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Crescimento e composição corporal de crianças nascidas muito pré-termo: comparação entre duas coortes alimentadas com leite humano, respetivamente com fortificação alvo e padrão

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Growth and body composition of very preterm infants: comparison between two human milk-fed cohorts, respectively with target and standard fortification

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Preface

This study assessed whether a newly implemented fortification method based on measured human milk (HM) macronutrient content improved the energy and macronutrient intake and the quality of growth in infants born at less than 33 weeks' gestation, compared with HM fortification based on assumed HM macronutrient content.

The original PhD project, submitted in 2019, was entitled "Growth and body composition of children born very preterm: comparison between two human milk-fed cohorts, respectively with target and pattern fortification".

Meanwhile, two non-expected difficulties led to deviations to the original study protocol, in relation to what is stated in the ClinicalTrials.gov NCT04400396 registry and published protocol (Cardoso M, et al. *Int J Environ Res Public Health*. 2021;18:823):

I. Change from an intervention to an observational design

Although the original design intended to compare, 'per protocol', the effects of 'standard' and 'target' HM fortification methods, for clinical and logistical reasons, the addition of modular protein and fat supplements to fortified HM has to been left to clinical discretion, and the study design became observational rather than interventional. As such, comparisons were made 'per intention to treat', following the contemporary national and international guidelines. Therefore, instead of strictly following the 'standard' fortification method by simply adding a fixed dose of the fortifier to the HM, physicians were allowed to further add modular supplements of protein and mid-chain triglycerides (MCT) to the fortified HM, based on assumed HM macronutrient content according to the reported preterm breastmilk composition. Instead of 'target' fortification, physicians were asked to customize the HM fortification according to the measured HM macronutrient content, by adding modular supplements of protein and MCT to the fortified HM, when necessary.

II. Change in periods of study

The recruitment for the contemporary cohort of infants fed fortified HM based on measured HM macronutrient content was originally planned to start in February 2020 in order to compare with the historical cohort (2014–2015) of infants fed fortified HM based on assumed HM macronutrient content.

However, a shortage in modular protein supplement from 1 February to 13 July 2020 precluded the adoption of the new institutional nutrition protocol, which implied the possible addition of modular protein to the fortified HM guided by the measured HM macronutrient content. Meanwhile, fortification continued to be based on assumed HM macronutrient content, and the arm of fortification based on assumed HM macronutrient content included not only the historical cohort, but also part of the contemporary cohort recruited from 1 February to 13 July 2020.

The arm of fortification based on measured HM macronutrient content was only recruited from 14 July 2020 to 31 May 2021.

For this reason, in relation to the title of the PhD project, and that stated in the ClinicalTrials.gov registry and the published protocol, the paper containing the main results was renamed “Individualized fortification based on measured macronutrient content of human milk improves growth and body composition in infants born less than 33 weeks: a mixed-cohort study” (Cardoso M, et al. *Nutrients* 2023;13;1533).

"The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny…'."

Isaac Asimov

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My first acknowledgement words go to parents of preterm infants who participated in this study. Without their trust, I wouldn't even have started. More than my gratitude, their children deserve my immense admiration and respect. All they show me daily, the meaning of resilience.

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The completion of this study would have been impossible without the invaluable support of Professor Ana Luísa Papoila and Dr Marta Alves in the statistical analysis. I am grateful to them for their knowledge, scientific rigor, ability to make things simple, and unlimited patience.

The nurses from the Human Milk Bank at Maternidade Dr Alfredo da Costa were trusted partners in some very difficult moments through this study. Their dedication was unparalleled, and I deeply thank them for that.

Since the beginning of my project, I had the privilege of having the full support of Dr Célia Neves, who never let me give up in the face of adversities. I am very grateful for that.

Despite the geographical distance, my co-supervisor Professor Diana e Silva was always present. I am grateful for her interest in my PhD project, constant words of support and expressions of confidence.

Last but not least, I would like to express my deep gratitude to my supervisor Professor Luís Pereira da Silva, for these 4 years: for believing in me, for what he taught me, for all the times he brought me back to reality, for staying with me in the most difficult moments of this path. His ability to share knowledge and experience inspired me to grow personally and professionally. Thank you so much for being a mentor, a friend, and an inspiration to me.

Abstract

Background

The human milk (HM) is recommended as the first choice for feeding preterm infants but it does not fulfill the needs of growing preterm infants from a nutritional point of view. HM multinutrient fortifiers are used to prevent nutritional deficits, according to different fortification methods. The "target fortification" has been advocated as a superior method than "standard fortification" as it allows for more accurate achievement of nutritional targets, despite of disadvantages of requiring an HM analyzer and being time consuming and labor intensive.

The current literature reports that very preterm infants at term age have a lower fat-free mass and a greater adiposity when compared with term equivalent-age (TEA) infants.

Objectives

This study aimed to assess in HM fed preterm infants, whether a fortification method newly implemented in our unit, based on measured HM macronutrient content, compared with other based on assumed HM macronutrient content, improved the energy and macronutrient intake, in-hospital weight gain (primary outcome), and in-hospital linear and head growth as well as body composition at late preterm or TEA as secondary outcomes.

Methods

This single-center, observational mixed-cohort study of preterm infants, was conducted at the Centro Hospitalar Universitário de Lisboa Central, in the Neonatology Unit and in the Human Milk Bank of Maternidade Dr. Alfredo da Costa and in the Nutrition Laboratory of the Hospital Dona Estefânia. The study was approved by the hospital ethics committee and registered in the ClinicalTrials.gov NCT04400396.

Consecutive infants born with less than 33 weeks of gestation, appropriate for gestational age, and exclusively or predominantly HM-fed were eligible. The exposure period was defined by feeding fortified HM.

Energy and macronutrient intake, growth, and body composition were compared in infants fed fortified HM, based either on assumed (Group 1) or measured (Group 2) HM macronutrient content.

Guidelines in effect for enteral nutrition of preterm infants (ESPGHAN 2010) were followed. Using any nutritional regimen, 'standard fortification' was started by adding a fixed dose of multinutrient fortifier to HM (4.4 g/100 ml). Then, modular protein and/or medium-chain triglycerides were added to fortified HM, left to clinical discretion. In Group 1, the addition of modular macronutrient supplements to fortified HM was based on assumed HM macronutrient content reported in literature. In Group 2, this addition was more accurately oriented by measured MH macronutrient content. A real-time MH analyzer (Miris AB, Uppsala, Sweden) was used to measure the HM macronutrient content. An Excel program was developed to facilitate the calculation of the amounts of modular supplements to be added to the fortified HM.

Body weight was measured by attending nurses and body length and head circumference were measured by the same observer, according to recommended techniques. Z-score differences (Δ) of the three anthropometric measurements as well as their gain velocities were calculated and compared between groups, from birth to discharge. In the case of weight gain velocity, it was also calculated from the beginning of the exposure period to discharge.

A single body composition assessment using air displacement plethysmography (Pea Pod, Cosmed, Italy) was measured up to one week after discharge.

The demographic and clinical characteristics of infants are described with frequencies, mean and standard deviation or median and interquartile, as appropriate. Nonparametric Chi-Square test, Fisher's exact test, median test, and Mann–Whitney test were used. Variables that attained a *p*-value ≤0.25 in the univariable analysis were candidates for the multivariable models.

Results

One hundred and fifteen infants were included, 57 Group 1and 58 in the Group 2.

Fortification in Group 2 provided significantly higher mean energy intake, median fat, and carbohydrate intakes, although with significantly lower median protein intake in

infants weighing ≥1 kg and significantly lower median protein-to-energy ratio in infants weighing <1 kg.

Δ z-scores: in both groups, children were born with negative mean weight, length and head circumference (HC) z-scores. Weight and length Δ z-scores were negative in both groups; while the weight Δ z-scores did not differ between groups, a significantly smaller decline in Δ length z-score in Group 2 (p=0.005) occurred. The HC Δ z-scores differed significantly (p<0,001), the HC z-score becoming positive at discharge in Group 2, while remaining negative in Group 1.

Growth velocities: using the Patel method, the median weight gain velocity was significantly higher in Group 2, both from birth to discharge (10.8 vs. 9.7 g/kg/d, p=0.023) and from beginning of fortification to discharge (15.3 vs. 13.0 g/kg/d, p<0.001). However, using a linear mixed effects model, that considered daily estimates of weight gain velocity, no significant association was found between them and the method of fortification.

Fortification based on measured HM macronutrient content was significantly positively associated with both length gain and head circumference gain velocities, with faster mean length increase of 0.20 cm/week (β-estimate=0.20; 95% CI 0.126, 0.280; p <0.001) and mean head circumference increase of 0.18 cm/week (β-estimate=0.178; 95% CI 0.111, 0.246; p <0.001).

Body composition was assessed in 74 (64.3%) infants after discharge (38 in Group 1 and 36 in Group 2), between 35 and 41 postmenstrual age. Infants of Group 2 had significantly lower adiposity indicated by both percentage of FM (p= 0.021) and fat mass index (p= 0.004), as well as higher percentage of fat-free mass (p=0.012).

Conclusions

In this cohort study of infants born at less than 33 weeks of gestation, those fed fortified HM based on its measured macronutrient content globally had significantly better weight gain, length, and head growth at discharge, and lower adiposity and greater lean mass at or near TEA. In Group 2, a deviation in fat intake and PER existed in relation to the ESPGHAN 2010 guidelines but became within the range of the updated ESPGHAN 2022 guidelines, pubished after the conclusion of the study.

Keywords: adiposity; body composition; growth velocity; human milk fortification; preterm infants

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Resumo

Introdução

O leite humano (LH) está recomendado como primeira escolha na alimentação de crianças nascidas pré-termo, mas não supre as suas necessidades de crescimento sob o ponto de vista nutricional. Os fortificantes multi-nutriente para o LH são utilizados para prevenir défices nutricionais, havendo diferentes métodos de fortificação. A "fortificação alvo" tem sido considerado um método com vantagens em relação à "fortificação padrão", pois permite de forma mais precisa atingir os alvos nutricionais, apesar das desvantagens de requerer um analisador de HM e ser um processo demorado e laborioso.

A literatura recente refere que crianças nascidas muito pré-termo ao atingirem a idade de termo, apresentam menor proporção de massa livre de gordura e maior adiposidade, quando comparados com as crianças nascidas de termo.

Objetivo

Este estudo teve como objetivo avaliar em crianças nascidas pré-termo alimentadas com LH, se um método de fortificação recentemente implementado na nossa Unidade, orientado pela medição de macronutrientes do LH, melhora o suprimento de energia e macronutrientes.

O aumento ponderal durante o internamento foi considerado a medida de efeito primária e os crescimentos linear e cefálico durante o internamento, assim como a composição corporal na idade próxima à de termo, constituíram as medidas de efeito secundário.

Métodos

Este estudo observacional de coorte mista, unicêntrico, em crianças nascidas pré-termo, foi realizado no Centro Hospitalar Universitário de Lisboa Central, na Unidade de Neonatologia e no Banco de Leite Humano da Maternidade Dr. Alfredo da Costa e no Laboratório de Nutrição do Hospital Dona Estefânia. Foi aprovado pela Comissão de Ética do Centro Hospitalar e registado no ClinicalTrials.gov NCT04400396.

Foram elegíveis crianças nascidas consecutivamente com menos de 33 semanas de gestação, com peso adequado para a idade gestacional e exclusiva ou predominantemente alimentados com LH. O período de exposição foi definido pelo período de alimentação com LH fortificado.

O suprimento de energia e macronutrientes, o crescimento e a composição corporal foram comparados entre crianças alimentadas com LH fortificado baseado na sua composição estimada (Grupo 1) e medida (Grupo 2).

Foram seguidas as recomendações vigentes para nutrição entérica de crianças nascidas pré-termo (ESPGHAN 2010). Usando quaisquer dos regimes nutricionais, foi iniciada a 'fortificação padrão' adicionando uma dose fixa de fortificante multi-nutriente ao LH (4,4 g/100 ml), independentemente da sua composição. De seguida, de acordo com o critério clínico, foram adicionados ao LH fortificado proteína modular e/ou triglicerídeos de cadeia média quando necessário. No Grupo 1, a adição de suplementos modulares de macronutrientes baseou-se no teor estimado de macronutrientes do LH descrito na literatura. No Grupo 2, essa adição foi orientada com maior precisão pelo teor de macronutrientes medido no LH, usando um analisador de LH (Miris AB, Uppsala, Suécia). Foi desenvolvido um programa Excel para facilitar o cálculo das quantidades de suplementos modulares a serem adicionados ao LH fortificado.

A medição do peso foi realizada por enfermeiras e o comprimento e o perímetro cefálico foram medidos pelo mesmo observador, de acordo com a técnica recomendada. As diferenças (Δ) de *z-score* das três medidas antropométricas, bem como suas velocidades de crescimento, foram calculadas e comparadas entre os grupos, desde o nascimento até à alta; no caso da velocidade de aumento ponderal, também foi calculada do início da fortificação à alta.

Foi realizada uma única avaliação da composição corporal, na semana imediatamente a seguir à alta, por pletismografia de deslocação de ar (Pea Pod, Cosmed, Itália).

As características demográficas e clínicas dos participantes estão descritas por frequências, médias e desvio padrão ou medianas e intervalo interquartil, conforme adequado. Foram usados testes não paramétricos Qui-quadrado, teste exato de Fisher, teste da mediana e teste de Mann-Whitney. Foram candidatas aos modelos multivariáveis as variáveis que obtiveram valor-p ≤0,25 na análise univariável.

Resultados

Foram incluídas 115 crianças, 57 do Grupo 1 e 58 do Grupo 2.

O método de fortificação no Grupo 2 proporcionou suprimento de energia, lípidos e hidratos de carbono significativamente maior, embora com menor suprimento de proteína nas crianças com peso ≥1 kg e menor rácio proteína/energia (RPE) nas crianças com peso <1 kg.

Δ *z-scores*: em ambos os grupos, as crianças nasceram com *z-scores* negativos de peso, comprimento e perímetro cefálico (PC). A mediana do Δ *z-score* do peso e comprimento foi negativo em ambos os grupos; enquanto o Δ *z-score* do peso não diferiu entre os grupos, houve um declínio significativamente menor do Δ *z-score* do comprimento no Grupo 2 (p=0,005). O Δ *z-score* do PC diferiu significativamente (p<0,001), tendo o *z-score* do PC tornado positivo no momento da alta no Grupo 2 e permanecido negativo no Grupo 1.

Velocidades de crescimento: usando o método de Patel, a mediana da velocidade de aumento ponderal foi significativamente maior nos lactentes do Grupo 2, tanto do nascimento à alta (10,8 vs. 9,7 g/kg/d, p= 0,023) como do início da fortificação à alta (15,3 vs. 13,0 g/kg/d, p< 0,001). No entanto, usando um modelo linear de efeitos mistos, que considerou as estimativas diárias da velocidade ponderal, não foi encontrada associação significativa entre as mesmas e o método de fortificação.

A fortificação baseada da medição de macronutrientes de LH teve uma associação positiva significativa com as velocidades de crescimento linear e cefálico. Houve um aumento médio da velocidade de crescimento linear em 0,20 cm/semana (β-estimativa =

0,20; 95% CI 0,126, 0,280; p <0,001) e de crescimento cefálico em 0,18 cm/semana (β-estimativa=0,178; IC 95% 0,111, 0,246; p <0,001).

A composição corporal foi avaliada em 74 (64,3%) crianças após a alta (38 no Grupo 1 e 36 no Grupo 2), entre as 35 e 41 semanas de idade pós-menstrual. As crianças do Grupo 2 apresentaram adiposidade significativamente menor, indicada pela percentagem de massa gorda (p= 0,021) e pelo índice de massa gorda (p= 0,004), assim como maior percentagem de massa livre de gordura (p=0,012).

Conclusões

Neste estudo de coorte de crianças nascidas com menos de 33 semanas de gestação, as alimentados com LH fortificado baseado na medição do seu teor em macronutrientes apresentaram globalmente melhor aumento ponderal, melhor crescimento linear e cefálico e adiposidade significativamente menor e massa magra significativamente maior na idade de termo ou próximo ao termo. Neste grupo, observou-se um desvio do suprimento de lípidos e da RPE em relação ao recomendado pela ESPGHAN em 2010, mas que se enquadram na amplitude do é recomendado pela ESPGHAN em 2022, atualização disponibilizada após a conclusão do estudo.

Palavras-chave: adiposidade; composição corporal; crianças pré-termo; fortificação de leite humano; velocidade de crescimento

Table of Contents

List of Tables

List of Figures

List of Abbreviations

%FM - fat mass percentage

ADP - air displacement plethysmography

ALA - α-linolenic acid

BPD - bronchopulmonary dysplasia

BUN - blood urea nitrogen

CRIB - Clinical Risk for Babies

CRIB-II - recalibrated versions Clinical Risk for Babies

DHM - donor human milk

ESPGHAN - European Society for Paediatric Gastroenterology Hepatology and Nutrition

FFM - fat-free mass

FM – fat mass

GA - gestational age

GR - gastric residuals

HC - head circumference

HM - human milk

HMOs - oligosaccharides

IVH - intraventricular hemorrhage

LA - linoleic acid

LCPUFA - long chain polyunsaturated fatty acids

LOS - late-onset sepsis

MBD - metabolic bone disease

MCT - mid-chain triglycerides

MOM - mother's own milk

NEC - necrotizing enterocolitis

NICU - Neonatal Intensive Care Unit

PER - protein:energy ratio

PMA - postmenstrual age

PN - parenteral nutrition

RCT - randomized controlled trial

SNAP - Score for Neonatal Acute Physiology

SNAPPE II - Score for Neonatal Acute Physiology Perinatal Extension-II

TEA - term equivalent-age

VLBW - very low birth weigh

WHO - World Health Organization

1 State of the Art

1.1 Prematurity – general aspects

Definition

The World Health Organization (WHO) defines preterm birth as any birth before 37 completed weeks of gestation, or fewer than 259 days since the first day of the last menstrual period. This is further subdivided based on gestational age (GA) in: extremely preterm (<28 weeks), very preterm (28 weeks to <32 weeks), and moderate or late preterm (32 weeks to <37 completed weeks). The limit of human viability in terms of extreme prematurity is defined as GA at which the chance of survival is 50% and until recently it was approximately 23–24 weeks in developed countries [1]. However, the limit of viability is a moving target and a dilemma concerning the quality of human life and sequelae of survivals [2].

Epidemiology

According to WHO 2022 data it is estimated that 15 million infants are born preterm every year, representing 5% to 18% of births, [3].

In Europe, the estimated rate of preterm births ranges from 5 to 10% [4,5].

In Portugal, between 2015 and 2020 there was a decrease in the percentage of preterm live births, from 8.0% to 6.8% [6].

1.2 Mortality and Morbidity related to prematurity

Currently, prematurity is the second leading cause of death in children under-5 years and the most important direct cause of death in the first month of life [7–9].

Despite recent technological advances, extremely preterm infants remain at high risk for death and disability. The mortality rate is around 30–50% and at least 20–50% of survivors are at risk of morbidity [1].

The main short-term and long-term morbidities in infants born preterm include [10] respiratory distress syndrome, patent ductus arteriosus, bronchopulmonary dysplasia (BPD) [11], necrotizing enterocolitis (NEC) [12,13], late-onset sepsis (LOS) [12], severe

intraventricular hemorrhage (IVH) [14,15], white matter injury (WMI) [16,17], retinopathy of prematurity (ROP) [13], and metabolic bone disease (MBD) [18–20].

1.2.1 Short-term morbidities affecting nutritional management

Some morbidities inherent to prematurity directly or indirectly affect the nutritional management in preterm infants. This occurs in NEC, in which intestinal ischemia limits enteral feeding [21] and in BPD, which forces fluid restriction, limiting provision of sufficient energy and nutrient intake [22].

Necrotizing enterocolitis

Necrotizing enterocolitis is one of the main causes for morbidity and mortality in preterm neonates, affecting 5–12% of very low birth weight (VLBW) infants (born < 1500 g) [23–28]. The mortality rate in infants with NEC varies from 10% to 30% [29].

Postnatal risk factors for NEC include intestinal dysbiosis, low hemoglobin concentration, conditions reducing intestinal perfusion as large patent ductus arteriosus and congenital heart disease [27,29].

In more immature infants the gastrointestinal barrier is impaired during the first postnatal weeks, associated with less milk-degrading microbes and more bacterial oxidative stress proteins [30]. These factors associated to reduced mucus thickness, lower intestinal alkaline phosphatase secreted by enterocytes, and diminished secretion of lysozymes by Paneth cells, increase the risk of inflammation, dysbiosis, and development of NEC [31].

Formula feeding is also an important risk factor for NEC, compared with mother's own milk (MOM) or donor human milk (DHM) feeding [32]. In fact, human milk (HM) has major immunologic components that attenuate inflammatory responses and/or play a role in regulating gut barrier integrity and microbial colonization, thereby protecting against NEC [28,33,34].

Bronchopulmonary dysplasia

Bronchopulmonary dysplasia, also named chronic lung disease of prematurity, is a lung disease that causes dependence on oxygen for at least 36 weeks postmenstrual age (PMA) [35](Bancalari et al. 2019). Extreme prematurity and extremely low birth weight (< 1000 g)

are risk factors for BPD, with GA and birth weight being inversely proportional to the incidence and severity of BPD [35,36].

Among postnatal factors, nutrition plays a central role in lung growth and repair [35,37,38]. The association of BPD development with deficit of energy and nutrient and growth restriction has been reported in preterm infants [39,40]. In these infants, a vicious cycle may occur. Growth restriction is predominantly due to malnutrition. Malnutrition, in turn, may worsen BPD probably by compromising lung development and function, and feeding difficulties can further affect nutrition [37,39,41].

In infants with BPD, a status of increased respiratory work and inflammatory response, along with the lung damage/repair process, is characterized by higher energy consumption [42]. In these infants, high energy should be provided in restricted fluid intake, since fluid overload may cause pulmonary edema, which can decrease lung compliance and increase airway resistance [43].

1.2.2 Long-term morbidity related to prematurity

The implications of morbidity resulting from prematurity extend well beyond the neonatal period throughout the life cycle, leading affected individuals needing future special care and face greater risks of serious health problems, including short bowel syndrome, decrease in brain volume, cerebral palsy, intellectual impairment, vision and hearing loss, chronic lung disease, lower bone mineral density, alterations in cardiac structure and function, increased arterial stiffness, high blood pressure, low insulin sensitivity, and increased intra-abdominal fat [44–48].

Prematurity not only affects a newborn infant directly but can also result in a vicious intergenerational cycle of risk, since preterm females are at risk of giving birth preterm offspring [49,45].

Preterm birth and programming of non-communicable diseases

The enhanced nutrition in early life in an attempt of optimize growth in preterm infants may result in a rapid catch-up growth that is associated to an increased risk of developing non-communicable diseases, including cardiovascular diseases and metabolic syndrome in later life [5,50].

Metabolic syndrome is a cluster of risk factors that include high blood pressure, excess body fat, elevated glycemia and abnormal cholesterol levels that increase the risk for cardiovascular disease and stroke. Co-existence of more than one factor results in evolution of systemic inflammation [48,51] or ongoing oxidative stress [48,52], further increasing the risk for cardiovascular disease. A marked increase in risks factors such as hypertension, airflow obstruction and glucose intolerance has been noted in young adults born preterm [48,53].

In infants born moderately preterm, a retrospective cohort study assessing waist circumference, blood glucose level, systolic blood pressure, and high-density lipoproteins and triglyceride levels, fund higher mean cardiometabolic risk scores at age 3 to 12 years compared to full-term infants [48,54]. Interestingly, each additional week of gestational age was associated with lower cardiometabolic risk score in preterm infants [48,54].

In very preterm infants, a systematic review found higher systolic blood pressures when these individuals reached at 18 years of age compared to those born at term [48,55]. Similarly, adults born with VLBW had higher systolic and diastolic blood pressures, with female gender and preeclampsia being additional risk factors [48,56].

It is described that in very preterm infants the postnatal age and PMA are the strong predictors of fat mass (FM) and percentage of FM (%FM) at 34–37 weeks PMA [57]. As the rapid accumulation of fetal fat begins by 32–33 weeks gestation, after a preterm birth interrupting the constant nutrient supply by the placenta, the ability for fat accretion may be an adaptive postnatal mechanism protecting against the risk of low nutrient availability [57,58].

In a systematic review it was found that preterm infants at term equivalent age (TEA) have greater %FM and less fat-free mass (FFM) than that of infants born at term [59]. It is possible that this peculiar pattern results from growth that occurs during the first postnatal weeks of life after preterm birth, in consequence of complex metabolic and endocrine adaptations at extrauterine life as well as of early nutrition [5].

Neurocognitive impairment related to early undernutrition

The third trimester, during which very preterm infants are born, is a period vulnerable to nutritional deficits in the extrauterine environment as placental supply is abruptly

discontinued [60]. In addition to suboptimal nutrition, immature brains are particularly susceptible to inflammation and oxidative stress [61,62].

Very preterm infants, in whom energy, protein and lipid intakes comply with enteral feeding recommendations during the first postnatal month, are reported to have white matter maturation improvement at 5 years of age, which in turn is related to better cognitive outcomes, in relation to those whose nutrient intakes did not achieve the recommendations [63]. The higher energy intake during the first postnatal week was found to mediate the better neurodevelopment [64]. Later in the lifecycle, it was found that higher energy and macronutrients intakes during the first nine postnatal weeks, predict better neurocognitive abilities by 25 years of age [65]. Regarding the type of feeds, higher exposure to HM in preterm infants is associated with improved microstructural properties of white matter tracts and cerebral structural connectivity and better neurocognitive abilities in adulthood [61,62,65].

In addition to energy and macronutrients, it is known that provision of other specific nutrients, such as long chain polyunsaturated fatty acids (LCPUFAs), iron, zinc, cooper, iodine, vitamin B12, folate and choline, are essential for early neonatal brain development and affect brain function across the lifespan [66].

Although not a direct measure of brain development, HC is correlated with brain growth at term-equivalent age (TEA) and is associated with later neurocognitive outcomes in preterm infants [60,67].

1.2.3 Risk indices

The Clinical Risk for Babies (CRIB) [68] and the Score for Neonatal Acute Physiology (SNAP) [69], based on physiologic items, were originally developed from multicenter prospective data, to assess illness severity and mortality risk in neonatal intensive care.

To calculate these indices, the physiologic items are collected in a window from the first 12 hours to 24 hours after admission to the neonatal intensive care unit (NICU) in order to minimize the effects of early treatments [70].

The recalibrated versions Clinical Risk for Babies (CRIB-II) [71] and Score for Neonatal Acute Physiology Perinatal Extension-II (SNAPPE II) [70] were developed to be more simple, accurate, and robust tools. CRIB-II and SNAPPE-II were recently validated in

very preterm infants, and it was concluded that both indices have good predictive ability for mortality and short-term morbidities related to the prematurity [72].

1.3 Nutritional approach in preterm infants

In very preterm infants, nutrition support entails three stages [73]:

1. Early aggressive parenteral nutrition (PN), progressively replaced with enteral feeding during the first several postnatal weeks.
2. Fortified HM or preterm formula when infants are advanced to full enteral nutrition or are already under exclusively enteral feeding.
3. Post-discharge stage, a challenging task trying exclusive breastfeeding

Guidelines for pediatric PN recently updated by the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) [74], including recommendations for infants born preterm. Based on this ESPGHAN position paper, the Portuguese Neonatal Society have updated their own recommendations focused on preterm infants [75,76].

PN should be initiated preferably within the first 24 postnatal hours, primarily to provide adequate intake of glucose, calcium, and amino acids.

1.4 Enteral nutrition approach in preterm infants during the hospital stay

A main reason for heterogeneity in enteral nutrition practice in preterm infants is the fear of NEC related to the intestinal immaturity [77]. Improvement of nutrition support in these infants benefit if the approach to enteral nutrition is standardized based on current evidence, namely regarding when and how to start, how to administer (continuously or intermittently), what to administer (MOM, DHM or formula), how to progress (volume) and when to interrupt or reduce [78].

1.4.1 Recommended enteral nutrient intakes in preterm infants

Recommended intakes herein stated primarily concern stable full enterally fed preterm infants and are based on the updated ESPGHAN guidelines recently published [78].

As the nutritional intakes considered in this thesis were focused on energy and macronutrients, only intakes of these nutrients will be addressed.

Energy

Energy supply needs to meet resting energy expenditure (REE) plus the requirements of physical activity, diet induced thermogenesis, and tissue deposition (growth) [78,79]. Specifically for growth, the estimated average energy requirements are ~3.6–4.7 kcal/g [78,80], to achieve 17–20 g/kg/d weight gain velocity.

Total energy of 115–140 kcal/kg/d is recommended in most stable preterm infants, not considering changes in energy needs related to acute illness or chronic disease states [78].

In infants with BPD [39] and/or suboptimal growth, higher than 140 kcal/kg/d may be necessary, provided not exceeding 160 kcal/kg/d and guaranteeing the recommended protein-to-energy ratio [78] stated below.

Protein and protein-to-energy ratio

In very preterm infants, protein intake of 3.5-4.0 g/kg/d together with sufficient intake of other macro and micronutrients is recommended [78].

In infants with slow growth rate, the protein intake may be increased to 4.5 g/kg/d, provided concomitant energy and other micronutrient intakes are adequate and no other causes justify suboptimal growth [78].

In infants under exclusive enteral feeding, reduction of protein intake should be considered if serum urea exceeds 34 mg/dl or blood urea nitrogen (BUN) exceeds 16 mg/dl, in the absence of fluid or renal derangements [78].

A protein-to-energy ratio (PER) of 2.8-3.6 g/100 kcal is recommended if energy and protein intakes are within the recommended ranges [78].

Fat

A total fat intake of 4.8-8.1 g/kg/d is recommended in most preterm infants [78].

Medium chain triglycerides should be < 40% of total fat intake [78].

Fatty acids intake should be of: linoleic acid (LA) 385–1540 mg/kg/d, α-linolenic acid (ALA) > 55 mg/kg/d, LA:ALA ratio (in mass) 5–15:1, arachidonic acid 30-100 mg/kg/d, docosahexaenoic acid 30-65 mg/kg/d, and eicosapentaenoic acid < 20 mg/kg/d [78].

Carbohydrates

Carbohydrate intake of 11-15 g/kg/d is recommended in most preterm infants [78].

Higher carbohydrate intake may be considered for a short period of time to facilitate catch-up growth [78].

1.4.2 Type of feeds

There are infant formulas designed for preterm infants, but these will be not addressed, once this thesis is focused on HM fed infants.

Human milk is a dynamic biofluid with evolving nutrient and bioactive factors [81–84]. These non-nutritional factors, include oligosaccharides (HMOs), hormones, growth factors, enzymes, immunoglobulins, antioxidants, cytokines, cellular components, and beneficial microbes, providing relevant biologic benefits [78,85]. Several bioactive factors are higher in preterm breastmilk compared to full-term breastmilk [85], one of the reasons why fresh MOM provides higher protection to preterm infants [78].

Mother's own milk

Bioactive factors in breastmilk are specially highly concentrated during the first 28 days after birth, and for most components, there is an inverse relationship between concentration and duration of gestation [86–88].

Several studies have assessed the clinical effects of certain HM bioactive factors. The HMOs are especially important in infants born prematurely, who are not able to immediately tolerate enteral feeding and thus are susceptible to changes within their microbiome and metabolome early in their lives [89]. The HMOs produce a wide variety of metabolites, many of which are important for immune system development, further

development of the gastrointestinal tract, and neurodevelopment. MOM acts by increasing proteolytic enzymes and decreasing gastric pH, determining less pathogenic bacterial flora and improving epithelial membrane and tight junctions. In preterm infants, MOM lowers the extent of microbial dysbiosis by stimulating peristalsis and gut motility, together with the immune system that also includes secretory IgA, lactoferrin, and growth hormones [47,90]. Other potential advantage of MOM in preterm infants is the ability of attenuating the toll-like receptor 4 mediated pro-inflammatory response (typical hallmark in NEC pathogenesis) by activating the receptor for epidermal growth factor, resulting in enhanced mucosal healing, intestinal stem cell proliferation and decreased enterocyte apoptosis [47,91,92].

Other studies have assessed clinical outcomes in preterm infants fed MOM. At short-term, provision of MOM to preterm infants was shown to reduce rates of NEC, sepsis, and BPD, compared with feeding preterm infant formula [88,93–96]. The incidence of NEC was found to be 6–10 times higher in exclusively formula-fed infants compared to the exclusively fed MOM [47,97,98]. MOM was also shown to improve neurodevelopmental outcome in preterm infants [88,99–103]. In very preterm infants, recent data revealed that early high dose of MOM was associated to intra and interhemispheric connectivity [62,88]. Improvements in many of the aforementioned outcomes demonstrate a dose-response relationship, with increasing amount MOM correlating with larger effects [84,104]. [47,97,105].

The variability and concentration of macronutrients in MOM and pasteurized DHM is an important consideration when prescribing preterm infants' nutrition as this may alter calculations for nutrient provision, bioavailability, and absorption thus impacting growth [84]. A systematic review and meta-analysis of energy and macronutrient content of preterm MOM, at various lactation periods, found that protein content is reduced in half within 10-12 weeks of lactation and fat content increases over time [106].

Donor human milk

When MOM is not available, the second choice for preterm infants is fortified DHM, conditionally recommended over preterm formulas [78].

Donor human milk undergoes pasteurization, at least two freeze-thaw cycles, that decreases or eliminates bioactive compounds including neutrophils, commensal bacteria, anti-inflammatory factors, lactoferrin, and immunoglobulins that are known to be

associated with reductions in neonatal morbidity [88,107]. Pasteurization slightly decreases and freeze-thawing slightly increases the osmolarity [108].

Nevertheless, pasteurized DHM, compared to preterm formula, may reduce NEC rates in preterm infants, whereas other neonatal morbidity and mortality rates are unaltered [78,109,110].

Donor human milk is usually expressed from women who delivered term born infants several months before and is often pooled from several donors. In consequence, DHM has a suboptimal nutrient profile, with lower protein and lipid content, absence of lipase, and reduced amylases, and proteases, compared with MOM of preterm infants and milk expressed at earlier stages [78,88,107,111]. In particular at 4 weeks lactation, the protein content in single or multiple DHM pools is lower than that of MOM [112].

Human milk pasteurization

Pasteurization is essential for using DHM in preterm infants. The Holder pasteurization method [83,113] implies heating HM to 62.5 ºC for 30 min, followed by rapid cooling. This method eliminates bacteria and viruses, including cytomegalovirus, but inactivates or destroys major bioactive components (immunoglobulins, lactoferrin, lysozyme, α-lactalbumin, and enzymes), whereas not affecting HM oligosaccharides [83,114] and lactose [78,83,115]. Alternative pasteurization methods have been proposed to destroy pathogens while maintaining the structure and function of most bioactive molecules in milk [83,114]. Recently, a method of sterilizing HM has become available, based on retort processing that uses high heat for shorter treatment time (121ºC for 5 min) and high pressure of 15 pounds per square inch above atmospheric pressure [83]. Compared with pasteurization methods that destroy heat-labile microorganisms, this sterilization method decreases all microorganisms (including spores) to undetectable levels. Published data on the effects of retort sterilization on HM bioactive proteins or HMOs are still not available.

Human milk bank

Globally, in some settings DHM is produced by country-level milk banking networks that serve a conduit between the recipient infants and the donors who provide the milk [116–118].

In Portugal, there are currently two HM banks. The first was inaugurated in Lisbon, in 2009, at Maternidade Dr Alfredo da Costa, Centro Hospitalar Universitário de Lisboa

Central, and the second inaugurated in Porto, in 2022, at Centro Hospitalar Universitário de São João which is currently is fully operational. The milk donated by these milk banks is mainly intended for premature infants.

1.4.3 Starting volume of feeds

In a recent national multicenter cohort study in very preterm infants, investigating the optimal time point after birth at which enteral nutrition could be started, it was concluded that enteral feeding should be initiated preferably within 24 postnatal hours, since it may promote feeding tolerance, shorten the time to reach total enteral feeding, and reduce the incidence of growth restriction and LOS, without increasing the risk of NEC [119].

Studies conducted more than 20 years ago in very preterm infants have reported advantages on initiating feeding using 'minimal enteral feeding' (MEF) or 'trophic feeding', defined as nutritional insignificant small volumes of feeds (typically 12-24 ml/kg/day) without advancement for 3-7 days [78]. However, there is no current evidence of a beneficial effect of maintaining for any period the MEF volume intake compared to advancing feeds immediately after birth [78].

In most preterm infants it is recommended initiation of enteral feeding within the first 24 postnatal hours, with 12-24 ml/kg/day, preferably using MOM or DHM, and advanced as the infant tolerates [78,119].

1.4.4 Advancing volume of feeds

After 4 postnatal days, feeding advancement of 30 mL/kg/day do not significantly increase the incidence of NEC or mortality compared to slower 15-20 mL/kg/day advancement that was formerly used [78,120].

Therefore, in stable preterm infants, feeding advancement of 18-30 mL/kg/day is recommended, especially if MOM is used [78].

1.4.5 Bolus versus continuous feeding

Bolus feeding is more physiological, promoting the cyclical release of gastrointestinal tract hormones that stimulate gut maturation and motility [78]. However, compared with continuous feeding, bolus feeding may increase splanchnic perfusion and energy expenditure, potentially compromising growth [78,121].

A meta-analysis of randomized controlled trials (RCTs) comparing continuous feeding with intermittent bolus feeding in VLBW infants did not find significant differences in feeding intolerance, duration of parenteral nutrition, growth, necrotizing enterocolitis, and duration of hospitalization [122]. Nevertheless, using continuous feeding, the time to achieving full feeds was longer [78,122]. On the other hand, continuous feeding has the inconvenience of greater fat adherence to the inner wall of the tube compared to bolus feeding[123], with risk of losing energy and fat content [78].

1.4.6 Gastric residuals

Gastric residuals (GR) are commonly used to define feeding tolerance. The type of enteral feed and positioning of the infant have an impact on gastric emptying. Gastric emptying is almost twice as fast with breastmilk than with formula [78]. On the other hand, the prone position in the first half hour after feeding may promote a fastest emptying [78].

Randomized controlled trials have considered significant GR higher than 2-5 mL or higher than 30-50% of volume of the previous feed [78,124]. However, no data regarding the volume and/or color of GR are sufficiently reliable to indicate feeding intolerance or to predict NEC [78].

Routine monitoring of GR is reported to increase the risk of feed interruption episodes and time to reach full enteral feeds and does not have an impact on NEC incidence [125].

Therefore, routine monitoring of GR is not recommended in clinically stable infants [78]. Gastric residuals should be otherwise assessed when clinical signs of NEC are present, such as extreme abdominal distension, tenderness, emesis, bloody stools, apnea, and temperature instability [78].

Although fortification of HM is part of the nutritional approach in preterm infants during the hospital stay, this topic deserves special attention in this thesis and will be addressed in the next specific section.

1.5 Fortification of human milk

1.5.1 Nutritional insufficiencies of human milk in preterm infants

The protein content of HM is variable with ~1g/100 mL in mature breastmilk and 1.5–2.0 g/100 mL in colostrum [78,82,106,126]. This means that a typical intake of 150 to 180 mL/kg/d of unfortified HM in stable preterm infants will not meet protein requirements [78]. In addition, protein content of MOM decreases rapidly over time, from around 1.5–2.0 g/100 ml in the first 2 weeks of lactation to around 1.0–1.5 g/100 ml thereafter, while the DHM contains much less protein, around 0.9–1.0 g/100 ml [78].

The nutritional content of HM should be adapted to the high requirements of growth in very preterm infants [78]. In this regard, supplementation of HM with multinutrient fortifiers may prevent nutritional deficits, while taking advantage of HM biological properties [78].

While fortifying the HM, it should be kept in mind that energy and macronutrient content may vary greatly either in MOM, according to the lactation time [106], or in DHM, depending on being single or multiple pools [112]. DHM may require higher levels of fortification than MOM [78].

Multinutrient fortifiers

Bovine-based multinutrient fortifiers are commonly used, but more recently HM-based multinutrientfortifiers were developed. The HM-based fortifiers may reduce the risk of NEC compared with bovine-based fortifiers, but there are insufficient reliable data to determine the optimal strategy [78]. Most bovine-based multinutrientfortifiers provide approximately 1–1.1g of additional protein per 100 ml of HM [78].

Addition of multinutrientfortifier to HM increases the osmolality of feeds, 70% occurring just after the fortifier addition and a further rise due to hydrolysis of carbohydrates by amylase activity of HM [127,128]. To avoid this additional increase in osmolality, the addition of the fortifier just before feeding has been proposed [129], although this strategy may be laborious and time consuming.

Fortification of HM using multinutrientfortifier is recommended in preterm infants [78] born with < 1800 g [130].

1.5.2 When to start fortification

Although the optimal time to start fortification is not clear, earlier fortification seems to be as safe as delayed fortification, reducing cumulative nutrient deficiencies and be beneficial for the bone metabolism [78].

Initiation of HM fortification is recommended when HM intake reaches 40-100 mL/kg/d [78].

1.5.3 Methods of fortification

Standard fortification

The standard fortification is the commonest method currently used in most of the neonatal units, using powder or liquid HM multinutrientfortifiers [131]. According to standard fortification, a fixed amount of fortifier is added to HM, *as per* the manufacturer's recommendations [130].

The standard fortification overlooks the great variability of the nutritional composition of HM, increasing the risks of energy–protein malnutrition, that includes extrauterine growth restriction, poor neurodevelopment, and MBD [130].

The protein content of some HM multinutrient fortifiers may be insufficient to reach recommended protein targets [78].

Individualized fortification

To compensate for variation in HM macronutrient content, two alternative methods of individualized fortification were proposed: the adjustable fortification and the target fortification [130].

Adjustable fortification

Using the adjusted fortification, protein intake is adjusted to infant's metabolic response, using BUN as a surrogate for protein adequacy. To be adequate, serum BUN levels should vary between 10–16 mg/dL (blood urea 21.40–34.24 mg/dL) [130]. The adjustable fortification method usually is initiated with standard fortification, and as soon as full-strength fortification is tolerated, BUN is regularly assessed. BUN levels < 10 mg/dL indicate that extra protein should be added in the form of modular protein, and BUN levels > 16 mg/dL indicate that the level of fortification should be reduced [130].

Target fortification

Using this method, regular measurements of energy and macronutrient content of HM is performed, guiding the possible addition of modular supplements of protein, fat, and carbohydrates to fortified HM to reach the desirable nutrient targets in each infant [130,132,133]. This method has the inconvenient of requiring an expensive HM analyzer and being time-consuming and labor-intensive [130].

It has been reported that individualized fortification, compared with standard fortification, may improve weight, length, and head circumference [89,133,134].

Independently of weight gain, infants fed fortified HM based on measured HM macronutrient content improved body composition not increasing excessively the adiposity and/or increasing lean mass [89,133,135]. In other words, improvement of body composition reflects a better quality of growth [136].

Concerning addition of modular macronutrients to fortified HM, it should be kept in mind that addition of hydrolyzed protein and glucose polymers to fortified HM further increase the osmolality of feeds [137].

1.6 Assessment of nutrition status

Growth faltering has been extensively documented in infants born very prematurely [136,138,139], and poor postnatal growth is associated with adverse neurocognitive outcomes [136,140]. Recent evidence also indicates that not only growth but also body composition, namely the fat-free mass gain, is associated with the development of brain in infants born preterm [84,141,142]. Accordingly, early identification of malnutrition and poor growth allowing an adequate nutritional intervention can improve long-term neurodevelopment outcomes in these vulnerable infants [143].

Early rapid weight gain, however, may predispose to later obesity, high blood pressure, and adverse cardiovascular and metabolic outcomes [136,140,144,145].

Both undernutrition and overnutrition may predispose to obesity and cardiovascular disease, through different metabolic pathways [136,146,147].

A comprehensive approach for evaluation of nutritional status in infants born preterm includes nutrient intake assessment, anthropometry, biochemical markers, body composition assessment, and indirect calorimetry [136,148]. From these methods, nutrient

intake assessment, anthropometry, and body composition assessment will be herein addressed in more detail.

1.6.1 Nutrient intake assessment

Assessment of nutrient intake is based on knowledge of composition of ingested feeds, implying the assessment of volume intake and feeds' composition [149]. In breastfed preterm infants, it is difficult to assess both parameters, since the milk volume is not directly measurable, and the HM has a dynamic composition, with nutritional profiles adapted to the needs of the growing infant [81,106,112,150].

Human milk macronutrient content

It has been reported a great variation in HM macronutrient content, that depends on several factors, including genetic and environmental factors, but some of them are still unknown [106,151]. The HM macronutrient composition may vary with maternal age [106,152,153], gestational age [82,106,150], duration of lactation [82,106,154], time of the day [155–157], or even during feeds [158,159].

A metanalysis [150] showed marked differences in HM of mothers who gave birth preterm infants from that of mothers who gave birth at term. Differences in protein and energy concentration are presented in Table 1.

Table 1. Protein and energy concentrations in preterm and term human milk

	Protein (g/100ml)		Energy (kcal/100ml)	
	Day 7	Day 28	Day 7	Day 28
Preterm milk	2.0	1.48	73.86	73.73
Term milk	1.78	1.31	73.62	72.71

Adapted from Bhatia [150]

A great variability exists in nutritional density, not only in DHM available in milk banks, but also in MOM [112] as shown in Table 2. That variation decreases in DHM pooled from several donors, but it still exists, with a negative impact on the nutritional intake of preterm infants [112].

Table 2. Percentage of variability in protein, fat, and energy contents of own mother's milk, single and multiple-donor milk pools

	Percentage of variability		
	Own mother's milk	Single-donor milk pool	Multiple-donor milk pool
Protein	14.7 (10.6)	19.3 (19.4)	13.5 (9.9)
Fat	14.5 (12.7)	10.3 (8.4)	10.6 (9.4)
Energy	7.3 (6.26)	6.9 (6.0)	5.3 (4.7)

All values are mean (SD)

Adapted from de Halleux el al [112]

A technology for analysis of HM macronutrient and energy content at local settings [159–161] was developed and currently is considered a gold standard tool [84,133,162–164] as to support the nutritional intervention in preterm infants. However, routine HM analyses have the disadvantage of being time consuming and labor intensive [165]. Most clinical settings do not have access to HM analyzer and base the nutritional interventions on the assumed HM energy and macronutrient content reported in literature [106].

Milk volume intake

In infants fed by gastric tube or by bottle, enteral volume intakes can be accurately recorded, but in breastfed infants, indirect assessment techniques are needed [166].

Even small variations in milk volume intake can have a significant impact on the dietary adequacy of very preterm infants [149,166]. Test weighing is considered the gold standard for measuring volume taken during a breastfeed in a clinical setting [149,166–169]. This method implicates weighing infants before and after a breastfeed, considering that the change in weight (in grams) reflects approximately the milk volume (in milliliters) swallowed by the infant [149]. However, this method is not routinely used in practice because of the stress it can cause on mothers, impacting their own confidence in breastfeeding [166,170,171], and the overload that usually represents for the team. Therefore, this technique is replaced by the monitoring of daily weight gain, in the assessment of nutritional adequacy [166].

As concluded in a systematic review [172] evaluating several possible breastfeeding assessment tools [173–176], there is currently no gold standard method.

1.6.2 Anthropometry

The more accurate method to assess the nutritional status quality is body composition [136,177,178], but this method is not accessible to most clinical settings [136,178–180]. As an alternative, anthropometry is the best alternative, being a noninvasive and inexpensive method, suitable for bedside evaluation, despite limitations in the validity of several measurements in small infants [136,181,182]. Certain anthropometric measurements may be a proxy for body composition [136,180].

Direct anthropometric measurements commonly used in preterm infants include body weight, crown-heel length, and HC; mid-upper arm circumference and skinfolds have been also used [136,182,183].

Indirect anthropometric measurements are based on indices calculated from direct measurements, assuming that some may provide a better insight into nutritional status and body composition than the original direct measurements [136,181,182].

From the aforementioned anthropometric measurements, body weight, crown-heel length, and HC will be addressed in more detail below.

Body weight

Body weight measurement is the most used single parameter to monitor neonatal growth in clinical practice, having the advantage of being simple and reproductible [136,184]. However, body weight does not give any information on the quality of growth, since in some preterm infants, weight gain may not result from the accretion of fat or protein, but from a positive balance of water and sodium [136,184,185].

Body weight is a good proxy of lean mass particularly in very and extremely preterm infants since they are born with scarce fat mass [136,179,186].

Crown-heel length

Body length reflects skeletal growth and fat-free mass [136,187,188].

Length assessment is useful only if measurement has been accurately undertaken [136,187,189]. Length is frequently measured inaccurately or ignored in clinical practice because of the perceived difficulty in measurement of neonates [136,188,190]. The reluctance of the observer to extend the lower limbs forcefully is a factor affecting its accuracy in

full-term neonates, who are more comfortable in flexion [136,190–192]. The accuracy of measured length is of utmost importance when it is squared or cubed in indices, since a small error in its measurement amplifies the distortion of final results [136,182,193].

In preterm infants, recumbent crown-heel length should be measured weekly to the nearest millimeter with the infant in supine position, by two collaborators, preferably involving a parent [136,182,194]. One collaborator should hold the infant's head against the fixed headboard, aligned with the trunk; the other should extend completely the lower limbs by gently pressing the infant's knees down, holding the feet vertically at a right angle to the length board, and moving the footboard up against the heels [136,182].

Head circumference

An increase in occipitofrontal circumference reflects brain growth, although it is not a sensitive or specific measure [136,195]. Some factors should be considered when interpreting HC measurements in neonates. During the first postnatal week, HC may decrease by about 0.5 cm due to extracellular fluid space contraction [136,195].

In preterm infants, postdischarge head growth seems to be a better predictor of cognitive outcomes than intra-hospital head growth [136,196].

Head circumference should be measured weekly to the nearest millimeter, encircling the supraorbital ridges and occipital protuberance with a non-stretchable measuring tape [136].

Growth charts

To monitor growth, appropriate charts and standards relating body weight, crown-heel length, and head circumference with the infant's age have been used, depending on the purpose and age of the infant [136,197,198].

Growth assessment at birth

The Fenton 2013 sex-and gestational-age-specific cross-sectional charts [199] are appropriate for assessing intrauterine growth based at birth.

These charts are based on a meta-analysis of six large population surveys of size at preterm birth, representing almost 4 million births at 22 to 36 weeks of gestation [136]. These are cross-sectional preterm charts that were harmonized with the longitudinal WHO Growth Standards for infants born at term [136,200], smoothing the data between the preterm

and WHO estimates while maintaining integrity with the data from 22 to 36 weeks and at 50 weeks [136,199].

To classify intrauterine growth, birth weight be related with gestational age is commonly used. Accordingly, neonates are classified as large-, appropriate-, or small-for-gestational age [182,201]. There is no consensus regarding the cut-offs for this classification [201–203]. While some authors define the 10th and 90th percentiles as lower and higher thresholds, others consider as lower thresholds the 5th percentile, 3rd percentile, or -2 standard deviations (SD) to classify as small-for-gestational age, and the 95th percentile, 97th percentile or +2 SD as higher thresholds to classify as large-for-gestational age [201–203]. The ability of a chart to estimate statistically defined thresholds is dependent on the sample size for each gestational age group of interest, and only samples greater than 120 individuals have enough statistical power to define the 3rd or the 5th percentiles [201,204–206]. Hence, using the Fenton 2013 curves [199], the 3rd and 97th percentiles may be used as the statistical thresholds for small-for-gestational age and large-for-gestational age infants, respectively. While these are statistical definitions for deviations of size measurement, the definition of clinically significant thresholds requires prospective longitudinal studies rather than cross-sectional assessments [201–203]. Due to the skewed distribution of weight by gestational age, the use of reference curves based on percentiles is preferred to those based on average and standard deviations [201,207].

Growth assessment during hospitalization

Body weight

At birth, the Fenton 2013 [199] are the most appropriate curves to assess intrauterine growth. These are cross-sectional sex and gestational age specific charts, and include directly measured birth weight, length, and head circumference. Fenton 2013 charts are based on a meta-analysis of six large population-based surveys of size at birth, from 22 to 36 weeks gestation. They were harmonized with the longitudinal World Health Organization (WHO) growth standards for infants born at term [200] smoothing the data between the preterm and WHO estimates, while maintaining integrity with the data from 22 to 36 weeks and at 50 weeks [199].

For the neonatal period, the body weight z-score change (Δ z-score) and the weight gain velocity (in g/kg/day) are considered the most trustworthy metrics [143].

Δ z-scores calculated from birth weight-based charts were criticized for not accurately reflecting the growth rate of very preterm infants [207,208]. Otherwise, the weight gain velocity over the previous days is reported to be more sensitive with respect to identifying changes in growth, with a minimum of a 5–7-day period required for a precise calculation [209]. It was suggested that 15–20 g/kg/d is the optimal weight velocity in infants born at 23–36 weeks' gestation, approximating to the mean estimates of fetal growth [210,211].

The period allowing a precise calculation of weight gain velocity seems to be a time interval of at least 5–7 days [136,209].

Compared with the conventional two-point assessment method, an exponential model was proposed by Patel *et al* [212]. as a more accurate estimate of weight gain velocity, as it is not affected by decreasing birth weight and increasing length.

Length and head circumference

Contrarily to body weight, which is characterized by an initial postnatal physiological decrease, body length and HC continuously increase after birth. In absence of reliable longitudinal curves, the sex-and gestational-age-specific cross-sectional Fenton 2013 charts can an alternative to monitor linear and head growth from birth to 50 weeks of postmenstrual age [201].

Nevertheless, length and HC Δ z-scores and velocities (in cm/week) are more sensitive metrics to monitor linear and head growth [136,143,201].

Length velocity of approximately 0.9–1.1 cm/week and HC velocity of 0.9–1.0 cm/week are reasonable targets, particularly in the period between 23 and 30 weeks of postmenstrual age [136,182,195,209].

1.6.3 Body composition

The most accurate method to assess the quality of the nutritional status is body composition assessment [136,177,178], although this method is not available in most clinical settings [136,178–180].

Methods for body composition assessment

Human body composition may be assessed at several levels depending on the clinical concerns. Methods used are classified as direct, criterion, and indirect [213]. Direct methods can analyze from the atomic through the cellular levels, and those used for clinical purposes include isotope dilution and total body counting [213]. Criterion methods describe amounts and distributions of tissues or measure its density, and include air displacement plethysmography (ADP), magnetic resonance imaging, and dual-energy X-ray absorptiometry. Indirect methods, provide estimates or indices of body composition and are based on measurements from direct or criterion methods, and the most frequently used are anthropometry and bioelectrical impedance analysis.

Indirect methods have larger predictive errors than direct methods, since they depend on biological interrelationships among measured body components and tissues and are affected by clinical conditions [213].

It should be noted that data obtained from different body composition methods are not usually interchangeable [214].

As this study only the ADP was used, therefore this method will be addressed in detail.

Air displacement plethysmography

The ADP measures directly the body mass and the body volume through air displacement inside a sealed chamber. From these measurements, body density is computed using the known density of fat (0.9007 g/ml) and age- and sex-specific FFM density coefficients to determine the total body FM and FFM [178,183,215–217].

This is a non-invasive method, particularly convenient in infants because it is relatively rapid to perform and not affected by movements, thus not requiring sedation or immobilization [218–220]. There are two commercially available ADP systems (Cosmed, Rome, Italy), the Pea Pod designed for infants up to 6 months (or 8 kg), and the Bod Pod for children older than 5 years, adolescents, and adults [217].

The ADP (Pea Pod) was validated in infants against known bovine phantoms [221] and deuterium dilution [222]. In small infants and children aged 2-6 years, the ADP compared with 4-compartment model was found to be highly reliable and accurate for determining the %FM [218,223]. In a small group of preterm infants at 32–36 weeks, the ADP was

validated against a three-component model, and no significant difference was found in %FM measured by the two methods [58,219].

Some factors may affect in some extent the accuracy of ADP, including body humidity and temperature, objects attached to infants, extremes of body size, chest gas prediction equations, FFM density, and fluctuations in FFM hydration factor [224]. Nevertheless, despite the above concerns, ADP has been considered a valuable tool in infants [224].

Charts are currently available to assess ADP body composition in preterm and term infants, from birth to 6 months of age [225].

Adiposity can be accurately determined by ADP, using as indicators the %FM (given automatically by the equipment) or by fat mass index (FMI) calculated using the equation FM (kg)/ length^2(m) [226]. The FMI has been preferred to %FM, since the %FM has the disadvantage of being a ratio with fat included both in the numerator and in the denominator (as a component of body mass) [227]. In children, a good correlation was found between FMI and body fat assessed by deuterium dilution as a reference method [228]. In addition, there is a logic for using FMI to normalize body FM to height in pediatric age, since body lean mass is a linear function of height grow [229]. When using the FMI, the length or height must be measured accurately, since an inaccurate measurement when squared magnifies the error of the index while losing the ability to differentiate overestimation from underestimation [189].

1.7 Questions currently unanswered about preterm infants' nutrition

1.7.1 Optimal method for human milk fortification

Target fortification

Many authors [132,163,230,231] consider the target HM fortification to be the gold standard method to achieve suitable nutrition in preterm infants according to energy and protein intakes recommended by the ESPGHAN [78]. However, this opinion is not unanimous, as other authors found that the target HM fortification method, as compared to the standard HM fortification, did not meet the recommended protein intake [232] or resulted in better growth outcomes [233]. The ESPGHAN recommendations were recently updated [78], which modified some nutrient targets, but do not answer the aforementioned question.

Osmolarity of feeds

The fortification of HM leads to an increase in osmolarity that cannot be neglected, especially in very preterm infants, considering the risk of feeding tolerance and necrotizing enterocolitis [108,234,235]. This issue has been addressed by some authors [108,236–239]. However, more studies will be necessary to simultaneously define the safe maximum concentrations of multinutrient fortifiers and modular protein supplements in the prescribed fluid intakes and comply with the current ESPGHAN enteral nutrition recommendations for preterm infants [78] in clinical practice.

1.7.2 Optimal method for body composition assessment

The current literature indicates that extremely and very preterm infants at term-equivalent age have a lower FFM and a greater fat mass percentage (%FM), compared with term-equivalent-age infants [59,240]. Reference values for body composition have been described for preterm infants [183,225,241], but the optimal body composition evolution in those infants remains to be determined. This lack of knowledge relates not only to the difficulty in defining the optimal nutrition intervention in preterm infants, a major factor determining body composition, but also to the current unavailability of a non-invasive, accurate, and easy to perform method for measuring body composition in fragile preterm infants under intensive care, including soon after birth [178,242].

1.7.3 A contribution of this study to answer some unsolved questions

In a previous cohort study of preterm infants born less than 33 weeks' gestation and fed standard fortified HM, we have evaluated the association of energy and macronutrient intakes with weight gain velocity and body composition [243,244]. We found that the actual energy, protein, and fat intakes based on measured HM macronutrient content were significantly lower than the assumed intakes based on estimates of HM macronutrient content [243]. This nutritional strategy was associated with a suboptimal weight gain velocity and low adiposity [244].

From this evidence, following the ESPGHAN enteral nutrition recommendations for preterm infants in effect [245], a nutritional strategy towards HM fortification based on measured HM composition was introduced in our unit expecting to improve growth and growth quality reflected by body composition.

2 Objectives

The aim of this study was to assess in infants born at less than 33 weeks' gestation fed HM, whether a newly implemented fortification method based on measured HM macronutrient content improved the energy and macronutrient intake and the quality of growth compared with HM fortification based on assumed HM macronutrient content.

Weight gain during the hospital stay was the primary outcome.

In-hospital length and HC growth and body composition at late preterm or TEA were secondary outcomes.

3 Hypotheses

We hypothesize that in preterm infants, the customized HM fortification based on measured HM macronutrient content improve energy and macronutrient intakes, compared with the standard HM fortification.

We also hypothesize that better nutrition provided with this customized HM fortification improves the quality of growth, reflected by better weight, linear and head growth rates while achieving an adiposity at term-equivalent age like that of infants born at term.

4 Methods

4.1 Study design

This is a single-center, observational mixed-cohort effectiveness study in preterm infants with less than 33 weeks of gestation [20].

The study protocol was registered in the ClinicalTrials.gov NCT04400396 and published elsewhere [20].

Energy and macronutrient intake, growth, and body composition were compared in infants fed fortified HM, either based on assumed HM macronutrient content (Group 1) or based on measured HM macronutrient content (Group 2).

Although the original design intended to compare, 'per protocol', the effects of standard and target HM fortification methods, as stated in the ClinicalTrials.gov NCT04400396 registry and the published protocol [20], addition of modular protein and fat supplements to fortified HM was left to clinical discretion. As such, comparisons were made 'per intention to treat'. Overall, the neonatal unit nutritional policy followed the contemporary ESPGHAN 2010 enteral nutrition guidelines for preterm infants [245]. Instead of strictly following the 'standard fortification' method by simply adding a fixed dose of the fortifier to the HM, physicians were allowed to further add modular supplements of protein and mid-chain triglycerides (MCT) to the fortified HM, based on assumed HM macronutrient content, according to a reported longitudinal analysis of MOM macronutrient content [106]. Instead of 'target fortification', the physicians were asked to customize the HM fortification as guided by the measured HM macronutrient content, thereby adding modular supplements of protein and MCT to the fortified HM, if necessary.

The exposure period was defined by feeding fortified HM for at least 2 weeks. Recruited infants were dropped out if they were fed formula for two or more days with a > 12.5% daily volume intake, or if they were transferred or deceased before completing the exposure period.

4.2 Ethical and legal issues

The study was approved by the hospital ethics committee (Nr 558/2018) and informed written consent was obtained from the parents or legal representatives of each infant.

4.3 Settings

The study was conducted at the Centro Hospitalar Universitário de Lisboa Central, specifically in the Neonatology Unit and in the Human Milk Bank of Maternidade Dr. Alfredo da Costa, as well as in the Nutrition Laboratory of the Hospital Dona Estefânia.

4.4 Participants

The eligibility criteria [20] were the same used in a historical cohort study [244], with sampling of consecutive infants born with less than 33 weeks of gestation, singletons or twins (=2), appropriate for gestational age, and exclusively or predominantly (> 87.5% volume per day) HM-fed. Infants diagnosed with an innate error of metabolism were not recruited.

4.5 Study periods

The recruitment for the contemporary cohort of infants fed measurement-based fortified HM was initially planned to start in February 2020 (ClinicalTrials.gov NCT04400396) to compare them with the historical cohort (2014–2015) of infants, who were fed fortified HM based on its assumed macronutrient content [20,243]. However, a shortage in modular protein supplement from 1 February to 13 July 2020 precluded the adoption of the new institutional nutrition protocol, which implied the possible addition of modular protein to the fortified HM guided by the measured HM macronutrient content; meanwhile, fortification continued to be based on assumed HM macronutrient content. As shown in Table 3, the arm of fortification based on assumed HM macronutrient content included not only the historical cohort of 2014–2015 [20], but also part of the contemporary cohort recruited from 1 February to 13 July 2020. The cohort of fortification based on measured HM macronutrient content was recruited from 14 July 2020 to 31 May 2021.

Table 3. Periods of recruitment of the infants fed either fortified HM based on its assumed macronutrient content (Group 1) or fortified HM based on its measured macronutrient content (Group 2).

Group 1	Group 2
- Subgroup 1a: Historical cohort-recruitment from February 2014 to February 2015 - Subgroup 1b: Contemporary cohort - recruitment from 1 February to 13 July 2020	- Recruitment from 14 July 2020 to 31 May 2021

HM – human milk

4.6 Nutrition protocol

The institutional neonatal nutrition protocol [20,244] was followed considering the contemporary international and national guidelines for parenteral and enteral nutrition [74–76,245,246].

In brief, parenteral nutrition was initiated within the first 2 postnatal hours and early trophic feeding was initiated within the first 2 to 4 postnatal days preferentially using MOM, and DHM when MOM was insufficient [133].

Addition of a powdered, multi-component HM fortifier (Aptamil FMS; Danone GmbH, Friedrichsdorf, Germany), containing 3.47 kcal of total energy, 0.25 g of protein, and 0.62 g of carbohydrates per g of powder, was started at a concentration of 4.4 g/100 mL HM when the HM intake was at least 80 mL/kg/day. For fortified HM based on its measured macronutrient content, the MOM was analyzed once a week and the DHM was analyzed after pasteurization [20].

The addition of modular protein and fat supplements to fortified HM was prescribed per clinical discretion, whatever the fortification method. According to the HM macronutrient content, a powdered modular protein (Aptamil Protein Supplement; Danone GmbH, Friedrichsdorf, Germany), containing 3.38 kcal of total energy and 0.821 g of protein per g of powder, and/or medium-chain triglycerides (MCT Oil; Danone, GmbH, Friedrichsdorf, Germany), containing a total energy of 8.6 kcal and 0.95 g of fat per 1 mL, were added to the fortified HM, when necessary, in order to follow the ESPGHAN

2010 guidelines and reach the following target daily intakes for actual body weight: 110–135 kcal/kg of total energy, 4.0–4.5 g/kg of protein for infants with < 1 kg body weight and 3.5–4.0 g/kg of protein for ≥ 1 kg body weight, and a protein-to-energy ratio (PER) of 3.6–4.1 for < 1 kg body weight and 3.2–3.6 for ≥ 1 kg body weight [245]. Although the ESPGHAN 2010 guidelines for the intake of protein and PER was stratified by infant body weight < 1 kg or 1–1.8 kg [245], we used the convenience criterion of < 1 kg and ≥ 1 kg body weight instead.

4.7 Calculation of sample size

The study sample size was calculated to detect a difference of 2 g/kg/day in weight gain velocity, the main outcome, with a significance level of 0.05 and 80% power; a required sample of 68 infants was estimated, with 34 infants assigned to each group [20]. The assumption was based on the mean (SD) weight gain velocity of 10.1 (3.8) g/kg/d obtained in the historical cohort study [244] and 12.1 (1.6) g/kg/d reported by McLeod *et al*. [165] in a similar sample of preterm infants fed fortified HM based on HM measured macronutrient content.

4.8 Retrieved data

The recorded demographic variables included gestational age at birth, classified as ≥ 28 weeks or < 28 weeks (extreme preterm); sex; singleton or twin; birth weight - small, appropriate, or large for gestational age (< 3rd percentile, ≥ 3rd percentile and ≤ 97th percentile, and > 97th percentile, respectively) [199]; severity index (SNAPPE II) [70]; prenatal corticosteroids; diagnosis of LOS [247]; NEC (grade ≥ 3) [248]; intra- periventricular hemorrhage (grade ≥ 3) [249]; multicystic periventricular leukomalacia [250]; and BPD [35].

4.9 Measurements

4.9.1 Analysis of human milk composition

The HM samples were analyzed for macronutrient content using a real-time HM analyzer (Miris AB, Uppsala, Sweden) [251], following the same measurement procedures described in the historical study [243]. The standard calibration solution was used to calibrate the analyzer before each use [231]. To minimize the daily variability in breast milk macronutrient content, mothers were asked to add milk collected in the last 24 h to the

same container. The energy and macronutrient content are expressed in densities; that is, kcal/dL of energy and g/dL of fat, raw and true protein, carbohydrates, and ashes. In the fortification based on the measured HM macronutrient content method, the adjustment of the MOM fortification for the infants' nutritional needs was guided by a weekly analysis of MOM macronutrient content. The adjustment of the DHM fortification was based on its macronutrient content, which had been previously analyzed and indicated on the containers' labels.

4.9.2 Nutrient intake assessment

The daily record of nutritional intake refers to feeds administered in the last 24 h. For each infant, the volumes of LH administered (MOM or DHM) were accurately recorded, considering the amounts actually administered, taking into account possible interruptions and gastric residual volumes.

To calculate macronutrient (g/mL) and energy (kcal/mL) densities of fortified HM, the macronutrient content of HM administered was recorded daily, as well as the macronutrient content of the added HM fortifier, modular protein, and MCT oil.

The measured HM macronutrient content was used for calculations in Group 2 and the assumed HM macronutrient content based on literature data 106 was used in Group 1.

The macronutrient content of commercial products was based on information provided by manufacturers.

The body weight recorded daily was used to calculate daily energy and macronutrient intakes per kg of body weight.

An Excel program to facilitate the calculations of the amounts of modular protein and fat supplements that were to be added to the fortified HM was developed and registered (Nona R, Cardoso M, Portuguese Directorate of Intellectual Property Services, IGAC-DSPI nr 480/2020, 26 February 2020). Energy and macronutrient intakes were compared to the contemporary ESPGHAN 2010 guidelines [245].

4.9.3 Anthropometry

Anthropometric parameters were assessed from birth to discharge.

Body weight was measured by attending nurses at birth and daily during the hospital stay, with infants naked, using scales incorporated into the incubators or external automatic scales calibrated to the nearest gram [136]. The weight gain velocity (g/kg/day) was calculated using the Patel exponential model [212].

In the contemporary cohorts Subgroup 1b and Group 2 weekly crown–heel length and head HC measurements were undertaken by the same observer (MC) to the nearest millimeter, and each considered measurement resulted from the average of three consecutive measurements. Crown–heel length was measured via the collaboration of two observers, using a rigid recumbent length board, and HC was measured encircling the supraorbital ridges and occipital protuberance using a non-stretchable measuring tape [136]. These measurements were used to calculate the length and HC gain velocities (cm/week). In the historical cohort (Subgroup 1a), the length and HC values recorded during the hospital stay were not considered, since methods for assuring the accuracy of these measurements were not included in the historical study design [244].

4.9.4 Body composition assessment

A single body composition assessment using ADP (Pea Pod, Cosmed, Italy) was scheduled for up to one week after discharge for infants who maintained exclusive or predominant HM feeding.

The FM, FFM, %FM, and FFM percentage (%FFM) were provided automatically by the equipment.

Body length was measured by the same observer (MC), and this measurement was used to calculate the FM index (FMI) [252].

The main body composition outcome was adiposity, indicated by both the %FM and FMI. Excess adiposity was defined as %FM > 95th percentile for sex and PMA [225] and/or FMI > 90th percentile for PMA [252].

4.9.5 Statistical analysis

The demographics and clinical characteristics of infants were described with frequencies (percentages) and with mean (SD: standard deviation) or median and interquartile range (IQR: 25th percentile–75th percentile), as appropriate. Nonparametric Chi-Square test, Fisher's exact test, median test, and Mann–Whitney test were used, as needed.

Univariable and multivariable linear regression models were used to identify the variables which explained the body weight, length, and head circumference z-scores at discharge, and between birth and discharge (Δ z-scores), and the variability of body composition. Normality assumption of the residuals was verified using the Kolmogorov–Smirnov goodness-of-fit test with Lilliefors correction.

To study the association between independent variables and outcomes of excess adiposity (%FM > 95th percentile and FMI > 90th percentile), logistic regression models were used. For quantitative variables, logit linearity assumption (linear association with the logit of the outcome variable) was verified using generalized additive regression models.

Additionally, in order to investigate associations between independent variables and weight, length, and HC gain velocities, linear mixed effects regression models were applied. Interaction terms were considered in the multivariable study.

All the variables that attained a p-value ≤ 0.25 in the univariable analysis were candidates for the multivariable models.

A level of significance $\alpha = 0.05$ was considered. Data analysis was performed using Stata (StataCorp, 2017, Stata Statistical Software: Release 15. College Station, TX: StataCorp LLC) and R (R: A Language and Environment for Statistical Computing, R Core Team, R Foun- dation for Statistical Computing, Vienna, Austria, year = 2022, http://www.R-project.org, accessed on 5 January 2023).

5 Results

The results herein presented were published elsewhere [133].

5.1 Study flowchart

As shown in the flowchart (Figure 1), 275 eligible infants were identified, but only 185 fulfilled the inclusion criteria: 115 in Group 1 (33 in Subgroup 1a plus 24 in Subgroup 1b) and 70 in Group 2. The reasons for not being recruited were parental refusal or inability to consent, hospital transfer, death, and formula feeding.

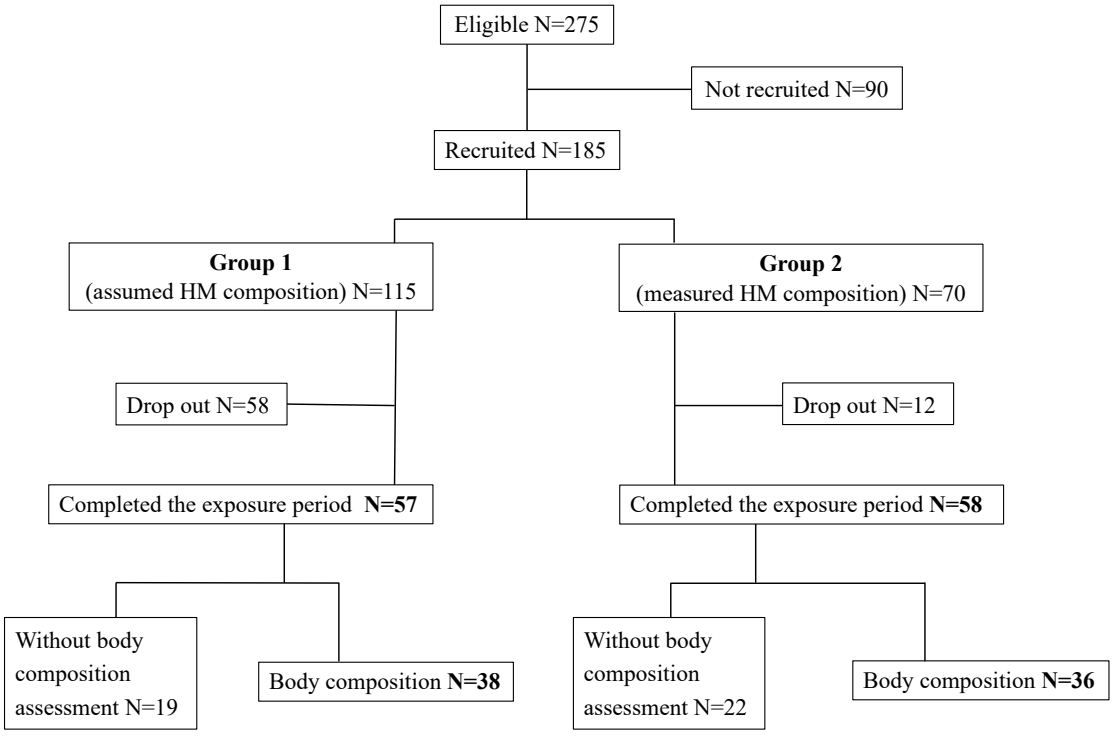


Figure 1. Study flowchart

No significant differences between recruited and not recruited infants were found regarding gestational age, birth weight z-score, twin prevalence, and prenatal steroids, as shown in the Table 4.

Table 4. Comparison between recruited and not recruited eligible infants

Eligible	Recruited	Not recruited	*p*-value
n=275	n=185	n=90	
Gestational age, weeks; mean (SD)	29.6 (2,19)	29.6 (2.52)	0.567
Birth weight z-score; median (P_{25}-P_{75})	-0.10 (-0.74-0.28)	-0.31 (-0.85-0.35)	0.489
Twins; n (%)	56 (30.3)	34 (37.8)	0.213
Prenatal steroids; n (%)	175 (94.6)	85 (94.4)	1.000
SNAPPE II; median (P_{25}-P_{75})	13 (0-25)	15 (5-30)	0.269
Extreme preterm; n (%)	40 (21.6)	25 (27.8)	0.260

Student-*t* test, Chi-Square test, Fisher's exact test, median test, or Mann–Whitney test, as appropriate.

From the 185 infants recruited, 58 (50.4%) dropped-out in Group 1 and 12 (17.1%) in Group 2 ($p < 0.001$). The reasons for lost to follow-up were formula feeding in 64 infants and hospital transfer before completing the study exposure period in 6 infants. Therefore, 115 completed the study exposure period, 57 in Group 1 and 58 in Group 2.

As shown in Table 5, no significant differences were found between infants completing the exposure period and those who were lost to follow-up regarding gestational age, birth weight z-score, twin prevalence, extreme prematurity, prenatal and postnatal steroids, severity risk, LOS, NEC, IVH, bronchopulmonary dysplasia, and hospital stay. An exception was a higher severity risk in infants who were lost to follow-up (median SNAPPE II 15 vs. 10, $p = 0.026$).

Table 5. Comparison between infants completing the exposure period of the study (N=185) and those lost to follow-up.

	Completed exposure period of the study	Lost to follow-up	*p*-value
	n=115	n=70	
Gestational age, weeks; mean (SD)	29.6 (2.15)	29.5 (2.27)	0.894
Birth weight z-score; median (P_{25}-P_{75})	-0.03 (-0.62-0.31)	-0.35 (-0.82-0.20)	0.171
Twins; n (%)	39 (33.9)	17 (24.3)	0.167
Extreme preterm; n (%)	26 (22.6)	14 (20.0)	0.338

SNAPPE II; median (P_{25}-P_{75})	10 (0-24)	15 (7-29)	0.026
Prenatal steroids; n (%)	108 (93.9)	67 (95.7)	0.316
Postnatal steroids; n (%)	5 (4.3)	3 (5.7)	0.642
Late-onset sepsis; n (%)	37 (32.2)	15 (21.4)	0.057
Necrotizing enterocolitis III; n (%)	0	2 (2.9)	0.142
Intraventricular hemorrhage IV; n (%)	2 (1.7)	4 (5.7)	0.147
Bronchopulmonary dysplasia; n (%)	10 (8.7)	5 (7.1)	0.354
Hospital stay, days; median (P_{25}-P_{75})	46 (34-64)	53 (29.5-71)	0.531

Student-*t* test, Chi-Square test, Fisher's exact test, median test, or Mann–Whitney test, as appropriate.

5.2 Characteristics of infants completing the study

No significant differences were found between groups regarding demographic characteristics, birth weight, and morbidity, with exception for the infants of Group 2, who had a significantly higher prevalence of twins and LOS, and were significantly shorter and had lower head circumference at birth (Table 6).

Table 6. The characteristics of infants that completed the exposure period (n = 115). Group 1—fortified HM based on its assumed macronutrient content. Group 2—fortified HM based on its measured macronutrient content

	Group 1 n=57	Group 2 n=58	*p*-value
Gestational age in weeks, mean (SD)	29.6 (1.99)	29.7 (2.31)	0.850
Females, n (%)	27 (47.4)	23 (39.7)	0.412
Twins, n (%)	15 (26.3)	24 (41.4)	0.047
Extremely preterm, n (%)	14 (24.6)	12 (20.7)	0.314
Prenatal steroids, n (%)	55 (96.5)	53 (91.4)	0.286
Birth weight z-score, mean (SD)	−0.11 (0.70)	−0.19 (0.66)	0.691
Birth length z-score, mean (SD)	−0.48 (0.512)	−1.07 (0.740)	0.001
Birth HC z-score, mean (SD)	−0.83 (0.90)	−1.60 (0.778)	< 0.001
Postnatal steroids, n (%)	1 (1.8)	4 (6.9)	0.102
Severity risk (SNAPPE II), median (P25; P75)	10 (0.22)	10 (0.27)	0.785
Late-onset sepsis, n (%)	12 (21.1)	25 (43.1)	0.001

Necrotizing enterocolitis III, n (%)	0	0	-
Intraventricular hemorrhage I, n (%)	2 (3.5)	0	0.128
Bronchopulmonary dysplasia, n (%)	4 (7.0)	6 (10.3)	0.264

HC—head circumference, HM—human milk, SD—standard deviation. Student-*t* test, Chi-Square test, Fisher's exact test, median test, or Mann–Whitney test, as appropriate.

Extremely preterm infants had a significantly higher severity risk, prevalence of LOS, and bronchopulmonary dysplasia than those who were born with more than 28 weeks' gestation (Table 7).

Table 7. Comparison between children born at gestational less than 28 weeks and greater than or equal to 28 weeks.

	Gestational age at birth < 28 weeks	Gestational age at birth ≥ 28 weeks	*p*-value
	n=26	n=89	
SNAPPE II; median (P_{25};P_{75})	20 (9;30)	13 (0;23)	0.033
Late-onset sepsis; n (%)	18 (69.2)	19 (21.3)	0.003
Necrotizing enterocolitis III; n (%)	0	0	-
Intraventricular hemorrhage IV; n (%)	1 (3.8)	1 (1.1)	0.410
Bronchopulmonary dysplasia; n (%)	6 (23.1)	4 (4.5)	0.018
Hospital stay, days; median (P_{25};P_{75})	71 (63;99)	40 (31;53)	< 0,001

HC—head circumference, HM—human milk, SD—standard deviation. Student-*t* test, Chi-Square test, Fisher's exact test, median test, or Mann–Whitney test, as appropriate.

5.3 Nutritional exposure

In both groups, the great majority of infants were fed MOM and a minority were fed DHM. The median (P_{25}; P_{75}) percentage of MOM volume administered during the hospital stay was 96.0% (71.2%; 100%) for Group 1 and 96.5% (80.8%; 99.2%) for Group 2.

No significant differences existed between the two groups in the postnatal age and PMA at the beginning or end of exposure, in time intervals before exposure, and from the end of exposure to discharge, in the percentage of days of exposure in relation to the total days of enteral feeding, and the total days of hospital stay (Table 8). No significant differences existed between Groups 1 and 2 in the median time of exposure to HM fortification (28.0 vs. 23.0 days, $p = 0.072$).

A significant negative correlation was found between gestational age at birth and the number of days of exposure ($r = -0.7$, $p < 0.001$).

Table 8. Comparison between groups, of infants' age before, during, and after exposure, time intervals of exposure, and percentage of exposure days during enteral feeding and hospital stay.

	Group 1	Group 2	***p*-value**
	N=57	N=58	
Gestational age (wks) at beginning of the intervention, in weeks; mean (SD)	31.5 (1.975)	31.45 (2.01)	0.412
Age at beginning of intervention, in days; median (Q1;Q3)	11 (9;13)	11 (8;14)	0.721
Postmenstrual age at the end of intervention, in weeks; mean (SD)	31.1 (1.98)	31.5 (2.01)	0.412
Postmenstrual age at hospital discharge, in weeks; mean (SD)	36.2 (1.21)	37.0 (2.40)	0.082
Period before intervention (days); median (IQR)	10.0 (9.0-12.0)	11.0 (8.0-14.0)	0.126
Period of intervention (days); median (IQR)	28.0 (17.5-50.0)	23.0 (16.0-36.0)	0.072
Period from end of intervention and discharge (days); median (IQR)	7.0 (3.0-12.0)	9.5 (5.0 19,3)	0.191
Percentage of intervention days during enteral feeding; mean (SD)	60.2 (20.83)	56.3 (18.82)	0.300
Percentage of intervention days during hospital stay; mean (SD)	53.4 (19.89)	50.9 (17.02)	0.588

Student-*t* test, Chi-Square test, Fisher's exact test, median test, or Mann–Whitney test, as appropriate.

The proportion of exposure days in which the nutrient intakes did not reach the minimum or exceeded the maximum ESPGHAN 2010 recommendations [245] is presented in Table 9. Regarding energy, PER, fat, and carbohydrate intakes, the proportion of days in which the maximum recommendations were exceeded was significantly higher in Group 2, while the proportion of days in which the minimum recommended intakes of the same nutrients were not reached was significantly higher in Group 1.

Table 9. Proportions of exposure days in which the intake of energy, P/E, and macronutrients did not reach the minimum recommended or exceeded the maximum recommended intakes (N=115). Group 1 - assumed-based fortified HM, Group 2 - measured-based fortified HM

	Recommended [245]	Group 1	Group 2	*p-value*
		n=57	n=58	
Energy intake, kcal/kg/d	110-135			
Proportion of days (%) in which the energy intake did not reach the minimum recommended		32.6	12.1	**< 0.001**
Proportion of days (%) in which the energy intake exceeded the maximum recommended		25.8	30.2	**0.002**
Protein intake, g/kg/d	< 1 Kg weight: 4.0-4.5 1-1.8 Kg weight: 3.5-4.0			
Proportion of days (%) in which protein intake	< 1 kg weight	31.3	31.4	0.494
	≥ 1 kg weight	25.0	15.5	**0.047**

did not reach the minimum recommended				
Proportion of days (%) in which the protein intake exceeded the maximum recommended	< 1 kg weight	51.5	46.4	0.235
	≥1 kg weight	51.9	54.2	0.372
Protein-to energy ratio	< 1 Kg weight: 3.6-4.1 1-1.8 g weight: 3.2-3.6			
Proportion of days (%) in which the protein-to energy ratio intake did not reach the minimum recommended	< 1 kg weight	34.3	1.3	**< 0.001**
	≥ 1 kg weight	27.8	0	**< 0.001**
Proportion of days (%) in which the protein-to energy ratio intake exceeded the maximum recommended	< 1 kg weight	54.5	95.4	**< 0.001**
	≥1 kg weight	56.8	99.3	**< 0.001**
Fat intake, g/kg/d	4.8-6.6 g/kg/d			
Proportion of days (%) in which the fat intake did not reach the minimum recommended		37.6	20.2	**< 0.001**
Proportion of days (%) in which the fat intake		23.3	46.5	**< 0.001**

exceeded the maximum recommended				
Carbohydrate intake, g/kg/d	11.6-13.2 g/kg/d			
Proportion of days (%) in which carbohydrate intake did not reach the minimum recommended		37.6	20.2	**< 0.001**
Proportion of days (%) in which the carbohydrate intake exceeded the maximum recommended		23.3	46.5	**< 0.001**

Chi-Square test

In Table 10, the compliance of energy and macronutrient intakes during the exposure period with the ESPGHAN 2010 recommendations [245] is presented for each group and compared between groups. During the exposure period, infants fed fortified HM based on its measured macronutrient content received significantly higher energy, fat, and carbohydrate intake than those fed fortified HM based on its assumed macronutrient content. On the other hand, infants weighing ≥ 1 kg fed fortified HM based on its measured macronutrient content received significantly lower protein intake and those weighing < 1 kg received significantly lower PER intake than those fed fortified HM based on its assumed macronutrient content (Table 10). In a secondary analysis, it was found that the mean (SD) carbohydrate content of MOM administered was significantly higher, and administered for a longer exposure period, in Group 2 than in Group 1: mean (SD) 6.7 (0.9) g/dL administered for 1226 days vs. 6.5 (1.1) g/dL administered for 1412 days, $p < 0.001$.

Table 10. Energy and macronutrient intake in infants completing the study exposure period (n = 115). Group 1—fortified HM based on its assumed macronutrient content. Group 2—fortified HM based on its measured macronutrient content

	Recommended [245]	Group 1	Group 2	*p-value*
		n=57	n=58	
Total energy intake, kcal/kg/d; mean (SD)	110–135 kcal/kg/d	121.3 (25.3)	127.9 (17.4)	**< 0.001**
Protein intake, g/kg/d; median (P_{25} ; P_{75})	All	4.2 (3.7; 4.7)	4.1 (3.7; 4.6)	0.109
	< 1 kg weight: 4.0–4.5 g/kg/d	3.9 (3.2;4.8)	4.1 (3.3; 4.9)	0.162
	1–1.8 kg weight: 3.5–4.0 g/kg/d	4.2 (3.8; 4.6)	4.1 (3.8; 4.5)	**0.004**
PER intake, g/100 kcal; median (P_{25} ; P_{75})	All	3.5 (3.2; 3.9)	3.4 (3.0; 3.8)	**0.001**
	< 1 kg weight: 3.6–4.1	3.5 (3.2; 4.0)	3.3 (3.0; 3.7)	**< 0.001**
	1–1.8 kg weight: 3.2–3.6	3.4 (3.2; 3.8)	3.5 (3.1; 3.9)	0.315
Fat intake, g/kg/d; mean (SD)	4.8–6.6 g/kg/d	5.5 (1.54)	6.7 (1.67)	**< 0.001**
Carbohydrate intake, g/kg/d; mean (SD)	11.6–13.2 g/kg/d	11.9 (1.93)	13.0 (1.90)	**< 0.001**

HM—human milk; PER—protein-to-energy ratio. Student-*t* test, median test, or Mann–Whitney test, as appropriate.

5.4 Anthropometry and its association with the human milk fortification method

5.4.1 Body weight z-score

In both groups, the infants were born with negative body weight z-scores (Table 6), whose deviation increased up to discharge, without significant differences between groups (Table 11).

In the multivariable analysis, no significant association was found between the body weight Δ z-scores from birth to discharge and the method of HM fortification ($p = 0.062$). Whatever the fortification method, an increase in length z-score from birth to discharge was significantly associated with an increase in body weight z-score from birth to discharge (β estimate = 0.759; 95% CI 0.561, 0.957; $p < 0.001$).

Table 11. Body weight, length, and head circumference Δ z-scores from birth to discharge (n = 115). Group 1—fortified HM based on its assumed macronutrient content. Group 2—fortified HM based on its measured macronutrient content.

	Group 1	Group 2	*p-value*
	n=57	n=58	
Body weight z-score at discharge, mean (SD)	−1.73 (0.92)	−1.73 (0.870)	0.693
Δ body weight z-score, median (P_{25}; P_{75})	−1.64 (−1.99; −1.28)	−1.47 (−1.79; −1.10)	0.565
Length z-score at discharge, median (perc. 25; perc. 75)	−1.47 (−1.685; −0.970) †	−1.49 (−2.135; −0.830)	0.744
Δ length z-score, median (P_{25}; P_{75})	−0.71 (−1.69; −0.37) †	−0.27 (−2.14; −0.04)	0.005
HC z-score at discharge, median (P_{25}; P_{75})	−1.17 (−1.70; −0.63) †	−0.80 (−1.390; −0.02)	0.102

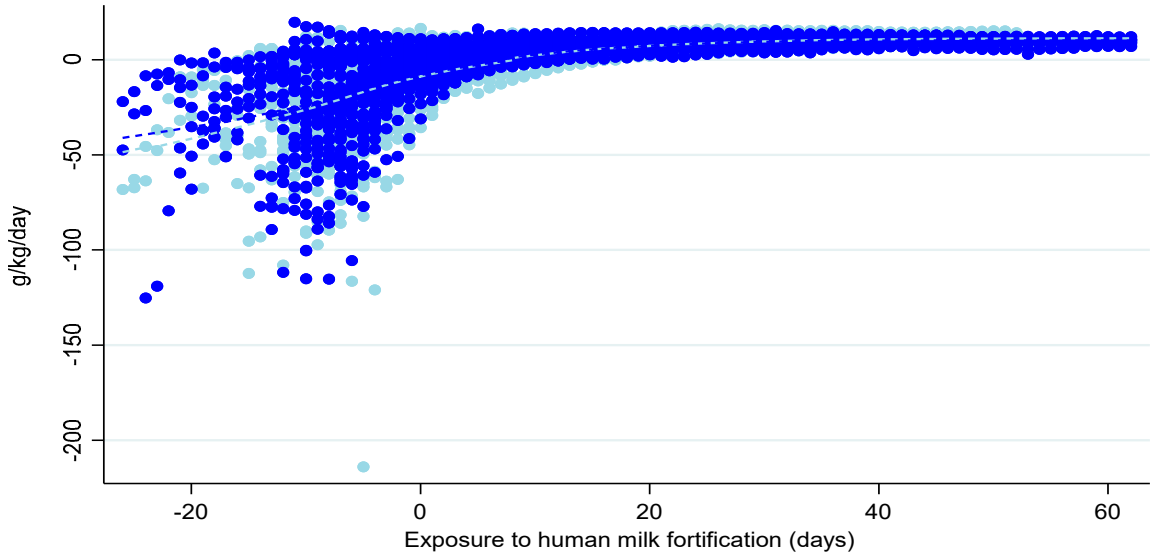
Δ HC z-score, median (P_{25}; P_{75})	−0.31 (−0.59; 0.04) †	0.75 (0.41; 1.17)	< 0.001

† Only assessed in 24 infants of the contemporary cohort fed assumed-based fortified human milk. *p*-values obtained via linear regression models; HC—head circumference, HM—human milk, SD—standard deviation. Student-*t* test, median test, or Mann–Whitney test, as appropriate.

5.4.2 Weight gain velocity

By using the Patel method, the median weight gain velocity was significantly higher in infants fed fortified HM based on its measured macronutrient content than those fed fortified HM based on its assumed macronutrient content, both from birth to discharge (10.8 vs. 9.7 g/kg/d, $p = 0.023$) and from beginning of fortification to discharge (15.3 vs. 13.0 g/kg/d, $p < 0.001$). This comparison considered only the weight gain velocity estimates at the time of discharge.

Using a linear mixed effects model that considered daily estimates of weight gain velocity from birth to discharge, no association was found between them and the method of HM fortification ($p = 0.158$). This result is in line with the graph shown in Figure 2, which depicts the evolution of weight gain velocity in both groups with a lowess smoother superimposed. Whatever the fortification method, the weight gain velocity was significantly slower with a longer exposure (β estimate = −0.070; 95% CI −0.120, −0.019; $p = 0.007$) and faster in infants born at or above 28 weeks' gestation (β estimate = 7.635; 95% CI 5.645, 9.624; $p < 0.001$). This means that for each additional day of exposure, a mean decrease of 0.070 g/kg/d in weight gain velocity occurred, and infants born at or above 28 weeks' gestation gained weight at a mean velocity of 7.6 g/kg/d faster than extremely preterm infants.



Day '0' indicates the starting day of exposure to human milk fortification

Fortification based on assumed composition

Fortification based on measured composition

--------- Lowess based on assumed composition

--------- Lowess based on measured composition

Figure 2. Weight gain velocity model in both groups, from birth to discharge, using a lowess smoother.

5.4.3 Length z-score

In both groups, infants were born with negative length z-scores (Table 6), whose deviations increased up to discharge. The infants fed fortified HM based on its measured macronutrient content, despite being born significantly shorter, had a significantly smaller z-score decline and attained similar length z-score at discharge than those fed fortified HM based on its assumed macronutrient content (Table 11).

The multivariable analysis showed that a smaller decrease in mean length z-score variation from birth to discharge was associated with the measured-based HM fortification method (β estimate = 0.423; 95% CI 0.181, 0.664; $p = 0.001$) and with the decrease in the deviation of the body weight z-score from birth to discharge (β estimate = 0.560; 95% CI 0.414, 0.706; $p < 0.001$).

5.4.4 Length gain velocity

In the univariable analysis, the length gain velocity had a significant positive association with the fortification method based on measured HM macronutrient content, gestational age, and birthweight, and a significant negative association with the number of days of exposure, LOS, and postnatal age.

In the multivariable analysis, adjusted for gestational age, length gain velocity from birth faster mean increase of 0.20 cm/week (β-estimate = 0.20; 95% CI 0.126, 0.280; $p < 0.001$) using the fortification method based on measured HM macronutrient content.

5.4.5 Head circumference z-score

In both groups, infants were born with negative HC z-scores (Table 6). The Δ HC z-score was negative from birth to discharge, in infants fed assumed-based fortified HM. In infants fed fortified HM based on its measured macronutrient content, despite being born with a significantly lower HC z-score, the Δ HC z-score was positive from birth to discharge, attaining a higher median HC z-score than those fed fortified HM based on its assumed macronutrient content (Table 11).

In the multivariable analysis, adjusting for the length z-score variation from birth to discharge (β estimate = 0.235; 95% CI 0.046, 0.425; $p = 0.016$), the fortification based on measured HM macronutrient content was significantly associated with a mean increase in the HC z-score from birth to discharge (β estimate = 0.955; 95% CI 0.672, 1.238; $p < 0.001$).

5.4.6 Head circumference gain velocity

In the univariable analysis, the HC gain velocity from birth to discharge had a significant positive association with the fortification method based on measured HM macronutrient content, gestational age, birthweight, length of stay, mean protein intake, and mean fat intake, and a negative association with the number of days of exposure and z-scores at birth of weight, length and HC.

In the multivariable analysis, the HC gain velocity from birth to discharge was significantly positively associated with the fortification method, with a faster mean increase of 0.18 cm /week (β-estimate = 0.178; 95% CI 0.111, 0.246; $p < 0.001$) using

the fortification method based on measured HM macronutrient content, adjusted for gestational age, HC z-score at birth and length of stay.

5.4.7 Body composition

Body composition was assessed in 74 (64.3%) infants after discharge, between 35 and 41 PMA: 38 in Group 1 and 36 in Group 2. The reasons that precluded body composition assessment were mainly due to the temporary closure of the Nutrition Laboratory, which occurred due to the COVID-19 pandemic, and parental refusal in the remaining cases.

No significant differences were found between infants with and without body composition assessment regarding gestational age, birth weight z-score, twin prevalence, extreme prematurity, prenatal and postnatal steroids, severity risk, IVH, BPD, NEC, and hospital stay. However, infants without body composition assessment had a higher occurrence of LOS (46.6% vs. 24.3%, $p = 0.015$), despite LOS not differing significantly between the HM fortification methods.

Infants fed fortified HM based on its measured macronutrient content, compared with those fed fortified HM based on its assumed macronutrient content, had significantly greater %FFM and lower FM and adiposity, indicated by both the %FM and FMI (Table 12).

Table 12. Single assessment of body composition after discharge, between 35–41 weeks postmenstrual age (n = 74). Group 1—fortified HM based on its assumed macronutrient content. Group 2—fortified HM based on its measured macronutrient content

	Group 1	Group 2	*p-value*
	n=57	n=58	
FM (g), median (P_{25}; P_{75})	360.1 (260.0; 483.3)	269.5 (169.1; 413.9)	0.026
FFM (g), median (P_{25}; P_{75})	2155.8 (1854.8; 2529.8)	2034.9 (1834.5; 2253.4)	0.173
%FM, median (P_{25}; P_{75})	14.5 (12.2; 17.3)	12.6 (8.3; 14.9)	0.021
FMI, median (P_{25}; P_{75})	1.8 (1.4; 2.3)	1.4 (0.9; 1.8)	0.004

%FFM, median (P_{25}; P_{75})	85.5 (82.7; 87.6)	87.4 (85.1; 91.0)	0.012

Mann–Whitney test; FM—fat mass; %FM—percentage FM; FFM—fat-free mass; %FFM—percentage FFM; FMI—fat mass index; HM—human milk. Student-*t* test, median test, or Mann–Whitney test, as appropriate.

Multivariable models were explored for body composition measurements, and specifically for adiposity excess.

The candidates for the body composition model (Table 12) were HM fortification method, number of exposure days, gestational age at birth, birth weight z-score, and sex, but no multivariable models were obtained.

The candidates for the adiposity excess model (%FM > 95th percentile and FMI > 90th percentile) were HM fortification method, number of days of exposure, gestational age at birth, and mean energy, protein, carbohydrate, and fat intakes.

In the univariable logistic regression for the outcome of excess adiposity (%FM > 95th percentile), considering the fortification based on assumed HM macronutrient content as a reference category, weak evidence was found that infants fed fortified HM based on its measured macronutrient content had 74% lower odds of excess adiposity (OR estimate = 0.26; 95% CI 0.06, 1.02; $p = 0.053$). No multivariable models were found for excess adiposity.

6 Discussion

In this cohort study, it was found that infants born at less than 33 weeks' gestation fed fortified HM based on its measured macronutrient content received significantly higher mean energy, fat, and carbohydrate intakes than those fed fortified HM based on its assumed macronutrient content. On the other hand, infants weighing ≥ 1 kg fed fortified HM based on its measured macronutrient content received significantly lower mean protein intake and those weighing < 1 kg received significantly lower mean PER than those fed fortified HM based on its assumed macronutrient content.

Infants of Group 2 had significantly better weight gain, length, and head growth at discharge. Body composition assessment at or close to TEA showed that infants who had been fed fortified HM based on its measured macronutrient content had significantly lower adiposity and greater lean body mass.

6.1 Nutrient intake according to the fortification method

In this observational study, the HM fortification prescription was left to clinical discretion. As such, a higher variability in nutrient intake would be expected than when following a fixed interventional study protocol.

The variability in energy and macronutrient intakes in the fortification based on assumed HM macronutrient content is in line with previous reports [112,132,165], and it was mainly due to intakes not achieving the minimum recommended amounts [245]. Overestimation of HM energy and macronutrient content was reported when fortification relies on assumed HM macronutrient content [132,135,243].

Individualized HM fortification was reported to provide the recommended nutrient intakes, with less macronutrient intake variability [112,253]. However, using fortification based on measured HM macronutrient content, we found an important variation in energy and macronutrient intakes, predominantly exceeding the maximum recommended amounts [245]. Rochow *et al.* [132] also reported excessive macronutrient intakes when using target fortification with added modular supplements, attempting to comply with ESPGHAN 2010 guidelines. Similarly, we found that infants fed fortified HM based on its measured macronutrient content received recommended mean intakes of energy, protein, and carbohydrates, but a mean fat intake (6.7 mg/kg/d) higher than the maximum

recommended (6.6 mg/kg/d). In particular, infants weighing < 1 kg fed fortified HM based on its measured macronutrient content have received a median PER intake lower than the minimum recommended (3.3 g/100 kcal) [245] due to disproportionately high energy intake in relation to the recommended protein intake. Nevertheless, the mean fat intake and the median PER intake were found to be within the range of 4.8–8.1 g/kg/d and 2.8–3.6 g/100 kcal, respectively, of the updated ESPGHAN 2022 guidelines for enteral nutrition in preterm infants, that became available after the study was concluded [78].

Since both fortification methods used the same fortifier without the addition of a modular carbohydrate supplement, a secondary analysis was performed to explain the differences in carbohydrate intake, and it was found that in the fortified HM based on its measured macronutrient content group, the carbohydrates were significantly denser in the MOM administered during the exposure period.

6.2 Body weight gain

In this study, to assess inadequate nutrition in preterm infants, we chose the body weight z-score decline and the weight gain velocity, since they are considered the most reliable metrics [143].

The infants of both groups were born with negative body weight z-scores, with a deviation that increased up to discharge, without significant differences between the groups. The multivariable analysis showed that the body weight Δ z-score between birth and discharge was not associated with the method of HM fortification; rather, it was associated with an increase in length z-score in the same period. In fact, body length reflects more specifically the skeletal growth and fat-free mass, while body weight is composed of the weight of multiple organs and tissues [143,254].

Z-score differences calculated from birth weight-based charts were criticized for not accurately reflecting the growth rate of very preterm infants [207,208]. Otherwise, the weight gain velocity over the previous days is reported to be more sensitive with respect to identifying changes in growth, with a minimum of a 5–7-day period required for a precise calculation [209]. It was suggested that 15–20 g/kg/d is the optimal weight velocity in infants born at 23–36 weeks' gestation, approximating to the mean estimates of fetal growth [210,211].

Compared with the conventional two-point assessment method, an exponential model was proposed by Patel *et al*. [212] as a more accurate estimate of weight gain velocity, as it is not affected by decreasing birth weight and increasing length. Using the Patel model, we found that the median weight gain velocity at discharge was significantly faster in Group 2 than in Group 1, either from birth (10.8 vs. 9.7 g/kg/d) or from the beginning of fortification (15.3 vs. 13.0 g/kg/d).

However, using linear mixed effects models that considered the correlation structure between the longitudinal estimates, no difference in weight gain velocity was found between the groups. In line with this, McLeod *et al*., using the Patel exponential model, did not find differences in weight gain velocity between the assumed- and measured-based HM fortification methods [165].

The multivariable analysis with stratification by gestational age showed that, whatever the fortification method, weight gain velocity was significantly faster in infants who were born at ≥ 28 weeks' gestation than in extremely preterm infants, an expected finding. The slower weight gain velocity in extremely premature infants may be related to significantly higher severity risk and LOS and higher incidence of bronchopulmonary dysplasia, morbidities that are inherent to extreme prematurity [255]. An association was also found between a longer exposure to fortification and a significantly slower weight gain velocity. The deceleration in weight gain velocity with the duration of fortification may reflect a progressive overcoming of cumulative nutrient deficits in malnourished infants, whose nutritional status improved as better nutritional support became possible on a regular basis [256].

6.3 Linear growth

Infants of both groups were born with negative length z-scores and had linear growth faltering up to discharge, indicated by a decline in length z-scores. Despite the fact that the infants fed fortified HM based on its measured macronutrient content were born significantly shorter than those fed fortified HM based on its assumed macronutrient content, they had a significantly smaller length Δ z-score decline, with both groups attaining similar mean length z-scores at discharge. This is consistent with a significant positive association between the fortification method based on measured HM macronutrient content and the length gain velocity, adjusted for gestational age, being 0.2

cm/week faster than using fortification based on assumed HM macronutrient content. In a systematic review comparing the effect of individualized fortification (targeted or adjustable) with standard non-individualized HM fortification on growth, infants fed individualized fortified HM had a length gain velocity 0.3 cm/week faster during the intervention [134]. This is clinically relevant, since superior linear growth was reported to be associated with better brain development [143].

6.4 Head growth

The HC measurements reflect brain size and head growth, which is an important indicator of neurodevelopment outcomes in preterm infants [67,143].

Infants in both groups were born with negative HC z-scores. In in Group 1, HC z-scores declined up to discharge. Despite the fact that the infants of Group 2 were born with significantly lower mean HC z-scores, their HC z-scores increased up to discharge, attaining a higher mean z-score than in the infants of Group 1. Consistently, the multivariable analysis revealed that the increase in HC z-scores from birth to discharge was significantly associated with the fortification based on measured HM macronutrient content, adjusted for length z-score variation in the same period.

The HC gain velocity was significantly associated with fortification based on measured HM macronutrient content, adjusted for gestational age, HC z-score at birth, and length of stay, and was 0.18 cm/week faster than using fortification based on assumed HM macronutrient content. In the aforementioned systematic review, preterm infants fed individualized fortified HM had, during the intervention, an HC velocity 0.10 cm/week faster than the standard non-individualized fortified HM [134].

6.5 Body composition

In a systematic review with meta-analysis, it was concluded that preterm infants at TEA have greater %FM and less FFM than those of infants born at term [59]. This increase in adiposity occurs even when nutrition is prescribed, according to the ESPGHAN 2010 guidelines [240]. The mentioned differences in body composition at TEA were reported to dissipate by 3–5 months' corrected age [257].

The body composition assessed after discharge at late preterm or term PMA (35 to 41 weeks) showed, via univariable analysis, that infants of Group 2 had significantly lower

fatness, as indicated by FM and adiposity (%FM and FMI), and greater leanness, as indicated by %FFM, compared with those of Group 1. In the univariable logistic regression, weak evidence was found that the infants of Group 2 had lower odds of excess adiposity; no multivariable models were obtained, rendering it impossible to control for other variables reported to affect fatness. This greater %FFM in infants of Group 2 is consistent with their faster linear growth, another indicator of lean mass 258. This is clinically relevant, since in preterm infants at TEA, it is reported that a greater leanness is associated with brain size 258,259.

These results for body composition raise two questions. First, why did infants of Group 1, receiving lower than recommended energy and fat intakes, had greater adiposity at near TEA compared with those of Group 2, who received higher energy and fat intakes? Second, why did infants of Group 2 had lower adiposity and greater leanness at near TEA after receiving in-hospital energy and protein intakes according to ESPGHAN 2010 guidelines [245], but mean fat higher than the maximum intake recommended, as well as a median PER (in infants weighing < 1 kg) lower than the minimum intake recommended?

Some mechanisms explaining these findings can be speculated.

First, it has been described that in very preterm infants, postnatal age and PMA are strong predictors of FM and %FM at 34–37 weeks PMA [57]. As the rapid accumulation of fetal fat begins by 32–33 weeks' gestation, after a preterm birth interrupting the constant nutrient supply of the placenta, the ability for fat accretion may be an adaptive postnatal mechanism protecting against the risk of low nutrient availability [57,58]. Using the fortification regimen based on measured HM macronutrient content, higher in-hospital energy, protein, and carbohydrate intakes were provided, which could have reduced excessive fat accumulation at this age [57].

Second, it is known that before 25 weeks of gestation, the human fetus accumulates only a small amount of lipids, with subsequent exponential accumulation of large amounts of fat [260,261]. We speculate that after a very premature birth, a lipid intake that we judge to be adequate, but is actually insufficient during this critical period, may trigger protective mechanisms (perhaps unknown) to save lipids for catching-up fat reserves, resulting in excessive fat accumulation. A parallel can be drawn with the birth cohort study by Rolland-Cachera [262]. in which early low-fat intake resulted in increased susceptibility to develop overweight via leptin resistance at later ages.

7 Limitations

Certain study limitations should be acknowledged.

First, this is an observational study that assessed the effectiveness of two methods of HM fortification in real-life clinical practice, where nutritional prescription was at the clinical discretion of physicians. The findings of this study do not substitute those of a randomized clinical trial assessing the efficacy of evaluated nutritional strategies.

Second, some differences between groups may have introduced biases. Infants lost to follow-up had significantly higher severity risk than those completing the study period. On the other hand, infants of Group 2 were born significantly shorter and with lower HC and had significantly higher severity risk, prevalence of twins, and LOS than infants of Group 1. Although these latter differences may be biases, they did not preclude the group fed fortified HM based on its measured macronutrient content from having better growth outcomes despite having more unfavorable factors.

Third, to accurately accomplish what the study set out to do, with recommendations for enteral nutrition in preterm infants, the ideal would have been to guide fortification based on daily analysis of MOM composition, but such frequency of analysis would be impractical as it would be overly laborious and time consuming [132,165]. Moreover, an excess of MOM volume required for frequent analyses would reduce its availability to feed the infants most in need. For these reasons, the reported weekly analysis of MOM [135] has been the better possible alternative to achieve the study's purpose.

Finally, body composition was based on a single assessment soon after discharge, rather than providing insight on previous changes and their relationship to in-hospital nutritional support. Nevertheless, our data can be compared with those from most other similar studies, which have focused on the body composition of preterm infants at TEA [5,59].

8 Strengths

Some strengths are also acknowledged.

In the contemporary cohort, nutrient intakes were recorded on daily basis, by the same observer, using a specifically developed software (IGAC-DSPI nr 480/2020, 26 February 2020) to calculate accurately the amounts of modular protein and fat supplements added to the fortified HM.

Growth assessment during the hospital stay relied on weight, length, and HC Δ z- scores and their velocities, which are currently considered the best metrics to evaluate growth in preterm infants [143].

To estimate weight gain velocity, we have used not only an accurate exponential method superior to the conventional two-point assessment method [212], but also a refined linear mixed effects regression models that consider the correlation structure between longitudinal measurements were used to analyze those estimates. This method provided differentiated results and may be used in future similar studies.

To the best of our knowledge, a better body composition profile was firstly described using intakes of fat higher than the maximum and of PER (in infants weighing < 1 kg) lower than the minimum contemporarily recommended [245]. Importantly, these intakes outside of that recommended turned out to be within the range of the recently updated ESPGHAN 2022 guidelines [78], supporting the advantages of these new enteral nutrition recommendations for preterm infants.

9 Conclusions

This cohort study of infants born at less than 33 weeks of gestation, evaluated differences between two methods of HM fortification on nutrient intakes, growth during hospital stay, and body composition soon after discharge, at or near TEA.

9.1 What was known

It was reported that target HM fortification, based on measured HM macronutrient content more accurately achieve the recommended nutrient targets in preterm infants. However, target fortification is excessively laborious method and time consuming, requiring the acquisition of a HM analyser and its maintenance. Therefore, is is of utmost importance an accurate assessment of the balance between costs and clinical effectiveness to prefer this method.

A more accurate evaluation of clinical benefits encompass not only conventional anthropometry, including sex and age specific z-scores of antropometric measurements, but also the quality of growth indicated by body compartments assessment (fat and lean mass).

9.2 What is added

This study showed that infants fed fortified HM based on its measured macronutrient content was associated with significantly better weight gain, length, and head growth during hospital stay. In addition, a better body composition profile was found at or near TEA, characterized by lower adiposity and greater lean mass, after receiving in-hospital fat and PER intakes outside of former contemporary guidelines, but within the range of the current recommendations, supporting their advantages.

10 Future directions

Results of this observational study provide data for future high-quality trials with long-term follow-up to confirm if HM fortification based on measured HM macronutrient content is the better enteral feeding regimen for growth and body composition in preterm infants.

This encompasses the use of accurate tools for monitoring growth and body composition not only during hospital stay but also during childhood and adolescence.

Better head growth associated with HM fortification based on measured HM macronutrient content deserves neurodevelopment assessment with longer-term follow-up as possible.

Only a cost-benefit analysis, evaluating the costs of fortification based on measured HM macronutrient content and the clinical and economical advantages, including possible short- and long-term neurodevelopmental benefits, can define the best HM fortification method to adopt in preterm infants fed HM.

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