NOTE TO USERS

COMPARISON OF THE EFFECTS OF TWO HUMAN MILK FORTIFIERS WITH DIFFERENT ENERGY SOURCES ON THE BODY COMPOSITION OF PREMATURE INFANTS

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

Your file Votre référence
ISBN: 0-612-98669-1
Our file Notre référence
ISBN: 0-612-98669-1

To my grandfathers Alexander Smith and Harold Kean

ABSTRACT

Human milk fortification is recommended to meet the nutritional requirements of preterm infants. Most human milk fortifiers (HMFs) contain non-protein energy (NPE) predominantly as carbohydrate which may lead to high fat deposition relative to lean mass accretion. We hypothesized that fortifying human milk with a HMF containing NPE predominantly as fat (fatHMF) would result in a higher 1) lean mass accretion (percent lean mass) and 2) growth (anthropometry), compared to fortifying with an isocaloric, isonitrogenous HMF containing NPE predominantly as carbohydrate (carbHMF). In a double-blind randomized trial, 29 infants ($\leq$ 32 weeks and appropriate for gestational age) admitted to the Neonatal Intensive Care Unit received either mother's milk fortified with the fatHMF (n=14) or the carbHMF (n=15). Body composition and growth measurements were performed at Baseline (at $\leq$ 10% of goal intake 150 ml/kg), Phase 1, and Phase 2 (3 weeks and 6 weeks, respectively, from starting HMF). Although neither percent lean (fat) mass nor growth were statistically different, by Phase 2 infants receiving fatHMF showed a 63% increase in percent fat mass, gained 1194 g in weight and 8.8 cm in length, whereas the carbHMF showed a 96% increase in percent fat mass, gained 1005 g in weight and 6.9 cm in length (p=0.3586, 0.3815, and 0.1851 respectively). By Phase 2, the fatHMF infants gained 128 g in absolute dry lean tissue, whereas the carbHMF infants gained 99 g (p=0.0362, Post hoc analysis). Differences of this magnitude are clinically important, but a larger study is required to demonstrate statistical significance.

RÉSUMÉ

La fortification du lait maternal est recommandée afin de répondre aux besoins de croissance des bébés prématurés. L'énergie non-protéine (ENP) inclus dans les fortifiants de lait maternal (FLM) est majoritairement sous forme de glucides ce qui peux entrainer une plus grande accumulation de la masse graisseuse par rapport à la masse maîgre chez les nouveau-nés prématurés. Nous avons posé l'hypothèse que la fortification du lait maternel avec un FLM ayant comme source d'ENP une prédominance de gras (grasFLM) favoriserait 1) une meilleur rétention de la masse maîgre (pourcentage de poid maîgre), et 2) une meilleur croissance anthropomètrique lorsque comparé à un FLM ayant comme source d'ENP une prédominance de glucides le tout en présentant un contenu azoté et calorique égale. Dans une étude randomisée à double-insu, 29 nouveau-nés prématurés ($\leq$ 32 semaines et approprié pour l'âge gestationnel) admis à l'unité des soins intensifs néonataux ont reçu leur lait maternel fortifié avec grasFLM (n=14) ou gluFLM (n=15). Des mesures de composition corporelle et de croissance ont été fait à la ligne de Base ($\leq$ 10% de l'apport prévue de 150 ml/kg), à la Phase 1 et à la Phase 2 (3 et 6 semaines depuis l'introduction du FLM, respectivement). Bien qu'il n'y a pas de différence statistiquement significative entre les groupes dans le pourcentage de la masse maîgre (ou masse graisseuse) et ni sur la croissance, les enfants ayant reçu grasFLM ont démontrés à la Phase 2 une augmentation de 63% dans le pourcentage de la masse graisseuse, gagnés 1194 g en masse corporel et 8.8 cm en longueur, pendant que le groupe carbFLM ont démontrés une augmentation de 96% dans le pourcentage de la masse graisseuse, gagnés 1005 g en masse corporel et 6.9 cm en longueur (p=0.3586, 0.3815 and 0.1851, respectivement). À la Phase 2, les enfants ayant reçu grasFLM ont gagnés 128 g en masse maîgre sèche, pendant que le groupe carbFLM a gagné 99 g (p=0.0362, l'analyse Post hoc). Des différences de cette magnitude sont cliniquement important mais une étude à plus grande échelle est nécessaire afin de démontrer leur différence statistique.

ACKNOWLEDGEMENTS

I dedicate this thesis to my family, specifically to my grandfathers, and to my boyfriend whose words of encouragement, love and understanding were invaluable.

I sincerely thank my supervisor, Dr. Louis Beaumier, for generously sharing his knowledge and providing support throughout my graduate studies, this research and thesis. I especially thank him for his confidence in me, and approach to teaching that gave me inspiration and enriched my graduate school experience.

I would like to thank and acknowledge the members of my supervisory committee, Dr. Linda Wykes and Dr. Keith Barrington for sharing their expertise, and providing advice and constructive feedback throughout my research and on this thesis.

I extend my appreciation to the staff of the McGill University Health Center: Dr. Margaret Berry for her thoroughness in reviewing my protocol and randomly assigning subjects; Xun Xhang and Elise Mok for statistical consultation; Dr. Robert Brouillette for statistical reference information; Dr. Annie Janvier for her help with recruitment; all the NICU medical staff for their cooperation and interest in this research; the NICU nurses for their assistance in the fortification of breast milk; Filo Monteleone and Anne-Marie Lanctôt for help with communication to all nursing shifts; the NICU Pharmacy, particularly Louis Chartier, for randomizing subjects and dispensing fortifiers; the NICU biochemistry lab, specifically Peter Moreau for performing blood analysis; Samir Mubarak for help with ordering supplies; Tom Kokkotis, Suzanne Lortie, and Michèle Fortin for their help with administrative details.

I am also grateful to the infants and parents of infants who consented to participate in this research. I hope that any knowledge gained from this work can be used to improve the nutritional care of future preterm infants.

I express my gratitude to the McGill University-Montreal Children's Hospital Research Institute for their studentship support and the opportunity to present this work.

I would like to thank Dr. Katherine Gray-Donald (Director), Faculty and Staff, and my fellow graduate students of the School of Dietetics and Human Nutrition, McGill University for a stimulating learning environment.

Finally, I would like to thank Mead Johnson Nutritionals (Ottawa, Canada) for their generosity in donating their human milk fortifier for use in this study.

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LIST OF ABBREVIATIONS

AAP	American Academy of Pediatrics
AGA	Appropriate for gestational age
Alb	Albumin
ALP	Alkaline Phosphatase
ANOVA	Analysis of Variance
BIA	Bio-electric Impedance Analysis
BPD	Bronchopulmonary Dysplasia
Ca	Calcium
CI	Confidence Interval
Cl	Chloride
carbHMF	Study HMF containing non-protein energy predominantly as carbohydrate (Similac® HMF, Ross Products)
CPS	Canadian Pediatric Society
DXA	Dual Energy X-ray Absorptiometry
DIT	Diet induced thermogenesis
EBM	Expressed breast milk
ECW	Extra-cellular water
EHMF[1] HMF	First formulation of Enfamil® HMF, Mead Johnson Nutritionals (no longer available)
EHMF[2] HMF	Second formulation of Enfamil® HMF, Mead Johnson Nutritionals (no longer available)
EN	Enteral nutrition
fatHMF	Study HMF containing non-protein energy predominantly as fat (most current Enfamil® HMF, Mead Johnson Nutritionals)
FM	Fat Mass
GA	Gestational age
HGB	Hemoglobin
HMF	Human milk fortifier
K	Potassium
LM	Lean Mass

MUHC	McGill University Health Center
Na	Sodium
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NEC	Necrotizing Entercolitis
NICU	Neonatal Intensive Care Unit
NPO	Nil per os
PDA	Patent Ductas Arterious
Phos	Phosphorus
PI	Ponderal Index
P-RNI	Preterm Infant Recommended Nutrient Intake
PUFA	Polyunsaturated fatty acids
RQ	Respiratory quotient
RVH	Royal Victoria Hospital, Montreal, Canada
SD	Standard deviation
SEM	Standard error of the mean
SHMF	Similac® HMF (Ross Products), same as study carbHMF
SNC	Similac® Natural Care liquid HMF (Ross Products)
TBW	Total body water
TOBEC	Total Body Electrical Conductivity
t0	Time of starting study HMF
TPN	Total Parenteral Nutrition
Vs.	Versus

CHAPTER 1. LITERATURE REVIEW

1.1 Overview

The generally accepted goal for the nutritional management of the premature low birth weight infant is the provision of sufficient quantity and quality of nutrients to support a rate of growth and tissue accretion similar to that found in-utero during the third trimester (CPS 1995, AAP 1997). Although human milk is recommended for term infants, it is well documented that exclusive feeding of human milk to premature infants is associated with slower rates of growth (Putet *et al.* 1984) and nutritional deficits (i.e. poor bone mineralization) during and beyond the period of hospitalization (Schanler 2001, Atkinson *et al.* 1981, Cooper *et al.* 1984). Despite the higher protein content of pre-term human milk compared to mature breast milk, particularly during the first postnatal month, it still has insufficient quantities of nutrients, specifically, calcium, phosphorus, iron, protein, and energy to meet the nutritional requirements of the pre-term infant (CPS 1995, Schanler 2001, Kuschel *et al.* 1998).

This has led to the development of human milk fortifiers (HMF(s)). Fortified human milk is the preferred feeding option recommended internationally by the Canadian Pediatric Society (CPS 1995), American Academy of Pediatrics (AAP 1997), and European Society of Pediatric Gastroenterology and Nutrition (ESPGN 1987). This is primarily due to the benefits of human milk that are not observed with use of pre-term formula, such as improved host defenses, digestion and absorption of nutrients, gastrointestinal function, neuro-developmental and cognitive outcomes, maternal/infant psychological well-being (Schanler 2001, Schanler[1] *et al.* 1999), lower incidences of Necrotizing Entercolitis and late-onset sepsis, as well as less morbidity and shorter hospitalization (Schanler[2] *et al.* 1999).

Surprisingly, available HMFs are quite different in many components and the formulation of HMF that results in the most optimal growth composition (i.e. achieves goal of growth and tissue accretion similar to that in-utero) of the premature infant is not well defined. Of interest, is that most HMFs contain carbohydrate as the predominant or sole non-protein energy source (Schanler *et al.* 1995, Sankaran *et al.* 1996, Porcelli *et al.* 2000, Barrett Reis *et al.* 2000) and growth composition may not be meeting in-utero goals. Although it is well established that the provision of adequate protein and total energy is

fundamental to optimizing growth (Micheli *et al.* 1993), the literature suggests that feeding neonates a higher proportion of non-protein energy as carbohydrate may promote high fat deposition relative to lean mass accretion (Kashyap[1] *et al.* 2001, Nose *et al.*1987). However, the literature on the effects of non-protein energy on growth composition is controversial, the mechanisms involved are not well established, and this has not been studied within the context of human milk fortification. So, there is insufficient evidence to make recommendations on the proportion of non-protein energy as fat or carbohydrate to include in HMFs.

1.2 Potential Mechanisms Related to Non-protein Energy and Growth Composition

The potential mechanisms involved in how the proportion of non-protein energy as carbohydrate or fat could affect the composition of growth in the neonate are unresolved. But, insulin is believed to play a role. As, under isocaloric conditions, insulin secretion increases in response to an increasing proportion of non-protein energy intake as carbohydrate, not fat (Bresson *et al.* 1989, Pineault *et al.* 1988). Also, insulin stimulates both protein synthesis (Davis *et al.* 2001, Davis *et al.* 2002, O'Connor *et al.* 2003) and lipogenesis (Geelen *et al.* 1978, Beyen *et al.* 1979, Witters *et al.* 1985, Gruppuso 1998, Hillgartner *et al.* 1995, Hunt *et al.* 1990). However, the extent of the influence of insulin, under conditions of different proportions of carbohydrate and fat intake, on postnatal protein and fat accretion in the neonate is unclear. The neonate is very sensitive to insulin (Wray-Cahen *et al.* 1997, Davis *et al.* 2001, Davis *et al.* 2002, O'Connor *et al.* 2003). So, conceivably, hyperinsulinemia (*i.e.* via increased proportion of non-protein energy intake as carbohydrate) may not equal higher net protein synthesis or better overall quality of growth and could favor high rates of *de novo* lipogenesis (Letton *et al.* 1995, Pierro *et al.* 1993, Bresson *et al.* 1989) and high fat deposition compared to lean mass accretion (Kashyap[1, 2] *et al.* 2001).

To understand the effects of insulin on net growth composition in the neonate, it is important to consider the relative effects of insulin on: 1) protein synthesis and proteolysis (as net protein accretion potentially can be accomplished by an increase in protein synthesis, a decrease in proteolysis, or both (Poindexter *et al.* 2001), and 2) *de*

novo lipogenesis (as this may affect, fat accretion, energy expenditure and protein oxidation (Bresson *et al.* 1991), therefore potentially affect energy and amino acid availability for growth).

1.2.1. Effects of Insulin on Protein Synthesis in the Neonate

The neonate is very sensitive to the effects of physiological levels of insulin on amino acid utilization (Wray-Cahen *et al.* 1997) and protein synthesis, particularly skeletal muscle synthesis (Davis *et al.* 2001, Davis *et al.* 2002, O'Connor *et al.* 2003). Furthermore, this sensitivity to insulin has been shown to decrease with development (Wray-Cahen *et al.* 1997, Davis *et al.* 2002).

For example, a study (Wray-Cahen *et al.* 1997) compared young neonatal animals (7 day old piglets) to older neonates (26 day old piglets) using hyperinsulinemic-euglycemic clamp techniques. When amino acids and glucose were clamped at basal fasting levels, circulating amino acids fell markedly as insulin concentration was increased within a physiological range, in the 7 day old piglets compared to the 26 day old piglets, with the plateau in amino acid concentration achieved at a lower insulin concentration in the younger piglets compared to the older piglets (20 μU/ml, a physiological concentrations of insulin characteristic of the fed pig, *vs.* 30 μU/ml, respectively). This shows that neonates are very sensitive to insulin and efficiently utilize amino acids at lower physiological insulin concentrations compared to older animals. As well, it suggests that an inadequate supply of amino acids would limit amino acid utilization, and alternatively, increasing amino acid substrate (i.e. higher than basal levels to the fed state) may increase protein utilization (i.e. for protein synthesis) (O'Connor *et al.* 2003).

This was shown in a recent study (O'Connor *et al.* 2003) in 7 day old neonatal pigs using pancreatic glucose-amino acid clamp techniques and a flooding dose of L-[4-^{3}H]phenylalanine to measure muscle protein synthesis. Endogenous insulin was suppressed while glucose and glucagon were maintained at fasting levels and insulin was infused to simulate either less than fasting, fasting, intermediate or fed insulin levels. At each insulin dose, amino acids were clamped at either the fasting or fed level. This showed : 1) a dose response effect of both insulin and amino acid on muscle protein synthesis and the effects of both insulin and amino acids were additive until maximal

rates of protein synthesis were reached at approximately 7μU/ml (intermediate insulin concentration between fasting and fed); 2) after the maximum protein synthesis was reached, there was a positive curvilinear relationship between skeletal muscle protein synthesis and increasing plasma insulin concentrations in fasted and fed neonatal pigs. Therefore, further increases in protein synthesis did not occur by increasing insulin levels beyond approximately 7 μU/L. This suggests a threshold effect and sensitivity of the young neonate to insulin, as relatively low levels of insulin, well within the physiological range (11-22 μU/ml) of human neonates (Poindexter *et al.* 1998), can maximize protein synthesis; 3) amino acid infusion at the fed level enhanced basal and maximal protein synthesis rates above that of the amino acid infusion at the fasted level, suggesting that protein synthesis is influenced by amino acid availability, and lack of amino acid can limit the effect of insulin; 4) increasing circulating amino acids to fed levels in the near absence of insulin increased muscle synthesis, but the increase in protein synthesis was less than that which occurred during fasting levels of insulin, as baseline protein synthesis was reduced; 5) also, the difference between the maximal rate of protein synthesis and baseline rate was the same for both the fasted and fed amino acid infusions, which suggested that amino acids do not increase the sensitivity or responsiveness of protein synthesis to insulin and that insulin and amino acids may act independently to stimulate protein synthesis in the young neonate. This is in contrast to more mature animals (Ballie *et al.* 1993, McNulty *et al.*1993) and humans (Denne *et al.* 1991, Tessari *et al.* 1996) in which insulin had little effect on increasing skeletal muscle protein synthesis without amino acid (O'Connor *et al.* 2003).

1.2.2. Effects of Insulin on Proteolysis in the Neonate

In human adults both physiologic and pharmacologic insulin concentrations suppress proteolysis in a dose dependent manner (Flakoll *et al.* 1989). In contrast, in neonates, it is unclear as to what extent physiological levels of insulin can suppress proteolysis (Poindexter *et al.* 1998). Neonates may be resistant to suppression of proteolysis at physiological levels of insulin. For example, in a study in term newborns (Denne *et al.* 1995), receiving infusion of glucose at 5.5 mg/kg/min (~7.92 g/kg/day), infusion of fat at 2.5 mg/kg/day, or the combined infusion of glucose at 5.5 mg/kg/min (~7.92 g/kg/day)

and fat at 2.5 mg/kg/day (with insulin concentrations of 6.2±3.7 μU/ml , 5.2±3 μU/ml, or 5.2±3 μU/ml, respectively) did not suppress proteolysis. However, no amino acids were provided and energy intake was low during this study and may have limited any potential anti-proteolytic effects of insulin. However, in another study involving preterm infants fed a similar intake of glucose (6 mg/kg/min or ~8.4 g/kg/day) and corresponding basal levels of insulin (~3.6 μU/ml), but with protein infusion of 2.4 g/kg/day, proteolysis was still not reduced (Poindexter *et al.* 2001). However, energy intake and amino acid intake did not meet recommended requirements for preterm infants (CPS 1995) and may have limited any potential anti-proteolytic effect of insulin (Poindexter *et al.* 2001). As, in contrast, infants born at term, studied using the same feeding protocol (Poindexter *et al.* 1997), proteolysis was reduced. This may be because of developmental reasons (Poindexter *et al.* 2001), or because protein and energy provided to the term infants more closely met growth requirements of the term infant (Institute of Medicine 2002). Furthermore, preterm infants provided with a higher intake of glucose (~9 mg/kg/min or 12.96 g/kg/day) which corresponded to a threefold increase in endogenous insulin concentration of ~13 μU/ml (within the higher physiological range of insulin of 11-22 μU/ml reported in this population (Poindexter *et al.* 1998)) did not change proteolysis from basal state (Hertz *et al.* 1993).

Suppression of proteolysis in the preterm infant may require pharmacological doses of insulin, but this may not create the desired net effect of net protein anabolism (Poindexter *et al.* 1998). As, when exogenous insulin was infused to a supra-physiological level (79 μU/ml), proteolysis was reduced by 20%, but protein synthesis was also reduced by the same magnitude. However, there were no amino acids provided and this may have attenuated the effect of insulin (Flakoll *et al.* 1989).

For instance, even if the premature neonate is resistant to proteolysis, it does not necessarily follow that protein sparing (Denne *et al.* 1995) cannot occur as a result of non-protein energy source. Potentially, there may be an optimal proportion of carbohydrate to fat, consequently insulin concentration to minimize amino acid oxidation and support protein reutilization. This may occur at a lower insulin concentration (hence, lower proportion of non-protein energy as carbohydrate). This was not addressed in the aforementioned studies, but is conceivable based on a study in human neonates by

Pencharz *et al.* (1989), as a glucose plus lipid fuel system enhanced amino acid reutilization of amino acid for protein synthesis compared to an isocaloric glucose only regime. A decrease in the rate of *de novo* lipogenesis associated with decreasing carbohydrate intake in the neonate (Chawls *et al.* 2000, Jones *et al.* 1993, Bresson *et al.*1989, Pierro *et al.* 1989, Letton *et al.* 1995, Sauer *et al.* 1986) may explain this, as this may spare amino acids from oxidation (Bresson *et al.* 1991).

1.2.3. Effects of Insulin on *de novo* Lipogenesis in the Neonate

Insulin is a potent stimulator of fat synthesis in liver and adipocyte cells, particularly in adipocyte cells as it stimulates adipocyte glucose transport, hence promoting uptake of glucose substrate for lipogenesis (Hillgartner *et al.* 1995). Moreover, insulin establishes a biochemical milieu favouring lipogenesis (Geelen *et al.* 1978, Beyen *et al.* 1979, Witters *et al.* 1985, Gruppuso 1998, Hillgartner *et al.* 1995, Hunt *et al.* 1990). As, it increases the activity of the enzymes which favour lipogenesis (i.e. phosphofructokinase, pyruvate kinase, and triacylglycerol synthetase) while decreasing the activity of the enzymes required for gluconeogenesis or lypolysis (i.e. fructose 1, 6 diphosphatase, phosphoenol pyruvate carboxykinase, and triacylglycerol lipase). Furthermore, in addition to stimulating the above lipogenic enzymes, insulin stimulates the activity of acetyl-CoA carboxylase and fatty acid synthetase and the enzymes involved in the generation of NADPH required for fat synthesis.

Of concern is that, given that human neonates have consistently been shown to be vulnerable to high rates of *de novo* lipogenesis with increasing carbohydrate intakes (Chawls *et al.* 2000, Jones *et al.* 1993, Bresson *et al.* 1989, Pierro *et al.* 1989, Letton *et al.* 1995), and protein synthesis in neonatal piglets can reach maximum rates of protein synthesis at relatively low insulin concentrations (O'Connor *et al.* 2003) within the physiological range of the human neonate (Poindexter et al. 1998), increasing insulin (i.e. via increasing carbohydrate intake) may favour a high fat deposition relative to protein accretion in the neonate. However, this issue is still unclear.

1.3 Effects of Non-Protein Energy Source on Growth Composition in the Neonate

It is well established that provision of adequate protein and total energy is fundamental to optimizing growth (Micheli *et al.* 1993). However, the literature suggests that fat and carbohydrate may have differential effects on *de novo* lipogenesis (Bresson *et al.* 1989, Chwals *et al.* 2000, Jones *et al.* 1993, Letton *et al.* 1995, Pierro *et al.* 1989; Sauer *et al.* 1986) and protein metabolism (Bresson *et al.* 1991, Salas-Salvadõ *et al.* 1993, Nose *et al.* 1987, Chessex *et al.* 1989, Kashyap [1, 2] *et al.* 2001). Consequently, the proportion of carbohydrate and fat provided to preterm infants to meet energy requirements for growth may have different net effects on the composition of growth achieved (specifically, fat and lean mass accretion) (Nose *et al.* 1987, Kashyap[1,2] *et al.* 2001). Moreover, there may be a specific ratio of carbohydrate and fat to optimize growth quality (Kashyap[1] *et al.* 2001, Nose *et al.* 1987).

Studies in human neonates (Bresson *et al.* 1991, Salas-Salvadõ *et al.* 1993, Nose *et al.* 1987, Chessex *et al.*1989, Kashyap[1, 2] *et al.* 2001), and growing animals (Harstook *et al.* 1973) support this concept. However, data is conflicting, which may be related to differences in experimental design, duration, and/or methodology, statistical power, infant pathology/condition (i.e. septic vs. stable), age of subjects (i.e. preterm *vs.* more mature infants/children or adults), protein and/or energy intake (i.e. for maintenance vs. growth), proportion of non-protein energy as fat and carbohydrate, and/or feeding route (enteral *vs.* parenteral) (Bresson *et a*l. 1991, Kashyap[2] *et al.* 2001, Salas-Salvadõ *et al.* 1993). Furthermore, the longer term effects of energy source on growth quality (i.e. fat and lean mass accretion) remains unclear as longitudinal studies in neonates that include appropriate body composition techniques are limited and inconclusive.

For instance, while these studies consistently report an increase in *de novo* lipogenesis with an increase in carbohydrate intake and a decrease in *de novo* lipogenesis with an increase in fat intake (Bresson *et al.* 1989, Chwals *et al.* 2000, Jones *et al.* 1993, Letton *et al.* 1995, Pierro *et al.* 1989, Sauer *et al.* 1986), the longer term cumulative or net effect on fat mass accretion is less clear.

In contrast to *de novo* lipogenesis, the effects of different proportions of carbohydrate and fat on protein metabolism/balance are quite inconsistent. Some studies support higher (Bresson *et al.* 1991, Salas-Salvadõ *et al.* 1993, Nose *et al.* 1987), lower (Chessex *et al.*

1989, Kashyap [1, 2] *et al.* 2001), or no difference (Pencharz *et al.* 1989, Rubecz *et al.* 1981, Pereira *et al.* 1993, Van Aerde *et al.* 1988, Pineault *et al.* 1988) in nitrogen retention with an increase in the proportion of non-protein energy intake as fat. But as with lipogenesis, the longer term net effects on lean mass accretion remains unresolved.

Furthermore, most studies have been in parenterally fed subjects, with limited studies in the enterally fed neonate, and none in infants fed fortified breast milk that have specifically examined the effects of using different ratios of carbohydrate and fat in HMFs to meet energy requirements for growth.

The studies that have investigated the differential effects of varying proportions of carbohydrate and fat on 1) *de novo* lipogenesis, 2) protein metabolism/balance, and 3) longer term effects on growth composition are discussed below.

1.3.1. Studies Addressing *de novo* Lipogenesis in Human Neonates

Increasing intakes of carbohydrate have consistently been associated with increasing *de novo* lipogenesis in neonates, with a net lipogenesis occurring when the oxidative capacity for glucose is exceeded (Bresson *et al.* 1989, Pierro *et al.* 1989, Sauer *et al.* 1986, Chwals *et al.* 2000, Letton *et al.* 1995). Furthermore, increasing the proportion of energy intake as carbohydrate, decreases fat oxidation and increases energy expenditure (Bresson *et al.* 1989, Pierro *et al.* 1989). Conversely, isocaloric replacement of carbohydrate with fat has been shown to decrease *de novo* lipogenesis from carbohydrate, increase fat oxidation, and decrease energy expenditure (Bresson *et al.* 1989, Pierro *et al.* 1989). There are various examples of studies in neonates which support this.

For example, one study (Bresson *et al.* 1989), evaluated energy substrate utilization (using the combined method of indirect calorimetry and chemical balance techniques) in 36 stable and steadily growing infants after receiving Total Parenteral Nutrition (TPN) regimens for at least 6 days. Infants received similar protein intakes (2.7-2.8 g/kg/day) and energy intakes (104.7-107.7 kcal/kg/d non-protein energy), but had non protein energy intakes either based on glucose alone, or different proportions of glucose and fat (85% glucose / 15% fat, 65% glucose / 35% fat, 50% glucose / 50% fat, 30% glucose / 70% fat). Glucose intakes in excess of ~18 g/kg/day exceeded glucose oxidative capacity and resulted in a respiratory quotient (RQ) greater than one, which is indicative of net

lipogenesis (Bresson *et al.* 1989). In contrast, net fat oxidation was achieved in infants fed less than ~18 g/kg/day of glucose. Notably, this was observed despite similar protein and caloric intakes, hence suggesting an independent effect of the ratio of carbohydrate to fat energy on *de novo* lipogenesis.

Another study (Pierro *et al.* 1989), examined in two phases: 1) the effects of carbohydrate intake on fat utilization during fat free parenteral nutrition (10% glucose / 2% amino acid) over a 24 hour period and 2) the isocaloric and isovolemic replacement of the intravenous (IV) glucose/amino acid with IV 10% fat emulsion over a 4 hour period. A combination of indirect calorimetry, chemical balance and linear regression analysis techniques were used in 21 stable appropriate for gestational age newborns (aged 35 weeks until term). In phase 1, a significant negative correlation between carbohydrate intake and fat utilization was found. Fat oxidation decreased with increased carbohydrate intake, with net lipogenesis when carbohydrate intake exceeded 15 g/kg/day, the oxidative capacity of glucose. In contrast, during the fat infusion phase of the study, there was a significant and progressive decrease of carbon dioxide production, RQ, and carbohydrate utilization (oxidation plus conversion to fat via *de novo* lipogenesis), suggesting that net lipogenesis ended and fat utilization increased (Pierro *et al.* 1989).

A further study (Jones *et al.* 1993) investigated the maximum oxidative threshold for intravenous glucose in 11 stable preterm and term surgical newborns. Infants received total parenteral nutrition containing constant amounts of amino acids (2.5 g/kg/day) and fat (3.0 g/kg/day), but different amounts of glucose (range, 10-25g/kg/day). After 3 days on feeding regimens, substrate utilization was measured using the combined method of urinary nitrogen excretion and indirect calorimetry. The study reported that the maximum oxidative capacity for glucose was 18 g/kg/day, because when this quantity of glucose was reached an RQ >1 was observed indicating that net fat oxidation ceased and net fat synthesis occurred (Jones *et al.* 1993).

In addition, Sauer *et al.* (1986) studied 16 term and preterm infants using the combined methods of indirect calorimetry with a primed constant infusion of [U-^{13}C] glucose, in order to measure glucose utilization and glucose oxidation, respectively. Infants were measured after receiving 36-48 hours of TPN as follows (mean± SEM): energy (75.5± 4.02 kcal/kg), glucose (16.8± 0.84 g/kg/day), and protein (3.12± 0.25 g/kg/day). A

significant difference was found between glucose utilization and oxidation, indicating increased glucose storage as fat (*de novo* lipogenesis) with increasing glucose intake.

Other studies (Chwals *et al.* 2000, Letton *et al.* 1995) show that the septic or acutely stressed infant may be particularly susceptible to increased lipogenesis with higher carbohydrate intakes. An abstract (Chwals *et al.* 2000) compared 4 septic and non-septic premature infants. RQ was measured using indirect calorimetry and percent *de novo* lipogenesis of palmitate was quantified using continuous infusion of 1-^{13}C acetate. Infants were fed ~58% of energy intake as carbohydrate (~11.3-13.8 g/kg/day), energy intake was ~70-80 kcal/kg/day. The septic infants had RQ >1 and percent *de novo* lipogenesis was on average 1.5 fold greater than non-septic infants. Therefore, increased *de novo* lipogenesis was observed despite carbohydrate and energy intakes well within the ranges currently recommended for preterm infants (CPS 1995). Also, this effect was demonstrated by Letton *et al.* (1995) in 7 newborns during the acute postoperative period. Seven infants were fed protein (2.0-2.5 g/kg/day), fat (1-2 g/kg/day), and carbohydrate (10 g/kg/day). An average RQ=1.05 was observed despite carbohydrate intravenous infusion rates of only 10 g/kg/day.

Furthermore, increasing *de novo* lipogenesis suggests a deterioration in the efficiency of glucose as an energy substrate, particularly when glucose is given in excess of its maximum oxidative capacity (Jones *et al.* 1993). This increase in energy expenditure associated with high carbohydrate intake and *de novo* lipogenesis could be due to an increase in Diet induced Thermogenesis (DIT) (Jones *et al.* 1993), which may mean an increased energy cost for growth (Reichman *et al.* 1982). Significant increases in DIT have been associated with net lipogenesis in stable post-surgical newborns given >18 g/kg glucose parenterally, compared to infants given < 18 g/kg/day (DIT=0.054 kcal/kg glucose or 1.3% of gross energy when glucose is preferentially oxidized compared to DIT=1.201 kcal/g glucose or 30% of gross energy with net lipogenesis) (Jones *et al.* 1993).

In summary, this increase in energy expenditure associated with high glucose intakes may represent a considerable disadvantage (Jones *et al.* 1993) to a growing pre-term infant, so warrants consideration when prescribing carbohydrate intake. Conceivably, isocaloric replacement of carbohydrate with fat may increase energy available for growth

(Jones *et al.* 1993). In addition, the increase in *de novo* lipogenesis and corresponding increase in energy expenditure associated with increasing carbohydrate intakes may be of particular concern in the metabolically stressed infant who may be more susceptible to carbohydrate overfeeding because of the acute metabolic stress response and corresponding decrease in energy requirements due to growth inhibition and inactivity (Chawls *et al.* 2000, Letton *et al.* 1995).

1.3.2. Studies Addressing Protein Metabolism in Human Neonates

The source of energy substrate may affect protein metabolism/balance, yet how remains controversial. As, studies have supported higher (Bresson *et al.* 1991, Salas-Salvadõ *et al.* 1993, Nose *et al.* 1987), lower (Chessex *et al.*1989, Kashyap[1, 2] *et al.* 2001), or no difference (Pencharz *et al.* 1989, Rubecz *et a*l. 1981, Pereira *et al.*1993, Van Aerde *et al.* 1988, Pineault *et al.* 1988) in protein retention in neonates fed with an increased proportion of non-protein energy intake as fat compared to carbohydrate. These studies are addressed in sequence below.

1.3.2.i. Studies reporting higher protein retention with an increased proportion of non-protein energy intake as fat: Bresson *et al.* (1991) studied the relative effects of glucose and fat on whole body protein metabolism kinetics in seven stable parenterally fed non-septic infants (aged 1.5 months to 8 months of age who had been stable and growing on TPN for approximately 3 weeks) using primed constant L-[^{13}C]leucine infusion combined with indirect calorimetry. It was a randomized cross over design, in which each infant received two randomly assigned 8 day periods of isocaloric isonitrogenous parenteral nutrition differing only in energy source (protein intake (2.8 ±0.2 g/kg/day) and non-protein energy as either solely glucose (28.5±0.9 g/kg/day; 106.6±4 kcal/kg/day) or 50% glucose / 50% fat (glucose 13.9±1 g/kg/day; fat 5.5±0.4 g/kg/day; 108.6±6 kcal/kg/day). Net protein synthesis was significantly higher on the glucose-lipid mixture than on the glucose regimen, as despite no difference in protein synthesis rates between regimes, protein turnover, protein breakdown, and amino acid oxidation rates were significantly higher for the glucose than the glucose/ lipid regime. Overall, despite the strength of the randomized cross over design involving stable and growing infants, this

was a short study in older infants. Therefore findings cannot determine a longer term effect and may or may not be valid for the less mature preterm infant.

Another study (Salas-Salvadõ *et al.* 1993) investigated 26 stable, parenterally fed term and preterm infants randomized to receive one of three isocaloric isonitrogenous (96.1-98.7 kcal/kg/day, protein 2.15 g/kg/day) parenteral regimes with non-protein energy as either A (82% glucose / 18% fat), B (71% glucose / 29% fat), or C (60% glucose / 40% fat). After 5 days on the regimens, substrate utilization was measured using combined indirect calorimetry and urinary nitrogen excretion. The higher fat regimes (B and C) significantly reduced protein oxidation and enhanced protein retention compared to highest carbohydrate regime (A). But, this was also a short protocol, and whether the difference is sustained over time cannot be confirmed.

A further study (Nose *et al.* 1987) also compared three parenteral feeding regimes differing only in the proportion of carbohydrate and fat (each 8% of total energy as amino acid (~1.82±0.1 g/kg/day) and ~91±5 kcal/kg/day), but either with carbohydrate/fat energy respectively as A (87%/5%), B (60%/32%), or C (34%/58%) of total energy. It was a random crossover design in older infants and children (aged 2 months-9 years). After 3-5 days on the regimes, substrate utilization was measured using combined indirect calorimetry and urinary nitrogen excretion. The solution B had significantly higher nitrogen retention. This supports, not only a beneficial effect of fat on nitrogen retention, but that there may be a more specific proportion of carbohydrate and fat to optimize nitrogen retention. However, this study was in older infants and children and may or may not be valid to less mature pre-term infants, and as with the above studies the protocol was short and the cumulative effects on growth cannot be determined.

1.3.2.ii. Studies reporting lower protein retention with an increase proportion of non-protein energy intake as fat: One study (Kashyap[1] *et al.* 2001) randomized 63 preterm infants to receive one of five enteral formulas differing only in the proportion of carbohydrate and fat energy. Groups 1, 2, and control received 130 kcal/kg/day with 35%, 65%, and 50% of non-protein energy as carbohydrate. Groups 3 and 4 received energy intakes of 155 kcal/kg/day with 35% and 65% of non-protein energy as carbohydrate. All groups received 4 g/kg/day of protein. Growth and nitrogen balance studies were carried out bi-weekly using a 72 hour urine and stool collection and indirect

calorimetry. Greater rate of weight gain and nitrogen retention were observed in the infants fed the high carbohydrate formula compared the groups fed the low carbohydrate (i.e. higher fat) formulas. Another part to this study (published in a different paper by Kashyap[2] *et al.* 2001) investigated substrate oxidation using indirect calorimetry and urinary nitrogen techniques. Protein oxidation was less in the infants receiving the high carbohydrate formula than in the groups receiving the high fat formula. Worth mentioning is that the infants in the high fat groups had steatorrhea, therefore, the metabolizable energy was significantly lower in the high fat feeding groups. This confounds the comparison of the specific effect of energy source on protein balance or oxidation. Therefore, the fact that the protein intakes were similar, suggests that the higher nitrogen retentions observed in the high carbohydrate feeding groups were merely because of the greater metabolizable energy intakes in these groups, not because higher carbohydrate intake is more effective in promoting nitrogen retention or lowering protein oxidation than fat. Whether or not a high fat formula that was better absorbed would have a similar effect or improved nitrogen retention cannot be clarified by this study.

Another study (Chessex *et al.* 1989) evaluated the effects of changing from a high glucose to a high fat parenteral nutrition regime in 11 term and preterm infants over a two day period. Urinary nitrogen excretion was measured. Two isocaloric (70 kcal/kg/day), isoproteinic (2.5g/kg/day) regimes were compared which differed only in the source of energy. On day 1, infants received a high glucose (12-17 g/kg/day), low fat (0-1 g/kg/day) regime. On the second day of the study the infants received a low glucose (4-8g/kg/day), high-fat (2.5-3g/kg/day) regime. During the high fat regimen, urinary nitrogen excretion increased significantly which led to a decrease in nitrogen retention. However, it is speculated that the rapid change from a high carbohydrate to a high fat regime may have caused poor fat utilization due to immature lipid clearing enzymes in the neonate (Hamosh *et al.* 1986), as high triglycerides were observed on day 2 during the high fat regime. This may have reduced metabolically available energy, and may explain the decreased nitrogen sparing effect after the high fat regime. In fact, triglyceride values were higher compared to values obtained after a gradual increase in triglyceride that may allow for metabolic adaptation and better fat utilization (Pineault *et al.* 1988). Yet, fatty acid oxidation was not measured, so it is difficult to reconcile poor

fat utilization (Chessex *et al.* 1989). Furthermore, the protocol was short (2 days) and the effects observed may have been a transient adaptation effect, as similar results were found in early studies in non-stressed orally fed humans and rats (Munro 1964) and parenterally fed adults with inflammatory bowel disease (Jeejeebhoy *et al.* 1976) after substituting lipid for carbohydrate, which resolved after 3-5 days on a higher fat regimen. As well, a carry over effect from the high glucose regimes may have occurred due to the short protocol (2 days) (Chessex *et al.* 1989), which adds to the difficulty of interpreting an independent effect of treatment regimen.

1.3.2.iii. Studies reporting no difference in protein retention with different proportions of non-protein energy source as fat and carbohydrate: One study (Pereira *et al.* 1994) compared premature infants with Bronchopulmonary Dysplasia (BPD) (28±1 weeks GA at birth, 9±1 weeks postnatal age). It was a crossover design and infants were randomly assigned to alternately receive for one week one of two formulas with different proportions of non protein energy as carbohydrate and fat (75% fat / 25% fat *vs.* 42% fat / 58% carbohydrate). Nitrogen balance was measured after 3 days on the feeding regimes. Despite similar gross caloric (124±2 *vs.* 120±2 kcal/kg/day) and protein (3.35±0.05 *vs.* 3.24±0.05 g/kg/day) intakes during both diets, when the infants were fed the higher carbohydrate formula, they had significantly higher weight gains, yet no difference was observed in nitrogen balance. However, as with the study discussed above (Kashyap[1] *et al.* 2001), fat absorption was significantly lower in the infants on the high fat formula. Therefore metabolizable energy intakes were not isocaloric, and this again confounds the diet comparisons. So, this study does not rule out the possibility that if fat had been better absorbed that nitrogen retention may have been enhanced during the high fat formula feeding. In addition, the study was very in short (1 week on each regimen) and nitrogen balance was initiated after only 3 days on each feeding regimen, therefore potential differences in nitrogen balance may not have been detectable after the short protocol.

Another study (Pencharz *et al.* 1989) investigated the effects of non-protein energy source on protein retention in 20 parenterally fed stable term infants using three constant infusion, end product isotope methods: enrichment of urinary urea and ammonia in response to a [^{15}N]glycine label and exhaled carbon dioxide enrichment in response to a

[1-^{13}C]leucine label. Infants were allocated (no randomization) to receive total parenteral nutrition as glucose only (17.6±1.1 g/k/day) *vs.* glucose plus lipid (14.5±0.8 g/kg/day and 2.24±0.1 g/kg/day respectively). Both groups received the same protein intake (3.1±0.2 g/kg/day), and similar energy intakes 81.4±4.7 *vs.* 90.5±4.0 kcal/kg/day in the glucose only and glucose plus lipid group respectively. Measurements were performed after 2-4 days on full parenteral nutrition. No differences in nitrogen retention were seen between the two groups. This is in contrast to the results of a comparable study discussed above (Bresson *et al.* 1991) which showed an increase in nitrogen retention in the glucose lipid regime compared to the glucose only. This discrepancy may be related to differences in fat availability (as fat intake was lower), fat oxidation, and energy expenditure (as suggested by differences in RQ (Jones *et al.* 1993)) between the infants receiving the glucose plus lipid regimens in each study (Bresson *et al.* 1991). For example, the fat infusion rate in the study by Bresson *et al.* (1991) was higher than in this study (5.5 ±0.4 g/kg/day *vs.* 3.9±1 g/kg/day), while the glucose and protein intakes were more similar (13.9±0.8 g/kg/day *vs.* 14.5±0.8 g/kg/day and 2.8±0.2 g/kg/day *vs.* 3.1±0.2 g/kg/day). Consequently, fat oxidation was higher and RQ was lower in the neonates of the Bresson et al. (1991) study compared to this study (3.5±0.6 g/kg/day and RQ= 0.859±0.02 *vs.* 0.65±0.28 g/kg/day and RQ=0.959±0.018, respectively). This may have contributed to a higher net protein synthesis via lower amino acid oxidation, and higher energy availability for protein synthesis in the Bresson *et al.* (1991) report compared to this study (Bresson *et al.* 1991). Another reason for the discrepancy between the two studies may be the longer time on treatment regimens before measurements (7 days *vs.* 2-4 days) in the Bresson *et al.* (1991) study compared to this study, which may have allowed more time for metabolic adaptation (Jeejeebhoy *et al.* 1976) and/or growth. Furthermore, this study was not randomized, thus a subject allocation bias may have occurred.

Another study (Van Aerde *et al.* 1989) investigated the effect of replacing glucose with lipid on nitrogen retention in 28 post-operative, AGA parenterally fed newborns (term and slightly preterm (35.9–36.9±1 weeks GA), 12-33 days postnatal age) using indirect calorimetry and a primed constant infusion of [U-^{13}C] glucose. Infants were randomized to receive glucose only (18.2 ±0.6 g/kg/day) or glucose plus lipid (14.2 ±0.3 and 1.96 ±0.12 g/kg/day). Both groups (glucose and glucose plus lipid group, respectively)

received similar intakes of protein (3.53±0.09 vs. 3.22±0.12 g/kg/day) and energy (87.9±2.9 kcal/kg/day vs. 89.4±1.9 kcal/kg/day). Measurements were taken after at least 36 hours on the feeding regimens. Nitrogen balance was similar in both groups. This study (as was the case with study just discussed above by Pencharz *et al.* (1989) is also in contrast to the results of the comparable study discussed earlier by Bresson *et al.* (1991) that showed an increase in nitrogen retention in the glucose lipid regimen compared to the glucose only. As with the study by Pencharz *et al.* (1989), the discrepancy may also be related to differences in fat availability (1.96±0.12 g/kg/day *vs.* 5.5 ±0.4 g/kg/day), fat oxidation (1.77± 0.11 *vs.* 3.5±0.6g/kg/day), energy expenditure (RQ=0.960±0.008 *vs.* RQ= 0.859±0.02) and duration of treatment (7 days *vs.* ~36 hours) with insufficient time for metabolic adaptation to treatment regimens (Jeejeebhoy *et al.* 1976), between the infants receiving the glucose plus lipid regimens in this study compared to the Bresson *et al.* (1991) study, respectively. Another reason for discrepancy could be the differences in the clinical condition of the infants just prior to treatment. The infants in this study were a minimum of 3 days post surgery for abnormalities of the gastrointestinal tract or after diagnosis with necrotizing entercolitis or sepsis. Whereas, the infants in the Bresson *et al.* (1991) study were stable and growing on parenteral nutrition for at least 3 weeks before study commencement and any infants with infectious or inflammatory syndromes or congenital abnormalities were excluded from the study. Hence, given that inflammatory response can suppress anabolism, an existing inflammatory response or short time in the anabolic phase may have overridden or restricted any potential effect of nutritional regimen on protein synthesis in this study (Bresson *et* al. 1991, Clowes *et al.* 1993).

A study by Pineault *et al.* (1988) examined the effect on non-protein energy source on protein balance in 16 AGA stable preterm newborns (GA 34.6± 0.7 weeks, post natal age 10± 1 days) using nitrogen balance techniques. In a latin square crossover design (in which each infant served as his/her own control), each subject received two 6 day periods of isocaloric (one period of 60 kcal/kg/day, the other 80 kcal/kg/day) and isonitrogenous (2.81 g/kg/day) parenteral regimens, differing only in the source of non-protein energy: high fat (3 g/kg/day) or low fat (1 g/kg/day). No differences in nitrogen balance were observed between regimens. A possible explanation for the findings could have been because protein and energy intakes were below those recommended for pre-term infants

(CPS 1995) and may have restricted any potential effect of regimen on protein accretion (O'Connor *et al.* 2003, Thureen *et al.* 2003, Micheli *et al.* 1991).

As well, an earlier study (Rubecz *et al.* 1981) compared the effect of non-protein energy source on protein balance in 10 postoperative infants (preterm and term (35-40 weeks GA), post natal age 2-60 days) using urine nitrogen balance techniques. Infants were fed isocaloric (55.2±2.5 and 56.2±1.7 kcal/kg/day), isonitrogenous (1.70±0.07–1.77±0.06 g/kg/day of amino acids) parenteral nutrition, differing only in non-protein energy source: glucose only (12.21±0.55 g/kg/day) or lipid only (4.12±0.13 g/kg/day). The glucose plus amino acid regimen was given first for 24 hours and followed by the fat plus amino acid regime for 24 hours. No differences were seen in nitrogen balance between the two regimens. This is not surprising given the extremely short duration that each regimen was fed (24 hours) and possible carry over effect of the glucose regimen on the fat regimen. Furthermore, as with the study just discussed above (Pineault *et al.* 1988), nutrient intake was low compared to recommended requirements for preterm infants (CPS 1995), therefore would limit growth (O'Connor *et al.* 2003, Thureen *et al.* 2003; Micheli *et al.* 1991). As well, these infants were 3 days postoperative and no information was provided in the study as to their clinical condition, particularly if or how long the inflammatory response to stress was resolved. Given that the inflammatory stress response attenuates protein synthesis (Bresson *et al.* 1991; Clowes *et al.* 1993), if unresolved it may have blunted any potential effect of nutritional regimen on protein synthesis and growth in this study.

1.3.3. Long Term Effect of Non-Protein Energy Source on Lean and Fat Mass

There is a paucity of data on the longer term net effect of non-protein energy source on growth composition in the preterm neonate. The aforementioned papers primarily studied the effects of non-protein regimes using parenteral feeding regimes, which may not be applicable to the enterally fed neonate. Furthermore, nitrogen balance or stable isotope techniques were used to study protein metabolism, so provide only a picture in time of what is metabolically occurring and does not measure the longer term cumulative effects of non-protein energy source on growth composition. There are only three relatively longer studies (Kashyap[1] *et al.* 2001, Pereira *et al.* 1994, Fomon 1976) (28-56, 7, or 8-111

days respectively) which have investigated the effects of non-protein energy on growth in the enterally fed neonate, and only two of these (Kashyap[1] *et al.* 2001, Pereira *et al.* 1994) have measured growth composition. But, still the longer term effects of energy source on growth quality are still unclear.

For example, in one study (described above in relation to protein metabolism) (Kashyap[1, 2] *et al.* 2001), higher nitrogen retention, skin fold thickness (1.70± 0.47 *vs.* 0.93±0.38 mm/week), and weight gain were observed in the infants fed the high carbohydrate formulas compared the groups fed the low carbohydrate (i.e. higher fat) formulas. However, as mentioned above, the infants in the high fat group had steatorrhea. Therefore, the metabolizable energy was significantly higher in the high carbohydrate feeding groups. This confounds the comparison of the specific effect of energy source on protein and fat balance. Therefore, the fact that the protein intakes were similar, suggests that the higher nitrogen and fat retentions observed in the high carbohydrate feeding groups may merely be because of the greater metabolizable energy intakes for growth in these groups, not necessarily because higher carbohydrate intakes promotes higher nitrogen and fat retention than higher fat intakes. Whether a high fat formula, that was better absorbed, would have had a similar effect or not remains unknown. As well, the high carbohydrate formula had higher fat deposition, but the anthropometric measurements of skin fold methods to measure body composition may lack precision and accuracy depending on the skill of the clinician doing measurements (Davies 1993, Lapillonne *et al.* 1999). Conversely, if the measurements were accurate, it does raise the concern that high carbohydrate intakes may promote high fat accretion in the preterm infant (Kashyap[1] *et al.* 2001). But, whether fat deposition would have been similar or different in infants fed a higher fat formula (i.e. and they had had better fat absorption) is also unclear.

Another study (Pereira *et al.* 1994) (also described above) compared two groups of premature infants, with BPD, fed formulas with different proportions of carbohydrate and fat. Despite similar gross caloric intakes, when the infants were fed the higher carbohydrate formula, there was no difference in nitrogen retention, yet they had significantly higher arm fat areas and weight gains and than when they were fed the lower carbohydrate formula. Yet, unlike the study just discussed above, no differences

were observed in nitrogen balance or skin fold measurements (despite higher arm fat areas reported in the high carbohydrate group) between feeding regimens. This supports the lack of precision and possibly accuracy of skin fold and arm fat to measure body composition, as they were not consistent with each other. As well, although suggestive of higher body fat gains, these measures may not necessarily reflect whole body fat composition, as body fat may not be uniformly distributed (Davies 1993, Lapillonne *et al.* 1999), but does raise the concern that high carbohydrate intakes may promote higher fat deposition (Pereira *et al.* 1994). Furthermore, as in the study of Kashyap[1] *et al.* (2000) low fat absorption was reported in the infants on the high fat formula, therefore metabolizable intakes were not isocaloric. Again, this confounds the diet comparisons and does not rule out the possibility that, if fat had been better absorbed, that enhanced nitrogen retention and/ or arm fat areas may have been observed during the high fat formula feeding as well. Furthermore, the study was very short duration (1 week on each regimen) and nitrogen balance was initiated after only 3 days on each feeding regimen, therefore differences in nitrogen balance may not have been evident after the short protocol. As well, a crossover design and a crossover affect may have occurred. Clearly, longer studies that involve more comprehensive body composition techniques are indicated to confirm results.

Lastly, an earlier study (Fomon, 1976) involved two groups of 15 male infants fed isocaloric diets made of infant formula and some strained foods which differed only in the percentage of non-protein energy as carbohydrate and fat (29% fat and 62% carbohydrate versus 57% fat and 34% carbohydrate). This was a longer study (term infants were followed from 8-111 days of age). But, body composition was not studied. Only weight and length were measured and there were no differences found between feeding regimes. Growth indices alone are not very informative of growth composition (Lapillonne *et al.* 1999). Hence, potential differential effects of non-protein energy source on body composition cannot be ruled out.

1.4 Differences in HMFs, Comparison to P-RNI, and Related Issues

Many clinically utilized HMF (past and present) are (have been) quite different in composition **(Table 1)**. The most recent HMFs available internationally (last six in Table 1) consist of varying amounts of energy, protein, minerals (i.e. calcium), and vitamins (Schanler[1] *et al.* 1999). Notably, most HMFs contain predominantly carbohydrate as the energy source, with little if any fat. Only one HMF Enfamil® (3rd) contains fat as the predominant source of energy. Another distinct difference among the HMFs includes the source of calcium used (soluble or insoluble). The differences in the contents of HMFs in comparison to the P-RNI and related issues will be discussed below.

TABLE 1.
HMF FORMULATIONS - COMPARISON OF SELECTED NUTRIENTS

Added per 100 ml milk)	**Enfamil** ® Mead Johnson (1st) 1	**Enfamil** ® Mead Johnson (2nd) 1	**Enfamil** ® Mead Johnson (3rd) 2	**SMA®** Wyeth 3	**Similac Natural Care®** Ross 4	**Similac** ®Ross 5	**Eo-Protein** ®Milupa 6	**FM 85®** Nestle 6
Energy (Kcal)	14	14	14	13	4	14	13	18
Protein (g)	0.7	0.7	1.1	1.0	0.2	1.0	0.8	0.9
Carbo-hydrate (g)	2.7	2.7	1.1	2.0	0.5	1.8	2.4	3.6
Fat (g)	0	0	0.65	0.05	0.18	0.36	0	0
Ca (mg)	60 in-soluble	90 soluble	90 (31.5 soluble, 58.5 in-soluble)	80 soluble	70	117 in-soluble	50	51

1. Schanler *et al.* 1995; 2. Mead Johnson Nutritionals 2000; 3. Porcelli *et al.* 2000;
4. Sankaran *et al.* 1996; 5. Ross. 2001; 6. Schanler[1] *et al.* 1999

1.4.1. Differences in the Energy Content of HMFs: Energy supplementation of breast milk is indicated for the pre-term newborn as it cannot always provide adequate energy for growth, particularly when a premature infant is fluid restricted. An energy intake of 105-135 kcal/kg/day is recommended (CPS 1995). However, preterm breast milk provides ~73 kcal/100ml (CPS 1995). Therefore, at typical intakes of unfortified breast milk of 140-160 ml/kg/day energy intake would be in the lower recommended range (~102-117 kcal/kg/day). If the infant is fluid restricted energy needs may not be met.

However, currently available HMFs vary in the supplemental energy they provide (**Table 1**) (Schanler 2001). Most would meet the P-RNI for energy at intakes of 140-160 ml/kg. However, FM85® (Nestle) may exceed energy requirements at higher intakes (i.e. 160 m/kg or 147 kcal/kg/day) as this level of intake is associated with high fat deposition (Putet *et al.* 1984, Reichman *et al.* 1981) in formula fed infants. In contrast, Similac Natural Care® (SNC, **Table 1**) (Schanler[1] *et al.* 1999), may provide sub-optimal energy intakes during lower intakes (i.e. 140 ml/kg/day or 108 kcal/kg/day).

1.4.2. Differences in the Non-Protein Energy Sources Used in HMFs: The use of protein as a source of supplemental energy is inappropriate given the possible adverse effects associated with higher intakes (>5g/k/d), such as metabolic acidosis, elevated blood urea concentrations, or adverse neuro-developmental outcomes (Goldman *et al.* 1974). Therefore, carbohydrate and fat are used in varying amounts as the non-protein energy source in HMFs (Kuschel *et al.* 1998, Schanler[1] *et al.* 1999).

All the HMF preparations (**Table 1**) contain carbohydrate (Schanler[1] *et al.* 1999), and all, except the Enfamil® (3rd), contain predominantly or solely carbohydrate as the non-protein energy source (i.e. ~1.8-3.6 g carbohydrate combined with breast milk to provide a total carbohydrate of 9.3-10.6 g/100ml fortified breast milk). The carbohydrate sources commonly used are glucose polymers alone or combined with small amounts of lactose (Schanler 2001, Kuschel[1] *et al.* 2000). In contrast, fat is rarely included as a component of HMFs currently available internationally. When fat is present, it is mostly in trace quantities, thus carbohydrate is the predominant non-protein energy source (Schanler[1] *et al.* 1999, Kuschel[2] *et al.* 2000). In fact, only one HMF currently available in Canada (Enfamil® HMF) (Mead Johnson 2000), contains fat as the predominant source of non-

protein energy. The fat source is a fatty acid blend of monounsaturated and polyunsaturated fatty acids (as linoleic and linolenic acid), and saturated medium and long chain fatty acids (Mead Johnson 2000).

There is insufficient evidence on which to make specific recommendations for the optimal proportion of carbohydrate and fat to include in HMFs for preterm infants (Kuschel[1, 2] *et al.* 2000). Yet, as discussed earlier, it is conceivable that the proportion of carbohydrate and/or fat may have effects on the growth composition achieved. Also important to consider is that the non-protein energy source used in HMFs may have other nutritional and metabolic effects.

1.4.2.i. Potential Effects of Energy Source contained in HMFs on Growth Composition, Energy Expenditure, and Respiratory Quotient: There have been no studies specifically comparing the effects of non-protein energy source in the context of HMFs, but high rates of lipogenesis, and associated increase in RQ and energy expenditure may be possible in pre-term infants fed breast milk fortified with HMFs containing non-protein energy predominantly as carbohydrate. For example, fortifying breast milk with commercially available (Schanler[1] *et al.* 1999) high carbohydrate HMFs will provide a total carbohydrate intake of 9.3-11.1 g/100ml fortified breast milk (1.8-3.6 g of carbohydrate from HMF plus ~7.5 g of carbohydrate per 100 ml of expressed breast milk). Thus, typical fortified milk intake volumes of 140-180 ml/kg/day would result in a total carbohydrate intake of 13.02-19.98 g/kg/day. This is comparable to the published values of ~15.4–18 g/kg/day in stable infants (Pierro *et al.* 1989, Bresson *et al.* 1989, Jones *et al.* 1993) and 10 and 13.8 g/kg/day in septic and post-operative infants (Letton et al. 1995, Chwals *et al.* 2000) shown to approach or exceed the oxidative capacity of glucose and be associated with high rates of *de novo* lipogenesis, increases in RQ and energy expenditure. Isocaloric replacement of the carbohydrate contained in HMFs with fat may decrease this affect, but to our knowledge, this has not been studied.

1.4.2.ii. Potential for the Carbohydrate and Fat Content in HMFs to Affect Osmolarity and Feeding tolerance: The addition of glucose polymers to human milk is associated with increases in osmolarity (De Curtis *et al.* 1999). This increase is larger than would be expected from the composition of the glucose polymers alone and may be a result of the hydrolysis of dextrins by human milk amylase (De Curtis *et al.* 1999). High osmotic

loads have been associated with diarrhea, feeding intolerance, and necrotizing entercolitis (NEC) in some infants (Kuschel[1] *et al.* 2000). Therefore, the potential for carbohydrate to increase osmolarity and contribute to osmolarity related feeding intolerance is important to consider when using HMFs containing high amounts of carbohydrate. But currently used HMFs containing higher amounts of carbohydrate (i.e. Similac® and SMA® HMFs) appear to be well tolerated (Barrett Reis *et al.* 2000, Porcelli *et al.* 2000). In contrast, addition of fat does not contribute as much to osmolarity (Clark *et al.* 1997), hence the potential for osmolarity related feeding intolerance, as carbohydrate (De Curtis *et al.* 1999). However, the effect of HMFs that contain higher amounts of fat (i.e. Enfamil, 3rd formulation) on gastrointestinal tolerance has not been studied.

1.4.2.iii. Potential Effects of Fat Contained in HMFs on Fat Availability for Energy and Polyuunsaturated Fatty Acid (PUFA) Status: Most HMFs contain carbohydrate as the sole or predominant non-protein energy source. Only one HMF (Enfamil® HMF) (Mead Johnson 2000) contains fat as the predominant energy source. However, inclusion of fat in HMFs seems supportable and may increase fat availability, as the process involved in fortification of mothers' milk may decrease fat availability. For instance, when infants are fed fortified human milk, mothers must express their milk, the milk is then fortified. Infants are then fed initially by orogastric tube and progressed to gavage and/or by bottle. The milk expression itself may introduce variability in the fat content of human milk due to timing and duration of milk expression, and there can be losses of fat due to adherence of fat to feeding equipment (Sankaran *et al.* 1996, Barrett Reis *et al.* 2000). Fat bioavailability may also be decreased by using HMFs containing 100% soluble calcium salts (Schanler 2001, Schanler[3] *et al.* 1999) because fortification of human milk with 100% soluble calcium (as calcium gluconate/calcium glycerophosphate) has been associated with a 50% reduction in human milk free fatty acids (Schanler[3] *et al.* 1999) and high fecal fat excretion (Schanler 1995). It is speculated that human milk fat undergoes saponification with the soluble calcium salts (Schanler[3] *et al.* 1999). So, fortification of human milk with 100% soluble may decrease the bioavailability, therefore metabolizable energy provided by human milk (Schanler *et al.* 1995, Schanler[2] *et al.* 1999, Schanler[3] *et al.* 1999).

Regarding the issue of the potential for saponification of human milk fat with soluble calcium, the mechanisms involved or whether or not there is an effect on the fatty acid profile of human milk have not been studied. Furthermore, this may or may not be an issue with HMFs containing lower amounts of soluble calcium. For instance, currently available HMFs in Canada which contain soluble calcium **(Table 1)** contain smaller amounts of soluble calcium. For example, the Enfamil® (3rd formulation) contains 31.5 mg or 35% of total calcium content as soluble calcium (Mead Johnson 2000) compared to the earlier formulation of Enfamil® (2nd formulation) which was associated with increased fecal fat excretion (Schanler *et al.* 1995) which contained 90 mg or 100% as soluble calcium (Schanler *et al.* 1995, Mead Johnson 2000).

Also important to consider is how the PUFA content of HMFs could affect the PUFA status of the preterm infant. Of the two most common HMFs used in Canada (Enfamil® and Similac® HMFs), only Enfamil® contains PUFAs (as linoleic and linolenic acid) (Mead Johnson 2000). However, the amounts present are low (90 mg linoleic acid and 11 mg of linolenic acid), so given that human milk already contain PUFAs (Heird 2001, Innis 1993), fortification changes the PUFA profile very little (Mead Johnson 2000). So, the PUFA content of human milk fortified with either Enfamil® or Similac® HMFs appears to be similar (Mead Johnson 2000). There is no direct evidence to suggest that enterally fed preterm infants would be deficient in these fatty acids if fed their mothers milk, despite the variability in the PUFAs in human milk due to differences in maternal diet and maternal fat/infant metabolism (Heird 2001). However, we speculate that there is potential for differences in the PUFA content of human breast milk fortified with these fortifiers, if soluble calcium created substantial losses of human milk fat or PUFAs. But, to our knowledge how soluble calcium affects the fatty acid profile using currently available HMFs that contain soluble calcium has not been studied.

1.4.3. Differences in Quantity of Protein Contained in HMFs: The current HMFs contain protein **(Table 1)**. The protein source used in HMFs is derived from modified bovine milk protein (Health Canada 1995, Mead Johnson 2000, Ross 2001, Boehm *et al* 1993). In Canada, the whey to casein ratio is 60:40 (Health Canada 1995, Mead Johnson 2000, Ross 2001) which more closely resembles that of human milk (Moro *et al.* 1989,

Pencharz *et al.* 1983). Whey dominant bovine protein sources are not considered ideal because of they contain high levels of threonine which can lead to high plasma threonine levels in infants (Rigo *et al.* 1980), so research is required to develop more suitable protein sources (Health Canada 1995).

The protein contents of the HMFs vary. However, there is consistent evidence to support the use of HMFs containing protein to allow a total intake (including the protein content of human milk of ~ 1.7 g/100ml) of 3-4g/kg/day (Schanler 2001, CPS 1995, Kuschel *et al.* 1999). This range of protein intake has been associated with improvements in linear growth and protein accretion and has not been shown to result in any metabolic disturbances (Schanler 2001, CPS 1995). But, higher intakes (>5g/kg/day) have been associated with metabolic acidosis, elevated blood urea concentration and adverse neurodevelopmental outcomes (Goldman *et al.* 1974). Currently available HMFs meet this recommended range at typical fortified milk intakes of 140-160 ml/kg/day, with the exception of Similac Natural Care® (SNC) **(Table 1)**.

However, there is a distinct interaction between protein and energy intakes (Micheli *et al.* 1991), therefore protein and energy cannot be considered independently. Energy intake in the range of 105-135 kcal/kg/day with the currently recommended protein intakes (3-4 g/kg/day) is associated with the highest protein gains (Micheli *et al.* 1991). However, given the potential effects of non-protein energy source on protein kinetics and accretion, there may be a ratio of carbohydrate to fat that may optimize protein retention (Nose *et al.* 1987).

1.4.4. Other Issues Related to HMFs

1.4.4.i. Differences in Calcium and Phosphorus Quantity and Source Used in HMFs: Exclusive feeding of unfortified human milk to very low birth weight infants is associated with poor bone mineralization, osteopenia and increased frequency of rickets and fractures early in life (Koo *et al.* 1985). So, fortification of human milk with calcium and phosphorus is advocated (CPS 1995, AAP 1997). Calcium and phosphorus are typically provided in their soluble (as calcium gluconate/calcium glycerophosphate) or insoluble forms (as calcium phosphate tribasic/calcium carbonate), or as a blend of both (Mead Johnson 2000, Ross 2001). Both of these sources appear to promote similar bone

mineralization (Chan *et al.* 2000). Calcium and phosphorus intakes in the range of 160-240 mg/kg/day and 77.5-117.8 mg/kg/day, with molar ratio of 1.6-2.0 of calcium to phosphorus, are recommended to achieve mineral retention approximating intrauterine rates (CPS 1995, Schanler[1] *et al.* 1988, Schanler *et al.* 1995). Fortification with HMFs commonly used in Canada meet these goals.

As discussed, it is speculated that when 100% soluble calcium (as calcium gluconate/calcium glycerophosphate) is used as the calcium source in HMFs, the soluble calcium may saponify with human milk fatty acids during fortification and lower the metabolizable energy available from fat in the fortified breast milk. (Schanler *et al.* 1988, Schanler[3] *et al.* 1999). However, the specific nature of this reaction is still unclear, but it should be considered when evaluating HMFs containing soluble calcium as it may impact energy bioavailability.

1.4.4.ii. Use of Iron in HMFs: Although the growing pre-term infant requires iron in excess of that provided by breast milk (Health Canada 1995, CPS 1995), few HMFs contain it. If they do the quantities vary, for example, there is a trace amount of iron contained in Similac® HMF (0.35 g to add per 100ml of breast milk), yet Enfamil® HMF (3rd formulation) contains 1.44 g (to add per 100ml of breast milk), which is similar to the iron content of iron fortified preterm formula (Ross 2001). This inconsistency may be explained by the controversy that exists over the optimal dosage and appropriate time to initiate supplemental iron. It is difficult to set a specific dosage for iron supplementation as there may be variability in infants' requirements. For example, infants with lower birth weights (<1000g) may have higher requirements than larger neonates (CPS 1995, Siimes *et al.* 1982). Furthermore, some infants may have higher iron needs than others because of blood loss (CPS 1995) that could not be replaced by erythrocyte transfusion (Health Canada 1995). Another reason for the inconsistent use of iron in HMFs could also be because of evidence to support the safety and effectiveness of iron supplementation (i.e. in preventing later iron deficiency) at two different time points: as early as 2 weeks post-natal age (Lundström *et al.* 1977, Melnick *et al.* 1988) and slightly later at 6-8 weeks post-natal age (Siimes *et al.* 1982). Therefore, attempts to provide the appropriate amount of additional iron by fortifying human milk would be difficult, not only because of the marked variability in infants' requirements, but also because of differences in opinions

regarding the best time to supplement. Therefore, it has become preferable to supplement iron separately, using liquid elemental iron, as it allows for more individualized iron supplementation compared to a set dosage of iron in a HMF (Health Canada 1995). However, it is considered acceptable by Health Canada (1995) to include a modest iron supplement in a HMF, but it is considered unnecessary, as additional supplements may be required. The currently accepted recommended intake for iron ranges from 2 mg/kg/day – 4 mg/kg/day. (CPS 1995, Siimes *et al.* 1982, Lundström *et al.* 1977, Melnick *et al.* 1988, Jansson *et al.* 1979). Furthermore, although the Canadian Pediatric Society (1995) recommends starting iron supplementation at 6-8 weeks post-natal age based on infant birth weight, given the evidence to support safe and effective supplementation at 2 weeks post natal age (Lundström *et al.* 1977, Melnick *et al.* 1988), some neonatal units have adopted this approach as standard practice.

1.4.4.iii. Vitamins and Other Minerals (besides calcium, phosphorus and iron as discussed above) Contained in HMFs: Unfortified human milk contains insufficient quantities of various vitamins and minerals to meet the P-RNI (CPS 1995). The two commonly used HMFs in Canada (Enfamil® and Similac® HMFs) contain vitamins and minerals and the P-RNI's (CPS 1995) are met after fortification at typical fortified milk intake volumes of 140-180ml/kg/day. An exception is vitamin D, as the P-RNI is not quite met for smaller infants (i.e. ≤1.5 kg) consuming lower volumes of fortified breast milk (i.e. ≤ 145 ml/kg), therefore, additional vitamin D may be necessary for some infants (CPS 1995). The lower and upper limits of tolerance for vitamins and minerals are not exceeded (Health Canada 1995) after fortification with either HMF.

1.5 Review of Clinical Studies Comparing the Effects of Different HMFs on Growth

Few studies exist that have compared the effects of different HMFs on growth, only two studied growth composition. These studies are discussed below.

Study 1 - Enfamil® HMF (EHMF[1], 1st powder formulation, no longer available) *vs.* Enfamil® HMF (EHMF[2], 2nd powder formulation, no longer available) (Schanler *et al.* 1995): This was a cohort study in 26 AGA (born ~28 weeks GA). Two cohorts were compared, one fed mother's milk fortified with the EHMF[1] and the other fortified with

the EHMF[2] (both from Mead Johnson Nutritionals, **Table 1**). Both HMFs were similar in all nutrients except for the type of calcium. EHMF[1] contained 100% insoluble calcium (as calcium phosphate tribasic/calcium carbonate). In contrast, EHMF[2] contained 100% soluble calcium (as calcium gluconate/calcium glycerophosphate). Infants were studied over 40 days and a nutrient balance study was conducted once during this period, bone mineral content of the radius was measured at the start and end of the study using single photon absorptiometry, and growth indices were measured throughout the study period. There was no difference observed in growth or percent calcium absorption between HMFs. However, the cohort fed the EHMF[2] (which contained 100% soluble calcium) had higher net calcium absorption and retention. Yet, this cohort of infants also required significantly more fortified milk volume to achieve the same growth. It follows that higher milk volume means increased nutrient intake including calcium. Thus, given that the percent calcium absorption between HMFs was similar, the increased calcium intake may have explained the higher net absorption and retention observed. Notably, the EHMF[2] cohort also had higher fecal fat excretion and fecal fat was significantly correlated with fecal calcium excretion ($r=0.68$, $p<0.001$). Given that this group of infants received only human milk fortified with soluble calcium, suggests that the soluble calcium may be interacting with the human milk fat (Schanler 2001, Schanler *et al.* 1995). As mentioned, it is speculated that soluble calcium salts, in contrast to insoluble calcium (Schanler[2] *et al.* 1988), may undergo saponification with human milk fatty acids and decrease human milk fat bio-availability (Schanler[3] et al. 1999). This may explain why the higher milk intakes were required in EHMF[2] cohort of infants in this study to reach the same growth rates. But, this is still speculation, as the mechanism has not been thoroughly studied. Yet, it should still be considered when evaluating HMF formulations containing soluble calcium.

Study 2 - Enfamil® HMF (EHMF[2], 2nd powder formulation, no longer available) *vs.* Similac Natural Care® (SNC) (Sankaran *et al.* 1996): This was a two center randomized clinical trial in premature infants (mean 30±0.3 weeks GA) fed either with the liquid HMF SNC (Ross Laboratories) mixed 1:1 with human milk or fed human milk fortified with the powder formulation EHMF[2] (Mead Johnson) (both HMFs in **Table 1**). Study

duration was unclear, but appeared to range from 10 days until 3 weeks. 60 infants were enrolled, however data was available for only 41 infants.

There were no significant differences in growth indices (weight, length, or head circumference). The lack of difference in growth is surprising since the HMFs were quite different in nutrient content, as since the SNC is mixed 1:1 with human milk, this dilutes the nutrient density of the fortified milk, particularly energy and protein. For example, the SNC provided only an additional 4 kcal and 0.2 grams of protein per 100 ml of breast milk, whereas the EHMF[2] provided an additional 14 kcal and 0.7 grams of protein per 100 ml of breast milk. However, it was the same EHMF[2] compared above that contained 100% soluble calcium that was associated with increased fecal fat excretion (Schanler *et al.* 1995). So, this may be an the reason for the similar growth observed between the HMFs. Fortification of breast milk with the 100% soluble calcium EHMF[2] may have resulted in a lower metabolizable energy intake and possibly may have resulted in fortified breast milk that was closer in energy content to that fortified with the SNC (Schanler[2] *et al.* 1999, Schanler[3] *et al.* 1999, Schanler *et al.* 1995).

However, even though no difference in growth was observed, the possibility of a difference in growth composition between the HMFs cannot be ruled out, as: 1) no body composition studies were performed to compare quality of weight gained associated with each fortifier; 2) the study was short and whether the same effects would have been observed over a longer time frame is unclear; 3) the data was only available for 41 of the 60 infants enrolled, so there may have been a loss of statistical power, so a difference was not detectable. Furthermore, there is a potential sample distortion bias as the reasons for incomplete data were not given.

Study 3 - Enfamil® HMF (EHMF[2], 2nd formulation, no longer available) *vs.* SMA® HMF (Porcelli *et al.* 2000): This was a randomized multi-center clinical trial which also evaluated EHMF[2]. However, this time it was compared to the HMF, SMA® (Wyeth Nutritionals). There were two groups of AGA premature infants (N=90) enrolled at 30 weeks GA and followed for 3 weeks. It was an intent to treat analysis; however, of the 90 infants enrolled, results were reported for only the 64 infants that strictly adhered to the protocol. Both HMFs were isocaloric, but the SMA HMF contained more protein, a trace amount of fat and less carbohydrate and soluble calcium than the EHMF[2] (**Table 1**, per

100 ml fortified milk: 1.0 g *vs.* 0.7 g protein, 0.05 g *vs.* 0 g fat, 2 g *vs.* 2.7g carbohydrate, and 80g *vs.* 90 grams soluble calcium).

The infants in EHMF^2 group received higher milk volumes than the SMA group (154 vs. 144 ml/kg/day), yet the SMA group demonstrated significantly higher rates of body weight gain and head circumference than the EHMF^2 fed infants. Yet, no differences in length were observed between groups.

This study raises a question regarding the quality of weight gain observed in the SMA group, as despite higher gains in weight and head circumference, there was no corresponding increase in length. This suggests an increase in Ponderal Index (an index of adiposity used in infants calculated as weight (g)/length (cm)^3 x 100 (Yau *et al.* 1992, Miller *et al.* 1971)), meaning that the SMA group of infants may have gained more body fat. So, if body fat gain is exceeding the goal of in-utero rates (CPS 1995), the SMA HMF may not be an optimal choice of HMF. The 100% soluble calcium content in the SMA HMF may have affected energy bioavailability from milk fat. As mentioned, in studies 1 and 2 above, it is speculated that 100% soluble calcium may undergo saponification with human milk fat, therefore may decrease the bioavailability of fat derived energy (Schanler^2 *et al.* 1999, Schanler^3 *et al.* 1999, Schanler *et al.* 1995).

However, the lower growth observed in the EHMF^2 compared to the SMA HMF, despite higher volumes of intake, may be explained by a lower protein intake or the presence of a higher amount of 100% soluble calcium in the EHMF^2 compared to the SMA (90 mg vs. 80 mg). In fact, greater fat absorption has been reported in studies evaluating HMFs containing lower quantities of calcium (Boehm *et al.* 1993, Schanler *et al.* 1997). Thus, the higher 100% soluble calcium content in the EHMF^2 may contribute to a lower metabolizable energy (Schanler *et al.* 1995) intake compared to the SMA HMF.

Study 4 - Enfamil® HMF (EHMF^2, 2nd formulation, no longer available) *vs.* Similac®HMF (SHMF) (Barrett Reis *et al.* 2000): Another study also evaluated the EHMF^2 formulation, but this time compared it to Similac® HMF (SHMF) (Ross 2000). It was a randomized trial in 119 AGA premature infants studied for 29 days.

Both HMFs were isocaloric, however the SHMF (like the SMA HMF discussed above) was higher in protein and contained a small amount of fat (thus, non-protein energy was also carbohydrate predominant) and contained less carbohydrate than the EHMF^2 (**Table**

1, per 100 ml fortified milk: 1.0 g protein *vs.* 0.7 g, 0.36 g fat *vs.* 0 g, 1.8 g carbohydrate *vs.* 2.7 g). In contrast, the SHMF contained insoluble calcium salts instead of soluble calcium salts contained in the EHMF^2. Both groups consumed similar fortified breast milk volumes (~150 ml/kg/day)

Infants fed the SHMF had significantly greater rates of weight gain, head circumference and length compared to infants receiving similar milk volumes fortified with EHMF^2 (Porcelli *et al.* 2000). However, despite the higher rates of growth indices observed in the SHMF group, the growth composition achieved may not be meeting in-utero goals of growth composition.

For example, closer analysis of the growth data for the SHMF group using standardized intra-uterine growth charts (Usher *et al.* 1969), suggests that growth may not have been as good as it first appears. This observation is based on the goal for growth in preterm infants, which is to attain and sustain a similar growth rate pattern for all growth indices (weight, length, and head circumference) (CPS 1995). For example, even though weight gain for infants in the SHMF group appears to be approaching the 25th percentile, length gain appears to be growing below the 3rd percentile. This suggests a substantial increase in Ponderal Index (an index of obesity used in infants, weight (g)/length (cm)^3 x 100 (Yau *et al.* 1992, Miller *et al.* 1971) from baseline in the SHMF group. Although fat accretion occurs during this neonatal period (Widdowson 1972), it does raise the question that fat accretion in the SHMF may be exceeding the goal of in-utero rates of fat accretion. As well, the lower increase in length compared to weight suggests stunting.

Also, as observed in the above studies involving the EHMF^2, the lower growth in the EHMF^2 may be due to less metabolizable energy in the EHMF^2 (potentially secondary to its 100% soluble calcium content (Schanler *et al.* 1995)) compared to the SHMF, lower protein content, or due to it having solely carbohydrate as its non-protein energy source. So due to confounding, we cannot determine the independent effects of these variables based on this study. Overall, neither HMF appears to meet current growth goals. Evaluation of body composition may have helped to clarify the quality of growth observed.

Study 5 - Enfamil® HMF (EHMF^2, 2nd formulation, no longer available) *vs.* Similac®HMF (SHMF) (Chan *et al.* 2000): An abstract comparing the same two HMFs as

above assessed body composition using the dual-energy x-ray absorption (DXA) method (although the study resembles the SHMF *vs.* EHMF[2] study discussed above it is unclear whether it is the same study as data was only reported for 43 infants). There were no observed differences in fat mass between groups. By day 15 of the study, the SHMF group was reported to have a higher lean mass than the EHMF[2] group. However, it is unclear whether this was still observed by the end of the 28 day study as these results were not specified. Moreover, the precision and accuracy of the DXA method has been questioned as it can be affected by infant size and hydration (Brunton *et al.* 1997, Butte *et al.* 1999, Picaud *et al.* 1996). Improvements in instrumentation and application of correction equations have improved estimations of body composition (Butte *et al.* 1999, Picaud *et al.* 1996) but are mainly applicable to older and larger infants, not smaller low birth weight preterm infants. However, the type of DXA instrumentation used and whether correction equations were applied during the study were not specified. Therefore, interpretation of the body composition measurements in this study should be made with caution. As well, even if the SHMF group had a higher lean mass than the EHMF[2] group, this does not mean that the SHMF optimizes growth composition.

Study 6 – HMF (designed specifically for study and not available) *vs.* fortification with soluble calcium only (Wauben *et al.* 1998):

This study compared 25 AGA preterm infants randomized to receive their mothers' milk Fortified with either the HMF specifically designed for the study (designed HMF, which added per 100 ml of EBM: 0.37 g protein, 3.47 g carbohydrate, 0 g fat, multivitamins, and minerals including soluble 100% soluble calcium glycerophosphate) or soluble calcium only (as 100% calcium glycerophosphate) for approximately 5 weeks until they reached term corrected age. Weight and length were measured weekly and body composition was measured at term corrected age using DXA. By term corrected age, the designed HMF group had significantly higher weight and length gain compared to the group receiving only fortification with soluble calcium, but there was no difference in the percent body composition as both had a mean 21% fat mass. So, the growth composition achieved by the designed HMF group may still not be meeting our goal (CPS 1995) of attaining growth composition similar to that in-utero, because compared to body composition published for infants born at term (~16% fat mass, Atkinson *et al.* 1994),

they were approximately 30% fatter. But, fat mass may have been overestimated as DXA can overestimate fat mass in small infants (Brunton *et al.* 1997). But excess fat accretion may have occurred because comparing the growth data to intrauterine growth standards (Usher *et al.* 1969), the infants had a higher rate of weight gain compared to length as their mean weight grew between the 25th and 50th percentile but their mean length was below the 25th percentile, which suggests stunting and an increase in fat mass (Miller *et al.* 1971). Overall, it is difficult to reconcile whether this growth was secondary to a low metabolizable energy intake (i.e. because of a low fat bioavailability due to its soluble calcium content (as explained above, Schanler *et al.* 1995), sub-optimal protein intake (as the HMF only added 0.35 g protein per 100 ml EBM), or because it contained predominantly carbohydrate as a non-protein energy.

Main Conclusions from HMF Studies:

1) 100% soluble calcium contained in HMFs may affect human milk fat bioavailability, and lower metabolizable energy intake (Schanler *et al.* 1995, Schanler[2] *et al.* 1999, Schanler[3] *et al.* 1999). There have been no HMF studies comparing the effects of HMFs containing lower amounts of soluble calcium to HMFs containing 100% insoluble calcium on fat bioavailability.

2) HMFs containing higher protein were associated with increased growth.

3) All the HMFs (except the mineral HMF in Study 6) studied contained non-protein energy predominantly as carbohydrate.

4) HMFs containing non-protein energy predominantly as carbohydrate may promote high fat mass deposition relative to lean mass. None of the above studies specifically compared the effects of using different proportions of carbohydrate and fat in HMFs on growth composition. For example, the studies above that did compare HMFs containing trace amounts of fat (i.e. SMA or SHMF), with a HMF containing solely carbohydrate as the energy source (EHMF[2]), the potential differences in metabolizable energy intake (Schanler *et al.* 1995, Schanler[2] *et al.* 1999, Schanler[3] *et al.* 1999) and differences in protein intake after fortification confounds the interpretation of a specific effect of non-protein energy source on growth. As well, the studies did not evaluate growth composition at all, or the two studies that did evaluate body composition, used the DXA method which can overestimate fat mass in small infants (Brunton *et al.* 1997).

1.6 Epidemiological Evidence to Support Concern for Excess Postnatal Fat Accretion in Low Birth Weight Infants

So why should we be concerned with excess postnatal fat accretion in low birth weight infants? This is of interest as infant or early age obesity increases risk of later age obesity (Parsons *et al.* 1999, Whitaker *et al.* 1997, Zack *et al.* 1979). Furthermore, obesity, particularly centrally distributed or abdominal obesity, is a well established independent risk factor for cardiovascular disease (Hubert *et al.* 1983, Rexrode *et al.* 1997), diabetes (Kahn *et al.* 1971, Larsson *et al.* 1981), and hypertension (Stamler *et al.* 1978, Dyer *et al.* 1989, Després *et al.* 1990). Paradoxically, low birth weight is associated with: 1) an increased risk of obesity in childhood (Walker *et al.* 2002) and adulthood (Schroeder et al. 1999, Law *et al.* 1992, Fall *et al.* 1995), particularly associated with stunting and with centrally distributed obesity; 2) cardiovascular disease in adulthood (Barker 1995, Godfrey *et al.* 2000); 3) type 2 diabetes in childhood (Wei *et al.* 2003) and adulthood (Barker *et al.* 1992, Lucas *et al.* 1999, Curhan[2] *et al.* 1996); and 4) hypertension in early childhood (Law *et al.* 1996) and adulthood (Barker *et al.* 1992, Law *et al.* 1996, Curhan[1, 2] *et al.* 1996).

These associations may be of fetal origins (Fetal Origin's or Barker Hypothesis), for example poor fetal nutrition may metabolically program a propensity to disease in later life (Barker *et al.* 1992, Lucas *et al.* 1999). Alternatively, it may be an interaction of low birth weight and nutrition and growth later in the postnatal period, or postnatal nutrition may have an independent effect (Post-Natal Hypothesis) (Lucas *et al.* 1999). The critical point or period of time and mechanisms involved are still unresolved (Lucas *et al.* 1999). However, given this knowledge, it seems prudent to take a preventative approach and prevent low birth weight infants from becoming obese and it warrants investigation into the impact of current feeding regimes, such as human milk fortification on growth composition and health outcome.

1.7 Technologies Available to Measure Body Composition in Neonates

There is no standard body composition method for use in preterm infants (Lafeber 1999, Roubenoff *et al.* 1993). Studies in neonates have mostly relied only growth indices to evaluate the effect of nutritional regimes. However, growth indices alone cannot quantify growth composition, so body composition techniques are needed (Lapillonne *et al.* 1999). Certain criteria are essential and must be considered before choosing a body composition technique appropriate for carrying out research involving premature neonates. These criteria include: validated for use in preterm infants with sufficient accuracy and precision to address the research question, non-invasive, safe (Lafeber 1999, Davies 1993) and practical for use in the neonatal unit setting, as well as technology that is accessible to the researcher. The technique found to satisfy the above criteria for our research was Bioelectric Impedance Analysis (BIA). The basis for BIA and the other body composition techniques reviewed are discussed below.

Bioelectric Impedance Analysis (BIA): This method is based on electrical theory (Nyboer *et al.* 1943). Water can conduct electricity within the body. Lean mass contains a high percentage of water, therefore conducts electricity. In contrast, fat is anhydrous and has a low electrical conductivity and high impedance relative to water. An advantage of BIA is that it is a rapid method (taking seconds to minutes to perform), non-invasive and portable, so can be performed even in the most fragile infant who cannot be mobilized or while infants are in incubators/isolettes. It involves distal placement of four electrodes on the skin of the infant. Then a portable BIA apparatus is used to measure body impedance by passing a source current (i.e. 200 Micro Amps, root mean square) between the surface electrodes and detector electrodes on the BIA apparatus (Raghavan *et al.* 1998, Tang *et al.* 1997, Mayfield *et al.* 1991). Total body water (TBW) can then be measured using previously validated regression equations for preterm infants (Tang *et al.* 1997, Mayfield *et al.* 1991, Raghavan *et al.* 1998). For example, using weight, foot-length and impedance measured a 50 kHz, 99.5% of variation in TBW was accounted for when compared to TBW measured using $H_2{}^{18}O$ dilution (Tang *et al.* 1997). TBW measured by BIA can then be used as a two compartment model to estimate lean mass (LM) and fat mass (FM) (Wells *et al.* 1998).

However, when using TBW to determine lean mass, it is important to consider that pre-term infants are susceptible to variations in intra-cellular water and extra-cellular water (ECW) compartments, particularly ECW (Tang *et al.* 1993, Mayfield *et al.* 1991, Bauer *et al.* 1991). For example, a physiological fluid redistribution occurs in the first few days of life (Bauer *et al.* 1991). Furthermore, depending on the clinical condition of the infant (i.e. sepsis, infection, respiratory insufficiency, and/or diuretic use) expansion or contraction of ECW can occur (Tang *et al.* 1993). Therefore, the potential for abnormalities in ECW volume need to be considered when measuring TBW, as including abnormalities could result in inaccurate calculation of LM based solely on TBW. BIA technology has the advantage that it can also measure ECW using the reactance component of impedance (as Impedance = $(\text{resistance}^2 + \text{reactance}^2)^{0.5}$ (Mayfield *et al.* 1991). Mayfield *et al.* (1991) hypothesized, that given that bioelectrical reactance is a measure of the effect of an insulating medium in an electrical field and that cell membranes have high lipid content, reactance may be correlated with fat free cell mass and ECW. They then compared ECW measured using bromide dilution and reactance at a frequency of 50 kHz in low birth weight infants and found reactance to be a good index of ECW, as a regression model including reactance, body weight and surface area as factors gave a good correlation (r=0.882). However, not all BIA units measure both resistance and reactance components of impedance separately, thus measure only total impedance.

Other techniques:

Stable isotope dilution methods to measure TBW: Stable isotopes of either hydrogen (deuterium or ^2H_2O) or oxygen ($H_2{}^{18}O$) can be used to measure total body water (Wolfe 1992, Bodamer *et al.* 2001). It is based on the Fick's principle, which states that the volume of a fluid space may be calculated after the administration of a marker into that space if the exact amount of the marker administered and its concentration in the fluid is known. So the stable isotope is administered to the infant and the body fluid volume is sampled over time using either blood or urine to determine maximum enrichment of the isotope (equivalent to TBW) using regression techniques. Lean mass and then fat mass can be calculated as a two compartment model (Wells *et al.* 1998). The major limitation of this method is the potential for abnormalities in ECW in preterm infants (Tang *et al.*

1993, Mayfield *et al.* 1991, Bauer *et al.* 1991). This should be considered when measuring TBW, as including abnormalities would result in inaccurate calculation of lean mass based solely on TBW. Other disadvantages are that it requires the use of isotopes, which can be expensive (particularly $H_2{}^{18}O$) and instrumentation methods such as isotope ratio mass spectrometry to measure isotopic enrichment, which may not be accessible. However, the technique is non-invasive and has been used with relatively good accuracy to measure TBW in premature infants (Tang *et al.* 1993, Jones *et al.* 1987).

DXA: This is an imaging approach involving dual photon absorptiometry. Briefly, it is based on the energy dependence of the attenuation coefficients for photon absorption of bone mineral, which contains the high atomic number element calcium, and soft tissue, which contains mostly the low atomic number elements hydrogen, oxygen, and carbon (Roubenoff *et al.* 1993). Analytical software programs designed for specific subject populations are then used to calculate total bone mineral content and total body soft tissue which is then compartmentalized into total body fat and total body lean mass (Brunton *et al.* 1997). However, the precision and accuracy of the DXA method for use in low birth weight preterm infants have been questioned, as both can be affected by infant size and hydration (Brunton *et al.* 1997, Butte *et al.* 1999, Picaud *et al.* 1996). For example, the precision and accuracy of DXA for body sizes comparable to infants has been evaluated using piglets as models for preterm and term infants (Brunton *et al.* 1997, Butte *et al.* 1999, Picaud *et al.* 1996). Poor precision of 16-20% (coefficient of variation in percent) in measurements of fat gains less than 500 g have been reported (Brunton *et al.* 1997, Picaud *et al.*1996). As well, fat mass was overestimated by 119% in small (1.58 kg) piglets and 29% in the large (5.89 kg) piglets compared with carcass analysis (Brunton *et al.* 1997). As, small errors in lean mass can result in quite large errors in the measurements of fat mass (Roubenoff *et al.* 1993, Brunton *et al.* 1997, Butte *et al.* 1999). Estimation of lean mass is complicated by the fluctuations in total body water, particularly ECW, and changes in percent lean mass as water for GA observed in early infancy (Tang *et al.* 1997, Bauer *et al.* 1991, Widdowson 1972) which the DXA method does not directly measure, as it assumes a uniform hydration factor (Roubenoff *et al.* 1993, Brunton *et al.* 1997, Butte *et al.* 1999). Improvements in instrumentation and application of correction equations have improved estimations of body composition

(Butte *et al.* 1999, Picaud *et al.* 1996) but are mainly applicable to older and larger infants, not smaller low birth weight preterm infants. Furthermore, this equipment is quite expensive, therefore not readily accessible. As well, it is not suitable for more fragile infants as it requires that infants be removed from isolettes and incubators for scanning.

Total Body Electrical Conductivity (TOBEC): This method is based on the property of lean tissue to conduct electricity. It involves placing the subject in a hollow cylinder containing an oscillating current. The perturbation of the electromagnetic field by the subject is an index of electrical conductivity (Forbes 1999). This is a two compartment model (Lapillonne *et al.* 1999). Lean mass is calculated based on validated regression equations using the TOBEC index of electrical conductivity and fat mass can be determined by subtracting lean mass from total body mass (Butte *et al.* 1998). This method has been validated for healthy term infants (deBruin[1] *et al.* 1995, Fiorotto *et al.* 1995), but not in preterm infants (Lapillonne *et al.*1999, Lafeber 1999). Also, it requires that infants be removed from incubators for measurement and is an expensive technology (Lapillonne *et al.* 1999).

Anthropometrics (Skin Fold Thickness)

This method is based upon the assumptions that the thickness of subcutaneous adipose tissue reflects a constant proportion of total body fat and that the sites selected for measurement represent the average thickness of the subcutaneous adipose tissue (Lapillonne *et al.* 1999). Skin fold measurements are used to predict total body fat based on data from post mortem chemical analysis of fetuses. An advantage of this method is that it utilizes a relatively simple and inexpensive technology of skin fold calipers to measure skin fold thickness. Major disadvantages are: 1) that the method is susceptible to inter-observer variation (de Bruin[2] et al. 1995); 2) it is difficult to measure in preterm infants (Lapillonne *et al.*1999), which may be due to the vulnerability of preterm infants to variations in hydration and skin fragility; 3) site skin-fold measurements may not reflect total body fat and the post mortem fetal data used to predict total body fat may not be accurate for live born preterm infants (Dauncey *et al.* 1977).

1.8 Summary and Rationale

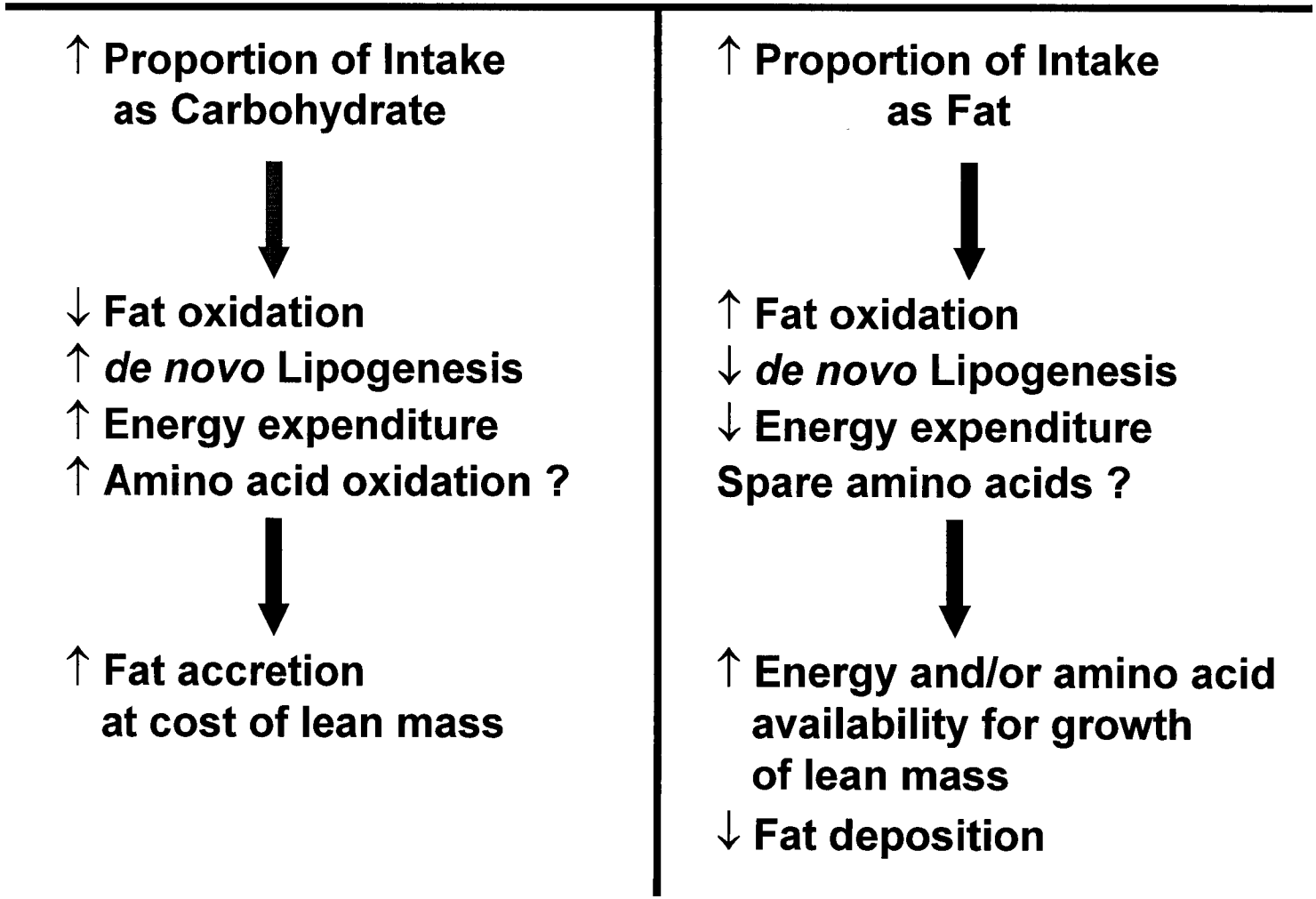
Although postnatal fat accretion is a normal part of neonatal development (Widdowson 1972) and a desirable goal for pre-term infants (CPS 1995), the potential for high fat accretion (exceeding in-utero rates of fat relative to lean mass accretion) is conceivable during high carbohydrate intakes (Kashyap[1] *et al* 2001, Pereira *et al.* 1994, Barrett Reis *et al.* 2000, Wauben *et al.* 1997). The mechanisms are not resolved.

This may be explained by a vulnerability of the neonate to *de novo* lipogenesis and increases in energy expenditure with increasing carbohydrate intake (Letton *et al.* 1995, Pierro *et al.* 1993, Bresson *et al.* 1989). For example **(Figure 1)**, increasing the proportion of non-protein energy as carbohydrate intake, is associated with decreased fat oxidation, and an increase in *de novo* lipogenesis, and an increase in energy expenditure in the neonate (Letton *et al.* 1995, Pierro *et al.* 1989, Bresson *et al.* 1989). This may promote high fat deposition (Kashyap[1] *et al* 2001) and/or decrease energy availability for growth processes, particularly lean mass accretion (Nose *et al.* 1987, Salas-Salvadõ *et al.* 1993). As, it follows that increasing the proportion of non-protein energy intake as carbohydrate would limit exogenous fat availability as an alternative non- protein energy source. So, available amino acids may be oxidized for energy (Bresson *et al.* 1991), which may limit amino acid availability, therefore limit the capacity of insulin to promote protein synthesis (O'Connor *et al.* 2003), and consequently, restrict protein accretion and growth, and/or favour fat deposition relative to lean mass accretion.

Alternatively **(Figure 1)**, increasing the proportion of non-protein energy intake as fat and reducing the proportion of carbohydrate intake can increase fat oxidation, decrease lipogenesis, and decrease energy expenditure (Letton *et al.* 1995, Pierro *et al.* 1989, Bresson *et al.* 1989), thus may increase exogenous energy source availability (i.e. from fat via fat oxidation), spare protein by decreasing protein oxidation (Bresson *et al.* 1991), hence increase energy and/or protein availability for protein accretion, and/or decrease fat deposition.

This hypothesis has not been adequately tested and to our knowledge has never been tested in the context of HMFs, so the proportion of carbohydrate and fat to include in HMFs optimize growth quality in the preterm infant is not known.

Figure 1. Research Rationale



Therefore, our research objective was to begin to address this hypothesis by comparing the effect of fortification of human milk using HMFs that contain either a fat predominant or carbohydrate predominant non-protein energy source on the body composition and growth of preterm low birth weight infants.

Measuring body composition overtime using the method of BIA will allow us to quantify growth composition and enable us to evaluate the impact of HMFs with two different non-protein energy sources on growth quality.

1.9 Statement of Purpose

Increasing the proportion of non-protein energy source as fat may promote better protein accretion and growth and more closely meet in-utero goals of growth composition.

We hypothesized that feeding preterm infants, human milk fortified with a HMF containing non-protein energy predominantly as fat would result in 1) a higher lean mass accretion (as percent lean mass) and 2) improved growth, compared to feeding human milk fortified with an isocaloric, isonitrogenous HMF containing non-protein energy predominantly as carbohydrate.

To determine the effect of the study HMF fortification regimens on these outcomes, our **specific aims** were:

AIM 1. To measure body composition, specifically as percent total lean and fat mass

AIM 2. To measure growth, specifically as total body weight, length, knee-heel length, and head circumference

AIM 1 was our primary outcome; AIM 2 was our secondary outcome.

CHAPTER 2. MATERIALS AND METHODS

2.1 Subjects and Recruitment

The Neonatal Intensive Care Unit (NICU) of the Royal Victoria Hospital (RVH) of the McGill University Health Center (MUHC) is a Level III, 26 bed unit admitting approximately 400 neonates annually (85% premature, 30-40 out born). The main reason for admission is prematurity.

Each infant admitted to the RVH NICU from January 12, 2002 until February 12, 2003 was screened by the investigator within 24-48 hours of birth for study eligibility according to inclusion and exclusion criteria for study entry defined *a priori*, which are listed in **Table 2**. The NICU admission list and medical records were used to obtain information to determine eligibility.

TABLE 2. INCLUSION AND EXCLUSION CRITERIA FOR STUDY ENTRY

INCLUSION CRITERIA	EXCLUSION CRITERIA
►Parental choice to feed mother's milk ►Gestational age (GA) at birth ≤ 32 weeks ►Appropriate for gestational age (AGA) (defined as birth weight between the 3rd and 97th percentile for gestation, according to intrauterine growth standards (Usher *et al.* 1969) ►Singleton, twin, or triplet infants	►Parental choice to feed exclusively formula ►GA at birth >32 weeks. ►Inappropriate for gestational age (defined as birth weight < 3rd or > 97th percentile for gestation, according to intrauterine growth standards (Usher *et al.* 1969) ►Major metabolic problems, congenital malformations, those who underwent or were scheduled for major surgery at time of recruitment

Consent: Subjects were recruited primarily by the investigators; however a staff physician assisted with the recruitment of four infants. Parents of all eligible infants were approached for informed written consent within 72 hours of birth. The study purpose and protocol were explained to the parents. All parents signed consent forms prior to enrollment of their infant into the study and received a copy of the protocol.

Medical Care: The medical care of all subjects was the sole responsibility of the medical treatment team under the direction of the primary attending physician(s). This included the decision of when to initiate or stop parenteral and/or enteral nutrition. The

investigators only had control of the human milk fortifier (HMF) as blindly randomized during the fortification of breast milk. All Infants were nursed in incubators/isolettes.

2.2 Experimental Design

2.2.1 Randomization

A double-blind randomized, controlled trial was conducted over a 15 month period from January 2002 until April 2003 in the Neonatal Intensive Care Unit (NICU), Royal Victoria Hospital (RVH), Montréal, Quebec.

Once it was confirmed that the mother of each enrolled infant had started to supply breast milk, infants were blindly and randomly assigned to one of two HMF groups **(Figure 2)**.

Group 1 - fatHMF: to receive their own mother's expressed breast milk (EBM) fortified with a HMF containing non-protein energy predominantly as fat (fatHMF) (Enfamil® HMF, Mead Johnson Nutritionals, Ottawa, Ontario)

Group 2 - carbHMF: to receive their own mother's EBM fortified with a HMF containing non-protein energy predominantly as carbohydrate (carbHMF) (Similac® HMF, Ross Laboratories, Saint Laurent, Quebec).

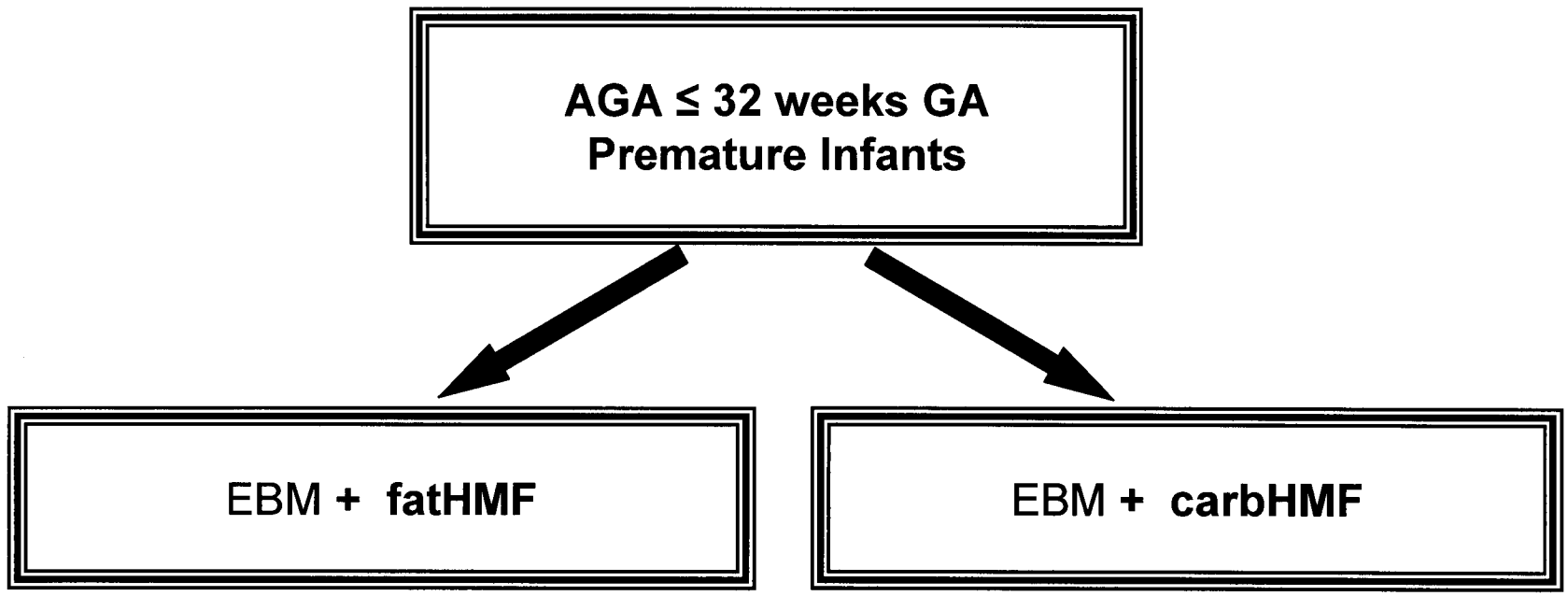
A placebo/control group was not possible as unfortified EBM is not recommended for preterm low birth weight infants (CPS 1995).

2.2.2. Assignment and Blinding

Assignment was prepared using computer generated random numbers, by a medical doctor with expertise in research methodology who was not otherwise involved with this study. The physician was instructed by the investigators of this study to generate 48 numbers (to allow for a total potential sample size of 48, based on a 50% padding of the calculated sample size (see Statistical Analysis) to account for anticipated protocol deviations or losses to follow-up in this high risk subject population). For each number generated, if the last digit was even, the assignment would be to the fatHMF, or if odd, the assignment would be to the carbHMF. The identity of each assignment was written on a card placed in an opaque envelope and sealed. A subject number, corresponding to the numerical order of the randomly generated number, was written on both the assignment

Figure 2. Study Design

Double-Blind, Randomized Clinical Trial



card and envelope. As each infant was randomized, the investigator provided the sealed assignment envelope in numerical order to the pharmacist on service, who then opened and signed each envelope, kept the assignment card and returned the empty envelope to the investigator for study records. A record of the code was kept by and the identity of the code was only known by the physician who prepared the random assignment and the RVH NICU pharmacy. The study was double-blinded, in that the investigators, members of the medical team in direct care of subjects, the subjects and their parents were blinded as to the identity of the code. The code was broken in April 2003 after the last subject completed the protocol.

To assure blinding, repackaging of the HMFs was necessary. Both HMFs were pale yellow powders of similar consistency, but packaging was different (for example, the fatHMF was in a cylindrical shaped sachet and the carbHMF was in a square shaped sachet). So, both HMFs were dispensed by the NICU pharmacy in identical sterile vials labeled with only the infant's name and study number, and the number of HMF sachets per vial.

Compliance Monitoring: Vials containing HMF were kept for both monitoring purposes and to be washed and sterilized for reuse. Compliance with fortification procedures and study feeding regimens were monitored daily by the investigators by checking the quantity of empty vials and sachets used (as indicated on the vials dispensed by the pharmacy for each infant) against the volume of EBM fortified and consumed (as recorded in each subjects chart by the NICU nursing staff). Any deviations from the allocated HMFs were recorded for each subject by the investigators and were defined as other intake during the calculation of nutrient intake.

2.3 Study HMFs

The fatHMF (Enfamil® HMF) is the most recent powdered HMF formulation from Mead Johnson Nutritionals (Ottawa, Ontario) and the carbHMF (Similac® HMF) is the powdered HMF from Ross Products (Saint Laurent, Quebec). The fatHMF (Enfamil® HMF) was donated by Mead Johnson Nutritionals (Ottawa, Ontario). The carbHMF (Similac® HMF) was the routine HMF in use in the RVH NICU at the time of our study. Both HMFs are used clinically and are based on the best of current knowledge.

Table 3 indicates the nutrient content of each study HMF to be added to 100 ml of expressed breast milk (EBM) (Mead Johnson 2000, Ross 2001). Both study HMFs are isocaloric and isonitrogenous but differ in the proportion of non-protein energy as fat or carbohydrate. The fatHMF contains non-protein energy predominantly as fat (58%). The carbHMF contains non-protein energy predominantly as carbohydrate (69%). The micronutrient contents of the study HMFs are similar, but not identical. But, as shown using **Table 4** and **Table 5**, after fortification all nutrients generally meet the P-RNI at the typical intake volumes of 140 ml/kg/day to 180 ml/kg/day and do not exceed the recommended upper or lower tolerable limits as published by Health Canada (1995). Exceptions are: 1) Vitamin D, which for both HMFs intakes are slightly lower than the P-RNI; and 2) iron for the carbHMF. However, these intake values do not fall beneath the lower limit recommended by Health Canada (1995) of 100 IU/ kcal for Vitamin D and 0.15 mg /100 kcal. As well, because nutrient requirements for these nutrients can vary, it is recommended that intakes of these nutrients be individualized (CPS 1995, Health Canada 1995). Also, the calcium sources used in each HMF are different, the fatHMF (Enfamil® HMF) contains 35% soluble and 65% insoluble calcium, the carbHMF (Similac® HMF) contains 100% insoluble. As mentioned, it is speculated that the 100% soluble calcium in HMFs may undergo saponification with human milk fat and lower energy bioavailability (Schanler[3] *et al.*1999, Schanler 2001). But, this has not been directly studied and may not apply to HMFs, like the fatHMF (Enfamil® HMF) that contain lower quantities of soluble calcium.

Standard Mixing of HMFs with EBM: EBM was fortified by the NICU nursing staff according to the manufacturer's directions and using a 24 hour standardized mixing protocol as follows:

1) frozen EBM was thawed in a tepid water bath (~21°C for 30-40 minutes)

2) an EBM volume sufficient for each subject for 24 hours (rounded to the nearest 25 ml) was pooled in one bottle and 1 sachet of HMF was added per 25 ml of EBM.

If the entire 24 hour volume of milk was not available, the available EBM was fortified, and the remaining volume required was prepared as soon as parents brought EBM and it was frozen (part of the routine infection control policy). All fortified EBM was refrigerated at ~4°C and discarded after 24 hours.

TABLE 3. COMPOSITION OF STUDY HMFS ADDED TO 100 ML OF EBM

Nutrient	Unit	fatHMF (Enfamil®) per 3.24g (4 sachets)	carbHMF (Similac®) per 3.60g (4 sachets)
Energy	kcal	14	14
Protein (whey:casein - 60:40)	g	1.1	1.0
Fat (% non-protein energy)	g	0.65 (58%)	0.36 (31%)
Linoleic Acid	mg	90	0
Linolenic Acid	mg	11	0
Carbohydrate (% non-protein energy)	g	1.1 (42%)	1.8 (69%)
Vitamin A	IU	950	620
Vitamin D	IU	150	120
Vitamin E	IU	4.6	3.2
Vitamin K1	µg	4.4	8.3
Thiamin	µg	150	233
Riboflavin	µg	220	417
Vitamin B6	µg	115	211
Vitamin B12	mcg	0.18	0.64
Niacin	mg	3	3.57
Folic Acid	µg	25	23
Pantothenic Acid	mg	0.73	1.5
Biotin	µg	2.7	26
Vitamin C	mg	12	25
Total Calcium -Soluble (Ca gluconate/ Ca glycerophosphate) -Insoluble (Ca phosphate tribasic/ Ca carbonate)	mg (% total)	90 31.5 (35%) 58.5 (65%)	117 -- 117 (100%)
Phosphorous	mg	45	67
Magnesium	mg	1	7
Iron	mg	1.44	0.35
Zinc	mg	0.72	1
Manganese	µg	10	7.2
Chromium	nmol	-	-
Copper	µg	44	170
Sodium	mg	11	15
Potassium	mg	20	63
Chloride	mg	9	38
Incremental Osmolality	mOsm/kg H20	63	90

TABLE 4. APPROXIMATE NUTRIENT COMPOSITION PER 100 ML OF EBM AND EBM FORTIFIED WITH STUDY HMFS

Nutrient	Unit	*EBM	**fatHMF (Enfamil®) plus EBM	**carbHMF (Similac®) plus EBM
Energy	kcal	73	85	85
Protein	g	1.7	2.75	2.65
Fat	g	4	4.55	4.27
Linoleic Acid (18:2n-6)	g	0.452	0.532	0.443
Linolenic Acid (18:3n-3)	g	0.044	0.055	0.044
Carbohydrate	g	7.5	8.43	9.1
Carbohydrate: Fat Ratio	g ratio	1.88	1.85	2.13
Vitamin A	IU	277	1203	879
Vitamin D	IU	40	186	157
Vitamin E	IU	0.675	5.12	4.46
Vitamin K1	µg	0.21	4.5	8.3
Thiamin	µg	---	147	228
Riboflavin	µg	40	255	448
Vitamin B6	µg	---	113	207
Vitamin B12	µg	---	0.18	0.63
Niacin	mg	---	2.9	3.5
Folic Acid	µg	3.3	27.9	25.8
Pantothenic Acid	mg	---	0.72	1.5
Biotin	µg	---	2.6	25.5
Vitamin C	mg	7.5	19.1	31.9
Calcium	mg	30	118	144
Phosphorous	mg	14	57.8	79.4
Magnesium	mg	2.92	3.84	9.73
Iron	mg	0.03	1.44	0.37
Zinc	mg	0.41	1.1	1.4
Manganese	µg	---	9.8	7.1
Chromium	nmol	***1.0-1.9/day	***1.0-1.9/day	***1.0-1.9/day
Copper	µg	59.7	102	225
Sodium	mg	25.3	35.6	39.5
Potassium	mg	55.6	74	116
Chloride	mg	39.1	47.2	75.6
Selenium	µg	2.4	2.4	2.4
Iodine	µg	16	16	16

* CPS 1995, ** corrected for 2% volume displacement, *** based on term milk

TABLE 5. INTAKE OF EBM FORTIFIED WITH STUDY HMFS AT VOLUMES OF 140-180 ML/KG BODY WEIGHT/DAY COMPARED TO PRETERM INFANT RECOMMENDED NUTRIENT INTAKE (P-RNI)

Nutrient	Unit	*P-RNI (per kg body weight unless otherwise noted)	fatHMF (Enfamil®) plus EBM (per kg body weight)	carbHMF (Similac®) plus EBM (per kg body weight)
Energy	kcal	105-135	119-153	119-153
Protein	g	3.0-4.0	3.85-4.95	3.71-4.77
Fat	g	4.5-6.8	6.37-8.19	5.98-7.69
Linoleic Acid	g	0.35-0.45	0.745-0.958	0.620-0.797
Linolenic Acid	g	0.082-0.105	0.083-0.099	0.061-0.079
Carbohydrate	g	7.5-15.5	11.8-15.2	12.74-16.38
Vitamin A	IU	600-1500	1684-2165	1230-1582
Vitamin D	IU	400/day	260-334	220-283
Vitamin E	IU	0.746-1.34	7.2-9.2	6.2-8.0
Vitamin K1	µg	N/A	5.4-8.1	11.6-14.9
Thiamin	µg	40-50	206-265	319-410
Riboflavin	µg	360-460	357-459	627-806
Vitamin B6	µg	45-60	158-203	290-373
Vitamin B12	µg	0.15	0.252-0.324	0.882-1.13
Niacin	mg	0.759-0.976	4.06-5.22	4.9-6.3
Folic Acid	µg	50/day	38.9-50.2	36.1-46.4
Pantothenic Acid	mg	0.8-1.3	1.0-1.3	2.1-2.7
Biotin	µg	1.5	3.6-4.7	35.7-45.9
Vitamin C	mg	6-10	26.7-34.4	44.7-57.4
Calcium	mg	160-240	165-212	202-259
Phosphorous	mg	77.5-117.8	80.9-104	111-143
Magnesium	mg	4.09-5.26	5.38-6.91	13.62-17.51
Chromium	nmol	1.0-1.9	~1.0-1.9	~1.0-1.9
Iron	mg	2.0-4.0	2.0-2.6	0.52-0.67
Zinc	mg	0.5-0.8	1.54-1.98	1.96-2.52
Manganese	µg	0.55-1.1	13.7-17.6	9.9-12.8
Copper	µg	69.8-121	142-183	315-405
Sodium	mg	57.5-92.0	49.8-64.1	55.3-71.1
Potassium	mg	97.5-136.5	103-133	162-209
Chloride	mg	88.8-142.0	66-85	106-136
Selenium	µg	3-6	2.5-3.2	2.5-3.2
Iodine	µg	31.8-63.5	19.6-32.0	19.6-32.0

* (CPS 1995), ** (CPS 1195, Health Canada 1995)

2.4 Experimental Protocol

The research protocol was scientifically reviewed by 3 staff physicians of the MUHC. Ethical approval was obtained from the RVH Research Ethics Board in October 2001 for a period of 12 months and was then renewed until October 2003 **(Appendices 1 and 2)**. The HMF companies had no jurisdiction over and the investigators were autonomous in regard to the study design, protocol, and analysis.

2.4.1. Feeding Protocol

All subjects were started on Total Parenteral Nutrition (TPN) via peripheral or central line on the first day of life and started enteral nutrition (EN) via orogastric tube within the first few days of life. TPN and when to start or stop enteral nutrition was prescribed by the attending physician(s) and not controlled by the investigator. Investigators only had control over the blinded randomization of HMFs used during breast milk fortification.

All infants were fed according to the feeding protocol in place in the RVH, NICU. TPN was infused continuously. TPN solutions used and progression to maximum parenteral plus enteral per kg body weight were as indicated in **Table 6**.

TABLE 6. TPN SOLUTIONS AND PROGRESSION TO MAXIMUM PARENTERAL PLUS ENTERAL PER KG BODY WEIGHT (AS PER NICU PROTOCOL)

	***Amino Acids** (Trophamine 10%, Braun, USA),	**Lipids** (Intralipid 20% Fresenius Kabi, Sweden)	**Dextrose** (Fresenius Kabi, Sweden)
Day 1	1.5 – 2.0 g/kg/day	nil	6.5 g/kg/day
Day 2 and Progression to Maximum Parenteral plus Enteral	Increase by 0.5-1.0 g/kg/day to a maximum of 3.5 g/kg/day of total protein	Start 1.0 g/kg/day, increase by 0.5 g/kg/day to maximum of 3.5-4.0 g/kg/day	Increase by 2 g/kg/day to a maximum of 12-15 g/kg/day

*L-cysteine hydrochloride (Abbott Laboratories, Chicago, USA) is routinely added to the amino acid solutions of all infants less than 1500 grams for a total dosage of 40 mg cysteine per gram of amino acid. Minerals and vitamin requirements were provided by adding Micro +4 and Multi-12/K_1 Pediatric (Sabex Inc., Quebec, Canada) to the amino acid solution.

Enteral Nutrition (EN) was started within the first week of life. All subjects were started on small volumes of 0.5 - 2 ml every 2-3 hours of EBM or full strength preterm formula (if EBM was not yet available) via an orogastric feeding tube. Fortification of EBM with the allocated study HMFs was started once the subject was tolerating 5 ml of EBM per feed (i.e. 60 ml/day). The first 24 hours of receiving 5 ml of fortified EBM per feed (i.e. 60 ml/day) (or preterm formula if mother's milk was not yet available for fortification) was considered **time zero (t0)** of the study. TPN was gradually reduced as EN was gradually increased by 1- 2 ml every 8-12 hours to reach a target of full enteral feeding of 150 ml/kg/day via orogastric feeding tube or orally (by bottle). EN was progressed from every 2 hours to every 3-4 hours as tolerated. All subjects received supplemental elemental iron (Fer in Sol®, Mead Johnson) typically starting at 3-4 mg/kg/day once they reached two weeks of life and were tolerating at least 5 ml per feed. However, supplemental iron or other vitamins were prescribed by the attending physician(s) and were individualized for each subject.

If human milk was not available at some point during the study, premature infant formula Similac Special Care ® (Ross Laboratories, Saint Laurent, Quebec), an acceptable alternative to fortified EBM (CPS 1995, AAP 1997), was substituted until mother's milk was available for fortification. However, every effort was made to support the mother's of all subjects to feed breast milk. Mother's were encouraged to practice skin-to-skin holding of their infants to help stimulate their breast milk production (Hurst *et al.* 1997), breast milk pumps were available to borrow and/or rent, and a lactation consultant was on staff to consult with and advise the mothers.

All sources of nutritional intake for each infant were recorded daily in each infant's medical record by the NICU nurses. This was part of the routine charting procedures that exist in the NICU. The investigator used each infant's charts to record intake for each 24 hour period (0700 one day until 0700 the next) per kg based on the infants morning weight recorded for that day. Total fluid, total energy, total enteral energy, total parenteral energy, total protein, total carbohydrate, total fat, and fraction of total EN intake from EBM fortified with randomized HMF, fraction of total enteral energy as other (i.e. infant formula), and fraction of total energy intake as parenteral nutrition was

calculated for each infant per kg per day from t0 until exit from the study (defined as Phase 1 or Phase 2 closest to discharge) by the investigators.

2.4.2 Outcome Measurements and Study Schedule

The primary outcome measurement was body composition (as percent total weight as lean and fat mass). The secondary measurements were: growth indices (weight, length, knee-heel length, and head circumference).

Figure 3 shows the study measurement points and how they relate to the postnatal period of the subjects. Body composition and growth measurements were taken at three time points: 1) **Baseline** (when infants achieved ≤ 10 % of their total goal intake of 150 ml/kg enterally, just prior to starting HMF (t0)), 2) **Phase 1** (3 weeks from starting HMF (t0) and 3), **Phase 2** (6 weeks from starting HMF (t0).

2.4.2.i. Primary Outcome - Body Composition (Percent Lean (Fat) Mass) (Aim 1)

BIA was carried out immediately post oral intake using a QuadScan 4000® unit (Bodystat Inc, Tampa, USA). Electrodes were cut to 1 cm diameter and attached to standard distal limb positions on the hand (one lead placed behind the knuckles and one lead on the wrist next to the ulnar head), and on the foot (one lead placed behind the toes and one lead placed on the ankle at the level of and between the medial and lateral malleoli) (Bodystat Inc. 2000). Triplicate measurements were performed (Tang *et al.* 1997), to determine impedance at the frequency of 50 kHz. Total body water (TBW) was calculated using a previously validated model (r^2=99.5) for premature infants (Tang *et al.* 1997):

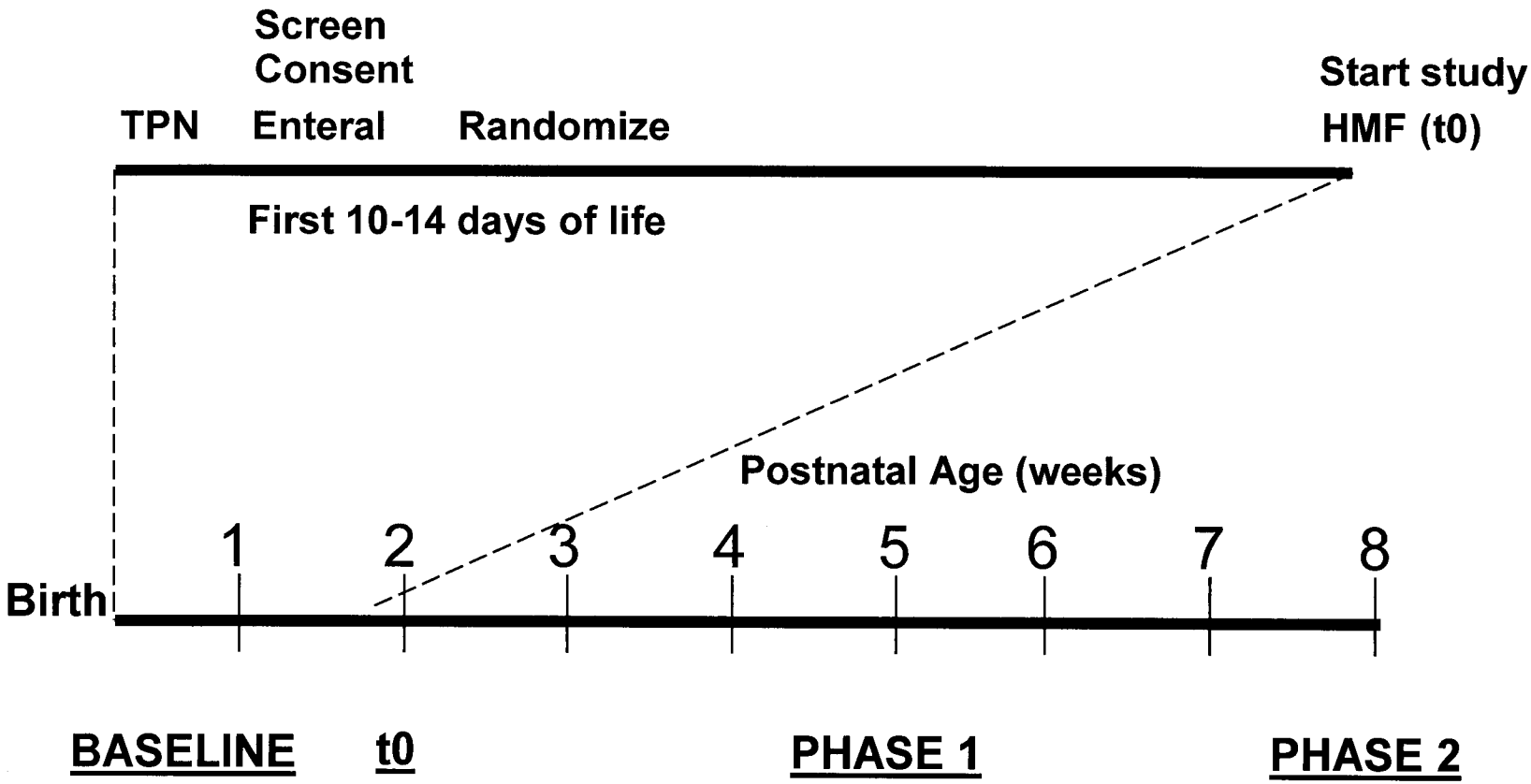
TBW (kg)

= 0.016 + 0.674(body weight in kg) – 0.038(body weight in kg)^2 + 3.84 (*foot length in cm)^2 / (impedance in ohms measured at a frequency of 50 kHz),

TBW (g) =TBW (kg) x 1000

*(Foot length was measured in triplicate immediately prior to BIA measurements (Hempe ® vernier caliper, New Berlin, WI) (precision ±0.254 mm) and the average measurement was used in the TBW calculation above.) Lean mass (LM) and Fat mass (FM) were determined based on TBW using the following calculations previously used in newborns (Wells *et al.* 1998):

Figure 3. Study Protocol



LM (g) = (TBW (g) – volume of feed in grams)/ Proportion of LM as water for GA

FM (g) = Total body weight (g) - LM (g)

The proportion of LM as water decreases with GA (Widdowson 1972, Ziegler *et al.* 1976). Therefore, values for the proportion of LM as water **(Figure 4)** were interpolated from the regression of previously published fetal reference data (Widdowson 1972).

Then, percent LM and percent FM were calculated as:

LM (g)/total body weight (g) x 100

FM (g)/total body weight (g) x 100.

2.4.2.ii. Secondary Outcome - Growth Indices (Aim 2)

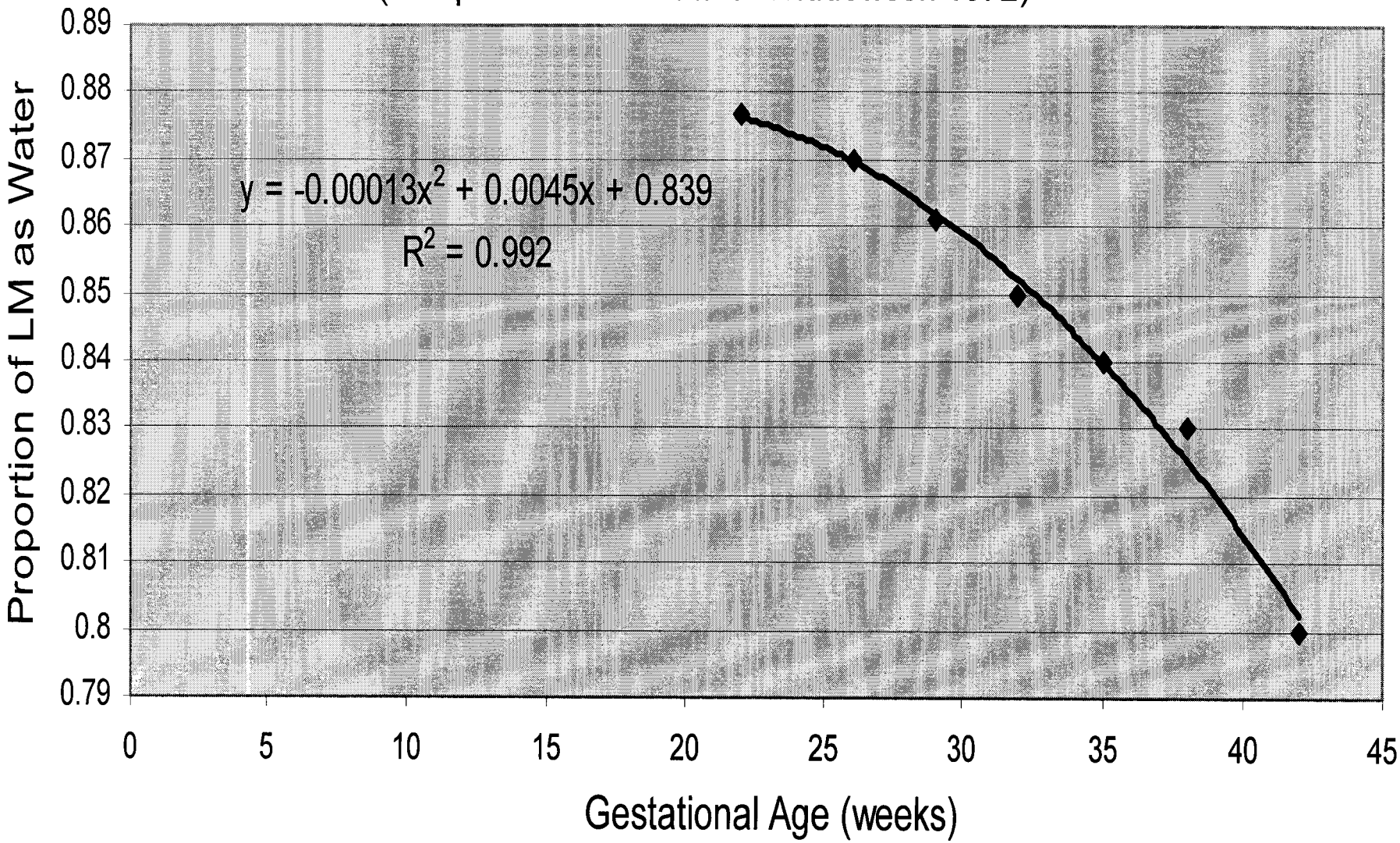
As per NICU schedule, nursing staff measured: daily, nude weights (Detecto®, Detecto Scales Co., Brooklyn, New York) (precision ±5g); weekly, recumbent length (Olympic Auto Length®, Olympic Medical, Seattle, U.S.A.) and occipital head circumference (disposable, non-extensible measuring tape, Ross Laboratories, Saint Laurent, Quebec) (precision ± 0.1 cm). The measurements corresponding to each measurement time (Baseline, Phase 1, and Phase 2) were used in the data analysis. The investigators measured knee-heel length (as a correlate of linear growth (Michaelson 1997, Skinner *et al.* 1997) in triplicate (Hempe ® vernier caliper, New Berlin, WI) (precision ±0.254 mm), at Baseline, Phase 1 and Phase 2. The technique was as described (Skinner *et al.*1997), but instead of placing the mobile arm of the caliper above the knee in line with the lateral head of the fibula, we marked the anterior portion of the knee joint with a non-toxic body marker and placed the mobile arm in line with the mark. This was to avoid the potential of the fat pad on the head of the fibula to contribute to an over estimation of knee-heel length.

If an anthropometric measurement was not completed as per the normal NICU schedule, the measure for the time point was interpolated based on the regression of known anthropometric measurements for the infant during the study time frame.

To interpret growth data in relation to intrauterine growth standards, mean length, weight, and head circumference for each group were compared against intrauterine growth standards developed specific to the RVH NICU population where our study was carried out (Usher *et al.* 1969). More recent intrauterine growth standards specific to this study

Figure 4. Proportion of Lean Mass (LM) as Water for Gestational Age

(Interpolated from Data of Widdowson 1972)



population are being developed by Usher and colleagues, but are not yet published and were not available for use in this study. Also, weight and length measurements for each subject were used to calculate Ponderal Index (PI or wt (in grams)/length (in cm)^3 x 100, an index of infant growth and adiposity (Yau *et al.*1992, Miller *et al.* 1971), for each group and this was compared using the intrauterine growth chart for PI of Lubchencho *et al.* (1963). Intrauterine growth charts do not exist for knee-heel length, so this data could not be compared to intrauterine growth standards.

2.4.3. Biochemical Markers of Nutritional Status at Baseline and Study Exit

Biochemical markers of nutritional status were measured as part of routine nutritional monitoring in the NICU and are recommended by Health Canada (1995) during the evaluation of HMFs. Serum albumin (Alb), blood urea (urea), alkaline phosphatase (ALP), calcium (Ca), phosphorus (Phos), sodium (Na), potassium (K), chloride (Cl), and hemoglobin (HGB) were compared at Baseline and at study exit (defined as Phase 1 or Phase 2 closest to infants discharge). All blood was taken by heel prick by the NICU lab technicians at time of each infant's routine blood analyses. So, no extra blood drawing was specifically required for our study. All blood analyses were performed by the NICU lab technician. Alb, urea, ALP, Ca, and Phos were analyzed using a Kodak Ektachem DT-60 II® (Johnson & Johnson, Division Orthochemical Diagnostics, Canada). Electrolytes were analyzed using a Ciba-Corning® Model 644 Electrolyte Analyzer (Bayer, Chiron Diagnostics, Canada). HGB was analyzed using an Advia 120® Hematologic Analyzer (Bayer Diagnostics, Canada). All laboratory values were obtained from the lab reports in each subject's medical record and compared against the normal reference ranges for newborns used by the RVH NICU laboratory **(Table 7).**

TABLE 7. NORMAL BLOOD TEST REFERENCE RANGES FOR NEWBORNS

Blood Test	Alb	urea	ALP	Ca	Phos	Na	K	Cl	HGB
Normal Range	26 -36 g/L	2.1-7.5 mmol/L	100-300 U/L	1.96-2.66 mmol/L	1.50-2.60 mmol/L	133-146 mmol/L	3.2-5.5 mmol/L	96-110 mmol/L	140-180 g/L

2.4.4. Subject Characteristics at Baseline and from Starting HMF (t0) until Exit

The preterm very low birth weight infant population can be heterogeneous (Sherry *et al.* 2003), so there is potential for confounding. A number of subject and clinical characteristics were compared that may affect growth outcome (Ehrenkranz *et al.* 1999, Schanler[2] *et al.* 1999, Berry[1] *et al.* 1997) and if any variable was found to be statistically different, it was planned *a priori* to be used as a covariate during statistical analysis

Subject characteristics were compared:

1) at Baseline for: birth weight, gestational age (GA), gender, percent lean and fat mass, weight, length, knee-heel length, head circumference, day of life of first enteral (EN) feeding, day of life at Baseline, time from Baseline to starting HMF(t0).

2) from t0 until exit from the study (defined as Phase 1 or Phase 2 closest to infants discharge) for the following clinical factors: day of life at t0, weight, length, knee-length, and head circumference at t0, number of days to reach full enteral (EN) feeding from t0, number of days NPO (nil per os), number of days of parenteral nutrition, the number of subjects with Necrotizing Entercolitis (NEC), sepsis, Bronchopulmonary Dysplasia (BPD, defined as oxygen dependency by 28 days of life (Merritt *et al.* 1991)), Intraventricular Hemorrhage (IVH, confirmed with head Ultrasound), and Patent Ductus Arteriosus (PDA, confirmed with Echocardiogram), number of subjects who had a PDA Ligation or other surgery, and number of days on diuretics.

All information was obtained directly from each subject's chart by the investigators.

2.5 Statistical Methods

Statistical consultation was obtained from the Montreal Children's Hospital-McGill University Research Institute Clinical Research Center.

Sample size was calculated based on the main outcome of interest of percent lean (fat) mass. Because no data was available to estimate sample size based on percent lean (fat) mass, specifically using BIA, the standard deviation (SD) of the population was first estimated as a SD=4 points in percent lean mass. This was estimated based on previous work (Chan *et al.* 2000) and later verified as the SD close to our study population at Phase 2, as SD increased slightly overtime (from SD=2.51 at Baseline to SD=3.79 at

Phase 2). Thus, based on the SD = 4 points in percent lean mass (or fat mass rounded to the nearest one), a total sample size (N) of 32 was calculated, as per Kramer (1988) to allow an 80% probability (β= 0.20) of detecting a clinically relevant difference of 4 points in percent lean mass (therefore, an overall difference in ~5% lean mass and 20% fat mass, which would result in a body composition more similar to the reference fetus of similar GA (Widdowson 1972)), a two sided hypothesis, and α=0.05. On the other hand, a smaller difference in percent fat mass would be clinically relevant, but would require a larger sample size to show statistical significance. To account for anticipated protocol deviations, a 50% padding was added (32 +16 = 48). Therefore, 48 assignment cards were prepared in an advance (as described above).

All data was entered into a data file (Excel® software, Microsoft Corporation, U.S.A.) and statistical analysis was performed using SAS® statistical software (SAS 8.2®, SAS Institute, Inc., Cary, NC). Continuous variables were compared between HMF groups by Repeated Measures ANOVA and Student's t-test. Categorical variables were compared by Chi-square and Fisher's Exact Test (if the expected value of any cell of the Chi-square table was < 5 (Cody[1] *et al.* 1997). It was planned *a priori* that if a subject characteristic (measured at Baseline or from t0-exit from study) was statistically different, the variable(s) would be used as a covariate in a Covariate ANOVA. The assumption of normality of data and homogeneity of variances for ANOVA was verified using the Shapiro-Wilk test for normality and the Levene's test for equality of variances (Cody[2, 3] *et al.* 1997).

All data was analyzed as intent-to-treat. Data values were expressed as mean ±SD. For the primary outcome of percent lean (fat) mass, p values < 0.05 were considered statistically significant. For secondary outcomes, to protect against a type 1 error (falsely rejecting the null hypothesis), p values less than the value calculated using the Bonferroni correction (threshold α= 0.05 for rejecting the null hypothesis divided by the total number tests performed in the set) (Kramer 1988) were considered statistically significant.

CHAPTER 3. RESULTS

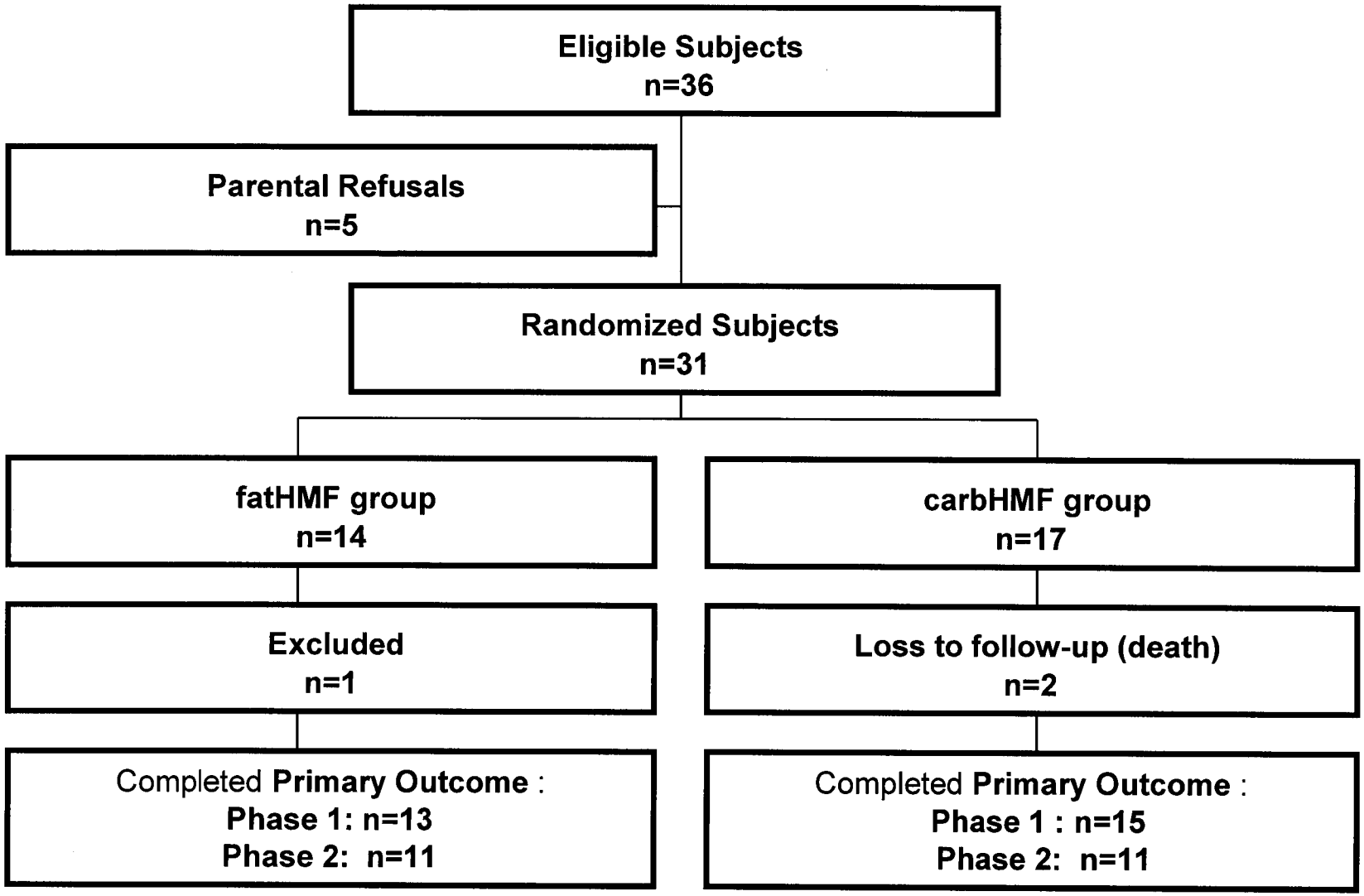
3.1 Subject Recruitment, Randomization, and Flow Through Study

Figure 5 illustrates the flow of subjects through the study between January 2002 and April 2003. 36 infants met inclusion criteria during this time period. Parents of all eligible infants were approached and consent was received for 31 infants (~86% consent rate). These 31 infants were enrolled and randomized to the study (14 to fatHMF and 17 to carbHMF). 2 of the 17 subjects (#15 and #24) randomized to the carbHMF were lost to follow-up because of death due to withdrawal of medical treatment, one infant (#15) died just after Baseline and before starting HMF (t0) and the other (#24) died at t0.

Flow of Subjects for the Primary Outcome of Percent Lean (Fat) Mass: **Figure 5** also describes the flow of subjects for the primary outcome. 28/29 subjects (~97%) completed Baseline and Phase 1 (13 fatHMF and 15 carbHMF). One of the 29 subjects (# 16) was not measured at Phase 1, as the infant was critically ill (sepsis) at the time and we considered it unethical to perform this measurement. However, this infant recovered and progressed to complete the Phase 2 body composition measurement. So, 23/29 subjects completed Phase 2 before discharge (12 fatHMF and 11 carbHMF). But, subject # 16 had to be excluded from analysis using Repeated Measures ANOVA, because it is inherent to this statistical design that only subjects that have completed all time points of interest can be analyzed (Cody[4] *et al.* 1997). So, 22/29 (~76%) subjects (11 fatHMF and 11 carbHMF) who completed all three measurement points (Baseline, Phase 1, and Phase 2) were analyzed.

Flow of Subjects for the Secondary Outcomes: **Table 8** indicates the flow of subjects through the study for the growth indices before discharge. For weight, 29/29 subjects completed Baseline and Phase 1, and 23/29 subjects (~79%) completed Baseline, Phase 1, and Phase 2; for length, 28/29 subjects (~97%) completed Baseline and Phase 1 (one subject (#25) was discharged before the scheduled measurement was completed at Phase 1 and there were no outpatient length measurements available to interpolate the data), and 23/29 (~79%) subjects completed Baseline, Phase 1, and Phase 2; for head circumference, 28/29 subjects (~97%) completed Baseline and Phase 1 (for the same reason as for length), and 23/29 subjects (~79%) completed Baseline, Phase 1, and Phase

Figure 5. Flow Of Subjects Through Study (January 2002 - April 2003)



2; for knee-heel length, 29/29 subjects completed Baseline and Phase 1, and 23/29 (~79%) subjects completed Phase 2.

TABLE 8. FLOW OF SUBJECTS THROUGH STUDY FOR GROWTH INDICES (JANUARY 2002-APRIL 2003)

	Completed Baseline and Phase 1		Completed Baseline, Phase 1, and Phase 2	
	fat HMF	**carbHMF**	**fat HMF**	**carbHMF**
Weight	14	15	12	11
Length	14	14	12	11
Head Circumference	14	14	12	11
Knee-heel Length	14	15	12	11

Flow of Subjects for Subject Characteristics: For subject characteristics at Baseline, 31 subjects were analyzed for all variables measured, except for time from Baseline – t0, 30 subjects were analyzed as one subject (#15) died before t0.

For subject characteristics from starting HMF (t0) until the exit from the study, 29 subjects were analyzed for all parameters, except, 30 subjects were analyzed for the following (as this was known for subject #24 prior to death): day of life at t0, weight, length, and head circumference at t0, and incidence of IVH.

For biochemical indices at Baseline and exit from the study, 28/29 of the infants who progressed through the study were analyzed for all the measured values, except 29/29 infants were analyzed for hemoglobin (HGB). Other blood tests besides HGB were not completed for one subject (#5) as there was not enough volume in the blood sample.

3.2 Subject Characteristics

3.2.1. Subject Characteristics at Baseline

Table 9 shows that there were no significant differences in characteristics between HMF groups at Baseline. Thus, a covariate analysis was not indicated.

TABLE 9. SUBJECT CHARACTERISTICS AT BASELINE

	fatHMF (±SD) (n=14)	**carbHMF**(±SD) (n=17) *(n=16)	**p**
Birth Weight (g)	1050 (±241)	1060 (±241)	0.9317
GA at birth (weeks)	27.45 (±1.86)	27.75 (±2.55)	0.7195
Gender: male **female**	8 6	6 11	0.2238
Lean Mass Baseline (%)	89.49 (±2.01)	90.18 (±2.89)	0.4594
Fat Mass Baseline (%)	10.51 (±2.01)	9.82 (±2.89)	0.4594
Weight Baseline (g)	989 (±239)	983 (±367)	0.9560
Length Baseline (cm)	36.3 (±2.8)	36.4 (±4.0)	0.8037
HC Baseline (cm)	25.0 (±1.9)	24.9 (±2.6)	0.8592
Knee-heel Length Baseline (mm)	78.8 (±7.5)	77.7 (±10.3)	0.7480
Day of Life of First EN Feeding (days)	2.93 (±1.39)	2.31 (±0.79)	0.1578
Day of Life at Baseline (days)	6.3 (±5.6)	4.3 (±1.5)	0.2172
Time from Baseline – t0 (days)	7.57 (±10.67)	*5.50 (±7.85)	0.5462

*(n=16)

3.2.2. Subject Characteristics from t0 – Study Exit

One length measurement (for subject #16) was not completed for as per the normal NICU schedule. Linear regression was used to estimate this length (r^2= 0.9927) and this was used in the analysis for length at t0. 7 subjects who had a PDA ligation and 2 subjects

who had other surgical procedures were transferred to the Montreal Children's Hospital for surgical treatment, however the same experimental protocol was followed as at the RVH and subjects remained on the study HMFs as randomized. **Table 10** indicates that there were no statistically significant differences between HMF groups for various clinical characteristics from starting HMF (t0) until the exit from the study. Thus, a covariate analysis was not indicated.

TABLE 10. SUBJECT CHARACTERISTICS FROM t0 – STUDY EXIT

	fatHMF (±SD) (n=14))	**carbHMF** (±SD) (n=15) *(n=16)	**p**
Day of Life Started HMF (t0)	13.9 (±15.8)	*9.8 (±8.3)	0.4013
Weight t0 (g)	1109 (±295)	*1174 (±314)	0.7616
Length t0 (cm)	37.3 (±2.9)	*37.1 (±3.6)	0.8944
HC t0 (cm)	25.8 (±2.0)	*25.6 (±2.5)	0.8259
Days to reach full EN feeding (#)	17.9 (±15.2)	10.1 (±10.3)	0.1144
NPO (# days)	4.9 (±6.5)	1.5 (±2.4)	0.0745
Parenteral Nutrition (# days)	21.5 (±15.1)	15.0 (±11.7)	0.2036
NEC (# subjects)	3	1	0.2493 **(0.3295)
Sepsis (# subjects)	2	3	0.6839 **(1.000)
BPD (# subjects)	6	7	0.8367
IVH (# subjects)	2	*2	0.8859 **(1.000)
PDA (# subjects)	8	*7	0.4642
PDA Ligation (# subjects)	2	5	0.2310 **(0.3898)
Other Surgery (# subjects)	***2	0	0.1292 **(0.2241)
Diuretics (# days)	5.71 (±7.74)	4.47 (±6.87)	0.6492

* (n=16), ** (Fisher's Exact Test), ***1 intraventricular drain, 1 ileosotomy

3.3 Nutritional Intake from t0 – Study Exit

Table 11 indicates nutritional intake for both HMF groups from t0 until the exit from the study. There was no difference in nutritional intake per kg per day in the fatHMF *vs.* the carbHMF group, respectively, for: total fluid intake, total energy intake, total enteral (EN) energy intake, total energy intake from parenteral nutrition (PN), fraction of total EN intake from EBM fortified with randomized HMF, fraction of total enteral energy intake from other formula sources, total protein, or total fat. However, as planned, carbohydrate intake was significantly higher in the carbHMF group (p=0.0012).

TABLE 11. TOTAL DAILY NUTRITIONAL INTAKE PER KG FROM t0 – STUDY EXIT

Nutrient (unit)	fatHMF (n=14) (±SD)	carbHMF (n=15) (±SD)	p
Fluid (ml/kg)	144.3 (±13.5)	149.7 (±9.2)	0.2085
Energy (kcal/kg)	115.4 (±13.4)	121.2 (±9.8)	0.1942
Total EN Energy(kcal/kg)	87.4 (±38.0)	105.9 (±25.0)	0.1276
Total PN Energy(kcal/kg)	28.0 (±25.2)	15.3 (±15.5)	0.1115
Fraction of Total EN Energy as EBM fortified with randomized HMF	0.733 (±0.326)	0.880 (±0.162)	0.1307
Fraction of Total EN Energy as Other (formula)	0.267 (±0.326)	0.120 (±0.162)	0.1438
Fraction of Total Energy as Parenteral	0.270 (±0.270)	0.136 (±0.144)	0.1129
Protein (g/kg)	3.66 (±0.40)	3.84 (±0.21)	0.1488
Carbohydrate (g/kg)	12.50 (±0.56)	13.39 (±0.74)	**0.0012**
Fat (g/kg)	5.63 (±1.14)	5.81 (±0.72)	0.6001

3.4 Biochemical Indicators of Nutritional Status at Baseline and Study Exit

Table 12 indicates that there were no significant differences between HMF groups for any blood values. All blood values were within the normal laboratory reference range for newborns, except HGB was lower and ALP was higher than the normal reference range for both groups.

TABLE 12. BIOCHEMICAL INDICATORS OF NUTRITIONAL STATUS AT BASELINE AND STUDY EXIT

Measure (unit)	Normal Reference Range for Newborns	**fatHMF** (n=14) **Baseline** (±SD) **Exit** (±SD)	**carbHMF** (n=14) *(n=15) **Baseline** (±SD) **Exit** (±SD)	**p**
Alb (g/L)	26-36	28 (±3) 28 (±4)	29 (±5) 29 (±3)	0.8487
Urea (mmol/L)	2.1-7.5	8.8 (±5.9) 2.8 (±2.3)	8.2 (±2.9) 2.3 (±1.3)	0.9250
HGB (g/L)	140-180	138 (±34) 104 (±16)	*161 (±31) *105 (±12)	0.0615
ALP (U/L)	100-300	264 (±100 399 (±172)	299 (±126) 335 (±123)	0.1306
Ca (mmol/L)	1.96-2.66	2.17 (±0.30) 2.44 (±0.17)	2.02 (±0.22) 2.44 (±0.14)	0.1109
Phos (mmol/L)	1.50-2.60	1.9 (±0.3) 2.0 (±0.3)	1.9 (±0.4) 2.0 (±0.3)	0.7409
K (mmol/L)	3.2-5.5	4.6 (±0.7) 4.4 (±0.4)	4.7 (±0.8) 4.9 (±0.6)	0.2349
Na (mmol/L)	133-146	140 (±5) 137 (±4)	139 (±3) 137 (±3)	0.8602
Cl (mmol/L)	96-110	107 (±7) 103 (±6)	106 (±5) 102 (±5)	0.7361

*(n=15)

3.5 Primary Outcome - Percent Lean (and Fat) Mass (Aim 1)

As mentioned above, several subjects (6) were discharged before Phase 2 was completed. Therefore, body composition was analyzed for all subjects in each group who completed Baseline and Phase 1 **(Figure 6)** and all subjects in each group who completed Baseline, Phase 1, and Phase 2 **(Figure 7)**. As expected, based on normal changes in neonatal body composition (Widdowson 1972, Ziegler *et al.* 1976) percent lean mass decreased and fat mass increased significantly over time ($p<0.0001$) for both HMF groups. But, there were no statistically significant differences between HMF groups by time and group interaction for percent lean mass (or fat mass) by Phase 1 ($p= 0.2259$) or by Phase 2 ($p=0.3586$). However, there was a suggestion of a clinical advantage of the fatHMF to promote a higher percent lean mass compared to the carbHMF by Phase 2. Because, as shown in **Figure 7**, the mean percent fat mass of the fatHMF group increased from 10.20 (±2.03, CI: 8.45-11.95) at Baseline to 16.60 (±3.60, CI: 14.68-18.52) by Phase 2 (a 63% increase), whereas the carbHMF group increased from 9.37 (±3.38, CI: 7.62-11.12) at Baseline to 18.45 (±2.39, CI: 16.53-20.37) (a 96% increase). Thus, the fatHMF group appeared to have a lower increase in percent fat mass by Phase 2 compared to the carbHMF group. To further explore the above observation, we performed a post hoc analysis **(Table 13)** using the same data as **Figure 7** (infants analyzed for percent lean and fat mass from Baseline to Phase 2) which included the:

1) change in absolute percent fat mass from Baseline to Phase 2

2) absolute fat and lean mass in grams (calculated as the fraction of lean or fat mass multiplied by total body weight in grams) at Baseline and Phase 2

3) change in absolute lean and fat mass from Baseline to Phase 2

4) absolute dry lean tissue in grams (absolute lean mass in grams minus TBW in grams as measured using BIA) at Baseline and Phase 2

5) change in absolute dry lean tissue in grams from Baseline to Phase 2.

Post Hoc analysis results were: 1) the fatHMF group gained 30 g more dry lean tissue from Baseline to Phase 2 compared to the carbHMF group (statistically significant, $p=0.0362$); 2) other post hoc values were not significant, however, despite HMF groups being similar at Baseline, the fatHMF group was 42 g higher than the carbHMF group in absolute dry lean tissue at Phase 2 (approached statistical significance, $p=0.0647$).

Figure 6. Percent Lean and Fat Mass at Baseline and Phase 1

p <0.0001 (time), p = 0.2259 (time*group)

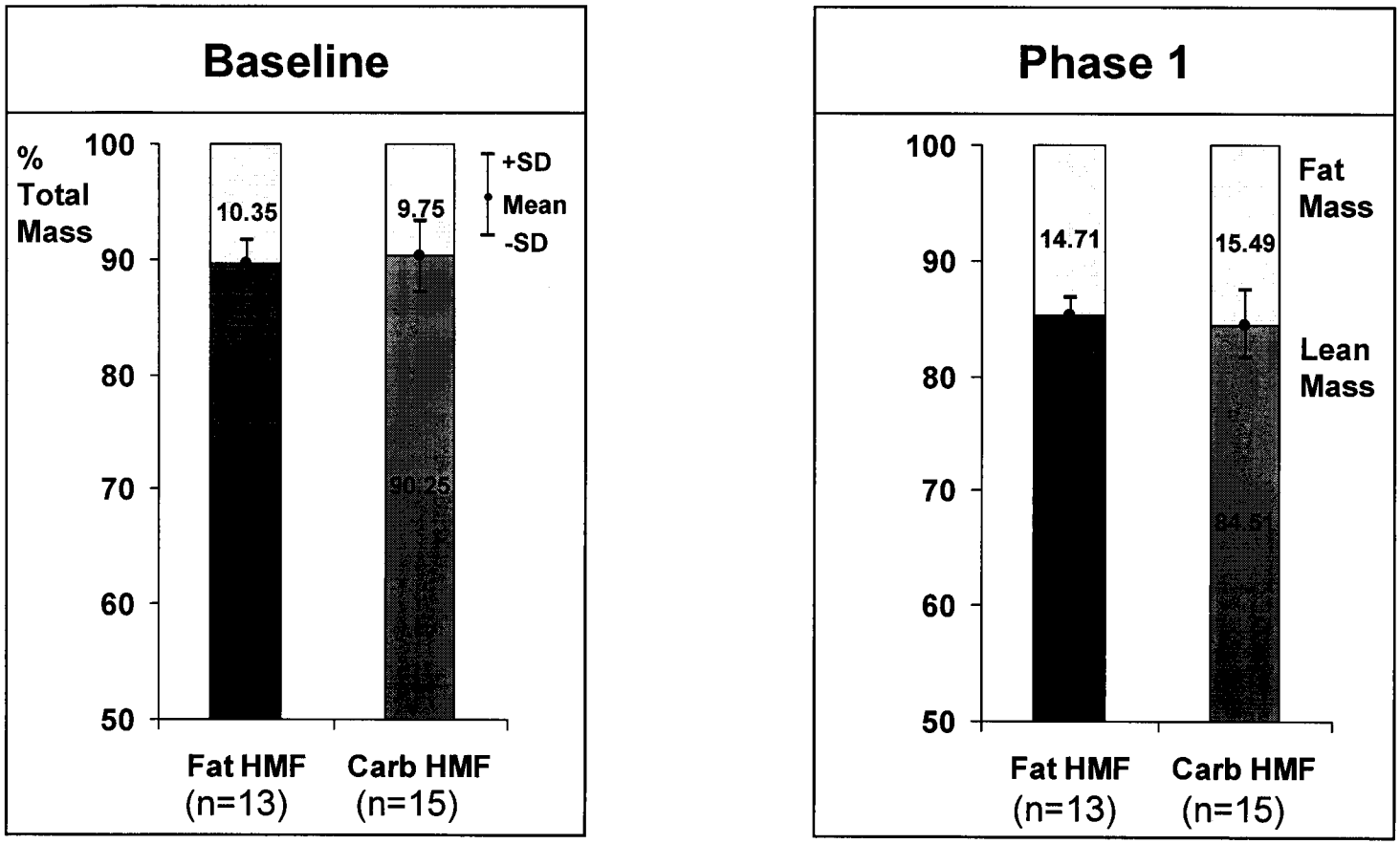


Figure 7. Percent Lean and Fat Mass at Baseline, Phase 1, and Phase 2

p <0.0001 (time), *p* = 0.3586 (time*group)

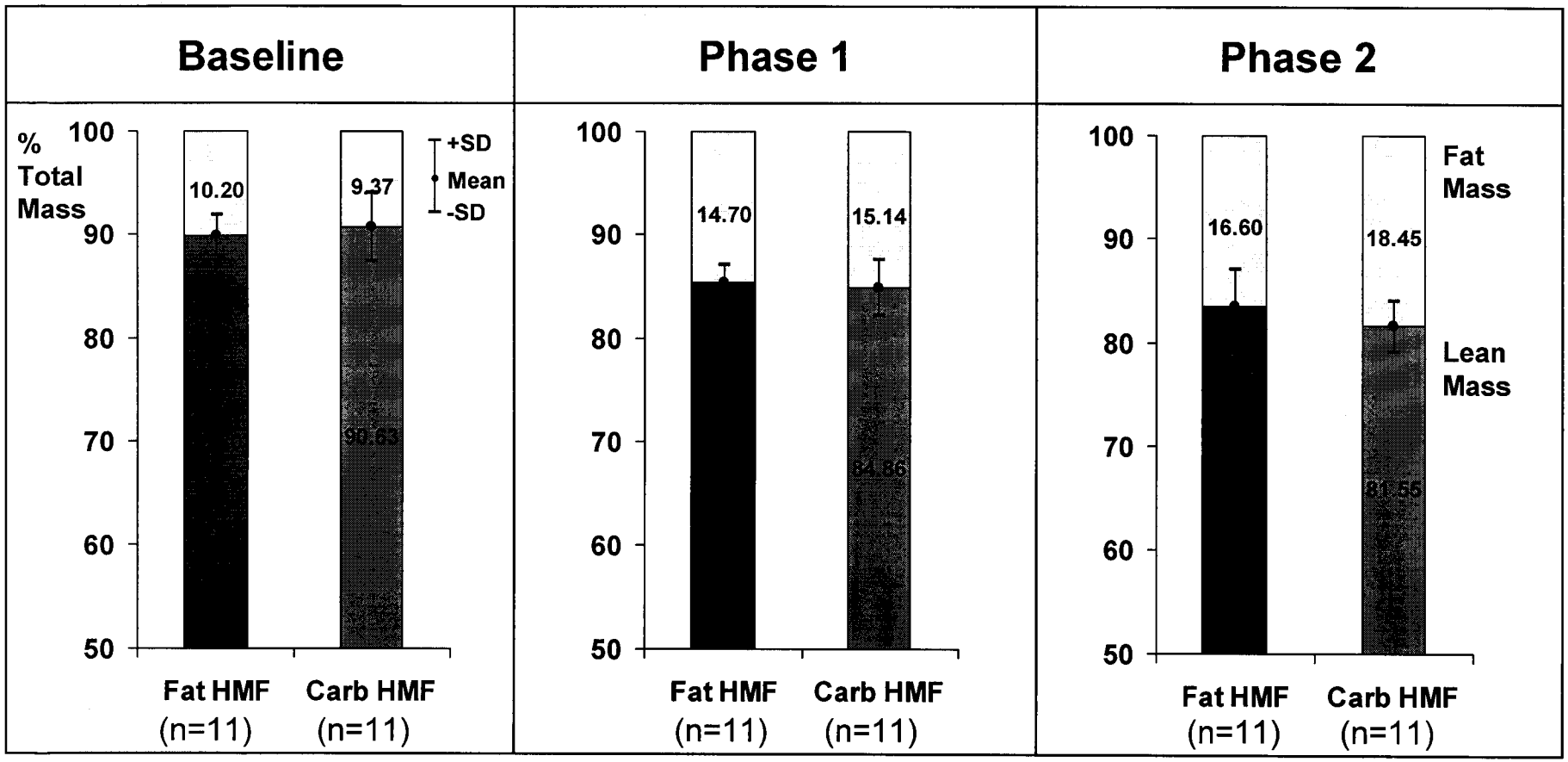


TABLE 13. POST HOC ANALYSIS OF CHANGE IN ABSOLUTE PERCENT FAT MASS FROM BASELINE TO PHASE 2; ABSOLUTE FAT MASS, LEAN MASS, AND DRY LEAN TISSUE AT BASELINE AND PHASE 2; AND CHANGE IN ABSOLUTE FAT MASS, LEAN MASS, AND DRY LEAN TISSUE FROM BASELINE TO PHASE 2.

Measure (unit)		fatHMF (±SD) (n=11)	carbHMF(±SD) (n=11)	p
Absolute Percent Fat Mass (%)	**Change Baseline to Phase 2**	+6.40 (±4.63)	+9.08 (±3.62)	0.1469
Absolute Fat Mass (g)	**Baseline**	98 (±30)	86 (±53)	0.5434
	Phase 2	356 (±144)	354 (±137)	0.9821
	Change Baseline to Phase 2	+258 (±132)	+267 (±103)	0.8447
Absolute Lean Mass (g)	**Baseline**	865 (±226)	777 (±257)	0.4005
	Phase 2	1731 (±360)	1531 (±371)	0.1768
	Change Baseline to Phase 2	+867 (±170)	+737 (±188)	0.1056
Absolute Dry Lean Tissue (g)	**Baseline**	116 (±32)	104 (±38)	0.4325
	Phase 2	244 (±53)	202 (±46)	**0.0647**
	Change Baseline to Phase 2	+128 (±33)	+99 (±29)	***0.0362**

*statistically significant based on $p<0.05$

3.6 Secondary Outcome - Growth Indices (Aim 2)

Several growth measurements were not completed as per the normal NICU schedule. This occurred for the same four subjects (for subject 13, 14, 16, and 25). For example, three length measurements (subjects 13, 16, and 25), 2 head circumference measurements (subjects 13 and 25), and two knee-heel lengths (subjects 14 and 16) were not completed for Phase 1. Two length and head circumference measurements (subjects 13 and 14) were not done at Phase 2. Linear regression was used to interpolate the measurements for subjects 13, 14, and 16, and these values were then used during analysis. However, subject 25 was discharged before the next scheduled measurement day and there was no outpatient growth data to interpolate these measures. The r^2 values for length for the subjects 13, 14, 16, were 0.9788, 0.9128, and 0.9825, respectively. The r^2 values for head circumference for the subject 13 was 0.9929 and for knee-heel length for subjects 14 and 16, 0.9762 and 0.9997, respectively. These growth measures were then used during statistical analysis.

As mentioned, several (6) subjects were discharged prior to reaching the Phase 2 measurement. Therefore, data was analyzed for all subjects in each group who completed Baseline and Phase 1, and who completed Baseline, Phase 1, and Phase 2 **(Figures 8–15)**. As expected weight, length, knee-heel length, and head circumference increased significantly over time ($p<0.0001$). But, there were no significant differences between HMF groups for growth indices (weight, length, knee-heel length, or head circumference) and time interaction by Phase 1 or by Phase 2. However, there was a suggestion of a clinical advantage of the fatHMF to increase weight and length. As shown in **Figure 9**, the fatHMF group increased in mean body weight from 952(±238) g at Baseline to 2146(±500) g by Phase 2 (a weight gain of 1194 g), whereas, the carbHMF group increased in weight from 863(±304) g at Baseline to 1868(±503) g by Phase 2 (a weight gain of 1005 g). This suggests that the fatHMF group gained ~189 g more in weight (1194 g – 1005 g) than the carbHMF group by Phase 2. Also, as shown in **Figure 11**, the fatHMF group increased in mean body length from 35.9(±2.8) cm at Baseline to 44.7(±3.5) cm by Phase 2 (a length gain of 8.8 cm), whereas, the carbHMF group increased in length from 34.9(±3.1) cm at Baseline to 41.8(±3.3) cm by Phase 2 (a length

Figure 8. Body Weight at Baseline and Phase 1

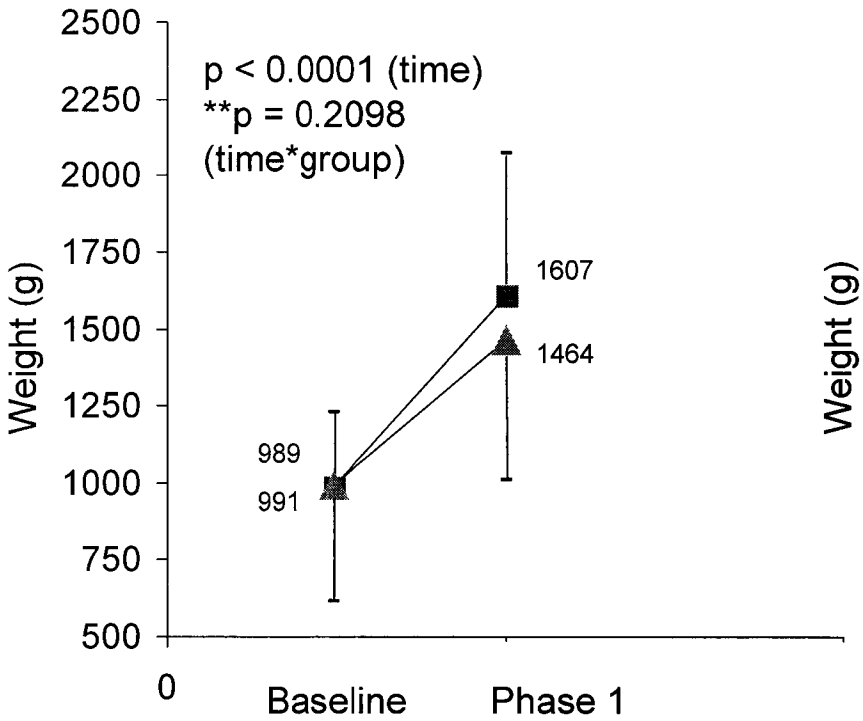
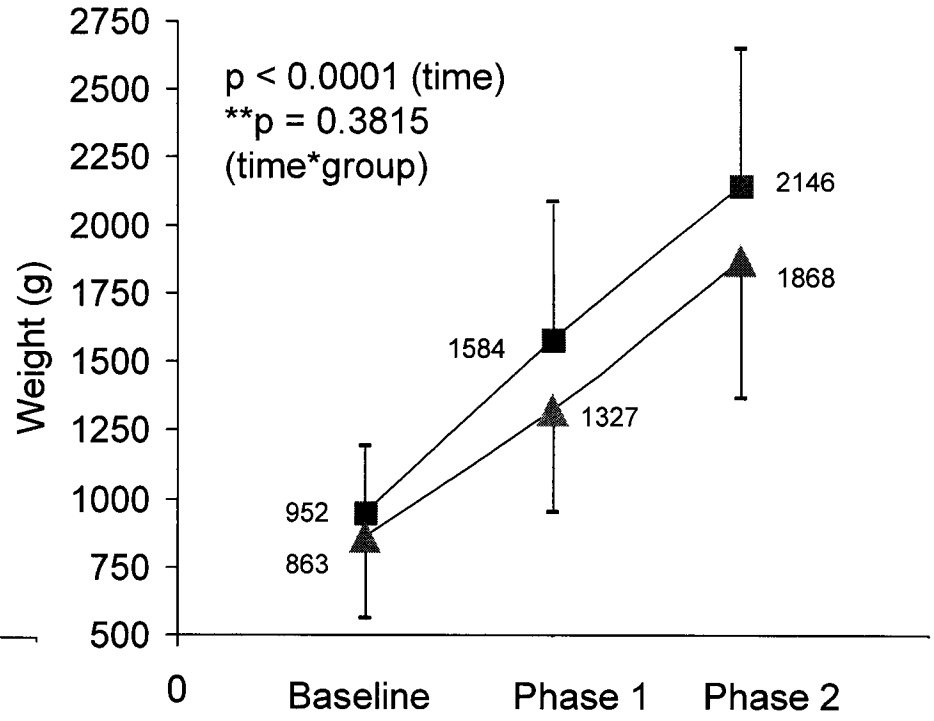


Figure 9. Body Weight at Baseline, Phase 1, and Phase 2



(n=14) ■ fatHMF (n=12)

(n=15) ▲ carbHMF (n=11)

**Values not statistically significant based on p value of 0.01
(calculated as per Bonferroni's correction: α of 0.05 divided by 5 secondary outcome measures)

Figure 10. Body Length at Baseline and Phase 1

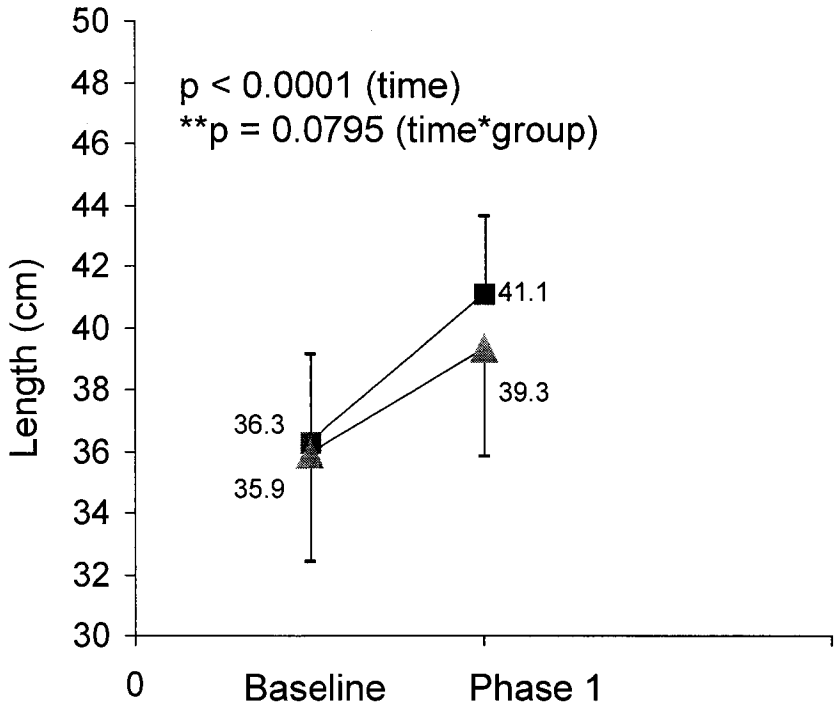
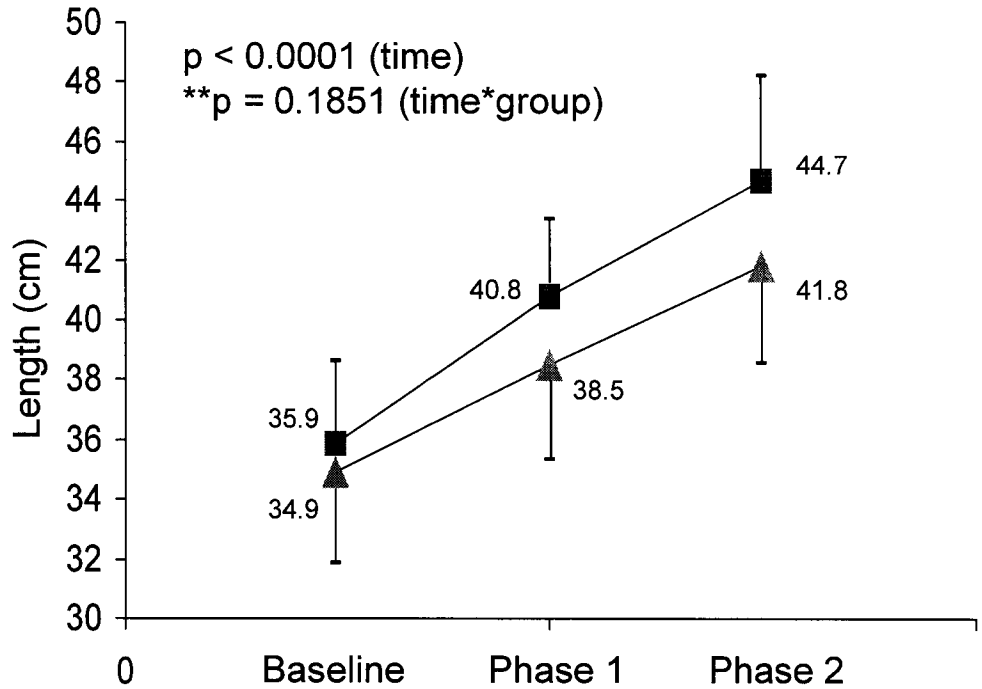


Figure 11. Body Length at Baseline, Phase 1, and Phase 2



(n=14) ■ fatHMF (n=12)

(n=14) ▲ carbHMF (n=11)

**Values not statistically significant based on p value of 0.01
(calculated as per Bonferroni's correction: α of 0.05 divided by 5 secondary outcome measures)

Figure 12. Knee-heel length at Baseline and Phase 1

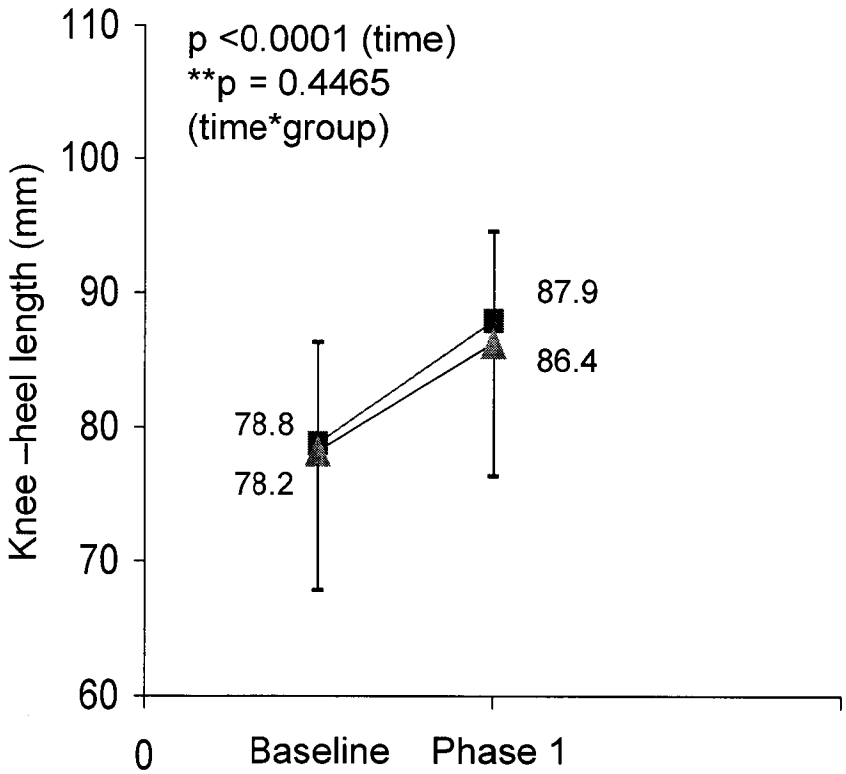
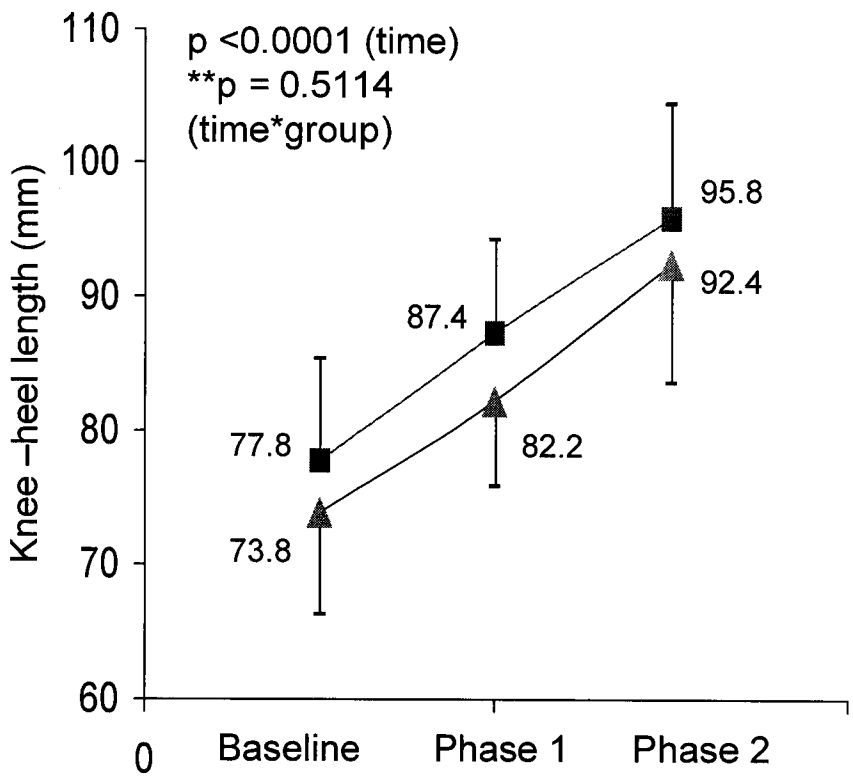


Figure 13. Knee-heel length at Baseline, Phase 1, and Phase 2



(n=14) ■ fatHMF (n=12)

(n=15) ▲ carbHMF (n=11)

**Values not statistically significant based on p value of 0.01
(calculated as per Bonferroni's correction: α of 0.05 divided by 5 secondary outcome measures)

Figure 14. Head Circumference at Baseline and Phase 1

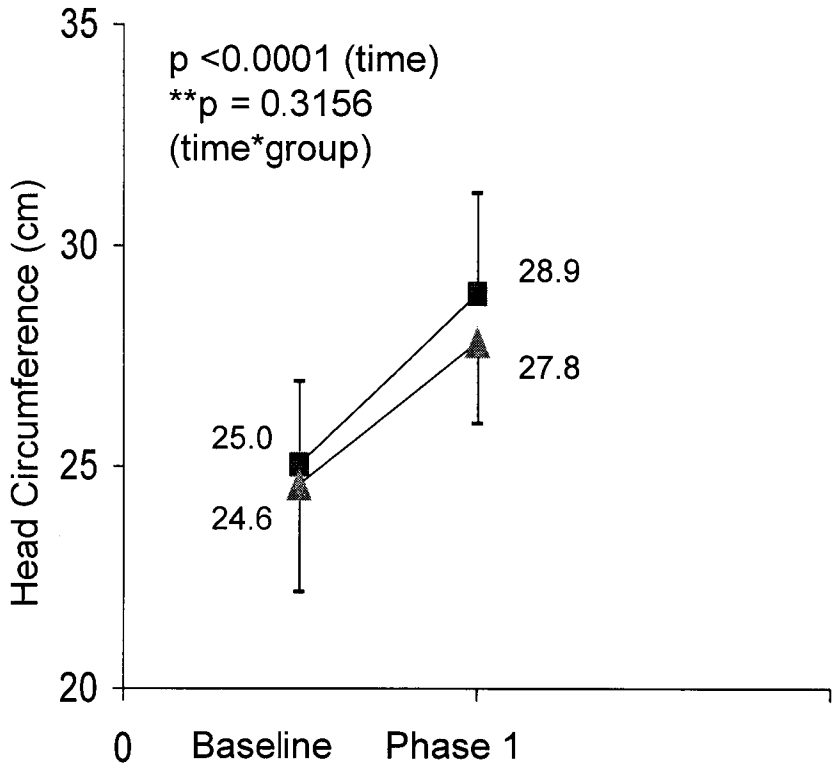
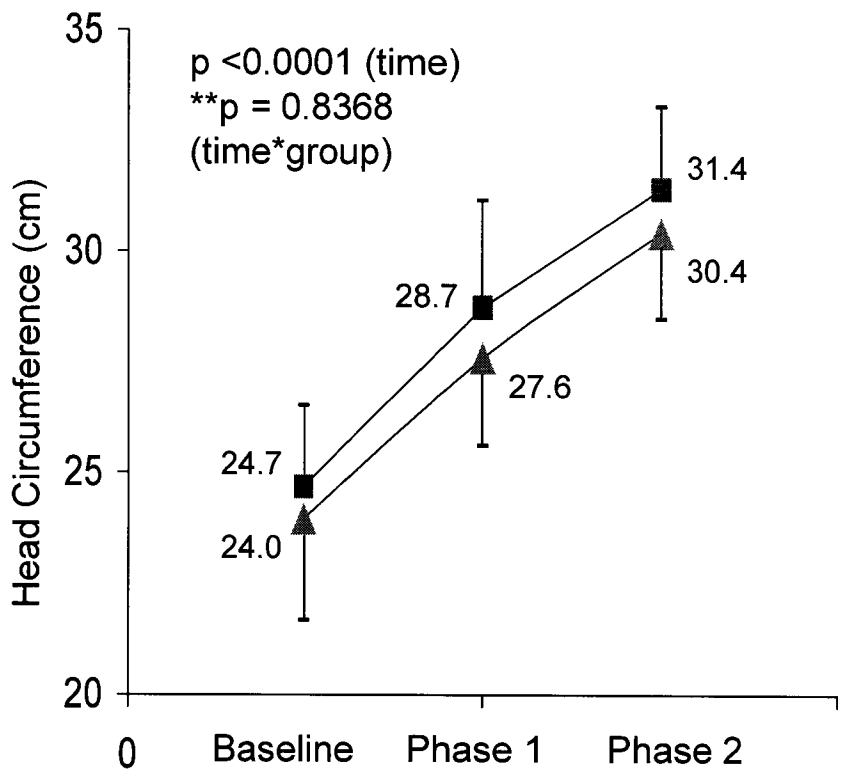


Figure 15. Head Circumference at Baseline, Phase 1, and Phase 2



(n=14) ■ fatHMF (n=12)

(n=14) ▲ carbHMF (n=11)

**Values not statistically significant based on p value of 0.01
(calculated as per Bonferroni's correction: α of 0.05 divided by 5 secondary outcome measures)

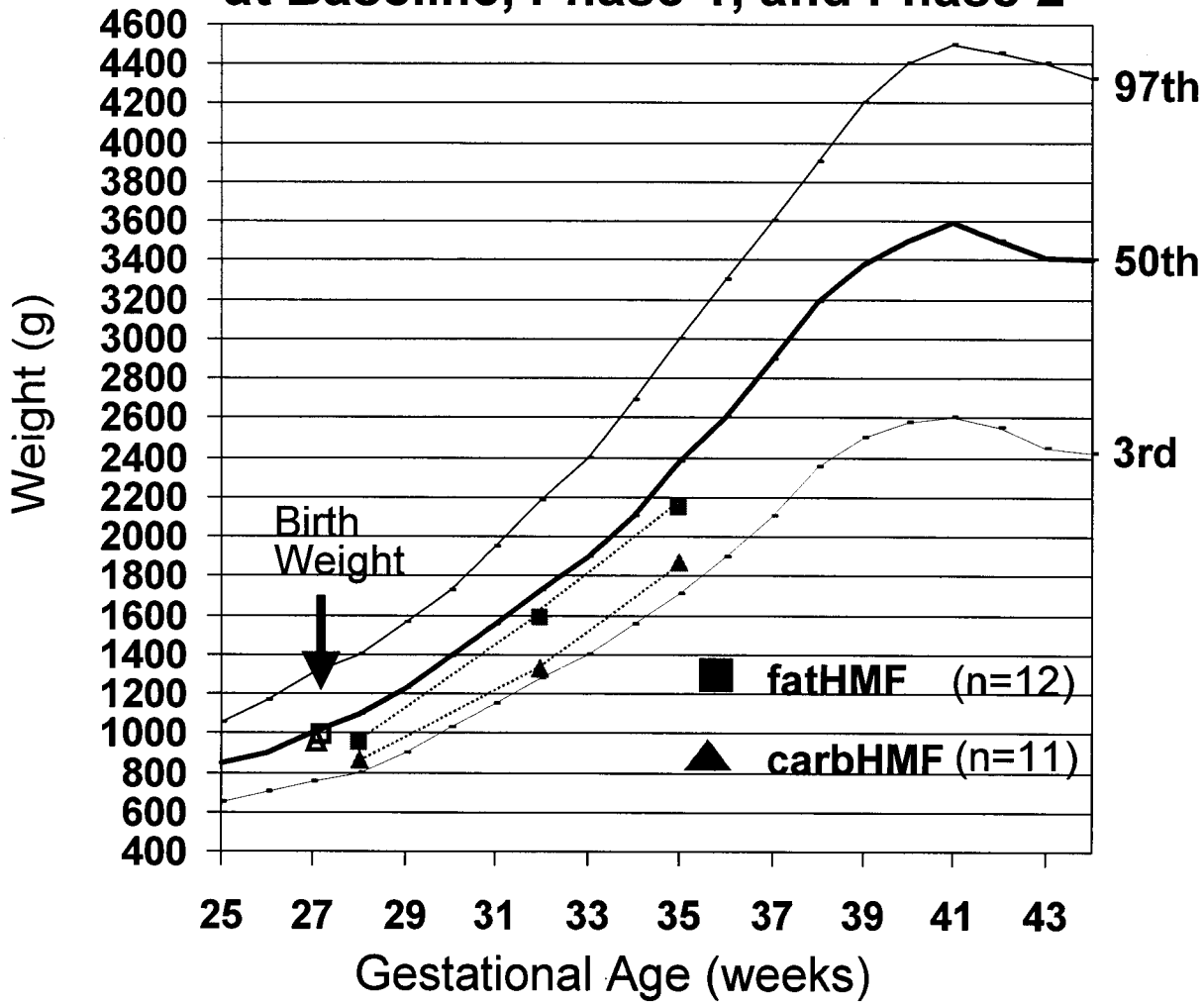
gain of 6.9 cm). This suggests that the fatHMF group gained ~1.9 cm (8.8cm – 6.9 cm) more in length by Phase 2.

The group means for weight, length, and head circumference at Baseline, Phase 1, and Phase 2 for the fatHMF (n=12) and the carbHMF (n=11) were compared using intrauterine growth charts.

Figure 16 shows mean weights. At Baseline the fatHMF group was growing at the 25th percentile and the carbHMF group was growing between the 3rd and 25th percentiles (*NOTE: At Baseline, mean weight percentiles were lower in both groups compared to the mean birth weight percentiles which were between the 25th and 50th percentile. This reflects the up to 10-15% weight loss due to the physiological body fluid redistribution which occurs in preterm infants during the first few days of life (Bauer *et al.* 1991). By Phase 2 the fatHMF group established growth for weight between the 25th percentile and 50th percentile. In contrast, the carbHMF group continued to grow between the 3rd and 25th percentile for weight. This suggests that the fatHMF group maintained growth velocity as expected based on birth weight, whereas the carbHMF group appears to have a slightly reduced growth velocity compared to that expected based on birth weight.

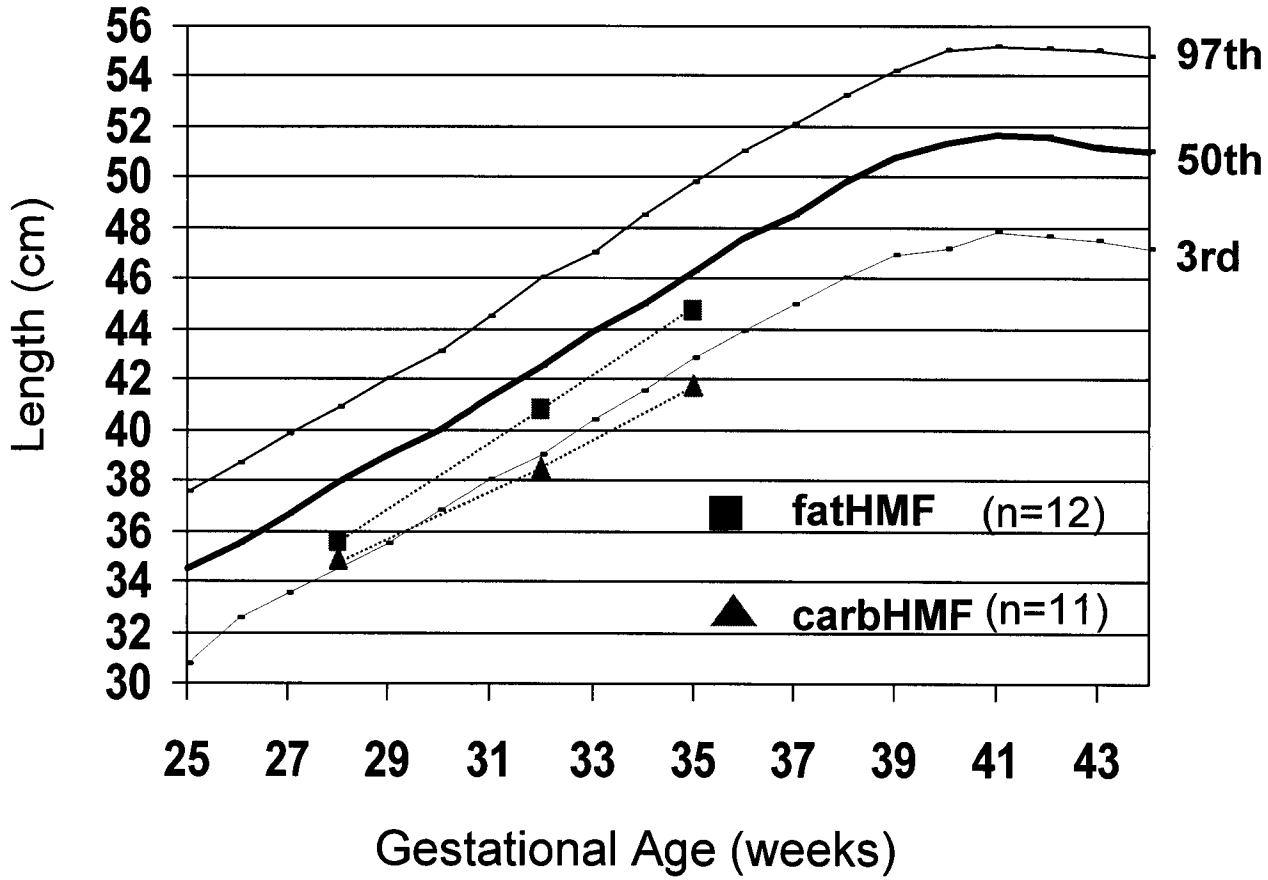
For mean lengths, **Figure 17** shows that at Baseline, the fatHMF group was growing between the 3rd and 25th percentiles and the carbHMF group was growing at the 3rd percentile (Birth length measurements were not available to compare to Baseline lengths as they are not routinely measured, but assuming that little linear growth is likely to occur during the first few days of life, we predict that mean birth length measurements would be at approximately the 25th percentile). By Phase 2, the mean length of the fatHMF group was growing along the 25th percentile, in contrast, the carbHMF infants were falling off the 3rd percentile. Falling off the curve suggests that the infants in the carbHMF group are growth stunted and not gaining length in proportion to weight. Also, this was further suggested by comparing the PI for both groups **(Figure 18)**. The fatHMF group grew from below the 25th percentile at Baseline to between the 25th percentile and 50th percentile by Phase 2. In contrast, the carbHMF group had a substantial shift in growth percentiles from below the 25th percentile at Baseline to above the 50th percentile by Phase 2. This corresponded to a change in PI from Baseline to Phase 2 that was 1.5

Figure 16. Mean Body Weight Percentiles at Baseline, Phase 1, and Phase 2



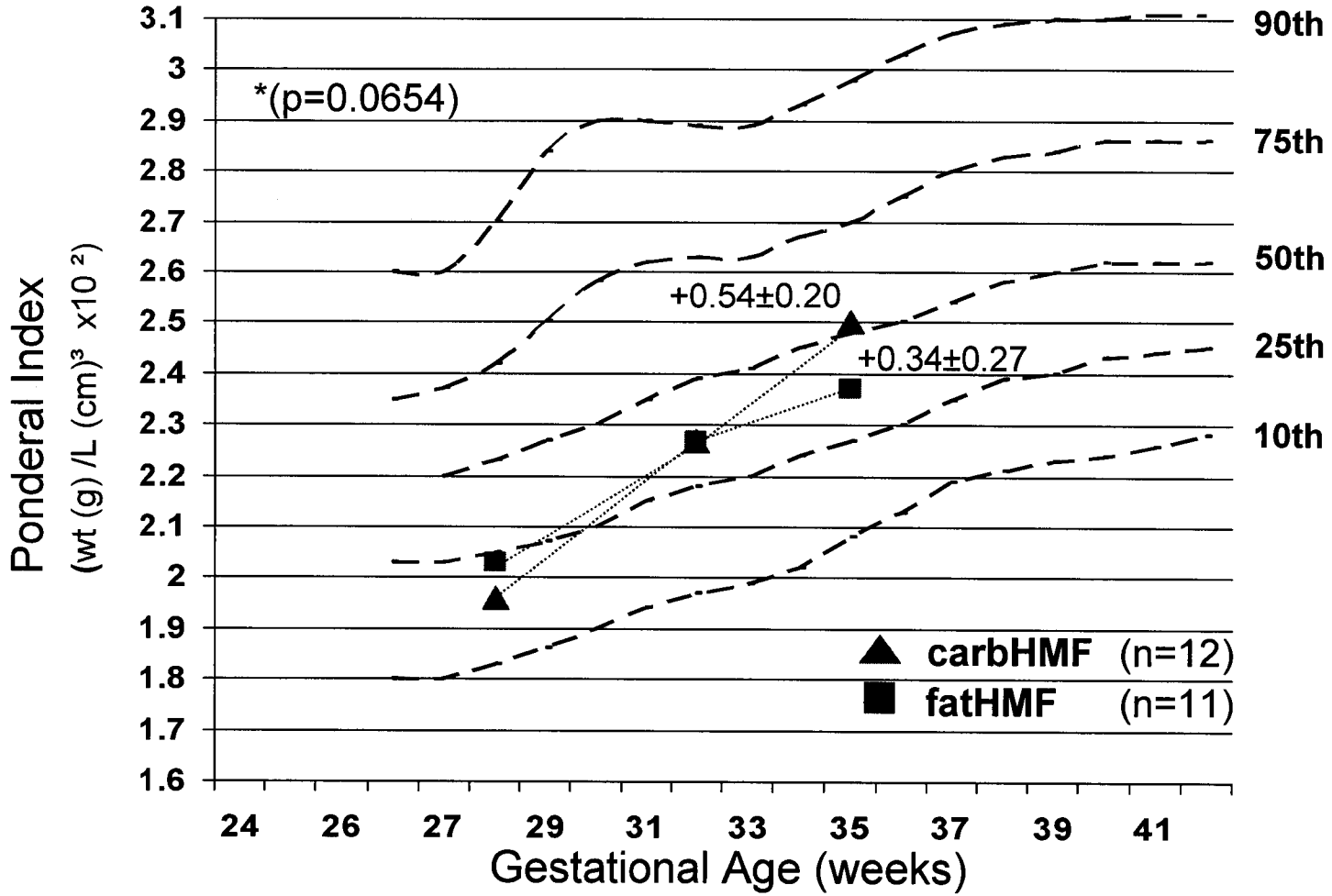
Intrauterine Growth Chart: Usher *et al.* 1969

Figure 17. Mean Body Length Percentiles at Baseline, Phase 1, and Phase 2



Intrauterine Growth Chart: Usher *et al.* 1969

Figure 18. Change in Mean Ponderal Index From Baseline to Phase 2



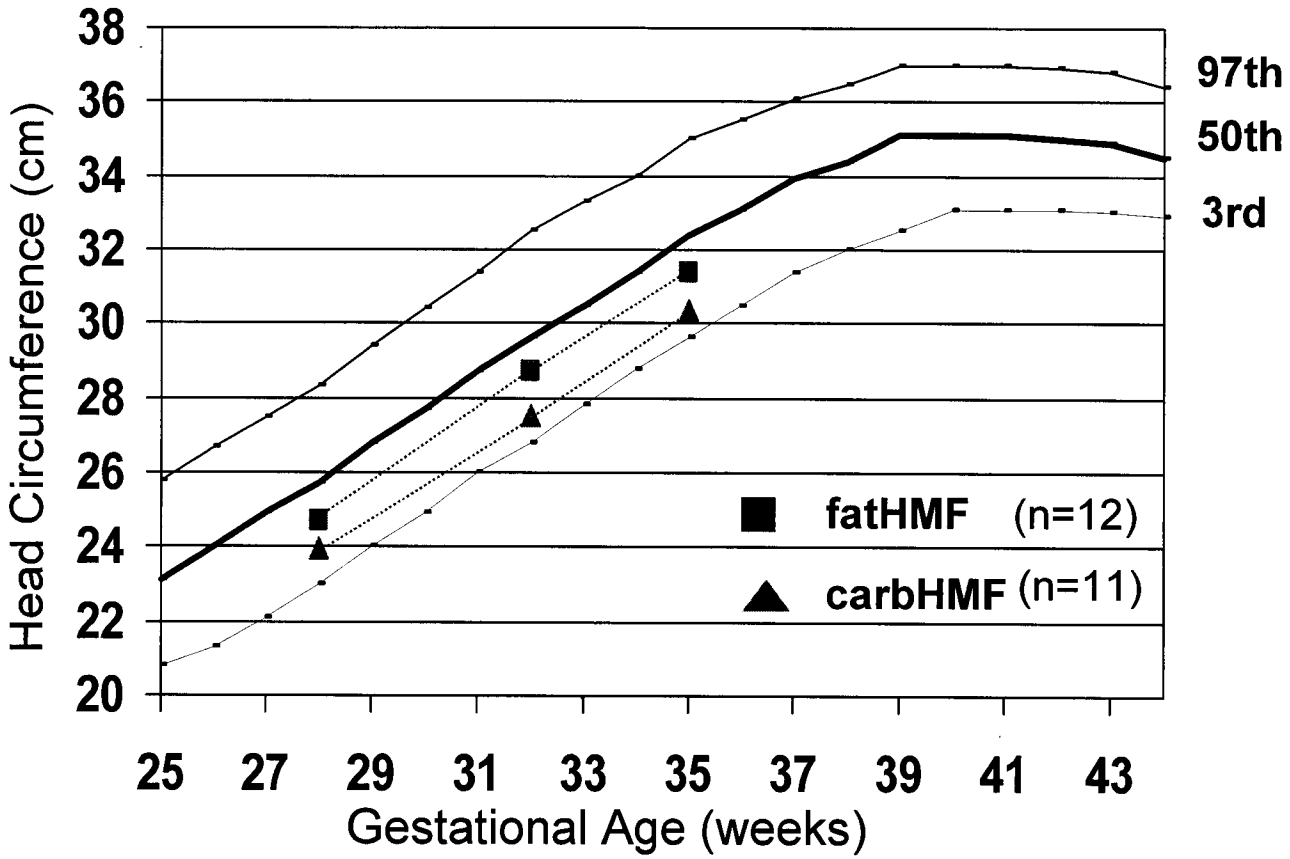
Intrauterine Growth Chart: Lubchencho *et al.* 1963

*Value not statistically significant based on p value of 0.01

(calculated as per Bonferroni's correction: α of 0.05 divided by 5 secondary outcome measures)

fold higher in the carbHMF group compared to the fatHMF group (+0.54 ±0.20 *vs.* +0.34±0.27 g/cm^3 x 10^2, respectively) that approached statistical significance (p=0.0654). For mean head circumferences, **Figure 19** shows a similar growth pattern for both groups. At Baseline, the fatHMF group was growing between the 25th and 50th percentiles and the carbHMF group was growing at the 25th percentile. By Phase 2 both groups were growing along the same percentile as they were at Baseline.

Figure 19. Mean Head Circumference Percentiles at Baseline, Phase 1, and Phase 2



Intrauterine Growth Chart: Usher *et al.* 1969

CHAPTER 4. DISCUSSION AND CONCLUSIONS

Although neither percent lean (fat) mass nor growth indices were statistically different, there was a suggestion that the fatHMF group had a lower increase in percent fat mass and a higher increase in weight and length by Phase 2. Results suggest a clinical advantage of the fatHMF to increase somatic growth in preterm infants. Supporting evidence for this conclusion, as well as, our study's potential limitations, strengths, and implications for future work are discussed below.

Primary Outcome (Percent Lean and Fat Mass): Our primary outcome was growth composition, as percent lean and fat mass. The mean percent fat mass of the fatHMF group increased from 10.20 (±2.03) at Baseline to 16.60 (±3.60) by Phase 2 (a 63% increase), whereas the carbHMF group increased from 9.37 (±3.38) at Baseline to 18.45 (±2.39) (a 96% increase). Thus, the fatHMF group appeared to have a lower increase in percent fat mass by Phase 2 compared to the carbHMF group, which suggests a clinical advantage of the fatHMF to promote higher lean mass accretion than the carbHMF.

We explored our body composition results further by doing a post hoc comparison of HMF groups based on: the change in percent fat mass from Baseline to Phase 2; absolute fat mass, lean mass, and dry lean tissue mass at Baseline and Phase 2; and the change in absolute fat mass, lean mass, and dry lean tissue mass from Baseline to Phase 2. Although the post hoc analysis should be confirmed with a larger study, it does further support the concept of a clinical advantage of the fatHMF. For example, the infants in the fatHMF group were 2.68 points higher in percent lean mass (consequently, lower in percent fat mass), or in other words, the fatHMF group were overall 15% lower in percent fat than the infants in the carbHMF group by Phase 2 (6 weeks after starting the HMF). Although not statistically significant, according to the calculation and classification of effect size by Cohen (1988), this difference in points of percent lean mass corresponds to an effect size of 0.87 (using the pooled SD=3.07 points in percent of both groups at Phase 2) and is considered large. In addition to the classification of the effect size in terms of size, it can be interpreted in terms of the percent non-overlap between the two HMF groups or can be thought of as the average percentile standing of the average infant in the fatHMF group relative to the average infant in the carbHMF

group (Cohen 1988). Therefore, the effect size of 0.87 can be interpreted as a non-overlap of 51.6% in the fatHMF and carbHMF group distributions or that the mean percent fat or lean mass of the fatHMF group is at the 82nd percentile of the carbHMF group. Moreover, based on the results for the change in absolute fat mass, absolute dry lean tissue at Phase 2, and change in absolute dry lean tissue, despite similar changes in absolute fat mass in both HMF groups, the fat HMF group: 1) showed a 42 g higher absolute dry lean tissue at Phase 2 than the carbHMF group (approached statistical significance, p=0.0647); 2) gained 30 g more in absolute dry lean tissue from Baseline to Phase 2 compared to the carbHMF group (p=0.0362). Together this data suggests that there was an overall higher lean mass accretion in the fatHMF group by Phase 2.

Furthermore, consistent with the suggestion of a higher percent lean mass and higher absolute dry lean tissue accretion observed in our post hoc analysis in the fatHMF group infants compared with the carbHMF group, was the larger (yet, statistically non-significant) increase in our secondary outcomes of weight and length seen in the fatHMF group compared to the carbHMF group. These growth indices will be discussed in more detail later, but are relevant here, as, weight and length are strong predictors of lean mass during infancy (Koo *et al.* 2000, deBruin[2] *et al.* 1995) with length becoming the dominant predictor with increasing postnatal age and weight remaining a significant predictor as weight and length are collinear (Koo *et al.* 2000).

Our growth composition data is clinically relevant. Firstly, it suggests that it may be possible to affect the body composition of the preterm infant over time with subtle changes in the proportion of non-protein energy intake as carbohydrate and fat during admission to the NICU (i.e. showing a cumulative effect). The mechanisms require further study. Although, insulin was not measured, we speculate that the carbohydrate intakes of both groups (12.50±0.56 and 13.39±0.74 g/kg/day in the fatHMF and carbHMF group respectively) would stimulate insulin secretion within the higher physiological range of serum insulin levels of 11-22 μU/ml published for human neonates (Poindexter *et al.* 1998). Based on work, previously reviewed by O'Connor *et al.* (2003) in neonatal piglets, this insulin range corresponds to the plateau in maximum rates of protein synthesis. So, assuming that human neonates would react similar to neonatal piglets, we expect that there would be no further increase in protein synthesis

stimulated by increases in insulin due to the higher carbohydrate intake in the carbHMF group, and that an increase in insulin would stimulate *de novo* lipogenesis, increase energy expenditure, therefore limit energy availability for protein synthesis. In contrast, the lower carbohydrate intake in the fatHMF group would lead to lower insulin levels, a lower rate of *de novo* lipogenesis, and increase energy available for protein synthesis, thus promote higher lean mass accretion. If we consider the energy required to deposit a higher lean mass in the fatHMF group, this seems possible. For example, based on our post hoc analysis of the change in dry lean tissue from Baseline to Phase 2, the fatHMF group gained 30 g more in absolute dry lean mass over about 50 days (approximate time from Baseline to Phase 2) or 0.6 g/day. Based on the published metabolic cost of protein gain of 10 kcal/g of protein deposited (Micheli *et al.* 1993), only 6 kcal/day more energy would need to be directed towards protein gain in the fatHMF group compared to the carbHMF group. Total energy expenditure data may help to clarify this. We included the measurement of total energy expenditure using the Doubly Labeled Water method validated for use in preterm infants (Jones *et al.* 1987, Jensen *et al.* 1992, Roberts *et al.* 1986) during our research and originally planned to include it in this thesis. However due to the length of time required for analysis of samples, this work will be reported elsewhere.

Our study demonstrates the importance of longer study periods to show a cumulative effect on growth composition, compared to other work reviewed (Bresson *et al.* 1991, Salas-Salvadõ *et al.* 1993, Nose *et al.* 1987, Chessex *et al.*1989, Kashyap[1, 2] *et al.* 2001, Pencharz *et al.* 1989, Rubecz *et a*l. 1981, Pereira *et al.*1994, Van Aerde *et al.* 1988, Pineault *et al.* 1988) that used nitrogen balance and stable isotope techniques to study protein metabolism. These methods study short time periods, therefore can be influenced by momentary changes in metabolism and cannot demonstrate a net effect on body composition.

In addition, the growth composition (percent fat/lean mass) achieved by the infants in the fatHMF group was closer to in-utero (Widdowson 1972), the generally advocated goal for the growth composition of preterm low birth weight infants (CPS 1995). Infants in the fatHMF group were 16.60± 3.60 % fat mass by Phase 2 of our study (at approximately 35 weeks corrected GA) compared to the fetus of similar GA of approximately 7-8 % fat

(Ziegler *et al.* 1976, Widdowson 1972) and the term infant at birth of approximately 15% (Widdowson 1972) to 16% fat (Atkinson *et al.* 1994). However, whether or not the growth composition of infants observed in our study is sustained by term corrected age, or into childhood, requires further study.

Furthermore, the lower percent fat mass observed in the fatHMF group compared to the carbHMF group may have metabolic implications later in life. For instance, studies in obese children (Sudi *et al.* 2001) and adults (Ross *et al.* 2000, Volek *et al.* 2002, Siervogel *et al.* 1998), have demonstrated that an approximately 7-10% reduction overall in percent fat mass is associated with a decreased risk for cardiovascular disease. This was indicated by a change in metabolic parameters, such as significantly decreased abdominal/truncal obesity (Sudi *et al.* 2001, Volek *et al.* 2002, Ross *et al.* 2000), insulin levels (Sudi *et al.* 2001), improved lipid profile (Sudi *et al.* 2001, Siervogel *et al.* 1998, Volek *et al.* 2002), glucose disposal and blood glucose levels (Ross *et al.* 2000). Notably, this decrease in overall percent fatness in those studies was of a lower magnitude than the approximately 15% lower absolute percent fat mass in the fatHMF group compared to the carbHMF group achieved in our study. This underscores the relevance of our study findings. As, low birth weight is associated with childhood (Walker *et al.* 2002) and adult obesity (Schroeder *et al.* 1999), particularly centrally distributed obesity. Moreover, obesity early in life has been shown to increase the risk of later life obesity (Zack *et al.* 1979, Roche *et al.* 1982, Charney *et al.* 1976, Garn *et al.* 1985, Freedman *et al.* 1987). Overall, the mechanisms involved are not clear (Lucas *et al.* 1999). So, based on the current state of knowledge, it seems prudent to take a preventative approach as early in life as possible to prevent excess adiposity in low birth weight infants. Feeding regimes for low birth weight infants that promote lean mass accretion during infancy, such as that suggested by the fortification of breast milk with the fatHMF in our study, seem fundamental to this and should be pursued.

Secondary Outcome (Growth Indices): Our secondary outcome of growth indices also suggests a clinical advantage of the fatHMF to promote somatic growth. As, although not statistically significant, the infants in the fatHMF group the fatHMF group increased in mean body weight from 952(±238) g at Baseline to 2146(±500) g by Phase 2 (a weight gain of 1194 g), whereas, the carbHMF group increased in weight from 863(±304) g at

Baseline to 1868(±503) g by Phase 2 (a weight gain of 1005 g). This suggests that the fatHMF group gained ~189 g more in weight (1194 g – 1005 g) than the carbHMF group by Phase 2. Also, the fatHMF group increased in mean body length from 35.9(±2.8) cm at Baseline to 44.7(±3.5) cm by Phase 2 (a length gain of 8.8 cm), whereas, the carbHMF group increased in length from 34.9(±3.1) cm at Baseline to 41.8(±3.3) cm by Phase 2 (a length gain of 6.9 cm). This suggests that the fatHMF group gained ~1.9 cm (8.8 cm – 6.9 cm) more in length by Phase 2. And as mentioned, weight and length are strong predictors of lean mass during infancy (Koo *et al.* 2000, deBruin[2] *et al.* 1995) with length becoming the dominant predictor with increasing postnatal age and weight remaining a significant predictor as weight and length are collinear (Koo *et al.* 2000).

We also compared growth indices using population specific intrauterine growth standards (Usher *et al.* 1969). This comparison suggests that the infants in the fatHMF group maintained growth velocity in weight and length, whereas the carbHMF group had a reduced growth velocity compared to that expected based on birth percentiles. For example, at birth, the mean weights of both groups of infants were between the 25th and 50th percentile and AGA. But, at the Baseline weight measurement (taken on average at 4-6 days of life), the mean weights of the infants in the fatHMF group was at the 25th percentile and the carbHMF groups was between the 3rd and 25th percentile (Note this lower percentile for weight at Baseline compared to weight at birth reflects the physiological weight loss due to fluid redistribution, that occurs in preterm infants during the first few days of life (Bauer *et al.* 1991). But, by Phase 2 (6 weeks after starting the study HMFs), the fatHMF group grew in weight between the 25th and 50th percentile, so maintained growth velocity for weight as expected based on birth weight. In contrast, the carbHMF group, by Phase 2, stayed between the 3rd percentile and 25th percentile for weight, so decreased more in percentile compared to its percentile for weight at birth.

Regarding mean length growth, the birth lengths of infants were not routinely measured for most infants. Thus, this does not allow comparison of later length growth to birth length. But, assuming that the length at birth was similar to that which we obtained within the first few days of life at Baseline, suggests that the mean birth length for both HMF groups were at approximately the 25th percentile. By Phase 2 of our study, the mean length of the fatHMF group was at the 25th percentile. In contrast, the carbHMF infants

were below the 3rd percentile by Phase 2, so appear growth stunted compared to the fatHMF group.

As well, analysis of the weight for length data using Ponderal Index (PI), an index of growth and adiposity used during infancy (Lubchencho *et al.* 1963, Yau *et al.* 1992, Miller *et al.* 1971), is consistent the suggestion from our body composition data that the fatHMF group was leaner compared to the carbHMF group. The infants in the carbHMF group showed a substantial change in PI from Baseline to Phase 2 compared to the infants in the fatHMF group (+0.54 ±0.20 vs. +0.34±0.27 $g/cm^3 \times 10^2$ in the carbHMF and fatHMF groups respectively), and this approached statistical significance (p=0.0654). When plotted on intrauterine growth charts for PI (Lubchencho 1963), this corresponded to a substantial shift in growth percentiles for PI in the carbHMF group from below the 25th percentile at Baseline to above the 50th percentile by phase 2. In contrast, the fatHMF group grew from between the 10th and 25th percentile at Baseline to between the 25th percentile and 50th percentile. So, although PI does not directly measure body composition, the larger increase in PI and the lower length gain in the carbHMF group compared to the fatHMF group suggest that the carbHMF group gained more fat mass relative to lean mass (Yau *et al.* 1992, Miller *et al.* 1971).

Our findings for mean weight, length, and PI plotted comparably to findings of the study of Barrett Reis *et al.* (2000) which studied the same carbHMF as used in our study. So, despite the relatively small sample size in our study, this adds credibility to our growth data for infants in the carbHMF group. However, we could not compare our findings for the infants in the fatHMF group, as there our no other studies that have investigated this HMF in relation to growth outcome. Whether, the differences in growth observed would be sustained requires a follow-up study.

We did not observe any differences or trends in our knee–heel length or our head circumference measurements. This may be because our sample size was not large enough to detect a difference, or alternatively for head circumference we may not have detected a difference between HMF regimes because of a head or brain sparing effect that has been described previously in neonates that demonstrated 1) a tendency for preterm infant head growth to grow along a higher growth percentile, despite poorer growth in weight and length (Berry[2] *et al.* 1997) and 2) a far smaller reduction in total brain weight (12%) than

expected, despite a marked deficit in body weight (50%) in undernourished neonatal rats (Freedman *et al.* 1980).

Potential Study Limitations: We recognize that there are potential limitations to our study and that our results must be interpreted in relation to these.

Firstly, our study sample size was small, so it limits our ability to detect a statistically significant difference (Kramer 1988, Browner *et al.* 2001). For example, the calculated total sample size for this study was 32. This was based on a standard deviation and difference of 4 points in percent fat or lean mass (i.e. an effect size of 1), α=0.05, ß=0.20, and two sided hypothesis. The effect size used was an estimate, as there were no studies that assessed body composition during NICU admission using appropriate body composition techniques in preterm infants fed fortified breast milk on which to base our calculation of sample size. However, as mentioned, the actual effect size observed in this study was 0.87. So, a total sample size of 66 subjects (α=0.05, ß=0.20, two sided hypothesis) (Kramer 1988) would be needed to show that this effect size was statistical significant. In our study, 29 subjects completed Phase 1 (3 weeks after initiating the study HMFs) before hospital discharge, yet only 23 of 29 reached Phase 2 (6 weeks after initiating the study HMFs) before hospital discharge. Therefore, our small sample size increases the probability that the observed clinical advantage of the fatHMF compared to the carbHMF occurred due to chance (random error) (i.e. increased the probability of committing a type I error (rejecting the null hypothesis that is actually true) (Browner *et al.* 2001), or falsely inferring that there is a difference between the HMF groups). Also, an inadequate sample size increases the probability of a type II error (ß) or failing to reject the null hypothesis of no difference (Browner *et al.* 2001). Our statistical power (1-ß) was reduced by 10% (from 80% to about 70%) by Phase 2 (Kramer 1988). In other words, we may not have been able to detect a statistically significant difference that could be actually true in this preterm infant population. However, despite the small sample size, our results, although not overall statistically significant, consistently support a higher lean mass accretion in the fatHMF group compared to the carbHMF (i.e. due to the higher percent lean mass, and significantly higher dry lean mass (shown in our post hoc analysis), as well as the higher weight and length in the fatHMF group). Therefore, we speculate that the observed clinical advantage of the fatHMF to increase lean mass

accretion was not due to chance, and it would have been considered statistically significant if the sample size was larger (i.e. around 66). But, this should be confirmed in a study with a larger sample size.

Furthermore, our study inclusion criteria included the infants ≤32 weeks GA at birth so only 23/29 completed Phase 2 before discharge. Including only infants ≤30 weeks GA in future studies may increase the sample size of infants completing the study protocol to Phase 2 (6 weeks after starting the HMFs) when we observed the largest difference in growth quality between the our study HMFs.

Another potential limitation of our study was related to the lack of investigator control over the feeding of formula and parenteral nutrition. While not statistically significant, the fatHMF group received proportionally less of total enteral energy intake per kilogram per day as fortified breast milk (0.733±.0326 *vs.* 0.880 ± 0.162), consequently proportionally more of their total enteral energy intake per kilogram per day from formula compared to the carbHMF group (0.267±0.326 vs. 0.120± 0.162). As well, the fatHMF group received a larger proportion of their total energy intake as parenteral nutrition than the carbHMF group (0.270±0.270 vs. 0.136± 0.144, respectively). This was beyond our control during our study and did not seem to be related to more intolerance to the fatHMF compared to the carbHMF, as none was reported. It seemed to be related to breast milk availability and variations in the duration of parenteral nutrition prescribed by the attending physician. For example, it is part of the NICU feeding protocol to feed all preterm infants, preterm infant formula when breast milk is not available. Also, parenteral nutrition is prescribed by the attending physician, so that when and how long an infant receives parenteral nutrition has the potential to be somewhat subjective. We expect that a larger sample size would have increased the likelihood of more comparable groups in this regard.

So what could be the possible effect of the fatHMF group receiving slightly more formula and parenteral nutrition than the carbHMF group? There are no published studies (to our knowledge) that have specifically compared the body composition and growth achieved with our current HMF formulations to that achieved with formula and/or parenteral nutrition in preterm infants. But there are studies in preterm formula fed infants that may provide insight. For instance, our data (unpublished, in progress) involving AGA preterm

infants (n=5) using the same feeding protocol as our study herein, but fed preterm formula, showed the infants to have a similar increase in percent fat mass yet lower overall growth gain than the fatHMF infants by Phase 2 (increase in percent fat mass of 62% *vs.* 63%, weight gain of 1114 g *vs.* 1194 g, and length gain of 6.2 cm *vs.* 8.8 cm, in the preterm formula group *vs.* fatHMF group, respectively). Despite the small sample size, our data for formula fed infants is consistent with a study by Wauben *et al.* (1998) involving AGA preterm infants (n=12) fed preterm formula. They showed that the infants at term corrected age had ~20% mean fat mass and at discharge (~37.5±1.7 weeks corrected age) mean weight was at the 3rd and mean length was below the 3rd, when plotted intrauterine growth standards (as per Usher *et al.* 1969). This contrasts our fatHMF group who had 16.60±3.60 mean percent fat mass and growth in weight and length at approximately the 25th -50th and 25th percentiles, respectively, by Phase 2 (~ 35 weeks mean corrected age). However, whether this would be maintained by term corrected age is unclear. Another study (Berry[2] *et al.* 1997) was carried out in the same NICU, involving a similar AGA preterm low birth weight infant population (n=109), who were followed for a similar period of time as our study (~8 weeks), but were fed a combination of parenteral nutrition and preterm infant formula. These infants were described, as having lower weight, length, and head circumference at 8 weeks of life, than at birth, with length being the lowest (mean Z scores of -0.32 at birth, but -2.24 by 8 weeks of life). Again, this data contrasts our AGA infants in the fatHMF group as they appeared to have similar growth percentiles at Phase 2 compared to birth. In summary, we reason that if our fatHMF infants had consumed less formula (i.e. the same formula intake as the carbHMF group) that the true increase in: 1) the percent fat mass for the fatHMF group is likely similar to what we observed, thus, the effect size should be similar and still in favour of the fatHMF group; 2) the true increase in weight and length growth for the fatHMF group is likely higher than what we observed, thus, the effect size should be even higher and still in favour of the fatHMF group.

Also, related to the slightly higher formula and parenteral nutrition intake of the fatHMF group compared to the carbHMF, is that although the HMF groups had similar mean total fluid intake per kilogram per day, the mean total energy intake was slightly lower (although not statistically significant) in the fatHMF group (115.42±38.04 kcal/kg/day)

compared to the carbHMF group (121.19±9.78 kcal/kg/day). This was due to the slightly lower energy content of preterm formula (81 kcal/100ml, Ross 2001) and the lower energy intake prescribed during parenteral nutrition compared to what we calculated for breast milk fortified with the fatHMF based on published values for the energy content of preterm breast milk (CPS 1995) and the fatHMF (Mead Johnson 2000). This may have led to some confounding, as, lowering energy intake can also decrease percent body fat in preterm infants compared to an isonitrogenous regime of higher energy (Van Goudoever *et al.* 2000). However, given that our data suggested that the fatHMF group, had higher weight and length gain, in addition to lower percent fat mass, compared to the carbHMF group does favour our study hypothesis that lean mass accretion would increase with consumption of a HMF containing non-protein energy predominantly as fat. We would expect that if the higher percent fat mass we observed in the carbHMF group was simply a matter of excess energy intake being deposited as fat, we would have at least observed a similar weight and length in the carbHMF group compared to the fatHMF group. Despite this, the carbHMF group was lower in weight and length than the fatHMF group.

Another issue to consider is that although comparable, vitamin and mineral contents of the HMFs were not identical. However, it is unlikely that this would have explained the observed higher percent lean mass and growth in the fatHMF group compared to the carbHMF group. Overall, both HMFs met the P-RNI (CPS 1995) after fortification with EBM. An exception is iron which was lower in the carbHMF and did not meet the P-RNI after fortification. However, both HMF groups received supplemental liquid iron to meet the P-RNI (CPS 1995) as part of the routine feeding protocol in the NICU. As well, all micronutrients did not exceed the lower or upper limits of tolerance for any micronutrient when fed at typical volumes of intake (Health Canada 1995). In fact, micronutrients, although not identical, were quite comparable (including iron as this was supplemented in both groups to meet the P-RNI), or on lower end of the P-RNI in the fatHMF group compared to the carbHMF group. So, if this had limited growth, we would expect to have seen poorer growth in the fatHMF group compared to the carbHMF group. In contrary, our data suggests that the growth was better in the fatHMF than the carbHMF group.

Also, important to consider are the different calcium sources contained in the fatHMF and the carbHMF and the potential to affect growth. The fatHMF contains 35% soluble

calcium and 65% insoluble calcium (Mead Johnson 2000), whereas the carbHMF contains essentially 100% insoluble calcium (Ross 2001). Schanler and colleagues (Schanler[1] *et al.* 1988, Schanler[2] *et al.* 1999) have proposed that soluble calcium contained in HMFs undergoes saponification with the human milk fat during fortification, and consequently decreases the fat and energy bioavailability in the fortified breast milk. However, this has not been directly studied and the specific nature of this reaction is not clear. As well, the speculation of the saponification of soluble calcium with human milk fat involved a HMF that contained 100% soluble calcium (Schanler *et al.* 1995). But, the fatHMF in our study contained 35% soluble calcium and if it had undergone saponfication with the human milk fat, we would expect to have observed poorer growth in the fatHMF group compared to the carbHMF group. Yet, the fatHMF group appeared to have better growth. Therefore, it seems that the fat and energy in the breast milk fortified with the fatHMF was very bioavailable. Our data (pending) involving total energy expenditure may help to confirm this.

Another issue to consider is that using a body composition method, such as BIA, which is based on total body water to estimate lean mass, could potentially over or under estimate lean mass. Although critically ill preterm infants are vulnerable to fluctuations in body water, particularly in the ECW compartment (Bauer *et al.* 1991) and the percent lean mass as water changes with GA (Widdowson 1972), both of our study groups appeared to be comparable. Both groups had similar total fluid intake, clinical status, and diuretic use. Also, a percent lean mass as water factor based on GA was included in the calculation of lean mass. Therefore, it is unlikely that over-hydration or errors related to differences in hydration in lean mass based on GA would explain why we observed a higher percent lean mass in the fatHMF group compared to the carbHMF group. Measurement of ECW, using either bromide dilution or BIA that uses the reactance component of impedance to measure ECW (Mayfield *et al.* 1991) would have been helpful to verify this, but these technologies were not available to us at the time of our study.

Another potential limitation is that we did not analyze the breast milk consumed by the infants in our study, but used published values for the nutrient content of preterm breast milk. The energy contribution of carbohydrate in human milk is fairly constant, but, the milk fat is more variable (Wang *et al.* 1999). Milk fat can vary between mothers and

within mothers (i.e. foremilk contains less fat than hindmilk) (Schanler[1] *et al.* 1999). As well, the protein concentration in preterm human milk decreases through lactation (Schanler *et al.* 2001). We acknowledge that it would have been optimal to analyze the breast milk; however, the challenges we encountered made it unethical and technically impossible at the time of our study. For instance, we attempted to sample the milk, but frequently there was just enough of the mothers' milk for the infants. So, taking breast milk samples, of the volume required (at least 15 ml) using currently available breast milk analysis techniques to analyze the protein (Bradford 1986), carbohydrate (Wang *et al.* 1999, Svennerholm 1956) and the fat content of breast milk (Swift *et al.* 1992), would have taken breast milk away from the infants and was not ethically feasible. We also investigated the possibility of alternative breast milk analysis techniques that required much smaller milk volumes of breast milk (i.e. 0.5-1mls) by consulting both the literature and experts in the field. But, at the time of our study there were no such techniques developed, so breast milk analysis was technically not possible. However, our study was blindly randomized. So, randomization should minimize the differences in the nutrient content of the breast milk between our HMF groups (Cummings *et al.* 2001).

Study Strengths: Despite the aforementioned limitations of our study, it has several distinct strengths. It was a double blinded, randomized clinical trial, so this minimizes confounding and prevents investigator bias during sampling or measurements (Browner *et al.* 2001).

Also, it was the first study to use an appropriate body composition method to assess the effects of HMFs on growth composition, specifically the first to study the effect of non-protein energy source in HMFs on the growth composition of preterm infants fed fortified breast milk. Also, our study spanned the NICU admission from near the first week of birth until discharge. Thus, this increases the likelihood of showing a net or cumulative effect of our nutritional regime. This contrasts other studies we reviewed Bresson *et al.* 1991, Salas-Salvadõ *et al.* 1993, Nose *et al.* 1987, Chessex *et al.*1989, Kashyap[1,2] *et al.* 2001, Pencharz *et al.* 1989, Rubecz *et a*l. 1981, Pereira *et al.*1993, Van Aerde *et al.* 1988, Pineault *et al.* 1988) which were quite contradictory in relation to their support for the effects of non-protein energy source on lean mass accretion, as they were quite short in comparison and were isotope studies or nitrogen balance studies, thus could only measure

a short period in time and could not demonstrate a cumulative net effect. Moreover, as discussed in detail above, although not statistically significant, our results are clinically relevant.

As well, despite the potential heterogeneity of the preterm infant population (Sherry *et al.* 2003, Ehrenkranz *et al.* 1999, Berry[1] *et al.* 1997) our study subjects were all AGA and fairly homogeneous based on the subject characteristics measured and biochemical indicators of nutritional status measured. Thus, our groups were clinically comparable and it is unlikely that any of these variables would have contributed to the differences in body composition and growth observed between the HMF groups.

Implications for Future Work: Future work should involve a larger sample size and investigate mechanisms involved. This could include measurement of various metabolic parameters associated with growth (i.e. insulin, insulin-like growth factors, growth hormone, glucagon (Davis *et al.* 1998), cortisol (Baxter *et al.* 1987, McMahon *et al.* 1988 and/or leptin (Ostlund *et al.* 1996), but not yet characterized in the context of non-protein energy source and the feeding of preterm infants. It would also be of interest, given the increase in energy expenditure associated with increases in *de novo* lipogenesis (Bresson et al. 1989, Pierro *et al.* 1989, Jones *et al.* 1993), to measure total energy expenditure on the larger sample size. The measurement of energy expenditure may also give us some indication of any differences in the bioavailability of the HMFs. A per protocol analysis of the data to test the efficacy of the treatment HMFs would also be of interest. As well, it would be relevant to track the cohort of infants in each HMF group to determine if, or how, the body composition differences observed in our study are sustained during infancy and childhood, and how metabolic parameters are affected. Finally, it would be interesting to pursue a study comparing HMFs of our own design that contain a higher proportion of non-protein energy as fat or as carbohydrate, as based on our findings, this may potentially show a larger treatment effect.

CHAPTER 5. FINAL SUMMARY AND SIGNIFICANCE

Although neither percent lean (fat) mass, nor growth indices, were statistically different, there was a suggestion that the fatHMF group had a lower increase in percent fat mass and a higher increase in weight and length by Phase 2. As, by Phase 2 infants receiving the fatHMF showed a 63% increase in percent fat mass, gained 1194 g in weight and 8.8 cm in length, whereas the carbHMF showed a 96% increase in percent fat mass, gained 1005 g in weight and 6.9 cm in length ($p=0.3586$, 0.3815, and 0.1851 respectively). Furthermore, post hoc analysis showed that the fatHMF group gained 30 g more in absolute dry lean tissue from Baseline to Phase 2 compared to the carbHMF group ($p=0.0362$). Differences of this magnitude are clinically important, but a larger study is required to demonstrate statistical significance. Our results favour our hypothesis to the extent that they suggest a clinical advantage of fortifying human milk with a HMF containing non-protein energy predominantly as fat (fatHMF) compared to a HMF containing non-protein energy predominantly as carbohydrate (carbHMF) to increase somatic growth closer to in-utero goals in AGA preterm low birth weight infants during admission to the NICU. Our preterm infant population was homogeneous in terms of clinical characteristics, so it is unlikely that this would have explained our findings.

Our work has clinical significance as it helped to show how a subtle decrease in the proportion of non-protein energy as carbohydrate contained in a HMF may promote, over time, growth composition that more closely meets in-utero growth goals of growth composition in preterm infants fed fortified breast milk. This helped to better define the HMF formulations that may optimize growth and to gain insight into the potential mechanisms by which growth can be optimized that could be pursued in future work. Our work has relevance to future research on the long term outcome of low birth weight infants, as breast milk fortification with a HMF containing non-protein predominantly as fat may help to decrease the risk of later adiposity and improve the metabolic risk profile of low birth weight infants. Further study involving a larger sample size is required and warranted to confirm our findings, explore the mechanisms involved, follow our study population to if, or how, the suggested differences in body composition and growth between the HMF regimens are sustained, and to investigate metabolic effects.

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APPENDICES