Examination of the relationship between blood urea nitrogen, macronutrient intake, and postnatal growth of body compartments in very low birth weight preterm infants

Examination of the relationship between blood urea nitrogen, macronutrient intake, and postnatal growth of body compartments in very low birth weight preterm infants

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TITLE: Examination of the relationship between blood urea nitrogen, macronutrient intake, and postnatal growth of body compartments in very low birth weight preterm infants

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Abstract

Background: Given that 43-97% of preterm infants face postnatal growth restriction by hospital discharge, monitoring of growth is challenging but critical for clinical management of preterm infants. Currently, serial anthropometric measurements of weight and height are used to monitor growth but lack sensitivity. Thus, by the time significant deviations in growth trajectory are identified, an infant has already reached sub-optimal growth. A biomarker that is predictive of sub-optimal growth can serve as a preventative tool in clinical decision making. Blood urea nitrogen (BUN) may be one such potential metabolic biomarker, as it has been used as a measure of protein adequacy and thus, may additionally indicate quality of growth. While protein intake has a well-established correlation with growth, it is currently unknown if BUN is correlated with postnatal growth and if it can be used as a biomarker for growth.

Objectives: 1) to examine the relationship between BUN and macronutrient intake factors such as protein intake, protein-to-energy (P:E) ratios, and carbohydrate to non-protein energy (CHO:NPE %) to better understand BUN response; 2) to examine the potential of using BUN as a predictive metabolic biomarker of growth status in a multiple linear regression. We hypothesize that BUN will positively correlate with protein intake, P:E and negatively with CHO:NPE ratio. It will also be positively correlated with growth parameters: growth velocity, length gain, head circumference gain and fat free mass.

Methods: Very low birth weight preterm infants (n=101) born ≤30 weeks of gestation at McMaster Children's Hospital's level III NICU were included. BUN was assessed at three time points: baseline (SDay1), study day 14 (SDay14) and study day 21 (SDay21). Intake of protein and energy were collected for the 24-hour period prior to the BUN measure, their averages computed over SDay14 and SDay21 and included as confounding predictor variables. Other confounding variables such as maternal characteristics and baseline study group characteristics were also considered. Growth velocity, length gain and head circumference gain at SDay14 and SDay21, and body composition (FFM%, FFMI) between 36-40 weeks were examined as dependent growth variables. After an initial univariate analysis of baseline and maternal confounders, multiple linear regression models were then developed in a block design as follows: for the analysis of BUN vs macronutrient factors- block 1: 24-hour macronutrient intake factors + relevant baseline and/or maternal confounders; block 2: average macronutrient factors; for the analysis of BUN vs growth- block 3: BUN.

Results: In the analysis of BUN and macronutrient intake, BUN was found to have a significant positive correlation with P:E ratio at all time points. Protein intake was positively correlated with BUN only at SDay1 and SDay21. CHO:NPE ratio did not correlated with BUN at any time point. The R^2 for the multiple regression of BUN and macronutrient factor analysis at SDay1, SDay14 and SDay21 was 0.19, 0.42 and 0.44 respectively. In the analysis of BUN vs growth, SDay1 BUN had a significant negative correlation with SDay21 growth velocity ($p=0.02$). The addition of SDay1 BUN to the model of SDay21 growth velocity was significant ($p<0.01$ of F change statistic, $R^2= 0.17$).

SDay21 BUN also had a significant negative correlation with SDay21 growth velocity ($p<0.01$) and its addition was significant to the model ($p<0.01$ of F change statistic, $R^2 =0.22$). BUN was not related to SDay 14 growth velocity, or to length gain, head circumference gain or any body composition estimates at any time point. Additionally, P:E was found to be significantly negatively correlated with growth.

Conclusion: BUN is a statistically and clinically significant marker of nutritional adequacy, both of protein intake and energy in relation to protein intake. Addition of BUN adds to the explanation of variation in growth, and this is statistically significant, however, the additional variation explained may be too small to be clinically significant. Additionally, we observed that P:E ratio was significantly negatively correlated with growth. Thus, it may be more clinically pertinent to use high BUN values as a marker of inadequate energy to protein intake to prevent future sub-optimal growth.

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List of Abbreviations

ADP:	Air Displacement Plethysmography
BIA:	Bioelectrical Impedance Analysis
BMI:	Body Mass Index
CHO:NPE:	Carbohydrate to non-protein energy (%)
DXA:	Dual Energy X-ray Absorptiometry
FM:	Fat Mass
FFM:	Fat Free Mass
PMA:	Post Menstrual Age
P:E:	Protein-to-energy ratio (g/100kcal)
TBW:	Total Body Water

1: Introduction

1.1 Overview

According to World Health Organization, premature birth, defined as a birth under 37 weeks of gestation, affects 15 million out of 135 million births globally. And the incidence of premature birth has increased worldwide[1]. In 1981, 6.4% of the live births were premature and peaked at 8.2% in 2004 and decreased again to 7.7% in 2010[2-4]. It is one of the main causes of perinatal mortality in industrialized nations. In Canada, 75%-85% of perinatal mortality is explained by premature birth[2]. Preterm birth is also a crucial determinant of infant morbidity such as acute respiratory failure, chronic respiratory illnesses, gastrointestinal complications, ophthalmologic issues, intraventricular haemorrhage, immunological deficiencies and neurodevelopmental impairment. Although the exact etiology of premature birth is not known, multiple births, increased maternal age, infections, inflammation, infertility treatments, diabetes and obesity have all been implicated as risk factors. Therefore, preterm birth has wide-ranging economic, medical, social and psychological implications.

1.2 Rationale for this work

Due to advances in neonatal medicine, preterm infant survival has improved markedly over the last few decades particularly for very low birth weight (VLBW, birth weight <1500 g) infants. However, morbidity rates still remain high. Prematurity exposes infants not only to neonatal complications such as respiratory distress syndrome, retinopathy and neurological insults but also adversely impacts long-term health outcomes such as cardiovascular disease, hypertension, insulin resistance, obesity and

neurodevelopmental delays. Current neonatal research focuses on improving long-term outcomes of surviving preterm infants. Epidemiological studies suggest that postnatal growth is associated with later-life health outcomes indicating that the postnatal period is a critical time of epigenetic modifications. Thus, monitoring of growth is critical for management of preterm infants. In current practice, anthropometric measurements are used to monitor growth. However, by the time trends in growth become apparent through the use of these measures, sub-optimal growth has already taken place. A sensitive biomarker that is predictive of poor growth can serve as diagnostic tool in clinical decision making. Blood urea nitrogen (BUN) may be one such potential metabolic biomarker, as it has been used as a measure of protein adequacy, and therefore, it may also indicate composition of growth. Additionally, it can be quickly measured in routine blood tests. As a metabolite, it is also affected by energy quality and quantity of energy in relation to protein, thus it is important to examine these factors in order to interpret BUN values. While protein intake has a well-established correlation with growth, it is currently unknown if BUN is correlated with postnatal growth and if it can be used as a biomarker for growth. It is also unknown how BUN responses to energy quality and quantity of energy in relation to protein.

1.3 Research Aims

1. Establish valid methods of assessing growth velocity, and body composition
2. Determine if BUN correlates with intake of protein, protein to energy (P:E) ratio, and carbohydrate to non-protein energy (CHO:NPE) ratio while accounting for maternal, and baseline study group characteristics as confounders.

3. Investigate if BUN can be used as a biomarker of growth by assessing the correlation between BUN and growth parameters, while accounting for protein intake, P:E and CHO:NPE ratio, maternal and baseline study group characteristics as confounders.

1.4 Research Hypotheses

We hypothesize that a high BUN will reflect a high protein intake, high P:E and low CHO:NPE ratio. Because of the association between protein intake and growth, we hypothesize that higher BUN will also reflect an increase in growth parameters: growth velocity, length gain, head circumference gain and fat free mass.

2: Literature Review

2.1 Fetal Growth and Nutrition

2.1.1 Fetal growth

Towards the end of the second trimester, specifically between 23-27 weeks of gestation, the fetus grows at a rate of 21 g/kg/day. Between 35-37 weeks of gestation, the fetus grows at a rate of 12 g/kg/day[5]. Early in its life, the fetus has a high water content and an abundance of extracellular sodium and chloride[6]. Throughout the second and third trimester, the fetus accrues lean mass at a steady rate, with an accretion of 2 g/kg/day at the beginning of the third trimester and decreasing to 1.8 g/kg/day towards the end of gestation[7]. Fat mass deposition is negligible prior to the third trimester, but daily accretion of fat increases rapidly in the last trimester, with deposition at 1.4 g/kg/day in the early part of the trimester. It even exceeds the protein accretion in the last few weeks of pregnancy at a rate of 1.9 g/kg/day[6-8]. The fetus derives 80% of its energy needs for growth and metabolism from glucose. The remaining energy is provided mainly by amino acid oxidation, but can also be obtained from lactate, ketoacid or fatty acid oxidization[9]. This rapid intrauterine growth of the fetus is sustained by the active and passive transport of nutrients by the placenta as well as the production of key hormones such as insulin and insulin like growth factors (IGF). Premature birth interrupts fetal nutrition, and the infant is deprived of the opportunity to grow at their intrauterine potential.

2.1.2 Regulators of fetal growth

Growth of the fetus is mainly regulated by a complex interplay between the supply of nutrients to the fetus by the placental unit, and the fetal endocrine system. Genetics plays little role in fetal growth. A well-known crossbreeding experiment of horses show that the fetus does not grow to its maximum genetic potential. Instead, a fetus of the same genetic make-up is smaller at birth if it gestates in the uterus of a smaller breed mare. Thus, fetal growth is constrained by maternal size[10]. This also holds true for humans; the birth weights of half siblings are more strongly correlated when the common parent is the mother[11]. In the cases of ovum donation, birth weight is closely associated with the weight of the recipient mother but unrelated to the weight of the ovum donor[12]. These associations suggest that maternal intrauterine environment has a more dominant role in fetal growth than the genetic potential of the fetus. Fetal growth then, is ultimately determined by non-genetic factors. It is regulated by fetal nutrition which is influenced by the size and transport capacity of the placenta. This in turn, is determined by maternal nutrition and uterine size[13].

In humans, maternal nutrition during late gestation does not appear to have a significant impact on fetal growth. Fetuses exposed to the Dutch famine during the first and second trimester had normal birth weights compared to fetuses exposed to famine during the second half of gestation[14]. The caloric intake was reduced to less than 50% the normal intake before a 10% reduction in birth weight was observed for those exposed to famine in late gestation. However, birth weight is a crude marker of fetal growth as birth weight can still be appropriate even if fetal growth is arrested for a period but is followed by an accelerated growth known as ‘catch-up’. Studies in sheep highlight this distinction as it is

possible to measure fetal growth by assessing fetal length experimentally, with an implanted measuring device. Undernutrition of ewes in late gestation in a study by Oliver et al.[15] showed a reduction in fetal growth rate, which resumed upon maternal refeeding. However, this trend was dependent upon periconceptional and early gestational maternal nutrition. If the fetuses were undernourished before mating and during early gestation, they grew slowly in late gestation and did not exhibit a further decrease in growth rate upon experiencing additional maternal undernutrition in late gestation. While fetal birth weight at 125 days of gestation were similar, this study indicated that periconceptional events can result in adaptation of fetal growth response to resist later nutritional insult.

The maternal, placental and fetal endocrine system also regulates fetal growth. Insulin-like growth factor-1 (IGF-1) and IGF-2 are produced by the fetal and maternal liver[16]. They signal cells to uptake glucose and amino acids, and are mitogenic. IGF-2 regulates early fetal growth whereas IGF-1 regulates fetal growth in late gestation[17]. In IGF-1 knockout mice, placental size is unaffected however, the fetuses are 60% smaller at birth[18]. IGF-2 knockouts are also 60% smaller at birth, however, placental size is limited implicating IGF-2 in placental development[19]. The endocrine system is also altered by nutrient supply. A study of maternal undernutrition of sheep for 20 days found a decrease in maternal and fetal IGF-1 and insulin levels[20]. Similarly to findings of Oliver et al.[15], fetal birth weights were unaltered, however organ size was affected. Upon maternal refeeding, or glucose or insulin infusion, fetal IGF-1 levels were restored[21]. This indicated that nutrient supply regulates IGF-1 through insulin response which in turn, regulates fetal growth. Additionally, while the placenta regulates fetal growth mainly through nutrient supply, it

also produces its own hormones. Of interest, is the placental growth hormone as it also regulates maternal IGF-1 and insulin levels[22]. The placenta also modulates fetal IGF-1 levels[23] while both fetal[24] and maternal IGF-1[25] influence placental function.

In summary, fetal growth is regulated by interactions between maternal, placental and fetal factors. Maternal nutrition affects the supply to the fetus, as well as maternal and fetal endocrine responses which regulate fetal growth. The placenta acts as an interface between the fetus and the mother, responding to factors from both to regulate growth. The interplay between the maternal-fetal-placental units is dynamic as they modify, and are modified by, one another.

2.2 Postnatal Growth and Nutrition

2.2.1 Postnatal growth

The transition from a fetus to a newborn is the most complex physiologic adaptation in human experience involving both physiological and metabolic changes[26]. In the last trimester, adipose tissue accretion, fetal liver glycogen stores and cortisol levels increases in order to prepare for extrauterine adaption[26]. Cortisol assists in the maturation of lungs, gastrointestinal tract, and also induces changes in substrate metabolism associated with parturition. As the fetus transitions from a continuous intravenous nutrition to an intermittent one, cortisol and glucagon surge with parturition and induce a postnatal catabolic state to produce glucose by breakdown of glycogen and gluconeogenesis. Fat is metabolized within few hours and serves as the main source of energy until feeding is established[27]. This transition is particularly challenging for the preterm infant as they do not have sufficient energy stores to sustain a catabolic state[28], and establishing feeds can

pose challenges. In addition, preterm infants have a reduced cortisol response, and an immature metabolic response leaving the preterm infant unable to maintain glucose homeostasis[26, 27]. Furthermore, while fetal fuel is predominately glucose, the preterm infant eventually establishes a diet based mainly in lipids and glucose as it is the composition of most enteral nutrition. In fact, the lipid and glucose intake rates in preterm infants exceed the fetal delivery rates, whereas amino acid delivery are far below fetal accretion rates[29]. The term infant also must regulate its own body temperature. This is accomplished through the metabolism of brown adipose tissue accreted during the last trimester. Preterm infants do not accrue sufficient quantity of brown adipose tissue for thermogenesis[26]. Preterm infants also have difficulties sucking, swallowing and digesting oral feeds. Additionally, due to structural and functional immaturity of most organs, preterm infants require respiratory support, nutritional support, and added medical interventions for gastrointestinal, metabolic and immunological issues exposing them to frequent stressful states. Thus, the extrauterine adaptation is already complex in the term infant with increased energy expenditure associated with gluconeogenesis and thermogenesis; it is uniquely challenging for the preterm infant who has additional energy costs associated with medical interventions and maintaining vital processes[30]. As a result of these challenges and inadequate protein to energy balance, preterm infants accrue significant protein deficits over their hospital stay[31]. And because of these distinct challenges of the preterm infant environment, the rate of growth that is appropriate for extrauterine life is not known. However, the current practice objectives for the postnatal period is to attain intrauterine

growth velocities and body composition comparable to a healthy fetus of the same gestational age[32].

2.2.2 Regulators of postnatal growth

In postnatal growth, unlike fetal growth, genetics play a much more significant role[21]. Nutritional intake during the postnatal period may be responsible for 45-55% of variation seen in growth[31, 33]. IGF-1 responds to nutritional intake through insulin to regulate growth[34], much like in the fetus. IGF-1 levels in term infants surge after one week, while IGF-1 levels remain low in preterm infants for several weeks suggesting low endogenous production in preterm infants[35]. Postnatal growth in a term infant is regulated by IGF-1 and insulin until 6 months, beyond which growth is regulated through the pituitary growth hormone (GH)[36]; this also occurs but at 6 months corrected age[37]. GH regulated growth allows the infant to reach its genetic potential. Additionally, periconceptional nutritional insults, as noted in studies by Oliver and Bauer[15, 20], alters endocrine response thereby affecting postnatal growth. This altered endocrine response also persists into adulthood[38] highlighting an adaptive developmental origin of adult phenotype.

2.2.3 Origins of Adult Disease

Aberrant pattern of growth during fetal life, as well as infancy have significant implications for long-term health outcomes. The first associations came to light from early epidemiological studies noting stark geographical association in infant mortality rates and cardiovascular disease decades later in England and Wales[39]. It was proposed that the environment in early life may play a critical role in the development of adult disease. This was later confirmed by epidemiological studies that showed an association between term

born low birth weight (LBW) adults and cardiovascular disease risk (CVD)[40], type 2 diabetes, hypertension, and obesity[41]. This came to be known as the Barker hypothesis and later, DOHaD (Developmental Origins of Health and Disease). The Dutch Hunger winter birth cohort lend support to the DOHaD hypothesis. Those exposed to famine in early gestation (born normal weight at birth) had an increased risk of coronary heart disease and higher rates of obesity, while those exposed in late gestation (born approximately 300g smaller at birth) had an increased risk of glucose intolerance and lower rates of obesity[42]. A similar situation was posed by the siege of Leningrad, however, those exposed to this famine did not exhibit higher rates of obesity or cardiovascular disease as adults[43]. It was proposed that this may be because the Dutch Hunger winter was 5 months long while the Leningrad siege lasted 2 years; thus the Dutch infants experienced a mismatch in their postnatal environment while the Leningrad infants did not[44]. This indicated that nutrition during prenatal and postnatal period was key in determining later life outcomes. It also implicated postnatal catch-up due to nutrition in the programming of long-term outcomes.

'Catch-up' growth describes the phenomenon of an accelerated growth after a period of growth retardation, with the effect of taking the infant towards the original pre-retardation growth trajectory and erasing the growth deficit[45]. In LBW term infants, catch-up of weight by 2 years of age is observed ubiquitously[46]. Most of this catch-up occurs within the first 6-12 months of postnatal life with earlier catch-up seen in AGA LBW infants than SGA LBW infants[47]. In preterm infants, the more mature infants (gestational age above 29 weeks) experience catch-up within the first year of postnatal-life with

continued catch-up to 3 years. More immature preterm infants continue to experience growth restriction up to 2 years, with catch-up occurring later and over a longer period[48].

Epidemiological data from Helsinski cohort showed that CVD risk in term born men is highest when fetal growth restriction (as measured by low weight at birth) is followed by catch-up in childhood[49]. This pattern of catch-up growth in childhood has also been associated with obesity, insulin resistance and hypertension in later life[50-53]. A systematic review found an association between catch-up in infancy, in the first 2 years of life, and later obesity[54]. Since most LBW infants also catch-up, it is difficult to separate the independent consequences of each on later life outcomes. However, experimental studies in rats are able to clearly discern the effect of postnatal catch-up growth from effect of low birth weight. LBW rats that catch-up develop obesity and insulin resistance, while prevention of catch-up reverses this phenotype[55]. Additional studies in rats show that early postnatal catch-up programs obesity and glucose intolerance, while later catch-up does not affect adult phenotype[56]. Thus, LBW and subsequent catch-up, rather than LBW alone may be associated with metabolic syndrome in animal models. Nevertheless, weight catch-up in term born SGA infants may confer a benefit against infection in early life[57], while catch-up in length confers cognitive benefits[58]. However, the benefits of weight catch-up towards cognition for the term SGA infant remains inconclusive as there are only two studies examining such an association, both with contradictory findings[59, 60]. Hence, promoting weight catch-up in term SGA remains controversial.

The findings regarding long term consequences in preterm infants come from markers of metabolic syndrome measured in adolescence as survivors of preterm birth are

still young. Similarly to the term born SGA adult, preterm born adolescent are also at risk for metabolic syndrome[61]. A follow-up of randomized nutritional trials in preterm infants show that diets promoting faster early postnatal growth (within first 2 weeks of life) elevate markers of CVD risk and diabetes in children[62, 63], while another reported similar outcomes in adolescents but for rapid gain between preterm birth and 3 months corrected age[64]. These studies implicate early postnatal nutrition in programming of later outcomes.

A recent longitudinal cohort study in 153 preterm infants analyzed pre and post discharge weight gain[65]. In contrast to the aforementioned studies, this study found no association between blood pressure, insulin sensitivity and fat mass in childhood, and rapid pre- or post-discharge weight gain up to 1 year of age. However, rapid weight gain after 1 year of age, was strongly correlated with adverse outcomes. While it is still unclear whether catch-up in early infancy in preterm infants manifests as adverse metabolic outcomes in adulthood, the cognitive benefits of catch-up in preterm infants are more established. A randomized nutritional study found improved cognitive outcomes in children who were fed enhanced diets as preterm infants[66]. Additionally, a long term prospective follow-up in 495 ELBW preterm infants found that accelerated weight gain was associated with improved motor developmental index (MDI) and psychomotor developmental index (PDI)[67] at 18 to 22 months corrected age. Another study showed that this effect lasts into young adulthood, noting that a higher catch-up growth in the first year of life was associated with better cognition and reduced disability at 19 years of age in preterm SGA[68]. Thus, unlike the term SGA infant, enhanced nutrition to promote early catch-up growth in the preterm infant even if it may be at a potential later cost to cardio-metabolic health, has been advised[65].

2.3 Postnatal growth retardation and long-term outcomes

The association between LBW, as an indicator of impaired fetal growth, and adult disease outcome as posed by DOHaD hypothesis has implications for preterm infants, who are commonly growth restricted at birth and by discharge. Slow growth rates during the postnatal period leads to postnatal growth retardation, defined as body weight below the 10th birth weight percentile at discharge, is a widespread concern in current neonatal care. Between 43 and 97% of preterm infants experience postnatal growth restriction and do not follow intrauterine weight gain trajectories after birth[69]. Preterm infants have significantly stunted linear growth[70] at term correct age compared to infants born at term; linear growth is associated with lean body mass. Similarly, preterm infants also have smaller head circumferences at term corrected age[70]. The composition of weight gain also does not follow the pattern of term born infants. A meta-analysis and systematic review of literature by Johnson and colleagues[71] found that preterm infants at term corrected age are dissimilar to term infants in both body size and composition. Eight studies that directly compared body composition between AGA preterm infants and term infants were reviewed. Each study either used dual x-ray absorptiometry (DXA), air displacement plethysmography (ADP) or magnetic resonance imagining (MRI) for body composition assessment. The meta-analysis therefore included 388 preterm and 345 full term infants (mean PMA at assessment: 39.5 weeks). The review found that preterm infants had a greater percent fat-mass (%FM), however absolute FM was similar between term infants and preterm infants at term. Fat-free mass (FFM) was also significantly reduced for preterm infants, and so the increase in %FM is better explained by the reduction in FFM, than a gain in absolute FM.

There is evidence that the pattern of fat mass accretion is also different in preterm infants. A study by Uthaya and colleagues found that preterm infants had elevated intra-abdominal adiposity compared to term infants, who have mostly subcutaneous fat mass[72]. However, this observation was correlated with illness severity. In contrast, a recent study by Roggero et al. reported that preterm infants did not exhibit increased intra-abdominal adiposity when co-morbidities associated with prematurity were accounted for[73]. There is scientific consensus that intra-abdominal or visceral fat is a marker for insulin resistance, cardiovascular disease and hypertension[74]. Hence there is concern that postnatal adiposity patterns can affect preterm born adults but studies investigating pattern of adiposity or body composition in preterm infants are scarce.

To prevent the adverse long-term health outcomes of inadequate growth, neonatologists promote rapid growth to meet intrauterine growth trajectories and ameliorate the postnatal growth deficits. The best source of nutrition to promote such growth, is breast milk.

2.4 Preterm Infant Nutrition, Dietary Protein and Growth:

Breast milk is currently considered the best source of nutrition for the preterm infant, but it was not always the case[75]. Mounting research evidence and increased advocacy led American Academy of Pediatrics took a position statement in 1997 acknowledging that human milk was beneficial for the clinical management of premature infants[76] and in 2012, recommending its use in all preterm infants[77]. Fortified breast milk is now the main source of enteral nutrition for preterm infants.

Human milk is unique in its composition and is nutritionally rich. Of the total nitrogen in human milk, non-protein nitrogen, such as free amino acids, urea, uric acid and ammonia make up 20-25%[78, 79]. Beside conferring passive immunity, breast milk contains other bioactive factors that aid in digestion and absorption of nutrients by promoting the maturation of the gastrointestinal tract[80]. Particularly, breast milk after premature birth is initially higher in fat, protein and non-protein nitrogen[81]. Furthermore, human milk compared to cow milk has higher levels of whey protein, as opposed to casein, and is more easily digestible[82]. Nevertheless, human milk alone does not meet the protein and energy needs of a preterm infant and must be fortified[83].

Protein synthesis and accretion is central to growth as it provides the structural matrix to build tissues and organs. While protein tissues are continually in a state of synthesis and breakdown- referred to as protein turnover, growth occurs when the rate of protein synthesis exceeds that of protein degradation resulting in net protein accretion[84]. Because skeletal muscle is the largest organ in the body[85], it is a major site of protein synthesis and degradation. In a growing mammal, feeding stimulates protein synthesis and inhibits protein breakdown[86] in all tissues of the body, however the magnitude of increase in the neonate, is the greatest in skeletal muscle[84]. This indicates that preterm infants cannot grow without increased protein intake[87]. Thus, protein deficits that accrue during first weeks of life are strongly associated with postnatal growth retardation[31].

Increased protein intake during the postnatal period is documented to improve growth outcomes in preterm infants. Studies have reported increased weight, length, head circumference [88-90] and growth velocity[91] up to term corrected age with increased protein

intake. A high protein intake during hospital stay is also related to decreased fat mass and increased lean mass up to 6 months corrected age[90, 92]. It is currently not known whether this trend in body composition persist into adulthood. However, due to the relationship between inappropriate growth and later life metabolic risk factors as established under the DOHaD principle, the importance of adequate growth and appropriate nutritional intake in the early postnatal life cannot be understated.

2.5 Current Routine Assessment of Growth

2.5.1 Anthropometric measurements

In current practice, serial measures of weight, length and head circumference are used to monitor and identify growth retardation. Measures related to weight are sensitive to fluid fluctuations and can be confounded by non-nutritional weight gain such as in the case of edema. It therefore gives an incomplete picture of the nutritional state of the preterm infant. Length and head circumference may also be used in conjunction with weight gain as surrogate indicators of body composition. Occipitofrontal circumference correlates with brain volume which reflects dendritic and synaptic proliferation, and therefore, is an indicator of brain growth[93]. Sub-optimal head circumference growth is associated with cognitive and motor impairment in school age children born preterm[94, 95]. Thus, achieving intrauterine head circumference velocity of 1cm/week is a current goal[96]. However, occipitofrontal circumference measurements can also be confounded by the moulding of the head due to respiratory apparatus. While length is not routinely used in clinical assessment and decision making, it may reflect actual growth better than weight as linear growth continues after birth whereas weight decreases due to fluid loss[97]. Crown-heel

length is an indicator of lean body mass[98] and protein accretion[99, 100]. Slow linear growth is also associated with decreased neurodevelopmental outcomes [101, 102]. Length is accurately measured on a length board and requires two people for assessment. However, most neonatal units employ simple measuring tapes for length assessment, which does not provide a consistent accurate measure.

Anthropometric measures are conducted at the bedside, can be quickly obtained, and shed some insight into the growth of the infant's body compartments. However, these measures indicate the quantity of growth and do not provide much insight into the quality of the growth.

2.5.2 Growth velocity

Assessment of absolute weight gain is common both in the NICU, and research. However, expression of growth as an absolute weight gain is not sufficient. A total weight gain of 30g over a day in a 1kg baby has different clinical implications than the same weight gain in a 2kg baby, as the former indicates a high growth rate depending on the gestational age. Hence, growth velocity should be normalized for body weight, which is expressed as g/kg/day. An increase in normalized growth velocity is shown to have improved neurodevelopmental outcomes, such as lower incidence of cerebral palsy, and improved MDI and PDI scores[67].

A systematic review conducted by Fenton and colleagues, showed that of the 1542 studies examined, only 366 quantified growth as a change over time. Of these studies, 40% used a growth velocity normalized for body weight (g/kg/day) while the rest used weight

gain (g/day) or change in z-scores. Among the studies that reported growth velocity normalized for body weight, there was a considerable variation in calculation methods. 63% calculated growth velocity beginning at birth or admission, 39% began after birth weight was regained, and 16% used a unit-less exponential formula. Additionally, 59% did not define the denominator used for normalization by body weight, 34% used an average weight for normalization, 19% used an initial weight at the beginning of study and 12% used birth weight[103]. Not only is growth quantified differently, but studies by Patel and colleagues[104] showed that there is a heterogeneity in the use of formula for calculation of growth velocity. Studies employ either linear or exponential formulas that model growth in a linear or exponential manner. Each of the models are further subdivided into two types: regression methods which incorporate all weight data, and 2-point methods which use only 2 data points to estimate growth velocity. Patel et al. compared linear and exponential 2-point methods against a reference method, which was calculated as a daily growth velocity in g/kg and then averaged over the study period resulting in g/kg/day. The 2-point exponential method yielded the lowest mean absolute difference in growth velocity estimate compared to the reference method. Patel et al., have suggested use of a 2-point exponential model as they found this calculation model to have the lowest mean absolute difference from the reference method[104]. To date, no studies have examined growth velocity estimates from regression methods which would have the advantage of incorporating all weight data, and may be a better reflection of growth velocity over a period. In summary, there is a great need for standardization of growth velocity calculation as the lack of

standardization makes comparisons between studies difficult and poses a challenge in comparing the efficacy of different nutritional interventions.

2.6 Body Composition

Body composition in preterm infants is an emerging field that might offer a better assessment of both the quantity and quality of growth than serial anthropometric measurements. The sole direct method of measuring body composition is by performing chemical carcass analysis. This involves drying to a constant weight to determine water content, ether extraction for fat content, and ashing for determination of mineral composition. Current indirect body composition techniques divide the body into several compartments. A weight measure represents a single compartment model of the body.

2.6.1 Compartmental Models

The two-compartment model (2C) is the earliest model of body composition. It divides the total body weight into just two compartments: fat mass and fat free mass. 2C model assumes that fat free mass constituents are have a constant composition and density[105]. Traditionally, hydrodensitometry or underwater weighing techniques were used for assessment. However, new techniques such as air displacement plethysmography have since emerged.

The three-compartment model (3C) expands on the 2C model and reduces the limitations inherent to the two compartment model[106]. The body is divided into fat mass and fat-free mass, which is further divided into total body water and remaining fat-free dry mass. This method employs not only hydrodensitometry but deuterium dilution as well, to

discern the total body water component. 3C model overcomes the assumptions of the hydration fraction of fat-free mass but does assume that the ratio of protein to minerals in the fat-free dry is constant[107].

The four-compartment model (4C) is the most widely used gold standard method available. It is an improvement upon the 3C model as it assumes that total body weight is composed of total body water, protein, mineral and fat mass and requires three independent measurements[105]. In this model, total body water is determined by deuterium dilution. Protein is often determined using a radioactive potassium tracer (^{40}K) in a whole-body counting machine. And mineral content is determined using dual x-ray absorptiometry (DXA). The total of these components represents fat-free mass. Fat mass is then calculated as a difference between fat free mass and the total body mass. Since this model further distinguishes dry fat-free mas into its mineral and protein components, it reduces the need for some of the assumptions about the densities and proportions of the different fat free mass components made by the other models[107]. However, 4C method is time consuming and invasive.

2.6.2 Body Composition Measurement Techniques

As mentioned in a previous section, one of the most basic indirect method of assessing body composition are anthropometric measures such as weight, length, abdominal circumference and associated indices such as BMI as there is an associative power between these measures and lean mass[108]. Skinfold thickness (SFT) is another type of anthropometric measure where calipers are used to assess subcutaneous fat thickness at various sites of the body. Raw values can then predict total body fat with the assumption

that subcutaneous fat reflects a constant proportion of total body fat. While SFT is a quick bedside method, it limited in its predictive potential of whole body lean mass and has errors associated with the use of population specific equations as well as observer bias[109]. Furthermore, the rapid and changing nature of fat accretion in the preterm infant makes it difficult to develop a consistent formula for this population[110].

Bioelectrical impedance analysis (BIA) is also a non-invasive beside indirect method for body composition assessment. It utilizes the electrical conductivity of the lean mass compartment to estimate total body water. Lean body mass is than derived from total body water by assuming a constant hydration percentage of the lean body mass[111]. However, errors are introduced due to hydration status changes in the preterm infant as well as their low fat content[110]. These factors make BIA insufficient tool for measuring body composition in preterm infants.

Dual x-ray absorptiometry (DXA) is a non-invasive in vivo measurement of whole-body composition. It uses photons of two different energy levels and determines composition of the body based on differential absorption. The scan takes approximately 2 minutes in infants and they must remain still. This technique, like others, assumes constancy of hydration, potassium content or tissue density. However, DXA estimates are affected by differences among manufacturers' technology, software and density assumptions[108].

Since its first commercial introduction 2004 for infants, air displacement plythesmography (ADP) has quickly become a reference method for body composition in

infants. It uses the same principles as whole body hydrodensitometry or underwater weighing, but with air as the medium. To reiterate, assumptions regarding tissue density exist- a fact which is inherent to all methods to a varying degree. The measurement device called PEA POD (COSMED, USA Inc) can be used beside and has been validated using volume phantoms[112], bovine tissue phantoms[113] as well as gold standard 4-compartment[114] and deuterium dilution[115] model in neonates.

Other methods include deuterium dilution method which measures TBW by using a stable isotope, and assessing blood and urine samples. Like BIA, it assumes that total body water to lean body mass ratio is a constant[110]. Imaging techniques such as magnetic resonance imaging, ultrasonography, and computed axial tomography (CT) have also emerged. However, these methods are invasive, expensive and not clinically feasible for preterm infant population.

A concern common to all assessment of growth is that all measurements are 'post hoc', and by the time growth retardation is identified by assessment of growth, deficits have already accumulated and are difficult to reverse without significant catch-up.

2.7 Protein Metabolism

Dietary proteins are broken down into amino acids and transported to the liver[116]. Liver plays a central role in maintaining metabolic homeostasis. Because amino acids have no special storage, dietary amino acids have only two metabolic fates: they're either oxidized as a source of energy or synthesized into body protein. The metabolic fate of dietary amino acids is determined primarily by diet and hormones[84, 117, 118]. In any growing

organism, if energy intake is adequate, then the fate of amino acids is to be synthesized into body protein.

The liver is the main site of amino acid catabolism, and also regulates amino acid supply to the rest of the body. It is also the only organ capable of urea synthesis, and synthesizes non-essential amino acids as well as plasma proteins such as albumin and clotting factors[119]. The oxidation of amino acid results in ammonia, and a carbon backbone which is transformed into intermediates that either generate glucose or enter the citric acid cycle where ATP is generated. The amino acids not taken up by the liver for oxidation enter into the systemic circulation and are transported to other tissues for protein synthesis. When these amino acids enter the systemic circulation, they have a profound regulatory effect on both whole-body and skeletal muscle protein synthesis, and also act as a physiologic stimuli for insulin secretion which stimulates protein synthesis[120] and inhibits breakdown. In the neonatal pig model, postprandial increase in amino acids stimulates protein synthesis in liver and most other tissues in the body, whereas skeletal muscle protein synthesis is increased independently both by amino acids and insulin[121]. Thus the skeletal muscle in the neonate is unique in that it responds to two anabolic stimuli that increase amino acid uptake, nutrient utilization and protein accretion[122].

2.7.1 Urea cycle

Urea is a major end product of oxidative protein metabolism and is chemically inert. Estimates of urea excretion have been used as indicators of protein catabolism. The primary function of the urea cycle is to remove ammonia, a toxic by-product of amino acid oxidation, by converting it to a less toxic product- urea. In mammals, urea synthesis takes

place chiefly in the liver[123]. Urea is then released into the systemic circulation (blood urea nitrogen) and excreted in urine (urinary urea).

Blood urea nitrogen (BUN) is a biochemical marker that is used in routine clinical care as an indicator of hepatic and renal function. However, unlike other biomarkers such as creatinine, BUN also responds significantly and rapidly to changes in dietary protein making. For this reason, BUN has long been used as marker of adequacy of protein intake in ruminants in agriculture research [124]. Later studies showed this to be true in monogastric animals such as piglets and growing rats; a significant negative correlation was found between the quality of protein and BUN, and a significant positive correlation between dietary protein content and BUN [124, 125]. In healthy human adults, increasing protein intake is also related to an increase in BUN, such that quantity of protein intake is considered an important variable for interpreting clinical BUN results for management of patients with renal disease[126].

In the human fetus, all the enzymes necessary for urea synthesis are present by 50th day of pregnancy and the human fetal liver has been shown to synthesize urea as early as 13th week gestation[127]. This indicates that not only is the fetus capable synthesizing urea and detoxifying ammonia from an early gestation, but that the fetus also uses amino acids for oxidative fuel. As such, it can be said that preterm infants have a functional urea cycle.

2.7.2 Blood urea nitrogen as a marker

Use of BUN has been proposed as indicator of adequacy of protein intake for preterm infants. Polberger and colleagues found that BUN was strongly positively

correlated with mean protein intake in AGA VLBW preterm infants (r= 0.85, n= 24, p<0.001) up to mean protein intake of 3 g/kg/day. BUN values at protein intakes above 3 g/kg/day were still higher than at intakes below, however, the correlation disappeared beyond an intake of 3 g/kg/day[128]. Arslanoglu and colleagues used BUN as a marker of nutritional adequacy to administer a nutritional intervention. They adjusted enteral protein intake of preterm infants based on twice weekly BUN assessment, increasing protein fortification if BUN was below 3.2 mmol/L, no adjustment if it was between 3.2-5.0 mmol/L, and reduction if BUN was above 5.0 mmol/L. They found that the protein adjustment group had an overall increase in mean protein intake and BUN over time compared to the routine group[129]. Kim et al. found similar results in a recent study investigating liquid fortifiers that have the same energy content but higher protein content than conventional powder fortifiers,. They found that BUN levels dropped over the study period in the powdered fortifier group, where as it remained high in the liquid fortifier group[130]. In addition, the liquid fortifier group achieved greater weight and linear growth. The studies indicate that BUN elevates proportionally in response to protein intake, and can be seen as a marker of protein adequacy. Since BUN correlates with protein adequacy, and protein intake is associated with more optimal growth outcomes, particularly lean mass growth, it may be possible to use BUN as a surrogate marker of growth.

2.8 Protein-energy interactions, and blood urea nitrogen:

The quality and quantity of energy plays an important role in the utilization of protein towards growth, which is key in understanding BUN as it is a metabolite. No studies have directly assessed the relationship between energy and BUN, though some studies have

reported BUN as biochemical outcome in nutritional studies with groups of different energy intakes. Quality of energy is classified as a percentage of the carbohydrate energy to non-protein energy (CHO:NPE%). Non-protein energy is the energy from fat and carbohydrate. Quantity of energy with respect to protein utilization is classified as a ratio of protein intake in grams to 100kcal of energy (P:E). A series of studies by Kashyap and colleagues[131] elucidated a differential effect of quality of non-protein energy on substrate oxidation. They studied 62 LBW infants fed iso-nitrogenous (4g/kg/day) diet with an energy intake of either 130 or 155 kcal/kg/day (i.e., P:E of 3.2 or 2.7 g/kg/day respectively) and found reduced protein oxidation in the group receiving 65% of non-protein energy as carbohydrates compared to the group receiving only 35%. In a regression analysis, increased carbohydrate intake was related to increased carbohydrate oxidation ($r = 0.71$, $p < 0.01$) and decreased protein oxidation ($r = –0.42$, $P < 0.01$)[131]. In a second study, the group investigated the impact of non-protein energy quality on growth and metabolic response. They found that high CHO:NPE groups were reported to have significantly lower urinary urea output ($p<0.01$), which was further reduced in the higher overall energy intake group (155kcal, i.e., a low P:E intake as the diet was iso-nitrogenous)[132]. Taken together with the findings of the previous study, this study indicated that quality of energy (CHO:NPE) was associated with reduced protein oxidation which may be reflected in lower BUN values. Furthermore, a high energy in comparison to protein also reduces protein oxidation lowering BUN. The group also found increased weight gain and protein accretion in the high carbohydrate groups at both energy intakes. However, composition of weight gain was affected by P:E ratios, with a significant increase in fat and protein stores in group receiving

highest CHO:NPE and lowest P:E ratio compared to the other groups. These studies demonstrate that energy supplied as carbohydrates has a nitrogen sparing effect and improves protein utilization leading to better composition of growth[132], which is reflected in lowered BUN values. The higher carbohydrate fraction possibly induces protein synthesis by inducing insulin secretion[132], reducing protein oxidation resulting in lower BUN values. In conjunction with the quality of energy in the form of higher percentage of carbohydrates, a higher ratio of energy to protein intake (low P:E ratio values) appears to improve growth and is reflected in lower BUN values. This may be because of the metabolic costs associated with protein metabolism. Currently, no studies have examined the direct relationship between BUN and macronutrient intakes, or the potential for BUN to be used as a surrogate marker of growth.

2.9 Summary of the problem

Neonatologists face a challenging task of optimizing preterm infant nutrition, with slow growth rates favouring later cardiovascular and metabolic health, while faster growth rates favouring neurodevelopmental outcomes. Given the long-term health and economic ramifications of preterm birth, current research trends focus on characterizing the quality of growth achieved with current nutritional practices. Changes in anthropometric measures are the most common measure of growth in preterm infants as they track an infant's progress over time. However, because these measures are performed post hoc, their usefulness as a preventative diagnostic tool is limited. By the time a reduction in growth is observed, the opportunity to prevent it has been lost and deficits may have already accrued. This is because opportunity to make meaningful changes occurs days to weeks after the

initiation of a nutritional plan[133]. Indirect methods of body composition such as ADP or DXA, while increasingly promising as a method of nutritional assessment, have their practical limitations as well. For example, ADP cannot be used in most extremely preterm infants until after 33 weeks of gestational age as infants rely on respiratory support. Similarly, it is not feasible to perform DXA scans for preterm infants still on respiratory support. There is a great need for a sensitive clinical biomarker reflecting growth of body compartments so that it may be used as a tool to prevent growth deficits from accumulating. There has been an increasing interest in the 'omics' fields in search of short-term biomarker for growth, but these methods are still in their infancy. BUN, as a metabolic end product of protein metabolism, may hold promise as a non-invasive serum marker that is both economical and is not time consuming. There appears to be no correlation between BUN and parental amino acid intake[134]. This may be because in the early postnatal period, amino acids are oxidized irrespective of intake and used for energy as adequate energy balance is achieved gradually. In contrast, several studies have shown a correlation between enteral protein intake and BUN. Thus, BUN may be predictive of growth in a clinically stable preterm population during enteral nutrition[135]. However, in order to understand the relationship of BUN with growth, it is important to understand how BUN responds to quality and quantity of energy intake. To our knowledge, no studies have assessed direct correlation between BUN and other nutrient intake factors (CHO:NPE% and P:E ratios). Secondly, it is unknown if BUN relates to growth and can be used to predict growth.

3: Materials and Methods

3.1 Method development

3.1.1 Growth velocity

Six methods of growth velocity calculation were compared in 94 preterm infants born ≤30 weeks at McMaster Children's Hospital in order to determine which method to employ for the outcome parameter. Of the six methods, three were regression models that incorporated 21 day study period weight data points. The other three were 2-point methods that incorporated only the beginning and the end weights of the study period in the calculation. Furthermore, each calculation method either assumed that growth is linear or exponential. The six methods were as follows: 2-point linear, linear regression and daily average (linear models), 2-point exponential, exponential regression, and generalized reduced algorithm (exponential models). The generalized reduced algorithm method was based on the work of Lasdon et al[136] while the rest were derived and extension of the work by Patel et al[104]. Below are the equations and method of each calculation:

Table 1: Growth velocity equation notations

Notations	Meanings
A	intercept parameter
b_i	growth velocity
W_t	weight measured at time t
$\hat{W}_t$	weight predicted at time t
t	time
T	time at study end point
argmin	argument to minimize residual sums

2-point linear:

This calculation connects the first and last points by a simple straight line.

$\hat{W}_t = (A + b * t)$ such that $\hat{A} = W_0$ and $\hat{b} = (W_T - W_0)/T$

Linear regression:

This calculation estimates the parameter b (the growth velocity) in the equation $\hat{W}_t = (A + b * t)$ by minimizing the sum of squared residuals.

Daily Average:

This method calculated a daily growth velocity (GV), which was then averaged over the study period.

$$GV = \text{Average}\left(\left(\frac{W_T}{W_t}\right) - 1\right) \text{x } 1000$$

2-point exponential:

This calculation connects the first and last points by a simple exponential curve.

$\hat{W}_t = (A * b^t)$ such that $\hat{A} = W_0$ and $\hat{b} = (W_T/W_0)^{1/T}$

Exponential regression:

This method estimates the parameter b (the growth velocity) in the equation $\hat{W}_t = A \exp(b * t)$ such that the squared difference of the log-transformed observed values and the predicted values is minimized,

$$(\log(\hat{A}_2), \hat{b}_2) = \text{argmin}(\sum(\log(W_t) - \log(A_2) + b_2 * t))^2).$$

It is equivalent to the linear regression on the log-transformed data.

Generalized Reduced Algorithm (GRM):

This calculation method estimates the parameter b (the growth velocity) in the equation $\hat{W}_t = A \exp(b * t)$ such that the sum of squared difference of the observed values and the predicted values is minimized:

$$(\hat{A}, \hat{b}) = \text{argmin}(\sum(W_t - A\exp(b * t))^2).$$

These equations were used to calculate growth velocity from weights over a 21 day study. First, GRM method was used as a reference method and the average mean difference between GRM and other methods was calculated. Magnitude of error from the GRM method was also calculated as the percent absolute difference (=[absolute ($GV_{METHODX}$ - GV_{GRM})/(GV_{GRM}) x100%]). However, since growth velocity is a parameter that is estimated from weight, and no true physiological measure of growth velocity exists a mean of these methods will be the closest to the true mean. Hence, in the last step of the analysis, the mean growth velocity from six methods was used as the reference method. The maximum difference in growth velocity estimates was calculated for each infant. This was averaged and reported as mean variation between the methods.

3.1.2 PEAPOD vs DXA Comparison

3.1.2.1 PEAPOD device

Body composition was assessed using PEA POD (Cosmed, USA). The device employs whole body densitometry using air displacement plethysmography (ADP). This is achieved by calculating body density by measuring body mass, measuring volume displaced by the infant, assessing length, and applying assumed fat and lean mass densities to the whole body to calculate total fat and lean mass. Volume of the subject is

measured in the test chamber by detecting pressure changes in the volume of air by applying Boyle's and Poisson's Laws. The mass is measured on the electronic scale built into the device which has a maximum capacity of 12kg and a sensitivity of 0.1g.

Prior to measurement, the PEA POD system is turned on for 2 hours to allow the test chamber to warm up to 31ºC to ensure patient comfort, as well as ideal performance under gas laws. Daily quality control tests were performed for the volume chamber as well as the weight scale using the 3L metal volume phantom and 3kg weight provided by COSMED.

The infant was assessed for eligibility for measurement with the PEA POD device. For this, an eligibility criteria was developed. Infants had to be on room air (21% oxygen), with respiratory support no greater than CPAP of 6cm H20 or HFNC < 6 L/min. If infants had respiratory support, they were removed from it for 5 minutes and assessed using the Silverman scoring system- a measure of respiratory distress (See Appendix).

Once the infant was considered eligible for measurement, they were prepared by being undressed and monitored with a wireless pulse oximeter. Irremovable items such as hospital ID bracelets, nasogastric tubes and electrodes were accounted for by tarring weight on the scale and for volume in the chamber. Infants were completely nude but wore a thin stocking cap, as hair adds surface area influencing the volume measurement. The infant was first placed on the weigh scale, while the volume chamber calibrated the irremovable items. The infant was then placed in the volume chamber for 2.5 minutes with the chamber door closed forming a pressure seal using an electromagnet system.

Once the volume measurement was completed, the length of the infant was measured with a stadiometer on a flat surface and input into the PEA POD system which then calculates the fat and lean mass based on assumptions of density of those compartments

Estimates were calculated by the device were available as absolute FM (g), FFM (g), body volume (L), body mass (g), and relative FM and FFM (%).

3.1.2.2 DXA device

Body composition was also measured with DXA for the purpose of validation of the PEA POD device. DXA measurement was performed with Hologic Discovery ADR 4500 System (Hologic Inc., Waltham, MA, USA). Scans were performed in pediatric whole-body mode. Weekly and daily quality control were performed with the manufacturer provided phantom, as per protocol. Infants were undressed, wore clean diapers and were swaddled tightly with a cotton blanket to restrict movement and were laid supine on the scanning bed. Infants were often fed prior to measurement in an attempt to make them fall asleep. A maximum of two scans was attempted on any infant to accommodate movement artifacts. Only scans without any movement artifacts were analyzed.

3.1.2.3 Comparison of Body Composition devices

PEAPOD is now considered a gold standard in measuring infant body composition. It has been validated against a 4-compartment reference method in older healthy term born infants (1 week old to 5 months age)[114]. It has also been validated in both preterm and term infants using a 2-compartment reference method[137]. The DXA device has been the

gold standard in body composition, and has been validated in the piglets[138] but it remains unclear if it can be used in preterm infants.

A large discrepancy in measures was observed between our two devices and cast doubt on their validity. However, it was not feasible to validate our devices using 4-C or 2-C methods in preterm infants, as they are time consuming and invasive. Hence, validation was accomplished by comparing the PEA POD and DXA mass estimates with an independent weight measurement using an electronic scale (Smart Scale Model® 65). The body composition estimates from PEA POD and DXA were also compared against each other.

72 preterm infants born <30 weeks of gestation were measured with PEA POD and DXA at three time points: <36 weeks of corrected gestational age, term corrected age and 3 months corrected age (n= 21, 33, and 18 respectively). The measurements were performed concurrently with both devices. Correlation was assessed using simple regression analysis, while bias and agreement was tested using Bland-Altman analysis.

3.2 Analysis of BUN, growth and macronutrient intake

3.2.1 Study Setting

This was a single centre prospective observational study conducted at McMaster Children's Hospital Level III Neonatal Intensive Care Unit (NICU). Infants in this study were participating in a randomized controlled clinical trial (TFO: Target Fortification of Breast Milk) between January 2013- February 2016. The study individually adjusted

macronutrient intake based on breast milk analysis to ESPGHAN 2010[139] recommendations for the infants randomized to the intervention arm.

3.2.2 Study Population

Preterm infants born at <30 weeks of gestational age birth, receiving breast milk and tolerating an enteral intake of ≥100 ml/kg/d for ≥24h were included in the study by written informed consent. Infants were excluded for the following criteria: gastrointestinal malformation, congenital anomalies or chromosomal abnormalities, short gut syndrome, sepsis, NEC, renal disease, and hepatic dysfunction.

3.2.3 Clinical Procedure

All infants in the NICU were assessed for eligibility. Informed written consent was obtained well in advance of the infant starting routine standard fortification practice. Once the infant started receiving full enteral nutrition (100 mL/kg/day), the breast milk was supplemented with a commercial fortifier (Enfamil) for 4 days. The study intervention was then introduced on the 8th day from when full enteral intake was achieved. Once informed consent was obtained, a standing bloodwork order was printed and signed by a physician or nurse practitioner. It outlined the measurement time points that BUN and creatinine parameters were assessed: baseline (SDay1) (1-3) days prior to the start of the study), day 14 (SDay14) and day 21 (SDay21) of the study. The study bloodwork parameters were added to routine nutritional labs measures that were performed on Monday mornings at 8am prior to feeding, by heelstick. The sample was sent to McMaster Core Laboratory and Specimen Collection Centre. The imprecision of the assay employed by the Core Laboratory for urea assessment was reported as <4.5% of

total CV. The overall CV of the assay was 1.5%. The imprecision of the creatinine assay was reported as <6% of the total CV. The total CV of the assay was 4.95%.

BUN values were corrected by using creatinine as an indicator of low glomerular filtration rate in preterm infants. A low glomerular filtration rate would lead to elevation of BUN as kidneys in preterm infants are not able to filter out blood at an appropriate rate. Correcting BUN would account for this 'renal' inflation of BUN. BUN was calculated as follows: BUN x 0.5/Creatinine where 0.5 is considered to be the normal serum creatinine concentration[140].

3.2.4 Clinical Data Abstraction

All infants were assigned a study identification number. The following data were abstracted from the medical charts of each patient:

- Macronutrient intake: daily native milk protein, fat, and lactose intake as measured by near infrared milk analyzer (SpectraStar, Unity Scientific) for all days of the intervention for all infants. Total intake was calculated with standard fortification plus additional study fortifiers.
- Infant characteristics: birth date, gestational age at birth and day of measurement
- Bloodwork: urea and creatinine values and date of measurement were abstracted from Meditech
- Maternal characteristics: maternal age, weight gain during pregnancy, BMI, ethnicity and presence of hypertension or diabetes were abstracted from antenatal visit 1 and 2 records in Sovera.

Additional variables such as, day of life, energy intake, protein:energy (PE) ratio, and carbohydrate:non-protein energy (CHO:NPE)% were calculated from abstracted values. Only 24 hour prior intake values were available at SDay1.

3.2.5 Anthropometric Measurements

Body weight was measured to the nearest 10 g every other day as part of clinical routine using an electric scale (Smart Scale Model® 65). Length was measured once weekly from the crown to heel using a length board (Preemie Stadiometer, Ellard Instrumentation Ltd) with a fixed headboard and moveable foot to the nearest 0.1cm. Occipitofrontal head circumference was measured once weekly to the nearest 0.1 cm using a non-stretchable paper tape to the nearest 0.1cm. Growth velocity in g/kg/day was calculated using the generalized reduced algorithm[141] (exponential regression model) from day 1 to day 14, and day 1 to day 21 of the study . Head circumference and length gains were also calculated for a similar period and were reported as cm/week.

3.2.6 Statistical Analysis

All statistical analysis were performed using SPSS 22.0 (IBM, Armonk, NY).

The analysis of patient characteristics and outcome variables were summarized using descriptive summary measures: mean (SD) for continuous variables and number (percentage) for categorical variables.

A bivariate analysis was performed using Pearson correlation between continuous variables that were assumed to be potential confounders. For dichotomous confounders such as ethnicity, gender, diabetes and hypertension, an ANOVA was performed.

Potential variables with $p<0.10$ were included in the subsequent multiple regression models.

For the multiple linear regression model, a hierarchical regression analysis using a 2 or 3-block design was performed. For the relationship between BUN and intake factors, the first of 2 blocks contained 24 hour confounding variables of protein intake, P:E ratio and CHO:NPE% as well as variables identified as confounders in the univariate analysis. The 24-hour intake factors were included to account for the immediate effect of these factors on BUN values. The second model contained all of the previous variables, as well as the intake factors averaged over the period relevant to the BUN marker.

To assess the association between BUN and growth, the first of 3 blocks contained 24 hour intake factors and confounders identified in the univariate analysis. In the second block, the average of intake factors over the relevant period were added. The BUN variable of interest was always entered in the last block. Unstandardized coefficients and their p value, R^2 and p value of the regression ANOVA were reported. In addition, p value of the change of the F statistic was also reported. This allowed for assessment of significance of the change in the model.

3.2.7 Sample Size

Sample size calculation was performed using *PS: Power and Sample Size v. 3.0.43.* It was used to calculate a sample size for a linear regression analysis with $\alpha= 0.5$ and power= 0.8. The standard deviation of the independent variable σ_x (BUN) was 1.7, and the standard deviation of the dependent variable σ_y (weight gain) was 6.4. The minimum

slope λ we wished to detect was 1.8. All values were obtained from pilot data. We require 36 subjects to reject the null hypothesis that the slope equals zero with probability (power) 0.8. The type I error probability associated with the test of the null hypothesis was 0.05. In order to account for an attrition rate of 30%, at least 47 subjects were required for analysis.

3.2.8 Outlier Analysis

Since regressions are sensitive to outliers, outlier was analysis was performed as described by Hoaglin and colleagues[142]. The formula is as follows:

Upper limit: Q3 + (IQR * 2.2)

Lower limit: Q1 – (IQR * 2.2)

3.2.9 Missing Data

In order to deal with missing data, multiple imputation with 5 iterations was performed using SPSS. The average of these 5 iterations was taken as the final value.

4.0 Results of method comparison

4.1 Growth velocity

The use of generalized reduced algorithm method (GRM) as the reference method yielded different estimates of growth velocity (**Table 2)**.

Table 2: The mean difference and absolute magnitude of error calculated using the GRM method in 94 infants.

Method	Mean Difference (g/kg/day)	P value of mean difference from 0	Average Absolute magnitude of error (%)
Generalized reduced algorithm			
Daily average	-.55± 2.21	***02***	9.91
Exponential regression	-.20± 0.49	***.00***	2.47
Linear regression	.05± 0.38	.18	1.77
2-point linear	-.03 ±1.85	.88	8.24
2-point exponential	-.30 ±1.94	.14	8.81

The mean difference between GRM and daily average and exponential regression methods was significantly different from 0 ($p< 0.05$). The absolute magnitude of error was largest between GRM and the daily average method. The regression methods had a smaller magnitude of error than the 2-point methods. Previous findings have suggested the use of the daily average or the 2-point exponential method[104]. In contrast, our findings

show that both these methods have the largest magnitudes of error when compared to the GRM method.

The GRM method had the highest correlation with the reference method among the other 5 methods. **Figure 1** shows a correlation between all six methods against the reference method in 94 infants.

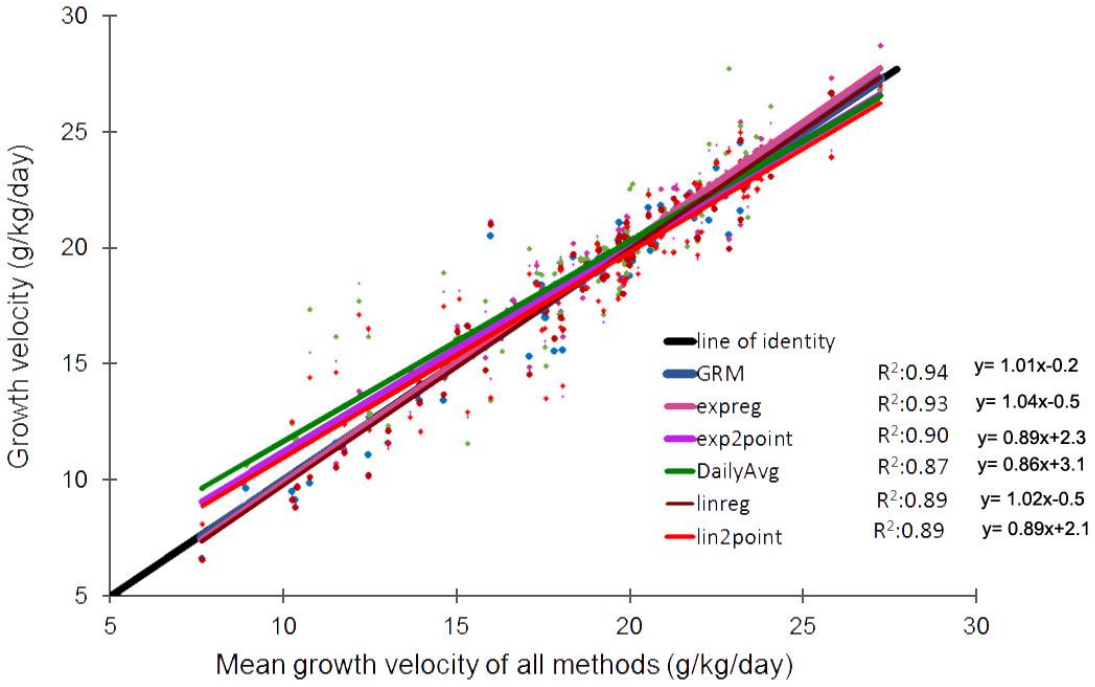

Figure 1: The correlation between the reference method growth velocity, on the x axis, and the growth velocity from six methods in 94 infants. The average variation between methods was 3.7 ± 2.2 g/kg/day

The average variation in growth velocity estimates from different methods was 3.7 ± 2.2 g/kg/day, The GRM method overlapped the line of equality, had the highest R^2, slope closest to 1 and intercept closest to 0 among all methods. Overall, the regression methods

were closer to the line of identity, while the different 2 point methods have a higher offset at the lower and upper ends of growth. In the range of normal growth velocity of 18-22 g/kg/day, all methods intersect the line of equality.

Based on these results, GRM was chosen as the calculation method for the purposes of this study.

4.2 Comparison of Body Composition devices

Total body mass estimates from ADP and DXA were both highly correlated with the independent electronic scale. But only DXA estimates showed a significant bias (**Figure 2a, 2b)**.

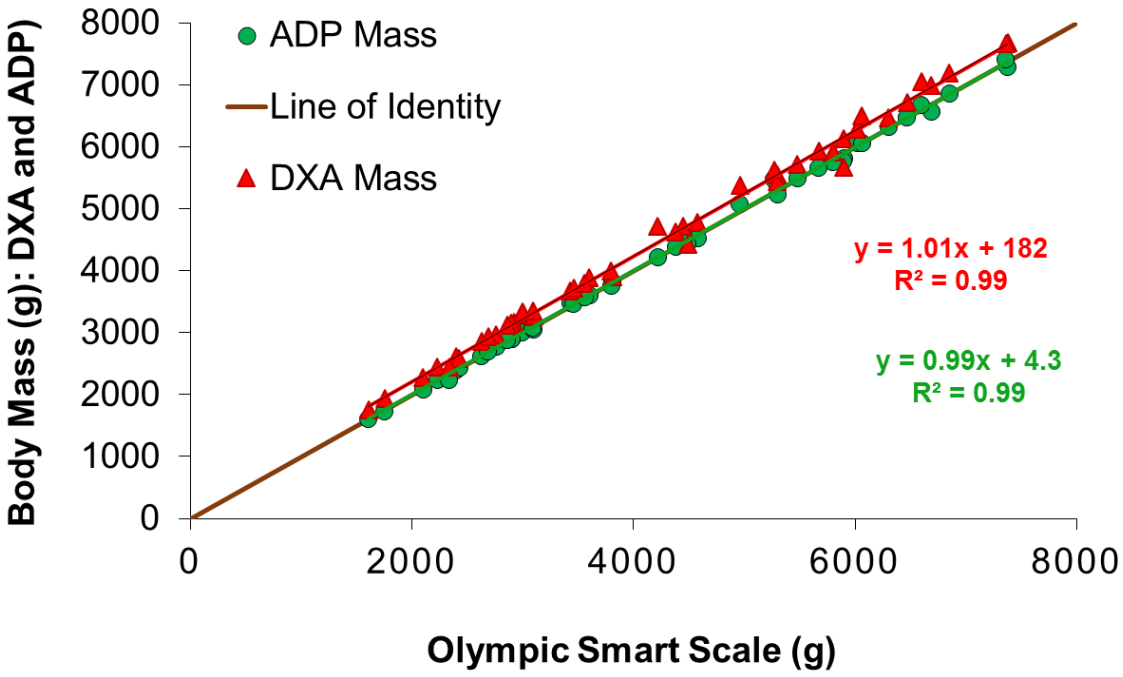

Figure 2a: Correlation between independent scale (reference weight method) and body mass from DXA and ADP (R^2= 0.995 and 0.999 respectively).

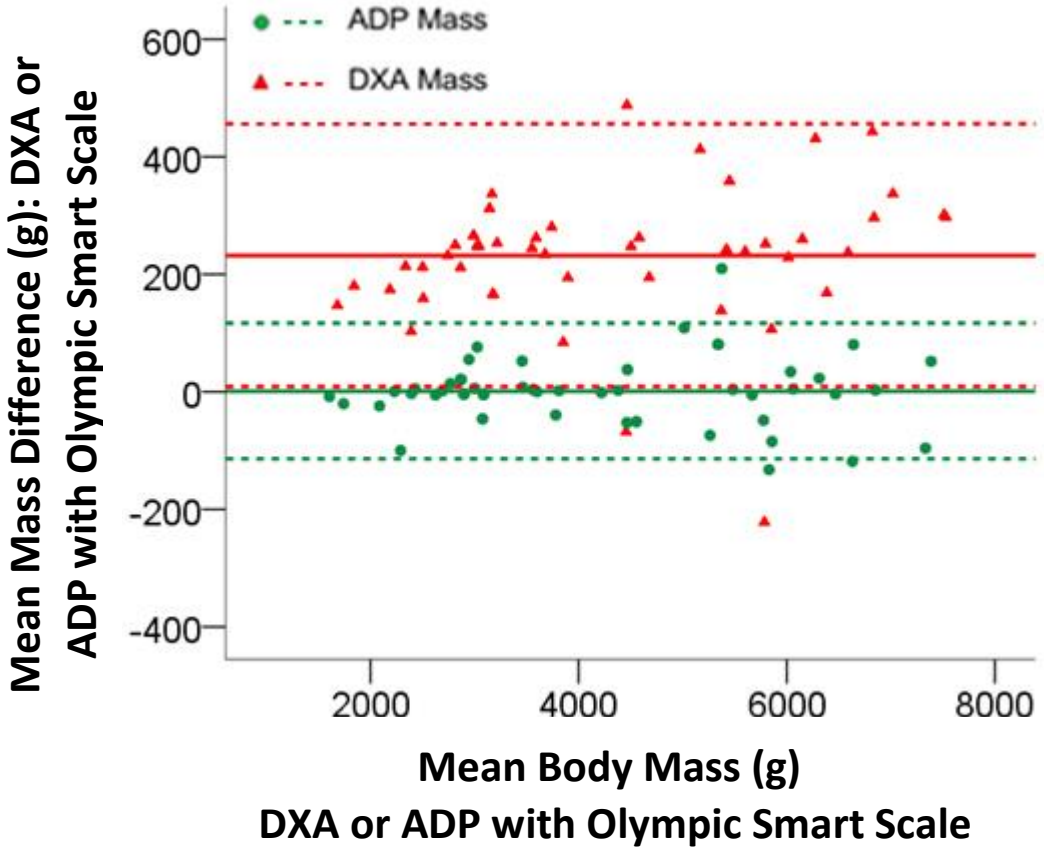

Figure 2b: Corresponding Bland-Altman, mean difference is 231 ±115 g and 1.23 ± 58.9g respectively) ($p < 0.001$ for DXA, $p = 0.884$ for ADP). n= 72.

The weights from the two methods are highly correlated with the reference mass measurement method, however, the Bland-Altman analysis revealed significant bias. The DXA method overestimated mass as the method had a mean difference of 231 ±115 g ($p < 0.001$ for difference from mean of 0) whereas ADP had a mean difference of 1.23 ± 58.9g which was not statistically different from 0 ($p = 0.884$).

FM% comparison between ADP and DXA are also highly correlated but revealed a significant bias (**Figure 3a, 3b)**.

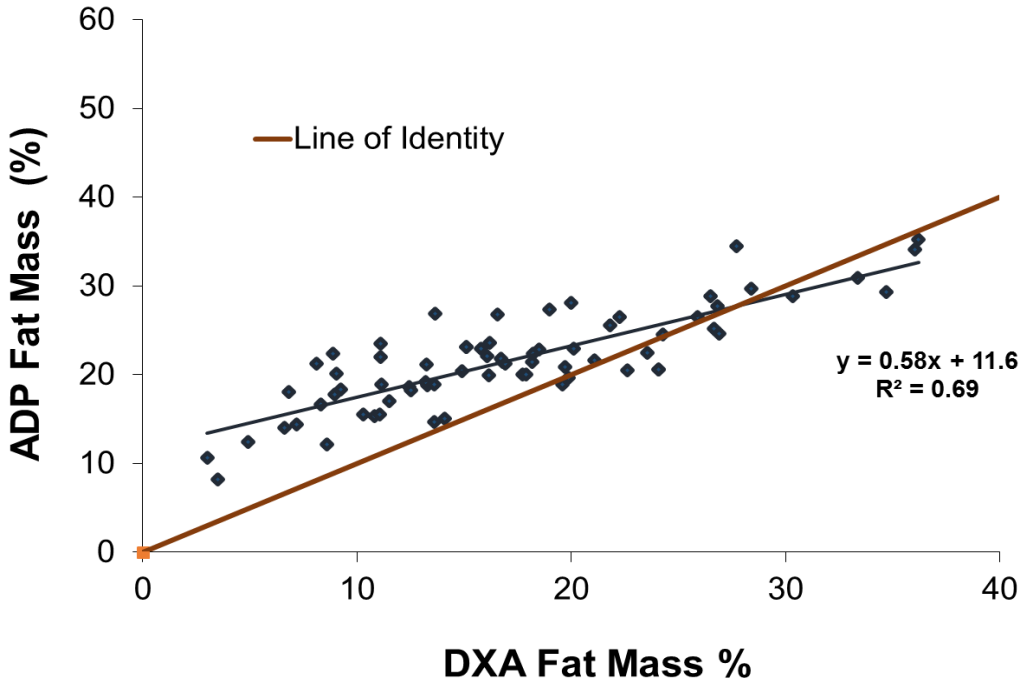

Figure 3a: Comparison of %fat mass from ADP and DXA. Measures are significantly correlated for % fat mass (R^2= 0.696)

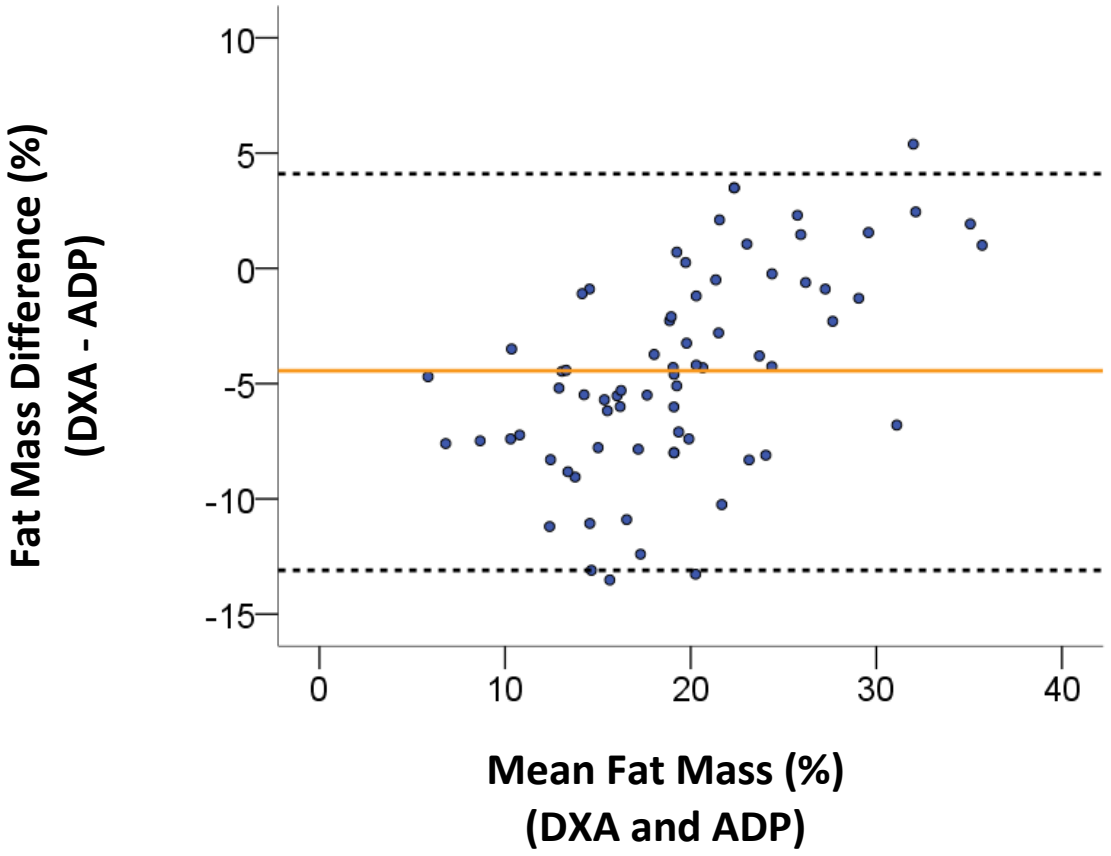

Figure 4b: Corresponding Bland-Altman for ADP and DXA %fat mass, mean difference is -4.4 ±4.4 % ($p < 0.001$)

The estimates were moderately correlated, FM% estimates were lower with DXA compared to ADP.

As the weight estimates were biased only for DXA and not for ADP, it showed that this disparity affected the %FM measures by DXA. Based on these results, ADP measures were considered appropriate for assessment of body composition for the purposes of this study.

5.0 Results of BUN, growth and macronutrient intake analyses

A total of 155 infants were consented and enrolled in the RCT, 54 infants did not complete the study. Reasons for this were: discharge or transfer before 21 days of intervention, withdrawals or exclusion due to medical reasons. The final sample size consisted of 101 AGA preterm infants born ≤30 weeks of gestation.

5.1 Study Population Characteristics

Table 3: General study population and maternal characteristics.

	Minimum	**Maximum**	**Mean**	**n**
Infant Characteristics				
Gestational age at birth (weeks)	23.4	30.1	26.86 ± 1.6	101
Day of life at start of intervention (days)	10	44	22 ± 7	101
Birth weight (g)	490	1490	928 ± 236	101
Head circumference at birth (cm)	19.50	38.50	24.61 ± 2.94	87
Length at birth (cm)	26.50	45.00	34.20 ± 3.62	58
Sex (n, %)				F(48, 47.5 %) M(53, 52.5)
Maternal Characteristics				
Maternal age (years)	18	41	30 ± 6	101
BMI (kg/m^2)	16.62	38.67	25.28 ± 5.15	67
Gestational weight gain (kg)	-1	23	8 ± 5	60
Hypertension (n, %)				Y(16, 15.8%) N(67, 66.3%)
Diabetes (n, %)				Y(4, 4%) N(79, 78.2%)

5.2 Analysis of confounders

Assessment of potential baseline study population and maternal characteristics showed no correlation between the majority of the variables and the parameters of interest in the study (Table 2). For the independent variable BUN, the value at baseline was significantly positively correlated with BUN at day 21 of study. However, day of life, birth, measurement, and maternal characteristics were not correlated. The dependent variable, growth velocity was not influenced by any of the considered variables, whereas FFM% and FFMI were influenced by maternal BMI and gender. This led to the building of the regression model, as only the confounders that were found to be significant were included in the main regression analysis.

Table 4: Univariate analysis of the main variables of interest and potential confounding variables.

Potential Confounders		BUN SDay1 (mmol/L)	BUN SDay14 (mmol/L)	BUN SDay21 (mmol/L)	GV by SDay14 (g/kg/day)	GV by SDay21 (g/kg/day)	Length Gain by SDay14 (cm/wk)	Length Gain by SDay21 (cm/wk)	HC gain by SDay14 (cm/wk)	HC gain by SDay21 (cm/wk)	FFM % at 36-40 weeks	FFMI at 36-40 weeks
SDay1 BUN (mmol/L)	r		***0.36***	***0.34***	-0.11	-0.13	-0.08	-0.01	0.18	0.13	0.05	0.01
	p		***0.00***	***0.00***	0.28	0.21	0.59	0.93	0.07	0.21	0.65	0.91
DOL	r	-0.13	-0.11	0.07	-0.20	-0.18	-0.01	0.14	-0.07	0.00	-0.12	-0.11
	p	0.22	0.41	0.95	0.12	0.13	0.95	0.20	0.53	0.98	0.84	0.82
GA at measurement (weeks)	r	-0.07	0.19	-0.01	-.04	-0.05	0.11	-0.01	-0.12	-0.21	-0.01	-0.01
	p	0.68	0.63	0.43	0.85	0.67	0.44	0.92	0.22	0.04	0.91	0.94
GA at birth (weeks)	r	0.09	0.17	0.04	0.05	0.15	0.13	-0.05	-0.04	-0.11	0.18	0.09
	p	0.36	0.13	0.68	0.62	0.13	0.35	0.60	0.70	0.28	0.14	0.45
Birth weight	r	0.28	0.21	0.19	0.04	0.08	-0.09	-0.05	0.04	-0.18	0.08	0.03
	p	***0.00***	***0.04***	***0.07***	0.71	0.46	0.55	0.65	0.67	0.07	0.50	0.83
Maternal age (years) n=(76)	r	-0.08	0.07	0.12	-0.10	-0.04	-0.19	-0.17	-0.14	-0.02	-0.01	0.10
	p	0.49	0.55	0.31	0.38	0.74	0.22	0.15	0.23	0.90	0.93	0.48
Maternal weight gain (kg) n=(60)	r	0.01	0.00	0.05	-0.22	-0.23	-0.19	-0.17	-0.14	-0.02	0.02	0.12
	p	0.92	0.98	0.72	0.11	0.10	0.22	0.15	0.23	0.90	0.92	0.47
Maternal BMI (n=67) (kg/cm$^{2)}$	r	-0.05	-0.07	-0.04	0.00	-0.04	-0.06	-0.09	0.12	0.19	0.29	-0.33
	p	0.67	0.56	0.74	0.99	0.73	0.71	0.47	0.33	0.13	***0.09***	***0.06***
Ethnicity (n=85)	p	0.37	0.25	0.35	0.53	0.69	0.95	0.68	0.77	0.82	0.48	0.48
Hypertension (n=83)	p	0.75	0.60	0.03	0.07	0.20	0.79	0.32	0.23	0.44	0.78	0.25
Diabetes (n=83)	p	0.23	0.90	0.90	0.14	0.20	0.77	0.71	0.90	0.60	0.23	0.26
Gender	p	0.59	0.89	0.40	0.41	0.12	0.56	0.96	0.69	***0.03***	0.55	***0.00***

5.3 Multiple Linear Regression Analysis: BUN vs intake factors

The 2-block design hierarchical regression analysis contains main variables of protein intake, P:E ratio and CHO:NPE% as well the confounders identified by the univariate analysis at the three study time points.

Table 5: Summary of multiple regression analysis of BUN (mmol/L) at baseline (SDay1)

Model	Variables	β	*p*	R^2	p of Model	p of F statistic change
1	Intercept	-5.46	.03	0.19	0.001	
	Protein intake 24 hr before SDay1 (g/kg/day)	1.39	<0.01			
	P:E ratio 24 hr before SDay1 (g/100kcal)	0.42	.02			
	CHO:NPE 24 hr before SDay1 (%)	0.06	.12			
	Birth weight (g)	0.002	.01			

All explanatory variables were positively correlated with BUN values. However, CHO:NPE% was not a significant predictor of BUN at SDay1. Infants with a higher protein intake tend to have higher BUN values 24 hours later. Similarly, higher birth weight predicts higher BUN values at the start of the study. The overall model was highly significant, and the predictors explained 19% of the variation seen in BUN at baseline, of which the most significant contributors to this model were birth weight and protein intake. The positive correlation observed between protein intake and BUN is consistent with previous literature[124, 128, 129].

Table 6: Summary of hierarchical multiple regression of BUN (mmol/L) at SDay14.

Model		B	*p*	R^2	Model p	p of F statistic change
1	Intercept	-6.33	0.00	0.32	<0.001	
	Protein intake 24 hr before SDay14 (g/kg/day)	0.99	0.02			
	P:E ratio 24 hr before SDay14 (g/100kcal)	0.76	0.38			
	CHO:NPE 24 hr before SDay14 (%)	0.05	0.28			
	Birth Weight (g)	0.00	0.27			
	BUN at SDay1 (mmol/L)	0.36	0.01			
2	Intercept	-6.13	0.03	0.42	<0.001	<0.01
	Protein intake 24 hr before SDay14 (g/kg/day)	0.44	0.47			
	P:E ratio 24 hr before SDay14 (g/100kcal)	0.38	0.05			
	CHO:NPE 24 hr before SDay14 (%)	-0.04	0.49			
	Birth Weight (g)	0.00	0.15			
	BUN at SDay1 (mmol/L)	0.41	0.00			
	Protein intake average by SDay14 (g/kg/day)	0.55	0.46			
	P:E Ratio average by SDay14 (g/100kcal)	3.55	0.00			
	CHO:NPE average by SDay14 (%)	0.03	0.62			

Significant predictors of the BUN value at SDay14 were protein intake 24 hours before the BUN value was taken as well as the BUN value at baseline. All predictors in the model explained 32% of the variation seen in BUN at SDay14. Block addition of the average macronutrient variables (protein intake, P:E ratio, and CHO:NPE%) explained an additional 10% of the variance in BUN. This change was significant ($p<0.01$). In the

second model, only baseline BUN and average P:E were significant predictors of BUN, average protein intake was not significantly correlated.

Table 7: Summary hierarchical multiple regression of BUN at SDay21

Model		B	*p*	R^2	Model p	p of F statistic change
1	Intercept	-9.17	0.00	0.37	<0.001	
	Protein intake 24 hr before SDay21 (g/kg/day)	1.06	0.01			
	P:E ratio 24 hr before SDay21 (g/100kcal)	0.70	0.36			
	CHO:NPE 24 hr before SDay21 (%)	0.12	0.01			
	Birth weight (g)	0.00	0.67			
	BUN at SDay1 (mmol/L)	0.52	0.00			
2	Intercept	-5.31	0.09	0.44	<0.001	0.01
	Protein intake 24 hr before SDay21 (g/kg/day)	0.50	0.25			
	P:E ratio 24 hr before SDay21 (g/100kcal)	1.53	0.07			
	CHO:NPE 24 hr before SDay21 (%)	-0.07	0.13			
	Birth Weight (g)	0.00	0.40			
	BUN at SDay1 (mmol/L)	0.47	0.00			
	Protein intake average by SDay21 (g/kg/day)	1.71	0.00			
	P:E Ratio average by SDay21 (g/100kcal)	.82	0.01			
	CHO:NPE average by SDay21 (%)	0.02	0.83			

Both protein intake and CHO:NPE% 24 hours prior to the urea value being taken at SDay21 had a significant positive affect on BUN values 24 hours later. Baseline BUN also had a significant impact on BUN values at this time point. The significant effect of 24 hour intake factors disappeared with the addition of average intake factor block. The second model explained an additional 7% of the variation in BUN. This change was statistically significant. An increase in average protein intake reflects a proportional

significant increase in BUN values. An increase in the average of P:E ratio by SDay21 reflects a decrease in BUN value.

Summary of results:

Tables 5-7 show that birth weight was a significant predictor of BUN only at baseline. BUN consistently had a high correlation with protein intake 24 hours prior at all study time points. However, this correlation became non-significant when average macronutrient intake variables were added in a block to model 2. Average protein intake variables were positively correlated with the BUN value at SDay1 and SDay21, but not at SDay14. 24 hour P:E ratios were not significantly correlated with BUN any of the time points. However, the average of P:E ratios were significant and positively correlated with BUN at all time points, indicating that an increase in average P:E intake ratio resulted in an increase in BUN levels. CHO:NPE was not a significant predictor of BUN at any time point.

5.4 Multiple Linear Regression Analysis: Growth vs BUN

5.4.1: Growth Velocity

The 3-block design hierarchical regression analysis contains main variables of protein intake, P:E ratio and CHO:NPE% as well the confounders identified by the in the univariate analysis at three study time points. The dependent variables of growth velocity, length gain, head circumference gain, FFM%, and FFMI were analyzed separately at each time point. BUN, the main predictor of interest, was added alone in block 3. The full model is shown only for the first table (table 8). Only the final model (model 3) is shown thereafter. However the change in model statistics is still reported on the right side of the table for all models.

Table 8: Summary of hierarchical regression of growth velocity by SDay14. Main predictor of interest, BUN at SDay1, is added individually in model 3.

Model		B	p	Model statistics	R^2	p of Model	p of F statistic change
1	Intercept	16.90	0.07	1	0.03	0.65	0.65
	Protein intake 24 hr before SDay1 (g/kg/day)	-0.94	0.58	2	0.05	0.71	0.55
	P:E ratio 24 hr before SDay1 (g/100kcal)	1.84	0.41	3	0.07	0.54	0.13
	CHO:NPE 24 hr before SDay1 (%)	0.03	0.86				
	Birth weight (g)	0.00	0.94				
2	Intercept	15.95	0.11				
	Protein intake 24 hr before SDay1 (g/kg/day)	-1.95	0.29				
	P:E ratio 24 hr before SDay1 (g/100kcal)	2.76	0.25				
	CHO:NPE 24 hr before SDay1 (%)	-0.01	0.97				
	Birth weight (g)	0.00	0.99				
	Protein intake average by SDay14 (g/kg/day)	1.71	0.22				
	P:E Ratio average by SDay14 (g/100kcal)	-1.07	0.69				
	CHO:NPE average by SDay14 (%)	0.01	0.97				
3	Intercept	12.40	0.22				
	Protein intake 24 hrs before SDay1 (g/kg/day)	-1.49	0.42				
	P:E ratio 24 hrs before SDay1 (g/100kcal)	3.13	0.20				
	CHO:NPE 24 hrs before SDay1 (%)	0.02	0.89				
	Birth weight (g)	0.00	0.71				
	Protein intake average by SDay14 (g/kg/day)	2.17	0.12				
	P:E Ratio average by SDay14 (g/100kcal)	-1.49	0.58				
	CHO:NPE average by SDay14 (%)	0.00	0.99				
	BUN at SDay1 (mmol/L)	-0.55	0.11				

Growth velocity at SDay14 is not explained significantly by any of the predictor variables examined. With the addition of BUN at SDay1 in block 3, the model explained a total of 7% of the variation seen in growth velocity by SDay14. However, neither the model nor the change to the variance was significant (Table 8).

Table 9: Summary of hierarchical regression of growth velocity at SDay21. Main predictor of interest, SDay1 BUN, is added individually in model 3.

Model		B	p	Model Statistics	R^2	p of Model	p of F statistic change
3	Intercept	21.7	0.02	1	0.01	0.98	
	Protein intake 24 hr before SDay1 (g/kg/day)	-0.57	0.65	2	0.12	0.11	0.01
	P:E ratio 24 hr before SDay1 (g/100kcal)	2.28	0.16	3	0.17	<0.01	0.02
	CHO:NPE 24 hr before SDay1 (%)	0.01	0.96				
	Birth weight (g)	0.00	0.32				
	Protein intake average by SDay21 (g/kg/day)	2.76	<0.001				
	P:E Ratio average by SDay21 (g/100kcal)	-3.63	0.01				
	CHO:NPE average by SDay21 (%)	0.03	0.82				
	BUN at SDay1 (mmol/L)	-0.47	<0.01				

Growth velocity at SDay21 was significantly explained by the average P:E ratio and protein intake over the corresponding period. Infants with increased protein intake had a higher growth velocity. An increase in P:E ratio reflected a significant decrease in growth velocity. Addition of average macronutrient variables of respective time points (protein intake, P:E and CHO:NPE) in block 2 resulted in a significant increase ($p<0.01$ of F statistic change) in R^2. However, the overall model was not statistically significant. The addition of SDay1 BUN had a statistically significant improvement on the overall model

(p=0.02 of F statistic change). BUN had a significant negative correlation with growth velocity at this time point. With the addition of BUN, the overall model explained 17% of the variation seen in growth and the overall model is significantly non-zero (p<0.01).

Table 10: Summary of hierarchical regression of growth velocity at SDay14. Main predictor of interest, BUN at SDay14, is added individually in model 3

Model		B		Model Statistics	R^2	p of Model	p of F statistic change
3	Intercept	21.76	0.00	1	0.06	0.26	
	Protein intake 24 hr before SDay14 (g/kg/day)	2.24	0.16	2	0.09	0.38	0.46
	P:E ratio 24 hr before SDay14 (g/100kcal)	-3.73	0.06	3	0.09	0.47	0.55
	CHO:NPE 24 hr before SDay14 (%)	0.28	0.08				
	Birth weight (g)	0.00	0.35				
	BUN at SDay1 (mmol/L)	-0.67	0.06				
	Protein intake average by SDay14 (g/kg/day)	0.27	0.89				
	P:E Ratio average by SDay14 (g/100kcal)	3.38	0.27				
	CHO:NPE average by SDay14 (%)	-0.32	0.14				
	BUN at SDay14 (mmol/L)	0.17	0.55				

Growth velocity at SDay14 was not explained by any of the variables examined. The addition of average macronutrient variables of respective time points (protein intake, P:E and CHO:NPE) in model 2 explained an additional 3% of the variance observed in the growth velocity. However, this change was not significant (p=0.5 of F change statistic). The addition of BUN to the model (model 3) was also not significant.

Table 11: Summary of hierarchical regression of growth velocity at SDay21. Main predictor of interest, BUN at SDay21, is added individually in model 3

Model		B		Model Statistics	R^2	p of Model	p of F statistic change
3	Intercept	19.58	0.00	1	0.07	0.26	0.26
	Protein intake 24 hr before SDay21 (g/kg/day)	-0.21	0.78	2	0.16	0.04	0.01
	P:E ratio 24 hr before SDay21 (g/100kcal)	1.56	0.24	3	0.22	<0.01	<0.01
	CHO:NPE 24 hr before SDay21 (%)	0.11	0.18				
	Birth weight (g)	0.00	0.13				
	SDay1 BUN (mmol/L)	-0.17	0.48				
	Protein intake average by SDay21 (g/kg/day)	3.59	<0.001				
	P:E Ratio average by SDay21 (g/100kcal)	-5.30	<0.01				
	CHO:NPE average by SDay21 (%)	-0.11	0.37				
	BUN at SDay21 (mmol/L)	-0.47	<0.01				

Growth velocity at SDay21 was significantly predicted by average protein intake and P:E ratio over the respective 21 day study period. The addition of average intake factors in model 2 explained an additional 9% of the variance observed in the growth velocity. This change was statistically significant (p=0.01 of F change statistic). Addition of BUN at SDay21 to model 3 was significant (p<0.01 of F statistic change). BUN was statistically negatively correlated with growth velocity.

Table 12: Summary of hierarchical regression of growth velocity by SDay14. Main predictor of interest, BUN at SDay21, is added individually in model 3

Model		B	p	Model Statistics	R^2	p of Model	p of F statistic change
3	Intercept	14.68	0.06	1	0.04	0.56	0.57
	Protein intake 24 hr before SDay21 (g/kg/day)	0.30	0.79	2	0.05	0.81	0.87
	P:E ratio 24 hr before SDay21 (g/100kcal)	0.31	0.87	3	0.05	0.86	0.71
	CHO:NPE 24 hr before SDay21 (%)	0.09	0.47				
	Birth weight (g)	0.00	0.57				
	Baseline BUN (mmol/L)	-0.39	0.28				
	Protein intake average by SDay14 (g/kg/day)	1.10	0.50				
	P:E Ratio average by SDay14 (g/100kcal)	-0.03	0.99				
	CHO:NPE average by SDay14 (%)	-0.10	0.57				
	BUN at SDay21 (mmol/L)	-0.10	0.71				

Growth velocity at SDay14 was not explained by any of the variables examined. The addition of average macronutrient variables of respective time points (protein intake, P:E and CHO:NPE) in model 2 explained only 1% of the variance observed in the growth velocity. This change was not significant (p=0.5 of F change statistic). The addition of SDay21 BUN to the model (model 3) was not significant.

Summary of results:

BUN at SDay1 and SDay14 does not add to the model of growth velocity by SDay14. BUN at SDay1 and SDay21 had a significant negative correlation with growth velocity by SDay21. The independent addition of BUN significantly improved the variability in growth velocity that could be explained by the model. Addition of SDay1 BUN to the model of growth velocity over SDay21 increased the R^2 by 5%, which was statistically significant change ($p=0.02$) Addition of SDay21 BUN increased the R^2 by 6%. This change was statistically significant ($p<0.01$).

5.4.2 Length Gains

Table 13-17 show multiple linear regression analysis of length gains by SDay14 and SDay21 with macronutrient intake factors and BUN at the 3 study time points.

Table 13: Summary of hierarchical regression of length gain (cm/day) by SDay14. Main predictor of interest, BUN at SDay1, is added individually in model 3

Model		B	p	Model Statistics	R^2	p of Model	p of F statistic change
3	Intercept	0.11	0.71	1	0.02	0.80	
	Protein intake 24 hr before SDay1 (g/kg/day)	-0.01	0.85	2	0.05	0.89	0.73
	P:E ratio 24 hr before SDay1 (g/100kcal)	0.05	0.57	3	0.07	0.91	0.55
	CHO:NPE 24 hr before SDay1 (%)	0.00	0.77				
	Protein intake average by SDay14 (g/kg/day)	-0.01	0.80				
	P:E Ratio average by SDay14 (g/100kcal)	0.07	0.50				
	CHO:NPE average by SDay14 (%)	-0.01	0.28				
	BUN at SDay1 (mmol/L)	-0.01	0.55				

Table 14: Summary of hierarchical regression of length gain (cm/day) by SDay21. Main predictor of interest, BUN at SDay1, is added individually in model 3

Model		B	p	Model Statistics	R^2	p of Model	p of F statistic change
3	Intercept	0.21	0.15	1	0.04	0.32	
	Protein intake 24 hr before SDay1 (g/kg/day)	0.00	0.98	2	0.05	0.61	0.81
	P:E ratio 24 hr before SDay1 (g/100kcal)	0.03	0.40	3	0.05	0.71	0.74
	CHO:NPE 24 hr before SDay1 (%)	0.00	0.09				
	Protein intake average by SDay21	0.00	0.82				
	P:E Ratio average by SDay21 (g/100kcal)	-0.02	0.57				
	CHO:NPE average by SDay21 (%)	0.00	0.34				
	BUN at SDay1 (mmol/L)	0.00	0.74				

Table 15: Summary of hierarchical regression of length gain (cm/day) by SDay14. Main predictor of interest, BUN at SDay14, is added individually in model 3

Model		B	p	Model Statistics	R^2	p of Model	p of F statistic change
3	Intercept	0.23	0.29	1	0.02	0.94	
	Protein intake 24 hr before SDay14 (g/kg/day)	0.00	0.99	2	0.06	0.91	0.60
	P:E ratio 24 hr before SDay14 (g/100kcal)	-0.03	0.76	3	0.07	0.93	0.60
	CHO:NPE 24 hr before SDay14 (%)	0.00	0.96				
	BUN SDay1 (mmol/L)	0.00	0.90				
	Protein intake average by SDay14	-0.01	0.90				
	P:E Ratio average by SDay14 (g/100kcal)	0.09	0.48				
	CHO:NPE average by SDay14 (%)	0.00	0.53				
	BUN at SDay14 (mmol/L)	0.00	0.59				

Table 16: Summary of hierarchical regression of length gain (cm/day) by SDay21. Main predictor of interest, BUN at SDay21, is added individually in model 3

Model		B	p	Model Statistics	R^2	p of Model	p of F statistic change
3	Intercept	0.18	0.09	1	0.00	1.00	
	Protein intake 24 hr before SDay21 (g/kg/day)	0.00	0.76	2	0.01	1.00	0.95
	P:E ratio 24 hr before SDay21 (g/100kcal)	0.00	0.95	3	0.02	0.99	0.34
	CHO:NPE 24 hr before SDay21 (%)	0.00	0.75				
	BUN SDay1 (mmol/L)	0.00	0.56				
	Protein intake average by SDay21	0.00	0.96				
	P:E Ratio average by SDay21 (g/100kcal)	0.00	0.93				
	CHO:NPE average by SDay21 (%)	0.00	0.66				
	BUN at SDay21 (mmol/L)	0.00	0.34				

Table 17: Summary of hierarchical regression of length gain (cm/day) by SDay14. Main predictor of interest, BUN at SDay21, is added individually in model 3

Model		B	p	Model Statistics	R^2	p of Model	p of F statistic change
3	Intercept	0.24	0.28	1	0.02	0.92	
	Protein intake 24 hr before SDay21 (g/kg/day)	-0.04	0.30	2	0.06	0.89	0.59
	P:E ratio 24 hr before SDay21 (g/100kcal)	0.00	0.99	3	0.07	0.92	0.60
	CHO:NPE 24 hr before SDay21 (%)	0.00	0.94				
	Baseline BUN (mmol/L)	-0.01	0.53				
	Protein intake average by SDay14	0.00	0.95				
	P:E Ratio average by SDay14 (g/100kcal)	0.10	0.30				
	CHO:NPE average by SDay14 (%)	-0.01	0.30				
	BUN at SDay21 (mmol/L)	0.01	0.60				

Length gain was not related to BUN, protein intake, P:E or CHO:NPE ratio at any of the study time points (Table 13-17). None of the models were significant overall ($p>0.05$).

5.4.3 Head Circumference Gains

Table 18-22 show multiple linear regression analysis of head circumference gains by SDay14 and SDay21 with macronutrient intake factors and BUN at the 3 study time points.

Table 18: Summary of hierarchical regression of head circumference gain (cm/day) by SDay14. Main predictor of interest, BUN at SDay1, is added individually in model 3

Model		**B**	**p**	**Model Statistics**	**R^2**	**p of Model**	**p of F statistic change**
3	Intercept	0.39	0.06	1	0.05	0.29	0.29
	Protein intake 24 hr before SDay1 (g/kg/day)	-0.02	0.65	2	0.11	0.15	0.13
	P:E ratio 24 hr before SDay1 (g/100kcal)	0.00	1.00	3	0.11	0.21	0.62
	CHO:NPE 24 hr before SDay1 (%)	-0.01	0.05				
	Protein intake average by SDay21	0.02	0.40				
	P:E Ratio average by SDay21 (g/100kcal)	-0.01	0.83				
	CHO:NPE average by SDay21 (%)	0.00	0.75				
	Gender	-0.04	0.08				
	BUN at SDay1 (mmol/L)	0.01	0.22				

Table 19: Summary of hierarchical regression of head circumference gain (cm/day) by SDay21. Main predictor of interest, BUN at SDay1, is added individually in model 3

Model		B	p	Model Statistics	R^2	p of Model	p of F statistic change
3	Intercept	0.33	0.10	1	0.03	0.48	0.48
	Protein intake 24 hr before SDay1 (g/kg/day)	-0.02	0.58	2	0.07	0.39	0.28
	P:E ratio 24 hr before SDay1 (g/100kcal)	0.01	0.78	3	0.00	0.26	0.11
	CHO:NPE 24 hr before SDay1 (%)	0.00	0.32				
	Protein intake average by SDay14 (g/kg/day)	0.03	0.34				
	P:E Ratio average by SDay14 (g/100kcal)	0.02	0.69				
	CHO:NPE average by SDay14 (%)	-0.01	0.17				
	BUN at SDay1 (mmol/L)	0.01	0.11				
	Protein intake 24 hr before SDay1 (g/kg/day)	0.33	0.10				

Table 20: Summary of hierarchical regression of head circumference gain (cm/day) by SDay14. Main predictor of interest, BUN at SDay14, is added individually in model 3

Model		B	p	Model Statistics	R^2	p of Model	p of F statistic change
3	Intercept	0.10	0.45	1	0.01	0.29	0.29
	Protein intake 24 hr before SDay14 (g/kg/day)	-0.01	0.63	2	0.04	0.15	0.13
	P:E ratio 24 hr before SDay14 (g/100kcal)	0.00	0.98	3	0.03	0.21	0.62
	CHO:NPE 24 hr before SDay14 (%)	0.00	0.42				
	BUN SDay1 (mmol/L)	0.01	0.12				
	Protein intake average by SDay14	0.04	0.23				
	P:E Ratio average by SDay14 (g/100kcal)	0.02	0.77				
	CHO:NPE average by SDay14 (%)	-0.01	0.13				
	BUN at SDay14 (mmol/L)	0.00	0.62				

Table 21: Summary of hierarchical regression of head circumference (cm/day) gain by SDay21. Main predictor of interest, BUN at SDay21, is added individually in model

Model		B	p	Model Statistics	R^2	p of Model	p of F statistic change
3	Intercept	0.19	0.20	1	0.08	0.09	
	Protein intake 24 hr before SDay21 (g/kg/day)	0.03	0.23	2	0.11	0.15	0.43
	P:E ratio 24 hr before SDay21 (g/100kcal)	0.01	0.79	3	0.11	0.19	0.45
	CHO:NPE 24 hr before SDay21 (%)	0.00	0.74				
	Baseline BUN (mmol/L)	0.01	0.18				
	Protein intake average by SDay14	-0.01	0.86				
	P:E Ratio average by SDay14 (g/100kcal)	0.02	0.72				
	CHO:NPE average by SDay14 (%)	-0.01	0.11				
	BUN at SDay21 (mmol/L)	0.00	0.45				

Table 22: Summary of hierarchical regression of head circumference gain (cm/day) by SDay14. Main predictor of interest, BUN at SDay21, is added individually in model

Model		B	p	Model Statistics	R^2	p of Model	p of F statistic change
3	Intercept	0.09	0.57	1	0.10	0.04	
	Protein intake 24 hr before SDay21 (g/kg/day)	0.00	0.92	2	0.17	0.02	0.10
	P:E ratio 24 hr before SDay21 (g/100kcal)	-0.01	0.10	3	0.18	0.03	0.32
	CHO:NPE 24 hr before SDay21 (%)	0.01	0.06				
	Baseline BUN (mmol/L)	0.00	0.77				
	Protein intake average by SDay21	0.01	0.63				
	P:E Ratio average by SDay21 (g/100kcal)	0.09	0.13				
	CHO:NPE average by SDay21 (%)	0.00	0.29				
	Gender	-0.04	0.04				
	BUN at SDay21 (mmol/L)	0.01	0.32				

Head circumference gain is explained only by gender, and only at SDay21 (p=0.04). Males had decreased head circumference gains compared to females. BUN did not add any explanatory power to the model as the F change statistic is not significant for any of the regression analyses (Table 22).

5.4.4 Body Composition

Table 24-30 show multiple linear regression analysis of body composition at 36-40 weeks PMA with BUN at the 3 study time points. In addition, a change in BUN from SDay21-SDay1 was also examined as an independent predictor variable.

Table 23: Hierarchical multiple regression of FFM% (between 36-40 weeks PMA, n=56). The main predictor of interest, BUN at SDay1 is added in model 3.

Model		**B**		**Model statistics**	**R^2**	**p of Model**	**p of F statistic change**
3	Intercept	52.0	0.0	1	0.07	.7	
	Protein intake 24 hr before SDay21 (g/kg/day)	1.4	0.70	2	0.27	.41	0.21
	P:E ratio 24 hr before SDay21 (g/100kcal)	0.1	0.78	3	0.32	.50	0.30
	CHO:NPE 24 hr before SDay21 (%)	-0.2	0.17				
	Protein intake average by SDay21 (g/kg/day)	1.3	0.36				
	P:E Ratio average by SDay21 (g/100kcal)	3.5	0.58				
	CHO:NPE average by SDay21 (%)	0.3	0.91				
	Maternal BMI (kg/cm^2)	-0.2	0.35				
	BUN at SDay21 (mmol/L)	-0.4	0.30				

The addition of SDay21 BUN in model 3 did not statistically improve the model. None of the other explanatory variables were significant predictors of FFM%.

Table 24: Hierarchical multiple regression of FFMI. The main predictor of interest, BUN at SDay1 is added in model 3.

Model		B	p	Model statistics	R^2	p of Model	p of F statistic change
3	Intercept	14.5	0.01	1.00	0.02	0.88	
	Protein intake 24 hr before SDay1 (g/kg/day)	0.28	0.74	2.00	0.33	0.31	0.04
	P:E ratio 24 hr before SDay1 (g/100kcal)	0.04	0.97	3.00	0.33	0.42	0.97
	CHO:NPE 24 hr before SDay1 (%)	-0.04	0.62				
	Protein intake average by SDay21 (g/kg/day)	0.48	0.53				
	P:E Ratio average by SDay21 (g/100kcal)	-0.32	0.80				
	CHO:NPE average by SDay21 (%)	-0.05	0.62				
	Maternal BMI (kg/cm^2)	-0.09	0.04				
	Gender	1.14	0.03				
	BUN at SDay1 (mmol/L)	-0.01	0.98				

BUN at SDay1 did not predict FFMI of infants between 36-40 weeks PMA. BMI of mothers was a significant negative predictor of FFMI. Males had higher FFMI values than females, this difference was significant. Addition of the average macronutrient intake as well as maternal BMI and gender in the second block explained an additional 31% of the variation in FFMI and this change was significant. Addition of SDay1 BUN did not improve the model (p=0.97 of F statistic change).

Table 25: Hierarchical multiple regression of FFM.% The main predictor of interest, BUN at SDay21 is added in model 3.

Model		B		Model statistics	R^2	p of Model	p of F statistic change
3	Intercept	39.30	0.03	1.00	0.09	0.74	
	Protein intake 24 hr before SDay21 (g/kg/day)	1.60	0.63	2.00	0.32	0.31	0.12
	P:E ratio 24 hr before SDay21 (g/100kcal)	6.15	0.29	3.00	0.35	0.31	0.27
	CHO:NPE 24 hr before SDay21 (%)	0.43	0.12				
	Baseline BUN (mmol/L)	-0.52	0.54				
	Protein intake average by SDay21	1.58	0.62				
	P:E Ratio average by SDay21 (g/100kcal)	-9.45	0.14				
	CHO:NPE average by SDay21 (%)	0.04	0.90				
	Maternal BMI (kg/cm^2)	0.21	0.23				
	BUN at SDay21 (mmol/L)	-0.61	0.28				

FFM% was not significantly explained by any of the variables examined. The addition of SDay21 BUN did not a significantly improve the model.

Table 26: Hierarchical multiple regression of FFMI. The main predictor of interest, BUN at SDay21 is added in model 3.

Model		B		Model statistics	R^2	p of Model	p of F statistic change
3	Intercept	13.90	0.00	1	0.06	0.99	
	Protein intake 24 hr before SDay21 (g/kg/day)	0.86	0.14	2	0.37	0.31	0.04
	P:E ratio 24 hr before SDay21 (g/100kcal)	-0.52	0.67	3	0.37	0.38	0.60
	CHO:NPE 24 hr before SDay21 (%)	0.00	0.95				
	Baseline BUN (mmol/L)	0.00	0.99				
	Protein intake average by SDay21 (g/kg/day)	0.07	0.93				
	P:E Ratio average by SDay21 (g/100kcal)	-0.29	0.86				
	CHO:NPE average by SDay21 (%)	-0.07	0.46				
	Maternal BMI (kg/cm^2)	-0.09	0.04				
	Gender	1.30	0.02				
	BUN at SDay21 (mmol/L)	.074	.601				

Addition of SDay21 BUN did not significantly improve the model to predict FFMI between 36-40 weeks PMA. Maternal BMI and gender were still significant as they were in Table 25.

Table 27: Hierarchical multiple regression of FFM%. The main predictor of interest, change in BUN (BUNSDay21-BUNSDay1), is added in model 3.

Model		B		Model statistics	R^2	p of Model	p of F statistic change
3	Intercept	39.25	0.02	1	0.08	0.75	
	Protein intake 24 hr before SDay21 (g/kg/day)	3.51	0.63	2	0.32	0.31	0.12
	P:E ratio 24 hr before SDay21 (g/100kcal)	8.85	0.29	3	0.35	0.31	0.28
	CHO:NPE 24 hr before SDay21 (%)	0.41	0.12				
	Baseline BUN (mmol/L)	-1.18	0.18				
	Protein intake average by SDay21	1.53	0.62				
	P:E Ratio average by SDay21 (g/100kcal)	-10.55	0.14				
	CHO:NPE average by SDay21 (%)	0.05	0.90				
	Maternal BMI (kg/cm^2)	0.18	0.23				
	Gender	-1.35	0.54				
	Change in BUN	-0.61	0.28				

Change in BUN over 21 days was not significantly correlated with FFM%. Addition of this term did not significantly improve the variation explained in FFM% by the model (model 3).

Table 28: Hierarchical multiple regression of FFMI. The main predictor of interest, change in BUN (BUN_{SDay21}-$BUN_{Baseline}$), is added in model 3.

Model		B		Model statistics	R^2	p of Model	p of F statistic change
3	(Constant)	13.90	0.00	1.00	0.01	0.99	
	Protein intake 24 hr before SDay21 (g/kg/day)	0.86	0.33	2.00	0.37	0.31	0.04
	P:E ratio 24 hr before SDay21 (g/100kcal)	-0.52	0.73	3.00	0.37	0.38	0.61
	CHO:NPE 24 hr before SDay21 (%)	0.00	0.95				
	Baseline BUN (mmol/L)	0.07	0.75				
	Protein intake average by SDay21 (g/kg/day)	0.07	0.93				
	P:E Ratio average by SDay21 (g/100kcal)	-0.29	0.87				
	CHO:NPE average by SDay21 (%)	-0.07	0.47				
	Maternal BMI (kg/cm^2)	-0.09	0.04				
	Gender	1.30	0.03				
	Change in BUN	.074	0.61				

Change in BUN was not correlated with FFMI at 36-40 weeks PMA. Addition of macronutrient intakes and BMI and gender in block 2 statistically improved the model and explained additional 36% of the variation seen in the FFMI. Within this block, BMI and gender contributed most significantly to the R^2. As in Table 25 and 27, the addition of block 2 with average macronutrient variables, BMI and gender still explained the most variation in FFMI, however, the overall model was not significant (model 2).

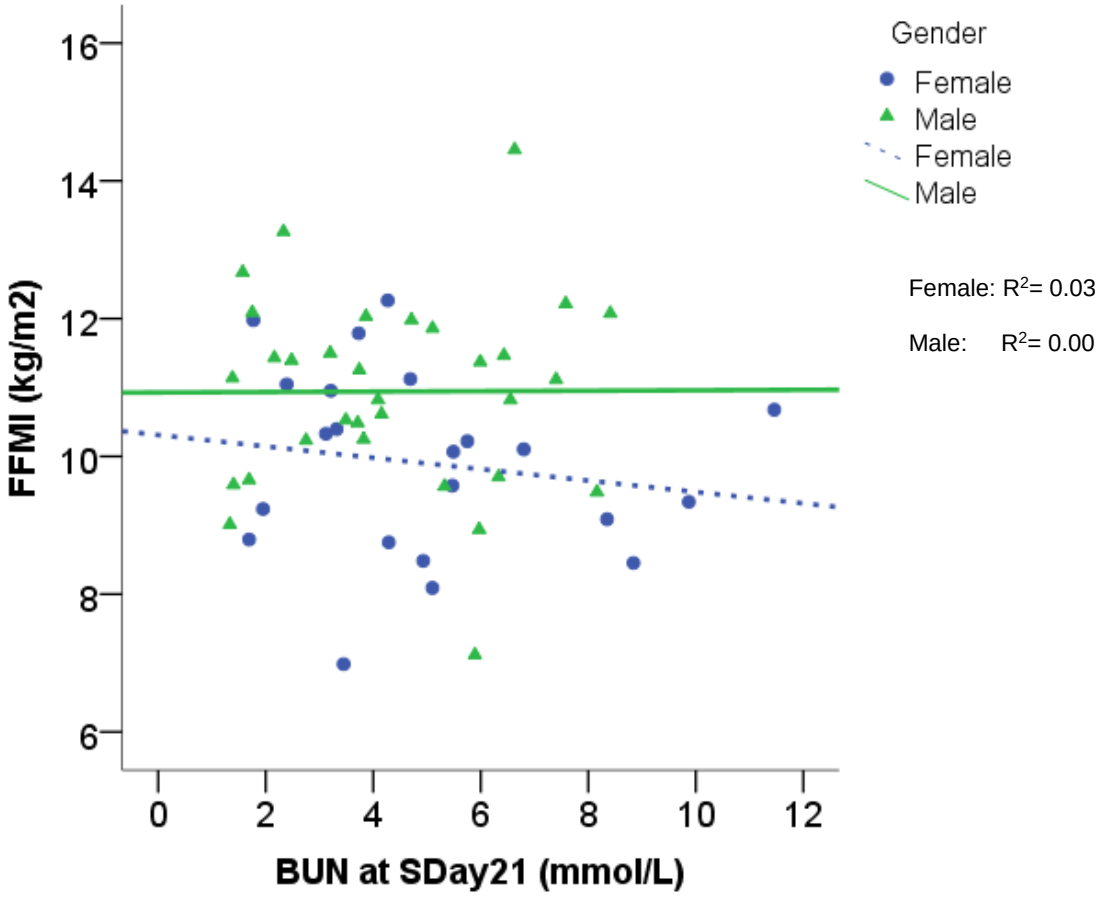

Figure 4: Relationship between SDay21 BUN and FFM% at 36-40 weeks PMA (n=56).

Figure 4 illustrates a simple linear regression analysis of FFMI vs SDay21 BUN with gender as an additional variable. There appears to be no relationship between FFMI and SDay21 BUN in the linear regression, as was observed in the multiple regression analysis. The relationship between FFMI and BUN does not appear to improve when regression lines are looked at by gender classification. However, the regression line of FFMI of males is higher than females.

Summary of results:

Prediction of body composition estimates was not improved by addition of any of the BUN values from any of the study time points, nor by examining a change in BUN over the study period. Addition of macronutrient intake factors increased the R^2, though this was not significant in the case of FFM%. However, for FFMI, the addition of block 2 (average macronutrient factors, maternal BMI and gender) resulted in a statistically significant change in R^2. Males had a higher FFMI compared to females, and maternal BMI was negatively correlated with FFMI in infants. The majority of this statistical significance can be attributed to maternal BMI and gender.

6.0 Discussion

6.1: Method development-Growth velocity

The growth velocity comparisons were performed in order to decide the best calculation method of growth velocity as it was the main outcome parameter in this study. The use of a 2-point exponential model for research and clinical use has been recommended[104], based on the use of the 'daily average' method as the reference. However, our study demonstrates that when GRM was used as a reference method, the daily average method had a significant bias (mean difference: -0.55g/kg/day, p=0.02) and the largest absolute percent error (9.91%) among the methods compared. Additionally, the 2-point exponential and the 2-point linear methods had the second and third largest absolute percent error, respectively, when the GRM method was used as the reference. Thus, the reference method chosen can impact the interpretation of the results. Since growth velocity is a parameter estimated from weight measurements, no true physiological reference method exists. Hence, using the average of growth velocity estimates by the six methods may be closest to the true mean. When the average growth velocity of all six methods is used as the reference method, all regression methods were closer to the line of identity than the 2-point methods, at the upper and lower ends of growth velocity indicating that regression methods are better at estimating growth velocity as they incorporate all data points. The GRM method showed the highest correlation and greatest agreement to the line of identity at all levels of growth. Our data also showed that the 2-point methods overestimate lower growth rates and underestimate high growth rates. This may be because

infants with suboptimal growth may be sick and may have perturbations in their growth curves resulting in an overestimation by the linear methods.

The average variation of 3.7 g/kg/day is clinically significant. Often 2-3 g/kg/day is chosen as minimum difference to power nutritional intervention studies. The existence of such a clinically relevant variation due to the method of measurement would have significant implications for comparisons between studies and recommendations drawn from comparisons of growth velocity between studies.

6.2 Method development: Body composition

In this study, it was important to establish the accuracy of the estimates from ADP as body composition was another outcome of interest. Due to the large differences observed between DXA and ADP measures that cast doubt on the validity of both devices, it was important to determine the best method for estimating body composition. DXA has not been validated against a 4-C or 2-C method in the preterm population, and though the PEAPOD has been validated with a 2-C model in the preterm population, we had previously noted that FM% estimates being given by our device were 3-5% higher than expected[118]. It was not feasible for us to perform 4-C or 2-C reference methods on our study population as these methods are invasive and time-consuming. Thus, we compared ADP and DXA mass estimates with a third independent scale measurement. Our results showed that ADP and DXA mass estimates are highly correlated to each other as well as the independent scale estimates, but DXA has a significant systematic overestimation bias whereas ADP does not. Since body composition is based on a 2-C model in both of these devices, it can be seen that this systematic bias in the estimation of mass translates to an underestimation

of FM% compared to ADP (Results: Figure 3). Since ADP measures mass accurately while DXA does not, it can therefore be inferred that estimates of FM% from ADP are more accurate.

Our findings our similar to a study by Fusch et al[138], where 23 piglets ranging from 848 to 7550 g were assessed for body composition with DXA and carcass analysis. It was found that DXA overestimated body weight by 1.2%[138]. These results have potential implications for the development of equations for corrections of the DXA algorithm for the preterm infant population.

6.3: Identification of confounders

In this study, BUN was not influenced by gestational age at birth or measurement, or day of life. This is in accordance with literature, where BUN is reported to decrease with day of life only up to the first two weeks of life[143]. The infants in our study were on average, 22 days old at the start of the observation period. Surprisingly, birth weight was positively correlated with BUN at all time points of the study and was included in the subsequent regression modelling. However, as seen in the regression models (Tables 5-7), the effect of birth weight on BUN disappeared by day 14 and other factors included became more important predictors of BUN. Since gestational age at birth was not correlated with BUN at study day 1, it cannot be said that the association between birth weight and BUN at SDay1 is a function of gestational age. While subjects in this study were all VLBW AGA infants, AGA infants are reported to have higher urea production compared to SGA infants where it reflects the functional maturity of the urea cycle[144]. It is possible that this effect also translates to birth weight alone and that preterm infants with higher birth weights

also have mature urea cycle capacity which may be reflected in increased urea production at SDay1. But the effect dissipates after this point and other factors, such as macronutrient intake became more important predictors of BUN.

Maternal characteristics also did not affect BUN. However, BUN at study day 14 and 21 was significantly influenced by BUN at baseline. This meant that infants with high BUN at study day 1 also had proportionately higher BUN at study day 14 and 21. Taken together with the findings mentioned above, this may indicate that infants with higher birth weights have higher BUN values at SDay1 which continue to remain high over time, though the effect of birth weight disappears. This may reflect that earlier high BUN may be caused by functional capacity of urea cycle enzymes, which increases over time due to maturation.

Growth velocity by study day 21 was not associated with any of the confounders assessed. It is surprising that neither birth weight nor gestational age at birth influenced growth velocity. This suggests that the *rate* of growth is influenced by factors other than birth characteristics or maternal characteristics. In contrast, maternal BMI was positively correlated with FFM% and negatively correlated with FFMI at $p<.0.10$. Gender also influenced FFMI in our study. In the literature, preterm males are reported to have higher FFM[145]. While differences in body composition by ethnicity, maternal hypertension and diabetes are reported in literature, none were observed in this study.

6.4: Identifying relationship between BUN and macronutrient intake & ratios

In order to understand the function of BUN as a response metabolite, it was important to examine its relation to macronutrient intake. The impact of protein intake on BUN has been examined, but little is known about the influence of energy intake in comparison to protein intake or quality of energy (as indicated by CHO:NPE%)[131, 132]. BUN at SDay1 remained a significant contributor and confounder in the BUN models examined. An increase in protein intake 24 hours before the urea value was taken reflected an increase in the value of urea. This was true at all time points. This effect disappeared with the addition of block two of average macronutrient variables which explained a significant additional amount of variation in BUN. This demonstrated that not only is BUN reflective of 24 hour-before protein intake, but it is also independently influenced by average intakes over a longer period. An increase of 1g in average protein intake over 21 day period reflects an increase of 1.71 mmol/L in BUN at that time point. Increased protein intake results in increased protein metabolism and thus, an increase in urea production. This confirms the findings in the literature that report an association between protein intake and BUN. Polberger et al[128], found a positive correlation between protein intake and blood urea in VLBW 26 AGA infants. Their findings were more highly correlated than ours with a reported R^2 of 0.77. We were able to confirm these findings in a 101 infants while demonstrating the importance of accounting for key confounding factors such as urea values at a starting time point. Our data would agree with existing suggestions that BUN may be used as a measure of protein adequacy. Our study adds that BUN may also be used as indicator of protein to energy ratio.

In our study, an increase in 1g/100kcal of P:E ratio can reflect upwards of 3.55 mmol/L increase in BUN values (Table 6). The trend is true for all BUN time points. Studies by Kashyap and colleagues[131, 132] reported BUN values when examining the effect of varying rates of protein, energy and CHO:NPE on growth. They found that when protein intake was equal, the group with a high P:E ratio indicating a limited energy supply, had a higher BUN compared to low P:E ratio groups[131]. Our study confirms this trend in a regression analysis as P:E is significantly positively correlated with BUN. This indicates that in the absence of sufficient energy supply (increasing P:E values), protein is oxidized to provide energy resulting in increased urea production.. Thus, our analysis shows that BUN is not only a marker of protein intake, but also of energy intake in relation to protein. For instance, a BUN of 3.3 mmol/L at SDay1 for an infant that weighed 1000g at birth may indicate sufficient protein intake of 4.0g/kg/day[87] and an adequate P:E ratio of 2.8 g/kg/day[146] (based on Table 5). Though it is important to note that only 19% of the variation in BUN is explained by these variables. For this same infant, a BUN of 5.1 mmol/L at SDay14 would indicate adequate P:E ratio with 42% of the variation explained by these variables (Table 6). However, protein intake would not be correlated with BUN based on our findings indicating that protein metabolism may be decoupled from protein intake at this time point. Analysis of the third aim (discussed in the next section: 6.5) also showed that growth is not related to protein intake at this time point. Collectively, this suggests that protein metabolism is altered at SDay14, though the reasons for this are unclear. There is evidence that a transitional state from parenteral to enteral nutrition is marked by suboptimal growth and changes in metabolism[147]. This cannot be true for infants in this

study as they had been on full enteral nutrition for 7 days prior to start of the study. At SDay21, for this same infant, a BUN of 5.3 mmol/L would indicate adequate nutritional intake, and 44% of the BUN value would be explained by the nutrient intake factors (Table 7).

Similarly, Kashyap et al., reported lower BUN values in groups receiving lower percentage of CHO:NPE[64]. Although the trend was positive in our study, it was not significant. This may be because in our study, no infants received a CHO:NPE% above 55% or below 35%. The mean intake was approximately 44% for all time points. This is in contrast to the studies by Kashyap and colleagues where the experimental groups were designed to receive either 35% or 65% CHO:NPE. As such, our study cannot draw inferences about the impact of CHO:NPE% on BUN as the intake ratios in our study were narrow and may have not been sufficient to examine trends in CHO:NPE%.

6.5: Regression models of growth using BUN as a predictor

The predictive capacity of BUN was examined by modelling BUN along with other predictor variables with growth velocity. Growth velocity at study day 14 could not be predicted by SDay1 BUN, SDay14 BUN or respective macronutrient intake factors. Interestingly, for the regression analysis between BUN and macronutrient factors (section 6.4), protein intake was also not significantly correlated with BUN at SDay14. However, P:E ratio was. This indicates that protein metabolism may be altered at this time point which disrupts the association between protein intake and growth (Table 8, 10) as well the association between protein intake and BUN (Table 6). Thus, SDay14 differs from the other study points. The reasons for this artifact are unknown.

BUN at study day 1 made a statistically significant impact on regression of growth velocity at study day 21 demonstrating a potential predictive capacity (Table 9). However, it only explained an additional 5% of the variation in growth which may not be clinically significant. In this study, a 1 mmol/L increase in BUN observed at SDay1 predicted a 0.5g/kg/day lower growth velocity 21 days later. An increase of 1g/kg/day in the average P:E ratio predicted a 3.6 g/kg/day decrease in growth velocity by SDay21, while an increase of 1g/kg/day in the average protein intake predicted a 2.76 g/kg/day increase in growth velocity by study day 21. Thus, for an infant to grow at 20 g/kg/day over a 21 day average while receiving 4.0 g/kg/day of protein at a 2.8g/100kcal P:E ratio, any 1mmol/L increase in BUN beyond 5.4 mmol/L at SDay1 may warrant an increase in energy intake to prevent future 0.5 g/kg/day drop in future growth rate (based on Table 9). This would mean reducing P:E by 0.04g/100kcal or increasing energy intake up to 145 kcal (based on Table 5). Though the predictive capacity of BUN towards growth may not be clinically strong, the relationship between BUN and macronutrient intake is both statistically and clinically relevant. Due to the strong negative correlation between P:E ratio and growth, and the strong positive correlation between BUN and P:E ratio, it may be clinically meaningful to use BUN as a marker of P:E ratio insufficiency rather than protein intake adequacy.

To our knowledge, this is the first study to quantify the relationship between BUN and growth. Our findings indicate that an increase in BUN reflects a decrease in growth velocity. It would appear to contraindicate the findings and suggestions of Arsangalou and colleagues[129], who adjusted protein intake based on low BUN values, and subsequently achieved greater weight gains and higher BUN values in the group with adjusted protein

intake[129]. However, our study highlights the importance of considering P:E ratio in such determinations as an increase in P:E ratio correlated with high BUN values (Tables 5-7), and it is simultaneously associated with significantly slow growth rate (Table 9 & 11). Our study indicates that at higher P:E intakes, the infant is not capable of synthesizing dietary protein into body protein due to low energy intake. Upon further examination, Arsangalou and colleagues reported higher energy intake in the adjusted group. This means that while they increased protein intakes, P:E ratio stayed at approximately 2.5 g/100kcal. This might explain the positive growth trends observed in their study. Although there may be a precedent in the literature to reduce protein intake on the basis of high urea values, our study showed that high urea values also reflect inadequate energy intake in relation to protein. Therefore, an appropriate clinical response to high urea values may be to increase energy intake which may result in increased growth velocity. The composition of this growth cannot be elucidated by this study as no correlations found in body composition at 36-40 weeks.

BUN at SDay21 had a significant negative correlation to growth by study day 21. This suggests that BUN has a potential to be reflective of growth that has occurred prior to the value being taken. While it may be useful to know that BUN can reflect growth velocity of a time period before, this may not be clinically relevant to ameliorate suboptimal growth as growth has already occurred. However, if this relationship can be established on a more frequent basis, for example, if BUN is also reflective of a growth period of 1 week before, then BUN may be used as a marker of poor growth that has already occurred. And may be used to clinically intervene going forward.

BUN did not explain head circumference and length gains at any of the time points. None of the other explanatory variables included, such as average macronutrient intake or gender, were significant predictors of head circumference and length gains. Head circumference measurement has a large error due to moulding of the head from respiratory support requirement. Therefore, it is difficult to conclude the lack of significant correlations as meaningful observation. Length measurement on the other hand, was performed with length boards by study personnel and were more reliable than the standard NICU practice of using measuring tapes. As such, it is possible that the macronutrient intake does not translate to linear growth, and that BUN is not predictive of linear growth. This would imply that the growth velocity changes observed reflect gain in fat mass over the 21 days of the study.

Body composition could not be predicted by BUN at SDay1, SDay21 nor the change of BUN over this period. It appears that predictive attributes of BUN noted with growth velocity do not translate to compartments of the body at a later time. It may be that there is a postnatal 'time limit' by which assessment of BUN has a meaningful impact. Body composition was measured between 36-40 weeks PMA, while the last study observation period for nutritional parameters and metabolic markers was SDay21 where infants were on average 33 weeks PMA. Additionally, the sample size in this regression was only 56 infants. Our study was unable to shed light on the association between body composition and BUN. Additionally, the predictor variables examined, such as macronutrient intake or maternal BMI, were not significant in predicting FFM%. However, FFMI was most explained by maternal BMI and gender. Males had an FFMI 1.2x higher

compared to females, indicating that they had increased fat free mass normalized for height compared to females. This would be consistent with previous observations, as males tend to be leaner at term and throughout life[148]. In addition, higher maternal BMI was related to decreased FFMI at 36-40 weeks PMA. This is consistent with observations in literature of pre-pregnancy maternal BMI influencing body composition of infants at birth and into childhood[149]. The correlation between body composition, and maternal health status in combination with the lack of correlation with macronutrient intake at 36-40 weeks PMA observed in our study is interesting. Collectively, with the other findings in this study, it indicates that postnatal macronutrient intake may influence *rate* of growth but has no bearing on body composition at 36-40 weeks PMA. Instead, maternal BMI and gender plays the most significant role in determining later body composition. This would consistent the DOHaD hypothesis of fetal programming, and would contradict suggestions that postnatal nutrition can modify body composition in preterm infants.

Our study suggests that while average intakes explain most of the variation in growth, there is merit to incorporating a metabolic response marker to explain growth as it statistically adds to the understanding of the variation observed. However, the additional variation may not be clinically significant in predicting growth. Still, due to the strong correlation between macronutrient intake and BUN, BUN may be a more relevant marker of P:E insufficiency. Our data indicates 3.3 mmol/L-5.4mmol/L as adequate ranges for a baby 1000g at birth, values beyond the upper limit may indicate inadequate energy in relation to protein.

6.6: Strengths and limitations

The present study is the only one to our knowledge that examined the relationship between BUN and energy intake with respect to protein, and quality of energy (measured as percentage of carbohydrates to non-protein energy). Previous studies have examined the relationship between protein intake and BUN, however, to date, no regression analysis between BUN and P:E and CHO:PE% have been performed. Additionally, to our knowledge no published studies exist examining the relationship between BUN and growth. In our study, the infant's period of analysis was rigorously screened to exclude any periods where antibiotics and dexamethasone, as they both influence growth and dexamethasone is known to reduce nitrogen metabolism, increasing BUN levels in piglets[150].

The screening of relevant baseline confounders was performed before the regression analysis was done using a systematic approach, where continuous variables were correlated using bivariate analysis and dichotomous variables are correlated using a t-test. This allowed for optimization of the regressors included in the main regression analyses.

Since a multiple regression analysis was performed, it was imperative that the design of model accounted for important covariates and that these were decided apriori. This is because multiple regression analyses are sensitive to the number of variables, and in order for the model to have clinical application, the variables of interest should be identified based on literature.

The hierarchical block design of this study allows not only the relationship between BUN and growth to be examined, but also allows conclusions to be drawn about the additional predictive capacity of BUN as a marker. An additional strength of this study is the stringent accounting of confounders. Nutrient intakes during the previous 24 hour were examined in the regression model to assess the immediate influences of nutrition on BUN.

The sample size of this study is another strength, as most literature that has examined association between BUN and protein intake has included between 20-30 infants. Our study examined not only additional variables, but also examined it in 101 infants. In addition, BUN values were corrected using creatinine values. This accounted for the low glomerular filtration rate of preterm infants which would elevate BUN values independent of nutritional intake. This has often not been accounted for in literature[128, 129].

One of the limitations of this study is generalizability. The study population was a very specific subset of preterm population, who were then studied only during the healthy periods of their NICU stay. In addition, our study design is based on day of a nutritional intervention as the metabolic and growth assessments were conducted at these study time points. While we included day of life as a potential confounder in the initial analysis and did not find a significant effect on our outcomes, it was not possible to do an analysis that was based on day of life as the three time points. This is because infant outcomes were not assessed by day of life as per the RCT study design. However, it is important to note that this ensured that infants were at similar time points with respect to their enteral nutrition intake, as SDay1 was equivalent to the 8th day of full enteral nutrition. While the inclusion of relevant covariates such as birth weight or maternal BMI would make the equations more

specific to individual infants, this analysis was conducted on infants VLBW infants born ≤30 weeks at birth and is only applicable to that population. Furthermore, because of the number of variables included in the model, beside utility is limited.

6.7: Implications

The different aspects of this study have many clinical implications. The method development of growth velocity sheds light on the need for standardization of such measures. This has vast implications for clinical studies that power to detect differences of 2-3 g/kg/day in nutritional interventions as the effect of the intervention may be underestimated or overestimated when compared with other studies, depending on the growth velocity calculation used.

The body composition comparison has utility in terms of development of correction equation for the DXA machine for the preterm population. This would expand the range of tools that can be used for assessment of body composition and nutritional adequacy. However, the limitations posed by neonatal respiratory apparatus remain when measuring body composition with the DXA machine.

Our study re-emphasizes the ability to use BUN as a marker of protein adequacy. However, we were able to highlight the importance of influence of P:E ratios on BUN levels. This has clinical implications as only when P:E ratios are considered, does BUN reflect the full nutritional status of the infant. An infant with high BUN level may have sufficient protein intake but may also reflect an insufficient ratio of energy intake in relation to protein. It may be more clinically useful to consider BUN as an indicator of P:E ratio or

utilization of protein, within groups of infants e.g., as an indicator of sufficiency of energy in groups already receiving at or above recommended intakes of protein, due to the negative correlation between P:E and growth.

6.8 Future Directions

In order to strengthen the current findings, future directions of research should focus on establishing frequent BUN assessments, and at day of life intervals. This would further highlight if BUN can be used on a more frequent basis to predict or reflect growth. Furthermore, to highlight association of BUN with body composition, it may be more relevant to use body composition assessment that can be performed more frequently and earlier to correspond to BUN values, such as skinfold thickness measurements. In order to elucidate the effect of CHO:NPE% on BUN or growth, a larger more variable intake of CHO:NPE% would need to be examined. Mostly importantly, the regression analysis would need to be validated in another dataset where multiple infants are receiving varying but fixed combinations of P:E and protein intake. This would allow for a suggestion of more firm BUN cut-offs.

6.9 Conclusion

BUN responds to changes in protein intake indicating an increase protein metabolism. It also responds to energy intake in relation to protein, indicating an increase in protein metabolism when energy intake is low compared to protein. Increases in BUN also indicate a statistically significant decrease in future growth, though the strength of this relationship may not be clinically significant. However, because BUN correlates strongly with P:E ratio and, as we have shown that protein to energy ratio is negatively associated

with growth, it may be clinically more meaningful to treat increases in BUN as indicator of inadequate protein to energy intake, as opposed to adequate protein intake. This may translate to increased future rate of growth. However, as we did not observe any influence on body composition, an increased rate of growth may not dictate improved future body composition. In summary, by examining how BUN responds to macronutrient intake, and how growth relates to BUN as well as macronutrient intake, we were able to highlight importance of using BUN as an indicator of protein energy adequacy. Our data indicates that a BUN above 5.4mmol/L in a baby 1000g at birth indicates an adequate protein to energy intake. However, appropriate cut-off levels cannot yet be suggested with our analysis. Future research will need to validate these equations in groups of infants at various fixed combination of P:E and protein intake before concrete cut-offs can be suggested.

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Appendix 1

PEAPOD ELIGIBILITY ASSESSMENT

INCLUSION CRITERIA:

1) Any gestation (including those on IV fluids)
2) In room air
3) On CPAP $\leq$ 6cm H20 or HFNC $\leq$ 6 L/min
4) No episode of desaturation/bradycardia (definitions mentioned at the end of the document) requiring positive pressure ventilation in past 1 week
5) No episode of desaturation/bradycardia requiring stimulation in last 48 hrs
6) Number of self-resolving episodes of desaturation/bradycardia $\leq$ 12 in last 24 hours

Those babies who meet ALL the above criteria would be assessed for eligibility to be measured with Peapod.

ASSESSMENT FOR ELIGIBILITY

1) In presence of MD/NNP, the nurse would take the baby off CPAP/HFNC – observe for 5 mins – put the baby back on CPAP/HFNC (as the case may be)
2) This observation would be done once in during morning handling
3) The following data should be recorded pre & post assessment:
    a. Heart rate, spO_2, respiratory rate, work of breathing (Silverman score) prior to assessment
    b. Post assessment: Heart rate, spO_2, respiratory rate, work of breathing (Silverman score)
    c. Any episode of desaturation or bradycardia during the assessment
4) In case of any episode of apnea/bradycardia/desaturation – stop assessment immediately and put the baby back on the CPAP/HFNC (as the case may be).

Following assessment, those babies who meet the following the eligibility criteria would be assessed on peapod.

ELIGIBILITY CRITERIA:

The baby is eligible to be examined with peapod if during assessment the baby meets the following:

1) No episode of apnea/bradycardia/desaturation during the 5 mins of assessment
2) Post assessment Silverman score is not more than 2 points above pre-assessment score
3) Post assessment HR (measured at least 5 mins after the baby is put back into the isolette) not more than 20% above baseline and post assessment RR not more than 40% above baseline

DEFINITIONS:

APNEA: Cessation of spontaneous breathing for > 20 secs

BRADYCARDIA: HR< 100/min

DESATURATION: spO_2 < 84% with proper probe attachment being observed by the nurse

Rationale for 5 mins assessment: The peapod measurement would require the baby to be off respiratory support for approximately 2.5mins. Hence we have taken 5 mins as time of assessment in our eligibility criteria to ensure safety of the assessed babies

Appendix 2

SILVERMAN ASSESSSMENT: