

TARGET FORTIFICATION FOR PRETERM INFANTS

TARGET FORTIFICATION OF BREAST MILK WITH PROTEIN, CARBOHYDRATE, AND
FAT FOR PRETERM INFANTS IMPROVES GROWTH OUTCOMES:
A DOUBLE-BLIND RANDOMIZED CONTROLLED TRIAL

By: AKSHDEEP SINGH BHATIA, BHSc

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TITLE: Target fortification of breast milk with protein, carbohydrate, and fat for preterm infants
improves growth outcomes: A double-blind randomized controlled trial

AUTHOR: Akshdeep Singh Bhatia, BHSc (McMaster University)

SUPERVISOR: Dr. Christoph Fusch

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Abstract

Background: Breast milk is an ideal source of nutrition for newborns as it provides nutrients required for growth in addition to numerous bioactive factors which help to develop an infant's immune system. However, the macronutrient content of breast milk alone is not able to support preterm infant's rapid growth needs and requires supplementation with fortifiers. There is strong evidence that the current practice of standard fortification (SF) may lead to nutritional deficits and consequently increases an infant's risk of inadequate postnatal growth. Furthermore, the natural variation of breast milk composition makes it increasingly difficult to provide recommended macronutrient intakes with the SF. Individualized approaches, like adjustable fortification or target fortification (TFO), have been proposed to improve growth during hospitalization. A recent pilot trial demonstrated that TFO, which individually adjusts deficient macronutrient content after SF by analyzing the breast milk for native protein, carbohydrate and fat, is feasible in clinical practice and significantly reduces variation of macronutrient intakes.

Objectives: To compare the response of preterm infants to feedings of breast milk with either SF or SF+TFO with respect to: 1) weight at 36 weeks' post-menstrual age and growth velocity during hospitalization; 2) head circumference, length and body composition; and 3) the relationship between preterm infant's weight or growth velocity and their macronutrient intake factors including protein intake and protein:energy (P:E) ratio.

Methods: This was a single-center, double-blind randomized controlled trial completed at McMaster Children's Hospital's Level III NICU with a study period of at least 21 days. Preterm infants (n=103) born at <30 weeks of gestation and tolerating full enteral intakes of breast milk were enrolled and randomized to the Control (SF only) or Intervention (SF+TFO) groups. Native

breast milk samples were collected for all infants on each study day and were analyzed for protein, carbohydrate and fat content. In the Control group, SF was provided using Enfamil (Mead Johnson, IL) human milk fortifier at the recommended dosage. In the Intervention group, after the addition of SF, modular macronutrient fortifiers were added based on analysis of the mother's milk to reach target values based on ESPGHAN recommendations. Adjustment of the modular fortifiers was done three times per week. The primary outcomes were weight at 36 weeks' PMA and growth velocity during the study period. Head circumference, length and body composition were also assessed at term-equivalent age. Subgroup analysis, stratified around the median protein levels after SF, also compared the growth outcomes between Control and Intervention groups. Multiple regression analysis models examined the effect of macronutrient intake factors and infant characteristics on weight, average growth velocity and daily weight gain.

Results: Infants fed with SF+TFO had significantly higher protein ($p<0.001$), carbohydrate ($p<0.001$) and fat intakes ($p<0.01$) in addition to higher protein:energy and carbohydrate:non-protein energy (CHO:NPE) ratios ($p<0.001$) compared to those fed with SF alone. The average weight at 36 weeks' PMA and growth velocity during the 21-day study period were higher for infants in the Intervention group ($p<0.001$). The Intervention group had significantly higher fat-mass ($p<0.05$) as well as more fat-free mass than the Control group at term-equivalent age (TEA), but were still within normal limits when compared to normative data from our NICU. At TEA, infants fed with TFO also showed significantly higher change in z-scores from birth for length when compared to infants fed SF with low-protein intakes ($p<0.05$). Change in head circumference z-scores were not statistically significant between groups. Higher average protein intakes and P:E ratios were each positively associated with higher weight at 36 weeks' PMA

($p < 0.05$). Moreover, higher daily weight gain was positively associated with higher daily protein intake from the previous study day ($p < 0.05$). The absolute difference in day-to-day macronutrient intakes, however, were not significant predictors of daily weight gain.

Conclusions: This study shows that target fortification of breast milk is promising as an individualized approach to improve the quality of nutrition for preterm infants. By addressing the variation and deficits of macronutrients that occur after standard fortification, infants were able to achieve higher body weight and faster weight gain. In the short term, target fortification may reduce the preterm infant's risk for sub-optimal postnatal growth. These improved growth outcomes also have positive clinical implications on infant's long-term health and development. Protein intake and the P:E ratio were identified as important factors for growth and should be considered in nutritional management and future fortification strategies for breast milk fed preterm infants.

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Table of Contents

Chapter 1. Introduction	1
1.1 Overview.....	1
1.2 Rationale	1
1.3 Objectives	2
1.4 Research Hypotheses	3
Chapter 2. Background	4
2.1 Fetal Growth and Nutrition.....	4
2.2 Postnatal Adaptations and Challenges	5
2.3 Preterm Infant Growth	6
2.3.1 <i>Anthropometrics</i>	7
2.3.2 <i>Body Composition Compartments</i>	8
2.3.4 <i>DXA and ADP for assessing Preterm Infant Body Composition</i>	9
2.3.5 <i>Clinical importance of body composition</i>	10
2.4 Preterm Infant Feeding Practices	10
2.4.1 <i>Parenteral Feeding</i>	10
2.4.2 <i>Enteral Feeding: Advantages of Breast Milk</i>	11
2.4.3 <i>Limitations of Breast Milk and Standard Fortification</i>	12
2.4.4 <i>Individualized Fortification: Adjustable vs Target</i>	13

Chapter 3. Methods	15
3.1 Study Design.....	15
3.2 Study Population.....	15
3.3 Randomization and Blinding	15
3.4 Milk Preparation	16
3.5 Fortifier Products and Dosage	17
3.5.1 Control Group Fortification	17
3.5.2 Intervention Group Fortification	17
3.6 Data Collection and Measurements	18
3.6.1 Study and Chart Data	18
3.6.2 Anthropometric Measurements	19
3.6.3 Body Composition Measurements.....	19
3.7. Analysis of Macronutrient Intakes and Growth Outcomes.....	20
3.7.1 Statistical Analyses	20
3.7.2 Regression Analyses	21
3.7.3 Sample Size.....	22
3.7.4 Missing Data	22
3.7.5 Subgroup Analysis.....	22

Chapter 4. Results	23
4.1 Study Population.....	23
4.2 Breast Milk Composition Analysis and Macronutrient Intakes.....	25
4.3 Weight and Growth Velocity comparisons.....	30
4.3.1 <i>Weight at 36 Weeks' PMA</i>	30
4.3.2 <i>Growth velocity over 21-day study period</i>	32
4.4 Anthropometric measures at term equivalent age.....	34
4.5 Body composition assessments.....	39
4.6 Regression Analysis.....	41
4.6.1 <i>Multiple Linear Regression Analysis</i>	41
4.6.2 <i>Repeated Measures Regression Analysis</i>	43
Chapter 5. Discussion	45
5.1 Target fortification improved macronutrient intakes and growth outcomes	45
5.2 Growth outcomes from the subgroup analyses.....	49
5.3 Other anthropometric measures	50
5.4 Regression models and the significance of protein on growth	51
5.5 Strengths and Limitations	53
5.6 Future Directions	55
5.7 Conclusion	55
References	57
Appendix 1. CONSORT Diagram	63
Appendix 2. Sample Fortification Recipe Sheet	64

List of Figures

Figure 1: Distribution of infants' average fat intakes during the study period – page 27

Figure 2: Distribution of infants' average carbohydrate intakes during the study period – page 28

Figure 3: Distribution of infants' average protein intakes during the study period – page 29

Figure 4: Comparison of mean weight at 36 weeks' PMA between Control and TFO groups – page 30

Figure 5: Comparison of weight at 36 weeks PMA between high-protein subgroups of the Control and TFO groups – page 31

Figure 6: Comparison of weight at 36 weeks PMA between high-protein subgroups of the Control and TFO groups – page 31

Figure 7: Comparison of average growth velocity over 21 study days between Control and TFO groups – page 32

Figure 8: Comparison of average growth velocity over 21 study days between high-protein subgroups of the Control and TFO groups – page 33

Figure 9: Comparison of average growth velocity over 21 study days between low-protein subgroups of the Control and TFO groups – page 33

Figure 10: Comparing the difference in weight z-scores for the Control and TFO groups – page 35

Figure 11: Comparing the difference in head circumference (HC) z-scores for the Control and TFO groups – page 35

Figure 12: Comparing the difference in length z-scores for the Control and TFO groups – page 36

Figure 13: A) Comparing the difference in weight z-scores for the Control and TFO low-protein subgroups. B) Comparing the difference in weight z-scores for the Control and TFO high-protein subgroups. – page 37

Figure 14: A) Comparing the difference in head circumference (HC) z-scores for the Control and TFO low-protein subgroups. B) Comparing the difference in head circumference (HC) z-scores for the Control and TFO high-protein subgroups. – page 37

Figure 15: A) Comparing the difference in length z-scores for the Control and TFO low-protein subgroups. B) Comparing the difference in length z-scores for the Control and TFO high-protein subgroups. – page 15

List of Tables

Table 1: Characteristics of study population groups – page 24

Table 2: Comparison of native breast milk content and fortified macronutrient intakes – page 25

Table 3: Comparison of body composition assessments – page 39

Table 4: Comparison of body composition assessments for the low-protein subgroups – page 40

Table 5: Comparison of body composition assessments for the low-protein subgroups – page 40

Table 6: Linear model predicting weight at 36 weeks' PMA with macronutrient intakes – page 41

Table 7: Linear model predicting weight at 36 weeks' PMA with macronutrient ratios – page 42

Table 8: Linear model predicting average growth velocity with macronutrient intakes – page 42

Table 9: Linear model predicting average growth velocity with macronutrient ratios – page 43

Table 10: Repeated measures model predicting daily weight gain – page 44

List of Abbreviations

ADP	Air Displacement Plethysmography
CHO:NPE	Carbohydrate to Non-Protein Energy Ratio
DXA	Dual-Energy X-Ray Absorptiometry
ESPGHAN	European Society for Pediatric Gastroenterology Hepatology and Nutrition
FMI	Fat Mass Index
FFMI	Fat-Free Mass Index
NICU	Neonatal Intensive Care Unit
P:E	Protein to Energy Ratio
PMA	Post Menstrual Age
SF	Standard Fortification
TEA	Term-Equivalent Age
TFO	Target Fortification
UWW	Under Water Weight

Declaration of Academic Achievement

Niels Rochow and Christoph Fusch: Developed the study design and analyzed and interpreted the data

David Pogorzelski and Geoffrey Travis: Performed recruitment and enrolled infants into the study

Akshdeep Singh Bhatia: Collected nutrition and study data, performed anthropometric and body composition measurements, analyzed and interpreted the data and wrote the thesis

Anaam Ali: Contributed to the collection of study data and performed anthropometric and body composition measurements

Augustine Nguyen and Aldin Bahonjic: Contributed to the collection of study data and performed analysis of native breast milk samples

Gerhard Fusch: Was involved in the interpretation of data and provided study support

Alex Kiss: Provided statistical support for regression models

Chapter 1. Introduction

1.1 Overview

Premature birth, defined as a gestational age at birth of less than 37 weeks, affects 15 million newborns around the world annually¹. Extreme (<28 weeks) and very preterm (28-32 weeks) account for 27% of neonatal intensive care unit (NICU) admissions in Canada². The rate of mortality of infants born prematurely has decreased markedly in the past years due to improved clinical care. However, these infants still face challenges like chronic lung disease, retinopathy of prematurity, impaired neurodevelopment and increased risk for cardiovascular and metabolic diseases in adulthood³⁻⁵. As such the focus of clinical management and research has shifted towards addressing these post-discharge and long-term morbidities. In addition to trying to improve quality of life, there is also significant economic interest in trying to prevent or manage these morbidities⁶. Improving the quality and quantity of nutrition provided during the postnatal period has become of increasing interest as strong associations have been made between inadequate nutrition, poor growth and poor long-term development⁷⁻¹⁰.

1.2 Rationale

Breast milk is an ideal source of nutrition for term born neonates as it is able to provide both macronutrients for growth and energy requirements as well as bioactive factors, like immunoglobulins and oligosaccharides, which help to confer immune protection¹¹. However, there are two major disadvantages in feeding preterm infants breast milk. First, the macronutrient content of breast milk, especially protein, is not sufficient to support the high growth rates that preterm infants should achieve. To address this deficiency, fortifiers must be added to increase the macronutrient content. Commercially available standard fortifiers are used in current routine practice and provide a fixed amount of additional macronutrients to each feed. Available

research indicates that despite providing additional macronutrients, standard fortification still fails to provide sufficient protein and energy for the preterm infant^{12,13}. The second disadvantage of breast milk is the significant inter- and intra-mother variation of macronutrient content. The standard fortification practice is also limited in overcoming this issue because it assumes a standard or an average composition of native breast milk. As such, there have been calls for alternative and individualized strategies for breast milk fortification¹⁴. Target fortification (TFO) is a novel approach where native breast milk is first analyzed in order to tailor the amount of individual macronutrient fortifiers required to supplement standard fortification. A pilot trial by Rochow and colleagues demonstrated feasibility of the method in a clinical setting and showed that infants fed with TFO experienced improved nutritive efficiency (growth per volume of intake) compared to control infants fed standard fortification¹⁵. Following the success of the pilot trial, the present study sought to compare the effect of standard fortification to target fortification on the growth of extremely premature infants in a double-blind randomized controlled trial.

1.3 Objectives

Primary Objective

The primary objective was to investigate the effect of the TFO nutrition on growth outcomes during the intervention period. This was achieved by comparing 1) the weight achieved at 36 weeks' post-menstrual age (PMA) and 2) the growth velocity (g/kg/d) experienced during the study period while also accounting for baseline study group characteristics. Comparisons were made between the control group receiving standard fortification (SF) and the intervention group receiving TFO. Comparisons were also made within low- and high-protein subgroups between infants who received SF or TFO.

Secondary Objective

The secondary objective was to investigate the effect of the TFO nutrition on anthropometrics and body composition measurements at Term Equivalent Age (TEA). This was achieved by comparing the length, head circumference, weight and results of body composition assessments. The Air Displacement Plethysmography (ADP) assessment for body composition was used with the PEAPOD device. Comparisons were made between the control group receiving standard fortification (SF) and the intervention group receiving TFO. Comparisons were also made within low- and high-protein subgroups between infants who received SF or TFO.

Tertiary Objective

The tertiary objective was to investigate the relationship between the quality of nutrition and growth outcomes during the intervention period. This was achieved by determining if the weight achieved at 36 weeks PMA and growth velocity experienced during the study period were associated with infants' energy intake, protein to energy (P:E) ratio, carbohydrate to non-protein energy ratio (CHO:NPE), birth and baseline study characteristics. The effect of day-to-day variation of nutritional intakes on growth velocity was also assessed.

1.4 Research Hypotheses

- 1) TFO will lead to higher weight at 36 weeks PMA and increased growth velocity during the study period
- 2) TFO will lead to higher length and head circumference outcomes at TEA
- 3) TFO will lead to similar distribution of fat-mass and fat-free mass at TEA
- 4) Nutritional intakes, and their variation, during the study period are predictive of the infants' weight gain

Chapter 2. Background

2.1 Fetal Growth and Nutrition

As the fetus transitions from the end of the second trimester into the third trimester, the intrauterine growth rate decreases from approximately 21 g/kg/day between 23-27 weeks' gestation to 12 g/kg/day between 35-37 weeks¹⁶. Chemical analysis from stillborn preterm infants indicates differences in lean mass and fat mass growth in late gestation. Lean mass accretion decreases from approximately 2.0 g/kg/day at the beginning of the third trimester to 1.8 g/kg/day by the end of the gestational period¹⁷. Fat mass deposition increases exponentially in preparation for birth and reaches rates of up to 1.9g/kg/day by the end of the third trimester¹⁸. Fetal energy and metabolic needs are primarily fulfilled by maternally derived glucose as well as some amino acid oxidation. These nutrients are transported to the fetus, by way of the placenta, through active and passive mechanisms.

Fetal growth is controlled by a complex exchange between the placenta's ability to supply nutrients to the fetus and the interaction between endocrine hormones in a maternal-placental-fetal axis. In this axis, insulin-like growth factors (IGFs) in maternal and fetal circulation interact to regulate the supply of glucose and amino acid uptake in response to changes in nutrient availability¹⁹. Specifically, IGF-1 has been implicated in regulating growth in late gestation and IGF-2 is thought to be involved in regulating early embryonic development²⁰. In the event of adverse conditions, the placenta itself will attempt to adapt in an effort to maintain nutrient supply for the fetus. For example, in response to undernourishment, the placenta can enlarge its surface area in order to enhance its' ability to extract nutrients from the maternal circulation²¹. Maternal nutrition is also associated with fetal growth. Analysis of birth weights of infants whose mother's were developing *in utero* during the Dutch famine of 1944-1945 and experienced severe energy restriction suggests that maternal nutrition is a factor for fetal growth

in late gestation²². Furthermore, birth weight is more closely associated with the weight of the recipient mother compared to the donor in cases of embryo transfer and the birth weights of half siblings are more correlated if the common parent is the mother^{23,24}. Together, these findings indicate that maternal size can also have a strong influence on the extent of fetal growth. In summary, the interplay between maternal, placental and fetal factors contributes to the regulation of healthy fetal growth. The event of preterm birth, however, disrupts fetal nutrition and consequently the opportunity to grow at the intrauterine potential is diminished.

2.2 Postnatal Adaptations and Challenges

The event of birth requires adaptations from multiple organ systems for the neonate to survive the transition to extrauterine life. Major postnatal adaptations that should occur include opening of the airways, decreasing pulmonary vascular resistance, as well as switching from fetal to human circulation through closure of fetal intravenous channels or shunts²⁵. Moreover, there must also be changeover from a continuous intravenous supply of glucose-based nutrition from the placenta to variable cue-based enteral feedings which are primarily composed of fats²⁶.

Cortisol, produced by the fetal adrenal glands around 30 weeks' gestation and onwards, is a key hormone which is involved in preparation for the transition²⁷. Some of the major physiological changes that are regulated by cortisol in the fetal-to-newborn transition include lung tissue maturation and the production of surfactant, clearance of fetal lung fluid and increasing the gut's digestive capacity²⁸. Preterm infants face additional challenges during their extrauterine transition because they are often born before their organ systems are prepared for these complex adaptations. For example, the immaturity of the adrenal gland leads to an attenuated cortisol activity and increased dependence on ventilation support²⁹.

The preterm neonate will also face difficulty with thermoregulation because they are lacking in

white adipose tissues, which is hypothesized to provide some insulation, and brown adipose tissue which generates heat through non-shivering thermogenesis^{30,31}. Both of these adipose tissues develop in quantity and response during the late gestational period¹⁸. Limited fat deposits and immature gluconeogenic abilities of the liver also strain the adaptation process as energy stores are rapidly depleted after birth³². Furthermore, the immaturity of a preterm infant's nervous system delays their ability to establish oral feedings and therefore places additional demands for nutritional management. The well established pattern of sucking and swallowing reflexes in coordination with unassisted breathing may not emerge until 37 weeks' PMA³³. These challenges are compounded by stressors faced by preterm infants during routine clinical care and may involve invasive respiratory and ventilation support, monitoring gastrointestinal and neurological development and tracking metabolic parameters³⁴. It is therefore of paramount importance to track their growth and development to minimize the risk and severity of long-term morbidities.

2.3 Preterm Infant Growth

Growth for preterm infants is an important clinical marker of overall health. The goal of the healthcare team is to provide support so that the preterm infant can achieve growth rates and composition similar to a fetus of similar age³⁵. Preterm infants will experience different patterns of weight gain after birth. All preterm infants, like term-born infants, experience a physiologically normal weight loss after birth due to the contraction of extracellular spaces and excretion of water³⁶.

After the birth weight is regained, there is a period of rapid weight gain that should reflect intrauterine growth rates, followed by a period of slower weight gain as term-equivalent age nears¹⁶. The quantification of weight growth over time is calculated using several different

methods throughout literature. A review of how preterm infant weight gain is being reported in literature found that some studies calculate change in z-scores, others will use absolute weight gain (g/day) and some use a growth velocity where weight gain is normalized for body weight (g/kg/day)³⁷. The review also indicated there was limited consistency of the time period for which the weight gain was calculated. The use of absolute weight gain in clinical practice and research is limiting as, for example, a weight gain of 20 grams over one day has different implications for an infant weighing 1 kg compared to an infant weighing 2 kg. On the other hand, while growth velocity normalized for body weight may be more useful, the lack of standardized calculations makes it difficult to make comparisons across studies³⁷. The quality and rate of growth during the NICU stay is important to monitor as it has been associated with long-term outcomes. For example, one study found preterm infants with higher growth velocities before discharge have a lower incidence of poorer neurodevelopmental outcomes at 18 to 22 months corrected age¹⁰.

2.3.1 Anthropometrics

Weight, head circumference and length are anthropometric measures clinicians routinely use to assess growth of preterm infants by plotting these outcomes on age and gender appropriate growth charts³⁸. The weight of the preterm neonate can be measured with precision and accuracy with relative ease on the electronic scales available in most NICUs. This information is valuable if the infant is weighed consistently and in the same manner (i.e. naked and without any attached equipment) because weight gain reflects the nutritional status³⁹. There is a positive association between the amount of protein and energy intake through fortified breast milk feeds and the rate of weight gain in the NICU^{40,41}.

Head circumference, measured as the occipito-frontal circumference, represents overall head size and is therefore a surrogate for brain volume. This measurement is important to track as brain volume is a principal determinant for future cognitive development⁴². Length measurements provide an indication of skeletal growth and lean mass deposition of the preterm infant⁴³. Length-normalized indices, like body-mass index (BMI), can also be calculated to see if the distribution of an infant's weight is appropriate for their size as well as tracking an infant's growth overtime^{44,45}.

2.3.2 Body Composition Compartments

Non-invasive techniques employed for measuring body composition compartmentalize and make use of assumptions about different tissues of the body. The total body weight is the most basic model and represents a single-compartment measurement. This is followed by the classical two-compartment model (2C) which divides the total body weight into fat mass and fat-free mass. Hydrodensitometric principles, used as part of under water weighing (UWW), were previously employed, however now air displacement plethysmography (ADP) techniques are preferred, especially for infants and toddlers⁴⁶. As part of the 2C model, these techniques assume a constant density of the tissues which make up the fat and fat-free mass⁴⁷.

The three-compartment model (3C) further differentiates the fat free mass into total body water content and the remaining dry tissue masses by adding an isotopic dilution measurement in conjunction with the hydrodensitometry⁴⁷. While the 3C model still includes an assumption about a constant ratio between protein and minerals in the dry tissues fraction, it does overcome the assumption of the hydration fractions of fat-free mass present in the 2C models⁴⁸.

The four-compartment molecular model (4C) assumes the body is composed of fat mass, water, minerals and protein. To employ this model, dual-energy x-ray absorptiometry (DXA) is used to

measure bone mineral content in addition to total body weight, hydrodensitometry and isotopic dilution described in the previous compartment models⁴⁹. In this manner, the protein content is assumed to be proportional to the bone mineral mass^{47,49}. Despite the time-consuming nature of the 4C model, it is considered to be an improvement over the 3C model because it is distinguishing the protein and mineral content of the dry tissues.

2.3.4 DXA and ADP for assessing Preterm Infant Body Composition

Techniques ranging from the use of magnetic resonance imaging or bioelectrical impedance analysis have been studied as ways to assess body composition of neonates, yet DXA and ADP continue to be the most commonly used in research related to preterm infants⁵⁰. Both these techniques offer relatively quick measurements, are non-invasive and produce reliable assessments.

The DXA scan measures the differential absorption of two low-energy photons between adipose and soft tissues as well as bone mineral content⁴⁸. The average scan with a device using a fan-beam is two minutes in length and there are limited radiation exposure concerns. While the use of this method has been validated in the preterm population, the accuracy is dependent on the infant remaining completely still during the scan. Additionally, the assumptions for tissue density or hydration level used by software can vary between different device manufacturers and lead to differences in measured values^{50,51}.

ADP uses similar densitometry principles as UWW to distinguish between fat mass and fat-free mass. Rather than submerging the subject under water, this technique will measure the mass in addition to the volume of air displaced by the infant in an environmentally controlled chamber in order to calculate total body density⁴⁶. An ADP device designed to measure infants up to six months, called the PEA POD (COSMED, USA) has been successfully validated against isotopic

dilutions and the 4C model^{52,53}. The device is mobile and allows for bedside measurements in the NICU and will also tolerate infant movements during the measurement. However, preterm infants cannot be assessed with intravenous lines or respiratory support equipment in place because these items would not allow the volume chamber to be sealed during measurements. Furthermore, after discharge, the PEA POD device can accommodate infants with weights up to 12kg. Assumptions of constant hydration status and tissue densities are applicable to both DXA and ADP^{46,50}.

2.3.5 Clinical importance of body composition

Early epidemiological research has raised the concern that rapid early weight gain, which is important for optimal neurodevelopmental outcomes, may consequently increase the risk of future cardiovascular and metabolic diseases⁴. As such, the importance of assessing body composition of preterm infants has gained traction in research. These assessments provide further details about the quality of growth by differentiating between the accumulation of body fat, lean tissue and even bone mass⁵⁰. Furthermore, serial body composition measurements can provide enhanced monitoring of nutritional status as fat and lean-mass accretion are reflections of energy storage and protein deposition respectively⁵⁰.

2.4 Preterm Infant Feeding Practices

Unlike term-born infants, preterm infants are completely dependent on the nutrition provided to them after birth to support their growth needs. In the NICU, this can take the form of parenteral nutrition followed by a gradual transition to enteral feeds as the infant becomes clinically stable.

2.4.1 Parenteral Feeding

Solutions of amino acids, dextrose, and other micronutrients as well as lipid emulsions are provided intravenously for parenteral nutrition⁵⁴. It is important that these are initiated within the

first hours of life because the preterm infant has extremely limited glycogen and fat stores it can draw on for energy and thermoregulatory needs^{26,28}. A study by Stephens et al. found that higher protein and energy intakes in the first week of life was associated with lower risk of length growth restriction and higher scores on neurodevelopmental assessments⁵⁵. Parenteral nutrition is continued until the preterm neonate is clinically stable and has successfully tolerated sufficient enteral feeds.

2.4.2 Enteral Feeding: Advantages of Breast Milk

Recent studies have proposed that preterm infants may benefit from early “aggressive” enteral nutrition without a significant increase in the risk of adverse metabolic outcomes^{56,57}. This involves provided enteral feeds sooner and advancing volumes at a higher rate⁵⁴. Early research in preterm enteral nutrition showed that infants achieved higher growth velocities for weight, when fed exclusively formula-based diets compared to those fed with breast milk alone⁵⁸.

Despite the improved growth outcomes seen with formula feedings, a breast-milk based diet is the preferred choice of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) and the American Academy of Pediatrics (AAP) because of the numerous advantages breast milk confers to the infant^{59,60}. In addition to being nutritionally diverse in micro- and macronutrients, breast milk contains bioactive molecules like growth and immunological factors. For example, epidermal growth factor (EGF), which is found in significant quantities during early lactation, helps to promote intestinal development and healing of the intestinal mucosa and as a result provides protection against necrotizing enterocolitis (NEC) of the preterm infant^{61,62}. Immunoglobulins (i.e. IgA, IgG), cytokines (i.e. IL-8, TNF- α , IFN- γ) and chemokines can also be found in breast milk and are valuable for the preterm infant in the short term as they provide protection against pathogens and stimulate development of the

immature immune system^{11,63}. A prospective study of extremely low birth weight infants found that ingestion of breast milk during the NICU was also associated with persistent positive long-term effects at 18 and 30 months corrected age including higher scores on neurodevelopmental assessments and fewer rehospitalizations after discharge^{64,65}. Initially, preterm infants will be provided with small volumes of breast milk in a practice referred to as either minimal enteral feeding or trophic feeding. The goal of these feeds are to prime the immature gut by promoting villi growth and developing the microbiome of the intestinal tract^{11,54}. As the enteral feeding volumes increase, the limitations of breast milk as a nutrition source come to light.

2.4.3 Limitations of Breast Milk and Standard Fortification

Breast milk from mothers after premature birth will initially contain higher protein and fat content relative to mothers who produce milk after a term birth⁶⁶. Longitudinal analyses of the preterm breast milk indicate that as the lactation continues to progress, the protein concentration decreases and is therefore not sufficient to meet the growth needs of a preterm infant^{66,67}. These studies have also found that the fat content is the most variable, the primary carbohydrate, lactose, remains relatively stable and that the concentration of one macronutrient is not a strong predictor of the others. Moreover, there is also significant variation in the macronutrient content of breast milk both between and within mothers influenced by maternal characteristics like diet, BMI and the quantity of the milk produced¹¹. This variation is not ideal for the preterm infant who is expected to maintain intrauterine growth rates without the constant flux of nutrients offered by the placenta. Therefore, recommendations state that once full enteral feedings are reached, approximately 120mL/kg/day or more, the breast milk must be supplemented with fortifiers before feeding a preterm infant^{59,60}.

The current routine fortification practice involves the addition of a standard commercial fortifier. When added to breast milk, these fortifiers provide additional protein, carbohydrates, fats as well as micronutrients like vitamins and minerals. Even with the standard fortification, the protein and energy intakes are insufficient for preterm infants^{12,13}. A study of 127 infants found that the proportion of growth restriction, defined as weight less than the 10th percentile, significantly increased from 33% at birth to 58% at discharge for preterm infants fed breast milk with standard fortification⁶⁸. Another criticism of this practice is that the various commercially available standard fortifiers assume average macronutrient contents of breast milk and therefore also fail to address the inherent variation as lactation progresses and between or within mothers. Given these limitations of the current practice, there is interest for an improved and individualized approach to breast milk fortification^{14,63,66}.

2.4.4 Individualized Fortification: Adjustable vs Target

Both adjustable and target fortification have been proposed as alternatives or supplements to the standard fortification approach to reduce the risk of nutritional deficiencies. The concept of adjustable fortification attempts to make changes to protein intake, by way of fortification, on the basis of an infant's metabolic response. This is determined through periodic measurements of blood urea nitrogen, which is positively correlated with dietary protein intake, even in preterm infants⁶⁹. A trial by Arslanoglu and colleagues in 32 preterm infants found that the adjustable fortification regimen, on average, led to increased protein intakes, up to 3.4g/kg/day, and higher weight gain, up to 17.4 g/kg/day compared to standard fortification⁷⁰. This method takes into account the actual protein status of each infant and as a result can minimize the risk of excessive protein intake. However, adjustable fortification has not yet been applied to adjust fortification of fat or carbohydrates and also requires more frequent blood sampling.

The concept of target fortification is to analyze the breast milk and individually adjust the fortification such that an infant receives a consistent macronutrient intake. Target fortification, initially introduced by Polberger et al. with protein adjustments only⁷¹, can be applied to all three macronutrients. Small samples of native breast milk can be used to accurately and reliably assess macronutrient content in real-time⁷². A pilot trial by Rochow and colleagues fed 10 preterm infants with target fortified breast milk for a three-week period and observed a linear relationship between milk intake and weight gain that was not seen in matched pairs fed with standard fortification¹⁵. In this study, native breast milk samples were analyzed daily and, after applying the standard fortifier, modular protein, fat and carbohydrate products were added as necessary to meet the target intakes.

The aim of the current study was to expand on the pilot trial and compare the effect of standard fortification to individualized target fortification with protein, carbohydrates and fats on the growth of preterm infants in a randomized controlled trial. Growth was assessed by examining the weight achieved at 36 weeks' PMA, the growth velocity achieved during the study period as well as length, head-circumference and body composition assessments at term equivalent age. An additional analysis compared the growth outcomes between the subgroup of infants in the Control and Intervention groups who had low protein intakes (<3.5g/kg/day) after standard fortification. Lastly, this study sought to also explore the association of day-to-day variation in macronutrient intakes and daily growth rates.

Chapter 3. Methods

3.1 Study Design

This was a prospective, single-centre, double-blind randomized controlled trial in the NICU (Level III) of McMaster Children's Hospital (Hamilton, Ontario, Canada). Infants were enrolled in the study from January 2013 to September 2016. The study was approved by the Research Ethics Board of McMaster University. Informed written parental consent was obtained from parents by the study coordinators. The study period was a minimum of 21 consecutive days up to a postmenstrual age (PMA) of 36 weeks.

3.2 Study Population

Infants with gestational age < 30 weeks at birth, tolerating breast milk feeds and who were anticipated to receive fortified breast milk (≥ 150 mL/kg/day) for 21 consecutive days were eligible. The exclusion criteria included: gastrointestinal malformation, major congenital anomalies, confirmed intraventricular hemorrhage (Grade >2), necrotizing enterocolitis (Bell stage ≥ 2), abdominal surgery, renal or hepatic dysfunction and gram-negative sepsis prior to recruitment. All infants admitted to the NICU who met the criteria and had reached a breast milk intake >100 mL/kg/d were approached for recruitment by the study coordinator.

3.3 Randomization and Blinding

Infants were randomized to receive either standard fortification in the Control group, or target fortification in the Intervention group. Randomization was stratified by gestational age (<28 weeks and ≥ 28 weeks). For each stratum a series of opaque, sealed, consecutively numbered envelopes were prepared. As each infant was enrolled, the dietary assistants responsible for milk preparation would open the next envelope in their offices. The investigators, research assistants, parents and all health care providers, except the dietary assistants, were blinded to the

randomization. Participant's allocation to receive either the control or intervention feeds were un-blinded after the last infant had completed the study period and the recruitment goal was met.

3.4 Milk Preparation

Breast milk was defined as milk of human origin. In our study this included mother's own milk provided by an infant's birth mother, or donor milk sourced from the Rogers Hixon Ontario Milk Bank (Toronto, Canada). Donor milk was used when mother's own milk was not available in sufficient quantity for an infant's feeding needs. All feeds were prepared outside the NICU in the adjacent Mother's Milk room that was accessible only by the trained dietary assistants. Feeds were prepared in pooled batches of breast milk with sufficient volumes to feed infants for 24-hour periods (14:00 to 13:59 of the next day).

Once the infant had reached full enteral intake (>100 mL/kg/day), the breast milk was gradually supplemented with a standard fortifier over four days to assess tolerance. The study period began after the infant received two full days at the full standard fortifier dosage. During the study period, native (unfortified) breast milk or donor milk samples were collected from the pooled volume that was chosen to prepare fortified feeds for the 24-hour period. These samples were analyzed by the research assistants for their protein, carbohydrates and fat concentration (g/dL) using a near-infrared spectrometer (Spectrastar, Unity Scientific, USA). The milk analysis provided the basis for a fortification recipe followed by the dietary assistants which was adjusted three times per week (Monday, Wednesday, Friday).

This frequency of recipe-adjustment was used to balance the increased workload with reducing macronutrient intake variation as proposed in a recent study⁷³ using data from a pilot trial¹⁵. In the intervention group, the amount of additional fortification required for each macronutrient after standard fortification was calculated using a standardized study recipe sheet (Appendix 2).

On days where the the fortification was not adjusted, a native milk sample was still collected, analyzed and then most recent fortification recipe was applied to the pooled milk batch.

3.5 Fortifier Products and Dosage

3.5.1 Control Group Fortification

After milk analysis, native breast milk batches were fortified only with the commercially available standard fortifier, Enfamil (Mead Johnson, OH, USA), at the recommended dosage of 1 package per 25 mL of native breast milk. At this dose, the fortifier provides an additional 1 g of fat, 1.1 g of protein and 0.4 g of carbohydrates per 100 mL of breast milk. When donor milk was used, an additional 0.4 g of Beneprotein (Nestle Health Care Nutrition, USA) per 100 mL of milk was added as per McMaster NICU guidelines.

3.5.2 Intervention Group Fortification

After milk analysis, the fortification recipe was adjusted to maintain macronutrient content of breast milk for fat (4.3 g/100mL), carbohydrates (8.5 g/100mL) and protein (3.0 g/100mL).

These concentrations would allow an infant to achieve the recommended ESPGHAN intakes for fat (6.6 g/kg/d), carbohydrates (13.2 g/kg/d) and protein (4.5 g/kg/d) assuming an average fluid intake of 150 mL/kg/d⁵⁹. To prepare feeds, first, the Enfamil standard fortifier used in the Control Group was added to native breast milk at the recommended dosage. Similar to the Control group, when donor milk was used, an additional 0.4 g of Beneprotein (Nestle Health Care Nutrition, USA) per 100 mL of milk was added as per McMaster NICU guidelines. Then, individual macronutrient fortifier products were added to achieve the target concentration according to the fortification recipe. The following commercially available products were used: Microlipid (Nestle Heath Care Nutrition, USA), a fat emulsion for enteral feeds (0.5g fat/mL);

Beneprotein (Nestle Health Care Nutrition, USA), a whey protein powder (0.86g protein/g); and Polycal (Nutricia, UK), a glucose polymer powder (0.94g carbohydrate/g).

3.6 Data Collection and Measurements

3.6.1 Study and Chart Data

All infants were assigned a unique study identification number. Data were collected from study records or patient records (bedside or electronic charts) as follows:

- *Infant characteristics at birth*: Date, gestational age and anthropometrics (weight, length and head circumference). Anthropometric values were converted to z-scores (z_{birth}) using Fenton growth charts⁷⁴.
- *Enteral feeding volumes*: Daily enteral feed volumes were recorded from patient charts to calculate total fluid intake (mL/kg/day)
- *Macronutrient intakes*: Native breast milk samples collected for each study day were used to determine protein and fat content (using a validated near-IR spectrometer⁷²) and native lactose content (using an established Ultra-performance liquid chromatography-tandem mass spectrometry method⁷⁵). Daily macronutrient intakes could then be calculated with standard fortification and additional target fortifiers.
- *Metabolic Parameters*: Results of routine blood panel were recorded weekly from patient charts to assess and monitor metabolic outcomes. Parameters included blood urea nitrogen, blood glucose, serum triglycerides and acid/base status. Standard protocols were established to handle events of hyperglycemia (serum glucose >12mmol/L), hypertriglyceridemia (serum triglycerides >2mmol/L), and elevated urea (BUN >7mmol/L).

- *Additional variables:* Day of life, energy intake, protein:energy (P:E) intake ratio and carbohydrate:non-protein energy (CHO:NPE) ratio were calculated using the collected data described above.

3.6.2 Anthropometric Measurements

Weight was recorded by NICU nursing staff to the nearest 10g, every second day as per the current NICU practice, using an electronic scale (Smart Scale Model® 65, Natus Medical Inc., USA). The growth velocity was calculated as the average rate of weight gain (in g/kg/day) during the 21-day study period using an exponential regression model⁷⁶.

The following anthropometric measurements were performed weekly in the NICU by the author:

Length, from crown to heel, was measured in triplicate using a length board (Premie Stadiometer, Ellard Instrumentation Ltd, USA) and the average was recorded to the nearest 0.1cm. Similarly, the occipitofrontal head circumference was measured in triplicate using a non-stretchable paper tape and the average was recorded to the nearest 0.1cm.

Weight, length and head circumference were also measured in a similar fashion at discharge or at a return follow-up visit at term-equivalent age. These values were also converted to z-scores (z_{TEA}) using the Fenton growth charts⁷⁴.

3.6.3 Body Composition Measurements

A body composition assessment was attempted using a PEA POD device (COSMED USA Inc, USA), which employs the ADP method, either once prior to discharge in the NICU or at a return follow-up visit at term-equivalent age. If the infant was measured in the NICU, the bedside nurse assisted in handling and monitoring the infant. The complete protocol for the measurement procedure has been previously described⁷⁷. In summary, the PEA POD device determines body density by measuring body mass on a scale, the volume displaced by the infant in a pressure controlled chamber and accounts for the infant's length (measured on a length board). Then by

incorporating assumptions of fat and fat-free tissue densities, estimates for absolute fat mass (g), fat-free mass (g), whole body mass, body volume (L) as well as relative fat-mass (%) and fat-free mass (%) are calculated by the device. Quality control tests for the scale and volume chamber were performed prior to assessing an infant using the calibration weight and phantom volume provided by the manufacturer. Infants were measured completely nude, except for a thin elastic cap to minimize the influence of the hair's surface area on the volume measurement. Irremovable items, including gastric feeding tubes, patient ID tags and a wireless pulse oximeter, were accounted for during the tarring and calibration steps for the scale and volume chamber respectively. After all quality control and calibration steps, each measurement takes approximately 3 minutes and can be repeated with little risk to the infant if needed.

3.7. Analysis of Macronutrient Intakes and Growth Outcomes

An intention-to-treat analysis was performed for the primary outcome (weight at 36 weeks' PMA) and included all infants who were enrolled and received any amount of study feeds. In a per protocol analysis of the primary and secondary outcomes, all infants who completed a minimum of 21 consecutive days in the study were included. Outcomes for infants who received 14-20 consecutive days of study feeds were also included for analysis as no bias was introduced when plotting their outcomes with infants who completed the full study period. Details are described in the missing data section below (3.7.4). Those infants who completed fewer than 14 days in the study, were treated for gram-negative sepsis or received steroids or diuretics for more than 48 hours were excluded from the final evaluable group analysis.

3.7.1 Statistical Analyses

Statistical analyses were performed using SPSS v20.0 (IBM, USA) and SAS v9.4 (SAS Institute, USA). Statistical significance, or the type I error associated with the null hypothesis, was set at

5% probability. Infant's characteristics and outcomes for both groups were reported using descriptive summary measures: mean (standard deviation) for continuous measures and count (percentage) for categorical measures. For comparative analyses of the growth and body composition outcomes, Student's t-test was used to assess if the mean difference between the Control and Intervention groups was statistically significant.

3.7.2 Regression Analyses

For each regression analysis, multicollinearity was assessed to ensure that multiple variables that are highly correlated with each other were not included in the same model and adding unnecessary "noise". This was assessed using tolerance statistics with a tolerance value <0.2 taken to indicate multicollinearity. Should multicollinearity exist, only one member of a correlated set of variables would be retained for the final model. Infant characteristics included birth weight percentile (%), the PMA (weeks) at which the study period began and the length of the study period (days).

In the multiple linear regression models the average macronutrient intakes (g/kg/day) during the study period and infant characteristics were assessed as potential predictors for the outcomes of weight at 36 weeks' PMA (g) and 21-day growth velocity (g/kg/day). A repeated measures linear regression model was also run to assess changes in daily weight gain over the entire period of the study. Here, the daily intake macronutrient intakes (g/kg/day), their absolute day-to-day variation (g/kg/day) in addition to infant characteristics were included as potential predictors. Two binary groups were created for each of the following variables that remained constant over time: gestational age at birth (<28 weeks and ≥ 28 weeks) and birth weight percentile ($<50^{\text{th}}$ percentile and $\geq 50^{\text{th}}$ percentile). A one-day lag-effect on macronutrient intakes was introduced here. This

would take the previous day's value, for example the previous day's protein intake, to predict the weight gain between the current and previous study days.

3.7.3 Sample Size

Based on a previous trial investigating the effect of different fortifiers on weight of preterm infants, it was determined that a mean difference of 180g between groups would be clinically meaningful and achievable in preterm infants after a 3-week nutrition-based intervention⁷⁸. To detect such a difference with a type I error rate (α) of 5% and with 80% power, a minimum of 38 infants per group was needed. Accounting for an estimated 30% attrition rate, due to potential dropouts, deviations from the protocol and loss to follow-up, a target recruitment goal was set at 112 infants or 56 infants per treatment arm.

3.7.4 Missing Data

Missing weight measurements during the study period were imputed by exponential regression model to achieve daily weights. If the infant was transferred out of the NICU before 36 post-menstrual weeks, the weight for the primary outcome was extrapolated by following the infant's growth curve for the weight percentile achieved at discharge. Infants with a study period below 21 days had their growth velocity (GV21) estimated using their 14-day growth velocity (GV14) in the linear regression model ($GV21 = 0.633 * GV14 + 6.89$, $R^2 = 0.62$, $p < 0.001$)

3.7.5 Subgroup Analysis

Infants from the Control and Intervention groups were further divided into either a low-protein or high-protein subgroup using the median protein level of all study infants after the standard fortification step. Those infants with protein levels below the median (<50th percentile) were classified as low-protein whereas those with levels above the median (>50th percentile) were classified as high-protein. Comparative analysis was then completed between the low-protein

subgroups for Control and Intervention infants and between the high-protein subgroups for the Control and Intervention infants.

Chapter 4. Results

4.1 Study Population

A total of 179 infants were consented and randomized to a study group; 90 were assigned to the Intervention (TFO) group to receive standard and target fortification, and 89 were assigned to the control group to receive standard fortification only. As a result of dropouts, early transfer out of the NICU and violations of the feeding protocol, 76 infants did not complete the study protocol. The CONSORT flow diagram in Appendix 1 details the full study enrolment. The remaining 103 infants were included for final analysis. In the control group, 12 infants received at least two weeks and 39 infants received a minimum of 21 days of study feeds. In the TFO group, 11 infants received at least two weeks and 41 infants received a minimum of 21 days of study feeds. There were no differences in demographics between infants excluded and included for the analysis. All birth and study characteristics for the excluded and included infants were also similar between the intervention and control groups (Table 1). None of the infants from both groups experienced adverse metabolic events including elevated BUN, hyperglycemia or hypertriglyceridemia during the study period.

Table 1: Characteristics of study population groups.

Infants Randomized to Study Groups		
Birth Characteristics		
	Control Group (n=89)	TFO Group (n=90)
Gestational age at birth (weeks)	27.0 ± 1.9	27.1 ± 1.6
Birth weight (g)	960 ± 310	980 ± 250
Length at birth (cm)	34.4 ± 4.6	35.3 ± 3.2
Head Circumference at birth (cm)	24.3 ± 4.6	25.0 ± 2.3
Sex (number of males)	49 (55%)	45 (50%)
Infants Excluded for Analysis		
Birth Characteristics		
	Control Group (n=38)	TFO Group (n=38)
Gestational age at birth (weeks)	26.6 ± 2.1	26.9 ± 1.9
Birth weight (g)	940 ± 360	990 ± 290
Length at birth (cm)	34.1 ± 9.1	35.6 ± 2.9
Head Circumference at birth (cm)	24.0 ± 6.6	25.3 ± 2.9
Sex (number of males)	22 (58%)	17 (45%)
Infants Included for Analysis		
	Control Group (n=51)	TFO Group (n=52)
Birth Characteristics		
Gestational age at birth (weeks)	27.2 ± 1.7	27.2 ± 1.2
Birth weight (g)	980 ± 270	980 ± 210
Length at birth (cm)	35.0 ± 3.6	34.9 ± 2.5
Head Circumference at birth (cm)	24.5 ± 1.9	24.8 ± 1.8
Sex (number of males)	27 (53%)	28 (54%)
Study Characteristics		
PMA at start of study (weeks)	30.7 ± 1.4	30.5 ± 1.0
Day of life at start of study (days)	19 (12,44)	19(12,42)
Length of study period (days)	28 ± 10	27 ± 9
Total fluid intake (mL/kg/day)	154 ± 5	153 ± 5

Values presented as count(%), mean±sd or median(min, max).

4.2 Breast Milk Composition Analysis and Macronutrient Intakes

For those infants included in the analysis, 2376 native breast milk samples and 434 donor milk samples were analyzed. Table 2 shows that the concentrations of the measured fat, lactose and protein of the native milks were similar for both groups.

Table 2: Comparison of native breast milk content and fortified macronutrient intakes.

	Control Group (n=51)	TFO Group (n=52)
Native Breast Milk Content		
Fat (g/100mL)	3.6 ± 0.6	3.6 ± 0.6
Lactose (g/100mL)	6.6 ± 0.5	6.7 ± 0.5
Protein (g/100mL)	1.2 ± 0.2	1.2 ± 0.2
Fortified Breast Milk Intakes		
Fat (g/kg/d)	7.1 ± 0.9	7.6 ± 0.9**
Lactose (g/kg/d)	10.8 ± 0.8	13.6 ± 0.8***
Protein (g/kg/d)	3.6 ± 0.4	4.5 ± 0.3***
Calories (kcal/kg)	121 ± 10	140 ± 10***
Protein:Energy (g/kcal)	3.0 ± 2.6	3.2 ± 2.5***
CHO:NPE (%)	41 ± 3	45 ± 3***

Values presented as mean±sd. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

The average macronutrient intakes for the 21 consecutive study days are also presented in Table 2. These values take into account the macronutrient content of native breast milk, the amount of fortifier products added and the total breast milk intake of the infants. The average intake of fat, lactose and protein were all significantly higher in the TFO group. Furthermore, the average caloric intake, P:E ratio and CHO:NPE ratio were also significantly higher for infants in the TFO group.

The distribution of the average macronutrient intakes compared between the Control and Intervention groups, relative to the ESPGHAN recommendations, is shown in Figures 1-3. These figures show final intakes after standard fortification for the Control group (A), for comparison

intakes after standard fortification alone for the Intervention group (B), and the final intakes after target fortification for the Intervention group (C) for each macronutrient.

In the Control group, average protein or carbohydrate intake was below the minimum recommended intake for 47% (n=24) and 88% (n=45) of the infants, respectively. If the TFO group would have received standard fortification only, intakes below the minimum recommended level would have occurred for 60% of infants (n=31) for protein and 88% (n=46) for carbohydrate. However, after the addition of the modular target fortifiers, all infants in the TFO group had average protein and carbohydrate intakes above the minimum recommendation. The average fat intake after standard fortification for all infants met or exceeded the minimum recommendation. For the subgroup analysis, stratified for protein content, the median protein level after standard fortification was 3.5g/kg/day.

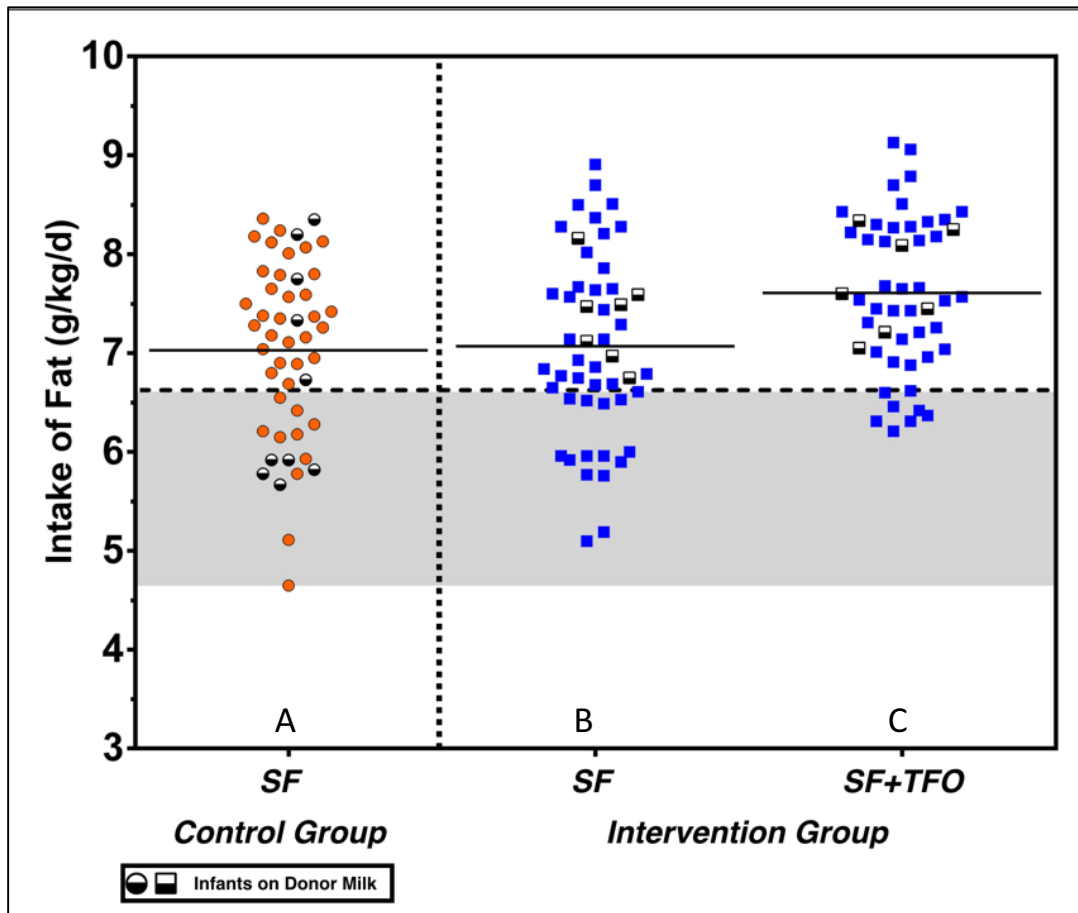


Figure 1: Distribution of infants' average fat intakes during the study period.

The grey shaded region represents the ESPGHAN recommended intake range for the macronutrient.

The horizontal dashed lines (---) represent the target intake for the Intervention group.

The horizontal solid lines (—) represent the group means.

Each marker represents one infant in the study. Infants fed fortified donor milk are indicated by half filled markers.

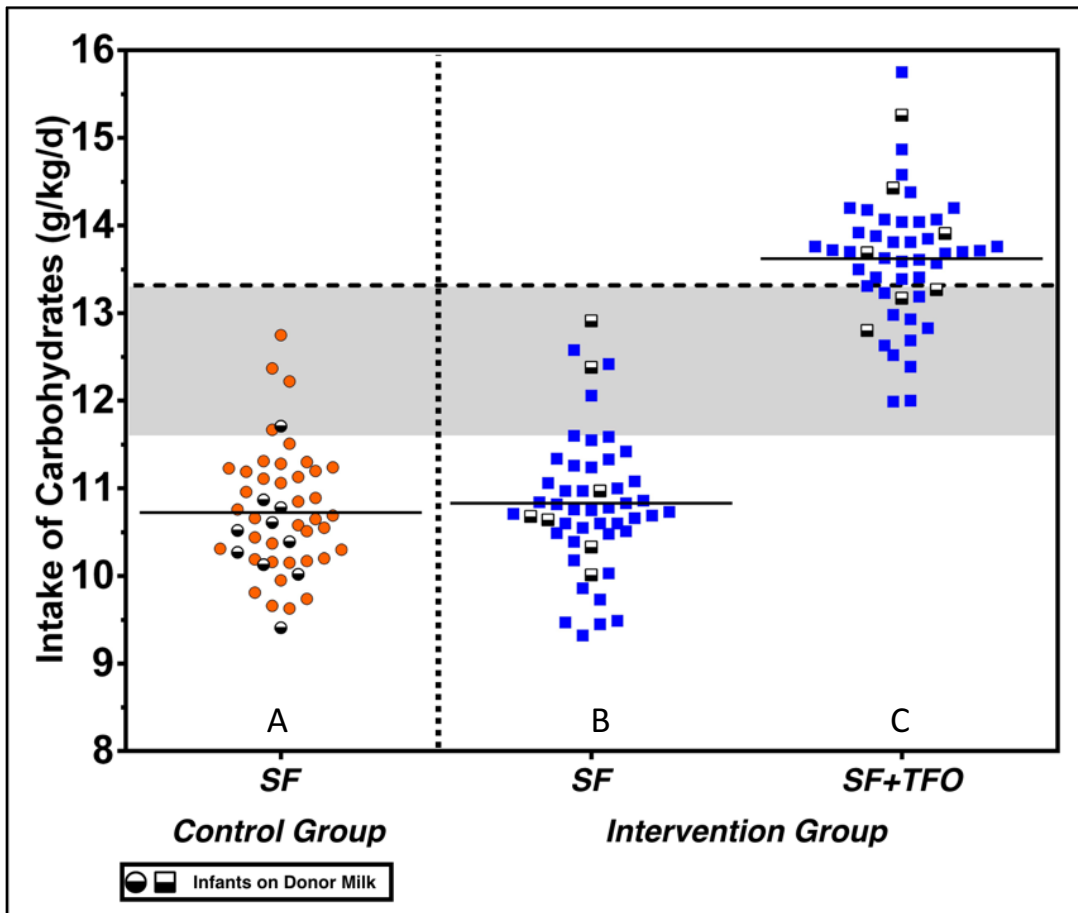


Figure 2: Distribution of infants' average carbohydrate intakes during the study period. The grey shaded region represents the ESPGHAN recommended intake range for the macronutrient. The horizontal dashed lines (---) represent the target intake for the Intervention group. The horizontal solid lines (—) represent the group means. Each marker represents one infant in the study. Infants fed fortified donor milk are indicated by half filled markers.

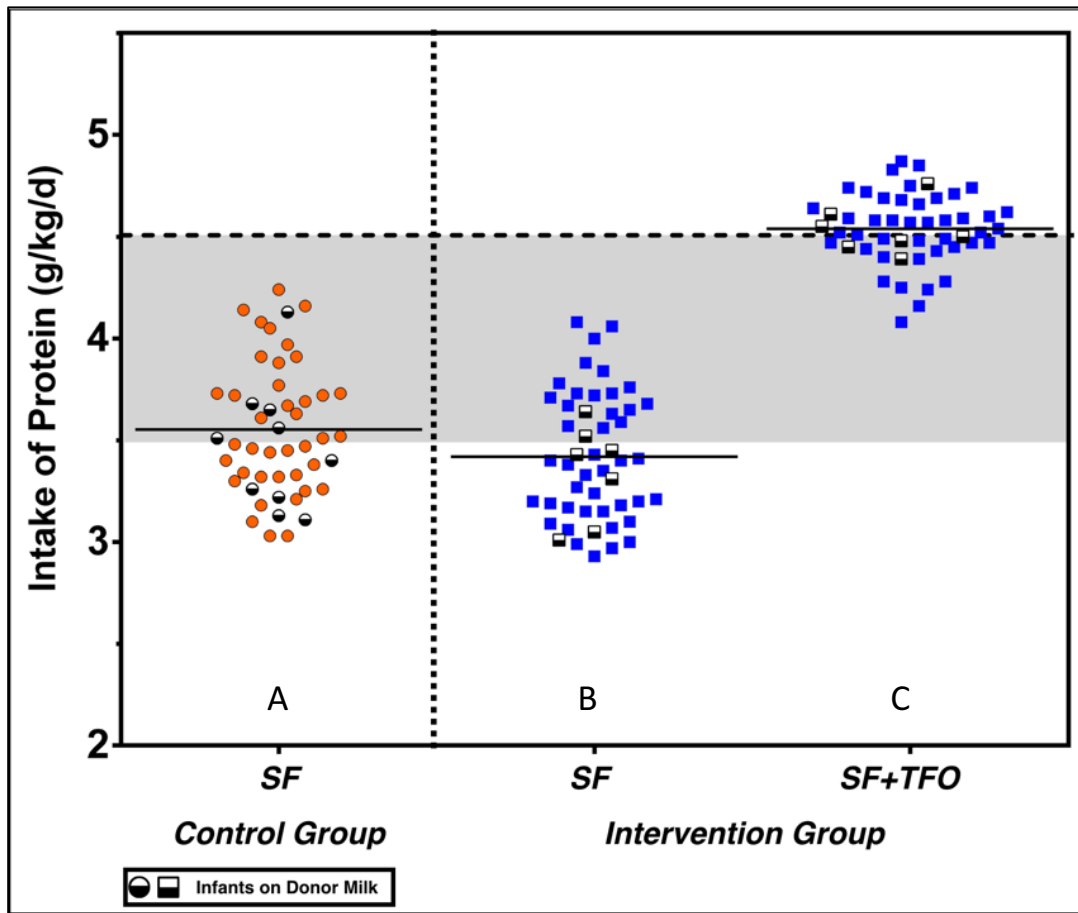


Figure 3: Distribution of infants' average protein intakes during the study period. The grey shaded region represents the ESPGHAN recommended intake range for the macronutrient. The horizontal dashed lines (- - -) represent the target intake for the Intervention group. The horizontal solid lines (—) represent the group means. Each marker represents one infant in the study. Infants fed fortified donor milk are indicated by half filled markers.

4.3 Weight and Growth Velocity comparisons

4.3.1 Weight at 36 Weeks' PMA

In the intention-to-treat analysis, the average weight at 36 weeks' PMA of infants randomized to the TFO group ($2430 \pm 350\text{g}$) was significantly higher than the infants randomized to the Control group ($2310 \pm 350\text{g}$), $p < 0.05$.

For the 103 infants included for the per protocol analysis, the TFO group achieved a significantly higher average weight ($2510 \pm 290\text{g}$) than infants in the Control group ($2290 \pm 330\text{g}$) at 36 weeks' PMA, $p < 0.001$. The average weight of infants in the TFO high-protein subgroup (2490 ± 290) was 100g higher than those in the high-protein Control group (2390 ± 340), though not significantly different (Figure 5). When comparing within the low-protein subgroups (Figure 6), infants receiving TFO also had a significantly higher weight ($2520 \pm 290\text{g}$) compared to infants in the Control group ($2180 \pm 300\text{g}$) at 36 weeks' PMA, $p < 0.001$.

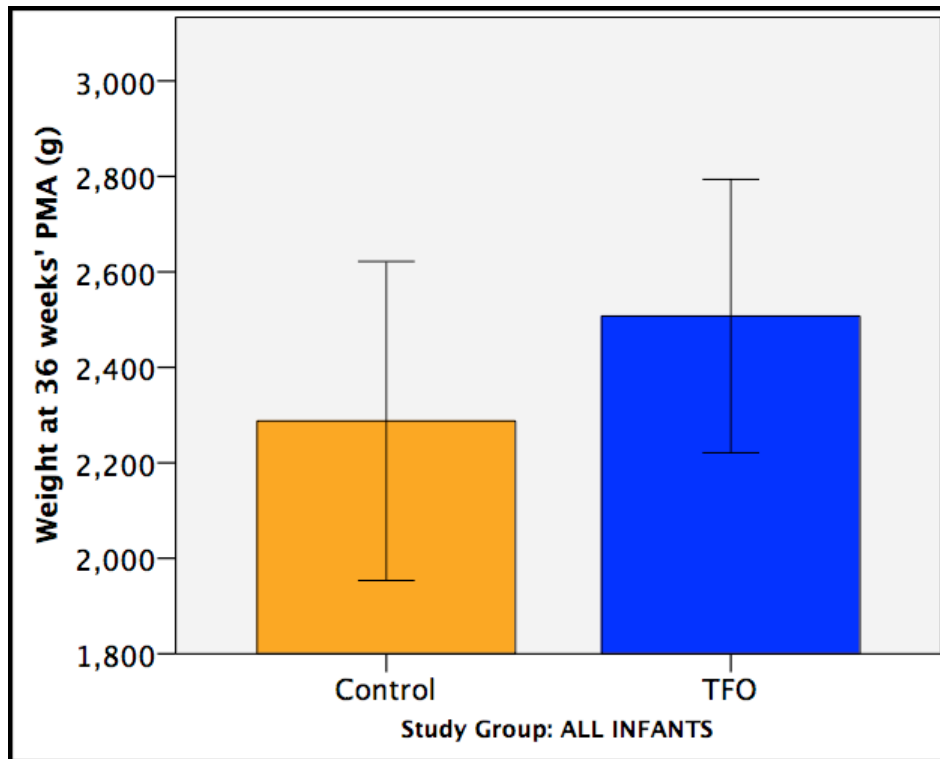


Figure 4: Comparison of mean weight at 36 weeks PMA between Control (n=51) and TFO (n=52) groups. Error bars represent standard deviation of the group.

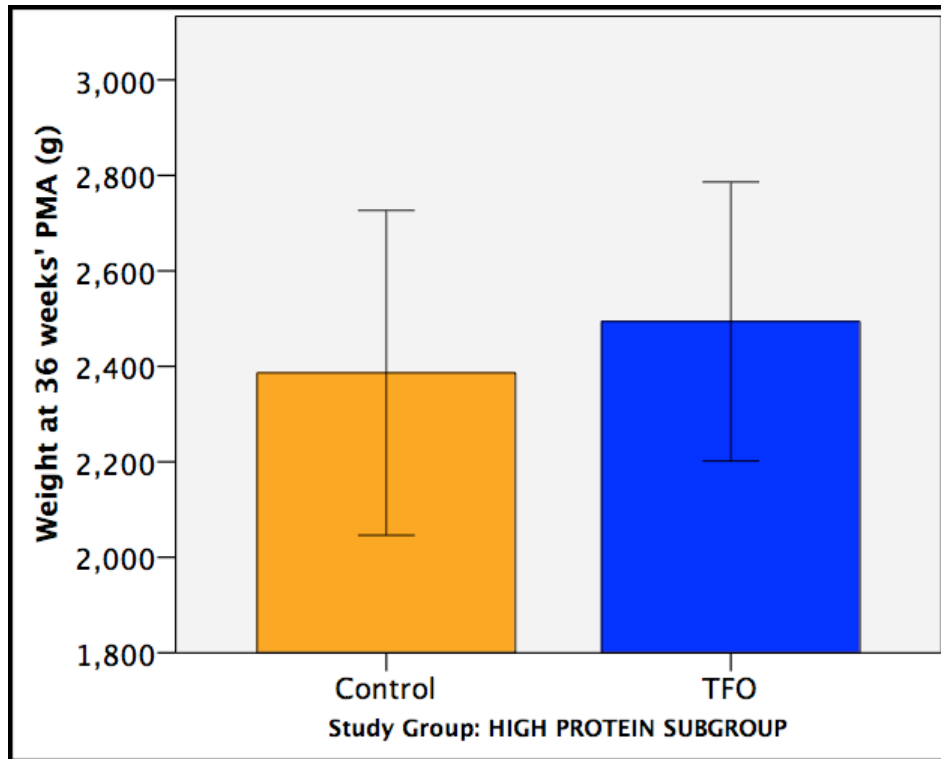


Figure 5: Comparison of weight at 36 weeks PMA between high-protein subgroups of the Control (n=27) and TFO (n=21) groups. Error bars represent standard deviation of the group.

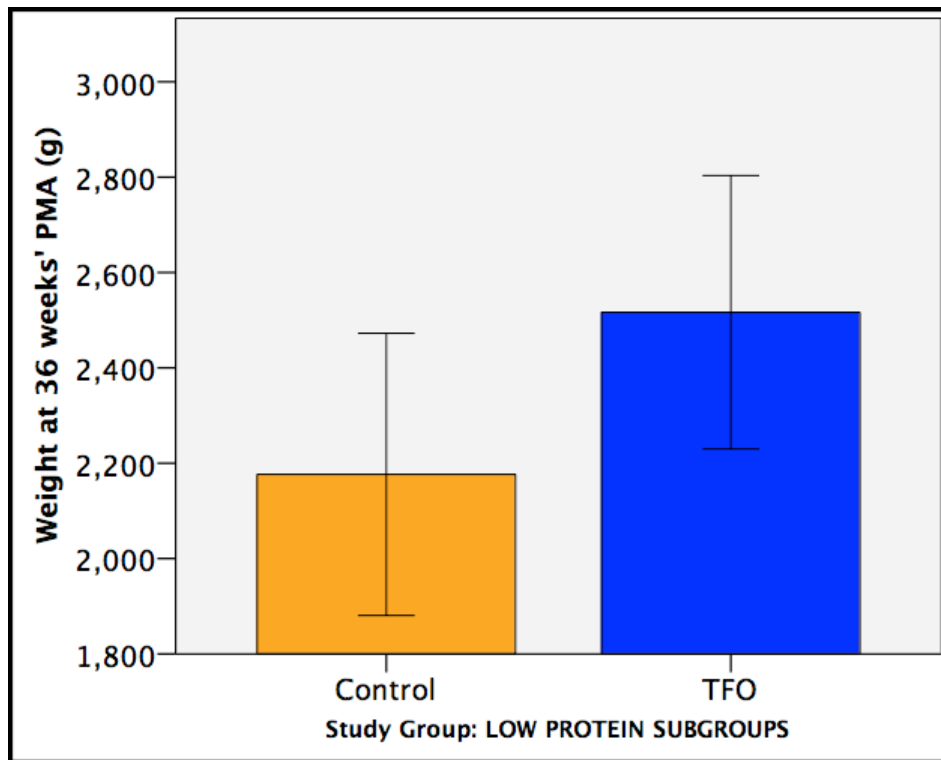


Figure 6: Comparison of weight at 36 weeks PMA between low-protein subgroups of the Control (n=24) and TFO (n=31) groups. Error bars represent standard deviation of the group.

4.3.2 Growth velocity over 21-day study period

Figure 7 compares the growth velocity experienced during the 21-day study period between the Control and TFO groups. Infants in the TFO group, grew on average at a significantly faster rate (21.2 ± 2.5 g/kg/day) during the study period compared to infants in the Control group (19.3 ± 2.4 g/kg/day), $p < 0.001$. Growth velocity in the high-protein subgroups (Figure 8) was higher in the TFO infants (21.6 ± 2.3 g/kg/day) than in the Control infants (19.3 ± 2.4 g/kg/day), $p < 0.01$ and similarly in the low-protein subgroup (20.9 ± 2.6 vs 19.2 ± 2.6 g/kg/day, $p < 0.05$) (Figure 9).

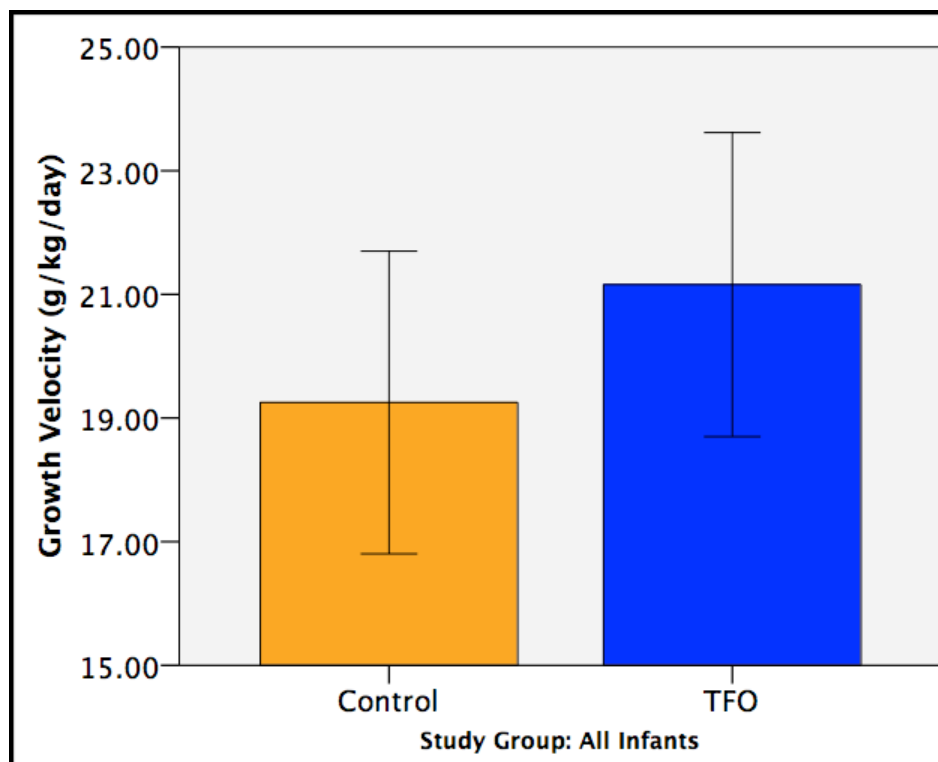


Figure 7: Comparison of average growth velocity over 21 study days between Control (n=51) and TFO (n=52) groups. Error bars represent standard deviation of the group. The average growth velocity is 1.9g/kg/day higher in TFO group ($p < 0.001$).

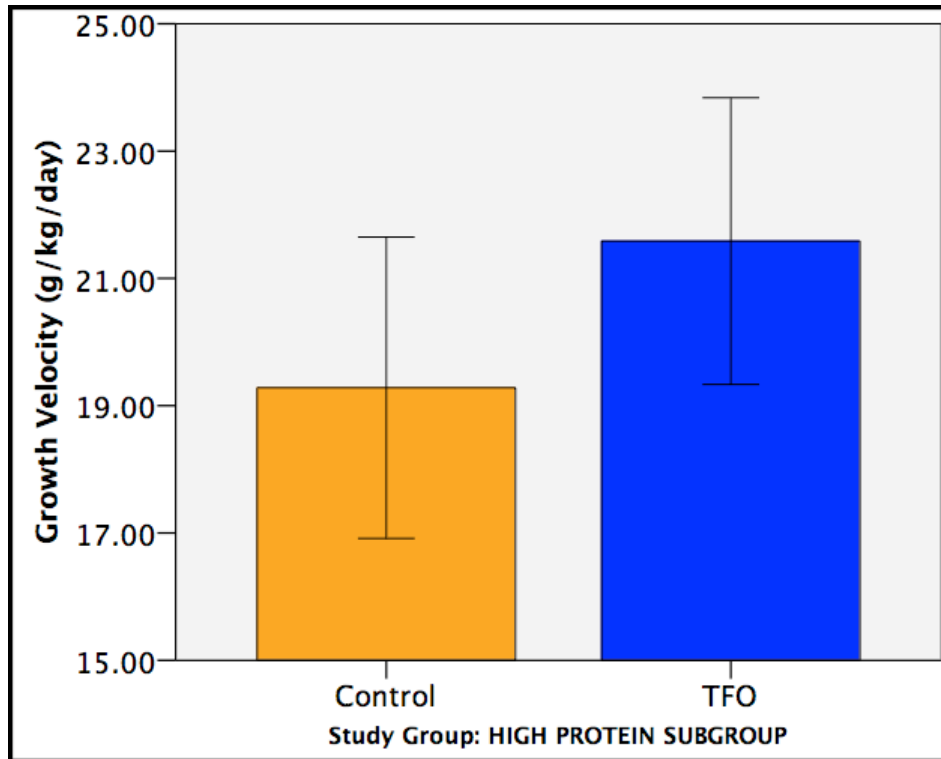


Figure 8: Comparison of average growth velocity over 21 study days between high-protein subgroups of the Control (n=27) and TFO (n=21) groups. Error bars represent standard deviation of the group.

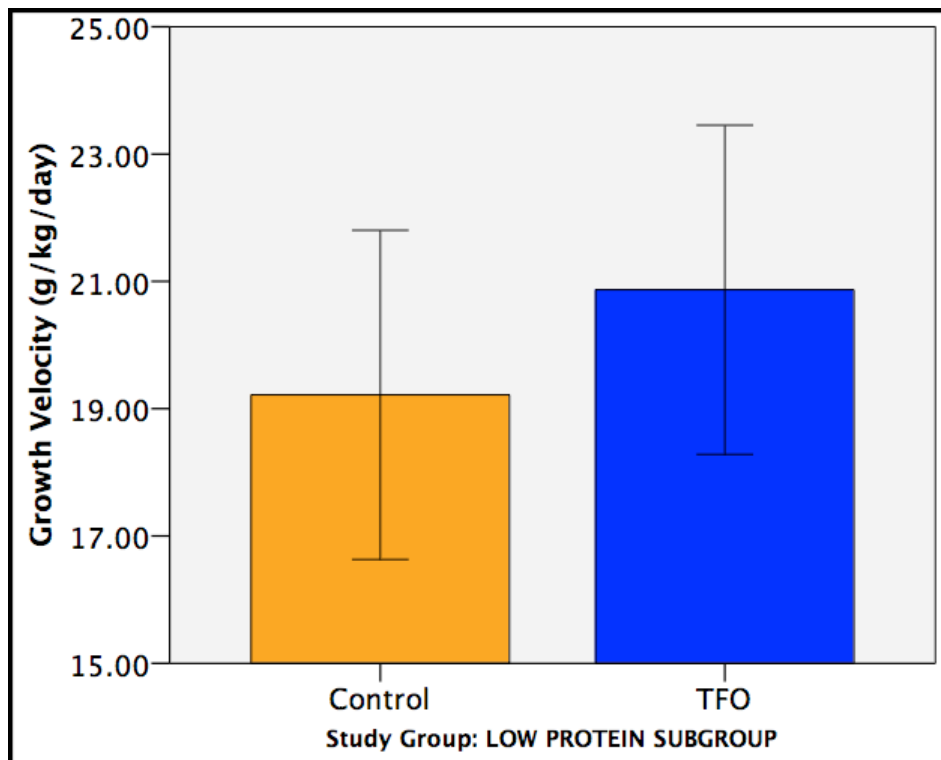


Figure 9: Comparison of average growth velocity over 21 study days between low-protein subgroups of the Control (n=24) and TFO (n=31) groups. Error bars represent standard deviation of the group.

4.4 Anthropometric measures at term equivalent age

Figures 10-12 show the distribution of the differences between z-scores from birth to TEA, that is $z_{TEA} - z_{birth}$, for the control and TFO groups. A positive difference indicates the infant achieved a higher z-score for the anthropometric measure at TEA compared to birth. A negative value indicates the infant achieved a lower z-score for the anthropometric measure at TEA compared to birth. The average change in weight z-score from birth was significantly greater in the TFO group (-0.49 ± 0.75) than the Control group (-0.82 ± 0.71), $p < 0.05$. The average change in head circumference z-score from birth for the TFO group (0.31 ± 0.83) was similar to the Control group (0.19 ± 0.88). Similarly, the average change in length z-score was higher in the TFO group (-0.84 ± 1.07) compared to the Control group (-1.35 ± 1.20), though not statistically different.

Figures 13-15 show the distributions of the changes in z-scores for the anthropometric measures between the low-protein subgroups (A) and the high-protein subgroups (B). In the high-protein comparison, infants in the TFO group experienced similar changes in weight (-0.47 ± 0.76), head circumference (0.65 ± 0.64), and length (-1.13 ± 1.07) z-scores compared to the changes in weight (-0.84 ± 0.75), head circumference (0.44 ± 1.21) and length (-1.21 ± 1.42) for the control group respectively. In the low-protein comparison, infants in the TFO group had a similar average change in weight z-score (-0.49 ± 0.75) compared to infants in the Control group (-0.80 ± 0.68). The low-protein subgroup of TFO infants, on average, experienced a significantly higher change in length z-score (-0.63 ± 1.05) compared to the Control infants (-1.46 ± 1.05), $p < 0.05$. The average change in head circumference z-score for the low-protein Control group (0.22 ± 0.93) was higher than the TFO group (0.10 ± 0.87), though not statistically different.

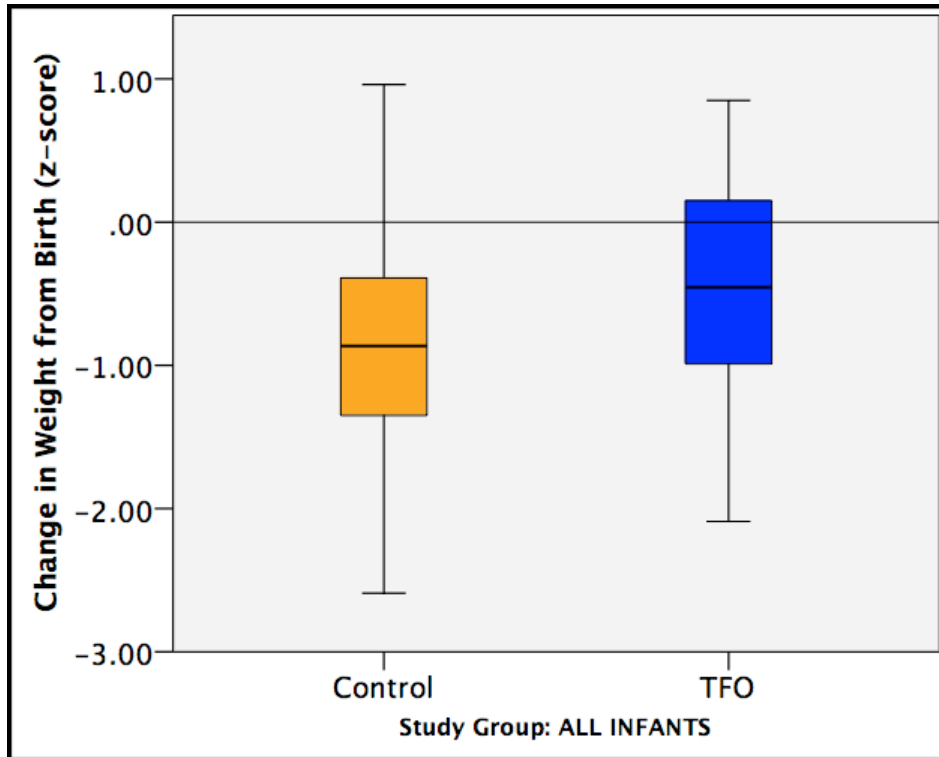


Figure 10: Comparing the difference in weight z-scores for the Control (n=46) and TFO (n=44) groups.

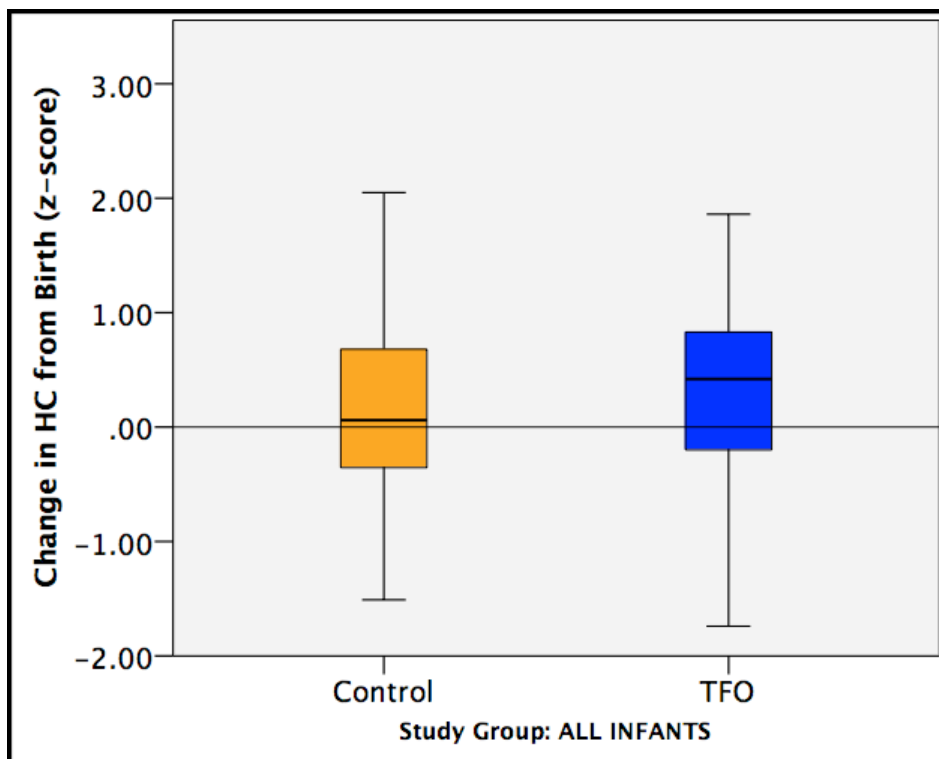


Figure 11: Comparing the difference in head-circumference (HC) z-scores for the Control (n=41) and TFO (n=37) groups.

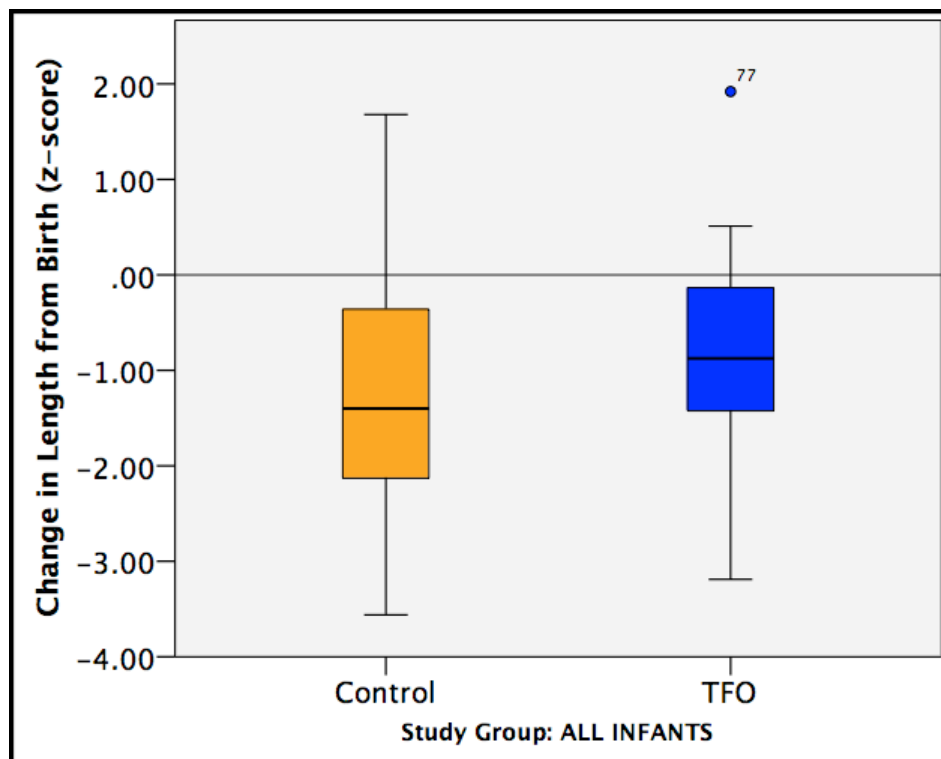


Figure 12: Comparing the difference in length z-scores for the Control (n=25) and TFO (n=28) groups.

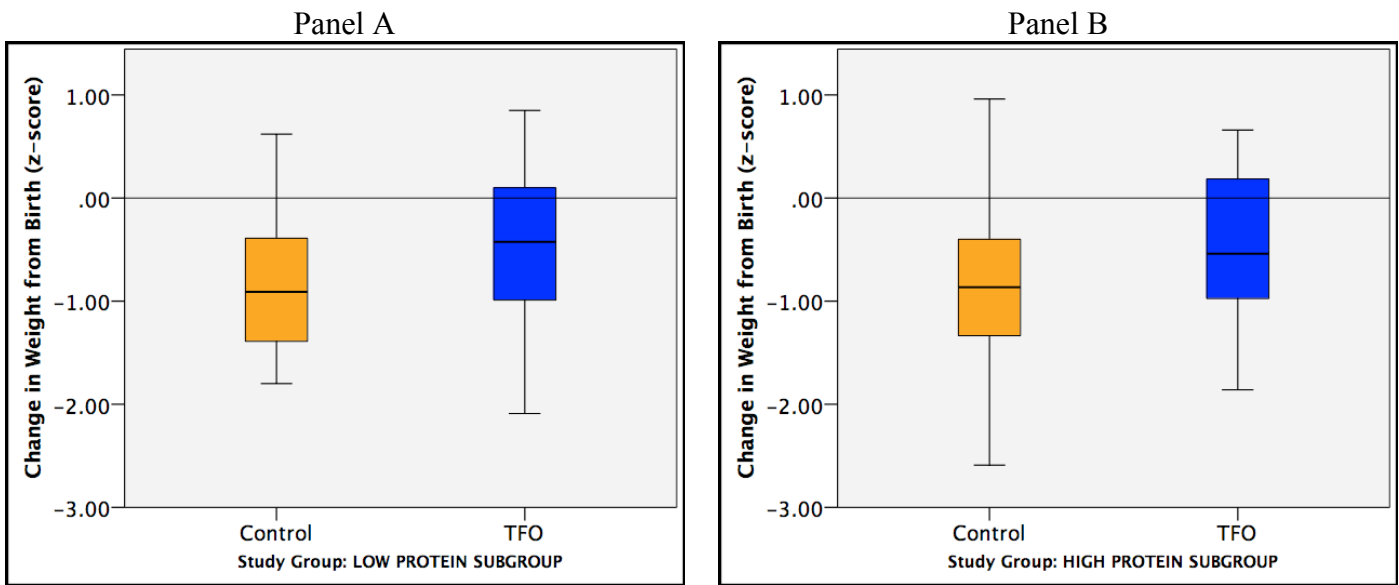


Figure 13

A) Comparing the difference in weight z-scores for the Control (n=22) and TFO (n=28) low-protein subgroups.
 B) Comparing the difference in weight z-scores for the Control (n=24) and TFO (n=16) high-protein subgroups.

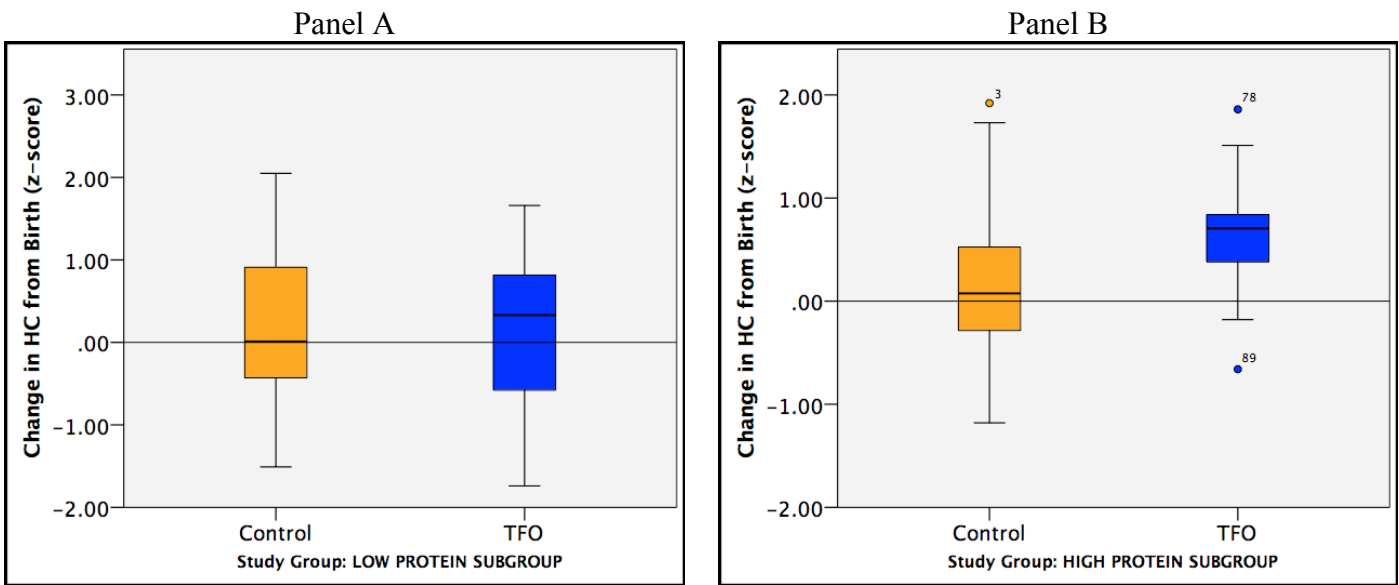


Figure 14

A) Comparing the difference in head circumference (HC) z-scores for the Control (n=19) and TFO (n=23) low-protein subgroups.
 B) Comparing the difference in head circumference (HC) z-scores for the Control (n=22) and TFO (n=14) high-protein subgroups.
 Outliers are indicated by filled circles. Removing outliers did not change outcome of statistical comparison.

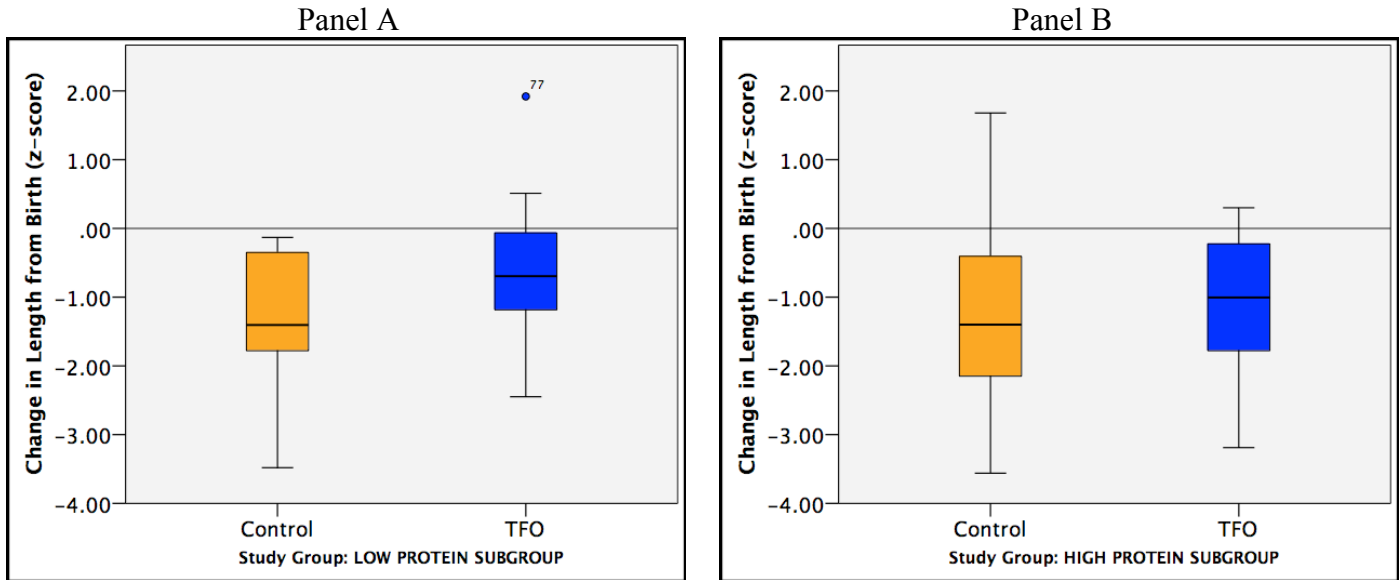


Figure 15

A) Comparing the difference in length z-scores for the Control (n=14) and TFO (n=16) low-protein subgroups.

B) Comparing the difference in length z-scores for the Control (n=11) and TFO (n=12) high-protein subgroups.

Outliers are indicated by filled circles. Removing outliers did not change outcome of statistical comparison.

4.5 Body composition assessments

Table 3 shows the comparison of body composition assessments completed between 35-43 week's PMA for 68 infants included in the analysis.

Table 3: Comparison of body composition assessments.

Outcome	Control Group (n=39)	TFO Group (n=29)
PMA [weeks]	38.9 ± 2.2	39.0 ± 2.4
Total Body Mass [g]	2890 ± 630	3070 ± 630
Fat Mass [g]	540 ± 220	670 ± 250*
Fat-Free Mass [g]	2350 ± 470	2390 ± 440
Fat %	18.1 ± 4.9	21.4 ± 4.8**
Fat Free %	81.9 ± 4.9	78.6 ± 4.8**
Fat Mass Index [kg/m ²]	2.5 ± 0.8	3.1 ± 0.9**
Fat-Free Mass Index [kg/m ²]	10.9 ± 1.2	11.2 ± 1.3

Values presented as mean±sd. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

The total body mass and fat-free mass, were similar between both groups. On average, the TFO group had 135g more fat mass than the Control group. When considering the distribution of the different masses as a percentage of total body weight, the TFO group had 3.3% more fat, and were consequently 3.3% less lean on average. Of the length normalized indices, TFO infants presented with 0.6kg and 0.3kg more fat mass and fat-free mass, respectively, per meter-squared of length. However, only the difference in the fat mass index was found to be significant. Tables 4 and 5 show the comparison of body composition assessments for the low- and high- protein groups respectively between the control and TFO groups.

Table 4: Comparison of body composition assessments for the low-protein subgroups.

Outcome	Control Group (n=20)	TFO Group (n=14)
PMA [weeks]	38.4 ± 2.4	39.1 ± 2.4
Total Body Mass [g]	2770 ± 740	3200 ± 730
Fat Mass [g]	510 ± 240	710 ± 280*
Fat-Free Mass [g]	2260 ± 560	2490 ± 470
Fat %	17.6 ± 5.4	21.5 ± 3.9*
Fat Free %	82.4 ± 5.4	78.5 ± 3.9*
Fat Mass Index [kg/m ²]	2.3 ± 0.9	3.2 ± 0.9*
Fat-Free Mass Index [kg/m ²]	10.6 ± 1.4	11.4 ± 0.8*

Values presented as mean±sd. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Table 5: Comparison of body composition assessments for the high-protein subgroups.

Outcome	Control Group (n=19)	TFO Group (n=15)
PMA [weeks]	39.4 ± 2.0	38.9 ± 2.4
Total Body Mass [g]	3010 ± 460	2940 ± 530
Fat Mass [g]	570 ± 200	640 ± 220
Fat-Free Mass [g]	2440 ± 340	2310 ± 390
Fat %	18.6 ± 4.5	21.3 ± 5.6
Fat Free %	81.4 ± 4.5	78.7 ± 5.6
Fat Mass Index [kg/m ²]	2.6 ± 0.7	3.9 ± 0.9
Fat-Free Mass Index [kg/m ²]	11.2 ± 0.8	10.7 ± 0.9

Values presented as mean±sd. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

From the low-protein subgroup analysis, the total body mass and fat-free mass were similar between the control and TFO groups. Infants in the TFO group had 3.9% more fat and consequently an equal proportion of less fat-free mass. Both the FMI and FFMI values were significantly higher for the TFO group. When analyzing the results of the body composition assessments from the high-protein subgroups, no differences were found between the control and TFO groups.

4.6 Regression Analysis

The tolerance tests for multicollinearity revealed that the protein intake and P:E ratio variables were correlated and that carbohydrate intake and CHO:NPE ratio variables were also correlated.

These correlated pairs could not be included in the same models and therefore in the multiple linear regression analysis, two sets of models were created for each growth outcome. The first set included the average macronutrient intakes and the other set included the macronutrient ratios.

4.6.1 Multiple Linear Regression Analysis

The results of the four multiple linear regression models are summarized in Tables 6-9.

Table 6: Linear model predicting weight (g) at 36 weeks' PMA with macronutrient intakes

Variable	Estimate	Standard Error	DF	t Value	p
Intercept	692	741	96	0.93	0.35
Fat intake (g/kg/day)	0.03	27.3	96	0.00	0.99
Carbohydrate intake (g/kg/day)	8.00	26.2	96	0.31	0.76
Protein intake (g/kg/day)	174	78.1	96	2.22	<0.05
Birth weight percentile (%)	8.18	0.98	96	8.38	<.0001
PMA at start of study (weeks)	9.25	20.7	96	0.45	0.66
Length of study period (days)	6.42	2.70	96	2.38	0.02

The overall model in Table 6 is statistically significant ($p < 0.001$) as there were variables with estimates that differed significantly from 0. The estimate for birth weight percentile was significant such that each unit increase in an infant's weight percentile at birth was associated with an 8.18 g increase in their weight at 36 weeks' PMA. Each 1-day increase in the length of the study period was also significantly associated with a 6.42 g increase in weight. The model also indicated that an infant's protein intake was a significant predictor of weight such that each 1g/kg/day increase in average intake was associated with a 174g increase in weight.

Table 7: Linear model predicting weight (g) at 36 weeks' PMA with macronutrient ratios

Variable	Estimate	Standard Error	DF	t Value	p
Intercept	1120	790	97	1.43	0.16
CHO:NPE (%)	-0.9	8.1	97	-0.11	0.91
P:E (g/100kcal)	244	116	97	2.10	<0.05
Birth weight percentile (%)	8.2	1.1	97	7.97	<0.001
PMA at start of study (weeks)	-2.4	21.6	97	-0.11	0.91
Length of study period (days)	6.7	2.84	97	2.36	<0.05

The overall model in Table 7 is statistically significant ($p < 0.001$) as there were variables with estimates that differed significantly from 0. The estimate for birth weight percentile was significant such that each unit increase in an infant's weight percentile was associated with an 8.2g increase in their weight at 36 weeks' PMA. Each 1-day increase in the length of the study period was also significantly associated with a 6.7g increase in weight. The model also indicated that an infant's P:E ratio was significant such that each 1g/100kcal increase in average intake was associated with a 244g increase in weight.

Table 8: Linear model predicting growth velocity (g/kg/day) with macronutrient intakes

Variable	Estimate	Standard Error	DF	t Value	Pr > t
Intercept	22.3	7.6	96	2.95	<0.01
Fat intake (g/kg/day)	-0.1	0.3	96	-0.26	0.79
Carbohydrate intake (g/kg/day)	0.5	0.3	96	1.70	0.09
Protein intake (g/kg/day)	0.2	0.8	96	0.25	0.80
Birth weight percentile (%)	-0.02	0.01	96	-2.09	0.04
PMA at start of study (weeks)	-0.2	0.2	96	-1.12	0.26
Length of study period (days)	0.0	0.0	96	0.68	0.50

The overall model in Table 8 is statistically significant ($p < 0.001$) because there is one variable with an estimate that differed significantly from 0. The estimate for birth weight percentile was significant and suggests that for each unit increase in the birth weight percentile, an infant's average growth velocity will decrease by 0.02g/kg/day. The key finding from this model was that the average macronutrient intakes were not significantly associated with an infant's average growth velocity during the study period.

Table 9: Linear model predicting growth velocity (g/kg/day) with macronutrient ratios

Variable	Estimate	Standard Error	DF	t Value	p
Intercept	25.0	7.8	97	3.2	<0.01
CHO:NPE (%)	0.1	0.1	97	1.44	0.15
P:E (g/100kcal)	0.2	1.2	97	0.13	0.89
Birth weight percentile (%)	-0.02	0.01	97	-2.03	<0.05
PMA at start of study (weeks)	-0.3	0.2	97	-1.47	0.14
Length of study period (days)	0.0	0.0	97	0.73	0.47

The overall model in Table 9 is statistically significant ($p < 0.001$) because there is one variable with an estimate that differed significantly from 0. The estimate for birth weight percentile was significant and suggests that for each unit increase in the birth weight percentile, an infant's average growth velocity will decrease by 0.02g/kg/day. The key finding from this model was that an infant's average macronutrient ratios were not significantly associated with an infant's average growth velocity during the study period.

4.6.2 Repeated Measures Regression Analysis

The overall model presented in Table 10 is statistically significant ($p < 0.001$) as there were variables with estimates that differed significantly from 0. The estimate for PMA was significant and indicates that, on average, each one-week increase in an infant's age during the study period was associated with a 0.8g/kg/day in daily weight gain. Of the binary characteristics, infants with a birth weight below the 50th percentile had, on average, a 1.84g/kg/day higher weight gain compared to those infants above this cutoff. From the macronutrient intake predictors, the "lagged" protein intake was significant such that a 1g/kg/day increase in the previous day's intake was associated with a 1.6 g/kg/day increase in daily weight gain. The magnitude of the day-to-day changes in protein, fat and carbohydrate intakes were not associated with the daily weight gain.

Table 10: Repeated measures model predicting daily weight gain (g/kg/day).

Variable	Estimate	Standard Error	DF	t Value	p
Intercept	35.6	12.7	100	2.80	<0.01
PMA during the study period (weeks)	-0.8	0.4	2267	-1.96	<0.05
Gestational age at birth group					
<28 weeks	-0.8	1.0	100	-0.80	0.42
≥28 weeks					
Birth weight percentile group					
<50 th percentile	1.8	0.7	100	2.56	<0.05
≥50 th percentile					
Daily macronutrient intakes with 1-day lag-effect					
Fat intake (g/kg/day)	0.0	0.3	2267	-0.01	0.99
Carbohydrate intake (g/kg/day)	0.3	0.2	2267	1.37	0.17
Protein intake (g/kg/day)	1.6	0.7	2267	2.50	<0.05
Absolute difference in day-to-day macronutrient intake					
Fat (g/kg/day)	-0.2	0.3	2267	-0.62	0.53
Carbohydrate (g/kg/day)	0.0	0.3	2267	-0.14	0.89
Protein (g/kg/day)	-0.4	0.9	2267	-0.39	0.69

Chapter 5. Discussion

In this double-blind RCT, we were able to show for the first time that infants receiving target fortified breast milk achieved higher macronutrient intakes and better growth outcomes when compared to those infants fed only standard fortified breast milk. Inadequate protein concentration in mother's native breast milk was identified as a determinant of infants with sub-optimal growth in weight and length. This was supported by the observation that daily weight gain was positively associated with protein intake from the previous day. Together, these findings provide practical solutions for individualizing breast milk fortification strategies, which can help to reduce the risk of restricted postnatal growth of preterm infants.

5.1 Target fortification improved macronutrient intakes and growth outcomes

Estimation of true macronutrient intakes based on analysis of native breast milk samples from each study day revealed that about 50% of infants did not achieve minimum ESPGHAN protein intakes after standard fortification. This supports previous observations that current routine fortification practices fail to provide sufficient intakes for all preterm infants⁷⁹. Use of an assumed average composition of native breast milk as a basis for fortification of macronutrients is the key factor contributing to nutrient inadequacy. If the macronutrient concentration in the native breast milk is higher or lower than the reference milk composition, standard fortification can lead to inappropriate intakes. Variations in the concentration of macronutrients in breast milk occurs between mothers and over the duration of lactation. Target fortification can overcome this limitation of breast milk as it introduces measurement of native breast milk content, adding a standard fortifier and then individually adjusting modular fortifiers to achieve a target concentration. In a previous study we were able to show that variation in macronutrient intakes was minimized when the target fortification recipe was adjusted only three times per

week⁷³.

The significantly improved macronutrient intakes in the TFO group, led to infant weight at 36 weeks' PMA that averaged 220g higher compared to the Control group. To put this into clinical context, using the Fenton growth curves, a male infant who achieved the average weight from the TFO group at 36 weeks' PMA would be at the 31st percentile compared to the 16th percentile for an infant who achieved the average weight from the Control group. The higher growth velocities in infants in the TFO during the study period were likely responsible for the greater achieved weight. Such higher growth rates as observed in the TFO group may have clinical relevance since Ehrenkranz demonstrated that preterm infants which averaged similar growth rates of 21g/kg/day during their NICU stay had the lowest risk for neurological impairment later in infancy¹⁰. Moreover, the method used to calculate growth velocities can lead to different results. The use of various calculation methods for growth velocities, which can lead to different results, has been previously discussed by both Patel and Fenton^{80,81}. We chose the exponential method for calculating the average growth velocity during the 21-day study period because it has been validated and was found to be more accurate for tracking the non-linear growth pattern of preterm infants in the NICU before discharge⁸².

While previous studies suggested that using target fortification based on frequent milk analysis significantly increases the macronutrient intakes and reduces their day-to-day variation^{79,83}, the results have not been consistent. An interventional study by Morlacchi and colleagues found that preterm infants fed with target fortification experienced daily growth rates that were, on average, 3.4g/kg/day higher than those fed with standard fortification⁸⁴. The generalizability of these findings are limited however as only 10 infants per group were included in their study and nutritional data for their control group were calculated retrospectively based on assumed breast

milk composition. A randomized, but not blinded, trial by McLeod and colleagues compared macronutrient intakes and weight gain in 40 preterm infants who received either routine fortification based on assumed breast milk macronutrient content (standard fortification) to target fortification based on measured breast milk macronutrient content⁸⁵. Given that their approach led to protein and energy intakes that were below ESPGHAN recommendations and similar between their study groups, it is not surprising that no group differences were observed in growth velocity or weight, head circumference and length achieved at discharge. The inconsistency in results with our present study may be attributable to a number of factors. First, the milk-analyzer used was not yet been validated for measuring macronutrient content in breast milk. Infrared milk analyzers, have significant measurement variation between devices, including between those from the same manufacturer^{72,86}. As such, it is recommended that each device is validated by calibrating results relative to established reference methods for determining the fat, lactose and protein content in breast milk. The second concern was that the authors only adjusted the target fortification once per week based on the average protein, carbohydrate and fat content in native breast milk from the previous study week. Adjusting the fortification only weekly is not sufficient to improve macronutrient intakes and reduce variation relative to standard fortification⁷³.

Lack of growth benefit with target protein fortification compared to standard fortification was also observed in a recent blinded randomized trial by Maas and colleagues in which 30 infants per group were followed until discharge⁸⁷. Due to their ordered hypothesis study design, the authors were not able to make further conclusions for comparisons between the target fortification group and high-protein standard fortification group. To make such a comparison, these authors stated that larger trials with daily milk analysis from 24-hour pooled feeds would

be required as opposed to the milk analysis completed twice per week in their study. Our study meets these design criteria and we have demonstrated that target fortification with all three macronutrients leads to improved nutritional intakes and has a positive effect on weight gain. The quality of weight gain is also an important factor to consider for preterm infants. In the present study, infants receiving TFO feeds had a higher fat mass, body fat percentage and FMI than infants in the Control group. Two previous studies which also assessed body composition of preterm infants around 40 weeks' PMA with a PEA POD device found that average body fat percentage was 14.8%⁸⁸ and 16.7%⁸⁹. These values are surpassed by the average adiposity values of infants in both Control and TFO groups in our study. However, the optimal distribution between fat and fat-free mass gains in preterm infants is currently unknown and requires further research. It is possible that infants in our study are experiencing a greater effect of postnatal adaptation whereby they achieve similar body composition at TEA compared to a term-born infant of similar chronological age of approximately three months⁹⁰. We also observed that all infants had an average fat intake that met the minimum recommended level after standard fortification. Therefore, the higher fat outcomes could be the result of the standard fortifier product used in our study that provides a larger proportion of its energy from fat. It would be of interest to study the hypothesis of whether the composition of the standard fortifier is associated with the relatively higher adiposity values of preterm infants observed in our study.

One factor that requires consideration in comparing body composition outcomes across studies is that absolute body composition values vary among different devices and NICUs. Considering potential inter-device and inter-observer variation, we compared our results to normative body composition data of 475 term and preterm infants measured in our own unit⁹¹. We found that the fat-mass and fat-free mass outcomes of TFO and Control infants around TEA were within the

range of values for infants born both below and above 36 weeks' gestation. This suggests that body composition outcomes at either extreme are not observed with either nutritional regime.

5.2 Growth outcomes from the subgroup analyses

Our results show significant improvements in weight and growth velocity outcomes for infants receiving TFO feeds in the low-protein subgroup, whereas, improvements in same outcomes were less prominent in the high-protein group comparison. To perform a subgroup analysis in this RCT is justified as it was based on previously established understanding of nutritional physiology and growth. Our finding can be directly linked to the concept that the degree of the TFO intervention is larger for infants who received breast milk with below average macronutrient content. The effect of adding TFO modular products on growth is relatively smaller for infants in the high-protein group because after standard fortification only, infants in the high-protein would have intakes close to the ESPGHAN recommendations. As such, the effect of the intervention is limited in this group and may contribute noise to the overall impact of TFO on growth.

The median protein level after standard fortification (3.5 g/kg/day) used to stratify infants into the subgroups also has clinical relevance as it coincides with the minimum protein intake recommended by ESPGHAN to achieve normal intrauterine growth rates. This also confirms the underlying assumption for the development of standard fortification which uses the average composition of breast milk. Therefore, infants in the low-protein group represent the portion of the preterm population where the most significant benefit of TFO would be observed.

This is especially concerning for preterm infants given that other studies and our regression models, discussed below, indicate increased protein intake is significantly associated with higher weight and higher daily growth rate. Without the TFO intervention, infants in the TFO low-

protein subgroup would have experienced insufficient protein intakes after standard fortification. We observed that the weight achieved by these infants was, on average, 340g higher than infants in the low-protein Control subgroup. As a result, a higher proportion of infants in the low-protein Control group would be below the 10th percentile compared to the TFO group. Other measures of growth, like average growth velocity during the study period, average change in length z-score and fat-free mass index, also significantly favoured those in the TFO low-protein subgroup. Infants in both the TFO and Control groups who had a protein concentration after standard fortification above the median (high-protein subgroups) had similar weight, change in anthropometric z-scores at TEA and body composition outcomes. As predicted, for these infants, individualized target fortification may not confer as significant of a benefit for their growth. Our overall findings from this additional analysis are similar to what previous studies have found and provides further evidence that protein deficits following standard fortification can lead to slower growth^{13,68,92}.

5.3 Other anthropometric measures

In our trial, TFO infants overall showed more positive changes in z-scores for weight compared to the Control group, while changes in head circumference, and length z-scores were similar. Changes in z-scores for growth outcomes that are closer to zero are indicative of stable growth. Some decrease in weight z-scores is expected as all neonates, including those born at term, undergo contraction of extracellular spaces and lose some weight shortly after birth⁹³. Infants in the TFO group had a significantly smaller decrease in weight z-scores at TEA, which suggests that they may gain back more weight after accounting for the initial amount lost after birth. In our study, although we were not able to show significant differences for positive head circumference growth, previous studies have found that improved nutritional intakes have been

positively associated with improved length and head circumference outcomes. These measures have subsequently been positively associated with improved neurodevelopmental outcomes later in infancy^{92,94}. We may not have observed similar improvements in HC growth because of issues that limit the accuracy of measurements. In the NICU, the increasing use of non-invasive ventilation which requires equipment that places continuous pressure on the infant's head and causes mild deformation of their soft skull. Therefore, measurements afflicted by this issue may have confounded potential differences resulting from the Control or TFO nutrition. Long-term follow-up of our cohort with anthropometric measures and neurodevelopment assessments, using the updated Bayley-III assessment, for our study population up to 18-22 months corrected age are being collected.

5.4 Regression models and the significance of protein on growth

In this study, we were able to confirm that higher average protein intake and the average P:E ratio were positively associated with greater weight. A significant new finding was that the daily growth rate was positively influenced by the protein intake from the previous study day. Our study design, which included accurate daily macronutrient intakes for each infant, allowed us to analyze variation of intakes and to quantify their effect on growth. For example, our results show that the average P:E ratio of all infants had a range of 1.6 g/100 kcal, which, according to the model presented in Table 8, would account for a 390g difference in weight. The repeated measures regression analysis allowed us to investigate the effect of daily macronutrient intakes on the weight gain observed over the following study day. In this model, the significance of the 1-day lag-effect might be due to the time it takes for the breast milk to be digested, absorbed and then utilized for energy and synthesized for tissue growth.

In contrast to our model's results for weight at 36 weeks PMA, the average growth velocity in our study was not influenced by either the average macronutrient intakes or the macronutrient ratios. A randomized trial by Kashyap and colleagues that fed fortified breast milk to three groups of preterm infants with either 35%, 50% or 65% CHO:NPE, while controlling for protein and total energy intakes, found different results. In their study, infants with 65% CHO:NPE experienced significantly higher growth velocity compared to infants with 35% CHO:NPE⁸. This suggests that, at a controlled protein intake, a higher amount of energy from carbohydrates relative to the energy provided by fats is associated with a faster growth rate. It is possible a similar trend was not observed in our study because both protein and energy intakes varied between infants. Additionally the average CHO:NPE ratio during the study period only ranged from 35% to 52% which may not be wide enough to capture differences in growth velocities with our sample size.

In our repeated measures model we did not find that the magnitude of day-to-day changes in the macronutrient intakes were associated with weight gain. The natural variation in breast milk can lead to variation in macronutrient intakes but the effect on growth and metabolic response of preterm infants is not fully known. From literature examining nutrition in adults, we see that variation induced by adding or omitting a meal were correlated with changes in weight and adiposity⁹⁵. Our findings may differ, in part, because we purposely attempted to minimize the variation in infants who received target fortification and the regression analysis could not detect the effect of small differences with our sample size. Alternatively, this result could also indicate that if a preterm infant is provided with an appropriate daily protein intake, for example, variation around that amount may not have a significant effect on the daily growth rate.

5.5 Strengths and Limitations

The present study, to our knowledge, is the first blinded randomized controlled trial with target fortification for all three macronutrients. This study design itself is a significant strength as it represents a gold standard in research trials. A unique feature of our trial is that native breast milk samples from each study day were collected for on-site analysis of protein, carbohydrate and fat concentration with validated methods. This allows us to accurately calculate daily macronutrient intakes rather than relying on assumed values of macronutrient content in breast milk. The adjustment of the fortification recipe in the TFO group was completed three times per week to balance the additional workload for NICU staff preparing feeds and the desire to minimize the variation in the intakes. This is a higher frequency of adjustment of milk nutrients than what others have attempted with target⁸⁵ and adjustable⁷⁰ fortification strategies, respectively. Furthermore, our study was sufficiently powered to detect a difference in weight of 180g which corresponds to a difference of 3g/kg/day over a 21-day study period which is clinically meaningful. Therefore our study group size of 103 infants is significantly larger than previous randomized or observational trials examining individual fortification options that included 40-50 subjects in total.

In the Control group, infants received standard fortified breast milk which only adds a fixed amount of macronutrients to each volume. Therefore, the existing variation in breast milk remains and, as expected, is reflected in the wide range of average intakes in the Control group. In comparison, infants in the TFO group experienced average protein and carbohydrate intakes with lower variation. However, one of the limitations of this study is that in the TFO group, the variation in protein, carbohydrate and fat intakes among infants could not be entirely eliminated. There are three factors which contribute to this. First, target fortification is not able to reduce fortification for breast milk samples that are exceeding recommendations after standard

fortification caused by the natural variation. As a result, variation from breast milk samples will remain present after standard fortification and may lead to intakes above the intended target. Secondly, in the intervention group, we adjusted the macronutrient content of breast milk to a target concentration three times per week. Because the native content was not constant and increased or decreased between the days when fortification was adjusted, the concentration of macronutrients, and consequently intakes, would have also increased or decreased, respectively. To precisely achieve the target concentration, daily analysis of native breast milk and adjustment of fortification would be required. Lastly, the final intake of nutrients also depends on the enteral volume of fortified breast milk fed to the infant. This volume is maintained at the discretion of the bedside nurses and the clinical team. If the total fluid intake was above or below the 150mL/kg/day level used when adjusting fortification, we would see macronutrient intakes that were above or below the intended target. It should also be noted that while some infants in the Intervention group had average protein and carbohydrate intakes slightly above the intended ESPGHAN target values, no metabolic adverse events were observed. Average fat intakes above the target were equally observed in both the Control and Intervention groups and is a result of the high fat content of the standard fortifier. To avoid this effect, the standard fortifier products should decrease the fat content and replace these calories with carbohydrates.

The generalizability of our results may also be considered as a limitation. Our study population included a relatively healthy subset of preterm infants whose study periods were generally free of extended use of medications like diuretics or steroids which can affect growth outcomes and this is not representative of all infants in neonatal care.

5.6 Future Directions

To expand on the current findings, future research should explore the effectiveness of individualized target fortification on growth outcomes in a multi-centre study. Particular focus should be on those infants who would face below average macronutrient intakes after standard fortification. Additionally, the efficacy of modular macronutrient fortifiers from other manufacturers should also be assessed to expand the applicability of the target fortification approach to NICUs with access to other products. Serial body composition assessments, when possible, and long-term neurodevelopmental assessments should also be done in trials to track the response to different nutritional regimens. This would provide insight into the quality of growth and address the uncertainties relating to balancing early rapid weight gain to optimize neurodevelopmental outcomes with the concerns about increased risk of future cardiovascular and metabolic diseases^{94,96}. Moreover, the regression analyses should be validated in another set of infants where accurate measures of enteral macronutrient intakes can be calculated based on daily breast milk composition measurements.

5.7 Conclusion

In order to meet the high growth requirements of the preterm infant, it has been suggested that approaches to optimization of the standardization of macronutrient content of expressed breast milk should be developed. The limitations of the current standard fortification practice, which jeopardize growth outcomes for preterm infants, can be overcome with individualized target fortification. The introduction of frequent breast milk measurements allows the deficiencies in protein, carbohydrate and fat content to be identified and corrected to recommended targets with modular fortifiers.

In our study, infants fed with this approach receive significantly improved macronutrient intakes and achieve higher weight and growth velocity outcomes compared to those on standard

fortification. The change in length, from birth to TEA, is also improved for infants fed target fortification compared to those who receive a low-protein intake with standard fortification. On the other hand, in this study we were not able to demonstrate improved head circumference outcomes at TEA. With respect to body composition outcomes, infants fed with target fortification experienced higher gains in both fat and fat-free mass at TEA. We have also demonstrated the significant importance of protein intakes on weight and daily weight gain outcomes in regression analysis. In summary, target fortification shows promise over standard fortification as a feasible way to improve the quality of nutrition and growth outcomes for preterm infants in the short-term.

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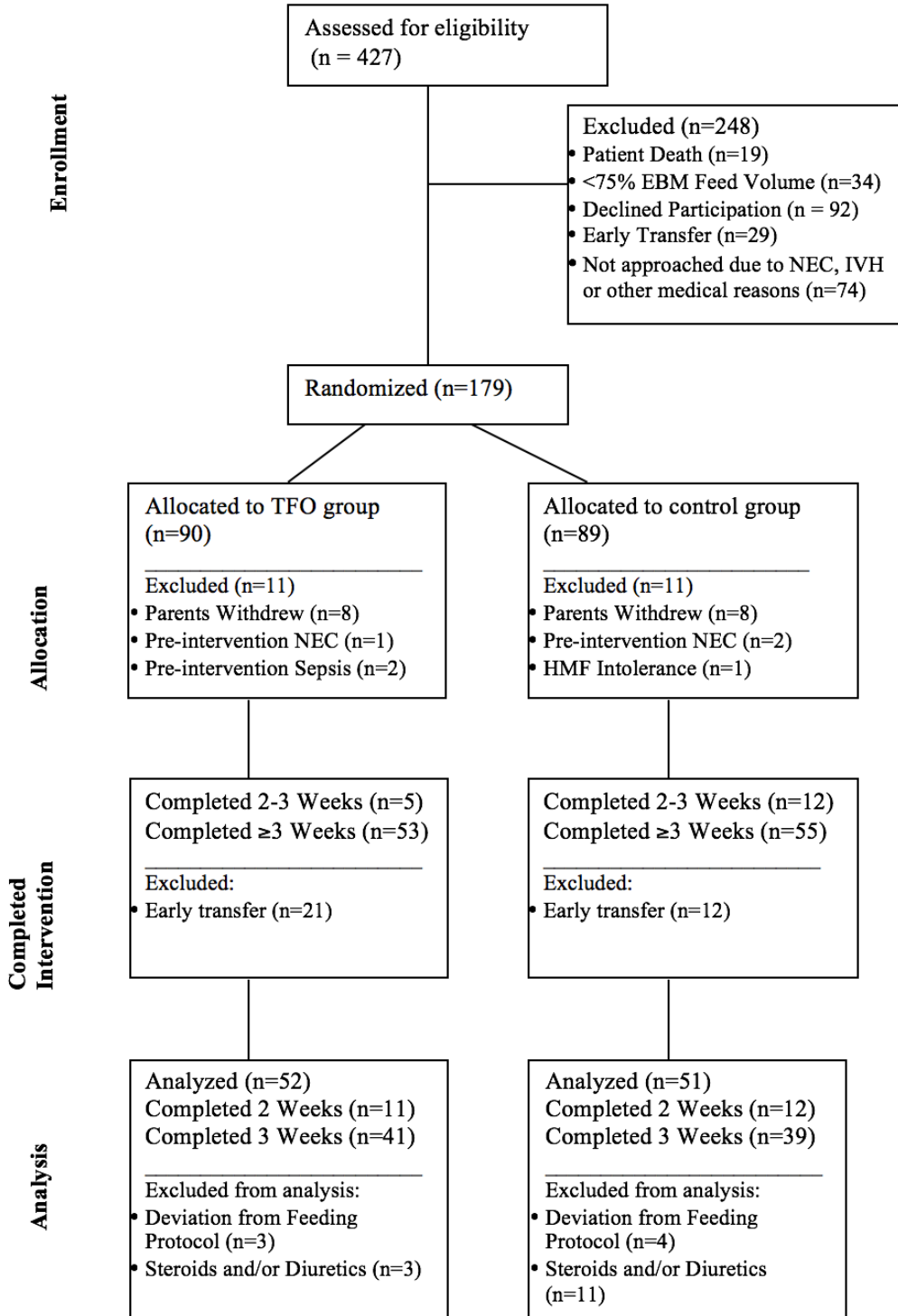
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Appendix 1. CONSORT Diagram



Appendix 2. Sample Fortification Recipe Sheet

Individualized Target fortification (TFO)

Infant name: **Sample Infant** Date: **2015-11-30**
 Infant number: **####-####**
 Start TFO: **2015-11-11** [mm/dd/yyyy]
 Prescription for: **2015-11-30** [mm/dd/yyyy] **Day 20**
 Total Milk batch: **300** Donor milk: **0** [mL]

	Milk measurement [g/100mL]	Milk composition corrected [g/100mL]	Final target composition [g/100mL]	Breast milk + Enfamil [g/100mL]	Amount to be fortified [g/100mL]	Final milk composition (breast milk + modulators + Enfamil) [g/100mL]	Amount of modulators to be added additionally per 100mL breast milk
Fat	2.90	2.36	4.40	3.36	1.04	4.40	Microlipid (0.1g Fat/0.2mL) 2.08 mL
Lactose	5.60	5.60	8.30	6.00	2.30	8.30	Polycose (0.1g Carb/0.11g) 2.53 g
Protein	1.42	1.59	3.00	2.69	0.31	3.00	Beneprotein (0.1g Prot/0.12g) 0.37 g

Feeding order

Date: **2015-11-30** [mm/dd/yyyy]

Prepare TFO fortification or routine fortification as randomized:

Add 302.5 mL Expressed Breast Milk into container

Draw 2.5 mL for milk analysis

Prescription for 300 mL batch	Prescription for 325 mL batch	Prescription for 350 mL batch
TFO Study Recipe 300 mL Breast / Donor Milk 12 packages Enfamil HMF 6.2 mL Microlipid 7.5 g Polycose 1.1 g Beneprotein	TFO Study Recipe 325 mL Breast / Donor 13.0 packages Enfamil HMF 6.8 mL Microlipid 8.0 g Polycose 1.2 g Beneprotein	TFO Study Recipe 350 mL Breast / Donor Milk 14 packages Enfamil HMF 7.3 mL Microlipid 9 g Polycose 1.3 g Beneprotein
Routine Fortification Recipe 300 mL Breast Milk 12 packages Enfamil HMF 0 mL Donor Milk 0 g Beneprotein (0.1g/25mL)	Please remember to add 0.1 g of Beneprotein per 25 mL of Donor Milk for TFO babies in routine arm. Babies in intervention arm always use Excel prescription.	

After the recipe is mixed, take a 1 mL sample for osmolality check and aliquot for individual feeds.