Long-chain polyunsaturated fatty acids and lipid peroxidation products in donor

human milk in the U.K.: Results from the LIMIT two-centre cross sectional study

Isabell Nessel, MSc[1*], Laura De Rooy, MB ChB, FRCPCH[2], Minesh Khashu, MBBS, MD,

FRCPCH[1,3], Jane L Murphy RD, RNutr, PhD[4], Simon C Dyall, PhD[5]

[1] Centre for Midwifery, Maternal and Perinatal Health, Faculty of Health & Social

Sciences, Bournemouth University, Bournemouth, U.K.

[2] Neonatal Unit, St George's University Hospitals NHS Foundation Trust, London,

U.K.

[3] Neonatal Unit, Poole Hospital NHS Foundation Trust, Poole, U.K.

[4] Ageing and Dementia Research Centre, Faculty of Health & Social Sciences,

Bournemouth University, Bournemouth, U.K.

[5] Department of Life Sciences, University of Roehampton, London, U.K.

Corresponding author:

Isabell Nessel

inessel@bournemouth.ac.uk

Bournemouth University, Bournemouth, BH13LT, U.K.

+441202965009

Abstract

Background: Donor human milk is increasingly used as alternative to mother's own milk to feed preterm infants, however, it may provide less long-chain polyunsaturated fatty acid (LCPUFA), and more oxidised lipids, which may be detrimental for preterm infant health and development. Levels have not been reported for donor human milk in the U.K.

Methods: Donor human milk (n=19) from two neonatal units, milk from preterm mothers from a neonatal unit (n=10), and term mothers from the community (n=11) were analysed for fatty acid, malondialdehyde, 4-hydroxy-2-nonenal, and hexanal content. Study registration: NCT03573531

Results: Donor human milk had significantly lower absolute LCPUFA content compared to term milk *(P<0.001)* and significantly lower omega-3 PUFAs than preterm milk *(P<0.05)*, although relative LCPUFA composition did not differ. Exclusive donor human milk feeding leads to significantly lower fat (3.7 *vs*. 6.7 g/d) and LCPUFA (DHA: 10.6 *vs*. 16.8 mg/d; ARA: 17.4 *vs*. 25.2 mg/d) intake than recommended by ESPGHAN, and provides only 17.3% and 43.1% of the *in utero* accreted ARA and DHA. Donor human milk also had the highest proportion of lipid peroxidation.

Conclusions: This study confirms that donor human milk in the U.K. has insufficient levels of LCPUFAs for preterm infants. It demonstrates for the first time that donor human milk has the highest level of lipid peroxidation, compared to preterm or term milk. This has important implications for preterm infant nutrition, as exclusive donor human milk feeding might not be suitable long-term, and may contribute to the development of major preterm neonatal morbidities.

Clinical Relevance Statement

Donor human milk is used to feed preterm infants; however, milk banking practices may negatively affect nutritional quality. This study measured for the first time fat, long-chain polyunsaturated fatty acid (LCPUFAs), and lipid peroxidation in donor human milk in the U.K., and compared them to breast milk from preterm and term mothers. The preliminary results reiterate that donor human milk as the sole nutritional source for preterm infants provides fat and LCPUFAs significantly below ESPGHAN recommendations. Furthermore, lipid peroxidation was significantly higher in donor human milk than term breast milk. This may be due to milk banking practices, and warrants further study, as it is well known that such peroxidation can contribute to neonatal morbidities like necrotising enterocolitis, and retinopathy of prematurity.

1. Introduction

Docosahexaenoic acid (22:6n-3, DHA) and arachidonic acid (20:4n-6, ARA) are omega-3 and omega-6 long-chain polyunsaturated fatty acids (LCPUFAs), respectively, essential for optimal visual and brain development, and immune system function[1]. The last trimester is the period of maximal brain growth, and an interrupted maternal-foetal LCPUFA transfer, low adipose tissue reserves, limited conversion of LCPUFAs from precursor fatty acids, and low provisions of preformed LCPUFAs put preterm infants at an increased risk of ARA and DHA deficiencies[2]. It is therefore essential to ensure adequate postnatal provision of LCPUFAs[3].

Breast milk (BM) provides ARA and DHA, and BM from mothers of preterm infants has higher DHA levels than that of term mothers[4]. However, producing an adequate BM supply is three times less likely in preterm mothers[5], and donor human milk (DHM) from a human milk bank is recommended[6]. The use of DHM is growing world-wide due to reported benefits, such as reduced necrotising enterocolitis[7] and bronchopulmonary dysplasia[8]. However, human milk banking practices, such as prolonged storage at -20°C, thawing in the refrigerator, and frequent container changes, may decrease total fat content and LCPUFAs[9]. The LCPUFA content of DHM is not well studied, and unknown in the U.K. Variations in LCPUFA content of DHM have been shown in the U.S.A. depending on the milk bank location, and levels may fall below preterm infant recommendations[10]. DHM in the U.S.A. is pooled from several donors[11], whereas in other countries, including the U.K., it is not pooled[12]. Since LCPUFA levels are highly dependent on maternal nutrition, age, length of lactation, and genetic variation, non-pooled DHM potentially has greater variability in LCPUFA levels[13].

LCPUFAs are susceptible to lipid peroxidation[14] by oxygen radicals. Lipid hydroperoxides are primary lipid peroxidation products, which react further to form secondary lipid peroxidation products. Malondialdehyde (MDA), the unspecific peroxidation product of PUFAs with more than two double bonds, as well as 4-hydroxy-2-nonenal (HNE), the specific peroxidation product of omega-6 PUFAs, and

hexanal, have been identified in BM[15]. Milk banking practices, such as storage and processing conditions, can increase lipid peroxidation[9]. Preterm infants are prone to oxygen radical associated diseases, such as necrotising enterocolitis (NEC), bronchopulmonary dysplasia and retinopathy of prematurity[16], which may be exacerbated by intake of lipid peroxidation products[17].

The aims of this study are to quantify for the first time absolute and relative LCPUFA content of BM from milk banks provided to neonatal units in the U.K., and compare these values with BM from mothers of preterm and term infants. MDA, HNE and hexanal levels were also measured to explore whether DHM is a source of lipid peroxidation products.

2. Material and Methods

2.1.   Ethics, participants, and samples

This cross sectional two-centre study was performed in the U.K. between December 2018 and February 2019 (www.ClinicalTrials.gov: NCT03573531). The protocol was approved by London-South East Research Ethics Committee (reference 18/LO/1330). 5 mL of unused, bedside DHM was collected in two neonatal units, served by two human milk banks, but as DHM is anonymised informed consent could not be obtained from donors. DHM in the U.K. is always from only one donor (non-pooled), Holder pasteurised, and frozen at -20°C for a maximum of six months from expression[12]. Preterm BM (5 mL) was collected at a neonatal unit, and term BM (5 mL) from the community. Eligible participants were ≥18 years, healthy, and delivered either before or after 37 weeks gestation for preterm and term BM samples, respectively. Exclusion criteria were not understanding study information in English, clinical chorioamnionitis or sepsis, smoking (including e-cigarettes), mastitis, or receiving treatment for (chronic) infection or inflammatory disease. Participants gave informed consent and the study was conducted according to the Declaration of Helsinki. A sample size of 10/20 per group was chosen as pragmatic approach, considering outcome measurements. After collection, samples were frozen at -70°C until analysis, which was within three months.

2.2.   Quantification of fatty acids and total fat content

Lipids were extracted and analysed as described previously[2]. Briefly, tricosanoic acid was added as internal standard to homogenised BM and fatty acids analysed by gas chromatography with flame ionisation detector (Agilent Technologies, 7820A). Since BM contains 98% triacylglycerols[18], the sum of the absolute fatty acid values was used to estimate total fat content.

2.3.   Quantification of MDA

MDA was analysed using a thiobarbiturc acid reactive substances assay (Cayman Chemical) following manufacturer's instructions. Samples were incubated with

trichloric acid and colour reagent and absorbance measured in duplicates at 540 nm. All samples were analysed on the same plate. Absolute MDA levels were adjusted according to previously determined recovery rate of 36.44% (unpublished data), and corrected for estimated total fat content, as fat content differed significantly between samples and may influence MDA concentrations.

2.4.   Quantification of HNE

HNE adducts were quantified using OxiSelect HNE Adduct competitive ELISA Kit (Cell Biolabs, Inc.) following manufacturer's instructions. After coating the plate overnight, samples were added and incubated, before adding anti-HNE antibody. After secondary antibody incubation absorbance was measured at 450 nm, and samples measured on the same plate. HNE adducts may depend on protein content; however, no significant differences were detected using Bradford Reagent, and no correction was performed. Values were corrected for estimated total fat content. Nine samples (six DHM, one preterm BM, two term BM) were above the limit of quantification (LoQ) (200 μg/mL); whereas 12 samples (six DHM, two preterm BM, four term BM) were below LoQ (1.56 μg/mL). To remove potential bias in the results, values above and below LoQ were set at the upper and lower LoQ, respectively, as recommended[19].

2.5.   Quantification of Hexanal

Hexanal was measured using solid phase micro-extraction (SPME)[20]. BM was homogenised and 25% NaCl (w/v) added. After equilibration, headspace volatiles were sampled using a polydimethylsiloxane fiber (100 μm, Supelco) before injection into a gas chromatograph with flame ionisation detector (Agilent Technologies, 7820A) fitted with a merlin micro seal, SPME liner, and J&W HP-5 column. Hexanal peak area was corrected for percentage recovery based on fat content, as suggested[20], and adjusted for estimated total fat content.

2.6.   Statistical analysis

Data is reported according to STROBE guidelines[21]. There was one missing value for hexanal in the DHM group, due to limited sample volume. Grubbs' test was used to detect significant outliers, which were excluded. Statistical analysis was performed using GraphPad Prism (Version 5.0). Data was tested for normality. Participant demographics where compared using a t-test. Pump expression was compared using Fisher's exact test. All other values were compared by One-Way ANOVA with Tukey's multiple comparisons test comparing all groups or Kruskal-Wallis test with Dunn's multiple comparisons test comparing all columns. For correlations, Spearman correlation was used.

Enteral fat, PUFA, and protein supply was calculated for hypothetical 30^{+6} week gestational age infant, weighing 1400 g and receiving full enteral feeding (150 mL/kg/day), and compared to European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) recommended intake levels[22] using a one sample t-test and compared between the groups using t-test or Mann Whitney test. LCPUFAs available for accretion were also calculated for this hypothetical infant as described previously[2], and compared to estimated accretion rates[23] by one sample t-test. For comparisons between groups, t-test or Mann Whitney test was used. Data presented as mean ± SD, and considered statistically significant at $P<0.05$. * indicates $P<0.05$, ** and *** P-values <0.01 and <0.001, respectively.

3. Results

3.1.   Participant and sample characteristics

40 samples were collected: 19 DHM, 10 preterm and 11 term BM samples (Figure 1). Most DHM samples (n = 15; 79%) were collected at St. George's University Hospitals NHS Foundation Trust, and the remaining at Poole Hospital NHS Foundation Trust, thse hospitals are served by two different human milk banks. DHM was pasteurised within three months of expression (81 ± 18.8 days), and stored for an average of 177 ± 5.6 days.

Preterm and term BM donor characteristics are shown in Table S1; DHM donor characteristics cannot be reported, due to anonymization. There was no statistical difference between preterm and term BM donors' age (33.1 ± 5.8 *vs.* 29.5 ± 5.6 years). Infants of preterm BM donors were significantly younger than infants of term BM donors (1.6 ± 1.4 *vs.* 5.6 ± 3.4 months; $P=0.0026$), and there was a significant difference in infants' gestational age at delivery (30^{+6} *vs.* 40^{+4} weeks; $P<0.0001$).

3.2.   BM fat content and composition

Estimated total fat content differed between the groups ($P<0.0001$). DHM had the lowest levels (1.74 ± 0.52 g/100 mL), followed by preterm BM (2.44 ± 0.65 g/100 mL), with both significantly lower than term BM (4.79 ± 1.60 g/100 mL; both $P<0.001$).

Levels of fatty acids expressed as percentage of total fatty acids are shown in Table S2. There were no significant differences in total saturated fatty acids (SFA) or monounsaturated fatty acids (MUFAs), or total omega-3 and omega-6 PUFAs. ARA was significantly higher in preterm than term BM (+0.11%; $P<0.05$), and preterm BM DHA was significant higher than both DHM and term BM (+0.24% $P<0.01$, and +0.22% $P<0.05$, respectively). 20:3n-6, 22:4n-6, and docosapentaenoic acid (22:5n-3, DPAn-3) were also significantly higher in preterm than term BM, and

eicosapentaenoic acid (20:5n-3, EPA), and DPAn-3 were also significantly higher in preterm BM than DHM.

In contrast to percentage composition, absolute total SFAs and MUFAs, and total omega-3 and omega-6 PUFAs were significantly lower in DHM than term BM (all *P*<0.001), Table 1. For individual fatty acids, all except 22:4n-6 were significantly lower in DHM than term BM. Total omega-3 PUFAs were significantly lower (*P*<0.05) in DHM than preterm BM. 18:3n-6, 20:3n-6, ARA, and 22:4n-6 were significantly lower in DHM than preterm BM, although total omega-6 PUFA content was not significantly different. Total SFAs and MUFAs, and total omega-6 PUFAs, but not total omega-3 PUFAs, were significantly lower in preterm than term BM. DHM had the lowest ARA, which was significantly lower than preterm and term BM (-6.24 mg/100 mL and -11.16 mg/100 mL; *P*<0.01 *and P*<0.001*, respectively*). DHA was significantly lower in DHM than preterm and term BM (-9.95 mg/100 mL and -13.69 mg/100 mL; *P*<0.05 and *P*<0.001, respectively).

3.3. Estimated PUFA and protein intake from DHM and preterm BM

Preterm infants in this study were born at 30^{+6} weeks gestational age, and to provide an estimate of potential daily enteral fat, PUFA, and protein intake from DHM and preterm BM, values were calculated for a hypothetical preterm infant of 1400 g bodyweight assuming full enteral feeding (210 mL), and values were compared to ESPGHAN recommendations[22]. DHM provided significantly lower fat than recommended by ESPGHAN (3.67 ± 1.06 g/d *vs.* 6.72 g/d; *P*<0.0001), whereas preterm BM achieved recommended levels (6.0 ± 2.92 g/d). Daily fat intake from preterm BM was significantly higher than from DHM (+2.33 g/d; *P*=0.0095). Similarly, DHM levels of LA, ALA, ARA, and DHA were all significantly below ESPGHAN recommendations (Table 2). Preterm BM levels were above the minimum recommendations, with DHA significantly above, although still below the upper daily limit (42 mg).

Feeding an infant exclusively DHM for seven days would result in ARA and DHA deficits of 55.05 and 43.29 mg/week, respectively, compared to ESPGHAN recommendations. Whereas feeding exclusively preterm BM would provide significantly more ARA (+97.1 mg/week; *P*=0.0012) and DHA (+141.07 mg/week; *P*=0.0048), and achieve recommendations[22].

DHM and preterm BM provide protein levels significantly below the ESPGHAN recommendations (1.96 ± 0.41 g/d and 2.46 ± 0.71 g/d *vs.* 4.9 g/d; both *P*<0.0001). Exclusive DHM or preterm BM for seven days results in deficits of 20.58 and 17.08 g/week protein, respectively. Protein intake from DHM is significantly below preterm BM (-0.5 g/d; *P*=0.0293).

3.4. Estimated LCPUFAs available for accretion

ARA and DHA available for accretion from DHM and preterm BM were calculated for the hypothetical infant. Full enteral feeding with DHM or preterm BM provides 51.44 ± 4.80 and 62.68 ± 11.25 mg/d of ARA, respectively, significantly below estimated *in utero* accretion rates[23] of 296.8 mg/d, by 82.7% and 78.9%, respectively (both *P*<0.0001). After one week of exclusively DHM or preterm BM feeding deficits of 1717.53 mg and 1638.12 mg, respectively, occur, with ARA significantly higher in preterm BM than DHM (*P*=0.0012). DHA from DHM and preterm BM was also significantly below estimated *in utero* accretion rate (25.97 ± 3.62 and 41.77 ± 13.28 mg/d, at 43.13% and 69.38% of *in utero* accretion of 60.2 mg/d; *P*<0.0001 and *P*=0.0024, respectively), producing deficits of 239.64 mg or 129.03 mg after seven days of full enteral feeding with DHM or preterm BM, respectively. DHA availability is significantly higher from preterm BM than DHM (*P*=0.0048).

3.5. Lipid peroxidation
3.5.1. MDA

Uncorrected MDA content was different between groups (Figure 2A). DHM levels were significantly lower than preterm BM (38.83 ± 11.05 µM *vs.* 55.32 ± 16.54 µM; *P*<0.05), but not than term BM (51.27 ± 19.66 µM). MDA is derived from PUFAs, and

MDA content was therefore corrected for estimated fat content (Figure 2B). After correction for total fat, MDA concentration remained significantly different between groups (P=0.0248); with both DHM and preterm BM levels significantly higher than term BM (23.46 ± 6.28 µM vs. 9.12 ± 2.66 µM, and 21.53 ± 6.83 µM vs. 9.12 ± 2.66 µM, respectively, both P<0.001).

3.5.2. HNE

HNE content did not differ significantly between groups (Figure 3A). When HNE was corrected for fat content, a significant difference was seen (P=0.0482; Figure 3B). DHM was significant higher than term BM (59.99 ± 75.13 µg/mL vs. 8.18 ± 13.14 µg/mL; P<0.05). Preterm BM was not significantly different from the other groups.

3.5.3. Hexanal

There was a significant difference in hexanal between groups (P=0.0091; Figure 4A). DHM was significantly higher than preterm BM (124.5 ± 90.86 mm^2 vs. 34.43 ± 32.85 mm^2; P<0.05), but not term BM. Following correction for fat, there were significant difference between groups (P=0.0009; Figure 4B). DHM levels were significantly higher than both preterm and term BM (67.68 ± 52.40 mm^2 vs. 16.12 ± 19.58 mm^2 and 9.47 ± 6.41 mm^2, respectively, both P<0.01).

3.5.4. Correlations of ARA and DHA with lipid peroxidation products

Absolute ARA and DHA were correlated with uncorrected MDA and hexanal, and ARA with uncorrected HNE content. There were significant positive correlations between both ARA and DHA and MDA (r=0.4700; P=0.0025, and r=0.3587; P=0.0249, respectively). There was no significant correlation between DHA and hexanal or ARA and hexanal and HNE.

4. Discussion and conclusion

This study investigated the LCPUFA and lipid peroxidation product content of DHM provided to two neonatal units in the U.K, and compared these values to preterm and term BM. Significantly lower fat, absolute ARA and DHA and increased lipid peroxidation product levels were identified in DHM, which can have important clinical implications for preterm infant nutrition and health outcomes.

The relative fatty acid percentage composition was consistent with reported values in the U.K.[24], and was not significantly different between DHM and term BM, suggesting milk banking practices do not affect levels. Similarly, a recent systematic review reported the fatty acid composition of DHM is not negatively influenced by milk banking processes[25]. However, when expressing fatty acids in absolute levels, significantly lower SFAs and MUFAs, and omega-3 and omega-6 PUFAs were found in DHM compared to term BM. This dissonance supports the recommendation that both relative and absolute fatty acid composition data should be reported[26].

Preterm BM had higher DHA than term BM, consistent with previous observations[4]. If maternal BM is unavailable, DHM is the recommended alternative[6]. However, significantly lower relative DHA and absolute ARA and DHA were found in DHM compared to preterm BM. Based on the estimated intake of a 1400 g infant this would lead to intake significantly below ESPGHAN recommendations, consistent with analyses of DHM in the U.S.A.[27]. ESPGHAN recommendations consider LA and ALA precursors of ARA and DHA, respectively; however, DHM levels of these were also below recommendations[22].

DHM and preterm BM were also estimated to provide ARA and DHA significantly below *in utero* accretion rates[23]. These deficits are of particular concern for preterm infants, where neurological development and immune system function are underdeveloped[3]. Low ARA and DHA intake are also associated with morbidities of prematurity, such as NEC, bronchopulmonary dysplasia, retinopathy of prematurity, and white matter injury[28]. DHM and maternal BM can be fortified with commercial

fortifiers. However, in the U.K., currently only bovine BM fortifiers for preterm infants are available. Of these, one contains no source of fat and is therefore not a source of LCPUFAs, whereas the other one contains LA, ALA, and DHA, but not ARA. This study strongly supports the use of a commercial fortifier providing both DHA and ARA. However, fortified BM will still provide LCPUFA levels below calculated *in utero* accretion rates, as also described by Harris and Baack[3]. When BM is expressed for donation, or expressed by mothers for their preterm infants, strategies to increase the overall fat content, such as hind milk expression, may also be helpful, since hind milk contains a higher fat content[29].

When controlling for the overall fat content of the different milk, significantly higher levels of MDA, HNE and hexanal were identified in DHM compared to term BM. This study cannot identify if milk banking practices contributed to these higher levels, although these practices have the potential to increase lipid peroxidation. For example, storage at 4°C increases HNE and MDA, storage at -20°C for 60 days increases MDA, and storage at -18°C for three months increases hexanal[9]. Not protecting BM from light has also been demonstrated to increase hexanal levels[30].

The highest proportion of lipid peroxidation products was identified in DHM. More research is needed to define safe upper intake levels for lipid peroxidation products. However, studies suggest ingesting lipid peroxidation products increases inflammation, potentially via activation of the nuclear factor κB signalling pathway[17]. Repeated intake of lipid peroxidation products has been shown to be carcinogenic, and associated with growth retardation, intestinal and cardiovascular diseases. The latest European Medicines Agency recommendation (EMA/PRAC/347675/2019) is to protect parenteral lipid solutions from light to avoid adverse outcomes in neonates due to toxic lipid degradation products, and a similar strategy should be used for DHM. Endogenous antioxidants in BM may provide protective effects; however, milk banking processes may have detrimental effects on their levels[9].

This is the first study to quantify relative and absolute LCPUFAs and lipid peroxidation in DHM, preterm and term BM in the U.K. DHM levels were measured at one time-point, and not over the journey from expression to administration, so the role of milk banking practices can only be inferred. It cannot be discounted that donor BM had low fat and LCPUFAs to begin with. Therefore, further research is needed to confirm our findings and clarify the relative contribution of milk banking to the observed differences between DHM and term BM. In the meantime, we have provided recommendations for human milk banking practices to maximise the LCPUFA levels and maintain nutritional quality of DHM[9].

A strength of the study is that DHM was measured from samples provided by two neonatal units, served by two different human milk banks, giving a clinically relevant perspective rather than mimicking processes in the laboratory. However, these were samples of surplus rather than fresh DHM, as DHM is rare in the U.K. and provision to the infant has priority. The study also used a range of endpoints to measure lipid peroxidation, providing a more complete assessment. A limitation is that the effects of milk banking practices on LCPUFAs and lipid peroxidation products can only be inferred; however, the results provide novel information on the actual levels of LCPUFAs and lipid peroxidation products in DHM that would have been provided to preterm infants.

In conclusion, the results suggest DHM has lower LCPUFA and higher lipid peroxidation than preterm or term milk. Estimated LCPUFA intake provided by DHM is significantly below ESPGHAN recommendations and far below foetal accretion rates. This implies that DHM is not suitable long-term as sole diet for preterm infants, and should be used in conjunction with preterm BM wherever possible. The lower LCPUFA and higher lipid peroxidation product levels may be due to milk banking practices. These results have important implications internationally, as milk banking guidelines and practices are often mirrored across countries. In addition, higher lipid peroxidation levels in DHM may leave preterm infants more vulnerable to common comorbidities. These results highlight a need for further research to

confirm our findings and explore interventions to increase and protect LCPUFAs in DHM and minimise lipid peroxidation.

Acknowledgements

5. References

1. Dyall SC. Long-chain omega-3 fatty acids and the brain: A review of the independent and shared effects of EPA, DPA and DHA. *Frontiers in Aging Neuroscience.* 2015-April-21 2015;7(52).

2. De Rooy L, Hamdallah H, Dyall SC. Extremely preterm infants receiving standard care receive very low levels of arachidonic and docosahexaenoic acids. *Clin Nutr.* 2017;36(6):1593-1600.

3. Harris WS, Baack ML. Beyond building better brains: bridging the docosahexaenoic acid (DHA) gap of prematurity. *J Perinatol.* 2015;35(1):1-7.

4. Bokor S, Koletzko B, Decsi T. Systematic review of fatty acid composition of human milk from mothers of preterm compared to full-term infants. *Ann Nutr Metab.* 2007;51(6):550-556.

5. Hill PD, Aldag JC, Chatterton RT, Zinaman M. Comparison of milk output between mothers of preterm and term infants: the first 6 weeks after birth. *J Hum Lact.* 2005;21(1):22-30.

6. World Health Organization. *Global Strategy for Infant and Young Child Feeding [online].* Geneva: WHO; 2003.

7. Quigley M, McGuire W. Formula versus donor breast milk for feeding preterm or low birth weight infants. *Cochrane Database Syst Rev.* 2014;4:CD002971.

8. Villamor-Martinez E, Pierro M, Cavallaro G, et al. Donor Human Milk Protects against Bronchopulmonary Dysplasia: A Systematic Review and Meta-Analysis. *Nutrients.* Feb 20 2018;10(2).

9. Nessel I, Khashu M, Dyall SC. The effects of storage conditions on long-chain polyunsaturated fatty acids, lipid mediators, and antioxidants in donor human milk - A review. *Prostaglandins, Leukotrienes and Essential Fatty Acids.* 2019;149:8-17.

10. Baack ML, Norris AW, Yao J, Colaizy T. Long-chain polyunsaturated fatty acid levels in US donor human milk: meeting the needs of premature infants? *J Perinatol.* 2012;32(8):598-603.

11. O'Hare EM, Wood A, Fiske E. Human milk banking. *Neonatal Network*. 2013;32(3):175-183.

12. National Institute for Health and Clinical Excellence. *Donor breast milk banks: the operation of donor breast milk bank services [online]*. London: National Institute for Health and Clinical Excellence; 2010.

13. Juber BA, Jackson KH, Johnson KB, Harris WS, Baack ML. Breast milk DHA levels may increase after informing women: a community-based cohort study from South Dakota USA. *Int Breastfeed J*. 2016;12:7.

14. Dyall SC. Methodological issues and inconsistencies in the field of omega-3 fatty acids research. *Prostaglandins Leukot Essent Fatty Acids*. Nov 2011;85(5):281-285.

15. Michalski MC, Calzada C, Makino A, Michaud S, Guichardant M. Oxidation products of polyunsaturated fatty acids in infant formulas compared to human milk--a preliminary study. *Mol Nutr Food Res*. Dec 2008;52(12):1478-1485.

16. Saugstad OD. Oxidative stress in the newborn-a 30-year perspective. *Biol Neonate*. 2005;88(3):228-236.

17. Awada M, Soulage CO, Meynier A, et al. Dietary oxidized n-3 PUFA induce oxidative stress and inflammation: role of intestinal absorption of 4-HHE and reactivity in intestinal cells. *J Lipid Res*. 2012;53(10):2069-2080.

18. Koletzko B. Human Milk Lipids. *Ann Nutr Metab*. 2016;69 Suppl 2:28-40.

19. Duval V, Karlsson MO. Impact of Omission or Replacement of Data Below the Limit of Quantification on Parameter Estimates in a Two-Compartment Model. *Pharm Res*. 2002;19(12):1835-1840.

20. Elisia I, Kitts DD. Quantification of hexanal as an index of lipid oxidation in human milk and association with antioxidant components. *J Clin Biochem Nutr*. 2011;49(3):147-152.

21. von Elm E, Altman DG, Egger M, et al. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement: guidelines for reporting observational studies. *Int J Surg*. 2014;12(12):1495-1499.

22. Agostoni C, Buonocore G, Carnielli VP, et al. Enteral nutrient supply for preterm infants: commentary from the European Society of Paediatric Gastroenterology, Hepatology and Nutrition Committee on Nutrition. *J Pediatr Gastroenterol Nutr*. 2010;50(1):85-91.

23. Lapillonne A, Jensen CL. Reevaluation of the DHA requirement for the premature infant. *Prostaglandins Leukot Essent Fatty Acids*. 2009;81(2-3):143-150.

24. Yuhas R, Pramuk K, Lien EL. Human milk fatty acid composition from nine countries varies most in DHA. *Lipids*. 2006;41(9):851-858.

25. Gao C, Miller J, Middleton PF, et al. Changes to breast milk fatty acid composition during storage, handling and processing: A systematic review. *Prostaglandins Leukot Essent Fatty Acids*. Jul 2019;146:1-10.

26. Brenna JT, Plourde M, Stark KD, Jones PJ, Lin YH. Best practices for the design, laboratory analysis, and reporting of trials involving fatty acids. *Am J Clin Nutr*. 2018;108(2):211-227.

27. Baack ML, Norris AW, Yao J, Colaizy T. Long-chain polyunsaturated fatty acid levels in US donor human milk: meeting the needs of premature infants? *J Perinatol*. Aug 2012;32(8):598-603.

28. Fares S, Sethom MM, Hammami MB, et al. Postnatal RBC arachidonic and docosahexaenoic acids deficiencies are associated with higher risk of neonatal morbidities and mortality in preterm infants. *Prostaglandins Leukot Essent Fatty Acids*. Nov 2017;126:112-116.

29. Mizuno K, Nishida Y, Taki M, et al. Is increased fat content of hindmilk due to the size or the number of milk fat globules? *Int Breastfeed J*. Jul 16 2009;4:7.

30. Vangnai K, Phamornsuwana T, Puhin K, Sribunsua R, Rathanachai S. Oxidative stability of human breast milk during freeze-storage. *Italian Journal of Food Science*. 2017:120-125.

Figures

Figure 1: Participant flow through the study

Preterm BM samples were collected at the Neonatal Unit at St. George's University Hospitals

NHS Foundation Trust, and term BM samples from the community at Bournemouth University.

Figure 2: Absolute MDA concentration of DHM, preterm and term BM (A) and corrected for fat content (B)

Figure 3: Absolute HNE concentration of DHM, preterm and term BM (A) and corrected for fat content (B)

Figure 4: Absolute hexanal concentration of DHM, preterm and term BM (A) and corrected for fat content (B)

Table 1: Absolute fatty acid composition of DHM, preterm and term BM

Fatty acids (mg/100 mL)	DHM	Preterm BM	Term BM	P-value
10:0	$26.53 \pm 9.09^{###}$	$38.19 \pm 11.81^{###}$	$78.05 \pm 29.55^{***}$	<0.0001
12:0	$112.0 \pm 41.09^{###}$	$179.8 \pm 65.90^{*\ ###}$	$315.20 \pm 96.89^{***}$	<0.0001
14:0	$115.1 \pm 37.23^{###}$	$239.1 \pm 125.9^{**}$	$310.3 \pm 111.1^{***}$	<0.0001
16:0	$378.5 \pm 119.2^{###}$	$498.8 \pm 152.2^{###}$	$1154 \pm 585.1^{***}$	<0.0001
18:0	$152.6 \pm 103.3^{###}$	$217.5 \pm 130.6^{#}$	$462.8 \pm 296.1^{***}$	0.0005
20:0	$2.03 \pm 1.36^{##}$	3.07 ± 1.87	$5.29 \pm 3.50^{**}$	0.0024
∑ SFAs	$809.5 \pm 293.2^{###}$	$1145 \pm 341.2^{###}$	$2198 \pm 822.7^{***}$	<0.0001
16:1n-7	$40.28 \pm 13.49^{###}$	$65.49 \pm 26.86^{##}$	$126.5 \pm 64.87^{***}$	<0.0001
18:1n-7/n-9	$610.5 \pm 173.7^{###}$	$910.2 \pm 367.0^{###}$	$1831 \pm 699.9^{***}$	<0.0001
20:1n-9	$9.47 \pm 2.97^{###}$	$14.41 \pm 4.43^{##}$	$34.15 \pm 23.29^{***}$	<0.0001
24:1n-9	$1.32 \pm 0.55^{###}$	$1.25 \pm 0.69^{###}$	$2.97 \pm 1.45^{***}$	<0.0001
∑ MUFAs	$664.2 \pm 190.7^{###}$	$993.8 \pm 396.8^{###}$	$2004 \pm 756.5^{***}$	<0.0001
18:2n-6 (LA)	$223.6 \pm 65.04^{###}$	$308.6 \pm 93.81^{###}$	$587.1 \pm 214.1^{***}$	<0.0001
18:3n-6	$2.26 \pm 0.69^{###}$	$3.36 \pm 1.10^{*\ ###}$	$6.80 \pm 1.70^{***}$	<0.0001
20:3n-6	$6.95 \pm 2.26^{###}$	$11.45 \pm 5.06^{*}$	$14.20 \pm 4.40^{***}$	<0.0001
20:4n-6 (ARA)	$8.26 \pm 2.90^{###}$	$14.86 \pm 6.97^{**}$	$19.78 \pm 5.97^{***}$	<0.0001
22:4n-6	1.87 ± 1.05	$3.53 \pm 2.09^{*}$	3.24 ± 1.34	0.0087
∑ Omega-6 PUFAs	$243.0 \pm 70.95^{###}$	$340.9 \pm 100.5^{###}$	$631.0 \pm 224.2^{***}$	<0.0001
18:3n-3 (ALA)	$22.28 \pm 9.49^{###}$	39.66 ± 28.81	$68.93 \pm 24.21^{***}$	0.0003
20:5n-3 (EPA)	$1.10 \pm 0.48^{###}$	$3.26 \pm 2.76^{*}$	$4.58 \pm 2.80^{***}$	0.0003
22:5n-3 (DPAn-3)	$2.85 \pm 1.37^{###}$	$6.49 \pm 4.00^{*}$	$9.38 \pm 6.07^{***}$	0.0001
22:6n-3 (DHA)	$4.70 \pm 1.67^{###}$	$14.65 \pm 8.50^{*}$	$18.39 \pm 14.34^{***}$	0.0005
∑ Omega-3 PUFAs	$31.39 \pm 12.41^{###}$	$64.07 \pm 40.67^{*}$	$97.21 \pm 38.59^{***}$	0.0001

* significant different to DHM, # significant different to term BM

Figure 2

Figure 3

A

B
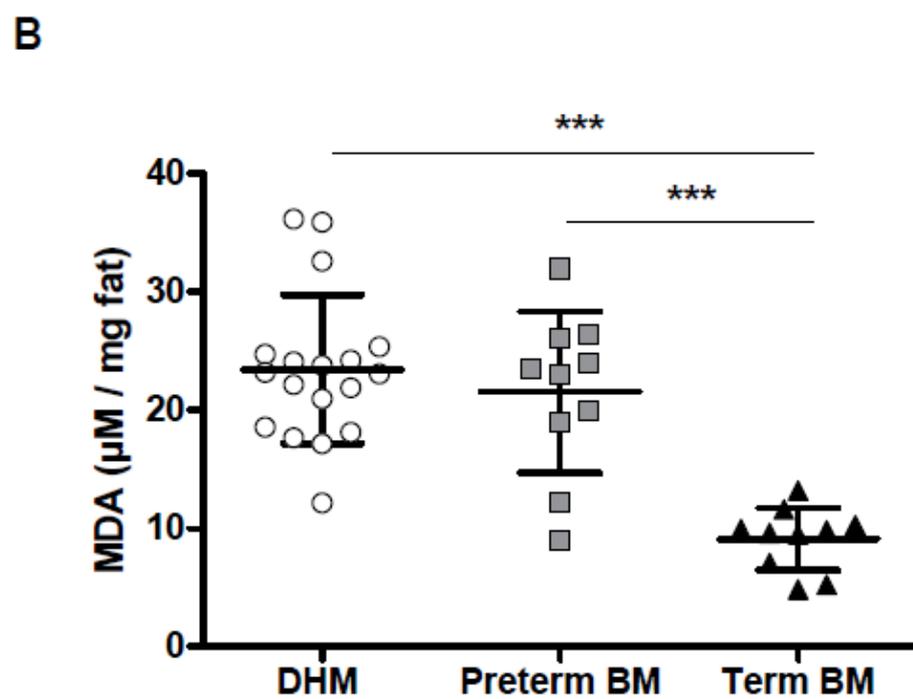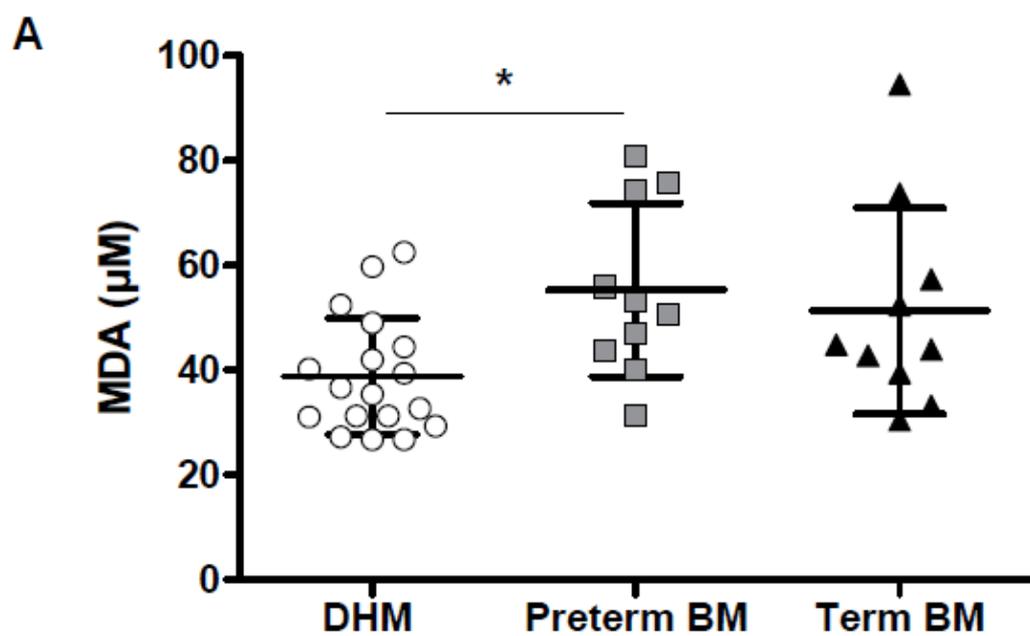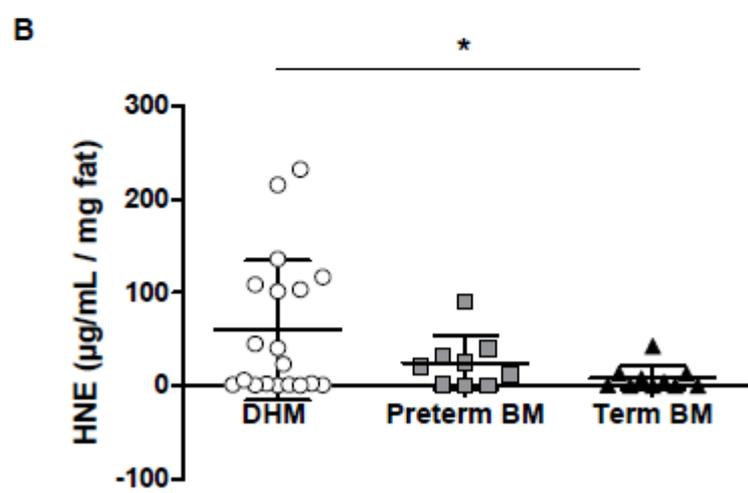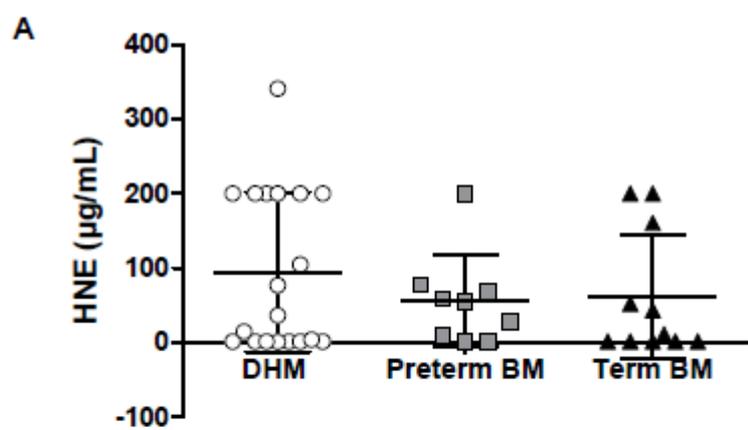
Figure 4
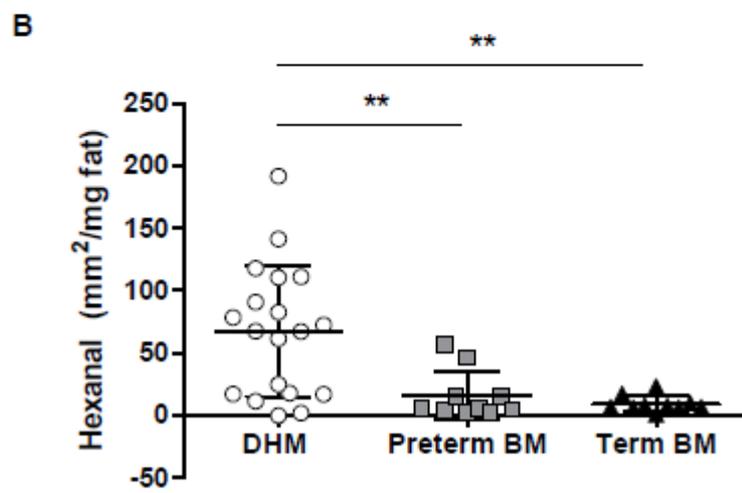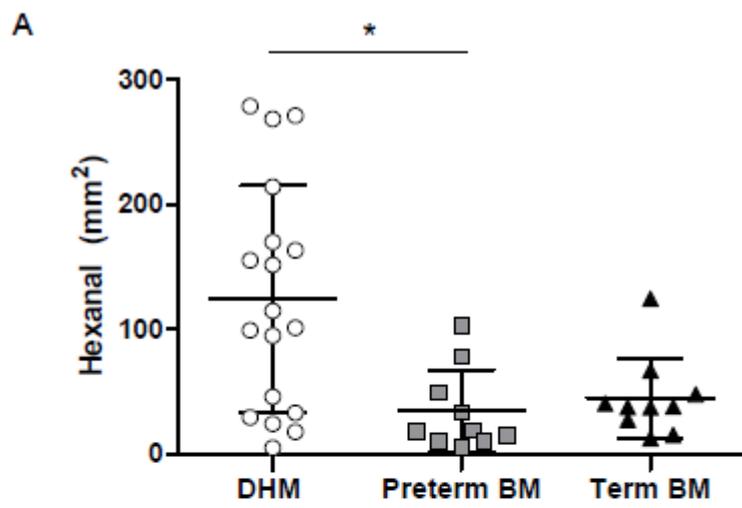
Table 2: Absolute fatty acid composition of DHM, preterm and term BM

Fatty acids (mg/100 mL)	DHM	Preterm BM	Term BM	P-value
10:0	26.53 ± 9.09[###]	38.19 ± 11.81[###]	78.05 ± 29.55[***]	<0.0001
12:0	112.0 ± 41.09[###]	179.8 ± 65.90[*] [###]	315.20 ± 96.89[***]	<0.0001
14:0	115.1 ± 37.23[###]	239.1 ± 125.9[**]	310.3 ± 111.1[***]	<0.0001
16:0	378.5 ± 119.2[###]	498.8 ± 152.2[###]	1154 ± 585.1[***]	<0.0001
18:0	152.6 ± 103.3[###]	217.5 ± 130.6[#]	462.8 ± 296.1[***]	0.0005
20:0	2.03 ± 1.36[##]	3.07 ± 1.87	5.29 ± 3.50[**]	0.0024
∑ SFAs	809.5 ± 293.2[###]	1145 ± 341.2[###]	2198 ± 822.7[***]	<0.0001
16:1n-7	40.28 ± 13.49[###]	65.49 ± 26.86[##]	126.5 ± 64.87[***]	<0.0001
18:1n-7/n-9	610.5 ± 173.7[###]	910.2 ± 367.0[###]	1831 ± 699.9[***]	<0.0001
20:1n-9	9.47 ± 2.97[###]	14.41 ± 4.43[##]	34.15 ± 23.29[***]	<0.0001
24:1n-9	1.32 ± 0.55[###]	1.25 ± 0.69[###]	2.97 ± 1.45[***]	<0.0001
∑ MUFAs	664.2 ± 190.7[###]	993.8 ± 396.8[###]	2004 ± 756.5[***]	<0.0001
18:2n-6 (LA)	223.6 ± 65.04[###]	308.6 ± 93.81[###]	587.1 ± 214.1[***]	<0.0001
18:3n-6	2.26 ± 0.69[###]	3.36 ± 1.10[*] [###]	6.80 ± 1.70[***]	<0.0001
20:3n-6	6.95 ± 2.26[###]	11.45 ± 5.06[*]	14.20 ± 4.40[***]	<0.0001
20:4n-6 (ARA)	8.26 ± 2.90[###]	14.86 ± 6.97[**]	19.78 ± 5.97[***]	<0.0001
22:4n-6	1.87 ± 1.05	3.53 ± 2.09[*]	3.24 ± 1.34	0.0087
∑ Omega-6 PUFAs	243.0 ± 70.95[###]	340.9 ± 100.5[###]	631.0 ± 224.2[***]	<0.0001
18:3n-3 (ALA)	22.28 ± 9.49[###]	39.66 ± 28.81	68.93 ± 24.21[***]	0.0003
20:5n-3 (EPA)	1.10 ± 0.48[###]	3.26 ± 2.76[*]	4.58 ± 2.80[***]	0.0003
22:5n-3 (DPAn-3)	2.85 ± 1.37[###]	6.49 ± 4.00[*]	9.38 ± 6.07[***]	0.0001
22:6n-3 (DHA)	4.70 ± 1.67[###]	14.65 ± 8.50[*]	18.39 ± 14.34[***]	0.0005
∑ Omega-3 PUFAs	31.39 ± 12.41[###]	64.07 ± 40.67[*]	97.21 ± 38.59[***]	0.0001

* significant different to DHM, # significant different to term BM

Table 2: Daily enteral PUFA supply compared to ESPGHAN recommendations

PUFA (mg/210 mL)	DHM	Preterm BM	Recommended minimum daily intake[21]
18:2n-6 (LA)	469.6 ± 132.94*	737.1 ± 319.76	539
18:3n-3 (ALA)	46.79 ± 19.4***	83.29 ± 57.4	77
20:4n-6 (ARA)	17.36 ± 5.92***	31.20 ± 13.88	25.2
22:6n-3 (DHA)	10.62 ± 4.62***	30.77 ± 16.94*	16.8

Enteral PUFA supply from DHM or preterm BM (210 mL) for a hypothetical 1400 g infant