EFFECT OF DIFFERENT COMPOSITION OF HUMAN MILK AND ITS FORTIFICATION ON BODY COMPOSITION AND NEURODEVELOPMENT IN A COHORT OF VERY PRETERM INFANTS

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Tese para obtenção do grau de Doutor em Medicina

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PREFACE

Early nutritional optimization is increasingly recognized as having a significant role in the short-, medium- and long-term outcomes of very preterm infants, prompting urgent attention and research in this field.

Our project aimed to determine the associations of in-hospital cumulative protein and energy intake with body composition at term corrected age and neurodevelopment at 18 months corrected age (CA).

The initially submitted project (October 2012) was a double-blinded randomized controlled trial, with the purpose of comparing the effect of the lowest with the highest recommended protein intake, without changes in the recommended energy intake on body composition at term CA and neurodevelopment at 18 months CA. Body composition was to be evaluated using the air displacement plethysmography method and neurodevelopment was to be evaluated at 18 months CA, using the Bayley Scales of Infant Developmental, version II. The study was to be performed at Maternidade Dr. Alfredo da Costa, specifically in its Neonatal Intensive Care Unit, Human Milk Bank, Pharmacy Service as well as at the Nutrition Lab of Hospital Dona Estefânia, both of which belong to the Centro Hospitalar de Lisboa Central, Portugal. The project was scheduled to start at beginning of 2013; however, the Ethical Committee and Administration Board approval of the Hospital was only given at June 2013.

Besides that, in 2012, there was a Government decision to close the Maternidade Dr. Alfredo da Costa (which did not happen) and merge all their services with Hospital Dona Estefânia services. Facing this decision, in 2013, more than half of the pharmaceuticals, laboratory personnel, and nurses left the Maternity. Unfortunately, many of them were committed to this research project, making the trial unfeasible. To overcome this constraint, we decided to redesign the project converting it into a cohort study that seemed feasible with the available resources.

The new study design was aimed to determine, in human milk-fed very preterm infants, the associations between estimated and measured in-hospital cumulative macronutrient intake and body composition at term CA and neurodevelopment at 18 months CA. The macronutrient intake would rely on measurements of human milk content.

In December 2013, the change in study design was submitted to the Scientific Board of the NOVA Medical School | Faculdade de Ciências Médicas and was approved in January 2014.

The recruitment of participants for the cohort study was initiated in February 2014. Facing a one-year delay in initiation of the redesigned study, due to the aforementioned reasons, and to comply with the fixed academic timeframe for the field work, the number of recruited participants was less than estimated, and the study was under powered. Nevertheless, the studied sample had sufficient dimension to provide interesting results.

This PhD research project was approved by Comissão Nacional de Proteção de Dados (autorização n.º 9767/2012), by the Ethics Committee of Centro Hospitalar de Lisboa Central (autorização n.º 116/2012), and by the Ethics Committee of NOVA Medical School/Faculdade de Ciências Médicas (autorização n.º 75/2014/CEFCM).

“It is not the answer which enlightens, but the question.”
Eugene Ionesco

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RESUMO

Introdução

Em recém-nascidos muito prematuros, o suporte nutricional inicial adequado é de extrema importância para a qualidade do crescimento e neurodesenvolvimento a curto, médio e longo prazo. O leite humano (LH) tem vantagens bem conhecidas em relação às fórmulas infantis, nomeadamente no desenvolvimento cerebral.

Objectivos

Determinar, numa amostra homogénea de recém-nascidos muito pré-termo, alimentados com leite humano, a associação do aporte proteico, energético e da relação proteína-energia (RPE) durante o internamento hospitalar, com a velocidade de aumento ponderal, a composição corporal e o perímetro cefálico (PC) na idade corrigida (IC) de termo, assim como o neurodesenvolvimento aos 18 meses de IC.

Métodos

Foi efetuado um estudo de coorte, sendo elegíveis recém-nascidos consecutivos nascidos na Maternidade com menos de 33 semanas de idade de gestação (IG), exclusiva ou predominantemente alimentados com LH (leite materno – LM e/ou leite de dadora).

O estudo foi aprovado pelas Comissões de Ética do Hospital e da Faculdade e está registado no ISRCTN (ID: 27916681). Foi obtido consentimento informado escrito dos pais ou representante legal de cada criança.

Foi seguido o protocolo de nutrição da nossa Unidade, baseado em recomendações internacionais e nacionais. Foi utilizado o método de fortificação padrão com adição de modo estimado de proteína e lípidos modulares, considerando o menor teor de proteína do leite humano descrito na literatura e as recomendações nutricionais mínimas recomendadas para o peso.

Para medir o conteúdo de macronutrientes do LH administrado, foi utilizado o analisador de LH por espectroscopia de infravermelhos. A antropometria foi efetuada utilizando as técnicas recomendadas. A avaliação da composição corporal, por

intermédio da pletismografia de deslocação de ar, foi agendada após a alta para a IC de termo; a percentagem de massa gorda (%MG) e o índice de massa de gorda (IMG) foram utilizados como indicadores da adiposidade. A avaliação do Índice de Desenvolvimento Mental (IDM) e do Índice de Desenvolvimento Psicomotor (IDP), utilizando as Escalas de Desenvolvimento Bayley versão II, foram agendadas para os 18 meses de idade corrigida.

Análise estatística: estimaram-se dimensões amostrais mínimas de 70 e 75 crianças para detetar, respetivamente, diferenças significativas na composição corporal e nos resultados do neurodesenvolvimento. Foi efetuada análise univariável, utilizando testes paramétricos ou não paramétricos adequados ao tipo de dados e distribuição encontrados, avaliando associações entre os aportes proteico, energético e da RPE, com a velocidade de aumento ponderal, a massa gorda (MG), a massa isenta de gordura (MIG), a %MG, o IMG, o PC, o IDM e o IDP. Os mesmos métodos estatísticos foram utilizados para avaliar as potenciais variáveis confundentes, usando $p<0,10$ para inclusão nos modelos multivariáveis. Foram utilizados modelos mistos lineares para calcular os valores da composição do leite materno nas amostras em não foi possível efetuar a sua medição e análises de regressão linear múltipla para avaliar o efeito ajustado entre variáveis independentes e dependentes. Utilizou-se uma análise caso-controlo anichada para determinar as associações entre os limites inferior (≤ -1 z-score) e superior (≥ +1 z-score) de adiposidade e os aportes proteico, energético e da RPE.

Resultados

Foram incluídas na coorte 33 crianças, com medianas (intervalos interquartílicos) de 30 (28-31) semanas de IG e peso ao nascer de 1175 (1010-1408) g. Comparando com os 56 lactentes excluídos do estudo, alimentados com formula, os 33 lactentes que completaram o estudo tinham IG significativamente menor, menor prevalência de gémeos e maior tempo de internamento.

Foram analisadas 832 amostras de LH, representando 65,0% do total das amostras administradas.

Desvendadas as medições de macronutrientes do LH, verificou-se que foram atingidos os aportes mínimos recomendados por peso em 63,6%, 15,2%, 93,9% dos lactentes em

relação às proteínas, energia e RPE, respetivamente. Os aportes diários de proteína, energia e RPE, do nascimento até às 35 semanas de IC, variaram entre 2,7-4,2 g/kg, 53,7-109,2 kcal/kg e 3,4-5,6, respetivamente.

A velocidade de aumento ponderal intra-hospitalar média (DP) foi de 10,1 (3,8) g/kg/dia. O peso médio (DP) foi de 2817,6 (504,3) g, a MG de 441,5 (184,0) g, a MIG de 2376,1 (376,0) g, a %MG de 15,3 (4,8) e o IMG de 2,0 (0,7).

O neurodesenvolvimento foi avaliado aos 20 meses de IC. Globalmente, o IDM médio (DP) foi 100,2 (11,5) e o IDP 97,4 (8,0). O IDM médio foi inferior ao normal em 6,2% dos lactentes, normal em 78,1% e acelerado em 15,6%; O IDP médio foi inferior ao normal em 6,2% dos lactentes e normal em 93,8%.

Na análise multivariável, apenas a IG se associou com menor velocidade de aumento ponderal ($p<0,0001$). Após ajustamento para a IG, apenas a MIG se associou com menores aportes proteico ($p=0,008$) e energético ($p=0,001$). Na análise caso-controlo anichada, nos lactentes com menor adiposidade, uma %MG ≤ -1 z-score associou-se a menores aportes de energia e proteína ($p=0,050$) e um IMG ≤ -1 z-score associou-se a menor aporte de RPE ($p=0,026$); em lactentes com maior adiposidade, um IMG ≥ +1 z-score associou-se a menor aporte de energia ($p <0,0001$) e maior aporte de RPE ($p <0,0001$).

Na análise multivariável, a IG e o sexo foram preditores de maior PC na IC de termo, ajustado para os aportes proteico ($p=0,010$), energético ($p=0,013$) e RPE ($p=0,013$). Os aportes intra-hospitalares cumulativos proteico, energético e da RPE não se associaram significativamente com as pontuações IDM e IDP na idade média de 20 meses de IC, nem reuniram os critérios para entrada na análise multivariável.

Conclusões:

Nesta coorte de crianças nascidas muito pré-termo exclusivamente ou quase exclusivamente alimentadas com LH, os aportes intra-hospitalares cumulativos proteico, energético e da RPE correlacionaram-se fraca a moderadamente com o aumento da velocidade ponderal, mas não com a composição corporal na IC de termo. Analisando os lactentes com extremos de adiposidade, os com menor adiposidade receberam significativamente menos proteína, energia e RPE, enquanto os com maior

adiposidade receberam significativamente menor aporte energético, mas maior RPE, do que a restante amostra. A IG e o sexo foram preditores significativos de maior PC na IC de termo, ajustado para os aportes proteico, energético e da RPE. Os aportes intra-hospitalares cumulativos proteico, energético e da RPE não se correlacionaram significativamente com o IDM nem com o IDP aos 20 meses de IC. O método de fortificação padrão com adição de modo estimado de proteína e lípidos modulares resultou no aporte insuficiente de energia e proteína.

A amostra subdimensionada pode ter sido insuficiente para testar as hipóteses admitidas de associação do aporte de macronutrientes com a composição corporal e o neurodesenvolvimento. Contudo, as nossas análises basearam-se na medição do conteúdo proteico e energético do LH e não na sua composição estimada, sendo um ponto forte do estudo.

Palavras-chave: aporte energético, aporte proteico, composição corporal, leite humano, neurodesenvolvimento, perímetro cefálico, recém-nascidos muito pré-termo, velocidade de aumento ponderal.

ABSTRACT

Background

In very preterm infants, adequate early nutritional support is of utmost importance for the quality of growth and neurodevelopmental outcomes in the short-, medium- and long-term. Human milk (HM) has well-known advantages over infant formulas, including for brain development.

Objectives

To determine, in a homogeneous sample of HM-fed very preterm infants, the associations of in-hospital measured protein, energy, and protein-to-energy ratio (PER) intake with weight gain velocity, body composition and head circumference (HC) at term corrected age (CA), and with neurodevelopmental outcome at 18 months CA.

Methods

A cohort study was conducted, being eligible consecutive inborn neonates with less than 33 weeks of gestation, who were exclusively or predominantly HM-fed (own's mother milk and/or donor human milk).

The study was approved by the Hospital and Medical School ethics committees and is registered at the ISRCTN (ID: 27916681). Informed written consent was obtained from the parents or legal representative of each infant.

Our unit nutrition protocol, based on international and national recommendations, was followed. A standard fortification method with the blinded addition of modular protein and/or fat supplements was used, considering the lowest reported HM protein content and the minimum recommended intake for weight. A mid-infrared analyzer was used to measure the macronutrients content of administered HM. Anthropometry was performed using the recommended techniques. Body composition assessment, using air displacement plethysmography (ADP), was scheduled after discharge, at 40 weeks CA; fat mass percentage (FM%) and fat mass index (FMI) were used as surrogates of adiposity. The assessment of the Mental Developmental Index (MDI) and Psychomotor

Developmental Index (PDI), using the Bayley Infant Development Scales version II, were scheduled at 18 months CA.

Statistical analysis: required samples of 70 and 75 infants were estimated to detect significant differences in body composition and neurodevelopmental outcomes, respectively. Univariate analysis, using parametric or nonparametric tests as adequate, assessed the associations of cumulative in-hospital protein, energy, and PER intake with weight gain velocity, fat mass (FM), fat-free mass (FFM), FM%, FMI, HC, MDI, and PDI. The same statistical methods were used to assess potential confounding variables, using $p<0.10$ for inclusion in models. Linear mixed models were used to input missing measured values of own's mother milk composition and linear multiple regression analyses were used to assess the adjusted effect between independent and dependent variables. A nested case-control analysis was used to determine the associations of lower (≤ -1 z-score) and higher (≥ +1 z-score) adiposity with protein, energy, and PER intake.

Results

Thirty-three infants were included in the cohort, with a median (interquartile range) gestational age of 30 (28-31) weeks and birthweight of 1175 (1010-1408) g. Compared with the 56-excluded formula-fed infants, the 33 infants who completed the study had significantly lower gestational age, lower prevalence of twins and stayed longer in hospital.

Eight hundred and thirty-two pooled HM samples were analyzed, representing 65.0% of the total administered samples. After disclosing the HM macronutrients measurements, it was found that the minimum recommended intake for weight were achieved in 63.6% of infants for protein, 15.2% for energy, and 93.9% for PER. The median daily protein, energy, and PER intake from birth to 35 weeks CA ranged from 2.7-4.2 g/kg, 53.7-109.2 kcal/kg, and 3.4-5.6, respectively.

The mean (standard deviation - SD) in-hospital weight gain velocity was 10.1 (3.8) g/kg/day. At mean (SD) 39.9 (1.9) weeks, body mass was of 2817.6 (504.3) g, FM of 441.5 (184.0) g, FFM of 2376.1 (376.0) g, FM% of 15.3 (4.8), and FMI of 2.0 (0.7).

Neurodevelopment was assessed at 20 months CA. Overall, the mean (SD) score for MDI was 100.2 (11.5) and for PDI 97.4 (8.0). The mean MDI score was below normal in 6.2% infants, normal in 78.1%, and accelerated in 15.6%; the mean PDI score was below normal in 6.2% infants and normal in 93.8%.

In multivariate analysis, only gestational age was associated with low weight gain velocity ($p<0.0001$). After adjustment for gestational age, only FFM was associated with low protein ($p=0.008$) and energy ($p=0.001$) intake. In the nested case-control analysis, in infants with lower adiposity, a FM% ≤ -1 z-score was associated with low energy and protein intake ($p=0.050$) and a FMI ≤ -1 z-score was associated with low PER intake ($p=0.026$); in infants with higher adiposity, a FMI ≥ +1 z-score was associated with low energy intake ($p<0.0001$) and high PER intake ($p<0.0001$).

In multivariate analysis, it was found that GA and sex were predictors of high HC at term CA, adjusted for protein ($p=0.010$), energy ($p=0.013$) and PER intake ($p=0.013$). In-hospital cumulative protein, energy, and PER intake were neither significantly correlated with any MDI or PDI scores at mean 20 months CA, nor met the defined criteria to enter multivariate analysis.

Conclusions

In this cohort of exclusively or almost exclusively HM-fed very preterm infants, the cumulative in-hospital protein, energy, and PER intake were weakly-to-moderately correlated with weight gain velocity, but not with body composition at term CA in the entire sample. Analyzing infants with extremes of adiposity, those with lower adiposity received significantly lower energy, protein, and PER intake, while infants with higher adiposity received significantly lower energy intake but higher PER intake, compared with the remaining infants. The GA and sex were significant predictors of high HC at term CA, adjusted for protein, energy and PER intake. In-hospital cumulative protein, energy, and PER intake were not significantly correlated with MDI or PDI scores at a mean of 20 months CA. The method of standard fortification with blinded modular protein and fat supplements resulted in insufficient energy and protein intake.

The undersized sample might be insufficient to test the hypothesized associations of macronutrient intake with body composition and neurodevelopmental outcome.

Notwithstanding, our analyses have relied on measured protein and energy HM content and not on its assumed composition, which is a strength of the study.

Key-words: body composition, energy intake, head circumference, human milk, neurodevelopmental outcome, protein intake, very preterm infants, weight gain velocity.

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ADP	Air displacement plethysmography
BMI	Body mass index
BSID-II	Bayley Scales of Infant Development, version II
BSID-III	Bayley Scales of Infant Development, version III
CA	Corrected age
DASII	Developmental Assessment Scale for Indian Infants
DEXA	Dual-energy x-ray absorptiometry
DHM	Donor human milk
FFM	Fat-free mass
FM	Fat mass
FM%	Fat mass percentage
FMI	Fat mass index
GA	Gestational age
HC	Head circumference
HM	Human milk
IPVH	Intra-periventricular hemorrhage
IQR	Interquartile range
MDI	Mental developmental index
MRI	Magnetic resonance imaging
NEC	Necrotizing enterocolitis
NICU	Neonatal intensive care unit
OMM	Own mother's milk
PDI	Psychomotor developmental index
PER	Protein-energy-ratio
PN	Parenteral nutrition
R	Correlation coefficient
R adjusted	Correlation coefficient adjusted for covariates in the model
r^2	Coefficient of determination

SD	Standard deviation
SNAPPE II	Score for neonatal acute physiology - perinatal extension-II
TBW	Total body water
VLBW	Very low birth weight

1. INTRODUCTION

1.1. Very preterm infant: the state of the art

Since the middle of the 20th century, preterm infants have survived at increasing rates, owing to the introduction of incubators and the provision of supplemental oxygen, facing pediatricians with the additional challenge of providing adequate nutritional support.(1)

Innovative neonatal ventilators, antenatal corticosteroids for lung maturation, surfactant for hyaline membrane disease and increasing non-invasive ventilation, further increased the survival of more premature infants by the end of the last century and the beginning of the 21st century.(1, 2)

In the last decades, several key innovations, including parenteral nutrition, central venous catheters, specially tailored neonatal amino acid and lipid emulsions, followed the nutritional improvements at the end of the 20th century and beginning of the 21st century.(2, 3) Tube enteral feeding, human milk banks, human milk fortifiers and preterm formulas further contributed to the improved survival.(1)

1.1.1. Mortality and morbidity: challenges

In infants with a birth weight of 501-1500 g, a 1% increase in survival was observed between the periods 1995-1996 and 1997-2000, reaching a plateau thereafter associated with an unchanged morbidity.(4, 5)

In Portugal, there has been a decline in the birth rate over the past 8 decades, but this trend seems to have slightly reversed, with a 3.8% increase in births in 2015 in relation to 2014 (INE 2016). Between 2005 and 2010, the percentage of preterm newborn infants ranged from 6.6% to 9.1%, and was 8.9% in 2016.(6)

Infant mortality is predominantly conditioned by prematurity. It accounts for 11% of births worldwide(7) and 6.2% in Europe(8), being responsible for about two thirds of all neonatal deaths. Very preterm infants, defined by birth before 32 full weeks of gestation, correspond to 0.8% of births in Portugal(9) and 1.0% in Europe, accounting

for about 60% of neonatal mortality.(8) Most of mortality in premature infants is due to the high mortality rate of extremely preterm infants, defined by birth before 28 full weeks of gestation; they constitute about 5% of all preterm infants, but contribute to about 35% of neonatal mortality.(10, 11) In Portugal, in 2015, the mortality rate in less than 33 weeks of gestation was 4.1% (19/463) and, in less than 28 weeks of gestation, was 3.7% (17/463). Severe intra-periventricular hemorrhage affected 9.3% of newborn infants aged less than 33 weeks of gestation in 2015.(9)

The difficulty in reducing neonatal mortality and major morbidity, among other factors, may be due to suboptimal nutrition in extremely preterm infants.(12) It is becoming evident that suboptimal nutrition in the perinatal period may have short-(13), medium- and long-term consequences, namely in neurodevelopment in infancy, school age, adolescence and adulthood.(14-16)

After a period of intense technical advances in neonatal intensive care, it seems that current research challenges in preterm infants are aimed at achieving optimal postnatal growth and maturation to promote healthier children and adults.(17, 18)

1.1.2. Development of the central nervous system

In the last 30 years, research into the brain and its role in psychological functions has provided new and deep insight in the development of the human brain, especially during the first 3 to 5 years of life, extending through adulthood. Much of this research has been performed in infants, but a lot of animal research also gave insight into the development of the human brain.(19, 20)

Neurulation starts at the beginning of conception; 2 weeks later, the embryo has a three-layered tubular structure. A **proliferation** phase then follows, where the cells of the innermost part of the tube proliferates at a logarithmic rate, forming the marginal zone, which will contain axons and dendrites. The highest growth rates are observed in the cortical grey matter and the cerebellum.(21) The number of neurons in the infant brain is much larger than in the adult brain. This overproduction of neurons is balanced by a process of programmed cell death, under genetic control.(19, 20)

At about 25 weeks of gestation, the neuronal cells travel to their final destinations, via **cell migration**, with an inside-out radial migration, beginning in the ventricular zone. The cells reach their destination on the outside of the developing brain. At its target destination, the neuron either undergoes **differentiation**, into a mature and complete neuron, with axons and dendrites, or can suffer apoptosis. With advancing gestational age, cortical folding progresses and gyrification becomes more complex.(19, 20)

Dendrite formation is thought to be driven by genes controlling calcium-regulated transcription factors.(22) Early dendrites appear as thick strands, with small protuberances from the cell body. When dendrites mature, the density of these protrusions or spines increases, as well as the chances of a contact with a neighboring axon.(19, 20)

These connections between dendrites and axons are the basis for synaptic connections between neurons, essential for brain function. **Synaptogenesis** begins about the 23rd week of gestation(23), reaching a peak by the first year of life, followed by a gradual reduction. This process is highly dependent on experience and is the basis of the learning process that occurs during the early years of life. The time at which the peak of synapse production in the brain is reached differs. For example, in the visual cortex, the peak is reached between the 4th and 8th postnatal month, while in the prefrontal cortex it is not reached before the 15th postnatal month.(19, 20)

After this overproduction of synapses, the unused synapses suffer a process called **synapse pruning**. During the synaptogenesis stage, the brain development is largely controlled by genes. After that stage, the process of synapses elimination is reached, and the brain development becomes largely experience driven. The timing of synapse elimination is also dependent on the area of the brain in which it occurs. In the visual and auditory cortex, for example, it is complete between the 4th and 6th year of life. In areas involved in higher cognitive functions (like inhibitory control and emotion regulation), it continues through adolescence.(24) This process of overproduction of synapses followed by their reduction, is essential for the flexibility and adaptive capabilities of the developing brain. While environmentally activated pathways are strengthened, unused pathways are eliminated. These networks of neurons involved in the development of behavior are modified and fine-tuned as needed.(19, 20)

The final process in the development of the brain is **myelination**. In this his process, the axons are wrapped by fatty cells, which facilitates neuronal activity and fastens the transmission of electrical signals. The timing of myelination is dependent on the region of the brain in which it occurs. Sensory and motor areas of the brain are myelinated earlier and are complete around the preschool age. Brain areas involved in higher cognitive abilities, such as the prefrontal cortex, are only complete near adolescence or early adulthood.(19, 20)

Although brain development is largely under genetic control during the prenatal months, the environment can clearly play a role, such as a lack of nutrition (e.g., folic acid, health and nutritional status of the mother) and the presence of toxins (e.g., alcohol), which can deleteriously affect the developing brain. Much of the postnatal brain development is experience-dependent and defined by gene-environment interactions.(19, 20) In a recent study, 151 children born with 25 to 41 completed gestational age (GA), apparently without brain lesions, were assessed at 10 to 13 years of age, using transcranial magnetic stimulation and functional assessments to examine corticomotor development. It was found that, for every week of reduced gestational age, there was an association with a reduction in corticomotor excitability that remained evident in late childhood.(25)

Very preterm infants are unexpectedly exposed to an extra-uterine environment in a period of critical brain development, rendering them susceptible to injury, especially of white matter structures, decreased microstructural connectivity, different patterns of neuronal activation and decreased cortical grey matter volumes.(26, 27)

Numerous factors, like the neonatal events, stresses, central nervous system lesions, and white matter injury secondary to intra-periventricular hemorrhages (IPVH) with subsequent hemorrhagic infarction or periventricular leukomalacia, are causes of brain injury following preterm birth. White matter injury is probably the most frequent lesion after preterm birth. The main pathogenic mechanisms are considered to be inflammation and ischemia, which are frequently coincident and potentiate each other.(28, 29) However, the developing brain is plastic and shows important compensatory abilities. Such lesions and activation differences do not necessarily result in cognitive delays later in life.(26, 27)

Nutrition that would reduce systemic infections and the inflammatory response may be able to alleviate white matter injury and promote brain development.(30, 31)

Another area of active research is immunomodulation, which may offer benefits to the developing brain through the microbiome-gut-brain axis, also affected by the enteral nutrition of the preterm infant.(17, 32)

1.2. Nutritional support

In very preterm infants, the prevention of in-hospital macronutrient deficits may be achieved through the optimization of nutritional policies.(33) This includes early high parenteral amino acid intake, the early introduction of parenteral lipids, early trophic enteral feeding, and the use of fortified human milk (HM), preferably the own mother's milk (OMM) or donor human milk (DHM).(34)

Details on each aspect are addressed in the following sections.

1.2.1. Recommendations for preterm infants

In the last decades, nutrition recommendations for very preterm Infants were subject to several significant changes. Parenteral solutions (PN), usually were started with low amounts of glucose and calcium gluconate alone. In the subsequent days, very modest protein intake was started, with very slow daily increases. Lipid emulsions (based on soy-bean oil) frequently were started only after several days.(35) In the beginning of the 21th century an evolution to a much more aggressive nutrition took place.(34, 36, 37)

The gold standard for parenteral and enteral nutrition was to achieve, as soon as possible, nutritional intake taking as reference the in-utero placental nutrition. The aim was to obtain body growth and a composition like that of term infants, also concerning bone nutrition, while avoiding the complications inherent of being premature.(36, 38-48)

International recommendations are available for neonatal parenteral (PN) and enteral nutrition(34, 38, 43), which are included in national guidelines.(45, 46) Briefly, in the more premature infants, PN is initiated within the first 2 postnatal hours with ≥2.5 g/kg/day of amino acids and glucose (4.5 to 5.0 mg/kg/min), which are increased in the

next days, respectively to 4.0 g/kg/day and ≤13 mg/kg/min; lipids are started within the first 24 postnatal hours with ≥1 g/kg/day and increased up to 3 g/kg/day, and the energy aim is 110 to 130 kcal/kg/day. Calcium, phosphorus and zinc are also provided from the first day of life. Careful fluid and electrolyte management are mandatory in the first days of life and in the more immature or sick newborns. The preterm fetus is in a state of relative total body water and extracellular fluid excess that must be mobilized and excreted. Increased aldosterone levels result in an impaired ability to excrete a large, or acute, sodium load; therefore, neither sodium nor potassium should be given until urine output and creatinine levels are within normal ranges and natremia is decreasing, in the face of some weight loss (up to 20% in the more immature infants).(49)

Early enteral trophic feeding (10-20 ml/kg/day), preferably with mother colostrum or OMM should be started within the first 2 to 4 postnatal days(50); if not available, the second choice should be pasteurized DHM, given by a gastric tube. Subsequently, enteral nutrition is increased as PN is proportionally reduced. Whenever possible, exclusive HM should be used.(51) When neither OMM nor DHM are available, formulas for preterm infants must be used.

1.2.2. Advantages of the human milk

Proteins in raw HM are an important source of amino acids for rapidly growing breastfed infants. Many HM proteins also play a role in facilitating the digestion and uptake of other macronutrients in breast milk. Bile salt–stimulated lipase and amylase, casein, lactoferrin, and haptocorrin, assist in the absorption of calcium, iron, and vitamin B-12, respectively. Human milk proteins have also numerous physiologic activities, including the enhancement of immune function, defense against pathogenic bacteria, viruses and yeasts, anti-inflammatory properties, development and maturation of the gut and its functions.(52)

In premature infants, intake of more than 50% of mother's milk was shown to be protective against late-onset sepsis and have an 83% reduction in necrotizing enterocolitis (NEC).(53)

In the medium and long-term outcome, an HM diet in the NICU has benefits reflected in fewer hospital readmissions for illness, improved growth and body composition, improved long-term sensory-neural development, and lower risk of metabolic syndrome.(54) The mechanisms for these long-term beneficial effects remain the subject of speculation and, more likely, are a consequence of the multiplicity of components in the milk acting together.(31, 54-61)

Nonetheless, OMM or DHM alone, are insufficient for the optimal nutrition of very preterm infants, unless a macronutrient supplement, or fortifier, is added to the HM.(48, 62)

1.2.3. Human milk composition and its assessment

Human milk composition

Human milk has macronutrients, micronutrients, functional components, human cells and bacteria. Its composition is dependent on the method of sampling, stage of lactation, GA, maternal diet, presence of maternal infection and parity. There is also a significant diurnal and inter-feed variation; thus, study designs that incorporate milk expressions collected over a 24-h period are preferred to ensure that analysis is being undertaken on a representative milk sample.(63, 64)

Human milk contains:

- Macronutrients:
    - Nitrogen compounds, most of them nutritional protein and enzymes - beta-casein, alpha-lactalbumin, lactoferrin, immunoglobulin IgA, lysozyme, bile salt-stimulated lipase, and serum albumin; 25% of total nitrogen of human milk represents non-protein compounds, including urea, uric acid, creatine, creatinine, and many amino acids. Of the latter, glutamic acid and taurine are prominent.
    - Carbohydrates (lactose, oligosaccharides).
    - Fat (palmitic, oleic acids, phospholipids).

- Mineral constituents, including sodium, potassium, calcium, magnesium, phosphorus, chloride, iron, copper, and zinc.
- All vitamins, except vitamin K, are found in human milk in nutritionally significant concentrations.
- Immune active molecules (epidermal growth factor, hepatocyte growth factor, transforming growth factors 1, 2 and 3) and cytokines (IL2, IL4, IL5, IL10, IFN, IL12, IL13) are also found.(65)
- Maternal cells: leukocytes, epithelial cells, stem cells, progenitor cells, lactocytes, and myoepithelial cells.(66)
- Bacteria: colostrum and milk from healthy women contain staphylococci, streptococci, corynebacteria, lactic acid bacteria, propionibacteria, and bifidobacteria.(67) Their impact on neonatal gut microbiota establishment, remains largely unknown.
- Human milk has better antioxidant protection than formulas, possibly due to the higher iron content and the presence of vitamin C.(68)

Human milk macronutrient composition assessment

To accurately measure the macronutrient composition of individual breast milk, 24-hour period feed samples are more representative for analysis, due to the diurnal and inter-feed variation in the composition of HM. The analysis process is difficult, costly and time consuming.(63, 64)

Colorimetric assay techniques are the reference laboratory techniques, but very expensive and time consuming.(69, 70)

Mid-Infrared spectroscopy analysis of HM has been developed and validated, and has been shown to be cheaper, easier, faster and more reliable, if properly used according to the manufacturer's instructions.(70, 71) Three-ml of frozen HM samples, after defrosting and heating to 40ºC, are homogenized by ultrasound before mid-infrared analysis. The results are displayed within 2 minutes, as g/dL of crude, true protein, carbohydrates, fat, kcal/dL of energy and g/dL of ashes. Batches of 10 analyses can be

performed in sequence, with cleaning and calibration (with solutions provided by the manufacturer) before a new batch sequence. Data is stored in the hardware of the analyzer and can be exported by an USB port.(72-75)

1.2.4. Human milk fortification

The American Academy of Pediatrics(76) and the European Society for Paediatric Gastroenterology, Hepatology, and Nutrition(38) recommend HM as the first choice for feeding preterm infants, provided that it is added with macronutrients necessary to meet requirements.(38) In this population, consensual strategies to prevent severe in-hospital macronutrient deficits encompass the multicomponent fortification of HM.(34) The widely used standard fortification, in which an empirical and fixed dose of macronutrients is added to HM, rarely meets the recommended intake of protein(38), with inherent risks of growth faltering and neurocognitive impairment.(34, 62, 77) As an alternative, two individualized methods were proposed, the targeted and adjustable fortifications.(78, 79) The targeted fortification is tailored to the individual infant's needs, based on previous analyses of HM macronutrient; however, this is time consuming, laborious, and analyzers are commonly unavailable.(62) In the adjustable fortification, fortifier and an extra amount of modular protein are added to HM, guided by changes in serial blood urea nitrogen measurements, assuming this is a surrogate of adequate protein nutrition(62); using this method, the adjustment of energy intake is not taken into account. In order to guarantee sufficient protein intake in very preterm infants, other strategies included the adjustable protein fortification by adding higher amounts of modular protein supplement to standard fortification(80, 81), an HM fortifier with higher protein content(82), and using concentrations of HM fortifier above that indicated by the manufacturer.(83)

1.3. Methods for assessment of nutritional status

The nutritional status of very preterm infants can be assessed by several methods, including laboratory assessment(84), anthropometry(85), body composition assessment(86), and indirect calorimetry.(87)

Laboratory assessment encompass indicators for protein nutrition(88), bone nutrition(89), and hematological markers.(90, 91)

Indirect calorimetry is not commonly available, is time consuming, requires training and is a method with several limitations in preterm infants.(92)

Anthropometry and body composition assessment are specially addressed, since these methods were used in this project.

1.3.1. Anthropometry

In newborn infants, and especially in preterm infants, anthropometry has the advantage of being easy to perform and convenient for bedside measurements. Unfortunately, in this age group, it is unreliable in untrained hands because of its inter-observer variation, and the fact that most of the measurements are regarded too inaccurate to be recommended in routine practice.(93)

Beyond the assessment of nutritional status, neonatal anthropometry may be a useful tool for the diagnosis of fetal malnutrition and prediction of long-term metabolic risk, dysmorphological characterization, and estimate of body surface.(85)

Direct measurements, such as body weight, length, and body circumferences are the most commonly used measurements for nutritional assessment in clinical practice.(85) Some indices and equations derived from direct measurements have been proposed and used in newborn infants for improving the accuracy of the anthropometry.(85, 93)

Accurate sequential measurements usually provide more information than single measurements.(85)

1.3.1.1. Reference and standard values

To determine whether an infant's measurements fall within normal ranges, an appropriate standard should be used. When compared with population-based standards, the population should have similar backgrounds and potential, including sex, race, gestational age, genetic background, altitude and type of feeding.(85)

To assess intrauterine growth - weight, length, and head circumference (HC) - the Fenton 2013 charts are adequate.(94) These are based on a meta-analysis on birth size of six large population-based surveys including almost 4 million neonates, of which 35000 were preterm infants <30 weeks.(94) In Fenton 2013 charts, smooth growth chart curves were developed, ensuring close agreement with the data between 24 and 36 weeks and at 50 weeks.(94)

Recently, the INTERGROWTH-21st charts were published, providing for the first-time standards for postnatal growth - weight, length, and HC - in preterm infants.(95) Unfortunately, very extremely premature infants were not included, and the charts cannot be accurately used in this population.

1.3.1.2. Body weight

Since the measurement of body weight is simple and reliable, it has been the most used isolated parameter for monitoring growth and nutritional status in neonates in clinical practice.(96)

Despite body weight being an independent predictor of body composition in preterm and term infants, it gives very limited information on body compartments and quality of growth.(97)

The weight gain velocity is considered a better weight-based measurement to accurately monitor in-hospital growth.(94, 98)

1.3.1.3. Body length

Length is considered a rough indicator of lean mass, reflecting the skeletal growth.(96, 97) Since linear growth continues after birth despite the acute loss of body weight, linear measurements may better reflect actual growth than body weight.(85, 99)

Some factors limit the accuracy and reliability of crown-heel length measurement in neonates.(100, 101) This is of utmost importance when length is included squared (body mass index - BMI) or cubed (ponderal index) in indices, since a small error in measurement causes an exponential distortion in the final results of the equations.(85)

The length velocity is considered an accurate measurement with which to monitor in-hospital growth.(102)

1.3.1.4. Head circumference

The increase in the occipito-frontal circumference reflects brain growth and is associated with both nutrition(103) and neurocognitive outcome in preterm infants.(104)

When interpreting HC measurements in preterm infants, some non-nutritional factors may confound, such as the presence of head molding or scalp edema in the first postnatal days and hydrocephaly secondary to severe intra-periventricular hemorrhage later on.(85)

1.3.1.5. Body mass index

The advantage of calculated anthropometric measurements, based on direct measurements, assumes that the association of various direct measurements may predict body composition better than each value on its own.(93) BMI is an example of a measurement that has been used to assess nutritional status in preterm infants.(105) Recently, validated reference sex-specific curves were published to track changes in BMI for prematurely born infants.(106)

However, in newborn infants, BMI was found to be poor a predictor of adiposity, compared with air displacement plethysmography (ADP) body fat measurements as a reference.(107, 108)

1.3.1.6. Other measurements

Other direct and calculated anthropometric measurements have limited accuracy in preterm infants or have not been validated yet.

Most of the body circumferences are difficult to interpret, since they include skin, subcutaneous tissue, muscle, and bone.(85) The mid-upper arm circumference reflects the combined arm muscle and fat and is a convenient bedside measurement to evaluate nutritional status in sick preterm infants. Compared with ADP measurements, the mid-

upper arm circumference was found to account for 60.4% of the variability of percent body fat in preterm infants.(109)

Sex-specific reference values of skinfolds for preterm infants have been published.(110) However, body water dilution and magnetic resonance imaging used as reference methods indicate that skinfolds produce inaccurate and biased estimates of total body fat.(111)

Upper arm cross-sectional areas, derived from mid-upper arm circumference and triceps skinfold thickness, have been used for the assessment of body composition and nutritional status(112), assuming that they represent a better indicator of the relative contribution of fat and muscle to the total arm area than the direct measurements.(85) Studies validating upper arm cross-sectional areas in term(113) and preterm(114) infants using imaging methods questioned their accuracy in predicting arm fat and muscle.

1.3.1.7. Growth pattern of preterm infants

Preterm infants, particularly VLBW, defined as birth weight less than 1500 g, are at considerable risk of extrauterine growth restriction during their hospitalization.(115, 116)

Extrauterine growth restriction is most frequently defined as a weight less than the tenth percentile for CA at the time of hospital discharge and is inversely related to GA.(115)

The reported incidence of extrauterine growth restriction is as high as 60% at 25 weeks GA, to 30% at 30 weeks GA, affecting weight gain velocity, length, and HC and commonly persisting during childhood.(116, 117) It is also associated with higher risk of neurodevelopmental impairment at 18 months CA and later on.(18, 118)

The mechanisms for extrauterine growth restriction are not completely explained, but there are probably periods of inadequate nutrition, feeding intolerance or critical illness, especially in the first postnatal weeks, responsible for this phenomenon.(18, 119)

Present efforts to decrease the incidence of extrauterine growth restriction and to mitigate length and HC growth faltering at discharge are focused on the accomplishment of early nutritional recommendations (especially protein and energy).(38, 48)

The gold standard for preterm growth velocity is in-utero growth velocity, estimated to be 15 to 20 g/kg/day, decreasing as GA increases.(94)

Even in the more premature or sickest newborns, these recommendations should be followed to achieve a growth matching intrauterine weight gain at discharge.(18, 120)

Postnatal growth curves were developed to serve as references to very preterm infants' growth.(94, 95) Recently, the first standard curves for preterm infants, from the consortium Interggrowth-21, were published (see section 1.3.1.1).(94, 95)

1.3.2. Body composition

1.3.2.1. Body composition levels and methods of assessment

The human body can be quantified at several levels, depending on clinical concerns. Body composition can be assessed at atomic, molecular, cellular, and tissue levels(121) including in neonates and infants.(86) These levels can be assessed by direct, criterion and indirect methods.

Analysis from the atomic through cellular levels is performed with direct body composition methods such as neutron activation, isotope dilution, and total body counting.(121)

Criterion methods measure a property of the body, such as its density, or describe amounts and the distributions of skeletal, muscle, and adipose tissues, for instance by magnetic imaging techniques. Criterion methods include densitometry, computed X-ray tomography, magnetic resonance imaging, and dual-energy x-ray absorptiometry (DEXA).(121)

Indirect methods, including anthropometry and bioelectrical impedance analysis, provide estimates or indices of body composition based on results from direct or criterion methods.(121) As a result, indirect methods tend to have larger predictive

errors than direct methods and are affected by sample specificity and disease conditions.(121)

The basic 2-compartment model, which assumes that body mass is composed of adipose and non-adipose tissue, that is, fat mass (FM) and fat-free mass (FFM) or lean body mass, is the most widely used. The three-compartment model adds a value for skeletal or bone mass, whereas in the multi-component model, body composition is obtained by integrating data from various techniques, such as whole-body density, total body water (TBW), bone mineral content, and anthropometry.(122)

1.3.2.2. Assumptions and limitations in estimating body composition

In neonates and small infants, the FM, FFM, and TBW are generally estimated by indirect methods, validated by the analysis of cadavers and living human adults.(123)

The relative reduction of total body water that occurs from birth influences the application of most methods related to hydration(123):

- While the FM density is constant (0.900 g/cm^3), the FFM density varies with age and sex (about 1.1 g/cm^3).(124)
- Estimates of the FFM calculated from measurements of TBW, assume that the lean mass contains a constant ratio of water: 732 g H_2O/kg lean mass.(125) However, a ratio similar to the adults is only achieved at three years of age, which may limit the estimation of lean tissue and fat by measuring the body water before this age.(124)
- In the neonate, the TBW varies with the gestational age and changes rapidly in the first weeks of life, and may lead to underestimation of FM.(122) To minimize this, Fomon et al.(123) and Butte et al.(126) proposed hydration coefficients to convert TBW into FFM in infants with rapid chemical maturation.
- It is assumed that bone represents a constant proportion of FFM.(124)

The fat content of adipose tissue is 60% in the newborn and gradually increases to reach 80% at 13 years, a level that is found in adults.(127)

The aforementioned and other constants extrapolated from adults such as potassium (68.1 mmol/kg) and nitrogen (33 g/kg) are often used for the calibration of indirect methods for assessing body fat and FFM. However, in neonates and small infants, the potassium content per unit of body weight is lower than that of adults, about 49 mmol/kg.(128)

Another limitation of most methods is measuring the FM without distinguishing different types of fat. Using magnetic resonance, it was found that "healthy" preterm infants have an acceleration of growth associated with increased total and subcutaneous fat, while those who suffered from severe disease had a significant increase in deep intra-abdominal fat, which is associated to insulin resistance and predisposition to the future metabolic syndrome.(129)

1.3.2.3. Air displacement plethysmography method

The ADP is a two-compartment model that measures body mass, FM, and FFM, assuming the density of fat to be 0.9007, and age- and sex specific densities of FFM are based on data of Fomon et al. (Fomon 2002). This method was validated in healthy infants, using the deuterium dilution method for body water and a four-compartment model as the references.(86, 130)

The ADP measurement is easily performed, and the infant is not restrained during the rapid procedure.(131) The measurement precision (<0.5%) for FM is excellent.(86, 130)

A systematic review and meta-analysis(132) assessing the relative accuracy and validity of 3 methods - ADP, DEXA, and magnetic resonance imaging (133) - found that they had similar precision (coefficients of variation ranging from 3% to 8%).

1.3.2.4. Body composition of growing preterm infants

The aforementioned systematic review and meta-analysis including eight studies using ADP, DEXA and MRI, found that preterm infants at term equivalent age have lower fat-free mass (FFM), greater body fat mass percentage (FM%), and are shorter compared with term infants.(132)

1.3.2.5. Indicators of adiposity and lean mass

The body composition assessment provides the quality of growth.(85) In a bi-compartmental model, appropriate indicators should be used to assess adiposity and leanness.

Adiposity assessment

Based on anthropometry, the BMI is commonly used to assess fatness in infants.(85) However, the BMI was found to be a poor predictor of adiposity compared with the FM% measured by ADP as reference method.(107)

Based on body composition measurements, the fat mass percentage (FM%) has been the more common indicator of adiposity used in neonates.(134)

The use of body weight for normalization of FM% (FM divided by body mass) fails to account for independent tissue accretion rates because FM% depends on the amount of FFM and vice versa.(135)

As the FM index (FMI) - based on the FM and length (kg/m^2) - in which the FM is adjusted to body length (reasonable surrogate of lean mass), seems to discriminate adiposity better than FM%.(135, 136)

Leanness assessment

Using a bicompartmental model, adiposity is more accurately estimated than leanness, because FM has a more constant density than FFM, which depends on its different water content.(136, 137) Moreover, FM is more homogenous, comprised predominantly of adipose tissue, while FFM is a complex compartment containing not only skeletal muscle, but also bone, organs, and blood.(136)

1.4. Methods of neurodevelopmental assessment

Four principal areas constitute the psychomotor development of a child: motor skills, cognition, language and social relationships.

As referred in Section 1.1.2, the human brain development starts in the first weeks of gestation and its' maturation has several determinants, such as genetic, perinatal, and environmental factors, which may affect mental and psychomotor development.(20)

Early evaluation and identification of children at higher risk of disability is important, because early intervention gives a chance of minimizing the severity of the neurological sequelae due to the plasticity of the immature brain.(138)

Several countries have screening and formal testing programs with this purpose.(139-141)

Screening tests may be applied by parents (like the Parents' Evaluation of Developmental Status(142, 143), Ages and Stages Questionnaires(144, 145), and Child Development Inventories(146), or by professionals (like the Bayley Infant Neurodevelopmental Screener or the Denver II test). Examples of **more detailed, professional, evaluation tests,** are the Peabody Developmental Motor Scale II, the Griffiths-II or BSID-II (developed in 1993) or BSID-III (developed in 2006).(147, 148) In the pre-school and school-ages other tests are used, for example, the Wechsler Intelligence Scale for Children, versions II and III.(149)

Nowadays, BSID-II is the preferred development screening test used from 1 to 42 months CA, validated for the North-American population, but not for the Portuguese population.

The Bayley Scale of Infant Developmental, version II, is still widely used by many developmental pediatricians and clinical psychologists to evaluate, from 1 to 42 months of CA, infants born preterm or term infants with perinatal events considered at high risk of neurodevelopmental delay.(150, 151) It takes 25 to 35 minutes to be applied in children under 15 months and up to 60 minutes for children over 15 months(152, 153), and consists of 2 quantitative scales, the Mental Developmental index (MDI) and the Psychomotor Developmental index (PDI), as well as a qualitative Behavior Rating Scale. The Mental Scale evaluates sensory, perceptual acuities, discriminations, acquisition of object constancy, memory, learning and problem-solving, vocalization, early verbal communication, abstract thinking, habituation, mental mapping, complex language and mathematical concept formation. The Motor Scale evaluates the degree of body control,

coordination of large muscles, fine manipulation skills, dynamic movement, postural imitation and stereo gnosis.(152-154)

Mental and Motor scales performance are classified as follows: ≤69 - significantly delayed, 70 to 84 - mildly delayed, 85 to 114 - within normal limits, ≥115 - accelerated.

The Behavior Rating Scale measures attention, arousal, orientation, engagement, emotional regulation and motor quality. It is scored in 3 levels: Not optimal (Perc. 1-10), Questionable (Perc. 11 - 25), Normal limits (Perc. 26 - 99).(155, 156)

The BSID-II scores are used to describe the current developmental functioning of infants and to assist in diagnosis and treatment planning for infants with developmental delays or disabilities.(154) The BSID-II has poor predictive value for cognition at school age, except in the very low scores.(157) It is considered a good screening device for identifying children in need of early intervention.(154) Although developed and validated to a healthy term population, they are the gold standard to normal neurodevelopment, frequently used for preterm and term infants with some risk of neurodevelopment delay.(158)

1.5. Effect of nutrition on growth and body composition

In very preterm infants, non-consensual results have been reported in studies that evaluated the effect of different in-hospital nutritional strategies on the body composition of preterm infants assessed by accurate methods, such as the dual energy X-ray absorptiometry and the ADP.

Some authors(159-161) found that, in preterm infants, higher estimated protein and energy intake were associated with better weight gain, but without significant differences in body composition. In contrast, other studies based on estimated or measured macronutrient intake found that, in preterm infants at term CA, specific body compositions were associated with different nutritional strategies. In these studies, higher protein(162-164) and PER(165) intake were associated with an increase in lean mass(164, 165) and a decrease in adiposity(162), while higher fat and energy intake were associated with increased fatness.(163)

1.6. Effect of nutrition on neurodevelopment

Neurodevelopment, as previously reported (Section 1.1.2) is a consequence of brain development, initially predominantly under genetic control, and subsequently in post-natal life, determined by gene-environment and experience-dependent interactions. It can be affected by factors such as disease, toxins, environmental stimuli, quantity and quality of nutrition provided during brain development.

Beneficial long-term effects of nutrition on neurodevelopment have been difficult to demonstrate, since nutrition is one of several factors influencing neurodevelopment.(3)

In very preterm infants, studies that evaluated the effect of different in-hospital nutritional strategies on brain growth and neurodevelopmental outcome, are scarce and with non-consensual results.

Extrauterine growth restriction has been used as a surrogate of malnutrition, and measures to mitigate it, as early adequate protein intake in preterm infants, were associated with better neurodevelopmental outcome.(14)

In preterm infants, a high macronutrient diet in the neonatal period, resulted in larger caudate volumes and higher verbal neurodevelopment in adolescence, compared with a low macronutrient diet group. This effect was more evident in males.(166)

Two other studies reported improved neurodevelopment at 12 and 18 months CA, when infants were provided with early recommended protein and energy intake.(167, 168)

The effect of high protein intake in the neonatal period resulted in contradictory neurodevelopmental outcome (BSID-III) at 18 and 24 months CA in two studies. One study did not find differences(169), while the other reported a positive association between enteral protein intake with neurodevelopmental outcome.(170)

A Swedish randomized controlled trial, compared estimated different macronutrient intake and found a significantly positive effect on head growth velocity and cerebral maturation, evaluated by imaging - magnetic resonance diffusion tensor imaging, near term age.(171)

To the best of our knowledge, no other studies have been published on the impact of nutrition during the neonatal period on the brain volume. Most studies have focused on

the relationship between postnatal nutrition and head growth as a surrogate measure of brain growth. When assessing the association between nutritional support and neurodevelopment, no study has measured the protein and energy intake administered. The existing studies correlating nutrition and neurodevelopmental outcome relied their calculations on reported estimates.(30)

2. OBJECTIVES

2.1. Nutritional support and growth and body composition

One of the primary objectives was to determine, in HM-fed very preterm infants, the associations of different in-hospital cumulative measured protein, energy, and protein-to-energy (PER) intake with in-hospital weight gain velocity and with body composition at term (40 weeks) corrected age (CA).

The secondary objective was to determine which protein, energy, and PER intake associate with lower and higher adiposity at term CA.

2.2. Nutritional support and neurodevelopmental outcome

Another primary objective was to determine in these infants, the associations of different in-hospital cumulative measured protein, energy, and protein-to-energy (PER) intake with neurodevelopment at 18 months CA.

The secondary objective was to determine which protein, energy, and PER intake associate with lower and higher Mental Developmental (MDI) and Psychomotor Developmental index (PDI) scores at 18 months CA.

3. HYPOTHESES

3.1. Nutritional support and body composition outcome

The primary hypothesis is that higher in-hospital cumulative protein, energy, and PER intake are associated with higher in-hospital weight gain velocity and greater FFM as a surrogate of lean body mass, at term CA.

The secondary hypothesis is that appropriate in-hospital cumulative energy intake, in presence of adequate protein and PER intake, are associated with an adiposity (indicated by FM% and FMI) at term CA, similar to that is reported in term infants of equivalent GA.

3.2. Nutritional support and neurodevelopmental outcome

The primary hypothesis is that is that higher in-hospital cumulative protein, energy, and PER intake are associated with higher MDI and PDI scores at 18 months CA.

The secondary hypothesis is that in infants with better neurodevelopmental, higher in-hospital cumulative protein, energy, and PER intake are associated with higher MDI and PDI scores at 18 months CA.

4. METHODS

4.1. Setting

This study was performed in the neonatal intensive care unit (NICU) of Maternidade Dr. Alfredo da Costa, Centro Hospitalar de Lisboa Central, Lisbon, Portugal.

The study period was from birth to 40 weeks CA. At 40 weeks CA, after discharge, body composition was measured at the Nutrition Lab of Hospital Dona Estefânia, Centro Hospitalar de Lisboa Central, Lisbon, Portugal.

4.2. Ethical and legal issues

The study was approved by:

- The National Data Protection Commission (9767/2012)
- The Hospital Ethics Committee (116/2012, 03/06/2013)
- The NOVA Medical School Ethics Committee (nº 75/2014/CEFCM, 01-10-2015)
- The study is registered at the International Standard Randomized Controlled Trials Number - ISRCTN (ID: 27916681).
- Informed written consent from parents or legal representative was required.

4.3. Study design and participants

Initially, this study was intended to be a randomized controlled trial to assess effects of high and low recommended protein intake associated with similar energy intake, on weight gain, body composition and neurodevelopmental in very preterm infants. Logistical constraints due to a sudden reduction of hospital technical personnel that was initially committed to the trial, precluded it and the nature of the study was re-designed to be a cohort study. This constraint forced a delay of 13 months in starting the study with consequent shortening of the enrolment period, considering the fixed age for neurodevelopmental assessment at 18 months CA to comply with the study protocol. This cohort study was conceived to assess the associations of different in-hospital cumulative protein, energy, and PER intake with in-hospital weight gain velocity, body composition at term CA and neurodevelopmental at mentioned 18 months CA.

Consecutive inborn neonates with <33 weeks of gestation exclusively HM-fed at least 80 ml/kg/day, were eligible. This minimum enteral intake was used as a convenience criterion for tolerance to enteral feeding.

Non-Inclusion criteria: Infants with major congenital malformations and triplets or more were not included.

Exclusion criteria: infants with diagnosed inborn errors of metabolism, and those who were subsequently formula-fed >12.5% of the enteral volume intake, transferred, deceased, or unavailable for body composition assessment. In our unit, enteral feedings are given every 3 hours (8 times per day); as a convenience criterium, the infants were considered predominantly HM-fed if no more one out of eight meals (12,5%) was replaced by formula.

4.4. Demographic and clinical variables

The demographic and clinical variables recorded were single or twin pregnancy, sex, gestational age(172), birth weight, small-for-gestational age (birthweight <10th percentile)(123), Neonatal Acute Physiology with Perinatal Extension-II (SNAPPE II) score(173), use of prenatal and postnatal corticosteroids, diagnosis of late-onset sepsis(174), worst grades of necrotizing enterocolitis(175), worst grades of intraventricular hemorrhage(176), multicystic periventricular leukomalacia(177), and chronic lung disease.(178, 179)

4.5. Nutrition protocol

Infants were managed according to our NICU nutrition protocol, based on international recommendations for neonatal parenteral nutrition (PN)(43) and enteral nutrition(34, 38), and the national consensus for neonatal parenteral and enteral nutrition.(45, 46) Briefly, PN was initiated within the first 2 postnatal hours with 2.5 g/kg/day of amino acids and was increased up to 3.8-4.0 g/kg/day; lipids were initiated within the first 24 postnatal hours with 1 g/kg/day and increased up to 3 g/kg/day. Early enteral trophic feeding (10-20 ml/kg/day) was initiated within the first 2-4 postnatal days using HM; subsequently, enteral nutrition was increased as the PN was proportionally reduced.

Until 35 weeks CA, exclusive HM (OMM or DHM) was used. If the OMM was not sufficient after 35 weeks CA, formula was used for preterm infants, owing to limited DHM stock. Nutrition was prescribed by physicians in collaboration with a nutritionist.

The minimum daily enteral intake according to body weight were classified as follows: energy 110 kcal/kg; protein (g/kg) 4.0 if <1000 g, 3.7 if <1200 g, 3.6 if <1800 g, and 3.4 if >1800 g; PER 3.6 if <1000 g, 3.2 if <1800 g, and 2.6 if >1800 g.(34, 38) Based on clinical evaluation, the prescriptions were adjusted to achieve these targets. Blood urea nitrogen was not routinely measured.

4.6. Measurement of human milk composition

Donor Human Milk and OMM were stored frozen in the maternity milk bank. Mothers were advised to collect milk every 3 hours, either in the hospital or at home, and identify the samples by date and hour of collection. For each infant, a daily pool of prescribed OMM was obtained from the sequentially collected samples. For the present study, a 3-ml sample was collected and homogenized for composition analysis using a mid-infrared HM analyzer (Miris AB, Uppsala, Sweden); the DHM composition was always measured and that of the pooled OMM was measured whenever available. The physicians and nutritionist were blinded to the HM composition during the entire study period. When breastfeeding predominated (unknown volume intake and composition), the OMM composition analysis was suspended.

4.7. Human milk fortification and modular supplementation

An HM fortifier (Aptamil FMS®; Milupa/Danone GmbH, Friedrichsdorf, Germany) was used when the HM intake was at least 100 ml/kg/day. The standard fortification method with modular protein(80) and fat supplements was used, considering an average low reported HM protein content; that is, 1.1 g/dL in preterm OMM during the first 1-3 postnatal weeks and 0.8 g/dL thereafter, and always 0.8 g/dL in DHM.(64)

When fortified HM was estimated insufficient to cover the estimated protein and energy requirements for CA, modular protein (Aptamil Protein Supplement®; Milupa/Danone GmbH, Friedrichsdorf, Germany) and modular medium-chain triglycerides (MCT oil; SHS

Nutricia/Danone Medical Nutrition®, GmbH, Friedrichsdorf, Germany) were added, respectively (Table 1).

Table 1. Energy and nutrient contents of the human milk fortifier (Aptamil FMS®), modular protein hydrolysate (Aptamil Protein Supplement®) and modular medium-chain triglycerides (MCT OIL SHS®) used.

Product	Energy (kcal)	Protein (g)	Lipids (g)
Aptamil FMS® (*per* 100 g)	347	25.2	0
Aptamil Protein Supplement ® (*per* 100 g)	328.4	82.1	0
MCT OIL SHS® (*per* 100 mL)	855	0	95

The administered volumes of OMM and DHM were used to estimate the energy, protein, and PER intake, according to the reported macronutrient content of preterm infants' OMM.(64, 180) The volumes and powder weights of PN solutions and commercial products were also accounted for in these estimates.

4.8. Anthropometry

Anthropometry was performed using the recommended techniques.(85) Body weight was measured daily using electronic scales from birth to discharge; the weight gain was expressed as the weight gain velocity (g/kg/day), calculated by an exponential model.(98)

4.9. Body composition assessment

Body composition assessment was scheduled after discharge at 40 weeks CA using air displacement plethysmography (Pea Pod; Cosmed, Ltd., Concord, CA, USA). This validated method for preterm infants(131) measures body mass (kg), FM, and FFM with a precision of 0.1 g. A constant fat mass density value of 0.9007 g/ml and age- and sex-specific FFM densities(123, 131) were used.

Concomitantly, the crown-heel length and HC were measured by the same trained observer. The FM%, based on the FM and body mass, and the FMI, based on the FM and

length (kg/m^2) were calculated.(135) Low and high adiposities(181), indicated by the FM% and FMI, were defined in this sample using the convenience thresholds of –1 and +1 z-scores, respectively.

4.10. Neurodevelopmental assessment

The BSID-II(147, 148) was used to assess neurodevelopment, scheduled to be applied at 18 months CA by the same trained clinical psychologist, that was not aware of the in-hospital nutrition support received by the infants. To assess neurodevelopment with infants in optimal conditions, dates different from the visits for other assessments were scheduled. In these cases, visits after 18 months CA had to be adapted to the parents' availability.

The BSID-II consists of 2 quantitative scales, the MDI and the PDI as well as a qualitative Behavior Rating Scale. Each BSID-II test takes about 1 hour to be applied to each child with more than 15 months of age.(147, 148)

As explained in section 1.4.:

The MDI evaluates sensory/perceptual acuities, discriminations, acquisition of object constancy, memory, learning and problem-solving, vocalization, early verbal communication, abstract thinking, habituation, mental mapping, complex language, and mathematical concept formation.(147, 148)

The PDI evaluates the degree of body control, coordination of large muscles, fine manipulation skills, dynamic movement, postural imitation, and stereo gnosis.(147, 148),

MDI and PDI performances are classified as follows: ≤69 - significantly delayed, 70 to 84 - mildly delayed, 85 to 114 - within normal limits, ≥115 - accelerated.(147, 148),

The Behavior Rating Scale measures attention, arousal, orientation, engagement, emotional regulation, and motor quality. It is scored in 3 levels: Not optimal (percentile 1-10), Questionable (percentile 11-25), and Normal limits (percentile 26-99).(155, 156)

The BSID-II was applied according to the CA of infants. BSID-II is reported as having a higher sensibility for handicapped children/low MDI/PDI scores then BSID III.

4.11. Statistical analysis

4.11.1. Sample size calculation

- For body composition assessment

The sample size was calculated to detect a difference of 3.0% in FM% with a standard deviation 4.4(182) for normally distributed variables, a significance level of 0.05, and an 80% power; thus, a required sample of **70 infants** was estimated.

- For neurodevelopmental assessment

The sample size was calculated to detect a difference between Mental Developmental Index or Psychomotor Developmental Index with a difference (δ) $\geq$ 11 and a standard deviation (σ) $\geq$ 8 points(183) in a normally distributed variable, a significance level of 0.05 and an 80% power; thus, a required sample of **75 infants** was estimated.

4.11.2. Univariate analysis

- Correlations of macronutrient intake with growth and body composition

To measure associations of daily cumulative protein, energy, and PER intake from birth to 35 weeks CA with in-hospital weight gain velocity and with FM, FFM, FM%, and FMI at 40 weeks CA, Pearson or Kendall-tau correlation coefficients were used.

The strengths of associations (positive or negative) were classified as follows(184):

- Weak, 0.10 to 0.29
- Moderate, 0.30 to 0.49
- Strong, 0.50 and above.

The coefficients of determination (r^2) were used to explain the percentage of the variation in the dependent variable, which is "explained by" the variation in the independent or predictor variable (not implying causality). The interpretation of the magnitude of r^2 depends on the research area. The adjusted β explains the variation in

the dependent variable by 1 unit of the independent variable, adjusted for covariates.(185, 186)

- Nested case-control analysis: association of macronutrient intake and extremes of adiposity

To determine the associations between lower and higher adiposity (normal distribution) and protein, energy, and PER intake, a nested case-control analysis was performed. Infants with measurements classified as lower and higher adiposity (as dummy variables) were compared with the remaining infants, using the Mann-Whitney U test.

- Correlations of macronutrient intake with neurodevelopmental outcome

Pearson or Kendall-tau correlation coefficients were used to measure the associations of daily protein, energy, and PER intake with BSID-II MDI and PDI scores, scheduled to be assessed at 18 months CA. The aforementioned classification of strengths of associations was used.(184)

- Associations of potential confounders with growth, body composition and neurodevelopment

Univariate analysis was performed to test associations of potential confounders with dependent variables (weight gain velocity, body composition and neurodevelopment). As the dependent variables were continuous, their normal or non-normal distribution was assessed by the Shapiro-Wilk test, and the Student T test or Mann-Whitney U test were used as appropriate.

No categorical variables were identified and therefore neither the Chi-square nor the Fisher tests were used. To consider independent variables as potential confounders, $p < 0.10$ was used. Some covariates, that is, clinically relevant variables, also entered in multivariate models, despite associations not being significant.

4.11.3. Mixed model and multivariate analysis

Linear mixed models and linear multiple regression analyses were used in multivariate analysis.

Linear mixed models were used to analyze the relation between independent and dependent variables, whenever random-effect factors had to be incorporated in the model, for example, for imputation of missing values in the independent variables.

Linear multiple regression analyses with the backward method, was used to identify interactions between independent variables, potential confounders and the outcome variables when no random-effect factors were present.

Human milk composition: imputation of missing measured values

As OMM composition measurements were not always possible, a *post-hoc* analysis for imputation of missing values was performed by referencing a meta-analysis of composition of preterm infants' OMM,(64) in which the true protein and energy changes were non-linear. Thus, logarithmic transformations of true protein or energy concentrations as dependent variables, using the postnatal days as the fixed effect and each case as a random effect, were used in the two mixed models to predict the missing measurements of OMM true protein and energy between birth and 35 weeks CA. Good agreements were found between the curves of reference data (meta-analysis), mixed model-predicted data, and measured *plus* estimated data for true protein (Figure 1) and energy (Figure 2).

5. RESULTS

The enrollment period was shortened owing to unexpected constraints that forced a change in the study design and the fixed age for assessment of neurodevelopment scheduled at 18 months CA (section 4.3). Therefore, the sample dimension was smaller than the estimated.

5.1. Characteristics of participants

During the enrolment period, 156 eligible infants were identified (Figure 1). Of these, 67 infants were not enrolled because the parents refused to participate in 38 cases, the parents were unable to give consent in 4 cases, and 25 cases were unavailable to follow-up. From the 89 enrolled infants, 56 were subsequently excluded because they became formula-fed >12.5% of total volume intake. Thus, only 33 infants completed the study and were analyzed, whose characteristics and clinical outcomes are summarized in Table 2.

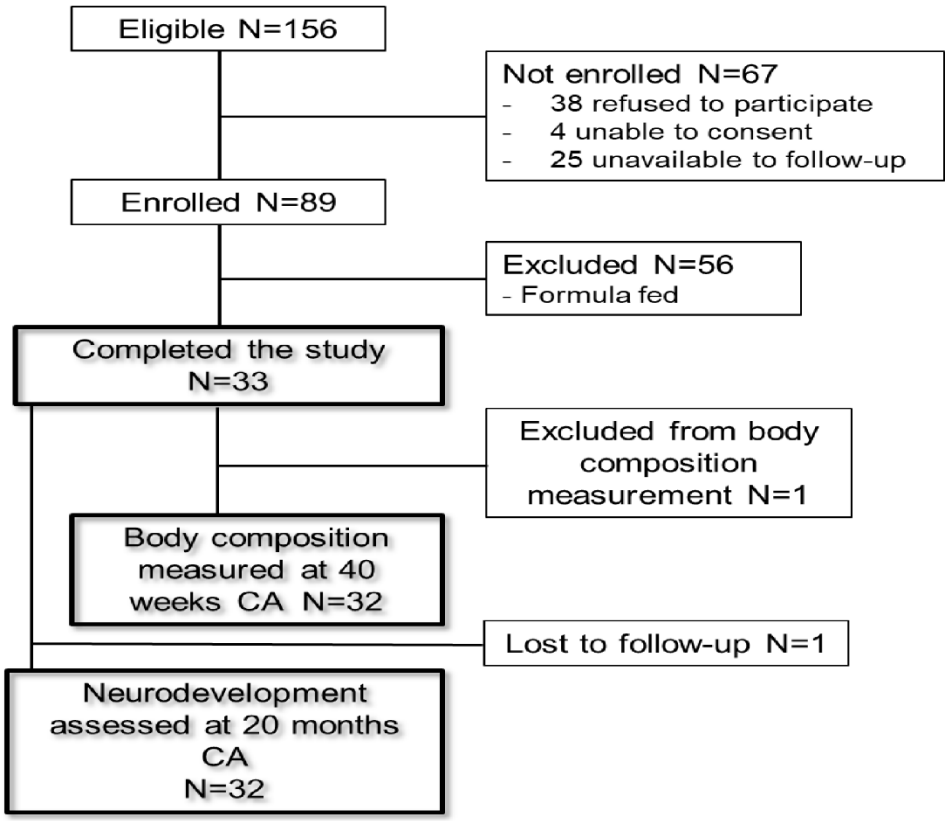

Figure 1. CONSORT flow chart for infants with measured body composition at 40 weeks corrected age and neurodevelopmental assessment at 20 months corrected age.

No cases of small-for-gestational age, severe necrotizing enterocolitis, multicystic periventricular leukomalacia, and transferred or deceased infants were recorded.

Table 2. Characteristics of participants (N=33).

Gestational age (weeks), mean (SD)	30 (1.8)
Birth weight (g), median (IQR)	1175 (1010-1408)
Twins, n (%)	4 (12)
Antenatal steroids, n (%)	33 (100)
Female, n (%)	11 (33)
Cesarean section, n (%)	25 (75.8)
SNAPPE II, median (IQR)	13 (0-21)
Small-for-gestational age n (%)	0 (0)
Late-onset sepsis, n (%)	4 (12.1)
Chronic lung disease n (%)	3 (9.1)
Steroids for chronic lung disease, n (%)	1 (3)
Severe necrotizing enterocolitis, n (%)	0 (0)
Severe intra-periventricular hemorrhage n (%)	2 (6.1)
Day of full enteral feeding, median (IQR)	12 (9-17)
Multicystic periventricular leukomalacia n (%)	0 (0)
Days with invasive ventilation, median (IQR)	0 (0-6)
Days with supplemental oxygen, median (IQR)	21 (5-42)
Length of stay (days), mean (SD)	48 (18)
Gestational age at discharge, median (IQR)	36 (35-39)

IQR interquartile range; SD standard deviation; BW birth weight

As compared with the 56-excluded formula-fed infants, the 33 infants who completed the study had significantly lower gestational age, lower prevalence of twins and stayed longer in the hospital (Table 3).

Table 3. Baseline characteristics of included versus excluded infants.

Baseline characteristics	Included (N=33)	Excluded (N=56)	p
Gestational age, weeks; mean (SD)	30 (28-31)	32 (30-32)	**0.002** †
Twins (%)	12	70	**<0.0001** ‡
Hospital stay (days); mean (SD)	51 (35-62)	39 (29-51)	**p=0.016** †

SD standard deviation; † t-Student test; ‡ Chi square

5.2. Human milk composition results

From 1281 daily pools of HM administered to each infant up to 35 weeks CA, macronutrients content was measured in 10.1% samples of DHM and 54.9% of OMM (Figure 2).

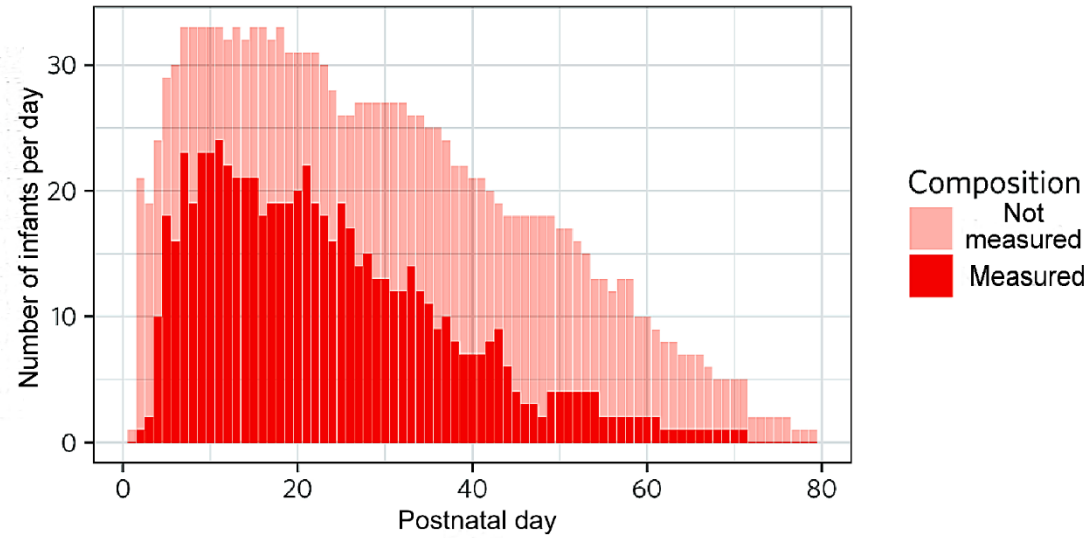

Figure 2. Proportions of human milk samples per day with measured and non-measured macronutrient content (N=832, 65%), up to 35 weeks corrected age.

Regarding the administered OMM, the measured true protein concentration decreased steeply from birth to the 10th postnatal day, after which it gradually decreased and stabilized after the 36th postnatal day (Figure 3).

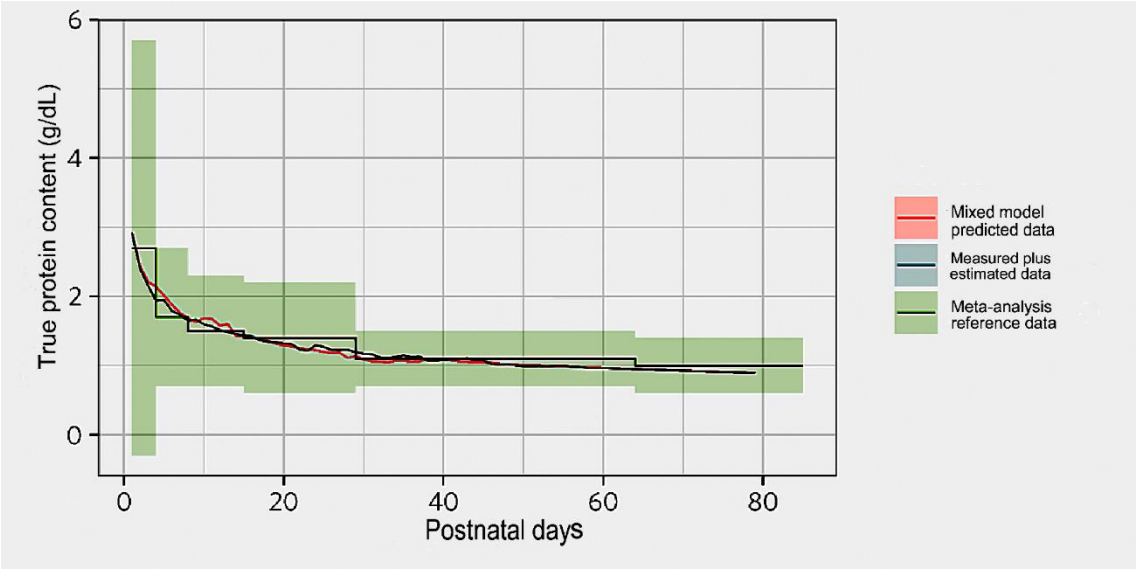

Figure 3. True protein concentration in own's mother milk: reference data(64) (green line), mixed model-predicted data (red line), and measured plus estimated data (blue line). Lines indicate mean values and shaded areas indicate ±2 standard deviations.

The measured energy concentration of administered OMM steeply increased within the first two postnatal weeks, after which it gradually increased (Figure 4).

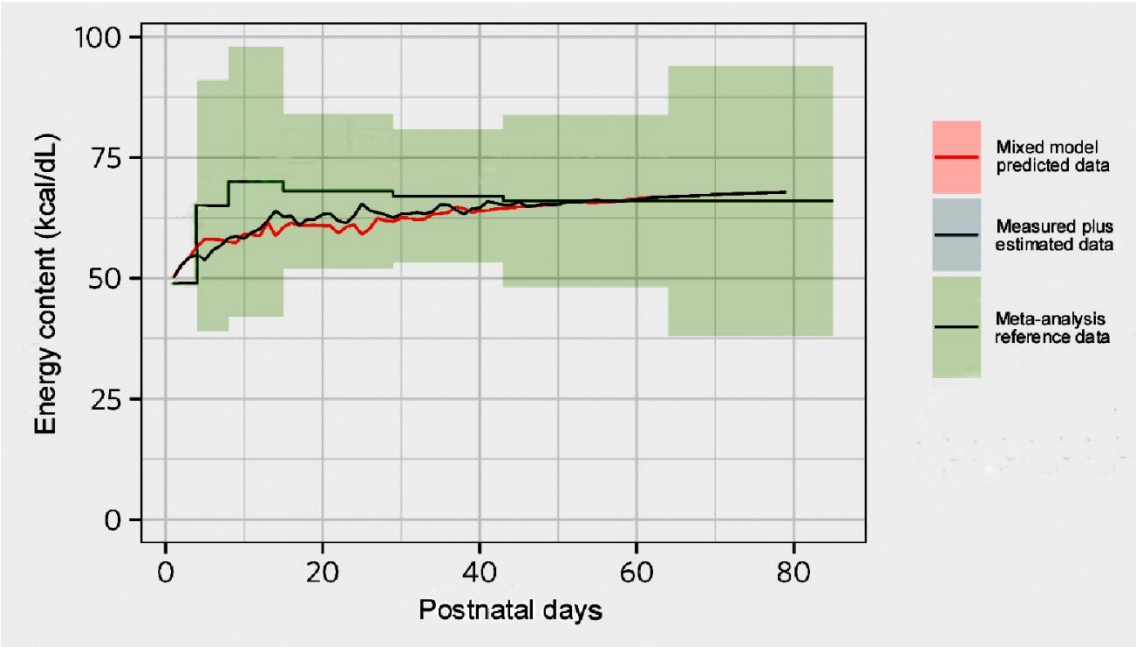

Figure 4. Energy concentration in in own's mother milk: reference data(64) (green line), mixed model-predicted data (red line), and measured plus estimated data (blue line). Lines indicate mean values and shaded areas indicate ±2 standard deviations.

5.3. Measured macronutrient intake

The studied infants received PN during a median (IQR) of 11 (8-16) days. On the 3rd postnatal day, 80% of infants had initiated HM, and on the 28th postnatal day, all infants were exclusively HM-fed. OMM was predominantly used up to 35 weeks CA, given by tube or mouth; subsequently, the infants were predominantly breastfed. Fortified HM was started on the 7th postnatal day and generalized to all infants by the 28th postnatal day (Figure 5).

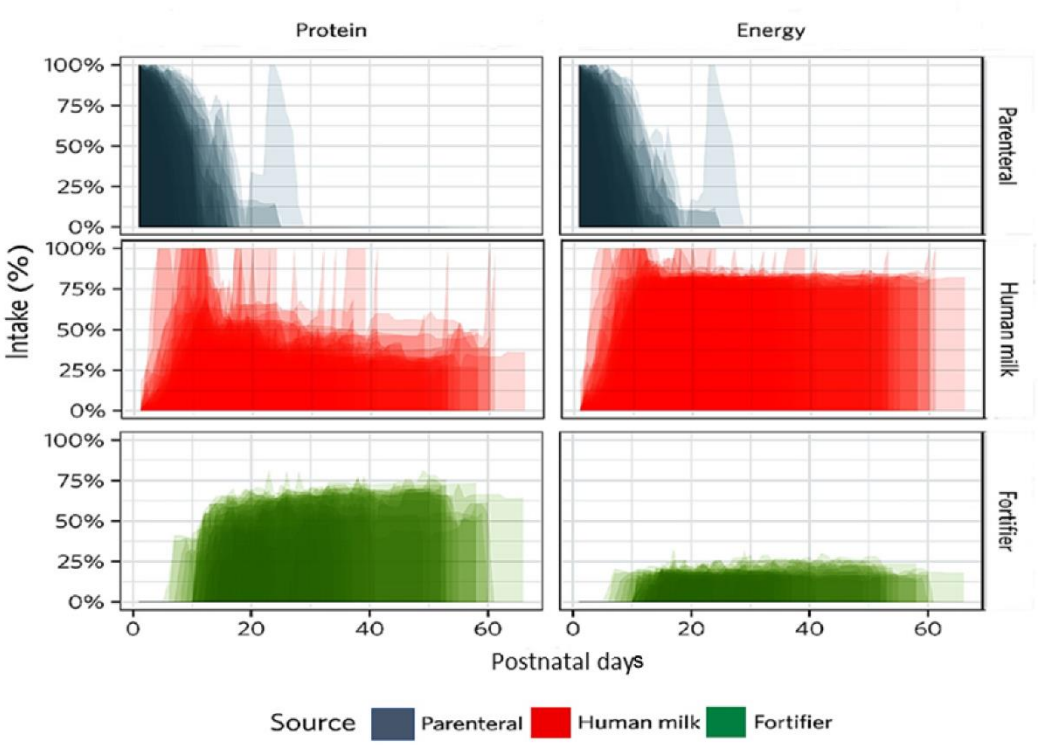

Figure 5. Sources of protein and energy, from birth to 35 weeks corrected age.

After disclosing the measured HM composition, the minimum recommended daily macronutrient intake achieved in at least 75% of the days between the 12th postnatal day (when the fluid intake reached a plateau) and 35 weeks CA, was assessed in each infant. In this period, the minimum recommended intake were achieved in (Figures 6 and 7):

- 63.6% of infants for protein
- 15.2% for energy
- 93.9% for PER

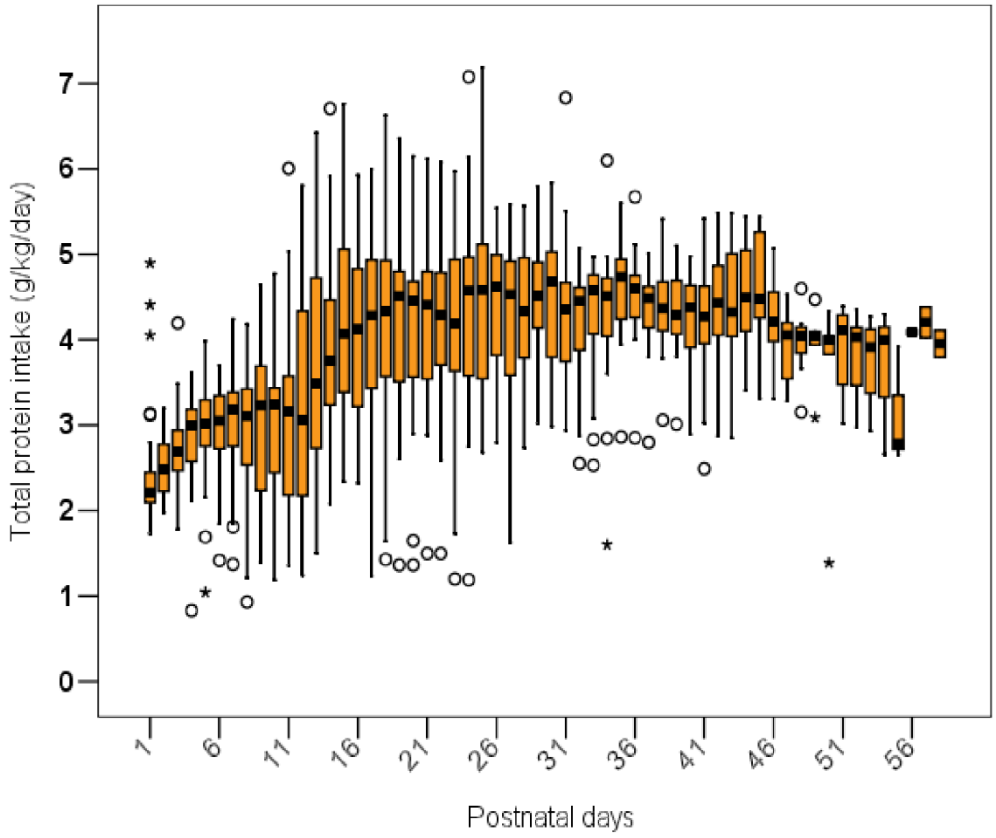

Figure 6. Daily total protein intake (parenteral plus enteral) from birth to 35 postnatal weeks.

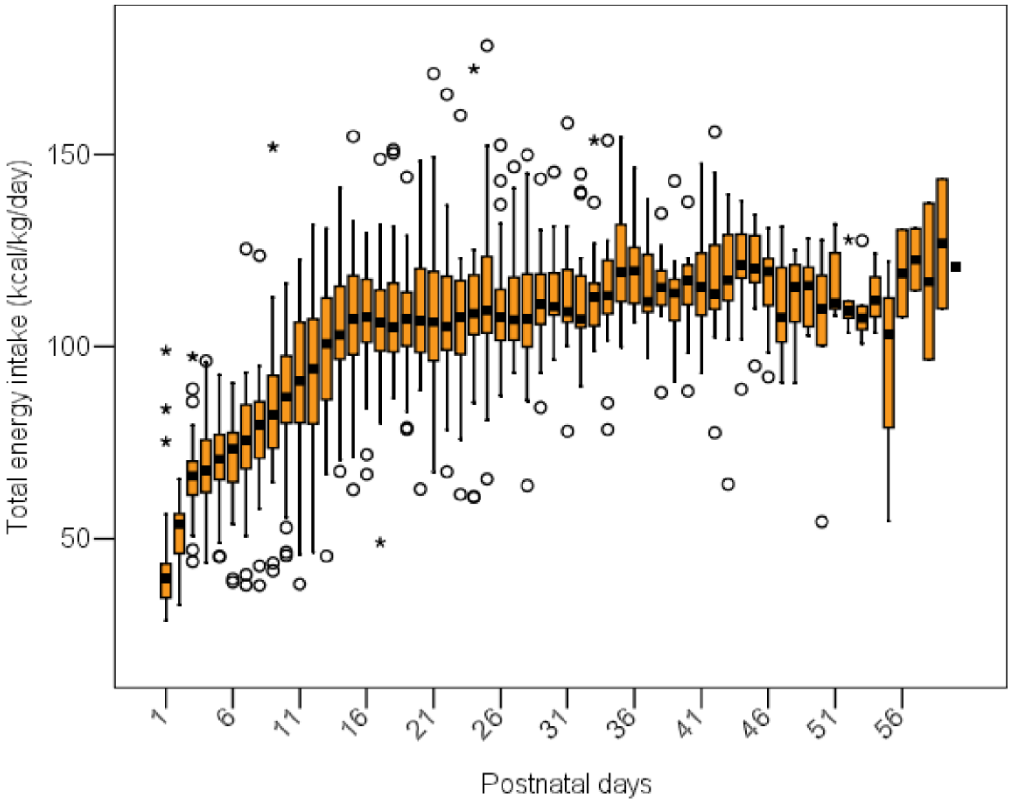

Figure 7. Daily total energy intake (parenteral plus enteral) from birth to 35 postnatal weeks.

In Table 4, the protein, energy, and PER intake from birth to 35 weeks CA are presented. The incidence of infants decreased with gestational age, thus nutrient intake were evaluated in small numbers of infants at lower gestational ages; at 35 weeks PMA nutrient intake were evaluated only in 28 infants, since in 5 the OMM composition was not measured because they were exclusively or predominantly breastfed. The median daily protein, energy, and PER intake ranged from 2.7-4.2 g/kg, 53.7-109.2 kcal/kg, and 3.4-5.6, respectively. Of note, higher PERs were recorded at lower corrected ages, reflecting that low protein intake were associated with very low energy intake.

Table 4. Daily median (interquartile range) of true protein (g/kg), energy (Kcal/kg), and protein-to-energy ratio intake, from birth to 35 weeks postmenstrual age (N=33).

Postmenstrual age (weeks)	N	Protein intake (g/kg/day), median (IQR)	Energy intake (Kcal/kg/day), median (IQR)	PER intake, median (IQR)
26	2	2.9 (2.4-3.5)	53.7 (39.5-67.9)	5.6 (5.1-6.0)
27	6	2.7 (2.5-3.6)	63.5 (54.9-66.6)	4.6 (4.3-4.9)
28	13	3.3 (2.7-3.5)	72.4 (54.8-86.4)	4.8 (4.0-5.0)
29	16	3.3 (3.1-4.0)	78.3 (72.8-99.4)	4.4 (4.1-4.6)
30	22	3.9 (2.7-4.7)	101.4 (58.1-106.3)	4.2 (3.8-4.6)
31	30	3.5 (2.8-4.7)	102.5 (63.2-110.8)	4.2 (3.9-4.5)
32	33	3.8 (2.9-4.5)	104.5 (81.8-109.9)	4.0 (3.6-4.4)
33	33	4.2 (3.4-4.6)	108.5 (99.1-108.5)	3.8 (3.2-4.1)
34	33	3.8 (3.3-4.4)	109.2 (103.1-119.5)	3.4 (2.9-4.0)
35	28	3.7 (2.6-4.3)	106.7 (99.2-116.9)	3.4 (2.5-3.9)

GA gestational age; IQR interquartile range; PER protein-to-energy ratio

After discharge, from 35 to 40 weeks CA, the macronutrient intake were neither measured nor estimated. During this period, 21 (63.6%) infants were exclusively breastfed, 10 (30.3%) were breastfed *plus* formula-supplemented, and 2 (6.1%) were formula-fed; in most cases, formula was initiated at or after 39 weeks CA.

5.4. In-hospital anthropometry

5.4.1. Descriptive analysis

In the whole sample, the recoup of birth weight occurred at a median (IQR) of 14 (11-17) postnatal days.

The median (IQR) body weight at birth was 1175 (1010-1408) and at 35 weeks CA was 2000 (1570-2105) g.

The mean (SD) weight gain velocity(98) from birth to 35 weeks was 10.1 (3.8) g/kg/day.

5.4.2. Univariate analysis: associations of macronutrient intake with weight gain velocity

Positive weak-to-moderate correlations of daily protein intake (r=0.651, p<0.001), energy intake (r=0.514, p=0.002), and PER intake (r=0.466, p=0.006) with weight gain velocity were found, with coefficients of determination of r^2=0.424, r^2=0.264, and r^2=0.217, respectively (Figure 8).

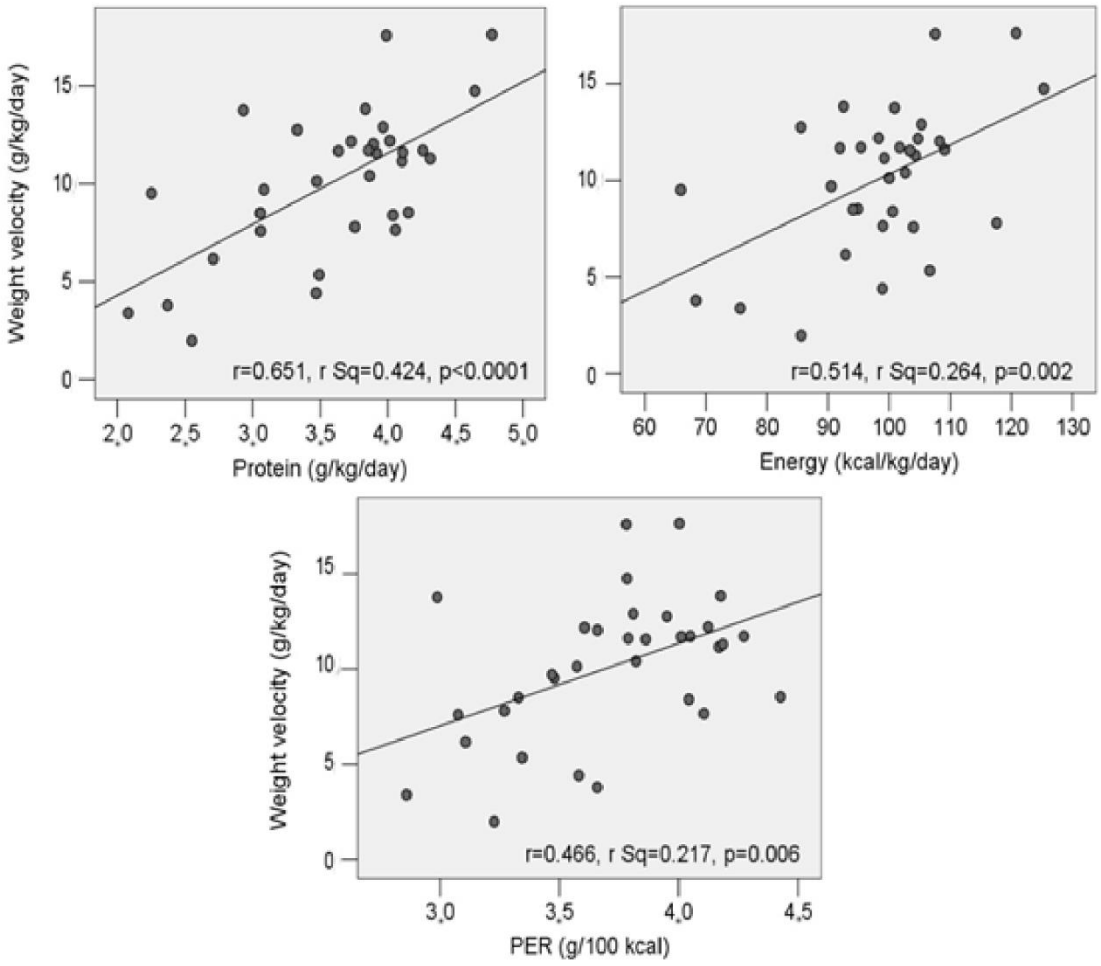

Figure 8. Correlation of weight gain velocity (g/kg/day) with daily protein (g), energy (Kcal) and protein-to-energy ratio (PER) intake per kg of body weight, from birth to 35 weeks corrected age.

5.4.3. Univariate analysis: associations of potential confounders with weight gain velocity

Among several potential confounders, only GA and birth weight had a prevalence allowing univariable analysis and had statistical significance:

- Gestational age was strongly negatively associated with weight gain velocity from birth to 35 weeks CA ($r=-0.662$, $p<0.0001$)
- Birth weight was moderately negatively associated with weight gain velocity from birth to 35 weeks CA ($tau=-0.450$, $p<0.0001$).

No associations were found between sex and twin prevalence and weight gain velocity.

5.4.4. Multivariate analysis: effect of macronutrient intake on weight gain velocity

To evaluate the adjusted effect of the median intake of each macronutrient on weight gain velocity, linear multiple regression was used, with GA as a co-variable. As GA had a normal distribution and collinearity with birth weight, that had a non-normal distribution, only the first was used as a co-variable in the models.

In the three models, only GA was negatively and significantly related with weight gain velocity ($p<0.0001$) (Table 5). The weight gain velocity significantly decreased as GA increased (Figure 9).

In other words, the weight gain velocity decreased in 1 g/kg/day for each 0.7-week (5 days) increase in GA, adjusted for nutrient intake; 41% to 43% of the variation of weight gain velocity is “explained by” the variation in GA, adjusted for nutrient intake (Table 5).

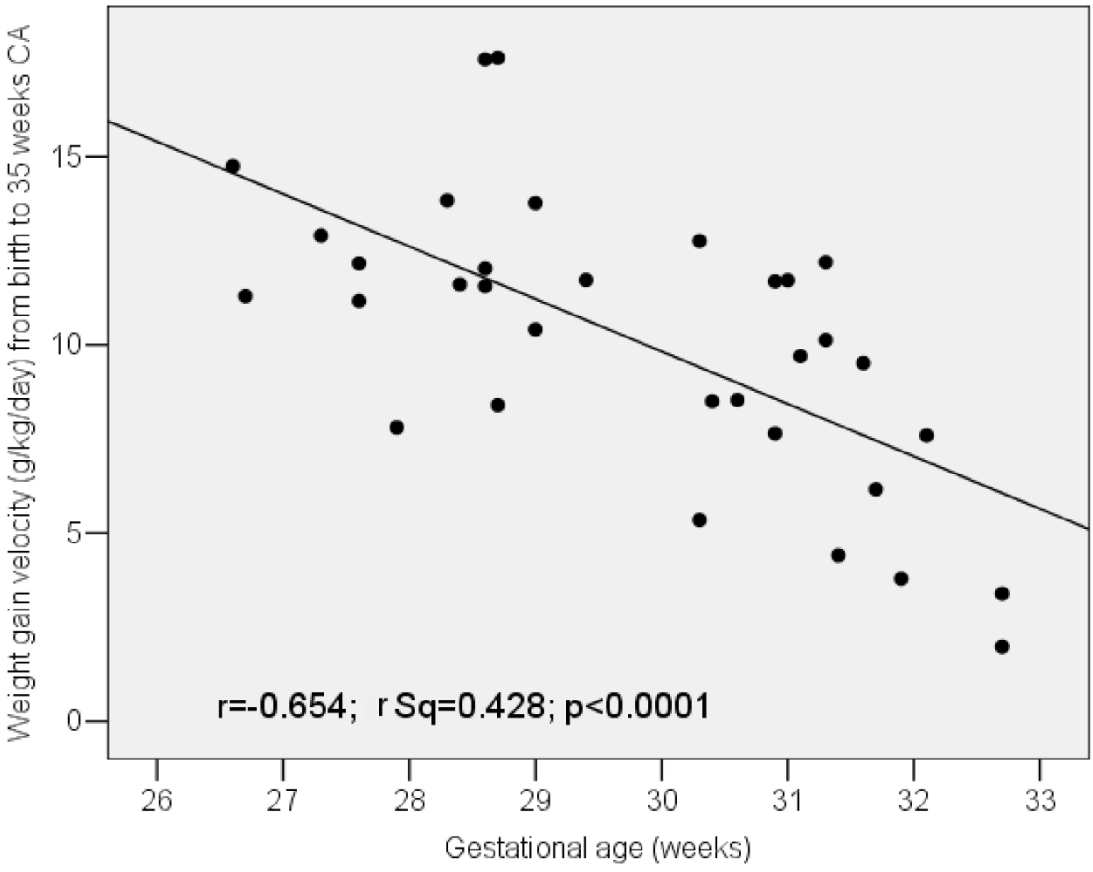

Figure 9. Weight gain velocity significantly decreased as gestational age increased.

Table 5. Adjusted effect of gestational age on weight gain velocity (g/kg/day), with macronutrient intake as covariables.

Variables	B	Adjusted r^2	β Coefficient	95% CI of B	*p*
GA (weeks)adjusted for protein intake (g/kg/day)	-1.39	**0.41**	**-0.65**	-0.20, -0.80	**<0.0001**
GA (weeks)adjusted for energy intake (kcal/kg/day)	-1.44	**0.43**	**-0.67**	-2.0, -0.83	**<0.0001**
GA (weeks) adjusted for PER intake	-1.44	**0.43**	**-0.67**	-2.0, -0.83	**<0.0001**

GA gestational age, PER protein-to-energy ratio

5.5. Body composition at term corrected age

5.5.1. Descriptive analysis

Body composition measured at a mean (SD) of 39.9 (1.9) weeks CA was analyzed only in 32 infants, because one infant was assessed at 46 weeks CA.

The body composition measurements showed a normal distribution, with a mean (SD) **body mass** of 2817.6 (504.3) g, **FM** of 441.5 (184.0) g, **FFM** of 2376.1 (376.0) g, **FM%** of 15.3 (4.8), and **FMI** of 2.0 (0.7).

5.5.2. Univariate analysis: associations of macronutrient intake with body composition

No correlations of daily protein, energy, and PER intake with body composition measurements were found.

5.5.3. Univariate analysis: associations of covariables with body composition

Among several covariables, only those with clinical relevance and prevalence allowing univariate analysis were selected. Associations between these covariables and body composition at term CA are shown in Table 6.

Table 6. Associations of covariables and body composition at term corrected age (n=32).

	Body composition at term CA			
	FM (kg)	FM (%)	FMI	FFM (kg)
GA (weeks)	r=0.245 p=0.177	r=0.039 p=0.834	r=-0.291 p=0.106	r=0.334 **p=0.062**†
Birth weight (kg)	tau=0.108 **p=0.068**	tau=0.326 p=0.389	tau=-0.004 p=0.974	tau=0.189 p=0.131
SNAPPE II	tau=-0.205 p=0.115	tau=-0.104 p=0.425	tau=0.037 p=0.778	tau=-0.218 **p=0.094**
Sex	p=0.487	p=0.579	p=0.273	p=0.675
Twin infants	p=0.655	p=0.646	p=0.439	p=0.508

† entered in multivariate models

5.5.4. Multivariate analysis: adjusted effect of macronutrient intake on body composition

The FFM was the only dependent variable used in the model adjusted to GA, because other covariates did not meet criteria to enter in the multivariate models. After adjustment, median protein (g/kg/day) and energy (kcal/kg/day) intake remained statistically significant in the linear multiple regression models (Table 7).

In other words, the FFM decreased in 1 kg for each 0.46 g/kg/day increase in protein intake and for each increase in 0.58 kcal/kg/day in energy intake, adjusted for GA; 21% and 32% of the variation in FFM is "explained by" the variation in protein and energy intake, respectively, adjusted for GA.

Table 7. Adjusted effect of macronutrient intake on fat-free mass, with gestational age as co-variable.

Variables	B	Adjusted r^2	β Coefficient	95% CI of B	*p*
Protein intake (g/kg/day) adjusted for GA (weeks)	-0.25	**0.21**	**-0.46**	-0.43, -0.07	**0.008**
Energy intake (kcal/kg/day) adjusted for GA (weeks)	-0.02	**0.32**	**-0.58**	-0.03, -0.01	**0.001**

GA Gestational age (weeks); FFM Fat free mass

5.5.5. Nested case-control analysis: association between macronutrient intake and extremes of adiposity

Seven, 7, 4, and 8 infants had a FM% ≤ -1 z-score, FM% ≥ +1 z-score, FMI ≤ -1 z-score, and FMI ≥ +1 z-score, respectively.

The nested case-control analysis, used to assess differences between infants with higher and lower adiposity, as compared to the remaining infants, showed some significant associations (Table 8).

- In infants with lower adiposity, a FM% ≤ -1 z-score was associated with lower energy and protein intake, while a FMI ≤ -1 z-score was associated with a lower PER intake.
- In infants with higher adiposity, an FMI ≥ +1 z-score was associated with a lower energy intake and a higher PER intake.

Table 8. Nested case-control study, comparing protein, energy and PER intake in infants with low adiposity (FM% and FMI ≤ -1 z-score) and high adiposity (FM% and FM ≥ -1 z-score) with the remaining infants.

FM%	**≤ -1 z-score**	**> -1 z-score**	*p*
Protein intake (g/kg/day), median (IQR)	3.8 (3.1-4.4)	4.0 (3.0-4.6)	**0.051**
Energy intake (Kcal/Kg/day), median (IQR)	103.3 (88.8-	107.4 (88.6-	**0.005**
PER intake, median (IQR)	3.8 (3.4-4.3)	3.8 (3.2-4.3)	0.73
FMI	**≤ -1 z-score**	**> -1 z-score**	*p*
Protein intake (g/kg /day), median (IQR)	4.0 (3.0-4.4)	4.0 (3.1-4.6)	0.12
Energy intake (Kcal/Kg/day), median (IQR)	108.0 (91.2-	106.3 (88.0-	0.16
PER intake, median (IQR)	3.7 (3.2-4.2)	3.9 (3.3-4.3)	**0.026**
FM%	**≥ +1 z-score**	**< +1 z-score**	*p*
Protein intake (g/kg/day), median (IQR)	4.0 (2.9-4.8)	4.0 (3.1-4.5)	0.542
Energy intake (Kcal/kg/day), median (IQR)	104.3 (81.5-	106.8 (91.1-	0.503
PER intake, median (IQR)	3.9 (3.3-4.2)	3.8 (3.3-4.3)	0.872
FMI	**≥ +1 z-score**	**< +1 z-score**	*p*
Protein intake (g/kg/day), median (IQR)	4.0 (3.0-4.8)	4.0 (3.1-4.5)	0.118
Energy intake (Kcal/kg/day), median (IQR)	103.3 (80.6-	107.9 (92.2-	**<0.0001**
PER intake, median (IQR)	4.0 (3.5-4.5)	3.8 (3.2-4.2)	**<0.0001**

FM% fat mass percentage; FMI fat mass index; IQR interquartile range; Mann-Whitney *U* test.

5.6. Neurodevelopmental outcome

5.6.1. Descriptive analysis

Due to one infant being lost to follow-up, neurodevelopment was assessed in 32 infants. To avoid hospital visits scheduled for other assessments coinciding with neurodevelopment assessment, this needed to be delayed to a mean (SD) of 20.2 (1.5) months CA.

Both MDI and PDI scores had a normal distribution. The mean (SD) score for MDI was 100.2 (11.5) and for PDI 97.4 (8.0).

The mean MDI score was below normal in 2 (6.2%) infants (boys), normal in 25 (78.1%) infants (8 girls and 17 boys), and accelerated in 5 (15.6%) infants (2 girls and 3 boys).

The mean PDI score was below normal in 2 (6.2%) infants (1 girl and 1 boy), and normal in 30 (93.8%) infants (8 girls and 21 boys).

The Behavior Scale rated as non-optimal in 2 (6.3%) infants (boys), questionable in 8 (25%) infants (2 girls and 6 boys), and normal in 22 (68.8%) infants (7 girls and 14 boys).

5.6.2. Univariate analysis: associations of macronutrient intake with head circumference

In-hospital cumulative energy intake was significantly and moderately correlated with low HC at term CA (r=-0.38, p=0.039).

5.6.3. Univariate analysis: associations of potential confounders with head circumference

Associations of potential confounders and HC at term CA are shown in Table 9. Only GA and sex had clinical relevance and prevalence to be selected for multivariate analysis.

Table 9. Associations of potential confounders with head circumference at term CA.

Variables	HC at term CA
GA (weeks)	r=0.460, **p=0.008**
Weight (kg)	tau=0.253, **p=0.047**
SNAPPE II	tau=0.266, **p=0.041**
Sex	**p=0.099**†
Twin infants	p=0.118†
Severe IPVH	p=0.531†
Late onset sepsis	p=0.164†

r Pearson correlation; tau Kendall's tau-B; † Student t test; CA corrected age; GA gestational age; HC head circumference

5.6.4. Multivariate analysis: effect of macronutrient intake on head circumference

To analyze the effect of macronutrient intake on head circumference, linear multiple regressions were used, adjusted to GA and sex, since another covariates did not meet criteria to enter in the multivariate models.

The GA and sex were significant predictors of higher HC at term CA (Figure 10); In other words:

HC at term CA increased in 1 cm:

- for each 0.43 week (3-day) increase in GA, adjusted for protein intake and sex;
- for each 0.45 week (3-day) increase in GA, adjusted for energy and PER intake.

These adjusted models "explain" 20% to 24% of the variation in HC by the GA.

In males, HC at term CA was 1.72 cm larger, when compared with females. In this adjusted model 25% of the variation in HC "is explained" by sex (Table 10).

Figure 10. The gestational age significantly predicted the head circumference at term age.

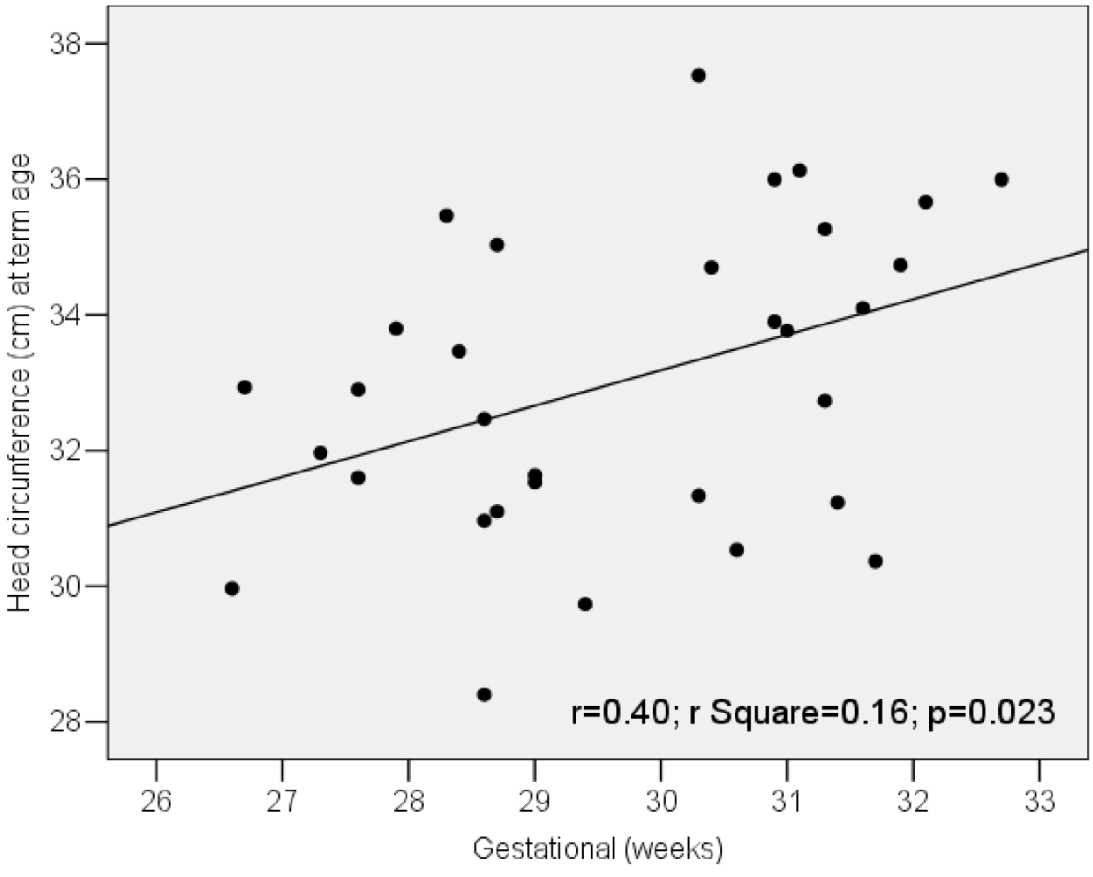

Table 10. Associations of macronutrient intake with head circumference adjusted for gestational age and sex.

Variables	B	Adjusted r^2	β Coefficient	95% CI for B	*p*
HC at term CA					
GA (weeks), adjusted for protein intake and sex	0.57	0.24	**0.43**	1.15, 1.00	**0.010**
Sex, adjusted for protein intake and CGA	**1.72**	0.25	0.36	0.20, 3.25	**0.028**
GA (weeks), adjusted for energy intake and sex	0.54	0.20	**0.45**	0.12, 0.96	**0.013**
GA (weeks), adjusted for PER intake and sex	0.54	0.20	**0.45**	0.12, 0.96	**0.013**

CA corrected age; GA gestational age; HC head circumference

5.6.5. Univariate analysis: associations of macronutrient intake with neurodevelopmental outcome

In-hospital cumulative protein, energy, and PER intake were neither significantly correlated with MDI or PDI scores at mean 20 months CA nor met the defined criteria ($p<0.10$) to enter in multivariate analysis.

6. DISCUSSION

6.1. Characteristics of the studied sample

This study was conceived to include a homogenous birth cohort of very preterm infants that were exclusively or almost exclusively HM-fed, to better evaluate the associations of in-hospital measured macronutrient intake provided by fortified HM with in-hospital weight gain velocity, body composition at term CA and neurodevelopment at 20 months CA.

The enrollment period was shortened owing to aforementioned reasons (section 4.3) and the sample dimension became smaller than estimated. Only 33 infants completed the study at discharge, and consequently the study may be under-powered to test the hypotheses.

6.2. Measured human milk composition

Cumulative in-hospital protein and energy intake were measured. This was based on the analysis of composition in macronutrient of HM as well as on compositions reported by manufacturers of the fortifier, modular protein and modular fat supplements. For the measurement of HM composition, samples of DHM and of pooled OMM were used.

As OMM composition measurements were not always possible, a post-hoc analysis for the imputation of missing values was performed taking as reference the reported data on composition of preterm infants' OMM.(64) Good agreements were found between the curves of reference data, mixed model-predicted data, and measured plus estimated data for true protein and energy, reflecting accuracy of the measuring model.

In preterm infants, some studies assessed the effective macronutrient intake provided by HM, analyzing the HM composition(187, 188), which allowed a more accurate targeted fortification method.(187)

Most studies assessing the effect of HM macronutrient intake on growth, body composition, and neurodevelopment in preterm infants, have relied on estimated rather than measured macronutrient intake from HM. Very few studies have assessed

the effect of effective macronutrient intake from HM on body composition of preterm infants.(162, 163) The majority of studies assessing the effect of nutrition on neurodevelopment in preterm infants have used growth as a surrogate of macronutrient intake.(34) The exception is the study by Stephens et al.(168), which linked neurodevelopmental outcome directly to estimated macronutrient intake rather than to growth failure. To the best of our knowledge, our study was the first to assess the association between measured macronutrient intake from HM and the neurodevelopmental outcome in preterm infants.

6.3. Measured macronutrient intake

We found that 36.4% and 84.8% of infants in our cohort did not receive the minimum targeted protein and energy intake, respectively, in at least 75% of in-hospital days. This may reflect a poor effectiveness of the fortification method we used, based on the standard fortification method with modular protein(80, 81) (80) and fat supplements.

In similar studies measuring(162, 163) or estimating(182) macronutrient intake provided by fortified HM and/or formula, insufficient intake to achieve the current recommended targets(38) were reported, suggesting the suboptimal effectiveness of other HM fortification methods as well.(162, 163) The macronutrient intake in our study are difficult to compare with data from most similar studies, as these were estimated, instead of measured macronutrient intake from HM.(159-163)

6.4. Measured macronutrient intake and weight gain velocity

The exponential weight gain velocity model has been reported to accurately monitor in-hospital growth, and is not affected by factors such as birth weight and length.(98)

In our cohort, the suboptimal nutritional support resulted in a mean weight gain velocity of 10.1 g/kg/day, which is lower than the velocities of 11.4 and 18.3 g/kg/day reported for very preterm infants in previous studies.(162, 164, 182)

We found significant positive weak-to-moderate correlations of macronutrient intake with weight gain velocity; specifically, protein, energy, and PER explaining 42.4%, 26.4%, and 21.7% of the weight gain velocity variation, respectively.

Despite of the low in-hospital weight gain velocity, the body weight (0.139, p=0.205), length (0.247, p=0.681), HC (-0.37 cm, p=0.358) of our infants at term CA were similar to the values reported by Roggero *et al.*(182)

6.5. Measured macronutrient intake and body composition

6.5.1. Measured adiposity

Despite of the low in-hospital weight gain velocity, the FM% (0.469, p=0.589) of our infants at term CA were similar to the values reported by Roggero *et al.*(182)

6.5.2. Association of measured macronutrient intake with body compartments

After adjustment for GA, only FFM was negatively and significantly associated with protein and energy intake; that is, a decrease of 1 kg of FMM was observed for each 0.46 g/kg/day increase in protein intake and 0.58 kcal/kg/day increase in energy intake, adjusted for GA. This means that 21% and 32% of the variation in FFM was "explained by" the variation in protein and energy intake, respectively, adjusted for GA.

Some other authors(159-161) found that, in preterm infants, higher estimated protein and energy intake were associated with better weight gain, but without significant differences in body composition.

In contrast, other studies based on estimated or measured macronutrient intake found that, in preterm infants at term CA, specific body compositions were associated with different nutritional strategies (Table 13). In these studies, higher protein(163, 164) and PER(165) intake were associated with an increase in lean mass(164, 165) and decrease in adiposity,(162) while higher fat and energy intake were associated with increased fatness.(163)

The unexpected association of decrease in FFM with increase in protein and energy intake in our cohort is difficult to explain. Using a bicompartmental model, adiposity is more accurately estimated than leanness, because FM has a more constant density than FFM, which depends on its different water content.(136, 137) Moreover, FM is more

homogenous, comprising predominantly of adipose tissue, while FFM is a complex compartment containing not only skeletal muscle, but also bone, organs, and blood.(136)

Table 11. Studies assessing the association between estimated or measured in-hospital macronutrient intake and body composition.

	N	GA (weeks)	HM macronutrients	CA (weeks) at body composition assessment	Outcome
Our study	32	≤32	Measured	40	Infants with lower adiposity (ADP) received lower energy, protein, and PER intake, than the remaining. Infants with higher adiposity lower energy intake but a higher PER intake, than the remaining.
Embleton et al. (2005)	77	≤34	Estimated	52	No differences in body composition (DEXA) with different macronutrient intake
Rochow et al. (2012)	239	≤32	Estimated	36	No differences in body composition (DEXA) with different macronutrient intake
Roggero et al. (2012)	171	≤32	Estimated	40	No differences in adiposity (ADP) with different macronutrient intake
McLeod et al. (2015)	17	≤32	Measured	31 to 40	Higher fat and total energy intake were associated with increasing FM (ADP), and higher protein intake was associated with increasing FFM (ADP)
Tremblay et al. (2017)	26	<29	Estimated	40	Comparison with 33 term infants: higher protein intake associated with a lean mass (DEXA) comparable to that of term infants
Simon et al. (2014)	141	<35	Estimated	36 to 38	Higher PER at 10-21 postnatal days was associated with decreased risk of FFM (ADP) deficit compared with reference values for term infants.
McLeod et al. (2016)		<30	Measured	38	A daily protein intake >3.4 g/kg reduced FM%(ADP) by 2 %

ADP Air displacement plethysmography; CA Corrected age; DEXA Dual-energy x-ray absorptiometry; FFM Fat free mass; FM Fat mass; FM% Fat mass percentage; GA Gestational age.

6.5.3. Associations between macronutrient intake and extremes of adiposity

We used a nested case-control analysis to explore the associations between cumulative in-hospital macronutrient intake and extremes of adiposity(181) at term CA.

In our cohort, infants with lower adiposity (FM% and FMI) received significantly lower energy, protein, and PER intake, than the remaining infants. It is expected that a low energy intake in the presence of very low protein intake (indicated by low PER) impairs lipogenesis and/or facilitates lipolysis, consequently resulting in low fat reserve.(189, 190) In surgical neonates, indirect calorimetry combined with body composition analysis showed that insufficient protein intake in the presence of poor energy intake resulted in preferential fat oxidation and decreased adiposity, suggesting increased lipolysis.(191, 192) Our infants with higher adiposity (FMI) received a significantly lower energy intake but a higher PER intake, than the remaining infants. This higher PER intake indicates that low protein intake was associated with very low energy intake; in this context, it may be speculated that preferential protein oxidation in relation to fat oxidation occurred in response to poor energy supply, leading to less lipolysis and sparing of the fat reserve.(189, 190)

6.6. Measured macronutrient intake, head circumference and neurodevelopmental outcome

6.6.1. Measured in-hospital macronutrient intake and head circumference

Regarding HC, in univariate analysis low HC at term CA was significantly and moderately correlated with in-hospital cumulative energy intake (r=-0.38, p=0.039). However, in multivariate analysis adjusted to GA and sex, GA was the only significant predictor of HC at term CA and its association with energy intake lost significance.

Head circumference has been used as surrogate of brain growth and associated with neurodevelopmental outcome.(193) Tan et al.(194) designed a randomized controlled trial to explore the relationships between early nutrition, post-natal head growth, quantitative magnetic resonance imaging and development outcome (BSID-II) in very preterm infants, assigned to receive either hyperalimentation or a standard feeding

regimen from birth to 34 weeks CA. A good correlation was found between HC and total brain volume. Energy deficit at 28 postnatal days correlated significantly with total brain volume at term CA and with MDI and PDI at 3 months CA. The authors concluded that improving early nutrition by reducing energy deficit in very preterm infants may improve brain growth and maturation.(193) Corroborating these results, we found a significant correlation of cumulative energy deficit at 35 weeks CA with low HC at term CA in univariate analysis, although this association lost significance in multivariate analysis.

6.6.2. Measured in-hospital macronutrient intake and neurodevelopmental outcome

Although the neurodevelopmental assessment was scheduled at 18 months CA, it was delayed to a mean of 20 months CA, to avoid hospital visits on the same day as other assessments. Despite the difference being only of two months, the assessment at a later age might be an advantage, since it was reported that the older the child is at the time of testing, the more robust the test is.(156)

At a mean of 20 months CA, most infants in our cohort were classified in the normal range, with 93.75% having normal or accelerated MDI, and the same proportion having normal PDI; the Behavior Scale rated normal in 68.8% and questionable in 25% of infants. Our results are in line with those by Coletti et al.(195), who found composite scores in the normal range in very preterm infants using the BSID-III.

In univariate analysis, in-hospital cumulative protein, energy, and PER intake were neither significantly correlated with any MDI or PDI scores at mean 20 months CA, nor met the defined criteria ($p<0.10$) to enter in multivariate analysis, so no further statistical analysis was performed.

A recent systematic review concluded that increased early nutrition may reduce neurodevelopmental impairment in preterm infants, although the relationship remains unclear.(196) One objective of our study was to contribute to clarify this issue, exploring the association of macronutrient intake and neurodevelopmental outcome in a homogeneous sample of HM-fed very low preterm infants. Although several micronutrients, total fat and carbohydrates contribute for adequate brain nutrition and

neurodevelopment(14, 30, 167), our study was only focused on protein and energy intake.

Most studies evaluating the association of macronutrient intake as independent variable with neurodevelopmental outcome as dependent variable, have used surrogates for both. Head growth has been used as reflection of good brain nutrition(197), and somatic growth as surrogate of macronutrient intake.(197) In other studies, macronutrient intake have been assumed from the type of feedings and when HM was used its macronutrient content was not quantified through analysis.(198, 199)

Regarding neurodevelopmental outcome, in univariate analysis, in-hospital cumulative protein, energy, and PER intake were not associated with any MDI or PDI scores at a mean of 20 months CA. Our hypothesis of association of higher protein and energy intake with better neurodevelopmental outcome was not demonstrated, probably due to an undersized sample.

Neurodevelopmental outcome in infants born very preterm is multifactorial. Beyond early nutrition, several factors influence neurodevelopment in these high-risk infants, including degree of immaturity, prenatal and perinatal insults, neonatal morbidities, and environmental stimuli.(32, 200) The independent effect of postnatal nutrition on neurodevelopmental outcome remains unclear in infants born preterm, due to the difficulty in adjusting for all concurrent factors.(196)

In preterm infants, controversial results have been reported on the effect of early energy and protein intake on the neurodevelopmental outcome. Beyond the study by Tan et al.(193), very few studies evaluated the direct effect of macronutrient intake on the neurodevelopmental outcome in preterm infants (Table 14).(168, 201, 202) Stephens et al.(168) performed a cohort study of extremely low birth weight infants and found that increased protein and energy intake in the first postnatal week are associated with higher MDI scores (BSID-II) at 18 months CA, after adjusting for confounding variables. More specifically, intake of each 10-kcal/kg/day and 1 g/Kg/day in protein were associated with 4.6-point and 8.2-point increases in the MDI, respectively. Christmann et al.(201) reported that in infants with less than 34 weeks of gestation, higher protein intake (adjusted for energy) in the first two postnatal weeks was associated with higher MDI scores in girls, and higher PDI scores especially in boys. On the other hand, Dogra

et al.(202) described that the fortification of HM with fortifier containing higher protein did not benefit neurodevelopment at 12 to 18 months CA. In these three studies(168, 201, 202), the macronutrient intake have been estimated, whereas in our study only including HM-fed very preterm infants, macronutrient intake have been measured relying on HM composition analysis.

Table 12. Studies assessing the association between estimated in-hospital macronutrient intake and neurodevelopmental outcome.

	N	GA (weeks)	HM macronutrients	CA at neurodevelopmental assessment	Outcome
Our study	32	≤32	Measured	20 months	
Stephens et al. (2009)	124	≤32	Estimated	18 months	Increased protein and energy intake in the first postnatal week, associated with higher MDI scores
Christmann et al. (2017)	112	<34	Estimated	24 months	Using the BSID-II and nutrient intake within the first two postnatal weeks: MDI ≥ 85 scores were associated with higher protein intake in girls; PDI ≥85 scores were associated with higher protein adjusted for energy intake, especially in boys.
Dogra et al. (2017)	120	<32	Estimated	12 to 18 months	Using the DASII, no benefit in neurodevelopmental was found with fortification of HM with fortifier containing higher protein

BSID-II Bailey Scales of Infant Developmental, version II; CA corrected age; DASII Developmentalal Assessment Scale for Indian Infants; GA gestational age, MDI Mental Developmental Index, PDI Psychological Developmental Index

6.7. Strengths and limitations

6.7.1. Strengths

The present study has strengths to be highlighted.

Body composition

First, we examined the association between macronutrient intake and body composition relying on measured protein and energy provided by the HM and not on its assumed composition. To the best of our knowledge, only two similar studies(162, 163) have measured the HM macronutrient content.

Second, a validated accurate method was used to assess body composition in infants.(86)

Neurodevelopment

To the best of our knowledge, this is the first study evaluating the association between the macronutrient intake and neurodevelopmental outcome in HM-fed preterm infants, with intake relying on the measured composition of HM. Other similar studies relied on estimated macronutrient intake, evaluating the intake indirectly, either assuming the composition of feedings, or using growth as a surrogate of nutritional support.

To evaluate neurodevelopment, we used the BSID-II, the most used test for infants aged 1 to 42 months, providing numeric continuous scores for mental and psychomotor developmental, with good reliability and validity. The mental and motor scales have high correlation coefficients (0.83 and 0.77 respectively) for test-retest reliability.(154, 157)

6.7.2. Limitations

Some limitations should be acknowledged in our study. The most important is that the study was interrupted before the estimated sample size was achieved, thus becoming under-powered.

Body composition

First, the under-powered study precluded the multivariate analysis of association of macronutrient intake and body composition.

Second, a bias of withdrawal exists because enrolled infants completing the study were significantly more immature, were more frequently singletons, and stayed longer in the hospital than those excluded. As the excluded infants were more mature, they needed shorter hospitalization. In addition, when OMM was unavailable they more frequently became formula-fed, owing to DHM being preferentially given to more immature infants in case there is a DHM shortage. In the case of twins, they also were more frequently formula-fed due to insufficient breastmilk for both.

Third, macronutrient intake was not assessed between 35 and 40 weeks CA; during this period, only 63.6% of infants were exclusively breastfed, although in partially or exclusively formula-fed infants, formula feeding was generally initiated within one week before the body composition was assessed.

Finally, convenience cut-offs of ≤ -1 z-score and ≥ +1 z-score were used as surrogates of low and high adiposity; however, these require validation.

Neurodevelopment

The BSID-II, although widely used as gold standard in neurodevelopmental outcome studies in term and preterm infants at risk of neurodevelopment impairment, was developed and validated for healthy term population and thus conceived to evaluate neurodevelopment of children born at term.(154, 158) In addition, BSID-II was validated in North-American population(154), and is not still validated in the Portuguese population.

7. CONCLUSIONS

This cohort study on a homogenous sample of exclusively or almost exclusively HM-fed very preterm infants was prematurely interrupted, becoming under powered to test the hypotheses.

A strength of this study was the assessment of associations of protein and energy intake with body composition and neurodevelopmental outcome relying on measured macronutrient HM content and not on its assumed composition.

Significant positive weak-to-moderate correlations of measured protein, energy, and PER intake with weight gain velocity were found.

The under-sized sample may explain the absence of the hypothesized correlations between cumulative in-hospital macronutrient intake and body composition at term CA. A nested case-control analysis showed that infants with lower adiposity received significantly lower energy, protein, and PER intake, while those with higher adiposity, received significantly lower energy intake but higher PER intake, than the remaining infants. The body composition of our infants did not differ significantly from that which was previously reported for very preterm infants. However, the measured macronutrient intake were much lower than those recommended. The method we used of standard fortification with blinded modular protein and fat supplements led to the minimum targeted protein and energy intake for weight not being achieved in 36.4% and 84.8% of infants, respectively. Further studies relying on measured HM macronutrient intake and using alternative HM fortification methods are needed.

At term CA, in univariate analysis, low HC at term CA was significantly correlated with in-hospital cumulative energy intake. However, in multivariate analysis adjusted to GA and sex, GA was the only significant predictor of HC at term CA and its association with energy intake lost significance.

In multivariate analysis, it was found that GA and sex were significant predictors of higher HC at term CA, adjusted for protein, energy and PER intake.

In-hospital cumulative protein, energy, and PER intake were neither significantly correlated with any MDI or PDI scores at mean 20 months CA, nor met the defined criteria to enter in multivariate analysis.

8. OPPORTUNITIES FOR FUTURE RESEARCH

This cohort study serves as a starting point for further studies aimed to assess the effect of early nutrition on body composition and neurodevelopmental outcome in very preterm infants.

A randomized trial using a large sample assessing the effect of early measured nutrition on body composition provides a higher level of evidence than a cohort study. A more insightful evidence will be obtained in a trial stratified by gestational age groups and controlled for covariates such as parental body composition, and micronutrients known to influence body composition. Long-term assessments of body composition and metabolic status may provide clues of early nutrition programming of late obesity and metabolic syndrome in preterm infants.

Similar to the effect of early nutrition on body composition, a randomized trial using a large sample would provide higher level of evidence than a cohort study on the neurodevelopmental outcome. More insightful evidence will be obtained in a trial stratified by gestational age groups and controlled for covariates such as sex, being twin, prenatal and perinatal insults, neonatal morbidities, micronutrients known to influence brain developmental, and environmental stimuli.

A randomized birth cohort also offers an opportunity to explore other outcomes, such as morbidities programmed in early life, long-term assessment of neurodevelopment at important stages of life, including scholar performance, rates of high school graduation, current educational achievement, social-emotional developmental, self-esteem, rates of permanent employment, independent living, and marital/cohabitating relationships. In other words, their health-related quality of life.(203, 204)

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