Studies on polyunsaturated fatty acid content and lipid peroxidation in preterm infant nutrition from non-maternal sources

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Abstract

Long-chain polyunsaturated fatty acids (LCPUFAs) are essential for infants' health and development. Preterm infants have particularly high requirements for LCPUFAs, due to premature interruption of the maternal-foetal transfer. Research suggests that current feeding practices are insufficient to meet preterm infants' LCPUFA requirements. Furthermore, LCPUFAs are highly susceptible to lipid peroxidation, giving rise to potentially harmful lipid peroxidation products (LPPs), which are related to major preterm morbidities. Donor human milk (DHM) and preterm formula milk (FM) are used when maternal breast milk is not available, sufficient, or suitable. Therefore, the first objective of this research was to quantify the LCPUFA intake of preterm infants from DHM and preterm FM and to explore how to increase these levels. The main focus of this thesis lies on LCPUFAs, however, other fatty acids, such as linoleic acid, were also considered. The second objective was to investigate the extent and nature of LPP formation in these non-maternal sources.

LCPUFA levels available to preterm infants from the non-maternal sources DHM and FM were measured, for the first time in the U.K., since this data is scarce in the U.K and Europe. DHM provided LCPUFAs below European Society for Paediatric Gastroenterology, Hepatology and Nutrition recommendations. Although, with fortification, recommended levels of docosahexaenoic acid (DHA 22:6n-3; 11.5 ± 2.3 vs. 8 mg/100 mL) but not arachidonic acid (ARA 20:4n-6; 8.3 ± 2.9 vs. 12 mg/100 mL) could be reached. Preterm FM provided LCPUFA levels in agreement with these recommendations. Results further suggest that preterm FM and DHM provided ARA and DHA levels of less than 27 and 67% of estimated *in utero* accretion rates, respectively. Current hospital storage conditions of preterm FM did not influence LCPUFA levels. Both DHM and preterm FM were found to be sources of LPPs (malondialdehyde, 4-hydroxy-2-nonenal HNE, hexanal), with the highest levels seen in DHM (23.5 ± 6.3 µM TBARS; 60.0 ± 75.1 µg/mL HNE). No detrimental effects of LPPs at levels expected in FM were detected in a preliminary *in vitro* model. However, as DHM and preterm FM may be the only nutritional source for an extended period, this may lead to a chronic intake of LPPs, which might contribute to the development of necrotising enterocolitis, bronchopulmonary dysplasia or retinopathy of prematurity.

Human milk banking practices that might alter LCPUFA and LPP content of DHM were identified through a nationwide survey of human milk banks; and recommendations for human milk banking, to improve quality and quantity of LCPUFAs in DHM, were provided. Some of the suggested improvements only require small changes in practice and could be implemented immediately. Recommendations should be translated into

(inter)national guidance, which generally need to be more specific to protect DHM nutritional quality.

Overall, LCPUFA quality and quantity in non-maternal nutritional sources in the U.K. can be improved. Especially exclusive, unfortified DHM feeding might not be suitable long-term and might contribute to the development of major preterm neonatal morbidities. Further research is needed to facilitate improvements in LCPUFA levels and the nutritional quality of DHM. Every effort should be made to promote and enable breast feeding.

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List of Abbreviations

2-AG	2-Arachidonylglycerol
AEA	Arachidonoyl ethanolamide (anandamide)
ALA	α-Linolenic acid (18:3n-3)
ARA	Arachidonic acid (20:4n-6)
BM	Breast milk
CV%	Coefficient of variation in percent
DHA	Docosahexaenoic acid (22:6n-3)
DHEA	Docosahexaenoyl ethanolamide
DHM	Donor human milk
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid (20:5n-3)
ESPGHAN	European Society for Paediatric Gastroenterology, Hepatology, and Nutrition
FA	Fatty acid
FAME	Fatty acid methyl ester
FID	Flame ionisation detector
FM	Formula milk
GC	Gas chromatography
HHE	4-Hydroxy-2-hexenal
HMB	Human milk bank
HNE	4-Hydroxy-2-nonenal
IL	Interleukin

IPEC-J2	Intestinal porcine enterocyte cell line from jejunum
LA	Linoleic acid (18:2n-6)
LCPUFA	Long-chain polyunsaturated fatty acid
LIMIT Study	Investigating **Li**pid Peroxidation Products in Donor Human **Mi**lk – a **T**wo-Centre Study
LPP	Lipid peroxidation product
MDA	Malondialdehyde
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
n-3	Omega-3
n-6	Omega-6
NEC	Necrotising enterocolitis
NF-$\kappa\beta$	Nuclear factor-$\kappa\beta$
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
PBS	Phosphate buffered saline
PUFA	Polyunsaturated fatty acid
SD	Standard deviation
SPM	Specialised pro-resolving mediator
SPME	Solid phase micro-extraction
TAC	Total antioxidant capacity
TBARS	Thiobarbituric acid reactive substances
UKAMB	United Kingdom Association for Milk Banking

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work has taken me, and of course, for care packages with all the German food I have missed! Without you, I would not be where I am now.

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Author's Declaration

I hereby declare that the work presented in this thesis has not been and will not be submitted in whole or in part to another University for the award of any other degree.

Signature:

1   General introduction

The essential role of omega-3 fatty acids (n-3 FAs) in the health and development of infants has been recognised for several years (Crawford 1993). Docosahexaenoic acid (22:6n-3, DHA), a long-chain polyunsaturated fatty acid (LCPUFA), is the most abundant n-3 FA in the brain and retina where it is needed for optimal development and function (Crawford et al. 1976b; Clandinin et al. 1980; Makrides et al. 1994; Birch et al. 2007). The quantitatively most important omega-6 (n-6) LCPUFA in the brain is arachidonic acid (20:4n-6, ARA) (Clandinin et al. 1980), which also plays an important role in regulating the inflammatory response (Bennett and Gilroy 2016). Other n-6 LCPUFAs in the brain are dihomo- γ-linolenic acid (20:3n-6), precursor for prostaglandin E1, which is regulating blood flow by dilating blood vessels and inhibiting platelet aggregation, and adrenic acid (22:4n-6), which is a major LCPUFA in myelin and also highly enriched in the adrenal gland (Kirtland 1988; Martínez and Mougan 1998; Wijendran et al. 2002). ARA is also a major component (around 20%) in the inner membranes of endothelial cells (Crawford et al. 1997).

The maternal-foetal DHA transfer has been interrupted prematurely in preterm infants. Amongst other things, like low adipose tissue levels and inefficient absorption rate of DHA, this contributes to the so termed "DHA gap of prematurity" (Harris and Baack 2015), which describes the DHA deficit after preterm birth. However, as described in more detail in Chapter 1.1.6, similar mechanisms also lead to an ARA deficit after preterm birth. Therefore, the concept has been broadened and termed "LCPUFA gap of prematurity". Consequently, dietary LCPUFAs are of great importance for preterm infants. Supplementing preterm infants with DHA (0.5-1.7% of total FAs) is associated with improved neurological outcomes at 18 months to two years, with higher problem-solving scores, better recognition memory, better sustained attention, higher mental development index scores and less mental development index scores below 70 (Lapillonne and Moltu 2016). These and other effects of LCPUFAs are mediated through their incorporation into cell membranes, where they can influence membrane fluidity, and cellular function, as well as being precursors for bioactive lipid mediators, which is described in more detail in Chapter 1.1.3 (Pike 2003; Dyall 2015; Bennett and Gilroy 2016). Furthermore, feeding infants FM without ARA and DHA resulted in significantly lower verbal IQ scores compared to infants receiving BM, whereas no difference was detected when a FM containing DHA and ARA was used (Birch et al. 2007). Despite their importance, research suggests that current postnatal preterm infant intake of LCPUFAs is inadequate, based on estimated *in utero* accretion rates (Lapillonne et al. 2010; De Rooy et al. 2017).

Survival rates of preterm infants are increasing due to improvements in care (Cooke 2006; Glass et al. 2015). Nevertheless, the percentage prevalence of preterm infants with severe disabilities, such as cognitive disabilities, has not changed over 10 years (Moore et al. 2012), potentially due to the low levels of LCPUFA intake. Other common co-morbidities of preterm infants, such as respiratory distress syndrome and intraventricular haemorrhage, are also associated with lower LCPUFA levels in red blood cells (Fares et al. 2017). The following chapter provides a general literature review of the metabolism and physiological functions of LCPUFAs, the relationship of LCPUFAs and co-morbidities of prematurity, and the implications of preterm delivery on (dietary) LCPUFA needs (Chapter 1.1).

The first 1000 days of life are of outmost importance for the development, and environmental factors like nutrition can have life-long consequences on morbidity and mortality later in life (Agosti et al. 2017). Therefore, it is essential to provide preterm infants with sufficient levels of LCPUFAs, and to ensure that LCPUFA levels are optimised in nutritional sources. For the first months of life, the main nutritional sources include parenteral nutrition, maternal breast milk (BM), donor human milk (DHM), and preterm formula milk (FM). The research presented in this thesis aims to investigate preterm infant LCPUFA intake in the perinatal period, and explore how these levels can be increased. Methods to increase BM LCPUFA levels have been researched extensively (Makrides et al. 1996; Jensen et al. 2000; Urwin et al. 2012). Therefore, this research will focus on the optimisation of LCPUFA provision from the non-maternal sources DHM and preterm FM.

Due to the presence of several double bonds in their chemical structure, LCPUFAs are particularly susceptible to oxidative damage and subsequent degradation (Cosgrove et al. 1987). This results in the formation of lipid peroxidation products (LPPs), which can amongst other things bind to proteins and DNA. This can lead to cell and tissue damage and has the potential to increase inflammation (Yadav and Ramana 2013). Increased LPPs in parenteral lipid emulsions due to light exposure for example increased the incidence of bronchopulmonary dysplasia in preterm infants (+32% vs. light protected lipid emulsion) (Chessex et al. 2007). Therefore, the second aim of this thesis is to investigate the extent and nature of LPP formation in preterm infant nutrition. Chapter 1.2 provides a general overview of LPPs, their role in comorbidities of prematurity, and their presence in non-maternal dietary sources of preterm infants.

LPPs have been measured in BM and FM, with higher concentrations found in the latter (e.g. 19.35 vs. 55.33 µg MDA/100 mL) (Martysiak-Zurowska and Stolyhwo 2006; Michalski et al. 2008; Almansa et al. 2013), and LPP levels increase in FM following

storage (e.g. 40% increase in MDA after 12 months storage at room temperature) (Almansa et al. 2013; Cesa et al. 2015). However, the influence of current storage conditions of preterm FM in neonatal units in the U.K. has thus far not been investigated. Therefore, the first specific objective of this research was to investigate the effect of different storage conditions on the LCPUFA and LPP content of preterm FM in the U.K. (Chapter 2).

In addition to identifying whether LPPs have the potential to increase in FM and DHM due to storage, it is important to establish the nature of their toxicity, especially considering that the average dietary 4-hydroxyalkenal intake can be up to ten times higher in infants than in adults (Surh and Kwon 2005). Ingested LPPs can be absorbed and act directly on intestinal tissue or elsewhere in the body (Awada et al. 2012). Preterm infants, in general, are more prone to inflammation, due to a decreased antioxidant defence system (Huertas et al. 1998) and their gastrointestinal tract is especially vulnerable to inflammation (Hackam and Sodhi 2018). The second specific objective of this research was to investigate the effects of LPPs on an *in vitro* model of the inflammation-sensitive intestine (Chapter 3).

Several studies have suggested that some milk banking practices may have negative effects on DHM LCPUFA levels (Wardell et al. 1981; Bertino et al. 2013; Garcia-Lara et al. 2013; Nessel et al. 2019). In the U.K., guidance for human milk banking can be found in the Clinical Guideline 93 "Donor Breast Milk Banks: The Operation of Donor Breast Milk Bank Services" issued by the National Institute for Health and Care Excellence (NICE). The guideline is mainly concerned with the safe operation of human milk banks (HMBs), not with the nutritional quality of DHM. Also, some of the guidance is vague and leaves room for interpretation; therefore, practices in the 16 HMBs may be very different. In order to gain a better understanding of current human milk banking practices, it was necessary to conduct a nationwide survey of U.K. milk banking practices, the first of its kind in the U.K. Therefore, the third specific objective of this research was to ascertain the current human milk banking practices in the U.K., with special interest in practices that may impact on LCPUFA levels (Chapter 4). Since there is no clear consensus in the literature as to the effects of human milk banking practices on the quantity and quality of LCPUFAs in DHM, Chapter 5 provides a detailed narrative review of the effects of storage conditions on LCPUFAs, lipid mediators, and antioxidants in DHM (Nessel et al. 2019).

During the literature search for this review, it was identified that LCPUFA levels of DHM had only been reported in the U.S.A. (0.40% ARA and 0.14% DHA) (Valentine et al. 2010; Baack et al. 2012) and in Sweden (0.30 % ARA and 0.19% DHA) (Ntoumani et

al. 2013), and only as percentage of total FAs, but not as absolute values. HMBs in America pool DHM from several donors (O'Hare et al. 2013), which is in contrast to HMBs in the U.K. (National Institute for Health and Clinical Excellence 2010). Furthermore, most studies investigating the effects of storage or processing on LCPUFA or LPP levels use BM or tried mimicking the milk banking process in the laboratory but did not analyse DHM that would be administered in a neonatal unit (Elisia and Kitts 2011; Garcia-Lara et al. 2013). Therefore, the fourth specific objective of this research was to quantify for the first time the absolute and relative levels of LCPUFAs in DHM provided by HMBs in the U.K. and measure LPP levels (Chapter 6). Due to the extremely low absolute LCPUFA levels in DHM, the enteral LCPUFA intake, as well as the LCPUFA levels available for accretion were also calculated and compared to levels provided by preterm BM, enteral intake recommendations from the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) (Agostoni et al. 2010), and published *in utero* accretion rates (Lapillonne and Jensen 2009) (Nessel et al. 2020). The thesis concludes with a General Discussion and Conclusion (Chapter 7).

This thesis is in the format of an integrated thesis, with Chapters 5 and 6 published in peer-reviewed academic journals, and the results of Chapter 4 form part of a publication on international human milk banking practices. This thesis format provided the opportunity to practice the specific writing style for publications, the major research output in the STEM field. Furthermore, preparing these chapters as publication and going through the submission process, as well as the peer-review process has provided a great learning experience for a future career in research.

1.1 Preterm infants and LCPUFAs

1.1.1 Preterm birth: Definition, prevalence, and impact

Preterm birth is defined as live birth before the completion of 37 weeks gestation (March of Dimes PMNCH Save the Children WHO 2012). Subcategories of preterm birth are defined by gestational age (Table 1-1).

Table 1-1: Sub-categories of preterm birth and their distribution

Subcategory	Gestational age	Proportion of preterm births[1]
Extremely preterm	< 28 weeks	5.2%
Very preterm	28 to < 32 weeks	10.4%
Moderate to late preterm	32 to < 37 weeks	84.3%

[1] *(Blencowe et al. 2013)*

Preterm infants are also often classified by their birth weight. Infants below 1000 g are classified as extremely low birth weight, very low birth weight ranges from 1000 to less than 1500 g and low birth weight infants weigh between 1500 and less than 2500 g.

Preterm birth remains a leading cause of neonatal mortality (GBD 2015 Mortality and Causes of Death Collaborators 2016), thus it is a major public health concern (Blencowe et al. 2012). In England and Wales, approximately 53,887 infants (7.9% of all live births) are born preterm every year, and the distribution in subcategories is very similar to worldwide numbers (84% moderate to late preterm, 10% very preterm, and 6% extremely preterm) (Office for National Statistics 2019).

Complications of preterm birth are the leading cause of neonatal deaths worldwide (Liu et al. 2012). Globally, 29.2% (765,900 of 2,621,500) of neonates below 1 months of age died due to preterm birth complications in 2015. In England and Wales, 50% (1.4 per 1000 live births) of neonatal deaths in 2018 were caused by conditions related to prematurity, including respiratory and cardiovascular disorders (Office for National Statistics 2020). Neonatal mortality within the first 28 days of life is inversely related to gestational age. 66.6% of infants born below 24 weeks gestation died on the day of delivery, and 80% of them died in the neonatal period. In infants weighting less than 2500 g, mortality was 32.5 deaths per 1000 live births, whereas it was close to 0 in normal birth weight babies. Stratified for preterm birth categories, 33.1% of extremely preterm infants, 3.6% of very preterm infants, and 2.6% of moderate to late preterm infants died in England and Wales in 2012 (Office for National Statistics 2014). These complications are mostly due to immaturity of the related organ systems, which are not prepared yet for extra-uterine life (Institute of Medicine (US) Committee on Understanding Premature Birth and Assuring Healthy Outcomes; Behrman RE 2007).

Common co-morbidities of prematurity include necrotising enterocolitis (NEC, 1.1%), bronchopulmonary dysplasia (5.8%), intraventricular haemorrhage (5.6%), and retinopathy of prematurity (19.2%) (Hakeem et al. 2012; Black et al. 2015). These conditions are classified as oxygen radical associated diseases (Saugstad 2005), which are caused by oxidative stress and inflammation, two common conditions in preterm infants, and mediated by free radicals. Therefore, it is believed that the diseases are manifestations of free radical damage in the respective affected organs.

Surviving infants, particularly extremely preterm infants, often face lifelong health consequences such as impaired neurodevelopment, leading to cognitive and learning impairments; cerebral palsy; visual and hearing impairments; chronic lung disease of prematurity; an increased risk of non-communicable diseases; and behavioural sequels (Blencowe et al. 2013; Platt 2014). The number of very and extremely preterm infants suffering from cognitive disabilities has not changed in in England between 1995 (13%) and 2006 (12%) (Moore et al. 2012). Similarly, a meta-analysis found no improvement in cognitive outcomes in preterm infants between 1990 and 2008, while their outcome was worse than in control infants (Twilhaar et al. 2018). Between 2011 and 2015, 19% of extremely preterm infants born in the U.S.A. suffered from severe neurodevelopmental impairment (Adams-Chapman et al. 2018). The total costs of preterm births to the public sector (health, social, and educational services) in England and Wales are estimated to be £2.9 billion for one birth cohort up to their 18th birthdays (Mangham et al. 2009). Therefore, preterm birth does not only affect the infant and their family, but also the health care system (National Health Service (NHS) in the U.K.) and the society (Petrou et al. 2011).

1.1.2 LCPUFAs and their metabolism

Polyunsaturated fatty acids (PUFAs) contain two or more double bonds in their structure. The omega nomenclature, introduced by Holman in 1964, classifies PUFAs depending on the position of the first double bond in relation to the methyl end of the FA. The schematic structures of ARA (n-6) and DHA (n-3) are shown in Figure 1-1.

Methyl end — Carboxyl end

n-6 position

Arachidonic acid 20:4n-6 (ARA)
5,8,11,14-Icosatetraenoic acid

n-3 position

Docosahexaenoic acid 22:6n-3 (DHA)
4,7,10,13,16,19-Docosahexaenoic acid
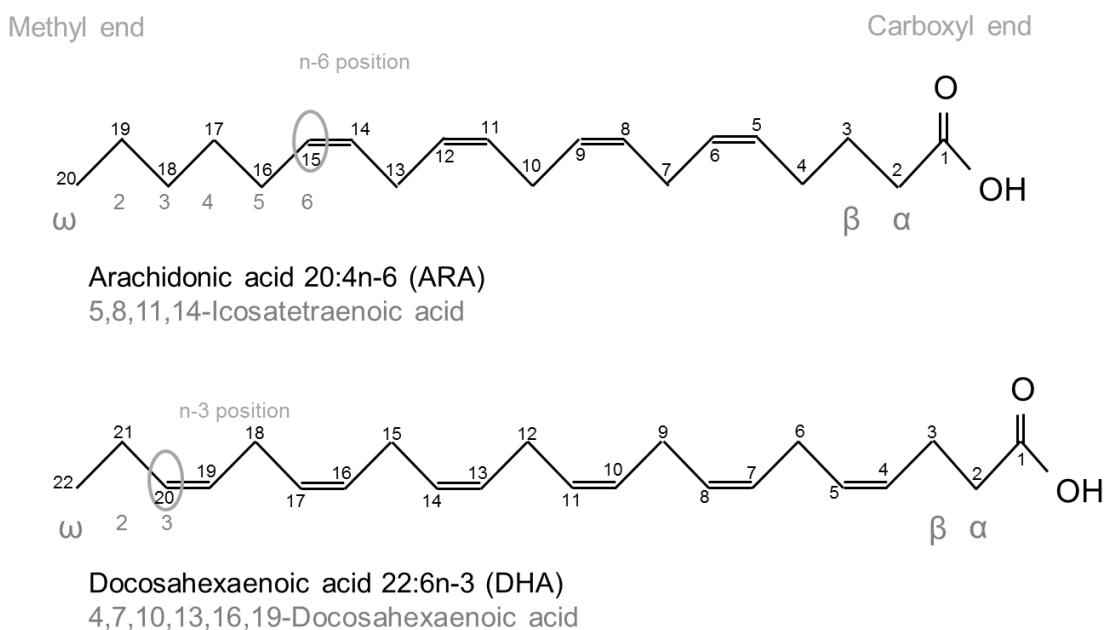
Figure 1-1: Schematic structure of ARA and DHA

LCPUFAs need to be provided through the diet, since humans are incapable of introducing double bonds beyond the carbon-9 position of FAs. Therefore, the LCPUFA precursor FAs linoleic acid (18:2n-6, LA) and α-linolenic acid (18:3n-3, ALA) are considered dietary essential. They can be converted into the LCPUFAs ARA and DHA via a series of desaturation and elongation processes (Figure 1-2) (Sprecher et al. 1995; Park et al. 2009; Gibson et al. 2011; Park et al. 2015). The conversion rates, however, are extremely limited. Although the conversion efficiency was observed to be higher in preterm infants than in term infants (Uauy et al. 2000), the absolute synthesis of endogenous ARA and DHA was estimated to be only 27 mg and 13 mg per kg bodyweight per day, respectively at 1 month in preterm infants, declining significantly over time to 12 mg and 2 mg per kg bodyweight per day, respectively at 7 months (Carnielli et al. 2007). Furthermore, it is to note that the Δ6 desaturase step is rate limiting in the conversion of precursor FAs to LCPUFAs, therefore, high levels of LA and ALA will reduce the conversion to DHA, which depends twice on the Δ6 desaturase activity (Nakamura and Nara 2004; Das 2006; Gibson et al. 2013). Although the Δ6 desaturase has a higher preference for n-3 FAs than for n-6 FAs (Das 2006), Western diets usually provide much higher levels of n-6 FAs (n-6 to n-3 FA ratio of 16:1) (Simopoulos 2002), limiting the ALA conversion. High levels of dietary LA can also reduce the incorporation of preformed n-3 LCPUFAs into tissue. Feeding marmosets with a diet containing preformed eicosapentaenoic acid, and either 7% or 33% LA, resulted in significantly lower eicosapentaenoic acid incorporation into erythrocyte phospholipids in the latter (McMurchie et al. 1990).

Omega-6 fatty acids | Omega-3 fatty acids

Endoplasmatic reticulum

Linoleic acid (18:2n-6) → α -Linolenic acid (18:3n-3)

Elongase — Δ6 Desaturase — Elongase

Dihomolinoleic acid (20:2 n-6) — γ-Linolenic acid (18:3n-6) — Stearidonic acid (18:4n-3) — Eicosatrienoic acid (20:3n-3)

Δ8 Desaturase — Elongase — Δ8 Desaturase

Dihomo- γ-linolenic acid (20:3n-6) — Eicosatetraenoic acid (20:4n-3)

Δ5 Desaturase

Arachidonic acid (20:4n-6) — Eicosapentaenoic acid (20:5n-3)

Elongase

Adrenic acid (22:4n-6) — Docosapentaenoic acid (22:5n-3)

Elongase

Tetracosatetraenoic acid (24:4n-6) — Tetracosapentaenoic acid (24:5n-3)

Δ4 Desaturase — Δ6 Desaturase — Δ4 Desaturase

Tetracosapentaenoic acid (24:5n-6) — Tetracosahexaenoic acid (24:6n-3)

β-oxidation

Osbond acid (22:5n-6) — Peroxisomes — Docosahexaenoic acid (22:6n-3)

Figure 1-2: Conversion of precursor FAs into LCPUFAs

Adapted from (Dyall and Michael-Titus 2008; Park et al. 2009; Gibson et al. 2011) The figure displays the Sprecher (black) and Δ4 (grey) LCPUFA biosynthesis pathways

1.1.3 Physiological functions of LCPUFAs

ARA in cell membranes contributes to membrane order. 15 - 25% of the phospholipid FAs in platelets, mononuclear cells, neutrophils, liver, and muscle cell are ARA (Calder 2007). LCPUFA incorporation into membrane phospholipids can influence membrane fluidity and cellular function (Niu et al. 2004), and ensures the correct environment for membrane proteins. DHA also influences the formation of lipid rafts and can therefore influence cell signalling (Pike 2003). Membrane LCPUFAs are precursors for a range of bioactive lipid mediators, which can influence various systems in the body, are neuroprotective, and regulate the inflammatory response (Serhan et al. 2008; Crean and Godson 2015; Bennett and Gilroy 2016; Jun et al. 2017). These include eicosanoids (prostaglandins, thromboxanes, leukotrienes) and specialised pro-resolving mediators (SPMs; lipoxins, resolvins, (neuro-)protectins, maresins, and elovanoids). Furthermore, LCPUFAs influence the immune function in preterm infants (Gottrand 2008), and LCPUFA derived endocannabinoids have an important role in neurodevelopment and neuroprotection in early life (Fride 2004; Dyall et al. 2016).

The brain consists of approximately 60% lipids, and of these 8-11% are ARA and 12-15% are DHA, thus they are the most abundant n-6 and n-3 PUFAs in the brain (Sastry 1985; Calder 2016). However, quantitatively, n-6 LCPUFAs are more important in the brain than n-3 LCPUFAs (25.5 vs 14.4%; phosphatidylethanolamine: 20:3n-6: 1.6%; 20:4n-6: 12.72%; 22:4n-6: 8.44%; 22:5n-6: 2.74%; 22:5n-3: 0.34%; 22:6n-3: 14.07%) (Martínez and Mougan 1998). During the last trimester, brain weight increases by five times and at the same time approximately 80% of the brain ARA and DHA accretion

occurs (Clandinin et al. 1980), which continues throughout the first two years of life, albeit at a slower rate, and levels are maintained throughout life (Makrides et al. 1994; Lauritzen et al. 2016). This rapid brain growth needs to be accompanied by an appropriate development of the vascular system to provide energy and nutrients (Crawford et al. 2003). Low LCPUFA provision can lead to functional abnormal cell membranes due to incorporation of other FAs such as mead acid (20:3n-9). Incorporation of mead acid and docosapentaenoic acid (22:5n-6) in cell membranes are signs of essential FA and DHA deficits (Rump et al. 2001). Mead acid incorporation could lead to cell membrane integrity loss and increased risk of membrane rupture. In the brain, 5-16% of ARA are converted into adrenic acid , the third most abundant PUFA in the brain (around 8%) (Martínez and Mougan 1998; Wijendran et al. 2002). Adrenic acid rapidly accumulates in the brain postnatally (Martínez and Mougan 1998), as part of myelin lipids, which facilitate faster signal transmission. Within the brain, LCPUFAs have a structural function, and DHA is highly enriched in synaptic membranes (Cotman et al. 1969). DHA promotes neurogenesis, neuronal differentiation, neurotransmission and synaptic function (Chalon et al. 1998; Cao et al. 2009; Katakura et al. 2009).

DHA is also the most abundant PUFA in the retina, contributing 50% of FAs in the rod outer segments (Anderson 1970). Besides the structural role, DHA also plays a role in photoreceptor differentiation during development (Garelli et al. 2006). Activation of the visual pigment rhodopsin by conformation change is possible due to the DHA molecules in the surrounding phospholipids (Mitchell et al. 2003), and rhodopsin regeneration is also affected by DHA content in the retina (Bush et al. 1994). In the DIAMOND study, term FM fed infants received either 0%, 0.32%, 0.64%, or 0.96% DHA and 0.64% ARA, and visual acuity was measured at 12 months. In the control group (0% DHA), infants had significantly poorer visual acuity than infants receiving DHA (Birch et al. 2010), demonstrating that DHA is essential for visual acuity. Similar results were obtained by other groups (Makrides et al. 1995a); and confirmed in meta analyses (SanGiovanni et al. 2000). Results persists into childhood (4 years) with DHA and ARA receiving term infants having better visual acuity than the control group, and similar visual acuity to breast fed infants (Birch et al. 2007).

1.1.4 Nutrition in the first 1000 days of life

The first 1000 days of life cover the period from conception to two years of life (Agosti et al. 2017). This is a sensible phase in life, in which stimuli, such as nutrition, can have life-long consequences for health outcomes, by affecting the development of important endocrine, metabolic, and immunological pathways. The underlying hypothesis was formed by the British epidemiologist David Barker in 1939, who postulated that

nutritional programming of organs in early life can determine the physiological and metabolic response later in life, and adverse nutrition early in life can lead to non-communicable diseases, including obesity, hypertension, coronary heart disease and stroke (Edwards 2017).

In the neonatal unit, it is aimed to provide preterm infants with nutrients similar to what would have been provided *in-utero* (Agostoni et al. 2010), aiming to achieve growth rates similar to intrauterine growth. Growth is an important measurement for clinicians. Improved infant growth during hospitalisation is significantly correlated to neurodevelopment and growth outcomes at 18-22 months corrected age (Ehrenkranz et al. 2006). This effect is long lasting, increased growth of preterm infants between birth and discharge was also associated with a reduced risk for abnormal neurological examination and impaired mobility at 5 years of age (Franz et al. 2009). Nevertheless, extrauterine growth retardation is common in, especially extremely, preterm infants (Poindexter 2014). Improved growth is achieved by better nutritional support, for example with higher amino acid and protein intake, as well as early lipid supply (Stephens et al. 2009; dit Trolli et al. 2012; Cormack and Bloomfield 2013), and a standardised feeding regime (McCallie et al. 2011).

Body growth mainly requires protein and preterm infants need high protein levels to achieve intrauterine growth rates (Hay and Thureen 2010). However, *in utero*, the foetus uses up to 70% of the energy for brain growth and development in the last trimester (Crawford 2000), the time in which the main LCPUFA accretion occurs (described in Chapter 1.1.5). The LCPUFAs ARA and DHA are the most abundant PUFAs in the brain (Sastry 1985; Calder 2016). Therefore, it is important to provide these building blocks to the developing preterm infants after birth (Makrides and Uauy 2014).

1.1.5   *In utero* accretion of LCPUFAs

In utero, FAs are transferred from the mother to the developing foetus. The placenta contains almost twice the amount of ARA and DHA than maternal plasma (Crawford et al. 1976b). Furthermore, DHA is selectively favoured for the transport to the foetal circulation during the last trimester (28 weeks gestation until birth), however, the selectivity for ARA increased with increasing maternal ARA concentrations (Haggarty et al. 1999). For example, an eight times increase of maternal DHA resulted in a 13 times increase in DHA in the foetal circulation, but a two times increase in maternal ARA resulted in an eight times increase in the foetal circulation. Data from midterm abortions also showed 1.9 times more ARA in cord blood than in maternal plasma in choline phosphoglycerides, while there was only 1.5 times more DHA (Crawford et al.

1976b). This was accompanied by a decrease in LA and ALA from maternal plasma to foetal cord blood. Similarly, at term, cord plasma ARA levels are 1.8 times higher than maternal plasma levels and cord plasma DHA levels are 1.3 times higher than maternal plasma levels (Olegard and Svennerholm 1970). Overall, this results in a bio-magnification of LCPUFAs in the foetus, with higher ARA and DHA levels in cord blood than maternal blood, providing it with building blocks for the developing brain (Crawford et al. 1976b; Kilari et al. 2009; Ogundipe et al. 2016).

Based on autopsy tissue analyses, the intrauterine accretion rates for LCPUFAs during the last trimester were calculated to be 212 mg ARA per kg bodyweight per day and 43 mg DHA per kg bodyweight per day (Lapillonne and Jensen 2009) (Table 1-2). The total accumulated n-3 FAs during the last trimester are distributed 65% to the adipose tissue, 30% into lean mass, 3.9% to the brain and 0.7% to the liver (Lapillonne and Jensen 2009). However, the calculations are based on estimations and animal studies due to limited available data for FA content and composition of organs at different gestational ages. Another study resulted in lower daily accretion rates (Kuipers et al. 2012), arguing that the previous study overestimates the LCPUFA content in adipose tissue and that there is too much uncertainty in the LCPUFA composition of lean body mass. However, accretion per kg bodyweight is not provided.

Table 1-2: Estimated n-3 and n-6 FA accretion rates during the last trimester

	per day	per kg bodyweight per day[1]
LA	184 mg	106 mg
ALA	7 mg	4 mg
ARA	368 mg	212 mg
DHA	75 mg	43 mg

[1] (Lapillonne and Jensen 2009)

1.1.6 LCPUFA gap of prematurity

If an infant is born preterm, the maternal-foetal LCPUFA transfer is interrupted prematurely and the infant misses out on the *in utero* accretion, which is highest in the last trimester and leads to a biomagnification of ARA and DHA in term infants (Crawford et al. 1976b). Therefore, preterm infants have higher (par)enteral LCPUFA requirements than term infants. Indeed, preterm infants have significantly lower LCPUFA blood levels than term infants, and LCPUFA blood levels are positively correlated with gestational age (Baack et al. 2015). As mentioned above, the majority of DHA is deposited in adipose tissue, however the white adipose tissue accretion of ARA is 4.7 times the DHA accretion during the last trimester (Clandinin et al. 1981). DHA stores will be exhausted within two months in term infants if dietary DHA levels

are insufficient (Farquharson et al. 1993), and both DHA and ARA will decline significantly after birth in preterm infants, even when fed maternal BM (Leaf et al. 1992a). In contrast, preterm infants have very little adipose tissue stores, which only provide negligible DHA and ARA levels. Part of the enterally provided DHA will also be β-oxidised for energy production and will therefore not be available for accretion (Cunnane et al. 2000; Lapillonne et al. 2013). The inefficient conversion rates, low levels of LCPUFAs in preterm nutrition (as further discussed in Chapter 1.1.8), the high levels of LA in preterm nutritional sources, an enteral absorption rate of only 80% (Carnielli et al. 1998), and that it might take several days until full enteral feeding is achieved, further limits the LCPUFA availability for preterm infants.

Harris and Baack have mentioned most of these points before, however, they have only focused on DHA and concluded in 2015 that taken together, these factors lead to what they called the "DHA gap of prematurity" (Harris and Baack 2015). However, as described above, most of these factors also contribute to lower ARA levels in preterm infants, which as described in Chapter 1.1.3, can also have detrimental effects on preterm infant development. Furthermore, it should be considered, that total n-6 LCPUFAs (20:3n-6, 20:4n-6, 22:4n-6, 22:5n-6) exceed total n-3 LCPUFAs (22:5n-3, 22:6n-3) in the brain (Martínez and Mougan 1998), and that ARA is an important precursor for adrenic acid (Wijendran et al. 2002). Often, studies focus solely on n-3 LCPUFAs and their role in brain development, however, n-6 LCPUFAs are as important. ARA is needed for the construction and function of the brain and its blood vessels, since it is a major component of endothelial cells (Crawford et al. 1997). Considering all this evidence, it is therefore proposed to broaden the concept of Harris and Baack, by considering ARA as well, and to rename the concept as the "LCPUFA gap of prematurity".

For the same reason, it has been recommended before that both LCPUFAs should be considered as conditionally essential in the first year of life (Cunnane 2003; Makrides and Uauy 2014), although they can be produced from the dietary essential precursor FAs LA and ALA. The LCPUFA gap of prematurity and low LCPUFA levels can have several health implications for preterm infants, as further detailed in the next section.

1.1.7 LCPUFAs and health outcomes of preterm infants

Neurodevelopmental outcomes

It is known that the developmental quotient of very preterm infants at school age (8 years) is lower than the one of term infants, with very preterm infants displaying cognitive, educational and behavioural impairments (Anderson et al. 2003). LCPUFA supplementation of BM (32 mg DHA and 31 mg ARA per 100 mL, resulting in a

combined intake of 59 mg/kg/day DHA and 47 mg/kg/day ARA) received by very low birth weight infants resulted in higher plasma LCPUFA levels (DHA: 70.1 ± 22.4 vs. 64.2 ± 23.5 mg/mL at baseline; ARA: 191.2 ± 58.9 vs. 205.6 ± 52.8 mg/mL at baseline; control group: 56.3 6 ± 15.3 mg/mL DHA and 155.9 6 ± 33.8 mg/mL ARA after intervention) and was positively correlated with cognitive development (Westerberg et al. 2011). Other studies found similar improvements in cognitive development of very low birth weight preterm infants receiving LCPUFA supplemented BM (0.86 vs. 0.35% DHA) (Henriksen et al. 2008). Recent research suggests better neurological outcomes for preterm infants at 18 month and 2 years when higher than routinely used amounts of DHA (e.g. BM supplementation to 1% vs. 0.35%; BM supplementation to 0.86% vs. 0.35%; or supplementation of FM to 0.5% vs. 0%, provided in conjunction with BM, resulting in intake levels of 0.5-1.7% DHA) are provided (Lapillonne and Moltu 2016). The DINO trial compared the neurodevelopmental outcome at 18 months corrected age of preterm infants (< 33 weeks gestational age) which were supplemented with DHA (1% of total FAs) to a control group receiving 0.3% DHA, resulting in less children with delayed cognitive development in the intervention group (Makrides et al. 2009). Girls in the intervention group also had a higher mean mental/cognitive development. Neurological improvements have also been seen in term infants receiving supplementary LCPUFAs. Receiving FM supplemented with ARA (0.72%) and DHA (0.36%) resulted in a mean increase of seven points on the mental development index of term infants at 18 months (Birch et al. 2010).

Oxidative stress

Evidence suggests that DHA and its metabolites can reduce oxidative stress (Fink et al. 2016). For example, preterm infants given a parenteral lipid emulsion containing n-3 LCPUFAs had reduced oxidative stress, indicated by a significantly increased total antioxidant potential (0.71 ± 0.18 mM uric acid at baseline vs. 1.09 ± 0.29 mM uric acid at 14 days), which was also significantly higher than in the control group receiving standard lipids (0.79 ± 0.16 mM uric acid) (Skouroliakou et al. 2010). Positive effects on oxidative stress and lipid peroxidation were also seen in very preterm infants given the SMOFlipid emulsion containing n-3 LCPUFAs compared to a lipid emulsion containing soy and olive oils (ClinOleic®) (Deshpande et al. 2014). In an animal model, Green and colleagues report a 58% reduction in lipid peroxidation compared to controls after intra amniotic injection of DHA to near term rat foetuses, and the authors speculate that DHA may actually be functioning as an antioxidant (2001).

Immunomodulation

Infections are common in preterm infants. Field and colleagues isolated peripheral blood lymphocytes from preterm infants fed with BM, FM, or FM containing LCPUFAs

(2000). FM with LCPUFAs resulted in lymphocyte populations, cytokine production and antigen maturity that was more closely related to that of BM fed infants, indicating that LCPUFAs status can affect the ability of the preterm infants to respond to immune challenges.

Blood pressure

Follow up of FM, FM with LCPUFA, and BM fed control infants at 6 years of life demonstrated beneficial effects of LCPUFA supplementation on blood pressure, with lower mean and diastolic blood pressure (-3.0 and -3.6 mm Hg, respectively) (Forsyth et al. 2003). No difference in diastolic pressure between BM fed and FM with LCPUFA fed infants could be detected (57.5 vs. 57.3 mm Hg). This indicates that LCPUFA provision during infancy can have long lasting effects on blood pressure and may reduce cardiovascular risk later on.

Co-morbidities of preterm infants

A Cochrane review concluded that preterm birth is reduced in women who increased their n-3 LCPUFA intake from supplementation or food during pregnancy (11.9% vs. 13.4% without n-3 LCPUFAs, risk ratio 0.89) (Middleton et al. 2018). After birth, low red blood cell ARA and DHA levels are associated with an increased risk of neonatal morbidities and mortality. Very low birth weight preterm infants with respiratory distress syndrome, sepsis, intraventricular haemorrhage and perinatal death had significantly lower DHA levels, and ARA was significantly lower in infants with respiratory distress syndrome, intraventricular haemorrhage, and perinatal death (Fares et al. 2017). Furthermore, the n6:n3 ratio was significantly higher in infants with respiratory distress syndrome, and perinatal death. Another study found postnatally decreased DHA and ARA levels in extremely and very preterm infants associated with an increased risk of chronic lung disease (OR 2.5) and late-onset sepsis (hazard ratio 1.4), respectively (Martin et al. 2011). Crawford and colleagues proposed that low ARA and DHA levels in preterm infants results in reduced vascular and endothelial integrity, predisposing them to the complications of prematurity (1997).

Feeding preterm infants with preterm FM containing egg phospholipids (containing choline, ARA and DHA), resulted in 16.5% less NEC stage II and III (Carlson et al. 1998), and the results of a systematic review indicate that DHA has a protective effect on NEC in extremely and very preterm infants (Zhang et al. 2014).

Retinopathy of prematurity is the main cause of visual impairment in preterm infants due to a disruption in the vascular development of the retina. Very low birth weight infants receiving intravenous fish oil emulsion required significantly less laser therapy for retinopathy of prematurity than infants receiving soybean oil emulsion (risk ratio

0.48) (Pawlik et al. 2014). Very low birthweight infants also had a significantly lower risk of stage 3 retinopathy of prematurity (risk ratio 0.66) after receiving 75 mg/kg/d DHA orally for 14 days (Bernabe-Garcia et al. 2019).

Makrides and colleagues increased maternal BM DHA from 0.3% to 1% using supplements. Less preterm infants (< 33 weeks gestational age) in this group required oxygen treatment at 36 weeks compared to the placebo group (Makrides et al. 2009). In preterm infants (< 33 weeks gestational age) boys and all infants < 1250 g birth weight also had a reduction in bronchopulmonary dysplasia (Manley et al. 2011). The results of a systematic review also indicate that DHA has a protective effect on bronchopulmonary dysplasia in extremely and very preterm infants (Zhang et al. 2014). The N3RO trial was conducted as fully powered study to determine the effect of enteral DHA supplementation on the incidence of bronchopulmonary dysplasia in preterm infants born < 29 weeks gestational age (Collins et al. 2017). Using a physiological disease definition, the risk of bronchopulmonary dysplasia was higher than in the control group, whereas no differences were seen when a clinical definition was used. It is noteworthy that the enteral emulsion in this trial only contained DHA, and no ARA.

Critique on the current evidence

Studies investigating the effect of DHA on neonatal outcomes vary extremely in the study design (Lapillonne et al. 2013). The dose, timing and duration as well as the source of LCPUFAs differ greatly between the studies. Furthermore, most trials have been performed on relatively healthy and mature preterm infants and had a small sample size. The outcome measures and the age of testing varied as well. And some of the tests might not be the most appropriate. The Bayley Scale of Infant Development is the most used general assessment of cognitive development (Georgieff 2007). However, it might be that specific neurobehavioral morbidities are not picked up with this tool, and therefore improvements will also not be noticed. Therefore, more specific and precise tests of e.g. memory or problem solving might be needed to capture small but clinical relevant changes after an increased LCPUFA intake (Cheatham et al. 2006). Therefore, it is not surprising that the authors of a Cochrane review analysing benefits of FM LCPUFA supplementation for preterm infants concluded that no differences exist in visual acuity, neurodevelopment and physical growth (Moon et al. 2016). The quality of the evidence was rated low and overall, as mentioned above, the included infants were relatively mature and healthy. Clearly there is the need for more studies with standardised doses and outcome measurements at similar time-points. Studies investigating the effects of LCPUFAs (DHA as well as ARA) on preterm infants' health and development should also include very and extremely preterm infants, since they most likely have the greatest benefit of an increased LCPUFA intake.

Overall, there are much more trials that have used DHA supplementation instead of LCPUFA supplementation with ARA and DHA. However, a recent study provided evidence that preterm infants have a much greater deficit in ARA intake than in DHA (De Rooy et al. 2017). Experts in the field recommend that preformed ARA should be provided to preterm infants, although more data are needed to know which amount would be beneficial (Lapillonne et al. 2013). Furthermore, in all the common comorbidities of preterm infants, inflammation plays a critical role and ARA and DHA are both needed for an appropriate inflammatory response. Future studies should therefore investigate the combined effect of ARA and DHA supplementation on common comorbidities of preterm infants.

1.1.8 Preterm nutrition and LCPUFAs

Based on the available evidence reviewed in the previous sections, it can be concluded that preterm infants should receive sufficient amounts of LCPUFAs from their diet to facilitate optimal health and development. Nutritional sources for preterm infants include parenteral nutrition and enteral nutrition.

Parenteral nutrition

Most extremely and very preterm infants will receive parenteral nutrition in the first days of life, which might further increase the LCPUFA gap. Parenteral nutrition is recommended for preterm infants (born before 30 weeks gestational age, or weighing less than 1250 g at birth, for infants who have failed to establish enteral nutrition with more than 100 ml per kg bodyweight per day by day 5 of life, or infants who fail to tolerate enteral nutrition) until the establishment of enteral feeding (British Association of Perinatal Medicine 2016a). Intravenous lipids (20% lipid emulsion, continuous infusion over 24 hours) should be provided within 24 hours of birth, since LA and ARA deficiencies in preterm infants have been reported as early as 2 days after birth, when feeding a fat free diet (Friedman et al. 1976). On the first day of life, 2 g/kg/day of intravenous lipids should be provided with a daily increase to a maximum of 3.5 – 4 g/kg/day.

In the U.K., two different intravenous lipid preparations are available for the use in neonates, Intralipid and SMOFlipid. Both provide LA and ALA through soybean oil (Intralipid 20%: 200 g/L soybean oil; SMOFlipid 20%: 60 g/L soy bean oil) (Tomsits et al. 2010). However, only SMOFlipid contains fish oil (30 g/L), and therefore provides eicosapentaenoic acid (20:5n-3, EPA) (4.8 g/L) and DHA (3.5 g/L). SMOFlipid can therefore significantly increase ALA and EPA, and the n-3 to n-6 ratio in red blood cells of preterm infants, compared to Intralipid (Tomsits et al. 2010). Nevertheless, SMOFlipid is more expensive and less used. None of the lipid emulsions provide

preformed ARA. Considering the above described inefficiency in the conversion of LA and ALA into ARA and DHA (Pawlosky et al. 2001; Plourde and Cunnane 2007), it is likely that preterm infants receiving parenteral nutrition do not meet their requirements based on *in utero* accretion rates (Lapillonne and Jensen 2009). Therefore, it is even more important to provide preterm infants with optimal enteral nutrition after the use of parenteral nutrition, to avoid deficiencies.

Enteral Nutrition

Mother's own BM is the accepted best practice and advisable way for feeding neonates (ESPGHAN Committee on Nutrition et al. 2009). Exclusive breast feeding for the first six months of life is recommended (WHO 2003). Extremely and very preterm infants often require feeding via a nasogastric tube, since the ability to suck, swallow and breathe only develops around 32 weeks gestation (Lau et al. 2003). In this case, expressed BM can be used. However, mothers of preterm infants often cannot express any or sufficient BM (Hill et al. 2005). Reasons include incomplete development of the mammary glands, or poor hormonal response, as well as psychological problems (Jones and Spencer 2005). If maternal BM is not available, DHM or FM can be used. Intake and LCPUFA levels of these are further discussed in Chapter 2.1,4.1.3 and 6.3.

The worldwide mean (± standard deviation, SD) LCPUFA concentrations in mature BM from mothers of term infants were reported to be 0.37 ± 0.11% for DHA (by weight percentage of total FAs) and 0.55 ± 0.1% for ARA (Fu et al. 2016). U.K. levels are below worldwide average (Table 1-3) (Yuhas et al. 2006). A meta-analysis of preterm and term BM revealed higher fat content in preterm BM during the first 2 weeks (Gidrewicz and Fenton 2014). A systematic review showed that relative DHA levels are higher in preterm BM, whereas data for ARA are inconclusive (Bokor et al. 2007).

Table 1-3: LCPUFA levels in term mature BM in the U.K. and worldwide

% of total FA	United Kingdom[1]	Worldwide[2]
LA	10.45 ± 0.41	
ALA	1.22 ± 0.06	
ARA	0.36 ± 0.01	0.55 ± 0.14
DHA	0.24 ± 0.01	0.37 ± 0.11

[1](Yuhas et al. 2006); [2](Fu et al. 2016)

BM FA composition has also changed in the last decades. For example, in 1976, n-6 LCPUFAs (20:2n-6 – 22:5n-6) made up 1.1% of BM FAs (average of four countries), and n-3 LCPUFAs 1.4% (20:3n-3 – 22:6n-3) (Crawford et al. 1976a). In 2006, this changed to 0.9% n-6 LCPUFAs in BM from nine different countries, and 0.85% n-3 LCPUFAs (Yuhas et al. 2006). It is noteworthy that in the same time, LA levels rose

from 9.97% to 12.95% (Crawford et al. 1976a; Yuhas et al. 2006), most likely due to a change in diet (Sanders 2000). Others similarly reported an increase of LA from 7-10% to 14% of FAs in human BM in the U.K. from 1977 to 1992 (Ghebremeskel et al. 1992). This might have affected the conversion of dietary ALA to n-3 LCPUFAs (Nakamura and Nara 2004), and therefore the n-3 LCPUFA levels in BM.For preterm babies born before 33 weeks gestation, fortification of maternal BM or DHM with BM fortifiers is recommended and most neonatal intensive care units in the U.K. use BM fortifiers (Klingenberg et al. 2012). In the U.K., Gow&Gate (Nutricia Ltd) and SMA (Nestlé UK Ltd) provide bovine BM fortifiers. Nutriprem Human Milk Fortifier contains no fat, whereas SMA BM Fortifier contains 0.18 g/g fat from rapeseed oil and fish oil (Danone Nutricia Early Life Nutrition 2019; SMA Nutrition 2019). Therefore, only SMA provides a BM fortifier in the U.K. that contains LCPUFA (LA 9.6 mg/g; ALA 4.2 mg/g; DHA 1.6 mg/g) for preterm infants. Prolacta Biosciences provides a human milk derived BM fortifier, containing DHA and ARA. However, information about the LCPUFA concentration is not available and they claim that levels may vary (Prolacta Bioscience Inc 2017).

1.1.9 Dietary LCPUFA guidelines

Lapillonne and Jensen defined the LCPUFA requirement of preterm infants as the amount of FAs that is needed to maintain an optimal LCPUFA composition of all tissues, as well as normal growth, and normal short- and long-term outcomes (Lapillonne and Jensen 2009). One straightforward to obtain indicator of LCPUFA status is the plasma FA composition. Leaf and colleagues have measured the FA composition of plasma phosphatidylcholine, the most abundant phospholipid in plasma accounting for around 70%, in preterm infants directly after birth (Leaf et al. 1992b). They reported significant correlations of ARA and DHA with birthweight (correlation coefficient 0.564, $p < 0.01$ and 0.496, $p < 0.05$, respectively), and of DHA with gestational age (correlation coefficient 0.638, $p<0.01$), indicating increasing plasma LCPUFA levels with increasing gestational age/birthweight. When feeding preterm infants, ideally similar plasma LCPUFA levels should be achieved, to allow for similar circulating levels of LCPUFAs as would occur in utero by placenta nutrition (Leaf et al. 1992a). Nevertheless, feeding preterm infants with BM, with FM (without LCPUFAs), or with a combination of both, in conjunction with parenteral feeding for the first 2 weeks after birth, resulted in a rapid decline of ARA and DHA within the first 2-6 weeks after birth (Leaf et al. 1992a). The decrease was less, but not significantly less, in BM fed infants. At the same time, a rapid increase in plasma LA occurred. This is not representing physiological levels that would have occurred in utero, since LA levels do not increase with gestational age (correlation coefficient 0.200) (Leaf et al. 1992b). This

is a further indicator that preterm infants have high LCPUFA requirements after birth, which should imitate the in utero accretion.

In the U.K., no specific for enteral nutrition recommendations for preterm infants are available. Therefore, clinicians follow European guidance. The ESPGHAN Committee on Nutrition issued the following recommendations for PUFA intake for infants weighing between 1000 and 1800 g (Table 1-4) (Agostoni et al. 2010). Extremely preterm infants, mostly weighing less than 1000 g, are excluded from the recommendations due to lack of evidence, although they are the most vulnerable group for LCPUFA deficits.

Table 1-4: PUFA intake recommendations by ESPGHAN

	mg per kg bodyweight per day[1]
LA	385 - 1540
ALA	55
ARA	18 - 42
DHA	12 - 30

[1](Agostoni et al. 2010)

Basing the dietary recommendations on the nutrient levels received *in utero* seems like a logic approach. Furthermore, early LCPUFA deficits should be considered. However, although the guideline was developed to achieve a growth rate similar to foetal growth, and satisfactory functional development (Agostoni et al. 2010), it is in huge discrepancy with the calculated intrauterine accretion rates for ARA and DHA (Lapillonne and Jensen 2009). Recommendations are based on suggested safety evidence and concentrations provided by BM and FM due to insufficient evidence. However, the evidence suggests that BM supplemented to 47 mg ARA per kg bodyweight per day and 59 mg DHA per kg bodyweight per day is safe for preterm infants (Henriksen et al. 2008). ESPGHAN also considers ALA as major source for the synthesis of DHA, despite the reported low conversion efficiency (Carnielli et al. 2007). Evidence from tracer studies suggests that preformed DHA is a 7-fold better source for brain DHA accretion than ALA-derived DHA (Su et al. 1999), and it is an overall consensus that preformed DHA can raise blood DHA status beyond what is achievable with ALA (Brenna et al. 2009). This is important since erythrocyte DHA status has been correlated to brain DHA status (Makrides et al. 1994).

The latest enteral nutrient intake recommendations for preterm infants were published by Koletzko and colleagues (2014). They recommend 35 to 45 mg ARA and 55 to 60 mg DHA per kg bodyweight per day. DHA intake in this range increases plasma concentrations by 12% over 9 weeks, whereas a concentration of 32 mg DHA per kg

bodyweight per day, which is greater than the ESPGHAN recommendations, was not sufficient to prevent a decline (Henriksen et al. 2008).

The incorporation of ARA and DHA into cell membranes is not only dependent on the ARA and DHA concentration, or the levels of precursor FAs, the ratio of LA to ALA is also important. In a study by Clark and colleagues, term infants were either fed a control FM A with 14% LA and 0.7% ALA (ratio 19:1), or experimental FMs with either B: 13% LA and 3.3% ALA (ratio 4:1) or C: 3.5% LA and 1.1% ALA (ratio 3:1) (1992). It is to note that at the time of the study, FM did not contain LCPUFAs. Decreasing the LA to ALA ratio increased the n-3 C20 and C22 incorporation into erythrocytes (A: 5.97 ± 0.76%, B: 8.98 ± 0.65% and C: 9.30 ± 0.95%) and did not change ARA levels in B (14.56 ± 0.76% vs. A: 14.81 ± 0.9%), but slightly decreased ARA in C (13.37 ± 1.05%). It is noteworthy that FM C with lower LA levels than FM B resulted in the best tissue incorporation of LCPUFAs (Clark et al. 1992), confirming earlier animal studies (McMurchie et al. 1990). Although FM provided very different amounts of FAs, the n-3 LCPUFA incorporation was similar, indicating the importance of the LA to ALA ratio (Clark et al. 1992). Nevertheless, the experimental FMs were not able to raise DHA levels to that found in BM fed infants, and ARA levels were also different compared to breast fed infants, stressing once again the need of preformed ARA and DHA for feeding preterm infants. The study further concluded that the LA to ALA ratio should not be lower than 4:1 due to potential undesirable physiological consequences, resulting from C20 and C22 PUFA levels distinctively different from that seen in BM fed infants. The recommended LA to ALA ratio described by ESPGHAN ranges from 7:1 up to 28:1, considering the lower and upper recommended intake for LA, and the minimum recommended intake for ALA (Agostoni et al. 2010). U.K. BM has a LA to ALA ratio of 8.5:1 (Yuhas et al. 2006). Considering the above mentioned study and the U.K. BM data, it might be advisable to not feed infants with a very high LA to ALA ratio of 28:1, although it falls into the ESPGHAN recommendations. Furthermore, the ratio of LA to ARA and DHA is very high in the ESPGHAN recommendations, and does not represent physiological ratios found in preterm BM or delivered by the placenta (Crawford et al. 1998). For example, the mid-range LA to mid-range ARA ratio is 32 in the recommendation, whereas it is 15 in mature preterm BM and 0.4 in umbilical cord plasma, and the mid-range LA to mid-range DHA ration is 54, whereas it is 41 in mature preterm BM and 0.8 in umbilical cord plasma (Genzel-Boroviczeny et al. 1997; Agostoni et al. 2010).

ESPGHAN recommendations were developed more than 10 years ago. Therefore, LCPUFA recommendations are due for an update based on latest evidence. However,

more research is needed to define adequate dietary LCPUFA requirements in the perinatal period of preterm infants.

1.1.10 Current dietary intake of LCPUFAs

Lapillonne and Jensen fed preterm infants (< 1500 g) with preterm BM containing 0.41% DHA and compared their blood to that of breast-fed term infants (2009). The preterm infants had 31% lower DHA red blood cell phospholipid content, indicating that preterm BM feeding alone leads to DHA deficiency of 56% at expected term. This also shows that functional development similar to *in utero* is not provided by exclusive BM feeding. Nevertheless, ESPGHAN recommendations are based on BM LCPUFA concentrations.

Lapillonne and colleagues have also conducted the first study to estimate the available DHA for accretion in the first month of life of extremely preterm infants receiving standard care in France (2010). They estimated that only 7, 21, 30 and 39% of the normally accreted DHA was available to these infants during the first, second, third, and fourth week of life, respectively, from their nutritional intake (parenteral and enteral). This related to a DHA deficit of 661 ± 100 mg per kg bodyweight four weeks after birth. The authors concluded that BM or FM DHA levels of 1.5%, similar to the recommendations by Koletzko *et al.,* are needed to compensate for the DHA loss during the first four weeks of life. However, they assumed that BM contains 0.42% DHA, whereas on average French BM more likely contains half of that (Brenna et al. 2007).

Following on from this study, De Rooy and colleagues calculated the intake of DHA as well as ARA from all sources of extremely preterm infants in the U.K. (2017) . Six weeks after birth, only 36.6% and 13.5% of the normally *in utero* accreted DHA and ARA, respectively was provided and the authors calculated that 183.4 mg ARA and 28.5 mg DHA per kg bodyweight per day would be needed to compensate for the deficits. The ARA levels were significantly below ESPGHAN recommendations in weeks one to five and DHA levels were below ESPGHAN recommendations in week one. Whether ESPGHAN recommended LCPUFA intake levels should be raised becomes a secondary question when the current recommendations cannot be reached with the presently available feeding methods. The question remains how to improve LCPUFA intake of preterm infants, to reduce the LCPUFA gap of prematurity.

1.2 Preterm infants and LPPs

1.2.1 Lipid peroxidation

N-3 and n-6 PUFAs are highly susceptible to autoxidation by oxygen radicals. There is a linear dependency between the number of double bonds and the peroxidisability of the PUFA, such that DHA has a five times greater peroxidisability than LA (Cosgrove et al. 1987). Relative rates of ARA, ALA, LA, and 18:1n-9 oxidation are 40:20:10:1 (Saxby 1996). One additional double bond in the FA structure at least doubles the rate of autoxidation (Holman and Elmer 1947).

Lipid peroxidation can be enzymatic or non-enzymatic. Enzymatic lipid peroxidation is part of normal metabolism (Benzie 1996), in which The cyclo-oxygenase and lipoxygenase metabolises PUFAs into eicosanoids and other lipid mediators. These are involved in inflammation and immunity (Massey and Nicolaou 2011). Non-enzymatic lipid peroxidation is an uncontrolled process that can have detrimental effects. It is a chain reaction process (Figure 1-3), initiated by a radical abstracting a hydrogen atom from a methylene group of an unsaturated lipid (Ayala et al. 2014), leading to the formation of a lipid radical. This is stabilised by double bond rearrangement to form a conjugated diene (Gutteridge 1995). During the propagation stage, the lipid radical rapidly reacts with molecular oxygen, producing a lipid peroxyl radical. In the next chain reaction stage, the lipid peroxyl radical reacts with another unsaturated lipid to form a lipid hydroperoxide and a new lipid radical, which starts the reaction again. The chain reaction process terminates when antioxidants, such as vitamin E, scavenge the lipid peroxyl radical, or when two radicals combine.

Initiation

$LH + R^{\bullet}$ $\qquad\qquad RH + L^{\bullet}$

Propagation

$L^{\bullet} + O_2$ $\qquad\qquad LOO^{\bullet}$

$LOO^{\bullet} + LH$ $\qquad\qquad LOOH + L^{\bullet}$

Termination

$LOO^{\bullet} + $ Antioxidant $\qquad LOOH + $ Antioxidant $^{\bullet}$
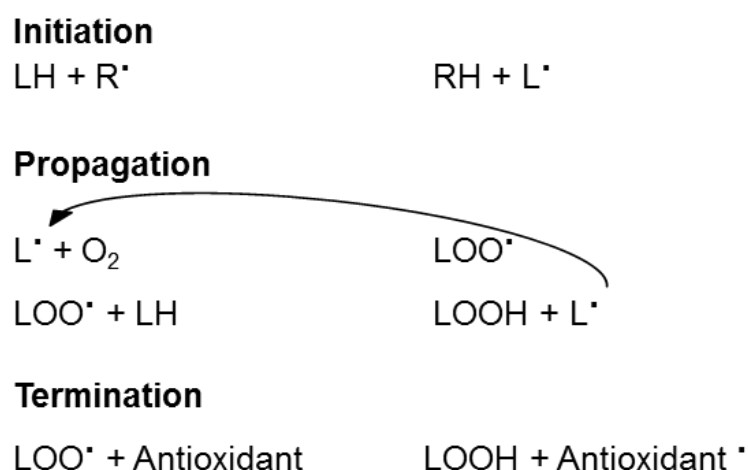
Figure 1-3: Lipid peroxidation chain reaction
Amended form (Ayala et al. 2014)

Lipid hydroperoxides are unstable primary LPPs, which rapidly decompose in the presence of transition metal ions, such as iron or copper (Esterbauer et al. 1990), to form secondary LPPs (Ayala et al. 2014). These include alkoxyl and alkyl radicals, aldehydes, ketones, and carboxyl compounds (Chávez-Servín et al. 2008). More specifically, these include the following aldehydes: malondialdehyde (MDA) which is an unspecific secondary LPP of PUFAs with more than two double bonds, as well as 4-hydroxy-2-nonenal (HNE) and 4-hydroxy-2-hexenal (HHE), resulting from n-6 and n-3 PUFA peroxidation, respectively (Figure 1-4) (Van Kuijk et al. 1990; Esterbauer et al. 1991; Ayala et al. 2014). Hexanal is the volatile secondary LPP of n-6 PUFAs (Romeu-Nadal et al. 2004). Further volatile secondary LPPs are propanal, pentanal, octanal and nonenal.
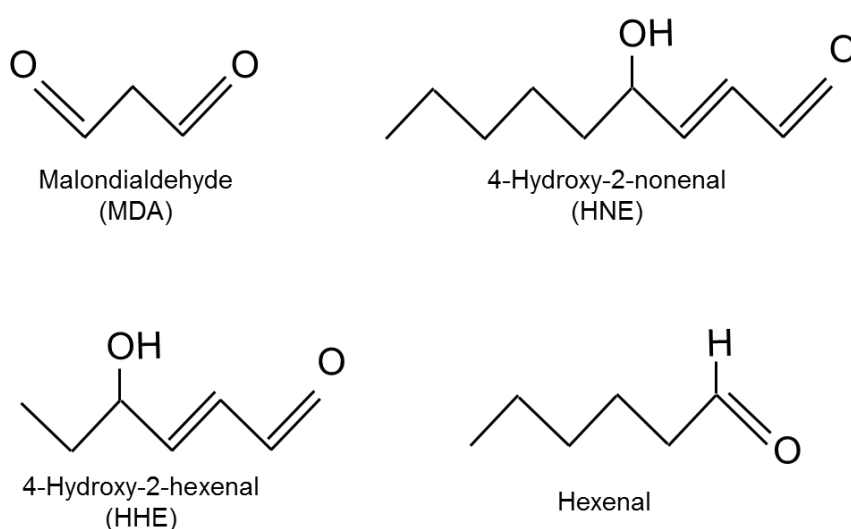
Figure 1-4: Schematic structure of LPPs

LPPs are highly lipophilic and therefore often accumulate in lipid bilayers such as in cell membranes. Effects of secondary LPPs are dose dependant (Riahi et al. 2010) and they have been described as second messengers of free radicals (Ayala et al. 2014). They can act as signalling molecules and at low physiological concentrations (around 1 µM, in humans and in cell culture) promote cell survival, for example via the up-regulation of stress response pathways (Niki 1987; Ayala et al. 2014). However, at higher concentrations HNE, HHE, and MDA can react with membrane phospholipids and proteins and alter the membrane structure, fluidity and membrane function (Riahi et al. 2010). They can also form adducts with proteins and DNA and influence the inflammatory signalling (Yadav and Ramana 2013). Therefore, high concentrations (above 10 µM) have been shown to be toxic in cell culture systems and lead to cell cycle arrest or cell death (apoptotic or necrotic), due to unrepairable cell damage (Esterbauer et al. 1991; Esterbauer 1993; Ayala et al. 2014). Secondary LPPs can be

measured in human plasma (Mori et al. 1999; Guichardant et al. 2006); however, they are metabolised in the liver as part of the xenobiotic metabolism and mainly excreted via urine (Alary et al. 1998; Guichardant et al. 2004; Yan et al. 2010). The major secondary PUFA LPPs are described in detail below.

MDA

MDA is a widely used and reliable marker of oxidative stress in clinical situations (Ayala et al. 2014). In healthy patients, the plasma concentrations range from 0.1 to 1 µM (Del Rio et al. 2005), with men having lower concentrations than women (Steghens et al. 2001). Extremely preterm infants have been shown to have plasma concentrations of 1-2 µM in the first week of life (Drury et al. 1997).

At physiological pH, MDA has a low reactivity, however at a lower pH, MDA is more reactive and rapidly attacks nucleophiles such as basic amino acid residues (lysine, histidine, arginine) or proteins (Ayala et al. 2014). Over 30 proteins, including enzymes, carrier proteins, cytoskeletal, mitochondrial and antioxidant proteins have been reported to be modified by MDA (Zarkovic et al. 2013). DNA damage induced by MDA includes mutations and strand breaks, which can lead to cell cycle arrest and apoptosis (Niedernhofer et al. 2003; VanderVeen et al. 2003; Ayala et al. 2014). Furthermore, MDA adducts can form intra or inter molecule crosslinks between proteins and/or DNA, which can lead to detrimental alterations in biochemical properties (Voitkun and Zhitkovich 1999; Niedernhofer et al. 2003).

HNE

HNE plasma concentrations in healthy men and women was 678.5 ± 421.2 nM and increased with age (Selley et al. 1989), these results were confirmed in another study with adults (Selley 1997). In moderate to late preterm neonates umbilical cord plasma levels of 100 nM have been reported (Schmidt et al. 1996). In tissue, HNE levels range from 0.1-3 µM under physiological conditions and can reach up to 12 mM under oxidative stress (Benedetti et al. 1984; Esterbauer et al. 1990).

HNE has physiological and protective functions at lower concentrations, but is cytotoxic at higher concentrations (Ayala et al. 2014). At physiological concentrations (below 10 µM), HNE can regulate transcription factors sensible to stress, such as nuclear factor erythroid 2-related factor (antioxidants and cytoprotective genes), activating protein-1 (glutathione), nuclear factor-$\kappa\beta$ (NF-$\kappa\beta$; inflammation, cell proliferation and apoptosis), and peroxisome proliferator activated receptors (lipid metabolism, mitochondrial biogenesis, antioxidant defence) (Ayala et al. 2014). However, HNE is more toxic than MDA (Michiels and Remacle 1991). At medium levels, HNE induces autophagy, senescence and cell cycle arrest, but at high levels induces apoptosis or

necrosis (Ayala et al. 2014). Concentrations above 10 µM are cytotoxic (Zarkovic et al. 1993). The toxicity can be explained by the rapid reaction with DNA and proteins. 4-Hydroxyalkenals can react with almost all amino acids (Esterbauer et al. 1991). These HNE adducts can modify enzymes, carrier proteins, membrane transporters, receptors, cytoskeletal proteins, transcription factors and antioxidants (glutathione) (Ayala et al. 2014). Similar to MDA, HNE can react with DNA and form crosslinks or DNA-protein conjugates. Genotoxicity is time and dose dependent (Eckl et al. 1993; Yadav et al. 2008) and can lead to apoptosis or necrosis (Ayala et al. 2014).

HHE

The plasma concentration of HHE is approximately twice the plasma concentration of HNE (Guichardant et al. 2006). However, the physiological reactivity is lower (Bacot et al. 2007) and therefore the higher concentrations are less damaging (Riahi et al. 2010). Of the two 4-hydroxyalkenals, HHE is much less studied than HNE. Nevertheless, HHE mediated activation of NF-$\kappa\beta$, was reported to lead to cellular dysfunction as well as apoptosis (Riahi et al. 2010).

Isoprostanoids

Isoprostanoids are LPPs that are formed non-enzymatically by free radical-catalysed peroxidation (Morrow et al. 1996). They are prostaglandin-like compounds which are widely used as markers of oxidative stress *in vivo*. F_2-Isoprostanes are derived from ARA, F_3-Isoprostanes from EPA, and DHA-derived compounds are termed neuroprostanes (Roberts et al. 2005). In situations with increased oxygen tension, e.g. in preterm infants with oxygen supplementation, isofurans and neurofurans, which have a substituted tetrahydrofuran ring in their structure, are increasingly formed (Fessel et al. 2002). 15-F_{2t}-Isoprostane is a well described biomarker for oxidative stress (Montuschi et al. 2004; Czerska et al. 2016). At birth, term infants have significantly higher 15-F_{2t}-isoprostane levels than healthy adults (approximately 2.3 vs. 9.9 pM), while preterm infants experience even higher oxidative stress and have significantly higher 15-F_{2t}-isoprostane levels than term infants (approximately 36.7 pM) (Comporti et al. 2004). Isoprostanoids are primarily produced from FAs esterified to phospholipids (Morrow et al. 1992). Since BM, DHM, and FM mainly contain triglycerides (around 98%), this thesis will focus on the toxic secondary LPPs MDA, HNE and HHE.

Table 1-5: Plasma LPP levels

	Plasma concentration	Population
MDA	0.1-1 µM; higher in women	Healthy adults
	1-2 µM	Extremely preterm infants
HNE	678.5 ± 421.2 nM; increase with age	Healthy adults
	100 nM	Moderate to late preterm infants
HHE	2x HNE levels → approximately 1.36 µM	Healthy adults
Isoprostanoids (15-F_{2t}-isoprostane)	2.3 pM	Healthy adults
	36.7 pM	Preterm infants

1.2.2 LPPs and preterm infants' health

Oxidative stress is defined as an imbalance between oxygen radicals and antioxidants, which can lead to oxidative damage (Betteridge 2000). The most common comorbidities of prematurity, retinopathy of prematurity, white matter damage, bronchopulmonary dysplasia, patent ductus arteriosus, and NEC, are classified as oxygen radical associated diseases (Saugstad 1990; O'Donovan and Fernandes 2004; Ozsurekci and Aykac 2016). Oxygen radicals, the consequential oxidative stress, and cellular as well as tissue injury play an important role in the development of these diseases (Saugstad 1990; Kelly 1993; O'Donovan and Fernandes 2004).

At birth, preterm infants have immature antioxidant systems, the enzymatic antioxidants are not fully matured yet and non-enzymatic antioxidant levels are low due to interruption of the maternal-foetal transfer (Qanungo and Mukherjea 2000; Robles et al. 2001; Georgeson et al. 2002; Lee and Chou 2005). At the same time, the transition of the preterm infant from the foetal relatively hypoxic environment to the neonatal environment outside the womb rapidly increases tissue oxygenation and increases reactive oxygen species levels (Kuligowski et al. 2014; Kuligowski et al. 2015). Due to under developed lungs, preterm infants often receive supplemental/high doses of oxygen, which can cause further oxidative stress (Tin and Gupta 2007; Ezaki et al. 2009), as oxygen is the precursor for oxygen radicals, for example produced by the hypoxanthine-xanthine-oxidase system (Saugstad 1988). Blood transfusions, which are common in preterm infants, can also increase oxidative stress (Wardle et al. 2002). Additionally, transferrin levels are low in preterm infants (Scott et al. 1975), leading to higher levels of free iron, which in turn can increase levels of hydroxyl radicals via the Fenton reaction (Saugstad 2003). Parenteral nutrition often contains peroxides, especially when not shielded from light (Laborie et al. 2002; Miloudi et al. 2012), and studies reported higher levels of lipid peroxides in FM than in BM (Michalski et al.

2008) (discussed further in Chapter 1.2.3 and 2.1.3). This large oxidative stress burden of preterm infants greatly increases the risk of endogenous lipid peroxidation and consequently tissue damage.

Indeed, significantly higher levels of MDA have been found in cord blood of preterm compared to term infants (e.g. 1.86 vs. 1.11 nmol/L) (Negi et al. 2012; Abdel Ghany et al. 2016). Furthermore, urinary MDA was twice as high in extremely low birthweight infants compared to low birthweight infants (Schlenzig et al. 1993). Urinary MDA levels in preterm infants were also significantly higher with the incidence of bronchopulmonary dysplasia (Schlenzig et al. 1993; Weinberger et al. 2004), patent ductus arteriosus (Schlenzig et al. 1993), retinopathy of prematurity, white matter damage, and NEC (Weinberger et al. 2004). Recently, a correlation of urinary isofurans in the first four days of life with the later incidence of bronchopulmonary dysplasia was reported (Kuligowski et al. 2015).

Activation of inflammatory pathways plays a crucial role in the aetiology of these diseases (Fink et al. 2016), and infection and inflammatory conditions in general are a major source of morbidity and mortality in premature infants. The generated LPPs can indirectly increase inflammation by damaging cells and tissues, but also have direct effects by activating inflammatory pathways, such as Toll-like receptors and NF-$\kappa\beta$ (Lavoie et al. 2010). Toll-like receptor activation plays a critical role in the development of NEC (Hackam and Sodhi 2018).

LPP induced inflammation might also lead to adverse cognitive development. The Extremely Low Gestational Age Newborns Study (ELGAN) explored the relationship between inflammation and risk of subsequent development of brain disorders in preterm infants born at less than 28 weeks gestation, (O'Shea et al. 2009). The time-course of inflammation (blood levels of 25 inflammation related proteins) was measured over two weeks after birth and was associated with a range of adverse cognitive outcomes, including impaired mental and motor development at two years of age (Kuban et al. 2015; Dammann et al. 2016; Kuban et al. 2017), and importantly these effects were shown to persist at 10 years of age (Kuban et al. 2017).

1.2.3   LPPs in dietary sources for preterm infants

Peroxidative damage of PUFAs in dietary sources for preterm infants not only decreases the PUFA levels, but also increases the LPP levels, which are ingested by the infants. Lipid peroxidation in dietary sources for preterm infants has been shown. For example, in BM the primary LPPs, lipid hydroperoxides were detected (Van Zoeren-Grobben et al. 1993). The presence of secondary LPPs has also been

demonstrated in several studies. Turoli and colleagues found conjugated dienes (24.33 µM), thiobarbituric acid reactive substances (TBARS; an indirect measurement of MDA, 3.17 µM), and lipid peroxides (6.15 µM) in fresh BM (2004). Michalski and colleagues measured MDA and 4-hydroxyalkenals in fresh BM and found higher levels of HHE/n-3 FA than HNE/n-6 FA (2008). Silvestre and colleagues found MDA concentrations of 0.72 µM in fresh BM (2010). Volatile LPPs can also be formed in BM. Pentanal, hexanal, octanal, nonenal have all been detected (Elisia and Kitts 2011). Lipid peroxidation in BM is life-style dependent, for example, BM from non-smoking mothers had the lowest MDA concentration (7.07 ± 1.2 µM), higher levels were found in BM from passive-smoking mothers (8.83 ± 1.91 µM), and the highest levels in active-smoking mothers (10.6 ± 2.02 µM), (Ermis et al. 2005).

BM storage leads to a time and temperature dependent increase in LPPs (Miranda et al. 2004; Michalski et al. 2008; Silvestre et al. 2010). For example, refrigerated storage for 48 hours almost doubled MDA in BM, and this increase was significant (Miranda et al. 2004). Frozen storage of BM at -20°C also significantly increased MDA in BM (+0.36 µM after 60 days storage) (Silvestre et al. 2010). DHM contains therefore higher levels of LPPs than fresh BM (Turoli et al. 2004). The effect of storage as well as other human milk banking practices on lipid peroxidation in DHM is described in greater detail in Chapter 5.3.4. However, specific levels of LPPs in DHM that would be fed to an infant in the neonatal unit have not been determined yet.

In (preterm) liquid and powder FM, lipid hydroperoxides, MDA, TBARS, and volatile peroxidation products were detected (Manglano et al. 2005; Michalski et al. 2008; Chávez-Servín 2009; Cesa et al. 2012). LPP content was higher in preterm FM than in fresh BM (Zoeren-Grobben et al. 1993; Raghuveer et al. 2002; Martysiak-Zurowska and Stolyhwo 2006; Michalski et al. 2008; Almansa et al. 2013), indicating a higher intake of LPPs from this non-maternal source. A significant time and temperature dependent increase in LPP content was also demonstrated in FM powder after storage for 21 days (Almansa et al. 2013; Cesa et al. 2015). Refer to Chapter 2.1.3 for a detailed overview of lipid peroxidation in FM. However, no study could be identified that reported the LPP levels of U.K. preterm FM after storage under current hospital storage conditions.

Although levels of LPPs in non-maternal sources for preterm infants in the U.K. are not know, evidence suggests that LPPs are present. LPP intake from parenteral nutrition was associated with a higher risk (+32%) for bronchopulmonary dysplasia in preterm infants (Chessex et al. 2007). Results from animal studies suggest that LPP ingestion can have adverse effects on the gut and also outside of the digestive system (Izaki et

al. 1984; Awada et al. 2012) (for a detailed review see Chapter 3.1.2). Therefore, it is important to understand the effects of LPP ingestion on the preterm gut, which is more prone to inflammation.

2 Influence of current storage practices on LCPUFA and LPP content of preterm FM in the U.K.

2.1 Introduction

Specialised FMs are available for the dietary management of preterm and low birth weight infants. Specialised FMs are foods for special medical purposes and must be prescribed and used under medical supervision (Crawley et al. 2019). In the U.K. these FMs are bovine milk based and have a higher energy density (ca 80 kcal/100 mL in first FM and ca 75 kcal/100/mL in post-discharge FM vs. 66 kcal/100 mL in term FM) and contain more macronutrients (carbohydrates and protein, but not fat), vitamins, and minerals than term FM (Crawley et al. 2019). Preterm infants, as well as low birthweight infants, and immunocompromised infants are at high risks for infections. Since powdered infant FM cannot be produced sterile, and might be contaminated with *Enterobacter sakazakii* and *Salmonella*, the WHO recommends using sterile liquid FM for the above mentioned infant groups (WHO and FAO 2007).

In the U.K., FMs for preterm infants (Nutriprem and Gold Prem) are provided by Cow & Gate (Nutricia Ltd) and SMA (Nestlé UK Ltd), respectively (Crawley et al. 2019). Both manufacturers provide ready to feed liquid FM for preterm/low birthweight infants (< 1800 g; Nutriprem 1 and Pro Gold Prem 1) and ready to feed liquid FM as well as powdered FM (Nutriprem 2 and Pro Gold Prem 2) for preterm infants post discharge or from 1800 g bodyweight onwards, up to six months corrected gestational age (infants age minus the number of weeks it was born early) (Figure 2-1).

Figure 2-1: U.K. preterm FM

Pro Gold Prem 1, Pro Gold Prem 2, Nutriprem 1, and Nutriprem 2 contain, 4.0, 3.8, 3.9, and 4.0 g fat per 100 mL FM, respectively (Danone Nutricia Early Life Nutrition 2017a, 2017b; SMA Nutrition 2017a, 2017b), more than preterm BM which contains around 3.4 g fat per 100 mL (Léké et al. 2019). Nutriprem FMs contain mainly vegetable oils (sunflower, rapeseed, coconut, palm, evening primrose), but also single cell oil, egg

lipids, and fish oil. The Gold Prem range contains vegetable oils (sunflower, palm, rapeseed, coconut), as well as single cell oils.

In the last decades, the fat composition of FM was greatly improved to more and more mimic the fat composition of human BM (Zou et al. 2016; Wei et al. 2019a). First, vegetable oils were added to the bovine milk based FM, to match the type and levels of FAs (Wei et al. 2019a). These include coconut, palm, high oleic safflower, sunflower, soy, and canola oil. BM contains around 43% saturated FA, mainly palmitic acid (20%); monounsaturated FA (38%), mainly oleic acid (30%); and PUFAs (16%), mainly LA (12%). Fish oil, single cell oil or egg phospholipids are added to provide LCPUFAs (Wei et al. 2019a). Addition of these is necessary since Cow's milk, for example, has one fourth of DHA compared to BM (0.09-0.11 vs. 0.37 ± 0.11 %) (Tripathi 2014; Fu et al. 2016). BM contains around 98% triacylglycerols, with a specific distribution of FAs. Saturated FAs including palmitic acid (16:0) are mainly attached in the *sn*-2 position (70%), and oleic acid (18:1n-9) and LA in the *sn*-1, 3 position (Qi et al. 2018). This FA distribution is beneficial for infants, it influences fat absorption and metabolism (Wei et al. 2019a). However, cow's milk fat and vegetable oil have a different triacylglycerol structure. Cow's milk contains a similar amount of palmitic acid, but only 40% is in the *sn*-2 position, and in vegetable oil palmitic acid is mainly attached in the *sn*-1,3 position, whereas oleic acid and LA are in the *sn*-2 position. During digestion, non-esterified saturated FAs can form insoluble soaps with minerals in the gut, which cannot be absorbed and result in hard faeces and loss of nutrients (Zou et al. 2016). Therefore, specific structured triacylglycerols which mimic the FA distribution of BM were developed. These resulted in reduced soap excretion and softer stools (Yao et al. 2014), as well as higher bone mineralisation (Beghin et al. 2019), due to better calcium absorption, in clinical trials.

Latest research is focusing on the milk fat globule membrane and its provision of phospholipids. The milk fat globule membrane is a trilayer membrane that surrounds the triacylglycerols of BM. Phosphatidylethanolamine (10-36%), phosphatidylcholine (19-38%), phosphatidylinositol (<10%), phosphatidylserine (<10%) and sphingomyelin (29-45%) can be found in BM fat, which contains around 1% phospholipids (Wei et al. 2019a). Cow's milk also contains a milk fat globule membrane, which however is damaged during dairy processing, and vegetable oil has no membrane. Generally, the amount of phosphatidylcholine is much higher and the amount of sphingomyelin lower in FM than in BM (Wei et al. 2019b). Researchers have added milk fat globule membranes extracted from bovine milk to FM in clinical trials and reported better performance in cognitive tests at 12 months of age in experimental FM fed infants than in standard FM fed infants (Timby et al. 2014). Performance of the experimental FM

group was not different from the breast fed infants. Better IQ scores for hand eye coordination, and performance, as well as general IQ in infants receiving the experimental FM compared to infants receiving the control FM were also reported (Gurnida et al. 2012).

2.1.1 LCPUFAs in preterm FM

Addition of LCPUFAs to FM started in 1994 in Europe (Hadley et al. 2016). The composition of infant FM is regulated by the Regulation (EU) No 609/2013, and Commission Delegated Regulation (EU) 2016/128 and 2016/127. In contrast to the former Commission Directive 2006/141/EC, which will only be repealed in February 2020, the new Delegated Regulation 2016/127 requires the addition of DHA but not ARA to infant FM and follow-on FM, whereas before, addition of both LCPUFAs was optional (Table 2-1).

Table 2-1: Comparison of legal LCPUFA requirements of (preterm) FM

	Commission Directive 2006/141/EC	**Delegated Regulation 2016/127**
LA	300 mg – 1200 mg / 100 kcal	500mg – 1200 mg / 100 kcal
ALA	not less than 50 mg / 100 kcal	50 mg – 100 mg / 100 kcal
ARA	may be added; max 1% of total fat content	may be added; max 1% of total fat content
DHA	may be added; shall not exceed that of n-6 LCPUFAs	20 mg – 50 mg / 100 kcal

The Commission Delegated Regulation (EU) 2016/127 is based on the latest "Scientific Opinion on the essential composition of infant and follow-on formulae", published by the European Food Safety Authority (EFSA) (EFSA Panel on Dietetic Products and Allergies 2014). The panel concluded "that there is no necessity to add ARA to infant formula even in the presence of DHA". Well-known researchers in the field of LCPUFAs and infant nutrition have criticised the EFSA statement and its translation into European law (Crawford et al. 2013, 2015; Koletzko et al. 2015; Brenna 2016). One major critique point was that the EFSA panel only reviewed publications from 2000 onwards. Crawford and colleagues have pointed out that this ignores the discovery of prostaglandins and related biologically active substances (a discovery that led to the 1982 Nobel Prize in Physiology and Medicine) (2015). These are ARA metabolites responsible for homeostatic functions, and mediation of pathogenic mechanisms like inflammation (Ricciotti and FitzGerald 2011). Additionally, the EFSA panel disregards human physiology, in which ARA levels in BM are very similar across the world, and normally occur in comparable or higher levels than DHA (Fu et al. 2016), and human BM should be regarded as best nutritional source for term infants. A systematic review

identified that ARA was either similar or higher than in term BM (Bokor et al. 2007). Furthermore, when DHA and DHA+ARA supplementation of FM was compared in clinical trials, none of the studies investigated ARA specific outcomes such as immune or vascular function (Brenna 2016). However, it has been shown that omitting ARA from FM will lead to decreased circulating ARA levels (Makrides et al. 1995b). Furthermore, the critiquing authors point towards the Retina Foundation Southwest study performed by Birch and colleagues, in which term infants were fed either 0.35% DHA, or 0.36% DHA in combination with 0.72% ARA (1998). Follow up at four years of age revealed significantly lower verbal IQ scores in the DHA only group compared to breast fed infants, whereas there was no difference to breast fed infants in the DHA+ARA group (Birch et al. 2007). Koletzko and colleagues also critique that there are no scientific studies using FM with 1% DHA and no ARA, and therefore there is lack of knowledge of the effects, suitability and safety of these FMs (2015). Regarding this, it was also pointed out that omitting ARA from FM might have a similar effect as COX-2 inhibitors (Crawford et al. 2015). COX-2 is the enzyme responsible for the conversion of ARA into prostaglandins, a precursor of prostacyclin. These inhibitors were developed to prevent inflammation and pain in rheumatoid arthritis, however, use resulted in more cardiovascular and stroke events, due to prothrombotic activity (Mukherjee et al. 2001). In the presence of reduced circulating ARA levels, DHA can act as COX-2 inhibitor, potentially leading to infant cardiovascular events, strokes or potentially deaths (Crawford et al. 2015). Overall, the effects might be even more dramatic in preterm infants, who haven't had the chance to accumulate large amounts of ARA during the last trimester.

After the Delegated Regulation 2016/2017 came into force in February 2020, SMA had to make changes to their preterm FM to be compliant. The DHA content of Pro Gold Prem 1 changed from 18 mg/100 kcal to 25 mg/100 kcal (SMA Nutrition 2020a). They have also increased DHA levels in Pro Gold Prem 2 to the same levels as Pro Gold Prem 1 (SMA Nutrition 2020b). Both manufacturers have not omitted ARA from their preterm FMs, and maintained ARA levels at the same amount per 100 kcal as DHA (Danone Nutricia Early Life Nutrition 2020b, 2020a; SMA Nutrition 2020b, 2020a). Experiments carried out in this chapter used FM from 2017, therefore the following section describes preterm FMs at that time. All preterm FMs available in the U.K. in 2017 contain both ARA and DHA (Danone Nutricia Early Life Nutrition 2017a, 2017b; SMA Nutrition 2017b, 2017a). LCPUFA levels in preterm FMs from 2017 are presented in Table 2-2.

Table 2-2: Absolute LCPUFA content of preterm FM in the U.K.

	Nutriprem 1[1]	Nutriprem 2[2]	Pro Gold Prem 1[3]	Pro Gold Prem 2[4]
	mg per 100 ml / mg per 100 kcal			
LA	data not available	data not available	561 / 701	573 / 785
ALA	data not available	data not available	77 / 96	75 / 103
ARA	20 / 20	20 / 20	15 / 18	14.4 / 20
DHA	10 / 20	10 / 20	15 / 18	14.4 / 20

Data from the U.K. as of March 2017

Amended from [1](Danone Nutricia Early Life Nutrition 2017a); [2](Danone Nutricia Early Life Nutrition 2017b); [3](SMA Nutrition 2017b); [4](SMA Nutrition 2017a)

These translate into the weight percentages (of total FAs) described in Table 2-3. Although preterm FMs contain more LCPUFAs than BM from women in the U.K., ARA levels are still below the worldwide levels of term BM (Yuhas et al. 2006; Fu et al. 2016; Danone Nutricia Early Life Nutrition 2017a, 2017b; SMA Nutrition 2017b, 2017a). In comparison, in Nutriprem products, DHA is similar to worldwide DHA term BM levels, and Pro Gold Prem products provide higher DHA levels, potentially taking into account that preterm BM contains more DHA than term BM (Bokor et al. 2007; Fu et al. 2016).

Table 2-3: Relative PUFA levels in BM and preterm FM in the U.K.

% of total FA	Term BM U.K.[1]	Nutriprem 1[2]	Nutriprem 2[3]	Pro Gold Prem 1[4]	Pro Gold Prem 2[5]
LA	10.45 ± 0.41	13.7	13.5	15.41	17.46
ALA	1.22 ± 0.06	1.97	1.86	2.11	2.28
ARA	0.36 ± 0.01	0.48	0.46	0.41	0.43
DHA	0.24 ± 0.01	0.37	0.35	0.41	0.43

Amended from [1](Yuhas et al. 2006); [2](Danone Nutricia Early Life Nutrition 2017a); [3](Danone Nutricia Early Life Nutrition 2017b); [4](SMA Nutrition 2017b); [5](SMA Nutrition 2017a)
Data from the U.K. as of March 2017

In term and preterm BM, phospholipids also contain ARA (2.0-3.1%) and DHA (0.5-1.6%). Nutriprem FMs contain soy lecithin and egg lipids, providing phospholipids. Composition of these are not provided by the manufacturer in the data cards. However, analysis of eight FMs in China revealed that the phospholipid composition was significantly different than that of BM, even though some of the FMs were supplemented with milk fat globule membrane, phospholipids, or lecithin (Wei et al. 2019b). FM phospholipids contained only 0.2-1.2% ARA and no DHA at all.

Besides all efforts, FM total LCPUFA composition is still very different from BM LCPUFA composition. Although ARA and DHA levels are similar to reported concentrations in human BM worldwide (Brenna et al. 2007), little attention is paid to other LCPUFAs, including dihomo-γ-linolenic acid, eicosapentaenoic acid, adrenic acid, and docosapentaenoic acid n-3 and n-6. For example, according to the datasheets, SMA's preterm range does not contain any of these LCPUFAs (SMA Nutrition 2020a, 2020b), whereas Cow and Gate's preterm range contains some of these LCPUFAs, albeit in very different concentrations compared to U.K. BM (dihomo-γ-linolenic acid: 0.04 vs. 0.33%; eicosapentaenoic acid: 0.08 vs. 0.11%; adrenic acid: 0 vs. 0.08%; docosapentanoic acid n-3: 0.02 vs. 0.18%; docosapentaenoic acid n-6: 0 vs. 0.03%) (Yuhas et al. 2006; Danone Nutricia Early Life Nutrition 2017a, 2017b). Normally, adrenic acid rapidly accumulates in the developing brain after birth (Martínez and Mougan 1998), therefore a lack of adrenic acid, together with low levels of its precursor ARA, are concerning. Low levels of these other LCPUFAs are not surprising, since they are not regulated in the current law. Concerning these LCPUFAs, the Commission Directive 2006/141/EC only states that C20 and C22 n-3 LCPUFAs shall not exceed 1% of total fat content, while the eicosapentaenoic acid content shall not exceed the DHA content. Furthermore, C20 and C22 n-6 LCPUFAs shall not exceed 2% of total fat content. Similarly, the Delegated Regulation 2016/127 states that other C20 and C22 LCPUFAs may be added, while the n-6 LCPUFAs shall not exceed 2% of the total fat content, and eicosapentaenoic acid content shall not exceed the DHA content.

2.1.2 Influences and alterations to LCPUFA content of FM

During shelf-life

FMs contain PUFAs as well as minerals such as iron, which in combination can lead to lipid peroxidation (Marshall and Roberts 1990), and loss of LCPUFAs. Studies have determined that storage of FM containing LCPUFAs for three, 12, or 18 months from production, had no significant effect on LCPUFA levels (Chávez-Servín 2009; Carmen García-Martínez et al. 2010), suggesting that LCPUFAs are stable in FM during the shelf-life. However, significant peroxidation can occur in FM without decreasing LCPUFA levels (Chávez-Servín 2009) (Chapter 2.1.3).

In FM that has been opened

The LCPUFA content of FM that has been opened for use was reported in one study, in which opening and subsequent storage for 30 days had no effect (Zunin et al. 2015). In another study, hospital/domestic use was mimicked, by opening the FM three times per day, stirring it each time in the original packet and removing two scoops (Chávez-Servín et al. 2008). Doing so for 70 days had no effect on LCPUFA percentage composition.

2.1.3 Influences and alterations to LPP content of FM

In general, FMs containing LPCUAs are more susceptible to peroxidation than FMs not containing LCPUFAs; LCPUFA containing FMs had higher MDA concentrations (e.g. 135 nmol/g vs. 35 nmol/g (Burg et al. 2010); 8.2 ppm vs. 2.8 ppm (Michalski et al. 2008); 1066 ppm vs. 590 ppm (Cesa 2004)) and peroxide values (0.98 vs. 0.52 mequiv O_2/kg) (Romeu-Nadal et al. 2007) than FMs without LCPUFAs. Furthermore, the MDA concentration correlated positively with the ARA, as well as the DHA concentration in FMs (Almansa et al. 2013).

During shelf-life

No differences in peroxidation products were detected when Spanish bovine-milk based infant FM was analysed three months after production (stored at 25°C) (Carmen García-Martínez et al. 2010). However, an increase of 40% in MDA has been recorded for FMs that were stored for 12 months after manufacturing (Cesa et al. 2015), and a significant time-dependent increase in peroxide value, hydroperoxide content, and TBARS was reported for FM stored for 17 months after manufacturing, although the shelf life of these products was three years (Manglano et al. 2005). Volatile aldehydes such as propanal, pentanal and hexanal, also increased during the shelf-life (18 months) of FM (Chávez-Servín 2009).

In FM that has been opened

Once FMs are opened, they are highly susceptible to lipid peroxidation (Chávez-Servín et al. 2008) due to increased oxygen exposure. Indeed, lipid peroxidation occurred in FM, with a significant increase of MDA and peroxide value over 21 days after opening of the box and storage at room temperature (Almansa et al. 2013; Ghazal et al. 2016). Similarly, FM storage at room temperature for 21 days increased MDA when FM was analysed six months after production, but did not change when FM was used directly after production (Cesa et al. 2015). This increase was also temperature dependent with higher MDA concentrations in samples stored at 28°C than at 20°C (75 vs. 25% increase in MDA, respectively) (Cesa et al. 2015). Surh and colleagues analysed the HHE and HNE content of Korean FM after opening and at 10 days (2007). The FM was opened for 3 minutes every day and stirred. They reported significant increases in the hydroxyalkenal content after 10 days. The tested term FMs contained similar levels of DHA to preterm FM in the U.K., but five times less ARA. Researchers in Spain opened 20 term FM three times per day and discarded two scoops (Chávez-Servín et al. 2008). Seven of the 20 FMs contained DHA and 12 of the 20 FMs contained ARA, all in varying levels which were lower than levels in preterm FM in the U.K. During the storage for up to 70 days, FM was kept at room temperature (25°C), and significant increases in propanal, pentanal and hexanal were detected over the storage time in all

LCPUFA containing FMs. Storage of liquid FM, containing LCPUFAs, for 1 and 24 hours at 4°C almost doubled and tripled the MDA concentration, respectively (Michalski et al. 2008).

2.2   Aim and objectives

As reviewed above, LCPUFA stability and lipid peroxidation have been studied over the shelf-life of FM. However, only one study has investigated the effect of clinical/domestic storage and use on LCPUFA content after opening of the FM (Chávez-Servín et al. 2008). Furthermore, although there have been more (five) studies investigating lipid peroxidation after FM opening, the majority of these studies have only opened the FM once and then analysed LPPs at various time-points afterwards. Only one study tried to actually mimic hospital/domestic use, in which FM powder is opened several times per day and FM is discarded (Chávez-Servín et al. 2008). Furthermore, all studies were performed on term FMs, which at least in the U.K. contain only around half as much LCPUFAs as preterm FM (Danone Nutricia Early Life Nutrition 2017b; SMA Nutrition 2017a; Danone Nutricia Early Life Nutrition 2018; SMA Nutrition 2018).

Manufacturers recommend preparing individual FM feeds when required, and the WHO guidance also recommends preparing feeds fresh as best practice. However, the WHO acknowledges that feeds may need to be prepared in advance for practical reasons in care settings or at home (WHO and FAO 2007). Therefore, it is recommended to store prepared, cooled feeds in a refrigerator (≤ 5°C) for a maximum of 24 hours. To the best of the authors' knowledge, there have been no studies looking at the effect of storing prepared FM powder and only one study has investigated the effect of storing opened liquid FM (Michalski et al. 2008).

Therefore, it was the aim of this study to investigate the effect of different storage conditions, which actually mimic the clinical care settings, on the LCPUFA and LPP content of preterm FM in the U.K.

- The first objective was to quantify the initial LCPUFA levels in liquid and prepared powder FM and to compare them to the levels stated on the FM labels
- The second objective was to quantify the absolute LCPUFA content in liquid and prepared powder FM, and to assess whether there was any decrease during FM storage
- The third objective was to calculate the daily LCPUFA intake from FMs for a 1 and 2 kg infant, and to compare it to ESPGHAN recommendations
- The fourth objective was to calculate the LCPUFAs available for accretion for a 1 and 2 kg infant and to compare it to estimated *in utero* accretion levels
- The fourth objective was to investigate lipid peroxidation in liquid and prepared powder FM during storage by measuring TBARS and HNE levels

2.3 Material and methods

Ethical approval for this project (Reference ID 12492) was obtained from Bournemouth University's Research Ethics (Appendix II.I). Two standard preterm FMs are available in the U.K., which were included in the analysis (Table 2-4). Data cards are provided in Appendix II.II. Since preterm FM is only available on prescription, samples were provided by the manufacturers (Nutricia Ltd, Nestlé UK Ltd). The manufacturers had no involvement in the experimental design, data analysis, and dissemination of the results.

Table 2-4: Preterm FMs included in the analysis

Category	Name	Company	Samples
Infant milk for preterm infants	Nutriprem 1 (liquid)	Cow & Gate, Nestlé UK Ltd	5
	Pro Gold Prem 1 (liquid)	SMA, Nutricia Ltd	3
Post-discharge infant milk for preterm infants	Nutriprem 2 (liquid)	Cow & Gate, Nestlé UK Ltd	5
	Nutriprem 2 (powder)	Cow & Gate, Nestlé UK Ltd	6
	Pro Gold Prem 2 (liquid)	SMA, Nutricia Ltd	3

Consultation with a Children's Dietitian at Poole Hospital NHS Foundation Trust identified that opened liquid FM and made-up powder FM might be stored for up to 12 hours and 24 hours, respectively, at 4°C in some hospitals. This was taken into consideration in the experimental design.

Liquid FMs were analysed for FA content at opening and after 4 hours storage at 4°C. Since only fresh FM samples were to be analysed, FA could not be analysed at 1 and 10 hours (Figure 2-2 A). Powder FMs were tested on day 0, 7, 14, 21, and 28 at 0, 4 and 24 hours storage at 4°C after preparation, respectively (Figure 2-2 B). These time-points were chosen to test the FM weekly after opening, for the recommended time of use by the manufacturers (28 days). To simulate normal use, powder was removed three times per day as it would be for feed preparation. Three times per day were chosen as pragmatic approach to represent top-up FM feeding. A similar approach has been used in other studies (Chávez-Servín et al. 2008), although in some cases in real life it would be more frequent.
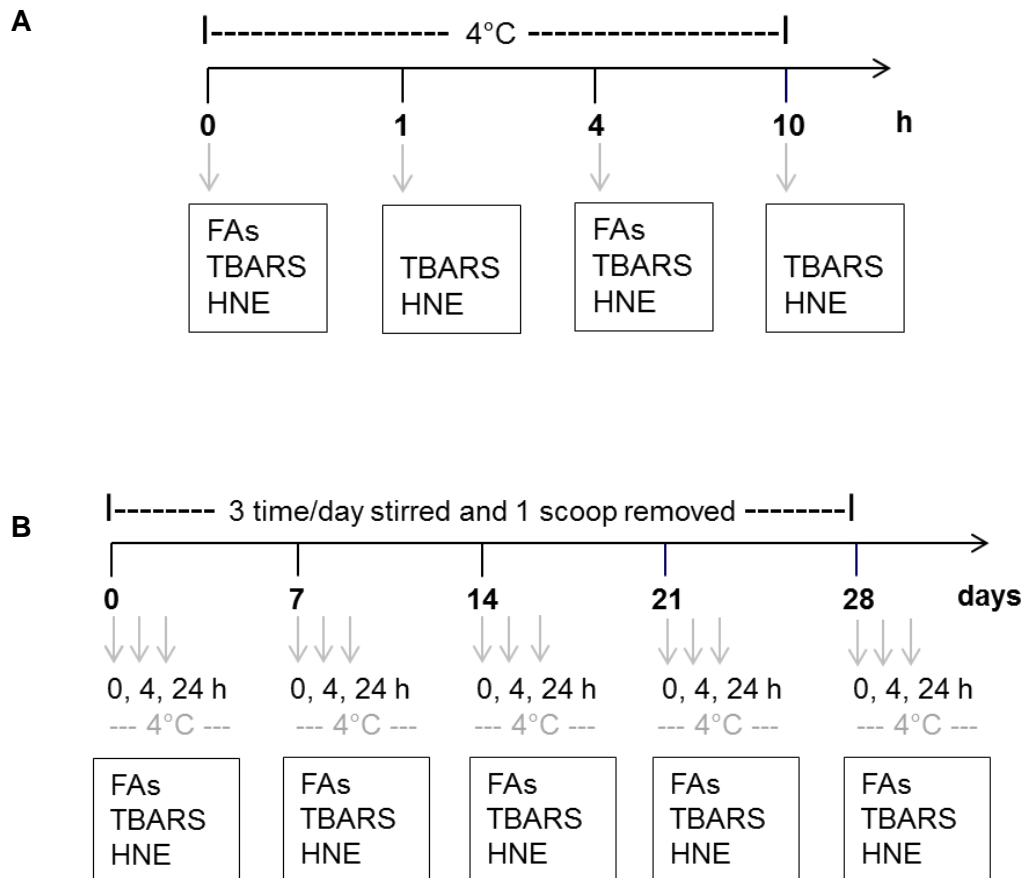
Figure 2-2: Time-line for preterm FM sampling

A: Liquid FMs were opened at 0 hours and afterwards stored at 4°C. FA content was analysed at 0 hours and 4 hours. Samples for LPP analysis were taken at 0, 1, 4, 10 hours and stored at -80°C until analysis
B: FM powder was prepared fresh at 0, 7, 14, 21, and 28 days and was then stored at 4°C. FA content was analysed at 0, 4 and 24 hours and samples for LPP analysis were taken at 0 ,4, 24 hours and stored at -80°C until analysis

It is recommended that FM powder should be prepared using 1000 mL H_2O, boiled and cooled for 30 min. It was determined that this temperature is 62°C. On the test day, FM powder (Nutriprem 2: 5.1 g/30 mL) was prepared using distilled H_2O (dH_2O) (62°C) and liquid FM was opened. Samples for FA analysis were processed directly, whereas samples for LPP analysis were stored under N_2 and frozen at -70°C until analysis.

2.3.1   FA quantification

FA extraction and transesterification

Lipids were extracted according to the Bligh and Dyer method (1959), which has been used previously in our laboratory to analyse FAs in BM (De Rooy et al. 2017), together with the transesterification according to Morrison and Smith (1964).

Extractions were carried out in a room without artificial light and solvents were kept on ice prior to the start. 2,[6]-Di-tert-Butyl-p-cresol (butylated hydroxytoluene, Sigma, U.K.) was added to the solvents (0.05%, w/v) as an antioxidant (Wren and Szczepanowska

1964), and tubes were flushed with N_2 (grade N4.8, BOC, U.K.) whenever caps were opened, to further protect FAs. Sample preparation was practiced until a coefficient of variation (CV%) of ≤ 15% was consistently achieved (ARA: 11.7%, DHA 11.4%). Thereafter, only single samples were analysed.

To 500 µL FM (liquid or powder prepared according to manufacturer's instructions), 700 µL dichloromethane/methanol/butylated hydroxytoluene (1:2, v/v, 0.05%, w/v) (Sigma Aldrich, U.K.), 400 µL C23:0 (25 mg/100 mL dichloromethane) as internal standard, and 800 µL methanol/butylated hydroxytoluene (0.05% w/v) were added and samples homogenised for 3 min. Next 625 µL dichloromethane/butylated hydroxytoluene (0.05% w/v) was added and samples homogenised for a further 3 min. In the next step, 625 µL dH_2O was added and the samples homogenised for a further 3 min, followed by a centrifugation step (2500 x g, 5 min, 10°C). The upper aqueous phase was discarded, and the organic phase filtered through Whatman phase separator filter paper (1PS, GE Healthcare Life Sciences, U.K.).

The organic phase was collected in 5 mL Reacti-Vials (Thermofisher, U.K.) and directly transesterified (Morrison and Smith 1964). Therefore, 1 mL BF_3-methanol solution (14%, Aldrich Chemistry, U.K.) was added and samples were placed in a Pierce Reacti-Therm Heating Module (Thermofisher, U.K.) for 20 min at 100°C. After 10 min cooling, 2 mL pentane (Sigma Aldrich, U.K.) and 1 mL dH_2O were added and the samples homogenised for 3 min, followed by a centrifugation step (2500 x g, 5 min, 10°C). The organic phase containing the FA methyl esters (FAMEs) was transferred to an amber gas chromatography (GC) vial (Agilent Technologies, U.K.), evaporated to dryness under a gentle stream of N_2 (SC-3 Sample Concentrator, Techne, U.K.), and re-suspended in 100 µL hexane/butylated hydroxytoluene (0.05% w/v) (Sigma Aldrich, U.K.). Samples were analysed within 3 weeks and stored until then at -20°C under N_2 (grade N4.8, BOC, U.K.).

FAME Quantification by GC

Quantification of FAMEs was performed by GC coupled with flame ionisation detector (FID). The GC-FID consists of an Agilent Technologies 7820A gas chromatograph, fitted with an Omegawax 100 fused silica capillary column (15 m x 0.1 mm x 0.1 µm film thickness, Supelco, Sigma Aldrich, U.K.; constant N_2 (grade N5.5, BOC, U.K.) flow at 0.2 mL/min, 39.5 psi pressure, average velocity 22.7cm/s). The temperature programme is described in Table 2-5.

Table 2-5: GC oven temperature programme for FAME analysis

	Increment (°C/min)	Temperature (°C)	Hold (min)
Initial		140	0
Ramp 1	60	200	0
Ramp 2	20	250	11.5
Total			15

Usually, 1 µL sample was injected in the split inlet (50:1 split, 250°C, 39.5 psi pressure, 10.7 mL/min). Detection was carried out with an FID (260°C, H_2 (grade N5.5, BOC, U.K.) flow 40 mL/min; air flow 450 mL/min (HT15-00137, Bambi Air Compressors Ltd, U.K.); constant makeup (N_2, grade N5.5) flow 30 mL/min). Identification of individual FAMEs was carried out in comparison to a range of analytical standards (Supelco PUFA No. 1 marine source; Supelco PUFA No. 2 animal source; Supelco 37 Component FAME Mix; all Sigma-Aldrich, U.K.; Figure 2-3), run on each analysis day under the same conditions as FAME quantification. Different retention times occurred due to column changes between experiments. The column has to be shortened before re-instalment, leading to a shorter run through the column and therefore, different retention times.

Analysis was performed using the ChemStation Software (Rev. C.01.04(35), Agilent Technologies, U.K.). Absolute values for FAs were calculated using the following formula (Benedetto 1987):

$$FA\,(mg/100\,ml\,FM) = aFA/aStd \times wStd \times RF \times 200$$

aFA = peak area of FA; aStd = peak area of internal standard; wStd = weight of internal standard per 0.5 mL sample; RF = detector response factor

The detector response factor was 1.1 for DHA.
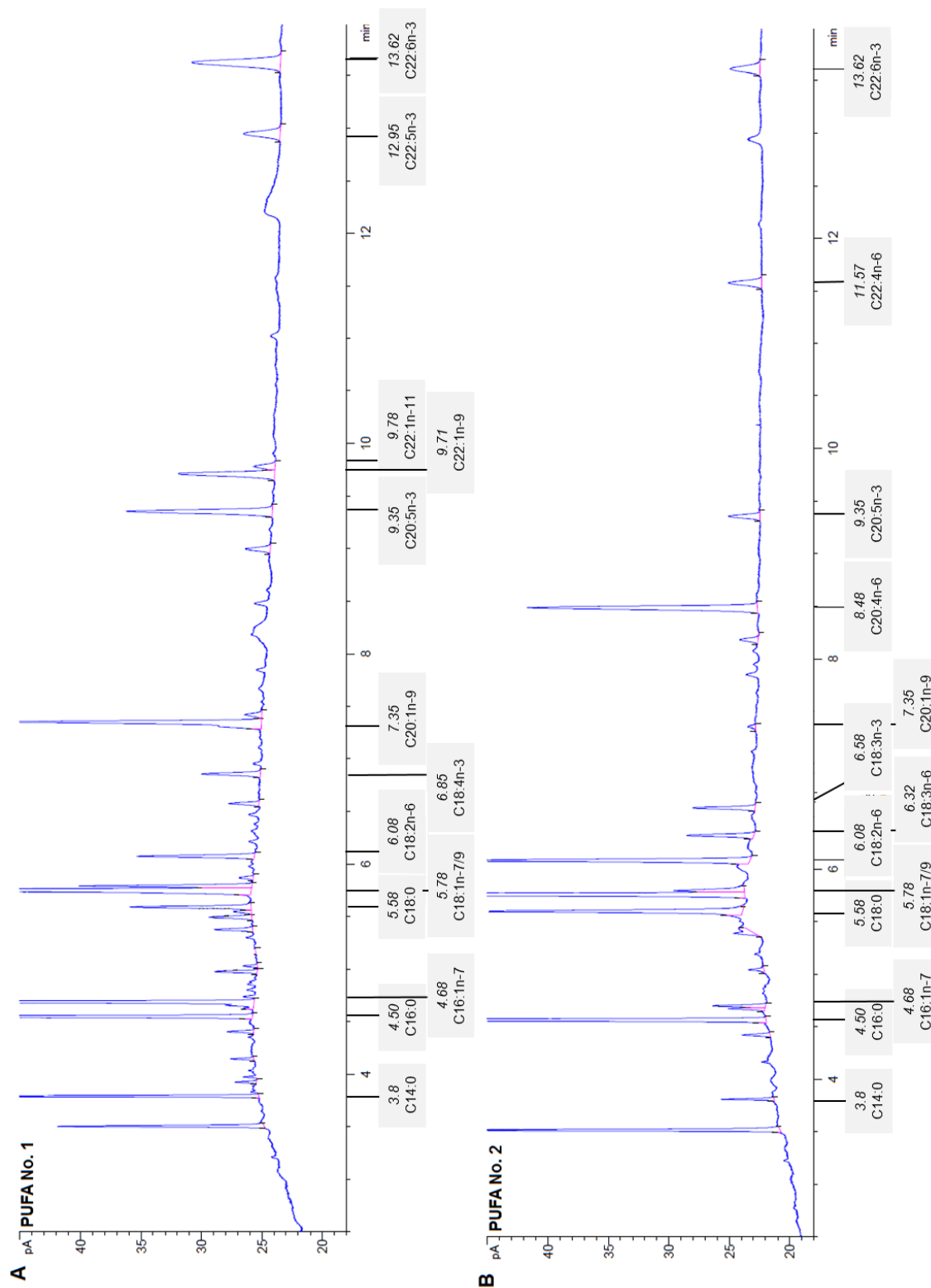
Figure 2-3: Representative chromatograms of analytical standards detected by GC-FID
A: PUFA No. 1 from marine source; B: PUFA No. 2 from animal source. The chromatograms have stable baselines and well resolved, sharp peaks, as well as a low signal to noise ratio. FAs of interest are labelled below the chromatogram.

2.3.2 TBARS quantification

MDA is one of the first stable molecules formed during lipid peroxidation and can be used as index for the beginning of the process (Cesa et al. 2012). MDA can form a coloured end-product with thiobarbituric acid under high temperature and acidic conditions. This product can be analysed photometrically to quantify MDA and other TBARS. This means that the TBARS assay is non-specific for MDA and might overestimate the MDA content. Other aldehydes, as well as amino acids, sialic acid, sugars and bilirubin can also react in this assay, even though MDA has the highest reactivity (Benzie 1996). Nevertheless, this assay is often used to measure lipid peroxidation in research. Analysis of this coloured end-product in FMs by third derivative spectrophotometry (Cesa et al. 2015), high-performance liquid chromatography (Almansa et al. 2013), or measuring fluorescent absorbance (Manglano et al. 2005) has been performed before.

First, analysis of TBARS was conducted using a Parameter TBARS Assay from R&D Systems Inc. according to the manufacturer's instructions. This assay was chosen as it requires a lower temperature than most other assays (45°C vs. 95°C), reducing the possibility of MDA production during the assay. However, due to technical issues encountered with this assay, the following assay was used for the final analysis.

TBARS were quantified using a TBARS (Trichloroacetic Acid Method) Assay (700870, Cayman Chemical) following manufacturer's instructions. Samples and standards (100 µL) were incubated with 100 µL TCA Assay Reagent (10%) and 800 µL Colour Reagent for 1 hour in a boiling water-bath (JB Nova, Grant). Next, samples and standards were placed in an ice bath for 10 min to cool. After centrifugation (1600 x g, 10 min, 4°C), supernatants were transferred to a 96-well plate in duplicates and absorbance was measured at 540 nm using an absorbance microplate reader (ELx800, BioTek). Results were analysed using the Gen5 software (Version 2.09.2 for Windows, BioTek). A standard curve was included to quantify TBARS concentration in µM (Figure 2-4).
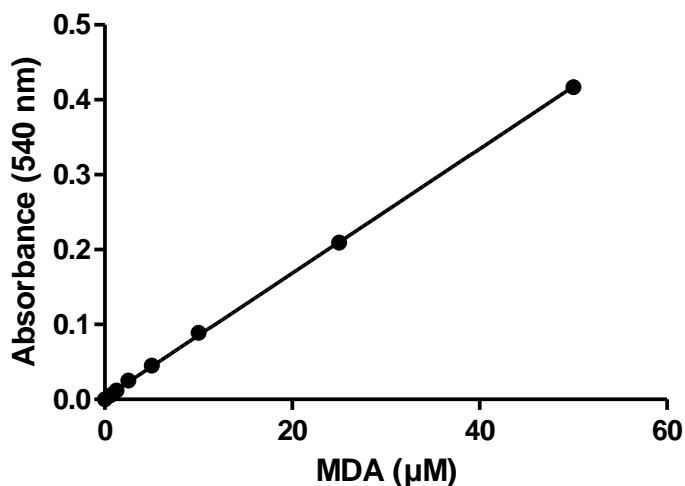
Figure 2-4: Standard curve for the determination of TBARS concentration in FM samples

The graph shows a representative MDA standard curve (R^2 = 0.9998; y = 0.0083x + 0.0024) determined by TBARS assay. Standards were analysed in duplicates.

Since the assay was not validated for the use with FM, intra- and inter-assay CV% were determined (Salimetrics Inc 2017). Inter-assay CV% of less than 15 and intra-assay CV% of less than 10 are generally defined as acceptable. The general formula used to calculate the CV% is as follows:

$$CV\ (\%) = \frac{standard\ deviation}{mean} \times 100$$

The intra-assay CV% was calculated for 48 samples Nutriprem 2 (duplicates) analysed on one plate. The intra-assay CV% was 7.8%. The inter-assay CV% was determined on four plates, using quadruplicates of Nutriprem 2. The inter-assay CV% was 6.1%. Both these values are considered acceptable (Salimetrics Inc 2017), and therefore demonstrate that this assay can be used to analyse TBARs in FM.

The FM matrix can have a significant effect on the recovery of MDA/TBARS (Cesa 2004). Therefore, the recovery rate was determined by spiking FM (5 samples in duplicates) with 12.5 µM MDA (Fenaille et al. 2001). Recovery was calculated using the following formula:

$$Recovery\ (\%) = \frac{(C\ spiked\ -\ C\ initial)}{C\ added} \times 100$$

C_{spiked}: TBARS concentration in spiked sample (µM); $C_{initial}$: TBARS concentration in unspiked sample (µM), C_{added}: MDA concentration added (µM)

Recovery was 39.0% for Nutriprem 1 (liquid), 61.0% for Nutriprem 2 (liquid), 49.11% for Nutriprem 2 (powder). Other researchers reported a recovery of 57 - 63% or 40 – 80% for MDA in different Italian FMs, respectively (Cesa 2004; Cesa et al. 2012). TBARS concentrations were adjusted accordingly and corrected for published fat content.

2.3.3   HNE quantification

HNE can form a stable adduct with proteins, called advanced lipid peroxidation end products. They can be quantified by comparison to an HNE-BSA standard curve using a competitive enzyme-linked immunosorbent assay (ELISA).

HNE adducts were quantified using the OxiSelect HNE Adduct competitive ELISA Kit (STA-838, Cell Biolabs, Inc.), according to the manufacturer's instructions. The 96-well plate was coated with 5 µg/mL HNE conjugate over night at 4°C. After washing with PBS, 200 µL Assay Diluent were added for 1 hour at room temperature. After removal, 50 µL standard or sample were added in duplicates and the plate was incubated (10 min, room temperature, orbital shaker), followed by an incubation with 50 µL anti-HNE antibody (1 hour, room temperature, orbital shaker). Next, plates were washed three times and incubated with 100 µL secondary Antibody-HRP Conjugate (1 hour, room temperature, orbital shaker). After three times washing, 100 µL Substrate Solution were added, and the plate was incubated for 10 min. The Stop Solution was added, and the absorbance was read at 450 nm, using an absorbance microplate reader (ELx800, BioTek). Results were analysed using the Gen5 software (Version 2.09.2 for Windows, BioTek). An HNE-bovine serum albumin (BSA) standard curve was included to quantify HNE adducts in µg/mL (Figure 2-5). Values were corrected for published fat content of FMs.
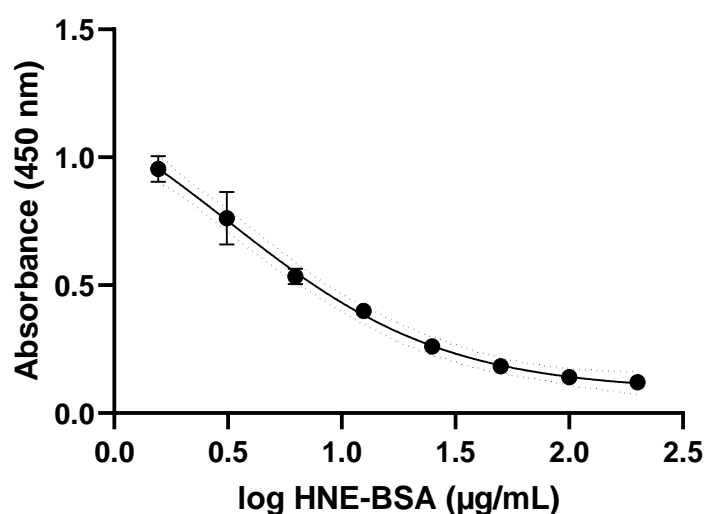
Figure 2-5: Standard curve for the determination of HNE-BSA in liquid FM samples
The graph shows a representative HNE-BSA four parameter logistics standard curve (R^2 = 0.9881) determined by ELISA. Standards were analysed in duplicates.

2.3.4 Statistics

It would be best practice to anonymise FMs in the results section. However, LCPUFA levels were compared to the levels provided by the manufacturers, and the antioxidant composition was also discussed. Since these differ between the different brands of FM, and since there are only two preterm FMs available in the U.K., FMs could be easily identified and it was decided not to anonymise the results.

Statistical analysis was performed using GraphPad Prism (Version 5.0). Due to small sample numbers, non-parametric tests were used. For comparisons between FMs, Mann Whitney test or Kruskal-Wallis test with Dunn's multiple comparisons test comparing all columns were used. For comparison over storage time, Wilcoxon matched pairs test or Friedman test with Dunn's multiple comparisons test comparing all columns was used. A one sample *t*-test was used to compare FA content to manufacturer's values. Enteral LCPUFA supply, based on the analysed FA content, was calculated for hypothetical 28 weeks gestational age infant, weighing 1000 g at birth and receiving full enteral feeding (150 mL/kg/day) with infant FM, and later on (2000 g) with post-discharge FM. Values were compared to ESPGHAN recommended intake levels (Agostoni et al. 2010) using a one sample *t*-test and compared between the FMs using Mann Whitney or Kruskal-Wallis test. LCPUFAs available for accretion were also calculated for this hypothetical infant as described previously (De Rooy et al. 2017), considering an absorption of 81.1% for ARA, and 78.4% for DHA (Carnielli et al. 1998), as well as an endogenous synthesis of 26.7 mg/kg/day ARA and 12.6 mg/kg/day DHA per day (Carnielli et al. 2007). Values were compared to estimated *in utero* accretion rates (Lapillonne and Jensen 2009) by one sample *t*-test. For comparisons between FMs, Mann Whitney or Kruskal-Wallis 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.

2.4 Results

2.4.1 LCPUFAs

LCPUFAs were analysed by GC-FID. Figure 2-6 shows a representative chromatogram of FAs detected in preterm FM.
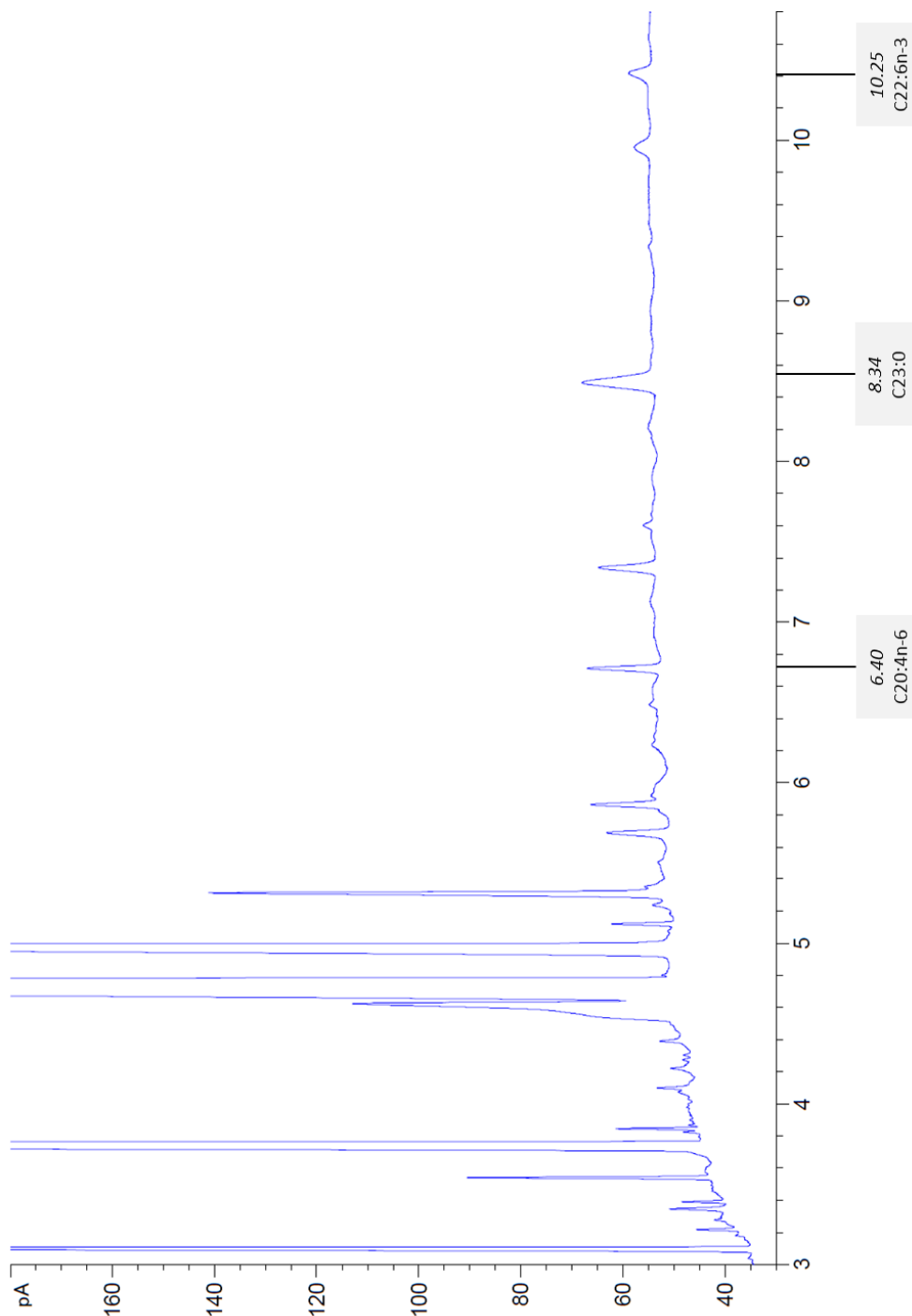
Figure 2-6: Representative chromatogram of preterm FM FAMEs detected by GC-FID

Nutriprem 2 day 0, 0 hours. The chromatogram has a stable baseline and well resolved, sharp peaks, as well as a low signal to noise ratio. LCPUFAs and the internal standard are labelled below the chromatogram. Peak identification was carried out in comparison to analytical standards.

LCPUFA levels compared to manufacturers' information

Measured LCPUFA levels at opening were compared to the information provided on the labels by the manufacturers. Liquid Nutriprem 1 had ARA (15.49 ± 5.52 vs. 20 mg/100 mL, $p = 0.2004$) and DHA (12.03 ± 4.45 vs. 10 mg/100 mL, $p = 0.4280$) levels comparable to the label. Liquid Nutriprem 2 had significantly higher ARA (23.77 ± 2.24 vs. 20 mg/100 mL, $p = 0.0197$) and DHA (13.44 ± 2.43 vs. 10 mg/100 mL, $p = 0.0338$) levels, than declared by the manufacturer. Nutriprem 2 powder had significant lower ARA levels (11.47 ± 2.10 vs. 20 mg/100 mL, $p = 0.0002$), but comparable DHA levels (8.24 ± 1.87 vs. 10 mg/100 mL, $p = 0.0692$). Liquid Pro Gold Prem 1 had significant lower levels of ARA (11.53 ± 0.79 vs. 15 mg/100 mL, $p = 0.0169$) and DHA (10.50 ± 0.95 vs. 15 mg/100 mL, $p = 0.00146$) at opening. Liquid Pro Gold Prem 2 levels were comparable to the label (ARA: 10.44 ± 2.06 vs. 14.4 mg/100 mL, $p = 0.0797$; DHA: 10.36 ± 1.86 vs. 14.4 mg/100 mL $p = 0.0639$).

There was no significant difference in the ARA ($p = 0.4000$) and DHA ($p = 0.8571$) concentration between the liquid preterm first FMs. Liquid Nutriprem 2 provided significantly more ARA than liquid Pro Gold Prem 2 and Nutriprem 2 powder (both $p < 0.05$). The DHA concentration was also significantly higher in liquid Nutriprem 2 than in Nutriprem 2 powder ($p < 0.01$). There was no significant difference in the DHA concentration between liquid Pro Gold Prem 2 and liquid Nutriprem 2.

LCPUFA levels during preterm FM storage

There was no effect of 4 hours refrigeration on the ARA and DHA concentration in liquid preterm first FMs (Nutriprem 1: $p = 0.8750$ and $p = 0.8750$, respectively; Pro Gold Prem 1: $p = 0.2500$ and $p = 0.2500$, respectively) (Figurer 2-7 A, B, E, F). There was no effect of refrigerated storage (4 hours) on the ARA and DHA concentration in liquid post-discharge FMs (Nutriprem 2: $p = 0.4375$ and $p = 0.8125$, respectively; Pro Gold Prem 2: $p = 0.5000$ and $p = 0.5000$, respectively) (Figure 2-7 C, D, G, H).

The ARA concentration in prepared Nutriprem 2 powder was 11.47 ± 2.10 mg/mL at opening (day 0, 0 hours) (Figure 2-8 A). Opened storage for 28 days did not result in a change in ARA concentration at 0 hours (directly after preparation; $p = 0.0700$). Refrigerated storage for up to 24 hours did not decrease ARA levels (Table 2-6).

The DHA concentration in prepared Nutriprem 2 powder was 8.24 ± 1.87 mg/mL at opening (day 0, 0 hours) (Figure 2-8 B). Opened storage for 28 days did not change the DHA concentration in prepared preterm FM at 0 hours (directly after preparation; $p = 0.0663$). Storing prepared preterm FM at 4°C did not decrease DHA levels at any time-point (Table 2-7).
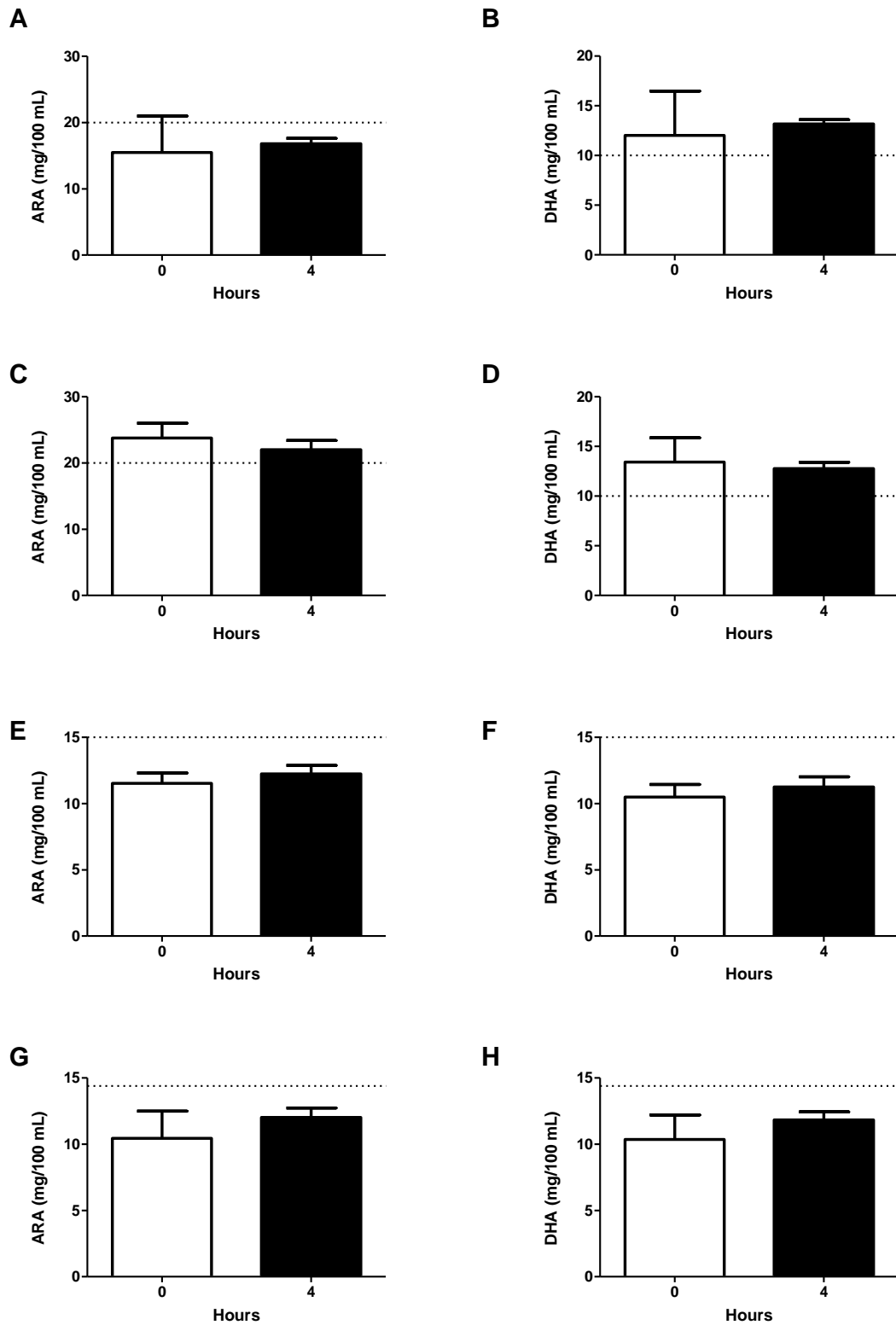
Figure 2-7: LCPUFA concentration in liquid preterm FM samples

ARA (left panel) and DHA (right panel) concentration in (A and B) Nutriprem 1 (n=4), (C and D) Nutriprem 2 (n=5), (E and F) Pro Gold Prem 1 (n=3), and (G and H) Pro Gold Prem 2 (n=3). Refrigerated storage for 4 hours had no influence on LCPUFA concentrations (p > 0.05, respectively). Dashed lines indicate ARA and DHA levels provided on the label.

A

B
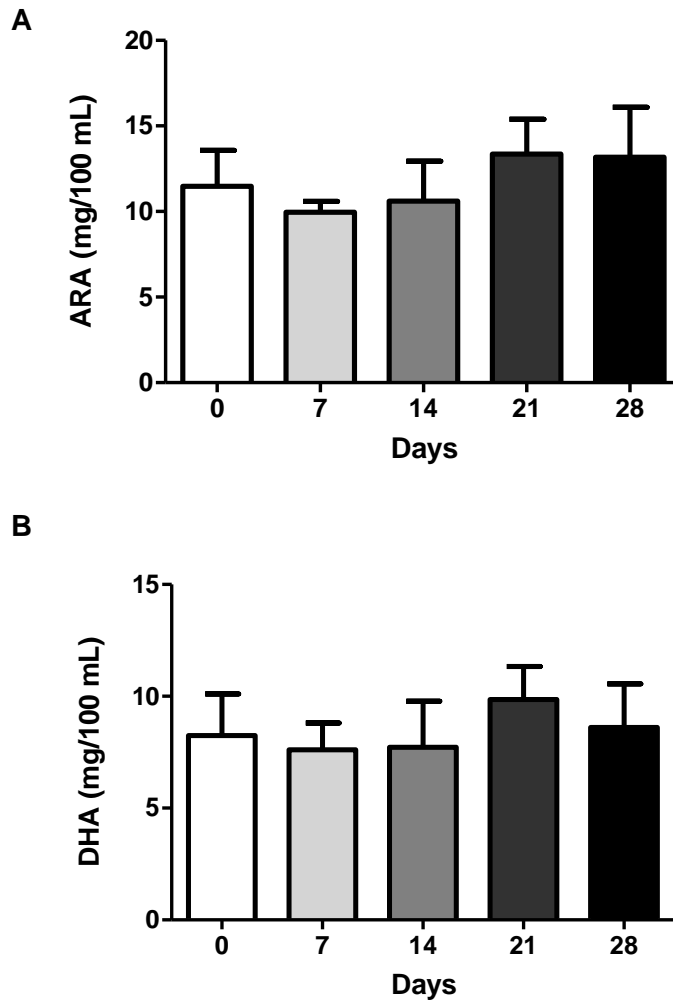
Figure 2-8: LCPUFA concentrations in Nutriprem 2 powder after preparation

(A) ARA (p = 0.0700) and (B) DHA (p = 0.0663) concentration in Nutriprem 2 samples (n=6) directly after preparation (0 hours) over the course of the experiment (28 days).

Table 2-6: ARA (mg/100 mL) in Nutriprem 2

	0 hours	**4 hours**	**24 hours**
Day 0	11.47 ± 2.10	11.75 ± 0.93	10.38 ± 2.46
Day 7	9.96 ± 0.63	11.63 ± 1.71	10.90 ± 2.53
Day 14	10.61 ± 2.34	13.58 ± 0.82	14.57 ± 1.60
Day 21	13.36 ± 2.04	13.43 ± 1.46	15.42 ± 2.13
Day 28	13.17 ± 2.92	13.54 ± 2.96	13.81 ± 1.66

ARA concentration (± SD) in Nutriprem 2 samples (n=6)

Day 0: p = 0.9563; day 7: p = 0.1416; day 14: p = 0.0055, significant differences in Dunn's multiple comparison test (0 vs. 24 hours, p < 0.05); day 21: p = 0.0289, significant differences in Dunn's multiple comparison test (0 vs. 24 hours, p < 0.05); day 28: p = 0.9563

0 hours: p = 0.0700; 4 hours: p = 0.1468; 24 hours: p = 0.0155, significant differences in Dunn's multiple comparison test (day 0 vs. 21, p < 0.05)

Table 2-7: DHA (mg/100 mL) in Nutriprem 2

	0 hours	**4 hours**	**24 hours**
Day 0	8.24 ± 1.87	7.91 ± 0.83	7.45 ± 1.34
Day 7	7.61 ± 1.20	7.84 ± 1.22	7.07 ± 1.36
Day 14	7.72 ± 2.05	8.81 ± 1.52	10.09 ± 1.46
Day 21	9.85 ± 1.48	10.24 ± 1.93	11.72 ± 2.28
Day 28	8.60 ± 1.96	8.17 ± 0.99	8.91 ± 1.13

DHA concentration (± SD) in Nutriprem 2 samples (n=6)

Day 0: p = 0.9563; day 7: p = 0.5705; day 14: p = 0.0289, significant differences in Dunn's multiple comparison test (0 vs. 24 hours, p < 0.05); day 21: p = 0.0289, significant differences in Dunn's multiple comparison test (0 vs. 24 hours, p < 0.05); day 28: p = 0.2522

0 hours: p = 0.0663; 4 hours: p = 0.0289, no significant differences in Dunn's multiple comparison test; 24 hours: p = 0.0049, significant differences in Dunn's multiple comparison test (day 0 vs. day 21, p < 0.05; day 7 vs. day 21, p < 0.05)

Estimated LCPUFA intake from preterm FM

Based on the analysed FA content at day 0, 0 hours, estimated daily LCPUFA intake from liquid Nutriprem 1 and Pro Gold Prem 1 was calculated for a hypothetical preterm infant of 1000 g bodyweight assuming full enteral feeding (150 mL), and values were compared to ESPGHAN recommendations. Nutriprem 1 and Pro Gold Prem 1 provided 23.23 ± 7.16 and 17.30 ± 0.97 mg/d ARA. These were not significantly different from the ESPGHAN minimum recommendation of 18 mg/d (ESPGHAN recommended range 18-42 mg/d) (*p = 0.2950* and *p = 0.4146*). DHA also reached the ESPGHAN minimum levels (18.05 ± 5.78 and 15.75 ± 1.17 vs. 12 mg/d, *p = 0.1673* and *p = 0.1123*, respectively) (ESPGHAN recommended range 12-30 mg/d). LCPUFA intake was not significantly different from Nutriprem 1 and Pro Gold Prem 1 (ARA: *p = 0.4000*; DHA: *p = 0.6286*)

Estimated LCPUFA intake from liquid Nutriprem 2, Pro Gold Prem 2, and Nutriprem 2 powder was calculated for a hypothetical preterm infant of 2000 g bodyweight, assuming full enteral feeding (300 mL), and values were compared to ESPGHAN recommendations. Liquid Nutriprem 2 provided ARA levels significantly above the ESPGHAN minimum intake level (71.32 ± 6.02 vs. 36 mg/d, *p = 0.0003*), however, the levels were still below the maximum of 84 mg/d. ARA levels in liquid Pro Gold Prem 2 and Nutriprem 2 powder were not significantly different from ESPGHAN recommendations (31.32 ± 5.05 and 34.41 ± 5.74 mg/d, *p = 0.3204* and *p = 0.563*, respectively). ARA levels differed significantly between the post-discharge FMs (*p = 0.0105*). Liquid Nutriprem 2 provided significantly more ARA than liquid Pro Gold Prem 2 and Nutriprem 2 powder (*p < 0.5)* (Figure 2-9 A).

Daily DHA intake was significantly above the minimum ESPGHAN recommendation in liquid Nutriprem 2 (40.33 ± 6.51 vs. 24 mg/d, $p = 0.0074$), but below the maximum of 60 mg/d. DHA levels in liquid Pro Gold Prem 2 and Nutriprem 2 powder were above the minimum ESPGHAN recommendation (31.08 ± 4.56 and 24.73 ± 5.11 mg/d, $p = 0.159$ and $p = 0.764$, respectively). The post-discharge FMs provided significantly different levels of DHA ($p = 0.0088$). Liquid Nutriprem 2 provided significantly more DHA than Nutriprem 2 powder ($p < 0.01$) (Figure 2-9 B).
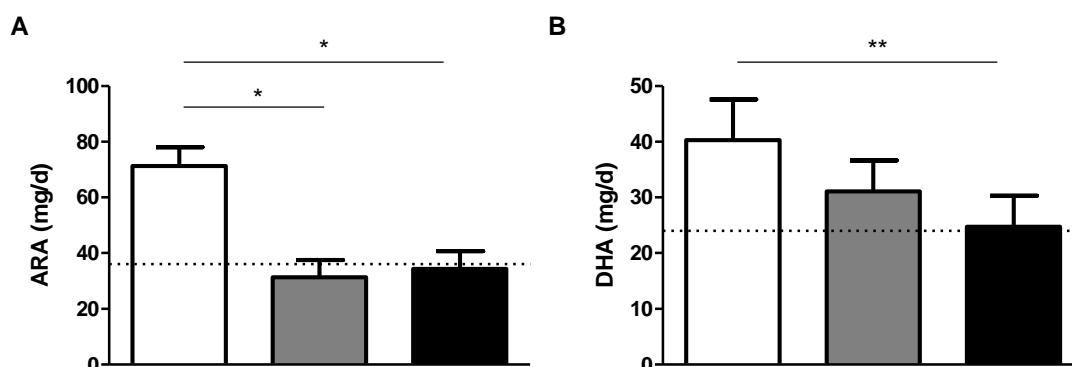
Figure 2-9: Daily enteral LCPUFA intake from post-discharge FMs

Daily enteral (A) ARA and (B) DHA intake from liquid Nutriprem 2 (white, n=5), liquid Pro Gold Prem 2 (grey, n=3), and Nutriprem 2 powder (black, n=6), for a 2000 g infant receiving full enteral feeding (300 mL). Dashed lines indicate minimum ESPGHAN intake recommendations.

Estimated LCPUFAs available for accretion

ARA and DHA available for accretion from the different preterm FMs were calculated for the hypothetical infants. Full enteral feeding with liquid Nutriprem 1 and Pro Gold Prem 1 provided 45.54 ± 5.81 and 40.73 ± 0.79 mg/d of ARA, respectively. These values represent accretion of only 21.48 and 19.21% of the *in utero* ARA accretion, respectively. Both provide ARA significantly below *in utero* accretion rates of 212 mg/d ($p < 0.0001$, and $p < 0.0001$, respectively). DHA from Nutriprem 1 and Pro Gold Prem 1 provided only 62.21 and 58.02% of the *in utero* DHA accretion. Therefore, DHA was also significantly below estimated *in utero* accretion rates (26.75 ± 4.53 and 24.95 ± 0.92 vs. 43 mg/d, respectively; $p = 0.0084$, and $p = 0.0013$, respectively). There was no significant difference in the ARA and DHA availability for accretion from the two preterm first FMs.

Liquid Nutriprem 2, Pro Gold Prem 2, and Nutriprem 2 powder provided 26.24, 18.59, and 19.18% of the *in utero* accreted ARA. These levels were all significantly below the estimated accretion rates (111.24 ± 4.88, 78.80 ± 4.10, and 81.31 ± 4.66 vs. 424 mg/d, respectively; $p < 0.0001$, $p < 0.0001$, and $p < 0.0001$, respectively). ARA available for accretion was significantly different between the post-discharge FMs ($p = 0.0105$). It

was higher in liquid Nutriprem 2 than in liquid Pro Gold Prem 2 and Nutriprem 2 powder (both *p < 0.05*). The post-discharge FMs provided 56.82 ± 5.11, 49.57 ± 3.57, and 44.58 ± 4.01 mg/d DHA for accretion. These levels were significantly below estimated *in utero* accretion rates (86 mg/d; *p = 0.0003*, *p = 0.0048,* and *p = 0.0001*, respectively) and represent 66.07, 57.64, and 51.84% of the *in utero* accretion. DHA availability was significantly different between the post-discharge FMs (*p = 0.0088*) and was significantly higher from liquid Nutriprem 2 than from Nutriprem 2 powder (*p < 0.01*).

2.4.2 TBARS

In the TBARS assay, samples are centrifuged to precipitate proteins. Pro Gold Prem products contain partially hydrolysed whey protein. This formulation affected the samples' ability to form precipitates, and therefore, the samples could not be transferred to the analysis plate without protein residue, interfering with the subsequent analysis. Therefore, the TBARS concentration for Pro Gold Prem products is not reported here.

There was no significant difference between the Nutriprem FMs at opening (*p = 0.0905*, Figure 2-10).
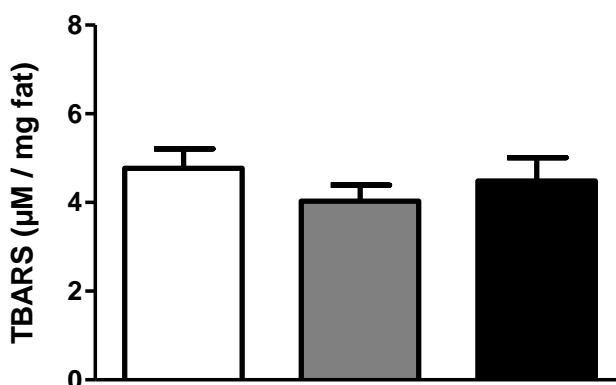
Figure 2-10: TBARS concentration in different Nutriprem FMs at opening
TBARS concentration in Nutriprem 1 (white, n=5) and 2 (liquid, grey, n=5; powder, black, n=6) samples directly after opening were not significantly different (p = 0.0905).

TBARS concentration was 4.77 ± 0.44 µM in freshly opened liquid Nutriprem 1, and did not change significantly when the FM was stored in the fridge for 1, 4 or 10 hours (*p = 0.5206*, Figure 2-11 A). The liquid Nutriprem 2 TBARS concentration at opening was 4.03 ± 0.36 µM (Figure 2-11 B). No significant changes occurred when the FM was stored in the fridge for 1, 4, or 10 hours (*p = 0.7709*).

A

B
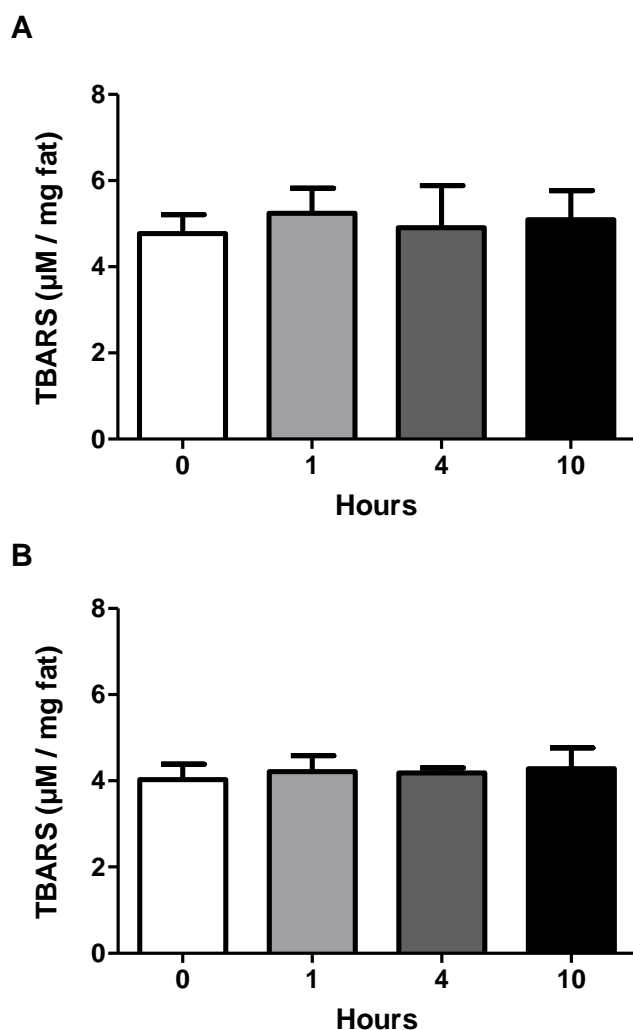
Figure 2-11: TBARS concentration in liquid Nutriprem FMs

TBARS concentration in (A) liquid Nutriprem 1 (n=5; p = 0.5206) and (B) liquid Nutriprem 2 (n=5, p = 0.7709) samples directly after opening.

The TBARS concentration in prepared Nutriprem 2 powder was $4.48 \pm 0.53\,\mu M$ at opening (day 0, 0 hours) (Figure 2-12). Opened storage for 28 days did not result in an increase of TBARS in the FM at 0 hours ($p = 0.6798$). Although there was a general increase in TBARS when prepared FM was stored in the fridge for 4 and 24 hours, the increase was only significant ($p = 0.0289$) between 4 and 24 hours at day 21 ($p < 0.05$) (Table 2-8).
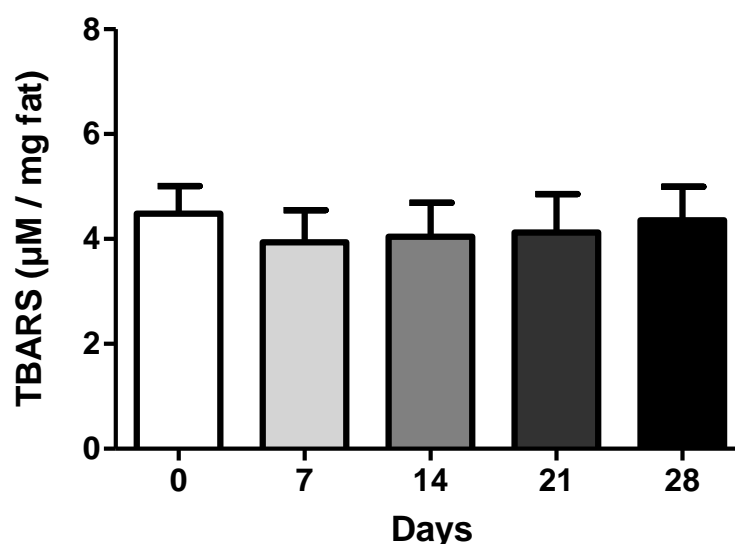
Figure 2-12: TBARS concentration in Nutriprem 2 powder after preparation

TBARS concentration in Nutriprem 2 samples (n=6) directly after preparing fresh FM (0 hours), over the course of the experiment (28 days). Friedman test p = 0.6798.

Table 2-8: TBARS (µM / mg fat) in Nutriprem 2

	0 hours	4 hours	24 hours
Day 0	4.48 ± 0.53	4.25 ± 0.33	4.64 ± 0.67
Day 7	3.93 ± 0.62	4.54 ± 0.77	4.63 ± 0.65
Day 14	4.04 ± 0.65	4.36 ± 0.47	4.69 ± 0.65
Day 21	4.12 ± 0.74	4.09 ± 0.58	4.85 ± 0.80
Day 28	4.36 ± 0.64	4.55 ± 0.63	4.77 ± 0.53

TBARS concentration (± SD) in Nutriprem 2 samples (n=6), determined by TBARS assay.
Day 0: p = 0.5705; day 7: p = 0.4297; day 14: p = 0.7402; day 21: p = 0.0289, significant differences in Dunn's multiple comparison test (4 vs. 24 hours, p < 0.05); day 28: p = 0.2522
0 hours: p = 0.6798; 4 hours: p = 0.4245; 24 hours: p = 0.7603

2.4.3 HNE

There was a significant difference between the Nutriprem FMs at opening (*p = 0.0094*, Figure 2-13). Liquid Nutriprem 2 had a significantly lower HNE concentration at opening than Nutriprem 2 powder (*p < 0.01*). There was no significant difference between liquid Pro Gold Prem 1 and 2 (*p = 0.2000*).
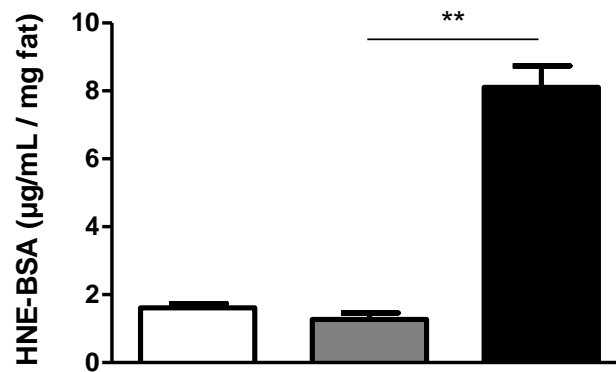
Figure 2-13: HNE concentration in different Nutriprem FMs at opening

HNE concentration in Nutriprem 1 (white, n=5) and 2 (liquid, grey, n=5; powder, black, n=3) samples directly after opening were significantly different (p = 0.0094).

HNE concentration was 1.61 ± 0.13 µg/mL in freshly opened liquid Nutriprem 1, and did not change significantly when the FM was stored in the fridge for 1, 4, or 10 hours ($p = 0.0755$, Figure 2-14 A). The concentration of HNE in Nutriprem 1 at opening was significantly higher than in Pro Gold Prem 1 (0.82 ± 0.11 µg/mL; $p = 0.0357$). Similar to Nutriprem 1, the concentration of HNE in Pro Gold Prem 1 did not change significantly when the FM was stored in the fridge for 1, 4, or 10 hours ($p = 0.9583$, Figure 2-14 B).

The liquid Nutriprem 2 HNE concentration at opening was 1.27 ± 0.19 µM (Figure 2-14 C). No significant changes occurred when the FM was stored in the fridge for 1, 4, or 10 hours ($p = 0.0548$). The HNE concentration was significantly higher in Nutriprem 2 than in Pro Gold Prem 2 (0.72 ± 0.06 µg/mL) at opening ($p = 0.0357$). As in the other FMs, cold storage (4°C) did not affect the HNE concentration in Pro Gold Prem 2 ($p = 0.1476$) (Figure 2-14 D).
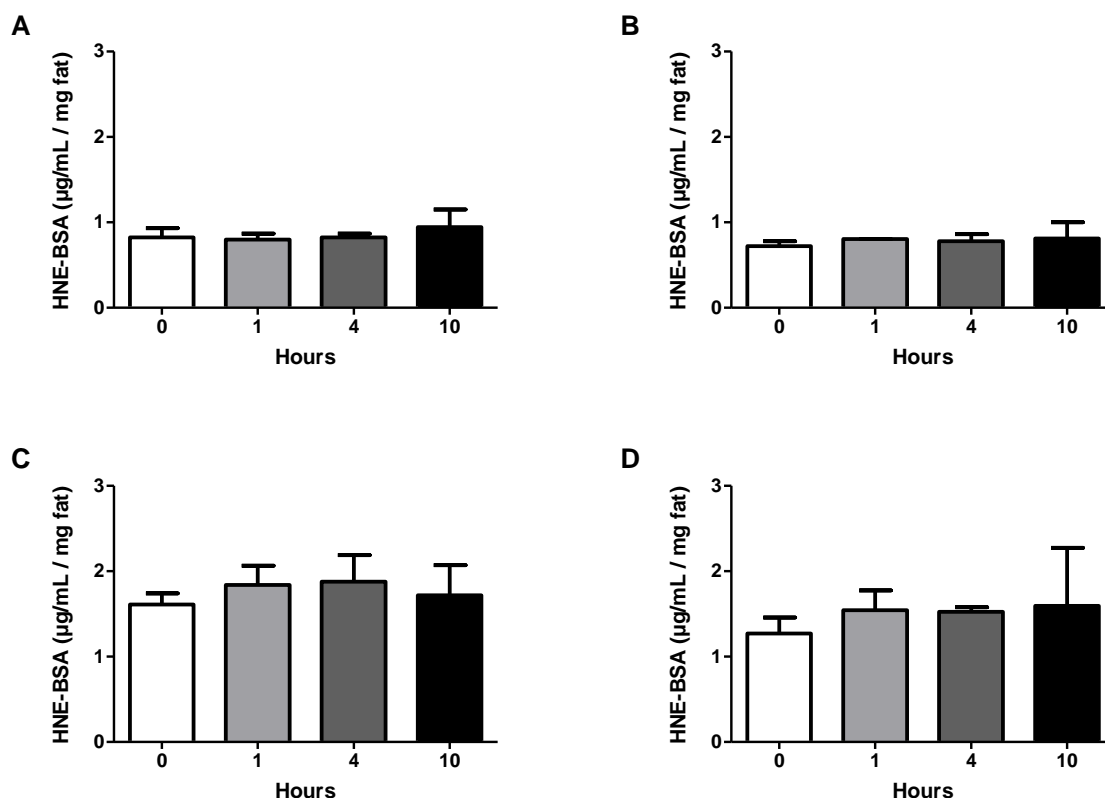
Figure 2-14: HNE concentration in different liquid preterm FMs

HNE-BSA concentration in (A) Nutriprem 1 (n=5, p = 0.0755), (B) Pro Gold Prem 1 (n=3, p = 0.9583), (C) Nutriprem 2 (n=5, 0.0548), (D) Pro Gold Prem 2 (n=3, p = 0.1476).

The HNE concentration in prepared Nutriprem 2 powder was 8.10 ± 0.64 µg/mL at opening (day 0, 0 hours) (Figure 2-15). Opened storage for 28 days did not result in an increase of HNE in the FM at 0 hours ($p = 0.1626$) (Table 2-9). There was an effect of storage days on HNE concentration at 4 hours ($p = 0.0172$), and at 24 hours ($p = 0.0009$), with significant differences between HNE concentration at day 14 and 21 ($p < 0.05$) (Table 2-9). The effect of storing FM in the fridge at opening (day 0) and at the end of the recommended opening time (day 28) is displayed in Figure 2-16. At day 0 a slight but not significant increase in HNE concentration was seen (Figure 2-16 A). This increase became significant at 28 days (9.47 ± 0.16 vs. 26.56 ± 4.01 µg/mL; p < 0.5) (Figure 2-16 B).
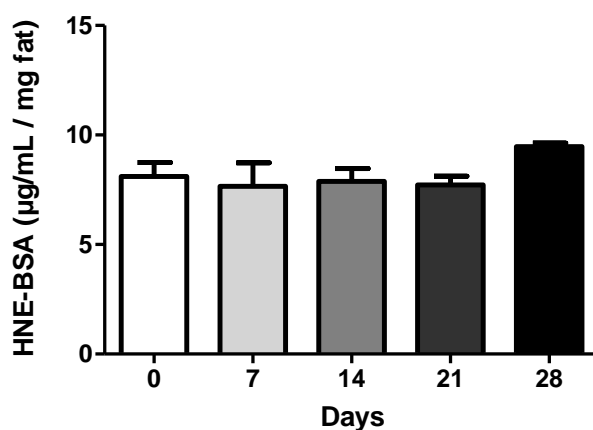
Figure 2-15: HNE concentration in Nutriprem 2 powder after preparation

HNE concentration in Nutriprem 2 samples (n=3) directly after preparing fresh FM (0 hours), over the course of the experiment (28 days). Friedman test p = 0.6798.

Table 2-9: HNE-BSA (µg/mL / mg fat) in Nutriprem 2

	0 hours	4 hours	24 hours
Day 0	8.10 ± 0.64	13.72 ± 1.36	18.20 ± 4.70
Day 7	7.66 ± 1.07	15.18 ± 0.34	15.33 ± 2.23
Day 14	7.88 ± 0.60	12.69 ± 0.42	11.23 ± 2.04
Day 21	7.72 ± 0.39	11.60 ± 0.34	30.0 ± 6.71
Day 28	9.47 ± 0.16	16.17 ± 2.02	26.56 ± 4.01

HNE concentration (± SD) in Nutriprem 2 samples (n=3), determined by ELISA.

Day 0: p = 0.1944; day 7: p = 0.1944; day 14: p = 0.0278, no difference in Dunn's multiple comparison test; day 21: p = 0.0278, no difference in Dunn's multiple comparison test; day 28: p = 0.0278, significant differences in Dunn's multiple comparison test (0 vs. 24 hours, p < 0.05)

0 hours: p = 0.1626; 4 hours: p = 0.0172, no difference in Dunn's multiple comparison test; 24 hours: p = 0.0009, significant differences in Dunn's multiple comparison test (day 14 vs. 21, p < 0.05)
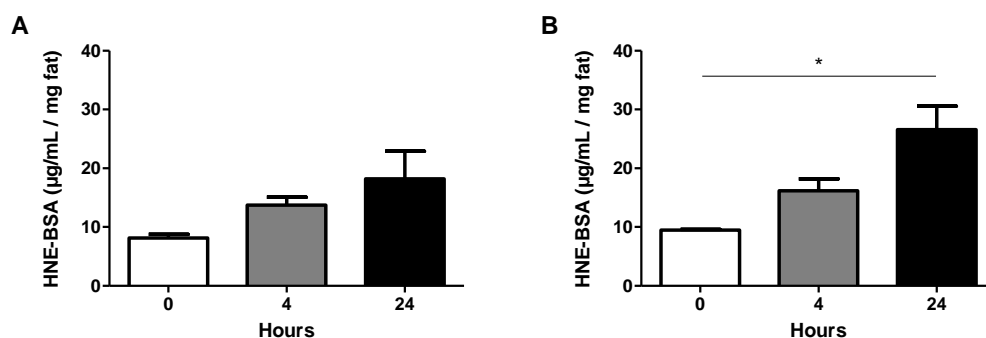
Figure 2-16: HNE concentration in Nutriprem 2 after refrigeration

HNE-BSA concentration in Nutriprem 2 samples (n=3) at (A) day 0 and (B) day 28 directly after preparing fresh FM (0 hours), and after storage for 4 or 24 hours at 4°C.

2.5   Discussion and conclusion

Although maternal BM is the first choice for feeding (preterm) neonates (ESPGHAN Committee on Nutrition et al. 2009), breast feeding rates in the U.K. are low, with 19% of all mothers not even initiating breast feeding (McAndrew et al. 2012). The initiation rate is higher in mothers of extremely preterm infants (92%). However, some mothers cannot breast feed at all, and DHM is not available in all hospitals and will mostly be used only for extremely preterm infants. Furthermore, at discharge, only 29% of mothers exclusively breast feed their preterm infant, and 6% of mothers combine breast feeding with FM feeding (Bonet et al. 2011). Hence, hospitals and parents rely on preterm FM for feeding preterm infants. This study has, for the first time, investigated the effects of current hospital storage methods on the LCPUFA and LPP levels of different preterm FMs available in the U.K.

No effect of refrigerated storage for 4 hours on the LCPUFA concentrations in liquid infant and post-discharge FMs from two different manufacturers was detected. Similarly, no differences in ARA and DHA concentrations were found when preterm powder FM was stored for 28 days after opening. There was also no difference in LCPUFA concentration when prepared powder FM was stored at 4°C for up to 24 hours at day 0 and day 28. These results are in line with findings of other researchers. Zunin and colleagues found no effect of storage for 30 days after opening on LCPUFA content (2015). Another study which also mimicked use of FM, by opening and stirring the FM three times per day, also found no differences in ARA, EPA, and DHA levels when FM was stored for up to 70 days (Chávez-Servín et al. 2008). However, a significant decrease in LA at the end of the study was reported. We could not detect a significant difference in LA between day 0, 0 hours and day 28, 0 hours (data not shown).

The preterm FMs used in this study were compliant with the Commission Directive 2006/141/EC, which states that the ARA concentration should maximum be 1% of total FA content, and that the DHA content shall not exceed the ARA content. Kus-Yamashita and colleagues found that some preterm and follow-on FM in Brazil contained more DHA than ARA, whereas the majority of FMs were compliant with the Codex Alimentarius (2016).

All infant and post-discharge preterm FMs tested also provided ARA and DHA daily enteral intake levels compliant with ESPGHAN recommendations. Nevertheless, the LCPUFA concentrations were different between the preterm FM, with liquid Nutriprem 2 providing more ARA than liquid Pro Gold Prem 2 and different concentrations between the preterm FM preparations, with liquid Nutriprem 2 providing more ARA and

more DHA than Nutriprem 2 powder. Likewise, another study found higher DHA levels in liquid ready to feed FM than in FM powder from the same brand (Loughrill and Zand 2016). Differences between the FMs can be explained by variations in raw ingredients, due to differences in soil, climate, or agricultural practices (Loughrill and Zand 2016). In algae, the lipid levels and composition can be altered by the season, the location and the growth conditions (Stengel et al. 2011). The nutrient information on the label is usually calculated from databases, which do not consider differences in raw ingredients. This might also be the reason for the differences between the measured LCPUFA levels and the concentrations provided on the label, found in this study. Both brands use single cell oil as LCPUFA source. Liquid Nutriprem 2 had higher ARA and DHA levels than provided by the manufacturer, whereas Nutriprem 2 powder and liquid Pro Gold Prem 1 had significantly lower ARA, and ARA and DHA, respectively, than on the label. Significantly lower levels of LA, ALA, and DHA than on the label have previously been reported for powder FM in the U.K. (Loughrill and Zand 2016). The researchers hypothesized that this could be an effect of preparing the powder with hot water. FM cannot be prepared with cold water, and for safety reasons, it should be prepared with water > 70°C to destroy harmful bacteria (WHO and FAO 2007), which is especially important for preterm infants with an underdeveloped immune system (Davis and Auten 2010). It is more likely that the used database overestimated the PUFA content, as suggested by other researchers (Fernandez et al. 2002). They found a 50, 60, 95, and 75% discrepancy between the actual and the calculated LA, ALA, ARA, and DHA levels in weaning foods. The authors also indicated that losses might occur due to processing of the raw ingredients.

All preterm FMs provided ARA and DHA levels significantly below the estimated *in utero* accretion rates (Lapillonne and Jensen 2009). Therefore, they contribute to the LCPUFA gap of prematurity. These low levels are of concern when feeding preterm infants, in which neurological development is ongoing (Harris and Baack 2015). Furthermore, low ARA and DHA intake is associated with several comorbidities of prematurity such as respiratory distress syndrome, sepsis, and intraventricular haemorrhage (Fares et al. 2017). ARA and DHA levels might be further decreased by tube feeding, which can result in a triacylglycerol loss of up to 33% in BM, although it did not decrease triacylglycerols in FM in the same study (Tacken et al. 2009). Nevertheless, enteral feeding via nasogastric tube is often necessary in preterm infants, since their ability to suck, swallow and breathe is not developed until 32 weeks gestation (Lau et al. 2003), and not fully matured until 35 weeks gestation (Gibson et al. 2006). This indicates that LCPUFA levels in preterm FM should be higher than they were in 2017. However, the new Delegated Regulation 2016/127 has not taken this into consideration. To be compliant with the new Regulation, only the amount of DHA in

liquid Pro Gold Prem 1 needs to be increased from 18 to 20 mg/100 kcal, whereas the DHA concentration in the other preterm FMs does not need to be altered and manufacturers could omit ARA from their FMs.

Analysis of the liquid and powder preterm FMs revealed LPPs directly after opening of the FMs. These initial levels of TBARS and HNE indicate that lipid peroxidation occurred before opening of the FMs. Additionally, differences between the two preterm FM brands in initial levels were detected and significantly higher HNE concentrations were identified in Nutriprem 2 powder, compared to liquid Nutriprem 2. These results were surprising; however, all FM HNE concentrations were within the assay's range of detection, and can therefore be considered as true representation of the HNE concentration in FM. Liquid and powder FM HNE concentrations were analysed with the same assay, with the same LOT number, within 5 days from each other. Nevertheless, these results should be confirmed in further studies, and more sensitive methods like high-performance liquid chromatography (Goldring et al. 1993) should be used. Similarly to our results, Fenaille and colleagues identified much higher hexanal content in powder FM than in liquid FM (2006). One reason for the initial LPPs and the differences might be FM processing. Spray drying of FM powder uses high temperatures (180 – 220°C), which may catalyse lipid peroxidation (Cesa et al. 2015). Liquid FM is sterilised in the bottle (120°C, 15 minutes). Although this treatment did not change the peroxide value of experimental FMs, secondary LPPs were not analysed in this study and the levels of tocopherols decreased significantly (García-Martínez et al. 2012). Another explanation is an increase of LPPs over the shelf life up to the point of opening. Several studies reported an increase of LPPs during closed storage, including MDA (Cesa et al. 2015), peroxide value, and TBARS (Manglano et al. 2005), and propanal, pentanal and hexanal (Chávez-Servín 2009). Long-term storage of liquid enteral formulas for adults also increased lipid oxidation significantly (Rufian-Henares et al. 2005).

FM is packed under a protective atmosphere. However, once FM powder is opened, it is exposed to oxygen and light, which renders the FM highly susceptible to lipid peroxidation (Chávez-Servín et al. 2008). Hence protective antioxidants are added. Preterm FM powder investigated in this study demonstrated no increase in TBARS or HNE over 28 days. Refrigerated storage of prepared powder FM for 4 and 24 hours increased TBARS concentration, however the increase was only statistically significant at 21 days from 4 to 24 hours. Similarly, refrigeration increased HNE concentrations, but the increase was only significant at 28 days. Refrigerated storage for up to 10 hours had no effect on the TBARS or the HNE content of liquid infant and post-discharge preterm FM. This indicates that the WHO recommendations as well as

clinical practice are acceptable and preterm infants can be fed with FM that has been opened or prepared and stored at 4°C.

All identified studies found significant increases in MDA (Almansa et al. 2013), TBARS (Cesa et al. 2015), and peroxide value (Ghazal et al. 2016) after opening and subsequent storage of FM. Similarly, studies mimicking normal use also found significant increases in hydroxyalkenal and volatile (propanal, pentanal, hexanal) content (Surh et al. 2007; Chávez-Servín et al. 2008). These differences might be due to different antioxidant composition of the preterm FMs investigated. The Codex Alimentarius permits addition of tocopherols, β-carotene, ascorbic acid, ascorbyl palmitate, and citric acid as antioxidants in milk based FM (FAO/WHO 2016). The amount and composition of antioxidants is important for the antioxidative and radical scavenging properties of FM. Ascorbyl palmitate and ascorbic acid had higher antioxidant effects than α-tocopherols, β-carotene, and citric acid in experimental liquid FM (Zou and Akoh 2015). An ascorbyl palmitate concentration of 0.005% also had a better protective effect than a higher concentration (0.02%) Additionally, synergistic effects might occur, e.g. the antioxidant capacity of α-tocopherol and β-carotene individually was lower in FM than when a combination was used. A mix of α-, γ-, and δ-tocopherols is also more effective as antioxidants than α-tocopherol alone (Liu et al. 2002). Turoli et al. also found lower TBARS and conjugated dienes in FMs with higher vitamin A and E content (Turoli et al. 2004). A negative correlation of the MDA content and the vitamin E content in FM was also noted (Burg et al. 2010). Differences in antioxidants might also explain the differences found between the preterm FM brands. Pro Gold Prem products list ascorbyl palmitate as ingredients, whereas Nutriprem products do not contain ascorbyl palmitate (Danone Nutricia Early Life Nutrition 2017a; SMA Nutrition 2017b, 2017a)(see also Appendix II.II). Furthermore, Pro Gold Prem products contain tocopherol rich extracts, whereas Nutriprem products only list vitamin E in their ingredients. Furthermore, citric acid was only added to all liquid preterm FMs but not to Nutriprem 2 powder, which might further explain the higher HNE concentration in FM powder.

Other researchers have also measured MDA levels in FM and compared them to levels in BM. Almansa and colleagues measured MDA using high performance liquid chromatography (2013). They detected initial MDA concentrations of 2.64 µM in FM, and significantly lower levels in BM (0.86 µM). The here reported TBARS concentrations where higher, however, this is not surprising as not only MDA but also other TBARS react in the assay. In the only study performed on preterm FM, significantly higher levels of TBARS and MDA were found in the FM compared to BM (Raghuveer et al. 2002). Levels of TBARS were similar to ours, however, data in this

study was corrected for fat content. Turoli and colleagues have measured TBARS in BM (3.17 µM) and FM (1.93 µM), and found significantly higher levels in the former (2004). Colostrum was used in this study, and the samples were standing at room temperature for three hours before analysis, which might have increased the concentrations. It remains to be tested whether preterm FM in the U.K. or preterm BM has higher LPP levels, as ideally it would be compared using the same assay.

As mentioned before, preterm infants often receive FM via a nasogastric tube. This can further increase LPPs. Significantly higher ratios of LA hydroperoxide/LA were found after tube feeding in FM (Van Zoeren-Grobben et al. 1993). Additionally, the FM is exposed to light during tube feeding. When commercial fluid (bovine) milk is stored under light exposure, hexanal increased significantly (Kim and Morr 1996). Peroxide value also increased in intravenous lipid emulsions, when exposed to ambient light and levels were further increased when phototherapy was used (Silvers et al. 2001). Similar results were obtained when triacylglycerol hydroperoxides were measured in parenteral lipid emulsion when administered at ambient light or with phototherapy light (Neuzil et al. 1995). Therefore, it has been recently recommended by the European Medicines Agency to protect parenteral lipid solutions from light to inhibit the formation of toxic lipid degradation products (EMA/PRAC/347675/2019). Similar precautions should be used when feeding FM to vulnerable preterm infants using a tube. Furthermore, preterm infants often receive supplemental iron, added to FM. This can further increase LPPs (Raghuveer et al. 2002).

LPPs in FM can be problematic, as they can increase the oxidative load of preterm infants. Preterm FM can be the only nutritional source for some of the infants for a prolonged period of time (up to six months), which can lead to chronic exposure to LPPs. Intake of LPPs has been associated with inflammation (Awada et al. 2012) and circulating MDA levels were correlated with development of retinopathy of prematurity, bronchopulmonary dysplasia, intraventricular haemorrhage, NEC and sepsis (Inder et al. 1996; Garg et al. 2012; Namdev et al. 2014; Abdel Ghany et al. 2016). Urinary MDA levels were also correlated to these common comorbidities of prematurity (Schlenzig et al. 1993; Weinberger et al. 2004). Protecting parenteral lipid emulsions from light, and thereby reducing the formation of LPPs, was associated with a 30% reduction in bronchopulmonary dysplasia in a clinical trial with preterm infants (Chessex et al. 2007). It also prevents hypertriglyceridemia in preterm infants (Khashu et al. 2009). Nevertheless, currently in the EU, there are no upper tolerable levels for LPPs defined in the Commission Directive 2006/141/EC, which has led to criticism from researchers in the field (Martysiak-Zurowska and Stolyhwo 2006; Cesa et al. 2012). No studies have tested the effect of feeding oxidised FM to preterm infants/a model animal for

preterm infants. Animal models testing the effect of oxidised parenteral nutrition showed detrimental effects, with lower glutathione levels (Elremaly et al. 2012), and disturbed lipid metabolism (Maghdessian et al. 2010). The effect of feeding oxidised FM should therefore be tested, since the antioxidant systems is still not fully developed in preterm infants (Davis and Auten 2010) and lipids are needed for brain development (Clandinin et al. 1980). Due to a lack of safety data, manufacturers should aim to a produce FM with LPP levels below the ones found in BM. Future studies should aim to determine upper tolerable levels of LPPs for FM.

Volatile compounds were not measured in this study. Yet, volatiles, including propanal, pentanal, and hexanal, were identified in FM, when it was stored during the shelf life (Romeu-Nadal et al. 2007; Chávez-Servín 2009), as well as when FM was stored open (Chávez-Servín et al. 2008). Therefore, it could be assumed that volatiles are also produced in the tested preterm FMs. Pentanal was described to have a woody, pungent, fruity flavour, and hexanal was described as green, grassy and fatty (Zou and Akoh 2015). A trained sensory panel described FMs directly after opening as significantly better smelling than FM that was stored and had increased volatile products (Chávez-Servín 2009). The latter was also described to have significantly more rancid flavour. These flavours might affect infants' acceptance of the FM, and could result in a shorter feeding time and lower intake (Mennella et al. 2004), as well as in oral aversion, which can be detrimental in preterm infants.

This is the first study investigating the effects of the real life storage conditions of preterm FM on the LCPUFA and LPP content. However, not all available preterm FMs could be tested, and sample numbers are relatively small, although they are comparable or higher than in other studies in this field of research (Michalski et al. 2008; Carmen García-Martínez et al. 2010; Almansa et al. 2013). Preterm FM is only available on prescription in the U.K. therefore, analysis was limited to samples provided by the manufacturers. Unfortunately, the TBARS concentration could not be analysed in Pro Gold Prem samples. TBARS development as well as LPP development in Pro Gold Prem 2 powder should be measured in the future, especially considering the differences between the brands in HNE content. Furthermore, during method development, an increase in TBARS over the storage times was identified in one box Gold Prem 2 powder, before the change in protein formulation. However, these are preliminary results that need to be confirmed in repeated testing. High performance liquid chromatography can be used as an alternative to specifically measure the MDA-TBA product in samples, which has been separated from other TBARS. This method has been used to analyse FM samples with partially hydrolysed protein before (Cesa

2004). Furthermore, antioxidant levels should be measured simultaneously in future studies.

In conclusion, the current FM storage conditions did not significantly affect LCPUFA and LPP content of liquid and powder infant and post-discharge preterm FM. Therefore, based on these results, the current FM storage conditions do not appear detrimental to preterm FM LCPUFAs. Although variations in LCPUFA levels were identified, it is possible to achieve ESPGHAN recommended levels with both brands. Nevertheless, LPPs have been detected in different levels in the analysed preterm FMs, and preterm FM contributes to the oxidative load of preterm infants. The effect of these levels on preterm infants, and especially their gut, should be investigated and upper tolerable levels of LPPs in FM should be defined, legislated and governed.

3 *In vitro* analysis of the effects of 4-hydroxyalkenals on a neonatal porcine intestinal cell line

3.1 Introduction

FMs containing LCPUFAs are also a source of LPPs. In Chapter 2, we reported TBARS and HNE-adducts in preterm FM in the U.K. Others have found further secondary LPPs in FM, including MDA (Cesa et al. 2015), HHE (Michalski et al. 2008), as well as the volatile secondary LPPs propanal, pentanal, and hexanal (Chávez-Servín 2009) (for a detailed overview see Chapter 2.1.3). Lipid peroxidation of commercially available freshly prepared FM in France has been shown to result in the daily ingestion of 1.36 ± 1.17 µg 4-hydroxyalkenals per kg bodyweight (Michalski et al. 2008). Research from Korea reports higher intakes of 4.2 µg per kg bodyweight per day (1.55 µg HHE and 2.66 µg HNE) (Surh et al. 2007). This is more than ten times higher than the average dietary 4-hydroxyalkenal intake of Korean adults (Surh and Kwon 2005). Upper tolerable levels for 4-hydroxyalkenals and other LPPs have not been defined for FM in the European Union. However, recently, the European Medicines Agency has acknowledged the risk of LPPs in parenteral lipid emulsions, and now recommends protecting parenteral nutrition from light, to avoid LPP mediated adverse outcomes for neonates (EMA/PRAC/347675/2019).

3.1.1 Effects of LPP ingestion

Metabolism

After ingestion, 4-hydroxyalkenals pass the mouth, stomach, and duodenum and reach the jejunum unaltered, as indicated by an *in vitro* digestion model, which uses a three step digestion, adding heated digestive juices and adjusting pH levels at each step, before incubation (Goicoechea et al. 2011). In the jejunum of rats, the mucus-associated glutathione-S-transferase has been shown to metabolise 4-hydroxyalkenals to less reactive products and minimise direct contact of the toxic compounds with the enterocytes (Samiec et al. 2000). 4-Hydroxyalkenals reaching the enterocyte are rapidly conjugated with glutathione (Grune et al. 1991), for excretion via the kidney and urine (Guichardant et al. 2004). Nevertheless, ingested 4-hydroxyalkenals can also be absorbed unaltered and have been observed in plasma of mice in a dose-dependent manner, with HHE plasma concentrations peaking (0.1% of fed dose) 2 hours post-feeding (Awada et al. 2012). Providing humans with highly oxidised oil also results in significantly higher conjugated dienes (4.7-fold increase) and TBARS content (not detectable vs. 0.14 ± 0.03 nmol/µmol triglycerides) in postprandial chylomicrons (Staprans et al. 1994). In the circulation, postprandial increased oxidised lipids were detected for seven hours.

General effects on the body

Adult mice received 500 µg MDA per g bodyweight per day in their drinking water for 90 days and weight loss was observed after 50 days. By 90 days, irregularities in hepatic nuclei, as well as pancreatic lesions were found (Siu et al. 1983). Five week old rats fed with oxidised rapeseed oil for 13 weeks had higher relative liver and kidney weights, higher TBARS levels and lower hepatic glutathione and tocopherol levels, and lower tocopherol content in the serum (Izaki et al. 1984). In mice fed for 8 weeks with an oxidised diet, proinflammatory markers (IL-6 and MCP-1) increased significantly in plasma (Awada et al. 2012). Overall this demonstrates that ingestion of LPPs over a long period can have detrimental effects outside of the digestive system.

Specific gastrointestinal effects

Studies specifically reporting effect of diets with oxidised lipids on the intestine are rare. *In vitro* incubation of Caco-2 cells with oxidised mackerel oil (100 µg/mL, 24 hours) resulted in significantly lower cell viability and induction of apoptosis (Alghazeer et al. 2008). Similarly, 50 µM MDA as well as 50 µM HNE reduced cell viability significantly. Furthermore, TBARS were significantly increased when cells were incubated with oxidised mackerel oil. In contrast, incubation of Caco-2/TC7 cells with 4-hydroxyalkenal concentrations up to 100 µM for 24 hours had no effect on cell integrity (Awada et al. 2012).

No human studies and only one animal study were identified by the author. Feeding mice with an oxidised high-fat diet (lipid blends in water (30% w/w) were kept at 50°C with continuous shaking until the α-tocopherol levels decreased by 50%, before incorporating into diet), for 8 weeks induced expression of glutathione peroxidase, as well as C/EBP homologous protein, a proapoptotic protein, in the intestine (Awada et al. 2012). Furthermore, a significant increase in phosphorylated NF-κβ, a transcription factor involved in the regulation of inflammation, together with a significant increase in phosphorylation of IκBα, its inhibitor, was seen in the jejunum. An increase in 4-hydroxyalkenal protein adducts was also seen in the duodenum and jejunum. Although not provided orally, intraperitoneal injection of HNE (5 mg per kg daily for 7 days) decreased tight junction proteins in the colon of mice, impairing the intestinal barrier, followed by toll-like receptor 4 activation and aggravated inflammatory bowel disease (Wang et al. 2019).

3.1.2 Higher intestinal susceptibility of preterm infants

Currently, the effects of 4-hydroxyalkenal ingestion on preterm infants are not known. It is particularly concerning that 4-hydroxyalkenal levels in FM are ten times higher than in an adult diet (Surh and Kwon 2005), since previously, LPP exposure form parenteral

lipid emulsion has been associated with bronchopulmonary dysplasia (Chessex et al. 2007). In general, infants up to approximately six months after birth, have a reduced metabolic activity, leading to higher and prolonged blood levels of toxins (Scheuplein et al. 2002). Indeed, preterm infants have significantly lower plasma glutathione levels than term infants (Jain et al. 1995). Glutathione plasma levels are gestational age dependent, with extremely preterm infants having three times lower plasma levels than term infants. This can potentially impair their ability to metabolise 4-hydroxyalkenals into less toxic compounds. Additionally, preterm infants are more vulnerable to oxidative stress and LPPs in general since their antioxidant defence systems are immature (Qanungo and Mukherjea 2000).

The immature gut of preterm infants is especially vulnerable to inflammation and is characterised by increased intestinal permeability and an immature mucosa barrier (Claud 2009). Nevertheless, the gut of viable preterm infants already consists of an intestinal epithelial monolayer that has formed in the first trimester, crypts and villi, which formed from 12 weeks gestation age onwards, which express microvilli on the apical surface, and are connected by tight junctions, formed from 10 weeks gestational age (Neu 2007; Halpern and Denning 2015). After the formation of tight junctions, chemical barriers are also expressed, like defensins, lysozyme and the mucus layer, although these mature with gestational age and are therefore immature at birth in preterm infants (Buisine et al. 1998; Halpern and Denning 2015). Additionally, decreased gut motility might result in longer exposure to 4-hydroxyalkenals from the diet. Induction of NF-$\kappa\beta$ and inflammatory cytokine production by 4-hydroxyalkenals (Yadav and Ramana 2013) might contribute to the development of NEC in these infants. Furthermore, the preterm gut also has higher toll-like receptor 4 expression, rendering it more prone to inflammation and NEC (Hackam and Sodhi 2018). HNE-induced activation of toll-like receptor 4 was reported in inflammatory bowel disease (Wang et al. 2019). Similarly, HNE-induced activation of toll-like receptor 4 might contribute to the development of NEC in preterm infants.

Inflammatory response in the intestine

It is not entirely clear yet, how 4-hydroxyalkenals stimulate an inflammatory response in the intestine, or elsewhere in the body (Yadav and Ramana 2013). It is known that 4-hydroxyalkenals activate the NF-$\kappa\beta$ pathway (Awada et al. 2012; Ayala et al. 2014), resulting in the release of inflammatory cytokines and inflammation. However, the upstream mechanisms are not clarified yet. It has also been shown that at least HNE can regulate many of the protein kinase C isozymes, which are upstream of NF-$\kappa\beta$ (Yadav and Ramana 2013). For example, PKK can phosphorylate IKKα and IKKβ, which are needed for NF-$\kappa\beta$ activation (Kim et al. 2014). However, the mechanisms

how HNE regulates protein kinase C have also not been identified yet (Yadav and Ramana 2013). It has been proposed that HNE could target upstream signals of protein kinase C, such as phospholipase C and diacylglycerol. Furthermore, it has also been shown that glutathione conjugated 4-hydroxyalkenals (part of the detoxification of 4-hydroxyalkenals (Samiec et al. 2000)), after being reduced by aldose reductase, can also activate signalling molecules, via not yet identified pathways, that will activate the NF-$\kappa\beta$ pathway (Ramana et al. 2006; Yadav and Ramana 2013). A second mechanism by which 4-hydroxyalkenals might mediate an inflammatory response in the intestine is by increasing the permeability of the gut epithelium. Previously, it has been shown that HNE can increase epithelial permeability in Caco-2 cells (Cindric et al. 2013). Similarly, increased apoptosis of enterocytes, induced by high concentrations of 4-hydroxyalkenals (Ayala et al. 2014), might also lead to a disruption of the intestinal epithelium (Claud 2009). Both can lead to bacterial translocation from the lumen to the lamina propria, initiating an immune response.

In the gut, a number of cells play a role in the regulation of the immune response. The intestinal epithelium forms the first line of defence against pathogens (Santaolalla et al. 2011). It consists of Paneth cells, enteroendocrine cells, absorptive enterocytes, goblet cells, and microfold cells. Chemokines and cytokines secreted by these cells will recruit and/or activate neutrophils, macrophages, dendritic cells, and T cells (Allaire et al. 2018). Paneth cells are located in the base of the crypts and can modulate the microbiome and mediate the inflammatory response (Lueschow and McElroy 2020). They secrete antimicrobial molecules, including α-defensins, lysozyme, TNFα, and phospholipase A_2. The main function of enteroendocrine cells is the production of hormones to mediate digestion, however, they can also sense microbial metabolites and, in response, release cytokines (Worthington et al. 2018). Goblet cells are distributed throughout the villi, between the enterocytes. They produce the mucus layer that covers the epithelium as a physical barrier against pathogens (Knoop and Newberry 2018). Furthermore, goblet cells secrete anti-microbial proteins, chemokines and cytokines. Recently, it has also been shown that goblet cells can form goblet cell-associated antigen passages to present luminal content to the antigen-presenting cells in the lamina propria. Enterocytes mainly facilitate nutrient absorption, however, they also play a role in inflammation. They express toll-like receptors, which, once activated by e.g. pathogens, can lead to the release of β-defensins and initiate a signalling cascade resulting in the activation of NF-$\kappa\beta$, and the release of cytokines and chemokines including TNF-α, IL-6, IL-8, IL-18, and CCL20 (Allaire et al. 2018). These can prime, and activate the underlying immune cells, and recruit further immune cells.

Macrophages and dendritic cells are located in the lamina propria underneath the epithelium (Santaolalla et al. 2011). Both macrophages and dendritic cells are largely responsible for the innate immune response in the intestine. The macrophages in the lamina propria can recognise bacteria or bacterial products that cross the epithelium or can take up dying epithelial cells that have previously acquired antigens (MacDonald et al. 2011). Activation of macrophages by phagocytosis, lipopolysaccharides, or cytokines, will lead to the release of proteases, collagenases, and other lysosomal enzymes (Pathmakanthan and Hawkey 2000). In response, they also produce proinflammatory cytokines, including IL-1, IL-6, IL-8 and TNF-α (MacDonald et al. 2011). These cytokines can also recruit activated T and B cells to the site of infection/lamina propria (Pathmakanthan and Hawkey 2000).

As mentioned above, cytokine and chemokine expression can recruit further immune cells. For example, IL-8 expressed by epithelial cells or macrophages can recruit neutrophils from the blood stream and also activate them (Claud 2009). Neutrophils are part of the innate immune system and can phagocytose pathogens and secrete anti-microbial factors (e.g. defensins). A novel function of neutrophils identified in 2004, is the formation of neutrophil extracellular traps, by the release chromatin fibres and granule derived antimicrobial peptides and enzymes, which help binding and killing pathogens (Kaplan and Radic 2012; Li et al. 2020). They can also release cytokines to amplify the inflammation. Neutrophils will be cleared by macrophages (Bratton and Henson 2011).

Microfold cells are located above Peyer's Patches. They can sample luminal contents and can present antigens via transcytosis, phagocytosis or microvesicle shedding to the underlying dendritic cells for antigen processing and presentation (Allaire et al. 2018). Dendritic cells are the link between the innate and adaptive immune response (Pathmakanthan and Hawkey 2000). Immature dendritic cells can then process the antigen and differentiate into mature dendritic cells, which in turn activate naïve T cells, either in the Peyer's patch or the mesenteric lymph nodes (MacDonald et al. 2011). Similarly, dendritic cells in the lamina propria can have cytoplasmic extensions that extend through the epithelial layer into the lumen to sample antigens (Santaolalla et al. 2011), or they can take up antigens from infected apoptotic epithelial cells. These dendritic cells can then migrate to the mesenteric lymph nodes and there, present antigens to naïve T cells to activate them. Depending on the major histocompatibility complex used to present the antigen, either CD8+ (T killer and T memory cells) or CD4+ (T helper) cells will be induced (Pathmakanthan and Hawkey 2000). Activated T helper cells can activate naïve B cells in the Payer's Patch or in the mesenteric lymph nodes. This will lead to a class switch and induce the expression of IgA antibodies after

homing to the side of inflammation (lamina propria), mediated by cytokines from activated T helper cells. The activated CD8+ and CD4+ T cells travel to the lamina propria as well, partially controlled by the epithelial derived chemokines. CD8+ T killer cells help for example in killing infected epithelial cells. Under normal conditions without antigens/pathogens, dendritic cells produce IL-10, to supress the action of T killer cells.

3.1.3   *In vitro* models of the intestine

The intestinal epithelium has a dual function: it acts as a barrier for pathogens and toxins and at the same time needs to allow for the absorption of nutrients (Cencic and Langerholc 2010). Therefore, *in vitro* models should ideally display both these functions. Primary intestinal epithelial cells isolated from human tissue mimic the *in vivo* situation closely (Cencic and Langerholc 2010). However, these are difficult to obtain and to culture *in vitro,* and usually divide only for a limited number of times before they die, making it difficult to use them for long-term experiments (Lea 2015b). Additionally, inter-individual differences between the human donors exist, leading to low reproducibility (Cencic and Langerholc 2010). Therefore, continuously growing cell lines are preferred *in vitro* models of the intestinal epithelium. The most common models include human Caco-2, HT-29, and T84 cells (Cencic and Langerholc 2010). All three of these cell lines are derived from the colon but are morphologically and functionally similar to enterocytes of the small intestine.

Caco-2 cells have many properties of small intestine enterocytes. They differentiate into polarized cells with microvilli expression on the apical site (Lea 2015b). Expression of tight junction proteins was reported as well as cytokine and digestive enzyme production (Lea 2015a). However, they do not produce mucin (Lea 2015b). Mucin production, however, is a property of HT-29 cells (Lea 2015b), which need to be differentiated by galactose in the culture medium (Martinez-Maqueda et al. 2015). This induces a polarised phenotype with tight junctions and hydrolases; however, their activity was reported to be lower than that *in vivo.* HT-29 cells are frequently used to study the intestinal immune response and can secrete a number of cytokines and growth factors. T84 cells are also frequently used as model for the epithelial barrier, albeit being less well characterised (Devriese et al. 2017). They form a polarised monolayer in culture, with tight junction expression and small microvilli at the apical site, resembling colonocytes more closely. Indeed, they also express more colonocyte-specific genes, and fewer brush border hydrolases than Caco-2 cells. HUTU-80 cells are the only human small intestinal cell line. However, similarly to T84, HT-29, and Caco-2 cells, they are established from a carcinoma and are therefore a tumorigenic cell line. This might affect their proliferation and behaviour in response to environmental stimuli (Cencic and Langerholc 2010).

Therefore, non-tumorigenic animal cell lines are often used. Among these, the rodent cell lines IEC-6 and IEC-18, derived from the small intestine of rats, are favoured. However, the intestine of pigs, amongst all non-primates, best resembles the human gut (Vergauwen 2015). Therefore, the intestinal porcine enterocyte cell line (IPEC-J2) was established to study transepithelial ion transport and enterocyte differentiation.

The IPEC-J2 cell line

The IPEC-J2 cell line was originally isolated from the jejunum of a neonatal unsuckled piglet by Helen Berschneider in 1989 (Berschneider 1989). It is a non-tumorigenic, non-transformed, permanent cell line that has been cultured continuously for up to 98 passages (Vergauwen 2015), indicating that the cells can divide up to 98 times under optimal culture conditions. The cell line was first characterized by Schierack and colleagues for its suitability as an *in vitro* intestinal model (2006). In culture, IPEC-J2 cells form a single cell monolayer. Cells rapidly differentiate (within 14 days), are polarised, and express microvilli of differing lengths and widths on the apical side. The tight junction proteins Claudin-3 and 4 as well as occludin were detected, whereas Claudin-14 and 16 could not be detected. Nevertheless, high transepithelial electrical resistance has been reported in these cells after 14 days of culture, indicating integrity of the cell monolayer. A thin, superimposed mucus layer on the IPEC-J2 cells has also been reported (Vergauwen 2015). IPEC-J2 cells also express and produce cytokines, defensins, and toll-like receptors. Expression of mRNA for Interleukin (IL)-1α, IL-6, IL-8, IL-12β, IL-18, transforming growth factor-β3, and tumour necrosis factor-α was reported (Brosnahan and Brown 2012). This resembles cytokine expression of human intestinal cells (Schierack et al. 2006). Of these cytokines however, only IL-6 and IL-8 have been confirmed at protein level (Brosnahan and Brown 2012).

IPEC-J2 cells mimic physiology and epithelial functionality of human enterocytes. Therefore, they have been used to study epithelial transport, interaction with enteric bacteria, as well as effects of probiotics and nutrients in the past (Vergauwen 2015). Furthermore, this cell line was characterized as a simple model for the inflammation-sensitive preterm intestine (Stoy et al. 2013). Since then, the cell line has been used to analyse the effects of amniotic fluid as well as human milk oligosaccharides on the inflammatory response of preterm intestinal tissue. (Siggers et al. 2013; Rasmussen et al. 2017). Additionally, the cell line has been used to investigate the effects of antioxidants on oxidative stress in the intestine (Vergauwen et al. 2015). Overall, this makes the IPEC-J2 cell line the preferred *in vitro* model to investigate the effects of LPP ingestion from preterm FM.

3.2   Aim and objectives

FM feeding will not only provide nutrients but also potentially harmful LPPs (Surh et al. 2007; Michalski et al. 2008). 4-Hydroxyalkenals in FM might increase HNE and HHE adducts, as well as inflammation in the intestine (Awada et al. 2012), and have the potential to induce apoptosis in enterocytes (Alghazeer et al. 2008). However, there is a paucity of research investigating the effects of LPP ingestion in infants, and particularly in preterm infants, who have a more immature gut (Claud 2009), and may subsequently be more sensitive to their toxic effects (Scheuplein et al. 2002). IPEC-J2 cells have previously been used as an *in vitro* model for the inflammation-sensitive preterm gut (Stoy et al. 2013), and have therefore been chosen as a model for this research.

Therefore, the aim of this project was to investigate the effects of the 4-hydroxyalkenals HNE and HHE on the integrity and metabolism of enterocytes, using IPEC-J2 cells as *in vitro* model of the preterm gut.

- The first objective was to identify the optimal treatment time-point for 4-hydroxyalkenal incubations
- The second objective was to characterize the effects of a single 4-hydroxyalkenal treatment, measuring cell viability, metabolic activity, cell cycle, cell migration, and inflammatory markers
- The third objective was to characterize the effects of repeated 4-hydroxyalkenal incubations, measuring cell viability, metabolic activity, cell cycle, cell migration, and inflammatory markers

3.3 Material and methods

Ethical approval for this research project was granted by Bournemouth University's Research Ethics Panel (ID 16130) (Appendix III.I). This project was carried out at the Human Nutrition and Metabolism Group, Department of Human Development and Health, Faculty of Medicine, University of Southampton, under the supervision of Dr Caroline E. Childs. A visitor's contract was obtained for the University of Southampton and local rules and regulations were followed.

3.3.1 Cell culture

The IPEC-J2 cell line was obtained from DSMZ (ACC 701, DSMZ, Germany) and cultured following the supplier's recommendations (Drexler et al. 2017). After thawing, IPEC-J2 cells were cultured in Dulbecco's Modified Eagle Medium (containing 4.5 g/L glucose, and 4 mM L-Glutamine, Gibco, U.K.), supplemented with 20% heat inactivated, sterile filtrated (0.2 μm Minisart Plus, Supelco, U.K.) foetal bovine serum (Gibco, U.K.) for 4 days, before changing to standard medium containing only 10% foetal bovine serum. Penicillin and streptomycin were not added to the culture medium, as they can significantly reduce proliferation (Schierack et al. 2006). Medium was changed every other day and IPEC-J2 cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C (Drexler et al. 2017). Experiments were normally carried out seeding $2x10^4$ cells/cm^2 on 12-well plates (Corning, Fisher Scientific, U.K.), unless otherwise stated. Cells were used between passage 12 and 22.

Sub-culturing

Cells were sub-cultured at a density of approximately 70%, using trypsin/EDTA (0.25 mg/mL; 0.5 mL/10 cm^2, 15 min, 37°C, Lonza, Belgium) after washing the cells with Dulbecco A Phosphate Buffered Saline (PBS, 2 mL/10 cm^2, Oxoid Limited, U.K.) (Thermo Fisher Scientific Inc. 2016). Trypsin was inactivated using double the amount of standard medium, before seeding $2x10^4$ cells/cm^2 in a new tissue culture flask to maintain cells.

Cell number determination

To determine the cell number, 20 μL of cell suspension were added to a cell counter cuvette and diluted with 10 mL Coulter Isoton II Diluent (Beckman Coulter, U.K.), before analysing 500 μL with a Coulter Counter Z1 (Beckman Coulter, U.K.). The cell number/mL was calculated as follows:

$$Cells\ per\ mL = \frac{(A + B)}{2} \times 500 \times 2$$

A: number of cells in first count; B: number of cells in second count

Trypan blue exclusion test

Before freezing, cell viability was assessed using the trypan blue exclusion test. In this test, non-viable cells are distinct from viable cells by their blue stained cytoplasm visible using a light microscope (Strober 2015). Therefore, cells in suspension were diluted 1:2 with trypan blue solution (0.4%, Sigma-Aldrich, U.K.) and counted twice in four corner squares of a Haemocytometer (Neubauer improved counting chamber, Marienfeld, VWR, U.K.), using a light microscope (10x magnification, SM LUX, Leitz, Germany). Percentage viability was calculated as follows:

$$Viability\ (\%) = \frac{Number\ of\ viable\ cells}{Number\ of\ total\ cells} \times 100$$

Cryopreservation

A stock of cryopreserved IPEC-J2 cells was collected after obtaining the cells (Yokoyama et al. 2012; Thermo Fisher Scientific Inc. 2016). Cells were cultured and detached as described above. Only cells with a viability greater than 70% were frozen. After centrifugation of the cell suspension (200 x g, 5 min, 4°C, 5810R centrifuge, Eppendorf, U.K.), the supernatant was discarded and the cells were dropwise re-suspended in Cell Culture Freezing Media (Merck, U.K.) to freeze $2x10^6$ cells/mL. The Cryo.S vials containing the cells (Greiner Bio-One, U.K.) were placed in a pre-cooled (4°C) CoolCell LX (biocision, U.K.) in the refrigerator for 30 min, before freezing at -80°C for at least 12 h. For long-term storage, vials were transferred to a liquid nitrogen storage tank.

3.3.2 Light microscopy

IPEC-J2 cells were seeded in different densities (0.9, 1.3, 1.6, and $2.1x10^5$ cells/well, respectively) on a 6-well plate for 5 days. Representative pictures of IPEC-J2 cells were obtained using an Axiovert 200 inverted light microscope (Carl Zeiss, Germany) with an AxioCam Mrm camera, using the Axio Vision software (version 4.7.2.0 for Windows, Carl Zeiss, Germany). Pictures were taken at 5x, 10x, 20x, and 40x magnification.

3.3.3 Treatment time-point determination

First, the optimal time-point for cell treatment needed to be specified. It was reported that IPEC-J2 cells show spontaneous differentiation within two weeks, with microvilli expression on the apical site, when grown on Trans-well collagen-coated PTFE filters (Schierack et al. 2006). To determine whether spontaneous differentiation also occurs on normal 12-well plates and how long IPEC-J2 cells can be cultured on these,

$1x10^5$ cells/well were seeded in triplicates on a 12-well plate and incubated for 14 days, with medium changed every other day. Cell number and cell viability were assessed at day 3, 5, 7, 10, 12, and 14 (n=3, respectively). Microvilli expression was quantified at day 2, 10, and 14 (n=1).

Cell viability

Cell viability was quantified by propidium iodide staining, using flow cytometry. Propidium iodide is a fluorescence dye, which is excited at 488 nm (R&D Systems 2018). It binds to double stranded DNA by intercalating between the bases. It cannot cross the cell membrane and therefore, only DNA of non-viable cells with damaged and/or permeable cell membranes will be stained.

After the incubation, cells were trypsinised and approximately $1x10^6$ cells in suspension were centrifuged (200 x *g*, 5 min, room temperature), re-suspended in 200 µL PBS and incubated with 5 µL propidium iodine solution (1 mg/mL sterile filtrated H_2O, Sigma-Aldrich, U.K.) for 1 min in the dark. Negative controls did not contain propidium iodide. Cells were analysed on a FACSCalibur flow cytometer (BD Biosciences, U.K.). Data acquisition was performed using the CellQuest Pro software (Version 5.2.1 for Mac, BD Biosciences, U.K.) and 10000 events were collected per sample. Forward scatter and side scatter were adjusted to display the population of interest. Data was analysed using the FlowJo software (Version 10 for Windows, FlowJo, LLC, U.S.A.). Gating was performed relatively to the negative control (Figure 3-1).

Scanning electron microscopy

IPEC-J2 cells ($1x10^5$ cells/well) were seeded on sterile 16 mm circular glass coverslips (Agar Scientific, U.K.) in a 12-well plate. Specimen preparation was carried out according to a protocol provided by the Biomedical Imaging Unit of the University of Southampton. Cells were grown for 2, 10 or 14 days before fixation (3% glutaraldehyde (Agar Scientific, U.K.), 4% formaldehyde (Fisher Scientific, U.K.) in 0.1 M 1,4-piperazinediethanesulforic acid buffer (Sigma-Aldrich, U.K.), pH 7.2, 20 min, room temperature). After rinsing the specimen twice for 10 min with 1,4-piperazinediethanesulforic acid buffer (0.1 M, pH 7.2), specimens were post-fixed with osmium tetroxide (1% in 0.1 M 1,4-Piperazinediethanesulforic acid buffer, pH 7.2 (Sigma-Aldrich, U.K.)) for 1 hour, followed by two more buffer washes. Specimens were gradually dehydrated using 30%, 50%, 70% and 95% ethanol (all Fisher Scientific, U.K.) for 10 min each, followed by two 20 min dehydration steps with absolute (100%) ethanol (Fisher Scientific, U.K.). Next, specimens were subjected to critical point CO_2 drying (31.1°C, 1072 P.S.I, 1 hour, Blazers CPD 030, Bal-Tec,

Liechtenstein) and mounted on 12.5 mm aluminium pin stubs (EM Resolutions, UK), and sputter coated with 5 nm platinum (Q150R ES, Quorum, U.K.).
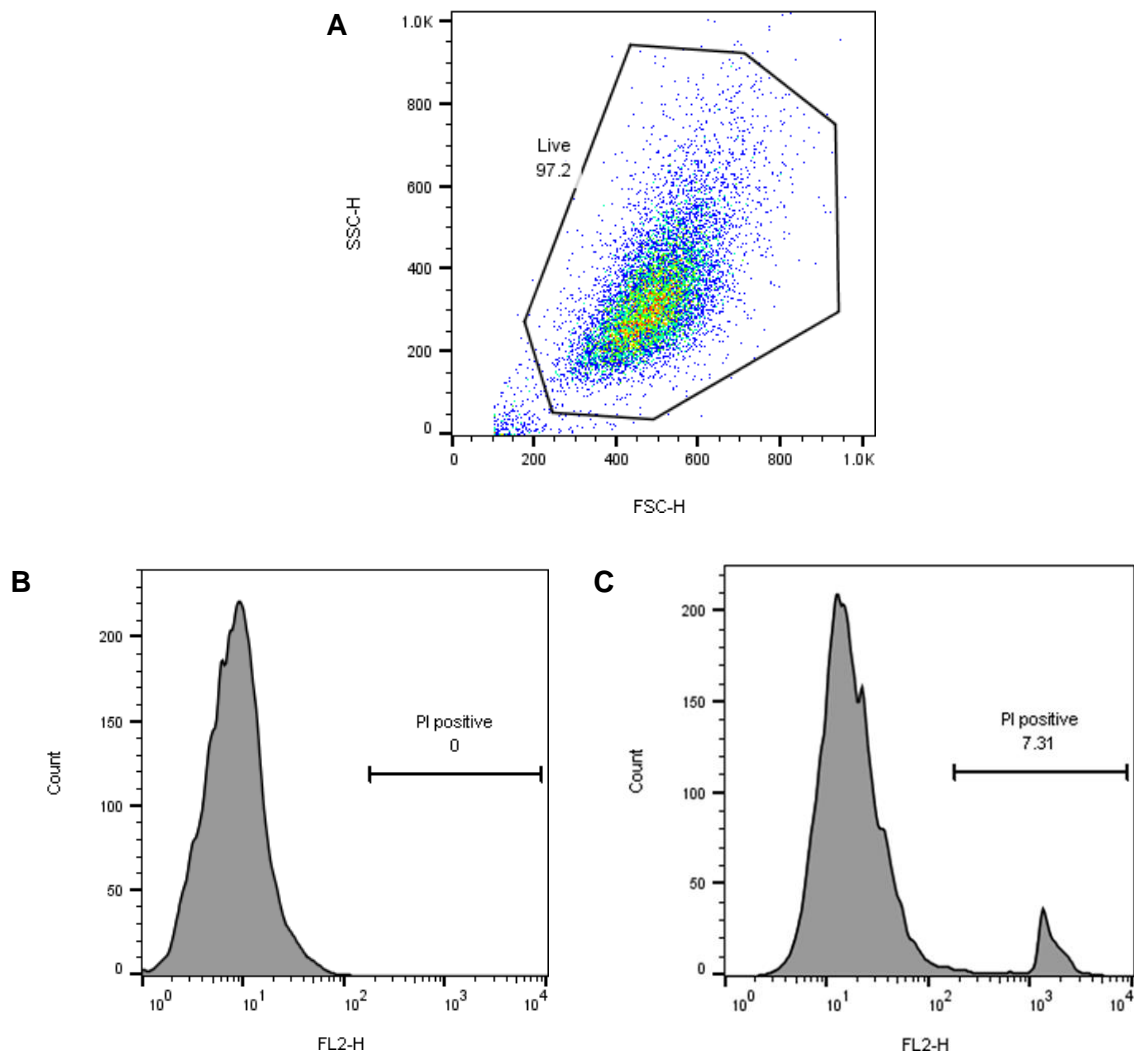
Figure 3-1: Analysis of flow cytometry data for propidium iodide staining

A: Gaiting for alive cells in the forward scatter (FWS) – side scatter (SSC) plot within the 10000 events collected.

B: Histogram of fluorescence signal (FL2 channel) of unstained cells (negative control) in the alive cell sub-population. This was used to set the gate for propidium iodide (PI) positive cells.

C: Histogram of fluorescence signal (FL2 channel) of stained cells in the alive cell sub-population with 7.31% of cells being PI positive.

Pictures were acquired at 1500x, 10000x and 25000x magnification using a FEI Quanta 250 FEG scanning electron microscope (High vacuum, Beam spot 2.5, high voltage 10.00 kV, 1.11 e-4 PA chamber pressure, 9.86e-8 Pa gun pressure, 254 μA emission current, Thermo Fisher, U.K.) and the xT microscope control software (Version 6.2.6 for Windows). Scanning electron microscope pictures were analysed using ImageJ (Version 1.51r for Windows, NIH, U.S.A.). Three pictures of day 2, 10

and 14 at 10000x magnification were analysed and in each, microvilli in six 10 μm^2 sized squares were quantified.

3.3.4 Hydrogen peroxide as positive control

H_2O_2 is a toxic reactive oxygen species that is produced physiologically during oxidative phosphorylation at the electron transport chain in mitochondria, or during oxidative stress, similar to lipid hydroperoxides (Sies 2017). Therefore, H_2O_2 was chosen as a positive control for the following experiments. In a first step, the optimal concentration had to be established. Extracellular H_2O_2 concentrations of >0.01 mM induce an oxidative stress response (Sies 2017).

IPEC-J2 cells were grown for 10 days, before incubation with H_2O_2 (0, 0.1, 0.2, 0.5, 0.75, 1, 1.5, 2, 2.5, 5, 10 mM, respectively, Sigma-Aldrich, U.K.) for 2 hours (n=3) to induce oxidative stress. H_2O_2 was diluted in sterile dH_2O and the final medium contained maximum 0.5% dH_2O_2. Control cells were incubated with medium only. Outcome measurements included cell number, and cell viability (as described in section 4.3.3.1).

3.3.5 Single exposure experiments

The 4-hydroxyalkenals HHE and HNE have been found in FM (Surh et al. 2007). Based on these reported concentrations, it was calculated that preterm FM in the U.K. could contain approximately 0.11 μM HHE and 0.14 μM HNE directly after opening, and approximately 0.48 μM HHE and 0.76 μM HNE after 10 days storage. The mean gastric half-emptying time in FM fed preterm infants was previously determined to be 65 min (Van Den Driessche et al. 1999).

To mimic the effects of a single feed, 10 days differentiated IPEC-J2 cells were incubated with 0.01, 0.1, 0.25, 0.5, 5, 50 μM HHE or 0.005, 0.05, 0.5, 1, 10, 100 μM HNE, respectively, or in combination 0.25 μM HHE + 0.5 μM HNE, 0.5 μM HHE + 1 μM HNE, 5 μM HHE + 10 μM HNE, 50 μM HHE + 100 μM HNE, for 2 hours. 4-Hydroxyalkenals were diluted in ethanol. Final ethanol concentration in the medium was 0.5%. Ethanol can have an influence on cell viability, however ethanol concentrations up to 1% for up to 18 hours have been shown not to affect IPEC-J2 cell viability (Vergauwen 2015). A solvent-carrier control consisting of 0.5% ethanol was included in all experiments. Outcome measurements included: cell viability (as described in 4.3.3.1, n=3), metabolic activity (n=3), cell cycle analysis (n=3), cell migration (n=1), IL-6 (n=1) and intracellular IL-8 (n=3) expression. Three hours after the 4-hydroxyalkenal incubations, cell supernatant was collected, centrifuged (200 x *g*, 5 min, room temperature), and stored at -80°C until analysis.

MTT assay

Cellular metabolic activity was measured using an MTT assay, in which the yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or MTT) is reduced by NAD(P)H-dependent oxidoreductases to purple, insoluble formazan (Sigma-Aldrich Co 2017). Absorbance of this product can be measured photometrically after dissolving in dimethyl sulfoxide. Darker colour indicates higher metabolic activity.

For this assay, IPEC-J2 cells were seeded on 96 well plates (5×10^3 cells/well). After the 4-hydroxyalkenal incubation, cells were washed with PBS, and medium containing 0.5 mg/mL MTT (Sigma-Aldrich, U.K.) was added, and cells were incubated for 4 h. Next, 75 µL medium were removed and replaced with dimethyl sulfoxide, plates were shaken and incubated for 30 min in the incubator, before measuring the absorbance at 540 nm using a MultiskanEx (Thermo Labsystems, U.K.) plate reader with the Ascent Software (Version 2.6 for Windows, 2002). Metabolic activity was expressed relatively to metabolic activity of control cells.

Cell cycle analysis

Cells in different stages of the cell cycle (G1, S, G2 phase) were quantified by DNA quantitation using propidium iodide staining and flow cytometry (Abcam 2018). For the dye to bind to the DNA of all cells, cells need to be permeabilised. Alcohol is a dehydrating fixative that also permeabilises cells. The DNA amount in cells doubles during the S phase of the cell cycle and propidium iodide binds stoichiometric to the DNA. Therefore, quantitation can be performed based on the fluorescent signal.

Directly after the 4-hydroxyalkenal incubation, cells were trypsinised and approximately 1×10^6 cells in suspension were centrifuged (200 x g, 5 min, room temperature), washed with 500 µL PBS and again centrifuged (200 x g, 5 min, room temperature). The supernatant was decanted and 500 µL ice-cold 70% ethanol were added drop-wise while vortexing. Cells were incubated for 30 min on ice and centrifuged (850 x g, 5 min, room temperature), washed with 500 µL PBS, centrifuged (850 x g, 5 min, room temperature) and re-suspended in 150 µL PBS/RNAse A (200 µg/ml, Sigma, U.K.) for 10 min at room temperature, before adding propidium iodide (25 µg/mL). Cells were analysed on a FACSCalibur flow cytometer (BD Biosciences, U.K.). Data acquisition was performed using the CellQuest Pro software (Version 5.2.1 for Mac, BD Biosciences, U.K.) and 10000 events were collected per sample. Forward scatter and side scatter were adjusted to display the population of interest. Data was analysed using the FlowJo software (Version 10 for Windows, FlowJo, LLC, USA). First, cell doublets were excluded, and single cells were identified using pulse processing (Figure 3-2 A), then obvious debris was excluded in the forward scatter - side scatter plot

(Figure 3-2 B). Cells in G1, S and G2 phase were identified using the Watson Pragmatic model in FlowJo, with the constraint 'Peak 2 = G1 x n', with n being adjusted to provide the best fit of the model (smaller Root Mean Square value).
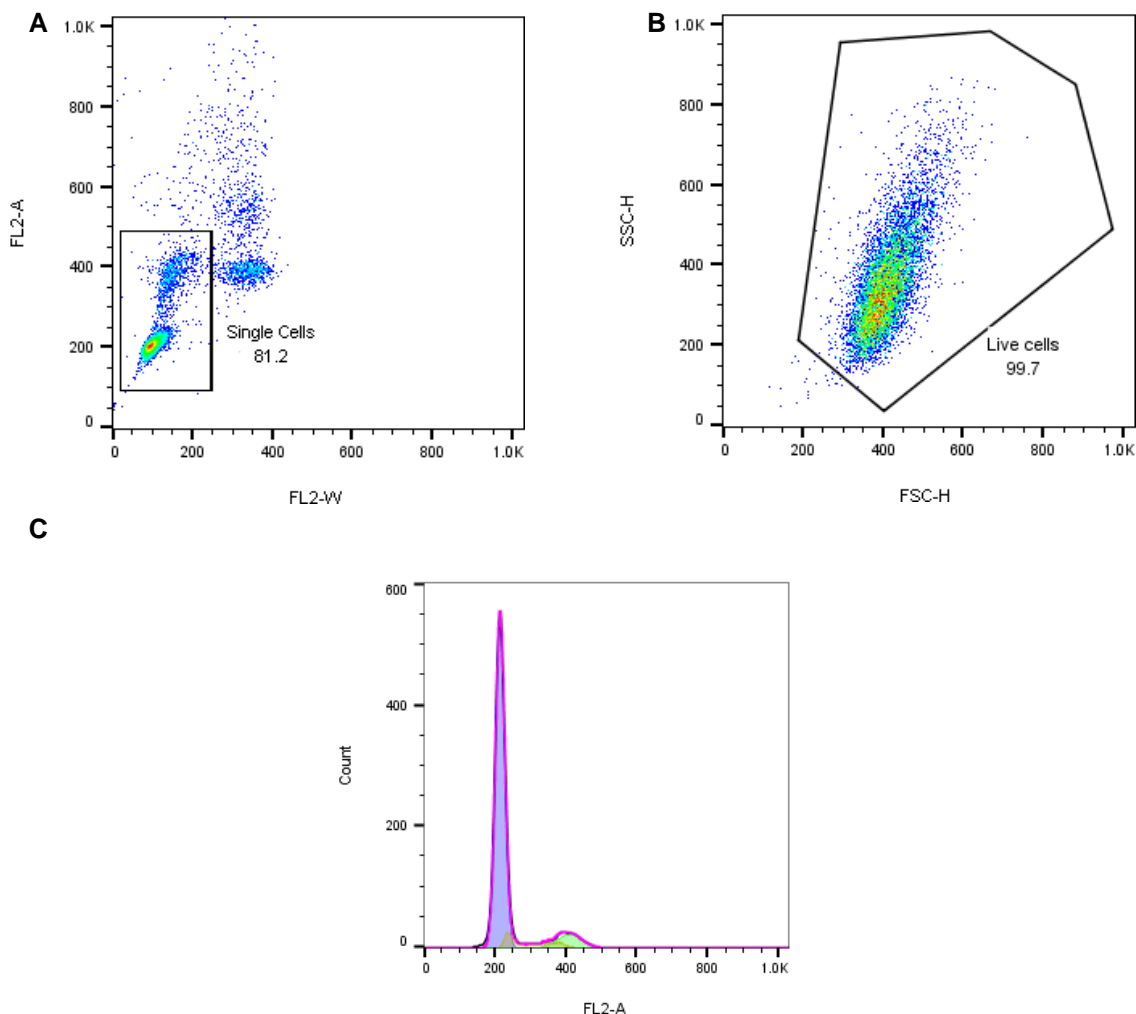
Figure 3-2: Analysis of flow cytometry data for cell cycle determination

A: Gaiting for single cells in the FL2-Width – FL2-Area plot within the 10000 events collected

B: Gaiting for alive cells in the forward scatter (FWS) – side scatter (SSC) plot in the single cell sub-population

C: Histogram of the Watson Pragmatic model of cell distribution in the G1 (blue), S (brown), and G2 phase (green) based on propidium iodide staining (FL2-Area).

Wound healing assay

A wound healing or scratch assay assesses cell migration *in vitro* after creating an artificial gap (scratch) in a cell monolayer and visualising its closing (Liang et al. 2007). After the 4-hydroxyalkenal incubation, media was removed, and cells were washed with PBS. PBS was mostly removed, and the cell monolayer was scratched from top to bottom of the 12-well, using a sterile 200 µL pipette tip, applying uniform pressure. To remove the scratched cells, the monolayer was washed twice with PBS and the medium was replaced. Wound closing was assessed using a Nikon Eclipse Ti inverted

microscope (Nikon U.K. Limited, U.K.), fitted with a Nikon Digital sight D2.U3 Camera, a heated stage (37°C), and 5% CO_2/ Air (BOC, U.K.) flow. Pictures were acquired using the NIS Elements (V 4.30.02, Nikon, Nikon U.K. Limited, U.K.) software. For 67 hours, three pictures per well were taken every 30.1 min (brightfield, 4x magnification). Pictures were analysed using the MRI wound healing tool in ImageJ (Version 1.51r for Windows, NIH, U.S.A.). The variance option was used, with the filter radius set to 5, threshold 100, radius open 7 and min size of 10000. Manual adjustments were made if necessary. Wound closure (%) at 5 hours was calculated using the following formula:

$$Wound\ closure\ (\%) = 100 - (\frac{100}{scratch\ area\ (0\ h)} \times scratch\ area\ (5\ h))$$

IL-6 ELISA

Porcine IL-6 expression was determined using the Quantikine sandwich ELISA (R&D systems, U.K.) following the manufacturer's instructions. Briefly, standards and samples (50 µL, in duplicates) were added to the IL-6 polyclonal antibody pre-coated plate containing 50 µL of Assay Diluent RD1-63. After incubation (2 hours, 500 rpm on a horizontal orbital shaker, room temperature), the plate was washed 5-times. Next, 100 µL of conjugated IL-6 antibody were added and the plate was incubated (2 hours, 500 rpm on a horizontal orbital shaker, room temperature), followed by 5 washes. Afterwards, 100 µL of Substrate Solution were added and the plate was incubated (30 min, room temperature, light protected). The Stop Solution (100 µL) was added and the absorbance was measured at 450 nm, using an absorbance plate reader (Multiskan Ex, Thermo Labsystems, U.K.). Absorbance was also measured at 550 nm for correction. Results were acquired using the Ascent software (Version 2.6 for Windows, Thermo Scientific, U.K.). A four-parameter logistic standard curve was calculated to quantify the IL-6 concentration in pg/mL (Figure 3-3). The sensitivity of the assay was 4.3 pg/mL. IL-6 concentration was only measured after incubation with 0.01, 0.1, 0.25, 0.5 µM HHE and 0.005, 0.05, 0.5, 1 µM HNE, respectively.
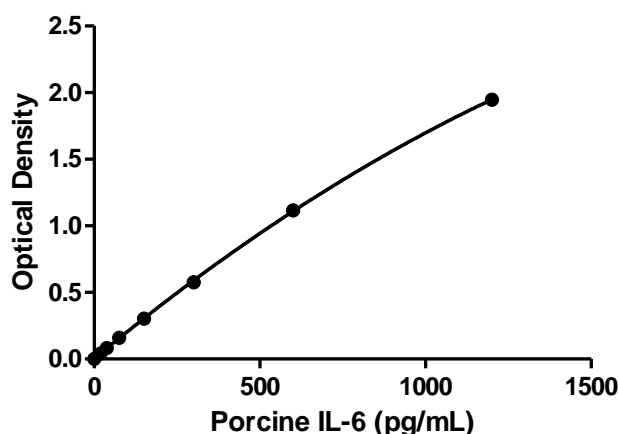
Figure 3-3: Standard curve for the determination of IL-6 in cell culture supernatant
The graph shows a porcine IL-6 four parameter logistics standard curve (R^2 = 0.9999) determined by ELISA. Standards were analysed in duplicates.

Intracellular IL-8 staining

IL-8 expression was analysed using intracellular cytokine staining and flow cytometry. To block cytokine release from IPEC-J2 cells, cells were incubated in media containing monensin. Monensin is a protein transport inhibitor, which interacts with the golgi transmembrane Na^{2+}/H^+ transport to prevent cytokine secretion into the medium, trapping them inside the cells (BD Biosciences 2018). For human IL-8, monensin is recommended over brefeldin A. Furthermore, cells treated with brefeldin A died within 12 hours. The BioLegend Intracellular Cytokine Staining Protocol was followed (BioLegend 2007).

Ten days differentiated IPEC-J2 cells were pre-incubated with monensin (final concentration 2 µM, BioLegend, U.K.) for 2 hours before the 4-hydroxyalkenal incubation. Cells were incubated with 4-hydroxyalkenals as described above, but the medium contained monensin all the time. After the 2 hour 4-hydroxyalkenal incubation, cells were washed with PBS and incubated for further 12 hours. Next, cells were trypsinised and centrifuged (350 $x\ g$, 5 min, room temperature) and the cell pellet was fixed (20 min, room temperature, in the dark) in 500 µL fixation buffer (BioLegend, U.K.), followed by a centrifugation (350 $x\ g$, 5 min, room temperature). Fixed cells were twice re-suspended in 500 µL 1x permeabilization wash buffer (BioLegend, U.K.), centrifuged (350 $x\ g$, 5 min, room temperature) and the supernatant was discarded. Cells were re-suspended in residual permeabilization wash buffer and incubated with primary antibody (mouse anti IL-8 IgG2a, ab34100, abcam, U.K. 1:10 in cell staining buffer, BioLegend, U.K.; 30 min, room temperature, in the dark). Cells were washed twice with permeabilization buffer, centrifuged (350 $x\ g$, 5 min, room temperature), and the supernatant was discarded, followed by secondary antibody incubation (goat anti mouse IgG H&L (Alexa Fluor 488), ab150113, abcam, U.K.; 1:2000 in cell staining

buffer, BioLegend, U.K.; 30 min, room temperature, in the dark). Next, cells were washed twice as above, and the pellet was re-suspended in 500 µL cell staining buffer, followed by immediate analysis. Cells were analysed on a FACSCalibur flow cytometer (BD Biosciences, U.K.). Data acquisition was performed using the CellQuest Pro software (Version 5.2.1 for Mac, BD Biosciences, U.K.) and 10000 events were collected per sample. Forward scatter and side scatter were adjusted to display the population of interest. No clear shift in the fluorescent signal between unstained and stained cells was detected. Therefore, data was analysed using a population comparison (SE Dymax %Positive cells) of the live cells in the FlowJo software (Version 10 for Windows, FlowJo, LLC, USA) (Figure 3-4).
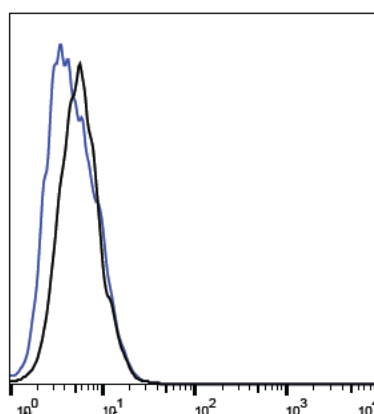
Figure 3-4: Population comparison for intracellular IL-8 staining

The graph shows the FL-2 Height signal for unstained, control (black), and stained, H_2O_2 incubated IPEC-J2 cells (blue). The signals were compared using the population comparison tool in FlowJo.

3.3.6   Repeated exposure experiments

Since infants receive FM several times per day (up to eight times for a 2000 g preterm infant), the effect of repeated 4-hydroxyalkenal exposure was investigated. Therefore, cells were incubated for 2 hours as described above. Next cells were washed with PBS and incubated for 1 hour with normal media. Incubation with 4-hydroxyalkenals was repeated two more times, intermitted by 1 hour normal media incubation (Figure 3-5). The same outcome measurements as described for single exposure experiments were used (Chapter 3.3.5).
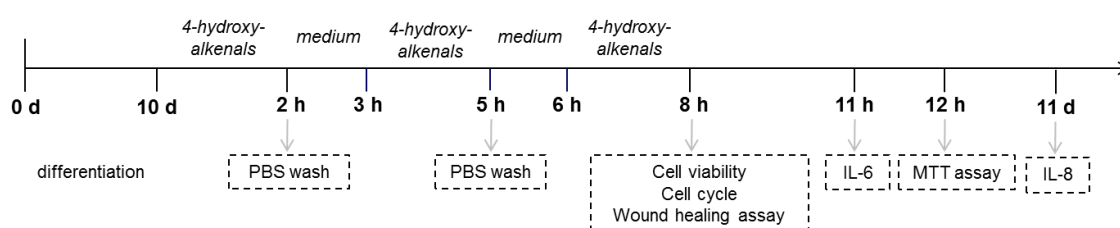
Figure 3-5: Time-line for repeated exposure experiments

3.3.7 Statistics

Sample size for most experiments was relatively low (n=3). Therefore, data was not tested for outliers and normality, and non-parametric tests were used throughout. To compare cell numbers for the growth curve, Friedman test with Dunn's multiple comparisons test comparing all columns to day 3 was used. For all 4-hydroxyalkenal treatment effects, Kruskal-Wallis test with Dunn's multiple comparisons test, comparing all columns to control was used. For comparison of control cells to solvent control and H_2O_2, respectively, 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.

3.4　Results

To investigate the effects of 4-hydroxyalkenals on preterm intestinal tissue *in vitro*, IPEC-J2 cells were used as experimental model (Figure 3-6).

| 100 % confluent | 70 % confluent | 50 % confluent |

| 5x magnification | 10x magnification |
| 20x magnification | 40x magnification |
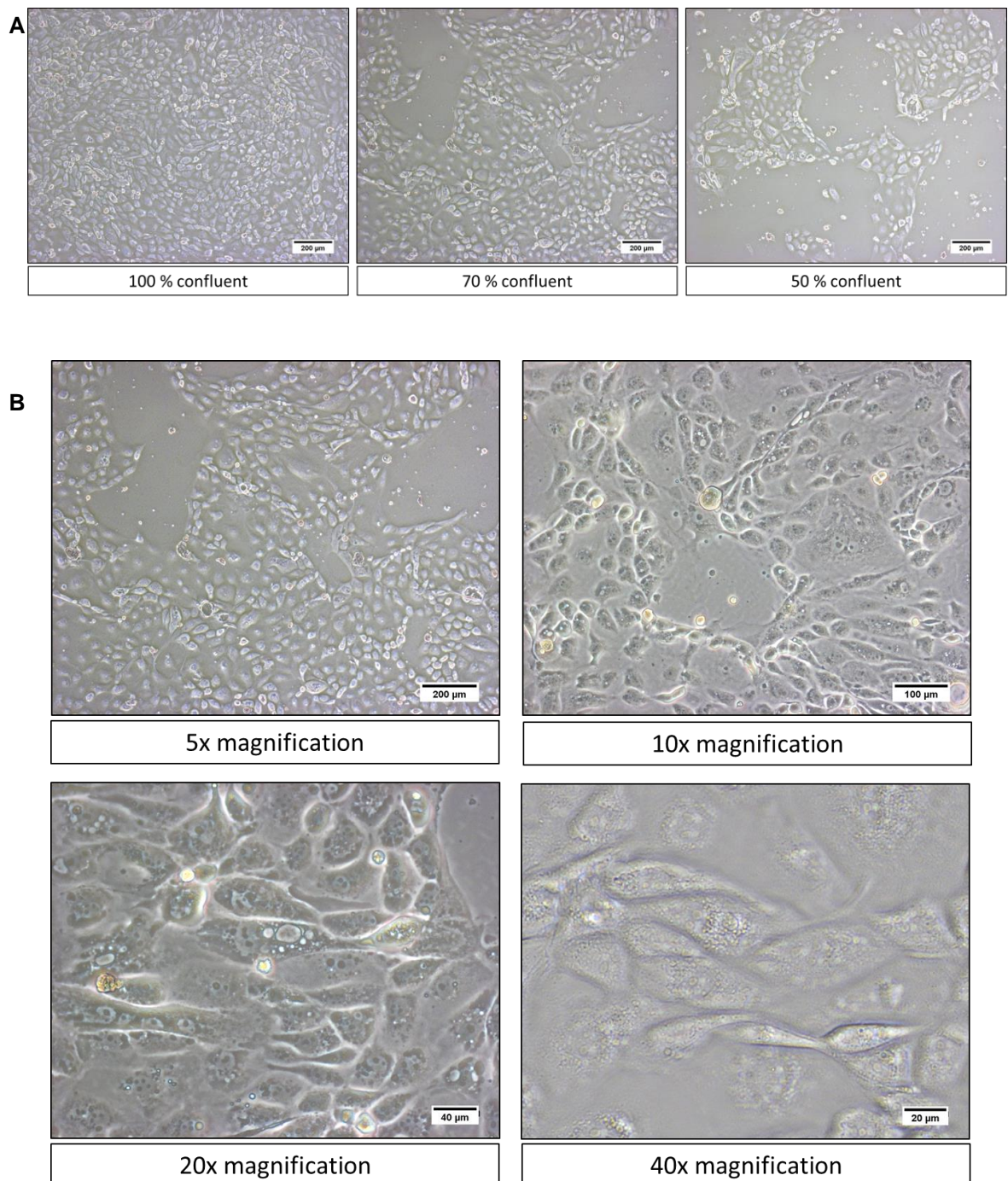
Figure 3-6: IPEC-J2 cells

A: Representative pictures of IPEC-J2 cells (passage 20) at different confluences. Cells were grown on cell culture plates and detected by light microscopy (5x magnification).

B: Representative pictures of IPEC-J2 cells (passage 20) at different magnifications. Cells were grown on cell culture plates imaged by light microscopy at different magnifications as indicated.

3.4.1 Time-point determination

To define the experimental conditions and to investigate whether IPEC-J2 cell could be cultured long-term on normal 12-well cell culture plates, cell growth was monitored over a period of 14 days (Figure 3-7 A). As described for this cell line, cell number almost doubled twice from seeding to day 3 (80000 cells vs. 316000 ± 34779 cells). Following this pattern, there was an increase in cell number over time until day 10 on which it reached significance (740333 ± 50897 cells, $p = 0.0003$, Dunn's multiple comparison test $p < 0.01$). Thereafter, cell number decreased. Viability of the cells, assessed by flow cytometry, was greater than 85% on all days (Figure 3-7 B). Splitting the total cell number into viable and non-viable cells, it was shown that on day 12, the number of non-viable cells from day 10 disintegrated, and that cell division decreased (Figure 3-7 C). Since these disintegrated cells are lost from the cell culture system, it could be misleading to only consider viability without looking at cell numbers. Furthermore, these disintegrated cells could not be considered in any subsequent analysis, indicating a limitation, and therefore, only cells until day 10 should be used.
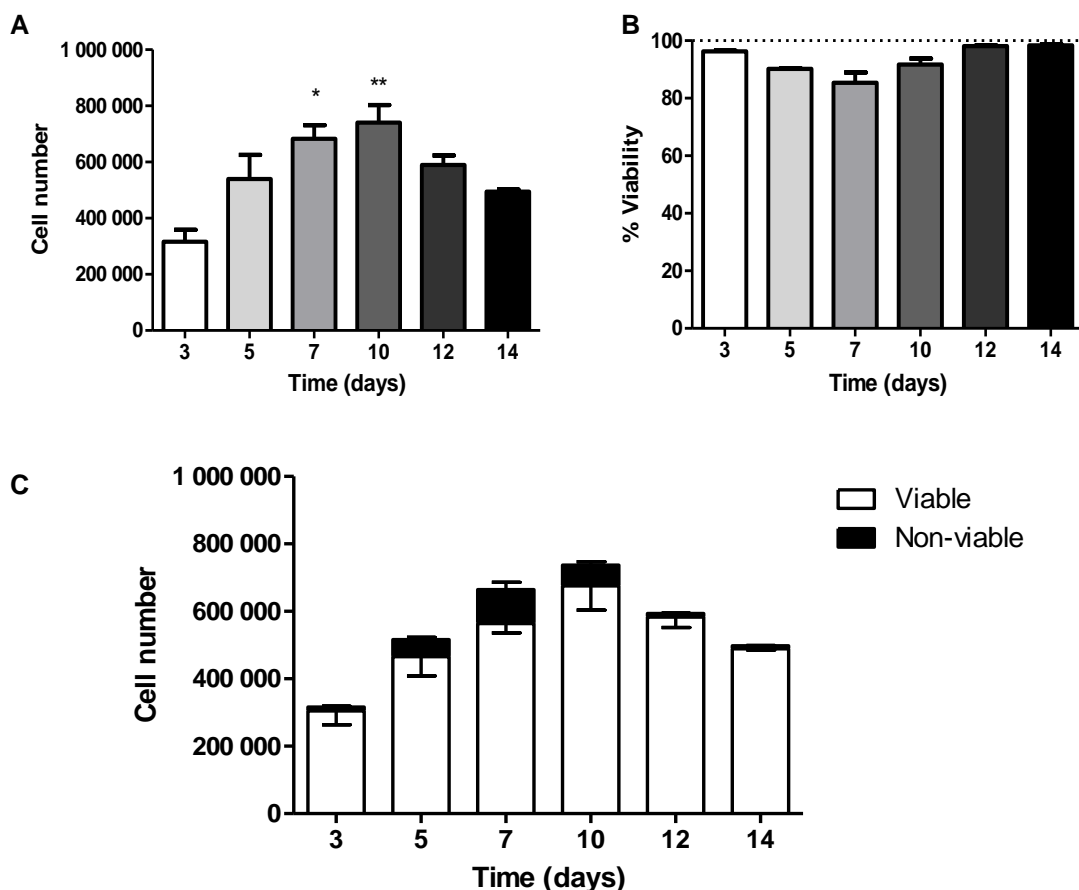
Figure 3-7: Cell growth and viability of IPEC-J2 cells over 14 days

A: Growth curve of IPEC-J2 cells cultured for 14 days on normal 12-well plates (p = 0.009)

B: Relative viability of IPEC-J2 cells cultured for 14 days on normal 12-well plates (p = 0.0073)

C: Absolute viability of IPEC-J2 cells cultured for 14 days on normal 12-well plates

n=3 respectively, Dunn's multiple comparisons test compared to day 3

IPEC-J2 cells have been reported to show spontaneous signs of differentiation, with microvilli expression on the apical site, within 2 weeks (Schierack et al. 2006). To investigate whether IPEC-J2 cells also show spontaneous differentiation with increased microvilli density when cultured on normal cell culture plates as opposed to trans-well plates, scan electron microscopy pictures were acquired on day 2, 10 and 14. Microvilli density increased significantly from day 2 to day 10 (Kruskal Wallis test *p = 0.0509*, Dunn's multiple comparison test *p < 0.05*) (Figure 3-8).

In summary, cell division slowed down after 10 days, cell viability was still greater 85% on day 10, and microvilli expression was significantly increased at day 10, indicating cell differentiation. Therefore, day 10 was chosen as treatment day for the following experiments.
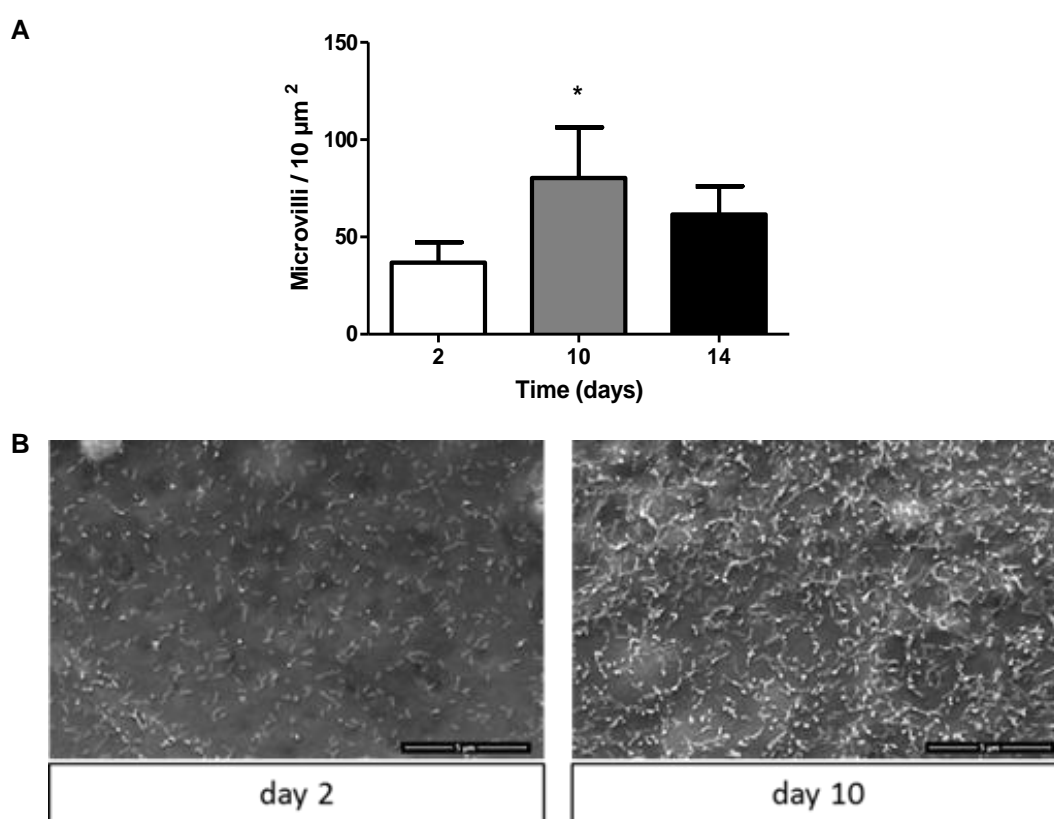
Figure 3-8: Microvilli expression of IPEC-J2 cells

A: Quantification of microvilli per 10 µm², n=3 pictures

B: Representative scanning electron microscopy pictures of microvilli on IPEC-J2 cells cultured on glass coverslips for 2 and 10 days, 10000x magnification

3.4.2 Hydrogen peroxide as positive control

H_2O_2 is a reactive oxygen species that can be produced under oxidative stress conditions. Therefore, H_2O_2 was determined as positive control for the experiments. In a first step, the optimal concentration of H_2O_2 was determined, using cell viability as an

outcome. A concentration dependent decline in cell viability was found for H_2O_2 (Figure 3-9). Incubation with 1.5 mM H_2O_2 lead to a significant decrease in cell viability (-17.4% compared to control, $p < 0.05$). This concentration was chosen as positive control for the following experiments.
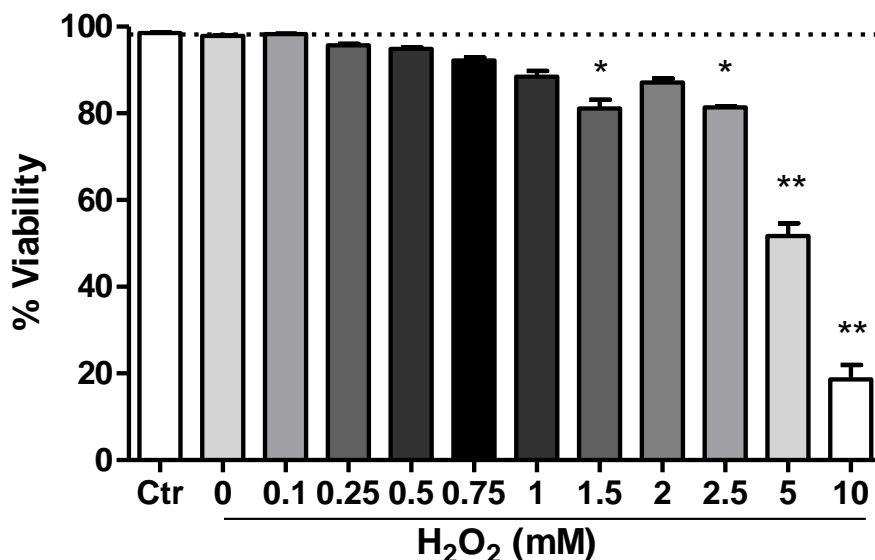
Figure 3-9: IPEC-J2 cell viability after H_2O_2 incubation

Relative viability of IPEC-J2 cells incubated with various concentrations of H_2O_2 for 2 hours after 10 days differentiation. n=3 respectively

3.4.3   Single exposure experiments

In the first series of experiments, the effects of a single 2 hour exposure of IPEC-J2 cells to various concentrations of 4-hydroxyalkenals, alone and in combination, were analysed, to mimic a single feed. All experiments were performed after 10 days of differentiation.

Cell viability

Cell viability was assessed by flow cytometry. Neither HHE (0.01, 0.1, 0.25, 0.5, 5, 50 µM) nor HNE (0.005, 0.05, 0.5, 1, 10, 100 µM) exposure for 2 hour reduced cell viability significantly ($p = 0.2119,$ and $p = 0.0648,$ respectively). However, Dunn's multiple comparison revealed a significant effect of 100 µM HNE *($p < 0.05$).* Combination of HNE + HHE (0.5 + 0.25, 1 + 0.5, 10 + 5, 100 + 50 µM) also had no effect on cell viability *($p = 0.2042$)* (Figure 3-10). The solvent had no effect *($p = 0.7483$)*, whereas H_2O_2 significantly decreased cell viability (-4%; *$p = 0.0022$*).

Cell viability was 98.31 ± 0.40% for the control cells and ranged from 98.08 ± 0.24% (0.01 µM) to 98.45 ± 0.16% (50 µM) for cells incubated with HHE, and 98.23 ± 0.73% (0.005 µM) to 97.16 ± 0.12% (100 µM) for cells incubated with HNE. The combination

of HNE + HHE resulted in cell viabilities of 97.42 ± 0.77% (0.5 + 0.25μM) and 96.50 ± 0.79% (100 + 50 μM).
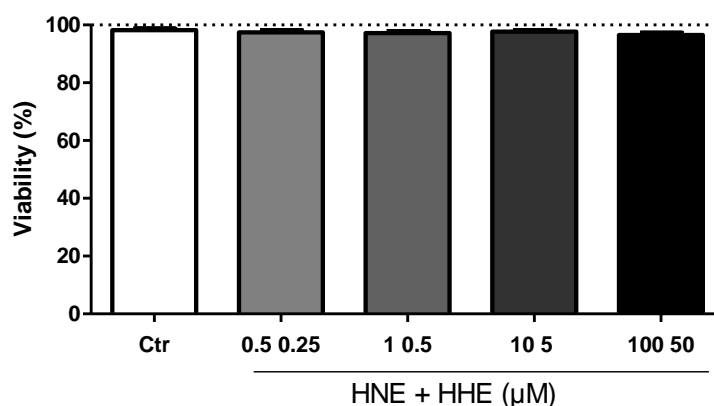
Figure 3-10: IPEC-J2 cell viability after single 4-hydroxyalkenal incubation

Relative viability of IPEC-J2 cells incubated with various concentrations of 4-hydroxyalkenals for 2 hours after 10 days differentiation. n=3

Metabolic activity

The metabolic activity was assessed using an MTT assay. Results are expressed in relation to control cells. There was no effect of HHE treatment ($p = 0.3644$) at any concentration tested (Table 3-1). The lowest metabolic activity was at 0.01 μM (87.3 ± 4.29%), and the highest at 50 μM (107.8 ± 28.02%). HNE had a general treatment effect ($p = 0.0070$), however, no statistical difference for any concentration was detected with Dunn's multiple comparison test (Table 3-1). A general effect of combined 4-hydroxyalkenals was seen ($p = 0.0212$); however, no statistical difference at any tested concentrations was detected (Figure 3-11). In general, lower concentrations decreased metabolic activity, whereas the highest concentration increased it, compared to control cells. No effect was identified for the solvent control ($p = 1.0000$) and H_2O_2 ($p = 0.5368$).

	Concentration (µM)	Metabolic activity (%)	P-value
HHE	0.01	87.3 ± 4.29	*0.3644*
	0.1	89.54 ± 11.17	
	0.25	107.0 ± 2.69	
	0.5	90.69 ± 16.81	
	5	89.69 ± 14.97	
	50	107.8 ± 28.02	
HNE	0.005	71.78 ± 6.96	*0.0070*
	0.05	69.93 ± 15.27	
	0.5	82.29 ± 5.57	
	1	71.08 ± 3.04	
	10	88.85 ± 15.76	
	100	152.0 ± 31.73	

Metabolic activity in IPEC-J2 cells relative to control cells (100%). n=3
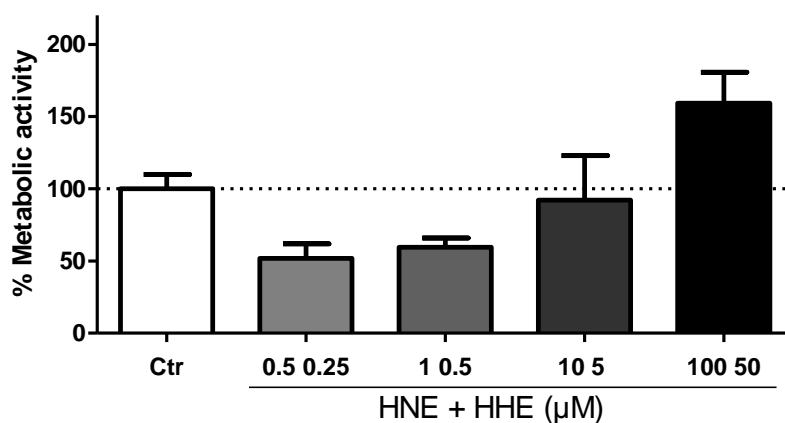
Figure 3-11: Metabolic activity of IPEC-J2 cells after single 4-hydroxyalkenal incubation
Relative metabolic activity of IPEC-J2 cells incubated with various concentrations of 4-hydroxyalkenals for 2 hours after 10 days differentiation. n=3

Cell cycle

Distribution of IPEC-J2 cells in the different phases of the cell cycle was analysed using flow cytometry. HHE incubation had an overall effect on cells in G1 phase ($p = 0.0284$), but no effect on S or G2 phase ($p = 0.0253$ and $p = 0.0635$, respectively) (Figure 3-12 A-C). Visually, an increase in cells in G1, together with a decrease in cells in S and G2 phase can be noted at a concentration of 5 and 50 µM, however this was not statistically different. A similar trend can be seen for HNE at 10 and 100 µM (Figure 3-12 D-E), although no statistical difference was seen (G1: $p = 0.1250$; S: $p = 0.0811$; G2: $p = 0.2098$).
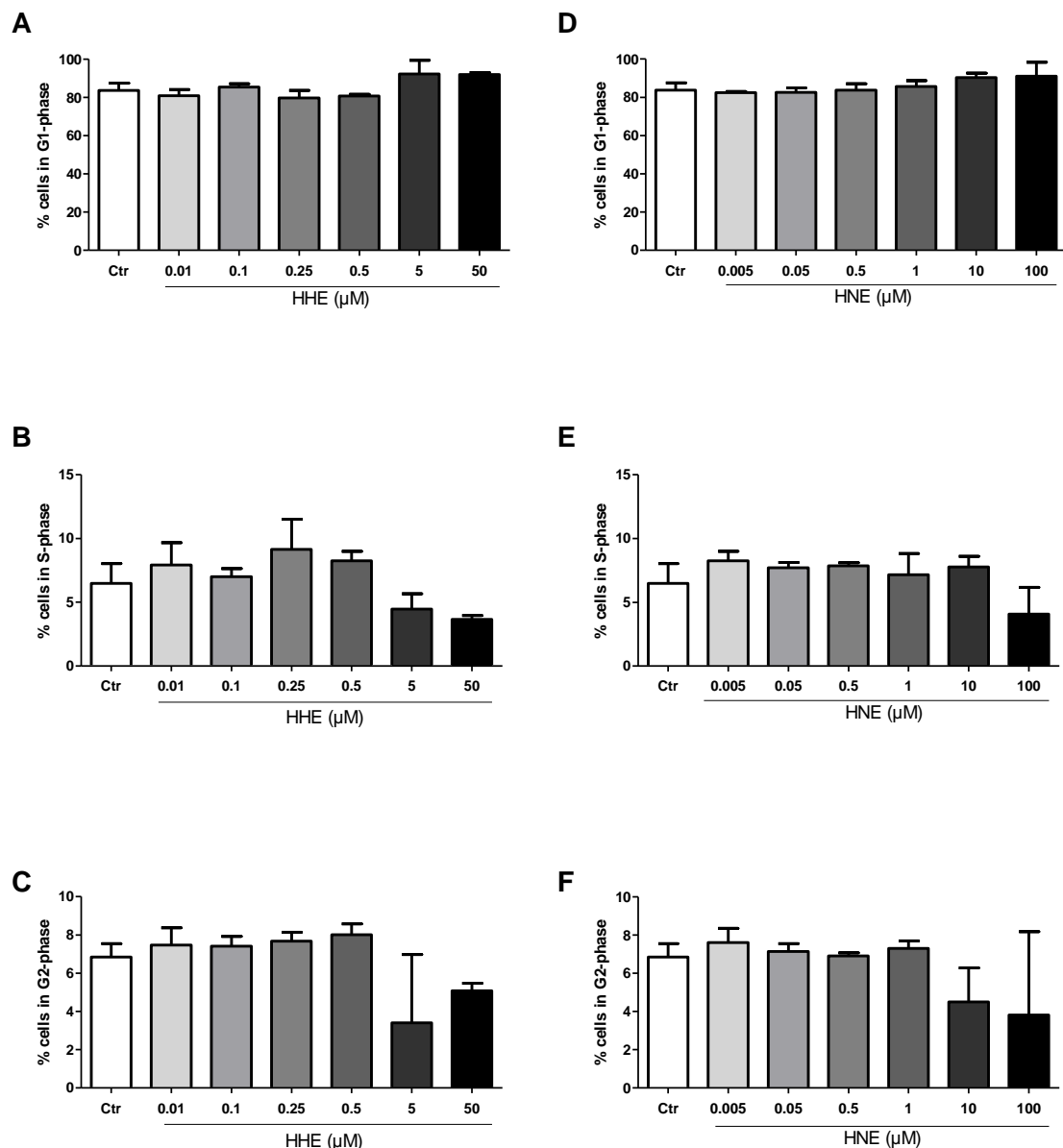
Figure 3-12: IPEC-J2 cells in different cell cycle phases after single 4-hydroxyalkenal incubation
IPEC-J2 cells in G1, S, and G2 phase after 2 hours HHE (A-C) and HNE (D-F) incubation, after 10 days differentiation. n=3

This effect was not seen when HNE and HHE were combined. No statistical difference was detected (G1: $p = 0.1396$; S: $p = 0.0631$; G2: $p = 0.2376$; Table 3-2). Neither solvent control, nor H_2O_2 had an influence on the cell cycle of IPEC-J2 cells ($p > 0.05$ for all).

Table 3-2: IPEC-J2 cells in different cell cycle phases after single 4-hydroxyalkenal incubation

	Concentration (µM)	G1	S	G2
Control	-	89.5 ± 0.2	4.19 ± 0.24	5.97 ± 0.68
HNE + HHE	0.5 + 0.25	94.6 ± 1.51	2.83 ± 1.03	2.23 ± 2.22
	1 + 0.5	89.87 ± 0.76	5.01 ± 0.28	4.09 ± 1.01
	10 + 5	89.33 ± 4.13	4.47 ± 0.36	4.76 ± 2.02
	100 + 50	93.77 ± 0.65	3.85 ± 0.72	2.77 ± 1.07

2 hours incubation after 10 days differentiation, n=3

Cell migration

Cell migration was assessed using a wound healing assay. There was no significant difference in cell migration after HHE and HNE exposure ($p = 0.1772$ and $p = 0.2775$, respectively). Combined HNE and HHE incubation showed no significant overall effect ($p = 0.0902$), however, significant lower cell migration was seen for 100 + 50 µM (100% vs. 16.92 ± 0.36% wound closure; $p < 0.05$) (Figure 3-13). Cell migration was not affected by the solvent ($p = 0.1000$). Although only one tenth of the wound closure was detected for H_2O_2, this decrease was not significant (57.4 ± 10.26% vs. 6.87 ±1.60%; $p = 0.1000$).
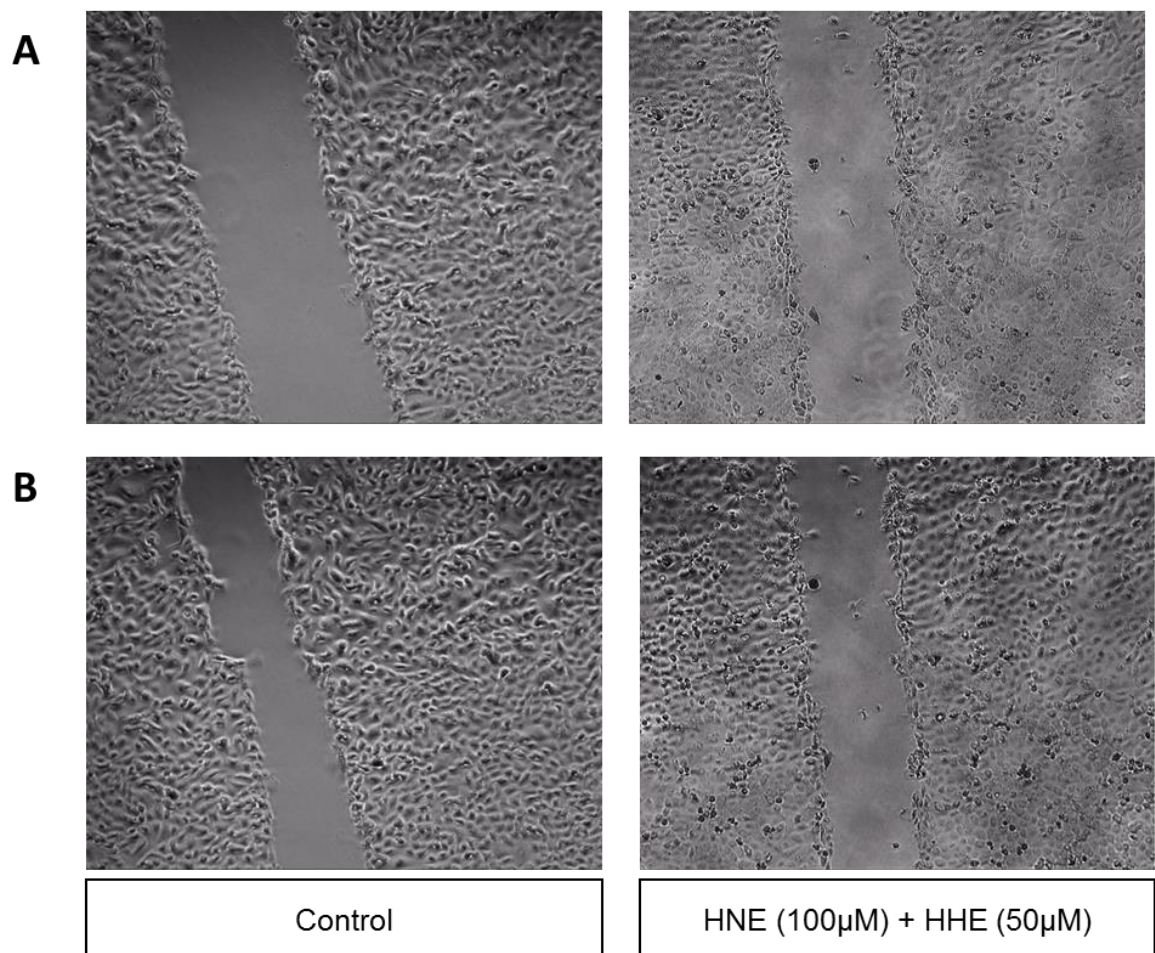
Figure 3-13: Cell migration of IPEC-J2 cells after 4-hydroxyalkenal incubation

The pictures display the scratch at (A) 0 hours and (B) 5 hours of control IPEC-J2 cells and cells incubated with 4-hydroxyalkenals for 2 hours after 10 days differentiation.

Inflammatory markers

IL-6 was measured using an ELISA. The IL-6 concentration in cell supernatant was below the limit of detection (4.3 pg/mL) under all conditions. IL-8 expression was not altered significantly when cells were incubated with HHE or HNE alone ($p = 0.0889$ and $p = 0.3058$, respectively). In combination, also no overall effect was seen ($p = 0.0874$), and no significant difference between the treatments was detected (Figure 3-14). In the HNE + HHE (100 + 50 µM) group, cells died and could therefore not be analysed. The solvent ($p = 0.4848$) and H_2O_2 ($p = 0.6623$) had no influence on intracellular IL-8 expression.
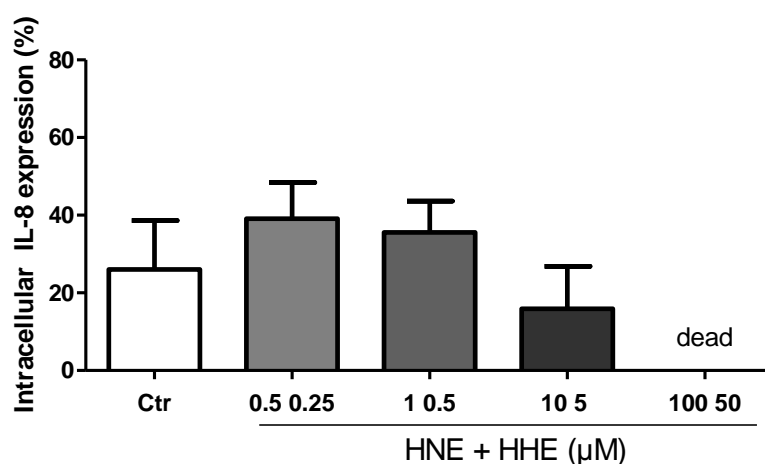
Figure 3-14: IL-8 expression in IPEC-J2 cells after 4-hydroxyalkenal incubation

Intracellular IL-8 expression in IPEC-J2 cells incubated with various concentrations of 4-hydroxyalkenals for 2 hours after 10 days differentiation. n=3

3.4.4 Repeated exposure experiments

In the clinical setting, or at home, preterm infants are fed several times per day. Therefore, the effects of repeated 4-hydroxyalkenal exposure on IPEC-J2 cells were investigated. In all experiments, cells were incubated three times for 2 hours with 4-hydroxyalkenals, with a 1 hour wash off in between incubations. All experiments were performed after 10 days of differentiation.

Cell viability

Repeated HHE (0.01, 0.1, 0.25, 0.5, 5, 50 µM) exposure had no significant effect on cell viability ($p = 0.0761$). Cell viability was ≥ 97% in all treatment groups. Repeated HNE incubation had a significant effect on cell viability ($p = 0.00362$). However, no significant difference between the groups was detected, although cell viability was only 43.97 ± 3.58% after incubation with 100 µM HNE. Cell viability in all other groups was ≥ 98%. Combined 4-hydroxyalkenal exposure also affected cell viability ($p = 0.0433$). A significant reduction in viability at the highest concentration (100 µM HNE + 50 µM HHE) was identified (98.74 ± 0.19% vs. 25.53 ± 19.52; $p < 0.05$) (Figure 3-15). Viability in all other treatment groups was ≥ 98%. Repeated solvent-carrier exposure had no effect ($p = 0.7922$), whereas H_2O_2 significantly decreased cell viability (-38%; $p = 0.0022$).
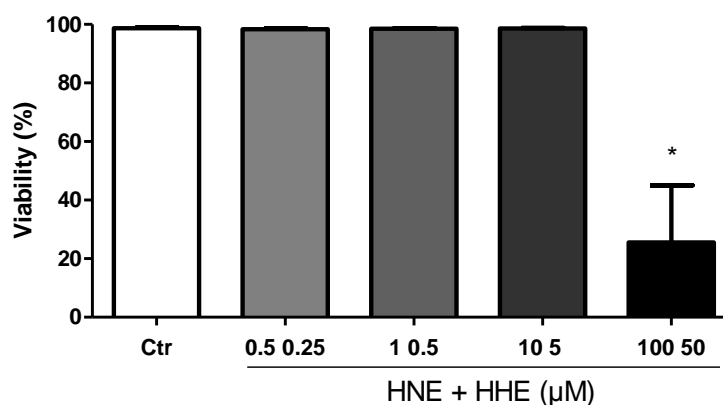
Figure 3-15: IPEC-J2 cell viability after repeated 4-hydroxyalkenal incubation

Relative viability of IPEC-J2 cells incubated with various concentrations of 4-hydroxyalkenals for three times 2 hours after 10 days differentiation. n=3

Metabolic activity

Repeated HHE exposure had an effect on the metabolic activity of IPEC-J2 cells ($p = 0.0174$). There was a significant increase in metabolic activity in the 50 µM treated cells (100% vs. 163.7 ± 14.65%; $p < 0.05$). Similarly, although repeated HNE exposure changed the metabolic activity ($p = 0.0131$), no significant differences between the treatment groups were detected. Slightly higher metabolic activity was seen for all concentrations but 10 µM, which showed a decreased metabolic activity. When cells were repeatedly incubated with HNE + HHE, no effect was seen (Figure 3-16). However, a similar dose-dependent increase as in the single exposure experiment was detected. Repeated solvent-carrier exposure had no detectable influence on the metabolic activity ($p = 0.3429$). No significant difference was seen when cells were incubated with H_2O_2 ($p = 0.3429$).
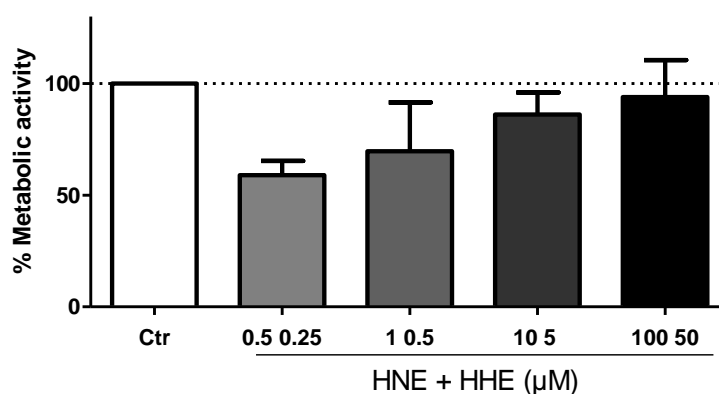
Figure 3-16: Metabolic activity of IPEC-J2 cells after repeated 4-hydroxyalkenal incubation

Relative metabolic activity of IPEC-J2 cells incubated with various concentrations of 4-hydroxyalkenals for three times 2 hours after 10 days differentiation. n=3

Cell cycle

Repeated HHE incubation had a general effect of altering cell cycle phase distribution of the IPEC-J2 cells (G1: $p = 0.0076$; S: $p = 0.0452$; G2: $p = 0.1582$). Although post-hoc tests revealed no significant differences between the concentrations, an increase in cells in G1 phase alongside a decrease of cells in the S phase was noted at 5 µM and 50 µM (Table 3-3). Repeated HNE incubation had a similar effect as HHE incubations (G1: $p = 0.0173$; S: $p = 0.0281$; G2: $p = 0.1114$). Cells in G1 phase increased and cells in S phase decreased when incubated with 10 or 100 µM HNE, although this was not significant (Table 3-3). Repeated solvent exposure did not influence the cell cycle ($p = 1.0000$). Also, H_2O_2 exposure had no effect on the cell cycle of IPEC-J2 cells.

Table 3-3: IPEC-J2 cells in different cell cycle phases after repeated 4-hydroxyalkenal incubation

	Concentration (µM)	G1	S	G2
Control	-	67.55 ± 7.14	15.70 ± 8.42	14.13 ± 1.65
HHE	0.01	62.90 ± 1.56	21.33 ± 1.95	13.97 ± 0.32
	0.1	62.07 ± 1.03	22.17 ± 1.82	12.60 ± 2.0
	0.25	61.73 ± 1.86	22.23 ± 0.64	13.17 ± 0.55
	0.5	59.17 ± 1.24	22.97 ± 3.37	13.87 ± 1.79
	5	80.40 ± 2.21	6.62 ± 1.84	11.44 ± 3.58
	50	92.73 ± 0.40	4.02 ± 1.29	5.91 ± 2.70
HNE	0.005	62.20 ± 1.78	22.30 ± 2.60	13.0 ± 1.57
	0.05	61.17 ± 3.0	19.93 ± 2.14	15.73 ± 1.15
	0.5	61.0 ± 0.79	21.27 ± 3.0	14.73 ± 2.15
	1	60.43 ± 1.10	21.50 ± 1.45	14.63 ± 0.64
	10	79.87 ± 1.60	4.85 ± 0.5	12.37 ± 1.84
	100	80.23 ± 2.23	4.61 ± 0.69	10.52 ± 3.05

Repeated 2 hours incubation after 10 days differentiation, n=3

When cells where repeatedly incubated with HNE + HHE, an effect on cell cycle could be seen, with significant differences in S phase ($p = 0.0337$; G1: $p = 0.1005$; G2: $p = 0.2092$) (Figure 3-17). Dunn's multiple comparisons test revealed significantly more

cells in G1 and significantly less cells in S phase after incubation with HNE (100 µM) + HHE (50 µM).
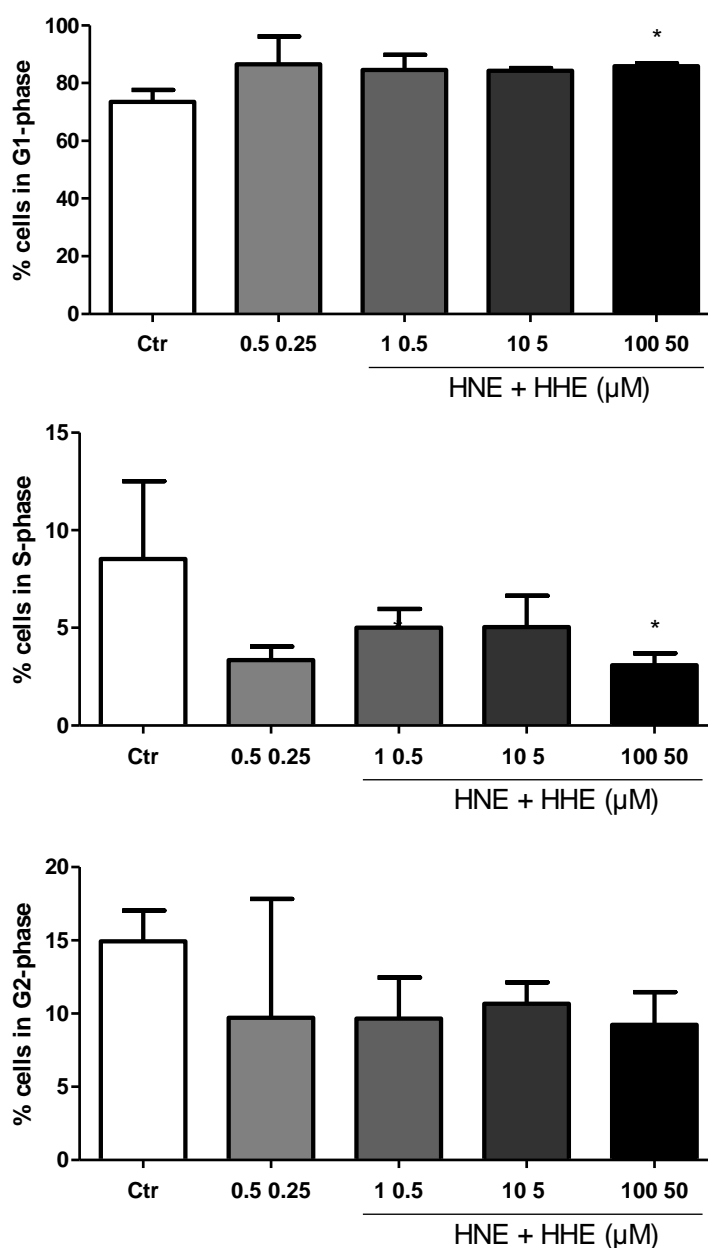
Figure 3-17: IPEC-J2 cells in different cell cycle phases after repeated 4-hydroxyalkenal incubation
IPEC-J2 cells in G1, S, and G2 phase after three times 2 hours HNE + HHE incubation, after 10 days differentiation. n=3

Cell migration

Repeated HHE exposure influenced cell migration ($p = 0.0425$), with a significant increase in cell migration detected at 5 µM HHE (Table 3-4). There was also an influence of repeated HNE exposure on cell migration ($p = 0.0125$), although no significant differences between the concentrations were detected (Table 3-4). The

lowest (0.005 µM) and highest (100 µM) concentration decreased cell migration, whereas 0.05, 0.5, 1, and 10 µM increased it. Repeated exposure of IPEC-J2 cells to HNE + HHE had no effect on cell migration ($p = 0.0984$) (Table 3-4).

	Concentration (µM)	Wound closure (%)	*P-value*
Control	-	53.96 ± 10.58	-
HHE	0.01	52.01 ± 3.74	*0.0425*
	0.1	75.4 ± 1.72	
	0.25	53.0 ± 12.7	
	0.5	49.05 ± 9.75	
	5	99.02 ± 1.7 *	
	50	51.67 ± 11.65	
HNE	0.005	37.63 ± 4.67	*0.0125*
	0.05	80.85 ± 14.26	
	0.5	61.42 ± 27.59	
	1	53.34 ± 21.08	
	10	81.36 ± 22.35	
	100	36.35 ± 3.96	
HNE + HHE	0.5 + 0.25	54.44 ± 19.27	*0.0984*
	1 + 0.5	90.62 ± 11.26	
	10 + 5	65.13 ± 26.78	
	100 + 50	-	

Table 3-4: Cell migration of IPEC-J2 cells after repeated 4-hydroxyalkenal incubation

* Significant different to control

n=3, three times 2 hours repeated incubations after 10 days of differentiation

Inflammatory markers

The IL-6 concentration in cell supernatants was below the limit of detection (4.3 pg/mL) under all conditions. Intracellular IL-8 expression in IPEC-J2 cells was not significantly different after repeated HHE ($p = 0.0609$), HNE ($p = 0.2942$), and combined HNE + HHE ($p = 0.0609$) exposures (Figure 3-18). At 100 µM HNE and at 100 µM HNE + 50 µM HHE cells died, and therefore, no intracellular IL-8 concentration can be reported. Similarly, H_2O_2 induced cell death in IPEC-J2 cells under these conditions. Repeated exposure to solvent did not change the intracellular IL-8 expression ($p = 0.8182$).
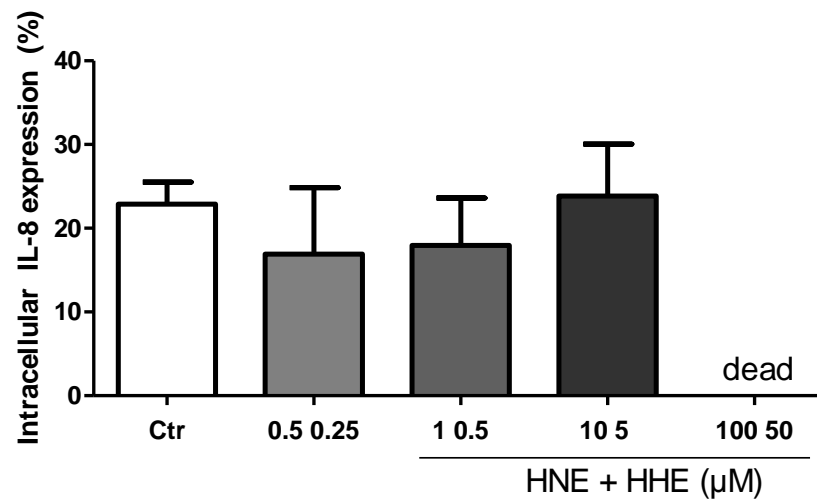
Figure 3-18: IL-8 expression in IPEC-J2 cells after repeated 4-hydroxyalkenal incubation
Intracellular IL-8 expression in IPEC-J2 cells incubated with various concentrations of 4-hydroxyalkenals
for three times 2 hours after 10 days differentiation. n=3

3.5   Discussion and conclusion

FM contains 4-hydroxyalkenals (Surh et al. 2007; Michalski et al. 2008). However, the effect of these on the integrity and metabolism of the gut, and particularly the immature preterm intestine, is not known. Therefore, this study investigated the effects of several 4-hydroxyalkenal concentrations, alone and in combination, on the immature intestinal epithelium, using the IPEC-J2 cell line as an experimental model.

The IPEC-J2 cell line was successfully established in the laboratory. Proliferation and differentiation of the cells was in line with previously reported data (Schierack et al. 2006), indicating that IPECJ-2 cells could be used as a model to analyse the effects of 4-hydroxyalkenal exposure on the preterm intestine.

Single and repeated exposure with 100 µM HNE, as well as repeated exposure with 100 µM HNE + 50 µM HHE decreased cell viability. Similarly, cell viability of Caco-2 cells was also significantly decreased after 50 µM HNE incubation for 24 hours (Alghazeer et al. 2008). Lower concentrations, similar to those in FM, had no effect on cell viability. Metabolic activity of IPEC-J2 cells was reduced at lower concentrations but increased at higher 4-hydroxyalkenal concentrations, although cells showed reduced viability. MTT reduction in the assay is associated with mitochondrial enzymes (Jo et al. 2015). However, intracellular reductases can also perform the MTT reduction to formazan. Dehydrogenases are partly responsible for HNE degradation (Hartley et al. 1995), and might be upregulated in response to high HNE concentrations. This, together with membrane damage caused by HNE (Bacot et al. 2007), could have led to false positive MTT results in non-viable cells. Similar effects have been reported for glioblastoma cells after ethanol incubation (Jo et al. 2015).

Surprisingly, neither the single, nor the repeated exposure experiments resulted in an increase of inflammatory cytokines, although 4-hydroxyalkenals have been shown to activate the NF-κβ pathway (Yadav and Ramana 2013). However, NF-κβ induction by HNE is cell specific. For example, in hepatocytes, cortical neurons, and colorectal carcinoma cells, HNE inhibited the activity whereas in vascular smooth muscle cells, fibroblasts, and macrophages HNE induced the activity of the transcription factor (Ayala et al. 2014). HNE-mediated activation of NF-κβ in IPEC-J2 cells has not been tested yet, and should therefore be clarified in the future, e.g. on mRNA level by quantitative polymerase chain reaction, and more importantly, on protein level e.g. by assessing nuclear translocation by western blotting, immunocytochemistry, or imaging flow cytometry.

Nevertheless, IL-6 and IL-8 expression was shown for this cell line after stimulation with lipopolysaccharides (*E. coli* O26:B6, 1µg/mL) for 24 hours in serum-free medium (Rasmussen et al. 2017) .Lipopolysaccharides can bind to toll-like receptor 4 and activate the NF-κβ pathway. Similarly, differentiated IPEC-J2 cells, cultured in trans-well plates, incubated for 2 hours with bacteria, showed increased IL-6 and IL-8 levels in the apical medium after 24 hours (Devriendt et al. 2010). In the future, it should be confirmed whether IPEC-J2 cells can, under the same culture conditions as used here, express IL-6 and IL-8, e.g. by treating them with lipopolysaccharide, as positive control. Since no shift in the fluorescent signal was detected and considering that golgi blocker can induce cell death (Hicks and Machamer 2005), ELISAs should be preferred over intracellular cytokine staining. Not knowing whether IPEC-J2 cells cultured under the here described conditions can produce cytokines in response to a pro-inflammatory stimulus is a limitation of this study.

Oxidative stress, induced by H_2O_2 did not change the relative gene expression of IL-6 in IPEC-J2 cells before, and relative gene expression of IL-8 was increased at 1 mM H_2O_2, but not at 2 mM H_2O_2 (Paszti-Gere et al. 2012). It may be that the incubation time was insufficient for protein transcription and translation. However, for IL-6 measurements, supernatant was collected three hours post-incubation and intracellular IL-8 was measured 12 hours post-incubation. The increased gene expression levels after 1 mM H_2O_2 incubation in the previous study translated into increased IL-8 levels in supernatant after 4 hours (Paszti-Gere et al. 2012), indicating that the sampling time was sufficient. The authors also speculated that higher levels of H_2O_2 might not lead to an increase in IL-8 due to the induced oxidative damage. Insignificant increases of intracellular IL-8 expression were noted in our experiments at the lower concentrations, whereas 10 and 5 µM HNE + HHE reduced the expression, and cells died when incubated with 100 µM HNE + 50 µM HHE after a single exposure, although other assays indicated cells were fine at this concentration. To stain for intracellular cytokines, their release from the cell needs to be blocked. Therefore, monensin, a protein transport inhibitor, which interacts with the golgi transmembrane Na^{2+}/H^+ transporter, was added to the medium (BD Biosciences 2018). Monensin was chosen over brefeldin A, another golgi blocker, since in contrast to the latter, it did not induce cell death in control cells in a pre-test. Nevertheless, monensin has been shown before to induce cell death (Verma and Das 2018), and it is known that block of the golgi complex can initiate cell signalling pathways to trigger apoptosis (Hicks and Machamer 2005). Therefore, there might have been synergistic effects of monensins and high doses of 4-hydroxyalkenals, which on their own have been shown to reduce cell viability before (Alghazeer et al. 2008), leading to the observed cell death.

Overall, these preliminary results suggest no detrimental effects of 4-hydroxyalkenals in concentrations found in FM on the gut. However, it is postulated that the experimental set up may have affected the results. Especially considering that repeated exposure to 4-hydroxyalkenals was expected to have an effect on IPEC-J2 cells. However, in these experiments, new media containing foetal bovine serum was provided to the cells after each incubation period. Cell culture medium, including Dulbecco's Modified Eagle Medium, provides a number of components, including cysteine, tyrosine, tryptophan and phenol red, that can contribute to the total antioxidant capacity (TAC) of media (Lewinska et al. 2007). Although TAC of media is lower than TAC of blood plasma, it needs to be considered that preterm infants in general have much lower antioxidative capacity than adults (Davis and Auten 2010). Addition of foetal bovine serum can further increase TAC of media (Lewinska et al. 2007), and has also been shown to decrease reactive oxygen species and to increase transcription of antioxidants in cell cultures of *in vitro*-produced porcine embryos (Mun et al. 2017). Therefore, TAC of our cell culture model could have been much higher than expected *in vivo* in preterm infants, and might have contributed to the results of this study.

Although 4-hydroxyalkenals were not detrimental in these *in vitro* experiments, this can only be used as a first indication. Stoy and colleagues have demonstrated major differences between gene expression, including immune response related genes, of IPEC-J2 cells and intestinal tissue of piglets after incubation / feeding with FM and colostrum, with two distinct groups separating the cell line and the tissue in a principal component analysis (2013). This demonstrates the limitations of extrapolation of results from the cell line to the i*n vivo* situation. Differences might be due to the heterogeneity of cells in the intestine (enterocytes, goblet cells, enteroendocrine cells, and Paneth cells) (Lea 2015b), compared to the homogenous cell line, which does not represent the physiological conditions (Stoy et al. 2013). Furthermore, the here described repeated exposure experiments consisted of three times two hour exposure during one day, whereas preterm infants receive FM up to eight times per day for up to six months. HNE alters proteasomal activity, which in turn leads to a decrease in protein turnover and an accumulation of HNE crosslinked proteins, resulting in cellular dysfunction (Riahi et al. 2010). This process happens over time, and it might be that repeated exposures over a longer time period are needed, to be able to quantify effects. Similarly, accumulation of HNE DNA adducts or HNE phospholipid adducts, influencing the cell membranes (Bacot et al. 2007), might need more time before detrimental effects are observed. It might also be that the 4-hydroxyalkenal levels in FM have no effect on IPEC-J2 cells, and on the intestine of preterm infants, although further research is needed to explore this. Even if 4-hydroxyalkenals would have no

detrimental effects on the intestine, they may still increase inflammatory cytokines in plasma (Awada et al. 2012), or affect the body elsewhere, as previously reported. Oral ingestion of LPPs over weeks affected the liver, pancreas, overall weight gain, and antioxidant levels in animal experiments (Siu et al. 1983; Izaki et al. 1984).

In general, the question remains whether IPEC-J2 cells are the ideal model for the immature intestine of preterm infants and for these experiments. Certainly, for non-primary cells and a non-primate cell line, IPEC-J2 cells resemble the human physiology closely (Vergauwen 2015). However, cells were differentiated for 10 days, whereas the preterm enterocytes are immature and resemble the foetal intestine. Also, the premature gut is characterised by increased intestinal permeability (Claud 2009). Addition of foetal bovine serum to the culture medium in our experiment, as recommended (Drexler et al. 2017), results in very high transepithelial electrical resistance values, as tested by others, indicating a high integrity of the cell monolayer (Zakrzewski et al. 2013). Using porcine serum, which is more physiologic for a porcine cell line, results in lower transepithelial electrical resistance values, closer to what is reported for human cell lines, and potentially resembling the *in vivo* situation better. Therefore, the choice of serum might have also affected the results.

The use of normal cell culture plates in contrast to transwell plates could be seen as limitation of this study, and might have influenced the results. Usually, IPEC-J2 cells are cultured on transwell collagen coated PTFE filters (Schierack et al. 2006; Vergauwen 2015) to mimic the intestine, and especially when studying nutrient absorption. This cell culture system mimics the intestinal situation more closely with an apical (luminal) and a basal (lamina propria) side, although our IPEC-J2 cells, cultured on normal cell culture plates, also differentiated into cells with microvilli expression on the apical side. However, amongst others, this transwell cell culture model could give an indication about 4-hydroxyalkenal absorption through the enterocyte. Furthermore, using this culture model, transepithelial electrical resistance can be analysed. It is a quantitative measurement of the integrity of a cell monolayer, provided by tight junctions (Srinivasan et al. 2015). Transepithelial electrical resistance can be measured in real time, without damaging cells. Therefore, using a transwell cell culture system, the effect of 4-hydroxyalkenals on epithelial integrity could be investigated. This could provide some further information about the effects of 4-hydroxyalkenals on the preterm gut, beyond inflammatory markers. Caco-2 cells treated with HNE (160 µM) showed significantly increased permeability, when cultured on transwell plates (Cindric et al. 2013), and similarly, HNE increased endothelial cell permeability in lung endothelial cells (Usatyuk and Natarajan 2004) and in a model of the blood brain barrier (Mertsch et al. 2001) *in vitro*. Increased intestinal permeability has also been shown in human

infants with NEC (Moore et al. 2016). 2Additionally, expression of tight junction proteins should be investigated after 4-hydorxyalkenal incubation. This could provide a second measure of the intestinal integrity. Previously, occluding as well as Claudin-3 and 4 have been detected in IPEC-J2 cells (Schierack et al. 2006). Incubating bovine lung microvascular endothelial cells with 25 µM HNE for 30 min resulted in redistribution of zonula occludens-1, a tight junction protein, and caused intercellular gap formation, visualised by immunostaining (Usatyuk et al. 2006). Further research is needed to understand the effects of 4-hydroxyalkenals on tight junctions in the intestine and especially in the preterm intestine.

Another limitation of this work was the use of only one cell type in our model system, which does not mimic the composition of the normal gut epithelial layer (Hidalgo 1996). The human intestinal epithelium is made up of several cell types, including enterocytes, goblet cells, Paneth cells, enteroendocrine cells and stem cells (Kleiveland 2015). The absorptive enterocytes and goblet cells make up the majority of the cells. Goblet cells are mucus producing cells, responsible for the mucus layer on the epithelial surface, which is part of the barrier function. Therefore, co-cultivation systems of enterocytes and goblet cells have been implemented (Kleiveland 2015). For example, Caco-2 cells can be cultured together with HT-29MTX cells (derived from intestinal goblet cells), in physiological relevant ratios between 90:10 and 75:25. This co-cultivation system and the resulting mucus layer should be considered when studying the effect of 4-hydroxyalkenals on the intestine in the future, since the mucus layer acts as barrier and has been shown to affect the absorption of lipophilic compounds (Behrens et al. 2001). However, the introduction of goblet cells, which do not form tight junctions, will increase the epithelial layer permeability. This will also mimic the physiological situation in the intestine more closely (Srinivasan et al. 2015). Especially when studying the inflammatory response and cytokine release, macrophages should be considered as well, since they produce the majority of cytokines in the gut (Pathmakanthan and Hawkey 2000). Therefore, Trapecar and colleagues have proposed an alternative co-culture model for studying immunomodulation in the developing small intestine (2014). This model consists of non-transformed polarised human neonatal small intestinal epithelial cells (H4-1) cultured on the apical side of a transwell, and non-transformed human macrophages (TLT) on the basal side, resulting in a reductionist 3D model of the human immature intestine. The model clearly indicated differences in the immune response (NF-$\kappa\beta$ p65 and STAT1 translocation) when cells were cultured alone or in combination, and the authors speculate that epithelial cells need soluble, macrophage-derived factors as feedback regulator for an appropriate, more physiological immune response, indicating the importance of co-culture models. Considering the above mentioned role of the mucus as well, it is proposed to establish a human three cell co-

culture model, consisting of absorptive enterocytes, cultured together with goblet cells on the apical side, and macrophages on the basal side of a transwell to investigate the effect of 4-hydroxyalkenals on the preterm gut in the future. Overall, an animal study might be more conclusive. As described above, 4-hydroxyalkenals might be influencing the intestinal integrity by influencing tight junctions (Usatyuk et al. 2006). It might be possible that the above described inflammatory changes in the jejunum and in plasma of mice seen by Awada and colleagues (Awada et al. 2012) have been mediated more indirectly, by decreasing tight junctions and increasing intestinal permeability, resulting in translocation of gut bacteria or bacterial products into the lamina propria and the systemic circulation. This can trigger the immune system and lead to an inflammatory response. Similarly, intraperitoneal injection of HNE in mice has been shown to decrease expression of the tight junction protein occludin in the colon, and therefore, to increase permeability, resulting in higher concentrations of toll-like receptor 4 ligands (lipopolysaccharides and bacterial products) in plasma, which in turn increased toll-like receptor 4 activation in the systemic circulation (Wang et al. 2019). In this model, increased expression of the IL-6 gene was also seen in the colon. The possibility of this mechanistic pathway should be explored in further animal studies. Both of the above mentioned studies have used mice as animal model. For investigating the effect of 4-hydroxyalkenals on the preterm intestine, it is suggested to use a preterm pig animal model.

The gut of pigs has high similarity with the gut of humans; therefore, preterm piglets should be used as appropriate animal model (Geens and Niewold 2011) in future studies. Preterm piglets have been used previously to study NEC (Stoy et al. 2015; Rasmussen et al. 2017). In a second step, oxidised FM should be provided to the preterm pigs, instead of 4-hydroxyalkenals alone, to truly investigate the effects of oxidised diets. 4-Hydroxyalkenals are most likely not the only LPPs in FM and there might be synergistic detrimental effects of LPPs. Furthermore, the antioxidant content of the FM should also be considered. Outcome measurements should cover the gut (inflammatory status, tight junction expression, LPP adducts), but also plasma levels of LPPs, ligands for toll-like receptor 4 (e.g. lipopolysaccharides) and inflammatory cytokines, as well as investigation of other tissues, including lung, eye and brain, since LPPs have been linked to the most common co-morbidities of preterm infants, which affect these tissues (Inder et al. 1996).

In conclusion, preliminary results from this experiment revealed no detrimental effects of HHE and HNE on intestinal cells at concentrations found in FM. The highest concentrations induced some negative effects, however, FM HHE and HNE concentrations would need to increase by 100 times to reach these concentrations.

Nevertheless, the *in vitro* model has several constraints and it needs to be considered that preterm infants are exposed several times per day to LPPs in FM, for an extended period of time. Further research, potentially in an animal model, is needed to fully understand the effects of 4-hydroxyalekenals and other LPPs in FM on not only the preterm intestine but the whole body.

4 Human milk banking practices in the U.K. – Potential influences on LCPUFA and LPP content of DHM

4.1 Introduction

4.1.1 DHM

DHM is defined as BM expressed by a mother and processed by a HMB for the use by a recipient that is not the mother's own baby (National Institute for Health and Clinical Excellence 2010). The World Health Organisation, as well as the ESPGHAN Committee on Nutrition and the American Academy of Pediatrics recommend DHM from a HMB as the preferred choice if mothers are unable to provide sufficient or appropriate BM for their preterm baby (WHO 2003; American Academy of Pediatrics 2012; Arslanoglu et al. 2013a; AAP Committee on Nutrition et al. 2017).

The idea of using BM from women other than the baby's own mother is not new. Before the introduction of FM, wet nurses were common when mother's own BM was not available (Stevens et al. 2009). Nowadays, DHM is provided by HMBs, and the first HMB was opened in Vienna in 1909. There are 15 NHS based HMBs and one non-NHS based HMB in the U.K. (Figure 4-1), which are in the United Kingdom Association for Milk Banking (UKAMB) (UKAMB 2017). In 2014, 7000 litres of DHM were distributed to 183 neonatal units in the U.K. and Ireland, whereas one neonatal unit may obtain DHM from more than one HMB (British Association of Perinatal Medicine 2016b). In 2013, 60.7% of neonatal units in the U.K reported the use of DHM, with a mean length of use of three weeks, and the main recipients being preterm infants (Zipitis et al. 2015). No national guideline is available to regulate the use of DHM, however most neonatal units agree that extremely preterm infants should receive it (British Association of Perinatal Medicine 2016b).

Figure 4-1: HMB locations in the UK

Adapted from (UKAMB 2017)

1: One Milk Bank for Scotland, Glasgow; 2: The Human Milk Bank, Irvinestown; 3: Calderdale and Huddersfield Donor Breast Milk Bank, Salterhebble; 4: Northwest Human Milk Bank, Chester; 5: The Birmingham Women's and Children's Hospital Milk Bank, Birmingham; 6: Neonatal Unit, Addenbrooke's NHS Trust, Cambridge; 7: Hearts Human Milk Bank, Hertfordshire; 8: Oxford Human Milk Bank, Oxford; 9: The Southwest Neonatal Network Donor Milk Bank, Bristol; 10: St Peter's Hospital Milk Bank, Chertsey; 11: Queen Charlotte's and Chelsea Hospital, London; 12: Guy's and St Thomas' NHS Foundation Trust, London; 13: St George's Hospital Milk Bank, London; 14: King's College Hospital Milk Bank, London; 15: Medway Maritime Hospital Milk Bank, Chatham; 16: Southampton Human Milk Bank, Southampton

DHM is preferred over FM due to its attributed benefits. A Cochrane review compared the effect of feeding preterm or low birth weight infants with FM compared to DHM (Quigley and McGuire 2014). Only nine trials were identified to be included in the analysis, and of these, only two trials were conducted after 2000, clearly demonstrating a recent lack of high quality studies in this field. Nevertheless, the systematic review demonstrated that DHM feeding of preterm infants significantly decreases the risk of feeding intolerance and developing NEC (Quigley and McGuire 2014), a severe gastrointestinal disease with a mortality rate of 30% in ELBW infants (Fitzgibbons et al. 2009).

The Cochrane review also assessed the effects of DHM feeding on neurodevelopmental outcomes (Quigley and McGuire 2014). Although no significant differences in neurodevelopment were found between the two groups, this conclusion is only based on two trials. Following from this, the DoMINO trial (Unger et al. 2014) was started in 2010. In this trial again no statistical differences in neurodevelopment at 18 months of age in very low birth weight infants was noticed when supplemental DHM was compared to supplemental preterm FM (O'Connor et al. 2016). However, the authors themselves noted that the Bayley Scales of Infant and Toddler Development III is a tool to assess early developmental delays, and not improvements in function. Therefore, it should probably not be used to test for improvements in function and these results should be viewed critically. Better outcome measurements, e.g. electrophysiological assessments instead of psychophysiological assessments (Cheatham et al. 2006), are needed to assess the effects of DHM feeding on neurodevelopment.

Bronchopulmonary dysplasia is one of the most common co-morbidities of preterm birth (Glass et al. 2015). A meta-analysis found a significant reduction in mean days on mechanical ventilation in randomised controlled trials and observational studies, as well as a significant reduction of bronchopulmonary dysplasia (oxygen dependency at 36 weeks post-menstrual age) in observational studies, when using DHM supplementation compared to preterm FM supplementation (Villamor-Martinez et al. 2018). Bronchopulmonary dysplasia however was not a primary outcome in any of the included trials. Based on these findings, there is a need for an adequately powered, randomised controlled trial to investigate the effect of DHM feeding on rates of bronchopulmonary dysplasia.

One recent study by Corpeleijn and colleagues found no benefit of supplementing mother's own milk with DHM compared to supplementation with FM on severe infections, NEC, or morality (2016). However, the supplementation period was only ten

days, with an intake of mother's own milk of > 89% in the DHM group and > 84% in the FM group, which might have masked the effect of DHM.

DHM feeding also resulted in a gut microbiota closer in composition as well as metabolic profile to the microbiota of a maternal BM fed preterm infant, when compared to a FM fed preterm infant (Parra-Llorca et al. 2018). This could lead to beneficial long-term health effects on for example intestinal functionality, immune system and metabolism. Follow-up of a study feeding preterm infants DHM vs. preterm FM revealed a significant, clinically relevant lower low-density lipoprotein cholesterol to high-density lipoprotein cholesterol ratio in adolescents (13-16 years) assigned to the DHM group. C-reactive protein was also significant lower in the same group and both outcomes remained significant after adjusting for confounding factors (Singhal et al. 2004). After adjusting, the apolipoprotein B concentration was also noted to be significantly lower in the DHM group. In general, these markers are associated with the development and severity of atherosclerosis. Follow-up of the same group of preterm infants at 13-16 years also revealed clinical relevant significant lower mean arterial blood pressure and diastolic blood pressure in the DHM group, indicating the potential benefit in terms of prevention of hypertension (Singhal et al. 2001). These studies demonstrate a long-term (more than 13 years) effect of early life DHM feeding (until 2000 g or discharge) on cardiovascular health, which is persisting into adolescence (13 to 16 years).

Use of DHM in neonatal units has also been associated with earlier initiation of enteral feeding and time to full enteral feeding, decreased use of FM, and increased breast feeding rates at discharge (Simmer and Hartmann 2009; Utrera Torres et al. 2010; Arslanoglu et al. 2013b). The absolute costs for DHM are higher than the cost of FM (£10-20 vs. £0.53 per 100 ml) (British Association of Perinatal Medicine 2016b; Crawley et al. 2019). Nevertheless, the use of DHM in neonatal intensive care units can lead to relative savings of $11 for every $1 spend on DHM due to a reduction in length of hospital stay, NEC cases and late-onset sepsis (Wight 2001). A recent economic model calculated that increasing the use of human milk, including DHM, from currently 35% to 100% in neonatal units could save the NHS £30.1 million during the first year of life of preterm infants, due to fewer episodes of NEC and sepsis, and the prevention of 190 deaths (Mahon et al. 2016). It is relevant to mention that this model did not include the cost of using DHM. The use of DHM can also have a positive impact on the environment. FM has excessive water, methane and carbon footprint, needs transportation at several stages during production and sales, and produces great amounts of waste (Joffe et al. 2019). Breast feeding for six months can save approximately 95-153 kg CO_2 per baby compared to FM use.

4.1.2 Human milk banking practices in the U.K.

Human milk banking practices in the U.K. are guided by the NICE Clinical Guideline 93 "Donor breast milk banks: the operation of donor milk bank services" (National Institute for Health and Clinical Excellence 2010), which provides best practice advice on the operation of HMBs. Figure 4-2 provides an overview of the milk banking process.
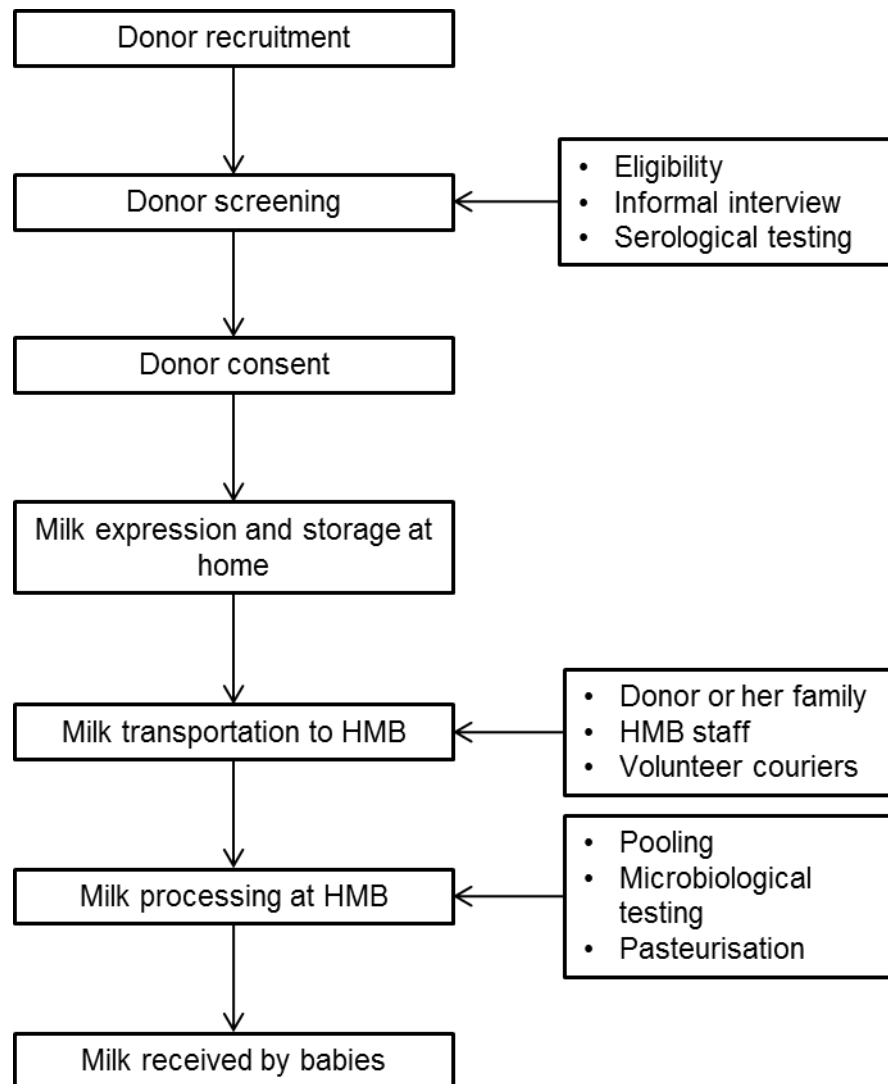
Figure 4-2: Human milk banking process in the U.K.
Adapted from (National Institute for Health and Clinical Excellence 2010)

Donors are recruited for example through leaflets, general practitioners, or staff at the antenatal and postnatal wards, (National Institute for Health and Clinical Excellence 2010). They are screened for eligibility and will have an informal interview with questions related to their health and life styles, and concerning the well-being of their babies. Serological testing is performed to avoid transmission of the human

immunodeficiency virus, hepatitis B and C, syphilis and the human t-lymphotropic virus. Afterwards, donors start expressing BM at home and store it for maximal 24 hours at 4°C, if needed, before transfer to the freezer (-18°C or below) for up to 3 months (Figure 4-3). The frozen BM is then transported to the HMB either by the donor or her family, milk banking staff (National Institute for Health and Clinical Excellence 2010), or by a volunteer courier service e.g. Service by Emergency Rider Volunteers (SERV) or Blood Bikes (personal communication, Gillian Weaver, International Milk Banking Consultant, 23 April 2016). At the HMB, DHM is stored frozen (-20°C) until pooling, microbiological testing and Holder pasteurisation (62.5 °C, 30 min, latest 3 months after expression) (National Institute for Health and Clinical Excellence 2010). Afterwards DHM is refrozen (-20°C) until it is cleared to be release to hospital neonatal units. DHM has an expiry date of six months, counted from the date of expression. At the neonatal unit, DHM needs to be used within 24 hours of defrosting.

Pre-collected BM can also be accepted from a mother whose baby has died, or who had collected expressed BM while her baby was on the neonatal unit (National Institute for Health and Clinical Excellence 2010). However, these donors must undergo the serological testing and answer the screening questions retrospectively.
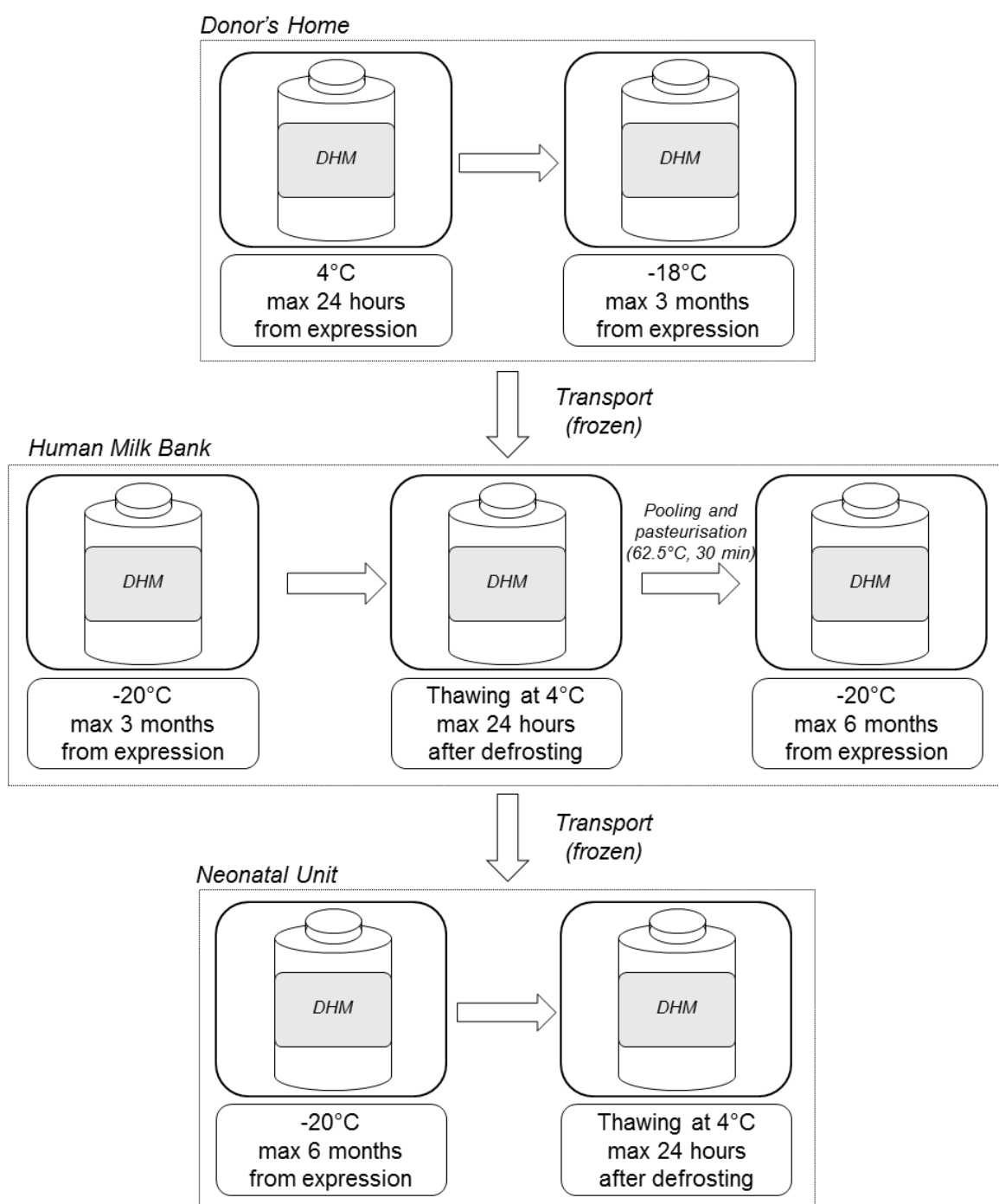
Figure 4-3: DHM storage and processing conditions

Donor human milk (DHM) is exposed to various storage conditions at donors' homes, the HMB, and the neonatal unit. This figure displays the recommended conditions for storage and use as per guidance of the National Institute of Health and Clinical Excellence Guideline 93 (National Institute for Health and Clinical Excellence 2010). Amended from (Nessel et al. 2019)

4.1.3 LCPUFA content of DHM

LCPUFA levels have not been reported for DHM in the U.K. One European study conducted in Sweden reported 0.30% ARA and 0.19% DHA in DHM, these levels were significantly lower than the levels in preterm BM (Ntoumani et al. 2013). Valentine *et al.* reported DHA levels of 0.1 molecular weight % of total FAs for DHM from the Mother's Milk Bank of Ohio, U.S.A (Valentine et al. 2010) and suggested that these levels are too low for preterm infants, considering the *in utero* accretion rates. Two years later, Baack *et al.* reported levels of ARA and DHA to be 0.40% and 0.14% respectively in DHM, with significant differences in DHA levels between the four studied American HMBs (Baack et al. 2012). ARA levels were relatively stable among the HMBs. These levels are noticeably below the reported worldwide ARA and DHA levels in BM (Fu et al. 2016), but only slightly below levels reported for the U.S.A. (0.45 ± 0.02% ARA; 0.17 ± 0.02% DHA) (Yuhas et al. 2006). Although it is good that levels were not far below average BM levels in the respective country, this does not mean that these LCPUFA levels are ideal for preterm infants. Furthermore, it is to consider that LCPUFA levels in BM have changed in the last decades due to a change in diet, with an increased intake of LA (Sanders 2000). Ways to increase the LCPUFA levels in DHM need to be explored, especially considering the LCPUFA gap of prematurity in the majority of the recipients, and the health benefits of LCPUFAs for preterm infants.

DHM in the U.S.A. is always pooled from several donors (O'Hare et al. 2013), whereas in the U.K., DHM is not pooled (National Institute for Health and Clinical Excellence 2010). Hence, LCPUFA levels might be more variable in U.K. DHM.

4.1.4 Influences and alterations to LCPUFA content of DHM

Several factors can influence the LCPUFA content of DHM, including diet, storage, and processing. The human BM LCPUFA composition is dependent on maternal adipose tissue composition, the LCPUFA synthesis from precursor FA, and the dietary intake of PUFAs (Demmelmair et al. 1998). Adipose tissue composition is reflective of the long-term (approximately 2 years) dietary fat intake (van Staveren et al. 1986; Hodson et al. 2008), which highlights the need of sufficient LCPUFA intake not only during pregnancy and lactation, but also preconception. The major dietary source of DHA is (oily) fish whereas ARA can be found solely in animal products such as meat and eggs (Meyer et al. 2003), which has implications for donors with a restricted diet, e.g. vegetarians or vegans.

BM DHA content is highly responsive to dietary intake and approximately 20% of dietary DHA is secreted into BM (Fidler et al. 2000). This leads to a high variability in BM DHA levels (Yuhas et al. 2006). For example, adding two n-3 PUFA enriched eggs

daily to the usual maternal diet significantly increases DHA, but not ARA levels, in BM (Cherian and Sim 1996). In contrast, 89% of BM ARA is derived from maternal adipose tissue, which reflects the dietary fat intake of the last 2 years (Del Prado et al. 2001; Hodson et al. 2008).

Furthermore, the human milk banking practices can have an influence on the LCPUFA levels of DHM. The effect of freezing on total fat content is inconclusive. Some researchers report freezing at -20°C does not affect the total fat content or triacylglycerol content, whereas others show a significant decrease after storage (Berkow et al. 1984; Romeu-Nadal et al. 2008b; Thatrimontrichai et al. 2012). A time and temperature dependent increase in free FAs has been reported (Dill et al. 1984; Lavine and Clark 1987). Repeated freezing and thawing can also decrease triacylglycerols, decrease the relative amount of LA, and further increase free FAs (Wardell et al. 1981; Berkow et al. 1984; Morera Pons et al. 1998). Pasteurisation does not affect the relative LCPUFA content, but might affect the total fat content and decreases total fat, and therefore LCPUFAs, absorption (Andersson et al. 2007; Garcia-Lara et al. 2013; Delgado et al. 2014).

4.2   Aim and objectives

Extremely preterm infants often receive DHM as supplementation to their mother's own BM, and they have higher LCPUFA requirements than term babies. Therefore, attention should be paid to provide DHM with the highest possible LCPUFA content. Several studies have demonstrated effects of storage time and temperature on the LCPUFA quantity and quality.

The NICE Clinical Guideline 93 focuses mainly on the safe and effective operation of HMBs, but not on the nutritional quality of DHM. Indeed, the research recommendations stated within the guideline, recommend assessing the effect of human milk banking practices on the nutritional and immunological components of DHM (National Institute for Health and Clinical Excellence 2010). However, before these effects can be assessed, the current human milk banking practices need to be ascertained.

The main aim of this study was to ascertain human milk banking practices in the U.K., with respect to international guidance and best practice recommendations, with special focus on LCPUFA content.

- The first objective was to ascertain the human milk banking practices in the U.K. with respect to LCPUFAs, using a questionnaire based survey. Additionally, the types of nutritional information or advice donors receive from HMBs with particular focus on LCPUFAs were investigated
- The second objective was to assess HMBs' adherence to the NICE Clinical Guideline 93
- The third objective was to compare practices between HMBs in the U.K. and to compare U.K. practices to international published guidance
- The comparison led to the development of recommendations that should be considered for inclusion in the NICE Clinical Guideline 93 and similar international guidance, to protect and preserve LCPUFAs in DHM

4.3   Material and methods

Ethical approval for this project (IRAS ID 205068) was obtained from the Health Research Authority for England (REC reference 16/HRA/5005), NHS Research Scotland for Scotland (R&D reference GN16HS659), and Research Gateway for Northern Ireland (HSC Trust ref: WT16/54). Hospital Research and Development Offices provided local approvals. Additionally, approval was obtained from Bournemouth University's Research Ethics Committee - Science, Technology and Health Panel. Confirmation letters are listed in Appendix IV.I-IV. This study was carried out in accordance with the Declaration of Helsinki (1964) and the Data Protection Act (1998).

To include all HMBs in the U.K., and considering the busy nature of Milk Bank Managers, an electronic questionnaire was chosen to collect data, to facilitate the most efficient form of response. Appendix IV.V provides an overview of all HMBs in the U.K., which were identified through the UKAMB website (http://www.ukamb.org/milk-banks/, accessed on 17/10/2017).

The questionnaire was designed to cover all aspects of human milk banking. Sections on donor selection, nutrition, DHM storage, transportation and handling by mothers and HMBs, as well as general questions about human milk banking were included. The questionnaire was specifically developed for this study, using relevant literature and knowledge about milk banking practices from a visit to the HMB in Southampton.

Face and content validity were established within the research team and through questionnaire testing (Bourke et al. 2016, pp.33-37). Questionnaire testing included pre-testing through expert review (Gillian Weaver, International Human Milk Banking Consultant), and field testing, including a pilot test with the Support Manager of the Northwest HMB. Feedback obtained through these measures further informed the questionnaire design. The final questionnaire can be found in Appendix IV.VI. The research proposal was also discussed at the UKAMB annual meeting (08/06/2016, London) and the obtained feedback further informed the study design.

All HMBs in the U.K. received an invitation e-mail, including the survey information sheet, the questionnaire and the optional spreadsheet. HMBs were asked to complete the questionnaire within three weeks, if no response was obtained in this time frame, reminders were sent. Returning the questionnaire was considered as "implicit" consent (as described in IRAS Question-specific guidance_Part A Version 2.3, June 2009).

Descriptive statistics were used to analyse the quantitative parts of the mixed-methods questionnaire. Analysis was performed using GraphPad Prism (version 5.0 for

Windows). Thematic analysis was used for the qualitative parts of the questionnaire (Guest et al. 2012) using and inductive semantic approach as described by Braun and Clarke (2006). Coding and themes can be found in Appendix IV.VII. Practices were compared to the NICE Clinical Guideline 93 (National Institute for Health and Clinical Excellence 2010) and other international human milk banking guidelines.

4.4 Results

The aim was to ascertain human milk banking practices of all HMBs in the U.K. The King's College Hospital Milk Bank was closed for refurbishment during the recruitment period (November 2016 to February 2018). Therefore, there were only 15 HMBs eligible to take part in the study. A response rate of 93% was achieved, one HMB declined participation due to staff shortage (Figure 4-4). No HMB completed the optional prospective spreadsheet detailing DHM storage at the HMB.
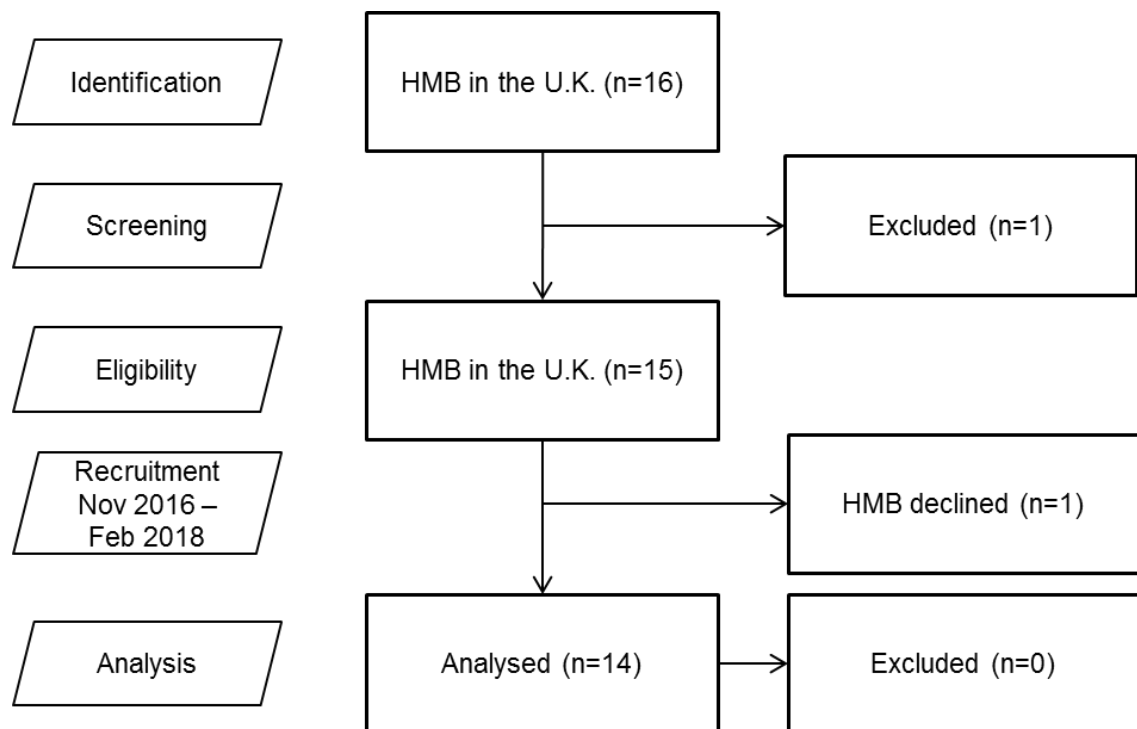
Figure 4-4: Consort flow chart of HMB recruitment and data analysis

One HMB was excluded from the study as it was closed for refurbishment. One HMB declined participation due to staff shortage. Response rate 93% (14 of 15 HMBs).

4.4.1 Donor selection

The majority of HMBs (12/14, 85.7%) allowed mothers to start donating up to six months after birth. 1/14 (7.1%) HMBs had no cut-off limit and another one (1/14, 7.1%) had three months as latest start point. Overall, reasons included a change in BM quality (6/14, 42.8%), including concerns about micronutrients such as iron and magnesium (1/6, 16.6%), trace elements (1/6, 16.6%), and energy content (1/6, 16.6%, HMB with three months cut-off). Further reasons included, BM contamination (1/14, 7.1%), cost efficiency (1/14, 7.1%), and that babies are usually weaned around this time (2/14, 14.3%).

Varying time-limits were in place for the last accepted donation (Table 4-1). HMBs with later time-limits noted that most mothers would have stopped donating before that time-

point, however, a cut off at twelve months may help to increase the amount of available DHM in times of low supply. Again, change in BM quality was the time determining factor, stated by 5/14 (35.7%) HMBs. 1/14 (7.1%) HMBs reported that an internal audit revealed that donations are often wasted due to declining donor hygiene when the baby gets older.

Table 4-1: Latest time-point for BM donation

Time after childbirth	HMBs (%)
6 months	2 (14.3)
7 months	2 (14.3)
6 to 8 months	1 (7.1)
9 months	2 (14.3)
9 to 10 months	1 (7.1)
12 months	4 (28.6)
No limit	2 (14.3)

Varying time-limits for last DHM donations. Responses to question1.6. Response rate 14 of 14.

All HMBs (14/14, 100%) accepted donations from women who had multiple pregnancies. Tandem feeding (simultaneously feeding a new-born and an older child) was allowed in 12/14 (85.7%) HMBs. 2/14 (14.3%) HMBs did not accept DHM from tandem feeding due to potential unsuitable BM composition for preterm neonates.

4.4.2 Nutrition and nutritional information

12/14 (85.7%) HMBs did not exclude mothers from donating if they follow a restricted diet, such as being vegan. 1/14 (7.1%) HMBs would potentially exclude donors after careful assessment of implications. 1/14 (7.1%) HMBs excluded vegans without vitamin B12 supplementation. Only 9/14 (64.3%) HMBs assessed donors' dietary habits. Of these nine HMBs, 4/9 (44.4%) assessed dietary habits before, and 5/9 (55.5%) before and during the period of donation. Dietary aspects of interest included following a vegetarian or vegan diet (5/9, 55.5%), an extreme exclusion diet (2/9, 22.2%), a dairy free diet (1/9, 11.1%), a nut free diet (1/9, 11.1%), a cow's milk protein free diet (1/9, 11.1%), a varied diet (1/9, 11.1%), caffeine intake (1/9, 11.1%) and oily fish intake (1/9, 11.1%).

Only 4/14 (28.6%) HMBs offered information about a healthy diet for breast feeding mothers to donors, 10/14 (71.4%) HMBs reported not to have any information available (Figure 4-5 A). It is important to note that in only 2/14 (14.3%) HMBs a registered dietitian was part of the team. In 8/14 (57.1%) HMBs, registered dietitians could be consulted at the hospital and 4/14 (28.6%) HMBs had no access to a registered dietitian (Figure 4-5 B).
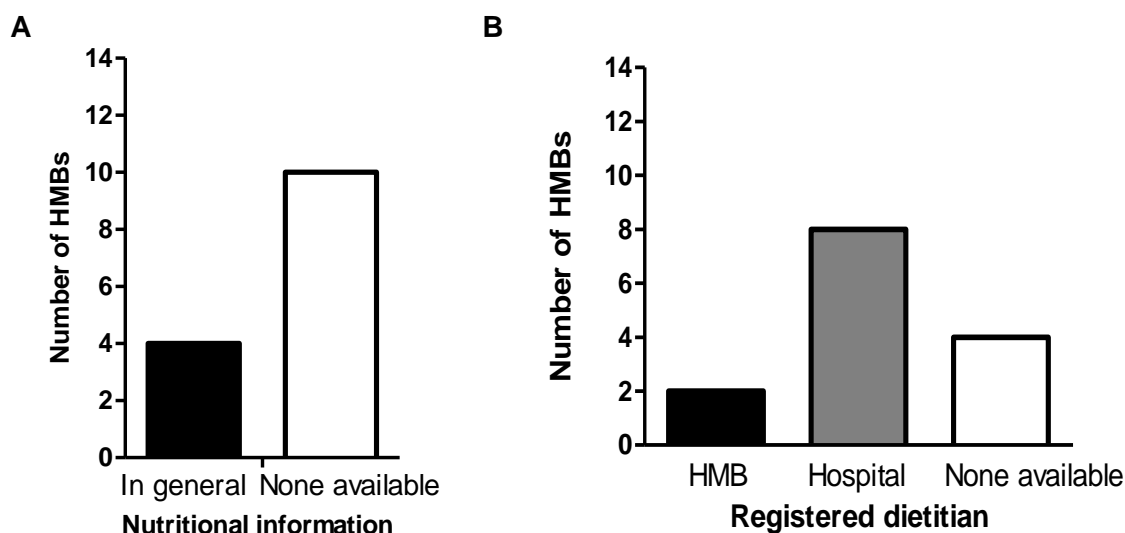
Figure 4-5: Provision of nutritional information and availability of registered dietitians
A: Nutritional information provided to donors. Responses to question 2.1. Response rate 14 of 14.
B: Availability of a registered dietitian for consultation. Responses to question 2.2. Response rate 14 of 14.

4.4.3 Handling of DHM at donor's home

DHM was collected in clear bottles in the majority of HMBs (13/14, 92.9%). 1/14 (7.1%) HMBs used clouded bottles. The bottles were provided by the HMBs (14/14, 100%), or by the hospitals which provided either HMB approved bottles (8/14, 57.1%) or own bottles (2/14, 14.3%). In 1/14 (7.1%) HMBs, mothers could donate BM providing their own containers.

Immediate freezing of expressed BM was requested by 4/14 (28.6%) HMBs, whereas pooling over 24 hours in the refrigerator was allowed by 10/14 (71.4%) HMBs. 8/10 (80%) HMBs further specified that BM should be stored on a shelf in the refrigerator. Table 4-2 displays the maximum storage time of DHM in donors' freezers. Based on estimations from two HMBs, the majority of DHM was stored for two to eight weeks before transportation to the HMB.

Table 4-2: DHM storage time in donors' freezers

Duration of storage	HMBs (%)
2 to < 4 weeks	1 (7.1)
1 to < 2 months	3 (21.4)
2 to < 3 months	10 (71.4)

Maximum storage time of DHM in donors' freezers. Responses to question 3.2. Response rate 14 of 14.

No minimum amount of DHM needed to be collected for donation in 5/14 (35.7%) HMBs. 1/14 (7.1%) HMB has no set minimum but expects 1000 mL. This was also the minimum in one other HMB (1/14, 7.1%). 4/14 HMBs (28.6%) expect at least 2000 mL, whereas one of them (1/4, 25%) would accept less from a bereaved mother. 2/14 (14.3%) HMBs specified a minimum of 3000 mL and one (7.1%) HMB specified that 3000 mL need to be collected over the whole time of donations. 6/9 (66.7%) HMBs indicated their limits were set to be cost effective (3x 3000 mL, 2x 2000 mL, 1x 1000 mL).

4/14 (28.6%) HMBs informed that DHM should be specifically labelled if the donor had delivered preterm (< 37 weeks gestational age). In 1/14 (7.1%) HMBs, DHM was labelled as cow's milk free and in another one (1/14, 7.1%) as dairy free. Other dietary habits were not specified. Colostrum was specifically labelled in two (14.3%) HMBs, one of those also specifically labelled transitional DHM (not further specified).

4.4.4 Transportation of DHM

The majority of HMBs (12/14, 85.7%) reported that they liaised with volunteer courier services for DHM collection. Five of them (35.7%) were exclusively using volunteer courier services. Staff collections were used in 7/14 (50%) HMBs, commercial couriers in 3/14 (21.4%) HMBs, and in 8/14 (57.1%) HMBs the donor or her family were transporting the DHM. Only 1/14 (7.1%) HMBs used dry-ice for transportation. 1/14 (7.1%) HMBs reported to sometimes use dry-ice. The general transportation time-limit policy varied greatly (Table 4-3).

Duration of transportation	HMBs (%)
30 minutes	2 (14.3)
1 hour	3 (21.4)
2 hours	1 (7.1)
4 hours	2 (14.3)
5 hours	1 (7.1)
No limit	5 (35.7)

Time-limit for frozen DHM transportation to the HMB. Responses to question 5.9. Response rate 14 of 14.

1/14 (7.1%) HMBs responded that a freezer vehicle will be used for any transportation over four hours. DHM was only accepted if it remained frozen during transportation by one of the HMBs without time-limit (1/5, 20%). 6/14 (42.9%) HMBs stated that DHM is examined for signs of defrosting on arrival and 3/14 (21.4%) HMBs reported that immediate pasteurisation is considered if there are concerns about DHM integrity.

4.4.5 Handling of DHM at the HMB

Unpasteurised DHM was stored at -20°C in 11/14 (78.6%) HMBs. 3/14 (21.4%) HMBs reported lower storage temperatures (one at -21°C; one at -20°C to -40°C; one at -20°C to -26°C). An estimation of DHM storage time until pasteurisation was provided by 3/14 (21.4%) HMBs. Accordingly, the majority of DHM was pasteurised between one and four weeks of arrival at the HMB. 1/14 (7.1%) HMBs prioritised DHM from bereaved mothers, to award the thank you card and donor badge faster to those mothers after distributing their DHM. Additionally, colostrum was pasteurised immediately upon arrival and preterm BM was prioritised for processing over term BM.

11/14 (78.6%) HMBs stated to pool unpasteurised DHM from one donor. 2/14 (14.3%) HMBs would only sometimes pool DHM from the same donor if they receive very small volumes, and 1/14 (7.1%) HMBs does not pool DHM. It was documented that DHM is thawed, pooled if applicable, and directly pasteurised after taking a sample for microbiological testing (12/14, 85.7%). The other 2/14 (14.3%) HMBs reported to refreeze the bottles for 48 hours or to store the bottles in the refrigerator for 24 hours, respectively, before pasteurisation (Figure 4-6). Pasteurisation frequency can be found in Table 4-4.
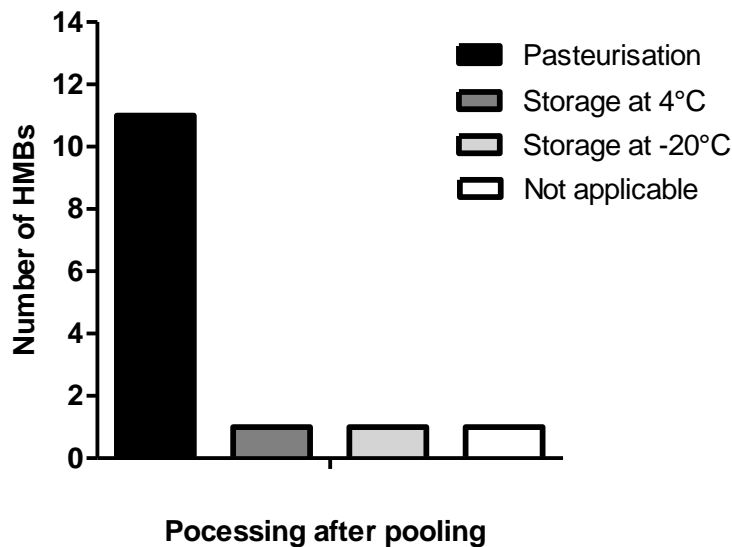
Figure 4-6: DHM processing after thawing and pooling

DHM is thawed, pooled (from one donor), and then either directly pasteurised, stored at 4°C for 24 hours, or stored at -20°C for 48 hours before pasteurisation. One HMB does not pool DHM from the same donor but would directly pasteurise. Responses to question 5.7. Response rate 14 of 14.

Table 4-4: Frequency of pasteurisation

Pasteurisation (per week)	HMBs (%)
Daily	1 (7.1)
3 times	4 (28.6)
2 to 3 times	1 (7.1)
2 times	3 (21.4)
1 to 2 times	1 (7.1)
1 time	1 (7.1)
Depending on staffing and amount of DHM	3 (21.4)

Pasteurisation frequency of DHM in HMBs. Responses to question 4.3. Response rate 14 of 14.

All HMBs (14/14, 100%) reported freezing DHM after pasteurisation. The freezing temperatures were -20°C in 10/14 (71.4%) HMBs and varied in the other 4/14 (28.6%) HMBs (one at -20 °C to -40°C, one at -21°C; one at -40°C, one at -20°C to -26°C). Distribution followed the "first in, first out" principle in 11/14 (78.6%) HMBs. 4/14 (28.6%) HMBs reported that preterm BM is distributed to preterm babies as preference, when available. Similar, 2/14 (14.3%) HMBs would provide preterm babies with DHM from a mother of a younger baby, if available. 3/14 (21.4%) HMBs try to provide DHM

from one donor for one recipient. 1/14 (7.1%) HMBs estimated the DHM storage time at the HMB to be one to two weeks for the majority of DHM.

Nutritional assessment of DHM for energy and macronutrient content was performed by HMB staff in 5/14 (35.7%) HMBs, of which 3/5 (60%) performed the analysis post-pasteurisation. Fortification of DHM with Cow and Gate BM fortifier was performed very rarely in 1/14 (7.1%) HMBs.

4.4.6 General questions and further comments

Five respondents described their HMB as working well. The incoming milk supply was also described as good in three HMBs. This is reflected by an increase of 51.5% of DHM provided by nine HMBs from 2014 to 2015 (4575.0 L vs. 6928.9 L), whereas the number of donors only increased by 6.6% (1135 vs. 1210) in the same period. 10/14 (71.4%) HMBs have never had to refuse the request for DHM from hospitals. One HMB reported that they are revising the criteria for DHM use, to make DHM available to a larger number of babies. DHM is for example used on the postnatal ward to support breastfeeding for small babies. One HMB placed emphasis on the donors' role:

> *"We look after our donors and really appreciate their time and effort to help these babies and try to make it as easy as possible to donate to us" (HMB #14).*

11/14 (78.6%) HMBs reported that they were never asked to provide DHM from donors with specific dietary habits. 1/14 (7.1%) HMBs provided cow's milk free diet DHM on request once in the last year. Another HMB was asked to provide high caloric DHM for infants with poor growth. Colostrum was provided regularly on request from the neonatal unit for extremely preterm infants in 1/14 (7.1%) HMBs. DHM for Muslim babies was provided by 1/14 (7.1%) HMBs. 1/14 (7.1%) HMBs was asked twice to provide low fat DHM. 2/14 (14.3%) HMBs regularly provided DHM from mothers of preterm babies.

Regular donations, where mothers start collecting BM for donation after their initial screening and training, accounted for the majority of BM donations (7/14 HMBs, 50%, ranging from 70 to 95% of donations). However, two HMBs described processing mainly pre-collected BM, e.g. expressed while the baby was on the neonatal unit (Figure 4-7).
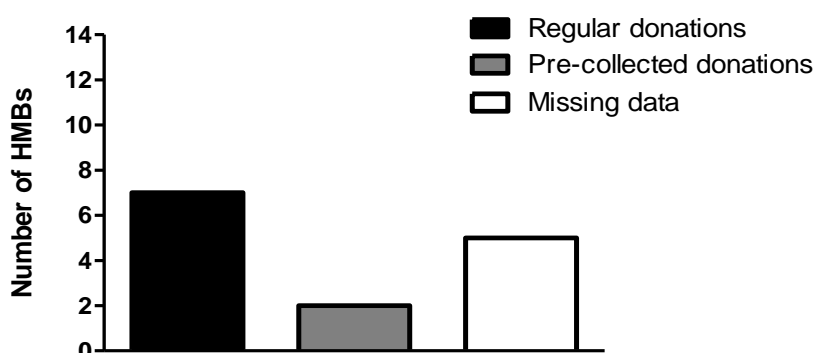
Figure 4-7: Predominantly processed DHM at HMBs

DHM can either be donated regularly after screening and training, or pre-collected milk can be donated. Donations were considered regular if more than 70% of the DHM came from regular donations. Responses to question 6.5. Response rate 9 of 14.

4.4.7   Areas for improvement

9/14 (64.3%) respondents suggested areas for improvement. The overall emergent theme was lack of resources. Indicative of this, three corresponding sub themes were identified: (1) lack of time, (2) staff training, (3) work environment. Lack of time was the most mentioned theme in the analysis, stated by four representatives. Staffs were working overtime in one HMB, and there was not enough time to pasteurise on more days in another HMB. In three HMBs, the milk bank managers had not enough time for the operation of the HMB due to too many additional responsibilities. This is underpinned by the following quote:

> *"I have to see breast feeders in difficulty, do breast feeding support groups and provide the training for both our staff and peer support mothers for UNICEF baby friendly. At present I am auditing this too. I really feel that a milk bank manager should have one sole role." (HMB #2).*

Time was also lacking to further staff knowledge in one HMB, leading to the second sub theme staff training. This was brought up by a second HMB which stated that more experienced staff would be needed to expand the HMB. Work environment was brought up by four representatives. One HMB needed more physical space, another one would like a dedicated space away from the neonatal unit. Furthermore, HMBs needed a new, more efficient pasteuriser, a bar coding system to save time and avoid human error, more computers and a dedicated HMB phone line and answer machine. Beyond that, one respondent stated that they were looking to streamline donor recruitment. One HMB struggled with organising phlebotomy for distant donors. One very important point for improvement was raised by an HMB manager:

> *"It would be ideal if every milk bank operated in the same way" (HMB #5).*

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4.5   Discussion

This study aimed to provide a representative summary of the human milk banking practices in the U.K., specifically focusing on factors that may affect LCPUFA content of DHM. An overall response rate of 93% was achieved (14/15 HMBs), hence the collected data is a highly representative summary of the human milk banking practices in the U.K. at the time of data collection. The results show great variation in practices amongst the different HMBs in the U.K., which can have a significant impact on the nutritional quality of DHM.

DHM might be the only enteral nutrition preterm babies receive for a prolonged period of time and it needs to provide all nutrients needed for infants' development. Therefore, it is imperative to provide preterm babies with the best possible quality of DHM. One key priority for the implementation of the NICE Clinical Guideline 93 was quality assurance. Part of the quality assurance is that DHM that will be administered in the NHS should be from HMBs that can demonstrate adherence to the Clinical Guideline 93 (National Institute for Health and Clinical Excellence 2010). Considerable variations were identified across all areas of human milk banking practices in this study. Therefore, it was analysed whether the variations are within the scope of the NICE Clinical Guideline 93. Furthermore, the different practices used across the U.K. were compared amongst each other and with practices used in other countries. Considering the available evidence, best practice recommendations for the handling of DHM with the aim to protect LCPUFAs were produced.

4.5.1   Donor inclusion and exclusion criteria

The NICE guideline does not regulate the latest starting point for donation, or the time-point for the last donation (National Institute for Health and Clinical Excellence 2010). Section 1.35 only states consideration of the number of babies receiving DHM, the DHM stocks, and donor's preferences when deciding how long women can donate BM (National Institute for Health and Clinical Excellence 2010). Therefore, it is not surprising that the HMBs had varying cut-off levels. Similarly, tandem feeding and accepting donations from donors with multiple pregnancies is not considered in NICE Clinical Guideline 93. Two HMBs did not accept tandem feeding, due to potential unsuitable BM composition. In relation to the latest starting point or the last time-point for donating, HMBs were mostly concerned about the nutritional quality of DHM, including micronutrient content.

Indeed, zinc and copper concentrations significantly decline during the first four months of lactation (Yalcin et al. 2015). A significant decline in copper and zinc during the first twelve months of lactation was also demonstrated, with significant lower concentrations

during months seven to twelve compared to months four to six (Rajalakshmi and Srikantia 1980). One more study showed significant reduced copper and zinc levels at one year postpartum (Vaughan et al. 1979). All HMBs accepted donations until at least six months after childbirth. Considering this evidence, cut off limits after six months should not be based on concerns for zinc and copper concentrations.

HMBs specifically mentioned concerns about the iron content of BM. Iron content was 20% greater at the beginning of lactation compared to one year postpartum (Vaughan et al. 1979). Another study found that BM iron levels decline by 47% during the first five months of lactation (Siimes et al. 1979). Iron concentrations are especially of concern when feeding preterm infants. They often have iron deficiencies due to prematurely interrupted placental iron transfer (Scott et al. 1975). Iron status in preterm infants is linked to their neuro-behavioural status, with lower levels correlating with poorer neuro-behavioural development (Armony-Sivan et al. 2004). Iron plays a role in neurodevelopment, more specifically in myelination, monoamine metabolism, energy metabolism, and dendritic growth in the hippocampus (Georgieff and Innis 2005; Georgieff 2008). Indeed, iron-deficiency at birth results in a poorer mental and psychomotor development of school aged children (Tamura et al. 2002). Iron also has a link to the LCPUFA metabolism, the FA desaturases have an active diiron centre (Shanklin et al. 2009).

HMBs were also especially concerned about the magnesium content of BM. Magnesium levels showed no consistent pattern of change during time of lactation in one study (Vaughan et al. 1979), whereas Rajalakshmi and Srikantia demonstrated a significant decrease in magnesium levels from colostrum to mature milk at one month of lactation, although no further decreases were recorded up to one year postpartum (Rajalakshmi and Srikantia 1980). Therefore, concerns about magnesium levels should not influence the cut off levels for donation.

HMBs did not raise concerns about LCPUFAs, however they should be considered when the DHM is intended for preterm infants, since they are prone to have a LCPUFA gap after birth. The LCPUFA composition of BM changes with the duration of lactation. DHM is mostly donated by mothers of term babies, who have established a sufficient and stable BM supply (Valentine et al. 2010; Valentine et al. 2013). Relative DHA and ARA concentrations have been shown to decline over the first 20 days (Genzel-Boroviczeny et al. 1997), and were significantly lower at three months (Nilsson et al. 2018), four months (Makrides et al. 1995c), and twelve months (Mitoulas et al. 2003) in studies. The absolute DHA and ARA levels also decline significantly within the first six weeks of lactation (De Rooy et al. 2017). However, during the first year of lactation, the

total fat content of BM increases (Bitman et al. 1983b; Mitoulas et al. 2003). The absolute amount of DHA and ARA in 150 mL BM at months 1, 2, 4, 6, 9, 12 of lactation were calculated, using the relative FA composition and absolute fat concentration at these times reported by Mitoulas *et al.* (2003). This shows that a preterm infant (28 weeks gestational age, 1000 g, assuming 150 mL per kg bodyweight per day as full enteral feeding) would receive similar amounts of DHA and ARA from DHM expressed during the first or the twelfth month of lactation (Appendix IV.VIII). However, this calculation is based on the values of five BM samples only. Larger studies are needed to confirm the results. Analysis of BM FA composition and fat content in the second year postpartum should also be performed. Perrin and colleagues have found no changes in fat content in BM samples from eleven to 17 months postpartum and based on this and their other results, they are recommending HMBs accept donations beyond one year postpartum to increase the DHM supply (2016).

The available evidence does not support the need to provide recommendations for the latest starting point or the time-point for the last donation with regards to LCPUFA levels. Other micronutrients need to be considered when informing guidelines, and as mentioned by Perrin *et al.* mineral fortification of DHM may be required (Perrin et al. 2016).

4.5.2 Nutritional status, dietary habits, and LCPUFAs

The NICE Clinical Guideline 93 recommends assuring that potential donors are in good general health (section 1.13) (National Institute for Health and Clinical Excellence 2010), which is not further defined. One HMB reported that dietary habits are discussed as part of the general health questions. Since maternal nutrition during lactation is not included within the remit of the NICE Clinical Guideline 93, there was wide variability in HMBs consideration of donors' diet. Five HMBs did not assess donor's dietary habits, whereas two HMBs excluded donors if they follow a restricted diet, although this is not specified in the exclusion criteria in the NICE Clinical Guideline 93 section 1.12 (National Institute for Health and Clinical Excellence 2010).

It is established that nutrition has an impact on LCPUFA levels and especially DHA levels of BM (Cherian and Sim 1996; Yuhas et al. 2006). Hence, it seems obvious that dietary habits of donors should be assessed. Swedish HMBs asses dietary habits and do not accept BM from donors who eliminate important staple foods (Polberger et al. 2016), although these are not further specified. The Swedish guideline also recommends that vegetarians should take food supplements to ensure a balanced diet. In the U.K., only one HMB excluded vegans who do not take vitamin B12 supplements. Vegan diets do not contain any natural vitamin B12, hence, their BM vitamin B12 levels

are lower (Specker et al. 1990), leading to vitamin B12 deficiency in infants. This can lead amongst others to irritability, failure to thrive, and developmental regression (Dror and Allen 2008). Therefore, the European Milk Bank Association recommends exclusion of donors that follow a vegan diet without supplementing vitamin B12 (Weaver et al. 2019).

Similarly, vegans and vegetarians also have significantly lower DHA levels in BM than omnivores (Sanders and Reddy 1992), whereas ARA BM levels are comparable to omnivores, although vegetarian and vegan diets provide little or no ARA (Sanders and Reddy 1992; Sanders 2009). This should especially be considered when DHM is intended for feeding preterm infants, due to the LCPUFA gap of prematurity.

BM LCPUFA levels are not only influenced by their respective dietary intake, but also by the levels of precursor fatty acids in the diet, which can influence the precursor conversion to LCPUFAs (Nakamura and Nara 2004), and therefore, the LCPUFA BM levels. High PUFA and especially LA intake can reduce DHA levels (Gibson et al. 2013). Hence, it is also important to consider C18 PUFA intake in donors. LA intake has significantly increased over the last decades. For example, in 1980, LA intake accounted for about 3% of the total energy intake, while this increased to 5% by 1992 (Sanders 2000). The increased LA intake has also been reflected in increased LA and decreased DHA levels in BM in the last decades (Crawford et al. 1976a; Yuhas et al. 2006). Increased use of vegetable fats with high LA content, e.g. sunflower and corn oils, and a decrease of animal fats are a main reason for this increase (Sanders 2000). The International Society for the Study of Fatty Acids and Lipids considers an LA intake of 2% of total energy intake as adequate (ISSFAL 2004). The high intake of LA is also reflected in the currently high n-6 to n-3 ration in Western diets (16:1) (Simopoulos 2002). In contrast to this, the hunter-gatherer diet in the Paleolithic period had a ratio of about 1:1 (Kuipers et al. 2010), while nowadays, a ratio of 5:1 is often recommended. **Recommendation 1:** Assess dietary habits of donors to ensure high nutritional quality of DHM.

The use of fish oil or DHA supplements can be useful to increase BM DHA levels in donors that eliminate fish from their diet, as well as in vegetarian and vegan donors. Although vegetarian diets can be high in ALA, preformed DHA is a better source for brain accretion (Su et al. 1999) and should therefore be provided to preterm infants. DHA supplementation of lactating mothers has been shown to dose-dependently increase BM DHA levels (Makrides et al. 1996), whereas flaxseed oil (high in ALA) supplementation does not increase DHA BM levels (Francois et al. 2003). Smithers and colleagues conclude from the DINO trial that BM exceeding 1% DHA may be

needed to increase the DHA status of preterm infants to levels of term infants (2008). Colostrum DHA levels of 1.15% were achieved after supplementing mothers with 2.2 g DHA from 20 weeks gestation until delivery (Dunstan et al. 2007). No adverse outcomes were reported using this dose.

Recommendation 2: Inform donors with restricted diets and/or no or low fish intake about the potential of DHA supplements to increase their BM DHA status and the likely benefits of this.

Changing donor's dietary habits could not only benefit the nutritional quality of the DHM but could also benefit the donor's own baby. Although ten HMBs reported having registered dietitians available for consultation, uptake of this service was not assessed by this survey. General nutritional advice for lactating women is not available to most HMBs. However, regarding fish and therefore DHA intake, the Scientific Advisory Committee on Nutrition's "Advice on fish consumption" is accessible, which states that lactating women should consume at least two portions of fish per week including one portion of oily fish, with restrictions on high methylmercury containing fish (marlin, swordfish, shark, and tuna) (Scientific Advisory Committee on Nutrition 2004). Methylmercury is a neurotoxin that can lead to neurological and developmental deficits (Farina et al. 2011). Nevertheless, several studies, summarised in a systematic review, show that there are no net adverse effects of seafood consumption on neurocognitive development, even in the highest intake group, consuming more than three portions of seafood (>340 g) per week (Hibbeln et al. 2019). Overall, the nutrients in seafood, including DHA, lead to a beneficial effect of seafood consumption on neurocognitive development, besides the presence of methylmercury (Spiller et al. 2019). Furthermore, results from the ALSPAC (Avon Longitudinal Study of Parents and Children) study indicate that children of women who consumed less than 340 g of seafood per week during pregnancy had an increased risk of being in the lowest quartile for verbal intelligence quotient (OR 1.09, 1-340 g vs. >340 g) (Hibbeln et al. 2007). This indicates that the current recommendations to limit seafood intake could even be detrimental (Hibbeln et al. 2007), and women should be encouraged to eat seafood/fish. Despite this, in the U.K., fish consumption recommendations are not met. The National Diet and Nutrition Survey defined a portion as 140 g of fish, however, the average intake of oily fish of women (19-64 years) was only 8 g per day or 56 g per week in 2012-2014 (Public Health England 2016). Furthermore, data from BM donors in the U.S.A. suggest that their dietary DHA intake is significantly less than recommended (Valentine et al. 2013). Therefore, fish intake could be improved in the U.K. to increase BM DHA, potentially by providing donors with this nutritional advice. Gibson and colleagues also recommend a blend of sunola oil with small amounts of

linseed and canola oil for cooking, resulting in a FA composition of 5% PUFAs with an LA to ALA ratio of around 2:1, which would be ideal to improve the n-3 status (Gibson et al. 2011), and therefore, most likely also the DHA content in BM.

Providing donors specifically with intake recommendations for lactating mothers might increase their BM DHA levels as demonstrated in a study by Juber *et al*. In this study, lactating mothers in the U.S.A. were informed about their BM DHA levels and provided with information on the recommended DHA intake for them (Juber et al. 2016). Within one month, the DHA BM levels increased significantly from 0.19% to 0.22% of total FA. Obviously, providing nutritional advice can only have an impact prospectively. Results from this questionnaire indicate that the majority of DHM in the U.K. is donated prospectively and not from pre-collected BM. Therefore, providing nutritional information to donors could be successful in the U.K.

Recommendation 3: Provide nutritional information to donors, especially the SACN recommendation of one portion (140 g) of oily fish per week and improve access to nutritional advice for lactating mothers.

4.5.3 Storage of DHM

Storage containers
Section 1.41 recommends that only BM collected in HMB provided or approved bottles can be accepted for donation (National Institute for Health and Clinical Excellence 2010). In two HMBs, mothers were able to donate BM, providing their own containers; however, this does not violate the guideline if the HMBs approve of the bottles. In all but one HMB, the bottles used for BM collection were clear. Chang and colleagues found that BM storage in light brown coloured polyether sulfone bottles led to the least fat loss compared to storage in other plastic or glass containers (1.2% less fat loss than in polyethylene bags) (2012). Similarly, BM storage in amber glass bottles for 4 months at -18 °C inhibits hexanal formation, and therefore lipid peroxidation, significantly (Vangnai et al. 2017). Protecting bottles from light exposure during collection, for example by wrapping collection bottles in foil, resulted in a 13% greater riboflavin (vitamin B2) content of BM (Bates et al. 1985). Since most HMBs provide the collection bottles, it would be a simple process to implement the use of different bottles.

Recommendation 4: Use light protective, e.g. amber coloured, bottles, or wrap bottles in aluminium foil, when collecting and storing DHM, to improve the nutritional quality.

Storage in the refrigerator
Section 1.38 recommends freezing expressed BM as soon as possible and allows pooling of BM from one donor over a 24 hour period in the refrigerator (National

Institute for Health and Clinical Excellence 2010). All HMBs complied with the guideline in this point.

Storage of BM at 4°C for up to 96 hours has been shown not to change the total lipid content (Slutzah et al. 2010). Similarly, LCPUFA content is not significantly altered when BM is stored for up to 96 hours at 4°C (Romeu-Nadal et al. 2008b). However, refrigerated storage for up to 24 hours increases free FAs significantly (Dill et al. 1983; Lavine and Clark 1987; Bertino et al. 2013). The highest increase was noted for n-3 PUFAs (Bertino et al. 2013). Free FA can bind to other components of BM, e.g. proteins or calcium, thereby decreasing the LCPUFA absorption (Dill et al. 1984; Lavine and Clark 1987). Free FAs in BM show cytotoxic activity, and high concentrations can lead to cytolysis of erythrocytes (Ogundele 1999), and leukocytes (Sakaguchi et al. 1995). Free FAs, and especially LCPUFAs, are prone to lipid peroxidation (Cosgrove et al. 1987; Silvestre et al. 2010). Indeed, storage at 4°C for 48 hours significantly increased MDA levels in mainly term BM (Miranda et al. 2004). However, similar storage did not increase MDA in preterm BM (Bertino et al. 2013), potentially due to higher antioxidant capacity (Turhan et al. 2011). LPPs can bind to DNA and proteins, potentially leading to cell and tissue damage and thereby increase inflammation (Yadav and Ramana 2013).There is vast evidence that refrigeration also decreases vitamin C levels (Buss et al. 2001; Rechtman et al. 2006; Romeu-Nadal et al. 2008b), whereas it does not have an effect on α- and γ-tocopherol levels (Moffatt et al. 1987; Romeu-Nadal et al. 2008b).

A recent publication from the United States recommended best practice for handling and storing DHM (Steele 2018). However, this review only summarised some current published guidelines, and recommended that fresh BM can be stored for 48 hours in the refrigerator. In Italy, BM for donation can be stored in the refrigerator at 4°C for up to 24 hours (Arslanoglu et al. 2010). If the donor's refrigerator has no temperature monitoring system, the BM needs to be transferred to the freezer within 12 hours. The Australian milk banking guideline recommends storing expressed BM immediately in the coldest part of the freezer, which is specified as in the lower part towards the rear of the freezer (Hartmann et al. 2007). The NICE guideline does not specify where BM should be stored in the refrigerator (National Institute for Health and Clinical Excellence 2010), and of the ten HMBs which allow pooling of BM in the refrigerator, only eight instruct their donors to store BM on a shelf in the refrigerator.

Recommendation 5: Freeze DHM immediately in a domestic freezer (≤ -18°C) after expression and do not allow pooling in the refrigerator over a 24 hour period.

One main reason for pooling DHM is to minimise the space used in the freezer. Providing donors with different sized containers could help to reduce the space used for DHM storage. The Italian milk banking guideline also specifies that the container should be stored in the coldest area of the refrigerator, distant from the door (Arslanoglu et al. 2010).

Recommendation 6: If pooling in the refrigerator for up to 24 hours is allowed, instruct donors to store BM in the coldest part of the refrigerator and not in the door.

Frozen storage

The majority of HMBs allowed frozen (< -18°C) storage of DHM at donors' homes for up to three months, which is compliant with section 1.40 (National Institute for Health and Clinical Excellence 2010). However, the same section also states that frozen BM should be transported to the HMB as soon as possible. Therefore, having wide variations regarding the minimum amount of DHM that needs to be collected before the first donation in nine HMBs disagrees with the guideline.

Data on the effects of frozen storage on the fat content and LCPUFA levels in DHM are inconclusive. Several researchers found that storage at -20°C for nine months does not affect total fat content (Ahrabi et al. 2016), and the absolute FA and relative FA content was not significantly altered in studies from 30 days storage (Lacomba et al. 2012) up to twelve months (Romeu-Nadal et al. 2008b). Storage at -18°C for 28 days does not change total triacylglycerols (Tacken et al. 2009). However, storage at -20°C leads to a significant loss of total fat after 48 hours (Chang et al. 2012), a significant decrease in total lipid concentrations after eight days (Pardou et al. 1994), a significant loss of total fat after 30 days storage (Thatrimontrichai et al. 2012) and resulted in a 10% decrease in triacylglycerols after five months (Berkow et al. 1984). Garcia-Lara and colleagues also found a significant reduction in fat content after seven days and up to 90 days after BM storage at -20°C, whereas the biggest decrease occurred during the first week (-0.027 g/dL/day) (2012). The variation in findings might be explained by the different analytical methods used (Silvestre et al. 2014). Additionally, BM might have not been homogenised sufficiently before analysis, BM storage in the fridge or on the bench for thawing after being frozen results in a rise of milk fat globules to the top, where they form a cream layer (Jensen and Jensen 1992). Other reasons might include differences in fat adherence to container walls (Chang et al. 2012; Lev et al. 2014), or differences in thawing methods which can influence fat content (Thatrimontrichai et al. 2012).

BM contains the bile salt-dependent lipase, which aids in the digestion of milk fat and compensates for the immature digestive system in new-borns (Hernell 1975; Watkins

1985). However, the bile salt-dependent lipase loses its bile salt-dependency during two weeks frozen storage at -10°C (Mehta et al. 1982), which can lead to lipolysis of triacylglycerols and increase in free FA levels. Longer-chain PUFAs seem to be more susceptible to hydrolysis than shorter-chain FAs and the degree of hydrolysis is temperature and time dependent (Lavine and Clark 1987). Indeed, free FAs in BM increase significantly after eight weeks storage at -11°C (Lavine and Clark 1987). An accumulation of free FAs was also already observed after 24 hours frozen storage (-20°C), further increasing after 180 days (Dill et al. 1983). Other studies also show significant increases in free FA after BM storage at -20°C for two to five months (Bitman et al. 1983a), four months (Morera Pons et al. 1998), or nine months (Ahrabi et al. 2016). Elevated levels of free FAs have the potential to increase lipid peroxidation, (Turoli et al. 2004; Martysiak-Zurowska and Stolyhwo 2006). Indeed, significant increases in MDA levels are seen after 60 days storage at -20°C, but not after 15 or 30 days (Silvestre et al. 2010). Storage at -18°C also increases hexanal significantly in pooled BM after three months storage, with a further significant increase after five and six months (Vangnai et al. 2017). Furthermore, the protective vitamin C content is significantly reduced after storage at -20°C (Bank et al. 1985; Marinkovic et al. 2016). However, storage at -18°C is the only option at donors' homes. This might be the reason why in Brazil, DHM storage in a freezer is allowed for no more than five days prior to processing (Gutierrez and de Almeida 1998). In Italy, the maximum total storage time for DHM that is intended to feed preterm babies is three months (Arslanoglu et al. 2010). To reduce pre-pasteurisation time, DHM should be transported to the HMB as soon as possible after expression. To facilitate this, ideally HMBs should not restrict the minimum amount of DHM that needs to be collected before the first donation. However, HMBs highlighted setting limits to be cost effective. One HMB mentioned that they are expecting the collection of 3000 mL over the course of donation.

Recommendation 7: Reduce DHM pre-pasteurisation storage time at -20°C to a minimum. Issues regarding cost effectiveness can be addressed by setting a minimum volume of DHM for the course of donation instead of a minimum for the first donation.

Storing DHM awaiting pasteurisation at -20 °C for a maximum of three months after the date of expression is recommended in section 1.53 (National Institute for Health and Clinical Excellence 2010). Based on the answers from three HMBs, DHM is normally stored for up to one month at the HMB. As described previously, the pre-pasteurisation storage time should be minimised. BM storage at -80°C for five or 12 months respectively, does not affect saturated or monounsaturated FAs, or PUFAs (Romeu-Nadal et al. 2008b) or triacylglycerols (Berkow et al. 1984). Another study found a

significant fat decrease after 44 days of storage at -80°C (Lev et al. 2014), although no comparison was made to storage at -20°C, so it is not known which storage condition would be better. BM storage at -80°C also prevented the formation of free FAs in several studies storing BM for up to five months (Bitman et al. 1983a; Lavine and Clark 1987; Morera Pons et al. 1998). Although it is not achievable to store unpasteurised DHM at -80°C at donors' homes, this would be feasible at HMBs.

Section 1.62 specifies storing frozen (-20°C) DHM for a maximum of six months after the date of expression (National Institute for Health and Clinical Excellence 2010). Frozen storage for three months at -20°C post-pasteurisation has no effect on total fat content (Lepri et al. 1997). Storage of pasteurised BM for one month at -25°C (Friend et al. 1983b), or for three months at -20°C (Lepri et al. 1997) resulted in no significant change in free FA content. One explanation for this is the inactivation of the bile salt-dependent lipase and lipoprotein lipase during Holder pasteurisation (Henderson et al. 1998). Therefore, post-pasteurisation storage at -20°C seems to have no negative effect on DHM lipid quantity and quality.

Recommendation 8: Store DHM at -80°C at HMBs, especially pre-pasteurisation.

Light protection

HMBs also prefer to have glass door freezers, which limit the opening time of the freezer (personal communication, Dr Natalie Shenker, Co-Founder Hearts Milk Bank, 31 January 2018). However, these freezers increase the exposure of DHM to room light and potentially sunlight. Similarly, DHM awaiting pooling or other handling at the HMB, is exposed to room light or potentially sunlight. Some components of DHM are light sensitive. Light exposure (five hours) results in a significant decrease of vitamins B6 and C in BM (Van Zoeren-Grobben et al. 1987). Bates and colleagues also found an approximately 40% decrease in riboflavin (vitamin B2) and an approximately 50% decrease in vitamin A content in BM after three hours of daylight exposure (1985). Phototherapy light decreases total antioxidant capacity (TAC) in BM (Unal et al. 2019). The effect of light exposure on LCPUFA levels in BM has not been described yet. Tacken *et al.* found no difference in triacylglycerol levels when BM was protected from light versus unprotected during tube feeding for six hours (Tacken et al. 2009). Another study also found no difference in LA hydroperoxides after six hours of light exposure (Van Zoeren-Grobben et al. 1993). Since LCPUFAs are more prone to lipid peroxidation than shorter chain PUFAs (Cosgrove et al. 1987), it would have been interesting to see the effects on other and longer-chain PUFAs. The effect of light exposure has been investigated on parenteral lipid emulsions. Total peroxides increased by approximately 500 µM after six hours exposure of Intralipid, an

intravenous fat emulsion, to ambient light (Silvers et al. 2001). Light protective tubing helped to reduce total peroxides, although it was not as effective as wrapping the tubing in foil. The Swedish human milk banking guideline explicitly recommends avoiding light exposure of BM outside of fridges and freezers, since light can impact on the nutritional quality of DHM (Polberger et al. 2016). The guideline recommends covering DHM with a towel. Similarly, the new European Milk Bank Association consensus statement recommends that HMBs should minimize the exposure of DHM to sunlight, as well as phototherapy lights, at all times (Weaver et al. 2019).

Recommendation 9: Protect DHM from light exposure in the freezers, on the bench, and during feeding.

4.5.4   Transportation of DHM

Five HMBs had no time-limit for DHM transportation, although section 1.44 of the NICE guideline states that critical transport conditions, including temperature- and time-limits, need to be defined by the individual HMBs (National Institute for Health and Clinical Excellence 2010). Varying transportation equipment, as well as different transportation modes might have resulted in the different time-limits applied by the different HMBs. The Human Milk Banking Association of North America further recommends transporting DHM on dry ice when mothers live out of town (Jones 2011; O'Hare et al. 2013). More research is needed to explore appropriate time-limits, based on transportation time, temperature and equipment.

Recommendation 10: There should be clear evidence based time-limits for transportation of DHM, considering outside temperature, mode of transportation, and transportation equipment, such as cool boxes.

4.5.5   Processing of DHM

Section 1.58 of the NICE guideline describes that a sample of each batch of pooled DHM should be tested for microbial contamination before pasteurisation (National Institute for Health and Clinical Excellence 2010). It is also recommended that DHM should be thoroughly thawed before testing and that it can be kept in the refrigerator for maximum 24 hours (section 1.55). Twelve HMBs followed the normal protocol of thawing the DHM, taking a sample for microbiological assessment and then pasteurising. One HMB however, described storing the bottle in the refrigerator for 24 hours after taking the sample and before pasteurisation and another described refreezing the DHM for 48 hours before pasteurisation, after taking a sample for microbiological assessment, which is a deviation of the NCIE Clinical Guideline 93. No reason was provided for these practices. Probably, HMBs are waiting for

microbiological clearance before pasteurisation to avoid unnecessary pasteurisation and to save costs and time. As described above, BM storage at 4°C does not seem to affect the total fat content or LCPUFA content, but it increases free FAs and LPPs (Miranda et al. 2004; Bertino et al. 2013). Therefore, storage time at 4°C should be kept to a minimum. Furthermore, freezing-and thawing can disrupt the milk fat globule membrane and further increase free FA concentrations (Morera Pons et al. 1998). Repeated freezing and thawing has also been shown to increase triacylglycerol losses (Berkow et al. 1984). In Sweden, the sample for microbiological testing is obtained by using a sterile device to scrape a sample from frozen DHM (Polberger et al. 2016).

Recommendation 11: Directly pasteurise DHM after taking a sample for microbiological testing to avoid repeated freeze-thaw cycles or prolonged storage at 4°C. If direct pasteurisation after sampling is not possible or not cost effective, a frozen DHM sample can be tested.

Pasteurisation

BM is a biological specimen also containing bacteria and viruses. Therefore, it is necessary to heat treat DHM to make it safe for the use in preterm or ill infants. Most, if not all, international milk banking guidelines recommend Holder pasteurisation (62.5°C, 30 minutes) (Hartmann et al. 2007; Arslanoglu et al. 2010; National Institute for Health and Clinical Excellence 2010; Jones 2011; Polberger et al. 2016). All HMBs in the U.K. pasteurise DHM. Holder pasteurisation is effective in destroying human immunodeficiency virus and cytomegalovirus (Orloff et al. 1993; Hamprecht et al. 2004). It can also destroy bacteria such as Staphylococcus, Enterococci, and Streptococcus (Landers and Updegrove 2010). However, pasteurisation also affects the anti-microbial properties of BM (Van Gysel et al. 2012). Furthermore, it reduces amongst others, vitamins (vitamin C and B6), immunoglobulin G4, cytokines (interleukin-7, macrophage inflammatory protein-1β, monocyte chemoattractant protein-1), and hormones (insulin and adiponectin) significantly (Peila et al. 2016). The effect of Holder pasteurisation on total fat content of BM is inconclusive, with studies reporting a significant decrease (Ley et al. 2011; Vieira et al. 2011; Garcia-Lara et al. 2013), or no change in fat content (Lepri et al. 1997; Fidler et al. 2001; Goes et al. 2002). Again, insufficient homogenisation or differences in adherence to container walls might be the reason for varying results (Jensen and Jensen 1992; Chang et al. 2012; Lev et al. 2014). Holder pasteurisation also affects the free FA concentration, which doubled after pasteurisation (Lepri et al. 1997). Nevertheless, Holder pasteurisation does not affect LCPUFA content (%weight) of BM (Henderson et al. 1998; Fidler et al. 2001; Romeu-Nadal et al. 2008a; Valentine et al. 2010; Moltó-Puigmartí et al. 2011; Baack et al. 2012; Delgado et al. 2014). Yet, Holder

pasteurisation decreases total fat absorption in infants by 17% (Andersson et al. 2007), most likely due to inactivation of the bile salt-dependent lipase and lipoprotein lipase (Henderson et al. 1998), which further limits the LCPUFA intake of preterm infants. Holder pasteurisation has no effect on MDA concentration of mature BM (Silvestre et al. 2008). However, the samples were immediately pasteurised after sampling, which is different from the milk banking setting, and no storage that could have increased the more susceptible free FAs had occurred. Similar, no increase in TBARS was found after pasteurisation of BM stored for 3 weeks at -80°C (Martysiak-Żurowska et al. 2017). Therefore, more research is needed to investigate the effects of Holder pasteurisation in a milk banking setting.

Currently research is focusing on processing techniques other than Holder pasteurisation to ensure not only safety of DHM, but also better preservation of nutritional, immunological and functional components (Peila et al. 2017). These techniques include high-temperature-short-time pasteurisation as well as the non-thermal methods of high pressure processing, ultraviolet irradiation, and ultrasonic processing. High pressure processing for example has shown to be superior to Holder pasteurisation in terms of Immunoglobulin G, adiponectin, growth factor and lactoferrin preservation (Wesolowska et al. 2018), however, Delgardo and colleagues found a significant decrease in DHA and ARA weight% after high pressure processing at 400 or 600 MPa for three or six minutes (2014). On the other hand, Moltó-Puigmartí and colleagues found no differences in LCPUFA weight% after high pressure processing at 400, 500 or 600 MPa for five minutes (2011). Results from cow's milk processing show no difference in ARA weight% after high-temperature-short-time (85°C 30 s) processing, whereas DHA levels were not measured (Rodriguez-Alcala et al. 2014). The effects on LCPUFA content of DHM following such methods should be assessed in future studies.

4.5.6 Areas for improvement

Nine HMBs suggested barriers to optimal practice and improvements among HMB practice. The predominant theme was lack of resources. In general, resources in the NHS are scarce (Howard 2010). Similarly to the HMBs, the Toolkit for High Quality Neonatal Services defines staffing, as well as professional competence, education, and training as key elements to deliver high quality neonatal services (Howard 2010). Understaffing is an overall problem in neonatal units in the NHS (Cole 2009; Pillay et al. 2012).

The international non-profit global health organisation PATH has defined four key principles for the establishment of effective HMBs. The first principle is a strong base or

foundation, which includes staff, appropriate space and facilities, equipment, as well as staff training (DeMarchis et al. 2017). Without these, HMBs cannot function to a high quality, safety, and ethical standard, and reach their full potential, as the base will link to the other three key principles of awareness and advocacy for breast feeding, networking, and developing key protocols. The potential for HMBs to have an impact is demonstrated by Brazil, which has the largest human milk banking system in the world. It is estimated that the provision of DHM to preterm and low birth weight babies saved the Brazilian Ministry of Health approximately $540 million in one year (Arnold 2006). This highlights the potential for benefit if the NHS invests more in HMBs. Some investments are relatively low cost and easy to implement e.g. a dedicated phone line or more computers as requested by HMBs in the U.K.

Improvements in care lead to increased survival of extremely preterm infants (Glass et al. 2015), and this will continue in the future. The need for DHM will therefore increase as mothers are less likely to be able to express BM for their extremely preterm infants (Jones and Spencer 2005). For vulnerable preterm infants Arnold declares DHM as human right (2006).

4.5.7 Data collection

This survey provided a first step to ascertain human milk banking practices in the U.K. and provides a highly representative summary of the current practices. The survey was sent out to all 15 HMBs in the U.K. Although this is in general a small number of questionnaires, data collection for this study took 15 months. A vast portion of the time was attributed to the ethical approval process and the Research and Development Office approval in the hospitals. Obtaining ethical approval for low-risk health service research in the U.K. can be bureaucratic and lengthy, as also noted by other researchers (van Teijlingen et al. 2008). Despite the fact that the Health Research Authority determined that participating NHS organisations in England are not expected to formally confirm capacity and capability, almost all Research and Development Offices wanted to assess the study and confirm capacity and capability. This took up to six months in several NHS Trusts. Delays in study set up at NHS Trusts are not unusual (Al-Shahi Salman et al. 2007).

However, waiting for participant responses also took more time than expected. Only three HMBs responded within the set three weeks' timeframe, and two HMBs answered within a week after the deadline. The average response time was 70 days. One HMB took nine months to respond. Lack of time with not enough dedicated time for the HMB operation was one of the identified sub-themes in the qualitative analysis. Therefore, the delayed responses are not surprising. Even though the period of data

collection was protracted, no major changes in practices are anticipated as the NICE Clinical Guideline 93 was not due to be updated before 2019 (National Institute for Health and Clinical Excellence 2010).

An overall response rate of 93% was obtained (14/15 HMBs). Several studies report that response rates for surveys completed by healthcare professionals are considerably lower (38% for online questionnaires (Cho et al. 2013), 60% for electronic surveys (Cook et al. 2009)). Having sent regular reminders might have helped to facilitate responses (Cook et al. 2009). Furthermore, using a personalised invitation letter and the statement that UKAMB was supporting the research might have further increased the response rate (VanGeest et al. 2007). Hence the collected data is overall a highly representative summary of the human milk banking practices in the U.K. at the time of data collection.

4.6 Conclusion

The current human milk banking practices in the U.K. were ascertained using an electronic questionnaire. A very high response rate (93%, 14/15 HMBs) was achieved, and great variations in all aspects of human milk banking, including donor selection and nutrition, as well as DHM storage, transportation, and processing were detected amongst the HMBs. Predominantly, the practices are within the scope of the NICE Clinical Guideline 93. The only deviation was extended cold storage of DHM after thawing before pasteurisation. Several practices that might influence DHM quality, such as inquiring about nutritional habits of donors, or time-limits for transportation, are not included in the Clinical Guideline 93. Additionally, the guideline is not specific enough, e.g. it advices immediate freezing of expressed BM, but also allows pooling BM from one donor over 24 hours in the refrigerator. This may contribute to the variations in practices found with this study. These results clearly indicate that the guidance needs to be more specific, to ensure that HMBs within the U.K. work towards the same standards and implement them effectively. This will ensure that future research can effectively address remaining questions such as clarifying the effects of human milk banking practices on the nutritional components of DHM, which is one of the aspects raised by the authors of the NICE Clinical Guideline 93.

Some of the identified practices, such as BM refrigeration, prolonged storage at -20°C, or light exposure, have the potential to decrease LCPUFA quantity and quality in DHM. This is especially of concern for preterm infants, who present with a LCPUFA gap of prematurity. The first 1000 days of life, from conception up to two years, are crucial for infants' development and health (Agosti et al. 2017) and environmental factors, such as nutrition can have life-long consequences on infants' morbidity and mortality. For example, good nutrition can reduce the risk for later malnutrition, and for chronic and non-communicable diseases such as heart diseases, cancer, and type 2 diabetes. Therefore, as a result of this study, recommendations for best human milk banking practice with focus on LCPUFAs were developed, based on available evidence, and under consideration of international human milk banking guidelines. The recommendations are summarised in Table 4-5. Some of these recommendations only require small changes in practice to potentially make a big difference in maintaining or protecting the LCPUFA content, as well as other nutritional components (e.g. vitamin C) of DHM, which will ultimately benefit the mostly preterm recipients and might help to reduce the potentially negative effects of the LCPUFA gap of prematurity.

Most studies investigating the effect of storage and processing conditions on the quality of DHM have only mimicked the HMB setting and investigated an isolated process. However, it might be the interplay of the various milk banking practices, e.g. the rise of

free FA concentrations after storage at 4°C in combination with the heat of Holder pasteurisation, that leads to a decreased nutritional quality of DHM. It is therefore extremely important that future research is conducted using actual DHM samples to ascertain the nutritional quality and the LCPUFA and LPP levels at bedside in a hospital setting. This will provide a realistic insight into the impact of current human milk banking practices. Overall, as also mentioned in the NICE Clinical Guideline 93, more research is needed to understand the effects of human milk banking practices on the quality of DHM, to provide further evidence-based guidance and improve practice and outcomes.

Table 4-5: Best practice recommendations to provide DHM with high LCPUFA levels

Recommendations
• Assess dietary habits of donors to ensure high nutritional quality of DHM
• Inform donors with restricted diets and/or no or low fish intake about the potential of DHA supplements to increase their BM DHA status and the likely benefits of this
• Provide nutritional information to donors, especially the SACN recommendation of one portion (140 g) of oily fish per week and improve access to nutritional advice for lactating mothers
• Use light protective, e.g. amber coloured, bottles, or wrap bottles in aluminium foil, when collecting and storing DHM
• Freeze DHM immediately in a domestic freezer (≤ -18°C) after expression and do not allow pooling in the refrigerator over a 24 hour period
• If pooling in the refrigerator for up to 24 hours is allowed, instruct donors to store BM in the coldest part of the refrigerator and not in the door
• Reduce DHM pre-pasteurisation storage time at -20°C to a minimum. Issues regarding cost effectiveness can be addressed by setting a minimum volume of DHM for the course of donation instead of a minimum for the first donation
• Store DHM at -80°C at HMBs, especially pre-pasteurisation
• Protect DHM from light exposure in the freezers, on the bench, and during feeding
• There should be clear evidence based time-limits for transportation of DHM, considering outside temperature, mode of transportation, and transportation equipment, such as cool boxes
• Directly pasteurise DHM after taking a sample for microbiological testing to avoid repeated freeze-thaw cycles or prolonged storage at 4°C. If direct pasteurisation after sampling is not possible or not cost effective, a frozen DHM sample can be tested

5   The effects of storage conditions on LCPUFAs, lipid mediators, and antioxidants in DHM – A review

Many of the processes involved in human milk banking can influence the nutritional quality and quantity of DHM. However, although this area has been widely investigated, studies have yielded conflicting results. Moreover, at the time of writing, no review had been published summarising the effects of human milk banking practices, including storage and processing, on the nutritional quality and quantity of DHM. Therefore, a narrative review was undertaken, investigating the effects of storage conditions on the fat and LCPUFA content of DHM. Human milk banking practices can also lead to lipolysis; therefore, the effect on free FAs was also reviewed. Since the lipid fraction of DHM comprises not only of triacylglycerols, but also lipid mediators such as eicosanoids, SPMs, and endocannabinoids, these were also included in the review. Antioxidants play a role in the protection of LCPUFAs from lipid peroxidation, and preterm infants also need enteral antioxidants since they have inadequate antioxidant capacity (Choe and Min 2009; Davis and Auten 2010). Whilst this manuscript was under review, Gao and colleagues published a systematic review on a similar topic (2019), highlighting some key issues in this area of study. Our review differs from the one by Gao and colleagues as it not only considers the fat content, but also lipid peroxidation, bioactive lipid mediators, and antioxidants. Thus, it provides for the first time an overall perspective of factors that affect DHM lipid quality.

Gao and colleague's review concluded that the fat content of expressed BM is not influenced by storage conditions and that the variations found are due to insufficient homogenisation or methodological variation, since most changes were less than 10%, or not significant (Gao et al. 2019). Indeed, our review also found discordant information on the effect of frozen storage on fat content (Nessel et al. 2019). As further detailed in the review, insufficient homogenisation and fat adherence to container walls could influence the results. However, seven studies were identified (three more than by Gao and colleagues) with three studies reporting effects ≥ 10%, which indicates an effect of storage at -20°C on fat content of DHM. Furthermore, Gao and colleagues themselves described in their review that a 20% fat loss results in a 10% energy loss for a 2 kg preterm infant receiving full enteral feeding (150 mL/kg/day) (2019), which may have significant deleterious effects on the infant, and should be considered.

The following section is the narrative review, published in the peer-reviewed journal *Prostaglandins, Leukotrienes and Essential Fatty Acids* (doi: 10.1016/j.plefa.2019.07.

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009) (Nessel et al. 2019). An author contribution statement can be found in Appendix V.I. Heading, figure, and table numbers have been adapted for this thesis.

5.1 Introduction

Mother's own BM is the accepted best practice for feeding neonates (ESPGHAN Committee on Nutrition et al. 2009) and exclusive breast feeding for the first six months of life is recommended (WHO 2003) for term infants. For preterm infants as well, mother's own BM is the favoured feeding choice. However, it may need to be fortified to accommodate the preterm infant's requirements (Agostoni et al. 2010). Producing an inadequate milk supply is nearly three times more likely in preterm mothers than in term mothers (Hill et al. 2005). Underlying reasons can be physiological, such as incomplete development of the mammary glands, or poor hormonal response, as well as psychological (Jones and Spencer 2005). In some cases, maternal BM might not be appropriate, due to illness or medication. In these instances, donor human milk (DHM) from a human milk bank is the best alternative (WHO 2003; American Academy of Pediatrics 2012; Arslanoglu et al. 2013a). Although, at least in the U.K., there are no clear guidelines regulating the use of DHM to a specific preterm gestation, most clinicians agree that extremely preterm infants (born at less than 28 weeks gestational age) should receive DHM (British Association of Perinatal Medicine 2016b). Similarly the Human Milk Banking Association of North America recommends the use of DHM for preterm infants or infants with a birth weight of less than 1750 g (Jones 2011). The American Academy of Pediatrics recommends use of DHM for all preterm infants, especially those weighing <1500 g, when mother's own milk is not available or sufficient (AAP Committee on Nutrition et al. 2017).

BM is generally the only food infants receive for the first few months of life. It provides macro- and micronutrients, immunological factors, hormones, enzymes, growths factors, essential fatty acids and other biologically active compounds, essential for the infant's development (Picciano 2001). Adequate dietary nutrient supply is especially important for preterm infants since their maternal nutrient supply has been interrupted prematurely. For example, during the last trimester, the brain weight increases approximately five-times and at the same time around 80% of the brain docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (ARA, 20:4n-6) are accumulated (Clandinin et al. 1980). Preterm birth also deprives the infant of enzymatic and non-enzymatic antioxidants that would have been matured or gained through maternal transfer in the third trimester, respectively (Qanungo and Mukherjea 2000; Georgeson et al. 2002).

DHM undergoes prolonged cold storage, freeze-thaw cycles, and processing, before it is fed to infants, which may negatively affect the BM composition. For example, in the U.K., expressed BM for donation can be stored for up to 24 h at 4°C, before

transferring to a -18°C (or below) freezer for up to three months (National Institute for Health and Clinical Excellence 2010). BM is then thawed, Holder pasteurised (62.5°C, 30 minutes) and refrozen for up to three months. Before feeding, thawed pasteurised DHM can be stored at 4°C for up to 24 h. These conditions have been summarised in Figure 5-1. Similar guidelines are followed widely, including in Australia, North America, Sweden, Italy, Spain, and India (Hartmann et al. 2007; Arslanoglu et al. 2010; Infant and Young Child Feeding Subspecialty Chapter 2013; O'Hare et al. 2013; Polberger et al. 2016; Calvo et al. 2018). Additionally, in Italy, BM undergoing direct pasteurisation after expression, can be stored for up to 72 h at 4°C (Arslanoglu et al. 2010). In Sweden, fresh and pasteurised DHM can be stored for 48 h at 4°C and can be kept for a maximum of two h at room temperature (Polberger et al. 2016). Lack of evidence is one reason for the different practices used for some aspects of human milk banking (Weaver et al. 2019).
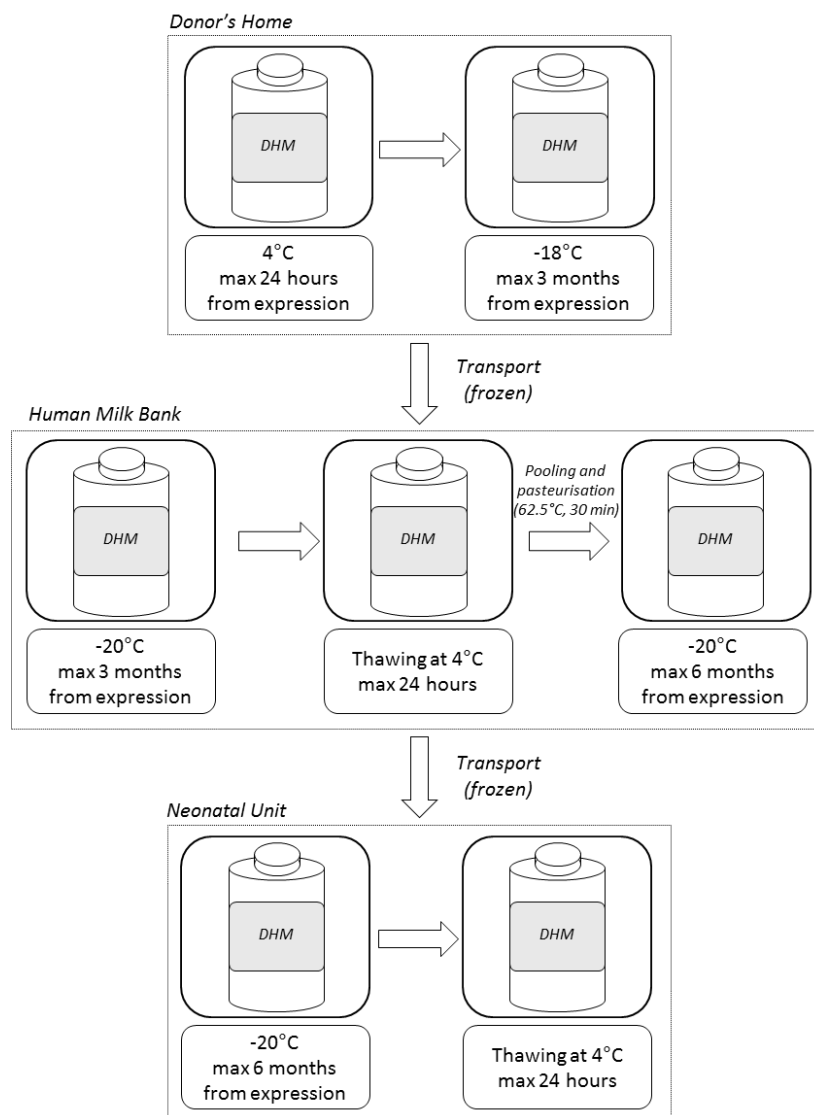
Figure 5-1: Donor human milk storage and processing conditions

Donor human milk (DHM) is exposed to various storage conditions at donors' homes, the human milk bank, and the neonatal unit. This figure displays the storage conditions allowable under the U.K. National

Recommendations for human BM storage conditions were predominantly developed to minimise bacterial growth, rather than to preserve nutritional components (Buss et al. 2001; Romeu-Nadal et al. 2008b). However, with the increasing demand for DHM, and improvements in neonatal care leading to even younger infants surviving, it is now imperative that the nutritional quality of DHM is prioritised. Therefore, this article reviews the effects of current storage and processing conditions on long-chain polyunsaturated fatty acid (LCPUFA) content, bioactive lipid mediators, and antioxidants in human BM. Gao and colleagues recently published a systematic review on the effects of storage, handling and processing on BM fatty acid composition (Gao et al. 2019). This present article compliments and extends these observations by also reviewing lipid peroxidation, bioactive lipid mediators and endogenous antioxidants. The effects of Holder pasteurisation on nutrients have been described elsewhere (Peila et al. 2016), and are not part of this review.

5.2   Search methodology

A search and discovery tool was used to search 80 databases, including Scopus, Web of Science, Medline, and Cinahl, using the following search terms: ((human milk) OR (donor milk) OR (donor human milk) OR (breast milk)) AND ((thaw* OR freeze* OR storage OR processing OR (cold storage) OR (-20°C) OR (-80°C) OR refrigeration) OR stability) AND ((fat OR lipid* OR triacylglycerol* OR triglyceride* OR (fatty acid) OR (long chain polyunsaturated fatty acid*) OR (polyunsaturated fatty acid*) OR (docosahexaenoic acid) OR (arachidonic acid) OR (free fatty acid*) OR lipolysis OR macronutrient* OR eicosanoid* OR leukotriene OR prostaglandin OR thromboxane OR (specialized pro resolving mediator*) OR (specialized pro-resolving mediator*) OR lipoxin* OR resolvin* OR protectin* OR maresin* OR endocannabinoid* OR (arachidonoyl ethanolamide) OR (docosahexaenoyl ethanolamide) OR arachidonylglycerol OR (lipid peroxidation) OR hexanal OR alkenal OR (lipid hydroperoxide*) OR malonyldialdehyde OR TBARS OR MDA OR hydroxynonenal OR hydroxyhexenal OR antioxidant* OR (vitamin C OR ascorbic acid) OR (vitamin E) OR tocopherol* OR (superoxide dismutase) OR catalase OR glutathione OR (glutathione peroxidase) OR (total antioxidant capacity)) OR (antioxidant capacity) OR (antioxidant status)). Furthermore, 'snowballing', searching the reference lists of the identified literature, was used (Greenhalgh and Peacock 2005), as well as searching google scholar. Studies were included when full text was available, the language of the publication was English and the publication date was before April 2019. Studies describing solely the effect of pasteurisation on nutrients were excluded.

5.3 Results

5.3.1 Total fat content and LCPUFAs

BM contains DHA and ARA, LCPUFAs of the omega-3 and omega-6 series, respectively (Brenna et al. 2007). DHA levels in BM are highly variable, ranging from 0.17% to 0.99% of total fatty acids, whereas ARA levels are more constant (0.36% to 0.49% of total fatty acids) (Yuhas et al. 2006). Intrauterine accretion rates for DHA and ARA peak in the last trimester (Lapillonne and Jensen 2009), a time when DHA is also selectively favoured for placental transport to the foetal circulation (Haggarty et al. 1999). This leads to a bio-magnification of LCPUFAs in the foetus, providing it with substrates for the developing brain (Crawford et al. 1976b). In preterm infants, maternal supply has been interrupted prematurely, and they therefore have an elevated requirement for enteral LCPUFA intake. Indeed, preterm infants have significantly lower DHA and ARA blood levels than term infants (Baack et al. 2015). Term infants fed with formula milk devoid of DHA will rapidly exhaust their adipose tissue DHA stores (Farquharson et al. 1993). This is also reflected by significant decreased erythrocyte DHA levels at day five of feeding formula milk devoid of DHA to term infants (Makrides et al. 1995b). Importantly, erythrocyte DHA status has been correlated with brain DHA status (Makrides et al. 1994). Preterm infants have in contrast to term infants very low adipose tissue stores (Clandinin et al. 1981; Lapillonne and Jensen 2009), which makes them even more dependent on adequate enteral LCPUFA intake. Inefficient conversion rates from precursor fatty acids (Uauy et al. 2000; Carnielli et al. 2007), as well as an enteral LCPUFA absorption rate of only 80% (Carnielli et al. 1998), and the prolonged period it may take until full enteral feeding is achieved, further limit the LCPUFA availability for preterm infants. However, it is critical to provide preterm babies with sufficient amounts of LCPUFAs optimal for brain and visual development, as well as cell and immune system function (Lapillonne et al. 2013; Smith and Rouse 2017). Although preterm BM may contain higher DHA levels than term BM, (Bokor et al. 2007), we have previously shown that extremely preterm infants under standard care receive very low levels of DHA and ARA, which are reflected in low blood fatty acid levels (De Rooy et al. 2017). Importantly, DHM is provided generally by mothers of term infants and consequently lower in LCPUFA levels (Valentine et al. 2013). It is therefore imperative that all appropriate steps are taken to maintain LCPUFA levels on the journey from donor to recipient. Since the total fat and LCPUFA content of DHM may be sensitive to human milk banking practices, the following section provides an overview of the literature investigating the effects of different storage conditions on human BM lipids, and is summarised in Table 5.1.

Storing BM at 4°C for 48 h has been shown to not significantly change the absolute or relative fatty acid content (Lacomba et al. 2012), or triacylglycerol content (Tacken et al. 2009). The latter was also not affected by refrigeration at 4°C for up to three days (Yuen et al. 2012). Total lipid content was also unchanged by refrigeration at 4°C for 24 h (Ezz El Din et al. 2004), or up to 96 h (Slutzah et al. 2010). Similarly, polyunsaturated fatty acid (PUFA) content (including linoleic acid (LA, 18:2n-6), α-linolenic acid (ALA, 18:3n-3), ARA and DHA), as well as saturated and monounsaturated fatty acid content was not significantly altered when stored for 96 h at 4°C (Romeu-Nadal et al. 2008b) or 6.8°C (Bertino et al. 2013).

Several studies show that total fat content is not significantly altered following storage at -20°C for nine months (Ahrabi et al. 2016), nor does absolute fatty acid and relative fatty acid content change significantly in studies ranging from storage for 30 days (Lacomba et al. 2012) to 12 months (Romeu-Nadal et al. 2008b). Furthermore, storage at -20°C for 3 days, or -18°C for 28 days does not change total triacylglycerols levels (Tacken et al. 2009; Yuen et al. 2012). Total fat and relative fatty acid levels were unaffected by storage at -25°C for three months, although these samples were refrigerated for up to 48 h before baseline analysis (Friend et al. 1983b). Consistent with these observations, storing BM for one week at -4 to -8°C did not change the fat content (Ezz El Din et al. 2004). However, others have found that storage at -20°C significantly decreases total fat after 48 h (Chang et al. 2012), 30 days (Thatrimontrichai et al. 2012; Janjindamai et al. 2013), and up to 24 weeks (Orbach et al. 2019). Similarly, total lipid concentrations (Pardou et al. 1994), and triacylglycerols (Berkow et al. 1984), significant decrease after eight days, and five months, at -20°C respectively. Significant reductions in fat content after storage at -20°C were also seen after seven days and up to 90 days, with the biggest decreases in the first week (-0.027 g/dL/day) (Garcia-Lara et al. 2012). Freezing at -80°C for five months did not affect saturated or monounsaturated fatty acids, or PUFAs (Romeu-Nadal et al. 2008b), or triacylglycerols for 12 months (Berkow et al. 1984). Although -80°C storage was shown to result in a significant decrease of fat, this was lower than the decreases seen at -20°C (Orbach et al. 2019). In contrast, significant decreases in fat content of 91% were seen after 44 days of BM storage at -80°C, which led to the conclusion of the authors that storage at -80°C should not be the gold standard as recommended by other researchers (Lev et al. 2014). This result is unexpected and since no comparison was undertaken with storage at -20°C, the results should be considered within the context of the wider literature. Discordant observations have also been seen post-pasteurisation, with storage at -20°C for 90 and 180 days resulting in 5.7% and 2.9% decreases in total fat content, respectively (Lepri et al. 1997; Garcia-Lara et al. 2013),

whereas, no differences in total fat content (Friend et al. 1983a; de Waard et al. 2018), or relative fatty acid concentrations (Friend et al. 1983a) of pasteurised BM stored for 1 month at -25°C or up to 12 months at -20°C, respectively were seen.

During human milk banking BM is thawed and then refrozen, which has the potential to affect the milk fat quality. Three-times freezing and thawing has been shown to lead to reductions in fresh BM triacylglycerols of up to 5% (Berkow et al. 1984). Freezing and thawing BM for three-cycles before storing it at -20 °C for five months resulted in an additional 3% triacylglycerol loss (13% in total, compared to 10% after freezing only). Relative amounts of saturated, and monounsaturated fatty acids of BM triacylglycerols did not change significantly after two freeze thaw cycles, whereas the relative LA content decreased by -65% (Wardell et al. 1981). It is noteworthy that thawing in the fridge (4°C) for 24 h, as recommended in the U.K. (National Institute for Health and Clinical Excellence 2010), significantly reduced total fat loss compared to thawing in a water-bath (37°C, 30 minutes) (Thatrimontrichai et al. 2012). Vieira and colleagues found no significant difference in total fat content when pasteurised BM was thawed in a water-bath (40°C, 10 minutes) or thawed in a microwave (45 s) (Vieira et al. 2011). No difference in fat content was found when thawing under tepid water and thawing by a waterless dry heat warmer were compared (Handa et al. 2014). Chang and colleagues also found that storage (-20°C, 48 h) in light brown coloured polyethersulfone bottles resulted in the least fat decreases (Chang et al. 2012).

In conclusion, the available evidence suggests that storage at 4°C is sufficient to minimise decreases in total fat and LCPUFAs in BM for up to 96 h. However, for longer-term storage the data for storage at -20°C is highly discordant, especially pre-pasteurisation. These differences may be related to variations in analytical methods used (Silvestre et al. 2014; Zhu et al. 2017), or methodological variations, such as not sufficiently homogenising the BM after storage (Jensen and Jensen 1992), differences in fat adherences to the container walls (Chang et al. 2012; Lev et al. 2014), particularly to polyethylene (Arnold 1995), and variations in fat loss due to different thawing methods (Thatrimontrichai et al. 2012), which are not specifically defined in the literature. Furthermore, BM is a complex biological matrix, and variations in unmeasured endogenous antioxidant levels or other components, may influence fat content stability during storage, discussed further in Section 5.3.5 below. However, overall the evidence suggests storage at -80°C is the best option for longer-term storage to maintain total fat and LCPUFA levels.

Table 5-1: Summary of studies investigating the effects of storage conditions on total fat and LCPUFA content

BM samples, storage temperature and duration		Study outcome
(Lacomba et al. 2012)	Fresh 4°C for 48 h, or -20°C for 30 days	No significant differences in absolute or relative fatty acid content between fresh, refrigerated, or frozen BM
(Tacken et al. 2009)	Fresh 4°C for 48 h, or -18°C for 28 days	No significant differences in triacylglycerol content between fresh, refrigerated, or frozen BM
(Yuen et al. 2012)	Fresh 4°C for 72 h, or -20°C for 72 h	No significant differences in triacylglycerol content between fresh, refrigerated or frozen BM
(Ezz El Din et al. 2004)	Fresh 4-6°C for 24 h, or -4 to -8°C for 1 week	No significant differences in fat content between fresh, refrigerated or frozen BM
(Slutzah et al. 2010)	Fresh 4°C for 24, 48, 96 h All samples were stored at -80°C until analysis	No significant differences in total lipid content between fresh, or refrigerated BM
(Romeu-Nadal et al. 2008b)	Fresh 4°C for 3, 6, 9, 12, 24, 48, 72, 96 h -20°C or -80°C for 3, 5, 8, 12 months	No significant differences in relative LA, ALA, ARA, DHA, saturated, monounsaturated, or polyunsaturated fatty acid content between the different storage times and temperatures
(Bertino et al. 2013)	Fresh (within 3 h of collection) 6.8°C for 24, 48, 72, 96 h	No significant differences in absolute saturated or monounsaturated fatty acids, PUFAs, LCPUFAs, or the saturated to unsaturated fatty acid ratio between fresh and refrigerated BM at any storage time
(Ahrabi et al. 2016)	Fresh 4°C for 72 h and then stored at -20°C for 1, 3, 6, 9 months All samples stored at -80°C after the initial storage time until analysis	No significant differences in total fat content between fresh, refrigerated and frozen, or directly frozen BM at the different storage conditions

(Friend et al. 1983b)	Fresh (up to 48 h at 4°C) -25°C for 1 week, 1, or 3 months	No significant differences in total lipid or relative fatty acid concentration between fresh or frozen BM
(Chang et al. 2012)	Samples stored in 9 different commercial milk containers for maximum 3 days at 4°C, before transfer to -20°C for 2 days	No significant differences in total fat content of BM stored in different containers Least fat loss in light brown coloured polyethersulfone bottles Significant total fat decrease in BM after freezing, storing and thawing (-0.27 to -0.30 g/dL, p = 0.02)
(Thatrimontrichai et al. 2012)	Fresh -20°C for 30 days, then thawed at 4°C for 24 h, or at 37°C for 30 minutes	Total lipids (g/100 mL): Fresh: 2.98 vs thawing at 4°C: 2.76 vs thawing at 37°C: 2.66 Significant less mean fat loss in frozen BM thawed at 4°C compared to thawing at 37°C (p = 0.02)
(Janjindamai et al. 2013)	Fresh -20°C for 30 days	Significant fat decrease after frozen storage Fat (g/100 mL): Fresh: 2.98 vs 30 days frozen: 2.66 (p < 0.001)
(Orbach et al. 2019)	Fresh (up to 24 h at 4°C) -20°C or -80°C for 4, 12, 24 weeks	Fat content was consistently higher in BM stored at -80°C than at -20°C (p < 0.0005) Difference in fat content between 4 and 24 weeks was 0.3 g/100 mL at -20°C (p = 0.001) and 0.14 g/100 mL at -80°C (p = 0.009)
(Pardou et al. 1994)	Fresh -20°C for 4, 8 days	Significant total lipid decrease in BM stored at -20°C Lipids (g/100 mL): Group 1 (no bacterial growth): fresh: 3.92 vs frozen for 4 days: 3.61 vs frozen for 8 days: 3.54, no significant differences Group 2 (containing saprophytes): fresh: 3.84 vs frozen for 4 days: 3.8 vs frozen for 8 days: 3.61 (p = 0.003) Group 3 (containing potential pathogens): fresh: 4.75 vs frozen for 4 days: 4.76 vs frozen for 8 days: 4.65, (p = 0.002)
(Berkow et al. 1984)	Fresh (up to 3 h before analysis) Analysed directly after 1, 2, or 3 freeze-thaw cycles (dry ice and acetone-cold water for thawing)	Maximum 5% decrease in triacylglycerols after freeze-thawing of fresh BM BM storage at -20 °C resulted in a 10% decrease of triacylglycerols, or a 13% decrease after 3 freeze-thaw cycles

		Storage at -20°C or -70°C for 5 months after 0, 1, 2, or 3 freeze-thaw cycles	Storage at -70°C resulted in no significant changes
(Garcia-Lara et al. 2012)		Fresh -20°C for 7, 15, 30, 60, 90 days	Significant total fat reduction in BM at each day after storage Fat (g/dL): Fresh: 4.88 vs 7 days frozen: 4.69 (p = 0.002) vs 15 days frozen: 4.54 (p = 0.001) vs 30 days frozen: 4.54 (p < 0.001) vs 60 days frozen: 4.37 (p < 0.001) vs 90 days frozen: 4.19 (p < 0.001)
(Lepri et al. 1997)		Fresh (4°C during same day transport to the laboratory) Pasteurised, then stored at -20°C for 35, 70, 90 days	Post-pasteurisation frozen storage for 90 days decreased total fat concentration by 5.7%, which was above the relative standard deviation of fresh BM
(Garcia-Lara et al. 2013)		Storage in donors' freezers until transfer to the hospital Storage at -20°C at the hospital Thawing, heating to 40°C and homogenisation, Holder pasteurisation, heating to 40°C and homogenisation Analysis day 0 (post-pasteurisation) Storage at -20°C for 30, 60, 90, 120, 150, 180 days	Significant total fat decrease over time (p = 0.001) in frozen BM Mean difference between 0 and 180 days was -0.13 g/dL
(Friend et al. 1983a)		Fresh (up to 48 h at 4°C) Holder pasteurised, then stored at -25°C for 1 month	No significant differences in total lipid or relative fatty acid concentration between fresh or pasteurised and frozen BM
(de Waard et al. 2018)		Pasteurised, then stored at -20°C for 1, 2, 3, 4, 5, 6, 8, 10, 12 months	No significant differences in total fat between any of the storage times
(Lev et al. 2014)		Fresh (up to 24 h at 4°C) -80°C for mean of 43.8 days (range 8-83 days)	Significant decrease of total fat concentration after frozen storage Total fat (g/100 mL): Fresh: 37.2 vs frozen: 3.36 (p < 0.001)
(Wardell et al. 1981)		Fresh (up to 3 h at 18-20°C) Frozen at -20°C, thawed at room temperature once, twice or three times	Freezing and thawing resulted in a loss of absolute milk triacylglycerols Relative amounts of saturated and monounsaturated fatty acids of BM triacylglycerols did not change significantly after two freeze-thaw cycles

		Relative LA concentration (%) changed significantly: Control: 6.64 vs freeze thaw 1: 4.71 vs freeze thaw 2: 2.35 (p < 0.01) vs freeze thaw 3: 2.60 (p < 0.002) ALA could not be measured accurately
(Vieira et al. 2011)	Frozen at -20°C, thawed in a water-bath (40°C, 10 minutes) or in a microwave (45 seconds)	No significant difference in fat content between the two thawing methods
(Handa et al. 2014)	Frozen at -20°C, thawed under tepid water or by waterless dry heat warmer	No significant differences in fat content between the two thawing methods

5.3.2 Free FA levels

The lipid portion of BM consists of approximately 98% triacylglycerols, 1% phospholipids, and 0.4% cholesterol and cholesterol esters (Lammi-Keefe and Jensen 1984). BM also contains the bile salt-dependent lipase, which aids in the digestion of milk fat and compensates for the immature digestive system in new-borns (Hernell 1975; Watkins 1985). However, the bile salt-dependent lipase loses its bile salt specificity during two weeks frozen storage at -10°C (Mehta et al. 1982), potentially resulting in lipolysis of triacylglycerols and an increase in free fatty acid levels. Freezing and thawing also damages the fat globule membrane, allowing the lipases greater access to triacylglycerols, thereby increasing free fatty acid levels (Berkow et al. 1984; Morera Pons et al. 1998). LCPUFAs appear more susceptible to hydrolysis than shorter-chain fatty acids and the degree of hydrolysis is temperature and time dependent (Lavine and Clark 1987). Additionally, elevated levels of free fatty acids have the potential to increase lipid peroxidation (Turoli et al. 2004; Martysiak-Zurowska and Stolyhwo 2006), discussed in Section 5.3.4.

Storing BM at 4°C increases the free fatty acid content significantly from 51% to 454% after 24 h (Dill et al. 1984; Lavine and Clark 1987; Bertino et al. 2013), from 76% to 502% after 48 h (Lavine and Clark 1987; Bertino et al. 2013), from 85% to 101% after 72 h (Lavine and Clark 1989) and by 265% after 96 h (Slutzah et al. 2010), although BM samples in the latter study were stored at -80°C after refrigeration, until subsequent free fatty acid analysis (Slutzah et al. 2010). In a time course experiment, higher free fatty acid levels were seen at 48 h compared to 24 h, although not statistically significant, whereas levels were significantly higher at 72 h, with the greatest increases seen with omega-3 PUFAs (Bertino et al. 2013). However, pasteurised, frozen, and thawed DHM samples stored at 4°C for up to 96 h show no change in free fatty acid levels following thawing (Slutzah et al. 2010).

Free fatty acids in BM increased significantly (+589% vs baseline) after eight weeks storage at -11°C (Lavine and Clark 1987), also, an accumulation of free fatty acids has been seen after 24 h at -20°C, increasing by 167% after 30 days, and 833% after 180 days (Dill et al. 1983). This is supported by other studies showing significant increases in free fatty acids after storage at -20°C for two to five months (Bitman et al. 1983a), four months (Morera Pons et al. 1998), five months (Berkow et al. 1984), and nine months (Ahrabi et al. 2016). No free fatty acids were detected after BM storage at -80°C for four months (Morera Pons et al. 1998). Storage for two months (Lavine and Clark 1987), two to five months (Bitman et al. 1983a), or five months (Berkow et al. 1984) at -70°C did also not increase free fatty acid concentrations significantly. Storage

of Holder pasteurised BM for one month at -25°C (Friend et al. 1983a), or for three months at -20°C (Lepri et al. 1997) did not significantly alter the free fatty acid content, and heating for 1.5 minutes at 80°C prevented the formation of free fatty acids in BM samples stored for four months at -20°C (Morera Pons et al. 1998).

Thoroughly thawing DHM and keeping it in the fridge for a maximum of 24 h is recommended by the U.K. guidelines (National Institute for Health and Clinical Excellence 2010); however, thawing BM at 4°C for 24 h resulted in 10% and 29% higher free fatty acid concentrations than thawing at room temperature for 2.5 to 4.25 h, or thawing in a water-bath (50°C, 12 to 30 minutes), respectively (Chan et al. 2011). A significant increase in free fatty acids was found after thawing (tepid water or waterless dry heater) BM (Handa et al. 2014). Furthermore, refrigeration of thawed BM for up to 24 h before warming and feeding further increased free fatty acids compared to only warming. Thawing and storing BM for 24 h at 4°C, after 30 days storage at -20°C, further increased the free fatty acid concentration by approximately 288%, compared to storage at -20°C for 30 days alone (Dill et al. 1983).

Overall, the evidence strongly suggests that storing BM at 4°C prior to pasteurisation significantly increases the free fatty acid levels, although these changes are not observed post-pasteurisation. These differences are potentially due to inactivation of the BM lipases (Henderson et al. 1998). Similarly, pre-pasteurisation storage at -20°C has been shown to increases free fatty acid levels, which are also not seen in post-pasteurisation BM. Therefore, in order to minimise increases in free fatty acids levels, it is recommended expressed BM should be frozen immediately and stored at the lowest possible temperature (ideally -70°C or below) prior to pasteurisation.

5.3.3 Lipid Mediators

Eicosanoids

Eicosanoids include the eicosapentaenoic acid (EPA, 20:5n-3) and ARA-derived thromboxanes, prostaglandins and leukotrienes, which are important mediators of the inflammatory response (Dyall 2017). Prostaglandins also modulate gastrointestinal function and may protect against gastrointestinal injuries (Ianiro et al. 2014). Eicosanoids (leukotriene E_4, prostaglandin E_2, cysteinyl leukotrienes, prostaglandins E and F, as well as the inactive thromboxane A2, prostacyclin, and prostaglandin F metabolites thromboxane B_2, 6-keto-prostaglandin $F_1\alpha$, and 13,14-dihydro-15-ketoprostaglandin) are secreted into BM (Lucas and Mitchell 1980; Laiho et al. 2003; Garcia-Ravelo et al. 2018). To the authors' knowledge, there have to date been no published studies looking at the effects of storage conditions or DHM processing on eicosanoid levels in BM. However, Lucas and Mitchel hypothesize that a low 13,14-

dihydro-15-ketoprostaglandin F:prostaglandin F ratio in BM suggests that prostaglandins are not rapidly metabolised in BM and may persist long enough to have an effect in the infant (Lucas and Mitchell 1980), and tritiated prostaglandins show minimal degradation after incubation in BM for 30 minutes at 37°C (Bedrick et al. 1989), suggesting that further work should seek to explore this area.

Specialised pro-resolving mediators

Specialised pro-resolving mediators (SPMs) facilitate the resolution of inflammation, are anti-inflammatory, reduce pain, and facilitate wound healing (Serhan et al. 2008; Serhan and Levy 2018). They include the ARA-derived lipoxins, EPA-derived resolvins, and docosapentaenoic acid (22:5n-3) and DHA-derived resolvins, (neuro)protectins and maresins (Dyall 2015). BM contains the SPMs resolvin D1, resolvin D2, resolvin D3, resolving D4, resolvin D5, resolvin D6, protectin 1, maresin 1, resolvin E2, resolvion E3, lipoxin A_4 and lipoxin B_4 in biologically relevant concentrations, which have shown to reduce the maximum neutrophil number and to shorten the resolution interval *in vivo* and to stimulate efferocytosis *in vitro* (Arnardottir et al. 2016). Resolution of inflammation is especially important for extremely preterm infants, in which sustained elevated inflammation in the first month of life is associated with cognitive impairment at ten years of age (Kuban et al. 2017). To the authors' knowledge, there are currently no studies that have investigated the effects of storage conditions on specialised pro-resolving mediator levels in BM. Interestingly, the BM samples in the above study (Arnardottir et al. 2016) were obtained from a commercial supplier, who stores BM at -20°C, and therefore, it is likely that specialised pro-resolving mediators tolerate some frozen storage; however further work should seek to extend these observations and investigate the effects of different storage and processing conditions on SPM levels.

Endocannabinoids

Endocannabinoids include the ARA derived compounds arachidonoyl ethanolamide (anandamide, AEA), 2-arachidonoylglycerol (2-AG), and the DHA-derived docosahexaenoyl ethanolamide (DHEA) (Dyall 2017), which have been identified in BM (Fride et al. 2001; Wu et al. 2016). The endocannabinoid system plays an important role in neuronal development and neuroprotection early in life (Fride 2004; Dyall et al. 2016). Animal studies showed that 2-AG and activation of the Cannabinoid Receptor 1 plays a critical role in milk suckling, holding on to the nipple, and therefore, growth and survival in the first week of life (Fride et al. 2001; Fride 2004). An analysis of BM found a non-significant increase of 503% in 2-AG levels after storage at 4°C for 24 h and a significant increase (1166%) after storage at -20°C for three months (Wu et al. 2016). Storage at -80°C for three months did not affect AEA and 2-AG concentrations. DHEA

was no longer detectable after storage at 4°C for one day, or storage at -20°C or -80°C for three months. The authors suggested that the concentrations of 12 endocannabinoid related compounds (2-AG, AEA, oleoylethanolamide, palmitoylethanolamide, *N*-arachidonoyl glycine, eicosapentaenoyl ethanoalmide, DHEA, *N*-palmitoleoyl-ethanolamine, dihomo-γ-linolenoylethanolamine, *N*-stearoylethanolamine, prostaglandin $F_{2\alpha}$ ethanolamide, prostaglandin E_2 ethanolamide) in BM are stable for a maximum of 24 h at 4°C, maximum one week at -20°C and that longer term storage requires temperatures of -80°C. The same group also demonstrated that two freeze-thaw cycles, as used in human milk banking, resulted in losses of 37% AEA, 49% 2-AG, and 36% DHEA in bovine milk (Gouveia-Figueira and Nording 2014). Additionally, it has been shown that 2-AG in culture medium and biological buffers adheres to glass and plastic surfaces (Rouzer et al. 2002), which could impact on their availability for the infant.

5.3.4 LPPs

Omega-3 and omega-6 LCPUFAs are highly susceptible to peroxidation by oxygen radicals (Dyall 2011). There is a linear dependency between the number of double bonds and the oxidisability of PUFAs (Cosgrove et al. 1987). Lipid hydroperoxides are unstable primary lipid peroxidation products, which react further to form secondary lipid peroxidation products (Ayala et al. 2014). For example, malondialdehyde (MDA) is produced from the unspecific peroxidation of PUFAs with more than two double bonds. At high levels, lipid peroxidation products can bind to DNA and proteins, which can lead to cell and tissue damage, and may thereby increase inflammation (Yadav and Ramana 2013). Repeated intake of lipid peroxidation products has been shown to induce growth retardation, intestinal irritation, cardiovascular diseases, and to be carcinogenic in animal studies (Esterbauer 1993). Direct activation of inflammatory pathways such as nuclear factor κβ has also been shown after feeding lipid peroxidation products to mice (Awada et al. 2012). More importantly, lipid peroxidation products do not only act locally in the intestine, but can also be absorbed and act elsewhere in the body (Awada et al. 2012).

Various lipid peroxidation products have been detected in BM, including MDA (Miranda et al. 2004; Martysiak-Zurowska and Stolyhwo 2006), the omega-6 and omega-3 PUFA derived 4-hydroxy-2-hexanal (4-HNE) and 4-hydroxy-2-nonenal (4-HHE), respectively (Michalski et al. 2008), lipid hydroperoxides (Van Zoeren-Grobben et al. 1993; Turoli et al. 2004), isoprostanes (Szlagatys-Sidorkiewicz et al. 2012), alkanals including pentanal, hexanal, octanal, nonanal, and 2-octanal (Elisia and Kitts 2011), as well as conjugated dienes (Turoli et al. 2004). Storage of fresh BM for 24 h at room temperature significantly increases the 4-HNE:omega-6 fatty acid ratio (Michalski et al.

2008). Storage at 4°C for 48 h was shown to significantly increase MDA content of BM (Miranda et al. 2004), whereas others found storage at 4°C for 96 h has no effect on MDA content of preterm milk (Bertino et al. 2013) potentially due to higher antioxidant capacity in the latter (Turhan et al. 2011). Although thiobarbituric acid reactive substances increased by 66% and conjugated dienes by 31% in the same samples, this was not statistically significant (Bertino et al. 2013). Storage at 4°C for four days increases LA hydroperoxides significantly (Van Zoeren-Grobben et al. 1993).

No significant increases in BM MDA levels were seen after storage at -20°C for ten days (Miranda et al. 2004), or 15 or 30 days, although increases were found after 60 days (Silvestre et al. 2010). Similarly, no increases in thiobarbituric acid reactive substances or conjugated dienes were seen after storage at -20°C for two months, although significant increases in precursor lipid hydroperoxides were found (Turoli et al. 2004). However, it should be noted that the fresh BM samples were from different donors than the frozen samples. MDA levels also significantly increased in term BM stored at -80°C for 60 days (Silvestre et al. 2010). Hexanal levels significantly increased after three months storage at -18°C, with further increases after five and six months (Vangnai et al. 2017). In this study, four months storage of BM in amber glass bottles also reduced the hexanal increase significantly compared clear glass bottles or low density polyethylene bags. Overall, the literature suggests an increase in lipid peroxidation when BM is stored at 4°C, with short-term storage at -20°C for maximal one month preferable, although there needs to be more research to clarify this, as well as whether storage at -80°C would be beneficial.

5.3.5   Antioxidants

Preterm infants have immature antioxidant systems and inadequate antioxidant capacity (Davis and Auten 2010) and there is a frequent requirement for blood transfusions, which increases oxidative stress (Wardle et al. 2002). Furthermore, the foetal to neonatal transition rapidly increases tissue oxygenation, thereby abruptly increasing the generation of reactive oxygen species (Kuligowski et al. 2015), and oxygen therapy as well as total parenteral nutrition expose the premature infant to further sources of oxidative stress (Lavoie et al. 2010). As a consequence, there is great potential for peroxidation of endogenous lipids and subsequent tissue damage. Bronchopulmonary dysplasia, retinopathy of prematurity, necrotising enterocolitis and peri-ventricular leukomalacia are common co-morbidities in preterm infants, which are classified as oxygen radical associated diseases (Saugstad 2005). Moreover, extremely and very preterm infants are not routinely supplemented with dietary antioxidants, as there has been limited research in this area and the outcomes of some trials have been equivocal (Brion et al. 2003). Therefore, BM, which includes enzymatic

(e.g. superoxide dismutase, glutathione peroxidase) and non-enzymatic (e.g. vitamin C, vitamin E, glutathione) antioxidants, is the only enteral source of antioxidants for preterm infants. However, DHM, compared to BM, has significantly lower concentrations of several antioxidants (Hanson et al. 2016).

Antioxidants are not only beneficial to the infant directly, but they also serve to protect PUFAs in the BM from lipid peroxidation and may subsequently decrease the levels of potentially toxic compounds (Shahidi and Zhong 2010). For example, vitamin C can directly prevent lipid peroxidation by scavenging free radicals, and thereby preventing the initiation stage of lipid peroxidation (Choe and Min 2009), and vitamin E can scavenge lipid peroxyl radicals and is then regenerated by vitamin C (Kamal-Eldin and Appelqvist 1996), which in turn is regenerated by glutathione (Wells et al. 1995). Glutathione and the glutathione peroxidase can form more stable lipid alcohols from lipid hydroperoxides (Niki 1987), and glutathione is also involved in the detoxification of MDA (Ankrah et al. 2000). Although evidence for the prevention of lipid peroxidation in human milk by antioxidants is limited, evidence suggests that the vitamin E content of formula milk is inversely related to thiobarbituric acid reactive substances and conjugated dienes (Marshall and Roberts 1990; Turoli et al. 2004), and lower glutathione peroxidase activity is associated with higher MDA concentrations in BM following refrigeration (Miranda et al. 2004). Due to the interplay and synergistic effects between antioxidants, antioxidant capacity should also be considered an appropriate measure of the antioxidant status of BM.

Vitamin C

Term BM contains around 34.7 ± 1.33 mg/L vitamin C (ascorbic acid + dehydroascorbic acid) (Romeu-Nadal et al. 2008b). Significant reductions in the vitamin C content of term BM have been reported after storage at 4°C for six h, and 24 h (Garza et al. 1982; Bank et al. 1985; Buss et al. 2001; Ezz El Din et al. 2004; Rechtman et al. 2006), and after one week at -4 to -8°C (Ezz El Din et al. 2004), as well as after two months at -16°C (Buss et al. 2001). Interestingly, significant decreases in vitamin C were seen after three months storage at -20°C in term, but not preterm BM (Bank et al. 1985), although, in another study significant decreases were seen in preterm BM after seven and 30 day storage at the same temperature (Marinkovic et al. 2016). However, others have reported that vitamin C levels are stable at -20°C in pooled BM for four week (Goldsmith et al. 1983), and up to three months, but significantly decrease after eight months (Romeu-Nadal et al. 2008b). Vitamin C content appears stable with storage at -80°C for eight months, although a significant decrease of 12% was seen at 12 months (Romeu-Nadal et al. 2008b). Overall, although the results are somewhat mixed, the evidence supports BM storage at lower temperatures to protect Vitamin C

content, with storage at both 4°C and -20°C leading to decreases, and storage at -80°C preferable, for the maximum recommended storage time of six months, although this is based on one publication.

Vitamin E

Vitamin E is a class of compounds including α-, β-, γ- and δ-tocopherol, with α-tocopherol being the main isomer in term mature BM, and one of the main contributors to antioxidant capacity of BM, which is found at concentrations of 2.32 ± 0.11 mg/L (Tijerina-Saenz et al. 2009). Storage of BM at 4°C for 24 h did not affect α- and γ-tocopherol levels in several studies (Moffatt et al. 1987; Ezz El Din et al. 2004; Romeu-Nadal et al. 2008b), likewise, no significant changes were found after 48 h for α-, β-, γ-, and δ-tocopherol (Lacomba et al. 2012), although others have reported significant reductions in α- and γ-tocopherol levels after 48 h (Romeu-Nadal et al. 2008b). Storing BM at -4 to -8°C for one week resulted in a significant decrease in vitamin E (Ezz El Din et al. 2004). Storage at -20°C did not affect vitamin E levels of BM stored for 30 days (Lacomba et al. 2012), 16 weeks (Moffatt et al. 1987), six months (Wei et al. 2018), or 12 months (Romeu-Nadal et al. 2008b), and no changes in vitamin E levels were seen after storage for 16 weeks (Moffatt et al. 1987), or six months at -70°C (Wei et al. 2018), or 12 months at -80°C (Romeu-Nadal et al. 2008b). Overall, the evidence suggests that current human milk banking storage processes are safe to protect the vitamin E content in DHM.

Superoxide dismutase, glutathione, and glutathione peroxidase

Superoxide dismutase is an enzyme involved in the dismutation of the superoxide radical. Its activity has been reported to be 36 U/mL in term mature BM (L'Abbe and Friel 2000). Although there is a paucity of research in this area, superoxide dismutase activity was reported to be significantly reduced after preterm BM was stored at -20°C for seven and 30 days (Marinkovic et al. 2016). Glutathione content of mature BM is approximately 163.9 µmol/L (Ankrah et al. 2000). A significant 79% loss of glutathione was noted after two h storage at 4°C as well as at -20°C (-81% vs baseline) (Ankrah et al. 2000). Glutathione peroxidase activity in mature term BM was reported as 38.8 U/mL (Hojo 1986). Significant reductions in activity were seen in term milk after 48 h at 4°C (Miranda et al. 2004), although these were not reported following storage of preterm BM for 30 days at -20°C (Marinkovic et al. 2016). Activity decreases were reported with increased storage time at -20°C, with activity completely lost after one week (Hojo 1986), and significant reductions in activity after 15, 30 and 60 days in another study (Silvestre et al. 2010). However, significant reductions were only shown after 60 days at -80°C, where the activity was not significantly different between the -20°C and -80°C conditions.

Total antioxidant capacity

Total antioxidant capacity (TAC) measures the additive effects of antioxidants and may provide a more useful measure than the assessment of individual antioxidants (Ghiselli et al. 2000). However, the different analytical methods for TAC have a weak or no correlations (Cao and Prior 1998), making it difficult to compare results between studies. Significant reductions in the TAC of preterm and term BM have been reported after storage at 4°C for 48 (Xavier et al. 2011), and 72 h (Aksu et al. 2015), although, others have shown that storing pooled preterm BM at 6.8°C for up to 96 h not affect TAC (Bertino et al. 2013). Freezing BM at -20°C shows significantly reduced antioxidant capacity after 48 h, which further decreased after one week (Hanna et al. 2004), and a significant decrease after one week with further decrease after one month (Paduraru et al. 2018), with similar effects seen at -8°C (Xavier et al. 2011). However, others report no changes in TAC after storing preterm milk for 30 days at -20°C (Marinkovic et al. 2016), or storing DHM at -20°C for two months (Turoli et al. 2004). Preterm colostrum stored for up to three months at -80°C did not show any change in TAC (Akdag et al. 2014), whereas term mature BM stored at -80°C showed significantly lower TAC after two months (Sari et al. 2012). Thawing BM at 4°C for 24 h (as recommended by the U.K. guideline (National Institute for Health and Clinical Excellence 2010)), as well as thawing at room temperature for 2.5 to 4.25 h did not change TAC (Chan et al. 2011), whereas using a water-bath for thawing (50°C, 12 to 30 minutes) resulted in a significant decrease in TAC. Overall, the evidence of different storage conditions on TAC is equivocal, potentially due to differences in analytical techniques, or it may be an indicator of potential variations in the antioxidant requirements of the different samples.

5.4 Conclusion and recommendations

Current human milk banking practices have been developed to provide microbiological safe DHM, with limited emphasis on the nutritional quality of DHM. There are currently no globally accepted guidelines for human milk banking practices, with wide variations in practices, regulations, and organization in each country, in part due to a lack of robust evidence (Weaver et al. 2019). However, more consideration must be given to the nutritional quality of DHM to ensure optimum nutritional intake for the infants. Specific focus should be given to components such as LCPUFAs, bioactive lipid mediators, and their supporting antioxidants, as their levels are essential for the health and development of preterm infants. The literature reviewed within this article clearly demonstrates that the quality of DHM can be influenced by the various storage and processing conditions used in human milk banking. The observations of minimal changes in fat composition are consistent with a recent systematic review (Gao et al. 2019); however, levels of lipid peroxidation products, and endogenous antioxidants appear more sensitive to the storage conditions, and when considering the effects of human milking banking practices on overall DHM lipid nutritional quality these aspects should also be considered, although further research is needed to understand these effects.

Due to the diversity of methodological approaches, and biological variability of the human BM samples, there remain many uncertainties and a general lack of consistency in the current literature around the optimal DHM storage conditions. Indeed, this is even more apparent when considering a range of different nutritional components, where there are different sensitivities to the storage conditions and processing. It is clear that further research is needed to improve the evidence base for human milk banking practices, particularly on the effects of storage conditions on bioactive compounds such as eicosanoids, SPMs and the TAC of DHM. However, in the interim, in order to maximise the LCPUFA content, and to ensure maintenance of supporting antioxidants we must accept a certain degree of uncertainty and adopt a precautionary approach. Therefore, we suggest considering the following recommendations where possible, to supplement current local and national guidelines:

- BM containers should protect the milk from exposure to light, either through the use of amber containers, or if unavailable, other approaches should be put in place, such as wrapping containers in aluminium foil, and putting covers over fridges and freezers with glass doors.
- DHM should be frozen at -20°C directly after expression, instead of pooling over 24 h in the fridge.

- Storage at 4°C at the human milk bank should be minimized wherever possible, and every effort should be made to transport DHM to the human milk bank as soon as possible after expression.

- At the human milk bank the DHM should ideally be frozen at -70°C or below, particularly prior to pasteurisation, although more research is needed to explore the effects of long-term storage of post-pasteurised DHM.

- Using different thawing methods (at room temperature, in the fridge or using a water-bath), affects BM components differently, and currently, the evidence suggests that thawing at 4°C is not detrimental to the fat content or TAC of the DHM.

6 LCPUFAs and LPPs in DHM in the U.K.: Results from the LIMIT two-centre cross-sectional study

The importance of LCPUFAs for preterm infant health and development has been long known (Crawford 1993) and led to the addition of LCPUFAs to FM in 1994 in Europe and in 2001 in the U.S.A. (Hadley et al. 2016). Regardless, DHM is preferred over FM for feeding preterm infants (WHO 2003) due to its multitude of benefits (Quigley and McGuire 2014). LCPUFA percentage composition of DHM in the U.S.A., Canada, and Sweden (Valentine et al. 2010; Baack et al. 2012; Pitino et al. 2019) are too low for preterm infants, who have higher LCPUFA requirements than term infants (as described in detail in Chapter 1.1.6). DHM LCPUFA levels might be even lower in the U.K., since U.K. BM LCPUFA levels are below the worldwide averages (Yuhas et al. 2006; Fu et al. 2016). Furthermore, DHM in the U.K. is not pooled from several donors during processing (National Institute for Health and Clinical Excellence 2010), which could lead to greater variability, and in some cases extremely low LCPUFA levels. Additionally, the total fat concentration of DHM is less than in preterm BM (Ntoumani et al. 2013).

Chapter 5 identified several human milk banking practices that might negatively affect the LCPUFA quality and quantity of DHM and might increase LPPs (Nessel et al. 2019). Additionally, the HMB Survey (Chapter 4) identified that these practices are used in HMBs in the U.K. However, the actual effects of the complete (U.K.) milk banking process on absolute LCPUFA and LPP content had not been investigated. Researchers have measured FA percentage composition (Valentine et al. 2010), used unprocessed DHM (Ntoumani et al. 2013), or mimicked the milk banking process (Garcia-Lara et al. 2013) in their studies. The effects of specific aspects of human milk banking, e.g. pasteurisation, were also investigated individually. For example, Holder pasteurisation, after short time storage at -80°C, did not increase hexanal or MDA levels (Elisia and Kitts 2011). However, the combination of the different processes, e.g. pooling of BM in the fridge over 24 hours, followed by prolonged frozen storage (-20°C) combined with Holder pasteurisation, may have additive negative effects on the nutritional quality. Consequently, it is important to investigate nutrient levels in DHM that has undergone the whole milk banking process. It was, therefore, the aim of the LIMIT study (Investigating **Li**pid Peroxidation Products in Donor Human **Mi**lk – a **T**wo-Centre Study) to quantify for the first time the relative and absolute levels of LCPUFAs, and LPPs in DHM provided for administration two two neonatal units in the U.K., from two different HMBs. Comparison was undertaken with BM from mothers of term infants (since the majority of DHM is provided by term mothers) and also with BM from mothers of preterm infants, the primary milk provision for the preterm infant. Total

enteral LCPUFA intake, as well as LCPUFA levels available for accretion from DHM were also calculated and compared to levels provided by preterm BM. This along with comparison to enteral intake recommendations form ESPGHAN (Agostoni et al. 2010), and published *in utero* accretion rates (Lapillonne and Jensen 2009), were utilised to provide information about suitability of current DHM for preterm infants, focusing on LCPUFA provision.

The following section is the original article, accepted for publication (10/12/2019) in the peer-reviewed *Journal of Parenteral and Enteral Nutrition* (Wiley, doi: 10.1002/jpen.1773) (Nessel et al. 2020). An author contribution statement can be found in Appendix VI.I. Heading, figure, and table numbers have been adapted for this thesis.

6.1   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 (Dyall 2015). 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 (De Rooy et al. 2017). It is therefore essential to ensure adequate postnatal provision of LCPUFAs (Harris and Baack 2015).

Breast milk (BM) provides ARA and DHA, and BM from mothers of preterm infants has higher DHA levels than that of term mothers (Bokor et al. 2007). However, producing an adequate BM supply is three times less likely in preterm mothers (Hill et al. 2005), and donor human milk (DHM) from a human milk bank is recommended (WHO 2003). The use of DHM is growing world-wide due to reported benefits, such as reduced necrotising enterocolitis (Quigley and McGuire 2014) and bronchopulmonary dysplasia (Villamor-Martinez et al. 2018). 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 (Nessel et al. 2019). 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 (Baack et al. 2012). DHM in the U.S.A. is pooled from several donors (O'Hare et al. 2013), whereas in other countries, including the U.K., it is not pooled (National Institute for Health and Clinical Excellence 2010). 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 (Juber et al. 2016).

LCPUFAs are susceptible to lipid peroxidation (Dyall 2011) 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 (Michalski et al. 2008). Milk banking practices, such as storage and processing conditions, can increase lipid peroxidation (Nessel et al. 2019). Preterm infants are prone to oxygen radical associated diseases, such as necrotising enterocolitis (NEC), bronchopulmonary dysplasia and retinopathy of prematurity

(Saugstad 2005), which may be exacerbated by intake of lipid peroxidation products (Awada et al. 2012).

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.

6.2 Material and Methods

6.2.1 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 (National Institute for Health and Clinical Excellence 2010). 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.

6.2.2 Quantification of fatty acids and total fat content

Lipids were extracted and analysed as described previously (De Rooy et al. 2017). 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 (Koletzko 2016), the sum of the absolute fatty acid values was used to estimate total fat content.

6.2.3 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.

6.2.4 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 (Duval and Karlsson 2002).

6.2.5 Quantification of Hexanal

Hexanal was measured using solid phase micro-extraction (SPME) (Elisia and Kitts 2011). 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 (Elisia and Kitts 2011), and adjusted for estimated total fat content.

6.2.6 Statistical analysis

Data is reported according to STROBE guidelines (von Elm et al. 2014). 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 (Agostoni et al. 2010) 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 (De Rooy et al. 2017), and compared to estimated accretion rates (Lapillonne and Jensen 2009) 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.

6.3 Results

6.3.1 Participant and sample characteristics

40 samples were collected: 19 DHM, 10 preterm and 11 term BM samples (Figure 6-1). 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, these 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.
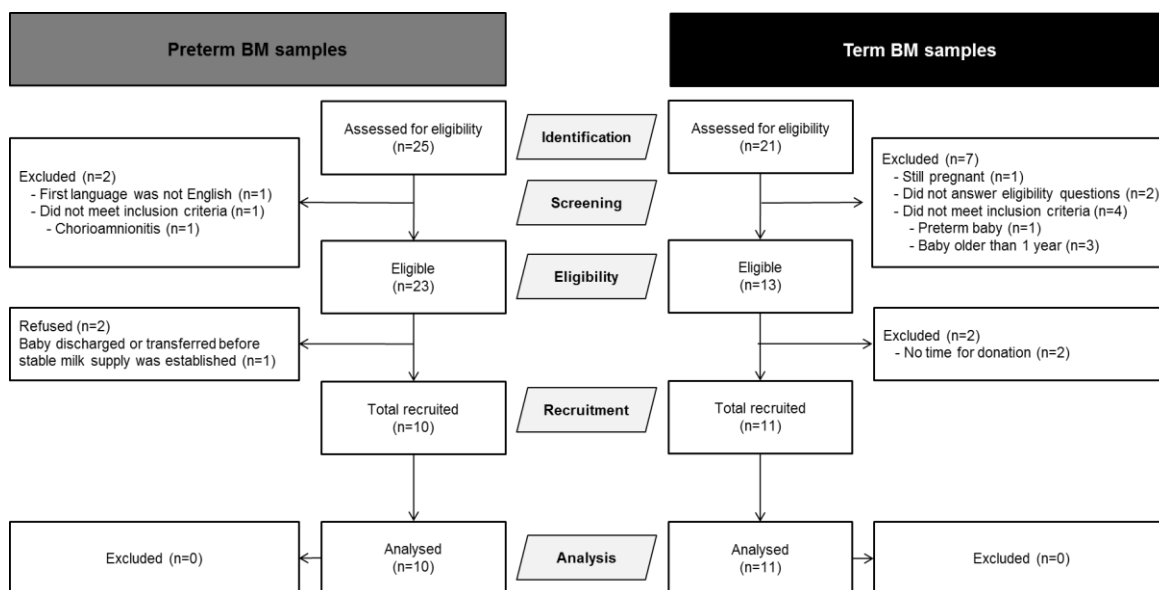
Figure 6-1: Participant flow through the study

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.

Preterm and term BM donor characteristics are shown in Table 6-1; 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).

Table 6-1: Preterm and term BM donor characteristics

Donor characteristics	Preterm	Term	p-value
Number (n)	10	11	N/A
Maternal age (years)	33.1 ± 5.8	29.5 ± 5.6	0.1809
Infant age (months)	1.6 ± 1.4	5.6 ± 3.4	0.0026
Delivery at (weeks + days)	$30^{+6} \pm 3^{+3}$	$40^{+4} \pm 0^{+5}$	<0.0001
Pump expression (n (%))	10 (100%)	6 (55%)	0.0351

6.3.2 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 6-2. 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 6-3. 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).

Table 6-2: Relative fatty acid composition of DHM, preterm and term BM

% of total fatty acids	DHM	Preterm BM	Term BM	*P*-value
10:0	1.48 ± 0.24	1.62 ± 0.42	1.68 ± 0.42	*0.2859*
12:0	6.50 ± 1.78	6.86 ± 1.30	7.05 ± 1.95	*0.6889*
14:0	6.71 ± 1.51	7.68 ± 2.00	6.59 ± 1.46	*0.4110*
16:0	21.58 ± 1.13	20.19 ± 2.68	20.70 ± 3.00	*0.2474*
18:0	8.16 ± 3.24	9.88 ± 5.97	10.71 ± 6.29	*0.6840*
20:0	0.11 ± 0.06	0.09 ± 0.05	0.12 ± 0.08	*0.6903*
∑ SFA	44.77 ± 3.56	47.83 ± 7.45	46.85 ± 6.37	*0.3466*
16:1n-7	2.45 ± 0.59	2.43 ± 0.71	2.30 ± 0.52	*0.8043*
18:1n-7/n-9	36.28 ± 3.55	32.96 ± 4.88	34.21 ± 6.16	*0.1927*
20:1n-9	0.53 ± 0.07	0.59 ± 0.12	0.58 ± 0.33	*0.2005*
24:1n-9	0.07 ± 0.02	0.05 ± 0.03	0.06 ± 0.02	*0.0672*
∑ MUFA	39.41 ± 3.83	36.02 ± 5.40	39.06 ± 4.27	*0.1432*
18:2n-6 (LA)	12.93 ± 1.64	12.74 ± 3.05	11.90 ± 1.70	*0.2868*
18:3n-6	0.13 ± 0.02	0.13 ± 0.04	0.14 ± 0.05	*0.7162*
20:3n-6	0.40 ± 0.08[#]	0.42 ± 0.11[#]	0.31 ± 0.09[*]	*0.0186*
20:4n-6 (ARA)	0.47 ± 0.09	0.53 ± 0.13[#]	0.42 ± 0.07	*0.0520*
22:4n-6	0.11 ± 0.05	0.13 ± 0.07[#]	0.07 ± 0.02	*0.0275*
∑ Omega-6 PUFAs	14.04 ± 1.80	13.94 ± 3.02	12.83 ± 1.73	*0.2626*
18:3n-3 (ALA)	1.25 ± 0.25	1.21 ± 0.37	1.42 ± 0.24	*0.1797*
20:5n-3 (EPA)	0.06 ± 0.02	0.11 ± 0.06[*]	0.09 ± 0.04	*0.0311*
22:5n-3 (DPAn-3)	0.15 ± 0.03	0.22 ± 0.07[**] [#]	0.17 ± 0.06	*0.0031*
22:6n-3 (DHA)	0.29 ± 0.08	0.52 ± 0.70[**] [#]	0.31 ± 0.15	*0.0018*
∑ Omega-3 PUFAs	1.76 ± 0.31	2.03 ± 0.43	1.98 ± 0.31	*0.0962*

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

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Table 6-3: 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

6.3.3 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 (Agostoni et al. 2010). 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 6-4). Preterm BM levels were above the minimum recommendations, with DHA significantly above, although still below the upper daily limit (42 mg).

Table 6-4: 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

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 (Agostoni et al. 2010).

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).

6.3.4 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 (Lapillonne and Jensen 2009) 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).

6.3.5 Lipid peroxidation

MDA

Uncorrected MDA content was different between groups (Figure 6-2 A). 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 6-2 B). 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 (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 $P<0.001$).
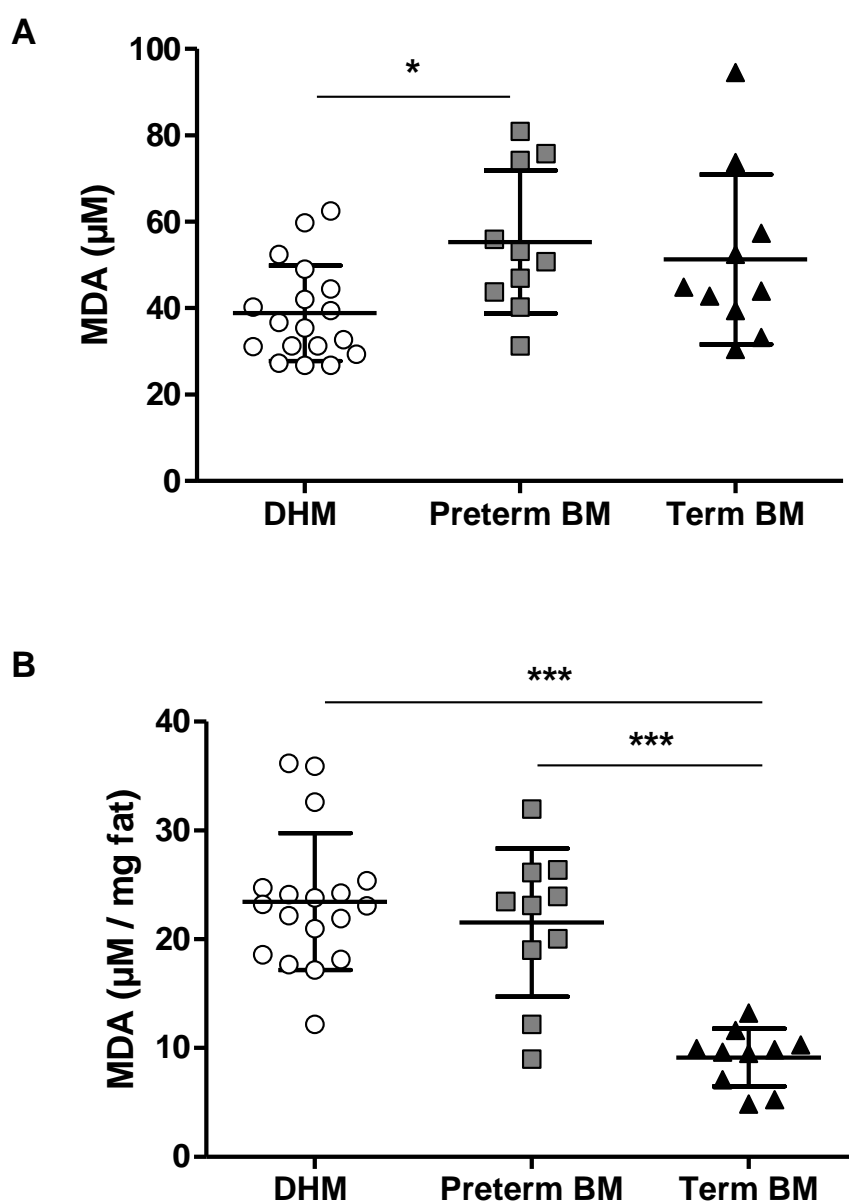
Figure 6-2: Absolute MDA concentration of DHM, preterm and term BM (A) and corrected for fat

content (B)

HNE

HNE content did not differ significantly between groups (Figure 6-3 A). When HNE was corrected for fat content, a significant difference was seen (P=0.0482; Figure 6-3 B). 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.
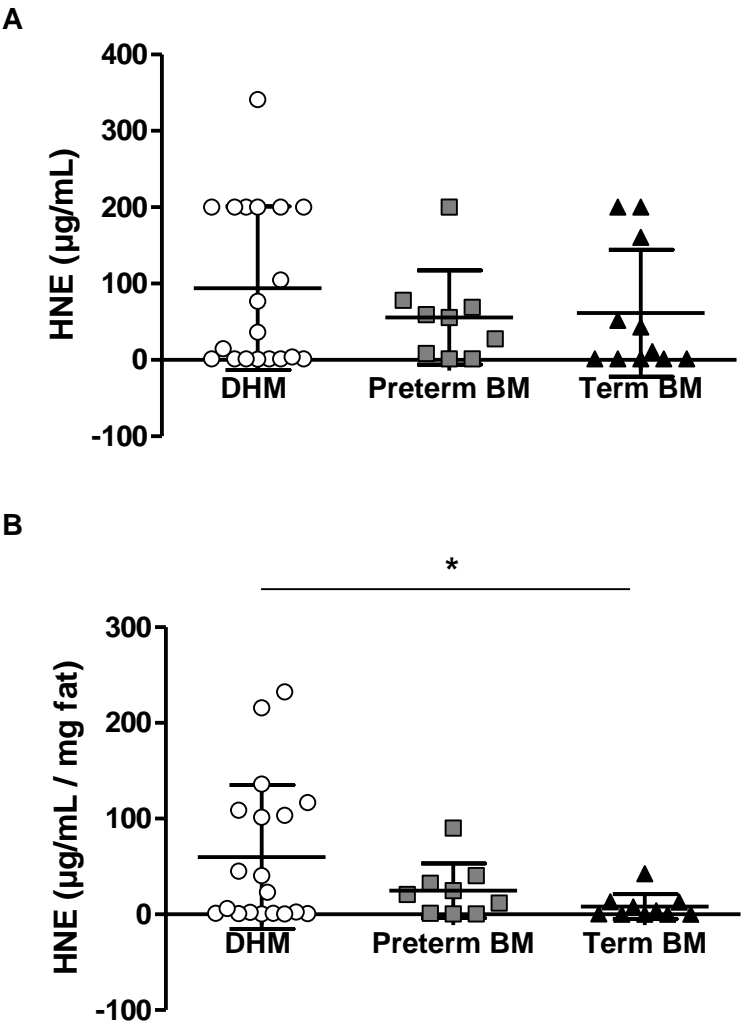
Figure 6-3: Absolute HNE concentration of DHM, preterm and term BM (A) and corrected for fat content (B)

Hexanal

There was a significant difference in hexanal between groups (P=0.0091; Figure 6-4 A). 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 6-4 B). 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).
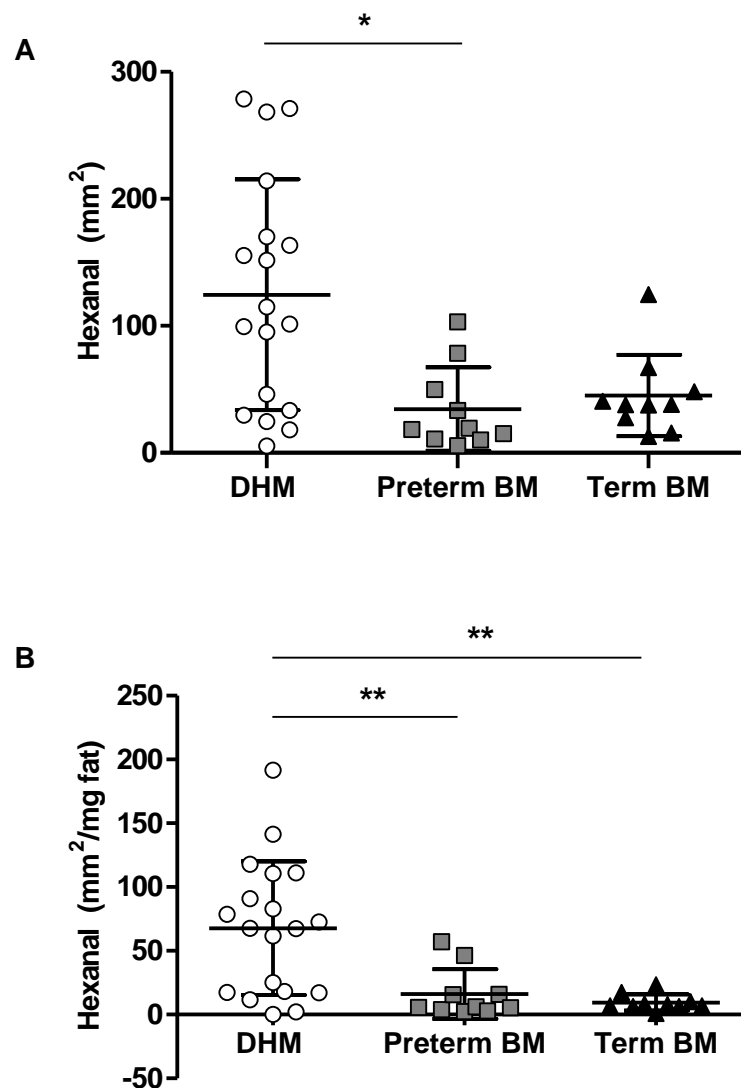
Figure 6-4: Absolute hexanal concentration of DHM, preterm and term BM (A) and corrected for fat content (B)

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.

6.4　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. (Yuhas et al. 2006), 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 (Gao et al. 2019). 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 (Brenna et al. 2018).

Preterm BM had higher DHA than term BM, consistent with previous observations (Bokor et al. 2007). If maternal BM is unavailable, DHM is the recommended alternative (WHO 2003). 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. (Baack et al. 2012). ESPGHAN recommendations consider LA and ALA precursors of ARA and DHA, respectively; however, DHM levels of these were also below recommendations (Agostoni et al. 2010).

DHM and preterm BM were also estimated to provide ARA and DHA significantly below *in utero* accretion rates (Lapillonne and Jensen 2009). These deficits are of particular concern for preterm infants, where neurological development and immune system function are underdeveloped (Harris and Baack 2015). Low ARA and DHA intake are also associated with morbidities of prematurity, such as NEC, bronchopulmonary dysplasia, retinopathy of prematurity, and white matter injury (Fares et al. 2017). 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 (Harris and Baack 2015). 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 (Mizuno et al. 2009).

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 (Nessel et al. 2019). Not protecting BM from light has also been demonstrated to increase hexanal levels (Vangnai et al. 2017).

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 $\kappa\beta$ signalling pathway (Awada et al. 2012). 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 (Nessel et al. 2019).

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 (Nessel et al. 2019).

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 co-morbidities. 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.

6.5   Supplementary material

The following supplementary information does not form part of the publication and is added only as part of this thesis to provide further information.

6.5.1   Study design

Ethical approval for this project (IRAS ID 221198) was obtained from the London-South East Research Ethics Committee (REC reference 18/LO/1330), the Health Research Authority, and Bournemouth University (ID 22015) (Appendix VI.II-VI.V). The study was sponsored by Bournemouth University and was performed in collaboration with Poole Hospital NHS Foundation Trust (local Principal Investigator: Prof Minesh Khashu) and St George's University Hospitals NHS Foundation Trust (local Principal Investigator: Dr Laura De Rooy). An Honorary Contract was obtained for Poole Hospital NHS Foundation Trust. Capacity and Capability to perform this research was confirmed by the local Research and Development Offices. Confirmation letters are listed in Appendix VI.VI-VIII. This study was carried out in accordance with the Declaration of Helsinki (1964) and the Data Protection Act (2018).

A sample size of twelve per group has been recommended for a pilot study (Julious 2005). Considering the outcome measurements, 10/20 samples per group were chosen as a pragmatic approach.

6.5.2   Recruitment

Potential preterm BM donors at the neonatal unit were identified and approached by the local Principle Investigator. Additionally, recruitment posters (Appendix VI.IX) were displayed on the neonatal unit. To recruit term BM donors, recruitment posters (Appendix VI.IX) were displayed at Bournemouth University and posted on the Bournemouth University Research Blog (https://blogs.bournemouth.ac.uk/research/2019/02/06/bu-research-into-breast-milk-quality-participants-needed-2/). Information was also spread by word of mouth.

Preterm and term BM donors received an invitation letter (Appendix VI.X) and the participant information sheet (Appendix IV.XI), and had the opportunity to ask questions before signing the consent form (Appendix IV.XII). All participants were reminded about their right of non-participation. Preterm BM donors were reminded that non-participation would not compromise their or their infants' clinical care. All participants were informed about their right to withdraw at any time, but were reminded that data and samples collected up to the withdrawal will be kept.

6.5.3 Outcome measurements

Quantification of FAs

FAs were analysed using GC-FID, as described in Chapter 2.3.1. Additionally to the absolute concentration, the percentage composition of FAs was analysed, using the following formula:

$$FA\,\%\,wt/wt = 100 \Big/ \sum_{i=1}^{19} aFA \times aFA \times RF$$

aFA = peak area of FA, RF = detector response factor

The detector response factor was 1.1 for C22:5n-3, C24:1n-9, and DHA.

FA data for this study was acquired and reported according to published best practice recommendations (Brenna et al. 2018). However, the proposed A list (21 FA in total, $\sum \geq 95\%$ of total plasma FAs; individual FA >30% of total FAs, or FAs critical for interpreting metabolism,) for FAs to be reported in studies is based on abundance of plasma FAs and some of these FAs are found in relative low levels in BM (C22:0: 0.08%; C24:0: 0.06%; C20:3n-9: not reported; C22:5n-6: 0.03% (Yuhas et al. 2006)), and were therefore not reported in our study. Furthermore, C20:2n-6, which has a relative abundance of 0.22% (Yuhas et al. 2006) could not be accurately identified using our available standards and is therefore also not reported. Figure 6-5 shows a representative chromatogram of DHM FAs.
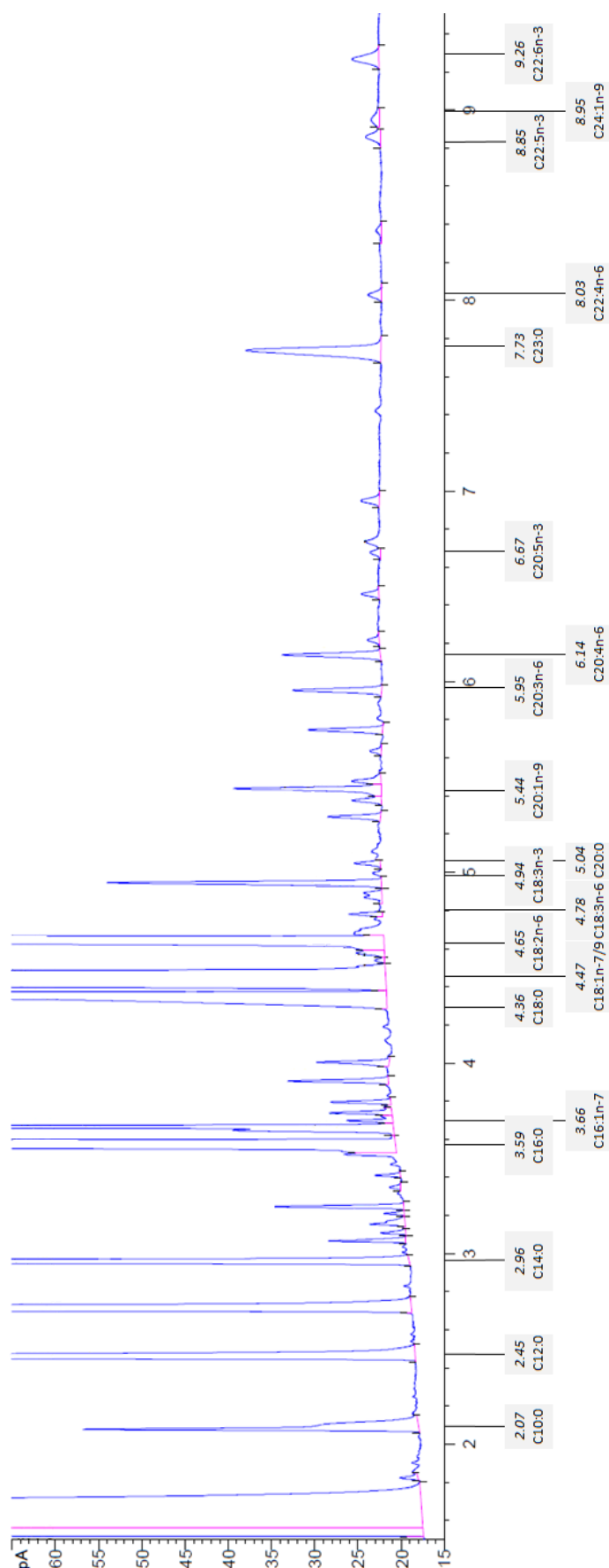
Figure 6-5: Representative chromatogram of DHM FAMEs detected by GC-FID

The chromatogram has a stable baseline and well resolved, sharp peaks, as well as a low signal to noise ratio. The 19 analysed FAs and the internal standard are labelled below the chromatogram. Peak identification was carried out in comparison to analytical standards.

Quantification of MDA

MDA was analysed using a TBARS (Trichloroacetic Acid Method) Assay (700870, Cayman Chemical) as described in Chapter 2.3.2. The standard curve is displayed in Figure 6-6.
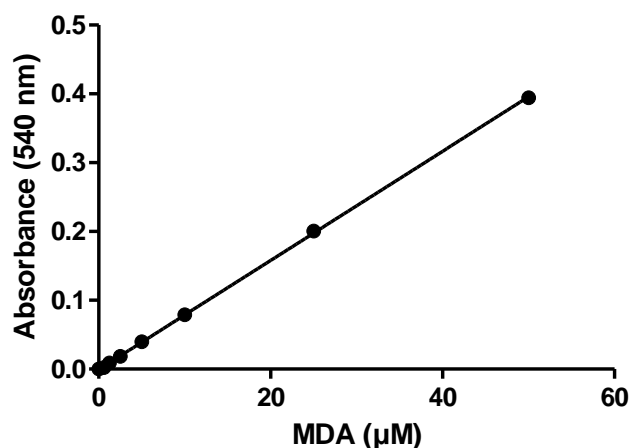
Figure 6-6: Standard curve for the determination of TBARS in BM samples

The graph shows a MDA standard curve ($R^2 = 0.9999$; $y = 0.0079x - 0.0006$) determined by TBARS assay. Standards were analysed in duplicates.

Quantification of HNE

HNE adducts were quantified using the OxiSelect HNE Adduct competitive ELISA Kit (STA-838, Cell Biolabs, Inc.), as described in Chapter 2.3.3. The HNE-BSA standard curve is displayed in Figure 6-7.
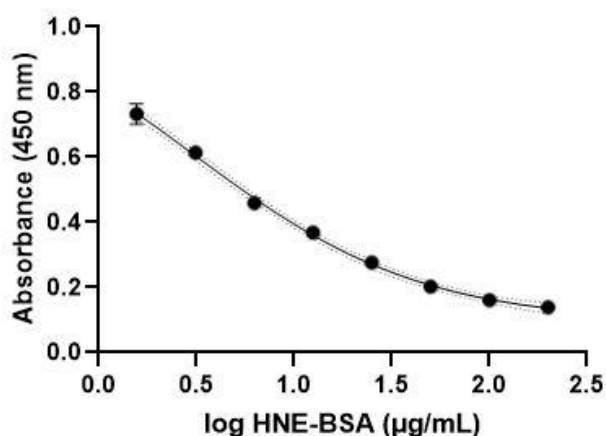
Figure 6-7: Standard curve for the determination of HNE-BSA in BM samples

The graph shows an HNE-BSA four parameter logistics standard curve ($R^2 = 0.9969$) determined by ELISA. Standards were analysed in duplicates.

Protein quantification

Since the HNE protein adducts may depend on the protein concentration, BM protein content was estimated using Bradford Reagent (Sigma Aldrich, U.K.), according to the manufacturer's protocol. During the assay, a complex forms between the Brillian Blue G dye and the proteins (Bradford 1976). Formation of the complex shifts the absorption maximum from 465 to 595 nm, and the absorption is proportional to the protein concentration.

The linear concentration range of this test is 0.1-1.4 mg/mL, therefore, BM samples were diluted 1:15 with dH_2O. Samples (5 µL) were transferred to a 96-well plate in duplicates, mixed with 250 µL Bradford Reagent, mixed for 30 s on a shaker, and incubated for 10 min, before the absorption was measured at 595 nm, using an absorbance microplate reader (ELx800, BioTek). Results were analysed using the Gen5 software (Version2.09.2 for Windows, BioTek). Protein content was determined using a bovine serum albumin standard curve (Figure 6-8) ranging from 0-1.2 mg/mL (Sigma Aldrich, U.K.).
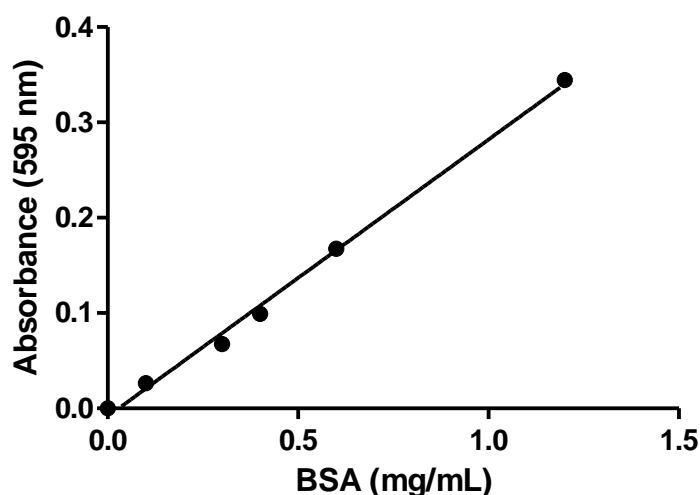
Figure 6-8: Standard curve for the determination of protein concentration in BM samples
The graph shows a BSA standard curve (R^2 = 0.9958; y = 0.2903x – 0.0083) determined by Bradford assay. Standards were analysed in duplicates.

Hexanal quantification

Hexanal is the major volatile aldehyde that is generated during lipid peroxidation in BM (Elisia and Kitts 2011). SPME coupled with GC-FID is more sensitive, and demonstrates better precision and accuracy in detecting hexanal than dynamic headspace analysis by GC (Marsili 1999).

Hexanal was measured using SPME, based on the method described by Elisia and Kitts (2011). BM (1.5 mL) was thawed, homogenised, and added to a vial containing 25% NaCl (w/v) (Sigma-Aldrich Co 1998). Samples were equilibrated to 25°C for 15

min with continuous stirring (Elisia and Kitts 2011). Headspace volatiles were sampled for 20 min using a preconditioned (250°C, 30 min) polydimethylsiloxane fiber (100 µm, Supelco).

Analysis was performed using an Agilent 7820A gas chromatograph (Agilent Technologies; constant N_2 (grade N5.5) flow at 9.9 mL/min, 27.9 psi pressure, average velocity 102.8 cm/s, holdup 0.48623 min), fitted with a merlin micro seal, SPME liner, and J&W HP-5 column (30 m x 0.32 mm x 0.25 µm). Oven temperature started at 40°C for 2 min, increased by 15°C/min to 60°C, further increased by 40°C/min to 300°C, and was held for 6 min.

The SPME fiber was immediately thermally desorbed in the gas chromatograph inlet (split-less mode, purge flow to split vent at 50 mL/min after 1 min, 250°C, 27.9 psi pressure). Detection was carried out with an FID (300°C, H_2 (grade N5.5) flow 40 mL/min, air flow 450 mL/min; constant makeup (N2, grade N5.5) flow 30 mL/min). Peak identification was carried out in comparison to a hexanal standard (Figure 6-9), an example chromatogram of DHM is shown in Figure 6-10. Hexanal peak area was corrected for percentage recovery based on fat content (Elisia and Kitts 2011), and was also adjusted for the estimated total fat content.
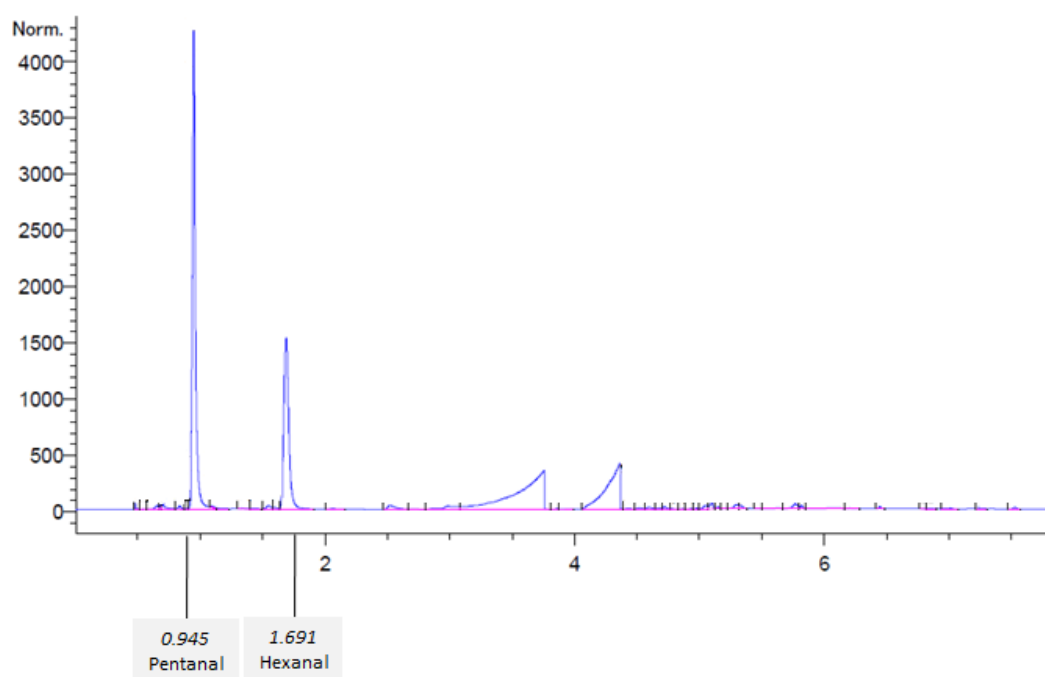
Figure 6-9: Representative chromatogram of hexanal standard detected by SPME GC-FID
The chromatogram has a stable baseline and well resolved, sharp pentanal and hexanal peaks.
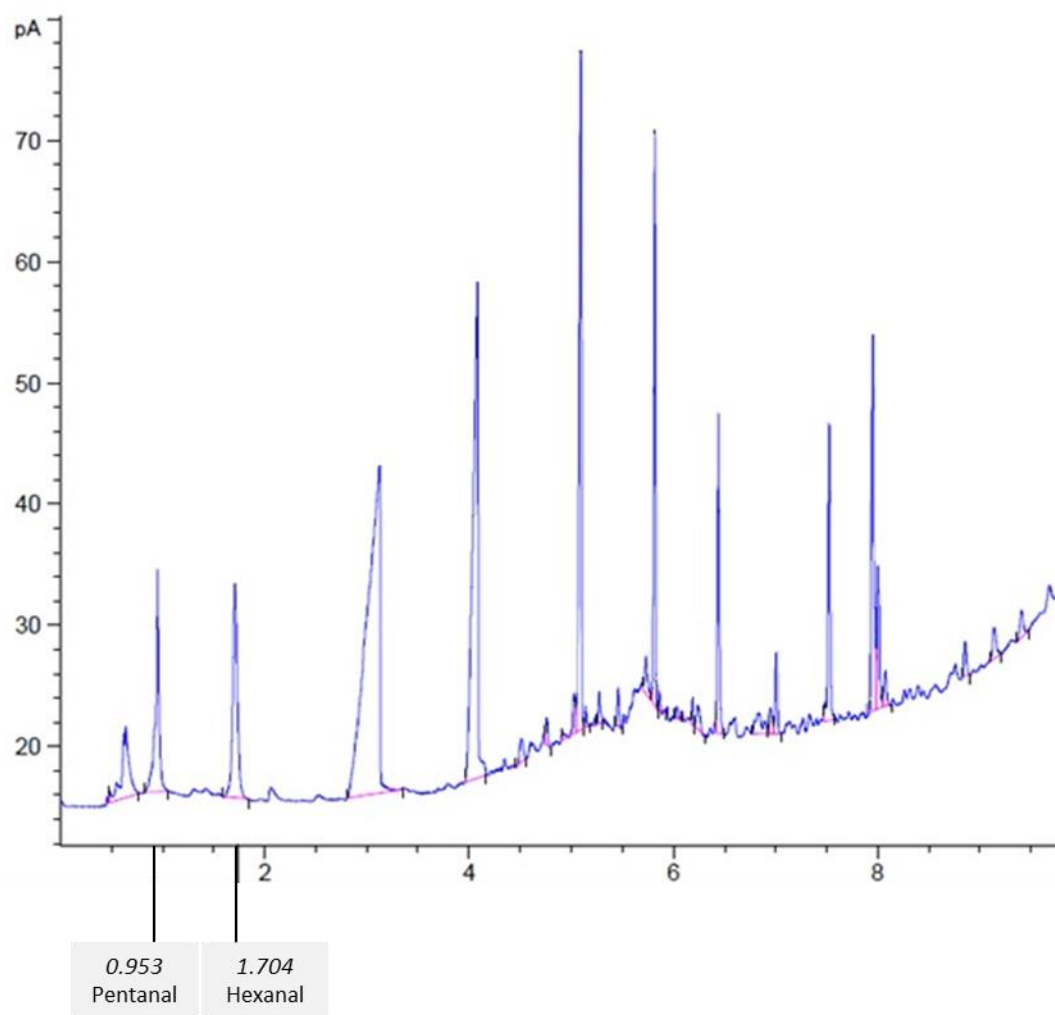
Figure 6-10: Representative chromatogram of hexanal in DHM detected by SPME GC-FID

The chromatogram has a stable baseline and well resolved, sharp peaks, as well as a low signal to noise ratio. Peak identification was carried out in comparison to an analytical standard.

6.6   Supplementary results and discussion

The following supplementary results do not form part of the publication and are added only as part of this thesis to provide further information. Following the LIMIT study, LCPUFA and LPP levels in the enteral non-maternal nutritional sources for preterm infants were compared against each other and to preterm BM.

LCPUFA levels of non-maternal sources for preterm infants

In this thesis, absolute LCPUFA levels of the two enteral non-maternal nutritional sources for preterm infants, which are preterm FM (Nutriprem 1 and Pro Gold Prem 1) and DHM have been measured (Chapter 2 and 6). For comparison, BM from mothers who delivered preterm infants was also analysed. A summary of LCPUFA levels in enteral nutritional sources for preterm infants in the U.K. is provided in Table 6-5. In most neonatal units, DHM would be used in conjunction with a BM fortifier (Klingenberg et al. 2012). Therefore, LCPUFA levels of DHM supplemented with SMA BM fortifier were calculated based on manufacturer's published LCPUFA values.

Table 6-5: LCPUFAs in enteral nutritional sources for preterm infants in the U.K.

	ARA (mg/100 mL)	DHA (mg/100 mL)
Nutriprem 1	15.49 ± 5.52	12.03 ± 4.45
Pro Gold Prem 1	11.53 ± 0.79	10.50 ± 0.95
DHM	8.26 ± 2.90	4.70 ± 1.67
DHM + SMA BM fortifier	8.26 ± 2.90	11.46 ± 2.26
Preterm BM	14.86 ± 6.97	14.65 ± 8.50

There was no significant difference between the LCPUFA content of the liquid preterm FMs, as described in Chapter 2.4.1. Nutriprem 1 had significantly higher levels of ARA ($p = 0.0316$) than DHM, and both preterm FMs provided significantly more DHA than DHM (Nutriprem 1: $p = 0.0134$ and Pro Gold Prem 1: $p = 0.0006$). When SMA's BM fortifier is added to DHM as recommended by the supplier, the DHA content of DHM increases to 11.46 ± 2.26 mg/100 mL. This is no longer significantly different from Nutriprem 1 and Pro Gold Prem 1 ($p = 0.4410$ and $p = 0.5030$). The BM fortifier does not contain ARA, so levels remain unchanged.

When compared to preterm BM, ARA levels in both FMs were not significant different. ARA levels were significantly lower in fortified DHM than in preterm BM ($p = 0.0031$), since the fortifier does not contain ARA. Both preterm FMs and fortified DHM had DHA levels that were not significantly different from preterm BM.

LPPs in non-maternal sources for preterm infants

LPP levels in non-maternal sources for preterm infants were also explored in this thesis (Chapter 2 and 6). For comparison, LPPs in preterm BM were also analysed. Table 6-6 provides a summary of the fat corrected LPP levels in preterm FM, DHM, and preterm BM.

Table 6-6: LPPs in nutritional sources for preterm infants

	TBARS (µM)	HNE (µg/mL)
Nutriprem 1	4.77 ± 0.44	1.61 ± 0.13
Pro Gold Prem 1	-	0.82 ± 0.11
DHM	23.46 ± 6.28	59.99 ± 75.13
Preterm BM	9.12 ± 2.66	24.77 ± 28.48

Due to the partially hydrolysed whey protein formulation, TBARS could not be measured in Pro Gold Prem 1

DHM had significantly higher TBARS content than Nutriprem 1 ($p = 0.0008$). The TBARS concentration of DHM and preterm BM was not significantly different, whereas Nutriprem 1 had significantly lower TBARS content ($p = 0.0007$) than preterm BM.

The HNE content of Nutriprem 1 was significantly higher than of Pro Gold Prem 1 ($p = 0.0357$). HNE in preterm FMs and DHM was not significantly different. When compared to preterm BM, preterm FMs had significantly lower HNE content (Nutriprem 1: $p = 0.013$ and Pro Gold Prem 1: $p = 0.0070$), whereas DHM was not significantly different ($p = 0.8702$).

Other researchers have also compared LPP levels in BM and FM. Michalski and colleagues found significantly higher 4-hydroxyalkena/related PUFA levels in FM powder as well as in liquid FM, compared to fresh BM (2008). Similarly, MDA/PUFA was also significantly lower in fresh BM. Martysiak and Stolyhwo found 19.35 µg MDA/100 mL BM, compared to on average 55.33 µg MDA/100 mL FM (2006). These studies report the opposite of our results. However, it is to note that in these studies MDA was measured by high-performance liquid chromatography, whereas we used a TBARS assay. One study that also reports higher LPP levels in BM than in FM has also used a TBARS assay (Turoli et al. 2004). As mentioned already in Chapter 2.3.2., the TBARS test is not specific for MDA and thiobarbituric acid can react with other molecules in the assay (Benzie 1996). BM has a much more complex matrix than FM and overestimation of TBARS in BM compared to FM might have occurred.

Recommendations and implications for clinical practice

For feeding preterm infants, maternal BM is normally the best choice, and is recommended by several organisations (WHO 2003; Agostoni et al. 2010). When maternal BM is not available or suitable, DHM remains the second-best choice. Undeniably, DHM, had lower ARA levels than preterm FM and preterm BM. When DHM was supplemented with BM fortifier, DHA levels were no longer different from DHA levels in preterm FM or preterm BM. Furthermore, HNE content was not significantly higher in DHM than in preterm FMs. It is recommended that ideally a BM fortifier is used that contains ARA and DHA, although, currently, only Prolacta's Human Milk Fortifier contains LCPUFAs, albeit in varying levels. When choosing a bovine BM fortifier, SMA should be preferred, since it can increase the DHA content of DHM. When neither maternal BM nor DHM is available, a preterm FM should be used. Preliminary results from this study indicate higher LCPUFA levels in the preterm FM that declared lower LCPUFA levels on the label. Further research is needed to confirm these results, to be able to make recommendations. Based on the here investigated parameters, the results indicate that current FM storage conditions are safe and do not need to be changed. However, care should be taken to not use powder FM longer than 4 weeks, which is the manufacturer recommended storage lengths for opened FM boxes.

7   General discussion and conclusion

The aim of this thesis was to investigate the quantity and quality of LCPUFAs provided to preterm infants in the U.K. from the non-maternal nutritional sources used in the perinatal period, and therefore also to investigate whether feeding DHM and preterm FM contributes to the LCPUFA gap of prematurity. The LCPUFA content, as well as a range of secondary LPPs, were measured in DHM, as well as in the available preterm FMs (April 2017), thereby covering the non-maternal enteral nutritional sources for preterm infants. The main findings from this PhD dissertation are discussed below.

Previous research from our laboratory has demonstrated that preterm infants receiving standard care in the U.K. receive very low levels of LCPUFAs (De Rooy et al. 2017). A limitation of this study was that LCPUFA intake from FM was calculated based on the manufacturers' published values. However, the present research found preliminary evidence that in two of the preterm FMs analysed ARA was significantly below the manufacturer's published values, and in one of the preterm FMs DHA levels were significantly below those published. These discrepancies are likely to lead to an overestimation of LCPUFA intake, not only in the research setting but also clinically. Furthermore, we measured for the first time in the U.K. absolute LCPUFA levels in DHM, and demonstrated that it provides significantly less LCPUFAs than preterm BM. This has previously been indicated by lower DHA weight% in DHM in the U.S.A. (Valentine et al. 2010; Baack et al. 2012). Since DHM LCPUFA levels were also not measured in the previous work from our laboratory (De Rooy et al. 2017), the LCPUFA intake deficit of preterm infants may be greater than reported, contributing to the LCPUFA gap of prematurity.

Although the low LCPUFA intake potentially contributes to the neurodevelopmental problems seen in preterm infants (Henriksen et al. 2008; Makrides et al. 2009; Westerberg et al. 2011), the optimal enteral level of LCPUFA intake is unknown (Lapillonne and Jensen 2009). LCPUFA levels from non-maternal sources were compared to ESPGHAN recommendations, which European paediatricians use as guidance for feeding preterm infants (Agostoni et al. 2010). Preterm FM in the U.K. contains LCPUFA levels that reach minimum ESPGHAN recommendations, whereas DHM provides LCPUFA levels significantly below ESPGHAN recommendations. When DHM is supplemented with a commercial bovine BM fortifier as suggested, it reaches ESPGHAN recommendations for DHA, but not ARA, since the fortifier contains only DHA, further contributing to the ARA/LCPUFA gap of prematurity. This clearly demonstrates the need for BM fortifiers containing not only DHA but also ARA. LCPUFA requirements of preterm infants can also be defined by the rate of LCPUFA

accretion *in utero* (Lapillonne and Jensen 2009). Results from this PhD indicate that both DHM and preterm FM provide LCPUFA levels significantly below the estimated *in utero* accretion rates.

Reaching ESPGHAN recommendations does not necessarily equal adequate LCPUFA supply for the preterm infants. Currently, LCPUFA levels in both non-maternal nutritional sources, and preterm BM are likely to lead to a decline in plasma LCPUFA concentrations, as shown for even higher LCPUFA levels in BM (Henriksen et al. 2008). This can predispose preterm infants to the development of multiple morbidities and perinatal death (Fares et al. 2017). This work provides, therefore, for the first time evidence that non-maternal nutritional sources for preterm infants in the U.K. provide insufficient LCPUFA levels. Additionally, it also confirms previous results from our laboratory (De Rooy et al. 2017), showing that maternal BM LCPUFA levels are also too low for preterm infants. FM manufacturers provide FM to several European countries, and human milk banking practices are similar worldwide (Hartmann et al. 2007; Arslanoglu et al. 2010; National Institute for Health and Clinical Excellence 2010). Therefore, our results are not only applicable to the U.K., but also to other countries. More research is needed to define the optimal LCPUFA levels for preterm infants, especially extremely preterm infants, who are currently not included in the ESPGHAN recommendations. Studies should test various dosages of DHA in combination with ARA, considering short and long-term health outcomes. In the meantime, the most recent LCPUFA intake recommendations from Koletzko and colleagues (35-45 mg ARA and 55-60 mg DHA per kg bodyweight per day) (2014) seem more suitable. These recommend DHA levels comparable to the *in utero* accretion rates. Similar concentrations (32 mg ARA and 59 mg DHA per kg bodyweight per day) have been shown to be safe in a clinical trial, and to increase plasma DHA and to decrease the decline in plasma ARA (Henriksen et al. 2008). Furthermore, these levels have been shown to significantly improve cognitive development at six months (higher score on the problem-solving subtest of the Ages and Stages Questionnaire).

With the LIMIT study, we provided preliminary evidence that absolute LCPUFA levels are currently very low in DHM and that DHM, therefore, is unsuitable long-term as an exclusive nutritional source for preterm infants. Although further work is needed to support these observations, addition of a BM fortifier may be prudent. A Human Milk Fortifier has recently been introduced to the U.K. market and is likely to be introduced in neonatal units in the future, due to the previously reported benefits over bovine BM fortifiers, including earlier discharge, lower mortality and lower incidence of NEC, late-onset sepsis, retinopathy of prematurity and bronchopulmonary dysplasia (Abrams et al. 2014; Hair et al. 2016a; Hair et al. 2016b). Based on the available evidence (Nessel

et al. 2019), and considering the low levels of LCPUFAs found in DHM in the U.K. (Nessel et al. 2020), it is likely that the current human milk banking practices influence LCPUFA levels. Since Prolacta's Human Milk Fortifiers are subject to similar storage and processing conditions as DHM (frozen storage at donor's home, pasteurisation, frozen storage and shipment) (Prolacta Bioscience Inc 2020), it could be speculated that they have low LCPUFA levels, and therefore, studies investigating the effects of these practices on their LCPUFA content are required. Practicality and safety of direct LCPUFA supplementation of DHM on the neonatal unit, for example by sonication of LCPUFA rich oil into DHM, as performed previously in clinical trials investigating the effects of LCPUFA supplementation (Henriksen et al. 2008), should be given consideration.

In this thesis, it was suggested that the DHA gap of prematurity concept, proposed by Harris and Baack in 2015, should be broadened into the "LCPUFA gap of prematurity", since several proposed mechanisms leading to the DHA gap also apply to ARA (see Chapter 1.1.6). There is now further evidence that current feeding practices in the U.K. might contribute to this LCPUFA gap, when feeding the non-maternal nutritional sources DHM and preterm FM. Besides not knowing optimal enteral LCPUFA intake levels, it is known that feeding maternal BM to preterm infants will result in a decline in ARA and DHA blood levels, while at the same time *in utero*, these levels would have increased until birth (Leaf et al. 1992a). This suggests that even preterm BM LCPUFA levels are not sufficient. Hence, although this PhD identified that ARA and DHA levels in preterm FM, and DHA levels in fortified DHM were not significantly lower than in preterm BM, both will still contribute to the LCPUFA gap. Furthermore, this research has demonstrated that ARA levels in DHM, fortified or not, are significantly below ARA levels in preterm BM, contributing even more to the LCPUFA gap, and potentially leaving preterm infants with a greater ARA than DHA gap of prematurity.

When talking about the LCPUFA gap of prematurity, the influence of precursor C18 PUFA levels, such as LA and ALA, should also be considered, since high levels of these can influence their conversion to LCPUFAs and inhibit the LCPUFA incorporation into tissue (McMurchie et al. 1990; Clark et al. 1992; Nakamura and Nara 2004; Gibson et al. 2013). Additionally, other LCPUFAs important for brain development and health, such as e.g. adrenic acid, should be considered as well in this concept, and further research is needed to ascertain the total LCPUFA needs of preterm infants.

Using a rough estimation based on an adrenic acid accretion of about half the ARA accretion in the last trimester (Martinez 1992), this would result in a need for 106 mg/kg/d of adrenic acid based on the calculated ARA accretion rates by Lapillonne

and Jensen (2009). Considering data from the LIMIT study, DHM and preterm BM provide much lower adrenic acid levels (2.8 ± 1.5 and 9.7 ± 5.7 mg/kg/d, respectively, considering 150 ml/kg/d) (Nessel et al. 2020), and according to the manufacturers' data sheets, preterm FM does not contain any adrenic acid (Danone Nutricia Early Life Nutrition 2017b; SMA Nutrition 2017a, 2017b). It should be considered that in a study in baboons, 17% of enteral ARA contributed to postnatal brain adrenic acid accretion. Nevertheless, even adding 17% of the enteral ARA (data from the LIMIT study: 2.1 and 3.8 mg/kg/d for DHM and preterm BM, respectively (Nessel et al. 2020)), this would only result in 4.9 and 13.5 mg/kg/d adrenic acid available for accretion, respectively. The endogenous synthesis of ARA form precursor FAs could also be considered. If 17% of endogenous synthesised ARA (27 mg/kg/d, (Carnielli et al. 2007)) are added, this increases the adrenic acid levels for accretion by 4.6 mg/kg/d, to 9.5 and 18.1 mg/kg/d in DHM and preterm BM fed infants, respectively. This accounts for only 8.9 and 17.1% of the *in utero* accreted adrenic acid. Therefore, the LCPUFA gap of other LCPUFAs than ARA and DHA might also be significant and should be investigated and considered in the future.

The LCPUFA gap of prematurity might also contribute to the common comorbidities of preterm infants. Not only are LCPUFA levels lower in red blood cells of infants with respiratory distress syndrome and intraventricular haemorrhage (Fares et al. 2017), Crawford and colleagues also provide a pathophysiological explanation, linking LCPUFA and antioxidant levels to these comorbidities of prematurity (Crawford et al. 1997; Crawford et al. 1998). Preterm infants have low LCPUFA levels at birth, which further decrease with the current feeding conditions (Leaf et al. 1992b; Baack et al. 2015), resulting in fragile cell membranes (Crawford et al. 1998). Furthermore, antioxidant levels are also low in preterm infants (Qanungo and Mukherjea 2000; Robles et al. 2001), while at the same time, tissue oxygenation increases rapidly after birth, although *in utero*, the environment would have been relatively hypoxic (Kuligowski et al. 2014). High oxygen levels combined with low antioxidant levels can lead to peroxidation of lipids/LCPUFAs in cell membranes. This damage can lead to leakage and rupture of the cell membrane, normally followed by vasoconstriction and cell adhesion for repair, mediated by the free ARA levels (Crawford et al. 1998). However, continuous damage, due to the fragile cell membranes, continuous peroxidation, and shortage of LCPUFAs for membrane repair, can lead to cell infiltration, oedema, haemorrhage, and ischaemia. These are common signs of the comorbidities of prematurity, e.g. intraventricular haemorrhage, or the ischaemic lesion of periventricular leukomalacia, retinopathy of prematurity and bronchopulmonary dysplasia (Crawford et al. 1997). Therefore, postnatal provision of LCPUFAs needs to be increased, to reduce the LCPUFA gap of prematurity and to improve preterm

infants' health and development. Brain DHA and ARA accumulation continues throughout the first two years of life (Lauritzen et al. 2016). Therefore, complementary foods, introduced after six months of exclusive BM/FM feeding, could potentially help to increase brain LCPUFA levels, and to mitigate the consequences of the LCPUFA gap of prematurity. Indeed, trial complementary foods containing oily fish increased red blood cell DHA and plasma DHA (Libuda et al. 2016), and visual acuity was better in term infants receiving DHA enrich complementary food after exclusive breast feeding (Hoffman et al. 2004). However, in the U.K., even fish-based complementary foods provide significantly less LCPUFAs than recommended (Loughrill and Zand 2016). This further indicates the need to provide preterm infants with sufficient levels of LCPUFAs in the perinatal period before complementary feeding is introduced.

For optimal brain development, further FAs besides the two LCPUFAs ARA and DHA are important. White matter, especially myelin, contains high levels of nervonic acid (24:1n-9) and a rapid increase in brain nervonic acid was reported after birth (Martínez and Mougan 1998). Recently, significantly lower levels of nervonic acid were reported for DHM in Sweden compared to preterm colostrum/transitional BM (Ntoumani et al. 2013). Our results do not indicate differences between relative or absolute levels of nervonic acid in DHM and preterm mature BM. Levels were similar to the reported levels in Swedish DHM. Other researchers have also reported significantly higher levels of nervonic acid in colostrum than in mature preterm BM (Thakkar et al. 2019). More research is needed to understand the role and need of nervonic acid in nutritional sources for preterm infants, especially in the first weeks after birth.

Since evidence suggests that LCPUFA intake levels are currently insufficient for preterm infants, this raises the question whether the LCPUFA levels can be optimised in non-maternal nutritional sources. We have investigated whether preterm FM storage conditions can lead to a decline in LCPUFA levels. Results indicate that the current clinical storage conditions of liquid and powdered preterm FM do not significantly decrease LCPUFA levels, and therefore, do not contribute to a further decrease to the LCPUFAs provided to preterm infants. Furthermore, the results also suggest that these clinical practices do not increase the secondary LPPs measured in this study (TBARS and HNE adducts).

A number of benefits of DHM feeding for preterm infants have been reported, including a reduction in NEC and bronchopulmonary disease (Quigley and McGuire 2014; Villamor-Martinez et al. 2018). Additionally, using DHM is associated with higher breast feeding rates at discharge (Arslanoglu et al. 2013b), which can result in life-long health benefits (Binns et al. 2016). Nevertheless, the issues of low LCPUFA levels and lipid

peroxidation (Nessel et al. 2020) need to be addressed in further studies. Based on the available evidence in the literature, we have provided a number of recommendations for human milk banking, focusing on optimisation of LCPUFA quality and quantity in DHM (Nessel et al. 2019). Due to the similarity of human milk banking practices worldwide, the recommendations are also applicable to HMBs outside the U.K. However, effectiveness of these recommendations has yet to be tested. The list of recommendations is by no means exhaustive. It would be useful to also understand the additive effects of human milk banking practices, as well as the most detrimental practices through rigorous testing of absolute LCPUFA levels at each step, to specifically focus on optimisation of these aspects. Furthermore, the effects of DHM storage at -80°C, especially pre-pasteurisation, on LCPUFAs, LPPs and antioxidants need to be better investigated.

Several studies have reported that refrigerated or frozen storage has no effect on LCPUFA levels in DHM (Friend et al. 1983a; Friend et al. 1983b; Romeu-Nadal et al. 2008b). All these studies based their observations on quantification of the LCPUFA levels by relative percentage FA composition. Results from our LIMIT study also showed that human milk banking does not affect the percentage composition of DHM (Nessel et al. 2020). However, we also reported the absolute levels of LCPUFAs, where significant differences in LCPUFA content were found between DHM and term BM. This shows the need to not only report FA percentage composition but to always include absolute amounts when reporting on the effect of human milk banking processes on LCPUFA levels in future trials. Reporting FAs in different units can lead to contradicting results (Brenna et al. 2018). Therefore, best practice recommendations for the reporting of FA data also stress the need to include sufficient data to convert relative to absolute FA concentrations.

The first U.K. national HMB survey by our group revealed that the majority of HMBs have no nutritional information available for donors and that only two HMBs have registered dietitians. Optimising donors' nutrition is an important way of increasing the LCPUFA levels of DHM. Indeed, providing donors with nutritional information was successful in increasing BM DHA levels in the U.S.A. (Juber et al. 2016). Unpublished data from our group also indicates that BM donors would appreciate nutritional information, preferably in form of a website (Casey 2018). Effectiveness of a website providing nutritional advice for BM donors in the U.K. should be investigated by measuring BM LCPUFA levels as well as other markers, for example antioxidants, before and after providing access to the website. The majority (89.5%) of donors would also consider taking supplements (Casey 2018). Providing donors with complementary supplements could be considered to increase LCPUFA levels. Acceptance, as well as

effectiveness of this should be tested in the U.K. Supplementation of mothers has been shown previously to dose-dependently increase BM DHA levels (Makrides et al. 1996). Supplementation of donors with 1 g DHA/day also increased their DHA levels significantly (Valentine et al. 2013).

LCPUFAs are needed for preterm infants health and development (Koletzko et al. 2001). However, LCPUFA administration is always coupled to the risk of lipid peroxidation (Dyall 2011). This PhD project has shown that the LCPUFA content of non-maternal nutritional sources is associated with lipid peroxidation. Several secondary LPPs in preterm FM as well as in DHM were identified.

Taking this into account, we performed a preliminary analysis of the effects of 4-hydroxyalkenal concentrations expected in preterm FM on neonatal porcine intestinal cells. Preliminary results of this study indicated no detrimental effect of these HHE and HNE concentrations *in vitro*. However, the study had several limitations, including no positive control that elicited a positive inflammatory response, no analysis of tight junctions or permeability of the monolayer, and the use of only one cell type. For future investigations into the effects of 4-hydroxyalkenals on the preterm gut, it is strongly suggested to use transwell plates and co-culture models *in vitro* (Trapecar et al. 2014; Kleiveland 2015), and to consider preterm piglets for *in vivo* studies (Geens and Niewold 2011). It is also suggested to not study HHE and HNE isolated, but to investigate the effects of feeding oxidised FM, which will provide not only further LPPs, but also antioxidants. Further investigations are required, considering that preterm infants receive preterm FM several times per day for an extended period of time, the increased susceptibility of FM fed preterm infants to NEC (Quigley and McGuire 2014), and the correlation of LPPs with the common comorbidities of preterm infants (Inder et al. 1996). Antioxidants can prevent lipid peroxidation and protect LCPUFAs. However, antioxidant levels in DHM are, similarly to LCPUFA levels, also low (Hanson et al. 2016). As mentioned above, milk donors would consider taking supplements, and antioxidant supplementation has been shown to increase BM antioxidant levels (Zarban et al. 2015). Interestingly, supplementation with probiotics can also increase BM antioxidant capacity and decrease MDA levels (Mahdavi et al. 2017). An increase in antioxidants levels, especially in vitamin E, would not only be beneficial for LCPUFA protection in DHM, but also for the preterm infants, which have lower antioxidant capacity (Davis and Auten 2010).

Existence of HNE and TBARS in preterm FM and DHM, as well as hexanal in DHM, can imply that further LPPs, such as lipid hydroperoxides, HHE, and pentanal are present as well, as described by others (Turoli et al. 2004; Michalski et al. 2008; Elisia

and Kitts 2011). Overall, this indicates that non-maternal nutritional sources have the potential to increase the oxidative load of preterm infants, which might contribute to the development of oxygen radical associated diseases (Saugstad 2001). These diseases have also all been associated with higher plasma and/or urinary LPPs (Schlenzig et al. 1993; Weinberger et al. 2004).

Deuterated PUFAs, in which hydrogen is replaced by deuterium at the bis-allylic sites, are less prone to lipid peroxidation and replacement of normal PUFAs in cell membranes with deuterated PUFAs can protect from oxidative stress (Hill et al. 2012). Currently, deuterated PUFAs are used in clinical trials of Friedreich's ataxia and infantile neuroaxonal dystrophy, two diseases related to lipid peroxidation (Adams et al. 2018; Zesiewicz et al. 2018). In the future, the possibility of including these deuterated lipids in preterm FM, or addition to DHM in the clinic, or direct supplementation of preterm infants should be explored, to reduce LCPUFA lipid peroxidation and to potentially provide protection from oxygen radical associated diseases in preterm infants.

In summary, although the optimal dose of LCPUFAs for preterm infants is still not known, our results show that ARA and DHA levels in non-maternal nutritional sources for preterm infants in the U.K., i.e. DHM and preterm FM, are too low and can contribute to the LCPUFA gap of prematurity. DHM should not be used exclusively long-term and use of a BM fortifier is recommended. We have also demonstrated that both non-maternal nutritional sources for preterm infants are a source of LPPs and might increase the oxidative load of preterm infants. Further research is needed to understand whether current LPP levels in nutritional sources are detrimental for preterm infants. We have started to provide evidence-based recommendations how to increase LCPUFA levels and also to prevent lipid peroxidation especially in DHM. Clearly, more research is needed to study and evaluate these recommendations and to fully understand the most detrimental current practices in human milk banking and provision of non-maternal BM to infants. When evaluating our results, we have highlighted the need to report absolute LCPUFA levels to truly capture the effects. Overall, LCPUFA quality and quantity can still be improved in non-maternal sources for preterm infants, which should only be used if maternal BM is unavailable or contraindicated. Every effort should be made to promote and enable lactation and breast feeding.

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9 Appendices

Appendix II.I: Bournemouth University Research Ethics approval – ID 12492

Research Ethics Checklist

Bournemouth University

Reference Id	12492
Status	Approved
Date Approved	22/07/2016

Researcher Details

Name	Isabell Nessel
School	Health and Social Care
Status	Postgraduate Research (MRes, MPhil, PhD, DProf, DEng)
Course	Postgraduate Research
Have you received external funding to support this research project?	No
Please list any persons or institutions that you will be conducting joint research with, both internal to BU as well as external collaborators.	S Dyall (BU), M Khashu (Poole Hospital), Nuffield Students

Project Details

Title	Lipid Peroxidation in Formula Milk in the UK
Proposed Start Date of Data Collection	01/08/2016
Proposed End Date of Project	30/10/2017
Supervisor	Simon Dyall

Summary - no more than 500 words (including detail on background methodology, sample, outcomes, etc.)

See attached document

External Ethics Review

Does your research require external review through the NHS National Research Ethics Service (NRES) or through another external Ethics Committee?	No

Research Literature

Is your research solely literature based?	No

Human Participants

Will your research project involve interaction with human participants as primary sources of data (e.g. interview, observation, original survey)?	No

Final Review

Will you have access to personal data that allows you to identify individuals OR access to confidential corporate or company data (that is not covered by confidentiality terms within an agreement or by a separate confidentiality agreement)?	No
Will your research involve experimentation on any of the following: animals, animal tissue, genetically modified organisms?	No
Will your research take place outside the UK (including any and all stages of research: collection, storage, analysis, etc.)?	No

Please use the below text box to highlight any other ethical concerns or risks that may arise during your research that have not been covered in this form.
The research team will obtain formula milk samples from SMA. However, SMA has no impact on the study design, the research methodology and execution or analysis and publication of the results.

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Appendix II.II: FM data cards

Cow & Gate Nutriprem 1 Low Birthweight Formula

Indications

Cow & Gate Nutriprem 1 Low Birthweight Formula is a food for special medical purposes, for the dietary management of preterm and low birthweight infants. It is part of the nutriprem range, the only complete range of feeds with prebiotic oligosaccharides (OS) specially formulated for preterm and low birthweight infants and has been specially developed and formulated in line with the latest guidelines in preterm nutrition and is compliant with ESPGHAN 2010 recommendations for infants weighing >1kg[1]. It provides energy and nutrient levels which support intrauterine growth rates with a lower volume intake than is required when either breast milk or infant milk is used. It is enriched with prebiotic OS, long chain polyunsaturated fatty acids, arachidonic acid and docosahexaenoic acid (AA & DHA), nucleotides, vitamins and minerals to meet the requirements of growing preterm infants, which should reduce the need for additional nutrient supplementation[1].

Contra-indications

I. Not suitable for intravenous use.

II. Cow & Gate Nutriprem 1 Low Birthweight Formula should only be used under the close medical supervision of a neonatologist or dietitian.

III. The levels of most vitamins and minerals in Cow & Gate Nutriprem 1 Low Birthweight Formula are higher than in infant milks and additional supplements are not usually necessary. If indicated, however, the increased nutrient density of Cow & Gate Nutriprem 1 Low Birthweight Formula should be considered.

IV. Not suitable for vegetarians.

Pack formats and product codes

Cow & Gate Nutriprem 1 Low Birthweight Formula is available in a ready-to-feed liquid format. 70 ml bottles are supplied to hospitals only in boxes of 24.

Preparation and storage

All Cow & Gate products carry preparation instructions. Please see packaging for further information. If fed from bottle discard contents within 1 hour of opening.

Nutricia Ltd, White Horse Business Park, Trowbridge, Wiltshire BA14 0XB.

1. Agostoni C et al JPGN 2010;50:85-91.

Ingredients

Demineralised water, Skimmed **milk**, Lactose (from **milk**), Vegetable oils (Sunflower oil, Rapeseed oil, Coconut oil, Palm oil, MCT oil, Evening primrose oil, Single cell oil) (contains **Soy** lecithin), Glucose syrup, Whey protein concentrate (from **milk**), Galacto-oligosaccharides (GOS) (from **milk**), **Milk** calcium enriched whey product, **Egg** lipids, **Fish** oil, Fructo-oligosaccharides (FOS), Sodium citrate, Sodium chloride, Inositol, Vitamin C, Acidity regulator (citric acid), Calcium phosphate, Calcium hydroxide, Choline chloride, Vitamin E, Iron lactate, Vitamin A, Taurine, Niacin, Cytidine 5'-monophosphate disodium salt, Zinc sulphate, Vitamin D_3, L-carnitine, Pantothenic acid, Uridine 5'-monophosphate disodium salt, Sodium selenite, Adenosine 5'-monophosphate disodium salt, Copper gluconate, Inosine 5'-monophosphate disodium salt, Folic acid, Potassium iodide, Biotin, Guanosine 5'-monophosphate disodium salt, Thiamin, Riboflavin, Vitamin B_6, Vitamin K_1, Manganese sulphate.

Allergy Advice. For allergens, see ingredients in **bold**.

PDS: 1006737

Healthcare professional helpline
0800 996 1234   eln.nutricia.co.uk

Cow & Gate Nutriprem 1 Low Birthweight Formula

Typical values	Units	/100ml	/100kcal
Major Nutrients			
Energy	kJ	335	420
	kcal	80	100
Fat	g	3.9	4.9
of which, saturates	g	1.5	1.9
of which, unsaturates	g	2.4	3
of which, LCPs†	g	0.04	0.05
of which, arachidonic acid (AA)	g	0.02	0.02
of which, docosahexaenoic acid (DHA)	g	0.01	0.02
Carbohydrate	g	8.4	10.5
of which, sugars	g	5.7	7.1
of which, lactose	g	4.7	5.8
Fibre	g	0.6	0.7
Protein	g	2.6	3.3
of which, whey	g	1.6	2
of which, casein	g	1	1.3
Salt	g	0.18	0.2
Vitamins			
Vitamin A	µg RE	361	450
Vitamin D₃	µg	3	3.7
Vitamin E	mg α-TE	3.5	4.3
Vitamin K₁	µg	6	7.5
Vitamin C	mg	17	21.2
Thiamin (B₁)	mg	0.14	0.17
Riboflavin (B₂)	mg	0.2	0.25
Niacin (B₃)	mg NE	3.2	3.99
Pantothenic acid	mg	0.88	1.1
Vitamin B₆	mg	0.12	0.15
Folic acid	µg	35	43.6
Vitamin B₁₂	µg	0.24	0.3
Biotin	µg	3.5	4.36
Minerals			
Sodium	mg	70	87.2
Potassium	mg	82	102
Chloride	mg	76	94.5
Calcium	mg	94	117
Phosphorus	mg	62	77.4
Magnesium	mg	8	10
Iron	mg	1.6	1.99
Zinc	mg	1.1	1.37
Copper	mg	0.08	0.10
Manganese	mg	0.01	0.013
Fluoride	mg	≤0.003	≤0.005
Selenium	µg	4.5	5.61
Iodine	µg	26	32.7
Others			
L-carnitine	mg	1.8	2.2
Choline	mg	17	21.2
Inositol	mg	24	29.9
Taurine	mg	5.5	6.85
Nucleotides	mg	3.2	3.99
GOS/FOS◊	g	0.8	
Osmolarity	mOsmol/l	315	
Osmolality	mOsmol/kg H₂O	375	
Potential renal solute load	mOsmol/l	239	

Fatty Acid Composition		g/100g fatty acids
Saturated		
Butyric acid	C4:0	0.02
Caproic acid	C6:0	0.17
Caprylic acid	C8:0	4.57
Capric acid	C10:0	3.56
Lauric acid	C12:0	10.2
Myristic acid	C14:0	4.23
Palmitic acid	C16:0	11.4
Stearic acid	C18:0	3.25
Arachidic acid	C20:0	0.31
Behenic acid	C22:0	0.35
Lignoceric acid	C24:0	0.07
Unsaturated		
Palmitoleic acid	C16:1 (n-7)	0.19
Oleic acid	C18:1 (n-9)	43.2
Linoleic acid	C18:2 (n-6)	13.7
Alpha-linolenic acid	C18:3 (n-3)	1.97
Gamma-linolenic acid	C18:3 (n-6)	0.21
Eicosaenoic acid	C20:1 (n-9)	0.4
Dihomo-gamma-linolenic acid	C20:3 (n-6)	0.04
Arachidonic acid	C20:4 (n-6)	0.48
Eicosapentaenoic acid	C20:5 (n-3)	0.08
Erucic acid	C22:1 (n-9)	0.09
Docosapentaenoic acid	C22:5 (n-3)	0.02
Docosahexaenoic acid	C22:6 (n-3)	0.37
LA:ALA		7:1

Amino Acid Composition	g/100g protein
L-alanine	4.2
L-arginine	3
L-aspartic acid	6
L-cysteine	2
L-glutamic acid	8.7
Glycine	2.1
L-histidine	2.3
L-isoleucine	5.7
L-leucine	11.1
L-lysine	8.8
L-methionine	2.3
L-phenylalanine	4.3
L-proline	7.7
L-serine	5.3
L-threonine	4.9
L-tryptophan	1.9
L-tyrosine	4.1
L-valine	6

†Long chain polyunsaturated fatty acids.
◊Galacto-oligosaccharides/Fructo-oligosaccharides.

Details correct as of November 2016.

Healthcare professional helpline
0800 996 1234 eln.nutricia.co.uk

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Cow & Gate Nutriprem 2 Post Discharge Formula

Indications

Cow & Gate Nutriprem 2 Post Discharge Formula is a food for special medical purposes. It is a nutrient-rich formulation, which has been developed to meet the increased nutritional needs of preterm and low birthweight infants prior to and once discharged from hospital. It is part of the nutriprem range, the only complete range of feeds with prebiotic oligosaccharides (OS) specially formulated for preterm and low birthweight infants. Cow & Gate Nutriprem 2 Post Discharge Formula has higher levels of nutrients than infant milks to enable optimum catch-up growth and development for preterm infants[1-3]. It can be used for infants until 6 months corrected age. It is nutritionally complete – enriched with prebiotic OS, long chain polyunsaturated fatty acids (LCPs), nucleotides, vitamins and minerals to meet the requirements of growing preterm bottlefed infants after hospital discharge.

Contra-indications

I. Not suitable for intravenous use.

II. Cow & Gate Nutriprem 2 Post Discharge Formula is not a low birthweight formula and should only be used during transitional care, and thereafter in the community.

III. Levels of most vitamins and minerals in Cow & Gate Nutriprem 2 Post Discharge Formula are higher than in infant milks used for term infants. Cow & Gate Nutriprem 2 Post Discharge Formula is nutritionally complete, therefore additional nutrient supplementation may not be needed for bottlefed preterm infants post discharge[4].

IV. Not suitable for vegetarians.

Prescribability

Cow & Gate Nutriprem 2 Post Discharge Formula may be prescribed for catch up growth in preterm (<35 weeks at birth) infants up to 6 months corrected age.

Pack formats and product codes

Cow & Gate Nutriprem 2 Post Discharge Formula is available in powder and liquid formats. 900g EaZypacks are supplied in cases of 6; the product PIP code is 272-4409. 200ml ready-to-feed bottles are available in cases of 12; the product PIP code is 312-6752. It is also available to hospitals only as a sterilised, ready-to-feed milk in a 90ml bottle, supplied in cases of 24.

Nutricia Ltd, White Horse Business Park, Trowbridge, Wiltshire BA14 0XB.

1. Cooke RJ *et al.* Ped Res 1998;43:355-60. 2. Cooke RJ *et al.* Ped Res 1999;46:461-4. 3. Cooke RJ *et al.* Ped Res 2001;49:719-22. 4. Tsang RC *et al.* (Eds). Nutritional needs of the preterm infant scientific basis and practical guidelines. 2nd ed. Cincinnati, OH: Digital Educational Publishing, Inc, 2005. p. 375.

Preparation and storage

All Cow & Gate products carry preparation instructions. Please see packaging for further information. EaZypacks should be stored in a cool, dry place; do not refrigerate, and use contents within four weeks of opening. Ready-to-feed 200ml bottles must be refrigerated immediately after opening and used within 24 hours. If fed from the 90ml ready-to-feed bottle, discard contents within 1 hour of opening. Store ready-to-feed bottles in a cool, dry place away from sunlight before opening.

Ingredients powder

Lactose (from **milk**), Vegetable oils (Sunflower oil, Coconut oil, Rapeseed oil, Palm oil, Evening primrose oil, Single cell oil), Skimmed **milk**, Whey protein concentrate (from **milk**), Glucose syrup, Galacto-oligosaccharides (GOS) (from **milk**), Medium chain triglycerides, **Egg** lipids, **Fish** oil, Fructo-oligosaccharides (FOS), Calcium carbonate, Calcium phosphate, Potassium citrate, Sodium chloride, Magnesium hydrogen phosphate, Inositol, Vitamin C, Choline chloride, Emulsifier (**Soy** lecithin), Taurine, Iron sulphate, Vitamin E, Zinc sulphate, Cytidine 5'-monophosphate, Niacin, Uridine 5'-monophosphate sodium salt, Inosine 5'-monophosphate sodium salt, Pantothenic acid, L-carnitine, Adenosine 5-monophosphate, Vitamin A, Guanosine 5'-monophosphate sodium salt, Copper sulphate, Thiamin, Vitamin B_6, Vitamin D_3, Vitamin B_{12}, Riboflavin, Folic acid, Potassium iodide, Manganese sulphate, Vitamin K_1, Sodium selenite, Biotin.

Ingredients liquid*

Demineralised water, Skimmed **milk**, Lactose (from **milk**), Vegetable oils (Sunflower oil, Rapeseed oil, Coconut oil, Palm oil, MCT oil, Evening primrose oil, Single cell oil) (contains **Soy** lecithin), Galacto-oligosaccharides (GOS) (from **milk**), Whey protein concentrate (from **milk**), Glucose syrup, **Egg** lipids, **Fish** oil, Fructo-oligosaccharides (FOS), Calcium phosphate, Acidity regulator (Citric acid), Potassium chloride, Sodium citrate, Calcium citrate, Vitamin C, Inositol, Calcium hydroxide, Magnesium carbonate, Choline chloride, Vitamin E, Iron lactate, Taurine, Sodium chloride, Potassium hydroxide, Zinc sulphate, Vitamin A, Cytidine 5'-monophosphate, Niacin, Uridine 5'-monophosphate disodium salt, Vitamin D_3, Pantothenic acid, L-carnitine, Inosine 5'-monophosphate disodium salt, Copper gluconate, Adenosine 5-monophosphate, Biotin, Guanosine 5'-monophosphate disodium salt, Potassium iodide, Sodium selenite, Folic acid, Thiamin, Riboflavin, Vitamin B_6, Vitamin B_{12}, Vitamin K_1, Manganese sulphate.

Allergy Advice. For allergens, see ingredients in **bold**.

PDS: 0128790 V2 & 1006738 V2
*Ingredients listed refer to 200ml ready-to-feed bottle.

DATACARD

Cow & Gate Nutriprem 2 Post Discharge Formula

Typical values	Units	Powder* /100g	Powder* /100ml pre-pared feed	Powder* /100kcal	Liquid* /100ml	Liquid* /100kcal
Major Nutrients						
Energy	kJ	2055	315	420	310	420
	kcal	491	75	100	75	100
Fat	g	26	4	5.3	4	5.3
of which, saturates	g	10.9	1.7	2.2	1.6	2.1
of which, unsaturates	g	15.1	2.3	3.1	2.4	3.2
of which, LCPs†	g	0.23	0.03	0.05	0.04	0.05
of which, arachidonic acid (AA)	g	0.11	0.02	0.02	0.02	0.02
of which, docosahexaenoic acid (DHA)	g	0.087	0.01	0.02	0.01	0.02
Carbohydrate	g	49.1	7.5	10	7.4	9.9
of which, sugars	g	41.3	6.3	8.4	6.4	8.6
of which, lactose	g	38.5	5.9	7.8	5.8	7.8
Fibre	g	3.7	0.6	0.7	0.6	0.8
Protein	g	13.4	2	2.7	2	2.7
of which, whey	g	8	1.2	1.6	1.2	1.6
of which, casein	g	5.4	0.8	1.1	0.8	1.1
Salt	g	0.46	0.07	0.09	0.07	0.09
Vitamins						
Vitamin A	µg RE	654	100	133	100	134
Vitamin D₃	µg	11	1.7	2.3	1.7	2.28
Vitamin E	mg α-TE	14	2.1	2.8	2.2	2.95
Vitamin K₁	µg	39	5.9	7.9	5.9	7.91
Vitamin C	mg	79	12	16	12	16.1
Thiamin (B₁)	mg	0.59	0.09	0.12	0.09	0.12
Riboflavin (B₂)	mg	0.98	0.15	0.2	0.15	0.2
Niacin (B₃)	mg	7.9	1.2	1.6	1.2	1.6
Pantothenic acid	mg	3.9	0.6	0.8	0.6	0.8
Vitamin B₆	mg	0.5	0.08	0.11	0.08	0.11
Folic acid	µg	131	20	27	20	26.8
Vitamin B₁₂	µg	1.4	0.22	0.29	0.22	0.3
Biotin	µg	20	3	4	3	4.02
Minerals						
Sodium	mg	183	28	37	28	37.6
	mmol	8	1.2	1.6	1.2	1.6
Potassium	mg	504	77	1.3	77	104
	mmol	12.9	2	2.6	2	2.7
Chloride	mg	360	55	73	52	70.1
	mmol	10.3	1.6	2.1	1.6	2
Calcium	mg	569	87	116	87	117
	mmol	14.2	2.2	2.9	2.2	2.9
Phosphorus	mg	308	47	63	47	63
Phosphate	mmol	9.9	1.5	2	1.5	2
Magnesium	mg	46	7	9.3	7	9.37
	mmol	1.9	0.3	0.4	0.3	0.4
Iron	mg	7.9	1.2	1.6	1.2	1.61
Zinc	mg	5.9	0.9	1.2	0.9	1.21
Copper	mg	0.39	0.06	0.08	0.06	0.08
Manganese	mg	0.05	0.007	0.009	0.01	0.01
Selenium	µg	11	1.7	2.3	1.7	2.28
Iodine	µg	131	20	27	20	26.8
Others						
L-carnitine	mg	5.9	0.9	1.3	0.90	1.2
Choline	mg	85	13	17	13	17.4
Inositol	mg	144	22	29	22	29.5
Taurine	mg	32	4.9	6.5	4.9	6.6
Nucleotides	mg	21	3.2	4.3	3.2	4.3
GOS/FOS◊	g		0.8		0.8	
Osmolarity	mOsmol/l		300		275	
Osmolality	mOsmol/kg H₂O		340		310	
Potential renal solute load	mOsmol/l		180		176	
Scoop weight**	g		5.1			
Standard dilution**	%		15.3			

Fatty Acid Composition**		g/100g fatty acids
Saturated		
Butyric acid	C4:0	0.02
Caproic acid	C6:0	0.2
Caprylic acid	C8:0	5.32
Capric acid	C10:0	3.85
Lauric acid	C12:0	10.9
Myristic acid	C14:0	4.37
Palmitic acid	C16:0	12
Stearic acid	C18:0	3.01
Arachidic acid	C20:0	0.28
Behenic acid	C22:0	0.36
Lignoceric acid	C24:0	0.11
Unsaturated		
Palmitoleic acid	C16:1 (n-7)	0.21
Oleic acid	C18:1 (n-9)	40.7
Linoleic acid	C18:2 (n-6)	13.5
Alpha-linolenic acid	C18:3 (n-3)	1.86
Gamma-linolenic acid	C18:3 (n-6)	0.2
Eicosaenoic acid	C20:1 (n-9)	0.39
Dihomo-gamma-linolenic acid	C20:3 (n-6)	0.04
Arachidonic acid	C20:4 (n-6)	0.46
Eicosapentaenoic acid	C20:5 (n-3)	0.07
Erucic acid	C22:1 (n-9)	0.08
Docosapentaenoic acid	C22:5 (n-3)	0.01
Docosahexaenoic acid	C22:6 (n-3)	0.35
Nervoic acid	C24:1 (n-9)	0.05
LA:ALA		7.3:1

Amino Acid Composition**	g/100g protein
L-alanine	4.2
L-arginine	3.1
L-aspartic acid	10.1
L-cysteine	2
L-glutamic acid	20.3
Glycine	2.1
L-histidine	2.6
L-isoleucine	4.9
L-leucine	11.2
L-lysine	9.3
L-methionine	2.4
L-phenylalanine	4.4
L-proline	7.2
L-serine	5.5
L-threonine	5
L-tryptophan	1.9
L-tyrosine	4.2
L-valine	5.6

*There are small nutritional differences between powder and liquid milks due to different processing methods. Liquid refers to 200ml ready-to-feed.
**Refers to powder.
†Long chain polyunsaturated fatty acids.
◊Galacto-oligosaccharides/Fructo-oligosaccharides.

Details correct as of November 2016.

Healthcare professional helpline
0800 996 1234   eln.nutricia.co.uk

FOR HEALTHCARE PROFESSIONAL USE ONLY

SMA® PRO Gold Prem 1– Preterm and low birthweight formula datacard

☑ Meets ESPGHAN 2010 guidelines[1]

70 ml

Suitable for:

SMA® PRO Gold Prem 1 is a nutritionally complete formula designed for preterm babies weighing less than 1.8 kg.
It is also suitable for those preterm babies requiring higher protein intakes.

Not suitable for:

1. Cows' milk protein intolerance/allergy.
2. Lactose intolerance.
3. Inborn errors of metabolism such as phenylketonuria, galactosaemia and galactokinase deficiency.
4. Vegetarians and Halal diets.

Shelf life:

SMA PRO Gold Prem 1 liquid has a shelf life of 9 months.

70 ml

Important notice:

The World Health Organisation (WHO) has recommended that pregnant women and new mothers be informed on the benefits and superiority of breastfeeding – in particular the fact that it provides the best nutrition and protection from illness for babies. Mothers should be given guidance on the preparation for, and maintenance of, lactation, with special emphasis on the importance of a well-balanced diet both during pregnancy and after delivery. Unnecessary introduction of partial bottle-feeding or other foods and drinks should be discouraged since it will have a negative effect on breastfeeding. Similarly, mothers should be warned of the difficulty of reversing a decision not to breast-feed. Before advising a mother to use an infant formula, she should be advised of the social and financial implications of her decision: for example, if a baby is exclusively bottle-fed, more than one can (400 g) per week will be needed, so the family circumstances and costs should be kept in mind. Mothers should be reminded that breast milk is not only the best, but also the most economical food for babies. **This product must be used under medical supervision. SMA® PRO Gold Prem 1** is a special formula intended for the dietary management of preterm and low birthweight babies who are not solely fed breast milk.

We're here for you
UK 0800 0 81 81 80
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SMA Nutrition, 1 City Place, Gatwick, RH6 0PA
In the Republic of Ireland: SMA Nutrition,
3030 Lake Drive, Citywest Business Campus,
Dublin 24, Ireland
ZTC1389/08/16

Updated August 2016
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Nutritional information for SMA® PRO Gold Prem 1

	Units	Per 100 ml	Per 100 kcal
Energy	kJ	335	419
	kcal	80	100
Fat	g	4	5
of which, saturates	g	1.7	2
of which, unsaturates	g	2.3	3
Carbohydrate	g	8.1	10.2
of which, sugars	g	3.7	4.6
of which, lactose	g	3.7	4.6
Protein	g	2.9	3.6
Salt* (=sodium x2.5)	g	0.13	0.16
Vitamins			
Vitamin A	µg	370	462
Vitamin D	µg	3.7	4.7
Vitamin E	mg	3.6	4.5
Vitamin K	µg	6.4	8.1
Vitamin C	mg	20.8	26
Thiamin	mg	0.14	0.17
Riboflavin	mg	0.2	0.25
Niacin	mg	1.6	2
Vitamin B$_6$	mg	0.09	0.12
Folic acid	µg	40.6	50.6
Vitamin B$_{12}$	µg	0.23	0.29
Biotin	µg	4	4.9
Pantothenic acid	mg	0.8	1
Minerals			
Sodium	mg	51	64
Potassium	mg	120	149
Chloride	mg	76	95
Calcium	mg	116	145
Phosphorus	mg	77	96
Magnesium	mg	8.3	10.4
Iron	mg	1.8	2.3
Zinc	mg	1.2	1.5
Copper	mg	0.08	0.1
Manganese	mg	0.01	0.02
Fluoride	mg	≤0.01	≤0.01
Selenium	µg	4.8	6
Chromium	µg	≤2.5	≤3.1
Molybdenum	µg	≤3.1	≤3.9
Iodine	µg	28	35
Others			
Taurine	mg	6.3	8
Choline	mg	20	25
Inositol	mg	20	25
L-carnitine	mg	3.1	4
Nucleotides†	mg	2.4	3
Omega 3:			
α-linolenic acid (ALA)‡‡	mg	77	96
Docosahexaenoic acid (DHA)∅	mg	15	18
Omega 6:			
Linoleic acid (LA)‡‡	mg	561	701
Arachidonic acid (AA)∅	mg	15	18
Osmolality	mOsmol/kg H$_2$O	308	
Potential Renal Solute Load	mOsm/L	256	
MCT (Medium chain triglycerides)	g	1.58	1.97
	39.5 % of total fat content		

Typical fatty acid profile of SMA® PRO Gold Prem 1

Fatty acid		Units	Per 100 ml
Saturated			
Caprylic	C8:0	mg	743
Capric	C10:0	mg	598
Lauric	C12:0	mg	9.4
Myristic	C14:0	mg	12.5
Palmitic	C16:0	mg	369
Stearic	C18:0	mg	60.4
Arachidic	C20:0	mg	8.34
Behenic	C22:0	mg	8.34
Lignoceric	C24:0	mg	3.13
Total saturated		g	1.8
Unsaturated/Monounsaturated			
Palmitoleic	C16:1	mg	4
Oleic	C18:1	mg	1177
Eicosanoic	C20:1	mg	16.7
Total monounsaturated		g	1.19
Polyunsaturated			
Linoleic	C18:2	mg	561
Linolenic	C18:3	mg	77
Arachidonic	C20:4	mg	15
Docosahexaenoic	C22:6	mg	15
Total polyunsaturated		g	0.65
Linoleic: linolenic ratio	7:1		

Typical amino acid profile of SMA PRO Gold Prem 1

Amino Acid	mg per 100 ml
Essential & Semi-Essential Amino Acids	
Arginine	118
Cystine	79
Histidine	117
Isoleucine	154
Leucine	354
Lysine	298
Methionine	63
Phenylalanine	99
Tryptophan	67
Tyrosine	95
Valine	142
Other Amino Acids	
Aspartic acid	338
Serine	117
Glutamic acid	498
Glycine	53
Proline	137
Alanine	155

SMA® PRO Gold Prem 1 ingredients

Liquid (bottle)

Water, maltodextrin, vegetable oils including medium chain triglycerides (rapeseed, sunflower, palm), lactose (**milk**), whey protein (**milk**), acidity regulators (phosphoric acid, citric acid), calcium hydroxide, potassium chloride, long chain polyunsaturated fatty acids (AA, DHA), potassium hydroxide, L-histidine, magnesium chloride, sodium hydroxide, L-arginine, choline bitartrate, potassium phosphate, vitamin C, inositol, ferrous sulphate, taurine, vitamin E, zinc sulphate, L-carnitine, nucleotides (cytidine-, disodium uridine-, adenosine-, disodium guanosine-5'-monophosphate), niacin, antioxidants (ascorbyl palmitate, tocopherol-rich extract), pantothenic acid, vitamin A, copper sulphate, thiamin, riboflavin, vitamin B$_6$, vitamin D, vitamin K, folic acid, vitamin B$_{12}$, biotin, potassium iodide, manganese sulphate, sodium selenate.

Reference:
1. Agostoni C et al. Enteral Nutrient Supply for Preterm Infants: Commentary from the European Society for Paediatric Gastroenterology, Hepatology and Nutrition Committee on Nutrition. J Pediatr Gastroenterol Nutr 2010; 50: 1–9.

* Salt is calculated as sodium x 2.5. Sodium is present for nutritional purposes.
† Nucleotides value represents innate and fortified levels.
‡‡ beneficial effect of essential fatty acids is obtained with a daily intake of 10 g of linoleic acid and 2 g of α-linolenic acid.
∅ LCPs= Long Chain Polyunsaturates.

FOR HEALTHCARE PROFESSIONAL USE ONLY

SMA® PRO Gold Prem 2 post-discharge formula data card

Suitable for:

SMA® PRO Gold Prem 2 is a nutritionally complete post-discharge formula for preterm and low birthweight infants on discharge from hospital, until 6 months corrected age. SMA PRO Gold Prem 2 is designed to bridge the gap between low birthweight and term infant formula. It may be used as the sole source of nutrition until complementary feeding commences.

Not suitable for:

1. Cows' milk protein intolerance/allergy.
2. Lactose intolerance.
3. Inborn errors of metabolism such as phenylketonuria, galactosaemia and galactokinase deficiency.
4. Vegetarians and Halal diet.

Shelf life:

SMA PRO Gold Prem 2 powder has a shelf life of 24 months.
SMA PRO Gold Prem 2 liquid has a shelf life of 9 months.

400 g   200 ml   90 ml

Scoop size – 4.8 g

✓ ACBS† approved (400 g and 200 ml available on prescription)

400 g   200 ml   90 ml

Important notice:

The World Health Organisation (WHO) has recommended that pregnant women and new mothers be informed on the benefits and superiority of breastfeeding – in particular the fact that it provides the best nutrition and protection from illness for babies. Mothers should be given guidance on the preparation for, and maintenance of, lactation, with special emphasis on the importance of a well-balanced diet both during pregnancy and after delivery. Unnecessary introduction of partial bottle-feeding or other foods and drinks should be discouraged since it will have a negative effect on breastfeeding. Similarly, mothers should be warned of the difficulty of reversing a decision not to breastfeed. Before advising a mother to use an infant formula, she should be advised of the social and financial implications of her decision: for example, if a baby is exclusively bottle-fed, more than one can (400 g) per week will be needed, so the family circumstances and costs should be kept in mind. Mothers should be reminded that breast milk is not only the best, but also the most economical food for babies. This product must be used under medical supervision. SMA PRO Gold Prem 2 is a special catch-up formula intended for the dietary management of preterm and low birthweight babies who are not solely fed breast milk. It is a nutritionally complete formula for use on discharge from hospital or when a low birthweight formula is no longer appropriate. It is suitable for use as the sole source of nutrition up to 6 months corrected age. SMA PRO Gold Prem 2 is not intended for use with newborn preterm babies, for whom fortified breast milk or a low birthweight formula such as SMA PRO Gold Prem 1 is more appropriate.

We're here for you
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ZTC1409/08/16

Updated August 2016
†Advisory Committee on Borderline Substances
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Nutritional information for SMA® PRO Gold Prem 2
(Information based on powder only)

	Units	Per 100 ml	Per 100 kcal	Per 100 g powder
Energy	kJ	305	418	2121
	kcal	73	100	507
Fat	g	3.8	5	26
of which, saturates	g	1	1.4	7
Carbohydrate	g	7.7	11	53
of which, sugars	g	5.5	7.6	39
of which, lactose	g	5.3	7.3	37
Protein	g	2	2.8	14
of which, whey	g	2	2.8	14
Salt* (=Sodium x 2.5)	g	0.09	0.13	0.6
Vitamins				
Vitamin A	µg	92	125	636
Vitamin D	µg	1.2	1.7	8.5
Vitamin E	mg	1.7	2.3	12
Vitamin K	µg	6.5	9	45
Vitamin C	mg	12.8	18	89
Thiamin	mg	0.1	0.15	0.8
Riboflavin	mg	0.19	0.3	1.3
Niacin	mg	0.7	1	5.1
Vitamin B₆	mg	0.07	0.1	0.5
Folic acid	µg	13	17.4	88
Vitamin B₁₂	µg	0.24	0.33	1.7
Biotin	µg	2.2	3	15
Pantothenic acid	mg	0.7	1	5.1
Minerals				
Sodium	mg	37	50	254
Potassium	mg	77	105	532
Chloride	mg	48	66	334
Calcium	mg	80	110	558
Phosphorus	mg	48	65	330
Magnesium	mg	8.6	12	60
Iron	mg	0.8	1	5.3
Zinc	mg	0.9	1.2	6.1
Copper	mg	0.06	0.08	0.4
Manganese	mg	0.01	0.02	0.08
Selenium	µg	2	3	14.5
Iodine	µg	17	24	120
Others				
Omega 3				
α-linolenic acid (ALA)	mg	75	103	522
Docosahexaenoic acid (DHA)†	mg	14.4	20	100
Omega 6				
Linoleic acid (LA)	mg	573	785	3980
Arachidonic acid (AA)†	mg	14.4	20	100
Taurine	mg	5.8	8	41
Choline	mg	13	18	90
Inositol	mg	14	20	100
L-Carnitine	mg	1.2	1.6	8
Nucleotides	mg	2.2	3	15.6

Typical fatty acid profile of SMA® PRO Gold Prem 2
(Information based on powder only)

Fatty Acid		Units	Per 100 ml
Saturated			
Caprylic	C8:0	mg	29.2
Capric	C10:0	mg	23.5
Lauric	C12:0	mg	174.2
Myristic	C14:0	mg	80.5
Palmitic	C16:0	mg	563
Stearic	C18:0	mg	117.2
Arachidic	C20:0	mg	10.7
Behenic	C22:0	mg	11.7
Total saturated		g	1.02
Unsaturated/Monounsaturated			
Palmitoleic	C16:1	mg	4
Oleic	C18:1	mg	1555.1
Eicosanoic	C20:1	mg	15.4
Total monounsaturated		g	1.58
Polyunsaturated			
Linoleic	C18:2	mg	573
Linolenic	C18:3	mg	75
Arachidonic	C20:4	mg	14.4
Docosahexaenoic	C22:6	mg	14.4
Total polyunsaturated		g	0.68
Linoleic: linolenic ratio			8:1

Typical amino acid profile of SMA PRO Gold Prem 2
(Information based on powder only)

Amino Acid	mg per 100 ml
Essential & Semi-Essential Amino Acids	
Arginine	63
Cystine	59
Histidine	42
Isoleucine	124
Leucine	256
Lysine	212
Methionine	45
Phenylalanine	73
Threonine	126
Tryptophan	42
Tyrosine	57
Valine	118
Aspartic acid	255
Serine	98
Glutamic acid	370
Proline	106
Glycine	39
Alanine	111

mmol information for SMA® PRO Gold Prem 2
(Information based on powder only)

Nutrient	Per 100 g powder (mmol)	Per 100 ml (mmol)
Calcium	14	2
Phosphate	11	1.5
Magnesium	2.5	0.4
Iron	0.09	0.01
Zinc	0.09	0.01
Sodium	11	1.6
Potassium	14	2
Chloride	10	1.4

SMA PRO Gold Prem 2 ingredients Powder

Lactose (**milk**), vegetable oils including structured vegetable oil (sunflower, palm, rapeseed, coconut), maltodextrin, partially hydrolysed whey protein (**milk**), calcium phosphate, long chain polyunsaturated fatty acids (AA, DHA), potassium hydroxide, magnesium chloride, potassium phosphate, sodium hydroxide, potassium chloride, choline bitartrate, calcium chloride, vitamin C, inositol, L-arginine, acidity regulator (citric acid), taurine, ferrous sulphate, nucleotides (cytidine-, disodium uridine-, adenosine-, disodium guanosine-5'-monophosphate), vitamin E, zinc sulphate, L-carnitine, antioxidants (ascorbyl palmitate, tocopherol-rich extract), pantothenic acid, niacin, riboflavin, copper sulphate, thiamin, vitamin A, vitamin B₆, manganese sulphate, folic acid, potassium iodide, vitamin D, vitamin K, vitamin B₁₂, biotin, sodium selenate.

Osmolality (powder): 290 mOsm/kg H₂O

Liquid (90 ml and 200 ml bottles)

Water, partially hydrolysed whey protein (**milk**), vegetable oils including structured vegetable oil (sunflower, palm, rapeseed, coconut), lactose (**milk**), maltodextrin, calcium phosphate, potassium hydroxide, long chain polyunsaturated fatty acids (AA, DHA), potassium chloride, magnesium chloride, acidity regulator (citric acid), vitamin C, sodium hydroxide, sodium chloride, choline bitartrate, L-arginine, inositol, taurine, ferrous sulphate, nucleotides (cytidine-, disodium uridine-, adenosine-, disodium guanosine-5'-monophosphate), zinc sulphate, vitamin E, L-carnitine, antioxidants (ascorbyl palmitate, tocopherol-rich extract), niacin, pantothenic acid, copper sulphate, vitamin A, thiamin, riboflavin, vitamin B₆, vitamin D, vitamin K, folic acid, vitamin B₁₂, biotin, potassium iodide, manganese sulphate, sodium selenate.

Osmolality (liquid): 296 mOsm/kg H₂O

* Salt is calculated as sodium x 2.5. Sodium is present for nutritional purposes.
† LCPs = Long Chain Polyunsaturates.

Appendix III.I: Bournemouth University Research Ethics approval – ID 16130

Research Ethics Checklist

Bournemouth University

Reference Id	16130
Status	Approved
Date Approved	24/08/2017

Researcher Details

Name	Isabell Nessel
School	Health and Social Care
Status	Postgraduate Research (MRes, MPhil, PhD, DProf, DEng)
Course	Postgraduate Research - HSC
Have you received external funding to support this research project?	No
Please list any persons or institutions that you will be conducting joint research with, both internal to BU as well as external collaborators.	Dr Simon Dyall (BU), Prof Minesh Khashu (Poole NHS), Dr Caroline Childs (University of Southampton), Prof Graham Burdge (University of Southampton), University of Southampton

Project Details

Title	Investigation of the effects of lipid peroxides on a neonatal jejunum cell line (IPEC-J2) in relation to necrotising enterocolitis
Proposed Start Date of Data Collection	10/07/2017
Proposed End Date of Project	30/11/2017
Original Supervisor	Simon Dyall
Approver	Research Ethics Panel

Printed On 24/08/2017 10:46:00

Summary - no more than 500 words (including detail on background methodology, sample, outcomes, etc.)
See attached form

External Ethics Review

Does your research require external review through the NHS National Research Ethics Service (NRES) or through another external Ethics Committee?	No

Research Literature

Is your research solely literature based?	No

Human Participants

Will your research project involve interaction with human participants as primary sources of data (e.g. interview, observation, original survey)?	No

Final Review

Will you have access to personal data that allows you to identify individuals OR access to confidential corporate or company data (that is not covered by confidentiality terms within an agreement or by a separate confidentiality agreement)?	No
Will your research involve experimentation on any of the following: animals, animal tissue, genetically modified organisms?	Yes
Will your research take place outside the UK (including any and all stages of research: collection, storage, analysis, etc.)?	No

Please use the below text box to highlight any other ethical concerns or risks that may arise during your research that have not been covered in this form.

Appendix IV.I: HRA approval letter – IRAS ID 205058

NHS
Health Research Authority

Miss Isabell Nessel
Room 304 Royal London House
Christchurch Road
Bournemouth
BH13LT

Email: hra.approval@nhs.net

03 November 2016

Dear Miss Nessel

Letter of HRA Approval

Study title: Exploring human milk banking practices in the U.K.
including provision of nutritional advice/information –
especially with respect to omega-3 and omega-6 fatty acids
IRAS project ID: 205068
Protocol number: N/A
REC reference: 16/HRA/5005
Sponsor Bournemouth University

I am pleased to confirm that **HRA Approval** has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications noted in this letter.

Participation of NHS Organisations in England
The sponsor should now provide a copy of this letter to all participating NHS organisations in England.

Appendix B provides important information for sponsors and participating NHS organisations in England for arranging and confirming capacity and capability. **Please read** *Appendix B* **carefully**, in particular the following sections:

- *Participating NHS organisations in England* – this clarifies the types of participating organisations in the study and whether or not all organisations will be undertaking the same activities
- *Confirmation of capacity and capability* - this confirms whether or not each type of participating NHS organisation in England is expected to give formal confirmation of capacity and capability. Where formal confirmation is not expected, the section also provides details on the time limit given to participating organisations to opt out of the study, or request additional time, before their participation is assumed.
- *Allocation of responsibilities and rights are agreed and documented (4.1 of HRA assessment criteria)* - this provides detail on the form of agreement to be used in the study to confirm capacity and capability, where applicable.

Further information on funding, HR processes, and compliance with HRA criteria and standards is also provided.

Page 1 of 9

It is critical that you involve both the research management function (e.g. R&D office) supporting each organisation and the local research team (where there is one) in setting up your study. Contact details and further information about working with the research management function for each organisation can be accessed from www.hra.nhs.uk/hra-approval.

Appendices

The HRA Approval letter contains the following appendices:

- A – List of documents reviewed during HRA assessment
- B – Summary of HRA assessment

After HRA Approval

The attached document *"After HRA Approval – guidance for sponsors and investigators"* gives detailed guidance on reporting expectations for studies with HRA Approval, including:

- Working with organisations hosting the research
- Registration of Research
- Notifying amendments
- Notifying the end of the study

The HRA website also provides guidance on these topics and is updated in the light of changes in reporting expectations or procedures.

Scope

HRA Approval provides an approval for research involving patients or staff in NHS organisations in England.

If your study involves NHS organisations in other countries in the UK, please contact the relevant national coordinating functions for support and advice. Further information can be found at http://www.hra.nhs.uk/resources/applying-for-reviews/nhs-hsc-rd-review/.

If there are participating non-NHS organisations, local agreement should be obtained in accordance with the procedures of the local participating non-NHS organisation.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please email the HRA at hra.approval@nhs.net. Additionally, one of our staff would be happy to call and discuss your experience of HRA Approval.

HRA Training

We are pleased to welcome researchers and research management staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

Your IRAS project ID is **205068**. Please quote this on all correspondence.

Yours sincerely

Miss Helen Penistone
Assessor

Email: hra.approval@nhs.net

Copy to: Dr Fiona Knight, Bournemouth University, (sponsor contact)
             fknight@bournemouth.ac.uk

             Participating NHS organisations in England

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Appendix IV.II: NHS GG&C (Scotland) approval letter – IRAS ID 205058

Administrator: Mrs Elaine O'Neill
Telephone Number: 0141 232 1815
E-Mail: elaine.o'neill2@ggc.scot.nhs.uk
Website: www.nhsggc.org.uk/r&d

R&D Management Office
West Glasgow ACH
Dalnair Street
Glasgow G3 8SW

9 January 2017

Miss Isabell Nessel
Postgrad Researcher
Bournemouth University
Royal London House, Room 304
Christchurch Road
Bournemouth
BH1 3LT

NHS GG&C Board Approval

Dear Miss I Nessel,

Study Title:	**Exploring human milk banking practices in the U.K. including provision of nutritional advice/information – especially with respect to omega-3 and omega-6 fatty acids**
Principal Investigator:	**No local investigator**
GG&C HB site	**Queen Elizabeth University Hospital**
Sponsor	**Bournemouth University**
R&D reference:	**GN16HS659**
REC reference:	**n/a**
Protocol no:	**V2; 23/08/16**

I am pleased to confirm that Greater Glasgow & Clyde Health Board is now able to grant **Approval** for the above study.

Conditions of Approval

1.   **For Clinical Trials** as defined by the Medicines for Human Use Clinical Trial Regulations, 2004

        a. During the life span of the study GGHB requires the following information relating to this site
            i. Notification of any potential serious breaches.
           ii. Notification of any regulatory inspections.

It is your responsibility to ensure that all staff involved in the study at this site have the appropriate GCP training according to the GGHB GCP policy (www.nhsggc.org.uk/content/default.asp?page=s1411), evidence of such training to be filed in the site file.
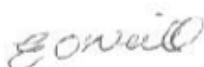
2.   **For all studies** the following information is required during their lifespan.
   a.   Recruitment Numbers on a monthly basis
   b.   Any change of staff named on the original SSI form
   c.   Any amendments – Substantial or Non Substantial
   d.   Notification of Trial/study end including final recruitment figures
   e.   Final Report & Copies of Publications/Abstracts

Please add this approval to your study file as this letter may be subject to audit and monitoring.

Your personal information will be held on a secure national web-based NHS database.

I wish you every success with this research study

Yours sincerely,

E O'Neill

Mrs Elaine O'Neill
Senior Research Administrator

Fiona Knight (Bournemouth University)

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Appendix IV.III: Research Gateway (Northern Ireland) approval letter – IRAS ID 205058

HSC Western Health and Social Care Trust

24 April 2017

Ms Isabell Nessel
Faculty of Health and Social Sciences
Bournemouth University
Room 304 Royal London House
Christchurch Road
Bournemouth
BH1 3LT

Dear Ms Nessel

Study Title: Exploring human milk banking practices in the UK
HSC Trust Ref: WT 16/54 (Please quote this number in all future correspondence)
IRAS Ref: 205068
REC Ref: n/a

I am pleased to advise that WHSCT has given Final Research Governance Permission for the above project to commence. Permission is granted for the duration of the project to 01/06/2017.

The following documents have been approved for use in the project:

Document	Version	Date
Invitation email	V 3	23/08/16
Human milk bank survey	V 7	22/08/16
Donor human milk storage time spreadsheet	V 1	25/08/16
Survey information sheet	V 5	02/11/16
Protocol	V 2	23/08/16
Protocol diagram	V 1	23/08/16

Research & Development Office, C-TRIC,
Altnagelvin Area Hospital, Londonderry, BT47 6SB
DDI 02871 611156
02871 345171 EXT 216603/4

Appendix IV.IV: Bournemouth University's Research Ethics approval – IRAS ID 205058

	Research Ethics Checklist
Bournemouth University	

About Your Checklist

Reference Id	13436
Status	Approved
Date Approved	18/05/2017 14:49:57
Date Submitted	02/05/2017 11:53:24

Researcher Details

Name	Isabell Nessel
Faculty	Faculty of Health & Social Sciences
Status	Postgraduate Research (MRes, MPhil, PhD, DProf, EngD, EdD)
Course	Postgraduate Research - HSS
Is This External Funding?	No
Please list any persons or institutions that you will be conducting joint research with, both internal to BU as well as external collaborators.	Dr Simon Dyall, Prof Minesh Khashu, Gillian Weaver

Project Details

Title	Exploring human milk banking practices in the U.K. including provision of nutritional advice/information - especially with respect to omega-3 and omega-6 fatty acids
End Date of Project	15/09/2017
Proposed Start Date of Data Collection	14/11/2016
Original Supervisor	Simon Dyall
Approver	Research Ethics Panel

Summary - no more than 500 words (including detail on background methodology, sample, outcomes, etc.)

Human breast milk is the first choice for feeding neonates, especially preterm infants, in whom, apart from other benefits, breast milk is known to decrease the risk of necrotising enterocolitis. If mothers are unable to provide sufficient breast milk for their babies, donor human milk (DHM) can be used. In 2013, 60.7% of the neonatal units in the U.K. reported the use of DHM. One of the most important components of human breast milk is fat. Apart from energy supply, the omega-3 and omega-6 long-chain polyunsaturated fatty acids (LCPUFA) docosahexaenoic acid (DHA) and arachidonic acid (AA) are essential for cognitive and visual development, infant growth and regulation of the inflammatory response. However, previous research has highlighted that processes involved in human milk banking, e.g. storage time and temperature, can significantly alter the fat content of DHM and may have an impact on the nutritional quality of DHM. Despite this, there is no specific national guidance focusing on the nutritional quality of DHM. Therefore, we aim to ascertain human milk banking practices in the U.K., through a mixed-method questionnaire, which will be sent to all human milk banks (HMB) in the U.K. Specific questions about donor selection, milk transportation and storage, and dietary advice are included, and it takes approximately 30 minutes to complete the survey. Quantitative aspects will be analysed using descriptive statistics, whereas the qualitative aspects will be explored by thematic analysis to create a representative national perspective. This will help to identify best practice as well as any variations in practice. Results will be shared with the United Kingdom Association for Milk Banking (UKAMB) and through conference presentations and publications as well as published as part of a PhD thesis. The study is sponsored by Bournemouth University and is being supported by UKAMB.

Page 1 of 2   Printed On 14/11/2019 09:17:07

External Ethics Review

Does your research require external review through the NHS National Research Ethics Service (NRES) or through another external Ethics Committee?	Yes

Please ensure that the researcher obtains external ethical approval before commencing research.

Attached documents

WT final permission letter. signed.pdf - attached on 02/05/2017 09:39:14

Board Approval GN16HS659.pdf - attached on 02/05/2017 09:39:14

205068_Letter_of_HRA_Approval 20161103.pdf - attached on 02/05/2017 09:39:14

DMB Invitation e-mail_V3.pdf - attached on 02/05/2017 10:36:56

Donor Milk Bank Survey _V7.pdf - attached on 02/05/2017 10:36:56

Spreadsheet V1 25082016.pdf - attached on 02/05/2017 10:36:56

Diagram of the protocol.pdf - attached on 02/05/2017 10:36:56

Research Protocol_Human milk banking pratices in the UK_V2 (2).pdf - attached on 02/05/2017 10:36:56

Survey Information Sheet V5.pdf - attached on 02/05/2017 10:36:56

Human Milk Banks_205068_V1.docx - attached on 02/05/2017 10:36:56

IRASForm_snapshot_205068_Human milk banking practices in the UK.pdf - attached on 02/05/2017 10:36:56

hra-schedule-events_205068_Human milk banking in the UK_Version 1 20161102.xls - attached on 02/05/2017 10:36:56

statement-activities_205068_Human milk banking in the UK_Version1 20161102.docx - attached on 02/05/2017 10:36:57

Appendix IV.V: List of Human Milk Banks in the U.K.

Human Milk Banks

#	Name	NHS Trust
1	NHS Greater Glasgow and Clyde Donor Milk Bank	NHS Greater Glasgow and Clyde, Queen Elizabeth University Hospital Maternity Unit, 1345 Govan Road, Glasgow G51 4TF
2	The Human Milk Bank	Western Health and Social Care Trust, Unit 2, The Cornsheads, Mill Street, Irvinestown, Co. Fermanagh BT94 1GR
3	Calderdale and Huddersfield Donor Breast Milk Bank	The Neonatal Unit, Calderdale Royal Hospital, Salterhebble, Halifax, West Yorkshire HX3 0PW
4	Northwest Human Milk Bank	(NoWFOOD Centre, University of Chester, Parkgate Road, Chester CH1 4BJ) Countess of Chester Hospital
5	Neonatal Unit – Addenbrooke's NHS Trust	Addenbrooke's NHS Trust, The Rosie Hospital, Robinson Way, Cambridge CB2 2SW
6	Birmingham Women's Hospital Milk Bank	Birmingham Women's NHS Foundation Trust, Mindelsohn Way, Edgbaston, Birmingham B15 2TG
7	Oxford Human Milk Bank	John Radcliffe Hospital, Oxford University Hospitals, Headley Way, Headington Oxford OX3 9DU
8	St George's Hospital	St George's Hospital, Neonatal Unit, Blackshaw Road, Tooting, London SW17 0QT
9	Guy's and St Thomas' NHS Foundation Trust	Guy's and St Thomas' NHS Foundation Trust Lambeth Palace Road, London, SE1 7EH
10	Queen Charlotte's and Chelsea Hospital	Imperial College Healthcare NHS Trust, Du Cane Road, London, W12 0HS
11	St Peter's Hospital Milk Bank	Ashford & St Peter's Hospitals NHS Trust NICU, St Peter's Hospital, Guildford Road, Chertsey, Surrey KT16 0PZ
12	King's College Hospital Milk Bank	King's College Hospital Milk Bank, Denmark Hill, London SE5 9RS
13	The Precious Drops Milk Bank (Bristol)	Southmead Hospital Bristol BS10 5NB
14	Southampton Human Milk Bank	Princess Anne Hospital, Coxford Rd, Southampton, Hants SO16 5YA
15	Medway Maritime Hospital Milk Bank	NICU, Medway Maritime Hospital, Windmill Road, Gillingham, Kent, ME7 5NY
16	The Hearts Milk Bank	N/A (Non-NHS Milk Bank) Biopark, Broadwater Road Welwyn Garden City Herts AL7 3AX

Appendix IV.VI: Human Milk Bank Survey

Bournemouth University

U.K. Human Milk Bank Survey

Identification Number for this survey

Thank you for taking your time to respond to this questionnaire. It will take you approximately 30 minutes to complete. Please tick the appropriate box(es) and use the free text space to provide further information, as appropriate. If you have any questions, please contact the research team. Contact details can be found at the end of the questionnaire.

The following questions are about the general selection criteria for donors at the human milk bank. We would like to learn about the process and procedures used for selecting donors at your human milk bank.

Selecting milk donors

1.1 **What is the latest starting point for mothers to donate milk after birth of their child at your milk bank?**

☐ Up to _____months

    ☐ Why? _____

☐ There are no set requirements for the starting point of milk donation

1.2 **Can mothers of multiple pregnancies donate their milk to your human milk bank?**

☐ Yes

☐ No

    ☐ Why? _____

☐ Mothers will not be asked if they have multiple children

1.3 **Can mothers who are tandem feeding (simultaneously feeding a new-born and an older child) donate their milk to your human milk bank?**

☐ Yes

☐ No

    ☐ Why? _____

☐ Mothers will not be asked if they do tandem feeding

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1.4 **Are potential donors asked about their dietary habits (besides alcohol consumption) at your milk bank?**

☐ Yes, before their first milk donation

☐ Yes, during the time of milk donations

☐ Yes, before and during the time of milk donation

☐ No, not at all

If donors are asked about their dietary habits, which aspects is your human milk bank interested in? Please provide details below.

1.5 **Does your human milk bank exclude mothers from donations if they follow a restricted diet?**

☐ No

☐ Yes

If Yes, please answer the following, if No please move to the next question

☐ Vegetarians without dietary supplementation will be excluded from donations

    ☐ Please specify which supplements they need to take to be eligible for donating milk: _____ _____

    _____ _____

☐ Vegetarians with dietary supplementation will be excluded from donations

☐ Vegans without dietary supplementation will be excluded from donations

    ☐ Please specify which supplements they need to take to be eligible for donating milk: _____ _____

    _____ _____

☐ Vegans with dietary supplementation will be excluded from donations

☐ Others will be excluded from donations

    ☐ Please specify:_____

1.6 **For how long can mothers donate milk to your human milk bank after giving birth to their child?**

☐ Up to _____months after the birth

   ☐ Why? _____

☐ There are no set requirements for the maximum period of milk donation

The following questions are about the nutritional information given to donors at human milk banks. We would like to learn about the nutritional information available at your human milk bank.

<u>Nutritional information</u>

2.1 **Does your human milk bank hand out leaflets/information to milk donors about a healthy diet for breastfeeding mothers?**

☐ Yes, in general

☐ Yes, if donor asks for information

☐ No, we don't have information material available

If your human milk bank provides information about a healthy diet for breastfeeding mothers please specify below which information or attach (a scan of) the leaflet and specify where you receive them from

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2.2 **Is there a registered dietician available for consultation?**

☐ Yes, we have a registered dietician at the human milk bank

☐ Yes, there is a registered dietician at the hospital which can be consulted

☐ No, there is no registered dietician available

☐ Other, please specify: _____

The following questions are about the general advice given by the human milk bank to mothers about handling expressed milk for donation.

Handling of donor human milk by mothers

3.1 **How does the human milk bank advise mothers to store their expressed milk? (please tick as many boxes as appropriate)**

☐ Expressed milk should be immediately frozen as best practice

☐ Expressed milk can be pooled over 24 hours in the refrigerator if necessary and should be frozen afterwards

☐ Expressed milk can be pooled up to 48 hours in the refrigerator if necessary and should be frozen afterwards

If pooling of milk over a certain period in the fridge is allowed, does the human milk bank give advice where to store the expressed milk or at what temperature?

☐ Yes

    ☐ In the door

    ☐ On a shelf

    ☐ Please specify the temperature: _____°C

☐ No

3.2 **How long does the human milk bank allow donors to store the frozen milk for donation at home, before the milk reaches the human milk bank?**

☐ Less than 1 week

☐ 1 to 2 weeks

☐ 2 to 4 weeks

☐ 1 to 2 months

☐ 2 to 3 months

☐ Over 3 months

3.3 **Is there a minimum amount of donor human milk which mothers need to provide before the milk can be donated to your human milk bank?**

☐ Yes

    ☐ Please specify: _____mL

    ☐ Why? _____

☐ No

3.4 Does your human milk bank record the storage time for frozen milk at mothers' homes, before the milk reaches the human milk bank? If so, could you provide a frequency distribution for the last year?

☐ Storage time is not recorded

☐ Storage time is recorded, but not consolidated. If so please give estimations below

☐ Less than 1 week_____% of the time

☐ 1 to 2 weeks_____% of the time

☐ 2 to 4 weeks_____% of the time

☐ 1 to 2 months_____% of the time

☐ 2 to 3 months_____% of the time

☐ 3 to 4 months_____% of the time

☐ Over 4 months _____% of the time

3.5 In which kind of bottles/bags is milk collected?

☐ Clear bottles/bags

☐ Brown/light protected bottles/bags

3.6 Who provides bottles for milk donations (please tick as many boxes as appropriate)

☐ Mothers

☐ Human Milk Banks

☐ Hospitals by themselves

☐ Hospitals will provide milk bank approved bottles

3.7 Is the donor human milk specifically labelled in terms of following donor characteristics: (please tick as many boxes as appropriate)

☐ Cow's milk free diet

☐ Vegetarian diet

☐ Vegan diet

☐ Mother of a preterm baby

☐ Other, please specify: _____ _____

☐ We don't record this

The following questions are about the transport of donated milk to the human milk bank. We would like to learn about your practices for transporting donor human milk.

Transportation of frozen donor human milk to the human milk bank

4.1 **Which of the following options for transportation are used in your human milk bank? (please tick as many boxes as appropriate)**

☐ The donor, or a member of her family, delivers the frozen milk to the human milk bank

☐ The frozen milk is collected by a commercial/medical courier service

☐ The frozen milk is collected by volunteer couriers (e.g. hospital volunteers or SERV and Blood Bikes)

☐ The frozen milk is collected by a member of staff from the human milk bank

4.2 **Does your human milk bank or the courier service use dry-ice for transportation of the frozen donor human milk?**

☐ Yes

    ☐ Always

    ☐ If the transportation takes longer than _____ min

☐ No

☐ I don't know

4.3 **Has your human milk bank a time-limit for transportation of frozen milk to the milk bank?**

☐ Yes, in general transportation shouldn't take longer than: _____ min

    ☐ What would happen if transportation would be longer? Please specify:

    _____

    _____

☐ Yes, transportations without dry-ice shouldn't take longer than: _____ min, transportations with dry ice can be longer

☐ No, there is no time-limit

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The following questions ask about the general handling of donor human milk at human milk banks. We would like to learn about your routines while handling donor human milk.

<u>Handling of the donated milk at the human milk bank</u>

5.1 At which temperature is the donated <u>unpasteurised</u> milk stored in your human milk bank?

☐ - 20°C

☐ Below - 65°C

☐ Other temperature, please specify: _____°C

5.2 At which temperature is the donated <u>pasteurised</u> milk stored in your human milk bank?

☐ - 20°C

☐ Below - 65°C

☐ Other temperature, please specify: _____°C

5.3 Does your human milk bank record the storage time at the milk bank until the frozen donor human milk will be pasteurised after arriving at the human milk bank? If so, could you provide a frequency distribution for the last year?

☐ Storage time is not recorded

☐ Storage time is recorded, but not consolidated. If so please give estimations below

☐ 1 to 3 days_____% of the time

☐ 3 to 5 days_____% of the time

☐ 5 to 7 days _____% of the time

☐ 1 to 2 weeks_____% of the time

☐ 2 to 4 weeks_____% of the time

☐ 1 to 2 months_____% of the time

☐ 2 to 3 months_____% of the time

☐ 3 to 4 months_____% of the time

5.4 Is unpasteurised milk from the same donor pooled together in one batch?

☐ Yes, always

☐ Yes, sometimes. Why sometimes? _____

☐ No, never

If Yes, please answer 5.5, if No, please go on to 5.6

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Bournemouth
University

5.5   **When is the donor human milk from one donor pooled?**

☐ Donor human milk will be thawed at arrival, pooled and refrozen

☐ Donor human milk will be thawed for microbiological tests and at this stage pooled

☐ Other, please specify: _____

5.6   **Is donor human milk thawed for taking a sample for microbiological tests?**

☐ Yes

☐ No

If Yes, please answer 5.7 and 5.8, if No, please go on to 5.9

5.7   **Is the bottle of pooled donor human milk refrozen after taking a sample for microbiological testing?**

☐ Yes, the bottle will be refrozen for approximately:_____ days, before all bottles
       undergo pasteurisation

☐ No, donor human milk will directly undergo pasteurisation and would be discarded if
       test results indicate high contamination

☐ No, donor human milk will be kept in the fridge for approximately:_____ hours
       until bottles undergo pasteurisation

5.8   **If donated milk is __not__ pooled together:**

☐ Only one bottle is thawed and tested for microbiological count

        ☐ This bottle will be discarded

        ☐ This bottle will undergo direct pasteurisation together with the other bottles

        ☐ This bottle will be refrozen for approximately:_____ days, until it
            undergoes pasteurisation

        ☐ This bottle will be kept in the fridge for approximately:_____ hours, until it
            undergoes pasteurisation

☐ All bottles will be thawed and tested

        ☐ Bottles will undergo direct pasteurisation after sample taking

        ☐ Bottles will be kept in the fridge for approximately:_____ hours, until test
            results are available and then pasteurised

        ☐ Bottles will be refrozen for approximately:_____ days before they
            undergo pasteurisation

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5.9 How often does your human milk bank pasteurise donor human milk?

☐ Daily

☐ 3 x per week

☐ 2 x per week

☐ 1 x week

☐ 3 x per month

☐ 2 x per month

☐ 1 x month

☐ Other, please specify:_____

5.10 Is all donor human milk frozen after pasteurisation?

☐ Yes

☐ No, a small amount of milk will be used directly in the hospital

5.11 How is the donor human milk distributed? (please tick as many boxes as appropriate)

☐ First in, first out (older milk will be used first)

☐ If the receiving baby is born preterm, it receives milk from mothers of preterm babies

☐ If the receiving baby is born preterm, it receives milk from mothers which have younger babies

☐ 1 baby mostly receives the milk from 1 donor

5.12 Does your human milk bank record the storage time at the human milk bank until the frozen donor human milk will be used in the hospital? If so, could you provide a frequency distribution for the last year

☐ Storage time is not recorded

☐ Storage time is recorded, but not consolidated. If so please give estimations below

☐ Less than 1 week _____% of the time

☐ 1 to 2 weeks _____% of the time

☐ 2 to 4 weeks _____% of the time

☐ 1 to 2 months _____% of the time

☐ 2 to 4 months _____% of the time

☐ 4 to 6 months _____% of the time

5.13 Is there a nutritional assessment of the donated human milk?

☐ Yes for...

    ☐ Energy content

    ☐ Macronutrients (fat, protein and carbohydrates)

    ☐ Polyunsaturated fatty acids

    ☐ Minerals

    ☐ Peroxidation products (e.g. peroxide value or malondialdehyde (MDA))

    ☐ Other, please specify: _____

☐ No

If Yes, please answer 5.14 and 5.15, if No, please go on to 5.16

5.14 Who does the nutritional assessment?

Please specify:_____

5.15 If there is a nutritional assessment of the donated human milk, when does it take place?

☐ Before pasteurisation

☐ After pasteurisation

5.16 Is the donated human milk fortified at the milk bank?

☐ Yes. If so, please specify with what: _____

☐ No

The following questions will ask about human milk banking in general. We would like to learn a bit more about your human milk bank.

General questions about human milk banking

6.1 How many donors and how much milk did your milk bank have in:

2015: donors _____, milk _____litres

2014: donors _____, milk _____litres

2013: donors _____, milk _____litres

6.2 Did your human milk bank ever get asked to provide milk in response to specific requests related to donor characteristics, and if so, how often?

☐ Cow's milk free diet _____ times per year

☐ Vegetarian diet _____ times per year

☐ Vegan diet _____ times per year

☐ Mother of a preterm baby _____ times per year

☐ Other, please specify: _____ _____

☐ We have never been asked to provide milk in response to specific requests

6.3 In the last year, how many times did your human milk bank provide milk in response to specific requests related to donor characteristics?

☐ Cow's milk free diet _____ times per year

☐ Vegetarian diet _____ times per year

☐ Vegan diet _____ times per year

☐ Mother of a preterm baby _____ times per year

☐ Other, please specify: _____ _____

☐ We don't record this, therefore, we can't provide milk in response to specific requests

6.4 Have you ever refused the request for donor human milk from hospitals in your region?

☐ Yes

    ☐ If so, do you monitor refusals? _____

    ☐ How often did that happen last year? _____ times

☐ No

6.5 Which percentage of donations comes from mothers becoming a donor before the first donation vs. mothers who donate pre-collected milk (e.g. milk collected while the baby was at a NICU)?

☐ Regular milk donations _____ %

☐ Donations of pre-collected milk _____ %

Is there anything else you wish to add about the practice of human milk donations in the UK?

From your perspective as a milk bank manager, what works well at your milk bank and what could be improved?

We are considering a survey of milk donors as well. Would you collaborate with us and distribute a questionnaire to the donors of your human milk bank on our behalf?

☐ Yes

☐ No

Do you have any other comments for us?

If you wish to receive a copy of the published results, please provide your e-mail address here:

_____

You have now reached the end of the questionnaire. Please return this questionnaire now.

Thank you for your time and participation!

Kind regards, *Isabell Nessel*

Contact details

Research team

Isabell Nessel
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Bournemouth, BH13LT, UK
Tel: +44(0)1202965009
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Christchurch Road,
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Prof Minesh Khashu
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Independent Contact

Prof Vanora Hundley,
Deputy Dean of Research and Professional Practice at Bournemouth University,
Room 118, Royal London House, Christchurch Road Bournemouth; BH13LT, UK
E-mail: vhundley@bournemouth.ac.uk

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Bournemouth University

Donor human milk storage time spread-sheet

Thank you for considering to fill in this additional spread-sheet adjunct to the U.K. Human Milk Bank Survey. Information about the frequency distribution of donor human milk storage time is of outmost interest for us. You are kindly asked to fill in this spread-sheet if you couldn't provide these information in the survey. If you have any questions or need further clarification please do not hesitate to contact us.
We would appreciate if you would send this spread-sheet back monthly to Isabell Nessel (inessel@bournemouth.ac.uk).

* **Mother, Milk Bank, Volunteer Courier, Medical/Comercial Courier**

Sample #	Your identification code	Date of expression From	Date of expression To	Transportation M, MB, VC, MC *	Arrival date	Pasteurisation date	Distribution date
Example	358/568/88	22/08/2016	25/08/2016	MC	28/08/2016	07/09/2016	27/09/2016
1							
2							
3							
4							
5							
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Appendix IV.VII: Coding and themes emerged from the open questions

HMB	Meaning Unit	Condensed Meaning Unit	Code	Sub-theme	Theme
2	"My staff are really hard working, and work beyond the hours that they have to."	Staff is working over hours, since there is too much work to do	Time	Lack of time	Limited resources
2	"I have to see breast feeders in difficulty, do breast feeding support groups and provide the training for both our staff and peer support mothers for UNICEF baby friendly. At present I am auditing this too. I really feel that a milk bank manager should have one sole role."	Too many roles, lack of time for HMB	Time	Lack of time	Limited resources
3	"More dedicated time for milk bank. Milk bank away from neonatal unit."	Not enough time for HMB	Time	Lack of time	Limited resources
10	"Only in post for last 6 month on part time basis - still reviewing."	Only part time role, not enough time for HMB	Time	Lack of time	Limited resources
8	"Would like to be able to pasteurise on more days."	Not enough time to pasteurise more often	Time	Lack of time	Limited resources
7	"Staff knowledge could be furthered but our contracts limit us to focus on the day to day running of the milk bank and providing as good a service as possible to our donor mums."	Not enough time for CPD CPD needed	Time CPD	Lack of time Staff training	Limited resources
15	"We would like more experienced staff in order to expand our milk bank."	Need more experienced staff	CPD Re-sources	Staff training Work environment	Limited resources
2	"We are desperately short of space as we issue 4 x the quantity of milk every year that we did when we moved here, we are hoping to have better premises soon."	Lack of physical space	Re-sources	Work environment	Limited resources
3	"Milk bank away from neonatal unit."	Lack of dedicated space	Re-sources	Work environment	Limited resources
3	"Dedicated telephone line/answer machine"	Lack of telephone	Re-sources	Work environment	Limited resources
2	"We have 1 computer, so this has to be answered out of work time."	Lack of computers	Re-sources	Work environment	Limited resources
14	"It would be lovely to have an up to date new pasteuriser so we could pasteurise more often with smaller amounts."	Lack of up to date pasteuriser	Re-sources	Work environment	Limited resources
4	"Service could be improved with a bar coding system to eliminate human error with batch numbers lots of time spent checking	Lack of bar-coding system	Re-sources	Work environment	Limited resources

dix IV.VIII: Calculated absolute DHA and ARA levels in a daily BM feed during the first year of lactation

DHA and ARA concentration received from BM at different time-points of lactation during the first year were calculated for a preterm baby (28 weeks gestational age, 1000 g), receiving full enteral feeding (150 mg per kg bodyweight per day) using the relative fatty acid concentrations and absolute fat content of BM reported by Mitoulas *et al.* (2003) (Table 9-1).

Table 9-1: Absolute LCPUFA concentrations in BM during the first year of lactation

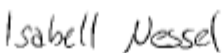|	DHA (mg/150 mL)	ARA (mg/150mL)
1 month	11.85	22.51
2 months	7.95	16.67
4 months	8.45	17.73
6 months	10.58	17.81
9 months	11.51	22.23
12 months	11.21	20.71

Appendix V.I: Authorship declaration PLEFA

Authorship Declaration

We hereby declare that Isabell Nessel, the lead author of the publication "The effects of storage conditions on long-chain polyunsaturated fatty acids, lipid mediators, and antioxidants in donor human milk – a review", accepted for publication in Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA) on the 12th of July 2019, contributed at least 75% of the substantive content of the paper.

This manuscript is original work resulting from the PhD work of Isabell Nessel, who prepared the first draft of the manuscript. The manuscript was prepared under the supervision of Dr Simon Dyall. Isabell Nessel, Prof. Minesh Khashu and Dr Simon Dyall reviewed the manuscript critically for important intellectual content and all authors provided final approval of the submitted manuscript.

Isabell Nessel

Isabell Nessel

Minesh Khashu

Dr Simon Dyall

Appendix VI.I: Authorship declaration JPEN

Authorship Declaration

We hereby declare that Isabell Nessel, the lead author of the publication "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", accepted for publication in the Journal of Parenteral and Enteral Nutrition (JPEN) on the 10th of December 2019, contributed at least 75% of the substantive content of the paper.

This manuscript is original work resulting from the LIMIT study, which was part of Isabell Nessel's PhD work. Isabell Nessel and Dr Simon Dyall contributed to the conception and design of the research; Prof Minesh Khashu and Dr Laura De Rooy contributed to the design of the research; Isabell Nessel, Dr Laura De Rooy, Prof Minesh Khashu, and Prof Jane Murphy contributed to the acquisition of the data. Isabell Nessel contributed to the analysis of the data. Isabell Nessel and Dr Simon Dyall contributed to the interpretation of the data. Isabell Nessel prepared the manuscript under the supervision of Dr Simon Dyall. All authors critically revised the manuscript, and provided final approval of the submitted manuscript.

Isabell Nessel   _____   12/12/2019

Dr Laura De Rooy   _____   12/12/2019

Prof Minesh Khashu   _____   18/12/2019

Prof Jane Murphy   _____   12/12/2019

Dr Simon Dyall   _____   13/12/2019

Appendix VI.II: REC approval letter – IRAS ID 221198

NHS
Health Research Authority

London - South East Research Ethics Committee
Barlow House
3rd Floor
4 Minshull Street
Manchester
M1 3DZ

> **Please note:** This is the favourable opinion of the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

18 July 2018

Dr Simon Dyall
Room 312 Royal London House
Christchurch Road
Bournemouth
BH13LT

Dear Dr Dyall

Study title:	Investigating Lipid Peroxidation Products in Donor Human Milk - a Two-Centre Pilot Study
REC reference:	18/LO/1330
Protocol number:	1718/IRASIN/1
IRAS project ID:	221198

The Proportionate Review Sub-committee of the London - South East Research Ethics Committee reviewed the above application.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact hra.studyregistration@nhs.net outlining the reasons for your request. Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

Ethical opinion

On behalf of the Committee, the sub-committee gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Conditions of the favourable opinion

The REC favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).

Guidance on applying for HRA and HCRW Approval (England and Wales)/ NHS permission for research is available in the Integrated Research Application System, www.hra.nhs.uk or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of management permissions from host organisations.

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database. This should be before the first participant is recruited but no later than 6 weeks after recruitment of the first participant.

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to request a deferral for study registration within the required timeframe, they should contact hra.studyregistration@nhs.net. The expectation is that all clinical trials will be registered, however, in exceptional circumstances non registration may be permissible with prior agreement from the HRA. Guidance on where to register is provided on the HRA website.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion").

Extract of the meeting minutes

The PR Sub-Committee agreed that this was a well presented study with no material ethical issues.

Approved documents

The documents reviewed and approved were:

Document	Version	Date
Copies of advertisement materials for research participants [Recruitment poster preterm mums]	V1	27 June 2018
Covering letter on headed paper [REC cover letter]	V1	11 June 2018
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [BU Insurance]	V1	15 July 2017
IRAS Application Form [IRAS_Form_05072018]		05 July 2018
IRAS Checklist XML [Checklist_05072018]		05 July 2018
IRAS Checklist XML [Checklist_13072018]		13 July 2018
Letter from funder [Funding letter]	V1	11 July 2018
Letter from sponsor [BU Sponsor letter]	V1	01 June 2018
Letters of invitation to participant [Invitation letter preterm mums]	V1	27 June 2018
Other [BU Indemnity]	V1	15 July 2017
Other [CV Minesh Khashu]	V1	18 June 2018
Participant consent form [Consent form preterm mums]	V1	08 March 2018
Participant information sheet (PIS) [Participate information sheet preterm breast milk]	V1	12 June 2018
Referee's report or other scientific critique report [Protocol review]	1	10 May 2018
Research protocol or project proposal [Protocol]	V1	08 June 2018
Summary CV for Chief Investigator (CI) [CV S Dyall]	V1	12 June 2018
Summary CV for student [CV Isabell Nessel]	V1	15 June 2018
Summary, synopsis or diagram (flowchart) of protocol in non-technical language [Trial Flow Chart]	V1	08 March 2018

Membership of the Proportionate Review Sub-Committee

The members of the Sub-Committee who took part in the review are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

<u>Reporting requirements</u>

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/
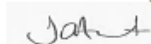
HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at
http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

18/LO/1330	Please quote this number on all correspondence

Yours sincerely

[signature]

pp
Ms Stephanie Cooper
Chair

Email: nrescommittee.london-southeast@nhs.net

Enclosures:	*List of names and professions of members who took part in the review*
	"After ethical review – guidance for researchers"
Copy to:	*Mrs Julie Northam*
	Suzy Wignall, Bournemouth University

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Ymchwil Iechyd
a Gofal Cymru
Health and Care
Research Wales

NHS
Health Research
Authority

Dr Simon Dyall
Room 312 Royal London House
Christchurch Road
Bournemouth
BH13LT

Email: hra.approval@nhs.net
Research-permissions@wales.nhs.uk

26 July 2018

Dear Dr Dyall

**HRA and Health and Care
Research Wales (HCRW)
Approval Letter**

Study title: Investigating Lipid Peroxidation Products in Donor Human
Milk - a Two-Centre Pilot Study
IRAS project ID: 221198
Protocol number: 1718/IRASIN/1
REC reference: 18/LO/1330
Sponsor Bournemouth University

I am pleased to confirm that HRA and Health and Care Research Wales (HCRW) Approval has
been given for the above referenced study, on the basis described in the application form, protocol,
supporting documentation and any clarifications received. You should not expect to receive anything
further relating to this application.

How should I continue to work with participating NHS organisations in England and Wales?
You should now provide a copy of this letter to all participating NHS organisations in England and
Wales, as well as any documentation that has been updated as a result of the assessment.

Following the arranging of capacity and capability, participating NHS organisations should **formally
confirm** their capacity and capability to undertake the study. How this will be confirmed is detailed in
the "*summary of assessment*" section towards the end of this letter.

You should provide, if you have not already done so, detailed instructions to each organisation as to
how you will notify them that research activities may commence at site following their confirmation of
capacity and capability (e.g. provision by you of a 'green light' email, formal notification following a site
initiation visit, activities may commence immediately following confirmation by participating
organisation, etc.).

It is important that you involve both the research management function (e.g. R&D office) supporting
each organisation and the local research team (where there is one) in setting up your study. Contact
details of the research management function for each organisation can be accessed here.

Page 1 of 7

How should I work with participating NHS/HSC organisations in Northern Ireland and Scotland?
HRA and HCRW Approval does not apply to NHS/HSC organisations within the devolved administrations of Northern Ireland and Scotland.

If you indicated in your IRAS form that you do have participating organisations in either of these devolved administrations, the final document set and the study wide governance report (including this letter) has been sent to the coordinating centre of each participating nation. You should work with the relevant national coordinating functions to ensure any nation specific checks are complete, and with each site so that they are able to give management permission for the study to begin.

Please see IRAS Help for information on working with NHS/HSC organisations in Northern Ireland and Scotland.

How should I work with participating non-NHS organisations?
HRA and HCRW Approval does not apply to non-NHS organisations. You should work with your non-NHS organisations to obtain local agreement in accordance with their procedures.

What are my notification responsibilities during the study?
The document "*After Ethical Review – guidance for sponsors and investigators*", issued with your REC favourable opinion, gives detailed guidance on reporting expectations for studies, including:
- Registration of research
- Notifying amendments
- Notifying the end of the study

The HRA website also provides guidance on these topics, and is updated in the light of changes in reporting expectations or procedures.

I am a participating NHS organisation in England or Wales. What should I do once I receive this letter?
You should work with the applicant and sponsor to complete any outstanding arrangements so you are able to confirm capacity and capability in line with the information provided in this letter.

The sponsor contact for this application is as follows:

Name: Mrs Julie Northam
Tel: 01202961208
Email: jnortham@bournemouth.ac.uk

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Who should I contact for further information?
Please do not hesitate to contact me for assistance with this application. My contact details are below.

Your IRAS project ID is **221198**. Please quote this on all correspondence.

Yours sincerely

Kevin Ahmed
Assessor

Telephone: 0207 104 8171
Email: hra.approval@nhs.net

Copy to: *Mrs Julie Northam, Sponsor Contact, Bournemouth University*
            Suzy Wignall, R&D Contact, Bournemouth University

Appendix VI.IV: Amendment I – REC approval letter – IRAS ID 221198

NHS
Health Research Authority

London - South East Research Ethics Committee

Barlow House
3rd Floor
4 Minshull Street
Manchester
M1 3DZ

Tel: 0207 104 8002

01 November 2018

Miss Isabell Nessel
PhD student
Bournemouth University
Room 304 Royal London House
Christchurch Road
Bournemouth
BH13LT

Dear Miss Nessel

Study title:	**Investigating Lipid Peroxidation Products in Donor Human Milk - a Two-Centre Pilot Study**
REC reference:	18/LO/1330
Protocol number:	1718/IRASIN/1
Amendment number:	Amendment 1
Amendment date:	10 October 2018
IRAS project ID:	221198

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

Approval was sought for a change in Chief Investigator.

The Sub-Committee noted that the amendment related to a change in Chief Investigator from Dr Dyall to Professor Murphy. They appreciated that the old Chief Investigator Dr Dyall remained on the research team, but was slightly confused, as they noticed that the invitation letters and PIS that were submitted do not have any reference made to the new Chief Investigator nor their contact details and still detailed the contact details for Dr Dyall. Therefore, the Sub-Committee requested that the invitation letters and PIS are revised to include the details of the new Chief Investigator Professor Murray and resubmit them for review.

You submitted revised documents.

The Sub-Committee was satisfied with the revised documents and no further ethical issues were raised.

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Letters of invitation to participant [Preterm Mums]	3	31 October 2018
Letters of invitation to participant [Term Mums]	3	31 October 2018
Notice of Substantial Amendment (non-CTIMP)	Amendment 1	10 October 2018
Other [GCP Certificate]		30 September 2018
Other [Original IRAS application signed by new CI (D1)]		15 October 2018
Participant consent form [Preterm Mums]	2	10 October 2018
Participant consent form [Term Mums]	2	10 October 2018
Participant information sheet (PIS) [Preterm Breast Milk]	4	31 October 2018
Participant information sheet (PIS) [Term Breast Milk]	4	31 October 2018
Research protocol or project proposal	2	10 October 2018
Summary CV for Chief Investigator (CI)		11 October 2018

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

Working with NHS Care Organisations

Sponsors should ensure that they notify the R&D office for the relevant NHS care organisation of this amendment in line with the terms detailed in the categorisation email issued by the lead nation for the study.
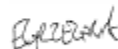
Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our Research Ethics Committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

18/LO/1330:	Please quote this number on all correspondence

Yours sincerely

[signature]

On behalf of
Ms Stephanie Cooper
Chair

E-mail: nrescommittee.london-southeast@nhs.net

Enclosures: List of names and professions of members who took part in the
review

Copy to: Suzy Wignall,
Bournemouth University

Miss Isabell Nessel,
Bournemouth University

Appendix VI.V: Bournemouth University Research Ethics – IRAS ID 221198

![Bournemouth University logo] **Research Ethics Checklist**

About Your Checklist

Reference Id	22015
Status	Approved
Date Approved	05/11/2018 08:47:02
Date Submitted	01/11/2018 14:41:18

Researcher Details

Name	Isabell Nessel
Faculty	Faculty of Health & Social Sciences
Status	Postgraduate Research (MRes, MPhil, PhD, DProf, EngD, EdD)
Course	Postgraduate Research - HSC
Have you received external funding to support this research project?	No
Please list any persons or institutions that you will be conducting joint research with, both internal to BU as well as external collaborators.	Dr Simon Dyall (University of Roehampton), Prof Minesh Khashu (BU/Poole Hospital NHS Foundation Trust), Dr Laura De Rooy (St George's Hospital NHS Foundation Trust), Poole Hospital NHS Foundation Trust, St George's NHS Foundation Trust, Dr Jane Murphy (BU)

Project Details

Title	Investigating Lipid Peroxidation Products in Donor Human Milk - a Two Centre Pilot Study – LIMIT Study
Start Date of Project	01/03/2018
End Date of Project	01/04/2019
Proposed Start Date of Data Collection	12/11/2018
Original Supervisor	Jane Murphy
Approver	Research Ethics Panel

Summary - no more than 500 words (including detail on background methodology, sample, outcomes, etc.)

Introduction: If maternal breast milk is unavailable, donor human milk (DHM) is the preferred alternative for feeding infants. Amongst other nutrients, it provides long-chain polyunsaturated fatty acids (LCPUFAs), which are essential for brain and visual development and immune system function. However, these LCPUFAs are also highly susceptible to lipid peroxidation (degradation). At high levels the resulting lipid peroxidation products are damaging to cells and may increase inflammation in the infant. Inflammation plays a central role in various diseases of prematurity like retinopathy of prematurity, bronchopulmonary dysplasia, and necrotising enterocolitis, and is implicated in poor neurological outcomes in preterm infants. We have recently identified that current human milk banking practices in the U.K. may have the potential to increase levels of lipid peroxidation products in DHM. It is therefore important to quantify the extent of this increase. This project aims to quantify for the first time the levels of lipid peroxidation products in DHM that is provided to infants on the neonatal unit.Methods and analysis: DHM samples will be collected from the Neonatal Unit at St George's University Hospitals NHS Foundation Trust and Poole Hospital Neonatal Unit, NHS Foundation Trust. Fresh mature term breast milk, collected in the community, and preterm transitional/mature breast milk, collected at the Neonatal Unit at St George's University Hospitals, NHS Foundation Trust, will be used for comparison. For each group, 10 x 5 mL samples will be analysed for the lipid peroxidation products, malondialdehyde, 4-

hydroxy-2-nonenal, hexanal, and 8-iso-PGF2α. The fatty acid content and total antioxidant capacity will also be measured. All analysis will be undertaken at Bournemouth University.Ethics and dissemination: The study will obtain ethical approval from the National Research Ethics Service and Bournemouth University. The results will provide information on the levels of lipid peroxidation products in DHM provided to infants on a neonatal unit. Results will be submitted for publication in a peer reviewed academic journal, presented at international conferences, and will be published as part of a PhD thesis.

HRA And NHS Approvals

Sponsorship	
Do you need to apply for Bournemouth University Sponsorship?	Yes

Risk Assessment	
Have you undertaken an appropriate Risk Assessment?	Yes

Attached documents
18 LO 1330 221198 _(PRS)_Favourable_opinion_18Jul18.pdf - attached on 20/07/2018 17:57:23
Trial flow chart V1 08032018.pdf - attached on 20/07/2018 17:57:23
IRASForm221198 20072018.pdf - attached on 20/07/2018 17:57:23
2018.11.01 Dyall 18-1330 (IRAS ID 221198) Amendment Favourable.pdf - attached on 01/11/2018 14:18:29
Consent form_Preterm mums V2 .pdf - attached on 01/11/2018 14:18:39
Invitation letter_Preterm mums V3.pdf - attached on 01/11/2018 14:18:42
LIMIT - Protocol V2.pdf - attached on 01/11/2018 14:18:46
Paticipat Information Sheet_Preterm Breast Milk V4.pdf - attached on 01/11/2018 14:18:50
IRASForm 221198 signed by JMurphy 15102018 .pdf - attached on 01/11/2018 14:20:42
Invitation letter_Term mums V3.pdf - attached on 01/11/2018 14:35:22
Consent form_Term mums V2 .pdf - attached on 01/11/2018 14:36:37
Paticipat Information Sheet_Term Breast Milk V4.pdf - attached on 01/11/2018 14:37:48
Recruitment flyer_Term mums V1.pdf - attached on 01/11/2018 14:38:23
Recruitment poster_Preterm mums V1.pdf - attached on 01/11/2018 14:38:26

Appendix VI.VI: Bournemouth University sponsor letter – IRAS ID 221198

Bournemouth University

To: HRA/NHS RESEARCH ETHICS COMMITTEE

Project Title: Investigating Lipid Peroxidation Products in Donor Human Milk - a two-centre pilot study (LIMIT Study)

As Project Sponsor, Bournemouth University agrees to ensure:

- The research proposal respects the dignity, rights, safety and well-being of participants

- The research proposal is worthwhile and of high scientific quality

- Arrangements proposed for the research are consistent with the UK Policy Framework for Health and Social Care

- That organisations and individuals involved in the research have or will agree the division of responsibilities between them

Signature of authorised signatory
on behalf of Bournemouth University: ....

Name: Mrs Julie Northam

Role: Head, Research & Knowledge Exchange Office

Date: 01/06/18 ....

Melbury House
1-3 Oxford Road
01202 961200
www.bournemouth.ac.uk

Appendix VI.VII: St George's University Hospitals – Confirmation of Capacity and Capability

Dear Sponsor Representative,

RE: IRAS 221198 Confirmation of Capacity and Capability at St Georges Healthcare NHS Foundation Trust

Full Study Title:	LIMIT study
R&D number	2018.0129
Site PI	Dr Laura de Rooey
Current Protocol version:	**V2.0 dated 10/10/2018**
Latest HRA Approval date:	**01/11/2018**

This email confirms that **St George's University Hospitals NHS Foundation Trust** has the capacity and capability to deliver the above referenced study. Please find attached the completed Statement of Activities.

The local research team must ensure that the participant/patient medical records are clearly marked to indicate their study participation. For electronic medical records you are advised to utilise the system research flags or alerts and for paper records to affix an alert sticker to the front cover. Alert stickers can be obtained from the JREO.

You are required to record all participant recruitment on the Trust's EDGE database. If you are unable to access this please contact the JREO.

If you wish to discuss further, please do not hesitate to contact us and local team (cc above).

Please note, in line with the national HRA approvals process, you will no longer receive a NHS R&D Approval/Permission letter.

Kind regards

Nadia Azzouzi

Nadia Azzouzi
Research Governance & Facilitation Officer
Joint Research & Enterprise Services
St George's, University of London and St George's University Hospitals NHS Foundation Trust
Cranmer Terrace
London SW17 0RE
Tel: 020 8266 6488
Email: nazzouzi@sgul.ac.uk

Appendix VI.VIII: Poole Hospital – Confirmation of Capacity and **Capability**

Dear Professor Murphy

RE: IRAS 221198 Confirmation of Capacity and Capability at Poole Hospital NHS Foundation Trust
Full Study Title: LIMIT - Investigating Lipid Peroxidation Products in Donor Human Milk - a Two-Centre Pilot Study

This email confirms that **Poole Hospital NHS Foundation Trust** has the capacity and capability to deliver the above referenced study. Please find attached agreed Statement of Activities as confirmation.

We agree to start this study on a date to be agreed when you as sponsor give the green light to begin.

If you wish to discuss further, please do not hesitate to contact me.

Kind regards,

Margaret Younger

Poole Hospital **NHS**
NHS Foundation Trust
Research & Innovation Department
1st Floor Cornelia House
Longfleet Road
Poole
Dorset
BH15 2JB

Margaret Younger
Research & Innovation Manager
☎ Tel: 01202 442982
✉ Email: Margaret.younger@poole.nhs.uk
🖱 Web: www.poole.nhs.uk

Core working hours: Monday – Thursday 8.30a.m. to 5.00 p.m.

Appendix VI.IX: Recruitment posters

Bournemouth University

St George's University Hospitals **NHS**
NHS Foundation Trust

Are you a mum expressing milk for your preterm baby?

We are looking for volunteers to take part in a **research study** about human milk conducted here at St George's Hospital, as well as at Poole Hospital and Bournemouth University.

We are looking for **Mothers (18+ years) of preterm babies** (born < 37 weeks gestational age) to donate a **small sample of breast milk** (maximum 5 mL, about a teaspoon).

We will analyse your sample of milk to look at the important fats contained within the milk, and compare this to the fats found in donor breast milk from a milk bank, to see if the way we process milk samples affects how much of the fats (the superfood in breast milk!) remain available.

If you are interested in helping us or if you would like more information please speak to **Dr Laura De Rooy** (Consultant Neonatologist) or one of the nurses caring for your baby.

Version 1   27/06/2018

Are you a breast feeding mum?

We are looking for volunteers to take part in a **research study** conducted at Bournemouth University.

We are looking for

Healthy **breast feeding mothers** (18+ years) **of term babies**

to

donate a **5mL breast milk** sample (about a teaspoon)

to

compare fat quality and quantity of breast milk and **donor human milk,** to see if processing of donor human milk samples affect the fat quality and quantity.

If you are interested in participating or would like more information please contact **Isabell Nessel** at Bournemouth University.

Isabell Nessel
Postgraduate Researcher
at Bournemouth University
01202965009
inessel@bournemouth.ac.uk

Version 1   27/06/2018

Appendix VI.X: Participant invitation letters

BU
Bournemouth
University

Investigating Lipid Peroxidation Products in Donor Human Milk - a Two-Centre Pilot Study (LIMIT Study)

Dear

We are a team of University researchers and Clinicians at St George's Hospital and Poole Hospital and we are analysing fat and fat degradation products in donor human milk and would need some preterm breast milk as comparison to see if the way donor human milk samples are processed affects the fat content. Therefore, we would like to invite you to participate in our study, which involves a one-time donation of 5 mL of your breast milk.

We enclose a participant information sheet which describes the study in more detail. This should help you to decide whether to take part in the study. If you would have any more questions, please do not hesitate to contact us using the details listed below.

The study was approved by a national Research Ethics Committee (ID: 18/LO/13303, 18.07.2018; IRAS Project ID 221198).

We thank you for your consideration and your potential help in this research study.

Yours sincerely,

Isabell Nessel

Isabell Nessel and the whole research team.

Research Team

Dr Laura De Rooy	Dr Simon Dyall	Isabell Nessel
Consultant Neonatologist	Principal Academic	Postgraduate Researcher
St George's University Hospitals NHS	University of Roehampton	Bournemouth University
Foundation Trust, Blackshaw Road,	Parkstead House 1063	Room 304, Royal London House,
Tooting, London, SW170QT, UK	Whitelands College	Christchurch Road,
Tel: +44(0)20 8725 2517	London, SW15 4JD, UK	Bournemouth, BH13LT, UK
E-mail:	Tel: +44(0)2083929598	Tel: +44(0)1202965009
laura.DeRooy@stgeorges.nhs.uk	E-mail:simon.dyall@roehampton.ac.uk	E-mail: inessel@bournemouth.ac.uk

Chief Investigator
Prof Jane Murphy, Professor of Nutrition, Bournemouth University, Room 104 Royal London House, Christchurch Road, Bournemouth, BH13LT, UK
E-mail: jmurphy@bournemouth.ac.uk

1

Version 3   31/10/2018

BU Bournemouth University

Investigating Lipid Peroxidation Products in Donor Human Milk - a Two-Centre Pilot Study (LIMIT Study)

Dear XXX,

We are a team of University researchers and Clinicians at St George's Hospital and Poole Hospital and we are analysing fat and fat degradation products in donor human milk and would need some breast milk as comparison to see if the way donor human milk samples are processed affects the fat content. Therefore, we would like to invite you to participate in our study, which involves a one-time donation of 5 mL of your breast milk.

We enclose a participant information sheet which describes the study in more detail. This should help you to decide whether to take part in the study. If you would have any more questions, please do not hesitate to contact us using the details listed below.

The study was approved by a national Research Ethics Committee (ID: 18/LO/13303, 18.07.2018; IRAS Project ID 221198).

We thank you for your consideration and your potential help in this research study.

Yours sincerely,

Isabell Nessel

Isabell Nessel and the whole research team.

Research Team

Isabell Nessel
Postgraduate Researcher
Bournemouth University
Room 304, Royal London House,
Christchurch Road,
Bournemouth, BH13LT, UK
Tel: +44(0)1202965009
E-mail: inessel@bournemouth.ac.uk

Dr Simon Dyall
Principal Academic
University of Roehampton
Parkstead House 1063
Whitelands College
London, SW15 4JD, UK
Tel: +44(0)2083929598
E-mail:simon.dyall@roehampton.ac.uk

Prof Minesh Khashu
Consultant Neonatologist
Poole Hospital NHS Foundation Trust
Longfleet Road,
Poole, BH152JB, UK
Tel: +44(0)1202 263057
E-mail: minesh.khashu@poole.nhs.uk

Chief Investigator
Prof Jane Murphy, Professor of Nutrition, Bournemouth University, Room 104 Royal London House, Christchurch Road, Bournemouth, BH13LT, UK
E-mail: jmurphy@bournemouth.ac.uk

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Appendix VI.XI: Participant information sheets

PARTICIPANT INFORMATION SHEET

Title: Investigating Lipid Peroxidation Products in Donor Human Milk - a Two-Centre Pilot Study

We would like to invite you to take part in our research study. Joining the study is entirely up to you. Before you decide, we would like to share with you why this research is being conducted and what it would involve. Discuss the study with friends or relatives if you wish. Please do not hesitate to ask us if there is anything unclear or if you need more information.

What is the purpose of this research study?
Through this study, we would like to investigate the amount of lipid peroxidation products in donor human milk which is used for feeding neonates on neonatal units in the U.K., if mums milk supply is insufficient. Lipid peroxidation products are fat degradation products, causing for example the rancid smell of old butter. Similar to butter, the fat contained in breast milk can get degraded and the resulting lipid peroxidation products may be harmful for neonates. The processes involved in human milk banking may increase the risk of fat degradation in donor human milk. So far, nothing is known about the concentrations of lipid peroxidation products in donor human milk. We will analyse donor human milk and would need some preterm transitional/mature breast milk samples as comparison for our results.

Why have I been invited to take part in this research study?
We are inviting 10 healthy, non-smoking breast feeding mothers (age > 18 years) of preterm babies (born < 37 weeks gestational age) to take part in our study.

Do I have to take part in this research study?
It is entirely up to you to take part. You don't have to take part. However, we would appreciate your invaluable help. You can withdraw from the study up to the point where you have donated your anonymised breast milk sample, without giving any reason. Neither your or your baby's medical care will be affected if you decline participation.

What is involved if I decide to participate and what will I have to do?
If you decide to take part you are asked to donate a breast milk sample (5 mL). A small sample from your expressed breast milk will be collected for the study. We will only collect a sample if there is a surplus to your baby's need. We will also collect some other basic information such as your age, and duration of breast feeding.

Version 4   31/10/2018   1

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What happens to my breast milk sample?

Your sample will be transported to Bournemouth University, where it will be analysed. The tests need to be performed on all samples at the same time, therefore, your sample will be stored until all samples are collected (approximately four months). Your sample will be destroyed during the analysis. If there should be any surplus, it will be destroyed latest three month after the study ends.

What are the possible advantages of taking part?

Whilst there are no immediate benefits for you, it is hoped that the outcome of the study will add to the scientific knowledge of lipid peroxidation product concentrations in milk sources of neonates.

What are the possible disadvantages and risks of participating?

There are no disadvantages or risks for you in participating in this study.

Will my taking part in this research study be kept confidential?

Yes, all information collected will be kept strictly confidential and will be stored securely. All results will be anonymised and you will not be identifiable in any report or publication. Only the research team will have access to the data. The procedure of handling, processing, storing, and destroying data related to the study will be in compliance with the Data Protection Act (1998).

What will happen to the results of this research study?

The results of this study will help to inform about the lipid peroxidation product levels of donor human milk, in comparison preterm transitional milk. Anonymised results will be published as part of a PhD thesis and might be shared nationally and internationally through conference presentations and publications in peer-reviewed academic journals.

Who is organising and sponsoring this research study?

The research is part of the PhD of IN at Bournemouth University, which is being supervised by SD and MK. The study is conducted in collaboration with Poole Hospital and St George's Hospital NHS Foundation Trusts.

Bournemouth University is the sponsor for this study based in the United Kingdom. We will be using information from you in order to undertake this study and will act as the data controller for this study. This means that we are responsible for looking after your information and using it properly. Bournemouth University will keep identifiable information about you for five years after the study has finished.

Your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. To safeguard your rights, we will use the minimum personally-identifiable information possible.

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You can find out more about how we use your information by contacting our Chief Data Officer on researchgovernance@bournemouth.ac.uk or for more general enquiries: DPO@bournemouth.ac.uk.

St George's Hospital will keep your name, NHS number and contact details confidential and will not pass this information to Bournemouth University. St George's Hospital will use this information as needed, to contact you about the research study, and make sure that relevant information about the study is recorded for your care, and to oversee the quality of the study. Certain individuals from Bournemouth University and regulatory organisations may look at your medical and research records to check the accuracy of the research study. Bournemouth University will only receive information without any identifying information. The people who analyse the information will not be able to identify you and will not be able to find out your name, NHS number or contact details.

St George's Hospital will keep identifiable information about you from this study for five years after the study has finished.

Who has reviewed and approved this research study?

All research in the NHS is looked at by an independent group of people, the Research Ethics Committee, to protect your safety, rights, wellbeing and dignity.

This study has been reviewed and approved by the Research Ethics Committee on 18.07.2018. (IRAS ID: 221198; REC reference 18/LO/13303)

Who can I contact for further information?

Dr Laura De Rooy
Consultant Neonatologist
St George's University Hospitals NHS
Foundation Trust, Blackshaw Road,
Tooting, London, SW170QT, UK
Tel: +44(0)20 8725 2517
E-mail:
laura.DeRooy@stgeorges.nhs.uk

Isabell Nessel
Postgraduate Researcher
Bournemouth University
Room 304, Royal London House,
Christchurch Road,
Bournemouth, BH13LT, UK
Tel: +44(0)1202965009
E-mail: inessel@bournemouth.ac.uk

Dr Simon Dyall
Principal Academic
University of Roehampton
Parkstead House 1063
Whitelands College
London, SW15 4JD, UK
Tel: +44(0)2083929598
E-mail:simon.dyall@roehampton.ac.uk

Chief Investigator
Prof Jane Murphy, Professor of Nutrition, Bournemouth University, Room 104 Royal London House, Christchurch Road, Bournemouth, BH13LT, UK
E-mail: jmurphy@bournemouth.ac.uk

Independent Contact
Prof Vanora Hundley, Deputy Dean of Research and Professional Practice at Bournemouth University
Room 118 Royal London House, Christchurch Road, Bournemouth, BH13LT, UK
E-mail: researchgovernance@bournemouth.ac.uk

Thank you for taking the time to read through this information sheet.
Yours sincerely,
Dr Laura De Rooy, Isabell Nessel, Dr Simon Dyall, and the whole research team.

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PARTICIPANT INFORMATION SHEET

Title: Investigating Lipid Peroxidation Products in Donor Human Milk - a Two-Centre Pilot Study

We would like to invite you to take part in our research study. Joining the study is entirely up to you. Before you decide, we would like to share with you why this research is being conducted and what it would involve. Discuss the study with friends or relatives if you wish. Please do not hesitate to ask us if there is anything unclear or if you need more information.

What is the purpose of this research study?
Through this study, we would like to investigate the amount of lipid peroxidation products in donor human milk which is used for feeding neonates on neonatal units in the U.K., if mums milk supply is insufficient. Lipid peroxidation products are fat degradation products, causing for example the rancid smell of old butter. Similar to butter, the fat contained in breast milk can get degraded and the resulting lipid peroxidation products may be harmful for neonates. The processes involved in human milk banking may increase the risk of fat degradation in donor human milk. So far, nothing is known about the concentrations of lipid peroxidation products in donor human milk. We will analyse donor human milk and would need some fresh breast milk samples as comparison for our results.

Why have I been invited to take part in this research study?
We are inviting 10 healthy, non-smoking breast feeding mothers (age > 18 years) of term babies (age < 1 year), to take part in our study.

Do I have to take part in this research study?
It is entirely up to you to take part to decide to join the study. You don't have to take part. However, we would appreciate your invaluable help. You can withdraw from the study up to the point where you have donated your anonymised breast milk sample, without giving any reason.

What is involved if I decide to participate and what will I have to do?
If you decide to take part you are asked to donate a breast milk sample (5 mL). In private, you will have to hand express the breast milk into a sterile container. You will obtain detailed instructions on how to collect the breast milk. We will also collect some other basic information such as your age, and duration of breast feeding.

What happens to my breast milk sample?

Your sample will be transported to Bournemouth University, where it will be analysed. The tests need to be performed on all samples at the same time, therefore, your sample will be stored until all samples are collected (approximately four months). Your sample will be destroyed during the analysis. If there should be any surplus, it will be destroyed latest three month after the study ends.

What are the possible advantages of taking part?

Whilst there are no immediate benefits for you, it is hoped that the outcome of the study will add to the scientific knowledge of lipid peroxidation product concentrations in milk sources of neonates. Furthermore, it will add to the knowledge whether human milk banking processes increase lipid peroxidation and may help us to look at ways to improving milk banking processes.

What are the possible disadvantages and risks of participating?

There are no disadvantages or risks for you in participating in this study.

Will my taking part in this research study be kept confidential?

Yes, all information collected will be kept strictly confidential and will be stored securely. All results will be anonymised and you will not be identifiable in any report or publication. Only the research team will have access to the data. The procedure of handling, processing, storing, and destroying data related to the study will be in compliance with the Data Protection Act (1998).

What will happen to the results of this research study?

The results of this study will help to inform about the lipid peroxidation product levels of donor human milk, in comparison to fresh breast milk. Anonymised results will be published as part of a PhD thesis and might be shared nationally and internationally through conference presentations and publications in peer-reviewed academic journals.

Who is organising and sponsoring this research study?

The research is part of the PhD of IN at Bournemouth University, which is being supervised by SD and MK. The study is conducted in collaboration with Poole Hospital and St George's Hospital NHS Foundation Trusts.

Bournemouth University is the sponsor for this study based in the United Kingdom. We will be using information from you in order to undertake this study and will act as the data controller for this study. This means that we are responsible for looking after your information and using it properly. Bournemouth University will keep identifiable information about you for six years after the study has finished.

Your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. To safeguard your rights, we will use the minimum personally-identifiable information possible.

You can find out more about how we use your information by contacting our Chief Data Officer on researchgovernance@bournemouth.ac.uk or for more general enquiries: DPO@bournemouth.ac.uk.

Who has reviewed and approved this research study?
All research in the NHS is looked at by an independent group of people, the Research Ethics Committee, to protect your safety, rights, wellbeing and dignity.

This study has been reviewed and approved by the Research Ethics Committee on 18.07.2018. (IRAS ID: 221198; REC reference 18/LO/13303)

Who can I contact for further information?

Isabell Nessel	**Dr Simon Dyall**	**Prof Minesh Khashu**
Postgraduate Researcher	Principal Academic	Consultant Neonatologist
Bournemouth University	Principal Academic	Poole Hospital NHS Foundation Trust
Room 304, Royal London House,	University of Roehampton	Longfleet Road,
Christchurch Road,	Parkstead House 1063	Poole, BH152JB, UK
Bournemouth, BH13LT, UK	Whitelands College	Tel: +44(0)1202 263057
Tel: +44(0)1202965009	London, SW15 4JD, UK	E-mail: minesh.khashu@poole.nhs.uk
E-mail: inessel@bournemouth.ac.uk	Tel: +44(0)2083929598	
	E-mail:simon.dyall@roehampton.ac.uk	

Chief Investigator
Prof Jane Murphy, Professor of Nutrition, Bournemouth University, Room 104 Royal London House, Christchurch Road, Bournemouth, BH13LT, UK
E-mail: jmurphy@bournemouth.ac.uk

Independent Contact
Prof Vanora Hundley, Deputy Dean of Research and Professional Practice, Bournemouth University, Room 118 Royal London House, Christchurch Road, Bournemouth, BH13LT, UK
E-Mail: researchgovernance@bournemouth.ac.uk

Thank you for taking the time to read through this information sheet.
Yours sincerely,
Isabell Nessel, Dr Simon Dyall, Prof Minesh Khashu, and the whole research team.

Appendix VI.XII: Consent forms

BU
Bournemouth
University

LIMIT Study

IRAS ID: 221198   **Participant Identification Number:**

CONSENT FORM

Title of the study: Investigating Lipid Peroxidation Products in Donor Human Milk - a Two-Centre Pilot Study

Chief Investigator: Prof Jane Murphy

Principal Investigator: Dr Laura De Rooy

Please initial inside each box: **INITIAL**

1.	I confirm that I have read and understood the information sheet (Version , / /2018) for the above study. I have had the opportunity to consider the information, to ask questions and have had these answered satisfactorily.	
2.	I understand that my participation is voluntary and that I am free to withdraw up to the point where I have donated my anonymised breast milk sample, without giving any reason, without my or my baby's medical care or legal rights being affected.	
3.	I understand that the data collected during the study may be looked at by members of the research team and by regulatory bodies, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	
4.	I understand that the information collected about me may be shared anonymously with other members of the research team.	
5.	I agree to take part in the above study.	

Signature: Date: / /2018

Name (in capitals):

I would like to receive publications arising from this study. E-mail:_____

Person taking consent

Signature: Date: / /2018

Name (in capitals):

When completed: 1 for participant; 1 for research team;

Version 2   10/10/2018   1

Bournemouth University

IRAS ID:	221198	**Participant Identification Number:**

CONSENT FORM

Title of the study: Investigating Lipid Peroxidation Products in Donor Human Milk - a Two-Centre Pilot Study

Chief Investigator: Prof Jane Murphy

Principal Investigator: Isabell Nessel

Please initial inside each box: **INITIAL**

1.	I confirm that I have read and understood the information sheet (Version , / /2018) for the above study. I have had the opportunity to consider the information, to ask questions and have had these answered satisfactorily.	
2.	I understand that my participation is voluntary and that I am free to withdraw up to the point where I have donated my anonymised breast milk sample, without giving any reason.	
3.	I understand that the data collected during the study may be looked at by members of the research team and by regulatory bodies, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	
4.	I understand that the information collected about me may be shared anonymously with other members of the research team.	
5.	I agree to take part in the above study.	

Signature: Date: /   /2018

Name (in capitals):

I would like to receive publications arising from this study. E-mail:_____

Person taking consent

Signature: Date: /   /2018

Name (in capitals):

When completed: 1 for participant; 1 for research team;

Version 2   10/10/2018   1

Appendix VII: Conference presentations

Oral presentations:

9th Annual Postgraduate Conference Bournemouth University, Bournemouth, U.K. - 8/03/2017

Lipid Peroxidation Products in Preterm Formula Milk

Isabell Nessel[1], Minesh Khashu[1,2], Simon C. Dyall[1]

[1] Centre for Midwifery, Maternal and Perinatal Health, Bournemouth University, Bournemouth
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Lipid peroxidation products are linked to several morbidities of prematurity eg bronchopulmonary dysplasia, retinopathy of prematurity and necrotising enterocolitis. Preterm formulas contain important omega-3 and omega-6 polyunsaturated fatty acids (PUFAs), which due to their high number of double bonds are particularly prone to peroxidation. Consequently, preterm formula may be a potential dietary source of oxidants. Furthermore, omega-3 and omega-6 PUFAs levels may also concomitantly decrease over time.

The aims of the study were to measure lipid peroxidation products and PUFA levels from both liquid and powdered preterm formulas. Liquid formulas were measured at opening and after 4 h storage at 4°C, whereas the powdered form was made-up and measured on days 0, 6, 14, 30 and 37, at time-points 0 h, 4 h and 24 h each day. The non-specific lipid peroxidation product malondialdehyde was measured colorimetrically; PUFA levels by gas chromatography. Results being collated to present at the conference.

Optimising long-chain polyunsaturated fatty acid content of donor human milk: a review of current milk banking practices and recommendations for improvement

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Extremely preterm infants have been shown to receive very low levels of docosahexaenoic acid and arachidonic acid, two long-chain polyunsaturated fatty acids (LCPUFAs) needed for optimal brain and visual development and immune system function (De Rooy et al. 2017). Donor human milk may be an important dietary source of LCPUFAs for many of these infants; however, processes involved in human milk banking, such as storage time and temperature, may negatively affect LCPUFA content of donor human milk.

To identify current milk banking practices and to develop best practice recommendations, a mixed-methods questionnaire was sent out electronically to all human milk banks in the U.K. between December 2016 and May 2017. The questionnaire covered donor selection, nutrition, and transportation, storage and handling of donor human milk.

The overall response rate was 81% (13 out of 16 human milk banks). This first national survey of human milk banking practices in the U.K. with respect to LCPUFAs, identified considerable variations in practice across all areas, including nutritional advice given to donors, maximum duration of milk donations, pooling of milk over 24 hours at 4°C, and same day pasteurisation of thawed samples.

Small changes in practices such as the provision of nutritional information to donors, direct freezing of expressed breast milk, reduced pre-pasteurisation storage time and direct pasteurisation after pooling could help to maintain or protect the LCPUFA content in donor human milk and may help to increase the LCPUFA intake of preterm infants. This analysis of human milk banking practices is currently being extended to Europe, North America and Australasia to identify if the recommendations would benefit other countries.

Poster presentations:

4th International European Milk Bank Association Congress, Glasgow, U.K. – 5 and 6/10/2017 and

10th Annual Postgraduate Conference Bournemouth University, Bournemouth, U.K. – 7/03/2018

Human Milk Banking Practices in the U.K.: Optimising LCPUFA content

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Fat intake for preterm infants comes from diverse sources, including maternal expressed breast milk, donor human milk, formula milk, and parenteral nutrition. We have previously shown that extremely preterm infants receive very low levels of omega-3 and omega-6 long-chain polyunsaturated fatty acids (LCPUFAs), which are essential for optimal brain and visual development and immune system function (De Rooy et al. 2017). Levels significantly below European Society for Paediatric Gastroenterology Hepatology and Nutrition guidelines and estimated *in utero* accretion rates were noted.

Previous research indicates that the processes involved in human milk banking, e.g. storage time and temperature, may negatively affect LCPUFA levels. Although operation of Human Milk Banks (HMBs) is covered by NICE Clinical guideline 93, this does not explicitly consider implications on LCPUFA content. Therefore, to identify the effects of current practices on LCPUFAs, an audit was conducted of all HMBs in the U.K. using a mixed-methods questionnaire, covering donor selection, nutrition, transportation, storage and handling. Questionnaires were distributed electronically between December 2016 and May 2017.

The overall responses rate was 86% (12 out of 14 HMBs). Considerable variations were identified across all areas of practice. Differences were observed in key practices such as nutritional advice given to donors, maximum duration of milk donations, pooling of milk over 24 hours at 4°C, and same day pasteurisation of thawed samples. The information gathered in this audit will be used to develop guidance for HMBs with specific regard to the optimisation of LCPUFA content in donor milk.

Human Milk Banking Practices in the U.K.: Optimising LCPUFA content

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Background

- Preterm infants receive omega-3 and omega-6 long-chain polyunsaturated fatty acid (LCPUFA) levels below international recommendations[1]

- LCPUFAs are important for optimal brain and visual development and immune system function

- Previous research suggests processes involved in human milk banking, such as storage time and temperature, may negatively affect LCPUFA content.

This study was the first national audit of human milk banking practices in the U.K. with respect to protecting and preserving LCPUFA levels. The results may be used to inform the development of best practice recommendations.

Study Design

- An audit of Human Milk Banks (HMBs) in the U.K. was conducted using a mixed-methods questionnaire, covering donor selection, nutrition, and transportation, storage and handling of donor milk.
- The questionnaire was distributed electronically to all 16 HMBs between Nov 2016 to Jul 2017, (IRAS ID 205068).

Results

- The overall response rate was 87% (13 of 15 HMBs) (Fig 1).
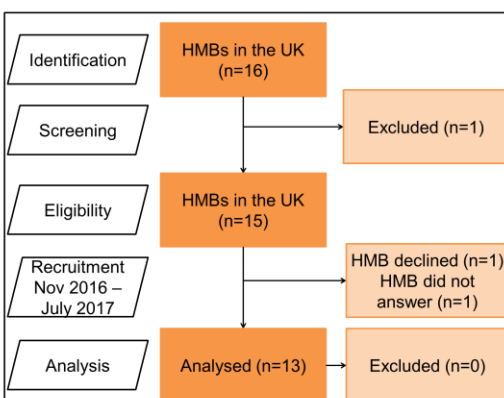
Fig 1: *Flow chart of HMB recruitment and analysis*

Results

- Considerable variations were identified across all areas of human milk banking practices (Table 1).

Table1: *Identified variations in human milk banking practices*

	Yes	No
Nutritional information offered to donors	3	10
Time limit for donor human milk transportation in place (Fig 2)	8	5
Donor human milk pooling at 4°C allowed for 24h	9	4
Storage of donor human milk for more than 2 months in donors' freezer	3	10
Direct pasteurisation of donor human milk after pooling	9	2

Legend: No limit, 30 min, 60 min, 120 min, 240 min, 300 min
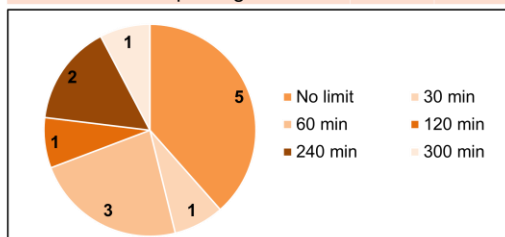
Fig 2: *Time-limits for donor human milk transportation*

Conclusions and Recommendations

- There is wide variability in current practices

- Some practices have the potential to decrease the quality and quantity of LCPUFAs

- Small changes, such as the provision of nutritional information, direct freezing of expressed donor human milk, reduced pre-pasteurisation storage time and direct pasteurisation after pooling could help to maintain/ protect the LCPUFA content of donor human milk.

Reference

[1] De Rooy, Hamdallah and Dyall (2016) *Clin Nutr [Epub ahead of print]*

Acknowledgements

Current storage practices of preterm infant formula milk do not increase lipid peroxidation as assessed by thiobarbitruic acid reactive substance levels

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Lipid peroxidation products are linked to morbidities of prematurity, such as bronchopulmonary dysplasia, retinopathy of prematurity, and necrotising enterocolitis. Docosahexaenoic and arachidonic acid, long-chain polyunsaturated fatty acids supplemented in preterm formula milk, are prone to lipid peroxidation due to their highly unsaturated nature. It is current practice in many neonatal units in the U.K. to pre-make powdered preterm formula milk and open liquid preterm formula milk and store it at 4°C for up to 24 and 12 hours, respectively.

To investigate whether these storage conditions increase the oxidative load of preterm infants, thiobarbituric acid reactive substances (TBARS) were measured at different time-points using a colourimetric assay. Preterm formula milk powder was opened and stirred 3 times per day and milk was prepared fresh at day 0, 7, 14, 21, and 28 and stored for 0, 4, or 24 hours at 4°C before analysis (n=3). Preterm liquid formula and preterm liquid post-discharge formula were analysed after opening and storing for 0, 4, or 10 hours at 4°C (n=5).

The TBARS concentration was 8.65 ± 0.48 µM at opening of the powder box and no significant changes were detected at subsequent time-points tested. Furthermore, no changes in TBARS concentration occurred after storage for 4 and 24 hours at 4°C. TBARS concentration in preterm liquid formula milk and preterm liquid post-discharge formula milk were 7.26 ± 0.6 µM and 9.86 ± 0.79 µM, respectively, with no changes occurring after storage. Nevertheless, these values are considerably higher than TBARS concentrations found in fresh human milk (Turoli et al. 2004).

For the first time, this work tested the effect of preterm formula milk storage conditions, as currently used in neonatal units, and demonstrated no effects on TBARS concentrations. However, further work is exploring the effects on other lipid peroxidation products.

Current storage practices of preterm infant formula milk do not increase lipid peroxidation as assessed by thiobarbitruic acid reactive substance levels

447006

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Background

- Lipid peroxidation products are linked to morbidities of prematurity e.g. bronchopulmonary dysplasia and retinopathy of prematurity (Weinberger et al. 2004)
- Docosahexaenoic acid (C22:6n-3) and arachidonic acid (C20:4n-6), ingredients of preterm formula milk, are prone to lipid peroxidation
- Powder and liquid preterm formula milk are prepared/opened and stored for up to 24 h or 12 h, respectively at 4°C in U.K. neonatal units

The aim of this study was to investigate whether current storage conditions of preterm formula milk increase lipid peroxidation products

Methods

- Thiobarbituric acid reactive substances (TBARS) were measured using a commercial colourimetric assay
- Liquid preterm formula milk (n=5) and liquid preterm post-discharge formula milk (n=5) were tested at opening (0 h) and after 4 h and 10 h storage at 4°C
- Powdered preterm formula milk (n=3) was tested as displayed in Fig 1
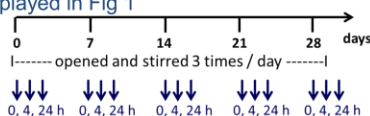
Fig 1: Time line for preterm powder formula milk sampling

- Friedman test and Dunn's post-hoc test for TBARS comparison at different times within one day; Kruskal-Wallis test and Dunn's post-hoc test for TBARS comparison on different days; Mean ± SD; * = p< 0.05

Results – Powder Formula Milk

- TBARS concentration in prepared preterm powder formula milk was 8.65 ± 0.48 µM at opening (Fig 3)
- No significant changes occurred over the 28 days
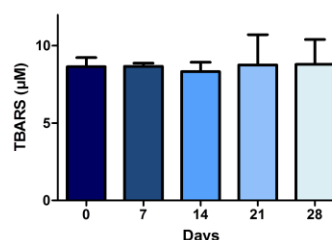
Fig 3: TBARS concentration in powdered preterm formula milk at 0 h

- TBARS concentration did not change significantly after 4 h and 24 h storage at 4°C at any time-point (Tab 1)

Tab 1: *TBARS (µM) in powdered preterm formula milk*

	0 hours	4 hours	24 hours
Day 0	8.65 ± 0.48	8.35 ± 0.64	8.47 ± 1.26
Day 7	8.67 ± 0.17	8.67 ± 1.75	8.33 ± 1.23
Day 14	8.33 ± 0.50	8.85 ± 0.36	9.76 ± 1.39
Day 21	8.76 ± 1.60	7.40 ± 0.29	8.23 ± 0.61
Day 28	8.79 ± 1.32	8.75 ± 1.14	9.01 ± 0.83

Results – Liquid Formula Milk

- TBARS concentration was 7.26 ± 0.6 µM in liquid preterm formula milk and 9.86 ± 0.79 µM in liquid preterm post-discharge formula milk (Fig 2).
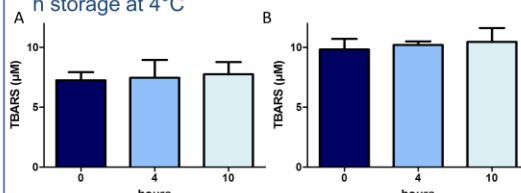- No significant differences occurred after 4 h and 10 h storage at 4°C

Fig 2: TBARS concentration in liquid preterm formula milk (A) and liquid preterm post-discharge formula milk (B)

Conclusions

- Levels were 2.7x higher than reported for fresh breast milk (Turoli et al. 2004) and 2.3x higher than reported for preterm formula milk (Raghuveer et al. 2002)
- Although current preterm formula milk storage conditions do not affect TBARs concentration, future work is exploring the effects on other lipid peroxidation products, with the aim to assess impact on oxidative load of preterm infants

Acknowledgements

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13th Congress Of The International Society For The Study Of Fatty Acids And Lipids (ISSFAL), Las Vegas, USA – 27-31/05/2018

and

Postgraduate Research Live Exhibition 2018, Bournemouth, U.K. – 5/12/2018

Effects of docosahexaenoic acid and arachidonic acid–derived lipid peroxidation products on porcine neonatal intestinal cells *in vitro*

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Long-chain omega-3 and omega-6 polyunsaturated fatty acids (LCPUFAs) are essential for optimal brain and visual development and immune system function. Therefore, preterm formula milk is currently supplemented with docosahexaenoic and arachidonic acids; however, these LCPUFAs are highly prone to lipid peroxidation and can give rise to the specific lipid peroxidation products 4-hydroxy-2-hexenal (4HHE) and 4-hydroxy-2-nonenal (4HNE), respectively. High concentrations of 4HHE and 4HNE can activate inflammatory pathways and exhibit cytotoxic effects. Such inflammatory cascades have been implicated in the development of necrotising enterocolitis, a severe gastrointestinal disease, mainly occurring in preterm infants. However, safe and toxic intake levels of 4HHE and 4HNE from preterm formula milk have not yet been established.

To investigate the effect of ingested 4HHE and 4HNE on the inflammation-sensitive preterm gut, IPEC-J2 cells were incubated in triplicates with 4HHE (0.01, 0.1, 0.25, 0.5 µM) and 4HNE (0.005, 0.05, 0.5, 1 µM) for 2 hours after 10 days of differentiation. Single exposure to 4HHE and 4HNE did not affect cell viability (98.5% ± 0.1% control vs 98.3% ±0.1 % 0.5 µM 4HHE vs 98.1% ± 0.5% 1 µM 4HNE) or cell cycle, measured by flow cytometry. Furthermore, cell migration was not affected in a wound healing assay and the metabolic activity did not differ significantly in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

These preliminary *in vitro* results reveal no detrimental effects of 4HHE and 4HNE on intestinal cells at concentrations found in preterm formula milk after single short exposure. Further work will investigate the effects of repeated exposures and explore effects on inflammatory and oxidative stress-related pathways.

Effects of docosahexaenoic acid and arachidonic acid derived lipid peroxidation products on porcine neonatal intestinal cells *in vitro*

443642

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Background

- Docosahexaenoic acid and arachidonic acid are ingredients in (preterm) formula milk
- Both are prone to lipid peroxidation → 4-hydroxy-2-hexenal (4HHE) and 4-hydroxy-2-nonenal (4HNE), respectively
- High concentrations activate inflammatory pathways and exhibit cytotoxic effects
- Inflammation plays a role in the development of necrotising enterocolitis, a preterm gut disease

The aim was to investigate the effect of ingested 4HHE and 4HNE on the inflammation-sensitive preterm gut *in vitro*

Methods

- IPEC-J2 cells were differentiated for 10 days and incubated with 0.01, 0.1, 0.25, 0.5 µM 4HHE or 0.005, 0.05, 0.5, 1 µM 4HNE for 2 h (n=3)
- Cell viability and cell cycle were measured by flow cytometry (PI staining)
- Cell migration (wound healing assay), metabolic activity (MTT assay) and IL-6 expression (ELISA) were assessed
- Kruskal-Wallis test and Dunn's post-hoc test were used for statistical analysis, data is presented as mean ± SD; * = p< 0.05

Results

- Single 2 h exposure to 4HHE and 4HNE did not affect cell viability of IPEC-J2 cells (Fig 1)
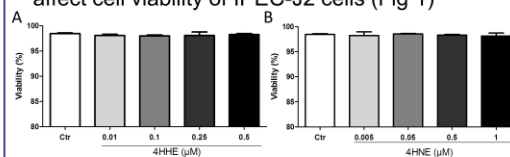
Fig 1: Cell viability (PI staining) after 2 h 4HHE (A) or 4HNE (B) exposure (n=3)

- Although differences were seen in metabolic activity, these did not reach statistical significance (p= 0.0572) for 4HNE
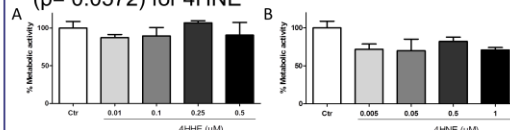
*Fig 2: Metabolic activity (MTT assay) after 2 h 4HHE (A) or 4HNE (B) exposure (n=3)*

Results

- Cell migration was not significantly different after 4HHE or 4HNE exposure (Fig 3)
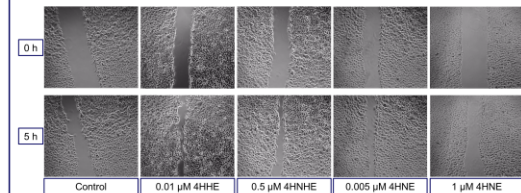
Fig 3: IPEC-J2 wound closure (cell migration) 5 h after a 2 h 4HHE or 4HNE incubation (4x magnification)

- IPEC-J2 cells did not demonstrate G1- or G2-phase arrest after 4HHE or 4HNE incubation (Fig 4). Cells in G2 phase were 9.44 ± 1.6% and ranged from 9.96 ± 1.4% to 11.28 ± 1.35% for 4HHE and from 9.62 ± 0.53% to 10.35 ± 0.41% for 4HNE
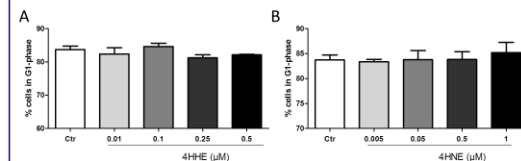
Fig 4: Cells in G1-phase (PI staining) 2 h after 4HHE (A) and 4HNE (B) exposure

- IL-6 expression was below the limit of detection (4.5 pg/mL) in the supernatant of control cells and all 4HHE and 4HNE treated cells

Conclusions

- Preliminary *in vitro* results reveal no detrimental effects of 4HHE and 4HNE on intestinal cells at concentrations found in preterm formula milk, after single 2 h exposure
- Nevertheless, preterm infants might be exposed repeatedly and over prolonged periods; therefore repeated exposures will be tested next

Acknowledgements

LCPUFAs and Lipid Peroxidation in Donor Human Milk in the U.K.: Results from the LIMIT Study

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Background

- Breast milk (BM) contains docosahexaenoic acid (DHA C22:6n-3) and arachidonic acid (AA C20:4n-6), which are prone to lipid peroxidation
- Donor Human Milk (DHM) is the preferred alternative if maternal milk is unavailable or insufficient
- Human milk banking processes may decrease LCPUFAs and increase lipid peroxidation, and these lipid peroxidation products can impact negatively on infant outcomes

Aim: Quantify for the first time the levels of LCPUFAs and lipid peroxidation products in DHM that is provided to infants in neonatal units

Study Design — NCT03573531

- Surplus DHM was collected from two neonatal units (n=19); preterm (n=10) and term BM (n=11) were collected from a neonatal unit and the community
- Fatty acids were analysed by GC-FID, and malondialdehyde (MDA) by colourimetric thiobarbituric acid reactive substances assay
- One-way ANOVA used to compare groups; Spearman correlation for correlations; t-test for enteral intake comparison; results expressed as means (SD); * $p < .05$, ** $p < .01$, *** $p < .001$

Results

- Samples were collected between Nov 18 and Feb 19

Table 1: Participant characteristics

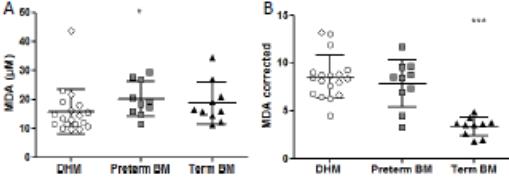|	Preterm BM	Term BM	p-value
Number	10	11	NA
Maternal age (years)	33.1 ± 5.8	29.5 ± 5.6	.181
Infant age (months)	1.6 ± 1.4	5.6 ± 3.4	.003
Delivery (weeks[+days])	$30^{+6} \pm 3^{+3}$	$40^{+4} \pm 0^{+5}$	<.0001
Pump expression	10 (100%)	6 (55%)	.035

- Linoleic acid (LA), α-linolenic acid (ALA), AA and DHA levels were all significantly lower in DHM (Table 2)

Table 2: Fatty acid content DHM, preterm, and term milk

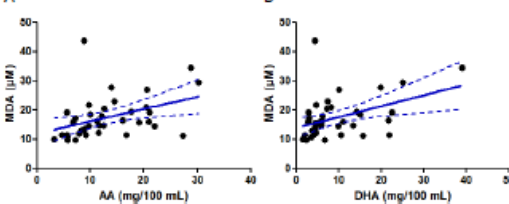| mg/100 mL [wt %]	DHM (n=19)	Preterm BM (n=10)	Term BM (n=11)
LA (18:2n-6)	223.6 (65.0) [12.9 (1.6)%]	351.0 (60.5) [12.8 (3.0)%]	587.1 (214.1)*** [12.7(3.0)%]
ALA (18:3n-3)	22.3 (9.5) [1.2 (0.2)%]	39.7 (28.8) [1.4 (0.6)%]	82.1 (49.5)*** [1.4 (0.2)%]
AA (20:4n-6)	8.3 (2.8) [0.5(0.1)%]	14.9 (6.6)* [0.5 (0.1)%]	23.7 (13.4)** [0.4 (0.1)%]
DHA (22:6n-3)	4.7 (2.0) [0.3 (0.1)%]	13.2 (7.3)** [0.5 (0.2)%]**	16.6 (12.3)*** [0.3 (0.1)%]

Results

- Absolute MDA content was significantly lower in DHM (Figure 1); however, fat content corrected DHM had significant higher MDA levels

Figure 1: MDA concentration in DHM, preterm, and term milk
A: Absolute MDA content; DHM vs preterm p<.05
B: MDA corrected for fat content, DHM vs term p<.001

- MDA concentration significantly correlated with absolute AA and DHA content (Figure 2)

Figure 2: Correlation of MDA and LCPUFAs
A: AA, n=38, r=.4385, p=.0059; B: DHA, n=38, r=.4969, p=.0015

- DHM provides AA and DHA levels below ESPGHAN recommendations and significant lower levels than human preterm BM, which provides sufficient levels (Table 3)

Table 3: Enteral LCPUFA intake from DHM and preterm BM for a preterm baby (28 weeks GA; 1kg) full enteral feeding (150 ml/kg/day) in mg/day. AA: p=.0012; DHA: p=.0001

Enteral intake	DHM	Preterm BM	ESPGHAN
AA (20:4n-6)	12.4 (4.4)	22.3 (10.5)**	18-42
DHA (22:6n-3)	7.3 (2.7)	22.0 (12.8)***	12-30

Conclusions

- DHM has lower fat and LCPUFA content and increased lipid peroxidation, which may be a consequence of current milk banking practices, which need reviewing.
- Full enteral feeding with DHM can lead to LCPUFA deficits in preterm infants, potentially leading to negative health outcomes.

Acknowledgements