neurological, genetic, or immune disorder likely to affect nutritional requirements; women dependent on medication that affects normal metabolism; women experiencing severe nausea, food avoidance, or vomiting throughout their pregnancy; women allergic to eggs or egg protein; women classified as underweight (<18.5 kg/m^2), overweight (25-30 kg/m^2) or obese (>30 kg/m^2); and women smoking cigarettes, taking illicit drugs, or consuming alcohol regularly during their pregnancy.

4.2. Experimental design

4.2.1. Pre-study day protocol

Participants were asked to meet for a brief (1 hour) pre-study assessment in preparation for the study day (Appendix B). Subjects arrived at the Child and Family Research Institute's Clinical Research and Evaluation Unit having fasted overnight, and in a calm physiological state (at least 8 hours since last caloric intake; no morning exercise, and assisted transportation to minimize stress). During this appointment, the following measurements were taken: height (Harpenden Stadiometer; Holtain Limited, UK) and weight (electric scale; HealthOMeter® Professional, Sunbeam Products, Inc.), bioelectrical impedance analysis (BIA; Q4 Bioimpedance Analyzer, RJL Systems, MI), 3 site skinfold (Harpenden Calipers; Baty International, UK), blood glucose (One Touch® Ultra®, LifeScan, Inc), and resting energy expenditure (REE; Vmax Encore, Viasys, CA). REE describes the caloric requirement for vital function in the absence of physical exertion, and was used to calculate the total energy content of the experimental diets in order to ensure that the specific caloric needs of each participant were met (Bross *et al.*, 1998). Blood

glucose was assessed to screen for gestational diabetes mellitus (GDM; fasting plasma glucose level of ≥6.7 mmol/L indicative of GDM; Metzger *et al*., 2007). Skinfold and BIA measurements were collected to determine maternal fat and fat free mass. Maternal body composition is difficult to determine as a result of pregnancy-related physiological changes (McCarthy *et al.*, 2004). Skinfold assessment is affected by changes to maternal hydration and skin contours, and tends to overestimate the subcutaneous fat content (McCarthy *et al.*, 2004). Therefore, we selected 3 sites minimally affected by hydration: the biceps, triceps and subscapular region. BIA is affected by changes in hydration and hematocrit concentration, but correlates well with other body composition approaches (i.e. deuterium water and hydrodensitometry), and is safe and easy to conduct (McCarthy *et al.*, 2004). Therefore, BIA was also used to determine body composition.

A questionnaire was completed that asked for information about recent medical history, pregnancy history, medication use, supplement use and exercise patterns (Appendix B). Prenatal vitamin use was requisite for participation in this study in order to ensure adequate vitamin and mineral availability. If necessary, prenatal vitamins (Materna®, Centrum) were available for the duration of the study at the request of the participant. Informed consent was obtained from each participant in accordance with the BC Women's and Children's Research Ethics Board guidelines (Appendix D). Lastly, subjects were asked to provide a 2-day food intake diary to reveal food preferences. These were used to inform food choices when designing the standardization diet (Appendix E).

4.2.2. Study day protocol

Each study day was carried out in the temperature-controlled Clinical Research and Evaluation Unit (Translation Building, CFRI; **Figure 3**). Subjects arrived fasted and in a calm physiological state. Subjects were assessed for abnormal fasting blood glucose levels using a glucometer (Brody *et al*., 2003). If blood glucose was detected at values ≥6.7mmol/L it was recommended that the participant contact their health care provider for further testing (Appendix G). Each study day proceeded in accordance with the minimally invasive IAAO model (**Figure 3**; Appendix F; Bross *et al.*, 1998). Meal consumption began at -240 minutes, followed by subsequent meals at -180, -120, -60, 0, 60, 120, and 180 minutes. Indirect calorimetry was performed at 0 minutes to determine the rate of CO_2 production (V_{CO2}), which was used to calculate ^{13}C enrichment. The first 4 meals did not contain our indicator amino acid, L-[1-^{13}C]phenylalanine. Urine and breath samples collected prior to the 5th meal were used to determine background ^{13}C enrichment. Continuous oral L-[1-^{13}C]phenylalanine infusion was introduced in the 5th meal along with a priming dose of L-[1-^{13}C]phenylalanine and $NaH^{13}CO_3$. Each subsequent meal (6th-8th) contained a constant dose of L-[1-^{13}C]phenylalanine. Urine and breath samples collected from 150-240 minutes post-infusion were used to determine L-[1-^{13}C]phenylalanine-derived ^{13}C enrichment. Following breath and urine sampling at 240 minutes post-infusion, the study was complete and participants were free to leave.

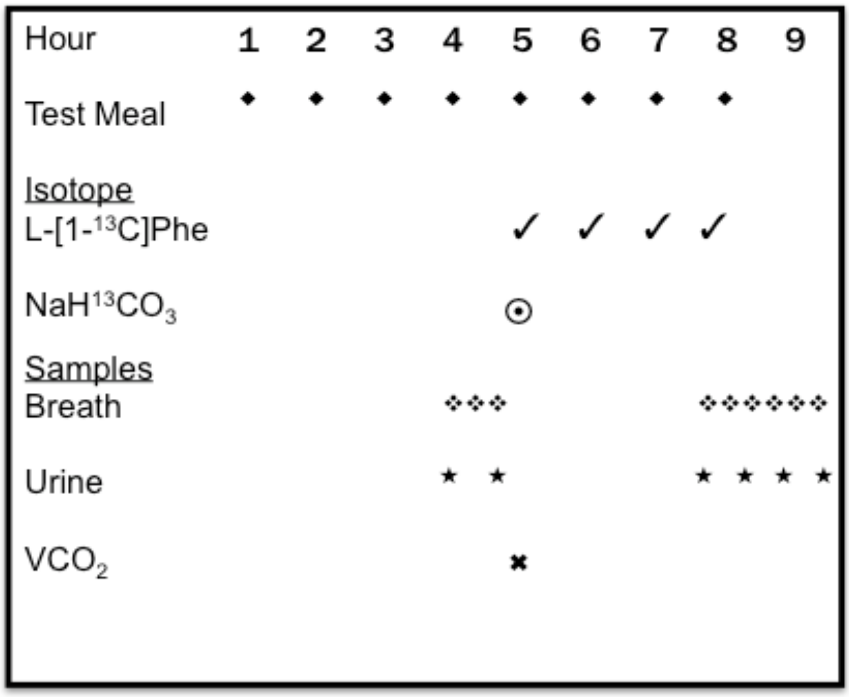


Figure 3. Study day protocol

4.3. Experimental diet

4.3.1. Standardization diet

Participants were asked to standardize their dietary protein intake to 1.5 g/kg/d during the 2 days before the study in order to reduce protein intake variability amongst subjects prior to IAAO analysis (Elango *et al.,* 2009). From food diaries collected during the pre-study assessment, each participant's 'normal' dietary protein intake was quantified using a nutrient analysis database (Food Processor SQL 11, ESHA Research). Participants were advised to increase, decrease or maintain their dietary protein intake in order to achieve a total intake of 1.5 g/kg/d. Food choice recommendations were provided based on individual food preferences in order to help participants consume the standardized protein intake.

4.3.2. Test protein intakes

To identify a breakpoint using the IAAO method, the test protein intakes must be varied enough to have sufficient data points above and below the requirement so that two distinct slopes are achieved upon statistical analysis. Conventionally, IAAO studies have selected ~7 protein or amino acid intake levels (3 above, 3 below and 1 at the predicted requirement), and performed IAAO analysis on each subject at all intake levels (Mager *et al.*, 2003; Turner *et al.*, 2006; Elango *et al.*, 2007; Humayun *et al.*, 2007; Pillai *et al.*, 2010). However, this approach was not possible in this study because of limitations posed by pregnancy. Within a matter of weeks, protein metabolism in the pregnant body can change through adaptive physiological strategies, negating the efficacy of the conventional IAAO experimental approach (Hadden and McLaughlin, 2009). To overcome this challenge, we provided a unique protein intake for each study day, as done recently in school-aged children (Elango *et al.,* 2011) and neonate (Chapman *et al.*, 2009, 2010) IAAO studies.

IAAO studies of protein requirements of healthy children and healthy adults have demonstrated that the current EAR for protein intake recommended by the DRI are 78% and 41% lower than IAAO-derived protein requirements, respectively (Elango *et al.*, 2011; Humayun *et al.*, 2007). A recent analysis of dietary protein intake patterns from pregnant women in British Columbia, Canada indicates that the DRI RDA for protein (1.1 g/kg/d) may be underestimated, given that women are consuming 1.5 g/kg/d in early gestation, and 1.3 g/kg/d in late gestation (Stephens, Innis, Elango *et al.*, unpublished data). Based on these findings, we predicted a protein requirement of approximately 1.3 g/kg/d for healthy, pregnant women.

With this estimated breakpoint in mind, subjects were randomly assigned to one of the following 40 test protein intakes (in g/kg/d): 0.22, 0.28, 0.34, 0.4, 0.46, 0.52, 0.58, 0.64, 0.7, 0.76, 0.82, 0.88, 0.94, 1, 1.06, 1.12, 1.18, 1.24, 1.3, 1.36, 1.42, 1.48, 1.54, 1.6, 1.66, 1.72, 1.78, 1.84, 1.9, 1.96, 2.02, 2.08, 2.14, 2.20, 2.26, 2.32, 2.38, 2.44, 2.50, 2.56. Participants were studied at the same 40 test protein intakes during both early and late gestation. However, in order to confirm the breakpoint analysis in late gestation, 3 additional test intakes were included: 0.84, 0.98, and 1.08 g/kg/d. A total of 37 and 44 studies were completed during early and late gestation, respectively.

4.3.3. Study day diet

Subjects were randomly assigned 1 of 40 test protein intakes comprised of a crystalline L-amino acid mixture consistent with egg protein amino acid composition, with the exception of phenylalanine and tyrosine. Phenylalanine intake was held constant at 30.5 mg/kg/d and tyrosine was held constant at 61 mg/kg/d. The test protein intake constituted 3-21% of the total energy provided by the experimental diet (total energy for the day calculated as 1.7 X REE). The remaining energy came from 37% fat and 42-60% carbohydrates, provided by corn oil, protein-free powder and protein-free cookies. The diet was consumed in 8 isocaloric and isonitrogenic meals, each representing $1/12^{th}$ the daily requirement. In addition to the experimental diet, subjects were allowed to consume only water prior to, and throughout, the study day.

4.4. Isotope tracer considerations

4.4.1. Indicator amino acid criteria

Selection of an indicator amino acid is based on specific criteria (Zello *et al.*, 1995). First, in order to measure changes in protein utilization in relation to amino acid intake, an indispensable amino acid must be use as the indicator. The body cannot synthesize indispensable amino acids, thus allowing for experimental control of indicator availability. Second, for IAAO to be measured from breath samples, the labeled carboxyl carbon of the indicator amino acid must be irreversibly oxidized to CO_2. Third, the indicator amino acid should not contribute significantly to any reactions within the body apart from protein synthesis or oxidation to CO_2. If an alternate biochemical pathway consumes the labeled carbon, tracer collected in breath samples will not reflect changes in whole-body protein utilization. These criteria leave phenylalanine (in the presence of excess of tyrosine), lysine, and the branched-chain amino acids as possible indicator amino acid candidates (Zello *et al.*, 1995; **Figure 4**). L-[1-^{13}C]phenylalanine was successfully applied for the determination of protein requirements of adults and children, and therefore was used in this study (Humayun *et al*., 2007; Elango *et al*., 2011).

4.4.2. Isotope tracer protocol

During each study day, a priming dose (0.66 mg/kg) and continuous dose (1.2 mg/kg/d) of L-[1-^{13}C]phenylalanine (99 atom percent excess, Cambridge Isotope Laboratories Inc., Andover, MA) was given orally with the 5th meal, followed thereafter by an hourly intake of 1.2 mg/kg/d (Humayun *et al.,* 2007). Isotopic steady state was achieved at this dose during each of the 81 study days. Isotopic

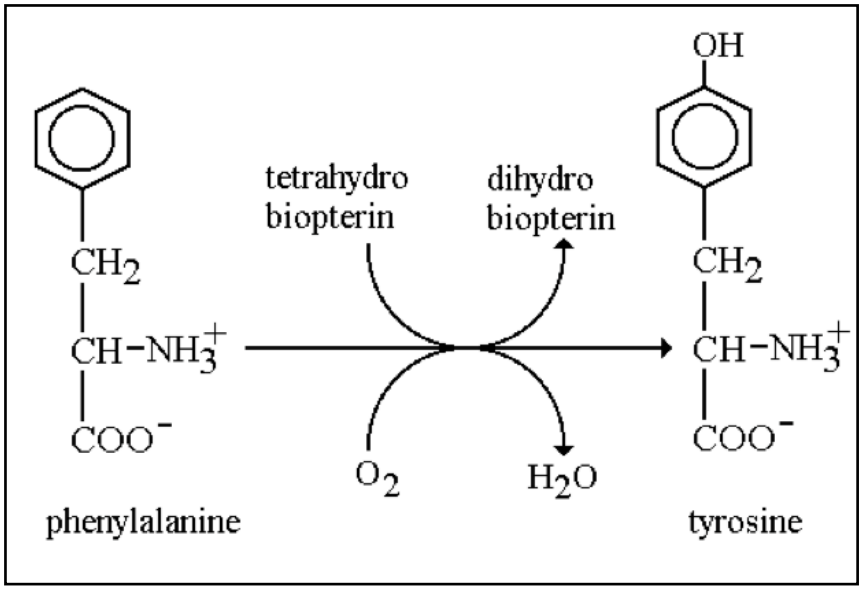


Figure 4. Biochemical conversion of phenylalanine to tyrosine

steady state was calculated as <10% coefficient of variation in ^{13}C enrichment during the 4hr tracer protocol.

Tracer phenylalanine intake was subtracted from the dietary phenylalanine content supplied by the experimental diet in order to keep the total phenylalanine intake constant. Because phenylalanine is normally metabolized by conversion to tyrosine, an excess of tyrosine (61 mg/kg/d) was provided in the experimental diet (**Figure 4**). This ensured that phenylalanine oxidation was sensitive to changes in protein intake (Pencharz and Ball, 2003).

A priming dose of $NaH^{13}CO_3$ (0.176 mg/kg; 99 atom percent excess, Cambridge Isotope Laboratories Inc., Andover, MA) was given orally with the 5th meal in order to increase the recovery of ^{13}C in breath (Hoerr *et al.,* 1989). $NaH^{13}CO_3$ increases the recovery of ^{13}C in breath based on the following relationship:

$$H+ + HCO_3^- \leftarrow\rightarrow H_2O + CO_2$$

CO_2 is transported in blood as HCO_3^- so by providing $NaH^{13}CO_3$ we primed the body pool to more rapidly achieve steady state in ^{13}C enrichment.

4.4.3. Sample collection and analysis

To measure the oxidation of L-[1-^{13}C]phenylalanine to $^{13}CO_2$ ($F^{13}CO_2$), V_{CO2} was measured once and breath samples were collected at 9 times points during each study day. Open-circuit indirect calorimetry was performed until ≥5 consecutive steady state data points were obtained to measure V_{CO2} (V_{MAX} Encore system by Viasys). 3 baseline and 6 isotopic steady state breath samples were collected at time -45, -30, -15, 150, 180, 195, 210, 225 and 240 minutes using disposable Labco exetainer tubes (Elango *et al.,* 2007). Breath samples were stored at room temperature in airtight bags.

In accordance with the minimally invasive IAAO model, urine samples were collected in place of plasma to assess whole-body amino acid pool L-[1-^{13}C]phenylalanine enrichment at isotopic steady state (Bross *et al.*, 1998). 2 baseline and 4 isotopic steady state urine samples were collected at time -45, -15, 150, 180, 210, and 240 minutes using sterile collection hats. Urine samples were preserved using 200 μL of 3.4 mol/L HCl per 10 mL sample, then stored at -80°C. Urine samples collected at time -45 minutes and 240 minutes were analyzed for the presence of protein using the Chemstrip 7® dipstick test by Roche®. If protein was detected in the urine at values ≥300 mg/24 hour (+1 or higher as indicated by

dipstick analysis) it was recommended that the participant contact their health care provider for further testing (Appendix G).

Breath $^{13}CO_2$ enrichment was determined using continuous-flow isotope ratio mass spectrometry (Isoprime Ltd, UK) and expressed as atom percent excess (APE) when compared against a reference standard of compressed CO_2 (Turner *et al.,* 2006; Elango *et al.,* 2007; Humayun *et al*., 2007). Urine sample [^{13}C]phenylalanine enrichments were analyzed by the SickKids (Toronto) mass spectrometry facility. Briefly, urine [^{13}C]phenylalanine enrichment was determined using a triple quadruple mass spectrometer (AB Sciex, ON) coupled to a high precision liquid chromatography system (Bross *et al.,* 1998; Turner *et al.,* 2006; Elango *et al.,* 2007; Humayun *et al*., 2007). Following mass spectrometry, select ion chromatograms were obtained by monitoring mass to charge (m/z) ratios of the product ions 165 and 166 for [1-^{13}C]phenylalanine, corresponding to unenriched (M) and enriched (M+1) peaks, respectively. The area under these peaks at baseline and plateau isotopic steady state was used to calculate [^{13}C]phenylalanine enrichment in urine (expressed as molecules percent excess; Turner *et al.,* 2006; Elango *et al.,* 2007; Humayun *et al*., 2007).

4.4.4. Calculation of isotope kinetics

To calculate L-[1-^{13}C]phenylalanine kinetics, the stochastic models described by Waterlow *et al.* (1978) and Matthew *et al.* (1980) were used. These models have been used to assess L-[1-^{13}C]phenylalanine kinetics in previous IAAO studies (Bross

et al., 1998; Kriengsinyos *et al.,* 2002; Mager *et al.,* 2003; Elango *et al.*, 2007; Pillai *et al.,* 2009). Whole body flux will be calculated as:

$$Q=i[E_i/E_u - 1]$$

Where *Q* represents phenylalanine flux (μmol/kg*h), *i* is the rate of L-[1-^{13}C]-phenylalanine infusion (μmol/kg*h), and E_i and E_u are the istopic enrichments as mole fractions (molecules percent excess) of the infusate and urinary phenylalanine, respectively, at plateau isotopic steady state.

The rate of phenylalanine oxidation was calculated using the rate of $^{13}CO_2$ released by L-[1-^{13}C]phenylalanine oxidation ($F^{13}CO_2$; in μmol/kg*h). $F^{13}CO_2$ is calculated as:

$$F^{13}CO_2 = (FCO_2)(ECO_2)(44.6)(60)/W(0.82)(100)$$

Where FCO_2 represents carbon dioxide production (mL/min), ECO_2 is the $^{13}CO_2$ enrichment in breath at plateau isotopic steady state (APE), W is the subjects weight (kg), 44.6 (μmol/L) and 60 (min/h) are constants used to convert FCO_2 to μmol/h, 0.82 is the correction factor for carbon dioxide conserved by the body due to bicarbonate fixation, and 100 is used to convert APE to a fraction (Hoerr *et al.,* 1989).

4.4.5. Statistical analysis

Subject characteristics are reported as the mean and standard deviation. A two-phase linear regression cross over model was used to analyze the effect of protein intake on $F^{13}CO_2$ (Proc Mixed, Statistical Analysis Systems – SAS/STAT version 9.0, SAS Institute, Cary, NC). A breakpoint was determined at the minimum residual standard error in a stepwise partitioning of data points between two regression

lines (Zello *et al*., 1995; Humayun *et al*., 2007; Elango *et al*., 2009). The model assumes that one regression line is sloped and the other is horizontal with minimal or no slope. Protein intake at the breakpoint is the estimated average protein requirement. The pre-study, study day, and protein intake data were compared using Student's t-test, and phenylalanine flux data were compared using one-way analysis of variance (Version 19, SPSS Inc, Chicago, IL). Significance was set at $P \leq 0.05$.

Chapter 5. Results

5.1. Subject characteristics

The majority of subjects enrolled in this study were in their late twenties or early thirties (Early Gestation, EG: 30.6±3.9 y; Late Gestation, LG: 30.3±2.8y), had a pre-pregnancy BMI of 18.5-25 kg/m^2 (EG: 22.1±2.9; LG: 21.8±2.9), and were pregnant with their first child (EG: 77%; LG: 74%; **Table 3**).

Due to recruitment difficulties the final sample included women ≤37 years of age, and women were not studied twice during each gestational period as expected. Participants were given the option of participating in a single study day or up to 4 study days per gestational period. This flexibility allowed us to enroll more women because it reduced the required time commitment to the study. Therefore, in the early gestation group 4 women were studied 4 times, 2 women were studied 3 times, 4 women were studied 2 times, and 7 women were studied once (n=37, N=17). In the late gestation group 3 women were studied 4 times, 3 women were studied 3 times, 10 women were studied 2 times, and 3 women were studied once (n=44, N=19).

None of the women studied were taking Diclectin® or any Anatomical Therapeutic Chemical (ATC) code A medications at the time of study. None of the women studied reported any previous or existing pregnancy complications, ongoing nausea or vomiting, or an allergy to eggs or egg protein. One participant reported the use of assisted reproductive therapy for conception (*in vitro* fertilization). None of the women studied reported consuming alcohol, cigarettes or illicit substances at any time during their pregnancy. 2 women reported using an antidepressant

Table 3. Subject Characteristics (N_{early} = 17, N_{late} = 19)[1]

Characteristic	Early Group	Late Group
Age (years)	30.6 (3.9)	30.3 (2.8)
Pre-pregnancy weight (kg)	60.2 (10.4)	59.0 (10.2)
Height (cm)	165.1 (7.2)	165.8 (7.3)
Pre-pregnancy BMI (kg/m^2)	22.1 (2.9)	21.8 (2.9)
Previous pregnancies	0 – 77 %	0 – 74%
	1 – 18%	1 – 21%
	≥2 – 5%	≥2 – 5%

[1]All values are mean (±SD) unless otherwise indicated

during their pregnancy (Venlafaxine, commonly referred to as Effexor®), one woman reported using a steroid inhaler, and one woman reported using an over-the-counter stool softener. No medications were taken on the study day. Although we did not inquire about the participants' ethnic background, participants were predominately Caucasian.

5.2. Pre-study assessment

5.2.1. Early gestation pre-study results

In the early gestation group, pre-study BMI was predominantly in the range of 18.5-25 kg/m^2 (23.1±2.8), with an average weight gain of 2.9±2.9 kg above pre-pregnancy weight (**Table 4**). Body composition analysis determined average percentage of FFM (kg) as 74.3%, 65.9%, and 70.2% via skinfold, BIA, and factorial

Table 4. Pre-study day results in early (N=17) and late (N=19) gestation

Variable	Early Group Mean (SD)	Late Group Mean (SD)	t value (df)[1]	*p*
Weight (kg)	63.2 (9.8)	70.5 (10.2)	-2.158 (34)	0.038
BMI (kg/m^2)	23.1 (2.8)	25.6 (3.0)	-2.554 (34)	0.015
Blood glucose (mmol/L)	4.85 (0.50)	4.58 (0.44)	1.722 (34)	0.094
Skinfold FFM (kg)	46.9 (7.2)	53.0 (7.3)	-2.438 (33)	0.020
BIA FFM (kg)	41.7 (5.3)	43.1 (4.8)	-0.815 (34)	0.421
Pipe *et al.* (1979) FFM (kg)	44.8 (6.9)	44.6 (6.5)	-0.081 (34)	0.936
Resting energy expenditure	1370 (180)	1480 (197)	-1.744 (34)	0.090
Respiratory quotient	0.90 (0.08)	0.88 (0.03)	1.003 (34)	0.323
VO_2 (L/min)	0.193 (0.027)	0.205 (0.038)	-1.091 (34)	0.283
VCO_2 (L/min)	0.173 (0.023)	0.181 (0.035)	-0.827 (34)	0.414
Protein intake before pre-study (g/kg/d)	1.38 (0.38)	1.35 (0.51)	0.215 (33)	0.831

[1]Reported values assume equal variances

calculation based on Pipe *et al.* (1979), respectively. Average fasted blood glucose levels were 4.85±0.50 mmol/L, and fasted indirect calorimetry revealed an average rate of oxygen consumption of 0.193±0.027 L/min, and rate of carbon dioxide exhalation of 0.173±0.022 L/min, to yield an average respiratory quotient of 0.90±0.08. Resting energy expenditure was 1370±180 kcal/d. Food record analysis indicated an average protein intake of 1.38±0.38 g/kg/d amongst participants prior to the pre-study.

5.2.2. Late gestation pre-study results

In the late gestation group, most participants had a pre-study BMI in the range of 18.5-25 kg/m^2 (25.6±3.0), with an average weight gain of 10.8±2.6 kg above pre-pregnancy weight (**Table 4**). Body composition analysis determined average percentage of FFM (kg) as 75.1%, 61.1%, and 63.3% via skinfold, BIA, and factorial calculation based on Pipe *et al.* (1979), respectively. Average fasted blood glucose levels were 4.58±0.44 mmol/L, and fasted indirect calorimetry revealed an average rate of oxygen consumption of 0.205±0.038 L/min, and rate of carbon dioxide exhalation of 0.181±0.035 L/min, to yield an average respiratory quotient of 0.88±0.03. Resting energy expenditure was 1480±197 kcal/d. Food record analysis indicated an average protein intake of 1.35±0.51 g/kg/d amongst participants prior to the pre-study.

5.3. Phenylalanine flux and protein requirement

Participants had study day blood glucose levels of 4.77±0.39 and 4.56±0.42 mmol/L in early and late gestation, respectively (**Table 5**). Participant protein intake standardization compliance in the 2 days preceding each study day was high, with early gestation group subjects consuming 1.44±0.30 g/kg/d and late gestation group subjects consuming 1.47±0.53 g/kg/d, on average.

Phenylalanine flux (EG: 45.72±7.04mol/kg/h; LG: 47.57±6.82 mol/kg/h) did not vary significantly (EG: p=0.212; LG: p=0.259) across protein intakes, as required by the IAAO technique (**Figure 5 and Figure 6**). Without a change in phenylalanine flux, changes in oxidation with protein intake reflect a shift in amino acid utilization, from amino acid oxidation to amino acid incorporation for protein synthesis.

L-[1-^{13}C]phenylalanine oxidation to $^{13}CO_2$ varied significantly with protein intake in both the early ($p<0.001$) and late ($p<0.001$) gestation group, declining with increasing protein intake to a threshold intake, then plateauing (**Figure 7 and 8**). Two-phase linear regression cross-over analysis determined a breakpoint in the L-[1-^{13}C]phenylalanine oxidation to $^{13}CO_2$ versus protein intake data at 1.22 and 1.52 g/kg/d protein intake in early and late gestation, respectively. These estimates represent minimum protein requirement required to meet the needs of 50% of the population (EAR).

Changes in L-[1-^{13}C]phenylalanine oxidation to $^{13}CO_2$ with protein intakes are modeled in SAS (SAS/STAT version 9) according to a protein intake cutoff. The

Table 5. Study day results in early (n=37) and late (n=44) gestation

Variable	Early group Mean (SD)	Late group Mean (SD)	t value (df)[1]	*p*
Weight (kg)	64.4 (10.5)	71.4 (9.1)	-3.260 (79)	0.002
Blood glucose (mmol/L)	4.77 (0.39)	4.56 (0.42)	2.307 (79)	0.024
Protein intake before study day (g/kg/d)	1.44 (0.30)	1.47 (0.53)	-0.317 (69)	0.752
Energy intake on study day (kcal/d)	2304.9 (291.9)	2482.5 (318.6)	-2.596 (79)	0.011
Energy intake on study day (kcal/kg/d)	36.1 (3.1)	34.9 (3.2)	1.727 (79)	0.088

[1]Reported values assume equal variances

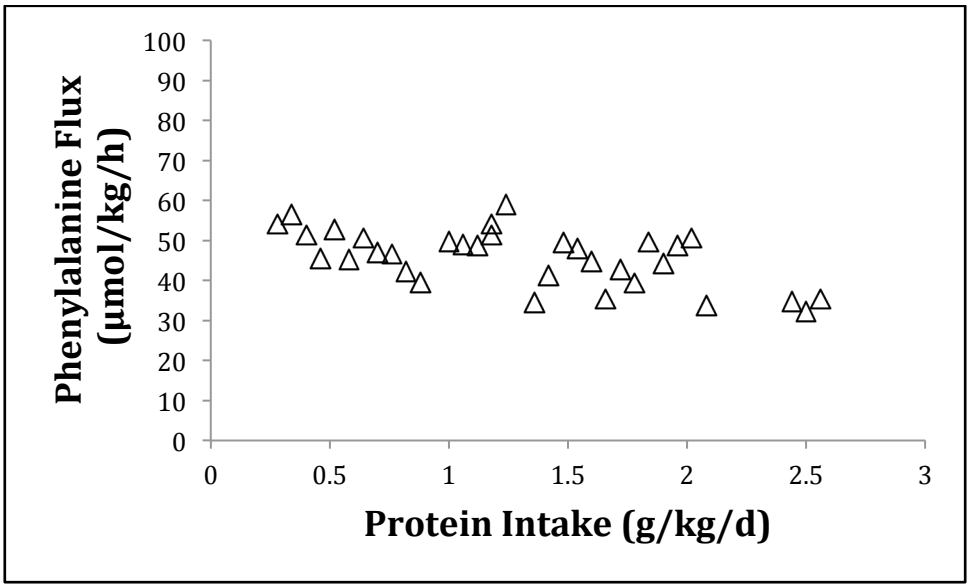


Figure 5. Variation in phenylalanine flux across protein intakes in early gestation group non-significant (p=0.212) as determined by one-way analysis of variance in SPSS (Version 19, SPSS Inc, Chicago, IL)

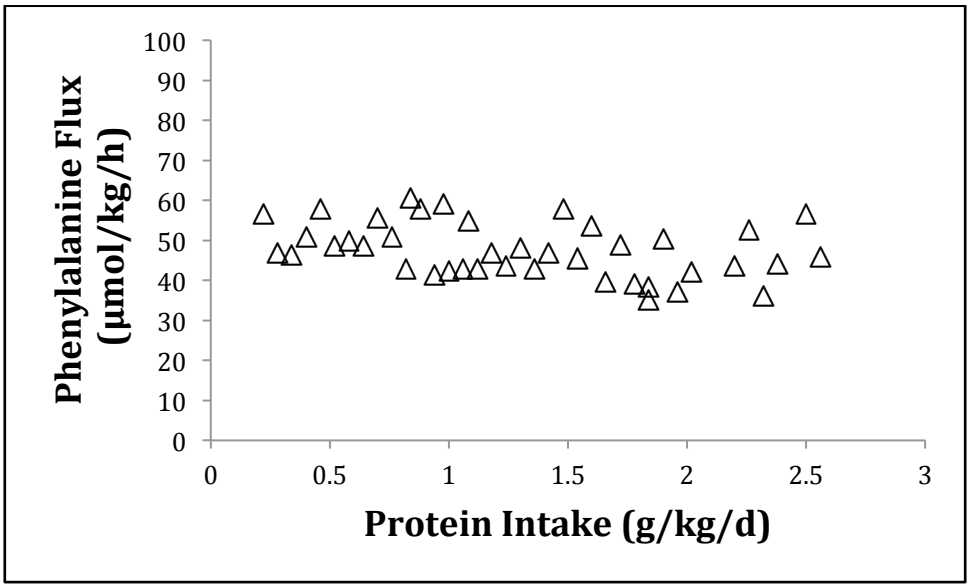
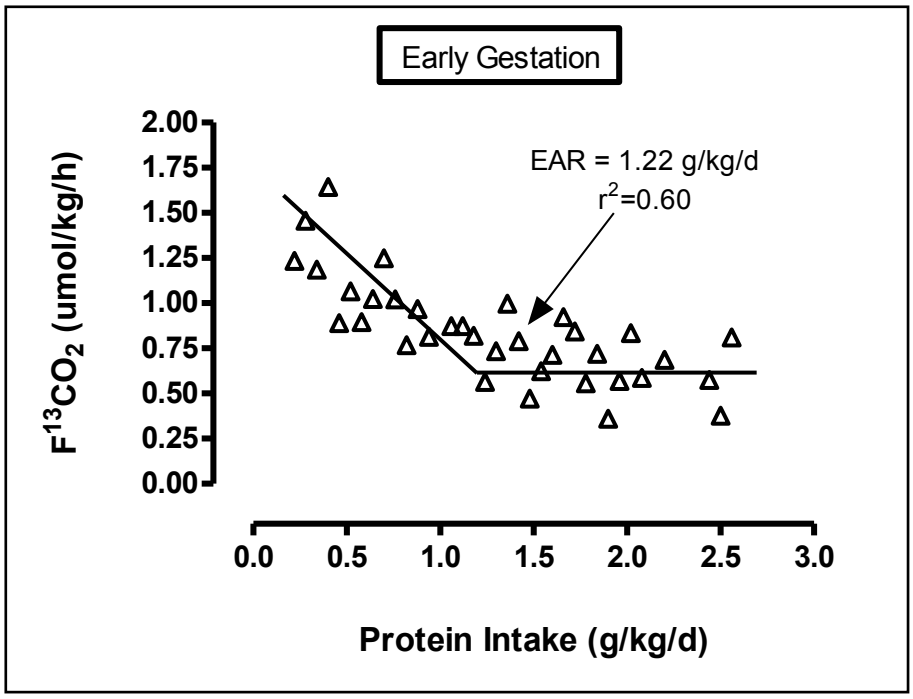


Figure 6. Variation in phenylalanine flux across protein intakes in late gestation group non-significant (p=0.259) as determined by one-way analysis of variance in SPSS (Version 19, SPSS Inc, Chicago, IL)



Figures 7. Estimated average protein requirement in early gestation derived by 2-phase linear regression cross-over analysis in SAS (Proc Mixed, Statistical Analysis Systems – SAS/STAT version 9.0 SAS Institute, Cary, NC)

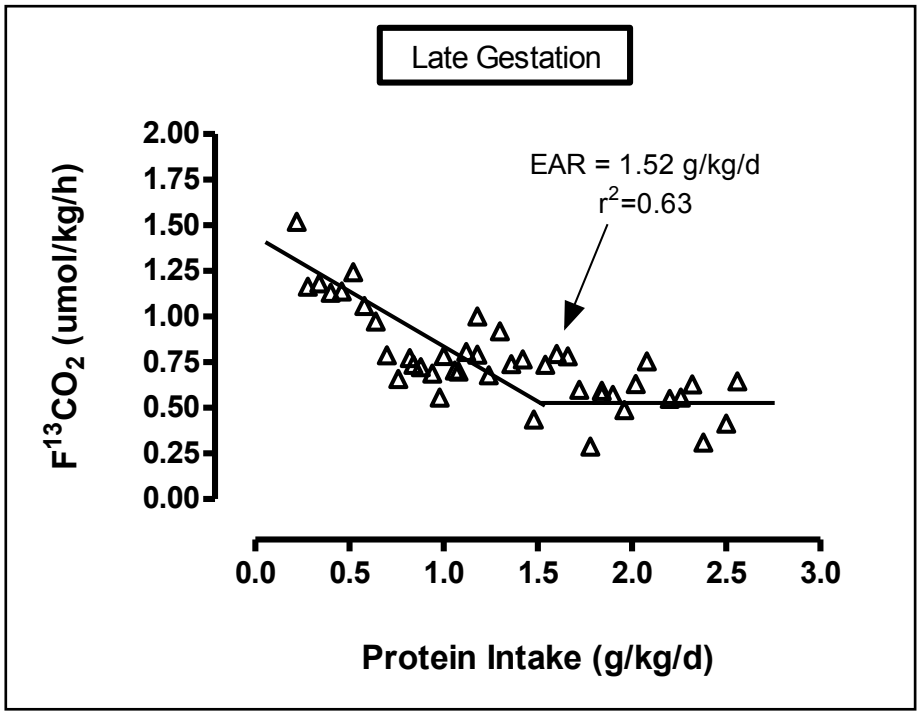


Figure 8. Estimated average protein requirement in late gestation derived by 2-phase linear regression cross-over analysis in SAS (Proc Mixed, Statistical Analysis Systems – SAS/STAT version 9.0 SAS Institute, Cary, NC)

cutoff separates the data into two groups: oxidation values associated with protein intakes below the cutoff, and oxidation values associated with intakes above it. With the oxidation data separated into 2 groups a regression is applied, producing 2 lines, which are analyzed using a two-phase linear regression crossover model. 4 different parameters are assumed for each cutoff: un-weighted where one line has slope, weighted where one line has slope, un-weighted where both lines have slope, weighted where both lines have slope. A breakpoint is determined from the model with the highest coefficient of determination (R^2), and the lowest coefficient of variation (CV) and root-mean-squared error (RMSE). Breakpoints determined for our early and late gestation groups came from models that assumed un-weighted data where one regression line was sloped and the other was not (EG: $R^2 = 0.60$; LG: $R^2 = 0.63$).

Chapter 6. Discussion

6.1. Subject characteristics

Women recruited for this study were healthy, 30 years of age on average, had a healthy pre-pregnancy BMI, and were predominately primigravida (**Table 3**). All of the women studied were taking prenatal vitamins prior to their first study day so protein metabolism is unlikely to have been affected by cofactor availability. 2 women in the early gestation group were included despite using Effexor® during their pregnancy. Effexor is a serotonin norepinephrine reuptake inhibitor that alters endocrine, gastrointestinal and metabolic function (Series, Micromedex Healthcare). Those taking Effexor® may experience weight loss (3-47% of users), constipation (8-15%), loss of appetite (8-22%), or sweating (6.7-25%), all of which may alter protein metabolism (Series, Micromedex Healthcare). Due to recruitment challenges in our early gestation group we decided to study these women. These women did not take their medications on the study day. Inspection of $F^{13}CO_2$ values from these studies revealed coefficients of variation less than 5%, and valid $F^{13}CO_2$ values. Therefore, these studies were included in our final breakpoint analysis. None of the other medications reportedly used by our participants significantly alter protein metabolism.

6.2. Indicators of metabolic adaptations to pregnancy

During the preliminary assessment we evaluated several metabolic indicators in each of the potential participants. Potential participants were instructed to arrive for this appointment having fasted overnight, and in a

physiologically calm state (e.g. no morning exercise; assisted transportation to minimize stress).

As expected, women in the late gestation group weighed more and had a higher BMI than women in the early gestation group (**Table 4**). Fat deposition occurs predominantly during the 2nd and 3rd quarters of pregnancy (Section 2.1.). Fat reserves are then used as a primary source of maternal energy during the 4th quarter of pregnancy, sparing glucose and amino acids for the fetal exponential growth phase (Pipe *et al.*, 1979; King, 2000). FFM is expected to increase during the last 10 weeks of pregnancy as the fetus more than doubles in size. However, only skinfold caliper analysis of body composition indicated a significant increase in FFM in the late versus early gestation groups (**Table 4**). BIA and a factorial calculation showed a trend toward higher FFM in late gestation, but this was not significant. Thus, our inability to detect a significant increase in FFM from early to late gestation using BIA and factorial estimate is likely due to the relatively small sample size.

Blood glucose measurements were collected from participants while fasted during the pre-study and on each study day (**Table 4 and 5**). None of the participants registered a blood glucose value ≥6.7mmol/L, and therefore are unlikely to have been experiencing GDM at the time of study. Cumulatively, these measurements revealed a significant decrease in fasting blood glucose levels from early to late gestation. Reduced circulating glucose in late gestation likely reflects increased placental uptake in order to accommodate the fetus's additional energy demands during this gestational period (King, 2000).

The resting energy expenditure, rate of O_2 inhaled and rate of CO_2 exhaled increased, and the respiratory quotient decreased, on average from early to late gestation (**Table 4**). However, these changes were not significant. Each of these measurements was collected once in the fasted state during the pre-study assessment, and thus detection of significant changes was likely obscured by small sample size. It is interesting to note that the average respiratory quotient in the early and late gestation groups were higher than that expected from a non-pregnant adult. During the fasted state, we expect the body to derive energy primarily from fat reserves, with an expected respiratory quotient closer to 0.7. The relatively high respiratory quotients observed in this study reflect maternal adaptations to pregnancy that alter energy metabolism to favor glucose oxidation, resulting in respiratory quotients closer to 0.9 (Butte, 2000).

6.3. Estimation of protein requirements

6.3.1. Protein requirement in early gestation

The estimated average protein requirement during early gestation was determined to be 1.22 g/kg/d (**Figure 7**). These results suggest that dietary protein needs are increased compared to non-pregnant adults from the first trimester onwards. As compared to the DRI recommended EAR (0.88 g/kg/d) for protein intake during pregnancy, our estimate is 39% higher (DRI, 2005). Compared to the IAAO-derived EAR for protein requirement in healthy, non-pregnant adults, our estimate is 32% higher (Humayun *et al.,* 2007). The increased need for dietary protein detected in this data set indicates that maternal adaptations to protein

metabolism are established early in pregnancy, and agree with previous findings that suggest changes to protein and nitrogen metabolism occur early in pregnancy, in anticipation of fetal needs (Section 2.4.; Kalhan, 2000; King, 2000).

Data used to derive this estimate was collected from women who adhered exceptionally well to our standardization diet recommendation of 1.5 g/kg/d in the 2 days preceding the study (**Table 5**). Liquid chromatography tandem mass spectrometry urine analysis revealed non-significant tracer phenylalanine flux (**Figure 5**). This is important because if phenylalanine flux varies proportionally with increasing intakes of protein then the measured $^{13}CO_2$ values are potentially erroneous. Therefore, we are confident that the appearance of tracer phenylalanine-derived $^{13}CO_2$ in breath reflects the partitioning of phenylalanine between protein synthesis and oxidation in response to protein intake.

The physiologic implications of these results are compelling. Despite minimal tissue accretion in the fetus before 20 weeks gestation, it appears that dietary protein requirements (on a body weight basis) are significantly increased. The additional need for dietary protein may be explained by a number of interrelated adaptations to pregnancy, as described below.

6.3.2. Sources of protein demand before 20 weeks gestation

The placenta is an essential extra-embryonic tissue that facilitates nutrient and waste exchange between the mother and fetus. In order to meet the demands of the fetus, the placenta must be fully formed before the fetus commences its exponential growth phase. Therefore, rapid growth of the placenta occurs in early gestation, with a fully functional placenta observed by 18-20 weeks gestation

(Porter RS and Kaplan JL, 2011). On average, human placenta has a mass of approximately 470g, suggesting that considerable protein deposition occurs in utero before 20 weeks gestation. As the placenta develops, its trophoblast cells invade the maternal decidua, eventually gaining access to the maternal blood supply via the spiral arteries (Espinoza *et al.*, 2006). By 10-12 weeks gestation maternal blood is in contact with the terminal villi of the placenta, allowing for gas and nutrient exchange between the mother and fetus, and increased metabolic activity in the feto-placental unit (Gude *et al.*, 2004). As previously stated (Section 2.3.2.), the placenta is not a passive conduit but rather a highly metabolically active organ that is also nourished by the maternal blood supply. In addition, the placenta has a particularly high affinity for amino acids, which concentrate here. Therefore, dietary protein requirements may increase at this time, as suggested by the current results.

Many maternal tissues, including uterine, breast, cardiovascular and kidney, undergo expansion in order to accommodate the physiological demands imposed by pregnancy. These changes occur progressively throughout gestation, in response to a progressive increase in progesterone and estrogen levels (**Figure 9**). However, human chorionic gonadotrophin (hCG) does not increase progressively, and may be implicated in contributing to muscle expansion during early gestation. HCG is produced by the syncytiotrophoblast layer of the placenta from approximately 2 weeks gestation until term (**Figure 10**). Unlike progesterone and estrogen, hCG production peaks during the first trimester, between 8-13 weeks gestation, then plateaus beyond 20 weeks gestation. While the common function of hCG is to maintain the corpus luteum, it may play a role in regulating protein turnover in

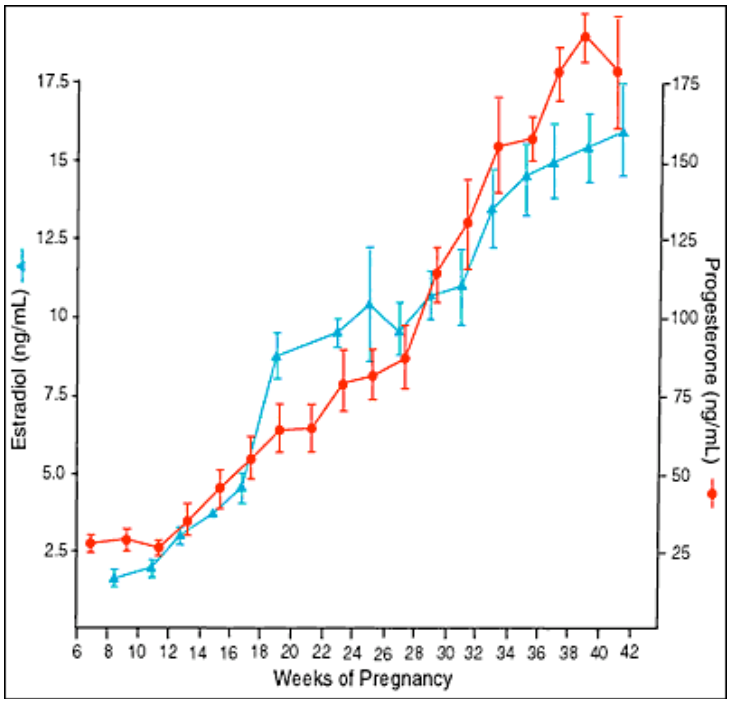


Figure 9. Plasma progesterone and estradiol levels throughout human pregnancy (adapted by Nieman L.K. for Endotext.org from Tulchinsky, D., *et al*. "Plasma estrone, estradiol, estriol, progesterone, and 17-hydroxyprogesterone in human pregnancy. I. Normal pregnancy." *American Journal of Obstetrics and Gynecology* 112.8 (1972): 1095)

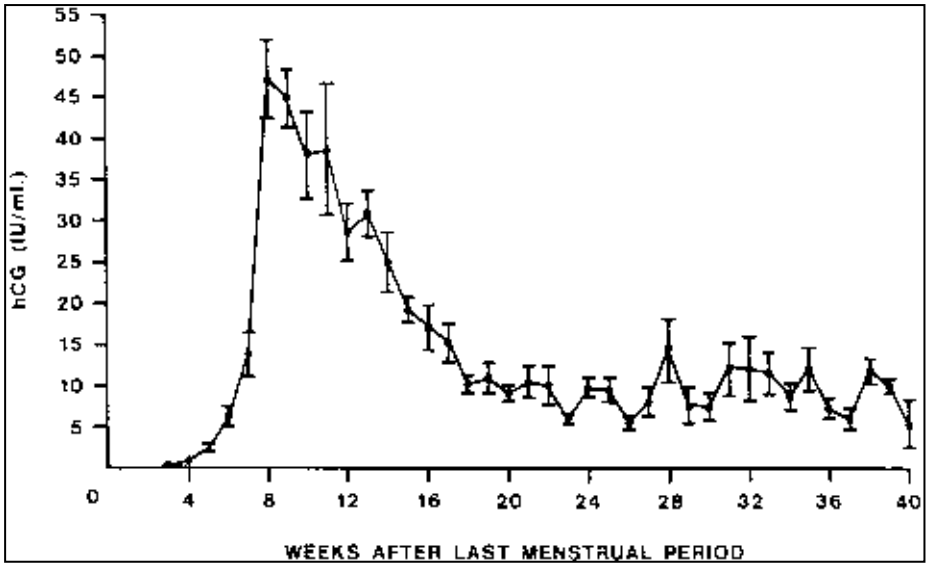


Figure 10. Mean serum hCG levels throughout normal pregnancy (from Braunstein, G. D., *et al*. "Serum human chorionic gonadotropin levels throughout normal pregnancy." *American Journal of Obstetrics and Gynecology* 126.6 (1976): 678)

muscle tissue. HCG has been shown to increase leptin release (Dos Santos *et al.*, 2007). A recent study conducted in mice found that leptin helped regulate muscle protein turnover by increasing protein synthesis and decreasing degradation (Mao *et al.*, 2012). HCG receptors are found in several tissues including the uterus (Rao, 2010). The uterus contains a substantial muscular layer, the myometrium, which undergoes expansion during pregnancy. If leptin released in response to hCG production stimulates protein synthesis in the myometrium, this activity would be most pronounced during peak hCG release, and taper off until 20 weeks gestation. A flurry of myometrium expansion during the 11-20 weeks period studied in our early gestation cohort would also help account for the increased protein requirement observed in this data set.

Pregnancy is characterized by hypoaminoacidemia (Kalhan, 2000). The decline in plasma amino acid concentrations is partially due to elevated progesterone levels (**Figure 9**). Progesterone increases amino acid catabolism at the liver for the production of new molecules. This is evidenced by a reduction in circulating amino acid concentrations without the concomitant increase in urinary amino acid nitrogen excretion, which is observed when protein catabolism is stimulated by adrenocortical or thyroid hormones (Landau and Lugibihl, 1961). Together with estrogen and insulin, progesterone also decreases lipolysis and increases lipogenesis in early gestation, facilitating maternal fat reserve formation (Butte, 2000). As discussed in Section 2.1., the first two-thirds of pregnancy are anabolic, and maternal energy demands are met primarily from carbohydrate and protein energy sources in order to conserve lipids for adipose tissue expansion. It is

possible the increased amino acid catabolism associated with progesterone secretion in early gestation facilitates fat reserve formation by favoring protein-derived energy. This too would help explain the increased need for dietary protein observed in our early gestation cohort.

6.3.3. Protein requirement in late gestation

The estimated average protein requirement during late gestation was determined to be 1.52 g/kg/d (**Figure 8**). These results suggest that dietary protein needs are increased above those of non-pregnant adults during the last quarter of pregnancy. As compared to the DRI recommended EAR (0.88 g/kg/d) for protein intake during pregnancy, our estimate is 73% higher (DRI, 2005). Compared to the IAAO-derived EAR for protein requirements of healthy, non-pregnant adults, our estimate is 63% higher (Humayun *et al.,* 2007).

Data used to derive this estimate was collected from women who adhered exceptionally well to our standardization diet recommendation of 1.5 g/kg/d in the 2 days preceding the study (**Table 5**). Liquid chromatography tandem mass spectrometry urine analysis revealed non-significant tracer phenylalanine flux (**Figure 6**). Therefore, we are confident that the appearance of tracer phenylalanine-derived $^{13}CO_2$ in breath reflects the partitioning of phenylalanine between protein synthesis and oxidation in response to protein intake.

Increased tissue accretion during the final quarter of pregnancy is essential for a healthy infant birth weight. Given the considerable difference between our estimate and that provided by the DRI, our results suggest that women who strictly adhere to current recommendations may not be consuming adequate protein.

6.3.4. Evidence that factorial calculation underestimates protein requirement

The discrepancy between our IAAO-derived EAR for late gestation and the DRI EAR for pregnant women (73%) is similar to that observed in a study by Elango *et al.* (2011) that examined protein requirements of school-aged children. Elango *et al.* (2011) determined that the IAAO-derived EAR for protein requirements of children was 78% higher than current DRI recommendations. The current method used by the DRI for estimating protein requirements of pregnant women and children is a factorial calculation that incorporates nitrogen balance data - derived primarily from non-pregnant adults - with mathematical constants that assume protein needs associated with growth. The protein needs of children and late gestation pregnant women are significantly influenced by rapid tissue accretion (e.g. juvenile growth phase and the fetal rapid growth phase). Thus, direct measurement of protein requirements is essential to identify the 'optimal' requirements in these unique populations.

6.4. Comparison of protein requirements to current reported dietary intakes

The 2003-2004 National Health and Nutrition Survey (NHANES), and the Canadian Community Health Survey (CCHS) Cycle 2.2 reported protein intakes in the United States of America and Canada, respectively, but excluded pregnancy-specific data from the analysis. We recently completed a prospective analysis of 270 pregnant women from British Columbia who were recruited for a separate study (Innis SM and Friesen RW, 2008; Stephens, Innis, Elango *et al*., unpublished). Dietary food frequency questionnaires were administered during early and late gestation in the same cohort of pregnant women. The results suggested that maternal protein

consumption adjusted for body weight (g/kg/day) was significantly higher at 16 weeks gestation than 36 weeks gestation, with median intakes of 1.5 (Interquartile Range, IQR: 0.61) and 1.3 (IQR: 0.56) g/kg/d respectively. The intakes on a g/d basis equate to 99.3 (IQR:34) and 100.5 (IQR:37) g/d, respectively. Since infant birth weight data was available, we compared protein intake to birth weight and found a significant negative correlation between protein intake and birth weight. The results from our Canadian cohort of pregnant women can be compared to Godfrey *et al.* (1996; n=538 British women) and Moore *et al.* (2004; n=429 Australian women). In contrast to our study, both of these studies found a positive correlation between protein intake and infant birth weight. The differing results may be explained by the substantial difference in protein consumption between the sample populations. In our study, the median total protein intakes at 16 and 36 weeks gestation were 99.3 and 100.5 g/d, respectively, which are considerably higher than the intakes of 84-87 g/d reported by Godfrey *et al* (1996) and Moore *et al* (2004). A quadratic relationship between maternal protein intake and infant birth weight has been proposed, which suggests that both insufficient and excessive protein intake is associated with LBW (Imdad and Bhutta, 2011; Sloan *et al.*, 2001). This suggests that 'optimal' or 'balanced' protein and amino acid intakes during pregnancy are beneficial, but excessive intake could have negative effects.

Thus, maternal protein intake patterns of women from British Columbia determined during early and late gestation compare favorably with our estimates of protein requirements of healthy pregnant women.

6.5. Comparison of protein requirement as a percentage of energy to DRI energy recommendations

Protein metabolism does not operate in isolation of other nutrients but rather is directly affected by carbohydrate, fat and total energy intake, particularly during pregnancy (Sec 2.1.). Thus, it is useful to consider protein requirements as a percentage of total energy. Energy was provided at 1.7 X REE for each participant. This meant that energy provisions were specific to the individual, and not biased by test protein intake. On average, we provided 2305 kcal/d and 2483 kcal/d of energy in early and late gestation, respectively (**Table 5**). Using the DRI formula to calculate estimated energy requirement (EER) for pregnant women weighing as much as our average participant in early and late gestation (EG: 64.4 kg; LG: 71.4 kg), we find that 2488 kcal/d and 2695 kcal/d is recommended by the DRI, respectively. Thus, the energy provided in this study is comparable to DRI energy recommendations. It should be noted that DRI recommendations for energy intake during pregnancy are unlike protein recommendations in that they are based on a substantial body of evidence derived from studies of pregnant women.

When DRI recommendations for protein intake during pregnancy are converted to a percentage of energy, we find that the DRI recommends 9% energy from protein in both early and late gestation. Our estimated protein requirements during early and late gestation represent 14% and 17.5% energy from protein, respectively. The current DRI recommends an acceptable macronutrient distribution range (AMDR) of 10-30% of energy from protein. Thus, despite our estimates of protein requirements being significantly higher than current DRI

protein intake recommendation for pregnancy on a g/kg/d basis, our estimates are more consistent with the AMDR than current DRI protein intake recommendations.

6.6. Strengths and implications of this study

Use of the minimally invasive IAAO method allowed us to study protein requirements of a healthy, adult, pregnant population for the first time. The rapid nature of IAAO protocol (8 hour studies) made it possible to study a wide range of test protein intakes and arrive at a strong intake versus response curve. The 'quick and easy' approach to our study days encouraged participant retention, accommodated participants who only wanted to enroll for a single study day, and allowed us to conduct more than 80 studies in less than 2 years.

Perhaps the most unique aspect of this experimental design was the inclusion of 2 gestational phases for analysis (i.e. early and late). The paucity of protein requirement data available for pregnancy was cited by the 2005 DRI. The early gestation data is particularly important given the relative absence of similar data available in the literature, and the critical importance of this stage to fetal development and placental sufficiency. With our data we are the first to provide pregnancy-specific dietary protein requirements for healthy women during early and late gestation.

Our results suggest that women require additional protein above non-pregnant adult requirements before 20 weeks gestation, and that protein requirements are significantly different during early versus late gestation. Thus, our

results support a stage-specific approach to protein intake recommendations for pregnant women.

These data suggest that current DRI and WHO/FAO/UNU recommendations for protein intake during pregnancy are significantly underestimated. Mothers in many regions around the world rely on government and non-government organizations to supplement their diet during pregnancy to ensure adequate nutrition. However, exactly what constitutes an 'adequate' amount of a particular nutrient is predominantly defined by DRI and WHO/FAO/UNU guidelines. These guidelines are used to allocate nutritional resources to individuals, and therefore directly impact the amount and type of food vulnerable populations have access to. Thus, these data indicate that current recommendations are inappropriate and may increase the risk of protein deficiency.

In North America the relative risk of protein deficiency is low (Fulgoni, 2008; Stephens, Innis, Elango *et al.*, unpublished). However, the popularization of high protein diets has increased the risk of excessive protein intake. As previously stated (Section 2.2.; Section 6.4.), both high and low protein diets during pregnancy have been associated with LBW infants. Therefore, by providing pregnancy-specific protein requirement estimates we can help North American mothers optimize dietary choices and improve pregnancy outcomes.

Chapter 7. Future directions

Using the IAAO technique to determine individual amino acid requirements throughout pregnancy is a natural step forward. Similar to protein requirements, very little pregnancy-specific data exists for individual amino acid requirements during this dynamic life stage. Currently, our first study of amino acid requirements is ongoing and focused on lysine requirements. Lysine is an indispensable amino acid, and the first limiting amino acid in plant-based diets. Therefore, women consuming diets devoid of animal protein are susceptible to deficiency. Women who fit this profile include those following vegetarian and vegan diets, as well as women with low food security that rely heavily on rice or grains as a primary food source. Lysine deficiency during pregnancy is associated with decreased immune function, increased diarrhea, and increased stress (Smriga *et al.*, 2004; Zhao *et al*., 2004; Ghosh *et al.*, 2010). Thus, we hope to provide pregnancy-specific lysine requirements in the near future.

Protein intakes in developing countries are very low during pregnancy (Swaminathan *et al*., 2012). Trends in protein intakes amongst pregnant women in India indicate that on average ~50 g/d is consumed, as compared to the 84-100 g/d reported by pregnant women in developed countries (Godfrey *et al.*, 1996; Moore *et al.*, 2004; Stephens, Innis, Elango *et al.*, unpublished). Furthermore, 60% of the protein consumed in developing countries arises from sources such as rice and grains, which are deficient in indispensable amino acids and thus are lower quality proteins. Findings of the current study will be significant in these settings, particularly in relation to the incidence/prevalence of LBW, which has been

implicated in adult onset chronic disease. Future studies will need to be conducted in pregnant women in settings where access to protein sources is limited in order to define the 'optimal' protein and amino acid requirements of these unique populations.

Chapter 8. Conclusion

In conclusion, we have determined that healthy, pregnant women during early and late gestation require 1.22 and 1.52 g/kg/d of protein, respectively. Each of these estimates is significantly higher than current DRI EAR and RDA recommendation for protein intake during pregnancy of 0.88 g/kg/d and 1.1 g/kg/d, respectively. The large discrepancy between our estimate of protein requirement and current DRI recommendations suggests that: 1) the factorial approach to determining protein requirements underestimates protein needs, and 2) emphasizes the importance of utilizing evidence derived directly from the population an estimate is meant to represent.

Our successful application of the IAAO method in pregnancy demonstrates the value of this novel technique for determining protein requirements of vulnerable populations. The IAAO method represents a new avenue for collecting data for these nutritionally unique populations, and should be utilized to identify protein and amino acid needs.

The results from our early gestation group suggest that maternal adaptations to pregnancy increase dietary protein needs before 20 weeks gestation, a gestational stage not addressed by the current DRI. In addition, the substantial difference between our early and late gestation estimates suggest that maternal protein needs increase as pregnancy advances. Taken together, these results indicate that protein intake recommendations should be provided in a gestational stage-specific manner, and current recommendations may be significantly underestimated.

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Appendices

Appendix A – DRI derivation of protein intake recommendations for healthy pregnant women

Tri-mester	Average additional body weight gained by end of trimester (kg)[a]	Total weight gained by end of tri-mester	[A] Additional protein to maintain increased body weight[b] (g/d)	Average protein deposition (additional lean tissue)[c] (g/d)	[B] Protein deposition corrected for conversion efficiency[d] (g/d)	[A+B] Average total additional protein required (g/d)[e]	RDA (g/d)[f]
1	Δ2.2	2.2	+1.4	-		-	
2	Δ7.3	9.5	+6.3	3.6	8.4	+14.7	
3	Δ6.5	16.0	+10.6	7.2	16.7	+27.3	
Average over 2nd and 3rd trimesters				5.4	12.6	+21.0	+25

a Carmichael *et al* (1997); average body weight gain by end of trimester; divided by 2 to get approximate increase mid-trimester
b End of trimester increase in body weight X 0.66 g/kg/d, the Estimated Average Requirement (EAR) for maintenance of protein in adults.
c From Table 10-15 where protein deposition = total potassium accumulated (mmol/day) / 2.15 (mmol potassium/g nitrogen) X 6.25; and assumption that nitrogen accretion during second trimester is ~50% that of third trimester
d Protein deposition / 0.43, slope of regression line of protein intake versus nitrogen balance (recalculated from King *et al*., 1973).
e Average required additional amount needed during pregnancy
f RDA is based on EAR + assumed variation in requirements; amount needed above nonpregnant needs

Appendix B – Pre-study questionnaire

Pre-Study Day Assessment
Protein Requirement: Healthy Pregnant Women

Principal Investigator: Dr. Rajavel Elango Student Investigator: Trina Stephens	[redacted] (office) (office) (cell)

PRELIMINARY ASSESSMENT

Subject ID:_____ Date:_____

Birthday: _____/_____/_____ Age:_____ Last Menstrual Period:_____

Ultrasound Dating: _____ Gestational Age: _____

Height (cm):_____ Weight (kg):_____ BMI:_____

Fasting Blood Glucose (mmol/L):_____ Pre-Pregnancy Weight (kg): _____

[Optional]
Primary Health Care Provider Details (name, phone, address):_____
_____

Skinfold measurement

MAC:_____
(mid-arm circumference)

TSF:_____
(tricep skin fold)

BSF:_____
(bicep skin fold)

SSSF:_____
(sub-scapula skin fold)

Bioelectrical Impedance Analysis

BIA:R_____
(resistance)

XC_____
(reactance)

Body Composition Profile

% Body fat (skinfolds):_____ Lean body mass (skinfolds):_____

% Body fat (BIA):_____ Lean body mass (BIA):_____

Indirect Calorimetry

Measured REE (kcal/day):_____ Estimated REE (kcal/day):_____

Daily energy requirement (kcal/day):_____

Medical History

Are you currently having vomiting episodes? Yes_____ How many/day _____ No_____

Are you currently taking Diclectin®? Yes_____ How many/day _____ No_____

Are you currently taking any other medications? Yes_____ No_____

List of medications:_____

Any preexisting health condition(s)? Yes_____ No_____

Details of health condition(s) _____

Do you smoke cigarettes? Yes_____ No_____

Are you consuming alcohol on a regular basis during your pregnancy? Yes___ No___

Have you ever been diagnosed with any of the following health conditions?

Gestational diabetes ☐ Pregnancy-associated or chronic hypertension ☐

Preclampsia/Eclampsia ☐ Pregnancy-related anemia ☐

Pregnancy-related jaundice ☐

Pregnancy Information

Is this your first pregnancy? Yes_____ No_____

If no, when did you last give birth (months)?_____

Were any assisted reproductive therapies (ARTs) used for conception? Yes____ No_____

Details of ARTs _____

Nutritional Supplement Intake

Are you currently taking prenatal vitamins? Yes_____ No_____

If yes, how long have you been taking them?_____

Which brand of prenatal vitamins?_____

Are you taking any other vitamins/ nutritional supplements? Yes_____ No_____

If yes, please list all vitamin/nutritional supplements:

1._____
2._____
3._____
4._____

Activity Level

Daily exercise (minutes)_____

Sedentary_____ Moderate_____ High_____

Availability for 4 studies

Yes_____ No_____

Comments:

Appendix C – Recruitment material

Research Study:
Dietary Protein in Pregnancy

Proteins from food are important for building strong, healthy bodies. During pregnancy, women need more high quality protein in their diet to support fetal development. At the Child and Family Research Institute, we are interested in determining how much dietary protein pregnant women need to ensure a healthy pregnancy.




You can help us!

We are looking for women in the 1st, 2nd, and 3rd trimester of pregnancy, aged 19-35 years.
This study involves an initial assessment followed by 4 separate visits to the BC Children's Hospital, each lasting about 8-9 hours. Each visit involves a special diet, collection of breath and urine, and measurement of blood glucose, body size, and muscle mass.

Compensation will be offered to participants.

If you would like more information about this study, **please contact us** today!

Principal Investigator: Dr. Rajavel Elango Office:

Primary Contact: Trina Stephens Office:

Email:

How Can You Help?

For our study we will need healthy, pregnant women who are more than 12 weeks gestation, or less than 36 weeks gestation. Participants will meet with us for an initial 1 hour assessment to participate in a 8-9 hour study day, during which we will provide a special diet containing protein and our tracer. Several breath and urine samples will be taken throughout the course of the day, and at the end of the 8-9 hours you can go home and eat whatever you like!

Each individuals is invited to participate in up to 4 separate study days.

Compensation will be provided for your time.

If you are interested, please contact:
Trina Stephens

CHILD & FAMILY RESEARCH INSTITUTE

For further details, please contact:

Trina Stephens

Principal Investigator:
Dr. Rajavel Elango

Email:

Dietary Protein in Pregnancy

The Importance of Protein

Protein is an essential part of our diet. It is used to build muscle and body tissue, to send signals throughout the body, and to support the immune system. During pregnancy, dietary protein becomes even more crucial, as it is needed for healthy development of the baby. Inadequate protein intake during pregnancy has been linked to a future risk of high blood pressure, heart disease and metabolic problems in the baby. For this reason, it is very important to understand how much protein we need to eat during pregnancy.

Understanding Protein Requirements in Pregnancy

Currently, it is recommended that women consume at least 0.88 grams of high quality protein for every kilogram of body weight per day. However, this estimate is based heavily on studies of non-pregnant adults. Up until now, studying protein requirements meant participants needed to eat an experimental diet low in protein for several days at a time. Obviously, this approach was not suitable for pregnant women, who must eat a balanced diet to support their developing baby. Thus, a new way of studying protein requirements was needed.

Our Study

To reduce the amount of time participants have to spend consuming the experimental diet, our study uses a stable isotope tracer. This tracer allows us to "see" how protein eaten in the experimental diet is used by the body. Because the tracer is rapidly expelled from the body in breath and urine, the entire study can be conducted in an 8 hour day. Since we are able to determine protein requirements by measuring the amount of stable isotope in breath and urine, we only need to collect breath and urine samples (no needles!). With this safe, non-invasive method of determining protein requirements, we are finally able to assess the dietary protein needs of pregnant women.

What is a Stable Isotope?

Isotopes are different types of atoms of the same element. For example, the element Carbon often appears as ^{12}C, ^{13}C, and ^{14}C atoms. While some isotopes are reactive and can be used to produce energy, many are completely stable. In our study we use ^{13}C, a naturally occurring, **stable** isotope which is completely safe.

Appendix D – Subject consent form

Department of Pediatrics
950 West 28th Avenue, Room 170A
Vancouver, BC, V5Z 4H4
Tel: [redacted]
Fax: [redacted]

INFORMED CONSENT AND SUBJECT INFORMATION

Determination of protein requirements in healthy pregnant women using the indicator amino acid oxidation technique

Principal Investigator: Dr. Rajavel Elango, PhD
Department of Pediatrics
Faculty of Medicine
The University of British Columbia
Telephone: [redacted]

Primary Contact: Trina Stephens, BScH (SSP), BA (Min), M.Sc candidate
Department of Pediatrics
Faculty of Medicine
The University of British Columbia
Telephone: [redacted]

Emergency Phone Number: Rajavel Elango [redacted]
Trina Stephens [redacted]
available 24 hours per day and seven days per week

Sponsors: **Canadian Institutes of Health Research (CIHR)**

Site: Oak Street Campus, UBC
Child & Family Research Institute

1. INTRODUCTION

Protein is an essential part of our diet. It is used to build muscle and body tissue, to send signals throughout the body, and to support the immune system. During pregnancy, dietary protein becomes even more crucial, as it is needed for healthy development of the baby. Inadequate protein intake during pregnancy has been linked to a future risk of high blood pressure, heart disease and other metabolic problems in the baby. For this reason, it is very important to understand how much protein we need to eat during pregnancy. Even though it is well known that pregnant women need more protein in their diet, it is not exactly known how much additional protein is required. The older techniques to measure how much protein we need require participants to eat a low protein diet for several days at a time. Because it is unethical to do this in a pregnant woman, there is very little information about protein requirements in pregnant women. To gain a better understanding of protein requirements throughout pregnancy, we plan to study pregnant women from all ethnic backgrounds, aged 19-35 years, in their 2nd and 3rd trimesters of pregnancy using a more quick and modern technique called the

indicator amino acid oxidation (IAAO) technique. This technique uses a test liquid meal with a specific amount of protein mixed with a stable isotope tracer. A stable isotope is a labeled amino acid (building block of protein). These labeled amino acids are colourless, odourless and tasteless, but can be measured in breath and urine, because it looks different than the rest of the amino acids in the body. This allows us to measure if you are eating enough protein for protein synthesis to take place, and we can study protein requirements in just one day. Stable isotopes are completely safe, because they are present in the air we breathe, water we drink and food we eat. This technique has been used to measure protein requirements in healthy babies, children and adult human beings. More details about the stable isotopes are available in point 4 below.

2. YOUR PARTICIPATION IS VOLUNTARY

You are invited to participate in our study. Your participation is entirely voluntary, so it is up to you to decide whether or not to take part in the study. Before you decide, it is important for you to understand what the research involves. This consent form will describe the study, why the research is being done, what will happen to you during the study and the possible benefits, risks, and discomforts. If you wish to participate, you will be asked to sign this consent form within 7 days. If you decide to take part in this study, you are still free to withdraw at any time and without giving any reasons for your decision(s). If you do not wish to participate, you do not have to provide any reason for your decision(s) nor will you lose the benefit of any medical care to which you are entitled or are presently receiving. Please take time to read the following information carefully and to discuss it with your family and friends before deciding.

3. WHO IS CONDUCTING THE STUDY?

The study is being conducted by the Nutrition and Metabolism Research Program of the Child and Family Research Institute, University of British Columbia. Funding has been received from the Canadian Institutes of Health Research (CIHR) to complete this study and you are entitled to request details about the research funding from the Principal Investigator.

4. WHAT IS THE PURPOSE OF THE STUDY?

This study is about how much protein pregnant women need in early (2^{nd} trimester) and late pregnancy (3^{rd} trimester). We will measure your protein requirements using the IAAO technique, which uses a ^{13}C stable isotope (labeled) amino acid to trace whole-body amino acid oxidation (^{13}C is a type of carbon; amino acids are made of mostly ^{12}C, so the ^{13}C can be detected in breath and urine samples with special equipment). The ^{13}C stable isotopes are completely safe, because they are present in the air we breathe, water we drink and food we eat. Oxidation of the labeled amino acid will be measured from breath and urine samples. This will allow us to determine the correct amount of dietary protein a pregnant women needs to

eat to maintain health in early and late pregnancy.

5. WHO CAN PARTICIPATE IN THE STUDY?

- ☐ Women who are 19 to 35 years-of-age and are pregnant with a single child

6. WHO SHOULD NOT PARTICIPATE IN THE STUDY?

- ☐ Women who are not pregnant or
- ☐ Women who are pregnant with more than one child (this changes protein demands)
- ☐ Women who are not 19-35y or
- ☐ Women not in good health or have a metabolic, neurological, genetic, or immune disorder, including gestational diabetes or anemia
- ☐ Women who smoke or consume alcohol during their pregnancy
- ☐ Women who are classified as underweight (<18.5 kg/m^2), overweight (25-30 kg/m^2) or obese (>30 kg/m^2**)**
- ☐ Women who are allergic to eggs and egg protein
- ☐ Women who have severe nausea/vomiting throughout their pregnancy

7. WHAT DOES THE STUDY INVOLVE?

This study will be conducted at the Oak Street Campus of UBC at the Child and Family Research Institute (CFRI). If you agree to participate in this study, then you will be asked to complete the procedures described below. You may participate in four separate study days, two in the 2nd trimester and two in the 3rd trimester.

Preliminary Study Day Procedures [Once during 2nd trimester and once during 3rd trimester]:

- ☐ The preliminary assessment is done to collect basic information about you, make sure you are informed about the study details, and to collect information about you to design the study diet specifically to meet your body needs.

- ☐ The preliminary assessment will be conducted at the Clinical Research Evaluation Unit (CREU) at the Child & Family Research Institute located in BC Children's Hospital. You will be asked to come at 8AM after having fasted overnight (10-12h). The whole procedure will take 1 hour to complete.

- ☐ During the preliminary assessment, a Research Assistant will measure your weight, height, blood glucose, body fat and muscle mass, and resting metabolic rate which tells us how much energy your body needs. Blood

glucose will be measured using a glucose meter that reads the amount of sugar in your blood by gently pricking the finger. Body fat will be measured using skin-fold thickness measured from the arm and shoulder using a caliper (a handheld instrument that gently pinches your skin between two moving arms). Body muscle will be measured using bioelectrical impedance which measures the passage of a small, safe amount of current (that cannot be felt) between four electrodes on the arms and legs while you lay still for a few minutes. The body fat and muscle measurements are completely safe and do not cause any discomfort or harm. Metabolic rate is measured using an indirect calorimetry machine, which consists of a clear hood that is placed over your head while you lay on a bed, breathing normally. You can see everything through the hood and breathe normally without any discomfort. This measurement takes about 20 minutes to complete.

- ☐ You will also be asked health related questions to assess your medical history. If you are not taking prenatal vitamins, we will provide you with some at this time.

- ☐ During the preliminary assessment you will meet with a Dietitian who will evaluate your normal dietary protein intake. The dietitian will give you recommendations on how you can meet the standard protein intake - required in the two days prior to each study day - from the foods you normally eat in your diet.

Study Day Procedures:

- ☐ The study day will be conducted in the Clinical Research Evaluation Unit (CREU) at the Child & Family Research Institute located in BC Children's Hospital. You will be asked to come at 8AM after having fasted overnight (10-12h).

- ☐ Only **water** may be consumed prior to arriving on the study day, and during the study day. The study day test diet as described below will provide your daily energy and nutritional needs. At the end of the study day, you are free to resume your normal food intake.

- ☐ On the study day a Research Assistant will again measure your weight, height and blood glucose. The Research Assistant will also measure the rate at which you are breathing out carbon dioxide (VCO_2) using the same indirect calorimetry machine that was used to determine metabolic rate in the preliminary assessment.

- ☐ You will eat the test liquid diet as eight small hourly meals on the study day. Each meal is made up of 1) a mixture of amino acids, 2) an amino acid-free flavored liquid and amino acid-free cookies that provide energy and

other nutrients, and 3) the labeled amino acid is added to the last four meals. The test meals will meet all your daily energy, vitamin and mineral needs, as they were determined during the preliminary assessment.

- ☐ To measure how your body responds to the test diet we will collect your breath sample 9 times and urine sample 6 times during the study day. To collect breath you will have to breathe into a container - just like blowing through a straw into a bag. To collect urine you will have to pass urine into a urine sample hat in the privacy of the washroom. The first and last urine samples of the day will be tested for protein in the urine using a dipstick test. When we are not collecting samples, you can watch television, listen to music, read or bring computer related work to complete.

- ☐ In total, you can expect to dedicate approximately 9 hours per study day you participate in. You are invited to participate in up to 4 studies over the course of your pregnancy. If you choose to participate in all 4 studies, you will be asked to dedicate approximately 36 hours to this project, plus an hour each during the 2 preliminary study visits.

8. WHAT ARE THE POSSIBLE HARMS AND SIDE EFFECTS OF PARTICIPATING IN THIS STUDY?

There are no known risks involved with participating in this research. Some women may find the finger prick used for blood glucose measurement uncomfortable. We recognize that the length of the study day, and travel to BC Children's Hospital might pose an inconvenience for you.

9. WHAT ARE THE BENEFITS OF PARTICIPATING IN THIS STUDY?

There are no direct benefits to you from taking part in this study. However, we hope that the information learned from this study can be used in the future to improve dietary protein recommendations during pregnancy.

10. WHAT HAPPENS IF I DECIDE TO WITHDRAW MY CONSENT TO PARTICIPATE IN THIS STUDY?

Your participation in this research is entirely voluntary. You may withdraw from this study at any time and without providing any reasons for your decision. If you decide to enter the study and then withdraw, there will be no penalty or loss of benefits, if any, to which you are otherwise entitled. If you choose to enter the study and then decide to withdraw at a later time, all data collected about you during the enrolment part of the study will be retained for analysis, after which the study information may be shredded.

11. WHAT WILL THE STUDY COST ME?

Participation in the study will not cost you anything. In appreciation of the time that it takes to complete this study you will receive $100 upon each study day completion to a maximum of $400 for 4 study days. Vehicle parking coupons for the pre-study duration at BC Children's Hospital will be provided.

12. WILL MY TAKING PART IN THIS STUDY BE KEPT CONFIDENTIAL?

Your confidentiality will be respected. No information that discloses your identity will be released or published without your specific consent to the disclosure. However, research records and medical records identifying you may be inspected in the presence of the Investigator or his or her designate by representatives of Health Canada and the UBC Research Ethics Boards for the purpose of monitoring the research. No records that identify you by name or initials will be allowed to leave the Investigators' offices. In this study your samples and questionnaires will be identified by a study code and any identifying information will be kept in a locked cabinet in a secure area. Analyzed sample data from the study will be shared with specific project collaborators at the University of Toronto and the University of Alberta. No information that identifies you will be allowed to leave the study center or be used in any reports or publications about the study. Signing this consent form in no way limits your legal rights against the sponsor, investigators, or anyone else.

13. WHO DO I CONTACT IF I HAVE QUESTIONS ABOUT THE STUDY DURING MY PARTICIPATION?

This study will be fully explained to you, and you will be given the opportunity to ask questions. If you have questions or want more information about the study procedures before or during participation, you may contact Dr. Rajavel Elango at any time at [redacted], Trina Stephens at any time at [redacted], and/or the toll free Research Subject Information Line at any time at [redacted]

14. WHO DO I CONTACT IF I HAVE QUESTIONS OR CONCERNS ABOUT MY RIGHTS AS A SUBJECT DURING THE STUDY?

Your rights to privacy are protected and guaranteed by the *Child, Family and Community Services Act*. This Act lays down the safeguards respecting your privacy and also gives you the right of access to the information about you that has been provided to the study, and if needed, you have the chance to correct any errors in the information. Further details about this legislation are available on request. If you have any concerns about your rights as a research subject and/or your experiences while participating in this study, contact the toll free Research Subject Information Line at any time at [redacted] or via e-mail to [redacted]

15. SUBJECT CONSENT TO PARTICIPATE

My signature on this consent form means that I:

- ☐ have had this study explained to me, read this form and understand the information concerning this study.
- ☐ have had sufficient time to consider the information provided, get advice and ask questions if necessary and I have received satisfactory responses to my questions.
- ☐ understand that all of the information collected will be kept confidential and that the results will only be used for scientific objectives.
- ☐ understand that my participation in this study is voluntary and that I am completely free to refuse to participate or to withdraw from this study at any time without giving any reason(s) and my decision to withdraw will not change in any way the quality of care that I receive.
- ☐ understand that signing this consent form in no way limits my legal rights against the sponsor, investigators or anyone else.
- ☐ understand that there is no guarantee that this study will provide any benefits to myself.
- ☐ understand that if I have any further questions or desire further information I should contact Dr. Rajavel Elango at [redacted]
- ☐ understand that if I have any concerns about my rights as a research subject or my experiences while participating in this study, I may contact the toll free Research Subject Information Line at any time at [redacted] or via e-mail to [redacted]
- ☐ have been told that I will receive a dated and signed copy of this form for my own record.

I, _____ voluntarily give consent for my participation in the
(Subject. Please print your name)

research study entitled:

Determination of protein requirements in healthy pregnant women using the indicator amino acid oxidation technique

_____ _____
Signature of Subject *Date*

_____ _____ _____
Investigator Signature *Printed Name of Principal Investigator/ Designated Representative* *Date*

Appendix E – Food record template

Dietary Record Name:_____

Date: ____/_____/_____ Mon Tues Wed Thu Fri Sat Sun

Item	Amount	Item	Amount
Breakfast		Snack	
		Dinner	
Snack			
Lunch			

Appendix F – Study day protocol

Study Day Schedule
Protein Requirement: Healthy Pregnant Women

Subject ID:_____ Date:_____

Height (cm):_____ Weight (kg):_____ Blood Glucose (mmol/L):_____

Protein intake (g/kg/d): _____ Energy intake (kcal/day): _____

Time	Sample Collection/ Anthropometry	Meals and isotope tracer	Comments
8:00		Meal #1	
9:00		Meal #2	
10:00		Meal #3	
11:00		Meal #4	
11:15	1st breath (3x) 1st urine		
11:30	2nd breath (3x)		
11:45	3rd breath (3x) 2nd urine		
12:00	VCO_2 measurement	Meal #5 – primer dose and 1st oral dose	
13:00		Meal #6 – 2nd oral dose	
14:00		Meal #7 – 3rd oral dose	
14:30	4th breath (3x) 3rd urine		
15:00	5th breath (3x) 4th urine	Meal #8 – 4th oral dose	
15.15	6th breath (3x)		
15:30	7th breath (3x) 5th urine		
15:45	8th breath (3x)		
16:00	9th breath (3x) 6th urine		

Chemstrip 7© Dipstick Test (Roche®)

Morning Urine		Afternoon Urine	
pH:	Nitrite:	pH:	Nitrite:
Glucose:	Protein:	Glucose:	Protein:
Ketones:	Blood:	Ketones:	Blood:
Leukocytes:		Leukocytes:	

Appendix G – Letters of contact to primary care provider

Department of Pediatrics
BC Children's Hospital
4480 Oak St., Room 2D19
Vancouver, BC Canada
Tel: [redacted] Fax: [redacted]

Date:_____

To the Health Care Provider of Ms._____

Ms. _____ volunteered to participate in a research study titled "Determination of protein requirements in healthy pregnant women using the indicator amino acid oxidation technique" being conducted at the Child & Family Research Institute, BC Children's Hospital and Department of Pediatrics, University of British Columbia.

As part of this study we measure fasting blood glucose using a finger prick glucometer to screen for normal glucose values (<6.7mmol/L).
Her fasting blood glucose on the date specified above was:_____mmol/L.
Her second measurement 5 minutes later was:_____mmol/L.

We have stopped her participation in our study and requested her to follow up this fasting glucose measurement values with you, as her primary health care provider.

If you require further information or any clarification regarding this please do not hesitate to contact me.

Sincerely,

Rajavel Elango Ph.D.
Assistant Professor, Department of Pediatrics
University of British Columbia
Scientist, Level 1, Diabetes, Nutrition & Metabolism
Child & Family Research Institute
BC Children's Hospital
Room 170A, 950 West 28th Avenue
Vancouver, BC, V5Z 4H4
Ph: [redacted]; Fax: [redacted]
Cell: [redacted]
Email: [redacted]

Department of Pediatrics
BC Children's Hospital
4480 Oak St., Room 2D19
Vancouver, BC Canada
Tel: [redacted] Fax: [redacted]

Date:_____

To the Health Care Provider of Ms._____

Ms. _____ volunteered to participate in a research study titled "Determination of protein requirements in healthy pregnant women using the indicator amino acid oxidation technique" being conducted at the Child & Family Research Institute, BC Children's Hospital and Department of Pediatrics, University of British Columbia.

As part of this study we conduct two dipstick tests to check for protein in the urine:

Chemstrip 7 Dipstick Test (Roche)

Morning Urine		Afternoon Urine	
pH:	Nitrite:	pH:	Nitrite:
Glucose:	Protein:	Glucose:	Protein:
Ketones:	Blood:	Ketones:	Blood:
Leukocytes:		Leukocytes:	

We have requested that she to follow up this urine analysis with you, as her primary health care provider.

If you require further information or any clarification regarding this please do not hesitate to contact me.

Sincerely,

Rajavel Elango Ph.D.
Assistant Professor, Department of Pediatrics
University of British Columbia
Scientist, Level 1, Diabetes, Nutrition & Metabolism
Child & Family Research Institute
BC Children's Hospital
Room 170A, 950 West 28th Avenue
Vancouver, BC, V5Z 4H4
Ph: [redacted]; Fax: [redacted]
Cell: [redacted]
Email: [redacted]

Appendix H – Protein turnover estimated from LC-MS analysis

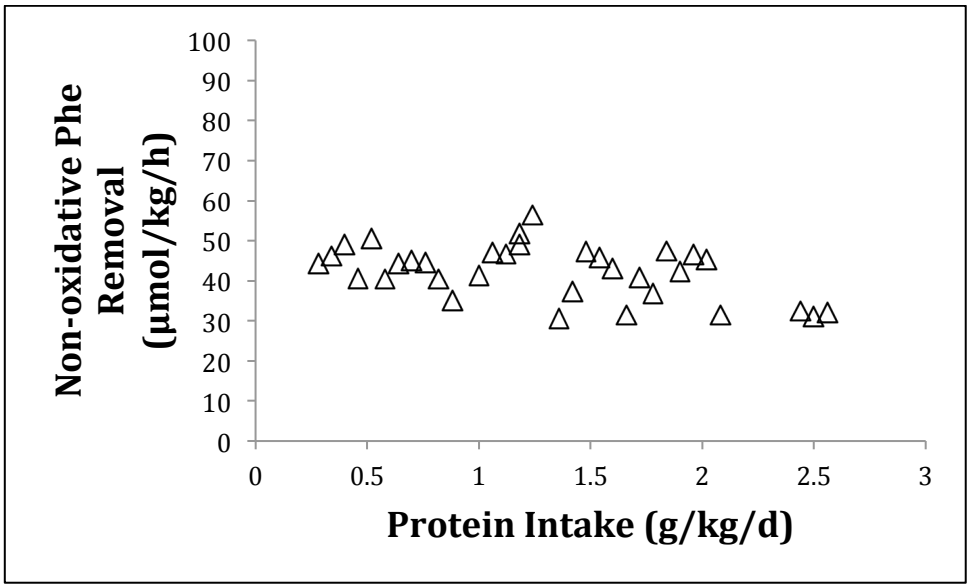

Figure 1. Protein synthesis versus protein intake in early gestation group

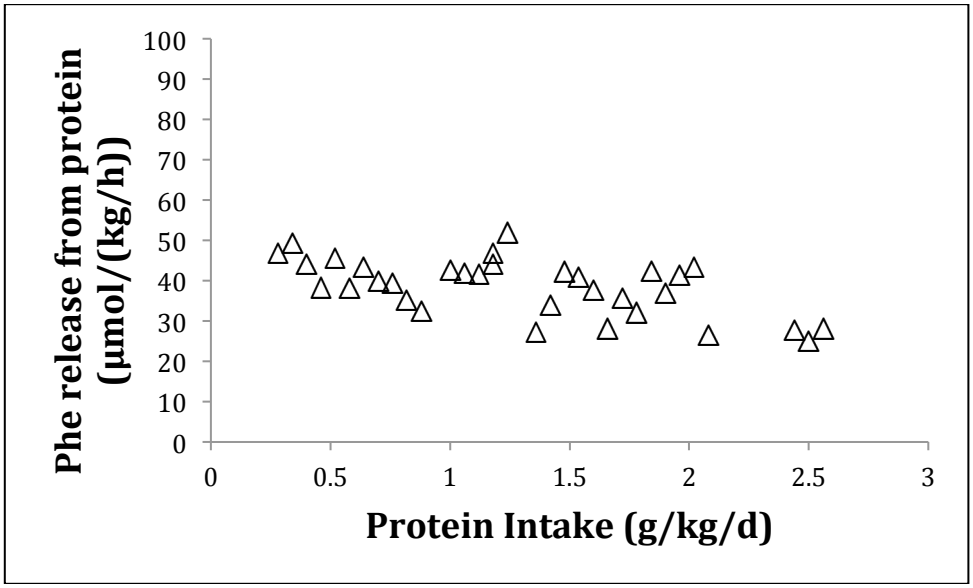

Figure 2. Protein breakdown versus protein intake in early gestation group

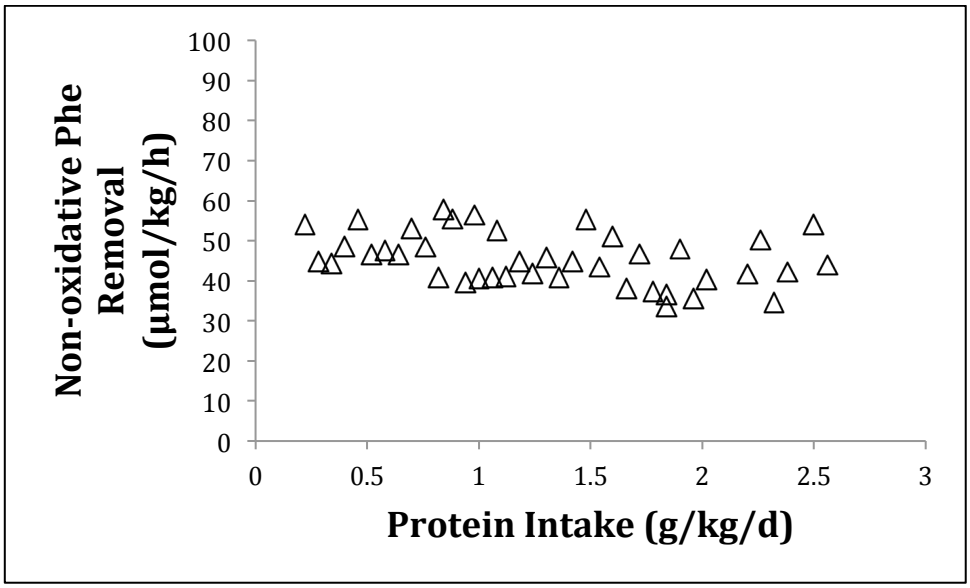

Figure 3. Protein synthesis versus protein intake in late gestation group

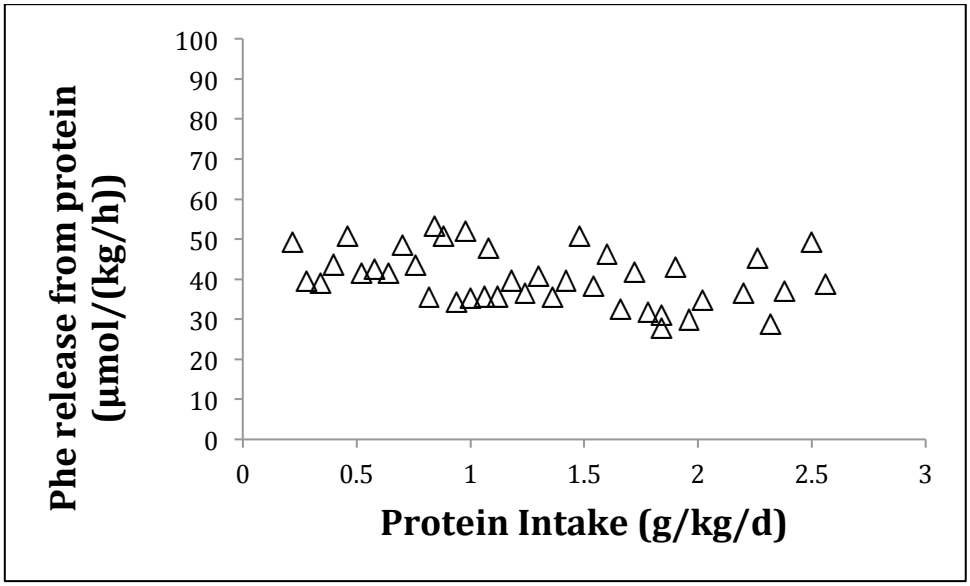

Figure 4. Protein breakdown versus protein intake in late gestation group

Table 1. Comparison of protein turnover estimates during pregnancy

Author	Gestational Age (weeks)	Sample size	State	Synthesis	Breakdown	Turnover
Stephens *et al.,* 2013	11-20	37	Fed	4.37 g PRO/kg/d[a]	3.98 g PRO/kg/d[a]	0.39
Stephens *et al.,* 2013	31-38	44	Fed	4.71 g PRO/kg/d[a]	4.17 g PRO/kg/d[a]	0.54
Thompson and Halliday 1992	35	6	Fasted	3.87 g PRO/kg/d	n/a	n/a
Jackson, Duggleby and Grove 2000	31	5	Fed	1.99 g N/hour	1.73 g N/hour	0.26
Willommet *et al.,* 1992	33	9	Fed	5.4 g PRO/kg/d	4.1 g PRO/kg/d	1.3
Wittaker, Lee and Taylor 2000	34-38	6	Fasted	4.81 g PRO/kg/d	5.39 g PRO/kg/d	-0.58

[a] Assuming 232μmol Phe/g muscle protein (Kaufman S. "A model of human phenylalanine metabolism in normal subjects and in phenylketonuric patients." *Proceedings of the National Academy of Sciences in the United States of America*, 96.6 (1999): 3160-3164)