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Abstract:	Background: Feeding supplemented mother milk during hospital stay improves neurodevelopment in preterm infants. Yet the composition of mother milk varies widely between subjects. The relationship between this variation and outcome is unknown. Objective: To determine whether the protein content in native breast milk (BM) correlates with 2-year infant outcome. Design: In a monocentric prospective observational study, LACTACOL, preterm infants born between 28 and 34 weeks of gestation, whose mothers decided to exclusively breastfeed, were enrolled during the first week of life. Samples of expressed breast milk obtained at several times of the day were pooled over a 24-hour period, and such pool was used for macronutrient analysis, using mid-infrared

	analyzer. Age and Stages questionnaire (ASQ) was used to assess 2-year neurodevelopmental outcome. We analyzed the relationship between protein content in BM, and (i) infant neurodevelopment at 2-year (primary outcome), and (ii) growth until 2-year (secondary outcome). Results: 138 infants were enrolled. The main analysis concerned 130 infants (including 40 twin infants) and 110 mothers with BM samples collected at week 3 after birth. Native BM samples were ranked in 3 tertiles of protein content (g/100ml): 0.91±0.09 (lower), 1.14±0.05 (middle) and 1.40±0.15 (upper); 48, 47 and 35 infants were ranked respectively in these 3 tertiles. Infants in the upper tertile were more often singleton (P = 0.012) and were born with lower birth weight and head circumference Z-scores (P = 0.005 and 0.002; respectively). Differences in weight and head circumference were no longer observed at 2-year. ASQ score at age 2 did not differ the 3 tertiles (P = 0.780). Sensitivity analyses with imputations, including all 138 infants, confirmed the main analysis as well as analyses based on fortified BM as exposure. Conclusions: Protein content of BM (native or fortified) is not associated with preterm infant neurodevelopment at 2-year . Higher protein content was associated with a lower birth weight.
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Breast milk protein content at week 3 after birth and neurodevelopmental outcome in preterm infants fed fortified breast milk

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Abbreviations:

ASQ, Ages and Stages Questionnaire

BM, Breast milk

HP, high protein

LP, low protein

MP, medium protein

Declarations

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The authors declare that they have no conflict of interest.

Ethics approval

This research study was approved on 2011 July 19th by the French Committee for the Protection of People Participating in Biomedical Research (CPP - Ouest I - Tours, France).

Consent to participate

All the parents signed consent form at enrolment.

Consent for publication (include appropriate statements)

The manuscript has been read and approved by all authors who have no conflict of interest to disclose.

Availability of data and material

The de-identified form data used in the manuscript will be available to editors upon request either before or after publication for checking.

Code availability (not applicable)

Authors' contributions

BOQUIEN, DARMAUN and ROZE designed research; LEGRAND, BILLARD, JORAM, SIMON, and ALEXANDRE-GOUABAU conducted research; BILLARD provided the database; MOYON and ROZE analyzed data; BOQUIEN and ROZE wrote paper; BOQUIEN had primary responsibility for final content. All authors read and approved the final manuscript.

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ABSTRACT

Background: Feeding supplemented mother milk during hospital stay improves neurodevelopment in preterm infants. Yet the composition of mother milk varies widely between subjects. The relationship between this variation and outcome is unknown.

Objective: To determine whether the protein content in native breast milk (BM) correlates with 2-year infant outcome.

Design: In a monocentric prospective observational study, LACTACOL, preterm infants born between 28 and 34 weeks of gestation, whose mothers decided to exclusively breastfeed, were enrolled during the first week of life. Samples of expressed breast milk obtained at several times of the day were pooled over a 24-hour period, and such pool was used for macronutrient analysis, using mid-infrared analyzer. Age and Stages questionnaire (ASQ) was used to assess 2-year neurodevelopmental outcome. We analyzed the relationship between protein content in BM, and (i) infant neurodevelopment at 2-year (primary outcome), and (ii) growth until 2-year (secondary outcome).

Results: 138 infants were enrolled. The main analysis concerned 130 infants (including 40 twin infants) and 110 mothers with BM samples collected at week 3 after birth. Native BM samples were ranked in 3 tertiles of protein content (g/100ml): 0.91±0.09 (lower), 1.14±0.05 (middle) and 1.40±0.15 (upper); 48, 47 and 35 infants were ranked respectively in these 3 tertiles. Infants in the upper tertile were more often singleton (P = 0.012) and were born with lower birth weight and head circumference Z-scores (P = 0.005 and 0.002; respectively). Differences in weight and head circumference were no longer observed at 2-year. ASQ score at age 2 did not differ the 3 tertiles (P = 0.780). Sensitivity analyses with imputations, including all

138 infants, confirmed the main analysis as well as analyses based on fortified BM as exposure.

Conclusions: Protein content of BM (native or fortified) is not associated with preterm infant neurodevelopment at 2-year. Higher protein content was associated with a lower birth weight.

INTRODUCTION

Preterm infants are a vulnerable population with a higher risk of neonatal mortality and impaired neurodevelopment [1]. The health benefits of breast milk (BM) for premature infants are well accepted [2]. Breastfed preterm infants are better protected from necrotizing enterocolitis [3], an often-fatal disease, and present improved tolerance to enteral feeding [4-6].

Breast milk composition varies with many parameters including gestational age at delivery, gestational diabetes, maternal obesity, stage of lactation, or mother's diet [7-9]. Multiple factors besides variation in breast milk composition, such as sepsis, lung immaturity, etc., obviously determine inter-subject variations in infant growth and neurodevelopment. Although still debated [3], there is overall general consensus regarding the benefit of BM for preterm infants on neurodevelopment. This is based on at least 3 observational studies comparing (i) 1035 extremely preterm infants receiving breastmilk *vs.* no breastmilk during hospitalization in an American multicenter study [10]; (ii) 180 extremely preterm infants receiving more than 50% enteral intake as breastmilk *vs.* less than 50% during the 28 first days after birth in a monocentric Australian cohort [11]; (iii) 2163 very preterm infants who were breastfed *vs.* not breastfed at discharge in a French nationwide population-based cohort [12].

An association between growth velocity and optimal infant neurodevelopment has long been demonstrated [13]. The association is, however, unclear for exclusively breastfed preterm infants. Due to their tremendous growth rate, preterm infants have higher protein requirements than term infants. Yet, despite standardized protein milk fortification [14], suboptimal growth during hospital stay is common among preterm infants receiving human milk, contrasting with improved neurodevelopment at 2 or 5

years. Such lack of apparent relationship between early growth velocity and long-term neurodevelopment was termed "*breastfeeding paradox*" [12].

The crucial question how best to manage human milk fortification remains debated among neonatologists, who must choose between: (i) standardized fortification, done regardless of milk composition, and commonly used in our hospital; (ii) targeted fortification, based on a human milk analysis prior to fortification so that each infant always receives the recommended amount of nutrients; or (iii) adjustable fortification, implying a monitoring of infant blood urea nitrogen assay as a surrogate for assessing infant's metabolic response and protein intake adequacy [15].

We hypothesized that preterm infant growth trajectory and neurodevelopment could be linked to native BM composition in macronutrients, particularly protein content. To test our working hypothesis, we set up an observational monocentric cohort of breastfed preterm infants. The aim of this study was to associate protein content of mother's own milk and (i) the 2-year neurodevelopment as the primary outcome, and (ii) growth trajectory (from 0 to 2 years) as the secondary outcome.

SUBJECTS AND METHODS

Cohort design

The LACTACOL cohort (registered as NCT01493063 on www.clinicaltrials.gov) is a single center prospective observational cohort supervised by Nantes University Hospital (Nantes, France). Inclusion criteria were: maternal decision of exclusively breastfeeding; infant born between 28 and 34 weeks of amenorrhea; no major congenital pathology. Clinical characteristics of infants and their mothers were recorded during hospital stay. Pre-gravid Body Mass Index (BMI) was calculated based

on declared maternal weight before gestation. Maternal education level was classified as low (below high school graduation), medium (2 years post high school graduation) or high (beyond 2 years of post-high school graduation).

Human milk collection and macronutrients analysis

Human milk sampling procedure has been previously described in detail by Alexandre-Gouabau *et al* [16,17]. Each mother expressed breast milk manually at home using a Medela manual breast pump (Medela Inc., Etampes, France) and brought back bottles of human milk to the Nantes University hospital where her infant was hospitalized. We tested several procedures to obtain a sample representative both of a 24-h mature milk expression and of the milk actually given to the infant. Several days per week, mothers brought 2 to 4 bottles of breast milk stored at 4°C to the hospital. Nurses sampled 2 mL from each bottle. We compared two procedures of sampling: taking one sample of human milk per day (one sample corresponding to one bottle) several times per week *vs.* one weekly single pool obtained by mixing the 2-4 daily samples (one 2 mL-sample per bottle, recording at what time each milk bottle had been expressed by the mother) for a better representation of a daily milk expression. The second procedure was chosen because the day to day variations were better smoothed. We therefore obtained once a week, a pool representative of a 24-h mature milk expression and of the milk administered to each preterm infant. The whole milk sample was heated for 10 minutes at 50°C in a hot water bath, homogenized with a disruptor (Polytron, Lucerne, Switzerland), aliquoted and kept frozen at -80°C at the human milk biobank (created and approved on 2010 June 24th by the Committee for the Protection of Persons Participating in Biomedical Research - CPP CB-2010-03) until analysis. After sample thawing at room temperature, human milk was sonicated using an ultrasonic vibrator (VCX500; Sonics Materials, Newton,

Connecticut, USA) and milk macronutrients concentration was measured using the MIRIS® analyzer (Miris AB®, Uppsala, Sweden) [18]. In order to standardize milk analysis, we made sure that: (a) calibration of the MIRIS® analyzer had been adjusted using reference methods [18] prior to each analysis; (b) an aliquot of the same Quality Control sample [18] was tested as a standard before each batch of analyses and used to assess the measures robustness; (c) all milk analyses (more than 500) were made by the same single assistant engineer. Protein content used in calculations is the true protein content given by the MIRIS analyzer.

Infant nutrition

Parenteral nutritional supply was recorded on the day of milk sampling (macronutrient intake in g/kg/day). Duration of parenteral nutrition was determined until central or peripheral perfusion withdrawal. For enteral supply, on the day of milk sampling, the nurse recorded on the Case Report Form: volume of each milk delivered per feeding session, nature of milk (mother's own milk, fresh or pasteurized at the local milk bank, or rarely donor BM when mother's own milk was not available at the beginning of hospitalization) and fortifiers used. The fortification was standardized (no human milk analysis prior to fortification) and followed the EPSGHAN recommendations [14]. Protein enrichment started at 2% as soon as the infant was fed with 50 ml/kg/day of BM. Depending on tolerance to enteral feeding, the protein fortifier was gradually increased in 6 days to a final level of 4%. Depending on their availability in our hospital, fortifiers used for protein were either Eoprotine® (Milupa, Domdidier, Switzerland) or FortiPré® (Guigoz, Marne-la-Vallée, France) for lipid, either Liprocil® (Nestlé clinical nutrition, Marne-la-Vallée, France) or Liquigen® (Nutricia, Saint-Ouen, France); and dextrin maltose for carbohydrate. On the day of milk sampling once a week, we calculated enteral intakes (expressed as

macronutrients intake in g/kg/day) representative of the corresponding week considering composition of mother milk, amount of fortifiers and volume of enteral feedings. Calculations were no longer performed when direct breastfeeding was initiated since the actual volume of breastmilk ingested was no longer known. Finally, the sum of enteral and parenteral intake was added to calculate total nutrient intake.

Infant growth

Infant weight, length and head circumference were monitored at weekly intervals from birth to discharge and then at one and 2 years. Z-score was calculated for weight, length and head circumference using LMS parameters derived from Olsen growth charts [19] (from birth to 40 weeks Corrected Gestational Age) and from WHO Child Growth Standards [20] thereafter. We also calculated the change in Z-score between birth and 2-years of corrected age to describe infant growth. The week before discharge, body composition was assessed using air-displacement plethysmography (PEA POD® Infant Body Composition System, COSMED, Rome, Italy) whenever PEA POD® was available. Data are expressed in absolute fat free mass (FFM) at discharge since in earlier work we found FFM to be better associated than fat mass percentage with classic clinical parameters reflecting perinatal growth [21].

Infant neurodevelopment

Infants were followed up until 2 years of age in the regional follow up network [22]. Neurodevelopment assessors were blinded to the protein content of native BM. Neurodevelopmental outcome was determined at 2 years using the Ages and Stages Questionnaire (ASQ) [23] which is a parent-completed questionnaire that assesses development in five domains, namely, communication, gross motor, fine motor,

problem solving, and personal-social skills. The maximal overall ASQ score is 300, a score of <185 is considered as non-optimal and a score > 220 as optimal. Infants with severe abnormal neurodevelopment as severe cerebral palsy leading to an ASQ score abnormally low were excluded from the ASQ analysis.

Study size

The objectives were to assess the impact of the protein content in native BM on (i) neuro-development of the preterm infants at 2 years of age as a primary outcome measure; (ii) growth trajectory of the preterm infants (from 0 to 2 years) as a secondary objective. First, native BMs were ranked in 3 tertiles of protein content. Then infants were attributed to the tertile corresponding to their mother's own milk. Power calculations determined *a priori* that 42 infants per tertile (for a total of 126 infants) would provide 80% power (given Type 1 error [α] of 5% and a 2-sided test) to detect a significant 25 points difference (*i.e*. 0.64 times the expected standard deviation) in the ASQ score between extreme tertiles. Considering potential failures in breastfeeding, missing data and losses to follow-up, the population to be approached was extended to 160 infants.

Statistical tests

First, we divided the population of mothers into 3 tertiles according to the protein content in native BM at week 3 after birth. We compared mother's and infant's characteristics between the 3 tertiles. Secondly, we compared ASQ scores (primary outcome), ASQ sub-scores and growth outcomes at discharge and 2-year (secondary outcomes), between the 3 tertiles of protein content in native BM. Estimated means were compared by ANOVA and percentages by chi-2. Thirdly, we used 2-levels hierarchized generalized linear model considering the exposure of twin

infants to the same mother's own milk when we assessed the relationship between protein content in native or fortified BM at week 3 as a continuous variable and outcomes (ASQ and growth outcomes at 2-year). The first level was represented by mothers, and the second one by infants. Gestational age, birth weight Z-score and sex were included in the model.

Finally, we performed sensitivity analysis using multiple imputations considering all the infants enrolled in our study including those for which data were lacking (either breast milk sample at week 3 or ASQ at 2-year). We generated 25 independent imputed datasets. Estimates were pooled according to Rubin's rule. All analyses were performed using SPSS software (V.25).

Ethics

This research study was approved on July 19th 2011 by the French Committee for the Protection of People Participating in Biomedical Research (CPP - Ouest I - Tours, France). All the parents signed consent form at enrolment.

RESULTS

Flow chart of the LACTACOL cohort

Among the 160 infants that could have been likely enrolled in LACTACOL cohort between October 2011 and April 2016 **(Figure 1)**, 22 infants were excluded: (i) 5 due to infant's disease severity (2 with severe neurological disorders, 1 with a genetic syndrome, 2 deaths), for which milk sampling was discontinued in view of infant's condition, (ii) one for error regarding the gestational age inclusion criteria, and (iii) 16 for lack of sampling (5 for discontinuation of breastfeeding or failure of breastfeeding, 11 for inadequate milk sampling). Among these 138 infants, none suffered

198 necrotizing enterocolitis, 4 infants had retinopathy of prematurity of mild severity, 3
199 presented intraventricular hemorrhage of grade 2, 8 displayed bronchopulmonary
200 dysplasia at 28 days and 6 at 36 weeks' postmenstrual age. One hundred and
201 eighteen mothers corresponded to these 138 infants (40 twin infants).

202 **Breast milk collection**

203 Due to the length of hospital stay, most milk was collected from week 2 to 6: less
204 than 50% of 118 human milks were collected after week 6. Week 3 corresponded to
205 the week where the majority of pools were collected and also the time period on
206 which we already focused our preliminary studies on milk composition [16,17]. At
207 week 3, only 8 milk samples were lacking, leading to a total of 110 milk samples
208 available. This week 3 therefore was selected to address the association between
209 protein content in native BM, infant nutrition, and infant growth and
210 neurodevelopment outcome. The 110 milk samples at week 3 corresponded to 110
211 mothers and 130 infants (including 40 twin infants).

212 **Clinical characteristics of the mother - infant dyads with known protein content**
213 **in native BM at week 3**

214 The 110 mothers were 30.7±4.9 (mean ± SD) years old at delivery and had a pre-
215 gestational BMI of 23.3±4.8 kg/m^2. Gestational age at birth was 31.3±1.7 weeks. The
216 130 infants, 59 female and 71 male, had a birth weight of 1494±336 g, a length of
217 39.9±2.9 cm, and a head circumference of 27.9±2.1 cm. Corresponding Z-scores
218 were -0.33 ± 1.05, -0.44±1.02, and -0.47±1.00, respectively.

219 **Native milk protein tertile**

220 All the milks were ranked in 3 tertiles according to the protein concentration at week
221 3. The lower, middle and upper tertiles corresponded to the following protein content:

low protein (LP) (1 g/100 mL or less), medium protein (MP) (above 1 g/100 mL and below or equal to 1.2 g/100 mL) and high protein (HP) (strictly higher than 1.2 g/100 mL). At week 3, the numbers of breast milks classified in LP, MP, and HP tertiles were 37, 41 and 32, respectively (Table 1). The numbers of breast milks were not equal between tertiles due to granularity of data.

We verified that the difference in protein content detected at week 3 was already observed at week 2 and persisted through weeks 4 to 6. **Figure 2** presents the time course of native milk protein content with lactation stage from week 2 to week 6. There are 3 parallel trajectories according to the tertile of protein content: although protein content was divided in 3 tertiles based on week 3 samples, the separation between tertiles persisted with time: LP tertile corresponded to the lower curve, MP to the intermediate curve and HP to the higher curve (differences between the 3 tertiles were significant: $P < 0.001$ at week 2, 4, and 5, and $P = 0.001$ at week 6). At week 6, mean protein content in native BM (±SD) was 0.8±0.2 g/100ml (n=16), 0.9±0.1 g/100ml (n=24), and 1.1±0.3 g/100ml (n=20) for LP, MP, and HP tertile, respectively.

Clinical characteristics of mother and infants dyads in the 3 tertiles of protein content in native BM

Main analysis of clinical outcomes was thus done based on a population of 130 infants including 40 twin infants (110 mothers). Infants were ranked in LP, MP, and HP tertiles according to the protein content of their mothers' own native milk at week 3. The numbers of infants classified in LP, MP, and HP tertiles were 48, 47 and 35, respectively.

Mothers did not differ in age, pre-gestational Body Mass Index, birth term or education level between the 3 tertiles (Table 1). Parity was not significantly different (P = 0.111). A higher percentage of twin infants was observed in LP tertile (P= 0.012). Remarkably, even though gestational age did not differ between tertiles of protein content in native BM, the LP tertile corresponded to infants with the highest values for birth weight while MP tertile corresponded to intermediate values and HP tertile to lowest values (P <0.001) (Table 1). Similarly, a gradual decrease was observed from LP to MP and then HP, regarding birth head circumference Z-score (P=0.002).

Infant nutrition depending on the 3 tertiles in protein content

After fortification, the difference in protein content between the LP and HP tertiles was still 0.5 g/100ml (Table 2). The duration of parenteral nutrition differed significantly between the 3 tertiles (P = 0.011), with a lower value for LP followed by MP and HP. We observed a gradual increase in total (parenteral and enteral) protein intake, from LP to HP tertile (P <0.001) (Table 1). Total energy intake and the total volume administered which was the sum of parenteral and enteral intake were similar between the 3 tertiles at week 2 3 (Table 1).

Growth trajectory and breastmilk protein exposure

At discharge, weight and length Z-score significantly differed with a gradual increase from LP to HP (P = 0.006 and 0.001, respectively) (**Table 2**). In contrast, head circumference Z-scores were not different anymore (P = 0.983). Similarly, fat free mass (g) did not differ between the 3 tertiles. The length of hospital stay differed significantly between the 3 tertiles (P = 0.004), with a gradual increase from LP to MP and HP (Table 2).

At 2-year, no difference was observed between protein tertiles whatever the growth outcome (Table 2). When calculating the change in Z-score between birth and 2-year of age, no difference was observed for weight and length. In contrast, the delta head circumference Z-score increased from LP tertile to HP tertile (P = 0.037) (Table 2). We then assessed the relationship between protein content as a continuous variable and outcomes. When delta Z-score for weight, length or head circumference between birth and 2-year was adjusted for gestational age, birth weight Z-score and sex, no association was found with protein content in human milk at week 3 before fortification (**Table 3**) or after fortification (**Table S1**).

Infant neurodevelopment at 2 years

ASQ was known for 115 infants. One patient had a severe cerebral palsy and was excluded from the analysis (Table 2). No significant difference in ASQ score was observed at 2 years between the 3 tertiles (*P* = 0.780 - Table 2). Suboptimal neuro-development was observed in one infant in LP tertile (none in MP and HP). Considering the 5 areas in the parent-completed questionnaire, no significant difference was observed between the 3 tertiles except a trend towards a difference in problem-solving abilities (*P* = 0.056 - Table 2).

When ASQ or the 5 domains of the parent-completed questionnaire were adjusted for gestational age, birth weight Z-score and sex, no association was found with protein content in human milk at week 3 before fortification (**Table 3**) or after fortification (**Table S1**).

Additional analysis

Two additional analyses were done. The first one consisted of performing analysis among the 90 infants from singleton pregnancy, among the 130 infants for which

protein content was known at week 3. The distribution was 26, 35, 29 infants in the LP, MP, and HP tertiles, respectively. Higher protein content in native BM was again associated with a lower birth weight: values of Z-score at birth were -0.08, 0.02, -0.95 for birth weight (*P*=0.001), -0.34, -0.12, -0.93 for length (*P*=0.006) and -0.20, -0.22, -1.03 for head circumference (*P*=0.002), for LP, MP, and HP tertiles, respectively. The second analysis consisted of a sensitivity analysis by performing multiple imputations enrolling all 138 infants, including the 8 infants who received a breast milk for which protein content was unknown at week 3 (Figure 1). Protein content at week 3 was imputed to 8 infants and ASQ score to 24 infants. These analyses confirmed the absence of a significant association between total ASQ score and protein content in native BM at week 3 (*P*=0.38 before adjustment, *P* =0.41 after adjustment for gestational age, birth weight Z-score and sex).

DISCUSSION

The primary aim of the current study was to determine whether the protein content in BM correlates with neurodevelopment and early growth in a cohort of preterm infants receiving their own mother's milk. The major findings of the current study are: 1) the lack of association of 2-year neurodevelopmental outcome with breastmilk protein content either before or after fortification; and 2) the higher protein content in native BM from mothers who delivered a baby with a lower birthweight, regardless of gestational age, sex and twin birth.

The inverse association between birthweight and protein content in native BM is intriguing. The percentage of twin infants, higher in the LP tertile (corresponding to the heaviest infants), cannot explain the inverse association between birthweight and protein content in native BM. Protein content has been negatively associated with

317 milk volume [24], which could be an effect of “milk dilution”. In term infants, milk
318 intake is significantly higher in neonates born to multiparous mothers than in those
319 born to primiparous mother [25] and this higher intake was suggested to compensate
320 for lower milk protein content. In contrast, during most of the hospital stay, early milk
321 intake in preterm infants is only determined by the volume of enteral fluid prescribed
322 and such compensation does not happen. Alternatively, fetal growth rate *per se* may
323 impact mammary gland development and early milk secretion. It is well-known that
324 several placental hormones mediate maternal adaptation to pregnancy but also to
325 lactation acting on mammary gland development and function [26]. Distinct hormone
326 profiles have been described between large and small for gestational age fetuses
327 [27]. Similarly, in term infants, fetal gender may “program” mammary gland
328 development *in utero*, resulting in a higher energy content in breastmilk obtained
329 from women delivering male infants than in those delivering female infants [28].

330 Two-year neurodevelopmental outcome was assessed by ASQ score. Our first
331 objective was to detect a difference of at least 25 points (0.64 SD) of this score
332 between extreme tertiles of protein concentration determined in native BM at the end
333 of hospitalization, when breastfeeding is exclusive. We observed a difference of only
334 2 points (*i.e.* 0.06 SD). We did not observe any significant impact of protein content in
335 native or fortified BM on neurodevelopment. ASQ score is based on a parent
336 questionnaire routinely used in our regional follow-up network [22] and validated by
337 comparison with developmental quotient at 2 years [23]. Moreover, it has been used
338 recently to evaluate neurodevelopmental outcome in a French nationwide cohort [29].

339 Growth parameters (weight, length, and head circumference) were similar between
340 the 3 tertiles at two years of age whereas at birth the premature infants of the HP

tertile were smaller for head circumference. Thus, the growth rate of head circumference was significantly higher in the HP tertile; this relationship, however, was no longer significant after adjustment for neonatal characteristics. Similarly, we did not observe any difference in fat free mass at discharge between tertiles. However, the size of these tertiles should make us cautious in the interpretation of this result. Overall, in this prospective study using standardized fortification, we therefore did not observe any significant relationship between growth and protein content in native or fortified BM.

To classify human milks, we used breast milk sample at week 3. This choice is relevant because (i) this week corresponded to the highest number of milk samples in our cohort; (ii) the time course of protein content over lactation time is parallel for the 3 tertiles (Figure 2); and (iii) since protein content declines until the end of third week after birth and then remains nearly constant between 4 and 9 weeks [30], the widest discrepancies in protein content are observed before week 3. When comparing our data to the meta-analysis of Gidrewicz and Fenton [7], the decline in protein content in the LACTACOL cohort was faster over lactation time. The reason for such discrepancy is unclear. In the LACTACOL cohort, all samples were analyzed by a single assistant engineer to avoid inter-observer variation. Moreover, calibration of the mid-infrared analyzer had been adjusted for human milk with both low and high protein content [18]. Regarding infant nutrition, our values of protein and calories intakes received during week 3 were also similar for infants of the HP tertile in our cohort to those reported in previous studies [31,8].

Limitations of this study include the population sample, as the proportion of mothers with a higher education enrolled in our cohort (42%) was higher than the equivalent

proportion for not breastfeeding mothers in the comparable nationwide French cohort EPIPAGE of preterm infants (24.5%) [12]. This was inevitable since only mothers who decided to breastfeed their infants were eligible for recruitment, and intent to exclusively breastfeed has long been shown to be positively associated with the level of maternal education [32]. Another limitation of the study is its limited power, although the observed power of the study was able to detect a difference of 21 points in the ASQ score (i.e. a higher power than initially calculated *a priori* due to a lower-than-expected rate of follow-up loss despite the lower number of infants enrolled in the study). A third limitation of the study is the reliance on ASQ at 2 years to assess neurodevelopmental outcome. Although the 2-year ASQ score is a significant predictor for 5-year school difficulties [33], it may be not precise enough to detect subtle differences in neurodevelopment. Poor postnatal head growth in preterm infants becomes more evident by 2 years and is strongly associated with poor neurodevelopmental outcome [34]. No significant difference in neuro-development scores was observed between the 3 tertiles. All the LACTACOL infants will be followed up until 6 years of age by our regional network LIFT of preterm infants [22] and this should facilitate the detection of subtle differences in neurological development in a cohort of children with a normal outcome at 2 years.

The current study focused on long term neurodevelopment as a primary outcome showed that protein content in native BM was higher in infants with a lower birthweight, who have the higher nutrient requirement. One could therefore argue that protein fortification of breastmilk may not need to be individualized. However, a better quantitative and qualitative growth during hospitalization was observed after individualized fortification in a double-blind randomized study [35]. This was not the case in an earlier study [36]. Neither of these assessed the effect on

neurodevelopment, as follow up was not extended beyond the end of neonatal hospitalization. Thus, multi-centric, randomized controlled trials with a long-term infant follow-up would clearly be warranted to conclude whether standard or individualized fortification of human milk is preferable.

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TABLE 1 - Clinical characteristics of mothers and infants at birth, according to the 3 native BM protein tertiles [a]

	Native Breastmilk Protein tertile			*P* [b]
	Low Protein	**Medium Protein**	**High Protein**	
Native Breast Milks	*n*=37	*n*=41	*n*=32	
Protein content at week 3 (g/100mL)	0.91±0.09	1.14±0.05	1.40±0.15	**<0.001**
Mothers	*n*=37	*n*=41	*n*=32	
Age	30.2 ± 4.3	30.2 ± 4.4	31.8 ± 6.0	0.166
BMI before pregnancy	23.1 ± 4.7	23.2 ± 4.0	23.6 ± 5.9	0.669
Gestational age (week)	31.6 ± 1.6	31.1 ± 1.9	31.1 ± 1.4	0.255
Primiparous [*n* (%)]	11 (30)	21 (51)	16 (50)	0.111
Education level [*n* (%)] [c]				0.473
Low Education level	9 (24)	11 (27)	4 (13)	
Medium Education level	12 (32)	17 (41)	15 (47)	
High Education level	16 (43)	13 (32)	13 (41)	
Infants	*n*=48	*n*=47	*n*=35	
Female [*n* (%)]	24 (50)	20 (43)	15 (43)	0.721
Twin infants	22 (45.8)	12 (25.5)	6 (17.1)	**0.012**
Birth weight (g)	1606 ± 297	1511 ± 371	1317 ± 263	**<0.001**
Birth length (cm)	40.6 ± 2.5	40.2 ± 3.2	38.6 ± 2.6	**0.003**
Birth head circumference	28.5 ± 1.8	28.0 ± 2.4	27.0 ± 1.6	**0.001**
Birth weight Z-score	-0.11 ± 0.89	-0.22 ± 1.06	-0.78 ± 1.12	**0.005**
Birth length Z-score	-0.33 ± 0.85	-0.29 ± 1.09	-0.78 ± 1.07	0.065
Birth head circumference Z-score	-0.25 ± 0.78	-0.34 ± 1.16	-0.94 ± 0.89	**0.002**

[a] Mean ± SD [*n*[(all such values)

[b] *P* calculated by ANOVA test for means and by chi-2 for percentages

[c] Low (below high school graduation), medium (2 years post high school graduation) or high (beyond 2 years of post-high school graduation);

TABLE 2 - Infant nutrition at week 3, growth outcomes at discharge and 2-year, and ASQ at 2-year of corrected age, analyzed by tertile of protein content in native BM at week 3 [a]

	Native BM Protein tertile			*P* [b]
	Low Protein	**Medium Protein**	**High Protein**	
Number of infants	n=48	n=47	n=35	
Estimated protein content at week 3 in fortified BM (g/100mL)	1.46 (0.34)	1.53 (0.34)	1.91 (0.38)	**<0.001**
Parenteral nutrition duration (days)	6.5±5.4	7.9±7.7	12.7±10.9	**0.011**
Infant nutrition at week 3 [c]	*n*=32	*n*=27	*n*=28	
Fluid volume ml/kg/day	154 ± 21.6	149 ± 21	150 ± 19	0.382
Total protein g/kg/day	2.4 ± 0.5	2.7 ± 0.5	2.9 ± 0.6	**<0.001**
Total energy kcal/kg/day	128 ± 21	124 ± 26	132.8 ± 19	0.430
At discharge	*n*=48	*n*=47	*n*=35	
Hospital length of stay (days)	42.8 ± 14.9	47.9 ± 14.6	53.2 ± 18.6	**0.004**
Fat free mass (g) [n]	2124 ± 297 [25]	2115 ± 266 [27]	2018 ± 19 [19]	0.202
Weight Z-score	-1.04 ± 0.68	-1.26 ± 0.84	-1.49 ± 0.60	**0.006**
Length Z-score	-1.52 ± 0.78	-1.73 ± 0.81	-2.13 ± 0.70	**0.001**
Head circumference Z-score	-0.44 ± 0.89	-0.36 ± 0.70	-0.45 ± 0.63	0.983
At 2-year of corrected age	*n*=45	*n*=42	*n*=31	
Post-natal age (months)	24.9 (1.9)	24.7 (3.7)	25.3 (0.7)	0.652
Corrected age (months)	22.7 (1.9)	22.5 (3.6)	23.0 (0.6)	0.709
Weight Z-score	-0.08 ± 0.96	-0.08 ± 0.90	-0.23 ± 1.05	0.775
Length Z-score	0.23 ± 1.06	0.38 ± 0.94	-0.07 ± 0.79	0.152
Head circumference Z-score	0.77 ± 1.12	0.75 ± 0.86	0.84 ± 1.06	0.930
Growth from birth to 2-year	*n*=45	*n*=42	*n*=31	
Delta weight Z-score	0.05 ± 0.92	0.14 ± 1.12	0.40 ± 1.38	0.406

Delta length Z-score	0.57 ± 1.12	0.66 ± 1.01	0.65 ± 1.26	0.935
Delta head circumference Z-score	1.05± 0.99	1.04± 1.04	1.66± 1.25	**0.037**
Neurodevelopment at 2-year	*n*=44	*n*=42	*n*=29	
Cerebral palsy	1 (2%)	0 (0%)	0 (0%)	_
ASQ questionnaire exploited	*n*=43	*n*=42	*n*=29	
ASQ total score	248 ± 28	246 ± 31	250 ± 23	0.780
Communication sub-score	49 ± 10	45 ± 15	48 ± 11	0.306
Gross motor skills sub-score	52 ± 9	51 ± 8	53 ± 6	0.744
Fine motor skills sub-score	53 ± 8	53 ± 7	56 ± 5	0.165
Problem solving abilities sub-score	46 ± 10	48 ± 10	51 ± 6	0.056
Personal social skills sub-score	48 ± 9	49 ± 9	47 ± 8	0.804

a Mean ± SD [n] (all such values)

[b] *P* calculated by ANOVA test for means and by chi-2 for percentages

[c] Intake in fluid volume (parenteral and enteral intakes), total protein, and energy was calculated as the sum of:

- Parenteral feeding as prescribed by the clinician;
- Enteral feeding which was calculated taking into account the volume of milk given, the fortifier added and its composition in macronutrient, and the milk composition in macronutrients. No calculation was done when direct breastfeeding began;

ASQ, Ages and Stages Questionnaire

TABLE 3 - Growth outcomes and ASQ at 2-year of corrected age, analyzed with protein content in native BM at week 3 as a continuous variable, after adjustment for gestational age, sex, and birth weight Z-score[a]

	n	*β*	*95% CI*	*P*
Growth from birth to 2-year of corrected age				
Delta weight Z-score	118	0.3	[-0.5, 1.2]	0.439
Delta length Z-score	118	0.2	[-0.9, 1.3]	0.732
Delta head Circumference Z-score	118	0.7	[-0.2, 1.5]	0.133
ASQ questionnaire at 2-year of corrected age				
ASQ total score	114	14.3	[-17.1, 45.8]	0.371
ASQ communication sub-score	111	-1.0	[-12.1, 10.2]	0.866
ASQ gross motor skills sub-score	111	5.1	[-2.7, 12.8]	0.197
ASQ fine motor skills sub-score	111	3.4	[-4.7, 11.5]	0.413
ASQ problem solving abilities sub-score	111	7.5	[-1.6, 16.6]	0.105
ASQ personal social skills sub-score	111	2.7	[-5.7, 11.3]	0.524

[a] We used a 2-levels hierarchized generalized linear model considering the exposure of twin infants to the same mother's own milk. The first level was represented by mothers, and the second one by infants.

ASQ, Ages and Stages Questionnaire

Legend for figures

Figure 1 - Flowchart of infants enrolled in the study (adapted from [16]). On each week during infant hospitalization, the sample of expressed breast milk was pooled over a 24-hour period, and used for macronutrient analysis.

Figure 2 - Mean (95% confidence interval of the mean) for true protein content (in g/100 mL) in native human milk for the 3 tertiles during lactation ($P < 0.001$ at each week except at week 6 where $P = 0.001$).

Supplementary Table S1 - Growth outcomes and ASQ at 2-year of corrected age, analyzed with estimated protein content in fortified BM (after supplementation) at week 3 as a continuous variable, after adjustment for gestational age, sex, and birth weight Z-score[a]

	n	*β*	*95% CI*	*P*
Growth from birth to 2-year of corrected age				
Delta weight Z-score	118	0.1	[-0.04, 0.2]	0.726
Delta length Z-score	118	-0.2	[-0.7, 0.3]	0.379
Delta head Circumference Z-score	118	0.4	[-0.2, 0.9]	0.212
ASQ questionnaire at 2-year of corrected age				
ASQ total score	114	4.7	[-8.1, 17.5]	0.468
ASQ communication sub-score	111	-1.8	[-8.0, 4.5]	0.578
ASQ gross motor skills sub-score	111	2.0	[-2.2, 6.2]	0.345
ASQ fine motor skills sub-score	111	3.3	[-2.8, 3.5]	0.839
ASQ problem solving abilities sub-score	111	2.4	[-2.7, 7.5]	0.362
ASQ personal social skills sub-score	111	-0.4	[-4.2, 3.5]	0.849

[a] We used a 2-levels hierarchized generalized linear model considering the exposure of twin infants to the same mother's own milk. The first level was represented by mothers, and the second one by infants.

ASQ, Ages and Stages Questionnaire

Figure 1

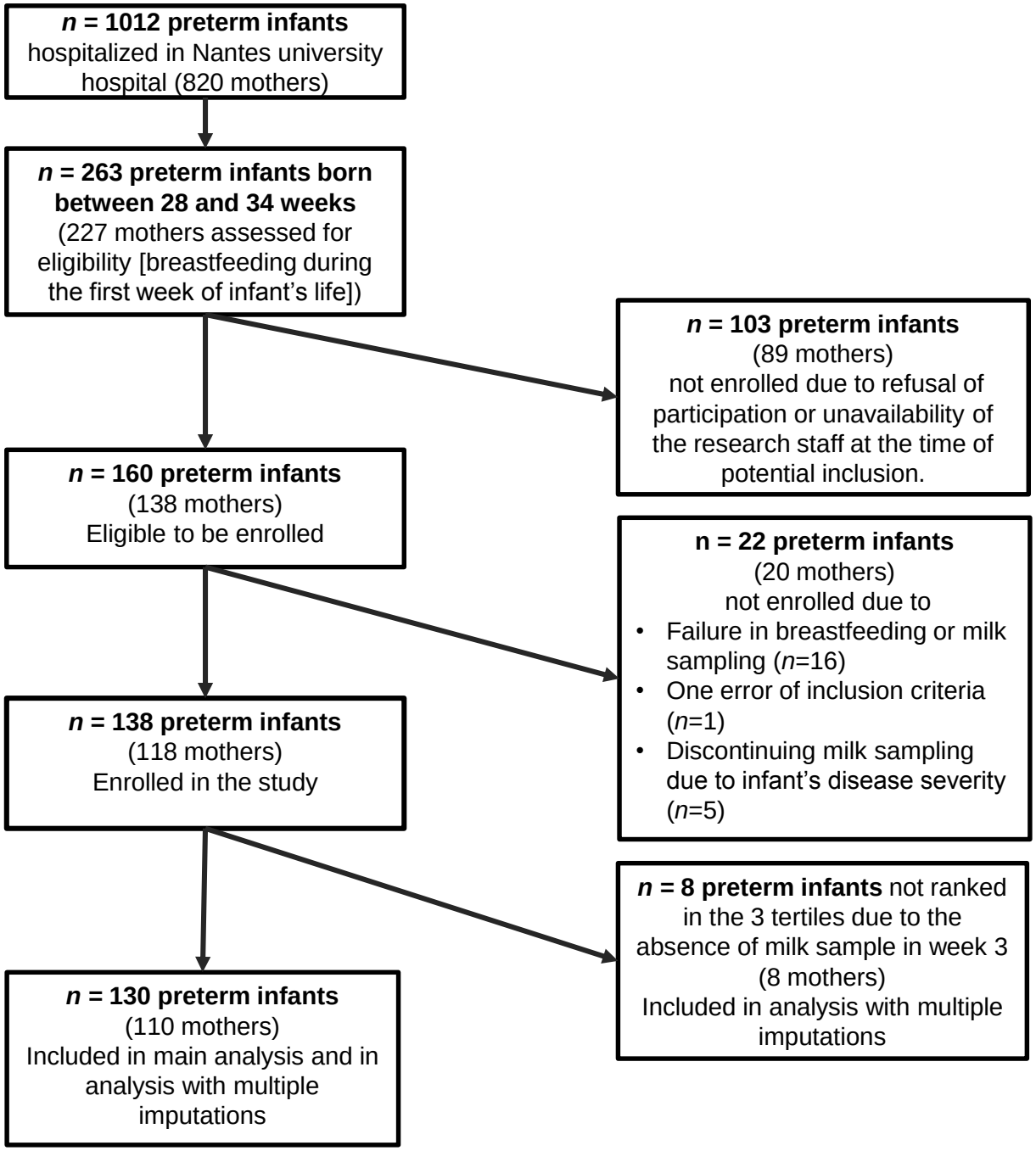
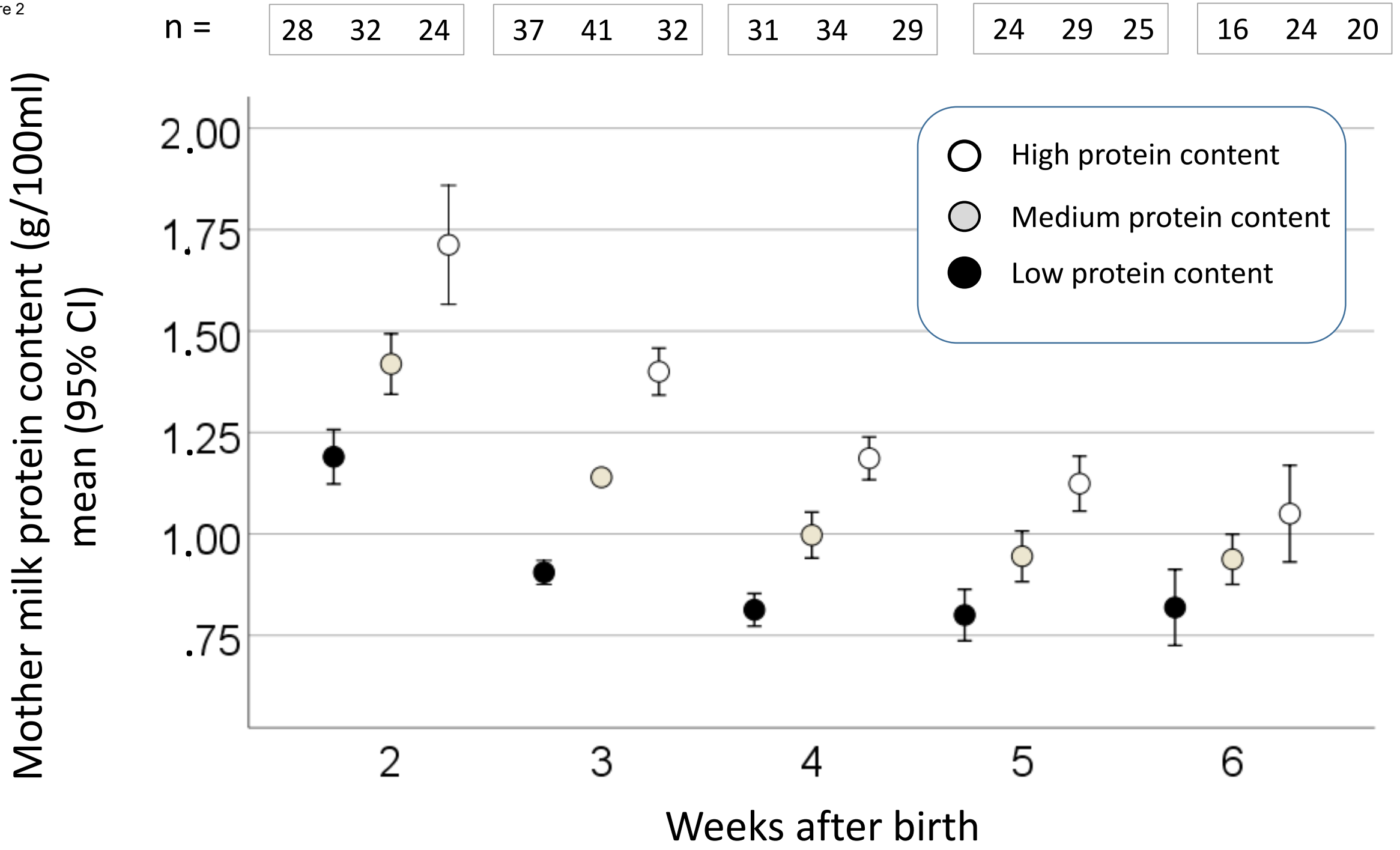

***n* = 1012 preterm infants**
hospitalized in Nantes university hospital (820 mothers)
***n* = 263 preterm infants born between 28 and 34 weeks**
(227 mothers assessed for eligibility [breastfeeding during the first week of infant's life])
***n* = 103 preterm infants**
(89 mothers)
not enrolled due to refusal of participation or unavailability of the research staff at the time of potential inclusion.
***n* = 160 preterm infants**
(138 mothers)
Eligible to be enrolled
n = 22 preterm infants
(20 mothers)
not enrolled due to
• Failure in breastfeeding or milk sampling (*n*=16)
• One error of inclusion criteria (*n*=1)
• Discontinuing milk sampling due to infant's disease severity (*n*=5)
***n* = 138 preterm infants**
(118 mothers)
Enrolled in the study
***n* = 8 preterm infants** not ranked in the 3 tertiles due to the absence of milk sample in week 3
(8 mothers)
Included in analysis with multiple imputations
***n* = 130 preterm infants**
(110 mothers)
Included in main analysis and in analysis with multiple imputations

Figure 2


n =
28 32 24
37 41 32
31 34 29
24 29 25
16 24 20
Mother milk protein content (g/100ml)
mean (95% CI)
2.00
1.75
1.50
1.25
1.00
.75
2
3
4
5
6
Weeks after birth
High protein content
Medium protein content
Low protein content

Cover letter

Version with apparent corrections