NUTRITION AND SENSORY PERCEPTION IN THE NEWBORN

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ABSTRACT

Nutrition is key to the care of preterm babies. Sensory cues from food can trigger physiological reflexes that assist with digestion and metabolism. However, most moderate-and-late preterm (MLP) babies receive some nutrition by gastric tube, bypassing the oronasopharynx, where smell and taste are perceived, thereby limiting sensory exposure.

The aims of this thesis are to: assess current evidence regarding effectiveness and safety of sensory stimulation with feeding for preterm babies; investigate the volatile compounds that potentially contribute to smell and taste of milks fed to preterm babies and maternal and infant factors that may influence these; and to investigate the role of smell and taste stimulation on blood flow to the orbitofrontal cortex (OFC).

A Cochrane review of three randomised trials suggested that exposure to smell and taste of milk with tube feedings decreased duration of hospital stay and possibly time to achieve full oral feedings in preterm babies. However, the quality of evidence is very low as trials were small and had significant limitations.

In 13 infant formulas and 34 human milk-based samples, we identified 121 volatile compounds, 40% of which varied significantly across milk type. Liquid and powder formulas exhibit volatile compounds originating from manufacturing processes. Fortification of breastmilk followed by prolonged refrigeration may alter its sensory properties. Volatile compounds in preterm breastmilk are influenced by maternal ethnicity, socioeconomic status, health and stage of lactation.

In a subset of MLP babies enrolled in a randomised, factorial, multicentre trial of nutritional support pending establishment of full enteral feeds (the DIAMOND trial) gastric tube feeding induced activation of the OFC, assessed by Near Infra-Red Spectroscopy, but sensory stimulation alone did not. Exposure to smell led to different pattern of oxygenation in OFC between girls and boys, suggesting sex-differences in the development of sensory perception.

Exposure to smell and taste of milk with tube feedings is a simple intervention that may reduce length of hospitalisation, but the effect on clinical outcomes remains unclear. Odour-active lipid oxidation products in milks fed to preterm infants are major contributors to olfactory cues and, therefore, differences in volatile compounds amongst products have the potential to influence feeding behaviour and metabolism, possibly in sex-specific ways. However, whether this is important in the clinical setting remains to be determined.

This thesis is dedicated to my parents, Monica and Jose Henrique Muelbert, and to my partner, Marcelo Neto Cabreira

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Mai i te kōpae ki te urupā, tātou ako tonu ai.

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From the cradle to the grave we are forever learning. Believe in yourself!

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ABBREVIATIONS

2CP	2-Chlorophenol
AF	Amniotic fluid
AH	Aromatic hydrocarbon
AMPK	Adenosine-monophosphate-activated protein kinase
ANOVA	Analysis of Variance
BM	Breastmilk
BMF	Bovine milk-based fortifier
BMI	Body mass index
CA	Corrected age
CI	Confidence Interval
DOL-II	Delayed onset Lactogenesis II
EIC	Extracted ion chromatogram
EN	Enteral nutrition
EP	Extremely preterm infants
ET	Early term infants
FA	Fatty acid
Fae	Fatty acid ester
FDR	False discovery rate
FFA	Free fatty acid
FFAR	Free fatty acid receptors
FT	Full term infants
GC-MS	Gas Chromatography Mass Spectroscopy
GDM	Gestational diabetes
GLP1	Glucagon-like polypeptide 1
Hb	Haemoglobin
HHb	De-oxygenated haemoglobin
HM	Human milk
HMB	Human milk bank
HMF	Human milk-based fortifier
HR	Hazard Ratio
HSD	Honest significant difference
ICC	Intra-cluster correlation coefficient

IGF	Insulin-like growth factor
IS	Internal standard
LCFA	Long-chain fatty acid
LC-PUFA	Long-chain polyunsaturated fatty acid
LF	Liquid formula
m/z	Mass-to-charge ratio
MCFA	Medium-chain fatty acid
MD	Mean Difference
MFG	Milk fat globule
MFGM	Milk fat globule membrane
MLP	Moderate and late preterm
MOB	Maternal breast odour
MOM	Mothers' own milk
MP	Moderate preterm
MUFA	Monounsaturated fatty acid
NEC	Necrotising enterocolitis
NG	Nasogastric tube
NICU	Neonatal Intensive Care Unit
NIRS	Near Infrared Spectroscopy
NNTB	Number needed to treat to benefit
NNTH	Number needed to treat to harm
NPY2R	Neuropeptide Y2 receptor
NZDep	New Zealand deprivation index
O_2Hb	Oxygenated haemoglobin
OAC	Oropharyngeal administration of colostrum
OAV	Odour activity value
OFC	Orbitofrontal cortex
OR	Odds Ratio
ORs	Olfactory receptors
PBM	Preterm breastmilk
PDBM	Pasteurised donor breastmilk
PF	Powder formula
PN	Parenteral nutrition
PTB	Preterm birth
PUFA	Polyunsaturated fatty acid
QC	Quality control
RD	Risk Difference
RR	Relative Risk

RTF	Ready-to-feed
SCFA	Short-chain fatty acid
SFA	Saturated fatty acid
SMD	Standardised mean difference
SPME	Solid phase micro extraction
TAG	Triacylglycerides
TAS1R	Taste receptor type 1
VOC	Volatile organic compound
VP	Very Preterm infants

CO-AUTHORSHIP FORMS

CHAPTER 1

GENERAL INTRODUCTION

Sections of Chapter 1 are published in

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1.1 Overview

Preterm babies are those born before 37 weeks of gestation and this definition can be divided further into extremely preterm (EP, birth before 28 weeks of gestation), very preterm (VP, birth between 28 and 31 completed weeks of gestation), moderate preterm babies (MP, birth between 32 and 33 completed weeks of gestation) and late-preterm babies (LP, birth between 34 and 36 completed weeks of gestation) (Blencowe et al., 2012). Globally, approximately 15 million babies are born preterm every year, which represents 11% of all birth worldwide (Blencowe et al., 2013).

While the definition of preterm birth as birth before 37 weeks' gestation is widely accepted (World Health Organization, 2004), the definition of term birth as birth between 37 and 42 completed weeks of gestation has been questioned given that maternal, neonatal and childhood outcomes vary considerably across this range. Recent recommendations are that births between 37^{+0} and 38^{+6} be designated "early term"(ET), births between 39^{+0} and 40^{+6} "full term" (FT) and births after 41^{+0} "late term" births (Spong, 2013). These definitions take into account the continuum of fetal maturation and that infant mortality rates and adverse health outcomes are lowest for births occurring at FT (Ananth et al., 2013).

Moderate- and late-preterm (MLP) and ET infants can be considered the "great dissemblers": some resemble healthy FT infants in appearance, but their immaturity places them at increased risk of poor short- and long-term outcomes. Nutritional requirements are greater than for FT babies, but there are few good data on the nutritional requirements for MLP and ET babies leading to substantial variation in practice. Feeding difficulties are common and the requirement of nutritional support may lead to longer hospitalisation.

Sensory cues from food can assist with digestion and metabolism of nutrients. Functional smell and taste receptors are present by the third trimester of gestation, and therefore there is growing interest in the effect of sensory stimulation with smell and taste of milk as a potential intervention to improve nutrition and other clinical outcomes in preterm infants.

1.2 Defining the problem: Trends in moderate and late preterm births

Prevalence of MLP birth is estimated between 3-6% in high-income countries, but MLP babies represent 65-75% of all preterm births (Delnord and Zeitlin, 2019). In New Zealand, 7.5% of all live births in 2017 were preterm, including 6.2% which occurred in the range of MLP births, representing almost 83% of births before 37 weeks (Ministry of Health, 2019). This proportion has remained relatively stable for the past decade and is higher than Canada (4.8%), Sweden (3.6%) and Denmark (3.3%) (Richards et al., 2016), but lower than Brazil (9.4%) (Ministerio da Saude, 2018), for example.

In the United States, there has been an estimated 25% increase in LP births between 1990 and 2006 (Engle, 2011); by 2016, LP births accounted for 7%, and ET births 25%, of all live births, equating to more than 275,000 and 1 million babies, respectively (Martin et al., 2018). Similar trends have been observed in Australia, where between 2001 and 2009 planned births (labour induction or prelabour caesarean delivery) have increased by 40% at <37 weeks' gestation and by 52.5% at 38 weeks' gestation (Morris et al., 2012).

Many factors have contributed to the increasing incidence of MLP birth, such as improved care for infants born very preterm, increased use of assisted reproduction, higher rates of multiple births and increased use of obstetric interventions (induction of labour and caesarean sections) (Petrou and Khan, 2012). Furthermore, the economic cost associated with MLP births is a significant burden to health care systems worldwide (Petrou, 2019), given that this population has greater mortality and morbidity than infants born FT.

1.2.1 Short and long-term health outcomes of MLP and ET births

Complications following preterm birth are the leading cause of neonatal mortality and deaths among children under age 5 (United Nations Inter-agency Group for Child Mortality Estimation, 2017). The risk of developing neonatal morbidities, including respiratory distress, temperature instability, hypoglycaemia, feeding problems and jaundice decreases significantly with increasing gestational age (Boyle et al., 2015; McIntire and Leveno, 2008). Despite an overall low incidence of mortality and neonatal morbidity in MLP babies, they still are more prone to health complications in the neonatal period and beyond compared to their FT counterparts (Kajantie et al., 2019; Muganthan and Boyle, 2019). This is particularly important given the large proportion of MLP births, their impact on early and long-term health outcomes and the substantial population-attributable fraction burden to healthcare system (Petrou, 2019).

1.2.1.1 Initial care, neonatal and childhood outcomes

Although MLP and ET babies are apparently "well" when compared to more preterm babies, as they often look like full-term babies and so are treated as such, in fact they have a degree of immaturity that places them at higher risk of many clinical problems, and these

outcomes are inversely correlated with gestational age (Barros et al., 2012; Engle, 2011; Mackay et al., 2010; Woythaler et al., 2011).

The way MLP and ET babies are cared for during the neonatal period may influence their health outcomes. While the vast majority of preterm infants born below 32 weeks or born weighing less than 1500 g are admitted to neonatal nurseries, there is currently no consensus on best practice for the care of MLP and ET babies. Therefore, variation in management of MLP and ET babies is likely to impact upon important health outcomes, such as weight gain and provision of adequate treatments, and on the economic impact depending on the use of hospital resources and length of hospitalization (McCormick et al., 2006; Petrou, 2019).

A survey of clinicians in Australia and New Zealand found wide variation in approach to the initial nutritional support of MLP babies whilst waiting for mother's own milk (MOM) to meet the baby's needs (Alexander and Bloomfield, 2019). In an hypothetical scenario of an MLP neonate born with appropriate weight-for-gestational-age, 61% and 53% of respondents, whilst waiting for sufficient MOM to meet the infant's needs, would initiate nutritional support with 10% dextrose for MP and LP respectively, with most of the remainder commencing infant formula. Of those providing 10% dextrose, almost 31% in MP and 50% in LP, would continue 10% dextrose as the only additional nutritional support for 3 or more days whilst waiting for MOM (Alexander and Bloomfield, 2019). This variation in practice may reflect the lack of high quality evidence around optimal nutritional support in the MLP population and the long-term outcomes of common complications of MLP and ET birth, such as neonatal hypoglycaemia, hypernatraemia and hyperbilirubinaemia.

There is an inverse association between initial hospitalisation costs and gestational age at birth; however, the hospital costs associated with MLP births are more than twice that of

FT births (Petrou, 2019). The estimated population attributable fractions for having at least 3 hospital admissions from birth to 9 months of age and from 9 months to 5 years is higher for children born MLP (17.7% and 5.7%, respectively) than very preterm babies (7.2% and 3.8%, respectively), mostly due to large number of MLP who require hospital re-admission for treatment of respiratory complications, gastrointestinal infections, viral infections or fever (Boyle et al., 2012).

1.2.1.2 Mortality

Compared to babies born FT, MLP babies present higher risk of early and late mortality (Kajantie et al., 2019; Muganthan and Boyle, 2019). A Swedish cohort study including more than 20,000 LP babies reported higher risk of mortality in the first 5 years of life (adjusted hazard ratio aHR= 1.53, 95% confidence interval (CI) 1.18 to 2.00) and early adulthood (aHR= 1.31, 95% CI 1.13 to 1.5) compared with FT babies (Crump et al., 2011a). Similarly, a Norwegian national registry linkage study of approximately 1.2 million births from 1967 to 1996 found that LP (*n*= 61,082) were 32% more likely to die between ages of 35-45 years (aHR= 1.32, 95% CI 1.10 to 1.58) than FT births (Risnes et al., 2016). In contrast, registry data linkage in a cohort of 700,000 births in Western Australia between 1980-2010 demonstrated that MLP were more likely to die in the first year of life, but there was no significant difference for mortality later in life (Srinivasjois et al., 2017).

1.2.1.3 Respiratory outcomes

Respiratory morbidities are one of the most common causes of hospital re-admission in the post-neonatal period and beyond for MLP (Boyle et al., 2012; Muganthan and Boyle, 2019). Results from several studies show that LP babies have higher odds of being diagnosed with asthma (Goyal et al., 2011), requiring asthma medication (Boyle et al., 2012; Goyal et al.,

2011), are more likely to experience chest wheezing at 3 and 5 years (Boyle et al., 2012) and have more visits to outpatient clinics for acute respiratory issues in the first 18 months of life compared to FT counterparts (Goyal et al., 2011).

1.2.1.4 *Neurodevelopmental outcomes*

Compared to term-born peers, LP and ET infants are at increased risk for requiring special education (adjusted odds ratio (aOR (95% CI) 1.16 (1.12–1.20) and 1.53 (1.43–1.63), respectively) (Mackay et al., 2010) and LP present twice the risk for neurodevelopmental disability (Relative Risk (RR) 2.19 (1.27–3.75)) (Johnson et al., 2015).

A recent review of early and later neurodevelopment outcomes in LP infants indicated that, on average, when compared with a group of FT infants, LP have an increased risk of mental, psychomotor and speech/language developmental delay at 2-3 years, worse school achievement and performance in reading and writing at 5-7 years, have a higher risk of an Intelligent Quotient (IQ) score <85 (considered a threshold for borderline intellectual functioning) and, later in life, LP babies may be at higher risk of mental retardation, psychological and behaviour disorders, blindness, hearing loss and receiving pension for disability (Woythaler, 2019).

Furthermore, data on mental health outcomes of young adults born MLP are concerning. Several studies using national registry linkage point in the same direction: compared to FT, those who were born MLP have increased odds of being diagnosed with psychiatric, psychotic, bipolar or mood disorders, depression and drug addiction in adulthood (Kajantie et al., 2019).

1.2.1.5 Metabolic and cardiovascular outcomes

Regarding metabolic health of MLP, a recent review of adults born MLP indicated that this population has higher risk of poor metabolic outcomes in adulthood (Kajantie et al., 2019). In a Finnish birth cohort, adults born LP had higher BMI, waist circumference and body fat and were 2.5 times more likely to have metabolic syndrome compared to adults born FT (Sipola-Leppänen et al., 2015). Similar findings were are also reported in Nordic studies using national registry linkage where MLP presented higher odds of being prescribed medication for diabetes and hypertension (Crump et al., 2011b; Engeland et al., 2017; Kajantie et al., 2010). Associations with other cardiometabolic parameters such as systolic/diastolic pressure, ischaemic heart disease or cerebrovascular events are less clear, although most studies have measured these outcomes before the 50th birthday, which might be too early to assess some cardiometabolic outcomes.

1.2.1.6 How robust is the evidence?

It is important to stress that most of the long-term outcomes of MLP birth are limited to a few studies in high-income, mostly Scandinavian, countries, reporting on births that occurred over three decades ago when estimation of gestational age and neonatal care were not as advanced as they are today. Therefore, data about some long-term health outcomes in MLP are still inconclusive and lack evidence from low- and middle-income countries, with high rates of MLP births. Furthermore, rates of MLP birth in Nordic countries are below 4% in contrast to 6% in USA and New Zealand (Delnord and Zeitlin, 2019; Ministry of Health, 2019) and even higher rates in middle- and low-income countries such as Brazil (9%) (Ministerio da Saude, 2018). Thus, the above mentioned short- and long-term outcomes of MLP births are

possibly very conservative estimates, that could mean the true population attributable risk elsewhere is even greater.

1.3 Nutrition of moderate and late preterm infants

As body stores of essential nutrients acquired via placental transfusion are dependent on the duration of gestation (Lapillonne et al., 2019), most preterm infants are born with few deposits of subcutaneous fat, glycogen and key micronutrients (e.g. iron, zinc, calcium and vitamins) that would have been provided by placental transfer during the last trimester of pregnancy (Harding et al., 2017).

Nutritional support for preterm infants often is targeted at supporting growth equivalent to intrauterine growth trajectories. However, there is inevitable weight loss after birth due to loss of extracellular fluid, and growth charts derived from cross-sectional data from infants born at different gestational ages are unlikely to represent optimal postnatal growth of preterm infants. Furthermore, preterm neonates are, as a population, relatively growth-restricted compared with their gestational-age matched *in utero* peers who go on to be born at term (Cooke, 2007).

Therefore, providing adequate nutrition after preterm birth to meet the rapid growth preterm babies would have had if still *in utero*, in addition to overcoming the physiological immaturity, are major challenges in the nutritional care of preterm infants (Embleton, 2013; Harding et al., 2017). The optimal target is to provide satisfactory nutrition to avoid short-term complications of undernutrition and support optimal brain development, without

promoting excessive growth that might predispose infants to increased adiposity in the future (Cormack et al., 2019; Giannì et al., 2012; Lapillonne and Griffin, 2013).

1.3.1 ***Nutritional recommendations***

Most nutrition guidelines provide recommendations for more preterm (<32 weeks) or very low birth weight neonates (<1500 g) but few provide nutritional recommendations for the MLP and ET baby (Lapillonne et al., 2013). The increased numbers of LP and ET births suggest that new research should focus on best nutrition practices among this growing population.

Current nutrition guidelines recommend that total energy intake for preterm infants should be 110-135 kcal/Kg/·day (Embleton, 2013), regardless of gestational age. This recommendation takes into account that resting energy expenditure does not vary with gestational age and is approximately 45 kcal/Kg/day; that requirements for new tissues are approximately 4.5-4.9 kcal/Kg and for fat and protein deposition are between 1.55-1.6 kcal/g and 5.5-7.75 kcal/g, respectively; and that an optimal intrauterine weight gain of 17 g/Kg/day will require 76-83 kcal/Kg/day of energy intake for preterm babies (Embleton, 2013).

Nutritional requirements of MLP babies are somewhere in between the recommendations for very preterm and full-term babies; however, MP babies have higher nutritional needs than LP and FT babies and are more likely to require early nutritional support (Lapillonne et al., 2019). Ideal fetal growth between 11 and 13 g/Kg/day can be achieved with energy intake between 127 and 115 kcal/Kg/day and a protein intake between 3.1 and 2.5 g/Kg/day for moderate (MP) and late preterm (LP) babies, respectively (Lapillonne et al., 2013). These lower recommendations for LP babies are consistent with the findings of

a multicentre RCT demonstrating that excessive protein intake in early life may increase adiposity in childhood among term-born children (Koletzko et al., 2009; Weber et al., 2014).

1.3.2 ***Nutritional support: What, when and how it should be provided?***

It is common for MLP babies to present with feeding difficulties, which place them at risk of postnatal growth restriction (Lapillonne et al., 2019). Therefore, until attainment of mature oral feeding skills, some LP babies will require enteral nutrition and approximately 60% of MP babies will need intravenous nutrition to support postnatal growth (Boyle et al., 2015; Brown et al., 2014; Iacobelli et al., 2015; Visruthan et al., 2015).

Intravenous nutrition may be required when transition to full enteral feeds is anticipated to take several days, especially in smaller babies at lower gestational ages and if associated with episodes of feed intolerance, making advancement of enteral volumes more difficult (Fanaro, 2013). Compared to LP, MP babies are four times more likely to present with feeding problems, defined as poor suck, abdominal distension or recurrent vomiting (OR= 4.29, 95% CI 2.91 to 6.32) and, in consequence, are five times more likely to require nutritional support, either intravenous infusion of dextrose alone and/or parenteral nutrition (OR= 5.42, 95% CI 3.88 to 7.57), increasing the duration of hospital stay (Visruthan et al., 2015).

Parenteral nutrition (PN) is usually considered when provision of nutrients via the enteral route is clinically contra-indicated or will result in nutrient insufficiency. The composition of PN varies from intravenous infusion of carbohydrates (mostly dextrose) alone, combinations of dextrose and amino acids in addition to minerals and vitamins, and complete PN with additional infusion of lipids. Currently, there is no robust evidence to indicate whether MLP babies should receive complete PN (with lipids, carbohydrates and amino acids)

or whether dextrose alone is sufficient to promote postnatal growth while waiting for maternal milk supply to meet demand and for full enteral feeds to be tolerated (Harding et al., 2017; Lapillonne et al., 2019) but use of complete PN in LP infants appears to be rare (Alexander and Bloomfield, 2019). Although it has been reported that 27% of LP infants require intravenous infusions compared to 5% of term babies, (Wang et al., 2004) very few LP and ET babies will receive parenteral nutrition support, with this usually reserved for babies with congenital malformations predicted to lead to delays in reaching full enteral feeds.

The optimal nutritional support for MLP is currently being investigated in the DIAMOND trial, a multicentre randomised factorial trial comparing different nutritional approaches to moderate- and late-preterm nutrition on feed tolerance, growth and neurodevelopment (Bloomfield et al., 2018). Although PN may improve nutrient intake in the first week after birth and increase body weight at discharge in MP babies (Smazal et al., 2016), duration of PN has been associated with an increased risk of infection, prolonged hospital stay and increased health costs (Sengupta et al., 2010; Zingg et al., 2012). Each day of parenteral support in LP infants has been reported to predict an increase in time to achieve full oral feeds of 2 hours (aHR= 0.92, 95% CI 0.89 to0.95) (Jackson et al., 2016).

In VP and EP babies, guidance is that enteral nutrition should start as early as possible with small volumes, such as 1 mL every 3-4 hours (or less than 24 mL/Kg/day) via oro- or naso-gastric tube and increase according to the infant's tolerance (Harding et al., 2017). This practice, called minimal enteral nutrition or trophic feeding, is associated with improved gut maturation, stimulation of gastrointestinal motility, hormone secretion and healthy microbial colonisation of the gastrointestinal tract (Harding et al., 2017). Recent data indicate that increasing feeds at up to 30 mL/Kg/day is safe (Dorling et al., 2019). However, there are no good data on initial volumes or speed of increase for enteral feeding of MLP babies.

In small-for-gestational-age MP babies, a proactive feeding regimen providing initial enteral feeds of 100 mL/Kg/day further increased to 200 mL/Kg/day within four days has been associated with several advantages compared to the standard practice (initial volume of 60 mL/Kg/day and increased to 170 mL/Kg/day within 9 days) (Zecca et al., 2014). These include: significantly higher nutritional intake; less weight loss and faster regain of birth weight; lower incidence of hypoglycaemia and late onset sepsis; less need for intravenous nutrition; shorter hospital stay, and more babies being fed with their mother's own milk (Zecca et al., 2014). These findings, despite coming from a small randomised trial (*n*= 72), suggest that nutritional intake of MP babies could be optimised and may improve short term outcomes. The ADEPT trial, which included many MLP babies, also demonstrated that earlier advancement of enteral feeds in growth-restricted babies is not associated with an increased incidence of necrotising enterocolitis (Leaf et al., 2012).

1.3.3 Transition from enteral nutrition via tube feeds to oral feeds

For many MLP babies, a brief requirement for gastric tube feeding is not uncommon. A retrospective review of 647 MLP infants in six New Zealand's NICUs between 2005 and 2011 reported that gestational age, birth weight, days of parenteral nutrition support and clinical condition were significantly associated with time to start oral feeds and time required to attain full oral feeding (Jackson et al., 2016). In this study, on average, LP infants had their first oral feed attempt in the first two days after birth, reaching full oral feeds by the 8th day. MP infants, however, had their first oral feed attempt 4–8 days after birth, reaching full oral feeds by the 10th day. Local practice impacted upon timing and the authors suggested that the lack of specialised services to support feeding may have contributed to differences in transition time (Jackson et al., 2016).

1.3.3.1 Role of smell and taste stimulation

Infants receiving tube-feeds may miss out on exposure to olfactory and flavour stimulation because tube feeds bypasses the nasal and oral cavities where these sensory perceptions mostly occur (Bloomfield et al., 2017). Smell and taste of food initiate a sequence of anticipatory physiological responses that prepare the body to digest, absorb and metabolise food, referred to as cephalic phase response (CPR) (Smeets et al., 2010), reviewed in detail in section 1.5.3. Evidence from a pilot randomised trial in very preterm babies (<29 weeks' gestation) indicates that exposure to taste and smell of milk before each feed may reduce time to full enteral feeds and improve weight gain, but the sample size was small (Beker et al., 2017). Despite limited evidence of benefit, in an electronic survey of neonatologists and paediatricians in Australia and New Zealand, 30% (*n*= 36) of respondents reported that smell or taste of mother's milk prior to tube feeds was provided routinely (Alexander and Bloomfield, 2019).

Two ongoing RCTs are investigating the role of smell and taste prior to tube feedings on time to full sucking feeds and body composition (Beker et al., 2019; Bloomfield et al., 2018). Results should shed light on whether this simple intervention to support early nutrition of LP babies is of benefit. This topic is addressed in greater detail in the Cochrane Review reported in Chapter 2.

1.3.4 Is breastmilk always best?

The short answer is 'Yes'. BM provides not only macronutrients essential for growth but also is rich in bioactive compounds that promote healthy development (Bertino et al., 2012). There are, however, many obstacles to providing an exclusive BM diet to preterm infants. Mothers of preterm babies are likely to experience factors that impact upon lactation,

including separation from their infant, medical conditions that may have contributed to the early birth such as diabetes, pre-eclampsia, multiple births and birth via caesarean section (Boies and Vaucher, 2016; Shapiro-Mendoza et al., 2008).

In some situations, however, BM alone is not enough to meet nutritional needs and, therefore, fortification of BM with extra protein, carbohydrate and micronutrients is recommended for some preterm infants (<32 weeks' gestation or birth weight < 1,800 g) (Arslanoglu et al., 2019). MP babies are more likely to have birth weight <1,800 g, and thus receive fortification of BM, than LP babies (Lapillonne et al., 2019). In addition, supplementation of minerals and vitamins is particularly important for preterm babies exclusively breastfed (Embleton, 2013). MLP babies are more likely to develop iron deficiency, iron-deficiency anaemia and vitamin D deficiency compared to babies born FT. Supplementation of iron and vitamin D are recommended but evidence regarding supplementation of other vitamins and trace elements in MLP babies is currently lacking (Lapillonne et al., 2019).

When maternal BM is not available, pasteurised donor breastmilk (PDBM) is preferred and only when both are not available are preterm or standard infant formulas recommended as an alternative feeding strategy (Arslanoglu et al., 2013; Embleton, 2013). There are few data on the benefits of PDBM in MLP and ET infants, although in more preterm infants provision of PDBM has been associated with a positive impact on any breastfeeding at hospital discharge (Williams et al., 2016) and a lower incidence of necrotising enterocolitis when compared to formula feeding (Quigley and Mcguire, 2014).

Growth is recognised to be slower in preterm infants fed PDBM, and most recommendations are that PDBM should be fortified to meet preterm infants' nutritional

requirements (Quigley and Mcguire, 2014). Pasteurisation can inactivate bio-active compounds present in fresh BM such as immunoglobulins, lactoferrin and lysozyme (Buffin et al., 2018; Paulaviciene et al., 2020). The large individual variability in BM macronutrient composition poses an additional challenge in the use of PDBM for preterm nutrition (for further review on BM composition see section 1.4). Combining BM from multiple donors might minimise individual variability in macronutrient composition of PDBM (John et al., 2019), but more research is needed to ensure optimal composition of PDBM and determine potential benefits of its use for nutrition of MLP infants. Further, the provision of donor BM can be limited by its availability. In New Zealand, for example, there is only one human milk bank in the entire country, meaning that neonatal units across the nation need to use informal donation with adequate donor screening as an alternative (Meeks et al., 2019).

Ultimately, mother's own BM remains preferable to PDBM and effort should be focused on supporting lactation and breastfeeding in mothers (Meier et al., 2017).

1.3.4.1 *Breastmilk substitutes*

When neither mother's own BM nor PDBM are available, then a variety of artificial formulas are available with differences in energy, protein and mineral content intended to mimic the nutritional content of human milk. Standard term formulas typically provide 68 kcal/100 mL of energy and 1.4-1.7 g/100 mL of protein in addition to calcium and phosphate, while preterm formulas are energy and protein-enriched, typically providing 80 kcal/100 mL of energy and 2.0-2.4 g/100 mL of protein.

Different protein concentrations in term infant formula were compared in an RCT in 5 European countries in which breastfed children served as reference group (Koletzko et al., 2009). During the first year of life, term babies were randomised to receive either low protein

formula (1.25 g/100 mL and 2.05 g/100 mL in initial and follow-on formula, respectively) or high protein formula (1.6 g/100 mL and 3.2 g/100 mL in initial and follow-on formula, respectively). Babies randomised to the higher protein content formula had higher weight at the age of two years (Koletzko et al., 2009), increased risk for excessive body fat in the second year that lasted until school age (Weber et al., 2014), and a greater than 2-fold increased risk of obesity by 6 years of age (OR 2.43 (1.12-5.27)) compared to the low protein content group (Totzauer et al., 2018). Whether there is a similar effect in MLP babies is unknown.

The fat components of infant formulas are also different from those in human milk. Lipids in human milk are essentially milk fat globules of triglycerides enveloped by a three-layer emulsifier membrane (phospholipids, proteins and cholesterol), whereas infant formulas have lipids with different molecule size and emulsifier membrane architecture. Palmitate in infant formula is low in the sn-2 stereoisomer compared with BM, which is high in this isomer. Novel infant formulas addressing fat structure (Gallier et al., 2015) and the proportion of sn-2 palmitate (Miles and Calder, 2017) suggest that current infant formulas can be improved substantially, resulting in better tolerance, stool composition and, potentially, other outcomes such as bone health. Future research should address the effect of formulas that reflect more closely the composition of human milk on later growth, body composition and neurodevelopment in MLP and ET formula-fed infants.

1.3.4.2 *Post-discharge formulas*

For formula-fed infants, nutrient-enriched post-discharge (or “follow-on”) formulas are available which have higher energy density, increased protein concentration and greater mineral and vitamin content compared with standard term formula. The evidence for the benefit from post-discharge formula in preterm babies is of moderate quality and inconsistent

and currently is insufficient to support their routine use (Teller et al., 2016; Young et al., 2016), although there may be benefit for infants who have been identified as growing poorly on standard formula or who have ongoing mineral or vitamin deficiencies. However, there are no data to support their use in MLP or ET infants.

1.3.5 ***Breastfeeding MLP and ET babies***

Coordination of sucking and swallowing reflexes are not fully developed until 34–35 weeks' gestation; thus, most MLP babies need time and support to develop the skills necessary for oral feeding (Embleton, 2013; Mally et al., 2010; Walker, 2008). These babies are usually less alert and have less stamina with poor head control to sustain a good latching position and less strength for adequate pressure to suck at breast (Boies and Vaucher, 2016; Eidelman, 2016; Walker, 2008). All these factors can lead to difficulties in breastfeeding, poor weight gain, dehydration and jaundice during early postnatal week and increasing the likelihood of rehospitalisation (Mally et al., 2010).

The Academy of Breastfeeding Medicine suggests that to support breastfeeding of LP infants there should be early initiation of breastfeeding (within the first hour where possible) and, should mothers and babies be separated, stimulation of milk production through regular expressing and frequent skin-to-skin contact or 'kangaroo cuddles' (Boies and Vaucher, 2016). This approach is also valid for MP and ET infants, and also improves mother-infant bonding, exclusive breastfeeding rates and reduces costs of care (Boies and Vaucher, 2016). Breastfeeding MLP infants successfully can be challenging as they are less alert, have poorer coordination of sucking-swallowing-breathing reflexes and have delayed maturation of the autonomic system that can predispose to cardiorespiratory instability (Hunt, 2006). Expressed BM may need to be given by gavage, cup, bottle, syringes or finger feeding (Boies and

Vaucher, 2016). These approaches also improve mother-infant bonding and exclusive breastfeeding rates (Boies and Vaucher, 2016). Furthermore, the presence of a dedicated lactation consultant in NICUs has been associated with increased feeds of mothers' own BM and improved breastfeeding outcomes in preterm babies (Mercado et al., 2019).

Cup feeding may improve breastfeeding rates up to six months among MLP babies when compared to bottle feeding, but compliance is problematic as it may increase feeding time (Boies and Vaucher, 2016) and require greater attention to possible adverse effects (choking, vomiting) that may be concerning for parents. Retrospective cohort data from the Pregnancy Risk Assessment Monitoring System (PRAMS) in the US found that LP babies are less likely to be initially breastfed and to be breastfed for 10 weeks or longer compared with FT infants (Hwang et al., 2013). Other data suggest this also is true for ET infants in the US, who are significantly less likely than full-term infants to be breastfed one month postpartum (OR= 0.77, 95% CI 0.60–0.99) (Hackman et al., 2016). Accordingly, robust evidence on how best to support successful breastfeeding in MLP and ET babies is needed.

Mothers who birth preterm are more likely to experience factors that may impact upon lactation, including separation from their infant, medical conditions that may have contributed to the early birth, multiple births and birth via caesarean section (Boies and Vaucher, 2016). A variety of breastfeeding assessment tools are available, although few have undergone adequate assessment and testing, with the Early Feeding Skills Assessment and the Bristol Breastfeeding Assessment Tool probably the most robust (Pados et al., 2016), and "lactation technology", such as nipple shields and hospital-grade breast pumps may also facilitate breastfeeding MLP and ET babies (Boies and Vaucher, 2016).

1.3.5.1 *Lactogenesis and production of human milk*

Lactogenesis is divided into two stages, based on the readiness of the mammary gland to initiate milk production, and its onset is highly hormone-dependent (Buhimschi, 2004; Neville et al., 2001). Reproductive hormones (oestrogen, progesterone, prolactin, insulin and placental lactogen) and metabolic hormones (growth factor, insulin and glucocorticoids) are required for secretory differentiation (Buhimschi, 2004; Pang and Hartmann, 2007). The differentiation of mammary epithelial cells into lactocytes (Lactogenesis I) takes place in mid-gestation and by the third trimester the mammary gland is fully differentiated to allow milk production, but high circulating levels of progesterone and oestrogen inhibit copious milk secretion (Buhimschi, 2004; Neville et al., 2001).

Following birth, circulating concentrations of progesterone and oestrogen decrease significantly and, coupled with high plasma concentrations of prolactin, insulin and cortisol, trigger the onset of milk production (Buhimschi, 2004; Neville et al., 2001; Pang and Hartmann, 2007). Secretory activation (also referred to as Lactogenesis II) usually occurs around 36-72 hours postpartum in response to the progesterone withdrawal that occurs following delivery of the placenta (Neville et al., 2001; Pang and Hartmann, 2007).

The initial lacteal secretion produced in low volumes has high concentrations of immune factors such as immunoglobulins and lactoferrin, which prime the gastrointestinal and respiratory tracts protecting against infections (Pang and Hartmann, 2007). The removal of milk from the alveoli triggers the production of more milk and this continuous cycle stimulates more milk production, in an “on-demand” basis (Neville et al., 2001; Pang and Hartmann, 2007). In the absence of such stimuli, the involution of the mammary gland occurs through cellular apoptosis, returning to non-lactating state (Neville et al., 2001; Pang and Hartmann,

2007). This is particularly important in mothers of preterm infants, given that these babies usually are less alert and have less stamina and strength, hence becoming more easily fatigued and less up to the task of milk extraction for the duration of a whole feed.

Delayed onset of Lactogenesis II (DOL-II) is commonly defined as secretory activation occurring after 72 hours postpartum (Huang et al., 2020; Neville et al., 2001; Preusting et al., 2017). The prevalence of DOL-II is about 20% (Huang et al., 2020; Rocha et al., 2020) and risk factors are maternal obesity and excessive pregnancy weight gain (Preusting et al., 2017), insulin-dependent diabetes mellitus (Neubauer et al., 1993), maternal stress (Neville et al., 2001), postpartum depression (Rocha et al., 2020), higher maternal age (Rocha et al., 2020), and preterm delivery (Cregan et al., 2002). A previous study found that 82% of mothers of preterm babies had compromised onset of lactation and low breastmilk production (Cregan et al., 2002). Mothers who experience DOL-II are less likely to exclusively breastfeed and are more prone to early cessation of breastfeeding (Huang et al., 2020; Preusting et al., 2017). Nevertheless, early initiation (in the first hours of birth) coupled with regular milk expression may be useful to prevent DOL-II in at-risk mothers (Fok et al., 2019; Parker et al., 2015).

1.4 Human milk and nutrition of preterm infants

Mothers' own milk (MOM) is considered the gold standard in neonatal nutrition (Bertino et al., 2012). Breastfeeding or provision of expressed BM has several nutritional and health benefits to neonates and mothers, leading to the general consensus that breastfeeding provides a series of advantages over any other feeding (Stuebe, 2020; Victora et al., 2016).

In addition to energy, proteins, fats, carbohydrates, minerals and vitamins, BM also provides immune protection and hormonal signalling that shape infant growth and behaviour in a very personalised and dynamic manner, contributing to the evolution of our species (Galante et al., 2020a; Stuebe, 2020). Although many efforts have been made to improve composition of BM substitutes (Gallier et al., 2015; Martin et al., 2016; Weber et al., 2014; Yuan et al., 2019), there is no formula that can replicate the intrinsic tailor-made composition of BM.

1.4.1 Human milk composition

Postnatal age, gestational stage, maternal nutrition and metabolism are important predictors for BM energy and nutrient content (Gidrewicz and Fenton, 2014; Lönnerdal et al., 2017). BM can be classified according to lactation stage as colostrum, the fluid secreted by the mammary gland in the first 5 days after birth; transitional milk, which is produced between 5 and 14 days after birth, and mature milk which is secreted from the third week after birth until breastfeeding is terminated (Ballard and Morrow, 2013). Further, BM secretion consists of two phases, fore- and hind-milk. Foremilk is rich in lactose and has a watery aspect, whereas hindmilk is thicker due to its higher fat content which can be 2 to 3 times higher than that of foremilk (Ballard and Morrow, 2013; Kociszewska-Najman et al., 2012). The adaptations in breastmilk composition within a feed, throughout lactation, with circadian rhythm (especially for fat concentration which is higher in BM produced at night than in the morning), and amongst individuals (Hahn-Holbrook et al., 2019; Moran-Lev et al., 2015; Pham et al., 2020) reflect the dynamic and unique interaction between the mother-infant dyad and environmental cues that are transmitted to the infant by mothers' milk, leading to a highly personalised nutritional content. One recent example of human milk

adaptive response is the identification of SARS-Cov-2 specific immunoglobulin A and G with neutralising activity against SARS-Cov-2 in BM produced by mothers diagnosed with COVID-19, which have not been identified in milk samples collected in the “pre-pandemic” world (Pace et al., 2021).

1.4.1.1 Length of gestation, postnatal age and breastmilk composition

When considering the changes in BM composition with advancing postnatal age, colostrum has a higher protein content which progressively decreases throughout lactation (Gidrewicz and Fenton, 2014; Lönnerdal et al., 2017; Mimouni et al., 2017). For example, in mothers of preterm babies, true protein content of colostrum is approximately 2.5 g/100 mL in the first days, decreasing to 1.9 g/100 mL in the second week postpartum, and decreasing further to 1.2 g/100 mL by 10-12 weeks postpartum (Mimouni et al., 2017). In mothers of term babies, however, the same longitudinal trend is observed but true protein content in colostrum is approximately 0.7 g/100 mL lower than colostrum produced by mothers of preterm babies, reaching similar levels by 10-12 weeks postpartum (Gidrewicz and Fenton, 2014). Colostrum is produced in low quantities and has low concentration of lactose; however, its high concentrations of immunologic components, human milk oligosaccharides (HMOs) and bioactive proteins, such as secretory immunoglobulin A (sIgA), immunoglobulin G (IgG), immunoglobulin M (IgM), lactoferrin, lysozyme and serum albumin suggest that the primary function of colostrum may be immunologic rather than nutritional (Ballard and Morrow, 2013; Coppa et al., 1993; Lönnerdal et al., 2017).

In contrast, content of energy, fat and lactose increase with progression of lactation (Gidrewicz and Fenton, 2014; Mimouni et al., 2017). In preterm BM, the energy content increases from about 60 kcal/100 mL in colostrum to 70 kcal/100 mL in transitional BM and

remains around 74 kcal/100 mL thereafter (Mimouni et al., 2017); fat content is about 2.5 g/100 mL in colostrum, 3.2 g/100 mL in transitional BM, reaching 3.8 g/100 mL in mature BM (Mimouni et al., 2017; Thakkar et al., 2019); lactose is about 6.2 g/100 mL in colostrum increasing to approximately 7.0 g/100 mL in mature BM (Mimouni et al., 2017).

1.4.1.2 Proteins in breastmilk

The dominant proteins in BM are casein and whey proteins, which include bioactive proteins such as lactoferrin, α-lactalbumin, lysozyme, sIgA, IgG and IgM (Lönnerdal et al., 2017). These bioactive proteins are essential to the development of the immune system and can help modulate the immune response through bacteriostatic and bactericidal properties, transfer of maternal antibodies and immunomodulatory activity (Lönnerdal et al., 2017). Furthermore, BM contains hormones that mediate metabolic function, stimulate growth and regulate appetite and energy intake, such as insulin-like growth factor 1 (IGF-1), leptin and adiponectin (Galante et al., 2020a, 2020b). Additionally, the unique variety of bioactive proteins in BM have enzymatic activity, stimulate growth and enhance micronutrient absorption (calcium, iron, zinc) (Lönnerdal et al., 2017).

1.4.1.3 Carbohydrates in breastmilk

The major carbohydrate in human milk is lactose, which is an important energy source and has low concentration in colostrum with a gradual increase over time (Gidrewicz and Fenton, 2014; Mimouni et al., 2017). In addition, complex bioactive oligosaccharides made of a combination of five monosaccharides (glucose, galactcose, N-ethylglucosamine, fucose and sialic acid), known as human milk oligosaccharides (HMOs), are the second group of carbohydrates present in human milk and are not fully digested by the infant (Azad et al., 2018; Thurl et al., 2010; Wiciński et al., 2020). HMOs are thought to function as prebiotics

assisting the development of healthy gut bacteria and protecting against pathogens binding to enterocytes (Underwood, 2013; Wiciński et al., 2020).

1.4.1.4 Lipids in breastmilk

Lipids provide up to 50% of the energy necessary for growth in addition to essential fatty acids and lipid soluble vitamins that promote visual acuity and neurodevelopment (Kociszewska-Najman et al., 2012). Fats in BM are available in the form of milk fat globules (MFG) (Lee et al., 2018). The MFG is a complex structure formed by a triacylglycerol (TAG) core, protected by a highly specialised layer, the milk fat globule membrane (MFGM) (Lee et al., 2018). The MFGM is composed mostly of phospholipids, sphingolipids, cholesterol, bioactive proteins and long chain polyunsaturated fatty acids (LCPUFAs) and its structure is unique to the human species (Demmelmair and Koletzko, 2018; Lee et al., 2018; Yuan et al., 2019). The TAG core provides about 98% of all milk fats and the remainder consists of free cholesterol, phospholipids and fatty acids (FA) in trace amounts (Demmelmair and Koletzko, 2018). The composition of the TAG core is approximately 42% saturated fatty acids (SFAs), 42% monounsaturated fatty acids (MUFAs) and 16% polyunsaturated fatty acids (PUFAs) (Bobiński and Bobińska, 2020).

1.4.1.5 Micronutrients in breastmilk

Whilst BM provides enough of most vitamins and minerals for optimal growth and development, vitamin D, iron and iodine can be deficient in some populations (Allen and Hampel, 2020). The concentration of some vitamins in BM are dependent on maternal dietary intake and body stores such as vitamins from the B complex (B1, B2, B6, B12), choline, vitamins A, D, E, K, iodine and selenium; thus, supplementation of maternal diet may improve BM concentrations (Allen and Hampel, 2020; Dror and Allen, 2018). On the other hand, folate,

calcium, iron, copper and zinc are unrelated to maternal status and supplementation of maternal diet has little impact on BM concentrations (Allen and Hampel, 2020). For preterm infants, supplementation of vitamin D and iron is common practice and breastmilk fortifiers provide adequate intake of most micronutrients (except vitamin D) (Oliver et al., 2020). Regardless, supplementation of with vitamin D, K and B12 are recommended for breastfed babies during early lactation and of iron after 6 months (Dror and Allen, 2018).

1.4.2 ***Sensory properties of breastmilk***

Volatile substances, called odourants, are small, organic and inorganic volatile molecules of several chemical structures. Odourants are produced in a variety of metabolic pathways by all living organisms (Dunkel et al., 2014). In BM, volatile compounds are likely to originate from endogenous metabolism and from maternal diet and contribute to the smell and flavour of breastmilk (Bingham et al., 2003b; Hausner et al., 2009; Mastorakou et al., 2019).

The odour of fresh breastmilk has been described as presenting subtle aroma notes of metallic, fishy, fatty and cooked milk by sensory panellists trained to recognise a diverse array of odourants (Spitzer et al., 2010). BM stored under refrigerated conditions for three days or stored frozen for 6 months (-20°C) can develop off-notes such as rancid, sweaty and buttery aromas (Spitzer et al., 2013; Spitzer and Buettner, 2013). However, if BM undergoes thermal treatment (i.e. pasteurisation), the development of off-notes is less intense than when prolonged storage of unheated BM occurs (Spitzer et al., 2010).

The development of unpleasant odours result mostly from lipid degradation, either through peroxidation of lipids yielding volatile compounds such as aldehydes, ketones and

alcohols; or from enzymatic lipolysis of untreated BM (by activity of human milk lipases), which releases free fatty acids and their esters from the milk fat globules (Spitzer et al., 2010, 2013; Spitzer and Buettner, 2013). In this sense, the presence of secondary products of lipids degradation are important indicators of BM integrity and oxidative status (Elisia and Kitts, 2011).

Previous studies have shown that modifications to maternal diet in pregnancy and lactation can influence infants' acceptance of certain foods (Mennella et al., 2001, 2017), but the flavour of BM has been less thoroughly investigated. Mastorakou et al (2019) investigated the flavour attributes of BM and found that most mothers perceived the flavour of BM as sweet, but bitter, vanilla and watery attributes were also reported (Mastorakou et al., 2019). Interestingly, positive correlations between BM macronutrients and BM flavour were identified, with fat and protein content in BM correlated with a perceived creamy flavour, carbohydrate content in BM with sweet flavour and bitterness of maternal diet with perceived bitter flavour of BM, which was estimated as relative bitterness scores from foods consumed by mother 24 hours before BM donation (Mastorakou et al., 2019).

Diet-induced flavour changes to BM can influence infants' feeding behaviour (Debong and Loos, 2020). In a study in which mothers consumed garlic extract capsules (Mennella and Beauchamp, 1991a) and vanilla-flavoured drink (Mennella and Beauchamp, 1996) after refraining from ingestion of these foods for three days before the test, infants spent more time breastfeeding, sucked more and consumed more milk (measured by infants' body weight before and after consumption) when consuming flavoured BM compared to breastfeeding trials in which the mother had not consumed these flavoured foods. Similarly, maternal ingestion of vegetables during late pregnancy and lactation has been associated with later child preference to cereals containing the same flavours. In contrast, when mothers

consumed orange juice with small amounts of ethanol (approximately 0.3 g per Kg of body weight), infants consumed significantly less BM compared to trials in which mothers drank orange juice without alcohol (Mennella and Beauchamp, 1991b).

These studies suggest that flavour and aroma of BM are determined by individual characteristics and can be modified by maternal diet, thermal treatment and length of storage. However, very little is known about sensory properties of preterm BM.

1.5 Sensory perception in the preterm infant

Smell and taste are intimately connected to nutrition and behaviour, directly influencing food preferences. Depending on the smell and taste of food, one can reject or accept a meal and this reaction has contributed with the survival of our species (Reed and Knaapila, 2010), by preventing the intake of harmful or poisonous food and assisting the search for energy-dense, pleasurable and beneficial foods. Maternal odours are paramount for kin recognition and nipple localisation by the newborn baby. Furthermore, smell and taste are essential for digestion and metabolism of food, triggering physiologic responses that allow individuals to better process nutrients ingested (Smeets et al., 2010).

1.5.1 Development of smell and taste

The ontogeny of the olfactory system is very complex, starting from as early as 16 weeks' gestation and is not complete until the second year of life (Sarnat et al., 2017). Differentiation of cells from the olfactory neuroepithelium occur by the end of the first trimester (Sarnat et al., 2017). The olfactory marker protein, which is involved in signal

transduction, is present in the primary receptor cells from about 28 weeks' post-conception and olfactory responses are present in fetuses after 30 weeks' gestation (Azoulay et al., 2006; Chuah and Zheng, 1987; Sarnat et al., 2010). Towards the end of gestation (around 36 weeks), the epithelial plugs that protect the fetal airway disintegrate exposing the olfactory receptors to chemicals present in the amniotic fluid (Lipchock et al., 2011). The final stage of olfactory development is the myelination of the olfactory bulb, which occurs after birth and is not complete until 2 years of age (Sarnat and Flores-Sarnat, 2017).

Similarly, ontogeny of the gustatory system starts around 8 weeks of gestation, with the appearance of taste receptors (Lipchock et al., 2011). By the end of the first trimester, these cells resemble mature receptor cells and they are considered functionally mature by 17 weeks (Lipchock et al., 2011). Swallowing reflexes appear at approximately 12 weeks of gestation (Lipchock et al., 2011), but active coordination between sucking and swallowing only develops later, after 34 weeks of gestation (Dasgupta et al., 2016; Underwood et al., 2005). By the last trimester of pregnancy the gustatory system is capable of conveying sensory information to the central nervous system and beginning of flavour learning occurs (Forestell, 2017).

Odours may be detected during inhalation by olfactory receptors located high in the nasal chamber (orthonasal route) and during tasting by receptors present in the oral cavity and nasopharynx (retronasal route). The sense of taste is mainly dictated by activation of taste receptors located in the mouth by molecules (nutrients, odourants, chemicals), but also can receive inputs from smell and texture of food, integrating the flavour perception (Lipchock et al., 2011).

Various nutrients and odourants can act as ligands to G-protein coupled receptor (GPCR) subunits within olfactory and taste cells and, once activated, these can mediate the

secretion of many hormones such as insulin, glucagon-like polypeptide 1 (GLP1) and ghrelin (Calvo and Egan, 2015; Ichimura et al., 2009). Examples include free fatty acid receptors (FFAR1 – 4) and taste receptors type 1 (TAS1Rs), which are activated by free fatty acids (FA) of different chain lengths and by umami and sweet flavours, respectively (Calvo and Egan, 2015; Ichimura et al., 2009; Teff, 2011), all of which are present in BM and infant formulas. Furthermore, odourants of different chemical classes are capable of binding to different GPCRs expressed within olfactory cells with odour-receptor specificity, allowing detection and discrimination of different smells (Johnson et al., 2005).

Once sensory information travels from peripheral sensory organs to higher brain regions, three main cortical areas are involved (de Araujo et al., 2003). The primary cortex is the first area of the cerebral cortex that receives the neuronal stimulation from peripheral smell and taste receptors. Next, the sensory information is directed to the secondary cortex where single sensations are processed (unimodal sensation), and finally, the integration of multimodal sensory information (flavour perception) occurs in the association cortices (de Araujo et al., 2003; Okamoto and Dan, 2007). The integration of sensory information rising from taste and smell receptors in the peripheral organs is believed to occur in the orbitofrontal cortex (OFC) (Avery et al., 2020; de Araujo et al., 2003; Small and Prescott, 2005). In addition, the OFC integrates information from all five sensory modalities (visual, auditory, somatosensory, gustatory and olfactory stimulations) forming a complex network with other brain areas, such as the insula, amygdala, piriform cortex and others (de Araujo et al., 2003; Small and Prescott, 2005).

The activation of specific olfactory and taste receptors allows the brain to discriminate smells and flavours (Bak et al., 2018; Calvo and Egan, 2015). Perception of odourants in humans is measured by the odour activity value (OAV), which takes into account the ratio of

the concentration of an individual substance in a sample and the threshold concentration of this substance (minimal concentration) that can be detected by the human olfactory receptors. Not all volatile compounds are odourants as compounds with OAV <1 cannot be perceived by the human nose (Andreas Dunkel et al., 2014). However, it has been suggested that babies might be able to detect odours at lower threshold concentrations than adults (Spitzer and Buettner, 2013).

Smell perception can evoke a variety of physiological responses (Power and Schulkin, 2008), including: head orientation towards the familiar smell of amniotic fluid and BM (Marlier et al., 1998); increase in non-nutritive sucking when smell of milk is provided with tube feeding in preterm babies (Bingham et al., 2007), with BM eliciting greater responses formula (Bingham et al., 2003a); greater cerebral oxygenation measured by near infrared spectroscopy (NIRS) in response to BM compared to smell of formula (Aoyama et al., 2010), and improved breastfeeding performance (Raimbault et al., 2007). Most of the studies have been conducted in controlled experimental settings; nonetheless, physiological responses to smell of different milks have been observed in preterm babies in a variety of experiments.

1.5.2 ***Flavour learning***

By the end of gestation, babies are actively swallowing around 250 mL of amniotic fluid (AF) per Kg per day (Dasgupta et al., 2016). A variety of chemicals present in mothers' diets, such as volatile compounds, can cross the placental barrier and diffuse into the fetal blood stream to stimulate the olfactory and taste receptors (Forestell, 2017; Lipchock et al., 2011). Thus, sensory learning initiates *in utero* (Schaal et al., 2020).

Odourants present in AF convey a variety of information from maternal chemical ecology including food odorants, which are later shared in BM (Schaal et al., 2020; Spahn et al., 2019). The intrauterine sensory environment prepares the fetus to the postnatal environment by facilitating recognition of familiar odours, attraction to maternal odours and facilitating postnatal adaptation (Schaal et al., 2020).

Right after birth, odour from the maternal breast elicits crawling towards the odour source in FT babies and they are more attracted to the nipple from the unwashed breast over the washed one (Varendi et al., 1994; Varendi and Porter, 2001), suggesting that natural odours emanating from the maternal breast may assist in nipple localisation and transition to nursing. It is noteworthy that for most preterm infants this "natural" transition from antenatal to postnatal sensory environment is disrupted by necessary medical interventions and skin-to-skin contact or early breastfeeding soon after birth is not always feasible. Whether this disruption may compromise postnatal adaptation or development of feeding skills is unclear.

Furthermore, antenatal flavour exposure may impact the development of feeding preferences (Spahn et al., 2019). Exposure to dietary flavours transmitted through BM can influence milk intake (as detailed above) (Mennella and Beauchamp, 1991a, 1991b, 1996), and may also shape later feeding preferences. During weaning, infants tend to exhibit better acceptance of foods that contain "familiar" flavours consumed by mothers during lactation, such as vegetables (Mennella et al., 2001, 2017), suggesting that early experiences with a variety of flavours can influence infants acceptance to weaning foods and reinforcing benefits of healthy and diversified maternal diet.

The evidence available for antenatal and postnatal sensory learning suggest that by the third trimester of gestation, fetus and preterm infants are capable of experiencing smell and

taste and this sensory experience may have lasting flavour impression, and hence this may be a period of great opportunity for establishing food preferences.

1.5.3 ***Cephalic phase response of digestion***

In response to sensory stimulation, such as the taste, texture, and sight of food, a cascade of preabsorptive physiological responses are activated by the brain, referred to as cephalic phase response (CPR) of digestion (Smeets et al., 2010). The CPR is an anticipation to food ingestion and prepares the body to digest, absorb and metabolise food by triggering peristaltic movements of gut, the secretion of digestive enzymes and the release of digestion-related hormones such as ghrelin, leptin, gastrin, insulin and others. These responses are responsible for about 30% of the gastric secretions associated with the ingestion of food (Power and Schulkin, 2008). For example, when individuals are asked to taste, chew and expectorate food, an increase in blood levels of insulin and pancreatic polypeptide can be detected within minutes (Teff, 2000). However, feeding in the absence of CPR led to elevated levels of both insulin and glucose, a metabolic state that if persistent might indicate impaired glucose metabolism (Power and Schulkin, 2008; Teff, 2000).

The first description of the CPR of digestion was made by Ian Pavlov in 1890, at the time referred to as *"psychic reflex"*. Experiments on dogs with an exteriorised oesophagus demonstrated that, when the food was placed in the dog's mouth without reaching the stomach, a great quantity of saliva and digestive secretions was produced. In contrast, when the food was placed inside the animal's stomach by tube-feeding without contact with the oral mucosa, it remained undigested for hours and few digestive secretions were produced. Similar responses were observed for the effect of sight and odour of food and production of saliva and digestive secretions. Thus, Pavlov concluded that the activity of the salivary gland

is always excited by some external phenomenon such as taste, smell or visual stimuli, characterising a conditional reflex (The Nobel Prize, 1904).

Later, the classical definition of CPR was proposed by Powley (1977), who stated "*the cephalic phase responses are autonomic and endocrine reflexes involved in the metabolism of food that are triggered by sensory contact with foodstuffs rather than by postingestional consequences of food (…) the onset of these reflexes anticipates the digestion and often even the ingestion of the food*" (Powley, 1977).

Smell and taste receptors are also found outside their primary sensorial location (nose and mouth) (Calvo and Egan, 2015; Maßberg and Hatt, 2018). Olfactory receptors (ORs) have been found throughout the gastrointestinal (GI) tract and in organs that assist digestion and nutrient metabolism such as the pancreas and liver. Receptors in these locations may be stimulated during feeding, mediating the secretion of digestive hormones (Braun et al., 2007; Maßberg and Hatt, 2018). Similarly, taste receptors are expressed throughout the GI epithelium and are involved in regulation of food intake and digestion, regulating the secretion of the digestive hormones such as ghrelin, leptin, glucagon and cholecystokinin (Calvo and Egan, 2015). Thus, sensory receptors located outside oral and nasal cavities may assist digestion and metabolism by mediating hormone secretion.

One important function of CPR is to modulate gastric phase processes such as the release of gastric and pancreatic juices and hormones (Smeets et al., 2010). Preterm infants are often unable to coordinate sucking, swallowing, and breathing; therefore, nutrition is provided through feeding tubes (nasogastric or orogastric tube) (Toce et al., 1987). In this feeding mode, milk deviates from the oral and nasal cavities limiting smell and taste experience in these infants (Bloomfield et al., 2017). It is possible, therefore, that in

exclusively tube-fed preterm infants the CPR is stimulated to a lesser degree, or not at all, and this may impact upon food transit time, absorption and digestion.

1.6 Cerebral activation measured by Near Infrared Spectroscopy

Brain activity is a process of intracellular and extracellular (intravascular) changes. Neuronal activity induces an increase in cerebral blood flow to supply the oxygen required to meet cellular metabolic demand (Fox et al., 1988). During sensory stimulation there is a rise in oxygen delivery to brain cells which leads to an increase in concentration of oxygenated haemoglobin (O_2Hb) in the cerebral cortex, including the orbitofrontal cortex (Kringelbach, 2005; Villringer and Chance, 1997). Oxygen is transported bound to haemoglobin and the oxygen carrying status of this molecule can be measured using near infrared spectroscopy as oxygenated and deoxygenated forms absorb light at different wavelengths (Villringer and Chance, 1997).

1.6.1 Near Infrared Spectroscopy

In order to understand cerebral haemodynamic and brain responses to different stimuli Near Infrared Spectroscopy has been used for clinical and research purposes (Aoyama et al., 2010; Aslin and Mehler, 2005; Frie et al., 2018, 2020; Harada et al., 2006).

This safe, non-invasive bedside technique uses an optical device that irradiates and detects light after it has been reflected and transmitted through tissue in a sender-receiver pair attached to the subject's head (Aslin and Mehler, 2005). Because biological tissues absorb light differently, it is possible to detect changes in natural chromophore concentrations, such

as the concentration of oxygenated and deoxygenated haemoglobin, in response to stimulation, through transillumination (Harada et al., 2006; Villringer and Chance, 1997).

Infrared light with wavelengths between 700-1000 nm can pass through body tissue and the absorption of light varies according to the quantity of oxygen present in haemoglobin, allowing the measurement of oxygenated (O_2Hb) and deoxygenated (HHb) and total changes in haemoglobin (Harada et al., 2006). The absorbance spectrum of haemoglobin depends on its oxygenation status. O_2Hb and HHb absorb near infrared light at different wavelengths, together accounting for total haemoglobin (THb= O_2Hb + HHb) (Dix et al., 2017). O_2Hb absorbs more infrared and less red light than HHb, allowing the differentiation between the two states of haemoglobin (Dix et al., 2017).

The NIRS devices applies this principle to measure the absorption of different wavelengths and to detect changes in the concentration of oxygen present in haemoglobin in cerebral blood flow (CBF), based in the modified Lambert Beer law (Nicklin et al., 2003). The general assumption is that the total haemoglobin concentration (HbT) remains constant, and a typical NIRS trace shows the impact of an episode of deoxygenation, where a decrease in O_2Hb concentration reflects an equal and opposite rise in HHb (Nicklin et al., 2003). Therefore, changes in oxygen concentration reflects brain activation, which can be distinguished based on the light absorption measurements of O_2Hb and HHb (Villringer and Chance, 1997).

Cerebral oxygenation is a highly relevant physiological parameter which can reflect the metabolic state of tissue and NIRS has been used in a variety of clinical and research settings due to its capacity to detect the oxygenation state of a particular region of the body in a continuous and non-invasive way (Brazy et al., 1985; Dix et al., 2017; Hyttel-Sørensen et al., 2013).

Recent observations of cerebral oxygenation monitored with NIRS have shown that preterm infants who develop NEC have lower cerebral oxygenation throughout hospital admission compared to those who do not develop NEC, potentially explaining the worse neurodevelopment outcomes of these babies (Howarth et al., 2020). Similarly, changes in cerebro-splanchnic oxygenation and perfusion during intermittent bolus feeding in preterm infants found that infants fed formula (*n*= 15) had higher regional splanchnic oxygenation during and after feeding compared to infants fed with BM (*n*= 15) (Grometto et al., 2018). Even though it is expected that the digestive process will induce increase in blood flow in the splanchnic region, the authors demonstrated this physiological redistribution in blood flow occurs at expenses of cerebral oxygenation (Grometto et al., 2018). Despite authors stating infants were matched by gestation and weight, it is noteworthy that formula-fed infants were on average born 2 weeks older, were heavier and assessed at later postnatal ages, although these differences were not statistically significant.

1.6.1.1 Advantages and limitations of NIRS

When comparing NIRS with other types of brain activity monitors like electroencephalography (EEG) and functional Magnetic Resonance Imaging (fMRI), NIRS has important advantages. First, NIRS is a non-invasive bedside measurement with no known side effects (Dix et al., 2017; Harada et al., 2006). Secondly, it achieves a high temporal resolution in less than one second and the wave-length can penetrate biological tissue, including bone (Harada et al., 2006). Third, while EEG is useful to record event-related electric potentials, it is restricted to detect cerebral activity, but the wavelength used in NIRS can detect cerebral perfusion and haemodynamic changes by measuring changes in the cerebral oxygenation status in relation to neural activity, providing important information on cerebral auto-regulation (Aslin and Mehler, 2005; Dix et al., 2017). Further, fMRI requires subjects to stay

still for long periods confined in a chamber and NIRS is relatively quick and possible to be done at the bed-side, which is more feasible for studies involving infants (Aslin and Mehler, 2005; Dix et al., 2017). Additionally, NIRS is a silent device in contrast to fMRI. Finally, NIRS uses two or more wavelengths that produce separate measures of O_2Hb and HHb and these separate measures allow better understanding of the oxygen consumption in the brain tissue (Aslin and Mehler, 2005).

In contrast, there are some limitations related to NIRS. Hyttel-Sørensen et al. (2013) have argued that long periods of cerebral blood flow monitoring can inflict burns from sensors due to heat provoked by infrared light (Hyttel-Sørensen et al., 2013). To overcome this possible hazard, researchers suggest that the sensors must be repositioned frequently. Further, influences from other physiological features, such as body movement, heart pulse, visual and hearing stimulation, can alter the interpretation of the measurements. Aslin and Mehler (2005) suggest that baseline measurements must be settled at the beginning of the analysis in addition to controlling environmental stimulation, such as light and noise which may activate different brain regions, and use of appropriate statistical analysis to try to eliminate signals with shared variance (Aslin and Mehler, 2005). Furthermore, light scattering also is an obstacle to NIRS measurements. The light loss can be significant, compromising the precision of the method (Nicklin et al., 2003). One solution to overcome this problem is to have an angular arrangement between emitting and receiving optodes and the use of multiple optode pairs placed over the surface of interest (Dix et al., 2017; Frie et al., 2018, 2020) to allow better spatial resolution.

1.6.2 ***NIRS and assessment of sensory stimulation***

During olfactory stimulation, oxygen delivery gradually exceeds oxygen consumption in the brain tissue, and therefore O_2Hb concentration is increased. These changes in cerebral blood flow (CBF) are a response to activation of the olfactory cortex (Aoyama et al., 2010; Bartocci et al., 2001; Harada et al., 2006).

Adults exposed to vanilla and strawberry essence, but not water as a negative control, demonstrate activation in the orbitofrontal cortex (OFC) (Harada et al., 2006) and similar activation of olfactory cortex, demonstrated by changes in O_2Hb concentration in the OFC, has also been detected in healthy full-term babies in response to the presentation of the smell of colostrum and vanilla (Bartocci et al., 2000), maternal breast odour (Frie et al., 2020) and nosocomial smells (detergent, adhesive remover) (Bartocci et al., 2001; Frie et al., 2018). In contrast, when babies were presented with a control stimulation (water), only minor fluctuations around baseline O_2Hb concentration was detected (Bartocci et al., 2000).

Differences in frontal lobe activation in healthy babies (mean gestational age of 37^{+5} weeks, range from 33^{+2} to 41^{+5} weeks) have also been investigated following exposure to smell of both breastmilk and infant formula in the first 10 days after birth (Aoyama et al., 2010), with odour of maternal breastmilk odour provoking a greater increase in O_2Hb concentration compared to odour of formula, irrespective of previous feeding experience (breastfeeding only compared to mixed feeding) (Aoyama et al., 2010).

More recently, NIRS has been used in studies involving VP, LP and FT infants (Frie et al., 2018, 2020). Functional NIRS (fNIRS) assessments were used to measure cortical processing of nosocomial odours from the neonatal intensive care (NICU) environment and found that

babies born as early as 31 weeks' gestation are capable of processing olfactory stimulation at the trigeminal and olfactory cortex level (Frie et al., 2018).

In a similar study, preterm and full term babies were exposed to maternal breast odour (MBO) during active sleep (Frie et al., 2020). The stimulus consisted of a cloth which mothers had worn over the night before the assessment and a clean cloth (laundered with perfume-free product) serve as control. No cortical response was observed for the VP group, however both LP and FT infants exhibited increase in O_2Hb concentration in response to control and MBO compared to baseline levels, but the brain regions recruited for cortical perception differ between both groups (Frie et al., 2020). Following presentation of MBO and control odour, FT infants displayed higher cortical activation in the left and right olfactory cortices and in the left frontal cortex, and LP infants displayed significant activation only of the right olfactory cortex for MBO. Interestingly, the authors also noted different patterns of cortical activation in boys compared to girls. On average, FT boys and girls presented activation of both left and right olfactory cortices; VP boys presented a weak activation of the left olfactory cortex and VP girls displayed no cortical activation whatsoever (Frie et al., 2020). The authors acknowledged that sample size was insufficient for a robust comparison between sexes but stressed that even VP neonates are aware of their olfactory environment.

Of note, all studies mentioned above have been conducted in controlled experimental settings, with most of babies asleep during smell presentation, so it is possible that these findings do not apply babies in the NICU environment. Additionally, no study has investigated the impact of taste stimulation in cerebral oxygenation of babies. Thus, we currently do not fully comprehend the impact of exposure to smell and taste of milk and of tube feeding itself on sensory processing and cerebral activation of the OFC in preterm infants.

1.7 Summary

Feeding MLP infants comes with many challenges that ultimately will impact their adaptation to postnatal environment. Despite the relationship between cephalic phase response and digestion being established over one century ago, these physiological reflexes are often underappreciated in nutrition of preterm infants. Since olfactory and taste receptors are functional by the end of gestation, it seems reasonable to assume that a simple intervention of providing smell and taste of milk could help tube-fed preterm babies to better digest their feeds.

Nevertheless, the clinical and safety implications of this intervention are still not clear; whether sensory stimulation provided with tube feeds effectively improves digestion and nutrition; and whether preterm babies can actually process sensory inputs from smell and taste in a clinical, rather than experimental, setting.

Breastmilk is a highly dynamic fluid and may provide sensory cues from maternal environment and metabolism to the infant. The sensory properties of preterm BM have not been investigated extensively and maternal and infant factors that can influence sensory cues conveyed by BM remain largely unknown.

1.8 DIfferent Approaches to MOderate & late preterm Nutrition: Determinants of feed tolerance, body composition and development: The DIAMOND trial

The work undertaken in this thesis is nested within a factorial multicentre randomised controlled clinical trial entitled "DIfferent Approaches to MOderate & late preterm Nutrition: Determinants of feed tolerance, body composition and development", the DIAMOND trial. This trial has been approved by The New Zealand Health and Disability Ethics Committee (16/NTA/90) and written informed consent were obtained from parents or legal guardians prior to enrolment.

Babies enrolled in the DIAMOND trial are MLP infants admitted into one of five neonatal care units in New Zealand, requiring insertion of intravenous nutrition for clinical reasons, and whose mother intended to breastfeed. Infants are randomised to receive a combination of three factors: (1) intravenous nutrition with amino acids or dextrose only; (2) supplemental milk (infant formula or donor milk) or exclusive own mothers' breastmilk; and (3) smell and taste of milk with all tube feeds or standard care (no smell and taste).

The primary outcome for factors (1) and (2) is body composition at 4 months' corrected age, and for factor (3) is time to reach full enteral feeds, defined as 150 mL/Kg/day or exclusive breastfeeding, whichever occurs first. Secondary outcomes include: time to full sucking feeds; number of days in hospital; body composition at discharge; growth: length, weight and head

circumference Z-scores and Z-score change from birth to 4 months' corrected age and at 2 years' corrected age; developmental assessment at 2 years' corrected age; breastfeeding rates; nutritional intake from birth to full enteral feeds or until 28 days of age. Additionally, breastmilk, stool and saliva samples were collected and measurements of cerebral oxygenation during tube feeding were performed in a subset of participants.

This research aims to address many knowledge gaps in the health outcomes of MLP infants by exploring the effects of different feeding strategies on nutrition, growth and neurodevelopment, human milk composition and microbiome.

This thesis investigates characteristics of breastmilk samples collected in the first ten days post-partum and at 4 months' corrected age for participants recruited from May 2017 to August 2019 in four of the research sites, and reports assessments of cerebral oxygenation using NIRS done twice in the first ten days of life while infants were receiving tube feeds. NIRS assessments occurred only for participants recruited at Auckland City Hospital from June 2018 to March 2020.

1.9 Aims

The main aims of this thesis are: to expand on current knowledge of sensory perception in tube-fed preterm infants; to characterise the profile of volatile compounds in milks commonly fed to preterm infants; to explore factors associated with the distribution of volatile compounds in preterm BM; and to determine whether tube-fed preterm infants can process sensory information in the OFC detected by NIRS.

1.9.1 Specific aims

1. Undertake a systematic review and meta-analysis to determine the clinical implications and safety of exposing preterm infants to smell and taste of milk with tube feeds.
2. Analyse the volatile compounds present in various milks commonly used for nutrition of preterm infants.
3. Analyse the volatile compounds in preterm BM and determine maternal and infant factors associated to the profile of volatile compounds in preterm BM.
4. Describe oxygenation changes in the OFC when infants are exposed to smell and taste of milk before tube feeds in comparison to when infants are not expose to sensory stimulation.
5. Describe oxygenation changes in the OFC while tube feeds are being administered.

CHAPTER 2

EXPOSURE TO THE SMELL AND TASTE OF MILK TO ACCELERATE FEEDING IN PRETERM INFANTS

Chapter published in Cochrane Database of Systematic Reviews

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2.1 Background

2.1.1 Description of the condition

Due to immaturity of neurologic and digestive systems, preterm infants (those born before 37 weeks' gestation) are often unable to co-ordinate sucking, swallowing, and breathing in order to feed. Initial nutrition is usually provided intravenously and via a tube which goes through the nose (nasogastric) or mouth (orogastric) into the stomach, with a gradual transition to sucking feeds as co-ordination improves (Toce et al., 1987). Usually, enteral feeds (feeds provided via the gut) start at small volumes and are increased slowly until full enteral feeds are tolerated.

Feeding intolerance is defined as the inability to digest enteral feedings in association with increased gastric residuals (fluid remaining in the stomach after tube feeds), abdominal distension, vomiting, or both (Moore and Wilson, 2011). It often leads to a delay in attainment of full enteral feeds and prolonged intravenous nutrition (Fanaro, 2013). Prolonged intravenous nutrition can increase the risk of: infection; cholestasis (impaired bile flow) (Gargasz, 2012); impaired development of the gut mucosa; necrotising enterocolitis (severe intestinal inflammation) (Fanaro, 2013); and morbidity and mortality (The SIFT Investigators Group, 2013).

Smell and taste are important for the appreciation of food, but also have a significant role in nutrition. In response to these sensory cues, a sequence of pre-absorptive physiological responses is triggered by the brain, collectively referred to as cephalic phase responses (Smeets et al., 2010). The cephalic phase response plays an important role in the activation of physiological processes at multiple sites to optimise digestion, including

increased salivation, increased peristaltic movements, and increased secretion of digestive enzymes and digestive-related hormones, all of which are active in the newborn (Lipchock et al., 2011; Mattes, 1997; Zolotukhin, 2013).

The pathways underlining the cephalic phase response to smell and taste stimulation are diverse and stimulate different parts of the digestive system. First, the increase in salivation starts the process of digestion as a result of the presence of salivary enzymes (such as -amylase and lingual lipase), salivary insulin, and the moistening of the digestive bolus to assist swallowing. Further down the gastrointestinal tract, the cephalic phase response initiates the release of gastric secretions containing gastrin, gastric acid, trypsin and gut peptides. It also initiates the release of hormones such as ghrelin, glucagon-like peptide-1, leptin and somatostatin, as well as increasing gut motility. Smell and taste also are known to stimulate gastric emptying by increasing contraction of segments of the gastrointestinal tract. Lastly, the release of pancreatic secretions that are rich in digestive enzymes such as lipase, amylase and cholecystokinin assist further digestion of nutrients. In addition to the pancreatic secretions released in the gut, the pancreas also releases insulin and glucagon into the bloodstream in response to sensory stimulation.

All of these responses contribute to food digestion and absorption (Mattes, 1997; Zolotukhin, 2013). However, little is known about the effects of smell and taste stimulation in preterm infants, despite the presence of functional taste receptors in the fetus from 18 weeks' gestation and flavour perception from around 24 weeks' gestation (Lipchock et al., 2011).

Distinct olfactory reflexes have been demonstrated in neonates after 32 weeks of gestation, with infants presenting different responses to the smell of substances (such as

amniotic fluid, colostrum or peppermint oil), varying from sucking response alone to a combination of sucking and arousal-withdrawal reflex (Bingham et al., 2003a; Marlier et al., 1998; Sarnat, 1978). These findings suggest that the olfactory system is fully functional in preterm infants after 32 weeks of gestational age.

Fetal swallowing of amniotic fluid starts by the end of the first trimester and reaches up to 750 mL/day by 34 weeks' gestation (Dasgupta et al., 2016). Thus, fetal smell and taste receptors are exposed to the components of amniotic fluid for many weeks before birth and at equivalent gestations to those of infants born preterm (Bloomfield et al., 2017), suggesting that the first sensory experiences happen *in utero.*

Tube feedings bypass the oral and nasal cavities, so tube-fed infants have limited exposure to the smell and taste of their feeds. Therefore, there is little stimulation of the cephalic phase response of digestion.

The provision of smell and taste exposure to preterm infants receiving tube feedings is currently being applied in the care of some preterm infants based on the assumption that there is biological plausibility for a possible benefit, despite lack of evidence to support this practice. More importantly, potential adverse effects have not been assessed; these could include risks such as aspiration, gagging or choking, bradycardia, desaturations or increase in oxygen requirement.

2.1.2 ***Description of the intervention***

The intervention consists of placing a cotton bud or gauze soaked with a few drops of milk with which the infant is being fed close to the infant's nostril to provide the smell of milk,

and placing a few drops of milk on the infant's lips and tongue in order to provide the taste of milk. The exposure should be done before starting the tube feeding in order to stimulate the cephalic phase response of digestion.

2.1.3 ***How the intervention might work***

Preterm infants being fed via orogastric or nasogastric tube have limited exposure to smell and taste stimulation which triggers the cephalic phase response of digestion, and this might contribute to feed intolerance and the need for prolonged intravenous nutrition.

Exposure to the smell and taste of milk before tube feeding may stimulate the cephalic phase response of digestion and assist digestion by increasing salivation, triggering peristaltic movements of the gut, secretion of digestive enzymes and release of digestion-related hormones such as ghrelin, leptin, gastrin, insulin and others (Power and Schulkin, 2008).

2.1.4 ***Why it is important to do this review***

Prolonged intravenous nutrition increases the risk of late-onset sepsis, prolonged hospital stay, and an increase in health costs. In addition, delayed enteral feeding can result in degeneration of the gastrointestinal mucosa and increase the risk of necrotising enterocolitis once the tube feedings start, significantly impacting infant survival and hospital costs (Johnson et al., 2014). Thus, any interventions that accelerate transition to enteral feeding, and then to sucking feeds, would be of considerable potential benefit to infants, their families, and the healthcare system.

It is increasingly common for staff in neonatal nurseries to include exposure to the smell or taste (or both) of milk in the process of tube feeding preterm infants. This is largely based on the belief that this must be beneficial, which could lead to performance bias when assessing the effects of the intervention. Furthermore, this additional intervention requires staff time (and therefore cost), and there is also the potential for adverse effects such as choking or aspiration. Reliable evidence is required on the clinical benefits and possible risks of this intervention.

2.2 Objectives

To determine whether exposure to the smell and taste (or both) of milk administered with tube feedings can accelerate progress to full sucking feeds without adverse effects in preterm infants.

We also planned to assess in subgroups the effects of different modes of administration of the intervention, gestational age, birthweight, and type of milk, but data were insufficient for these analyses.

2.3 Methods

2.3.1 Criteria for considering studies for this review

2.3.1.1 Types of studies

We included published and unpublished randomised or quasi-randomised trials where the unit of randomisation was the infant, or cluster-randomised trials where the neonatal unit

or hospital was the unit of randomisation. We excluded cross-over trials and non-randomised trials such as before-and-after studies.

2.3.1.2 Types of participants

We included preterm infants (born before 37 weeks' gestation) of both sexes and all ethnicities who were receiving any orogastric or nasogastric tube feedings and had not yet reached full sucking feeds.

2.3.1.3 Types of interventions

We included studies that reported exposure to the smell and taste (or both) of breast milk or formula milk, immediately before or at the time of tube feedings.

For smell stimulation, we included in this review studies that reported delivering the smell of milk to preterm infants using a gauze with a few drops of milk placed in the cot/incubator close to the infant's nose, or using a cotton bud soaked with milk, or other forms of administration of the smell of milk (e.g. using an olfactometer adapted to a pacifier).

For taste stimulation, we included in this review studies that reported placing a few drops of milk on the infant's lips or tongue using a syringe, or other forms of oral administration of a small amount of milk (e.g. using a pacifier or swab dipped in milk).

We planned to undertake subgroup analyses to explore the effects of different modes of administration of the intervention (smell of milk versus no smell exposure; and taste of milk versus no taste exposure). However, there were insufficient data to perform these analyses.

2.3.2 Types of outcome measures

2.3.2.1 Primary outcomes

1. Time to reach full sucking feeds (defined as the removal of the feeding tube), measured in days.
2. Adverse effects related to the intervention, such as aspiration, gagging/choking, bradycardia, desaturations or increase in oxygen requirement during the intervention period.

2.3.2.2 Secondary outcomes

1. Duration of parenteral nutrition (defined as the removal of intravenous nutrition line), measured in days.
2. Time to reach full enteral feedings (150 mL/Kg/day, or as defined by the trialists), measured in days.
3. Feed intolerance (resulting in cessation or reduction in enteral feeding), during the period of hospitalisation.
4. Necrotising enterocolitis (Bell's stage 2 or more) (Walsh and Kliegman, 1986), during the period of hospitalisation.
5. Late infection (bacterial or fungal infection confirmed by presence of blood or cerebrospinal fluid infection with initiation of symptoms beyond 48 hours after birth) (Australian and New Zealand Neonatal Network, 2016), during the period of hospitalisation.
6. Growth from birth to discharge (weight, height/length, head circumference and z-scores; gain in these parameters from birth to 36 weeks' postmenstrual age or to term equivalent age; body composition).
7. Exclusive breastfeeding at time of discharge (World Health Organization, 2008).

8. Time to first discharge home, measured in days.

2.3.3 Search methods for identification of studies

We used the criteria and standard methods of Cochrane and Cochrane Neonatal (see the Cochrane Neonatal search strategy for specialised register). We searched for errata or retractions from included studies published in full-text on PubMed (www.ncbi.nlm.nih.gov/pubmed) on 1 June 2018. We did not limit the search to any particular geographical region, language or timing of publication.

2.3.3.1 *Electronic searches*

We searched: the Cochrane Central Register of Controlled Trials (CENTRAL 2018, Issue 5) in the Cochrane Library; MEDLINE via PubMed (1966 to 1 June 2018); Embase (1980 to 1 June 2018); and CINAHL (1982 to 1 June 2018), using the following search terms: ((“Taste”[MeSH] OR “Taste Perception”[MeSH] OR “Smell”[Mesh] OR “Olfactory Perception”[Mesh] OR “Odourants”[MeSH] OR taste*[tiab] OR tasting[tiab] OR gustat*[tiab] OR smell*[tiab] or smelt[tiab] OR olfact*[tiab] OR odour*[tiab]) AND (“Milk, Human”[MeSH] OR “Infant Formula”[MeSH] OR “Colostrum”[MeSH] OR milk*[tiab] or breastmilk*[tiab] OR formula*[tiab] OR colostrum[tiab] OR colostral[tiab])), plus database-specific limiters for randomised controlled trials and neonates (see Search strategies for the full search strategies for each database).

We searched clinical trials registries for ongoing or recently completed trials (clinicaltrials.gov; the World Health Organization’s International Trials Registry and Platform, the ISRCTN Registry, and ANZCTR).

2.3.3.2 Searching other resources

We searched the reference lists of articles selected for inclusion in this review, in order to identify additional relevant articles. We also approached well-known researchers in this area to identify any unpublished or ongoing research.

2.4 Data collection and analysis

We used the criteria and standard methods of Cochrane and Cochrane Neonatal (see the Cochrane Neonatal search strategy for specialised register).

2.4.1 *Selection of studies*

Search results from different databases were merged and duplicates were removed using reference management software. Two review authors (MM and LL) independently assessed the retrieved studies, following the steps below.

1. Screened titles and abstracts to select relevant reports and excluded studies not relevant for this review.
2. Accessed the full text of potentially relevant reports.
3. Used a reference management software Covidence 2018 (*Covidence Systematic Review Software*, 2018) to combine search results and remove duplicate records of the same report and combine multiple reports of the same study.
4. Examined full-text studies for compliance with the eligibility criteria for this review.
5. Where appropriate, corresponded with study authors in order to request missing results or seek additional information.
6. Made final decisions on study inclusion and proceeded to data collection.

The review authors did not encounter disagreements when selecting reports to include in the review. Details of the selection process are shown in the PRISMA flow diagram (Moher et al., 2009) (Figure 2.1).

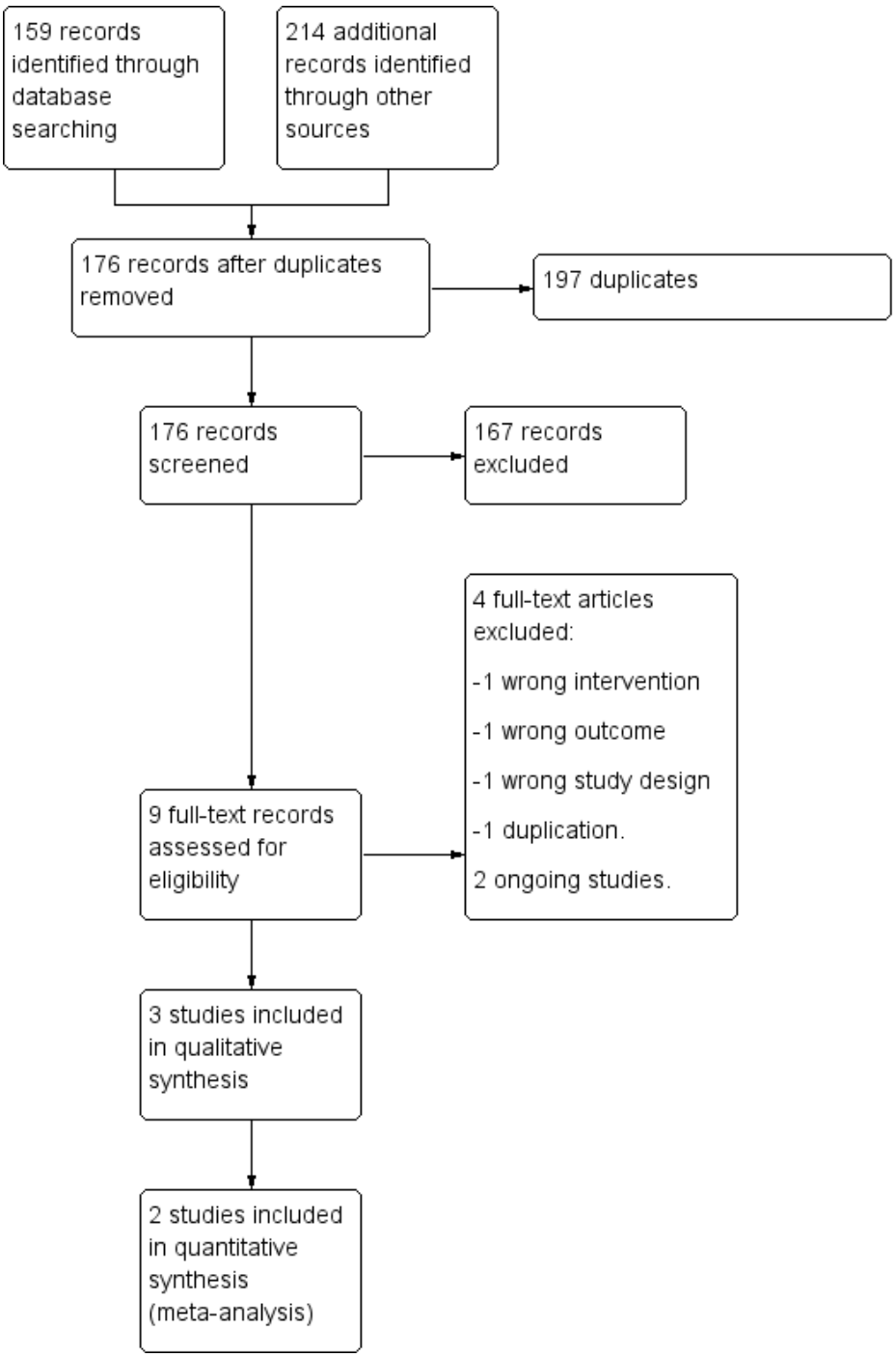

Figure 2.1: Study flow diagram

2.4.2 ***Data extraction and management***

We extracted data from included studies using a specially developed data extraction form. Information extracted included, but was not limited to: source details; eligibility assessment; methodological details; characteristics of participants; details of intervention, and outcomes reported. Disagreements were resolved by discussion with a third assessor (JH). Data from the included studies were entered into Review Manager 5 (*Review Manager*, 2014).

When review authors were authors of an included trial, we have ensured that those authors were excluded from any decision-making regarding inclusion of the trial in this review, and they were not involved in data extraction or quality assessment relating to that trial. We requested additional information from Beker et al (Beker et al., 2017) (mean and standard deviation for the outcomes of interest) and Davidson et al (Davidson et al., 2015) (only an abstract was published). The authors of Beker et al (Beker et al., 2017) provided the additional information requested.

2.4.3 ***Assessment of risk of bias in included studies***

Two review authors (MM and LL) independently assessed the methodological quality of all included trials to determine potential risk of bias (low, high, or unclear) using the Cochrane 'Risk of bias' tool (Higgins et al., 2017) for the following domains:

1. Sequence generation (selection bias);
2. Allocation concealment (selection bias);
3. Blinding of participants and personnel (performance bias);

4. Blinding of outcome assessment (detection bias);
5. Incomplete outcome data (attrition bias);
6. Selective reporting (reporting bias);
7. Any other bias.

Any disagreements were resolved by discussion or by a third review author (JH). See Appendix 2 for a more detailed description of risk of bias for each domain. We entered the assessed risk of bias into ReviewManager 5. See Figure 2.2 and Figure 2.3.

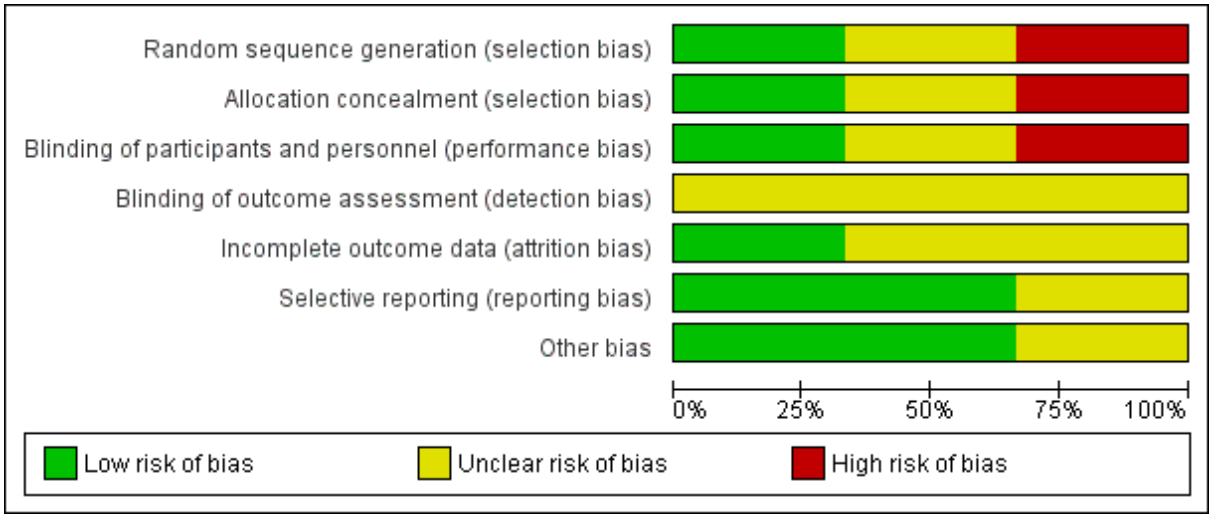

Figure 2.2: 'Risk of bias' graph

Review authors' judgements about each 'Risk of bias' item presented as percentages across all included studies.

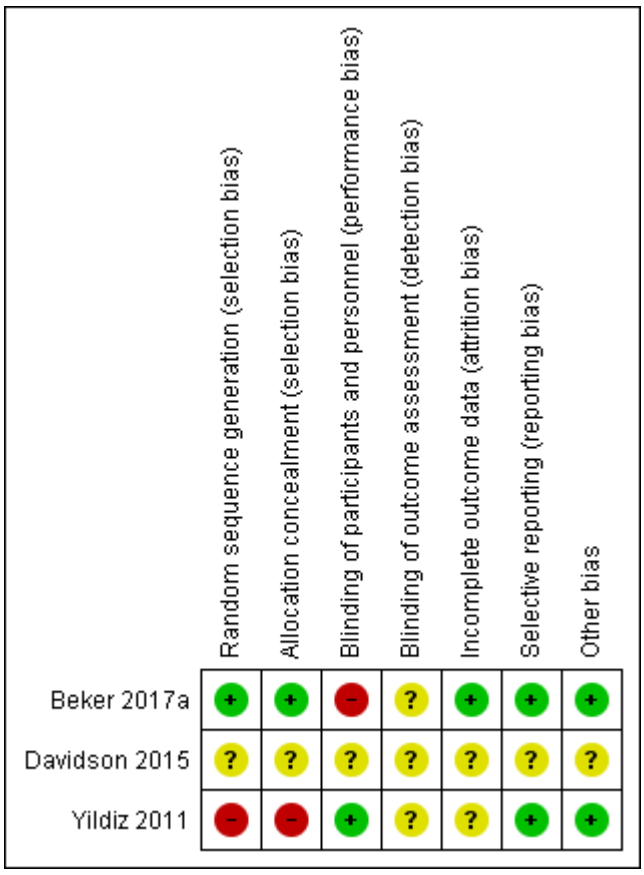

Figure 2.3: 'Risk of bias' summary

Review authors' judgements about each 'Risk of bias' item for each included study.

2.4.4 ***Measures of treatment effect***

We analysed treatment effects in individual trials by using ReviewManager 5. We used the numbers of events in the control and intervention groups of each study to calculate risk ratios (RRs) for dichotomous data. We calculated mean differences (MDs) for outcomes measured on a continuous scale.

Where outcomes were measured differently, we intended to report data as standardised mean differences (SMDs) and risk differences (RDs), and if a significant effect was found we planned to calculate the number needed to treat for an additional beneficial

outcome (NNTB) or the number needed to treat for an additional harmful outcome (NNTH). We reported 95% confidence intervals (CIs) for all outcomes.

The included studies reported data about infant growth differently and we were not able to combine these in a meta-analysis. However, from the reported mean weights and length of hospitalisation we were able to estimate growth rate using the exponential model (Patel, 2005), which uses difference in weights between two time points and elapsed time (in this case birth and discharge weights and duration of hospitalisation), allowing us to estimate mean growth velocity from birth to discharge in grams per Kilo per day (g/Kg/day).

2.4.5 ***Unit of analysis issues***

The unit of analysis was the participating infant in individually randomised trials. We planned to include cluster-randomised trials in analyses, along with individually randomised trials, but no cluster-randomised trials were identified.

If cluster-randomised trials were identified, the participating neonatal unit or section of a neonatal unit or hospital would have been the unit of analysis. We would have analysed them using an estimate of the intra-cluster correlation coefficient (ICC) derived from the trial (if possible), or from a similar trial or from a study with a similar population as described in Section 16.3.6 of the Cochrane Handbook for Systematic Reviews of Interventions (Higgins et al., 2011). If we used ICCs from a similar trial or from a study with a similar population, we planned to report this and conduct a sensitivity analysis to investigate the effect of variation in the ICC.

If we identified both cluster-randomised trials and individually randomised trials, we planned to only combine the results from both if there was little heterogeneity between the study designs, and the interaction between the effect of the intervention and the choice of randomisation unit was considered to be unlikely.

We planned to acknowledge any possible heterogeneity in the randomisation unit and perform a sensitivity analysis to investigate possible effects of the randomisation unit.

2.4.6 ***Dealing with missing data***

We contacted the investigators to request information on missing or unclear data for outcomes of interest. We analysed all participants in the treatment group to which they were randomised, regardless of the actual treatment received, where possible. We carried out analyses on an intention-to-treat basis for all outcomes, where feasible. If we had concerns regarding the impact of including trials with high levels of missing data in the overall assessment of treatment effect, we planned to explore this through sensitivity analysis, but no included studies had high levels of missing data.

2.4.7 ***Assessment of heterogeneity***

We planned to consider whether clinical and methodological characteristics of the included studies were sufficiently similar for meta-analysis to provide a clinically meaningful summary by assessing statistical heterogeneity using the Chi^2 test and the I^2 statistic. We used the guidelines recommended by Cochrane Neonatal for interpretation of results. We considered an I^2 value of less than 25% to represent no heterogeneity; 25% to 49% to

represent low heterogeneity; 50% to 74% to represent moderate heterogeneity, and more than 75% to represent high heterogeneity.

We considered an I^2 value greater than 50% and a low *p* value (less than 0.10) in the Chi^2 test for heterogeneity to indicate substantial heterogeneity (Deeks et al., 2017). Where substantial heterogeneity was detected, we planned to explore this through sensitivity/subgroup analyses, but there were insufficient data to perform these analyses.

We took statistical heterogeneity into account when interpreting the results, especially when variation in the direction of effect was detected and data were insufficient to carry out further assessment of heterogeneity.

2.4.8 ***Assessment of reporting biases***

For the included trials, two reviewers (MM and LL) examined the methods of each study for the prespecified outcomes. When all prespecified outcomes were reported in the results, the studies were considered to have a low risk of bias. When prespecified outcomes were not reported in the results, the study was considered to carry high risk of bias. If we identified that a trial carried reporting bias, with the potential to introduce serious bias, we planned to conduct a sensitivity analysis to determine the effects of including and excluding such a study in the analysis. However, due to the limited number of studies included in this review, and the small sample sizes, we did not perform sensitivity analyses.

2.4.9 ***Data synthesis***

We conducted meta-analyses using Review Manager version 5.3 (*Review Manager*, 2014), as supplied by Cochrane. We used a fixed-effect model to combine data when it was reasonable to assume that studies were estimating the same underlying treatment effect. Where moderate or high heterogeneity existed, we planned to examine the potential causes in subgroup and sensitivity analyses.

2.4.10 ***Quality of evidence***

We used the GRADE approach, as outlined in the GRADE Handbook (Schünemann et al., 2013), to assess the quality of evidence for the following outcomes:

1. Time to reach full sucking feeds (defined as the removal of the feeding tube), measured in days;
2. Adverse effects related to intervention such as aspiration, gagging/choking, bradycardia, desaturations or increase in oxygen requirement;
3. Time to reach full enteral feedings (150 mL/Kg/day, or as defined by the trialists), measured in days;
4. Feed intolerance (resulting in cessation or reduction in enteral feeding);
5. Duration of parenteral nutrition (defined as the removal of the intravenous nutrition line), measured in days;
6. Necrotising enterocolitis (Bell's stage 2 or more) (Walsh and Kliegman, 1986);
7. Late infection (bacterial or fungal infection confirmed by presence of blood or cerebrospinal fluid infection with initiation of symptoms beyond 48 hours after birth) (Australian and New Zealand Neonatal Network, 2016).

Two review authors independently used GRADEpro GDT to assess the quality of evidence for all the outcomes above, except for adverse effects and feed intolerance, for which no data were reported.

We considered evidence from randomised trials as high quality, but downgraded the evidence one level for serious (or two levels for very serious) limitations based upon the following: design (risk of bias); consistency across studies; directness of the evidence; precision of estimates, and presence of publication bias.

We also used GRADEpro GDT to create a 'Summary of findings' table (Table 2.1) to report the quality of the evidence. The GRADE approach results in an assessment of the quality of a body of evidence in one of the following four grades:

1. High: we are very confident that the true effect lies close to that of the estimate of the effect;
2. Moderate: we are moderately confident in the effect estimate: the true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different;
3. Low: our confidence in the effect estimate is limited: the true effect may be substantially different from the estimate of the effect;
4. Very low: we have very little confidence in the effect estimate: the true effect is likely to be substantially different from the estimate of effect.

2.4.11 ***Subgroup analysis and investigation of heterogeneity***

We had planned to perform the following subgroup analyses using a fixed-effect model. However, insufficient data were available to conduct any subgroup analyses:

1. Type of administration of smell exposure (cotton swab or similar soaked with milk placed close to infant's nostril versus placed by the infant's side);
2. Type of administration of taste (cotton swab or similar soaked with milk placed on infant's lips and tongue versus syringe administration of milk directly onto the infant's lips and tongue versus use of pacifier to deliver taste of milk);
3. Type of exposure (provision of smell and taste versus provision of taste only versus provision of smell only);
4. Gestational age (less than 28 weeks' versus 28 to less than 32 weeks' versus 32 to less than 37 weeks' postmenstrual age);
5. Type of diet (exclusively human milk versus formula versus human milk plus formula);
6. Intrauterine growth restricted or small for gestational age (less than 10th centile or as defined by the trialists) versus appropriately grown at birth.

2.4.12 ***Sensitivity analysis***

We had planned to conduct sensitivity analyses by examining only those trials considered to have a low risk of bias for allocation concealment and randomisation. We were unable to do this as only one of the included studies was judged to be of low risk of bias for both allocation concealment and randomisation.

2.5 Results

2.5.1 Description of studies

2.5.1.1 Results of the search

In total, 373 publications were identified by the search strategy for possible inclusion in this review. Of these, 197 were duplicates and were removed, and 176 studies were screened for eligibility. After title and abstract screening, 167 studies were considered irrelevant and were excluded. Nine studies underwent full-text screening, of which three met the inclusion criteria for this review. We identified two ongoing registered clinical trials. For a full description of our selection process, please refer to Figure 2.1.

2.5.1.2 Included studies

Three studies met the inclusion criteria for this review (Beker et al., 2017; Davidson et al., 2015; Yildiz et al., 2011). All included studies were published in English between 2011 and 2017. All were single-centre trials. One was a randomised controlled pilot trial with 51 preterm infants (Table 2.2) (Beker et al., 2017) and one was a quasi-randomised study in which 80 preterm infants were sequentially allocated to treatment and control groups (the first 40 infants comprised the control group and the next 40 infants comprised the intervention group) (Table 2.3) (Yildiz et al., 2011). One was a conference abstract and had limited information available (Table 2.4) (Davidson et al., 2015) It provided an overall sample size, without specifying the number of participants allocated to each group, so these data were not included in meta-analysis. In total, data on 161 preterm infants were included in this review (three trials) but only 131 infants (two trials) were included in meta-analyses. Refer to Characteristics of included trials for a summary of the included trials.

2.5.1.3 Participants

All trials included preterm infants admitted to a neonatal intensive care unit (NICU) at a tertiary hospital. One trial included infants born between 28 and 34 weeks' gestation born in a Turkish hospital between September 2007 and December 2008 (Yildiz et al., 2011), and one included extremely preterm infants (less than 29 weeks' gestation) born in a hospital in Melbourne, Australia between March 2014 and April 2015 (Beker et al., 2017). The conference abstract reported that preterm infants born between 28 and 34 weeks' gestation were included but no information was provided on setting and trial duration (Davidson et al., 2015). All infants were being tube-fed at the time of intervention and receiving either mother's milk, donor milk, or infant formula.

2.5.1.4 Intervention

In all three studies, the provision of the smell of milk was done at the time of tube feedings. Only one trial provided taste of milk as well as smell (Beker et al., 2017). Exposure to the smell of milk was achieved by placing a gauze or pad with drops of milk close to the infant's nostrils in all three trials. Exposure to the taste of milk was achieved by offering a cotton wool bud soaked in milk for sucking. While in the study of Beker et al (2017) the intervention was performed with all tube feeds, in the study of Yildiz et al (2011) smell stimulation was provided during three tube feedings each day, and in Davidson et al (2015) the smell stimulation was performed once a day for 15 minutes for at least four days each week. No information regarding duration (in minutes) of the intervention was provided in Beker et al (2017) or Yildiz et al (2011).

2.5.1.5 Comparators

All included studies reported a control group. In the study of Beker et al (2017), the control group consisted of infants who were not given any milk in the mouth until 32 weeks'

gestation (Beker et al., 2017). In the study of Yildiz et al (2011), the comparison group consisted of infants receiving routine orogastric or nasogastric tube feedings without provision of olfactory stimulation (Yildiz et al., 2011). In the study of Davidson et al, the comparison group received olfactory stimulation with water placed close to infants' nostrils (Davidson et al., 2015).

2.5.1.6 Outcomes

All included trials reported at least one of the pre-specified outcomes of this review. All three trials reported time to reach full sucking feeds. However, the abstract of Davidson et al (2015) provided no information about the number of participants allocated to treatment and control groups and results were descriptive without numerical values for outcomes of interest. Beker et al (2017) reported that no adverse effects related to smell and taste stimulation were observed. The other two trials did not provide any comments on adverse effects related to the intervention (Davidson et al., 2015; Yildiz et al., 2011). Growth from birth to discharge was reported in different ways by two studies (Beker et al., 2017; Yildiz et al., 2011), and therefore we were not able to combine data to perform meta-analysis, but we were able to estimate mean growth velocity by applying the exponential model of Patel (2005) (Patel, 2005). Duration of hospitalisation was reported by two studies (Beker et al., 2017; Yildiz et al., 2011). Only one trial, Beker et al (2017), reported duration of parenteral nutrition, time to reach full enteral feeds, incidence of necrotising enterocolitis and sepsis, and type of milk feeds at 36 weeks' postmenstrual age. Only one trial reported information on time to reach full sucking feeds, stratified by gestational age and gender (Davidson et al., 2015). However, we were not able to identify the groups to which participants were allocated to due to limited information available in the abstract (Davidson et al., 2015).

2.5.1.7 Excluded studies

See Table 2.5: Characteristics of excluded studies for details. We excluded four publications from this review: one because it was a crossover trial (Bingham et al., 2003b); one because the intervention was delivered during the first breastfeeding attempt and was not related to orogastric or nasogastric tube feeding (Raimbault et al., 2007); one because the trial was olfactory stimulation for pain relief and was not related to nutrition (Neshat et al., 2016); and one because it was an undetected duplication of a study already included in this review (Beker et al., 2017).

2.5.1.8 Ongoing studies

We identified two ongoing trials (see Characteristics of ongoing studies Table 2.6 and Table 2.7).

2.5.2 Risk of bias in included studies

Details of methodological quality of each trial are provided in the Characteristics of included trials, Figure 2.2: 'Risk of bias' graph and Figure 2.3: 'Risk of bias' summary.

2.5.2.1 Allocation

Risk of bias for allocation was low in one of the included studies (Beker et al., 2017). In one trial, the method of allocating participants was not clearly described and so we classified this as an unclear risk of bias (Davidson et al., 2015). In another trial, participants were sequentially allocated to treatment and control groups (the first 40 to control, and the next 40 to intervention) and we therefore classified the trial as having high risk of selection bias (Yildiz et al., 2011).

2.5.2.2 Blinding

Risk of performance bias was low in one trial as participants and clinicians were blinded to study group allocation (Yildiz et al., 2011), but high in one trial as blinding of participants and clinicians was not feasible (Beker et al., 2017). Despite lack of blinding in Beker et al (2017) and Yildiz et al (2011), we judged that the outcome assessors were unlikely to have influenced some of the outcomes reported, and in Davidson et al (2015) the blinding of outcome assessors was not clearly stated. Therefore, we considered all three trials to have an unclear risk of detection bias.

2.5.2.3 Incomplete outcome data

We considered two trials to have unclear risk of bias (Davidson et al., 2015; Yildiz et al., 2011). In Davidson et al (2015), there was limited information available in the abstract, meaning we were unable to determine if all outcomes were adequately addressed. In Yildiz et al (2011), the authors stated that infants were excluded when unexpected conditions emerged, but no data were provided on excluded participants. Only one study was considered to be at low risk of attrition bias as all prespecified outcomes were reported (Beker et al., 2017).

2.5.2.4 Selective reporting

We considered two trials to be free of reporting bias as all prespecified outcomes were reported (Beker et al., 2017; Yildiz et al., 2011). We deemed one trial to have unclear risk of reporting bias as no protocol was available for comparison and the abstract did not address this issue (Davidson et al., 2015).

2.5.2.5 Other potential sources of bias

Other potential sources of bias were considered unclear for one trial as the abstract provided limited information (Davidson et al., 2015), and low for two trials as there were no significant differences in baseline characteristics between groups (Beker et al., 2017; Yildiz et al., 2011).

2.5.3 Effects of interventions

See Summary of findings for the main comparison.

Exposure to smell and taste stimulation of milk with tube feeds versus no exposure

2.5.3.1 Time to reach full sucking feeds

Two studies contributed data for meta-analysis on time to reach full sucking feeds (Beker et al., 2017; Yildiz et al., 2011). There was no evidence of a clear effect of exposure to the smell and taste of milk with tube feedings on time to reach full sucking feeds (MD -2.57 days, 95% CI -5.15 to 0.02; I^2= 17%; 2 trials, 131 infants; very low quality evidence; Analysis 1.1 – Time to reach full sucking feeds). We downgraded the quality of evidence three levels for risk of bias (lack of blinding and lack of allocation concealment), imprecision (small sample sizes and large confidence intervals), and indirectness (trials had different inclusion criteria (less than 29 weeks' gestation versus 29 to 34 weeks' gestation) and different interventions (smell and taste of milk with all tube feeds versus smell of milk three times per day with tube feeds).

In Davidson et al (2015), it was reported that infants allocated to the control group attained full sucking feeds at an earlier postmenstrual age compared to the intervention

group (35^{+2} versus 36 weeks', respectively; *p*= 0.05). They also reported that infants in the intervention group born at earlier gestational age, and females, demonstrated a trend towards reaching full oral feeds in a shorter time, but associations were not statistically significant. No data on sample size per group were available to allow this trial to be included in the meta-analysis.

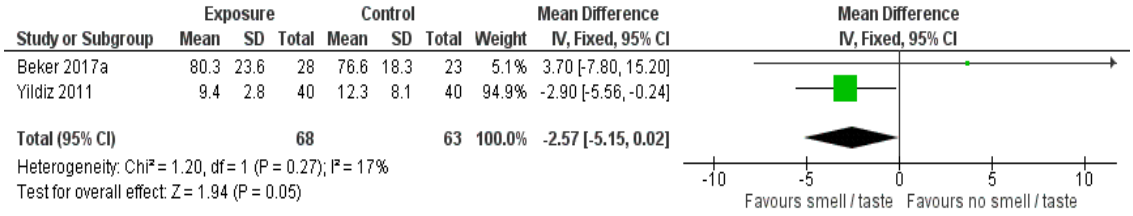

Figure 2.4: Analysis 1.1 - Time to reach full sucking feeds

Analysis 1.1 – Comparison 1: Exposure to smell and taste stimulation of milk with tube feeds versus no exposure. Outcome 1: Time to reach full sucking feeds (days).

2.5.3.2 Adverse effects related to the intervention

There were no data available on potential adverse effects related to exposure to the smell and taste of milk with tube feedings. However, Beker et al (2017) reported that no adverse effects related to the intervention were observed.

2.5.3.3 *Time to reach full enteral feeds*

One trial contributed data on time required to reach full enteral feeds, defined as 120 mL/Kg/day by the trialists (Beker et al., 2017). There was no evidence of a clear effect of exposure to the smell and taste of milk with tube feedings on time required to reach full enteral feeds (MD -1.57 days, 95% CI -6.25 to 3.11; 1 RCT, 51 infants; very low-quality evidence; Analysis 1.2 - Time to reach full enteral feedings). We downgraded the quality of evidence one level for risk of bias (lack of blinding) and two levels for imprecision (data derived from a single trial with small sample size and wide confidence intervals).

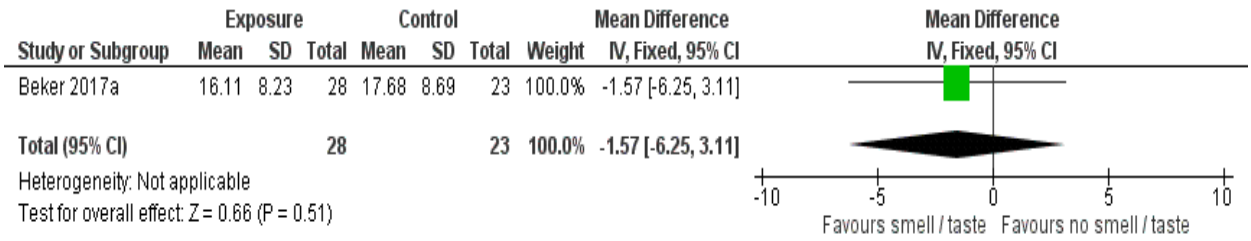

Figure 2.5: Analysis 1.2 - Time to reach full enteral feedings

Analysis 1.2 – Comparison 1: Exposure to smell and taste stimulation of milk with tube feeds versus no exposure. Outcome 2: Time to reach full enteral feedings (days).

2.5.3.4 *Duration of parenteral nutrition*

One trial (Beker et al., 2017) reported duration of parenteral nutrition. There was no evidence of a clear effect of exposure to the smell and taste of milk with tube feedings on the duration of parenteral nutrition (MD -2.20 days, 95% CI -9.49 to 5.09; 1 RCT, 51 infants; very low-quality evidence, Analysis 1.3 - Duration of parenteral nutrition). We downgraded the quality of evidence one level for risk of bias (lack of blinding) and two levels for imprecision (data derived from a single trial with small sample size and wide confidence intervals).

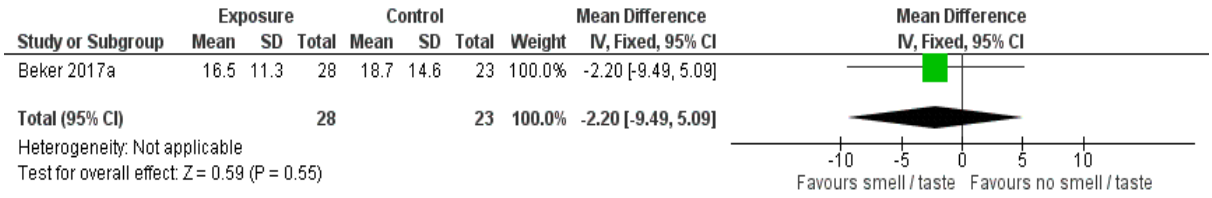

Figure 2.6: Analysis 1.3 - Duration of parenteral nutrition

Analysis 1.3 – Comparison 1: Exposure to smell and taste stimulation of milk with tube feeds versus no exposure. Outcome 3: Duration of parenteral nutrition (days).

2.5.3.5 *Incidence of necrotising enterocolitis*

One trial reported the incidence of necrotising enterocolitis (Beker et al., 2017). There was no evidence of a clear effect of exposure to the smell and taste of milk with tube feedings on the incidence of necrotising enterocolitis (RR 0.62, 95% CI 0.15 to 2.48; 1 RCT, 51 infants; low-quality evidence; Analysis 1.4 - Necrotising enterocolitis). We downgraded the quality of evidence two levels for imprecision (data derived from a single trial with small sample size, and wide confidence intervals). We judged that the lack of blinding was unlikely to have influenced the assessment of this outcome.

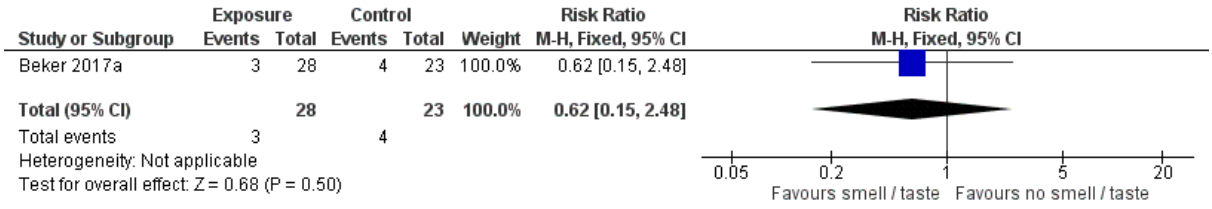

Figure 2.7: Analysis 1.4 - Necrotising enterocolitis

Analysis 1.4 – Comparison 1: Exposure to smell and taste stimulation of milk with tube feeds versus no exposure. Outcome 4: Incidence of necrotising enterocolitis.

2.5.3.6 *Incidence of late infection*

One trial reported the incidence of late infection (Beker et al., 2017). There was no evidence of a clear effect of exposure to the smell and taste of milk with tube feedings on the incidence of late infection (RR 2.46, 95% CI 0.27 to 22.13; 1 RCT, 51 infants; low-quality evidence; Analysis 1.5 - Late infection). We downgraded the quality of evidence two levels for imprecision (data derived from a single trial with small sample size, and wide confidence intervals). We judged that the lack of blinding was unlikely to have influenced the assessment of this outcome.

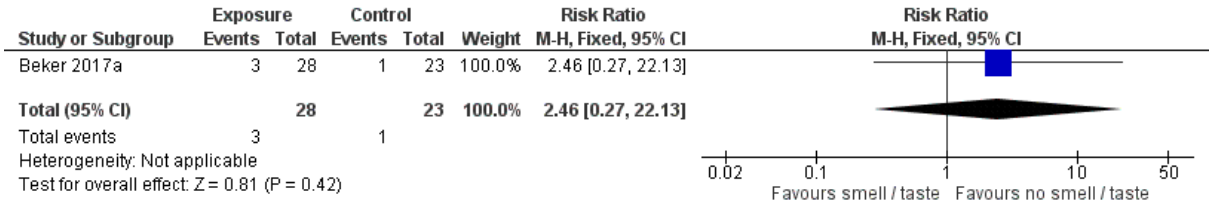

Figure 2.8: Analysis 1.5 - Late infection

Analysis 1.5 – Comparison 1: Exposure to smell and taste stimulation of milk with tube feeds versus no exposure. Outcome 5: Incidence of late infection.

2.5.3.7 *Growth*

Data on growth during hospitalisation were assessed differently in each of the included studies and we were not able to combine data to perform meta-analysis. However, we were able to estimate mean growth rates using exponential model estimates (Patel, 2005), and found that infants exposed to the smell and taste of milk with tube feedings had faster mean growth rates than infants in the control group (14.2 g/Kg/day versus 12.8 g/Kg/day in Beker et al (2017); 14.0 g/Kg/day versus 7.9 g/Kg/day in the study of Yildiz et al (2011)).

2.5.3.8 *Exclusive breastfeeding at time of discharge*

No data were available to assess the effect of exposure to the smell and taste of milk with tube feedings on rates of exclusive breastfeeding at time of hospital discharge.

2.5.3.9 *Episodes of feed intolerance*

No data were available to assess the effect of exposure to the smell and taste of milk with tube feedings on episodes of feed intolerance.

2.5.3.10 ***Time to first discharge home***

Two trials reported data on duration of hospitalisation (Beker et al., 2017; Yildiz et al., 2011). Infants exposed to the smell and taste of milk with tube feedings had a shorter hospital stay than infants not exposed to the intervention (MD -3.89 days, 95% CI -7.03 to -0.75; I^2= 51%; 2 trials, 131 infants; very low-quality evidence; Analysis 1.6 - Time to first discharge home). We downgraded the quality of evidence one level for risk of bias (lack of blinding and lack of allocation concealment), one level for imprecision (small sample sizes and wide confidence intervals), and one level for indirectness of evidence (trials had different inclusion criteria (less than 29 weeks' gestation versus 29 to 34 weeks' gestation) and differences in the provision of intervention (smell and taste of milk with all tube feeds versus smell of milk three times per day with tube feeds).

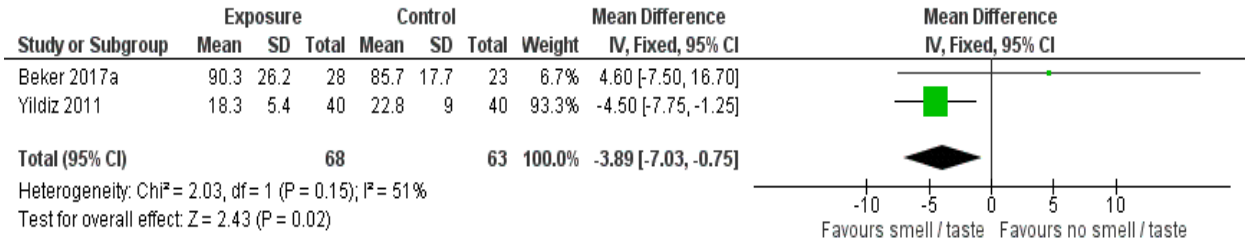

Figure 2.9: Analysis 1.6 - Time to first discharge home

Analysis 1.6 – Comparison 1: Exposure to smell and taste stimulation of milk with tube feeds versus no exposure. Outcome 6: Time to first discharge home (days).

2.6 Discussion

2.6.1 ***Summary of main results***

The evidence from three trials, involving 161 preterm infants, was judged to be of very low quality and the overall effect of provision of the smell and taste of milk to accelerate feeding in preterm infants is uncertain. There was no evidence of a clear effect of exposure to the smell and taste of milk during tube feedings on time to reach full sucking feeds, and there were no data available to assess potential adverse effects related to the intervention. There was no evidence of a clear effect on time to reach full enteral feeds, duration of parenteral nutrition, incidence of necrotising enterocolitis, late infection and growth. No data were available for the assessment of the effects of exposure to the smell and taste of milk on episodes of feed intolerance and prevalence of exclusive breastfeeding at discharge. However, very low-quality evidence demonstrated that exposure to the smell and taste of milk with tube feedings decreased duration of hospital stay by almost four days.

2.6.2 ***Overall completeness and applicability of evidence***

The trials included in this review had small sample sizes and did not provide data for all of the outcomes of interest. We were able to include data from two trials for two outcomes only: time to reach full sucking feeds (Analysis 1.1 - Time to reach full sucking feeds), and time to first discharge home (Analysis 1.6). Only one trial (Beker et al., 2017) contributed data for the other planned outcomes: time to reach full enteral feeds (Analysis 1.6); duration of parenteral nutrition (Analysis 1.3); incidence of necrotising enterocolitis (Analysis 1.4); and late infection (Analysis 1.5). In addition, the two trials that contributed data for the meta-

analyses included different populations of preterm infants, provided the intervention differently, and used different methods of allocation to the intervention groups. Thus, caution is needed when interpreting the results of this review.

2.6.3 ***Quality of the evidence***

We judged the overall quality of evidence for all reported outcomes to be of low to very low quality, due to a combination of factors that might influence the overall effect of the intervention. This means that we are very uncertain about the effect estimates and their precision. Firstly, we downgraded the quality of evidence for risk of bias, taking into account the lack of allocation concealment in the quasi-randomised trial (Yildiz et al., 2011) and lack of blinding in one trial (Beker et al., 2017). Secondly, we downgraded the quality of evidence for indirectness as trials used different inclusion criteria, provided different exposures to the intervention, and had differing estimate of effects, which could have influenced the overall effect of the intervention. Lastly, we downgraded our assessment of the quality of evidence for imprecision because of the small sample size, small number of trials included, and wide confidence intervals, which can also impact the estimation of effect. Only for two outcomes (incidence of necrotising enterocolitis and late infection) did we judge that the lack of blinding in Beker et al (2017) was unlikely to have influenced the assessment of these outcomes.

2.6.4 ***Potential biases in the review process***

Due to the small number of included studies, we were unable to create funnel plots to assess the potential risk of publication or reporting bias. We minimised bias by conducting a

systematic search of the literature, and data extraction was undertaken independently by two reviewers.

2.6.5 ***Agreements and disagreements with other studies or reviews***

Provision of smell and taste stimulation for preterm infants receiving tube feedings is a relatively new topic. Thus, we are not aware of any previous systematic reviews on this topic, nor of other trials not included in this review.

2.7 Conclusion

2.7.1 ***Implications for practice***

There is a lack of high-quality evidence on the effects and safety of provision of the smell and taste of milk with tube feedings on progress to reach full sucking feeds and other important clinical outcomes. We are currently unable to determine the overall effect of the intervention.

2.7.2 ***Implications for research***

Given the biological plausibility of exposure to the smell and taste of milk with tube feedings to improve feed tolerance, and the potential benefit on progression to full enteral feeds and then full sucking feeds, we consider that further randomised trials should be

conducted on this topic. Such trials should evaluate outcomes during hospitalisation, such as: time to reach full enteral and sucking feeds; episodes of feed intolerance (e.g. vomiting or gastric residual leading to cessation or reduction in enteral feed); incidence of infection, growth (e.g. Z-scores and Z-score change in growth parameters from birth to discharge, as suggested by Cormack et al (2016), or the exponential model estimates by Patel (2005)) (Cormack et al., 2016; Patel, 2005), and safety of the intervention (e.g. episodes of desaturation, aspiration or choking/ gagging at time of exposure to the smell and taste of milk).

Future research should be sufficiently powered to evaluate the effect of the intervention on infants of different gestational ages and sexes, as well as the optimal frequency and duration of the exposure to smell and taste of milk.

2.8 Differences between protocol and review

We added methods for dealing with the unit of analysis if cluster randomised studies had been found. We planned to analyse the quality of evidence for episodes of feed intolerance and include it in the “Summary of findings ” table; however, no data for this outcome were available. Therefore, we analysed the quality of evidence for time to first discharge home and included this outcome in “Summary of findings” table.

2.9 Tables & Supplementary material

2.9.1 *Summary of findings*

Table 2.1: Summary of findings for the main comparison

Exposure to the smell and taste of milk with tube feeds compared to no exposure in preterm infants

Patient or population: preterm infants
Setting: Neonatal Intensive Care Unit
Intervention: exposure to smell and taste of milk with tube feeds
Comparison: no exposure

Outcomes	Anticipated absolute effects* (95% CI)		Relative effect (95% CI)	N of participants (studies)	Certainty of the evidence (GRADE)	Comments
	Risk with no exposure	Risk with exposure to smell and taste of milk with tube feeds				
Time to reach full sucking feeds (days)	The mean time to reach full sucking feeds (days) ranged from 12.6 to 76.3 days	MD 2.57 days lower (5.15 lower to 0.02 higher)	-	131 (2 RCTs)	⨁OOO VERY LOW 1,2,3	
Adverse effects related to intervention (not reported)	--	--	- -	51 (1 RCT)	⨁OOO VERY LOW [4,5]	No data on adverse effects were reported. One trial stated that "No adverse events or side effects, no concerns with regard to acceptability to parents and no logistical implications for the delivery of smell and taste were

Outcomes	Anticipated absolute effects* (95% CI)		Relative effect (95% CI)	N of participants (studies)	Certainty of the evidence (GRADE)	Comments
	Risk with no exposure	Risk with exposure to smell and taste of milk with tube feeds				
						observed in this study"
Time to reach full enteral feedings (days)	The mean time to reach full enteral feedings (days) was 17.7 days	MD 1.57 days lower (6.25 lower to 3.11 higher)	-	51 (1 RCT)	⨁OOO VERY LOW [4,6]	
Duration of parenteral nutrition (days)	The mean duration of parenteral nutrition (days) was 18.7 days	MD 2.2 days lower (9.49 lower to 5.09 higher)	-	51 (1 RCT)	⨁OOO VERY LOW [4,6]	
Necrotising enterocolitis	Study population		RR 0.62 (0.15 to 2.48)	51 (1 RCT)	⨁⨁OO LOW [4]	
	174 per 1,000	108 per 1,000 (26 to 431)				
Late infection	Study population		RR 2.46 (0.27 to 22.13)	51 (1 RCT)	⨁⨁OO LOW [4]	
	43 per 1,000	107 per 1,000 (12 to 962)				
Time to first discharge home (days)	The mean time to first discharge home (days) ranged from 22.8 to 85.7 days	MD 3.89 days lower (7.03 lower to 0.75 lower)	-	131 (2 RCTs)	⨁OOO VERY LOW [1,2,3]	

* The risk in the intervention group (and its 95% confidence interval) is based on the assumed risk in the comparison group and the relative effect of the intervention (and its 95%CI).
CI: confidence interval; MD: mean difference; RCT: randomised controlled trial; RR: risk ratio

GRADE Working Group grades of evidence
High certainty: We are very confident that the true effect lies close to that of the estimate of the effect
Moderate certainty: We are moderately confident in the effect estimate: The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different

Low certainty: Our confidence in the effect estimate is limited: The true effect may be substantially different from the estimate of the effect
Very low certainty: We have very little confidence in the effect estimate: The true effect is likely to be substantially different from the estimate of effect

[1] Downgraded one level for risk of bias due to lack of blinding and lack of allocation concealment
[2] Downgraded one level for imprecision as included trials had small sample sizes and wide confidence intervals
[3] Downgraded one level for indirectness as trials presented different inclusion criteria, provided different exposures to the intervention and differing estimate of effects
[4] Downgraded two levels for imprecision as data derived from a single trial with small sample size
[5] Downgraded one level for indirectness as no data to assess potential adverse effects of the intervention were available
[6] Downgraded one level for risk of bias due to lack of blinding that could have influenced assessment of outcome

2.9.2 ***Characteristics of included trials***

Table 2.2: Characteristics of included trials - Beker et al (2017)

Methods	Randomised controlled pilot trial
Participants	Inclusion criteria: tube-fed infants with a postmenstrual age of less than 29 weeks, admitted to the Neonatal Intensive Care Unit and who had not yet received regular feeds (2-hourly) for more than 24 hours Exclusion criteria: any major congenital anomaly and infants with birth weight below the 10th centile measured on Fenton Growth Charts Sample size: 51 preterm infants (treatment group (n = 28) and control group (n = 23)) Setting: neonatal intensive care unit in Melbourne, Australia Timing: March 2014 to April 2015
Interventions	Intervention: smell and taste of human milk (own mother's milk or pasteurised donor breastmilk) before each tube feeding. Smell was provided by placing a gauze swab soaked with milk close to infants' nostrils. Taste was provided by offering a cotton wool bud soaked in milk for sucking Control: no oral administration of milk until 32 weeks' gestation
Outcomes	Primary outcome: time from birth to full enteral feedings (in days), defined as enteral volume of 120 mL/Kg/day sustained for at least 24 hours Secondary outcomes: death; type of milk feeds at 36 weeks' postmenstrual age; postmenstrual age at removal of nasogastric tube; necrotising enterocolitis; spontaneous intestinal perforation; duration of any parenteral nutrition in days; postmenstrual age at discharge to home; weight and weight z-scores at birth, 28 days, 36 weeks' postmenstrual age and at discharge; time with high-flow nasal prongs or nasal intermittent positive airway pressure and time with endotracheal ventilation in hours; any intraventricular haemorrhage and intraventricular haemorrhage higher than grade 2; any retinopathy of prematurity and retinopathy of prematurity higher than stage 2 in any zone; presence of chronic lung disease; persistent ductus arteriosus requiring treatment; bacterial sepsis diagnosed after 48 hours of life
Notes	Funding: pilot trial funded by Research Foundation for Women and Babies and Research grant from the Mercy Hospital for Women, Melbourne, Australia Conflict of interest: none declared.

Risk of bias

Bias	**Authors' judgement**	**Support for judgement**
Random sequence generation (selection bias)	Low risk	Sequence generation was determined using a computer-generated random-number table
Allocation concealment (selection bias)	Low risk	Treatment allocation was determined using sequentially numbered, opaque, sealed envelopes
Blinding of participants and personnel (performance bias)	High risk	Participants and personnel were not blinded

All outcomes		
Blinding of outcome assessment (detection bias) All outcomes	Unclear risk	Outcome assessors were not blinded but are unlikely to have influenced the outcomes
Incomplete outcome data (attrition bias) All outcomes	Low risk	One participant was randomised to the control group and later excluded because they did not meet the inclusion criteria for the trial. However, analysis was performed on intention-to-treat and therefore exclusion is unlikely to have influenced the outcome
Selective reporting (reporting bias)	Low risk	All outcomes have been reported
Other bias	Low risk	No significant differences for baseline characteristics between groups and no losses to follow-up

Table 2.3: Characteristics of included trials - Yildiz et al (2011)

Methods	Prospective experimental study (quasi-randomised)
Participants	Inclusion criteria: infants born after 28 and before 34 weeks' gestation, without sucking reflex (based on neonatologist evaluation), with birth weight approximately 1000 grams, "mean of Apgar scores >6," medically stable during the first 24 hours after birth, with no congenital malformation that could have caused asphyxia or otherwise affected respiration and spontaneous respiration at birth, receiving and tolerating tube feedings, receiving breast milk, mother literate in Turkish and willing to feed the baby Exclusion criteria: intraventricular haemorrhage grade III or IV, intracranial haemorrhage, periventricular leukomalacia, necrotising enterocolitis, chromosomal anomalies, craniofacial malformation, respiratory distress syndrome, bronchopulmonary dysplasia or other chronic lung disease, need for mechanical ventilation, neonatal seizures, culture positive sepsis or meningitis at study screening Sample size: 80 preterm infants: control group (n = 40) and treatment group (n = 40) Setting: neonatal intensive care unit in Turkey Timing: September 2007 to December 2008
Interventions	Treatment group: olfactory stimulation consisting of placement of a sterile pad soaked in breast milk approximately 2 cm from the infant's nose during three daily tube feedings in the incubator Control group: routine tube feeding without delivery of olfactory stimulation
Outcomes	Primary outcome: time for transition to total sucking feeds. Secondary outcomes: not stated in method section but data on weight gain and duration of hospital stay were available
Notes	Funding: experimental study funded by Ataturk University Scientific Research Project Funds

	Conflict of interest: none declared Infants were sequentially allocated to treatment and control groups: the first 40 participants were allocated into control group and the next 40 participants were allocated to the treatment group

Risk of bias

Bias	Authors' judgement	Support for judgement
Random sequence generation (selection bias)	High risk	Participants were sequentially allocated into treatment and control groups based on date of admission (the first 40 to control, and next 40 to treatment group)
Allocation concealment (selection bias)	High risk	No allocation concealment was used
Blinding of participants and personnel (performance bias) All outcomes	Low risk	Authors state that "Although study subjects and the neonatologist were blinded to the study groups, the investigator was not blinded". The exact method of achieving blinding was not reported
Blinding of outcome assessment (detection bias) All outcomes	Unclear risk	Investigators were not blinded but are unlikely to have influenced the outcome
Incomplete outcome data (attrition bias) All outcomes	Unclear risk	Authors state that when unexpected conditions emerged during the study (clinical conditions, or those induced by the mother, infant, or research conditions), those infants were excluded from the study. However, no data on excluded participants were reported
Selective reporting (reporting bias)	Low risk	All outcomes have been reported
Other bias	Low risk	No significant differences for baseline characteristics between groups

Table 2.4: Characteristics of included trials - Davidson et al (2015)

Methods	Prospective, placebo-controlled, partially-blinded, single-centre, pilot randomised trial
Participants	Inclusion criteria: infants born between 28 0/7 and 33 6/7 weeks' postmenstrual age to mothers who planned to breastfeed Exclusion criteria: not stated Sample size: 30 preterm infants (28 to 29 6/7 weeks' gestation (n = 8); 30 to 31 6/7 weeks' gestation (n = 13); and 32 to 33 6/7 weeks' gestation (n = 9)) Setting: not stated Timing: not stated
Interventions	Treatment group: olfactory stimulation with mother's own milk held 2 cm from the nares for 15 minutes during enteral feedings, once a day, for at least 4 days a week until transfer to a Level II nursery or attainment of full sucking feeds Control group: olfactory stimulation with water held 2 cm from the nares for 15minutes during enteral feedings, once a day, for at least 4 days a week until transfer to a Level II nursery or attainment of full oral feeds
Outcomes	Primary outcome: time to reach full sucking feeds Secondary outcomes: optimal timing and sex-specific responses to olfactory stimulation
Notes	Funding: not stated Conflict of interest: not stated

Risk of bias

Bias	**Authors' judgement**	**Support for judgement**
Random sequence generation (selection bias)	Unclear risk	Sequence generation was not stated
Allocation concealment (selection bias)	Unclear risk	Allocation concealment was not stated
Blinding of participants and personnel (performance bias) All outcomes	Unclear risk	Blinding of participants and personnel was not stated
Blinding of outcome assessment (detection bias) All outcomes	Unclear risk	Blinding of outcome assessors was not stated
Incomplete outcome data (attrition bias) All outcomes	Unclear risk	Limited information was available in the abstract to assess attrition bias
Selective reporting (reporting bias)	Unclear risk	No protocol was available to be compared with study's final report
Other bias	Unclear risk	Not possible to assess due to limited information in the abstract

2.9.3 ***Characteristics of excluded studies***

Table 2.5: Characteristics of excluded studies

Study	Reason for exclusion
Beker 2016	Duplication not detected previously
Bingham 2003	Wrong study design (cross-over design)
Neshat 2016	Wrong outcome (olfactory stimulation for pain relief)
Raimbault 2007	Wrong intervention (intervention given during breastfeeding trials and not related to tube feeds)

2.9.4 ***Characteristics of ongoing studies***

Table 2.6: Characteristics of ongoing studies - Beker et al. 2019

Trial name or title	The TASTE trial - effect of smell and taste to improve nutrition in very preterm babies
Methods	Randomised controlled clinical trial
Participants	Preterm infants born < 29 weeks' gestation and/or less than 1250 grams birth weight admitted to Neonatal Intensive Care Units in Queensland and Victoria, Australia
Interventions	Smell and taste of milk (mothers' breast milk, pasteurised donor breast milk or formula, whatever is fed at the time) given with every tube feeding and for the duration of the feed. For infants born before 32 weeks' gestation the intervention consists of providing a cotton bud soaked in milk, offered for sucking, and a drop of milk on a cotton pad placed close to the infants' nose until infants reach 32 weeks' gestation. Once infants are 32 weeks' gestation, and until removal of nasogastric tube or discharge, the intervention will consist of 0.2 mL of milk given orally with a feeding syringe with every tube feeding
Outcomes	Primary outcome: weight z-scores at discharge home Secondary outcomes: time (days) to full enteral feedings (120 mL/Kg/day for at least 24 hours); total duration of parenteral nutrition (days); duration of parenteral nutrition (until first episode of cessation of parenteral nutrition); total duration of antibiotics (days); episodes of late onset sepsis; postmenstrual age at discharge home from hospital
Starting date	8 May 2017
Contact information	Dr Friederike Beker Address: Neonatal Critical Care Unit, Mater Mothers' Hospital, Raymond Terrace, South Brisbane, QLD 4101, Australia Email: friederike.beker@mater.org.au
Notes	Funding: Mater Research Institute (Australia) and Royal College of Physicians and Paediatricians –Queensland Branch (Australia) Trial registration: ACTRN12617000583347 Conflict of interest: none declared

Table 2.7: Characteristics of ongoing studies - Bloomfield et al 2018

Trial name or title	The DIAMOND trial - DIfferent Approaches to MOderate & late preterm Nutrition: Determinants of feed tolerance, body composition and development: protocol of a randomised trial
Methods	Multicentre, factorial, randomised, controlled clinical trial
Participants	Moderate to late preterm infants (32+0 and 35+6 weeks' gestation) admitted to Neonatal Intensive Care Units in Auckland, New Zealand
Interventions	(i) Parenteral nutrition: intravenous amino acid solution versus intravenous dextrose solution until full milk feeds established (ii) Enteral nutrition: milk supplement versus exclusive breast milk (iii) Sensory stimulation: taste and smell given or not given before gastric tube feeds
Outcomes	For parenteral nutrition (i) and milk supplement interventions (ii), body composition at 4 months' corrected age For taste/smell intervention (iii), time to full enteral feeds (defined as 150 mL/Kg/day) or exclusive breastfeeding
Starting date	29 March 2017
Contact information	Professor Frank H. Bloomfield Address: Liggins Institute, University of Auckland. Private Bag: 92019. Auckland, 1142. New Zealand Email: f.bloomfield@auckland.ac.nz
Notes	Funding: Health Research Council of New Zealand and Counties Manukau Health Trial registration: ACTRN12616001199404 Conflict of interest: none declared

2.9.5 ***Search strategies***

PubMed:

(((((((((Taste[MeSH] OR Taste Perception[MeSH] OR Smell[Mesh] OR Olfactory Perception[Mesh] OR Odourants[MeSH])) OR ((taste*[tiab] OR tasting[tiab]))) OR gustat*[tiab]) OR ((smell*[tiab] or smelt[tiab]))) OR olfact*[tiab]) OR odour*[tiab])) AND (((((Milk, Human[MeSH] OR Infant Formula[MeSH] OR Colostrum[MeSH])) OR ((milk*[tiab] or breastmilk*[tiab]))) OR formula*[tiab]) OR ((colostrum[tiab] or colostral[tiab])))) AND ((((infant, newborn[MeSH] OR newborn OR neonate OR neonatal OR premature OR low birth weight OR VLBW OR LBW or infan* or neonat*) AND (randomised controlled trial [pt] OR controlled clinical trial [pt] OR randomised [tiab] OR placebo [tiab] OR drug therapy [sh] OR randomly [tiab] OR trial [tiab] OR groups [tiab]) NOT (animals [mh] NOT humans [mh]))))

Embase:

1	exp taste/
2	(taste$ or tasting).ti,ab.
3	gustat$.ti,ab.
4	exp odour/
5	exp smelling/
6	(smell$ or smelt).ti,ab.
7	olfact$.ti,ab.
8	odo?r$.ti,ab.
9	1 or 2 or 3 or 4 or 5 or 6 or 7 or 8
10	exp breast milk/
11	(milk$ or breastmilk$).ti,ab.
12	exp artificial milk/
13	formula$.ti,ab.
14	exp colostrum/
15	(colostrum or colostral).ti,ab.
16	10 or 11 or 12 or 13 or 14 or 15
17	(infan* or newborn or neonat* or premature or very low birth weight or low birth weight or VLBW or LBW).mp
18	exp infant/
19	17 or 18
20	(human not animal).mp.
21	(randomised controlled trial or controlled clinical trial or randomised or placebo or clinical trials as topic or randomly or trial or clinical trial).mp
22	19 and 20 and 21
23	9 and 16 and 22

CINAHL

S1	(MH “Taste”)
S2	TI (taste* OR tasting) OR AB (taste* OR tasting)
S3	TI gustat* OR AB gustat*
S4	(MH “Smell”)
S5	(MH “Odours”)
S6	TI (smell* OR smelt OR olfact* OR odour*) OR AB (smell* OR smelt OR olfact* OR odour*)
S7	S1 OR S2 OR S3 OR S4 OR S5 OR S6
S8	(MH “Milk, Human+”)
S9	(MH “Infant Formula”)
S10	(MH “Colostrum”)
S11	TI (milk* OR breastmilk* OR formula* OR colostrum OR colostral) OR AB (milk* OR breastmilk* OR formula* OR colostrum OR colostral)
S12	S8 OR S9 OR S10 OR S11
S13	(infan* OR newborn OR neonat* OR premature OR low birth weight OR VLBW OR LBW) AND (randomised controlled trial OR controlled clinical trial OR randomised OR placebo OR clinical trials as topic OR randomly OR trial OR PT clinical trial)
S14	S7 AND S12 AND S13

CRS Web:

1	MESH DESCRIPTOR Taste EXPLODE ALL AND CENTRAL:TARGET
2	MESH DESCRIPTOR Taste Perception EXPLODE ALL AND CENTRAL:TARGET
3	MESH DESCRIPTOR Smell EXPLODE ALL AND CENTRAL:TARGET
4	MESH DESCRIPTOR Olfactory Perception EXPLODE ALL AND CENTRAL:TARGET
5	MESH DESCRIPTOR Odourants EXPLODE ALL AND CENTRAL:TARGET
6	(taste* or tasting):ti,ab AND CENTRAL:TARGET
7	gustat*:ti,ab AND CENTRAL:TARGET
8	(smell* or smelt):ti,ab AND CENTRAL:TARGET
9	olfact*:.ti,ab AND CENTRAL:TARGET
10	odour*:ti,ab AND CENTRAL:TARGET
11	#1 OR #2 OR #3 OR #4 OR #5 OR #6 OR #7 OR #8 OR #9 OR #10
12	MESH DESCRIPTOR Milk, Human EXPLODE ALL AND CENTRAL:TARGET
13	MESH DESCRIPTOR Infant Formula EXPLODE ALL AND CENTRAL:TARGET
14	MESH DESCRIPTOR Colostrum EXPLODE ALL AND CENTRAL:TARGET
15	(milk* or breastmilk*):ti,ab AND CENTRAL:TARGET
16	formula*:ti,ab AND CENTRAL:TARGET
17	(colostrum or colostral):ti,ab AND CENTRAL:TARGET
18	#12 OR #13 OR #14 OR #15 OR #16 OR #17
19	(infan* or newborn or neonat* or premature or preterm or very low birth weight or low birth weight or VLBW or LBW) AND CENTRAL:TARGET
20	#11 AND #18 AND #19

2.9.6 ***Risk of bias tool***

We used the standard methods of Cochrane and Cochrane Neonatal to assess the methodological quality of the trials. For each trial, we sought information regarding the method of randomisation, blinding and reporting of all outcomes of all the infants enrolled in the trial. We assessed each criterion as being at a low, high, or unclear risk of bias. Two review authors separately assessed each study.

Disagreements were resolved by discussion. We added this information to the table Characteristics of included studies. We evaluated the following issues and enter the findings into the risk of bias table:

1. Sequence generation (checking for possible selection bias). Was the allocation sequence adequately generated?

For each included study, we categorised the method used to generate the allocation sequence as:

- low risk (any truly random process, e.g. random number table; computer random-number generator);
- high risk (any non-random process, e.g. odd or even date of birth; hospital or clinic record number); or
- unclear risk.

2. Allocation concealment (checking for possible selection bias). Was allocation adequately concealed?

For each included study, we categorised the method used to conceal the allocation sequence as:

- low risk (e.g. telephone or central randomisation; consecutively numbered sealed opaque envelopes);
- high risk (open random allocation; unsealed or non-opaque envelopes, alternation; date of birth); or

- unclear risk

3. Blinding of participants and personnel (checking for possible performance bias). Was knowledge of the allocated intervention adequately prevented during the study?

For each included study, we categorised the methods used to blind study participants and personnel from knowledge of which intervention a participant received. Blinding was assessed separately for different outcomes or class of outcomes. We categorised the methods as:

- low risk, high risk or unclear risk for participants; and
- low risk, high risk or unclear risk for personnel.

4. Blinding of outcome assessment (checking for possible detection bias). Was knowledge of the allocated intervention adequately prevented at the time of outcome assessment?

For each included study, we categorised the methods used to blind outcome assessment. Blinding was assessed separately for different outcomes or class of outcomes. We categorised the methods as:

- low risk for outcome assessors;
- high risk for outcome assessors; or
- unclear risk for outcome assessors.

5. Incomplete outcome data (checking for possible attrition bias through withdrawals, dropouts, protocol deviations). Were incomplete outcome data adequately addressed?

For each included study and for each outcome, we described the completeness of data including attrition and exclusions from the analysis. We noted whether attrition and exclusions were reported, the numbers included in the analysis at each stage (compared with the total randomised participants), reasons for attrition or exclusion where reported, and whether missing data were balanced across groups or were related to outcomes. Where sufficient information was reported or supplied by the trial authors, we re-included missing data in the analyses. We categorised the methods as:

- low risk (< 20% missing data);

- high risk (≥ 20% missing data); or
- unclear risk.

6. Selective reporting bias. Are reports of the study free of suggestion of selective outcome reporting?

For each included study, we described how we investigated the possibility of selective outcome reporting bias and what we found. For studies in which study protocols were published in advance, we compared prespecified outcomes versus outcomes eventually reported in the published results. If the study protocol was not published in advance, we contacted study authors to gain access to the study protocol. We assessed the methods as:

- low risk (where it is clear that all of the study's prespecified outcomes and all expected outcomes of interest to the review have been reported);
- high risk (where not all the study's prespecified outcomes have been reported; one or more reported primary outcomes were not prespecified outcomes of interest and are reported incompletely and so cannot be used; study fails to include results of a key outcome that would have been expected to have been reported); or
- unclear risk.

7. Other sources of bias. Was the study apparently free of other problems that could put it at a high risk of bias?

For each included study, we described any important concerns we had about other possible sources of bias (for example, whether there was a potential source of bias related to the specific study design or whether the trial was stopped early due to some data-dependent process). We assessed whether each study was free of other problems that could put it at risk of bias as:

- low risk;
- high risk; or
- unclear risk.

If needed, we explored the impact of the level of bias through undertaking sensitivity analyses.

CHAPTER 3

OLFACTORY CUES IN INFANT FEEDS: VOLATILE PROFILES OF DIFFERENT MILKS FED TO PRETERM INFANTS

Chapter published in Frontiers in Nutrition

Muelbert M, Bloomfield FH, Pundir S, Harding JE and Pook C (2021). Olfactory cues in infant feeds: volatile profiles of different milks fed to preterm infants. Frontiers in Nutrition, 7: 1–12. DOI: https://doi.org/10.3389/fnut.2020.603090.

3.1 Introduction

Smell and taste are critical to food appreciation and are determined, in part, by volatile substances called odourants. Odourants are small, organic and inorganic volatile molecules which bind to olfactory receptors, triggering electrical impulses in the olfactory centre. Odourants are produced in a variety of metabolic pathways by all living organisms. Examples include microbial fermentation or endogenous enzymatic activity (Dunkel et al., 2009).

The activation of specific olfactory receptors allows the brain to discriminate smells (Bak et al., 2018). Perception of odourants in humans is estimated by the odour activity value (OAV), which takes into account the ratio of the concentration of an individual substance in a sample and the threshold concentration of this substance in the respective matrix (minimal concentration) that can be detected by the human olfactory receptors. Not all volatile compounds are sensory active as compounds with OAV <1 cannot be perceived by the human nose (Dunkel et al., 2014). However, it has been suggested that newborn infants might be able to detect odours at lower threshold concentrations than adults (Spitzer and Buettner, 2013).

Olfactory and gustatory stimulation may be important for digestion and metabolism of feeds in the neonate by triggering reflexes in the brain that promote salivation, peristaltic movements and release of hormones and enzymes related to digestion, thereby initiating the digestive process even before food reaches the stomach (Smeets et al., 2010). These reflexes are referred to as the cephalic phase response (CPR). Observations of infants' physiological responses to breastmilk and infant formula demonstrate that they are capable of distinguishing between the smell of different milks, indicating that odourants present in breastmilk differ from those in infant formula (Aoyama et al., 2010; Bingham et al., 2003a;

Porter et al., 1991). Exposure to odour of breastmilk has been related to improved response to pain and enhanced sucking skills (Badiee et al., 2013; Bingham et al., 2003a, 2007; Raimbault et al., 2007), with babies exposed to the smell of breastmilk displaying more non-nutritive sucking bouts (Bingham et al., 2003a) and longer sucking bouts leading to greater milk consumption (Raimbault et al., 2007) compared to babies exposed to smell of formula or water respectively. Furthermore, recent evidence indicates that smell and taste of milk may contribute to early attainment of full oral feeds and shorter hospital stay, although the quality of evidence is low (Muelbert et al., 2019; Nasuf et al., 2018). Yet, sensory stimulation is often under-appreciated in the care of preterm infants, despite functional olfactory receptors being present from 28 weeks' gestation onwards (Bloomfield et al., 2017).

Research into human milk composition has demonstrated that breastmilk metabolic profile (i.e. concentration of different amino acids, fatty acids, sugars, etc.) is influenced by gestational age at birth and postnatal age (Longini et al., 2014; Marincola et al., 2012; Wu et al., 2016). In addition, maternal dietary intake contributes to flavour and nutritional composition of breastmilk (Hascoët et al., 2019; Mennella et al., 2001; Schaal, 2000), suggesting that the components in breastmilk responsible for flavour are not fixed. Positive correlations have been identified between macronutrient composition and breastmilk flavour, with fat and protein content correlated with perceived creamy flavour, carbohydrate content with sweet flavour and bitterness of maternal diet with perceived bitter flavour (Mastorakou et al., 2019). Thus, it has been suggested that, prior to weaning, formula fed infants are exposed to fewer flavours compared to breastfed infants (Lipchock et al., 2011; Mennella et al., 2009).

Different volatile compounds in infant feeds may contribute to feed tolerance and digestion, both through palatability and activation of the CPR. This could be important in

preterm babies, in whom establishment of full enteral feeding may be delayed and who may not receive olfactory stimulation when milk feeds are given via gastric tube. Previous studies using a variety of techniques for extraction of volatile compounds (such as purge-trap (Hausner et al., 2009), solvent-assisted extraction (Shimoda et al., 2000), solid-phase micro extraction (SPME) (Wang et al., 2019) and electronic nose (Jia et al., 2019)) have reported that aldehydes, ketones, fatty acids, alcohols and terpenoids are volatile compounds present in term breastmilk (Hausner et al., 2009; Shimoda et al., 2000) and infant formulas (Hausner et al., 2009; Jia et al., 2019; Wang et al., 2019). However, it remains unclear which volatile compounds in preterm breastmilk, breastmilk fortifiers and substitutes for mother's own milk that commonly are used for feeding preterm babies may contribute to olfactory stimulation. Therefore, the purpose of this study was to analyse the volatile compounds in a variety of different milks routinely fed to preterm infants, including expressed breastmilk, fortified breastmilk, pasteurised breastmilk, human milk-derived commercial products and several infant formulas. Volatile compounds in milk were extracted without solvent at the temperature at which they are fed to the infant, rather than at higher temperatures, in order to provide a better understanding of the compounds that might be biologically relevant and, perhaps, play a physiological role in olfactory perception in the newborn.

3.2 Methods

3.2.1 ***Samples***

3.2.1.1 *Human milk samples*

3.2.1.1.1 ***Preterm breastmilk***

Preterm breastmilk (PBM) samples were obtained from 15 mothers of participants in an ongoing randomised control trial investigating different nutritional support strategies for moderate-to-late preterm infant admitted to Neonatal Intensive Care Units (NICUs) in the city of Auckland, New Zealand (DIAMOND trial; ACTRN12616001199404). This study was approved by New Zealand's Health and Disability Ethics Committee (HDEC 16/NTA/90 and 18/CEN/256) and participants provided written informed consent. Information on the collection protocol can be found elsewhere (Galante et al., 2019). Briefly, samples were collected in the first 10 days after birth and mothers were requested to express milk from their right breast using an electronic breast-pump (Medela Symphony®, Switzerland) into disposable sterile bottles (Medela®) at least 2-3 hours after the previous milk expression. After the right breast was completely emptied, the total volume of expressed breastmilk was vortexed for 2 minutes at high speed to ensure homogeneity and 2 mL of breastmilk was collected using a sterile enteral syringe (BD, Singapore) and aliquoted in equal amounts into four low protein binding microtubes (Eppendorf, Germany), one aliquot of which was used in this study. After aliquoting, samples were frozen at -80°C and stored for 12 – 15 months until analysis.

3.2.1.1.2 ***Pasteurised breastmilk***

Pasteurised donor breastmilk (PDBM) was obtained from 4 different mothers who donated breastmilk to the Human Milk Bank in Christchurch, New Zealand. The milk bank

pools breastmilk from single donors to make up batches of milk for pasteurisation. More information about the Human Milk Bank protocols can be found elsewhere (Lamb et al., 2020). No maternal or infant information was available under the ethical approval for research involving these samples. De-identified samples were shipped frozen from the Human Milk Bank to our facility and stored at -20°C freezer until analysis, which took place within six months of pasteurisation.

3.2.1.1.3 ***Human milk-based formulas***

Samples of three human milk-based products were included. Two of the products are ready-to-feed (RTF) formulas designed to contain protein, fat and calories from pasteurised donor human milk with essential minerals added to deliver standardised caloric and micronutrient content. Prolact RTF 24 and Prolact RTF 28 (Prolacta Bioscience, Inc., California, USA) provide 40.5 calories and 1.1 g protein and 47.5 calories and 1.6 g protein per 50 mL, respectively. Additionally, we included samples of PremieLact (Prolacta Bioscience), nutritionally incomplete product designed for trophic feeding only and providing 0.7 calories per mL with no minerals added. Products were shipped frozen to our facility and stored for four months at -20°C until analysis.

3.2.1.2 *Fortified breastmilk*

3.2.1.2.1 ***Human milk-based fortifier***

Two human milk-based fortifiers (HMF) were added to pasteurised breastmilk from a single donor and prepared according to manufacturer's instructions. Human milk-based fortifiers are made from pasteurised breastmilk with minerals added. Products (Prolact Plus 4 H2MF and Prolact Plus 6 H2MF, Prolacta Bioscience) were shipped frozen to our facility and

stored for four months at -20°C until analysis. Just prior to analysis, products were added to pasteurised breastmilk to provide approximately 41 calories and 1.2 g protein (Prolact Plus 4 H2MF) and 45 calories and 1.4 g protein (Prolact Plus 6 H2MF) per 50 mL.

3.2.1.2.2 ***Bovine milk-based fortifier***

Breastmilk containing bovine milk-based fortifier (BMF) was obtained from 10 mothers of preterm infants (<32 weeks' gestation or with birthweight <1800 g) admitted to the Auckland City Hospital NICU who had fortification of breastmilk with bovine milk-based multi-nutrient fortifier (PreNan, FM 85, Nestlé, Vevey, Switzerland) prescribed for clinical reasons. This product is a powder made of extensively hydrolysed bovine milk protein, carbohydrates, lipids, trace elements and vitamins and fortification was done following manufacturer's instructions. This study was approved by New Zealand's Health and Disability Ethics Committee (HDEC 18/CEN/256) and participants provided written informed consent. No maternal or infant information was collected. Fortified breastmilk samples that had been stored at 4°C for >8 hours, the limit for clinical use in this hospital but still within the timeframe used by many nurseries (Steele, 2018), were collected between March and July 2019. Samples were de-identified and vortexed for 30 seconds at medium speed before a 2 mL volume was collected and stored at -80°C for six months until analysis.

3.2.1.3 *Infant formulas*

3.2.1.3.1 ***Liquid formulas***

Six liquid infant formulas (LF) were included in the analysis: two preterm infant formulas (Aptamil Preterm Gold Plus, Danone Nutricia NZ Ltd, Auckland, New Zealand; Pre Nan Gold, Nestlé); one low-birth weight infant formula (S-26 LBW, Wyeth Nutritionals Singapore Pte Ltd,

Singapore), and three term infant formulas (S26-Gold, Wyeth Nutritionals, Askeaton, Ireland; Aptamil Gold Plus, Danone Nutricia NZ Ltd; Similac Advance Pro, Abbot Laboratories, Columbus, Ohio, USA). All liquid formulas contained cows' milk protein and mixed vegetable oils containing long- and medium-chain polyunsaturated fatty acids. All samples were unopened prior to analysis and analysed within shelf-life stated in the product.

3.2.1.3.2 ***Powder formulas***

We analysed seven stage 1 (from birth to 12 months) powder infant formulas (PF) with different sources of protein: 4 cows' milk formulas (Similac Pro-Advance, Abbot Laboratories; SMA, Wyeth Nutritionals Singapore Pte Ltd; Nurture, Heinz Wattie's, Hastings, New Zealand; S-26 Gold, Wyeth Nutritionals Singapore Pte Ltd); one goat milk formula (Karicare Goats' Milk, Danone Nutricia NZ Ltd); one soy-based formula (Kericare+ Soy Milk, Danone Nutricia NZ Ltd) and one formula made of cow's milk with only A2 β-casein protein (S-26 A2 Milk, Wyeth Nutritionals Singapore Pte Ltd). All formulas were prepared using boiled tap water on the day of analysis following manufacturers' instructions and analysed within shelf-life stated in the product.

3.2.2 ***Sample preparation***

To minimise potential batch effects, samples were randomly allocated into batches for analysis. Frozen samples were thawed at 4°C for 4 hours. Samples were vortexed at low speed to ensure homogeneity and 400 µL of milk was transferred into 10 mL headspace amber vials with magnetic screw caps and polytetrafluoroethylene-lined, silicone septa (Thermo Fisher Scientific Inc., New Zealand). The same volume of milk was analysed for all samples to ensure standardisation and comparison among different milk types.

3.2.3 ***Quality control***

Procedural blanks contained 400 µL of the same boiled water used to prepare the powder infant formulas. Two types of Quality Controls (QC) were prepared: one contained a pool of all preterm and pasteurised breastmilk samples (PBM and PDBM) and another contained a pool of powder and liquid formula samples (LF and PF). Bovine milk-based fortified breastmilk (BMF) and human milk-based products were not included in QCs due to the limited volume available. Each type of QC was aliquoted into three vials each containing 400 µL. The procedural blanks and the human and formula milk QCs were placed at the beginning, middle and end of each batch. All samples, QCs and blanks were spiked with 10 µL of ultrapure water containing 2-chlorophenol at 20 µL/L as an internal standard (IS) (Sigma-Aldrich, Sidney, Australia). Data from the QC analyses were processed in parallel with the samples to confirm reproducibility of retention times and peak areas among batches.

3.2.4 ***Analysis of volatile compounds***

Analysis of volatile compounds was performed by headspace solid phase micro extraction gas chromatography with mass spectrometry (SPME-GC-MS). A Gerstel MPS2 autosampler was used to equilibrate samples at 37°C, with continuous stirring, for 10 minutes. The SPME fibre used for extraction of volatile compounds was divinylbenzene-carboxen-polydimethylsiloxane (DVB-CAR-PDMS) 50/30 µm (Supelco) measuring 20 mm in length. A 10 mL amber vial was used to prevent contact between the milk and the SPME fibre. Extraction of volatile compounds occurred for 10 minutes. The GC-MS instrument was a Thermo Fisher Trace GC Ultra with a Programmable Temperature Vapouriser connected to a

Thermo ISQ mass spectrometer. The carrier gas was zero grade helium (99.995%, BOC New Zealand) at a constant flow rate of 1.1 mL/minute. Upon injection the SPME fibre was simultaneously desorbed and conditioned in the GC injector in high pressure splitless mode using a low-volume SPME-specific deactivated liner (0.75 mm ID) at 250 °C for 10 min. The column was a Phenomenex 1701 capillary column (30 m x 250 µm x 0.25 µm). Oven temperature was set at initial temperature of 35°C and held for 4 minutes, followed by an increase of 5°C/minute up to 165°C followed by an increase of 50°C/minute up to 265°C. Data were acquired at a scan rate of 5 Hz in the range m/z 20-300.

3.2.5 ***Compound identification and statistical analysis***

Deconvolution and identification of features in the GC-MS data was performed using Agilent MassHunter Unknowns software (Agilent technologies) searching the 2017 version of the National Institute of Standards & Technology mass spectral library (NIST, USA) using retention time calibration by Kovats Index and a match factor threshold of 80%. Identities were filtered to exclude features with a signal-to-noise ratio <10 or that were identified in less than two samples (at least one sample and its QC). Authentic standards were run to verify the identities of short- and medium-chain fatty acids and their esters, as well as various alcohols, aldehydes and ketones for which standards were readily available. Compounds identified with authentic standards are indicated in Supplementary Table 3.1. These annotations comply with criteria for level 1 metabolite identification described by the Metabolomics Standards Initiative (Sumner et al., 2007). All other annotations complied with level 2 metabolite identification.

Automated integration of extracted-ion chromatogram (EIC) signals was carried out using MassHunter Quantitative Analysis software (Version 10.0; Agilent Technologies), with visual inspection and manual correction where necessary. Peak areas were normalised to the internal standard (2-chlorophenol) and subtracted from procedural blanks (boiled water). Unsupervised multivariate test (Principal Component Analysis, PCA) and one-way Analysis of Variance (ANOVA) with Tukey's HSD post-hoc were used to explore differences between volatile profiles among milk types. When assumptions for ANOVA were not met, pairwise multiple comparisons were analysed by non-parametric test (Kruskal-Wallis with Conover's post-hoc test). To account for multiple comparisons, false discovery rate (FDR = 0.05, Benjamini-Hochberg) adjusted p value of <0.05 was considered statistically significant. Relative abundance of identified features are presented as mean peak area and standard deviation. In order to improve visualisation and comparison of PCA and heatmap data, each compound's relative peak area was normalised (mean divided by standard deviation of each compound) and transformed using generalised logarithmic transformation. Statistical analysis was performed with statsmodels, scipy and scikit-posthocs packages in Python 3.6.5 (Anaconda 3 v5.2, Continuum Analytics).

3.3 Results

3.3.1 Volatile profile of included milk types

In total, 47 samples (13 infant formulas and 34 human milk-based samples) were analysed. Based on Kovats Retention Index and similarity with library spectra or confirmation with authentic standards, identities were assigned to 121 features: 24 alkyls; 17 fatty acids

(FA) and FA esters; 14 siloxanes; 12 aldehydes; 12 ketones; 12 aromatic hydrocarbons; eight terpenoids; six alcohols; four furans; three chlorination by-products; two sulphur compounds; one ether; one amide; one hydrocarbon derivate; one phenolic compound; one acrylate; one microbial metabolite (indole), and one pharmaceutical compound (chlorobutanol). One unknown feature was detected to which no identity could be robustly assigned (Supplementary Table 3.1). Where information is available, odour threshold, odour activity value (OAV) and odour description for compounds are presented in Supplementary Table 3.2.

Breastmilk containing bovine-based fortifier presented the highest number of compounds (109), followed by human milk-based ready-to-feed formula (94), preterm breastmilk (91), pasteurised breastmilk (83) and breastmilk containing human milk-based fortifier (81). Powder formula presented more compounds than liquid formula (88 and 70, respectively). Despite different sources of nutrients (soy, goat, and bovine milk), all powder formulas presented a similar profile of volatile compounds. The distribution of volatile compounds in each milk type is illustrated in Figure 3.1.

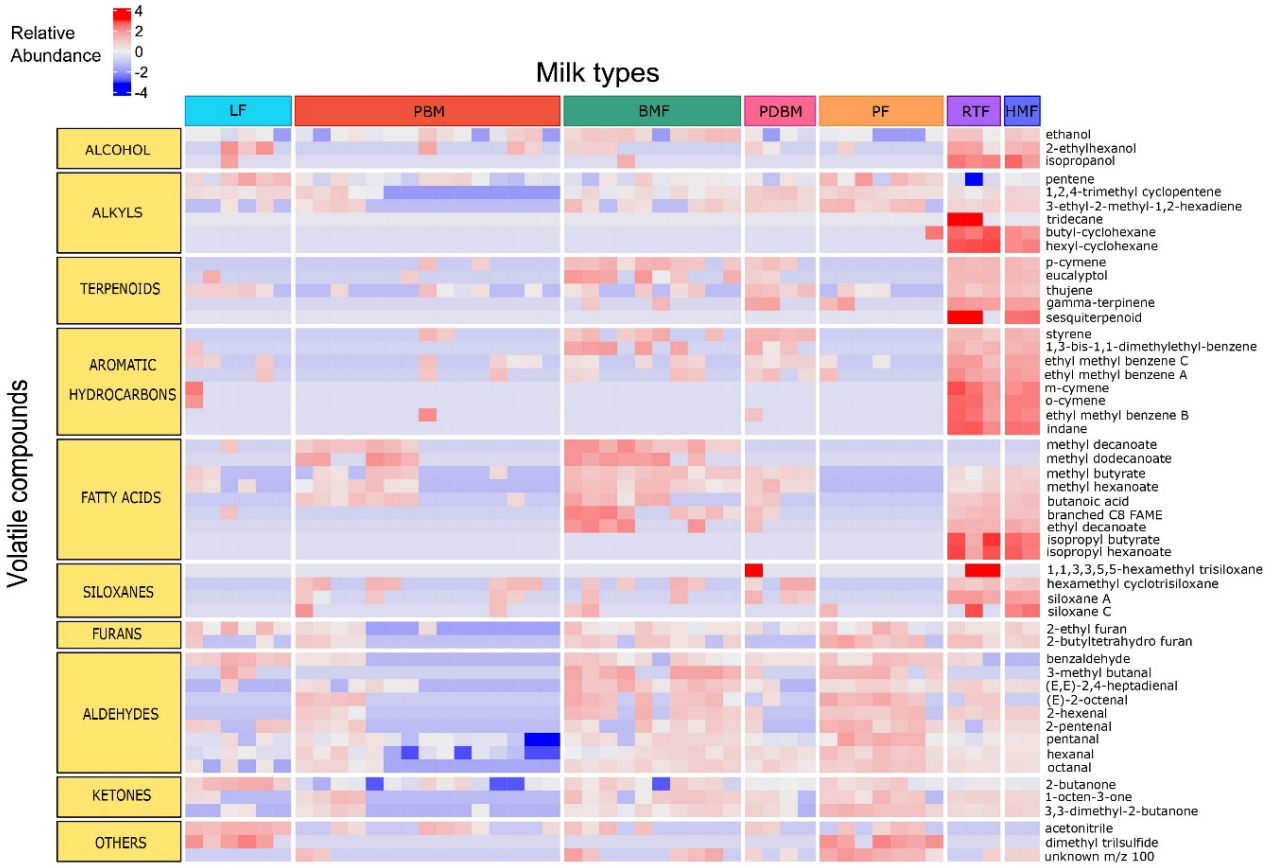

Figure 3.1: Relative abundance of compounds significantly different across milk types

Compounds represented in rows and samples represented in columns. Data are log-transformed and colour scale indicates intensity of relative abundance of a compound. Positive values (red) represent high abundance, negative values (blue) represent low abundance, and zero represents moderate abundance. LF, Liquid Formula; PBM, Preterm breastmilk; BMF, Bovine milk-based fortified breastmilk; PDBM, Pasteurised donor breastmilk; PF, Powder Formula; RTF, Human milk-based Ready-To-Feed formula; HMF, Human-milk based fortified breastmilk.

3.3.2 ***Comparison between different milk types***

The profile of volatile compounds varied significantly among the different milk types. Principal Component Analysis (PCA) explained 47% of the variance among volatile profiles in the first three components (PC1 24.1%; PC2 13.6% and PC3 9.1%), Figure 3.2. Human-milk based products (HMF and RTF) were clearly differentiated from the other milk types, indicated by a separate cluster in PC1. The loadings for this component reveal that the compounds driving differences between these milk types were aromatic hydrocarbons and specific FA esters and terpenoids, which were more abundant in HMF and RTF. Almost all preterm breastmilk (PBM) samples and human milk-based products (HMF and RTF) were separated from the other milk types in PC2, clustering in the lower portion of PC2 axis. The loadings show these differences result from elevated concentrations of aldehydes and ketones in infant formulas (LF and PF), bovine milk-based fortified breastmilk (BMF) and pasteurised breastmilk (PDBM) compared to PBM, HMF and RTF. Breastmilk samples (PBM, PDBM and BMF) were differentiated from infant formulas by PC3, with the infant formulas forming a cluster low on PC3 axis. These differences were mostly due to decreased concentrations of FA and FA esters in the infant formula samples.

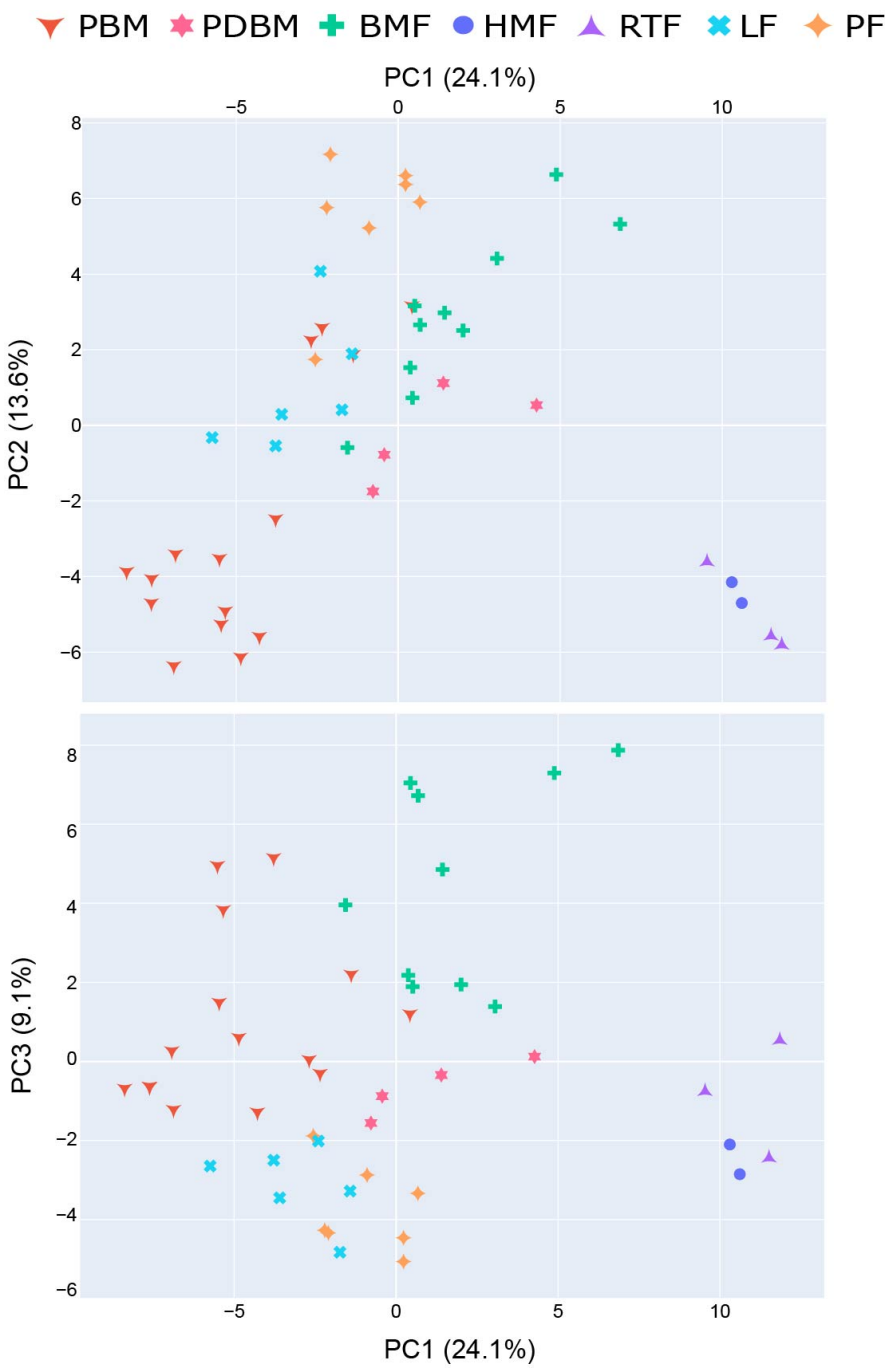

Figure 3.2: Principal Component Analysis (PCA)

PCA explained 47% of variability of different milk types in the first three components. HMF, Human-milk based fortified breastmilk; PBM, Preterm breastmilk; BMF, Bovine milk-based fortified breastmilk; RTF, Human milk-based Ready-To-Feed formula; PF, Powder Formula; LF, Liquid Formula; PDBM, Pasteurised Donor breastmilk.

3.3.3 ***Post hoc group comparisons***

The differences observed in the PCA were supported by post hoc group comparisons. The concentration of fifty-one compounds differed significantly (FDR adjusted p <0.05) across the different milk types (Figure 3.1). Compared to the other milk types, significantly higher mean peak areas of the following were detected in the human milk-based products (HMF and RTF): aromatic hydrocarbons indane, styrene, *meta* and *ortho* cymene and isomeric forms of ethyl methyl benzene (p <0.001); FA esters isopropyl butyrate and isopropyl hexanoate (p <0.001), and the unknown sesquiterpenoid (p <0.001). Compared to samples containing breastmilk, infant formulas presented higher abundance of aldehydes such as hexanal, octanal, pentanal and benzaldehyde (p <0.001), alkyls such as pentane and 3-ethyl-2-methyl-1,3-hexadiene (p <0.001), ketones such as 2-butanone and pinacolone (p <0.001) and furans such as 2-ethyl furan and 2-butyltetrahydro furan (p <0.001). Samples of breastmilk with bovine-based fortifier presented increased abundance of FA such as butanoic acid (p <0.01) and esters of FA such as methyl hexanoate, methyl butyrate and methyl decanoate (p <0.001) compared to the other milk types. The main differences among milk types with respective pairwise comparisons are illustrated in Figure 3.3.

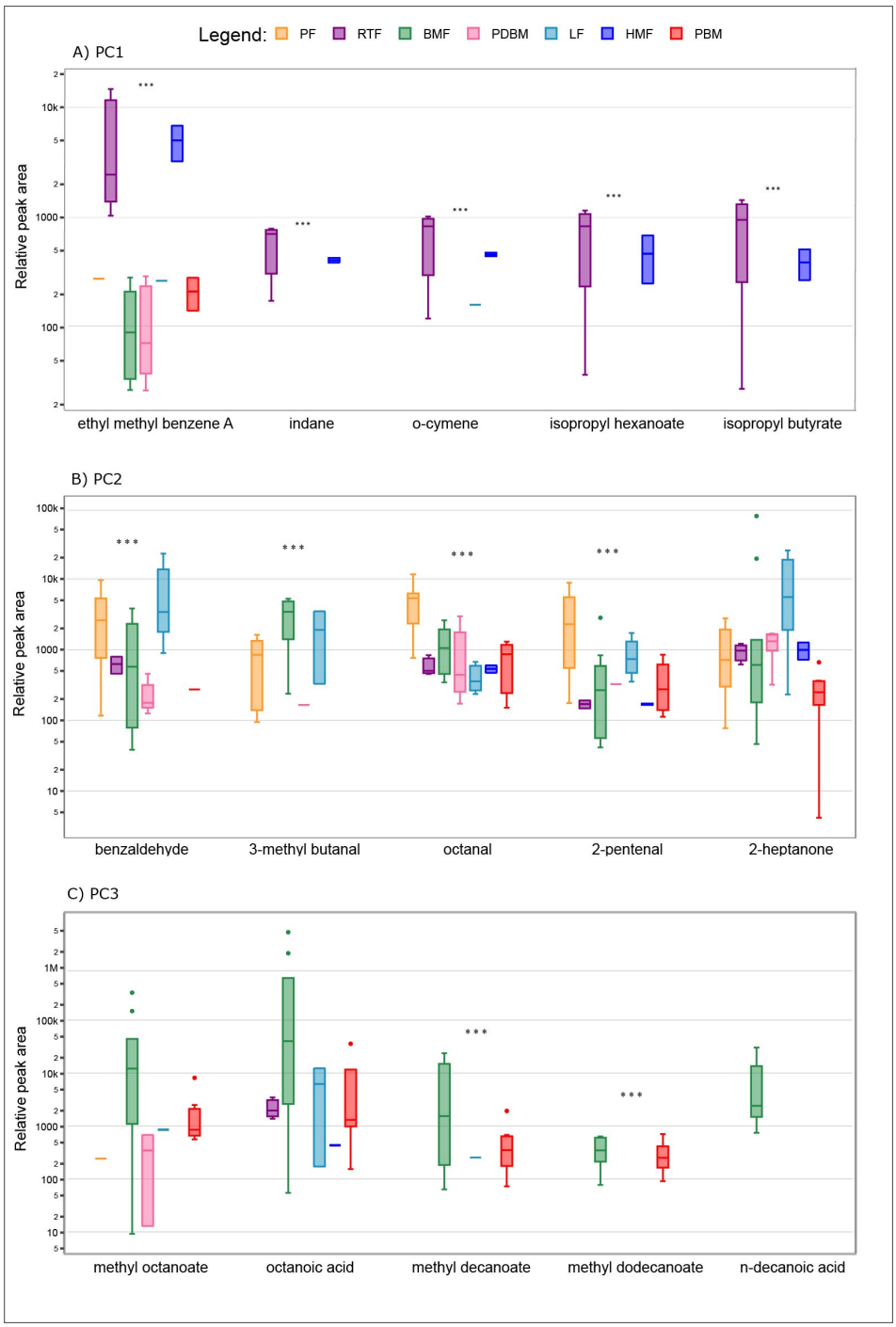

Figure 3.3: Relative peak area of the top 5 volatile compounds in different milk types contributing to Principal Component Analysis (PCA)

*Boxes represent median and interquartile range, whiskers represent highest and lowest peak area detected and dots represent outliers. Milk types are not shown when compound was not detected. (A) Aromatic hydrocarbons and specific fatty acids contributed to differences observed in PC1; (B) aldehydes and ketones contributed to differences observed in PC2; (C) fatty acids contributed to differences observed in PC3. PF= Powder Formula; RTF= Human milk-based Ready-To-Feed formula; BMF= Bovine milk-based fortifier; PDBM= Pasteurised donor breastmilk; LF= Liquid Formula; HMF= Human-milk based fortified breastmilk; PBM= Preterm breastmilk; *** p <0.001.*

3.4 Discussion

3.4.1 *Main findings*

The profile of volatile compounds is an important indicator of milk integrity (Clarke et al., 2020; Spitzer et al., 2010; Wang et al., 2019) and may also contribute to the palatability and digestibility of feeds. Our results demonstrate that the volatile profile of preterm breastmilk is different from other infant feeding options including pasteurised breastmilk, breastmilk with bovine milk-based fortifier, products made from pasteurised breastmilk and various liquid and powder infant formulas, and that these different types also differ from each other. These clear distinctions amongst milks result mostly from the varying concentration of aldehydes, ketones, aromatic hydrocarbons, terpenoids, fatty acids and fatty acids esters. Our results identified a more diverse profile of volatile compounds in breastmilk compared to infant formulas. In general, the profile of volatile compounds in breastmilk milk samples (PBM, PDBM, BMF) was marked by presence of fatty acids, ketones and some terpenoids. The profile of human milk-based products (RTF and HMF) was more similar to pasteurised breastmilk than to preterm breastmilk, but presented more aromatic hydrocarbons, terpenoids and specific fatty acids, whereas the profile of infant formulas was characterised by alkyls, aldehydes and furans.

Breastmilk is considered a bridge between the antenatal and postnatal chemosensory environment. Maternal diet and metabolism are known to influence compounds in amniotic fluid and breastmilk, suggesting that some substances might be common to both (Lipchock et al., 2011; Mennella, 1995; Mennella et al., 2001). The many chemical reactions that occur in foodstuffs involving metabolites, including proteins, sugars and lipids, contribute to the profile of volatile compounds and can potentially lead to formation of either pleasant or off-

flavours in the food matrix (Diez-Simon et al., 2019; Elisia and Kitts, 2011; Spitzer and Buettner, 2013). Previous studies reported that the volatile profile of breastmilk is more variable than of infant formulas due to individual contributions from endogenous metabolism and diet (Bingham et al., 2003b; Hausner et al., 2009; Shimoda et al., 2000). On one hand, the profile of volatiles in infant formulas is reported to be characterised by compounds developed during the manufacturing process (involving heat, light and storage) generating alkanes, aldehydes and furans and present less terpenoids (Hausner et al., 2009; Wang et al., 2019). On the other hand, it has been suggested that volatile compounds in breastmilk result from oxidation of lipids (yielding ketones, aldehydes, free fatty acids and fatty acid esters) and maternal diet (mostly terpenoids) (Bingham et al., 2003b; Hausner et al., 2009; Shimoda et al., 2000). Thus, our findings are largely in agreement with previous reports. Furthermore, we report for the first time the volatile profile of fortified breastmilk and of products made from pasteurised donor breastmilk, adding new information to human milk research.

The varying distribution of volatile compounds in milk might provide different cues related to feeding behaviour. It has been demonstrated that exposure to aroma of breastmilk and infant formula evokes different physiological reactions in infants (Aoyama et al., 2010; Bingham et al., 2003a). In a small study, Bingham et al (2003) investigated the impact of olfactory exposure during tube-feeding on non-nutritive sucking and reported increased sucking behaviour (measured by number of sucks and suck bursts) in preterm infants exposed to smell of fortified breastmilk compared to infants exposed to smell of infant formula (Bingham et al., 2003a). Aoyama et al (2010) reported a significant increase in oxygenation of the olfactory region of the brain following exposure to smell of breastmilk but not to formula (Aoyama et al., 2010). Preterm infants also have functional olfactory receptors (Bloomfield et al., 2017) and there is an emerging interest in potential health benefits related to sensory

exposure to smell and taste of milk in tube fed preterm infants (Muelbert et al., 2019). However, most studies investigating physiological and behavioural changes in response to smell of milk have not simultaneously assessed the volatile compounds that could be responsible for the effects seen, which prevents us from inferring which compounds detected in our study may have the potential to influence sucking behaviour or changes in cerebral oxygenation.

The volatile profile observed in fortified breastmilk retrieved from a hospital refrigerator is particularly important for nutrition in preterm infants. To ensure a continuous human milk-based diet with sufficient nutrition for their preterm infants, mothers often need to express their milk, fortify and refrigerate it (Steele, 2018). Even though changes in the chemical properties of breastmilk following fortification have been reported previously (Donovan et al., 2017; Kreissl et al., 2013; Pozzo et al., 2019), fortification of breastmilk is extremely important for nutrition and growth of some preterm infants (Brown et al., 2016). Compared to preterm breastmilk, our data demonstrate that the volatile profile of breastmilk with bovine milk-based fortifier (BMF) is marked by products generated through lipolysis (fatty acids) and lipid auto-oxidation (ketones and aldehydes), suggesting that changes in breastmilk properties occur within the recommended refrigerated storage period for fortified breastmilk (Steele, 2018). Additionally, volatile compounds in breastmilk with human milk-based fortifier breastmilk (HMF) differ from BMF and preterm breastmilk, with higher concentrations of aromatic hydrocarbons, terpenes, alkyls, alcohols and some fatty acids. Although no harm has been directly associated with fortification of breastmilk, some studies have suggested a relationship between bovine milk-based diet and milk-curd obstruction (Longardt et al., 2019; Wagener et al., 2009) and necrotising enterocolitis (Cristofalo et al.,

2013; Hair et al., 2016) in very preterm infants. However, the underlying mechanism leading to formation of curds in fortified breastmilk is not completely understood.

The majority of the volatile compounds detected in our study are oxidation products of the polyunsaturated fatty acid (PUFA) moieties of lipids. Hexanal and pentanal are products of the oxidation of linoleic acid and are considered markers of lipid oxidation in human milk and infant formula (Elisia and Kitts, 2011; Jia et al., 2019). Nonanal is a product of the oxidation of *n*-9 fatty acids such as oleic acid, the most abundant fatty acid in breastmilk (Elisia and Kitts, 2011). Relative abundance of hexanal, pentanal, octanal and benzaldehyde were significant higher in both powder and liquid formula, whereas nonanal, 3-methyl butanal and 2-hexenal were significant higher in breastmilk with bovine-milk based fortifier. Propanal, pentanal and hexanal previously have been linked to degradation of powdered infant formula, mostly due to thermal processing and storage conditions (i.e. light, temperature and length of storage) (Jia et al., 2019; Wang et al., 2019).

Increasing concentrations of aldehydes and free fatty acids following storage of breastmilk at 4°C and -19°C have been reported previously (Spitzer et al., 2013; Spitzer and Buettner, 2013), and a rise in concentration of free fatty acids (FFA) due to enzymatic lipolysis might be responsible for conferring an unpleasant odour to breastmilk stored under refrigerated conditions (Spitzer et al., 2010, 2013). In fact, Spitzer et al reported a fivefold increase in the concentration of octanoic acid after one day of refrigerated storage (Spitzer et al., 2013). Compared to pasteurised donor breastmilk and preterm breastmilk that had been stored frozen at -80°C, samples of BMF refrigerated for at least 8 hours presented a much higher concentration of all FA (including octanoic acid) and the majority of fatty acid esters and aldehydes detected in our study. One possible explanation is that FA and FA esters result from the activity of breastmilk lipase during refrigerated storage of untreated

breastmilk, which does not occur in pasteurised breastmilk due to inactivation of lipase during the pasteurisation process (Peila et al., 2016). Additionally, it is possible that the lower concentration of aldehydes in preterm breastmilk relates to the fact that samples were frozen immediately after collection. The impact of different fortification and storage practices for fortified breastmilk on feed tolerance and infant nutrition needs further investigation.

To the best of our knowledge, few studies previously have compared the volatile profile of liquid and powdered formulas (Hausner et al., 2009; van Ruth et al., 2006). In general, previous studies indicate that the volatile profile of liquid formulas is rich in terpenoids and ketones, whereas powder formulas are characterised by the presence of aldehydes resulting from lipid oxidation and thermal degradation generated during manufacturing and storage (Hausner et al., 2009; van Ruth et al., 2006), which might confer an unpleasant odour to milk (Elisia and Kitts, 2011; Jia et al., 2019).The extreme heat used for water evaporation during powder formula production might intensify lipid auto-oxidation and contribute to formation of aldehydes (Hausner et al., 2009). Our results substantiate this, showing higher abundance of 2-butanone, acetone, benzaldehyde, 2-ethyl-furan and pentane in liquid formula, whereas aldehydes such as hexanal, pentanal and octanal contributed significantly to the volatile profile of powder formulas. Except for terpenoids, which in our study were more abundant in powder compared to liquid formula, the differences observed between the two formula types are in line with previous reports (Hausner et al., 2009; van Ruth et al., 2006) and probably relate to different farming practices, formulation, manufacturing, storage and packaging processes in production of powdered and liquid infant formulas. Interestingly, and contrary to what might be expected, powder formulas made from different protein sources (soy, goat and bovine milk) exhibit similar volatile profiles.

The presence of terpenoids in breastmilk and formula has been reported previously (Hausner et al., 2009; van Ruth et al., 2006) and likely originates from maternal diet, skin absorption of cosmetics or inhalation of products containing terpenoid-based fragrancies (Vieira et al., 2018; Wolkoff and Nielsen, 2017), animal diet/pasture (Clarke et al., 2020; Faulkner et al., 2018), and from mixed vegetable oils added to infant formulas to enrich them with essential fatty acids and liposoluble vitamins. We detected eight different terpenoids, largely in breastmilk samples and especially among the human milk-based products (HMF and RTF) in which, compared to other milk types, significantly higher relative peak areas were observed for half of the terpenoids. In quantifying volatile compounds in breastmilk, liquid and powder formulas, Hausner et al (2009) concluded that the main source of terpenoids in breastmilk is direct transfer from maternal diet, with high individual variability (Hausner et al., 2009). It seems likely that, because human milk-based products are made from pooled donor breastmilk, there could be contributions from many different donors to the overall terpenoid content in these products.

Ketones are also generated from lipid oxidation, Maillard Reactions (non-enzymatic browning of carbohydrates) and thermal processing (Diez-Simon et al., 2019). In our study, ketones were detected in almost all milk types analysed, but acetone, pinacolone and 2-butanone were more abundant in infant formulas than in breastmilk samples, consistent with previous findings (Shimoda et al., 2000; van Ruth et al., 2006; Wang et al., 2019). These compounds are particularly odour active and are thought to contribute to the overall aroma of human milk and infant formulas (Buettner, 2007; Wang et al., 2019). Similarly, furans are formed by Maillard Reactions, thermal degradation of some amino acids, oxidation of PUFAs, carotenoids and ascorbic acid (Seok et al., 2015; Yoshida et al., 2007), from spray-drying and hydrolysis of milk for infant formula production (Yoshida et al., 2007). 2-ethylfuran and 2-

pentylfuran are known products of lipid oxidation and previously have been detected in both breastmilk and infant formula (Hausner et al., 2009). Furans have been described as 'possibly carcinogenic to humans' and their presence in food is monitored globally (European Food and Safety Authority, 2011; Knutsen et al., 2017). Some studies reported concentrations in infant formulas ranging from 0.02 to 36 µg/g (Lambert et al., 2018; Yoshida et al., 2007), but estimated margin of exposure through consumption of infant formula was not considered of high concern (Altaki et al., 2017). In our study furans were detected in most milk types but 2-ethyl-furan and 2-butyltetrahydro-furan were significantly more abundant in infant formulas.

Significant higher relative peak areas of the aromatic hydrocarbons styrene, indane, *ortho* and *meta* cymene, three isomers of ethyl methyl benzene and 1,3-bis(1,1-dimethylethyl)-benzene were observed in human milk-based products. These compounds have been previously found in samples of breastmilk and are likely to originate from exposure to pyrogenic and petrogenic air pollutants (Blount et al., 2010; Fabietti et al., 2004; Kim et al., 2007). Volatile organic compounds (VOCs) have been highly correlated with in-door air exposure to VOCs, and inhalation is thought to be the main entry route into the human body (Blount et al., 2010; Fabietti et al., 2004; Kim et al., 2007). However, the levels of VOCs reported were not deemed to be of health concern. As for terpenoids, a possible reason for the observed concentrations of specific VOCs in the human milk based products (HMF and RTF) could be the contribution of multiple donors living in areas of high pollution.

Liquid and powder formulas presented the highest relative abundance of aldehydes, consistent with previous reports (Jia et al., 2019; Wang et al., 2019), but also the lowest relative abundance of fatty acid esters and fatty acids. Aldehydes generally present lower odour thresholds compared to fatty acids (Spitzer et al., 2013). Even low concentrations of compounds with a low odour threshold may be sufficient to provoke odour detection and

confer off-notes to food (Jia et al., 2019), indicating that it is possible that the smell of infant formula is less pleasant to babies compared to the smell of fresh breastmilk. The odour activity values of several aldehydes found in fresh, refrigerated and frozen breastmilk have been reported to remain constant in breastmilk stored under refrigerated conditions (Spitzer et al., 2013) but to increase in breastmilk frozen for 6 months (Spitzer and Buettner, 2013). In contrast, the OAV of most fatty acids increased with refrigeration / frozen storage (Spitzer et al., 2013; Spitzer and Buettner, 2013). Most OAV in fresh breastmilk reported in these studies were <1 (Spitzer et al., 2013; Spitzer and Buettner, 2013), suggesting that smell of fresh breastmilk is not very intense. Nevertheless, it is worth mentioning that only an approximated OAV for breastmilk was reported, calculated based on odour threshold information from adults (Spitzer et al., 2013; Spitzer and Buettner, 2013), which might differ from that in babies. Further, odour threshold and odour release are highly influenced by the composition of the matrix under investigation (skim milk, whole milk, water, air) (Czerny et al., 2011). Thus, odour thresholds and OAV of volatile compounds in infant formula may differ from those in human milk, and remain to be determined.

The concentration and interactions of odour-active compounds is very relevant to the overall aroma of food, to palatability (Diez-Simon et al., 2019), and can even influence infants' acceptance of weaning foods (Mennella et al., 2011). From the compounds identified in our study, it seems likely that aldehydes and ketones are responsible for most of the olfactory cues in infant formulas whereas FA and FA esters contribute most to the smell of breastmilk. The aldehydes and ketones have a relatively low odour threshold, meaning that they can be perceived even at low concentrations, and are responsible for malty, almond, fatty and even fishy odour descriptions (Jia et al., 2019; Spitzer et al., 2013). Most fatty acids are important odour-active compounds but have high odour perception thresholds (at parts per million

level) (Poette et al., 2014). In contrast, esters of fatty acids present lower perception threshold (at parts per billion level) but their contribution to overall flavour perception can be potentiated by concentration and interaction with other esters and volatile compounds (Liu et al., 2004). For example, at low concentration, esters can contribute positively to aroma conferring fruit-like notes, but at high concentrations they may introduce an unpleasant flavour impression (Clarke et al., 2020). Thus, not only odour activity of a specific compound but also the interaction with other volatile compounds in the food matrix, play a role in overall olfactory perception.

Infants may have a greater sensitive olfactory perception than adults, which would allow detection of odours even at low concentrations (Spitzer and Buettner, 2013). Given that olfactory perception occurs retronasally in the oropharyngeal cavity, as well as nasally, the feeding experience *per se* might intensify the sensory perception of odourants in milk, even at low odour concentrations (Thierry et al., 2017). Our findings demonstrate that the compounds responsible for the smell of preterm breastmilk differ from those of other options commonly used for feeding preterm infants. Whether these differences have any biological or physiological effects upon preterm infant nutrition requires further investigation. Sensory stimulation with smell and taste of milk during tube feeds is a very simple intervention and might be an important factor in stimulation of the cephalic phase response, assisting with tolerance to tube feeds. The results from two ongoing randomised clinical trials will contribute to better understanding of the impact of exposure to smell and taste on nutrition of preterm infants (Beker et al., 2019; Bloomfield et al., 2018).

3.4.2 ***Strengths and Limitations***

Some limitations to this study should be mentioned. First, we were unable to analyse samples of preterm breastmilk in duplicate due to limited volume. However, triplicate measurements on other milk types were highly reproducible (data not shown) and suggest this limitation was minor. Secondly, samples of fresh fortified breastmilk were not obtained given the importance of even small amounts of this milk for preterm infants in the Neonatal Intensive Care Unit. Ethical approval was obtained only to retrieve samples that exceeded the unit's policy for refrigerated storage of fortified breastmilk and were therefore no longer clinically useful. Thus, despite differences observed between the profile of volatile compounds of BMF and unfortified preterm breastmilk, we are unable to comment on whether freshly fortified breastmilk contains different volatile components from fortified breastmilk that has been refrigerated for 8 hours. Additionally, no maternal nutritional information was collected, meaning we cannot relate maternal diet to the volatile profile found in breastmilk samples. Finally, we opted for an untargeted approach for the detection of volatile compounds; thus, quantification of specific compounds was only relative and could have been influenced by the matrix effects between the internal standard concentration and fat content of samples, as previously reported by Elisia and Kitts (2011) (Elisia and Kitts, 2011). We did not analyse macronutrient composition of breastmilk samples due to limited volume available and the incompatibility of human milk analysers with fortified breastmilk and infant formulas (Miris AB, 2017). Nevertheless, the volatile profile of a biological sample results from complex physical, chemical and biological interactions. An untargeted approach is therefore essential to investigate the contribution of diverse sensory active compounds to the organoleptic properties of matrices such as human milk, and may serve as screening step for compounds that require further investigation and absolute quantification.

Previous research into odourants of human milk has been carried out using techniques that require use of solvents (Spitzer et al., 2010, 2013; Spitzer and Buettner, 2013), distillation (Shimoda et al., 2000) or extraction of volatile compounds applying high temperatures (Romeu-Nadal et al., 2004) to increase volatility of compounds, all of which may alter the chemical properties of milks and, therefore, analyse odourants that do not reflect exactly what infants are exposed to through feeding. In contrast, head space SPME-GC-MS does not use solvent and allows the identification of volatile compounds without requiring the SPME fibre to come into contact with the actual sample matrix, but only with the volatile compounds trapped in the headspace above the sample (Blount et al., 2010; Wang et al., 2019). Thus, this approach that extracts volatile compounds in milk at temperatures similar to feeding temperature might provide a better understanding of sensory active compounds that could play a physiological role in sensory stimulation in the newborn. Moreover, for the first time the volatile compounds in human milk-based products and fortified breastmilk were analysed.

3.4.3 ***Implications / Future directions***

Given that provision of smell and taste of milk with tube feeds is a very simple and non-invasive intervention that might enhance metabolism of feeds, future research is needed to understand whether sensory active volatile compounds in milk can activate the cephalic phase response of digestion and hence improve feed tolerance in preterm infants. Furthermore, we have observed products from lipid oxidation and lipase activity in samples of breastmilk with bovine milk-based fortifier that had been refrigerated for at least 8 hours, suggesting that changes in breastmilk properties can occur during the recommended storage

of fortified breastmilk. Thus, how fortification and storage practices impact on digestion of fortified breastmilk merits further investigation. The detection of potential environmental contaminants such as aromatic hydrocarbons and furans in almost all milk types analysed is potentially of concern, particularly as these were highest in human milk-based products made from pooled donor breastmilk. The level of potential exposure of preterm infants receiving these products needs to be determined so that any risk can be assessed.

3.5 Conclusion

Sensory active products of fatty acids oxidation are the major contributors to olfactory cues in infant feeds. We have demonstrated that the profile of volatile compounds in preterm breastmilk differs from of other milks commonly used for infant feeding but the physiological impact of these differences in the nutrition of preterm infants requires further research. In addition, analysis of volatile compounds may be useful for monitoring oxidation in milk and detection of environmental contaminants.

3.6 Supplementary tables

Supplementary Table 3.1: Relative peak area of volatile compounds in different milk types

Compounds	RT (min)	Ref Ion (m/z)	PBM (*n*= 15)	PDBM (*n*= 4)	BMF (*n*= 10)	HMF (*n*= 2)	RTF (*n*= 3)	LF (*n*= 6)	PF (*n*= 7)	FDR *p*-value
Alcohol										
methanol*	1.82	31	24621 ±43118	20167 ±37469	7474 ±13854	-	-	1667 ±3727	-	ns
Ethanol	1.94	45	8367 ^{ac} ±17050	527 ^{ac} ±823	38791 ^{b} ±38316	33391 ^{ab} ±25381	42797 ^{abc} ±36554	799 ^{ac} ±1123	306 ^{ac} ±344	<0.001^{A}
isopropanol*	2.07	45	-	-	128 ^{a} ±406	93802 ^{b} ±123861	45369 ^{b} ±28743	728 ^{a} ±1628	-	<0.001^{B}
2-methylpropanol	2.15	59	31951 ±69016	295 ±344	3846 ±8156	89 ±126	69 ±77	637 ±757	5 ±6	ns
1-octenol	12.98	57	320 ^{a} ±572	1902 ^{ab} ±1785	1088 ^{ab} ±1751	2065 ^{ab} ±86	1006 ^{ab} ±650	265 ^{ab} ±314	3959 ^{b} ±3849	<0.001^{B}
2-ethylhexanol	14.51	57	1026 ^{abd} ±3121	54 ^{abd} ±100	57 ^{abd} ±94	5192 ^{cd} ±2534	16331 ^{c} ±14367	26692 ^{bc} ±36298	28 ^{bd} ±31	<0.001^{B}
Aldehyde										
3-methyl butanal*	3.11	43	-	41 ^{a} ±83	2473 ^{b} ±2107	-	-	763 ^{ac} ±1528	609 ^{bc} ±685	<0.001^{B}
pentanal*	3.9	44	6861 ^{ac} ±10498	3543 ^{ac} ±3512	31973 ^{bc} ±20282	12103 ^{abc} ±346	5326 ^{a} ±4601	11325 ^{ac} ±15754	380083 ^{bc} ±378430	<0.01^{B}
2-pentenal	5.98	83	101 ^{a} ±232	81 ^{a} ±162	469 ^{a} ±866	169 ^{ab} ±6	113 ^{ab} ±100	591 ^{ab} ±726	3060 ^{b} ±3156	<0.001^{A}
hexanal*	6.56	56	65621 ^{a} ±158777	100447 ^{ab} ±57284	105252 ^{ab} ±100365	60598 ^{ab} ±10180	55370 ^{ab} ±26068	17589 ^{a} ±27835	688518 ^{b} ±675367	<0.001^{A}
2-hexenal*	9.05	55	41^{a}	44 ^{ab}	731 ^{b}	237 ^{ab}	227 ^{ab}	-	485 ^{b}	<0.001^{A}

Compounds	RT (min)	Ref Ion (m/z)	PBM (*n*= 15)	PDBM (*n*= 4)	BMF (*n*= 10)	HMF (*n*= 2)	RTF (*n*= 3)	LF (*n*= 6)	PF (*n*= 7)	FDR *p*-value
			(96)	(88)	(887)	(6)	(92)		(344)	
heptanal*	9.82	43	799 ±1582	3038 ±1599	6561 ±8423	4932 ±814	2599 ±396	936 ±1851	4686 ±3235	ns
(Z)-2-heptenal	12.38	83	128 ±229	322 ±304	1123 ±1923	582 ±5	317 ±355	-	4542 ±5063	ns
Benzaldehyde	12.70	106	73 [a] ±150	233 [a b] ±150	1098 [b] ±1351	-	416 [a b] ±398	9016 [c] ±9109	3266 [b c] ±3350	<0.001[A]
Octanal	13.05	56	247 [a] ±453	1005 [a b] ±1317	1231 [b] ±808	535 [a b] ±92	597 [a b] ±209	181 [a b] ±292	4375 [c] ±4107	<0.001[A]
(E,E)-2,4-heptadienal	14.1	81	87 ±181	-	102 ±191	-	-	-	2372 ±2661	ns
(E)-2-octenal	15.45	83	102 [a] ±185	5 [a] ±10	590 [a] ±752	-	103 [a] ±90	-	982 [b] ±1082	<0.001[A]
Nonanal	16.08	82	137 [a] ±270	136 [a] ±200	9077 [b] ±11972	421 [a] ±114	1145 [a b] ±858	292 [a] ±652	545 [a] ±601	<0.001[A]
Alkyls										
Pentane	1.8	43	6078 ±7156	4228 ±3735	6033 ±8346	3722 ±1560	829 ±740	65510 ±68377	62545 ±66402	ns
undecane*	13.34	57	256 ±462	374 ±713	745 ±1186	-	374 ±648	150 ±336	198 ±223	ns
tridecane*	19.07	73	-	-	-	-	68±69	-	-	<0.001[B]
nonane*	7.09	57	-	48 ±57	434 ±734	-	-	129 ±199	111 ±125	ns
2-methyl pentane	1.92	71	10005 ±24710	651 ±782	2038 ±4291	-	-	31238 ±47953	564 ±634	ns
Cyclohexane	2.05	56	7667 ±17632	3203 ±5585	346 ±792	-	-	1721 ±2893	1408 ±1582	ns
hexyl-cyclohexane	17.54	83	-	-	-	129 [a]	612 [a]	-	45 [a]	<0.001[B]

Compounds	RT (min)	Ref Ion (m/z)	PBM (*n*= 15)	PDBM (*n*= 4)	BMF (*n*= 10)	HMF (*n*= 2)	RTF (*n*= 3)	LF (*n*= 6)	PF (*n*= 7)	FDR *p*-value
						±68	±326		±51	
butyl-cyclohexane	11.44	83	-	-	-	208 [a] ±71	1065 [a] ±160	-	-	<0.001[A]
1,2,4-trimethyl-cyclopentane	4.27	70	1268 [a] ±3287	9590 [b] ±5824	1070 [a] ±1123	3561 [a] ±366	1383 [a] ±1181	1865 [a] ±1356	3272 [a] ±3531	<0.001[A]
5-methyl-1-heptene	4.31	55	1494 ±3256	2890 ±1983	1540 ±1012	3911 ±2170	2651 ±2614	1873 ±2241	2011 ±2260	ns
2,4-dimethyl-1-heptene	5.6	43	5158 ±7923	1035 ±714	2452 ±5916	664 ±251	-	17609 ±27444	8053 ±8957	ns
2,3-dimethyl octane	9.02	57	9 ±36	46 ±59	269 ±520	297 ±261	2075 ±1198	219 ±269	7525 ±8381	ns
2-methyl nonane	9.1	57	-	366 ±342	812 ±1590	162 ±53	54 ±94	892 ±1318	288 ±323	ns
2,3-dimethyl-butane	1.916	43	18505 ±45450	957 ±1134	4869 ±7736	2712 ±2762	2289 ±3294	19149 ±25558	4023 ±4526	ns
2,3,5-trimethyl-hexane	4.78	43	4582 ±7300	2839 ±2334	25516 ±50745	585 ±347	64 ±110	3949 ±3599	9491 ±10507	ns
2,3-dimethyl-heptane	6	85	953 ±1571	1080 ±923	1549 ±2969	631 ±47	29 ±50	6405 ±6973	454 ±511	ns
3-ethyl-2-methyl-1,3-hexadiene	14.23	67	126 [a] ±271	463 [a b] ±208	509 [a b] ±995	519 [a b] ±28	358 [a b] ±175	79 [a b] ±144	2152 [b] ±2089	<0.01[A]
branched C9 alkane	7.00	113	-	-	-	-	-	-	345 ±388	ns
branched C10 alkane A	9.35	57	60 ±232	28 ±56	307 ±278	657 ±68	933 ±638	2289 ±4239	1522 ±1712	ns
branched C10 alkane B	9.85	56	84 ±243	1780 ±1194	1668 ±2398	2160 ±204	802 ± 452	3842 ±7455	3393 ±3611	ns
branched C10 alkane	10.00	57	44 ±135	48 ±86	84 ±110	-	173 ±291	-	315 ±354	ns

Compounds	**RT (min)**	**Ref Ion (m/z)**	**PBM (*n*= 15)**	**PDBM (*n*= 4)**	**BMF (*n*= 10)**	**HMF (*n*= 2)**	**RTF (*n*= 3)**	**LF (*n*= 6)**	**PF (*n*= 7)**	**FDR** *p*-value
branched C11 alkane A	10.91	57	10 ±40	461 ±481	414 ±781	290 ± 57	138 ±119	779 ±926	337 ±380	ns
branched C12 alkane	18.40	55	-	11 ±22	225 ±380	-	43 ±47	-	235 ±265	ns
Aromatic Hydrocarbon										
toluene	4.69	91	1462 ±5562	116 ±231	1674 ±4636	-	-	1740 ±3748	3372 ±3793	ns
m-xylene	7.32	91	113 ±122	28939 ±56445	1785 ±2621	2361 ±78	1829 ±824	2704 ±5517	114 ±128	ns
o-xylene	7.57	91	258 ±892	13592 ±25919	200 ±169	2073 ±1627	3974 ±1715	1852 ±3128	175 ±197	ns
p-xylene	8.46	106	91 ±351	313 ±81	94 ±219	653 ±335	884 ±652	542 ±869	46 ±52	ns
o-cymene	13.58	119	-	-	-	462 [a] ±24	657 [a] ±474	-	20 [b] ±23	<0.001[B]
m-cymene	14.48	119	-	-	-	237 [a] ±102	597 [a] ±589	-	43 [b] ±48	<0.001[A]
styrene	8.76	104	228 [a] ±768	3223 [b] ±1034	1620 [a b] ±3039	4101 [b] ±2310	1992 [a b] ±1432	-	-	<0.001[A]
ethyl methyl benzene A	10.65	105	28 [a] ±80	98 [a] ±133	49 [a] ±93	5021 [b] ±2529	6053 [b] ±7494	53 [a] ±119	35 [a] ±39	<0.001[A]
ethyl methyl benzene B	11.25	105	37 [a] ±144	5 [a] ±10	-	790 [b] ±307	1567 [b] ±1340	-	-	<0.001[A]
ethyl methyl benzene C	11.64	105	71 [a] ±219	107 [a] ±69	47 [a] ±74	7990 [b] ±2398	12611 [b] ±10222	89 [a] ±133	43 [a] ±48	<0.001[A]
indane	13.12	117	-	-	-	409 [a] ±28	559 [a] ±335	-	-	<0.001[B]
1,3-bis(1,1-dimethylethyl)-benzene	18.88	175	-	167 [a] ±256	673 [a] ±767	401 [a] ±109	347 [a] ±198	-	15 [b] ±17	<0.001[B]

Compounds	RT (min)	Ref Ion (m/z)	PBM (*n*= 15)	PDBM (*n*= 4)	BMF (*n*= 10)	HMF (*n*= 2)	RTF (*n*= 3)	LF (*n*= 6)	PF (*n*= 7)	FDR *p*-value
Fatty acids										
butanoic acid*	9.9	60	378 [a] ±559	268 [a] ±352	3953 [a b] ±6269	1431 [b] ±804	1014 [a b] ±870	-	-	<0.01[B]
hexanoic acid*	15.54	60	1406 ±4071	797 ±1587	2816 ±3827	2548 ±105	4509 ±3101	-	-	ns
octanoic acid*	20.71	60	2887 ±9442	-	792601 ±1495737	219 ±309	2337 ±1111	2564 ±5636	-	ns
nonanoic acid	23.13	129	-	-	268 ±490	-	-	-	-	ns
n-decanoic acid	25.43	73	-	-	4445 ±9722	-	-	-	-	ns
Fatty acid esters										
branched C8 fatty acid ester	18.06	88	-	236 [a] ±424	30973 [b] ±49605	625 [b] ±98	1087 [b] ±678	108 [a] ±243	-	<0.001[B]
dimethyl carbonate	2.72	59	284 ±972	24 ±42	121 ±267	71 ±101	60 ±75	122 ±185	1 ±1	ns
methyl butyrate*	4.04	74	292 [a] ±599	309 [a] ±152	1167 [b] ±799	354 [a b] ±148	136 [a b] ±120	35 [a] ±79	62 [a] ±69	<0.001[A]
ethyl butyrate*	6.03	88	-	176 ±353	1127 ±3061	473 ±162	552 ±483	-	-	ns
isopropyl butyrate*	7.15	89	-	-	-	391 [a] ±172	807 [a] ±717	-	-	<0.001[B]
vinyl caproate	12.81	43	931 ±1836	792 ±528	2030 ±2466	486 ±35	480 ±358	-	6974 ±7761	ns
methyl octanoate*	16.1	115	980 ±2158	178 ±347	60508 ±108415	-	-	173 ±386	31 ±35	ns
2-ethylhexyl acetate	16.86	70	-	-	70 ±101	360 ±153	1196 ±1447	270 ±605	56 ±62	ns
methyl decanoate*	21.48	143	276 [a]	-	6473 [b]	-	-	52 [a]	-	<0.001[B]

Compounds	RT (min)	Ref Ion (m/z)	PBM (*n*= 15)	PDBM (*n*= 4)	BMF (*n*= 10)	HMF (*n*= 2)	RTF (*n*= 3)	LF (*n*= 6)	PF (*n*= 7)	FDR *p*-value
			±524		±8922			±117		
ethyl decanoate	23.23	88	-	18 [a] ±36	633 [b] ±808	240 [bc] ±128	166 [c] ±15	-	-	<0.001[B]
methyl dodecanoate*	26.34	74	106 [a] ±202	-	277 [b] ±265	-	-	-	-	<0.001[B]
methyl hexanoate*	9.99	74	698 [ac] ±1997	3782 [ab] ±5896	4442 [bc] ±4483	1746 [ab] ±104	392 [abc] ±347	110 [ac] ±168	87 [ac] ±98	<0.001[A]
isopropyl hexanoate	13.34	99	-	-	-	469 [a] ±308	675 [a] ±575	-	-	<0.001[B]
Furans										
2-ethyl-furan	3.21	81	144 [a] ±237	214 [ab] ±202	168 [ab] ±202	1047 [ab] ±1047	429 [ab] ±306	4902 [b] ±7041	3044 [b] ±4662	<0.001[A]
2-pentyl-furan	11.32	81	169 ±377	830 ±451	536 ±308	2431 ±354	2348 ±1368	3493 ±2772	4934 ±1116	ns
2-propyl-furan	14.59	81	36 ±138	-	63 ±93	72 ±59	267 ±123	317 ±495	2711 ±4806	ns
2-butyltetrahydro-furan	16.82	71	35 [a] ±81	-	98 [ab] ±106	143 [ab] ±53	437 [b] ±341	5 [a] ±10	1360 [b] ±1879	<0.001[A]
Ketones										
acetone*	2.04	43	66133 ±99397	11019 ±8947	76902 ±100357	-	-	492890 ±774822	51702 ±51617	ns
2-butanone	2.55	43.1	9503 [a] ±20443	3278 [acd] ±455	44831 [c] ±52922	2180 [a] ±337	1909 [a] ±705	932182 [b] ±735935	55056 [cd] ±61772	<0.001[B]
2-(1,1-dimethylethyl)-cyclobutanone	3.01	83	3777 ±12147	318 ±180	338 ±825	125 ±9	163 ±129	2075 ±2114	964 ±994	ns
1-penten-3-one	3.80	55	5873 ±12823	9 ±17	5370 ±14180	584 ±24	394 ±621	-	4998 ±4653	ns

Compounds	**RT (min)**	**Ref Ion (m/z)**	**PBM (*n*= 15)**	**PDBM (*n*= 4)**	**BMF (*n*= 10)**	**HMF (*n*= 2)**	**RTF (*n*= 3)**	**LF (*n*= 6)**	**PF (*n*= 7)**	**FDR** *p*-value
3,3-dimethyl-2-butanone (pinacolone)	4.18	57	745 [a] ±1765	1090 [a] ±1271	1134 [a] ±1155	3230 [a b] ±1308	1199 [a b] ±404	97 [a] ±138	23814 [b] ±25072	<0.001[A]
2-heptanone	9.72	58	93 ±187	1313 ±664	10139 ±24397	992 ±384	931 ±298	11396 ±10251	956 ±1066	ns
6-methyl-2-heptanone	11.78	58	60 ±149	344 ±101	242 ±462	325 ±69	82 ±71	171 ±237	-	ns
1-octen-3-one	12.47	97	134 ±316	62 ±65	242 ±255	114 ±5	131 ±25	11 ±25	294 ±282	ns
3-octanone	12.6	43	152 ±320	182 ±204	359 ±470	60 ±84	9 ±16	-	58 ±65	ns
2-nonanone	15.94	127	-	-	9 ±24	-	-	-	-	ns
3,5-octadien-2-one	16.1	95	9 ±35	63 ±126	116 ±246	692 ±5	515 ±309	-	2055 ±2312	ns
acetophenone	16.18	105	8 ±20	28 ±33	155 ±236	147 ±45	261 ±208	219 ±165	87 ±98	ns
Siloxane										
hexamethyl disiloxane	2.5	147	380 ±772	-	35 ±111	578 ±176	269 ±239	-	-	ns
trimethyl-silanol	2.67	75	20605 ±58620	3943 ±4160	6533 ±8896	6404 ±6917	4390 ±2574	-	8 ±9	ns
1,1,3,3,5,5-hexamethyl-trisiloxane	3.98	193	-	393 [a] ±786	-	-	801 [b] ±1247	-	-	<0.001[B]
hexamethyl-cyclotrisiloxane	5.03	207	16641 [a] ±25189	93652 [a] ±107786	8477 [a] ±20017	7514 [a] ±5465	12090 [a] ±8777	-	-	<0.05[B]
tetramethyl- silane	8.3	77	44475 ±111006	-	1365 ±4317	-	17637 ±25004	-	34252 ±38533	ns
dimethyl- silanediol	8.6	77	3727 ±11994	-	1676 ±5300	-	24548 ±42519	-	14203 ±15978	ns

Compounds	**RT (min)**	**Ref Ion (m/z)**	**PBM (*n*= 15)**	**PDBM (*n*= 4)**	**BMF (*n*= 10)**	**HMF (*n*= 2)**	**RTF (*n*= 3)**	**LF (*n*= 6)**	**PF (*n*= 7)**	**FDR *p*-value**
siloxane A	9.26	267	62 [a] ±123	423 [a] ±581	140 [a] ±303	6069 [b] ±2545	6400 [b] ±3449	-	-	<0.001[A]
octamethyl-cyclotetrasiloxane	10.27	281	5102 ±11670	32051 ±63756	711 ±1724	31987 ±10800	58645 ±43021	-	-	ns
siloxane B	13.61	193	57 ±115	5 ±9	37 ±74	-	15 ±16	31 ±66	115 ±129	ns
siloxane C	14.17	267	5 [a] ±19	-	3 [a] ±7	180 [b] ±81	178 [b] ±309	-	2 [a] ±2	<0.001[A]
decamethyl-cyclopentasiloxane	14.96	267	351 ±831	-	28 ±88	-	4002 ±6932	-	-	ns
dimethoxydimethyl- silane	16.37	105	254 ±609	-	261 ±768	-	-	45 ±100	144 ±162	ns
siloxane D	19.6	207	8 ±29	-	4 ±12	-	-	-	101 ±114	ns
dodecamethyl-pentasiloxane	23.82	147	3 ±11	-	99 ±231	-	-	46 ±96	235 ±264	ns
Terpenoids										
Thujene	8.429	93	173 [a c] ±585	1440 [b] ±861	210 [a c] ±317	1672 [a c] ±932	1729 [b] ±1062	347 [a c] ±353	92 [a c] ±104	<0.001[A]
beta-pinene	10.06	93	201 ±771	312 ±334	585 ±830	1582 ±106	1444 ±575	-	234 ±264	ns
3-carene	11.1	93	179 ±242	292 ±163	141 ±213	356 ±71	368 ±297	440 ±715	159 ±179	ns
d-limonene	11.86	136	111 ±142	149 ±108	596 ±1117	520 ±67	1038 ±848	-	-	ns
gamma terpinene	12.94	93	-	64 [ac] ±75	6 [a] ±13	188 [b c] ±2	222 [a b c] ±76	-	33 [a] ±37	<0.001[B]
p-cymene	12.43	119	48 [a] ±149	265 [a b] ±268	906 [b] ±933	865 [b] ±322	1117 [b] ±250	-	-	<0.001[A]

Compounds	RT (min)	Ref Ion (m/z)	PBM (*n*= 15)	PDBM (*n*= 4)	BMF (*n*= 10)	HMF (*n*= 2)	RTF (*n*= 3)	LF (*n*= 6)	PF (*n*= 7)	FDR *p*-value
eucalyptol	12.49	108	2 [a] ±9	9 [a c] ±10	408 [b c] ±516	142 [b] ±21	160 [b] ±3	64 [a c] ±142	-	<0.001[B]
unknown sesquiterpenoid	22.4	161	-	-	-	48 [a] ±2	129 [a] ±114	-	-	<0.001[B]
Others										
Acrylate										
2-ethylhexyl acrylate	19.05	55	-	-	4 ±11	-	258 ±447	-	-	ns
Chlorination byproduct										
chloroform*	2.66	83	-	-	3549 ±5865	-	-	378 ±846	341 ±383	ns
bromodichloromethane	4.06	83	-	-	649 ±1540	-	-	983 ±2197	255 ±193	ns
bromochloronitromethane	6.58	129	-	-	84 ±265	-	-	85 ±189	263 ±259	ns
Ether										
2-ethoxy-2-methylpropane	2.26	59	21959 ±44068	-	750 ±864	-	-	-	89 ±100	ns
Microbial metabolite										
Indole	25.1	117	-	-	75 ±125	-	-	-	-	ns
Nitrogenous compound										
N-(but-2-enoyl)butanamide	1.95	69	2173 ±4695	-	16 ±41	-	-	76974 ±163490	693 ±780	ns
acetonitrile*	2.17	41	3010 [a] ±7349	791 [a] ±1101	3052 [a] ±5344	-	-	41583 [b] ±25763	6027 [a] ±5815	<0.001[A]
Pharmaceutical compound										

Compounds	**RT (min)**	**Ref Ion (m/z)**	**PBM (*n*= 15)**	**PDBM (*n*= 4)**	**BMF (*n*= 10)**	**HMF (*n*= 2)**	**RTF (*n*= 3)**	**LF (*n*= 6)**	**PF (*n*= 7)**	**FDR** *p*-value
chlorobutanol	14.09	59	575 ±1368	-	267 ±843	77 ±7	85 ±19	-	-	ns
Phenolic compound										
Phenol	17.08	94	0 ±1	1 ±1	17 ±33	-	207 ±359	-	9 ±10	ns
Sulphur compound										
dimethyl trilsulfide	11.43	126	-	-	-	-	-	484 [a] ±743	528 [a] ±573	<0.001[B]
methional	11.66	48	-	-	55 ±125	-	-	938 ±2098	1589 ±1674	ns
Unidentified										
unknown m/z100	4.15	100	66 [a] ±198	28 [a] ±39	1387 [b] ±2705	104 [b c] ±5	43 [a b] ±75	-	4140 [c] ±4489	<0.001[B]

Data are mean (± SD). Peak area reported is relative to internal standard. RT: Retention time; Ref Ion: Reference ion; SD: Standard deviation; PBM: Preterm breastmilk; PDBM: Pasteurised donor breastmilk; BMF: Bovine milk-based fortified breastmilk; HMF: Human-milk based fortified breastmilk; RTF: Human milk-based ready-to-feed formula; LF: Liquid Formula; PF: Powdered Formula. FDR: False discovery rate adjusted *p*-value; *n*: number of samples analysed; Annotations represent post hoc comparisons where groups with same letter are not significantly different at $p<0.05$; A: ANOVA/Tukey's post hoc; B: Kruskal-Wallis/Conover's post hoc; ns: not significant; ***Identification confirmed with authentic standard**.

Supplementary Table 3.2: Odour thresholds, odour activity value (OAV) and odour attributes of volatile compounds reported in the literature

Volatile compound	Odour threshold (µg/L)	OAV	Matrix	Odour attributes	Reference
hexanal	88	<1 – 2	Breastmilk	Grassy, green	1
octanal	25	<1	Breastmilk	Soapy	1
nonanal	174	<1	Breastmilk	Citrusy	1
decanal	865	<1	Breastmilk	Fatty, soapy	1
(e)-hex-2-enal	671	<1	Breastmilk	Green, grassy	1
3-methyl butanal	1.2	-	Water	Malty	2
benzaldehyde	0.3-4.6	-	Water	-	3
butanoic acid	4800	1.4 – 3.5	Breastmilk	Sweaty	1, 4
hexanoic acid	14,000	<1	Breastmilk	Musty, pungent	1, 4
octanoic acid	22,000	<1	Breastmilk	Musty, plastic-like	1, 4
decanoic acid	27,000	<1	Breastmilk	Fatty, rancid	1, 4
dodecanoic acid	108,000	<1	Breastmilk	Fatty, rancid	1, 4
methyl octanoate	0.2-0.9		Water	-	4
methyl decanoate	0.0043 - 0.009	-	Water	Fruity	4, 5
ethanol	990,000	-	Water	Alcoholic, ethanol, pungent, sweet	2, 6
toluene	9	-	Milk chocolate	-	3

Volatile compound	Odour threshold (µg/L)	OAV	Matrix	Odour attributes	Reference
limonene	6.5	-	Whole milk	-	3
(r)-α-pinene	4.6	-	Water	Rosiny, fir needle-like	2
acetone	16	-	Milk chocolate	-	3
oct-1-en-3-one	0.1	<1 – 47	Breastmilk	Mushroom-like	1
2-heptanone	62-98	-	Sunflower oil	Cheese, cured ham, fruity, toasted	3, 5
pentane	340	-	Mineral oil		3
o-cymene	0.004-0.005	-	Air		3

1. Spitzer and Buettner, 2013.
2. Czerny et al., 2011.
3. van Gemert, 2011.
4. Buettner, 2007.
5. Liu et al., 2004.
6. Jia et al., 2019.

CHAPTER 4

ODOUR ACTIVE VOLATILE COMPOUNDS IN PRETERM BREASTMILK

Chapter published in Pediatric Research

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4.1 Introduction

Breastmilk (BM) is considered the gold standard in neonatal nutrition (Lapillonne et al., 2019) and breastfeeding provides several nutritional and health benefits to infants and mothers, leading to the general consensus that BM has many advantages over other feeding methods (Bertino et al., 2012; Victora et al., 2016).

Volatile compounds originating from maternal diet and metabolism can contribute to the flavour and composition of amniotic fluid and BM (Mastorakou et al., 2019; Mennella et al., 2017; Schaal, 2000), influencing fetal and infant flavour learning and later infant feeding preferences (Lipchock et al., 2011; Mennella et al., 2009). For example, infants have been reported to demonstrate greater acceptance of weaning foods that mothers consumed during late gestation and lactation (Mennella et al., 2017; Mennella and Beauchamp, 1991a). Neonates are capable of recognizing odours from their own amniotic fluid and from their own mother's milk (Marlier et al., 1998; Schaal, 2000) and can distinguish between smell of BM and infant formula, demonstrated by increased sucking behaviour (Bingham et al., 2003a; Raimbault et al., 2007) and cerebral oxygenation in response to BM (Aoyama et al., 2010). Hence, BM may be a link between the antenatal and postnatal sensory environments (Lipchock et al., 2011).

Smell and taste of food are intimately related to appreciation of food and digestion. Sensory cues from food assist digestion and metabolism by triggering a cascade of physiological responses that lead to increased salivation and peristaltic movements and the release of hormones and enzymes related to digestion (Smeets et al., 2010). Promotion of these physiological responses has the potential to enhance tolerance and metabolism of

enteral feeds in preterm infants in whom establishment of milk feeds can be a major determinant of hospital length of stay. There is some evidence that exposure to smell and taste of milk may reduce duration of hospital stay in preterm infants (Muelbert et al., 2019), but the quality of evidence is very low.

The composition of BM is highly variable and can be affected by lactation stage (Gidrewicz and Fenton, 2014; Samuel et al., 2020), stage of a feed (fore- versus hind-milk) (Pham et al., 2020), circadian rhythm (Hahn-Holbrook et al., 2019; Moran-Lev et al., 2015), maternal diet, nutritional supplementation and medical conditions (Hascoët et al., 2019; Nasser et al., 2010; Samuel et al., 2020), maternal anthropometry, parity, socioeconomic status (Bahreynian et al., 2020; Lee et al., 2018) and infant sex (Samuel et al., 2020; Thakkar et al., 2013), although the strength of the evidence for maternal and infant factors is less strong. Length of gestation also influences composition of BM (Gidrewicz and Fenton, 2014) with mothers of preterm infants producing BM in the first 12 – 16 postnatal weeks with higher energy and protein content (Gidrewicz and Fenton, 2014), lower lactose (Léké et al., 2019) and a different profile of fatty acids (FA), especially medium-chain fatty acids (MCFAs) (Thakkar et al., 2019), compared to BM produced by mothers of full-term infants. Environmental contaminants and toxins (e.g. alcohol and recreational drugs) also have been detected in BM and have the potential to impact upon infant growth and development (Blount et al., 2010; Cano-Sancho et al., 2020). However, there is much less evidence around how the volatile compounds present in BM responsible for smell, taste and activation of the cephalic phase response vary according to different maternal, pregnancy and infant factors.

We recently demonstrated that the profile of volatile compounds in different milk types commonly fed to preterm infants (BM, fortified BM, pasteurised donor BM and a variety of

infant formulas) are markedly distinct (Muelbert et al., 2021). However, that study only analysed a small number of samples from preterm BM, all collected in the first two weeks postpartum, as the main purpose was to compare different types of milk. Therefore, this study aims to investigate maternal and infant factors that may be associated with the profile of volatile compounds in BM produced by mothers of moderate- and late-preterm (MLP) infants enrolled in a multicentre randomised controlled trial, the DIAMOND trial. We hypothesised that the profile of volatile compounds would be influenced by lactation stage, length of gestation, infant sex, birth weight and maternal characteristics such as age, ethnicity, socioeconomic status and medical condition.

4.2 Methods

4.2.1 Study population

This is a cohort study nested within the DIAMOND trial (ACTRN12616001199404) (Bloomfield et al., 2018), a multicentre, factorial, randomised controlled trial investigating the impact of different nutritional approaches on feed tolerance, body composition and neurodevelopment in MLP infants. The randomisation factors are: 1) provision of intravenous parenteral nutrition compared to intravenous dextrose alone; 2) provision of enteral nutrition with exclusive maternal BM compared to milk supplementation (with infant formula or donor breastmilk), and 3) exposure to smell and taste of milk before all gastric tube feeds compared to standard care (no exposure to smell and taste of milk prior to tube feeds). Eligible infants were born between 32^{+0} and 35^{+6} weeks' gestation, admitted to one of five neonatal care units (NCUs) in New Zealand, had intravenous access secured for clinical reasons, and whose

mothers intended to breastfeed. Infants with a congenital abnormality or for whom a particular mode of nutrition was clinically indicated were not eligible. This research was approved by the national Health and Disability Ethics Committee (HDEC 16/NTA/90) and written informed consent was obtained from parents or caregivers.

Maternal ethnicity, education, postcode and clinical information (maternal diabetes, antenatal steroid administration, delivery mode) and infant characteristics (sex, gestational age at birth, anthropometric measures) were collected prospectively. In New Zealand, postcode of domicile is used to generate a social deprivation index (the New Zealand Deprivation (NZdep) index) in the Classification Coding System from Statistics New Zealand (Atkinson et al., 2014) with a scale from 1 to 10, representing low to high social deprivation. NZdep index data were divided into quintiles, the first (Q1) and fifth quintiles (Q5) representing least and most socially deprived areas, respectively. Ethnicity was self-identified and prioritised according to New Zealand's Ministry of Health protocols (Ministry of Health, 2017). For analysis, ethnicity was grouped into: Caucasian/European; Asian (Asia, South East Asia and Indian subcontinent); Pasifika (South Western Pacific); Māori (New Zealand Māori); and Other (ethnicities not defined in the previously mentioned categories).

Gestational diabetes (GDM) was classified according to the New Zealand Ministry of Health criteria of fasting glucose ≥5.5 mmol/L (≥99 mg/dL) or glucose ≥9.0 mmol/L (≥162 mg/dL) 2 hours following a 75 g oral glucose tolerance test. Antenatal steroid course was classified as complete (more than 1 dose given, with the first dose >24 hours before birth), incomplete (1 dose given <24 hours before birth) or no antenatal steroid received.

4.2.2 Sample collection and preparation

Mothers of infants enrolled in the trial were asked to provide a sample of breastmilk (BM) at postnatal day 3 (+2), 5 (+2), 10 (±2), and at follow-up visit at 4 months (±2 weeks) corrected age (CA, counting from full term at 40 weeks). Lactation stage was defined as colostrum (samples collected at postnatal day 3 and 5), transitional BM (samples collected at postnatal day 10) and mature BM (samples collected at 4 months' CA). The cohort reported here includes mothers who provided at least one BM sample during the study period and their infants. More details on BM sample collection can be found elsewhere (Galante et al., 2019). Briefly, mothers were asked to express BM from their right breast until completely empty, using a hospital-grade electronic breast pump (Medela, Baar, Switzerland). Sample collection occurred in the morning at least 2-3 hours after the previous BM expression or breastfeeding. After the right breast was completely emptied, the total volume of expressed BM was vortexed for 2 minutes at high speed to ensure homogeneity and 2 mL were collected using a sterile syringe (brands varied according to collection site). Equal amounts of BM were aliquoted into four low protein binding microtubes (Eppendorf, Germany), one aliquot of which was used in this study. Samples were frozen immediately after collection and stored at −20°C for up to two weeks in a dedicated freezer in each NCU then transferred to the Liggins Institute and stored at −80°C freezer for 12–24 months until analysis.

Samples were randomly allocated into batches for analysis. Aliquots of BM were thawed at 4°C for 2 hours and vortexed at low speed to ensure homogeneity before 400 µL was transferred into 10 mL headspace amber vials with magnetic screw caps and polytetrafluoroethylene-lined silicone septa (Thermo Fisher Scientific Inc., New Zealand). As limited preterm BM was available, pasteurised BM (PBM) from a single deidentified donor

was used as quality control (QC) and processed in the same way as the study samples. PBM was obtained from Christchurch Milk Bank, New Zealand, shipped frozen and then stored at −20°C until analysis. More information about the Human Milk Bank protocols can be found elsewhere (Lamb et al., 2020). Procedural blanks consisted of 400 µL ultrapure water (MilliQ). The procedural blanks and QCs were placed at the beginning, middle and end of each batch. All samples, QCs and blanks were spiked with 10 µL of ultrapure water containing 2-chlorophenol at 20 µL/L as an internal standard (IS) (Sigma-Aldrich, Sydney, Australia). Data from the QC analyses were processed in parallel with the samples to confirm reproducibility of retention times and peak areas among batches.

4.2.3 ***Analysis of volatile compounds***

Untargeted analysis of volatile compounds was performed by headspace solid phase microextraction gas chromatography with mass spectrometry (SPME-GC-MS) using a Thermo Fisher Trace GC Ultra with a Programmable Temperature Vaporizer connected to a Thermo ISQ mass spectrometer as described previously (Muelbert et al., 2021). In brief, a Gerstel MPS2 autosampler was used to equilibrate samples at 37°C, with continuous agitation, for 10 min. The SPME fibre was divinylbenzene-carboxen-polydimethylsiloxane (DVB-CAR-PDMS) 50/30 µm (Supelco, Bellefonte, Pennsylvania) measuring 20 mm in length. A 10 mL amber vial was used to prevent contact between the milk and the SPME fibre. Extraction of volatile compounds occurred for 10 min. The carrier gas was zero grade helium (99.995%, BOC New Zealand) at a constant flow rate of 1.1 mL/min. Upon injection, the SPME fiber was simultaneously desorbed and conditioned in the GC injector in high pressure splitless mode using a low-volume SPME-specific deactivated liner (0.75 mm ID) at 250°C for 10 min. The

column was a Phenomenex 1701 capillary column (30 m × 250 µm × 0.25 µm). Oven temperature was set at initial temperature of 35°C and held for 4 min, followed by an increase of 5°C /min up to 165°C followed by an increase of 50°C /min up to 265°C. Data were acquired at a scan rate of 5 Hz in the range m/z 20–300.

Deconvolution, integration and identification of features in the GC-MS data were performed using Agilent MassHunter Unknowns software version B10.00 (Agilent Technologies, Santa Clara, California) searching the 2020 version of the NIST mass spectral library (National Institute of Standards & Technology, Gaithersburg, Maryland) using retention time calibration by Kovats Index and a match factor threshold of 80%. Identities were filtered to exclude features with a signal-to-noise ratio <10 or that were identified in less than 1% of samples. Authentic standards were run to verify the identities of short (SCFA) and medium-chain fatty acids (MCFA) and their esters, as well as various alcohols, aldehydes and ketones for which standards were readily available. These annotations comply with criteria for level 1 metabolite identification described by the Metabolomics Standards Initiative (Sumner et al., 2007). All other annotations complied with level 2 metabolite identification. Relative quantification of compounds was performed as function of the area under the peak for a compound in relation to the peak area of a known compound (internal standard). Identified peaks in each sample were blank-subtracted to remove background noise and quantified relative to the area of the internal standard peak in that sample (2-chlorophenol). This technique reveals differences in concentration of identified compounds between samples, expressed as relative peak area, but does not provide their absolute concentration.

4.2.4 ***Statistical analysis***

We investigated the associations between relative concentration of volatile compounds and variables of interest (lactation stage, ethnicity, NZdep, maternal diabetes, antenatal steroid course, infant sex and gestation at birth), using mixed model regression analysis. To account for within-subject variability and repeated measurements, participant ID and lactation stage were included as random effects. In all models, compound symmetry was assumed. A false discovery rate adjusted *p* value of <0.05 (FDR= 0.05, Benjamini-Hochberg) was considered statistically significant for multiple comparisons. Relative concentration of identified compounds are presented as mean peak area and standard deviation. Given the exploratory nature of this study and semi-quantitative approach, no formal sample size calculation was performed a priori. Statistical analysis were conducted using R programming environment version 3.6.1 (R Core Team, 2017).

4.3 Results

4.3.1 Study population

Between July 2017 and August 2019, 400 breastmilk samples were collected from 170 mothers who gave birth to 195 preterm infants recruited into the DIAMOND trial (Figure 4.1). Infants were born at a median gestational age of 33 (range 32-35) weeks and most were male (57%), singletons (74%), and recruited at the two Auckland NCUs (76%) (Table 4.1). Almost 80% of infants were exclusively breastfed at hospital discharge, but only 20% of infants were still being exclusively breastfed at 4 months CA. Most mothers were European or Asian, delivered by caesarean section, received antenatal steroids (57%), and almost 50% were from the two most deprived socioeconomic quintiles (Table 4.1).

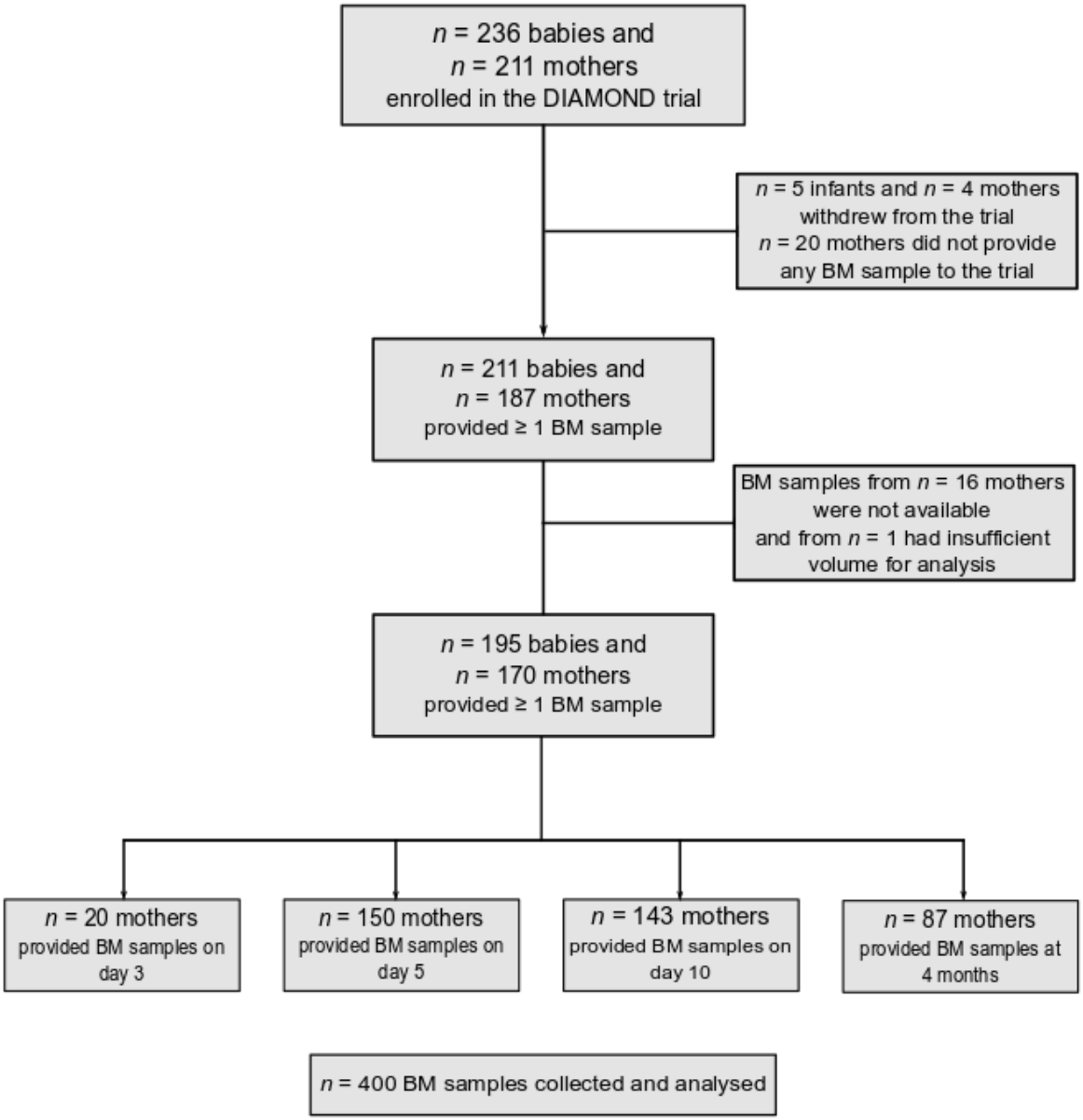

Figure 4.1: Study flowchart

BM= breastmilk. Numbers do not add up as some mothers provided more than 1 sample and some mothers gave birth to multiples (twins and triplets).

Table 4.1: Participant characteristics

Recruitment site	
Auckland	80 (41)
Middlemore	68 (35)
North Shore	39 (20)
Waitakere	8 (4)
Maternal characteristics (*n*= 170)	
Maternal age, years	31 (6)
Ethnicity	
European	60 (35)
Māori	23 (14)
Asian	58 (34)
Pasifika	26 (15)
Other	3 (2)
New Zealand Deprivation Index	
Q1 (1,2)	26 (15)
Q2 (3,4)	41 (24)
Q3 (5,6)	22 (13)
Q4 (7,8)	31 (18)
Q5 (9,10)	48 (29)
Education level	
No tertiary education	80 (47)
Complete tertiary education (bachelor, masters, doctoral degree)	90 (53)
Maternal diabetes	34 (20)
Caesarean section	100 (59)
Antenatal steroids course	
None	32 (19)
Incomplete (first dose <24h from birth)	41 (24)
Complete	97 (57)
Number of samples provided	
1	17 (10)
2	80 (47)
3	69 (40.6)
4	4 (2.4)

(Cont. Table 4.1)

Infant characteristics (*n*= 195)	
Boys	112 (57.4)
Gestational age, weeks	33 (32-35)
Birth order	
Singleton	145 (74)
Twins	47 (24)
Triplets	3 (2)
Duration of hospital stay, days	22 (11)
Anthropometric measures	
At birth:	
Weight, g	2091 (402)
Length, cm	44.5 (3.0)
Head circumference, cm	31.2 (1.5)
At hospital discharge:	
Weight, g	2488 (322)
Length, cm	47.4 (2)
Head circumference, cm	33.0 (1.2)
At follow up:	
Weight, g	6505 (846)
Length, cm	63.6 (2.6)
Head circumference, cm	41.6 (1.3)
Feeding practice	
At hospital discharge (n=194):	
Exclusively breastfed	151 (77.8)
Partially breastfed	37 (19.1)
Exclusively formula fed	6 (3.1)
At follow up (n=168):	
Exclusively breastfed	36 (21.4)
Partially breastfed	67 (39.9)
Exclusively formula fed	65 (38.7)

Data are presented as n (%), mean (standard deviation) or median (range).

4.3.2 ***Volatile compounds***

We identified forty volatile compounds (Table 4.2). Most compounds were volatile organic compounds (VOCs, *n*= 9), fatty acids and esters (FA and FAe, *n*= 9), followed by aldehydes (*n*= 7), terpenoids (*n*= 5), alcohols (*n*= 4), ketones (*n*= 3), alkyl, furan and sulphone (*n*= 1 each). Acetone was the most abundant compound, observed in almost 90% of the samples analysed, followed by octanoic acid methyl ester (56%), o-cymene (56%) and decanoic acid methyl ester (52%). The median number of compounds per sample was 4 (range 0-12). Seventeen compounds (VOCs (*n*= 4), terpenoids (*n*= 4), aldehydes (*n*= 4), alcohol (*n*= 2), sulphone (*n*= 1) and fatty acid (*n*= 1)) were detectable in fewer than 5 samples (<1%) and were not included in regression models.

Table 4.2: Summary of volatile compounds detected in preterm breastmilk

Class	Compound	RT	m/z	*n*	ND	Relative peak area mean (SD)
Alcohol	amylene hydrate	3.18	59	62	338	673 (345)
	2-ethyl-1-butanol	6.00	43	15	385	312 (198)
	ethanol*	1.95	31	4	396	44503 (68482)
	ethyl acetate*	2.55	43	3	397	215 (159)
Aldehyde	hexanal §	6.60	56	126	274	15617 (20758)
	butanal, 3-methyl- §	3.89	57	25	375	3044 (1880)
	octanal	13.09	56	15	385	599 (377)
	pentanal, 3-methyl-*	1.81	29	3	397	610 (189)
	formaldehyde*	6.60	56	3	397	304 (82)
	pentanal*§	3.89	41	3	397	898 (786)
	cyclohexane carboxaldehyde*	12.42	41	2	398	243 (37)
Fatty acid	octanoic acid §	20.74	73	67	333	1766 (2030)
	pentanoic acid 3-methyl	15.61	60	33	367	1299 (1363)
	hexanoic acid §	15.59	60	23	377	2821 (2244)
	butanoic acid* §	9.86	60	3	397	2160 (338)
Fatty acid ester	octanoic acid, methyl ester §	16.11	74	224	176	4347 (4290)
	decanoic acid, methyl ester §	21.50	74	209	191	2177 (1847)
	hexanoic acid, methyl ester §	10.03	74	118	282	723 (611)
	dodecanoic acid, methyl ester §	26.34	74	55	345	513 (367)
	butanoic acid, methyl ester §	4.06	74	36	364	555 (269)
Ketone	acetone §	2.02	58	351	49	5189 (7357)
	1-hepten-3-one	12.48	70	29	371	755 (611)
	cyclobutanone, 2,2,3-trimethyl-*	4.31	41	2	398	127 (102)
Terpenoid	eucalyptol	12.50	81	38	362	1158 (1941)
	d-limonene*	11.90	67	4	396	67 (57)
	camphor*	17.79	95	3	397	74 (34)
	fenchone*	15.71	81	3	397	304 (82)
	p-cymene*	12.50	119	3	397	898 (786)
VOC	o-cymene	12.50	119	224	176	4347 (4290)
	thrichloromethane	2.67	40	123	277	4577 (13508)
	toluene	4.68	91	36	364	2407 (57412)
	chloromethane	1.69	50	10	390	88 (56)
	methane, bromodichloro-	4.11	83	5	395	249 (113)
	methane, dichloronitro-*	2.67	40	4	396	302 (442)

Class	Compound	RT	m/z	*n*	ND	Relative peak area mean (SD)
	ethyl benzene*	7.62	91	4	396	406 (112)
	styrene*	8.80	104	3	397	213 (86)
	benzene*	2.84	78	3	397	147 (77)
Alkyl	pentane	1.80	41	26	374	1590 (1806)
Furan	furan, 2-methoxy-	3.04	83	37	363	59 (62)
Sulphone	dimethyl sulphone*	16.16	94	3	397	27 (9)

RT: Retention time (minutes). m/z: mass to charge ratio of detected ion. *n*= number of samples in which a compound is present. ND: number of samples in which a compound was not detected. VOC: volatile organic compounds. *compounds present in fewer than 5 samples and not included in regression models. § Identification confirmed with authentic standards.

4.3.3 ***Factors associated with distribution of volatile compounds in preterm breastmilk***

We explored the association between the distribution of volatile compounds in preterm breastmilk and infant age at sample collection. Twelve compounds differed significantly with infant age (p<0.05) (Supplementary Table 4.1): seven FA and FAe (Table 4.3); two aldehydes; one terpenoid; one ketone, and one VOC. Within the colostrum lactation stage, there was a significant increase in relative concentration of octanoic methyl ester (p<0.001), decanoic methyl ester (p<0.001) and o-cymene (p<0.001) from postnatal day 3 to 5. Butanal 3-methyl (p<0.01), dodecanoic acid methyl ester (p<0.05) and 1-hepten-3-one (p<0.05) were significantly higher in samples collected on postnatal day 10 compared to mature BM (4 month samples). In contrast, mature BM contained significantly higher relative concentration of butanoic acid methyl ester (p<0.001), hexanoic acid methyl ester (p<0.001), pentanoic acid 3 methyl (p<0.01), hexanoic acid (p<0.001), hexanal (p<0.01) and eucalyptol (p<0.05) compared to samples from colostrum and transitional BM (Figure 4.2).

BM samples from European mothers had higher relative concentration of o-cymene, octanoic, and decanoic acid methyl esters compared to BM samples from Pasifika (p<0.01) (Table 4.3 and Supplementary Table 4.2). Pentane was significantly higher in BM from Pasifika mothers compared to Māori and Asian mothers (p<0.01) (Figure 4.3).

The relative concentrations of three FA esters (octanoic, decanoic and dodecanoic acid methyl esters) (Table 3) and one VOC (o-cymene) were significantly lower in BM from mothers with diabetes compared to mothers without diabetes (all p<0.05, Figure 4.3 and Supplementary Table 4.3).

Eight compounds differed across NZdep quintiles (Supplementary Table 4.4). These were: hexanal (p<0.001); the methyl esters of hexanoic acid (p<0.05), octanoic acid (p<0.01), decanoic acid (p<0.01) and dodecanoic acid (p<0.05); hexanoic acid (p<0.01), octanoic acid (p<0.01), and o-cymene (p<0.01) (Table 4.3). BM samples from mothers of the most deprived quintile presented significantly lower relative concentration of o-cymene, octanoic and decanoic methyl esters compared to BM samples from mothers belonging to the 3rd quintile of deprivation (p<0.05). In contrast, hexanal was significantly higher in breastmilk of mothers from the most deprived quintile (Q5) in relation to mothers from least deprived areas (Figure 4.4).

There were no associations between the distribution of volatile compounds in preterm BM and other maternal characteristics such as delivery mode, antenatal steroids, education level and maternal age. There were no associations between the distribution of volatile compounds in preterm BM and infant sex, birth weight nor length of gestation.

Table 4.3: Association between maternal and infant characteristics and distribution of volatile fatty acids and fatty acid esters in preterm breastmilk

Characteristics	pentanoic acid 3-methyl	hexanoic acid	octanoic acid	butanoic acid ME	hexanoic acid ME	octanoic acid ME	decanoic acid ME	dodecanoic acid ME
Lactation stage	$F_{(3, 329.3)}$= 4.7 **	$F_{(3, 329.3)}$= 8.6 ***	$F_{(3, 303.1)}$= 2.0 p=0.12	$F_{(3, 329.3)}$= 29.6 ***	$F_{(3, 306.7)}$= 20.5 ***	$F_{(3, 297.9)}$= 11.8 ***	$F_{(3, 275.5)}$= 10.2 ***	$F_{(3, 267.2)}$= 3.6 *
Day 3	0 (0)^{AB}	0 (0)^{A}	0 (0)	0 (0)^{A}	0 (0)^{A}	592 (1007)^{AC}	365 (714)^{A}	20 (91)^{AB}
Day 5	12 (91)^{AB}	16 (912)^{A}	423 (1213)	8 (49)^{A}	64 (182)^{A}	3490 (4768)^{B}	1563 (2058)^{B}	106 (283)^{A}
Day 10	122 (465)^{A}	46 (202)^{A}	193 (1132)	13 (92)^{A}	217 (402)^{A}	1395 (2882)^{A}	791 (1525)^{A}	68 (213)^{AB}
4 months CA	270 (932)^{B}	555 (1558)^{B}	314 (672)	194 (319)^{B}	514 (740)^{B}	2746 (3382)^{BC}	1153 (1345)^{AB}	24 (92)^{B}
Ethnicity	$F_{(4, 157.6)}$= 0.42 p=0.8	$F_{(4, 157.7)}$= 1.14 p=0.34	$F_{(4, 160.8)}$= 1.4 p=0.23	$F_{(4, 157.6)}$= 0.54 p=0.71	$F_{(4, 160.4)}$= 1.3 p=0.26	$F_{(4, 160.9)}$= 3.5 **	$F_{(4 ,162.9)}$= 4.04 **	$F_{(4, 163.6)}$= 1.4 p=0.24
European	124 (594)	262 (1142)	405 (1363)	53 (176)	251 (477)	3094 (4300) ^{A}	1486 (1907)^{A}	93 (254)
Māori	95 (479)	88 (587)	87 (279)	39 (174)	183 (513)	1475 (2580)^{AB}	678 (1115)^{AB}	32 (103)
Asian	127 (564)	139 (686)	344 (1018)	61 (207)	238 (510)	2619 (3949)^{AB}	1237 (1844)^{AB}	85 (254)
Pasifika	30 (230)	32 (245)	94 (459)	30 (115)	102 (280)	1281 (3021)^{B}	469 (935)^{B}	15 (72)
Other	79 (224)	0 (0)	0 (0)	0 (0)	47 (133)	291 (723)^{AB}	144 (407)^{AB}	0 (0)
Maternal Diabetes	$F_{(1,164)}$= 2.52 p=0.11	$F_{(1,163.9)}$= 0.36 p=0.55	$F_{(1,167.8)}$= 2.54 p=0.11	$F_{(1,163.7)}$= 2.34 p=0.13	$F_{(1,166.8)}$= 3.14 p=0.08	$F_{(1,167.9)}$= 4.78 *	$F_{(1,169.5)}$= 5.9 *	$F_{(1,169.8)}$= 4.45 *
No	127 (580)	173 (898)	341 (1158)	56 (191)	235 (487)	2662 (4077)	1263 (1820)	84 (243)
Yes	23 (124)	114 (549)	104 (357)	26 (99)	119 (359)	1463 (2606)	602 (1069)	13 (66)
NZdep	$F_{(4, 153.1)}$= 1.03 p=0.4	$F_{(4,153.1)}$= 4.67 **	$F_{(4,157.4)}$= 4.57 **	$F_{(4,153.1)}$= 0.74 p=0.57	$F_{(4,156.9)}$= 2.75 *	$F_{(4,158.1)}$= 4.81 **	$F_{(4 ,161.1)}$= 4.33 **	$F_{(4 ,161.9)}$= 2.61 *
Q1	199 (782)	127 (589)^{A}	228 (519)^{A}	76 (214)	294 (592)^{A}	3076 (3672)^{AB}	1281 (1552)^{AB}	42 (130) ^{AB}
Q2	121 (611)	194 (838)^{A}	409 (1220)^{AB}	59 (206)	270 (527)^{A}	2921 (4437)^{AB}	1471 (1979)^{B}	94 (261) ^{AB}
Q3	104 (354)	604 (1880)^{B}	871 (2254)^{B}	40 (141)	300 (574)^{A}	4062 (5853)^{A}	1840 (2594)^{B}	172 (397) $^{\dagger A}$
Q4	36 (166)	63 (330)^{A}	88 (273)^{A}	45 (154)	141 (295) $^{\dagger B}$	1623 (2326)^{B}	816 (1137)^{AB}	39 (134) ^{AB}
Q5	77 (449.6)	43.7 (387.7)^{A}	130.2 (474.7)^{A}	34.3 (156)	123.1 (341) $^{\dagger B}$	1488 (2808)^{B}	678 (1243)^{A}	47 (158) $^{\dagger B}$

Characteristics	pentanoic acid 3-methyl	hexanoic acid	octanoic acid	butanoic acid ME	hexanoic acid ME	octanoic acid ME	decanoic acid ME	dodecanoic acid ME
Antenatal Steroids	$F_{(2,153.9)}$= 0.21 *p*=0.81	$F_{(2,154.5)}$= 0.1 *p*=0.9	$F_{(2,161.4)}$= 0.65 *p*=0.52	$F_{(2,153.6)}$= 2.01 *p*=0.14	$F_{(2,160.1)}$= 2.53 *p*=0.08	$F_{(2,162.1)}$= 1.71 *p*=0.18	$F_{(2,165.2)}$= 2.92 *p*=0.06	$F_{(2,166.1)}$= 0.96 *p*=0.38
None	100 (443)	148 (990)	149 (579)	50 (197)	128 (378)	1571 (3379)	582 (1025)	25 (95)
Incomplete	142 (624)	208 (835)	360 (1027)	83 (222)	305 (608)	2638 (3972)	1295 (1844)	77 (251)
Complete	94 (505)	147 (798)	314 (1177)	35 (146)	200 (414)	2613 (3941)	1241 (1808)	82 (236)
Tertiary education	$F_{(2,130.4)}$= 0.03 *p*=0.97	$F_{(2,131.4)}$= 0 *p*=0.99	$F_{(2,140.2)}$= 0.08 *p*=0.92	$F_{(2,130.7)}$= 0.02 *p*=0.98	$F_{(2,139.5)}$= 0 *p*=0.99	$F_{(2,142.5)}$= 0.36 *p*=0.7	$F_{(2,150.3)}$= 1.43 *p*=0.24	$F_{(2,153.2)}$= 0.99 *p*=0.37
Incomplete	100 (472)	146 (836)	298 (1239)	44 (164)	207 (489)	2263 (4258)	1015 (1761)	59 (216)
Complete	114 (571)	176 (855)	298 (895)	55 (190)	220 (453)	2563 (3543)	1214 (1675)	76 (226)
Delivery mode	$F_{(1,154.7)}$= 1.53 *p*=0.22	$F_{(1,154.6)}$= 2.09 *p*=0.15	$F_{(1,161.6)}$= 0.03 *p*=0.87	$F_{(1,154.5)}$= 2.8 *p*=0.09	$F_{(1,160.7)}$= 0 *p*=0.98	$F_{(1,162.3}$)= 0.13 *p*=0.72	$F_{(1,165.4)}$= 0.59 *p*=0.44	$F_{(1,166.3)}$= 0.25 *p*=0.62
Vaginal	70 (291)	234 (999)	310 (1121)	67 (217)	219 (438)	2410 (3786)	1077 (1667)	65 (216)
C-section	132 (639)	113 (713)	286 (1013)	38 (144)	209 (487)	2451 (3930)	1179 (1760)	74 (227)
Gestational age	$F_{(3,152.3)}$= 0.76 *p*=0.52	$F_{(3,152.4)}$= 1.27 *p*=0.29	$F_{(3,159.2)}$= 0.54 *p*=0.65	$F_{(3,152.3)}$= 0.11 *p*=0.96	$F_{(3,158.5)}$= 0.1 *p*=0.96	$F_{(3,160.1)}$= 0.52 *p*=0.67	$F_{(3,163.3)}$= 0.79 *p*=0.5	$F_{(3,164.1)}$= 1.26 *p*=0.29
32	104 (563)	179 (787)	233 (583)	49 (161)	224 (526)	2293 (3576)	1013 (1527)	37 (129)
33	51 (238)	170 (802)	387 (1469)	45 (160)	223 (456)	2749 (4197)	1367 (1911)	101 (258)
34	136 (681)	58 (447)	240 (945)	49 (177)	189 (445)	2125 (3755)	1022 (1662)	64 (209)
35	160 (506)	322 (1399)	338 (954)	63 (232)	225 (441)	2685 (3929)	1141 (1761)	82 (284)
Sex	$F_{(1,151.7)}$= 0.87 *p*=0.35	$F_{(1,152.1)}$= 0.42 *p*=0.52	$F_{(1,158.8)}$= 0.03 *p*=0.85	$F_{(1,151.8)}$= 0.77 *p*=0.38	$F_{(1,157.8)}$= 0.54 *p*=0.46	$F_{(1,159.3)}$= 0.78 *p*=0.38	$F_{(1,162.5)}$= 2.08 *p*=0.15	$F_{(1,163.4)}$= 1.03 *p*=0.31
Male	179 (1012)	15 (120)	303 (1233)	37 (155)	29 (261)	2124 (3850)	926 (1603)	54 (198)
Female	135 (657)	28 (144)	288 (926)	57 (190)	8 (118)	2589 (3843)	1250 (1769)	80 (237)

Relative peak area presented as mean (SD). ME= methyl ester. NZDep = New Zealand Deprivation index. Significance level taken as *p*-value <0.05, adjusted for comparison (Bonferroni).* *p<0.05*. ** *p<0.01*. ****p<0.001*. [†]adjusted *p*>0.05 (Bonferroni).

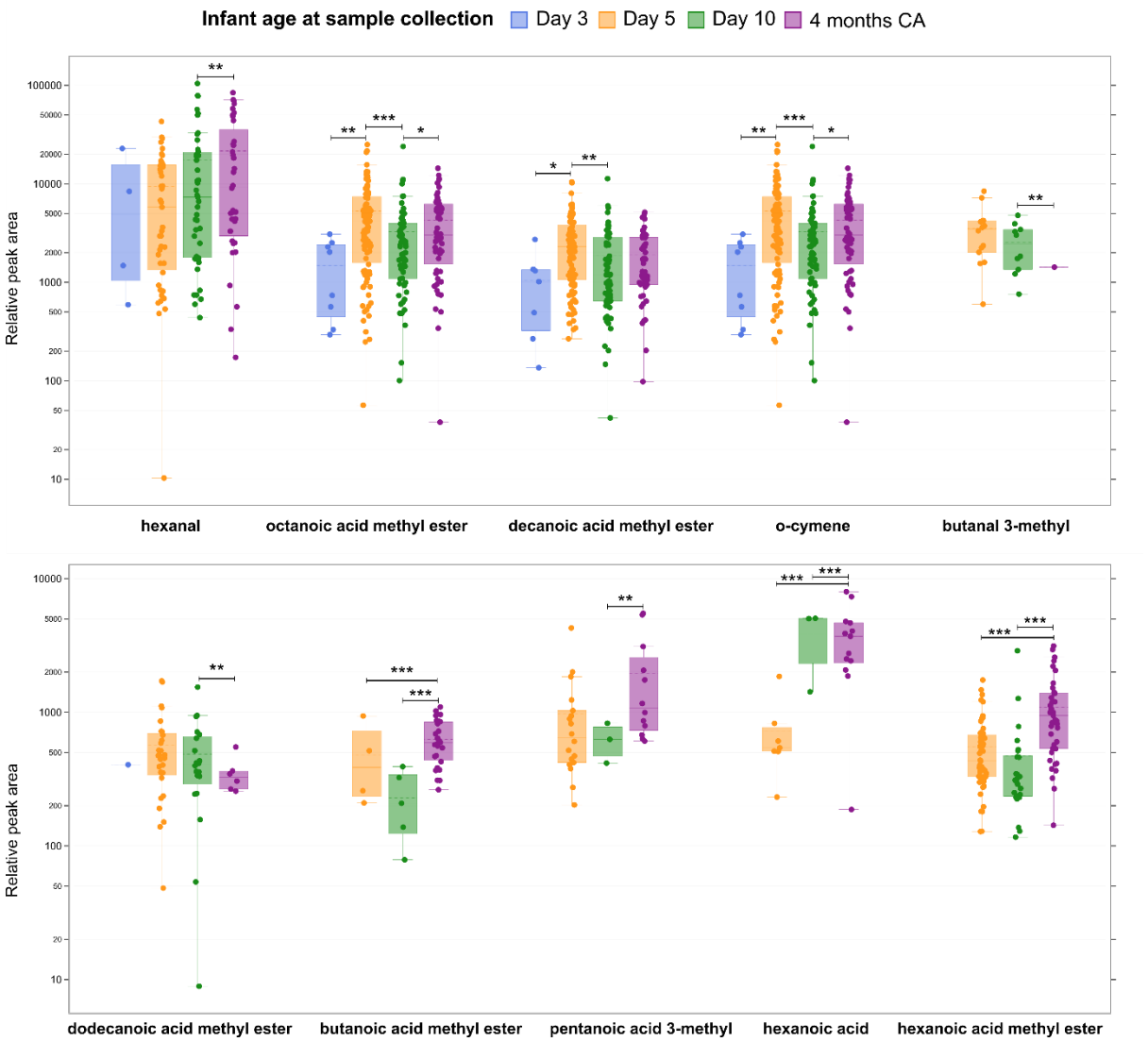

Figure 4.2: Relative peak area of volatile compounds in preterm breastmilk by infant age at time of collection

Boxes represent median and interquartile range, whiskers represent highest and lowest peak area detected and dots represent samples. Note logarithmic scale of the y axes. Groups not shown when compound was not detected. Significance level taken as p-value <0.05, adjusted for comparison (Bonferroni). p<0.05; ** p<0.01; *** p<0.001. CA, corrected age.*

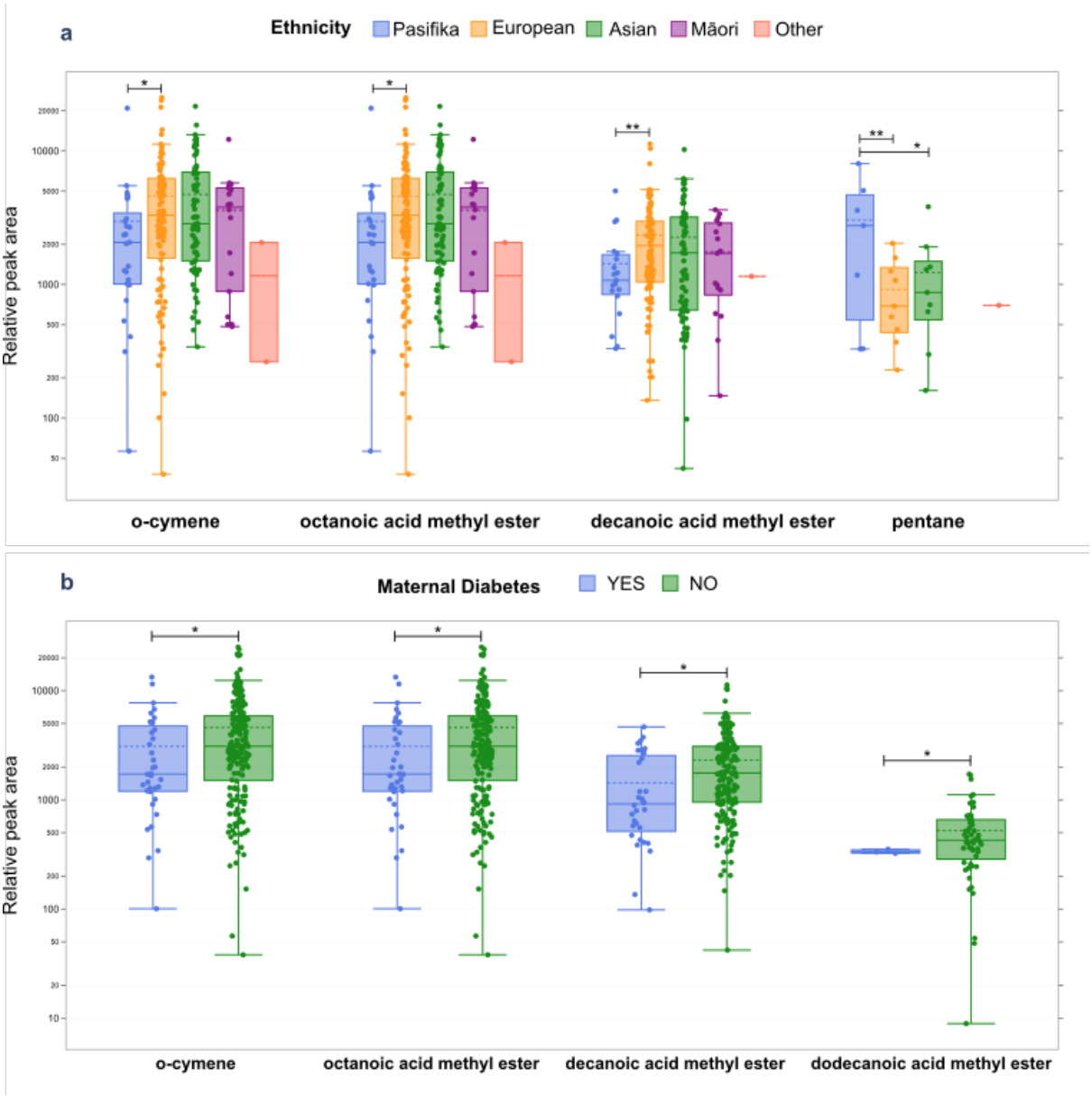

Figure 4.3: Relative concentration of volatile compounds in preterm breastmilk by (A) maternal ethnicity and (B) Maternal Diabetes

*Boxes represent median and interquartile range, whiskers represent highest and lowest peak area detected and dots represent samples. Note logarithmic scale of the y axes. Groups not shown when compound was not detected. Significance level taken as p-value <0.05, adjusted for comparison (Bonferroni). * p<0.05; ** p<0.01; *** p<0.001.*

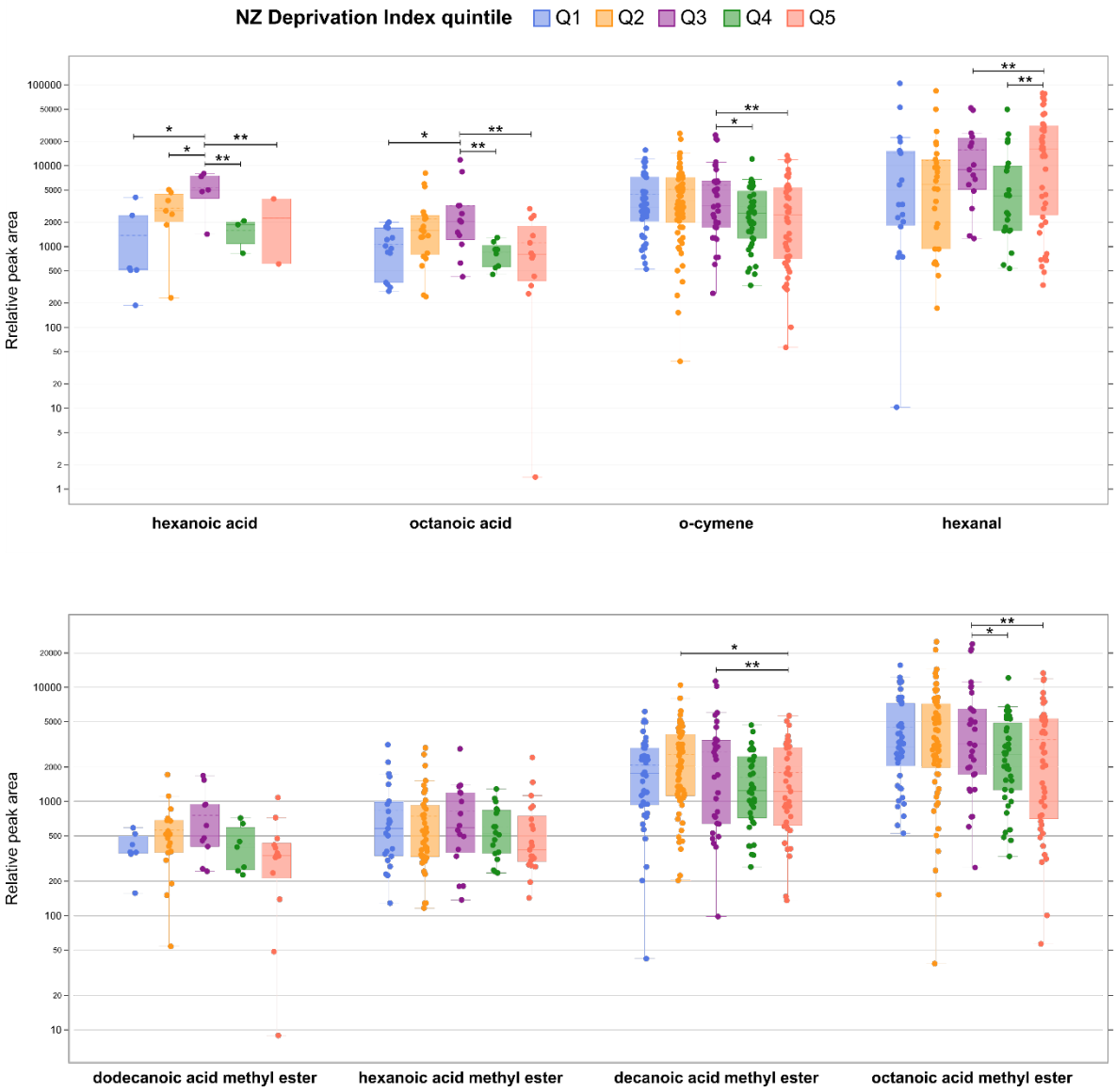

Figure 4.4: Relative concentration of volatile compounds in preterm breastmilk by quintiles of New Zealand Deprivation Index

*Boxes represent median and interquartile range, whiskers represent highest and lowest peak area detected and dots represent samples. Note logarithmic scale of the y axes. Groups not shown when compound was not detected. Significance level taken as p-value <0.05, adjusted for comparison (Bonferroni). Quintiles of deprivation index (Q1, low; Q5, high). * p<0.05; ** p<0.01; *** p<0.001.*

4.4 Discussion

Using an untargeted approach, we detected forty volatile compounds in preterm BM, the majority of which were fatty acids and their respective esters (FA and FAe) and VOCs, but aldehydes, ketones and terpenoids were also present. Semi-quantitative analysis demonstrated that the profile of volatile compounds in preterm BM is influenced by maternal, but not infant, characteristics. Stage of lactation, and even day of collection within the colostrum stage, maternal ethnicity, maternal diabetes and social deprivation index were all significantly associated with the profile of volatile FA and FAe.

Previous studies suggested that the profile of volatile compounds in BM results from degradation of milk lipids (yielding ketones, aldehydes, alcohols, free fatty acids, and fatty acid esters) and from maternal diet (mostly terpenoids) (Bingham et al., 2003b; Buettner, 2007; Hausner et al., 2009; Muelbert et al., 2021; Shimoda et al., 2000). BM from 10 mothers of full-term (FT) infants was found to contain higher amounts of terpenes, aldehydes and alcohols than infant formulas (Hausner et al., 2009) although, in contrast to our findings, that study only detected SCFA in the BM. However, samples analysed in this study were all from mature BM (>5 weeks postnatal) provided by mothers of FT infants, which may explain the different results (Hausner et al., 2009).

To the best of our knowledge, volatile compounds in preterm BM have been reported only twice previously and in small numbers. A study of four BM samples analysed by gas chromatography/olfactometry reported five volatile compounds (two ketones, two alcohols and one aldehyde) (Bingham et al., 2003b). We previously reported that volatile compounds in 15 samples of preterm BM differed from those in infant formulas largely due to the

presence of specific FA in BM that were absent from formulas (Muelbert et al., 2021). Therefore, our findings are largely in agreement with previous reports and expand on the current knowledge of volatile compounds in BM by demonstrating that a variety of compounds of different chemical classes can be found in preterm BM, including MCFA and their esters, terpenoids and VOCs.

The lipid fraction of human milk, in the form of milk fat globules (MFG) (Lee et al., 2018), is the most variable macronutrient in BM composition, responsible for provision of about 50% of total energy for the growing infant (Bobiński and Bobińska, 2020; Demmelmair and Koletzko, 2018). The MFG is a complex structure formed by a triacylglycerol (TAG) core, protected by a highly specialised membrane composed of phospholipids, sphingolipids, cholesterol, bioactive proteins and long-chain polyunsaturated fatty acids (LCPUFAs) that is unique to the human species (Bobiński and Bobińska, 2020; Lee et al., 2018; Yuan et al., 2019).

The FA composition of the TAG core is approximately 42% saturated FAs (mostly MCFAs), 42% monounsaturated FAs (MUFAs) and 16% polyunsaturated FAs (PUFAs) (Bobiński and Bobińska, 2020). Long-chain saturated FAs and PUFAs are mainly sourced from the maternal circulation and body stores; saturated MCFA are synthesized in the mammary epithelial cells and incorporated into TAGs for secretion into MFG (Bobiński and Bobińska, 2020; Demmelmair and Koletzko, 2018). The *de novo* synthesis of MCFA from glucose in the mammary epithelial cells is influenced by substrate availability (from circulation or body stores) and the action of hormones such as prolactin, growth hormone and insulin (Bobiński and Bobińska, 2020; Demmelmair and Koletzko, 2018). Thus, the composition of TAG can be modified by maternal factors which influence those hormones or the supply of FA and glucose precursors, such as diet and supplementation (Nasser et al., 2010; Sosa-Castillo et al., 2017;

Yahvah et al., 2015), length of gestation (Thakkar et al., 2019), lactation stage (Bobiński and Bobińska, 2020; Thakkar et al., 2019) and socioeconomic status (Al-Tamer and Mahmood, 2006).

Previous studies report an increase in the content of FA in BM as lactation stage progresses (Bobiński and Bobińska, 2020; Thakkar et al., 2019), consistent with our findings. Mothers of preterm infants have been reported to secrete BM with higher amounts of MCFA throughout lactation compared to BM from mothers of full-term infants (Thakkar et al., 2019). We observed greater relative concentrations of octanoic and decanoic methyl esters in colostrum, with a significant increase from postnatal day 3 to day 5. This may be advantageous to preterm infants as MCFA can be more easily converted into energy (Bobiński and Bobińska, 2020) and may exhibit antimicrobial activity (Huang et al., 2011). Unlike long-chain saturated FAs that are packed into chylomicrons and transferred to the liver via the lymphatic system for later utilisation, MCFAs are readily solubilised in the enterocyte, absorbed bound to albumin, and released into the portal system. Upon reaching the liver, they are either immediately oxidised (generating energy) or transferred to fat stores (Ramírez et al., 2001). This improved energy bioavailability in colostrum and possible protection against pathogens may be advantageous for transition to the postnatal environment in immature infants with high metabolic demand and limited endogenous lipase activity (Lindquist and Hernell, 2010).

Alternatively, the presence of volatile MCFA in preterm BM may relate to immaturity of the mammary gland and inefficient milk secretion following a shorter gestation period, resulting in temporary increased secretion of TAGs with higher content of MCFA, which may resolve as lactation progresses (Bobiński and Bobińska, 2020). It is notable that free FA can

bind to G-protein-coupled taste and smell receptors located throughout the gastrointestinal tract, influencing secretion of hormones associated with digestion and metabolism (Calvo and Egan, 2015; Maßberg and Hatt, 2018). Nevertheless, the reason for higher MCFA in colostrum compared to transitional BM is not yet clear.

We found that maternal factors were significantly associated with the distribution of MCFA volatile compounds in preterm BM. Recent meta-analysis of FA composition in BM from various countries found that saturated FA in BM, including MCFA, can vary between 39% and 50% of total milk fats (Bahreynian et al., 2020). One small study of 78 multi-ethnic lactating mothers conducted in New Zealand found that, although the composition of BM did not differ between mothers of different ethnicities, PUFA content in BM from Asian mothers was significantly higher than in BM from European and Māori / Pasifika mothers (Butts et al., 2018) even though maternal diets were similar in terms of energy and macronutrients. However, consumption of specific foods differed, with intake of chicken and legumes significantly higher in Asian mothers and consumption of dairy product significantly higher in European mothers (Butts et al., 2018).

It is known that the carbohydrate-to-fat ratio of the maternal diet modulates the profile of FA in BM (Demmelmair and Koletzko, 2018; Nasser et al., 2010). Therefore, the ethnic differences in profile of MCFA observed in our study may relate to maternal diet and habits. However, the lack of maternal dietary information to further assess this is a limitation of our study.

Maternal socioeconomic deprivation also was associated with the distribution of FA and FAe volatile compounds of preterm BM. An Iraqi study reported that mothers of low socioeconomic status produced mature BM with a significantly lower content of TAGs, total

cholesterol, phospholipids, and the saturated FAs decanoic and dodecanoic acid (smaller FA were not analysed) compared to mothers with a higher socioeconomic status (Al-Tamer and Mahmood, 2006). Our results are consistent with these findings, as we also found significantly lower relative concentrations of FA and FAe in BM of the most socially deprived mothers.

Interestingly, the relative concentration of hexanal was significantly higher in mature BM and in samples collected from most socially deprived mothers (Q5). Hexanal is a secondary product originating from oxidation of omega-6 FAs and its presence in BM and infant formula can indicate lipid degradation (Elisia and Kitts, 2011). It has been demonstrated that the formation of hexanal from lipid peroxidation in BM is inversely correlated with the concentration of vitamins E and C (Elisia and Kitts, 2011). Vitamins are powerful antioxidants and, therefore, their anti-antioxidant capacity may prevent lipid oxidation of BM (Elisia and Kitts, 2011). Thus, considering the influence of maternal diet on the distribution of volatile compounds in BM (Bingham et al., 2003b; Hausner et al., 2009; Muelbert et al., 2021), it seems possible that the elevated concentration of hexanal in BM of mother from most deprived socioeconomic quintile could be related to reduced anti-oxidant components in their BM. Further research is required to confirm this association.

We found that BM from mothers with diabetes in pregnancy had lower relative concentrations of methyl esters compared to mothers without diabetes. Several studies have suggested that gestational diabetes mellitus (GDM) influences the composition of BM, but evidence is conflicting (Peila et al., 2020). While higher concentrations of omega-6 LCPUFAs in colostrum of mothers with GDM (Chertok et al., 2017) has been reported, others found lower concentrations of organic acids and unsaturated FA in BM of mothers with GDM compared to mothers without GDM (Wen et al., 2019); others reported no differences in

colostrum and transitional BM macronutrient composition between mothers with and without GDM, except for a slightly higher fat and energy content in BM of mothers without GDM (Shapira et al., 2019). These studies raise the possibility that inappropriate glucose homeostasis may influence FA metabolism leading to altered profile of FA in BM (Chertok et al., 2017; Peila et al., 2020; Shapira et al., 2019). Obesity and insulin-dependency are known risk factors for delayed onset of Lactogenesis II (Jackson et al., 1994; Neubauer et al., 1993; Preusting et al., 2017). Hence, it is possible that varying levels of circulating glucose and insulin in mothers with diabetes in pregnancy disrupt *de novo* synthesis of MCFA in the mammary epithelial cells or delayed onset of Lactogenesis II, which in turn would lead to a TAG deficient in MCFA, consistent with our findings.

In addition to FA and FAe, we have also identified several compounds classified as VOCs. Presence of VOCs in BM, such as benzene, toluene, styrene, bromochloronitromethane and others, has been correlated with indoor exposure to pyrogenic and petrogenic air pollutants (Blount et al., 2010; Kim et al., 2007). The main entry routes into the human body are inhalation, ingestion and dermal absorption (Blount et al., 2010; Kim et al., 2007; Wolkoff and Nielsen, 2017). Alternatively, VOCs like o-cymene have been detected in kitchen cleaning agents and air fresheners (Nørgaard et al., 2014). We found that the relative concentration of o-cymene was more abundant in BM from European mothers than from Pasifika mothers, and significantly less abundant in BM from mothers with diabetes, those from the most socially deprived quintiles and in transitional BM compared to colostrum and mature BM samples. Similarly, toluene was also detected in our study and might originate from exposure to air pollutants (Fabietti et al., 2004) but may also originate from degradation of dietary beta-carotene (Garrido et al., 2015). Although previous studies have detected only low levels of

VOCs in BM, the lipophilic nature of these compounds suggests that bioaccumulation in fat tissue deposits may occur (Blount et al., 2010; Kim et al., 2007; Nørgaard et al., 2014; Wolkoff and Nielsen, 2017), and these could in turn be mobilised during BM fat synthesis. Nevertheless, the limited information available from maternal diet and exposure to environmental contaminants prevents us from determining the origin of these compounds in preterm BM.

We have demonstrated that the odour of preterm BM comprises a mixture of FA and their esters and ketones and, to a lesser extent, aldehydes and terpenoids. FAe have low perception threshold (at parts per billion level), meaning that even when present in low concentrations these compounds can contribute to the aroma of a food matrix (Clarke et al., 2020; Liu et al., 2004). Similarly, ketones also have low odour perception thresholds (Spitzer et al., 2013). The odour characteristics of both of these compounds are described as fruity, malty, almond and fatty (Liu et al., 2004; Spitzer et al., 2013). FAs also contribute to overall aroma of food; however, these compounds have higher odour perception thresholds (at parts per million level), compared to esters and ketones (Spitzer et al., 2013), meaning that higher concentrations are required for odour perception.

The concentration of specific volatile compounds and their interaction with other compounds present in the food matrix are important for overall aroma of food (Muelbert et al., 2021). For example, free FAs present in low concentration in the food matrix may contribute positively to the aroma of food; however, when present in high concentrations they may confer off-notes such as fishiness and rancidness (Clarke et al., 2020), influencing overall palatability of food. Similarly, FAe can contribute positively to the aroma of food in low concentrations but their contribution to overall flavour perception can be negatively

affected by interaction with other FAe present in the food matrix, leading to development of unpleasant smells (Liu et al., 2004). Accordingly, our results indicate that the odour of preterm BM is largely influenced by the concentration of FA and FAe, but ketones, aldehydes and terpenoids might also contribute to overall smell of BM.

4.4.1 ***Strengths and limitations***

Some limitations of our study should be acknowledged. First, no information on maternal diet or supplementation, anthropometry, smoking and exposure to environmental toxins was collected, preventing us from drawing further conclusions about the source of some volatile compounds identified in preterm BM. Secondly, as samples were collected during morning, it is possible that this resulted in our measuring lower fat content as BM produced towards the end of the day contains higher fat concentrations (Moran-Lev et al., 2015). The number of day 3 samples is low as collection so soon after preterm birth was challenging. Nevertheless, our findings of significant changes in the volatile profile between days 3 and 5 suggest that further investigation of changes in breastmilk composition during the colostrum stage is warranted. Samples were analysed up to 24 months after collection and frozen storage of BM for long periods may increase volatile products of lipid degradation, such as aldehydes and FA (Spitzer and Buettner, 2013). We did not find any association between the distribution of volatile compounds in preterm BM and time elapsed between sample collection and analysis (data not shown), perhaps because samples were stored at very low temperature (-80^{o}C) (Nessel et al., 2019; Ramírez-Santana et al., 2012). Thus, we are confident that lipid degradation has not biased the final results.

Our study also has several strengths. Sample collection followed a strict protocol to minimize technical variations (Galante et al., 2019). For the first time, volatile compounds have been analysed in a large number of samples from colostrum, transitional and mature BM, enabling assessment of longitudinal changes in the profile of volatile compounds. Further, an untargeted approach is a useful tool for discovery of volatile compounds in preterm BM (Gertsman and Barshop, 2018) and can serve as a screening method for future quantification studies. In addition, by extracting the volatile compounds at a temperature similar to feeding (37^{o}C) we were able to assess which compounds are likely to be perceived by infants during feeding, expanding current knowledge on sensory properties of preterm BM.

4.4.2 ***Future directions***

Considering that early life sensory exposure may impact on later life feeding behaviour and preferences (Lipchock et al., 2011), future studies should aim to determine the contribution of maternal diet and exposure to environmental contaminants to the profile of volatile compounds in BM. Quantification of environmental contaminants and determination of their origin in preterm BM from different populations may be warranted, as exposure to persistent organic pollutants may influence growth of preterm infants (Cano-Sancho et al., 2020). In addition, the association between various maternal characteristics and volatile compounds in BM and whether differences in volatile compounds in BM affect feeding behaviour or metabolism in preterm babies are yet to be determined.

4.5 Conclusion

Sensory active volatile fatty acids and their esters are the major contributors to smell of preterm breastmilk. Our findings demonstrate that the profile of volatile compounds in BM is influenced by maternal but not by infant characteristics. Lactation stage, ethnicity, maternal diabetes and socioeconomic status are associated with distribution of volatile FA and FAe in preterm BM. Future studies are required to investigate the origin of volatile compounds in preterm BM and their implications for nutrition of preterm infants.

4.6 Supplementary tables

Supplementary Table 4.1: Relative concentration of volatile compounds in preterm breastmilk by infant age at sample collection

		Day 3	Day 5	Day 10	4 months	Lactation stage effect			
	n	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Num. df	Denom. df	F	*p*
Chloromethane	10	4.5 (20.3)	0.6 (7.6)	1.8 (15.1)	5.1 (25.1)	3	309.3	1.4	0.24
Pentane	26	45.1 (144.3)	114.2 (581.2)	110 (716.7)	87.2 (473.4)	3	328.5	0.1	0.96
Thrichloromethane	123	1341.4 (5813.2)	840.2 (4235.7)	1983.2 (10980.5)	1453.8 (6391)	3	315.9	0.6	0.63
Furan, 2-methoxy-	37	4.6 (20.5)	5.2 (20.3)	5.5 (32.1)	6.2 (21.5)	3	329.3	0.0	0.99
Amylene hydrate	62	75.4 (248.4)	123.6 (309.5)	97.7 (252.3)	88.6 (272.7)	3	313.8	0.5	0.69
Toluene	36	186.6 (782.2)	94.8 (458.2)	378.2 (2962.3)	168.1 (751)	3	321.8	0.7	0.58
2-Ethyl-1-butanol	15	0 (0)	10.5 (51.7)	9 (61.2)	21.1 (108.7)	3	329.3	0.8	0.50
Acetone	351	3139.8 (3851.6)	4173.4 (7652.7)	4153.4 (6786.3)	6189.3 (7018)	3	317.5	2.1	0.09
1-Hepten-3-one	29	0 (0) ^{A}	62.2 (279.8)^{AB}	87.9 (310.1) ^{B}	0 (0) ^{A}	3	311.4	2.9	**<0.05**
Eucalyptol	38	229.8 (961.8) ^{AB}	74.3 (369.7)^{AB}	31 (160.9)^{A}	274 (1276.6)* ^{B}	3	310.7	2.8	**<0.05**
o-Cymene	224	592.5 (1006.8)^{AC}	3489.9 (4767.8)^{B}	1395.2 (2881.9) ^{A}	2746.4 (3382.2)^{BC}	3	298.0	11.8	**<0.001**
Butanal, 3-methyl-	25	0 (0)^{AB}	135.7 (654.7)^{AB}	379.8 (1264.8)^{B}	16.4 (152.9)^{A}	3	315.7	4.2	**<0.01**
Hexanal	126	1666 (5331.2) ^{AB}	2698.8 (6849.5)^{A}	5268.6 (15431.5)^{AB}	8922.4 (19048.3)^{B}	3	323.6	4.4	**<0.01**
Octanal	15	0 (0)	16.4 (91.8)	45.7 (201.8)	0 (0)	3	326.9	1.5	0.23
Butanoic acid, methyl ester	36	0 (0)^{AB}	7.9 (49.2)^{A}	13.2 (91.6)^{A}	194.4 (318.9)^{B}	3	329.3	29.6	**<0.001**
Pentanoic acid, 3-methyl-	33	0 (0)^{A}	12.5 (90.9)^{A}	122.2 (465.1)^{A}	270.5 (932.1)^{B}	3	329.3	4.7	**<0.01**
Hexanoic acid	23	0 (0)^{A}	43.2 (427.9)^{A}	70.7 (459.1)^{A}	555.1 (1558.2)^{B}	3	329.3	8.6	**<0.001**
Hexanoic acid, methyl ester	118	0 (0)^{A}	64.4 (182.4)^{A}	216.7 (402.4)^{A}	513.7 (740.3)^{B}	3	306.7	20.5	**<0.001**
Octanoic acid	67	0 (0)	422.6 (1213.4)	192.9 (1131.8)	314.2 (672)	3	303.1	2.0	0.12
Octanoic acid, methyl ester	224	592.5 (1006.9)^{AC}	3489.9 (4767.8) ^{B}	1395.2 (2881.9)^{A}	2746.4 (3382.2)^{BC}	3	298.0	11.8	**<0.001**

		Day 3	Day 5	Day 10	4 months	Lactation stage effect			
	n	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Num. df	Denom. df	F	*p*
Decanoic acid, methyl ester	209	365.16 (713.7)^{A}	1562.6 (2057.9)^{B}	790.6 (1525)^{A}	1152.9 (1345.1) ^{AB}	3	275.5	10.3	**<0.001**
Dodecanoic acid, methyl ester	55	20.3 (90.7)^{AB}	106.4 (283.2)^{A}	68.1 (213.3) ^{AB}	24 (92.5) ^{B}	3	267.2	3.6	**<0.05**

Compounds significantly different represented by different letters. Significance level taken as $p<0.05$. *Multiple comparison adjusted *p*-value >0.05 (Bonferroni). *n*= Valid observations.

Supplementary Table 4.2: Relative concentration of volatile compounds in preterm breastmilk by maternal ethnicity

		European	Māori	Asian	Pasifika	Other	Maternal ethnicity			
	n	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Num. df**	**Denom. df**	**F**	***p***
Chloromethane	10	3.7 (20.1)	4.5 (25.1)	0.9 (10.4)	0 (0)	0 (0)	4	160.3	0.97	0.43
Pentane	26	54.1 (258.5)^{A}	0 (0)^{A}	80.7 (408.7)^{A}	367.4 (1355.9)^{B}	87.3 (246.9)^{AB}	4	157.55	3.53	***<0.01***
Thrichloromethane	123	639.3 (3111.2)	1716.6 (6614.1)	2604.4 (12073.2)	517.5 (3027)	347.2 (409.4)	4	159.27	1.22	0.30
Furan, 2-methoxy-	37	6 (30.3)	6.4 (18.7)	5.7 (25.1)	3.8 (16.1)	0 (0)	4	157.55	0.19	0.94
Amylene hydrate	62	106.6 (272.1)	136.2 (344.6)	81.7 (238.2)	133.6 (338.5)	58.1 (164.3)	4	159.62	0.43	0.79
Toluene	36	144.4 (604.8)	91.6 (432.3)	423.7 (3051.8)	43.2 (262.9)	0 (0)	4	158.58	0.63	0.64
2-Ethyl-1-butanol	15	9.8 (59.9)	33.1 (139.7)	7.9 (52.5)	11.4 (55.4)	0 (0)	4	157.55	1.21	0.31
Acetone	351	4456.2 (6752.7)	5017 (6724.8)	4861.8 (8043.5)	3155.6 (3396.9)	8695.5 (14607.9)	4	159.26	1.3	0.27
1-Hepten-3-one	29	54 (262.7)	41.2 (173)	78.3 (311.3)	19 (108.3)	0 (0)	4	159.9	0.57	0.68
Eucalyptol	38	80.7 (445.1)	338.6 (1747.7)	58.5 (216.3)	139 (505.6)	86.9 (184)	4	159.96	1.51	0.20
o-Cymene	224	3094.3 (4299.8)^{A}	1474.7 (2580.5)^{AB}	2619.5 (3949.2)^{AB}	1280.7 (3021.1)^{B}	291.2 (723)^{AB}	4	160.95	3.52	***<0.01***
Butanal, 3-methyl-	25	206.1 (871.1)	144.3 (607.9)	197.1 (973.3)	193.2 (853.2)	0 (0)	4	159.56	0.15	0.96
Hexanal	126	4970.2 (14682.6)	5212 (15818.8)	4067.5 (10101.2)	7179.4 (17376.6)	547.9 (1549.6)	4	158.4	0.82	0.51
Octanal	15	19.3 (102.5)	28.1 (107.2)	35 (192.3)	0 (0)	0 (0)	4	158.14	0.78	0.54
Butanoic acid, methyl ester	36	53.3 (175.6)	38.8 (173.7)	61.3 (206.8)	29.7 (115.2)	0 (0)	4	157.6	0.54	0.71
Pentanoic acid, 3-methyl-	33	123.6 (594.5)	95.1 (478.8)	127.1 (563.6)	30.2 (229.6)	79.3 (224.2)	4	157.59	0.42	0.80
Hexanoic acid	23	262.3 (1141.6)	88.5 (586.8)	138.6 (685.7)	32.2 (245.5)	0 (0)	4	157.74	1.14	0.34
Hexanoic acid, methyl ester	118	250.6 (477.5)	183.2 (512.8)	238.1 (509.8)	102.5 (280.2)	47.1 (133.1)	4	160.37	1.34	0.26
Octanoic acid, methyl ester	224	3094.3 (4299.8)^{A}	1474.7 (2580.5)^{AB}	2619.5 (3949.2)^{AB}	1280.7 (3021.1)^{B}	291.2 (723)^{AB}	4	160.95	3.52	***<0.01***
Octanoic acid	67	405.1 (1362.8)	86.7 (278.8)	343.8 (1018.3)	93.6 (459.4)	0 (0)	4	160.79	1.41	0.23
Decanoic acid, methyl ester	209	1486.5 (1906.8)^{A}	677.9 (1115.4)^{AB}	1236.8 (1844.3)^{AB}	469.2 (935)^{B}	144 (407.2)^{AB}	4	162.9	4.04	***<0.01***
Dodecanoic acid, methyl ester	55	93.5 (253.7)	31.7 (102.6)	85 (254.5)	14.9 (72.1)	0 (0)	4	163.64	1.39	0.24

Compounds significantly different represented by different letters. Significance level taken as $p<0.05$. *Multiple comparison adjusted p-value >0.05 (Bonferroni). *n*= Valid observations.

Supplementary Table 4.3: Relative concentration of volatile compounds in preterm breastmilk by maternal diabetes mellitus

		No Diabetes	With Diabetes	Maternal diabetes effect			
	N	**Mean (SD)**	**Mean (SD)**	**Num. df**	**Denom. df**	**F**	***p***
Chloromethane	10	2.2 (15.5)	2.2 (18.8)	1	167.42	0.05	0.82
Pentane	26	96.1 (612.5)	134.3 (538.8)	1	164.94	0.24	0.62
Thrichloromethane	123	1541.3 (8392.1)	836.4 (4109.4)	1	166.66	0.41	0.52
Furan, 2-methoxy-	37	5.5 (25.5)	5.7 (24.5)	1	164.68	0	0.95
Amylene hydrate	62	102.5 (284)	111.8 (256.3)	1	166.94	0.09	0.76
Toluene	36	255.4 (2033.2)	51.5 (278.8)	1	165.8	0.67	0.41
2-Ethyl-1-butanol	15	11.4 (72.8)	13.3 (57.2)	1	164.68	0.05	0.83
Acetone	351	4364.4 (6442.9)	5356.9 (9403.5)	1	166.15	1.22	0.27
1-Hepten-3-one	29	53 (251.9)	62.1 (266.1)	1	166.79	0.09	0.76
Eucalyptol	38	120.8 (748.5)	64.1 (239.4)	1	166.89	0.38	0.54
o-Cymene	224	2662.3 (4077.2)	1463.1 (2606.2)	1	167.86	4.78	***<0.05***
Butanal, 3-methyl-	25	175.4 (769.8)	253.6 (1212.2)	1	166.24	0.32	0.57
Hexanal	126	4912.2 (13980.5)	4950.6 (12532.5)	1	165.03	0	0.98
Octanal	15	24.5 (143.3)	13.8 (84.2)	1	165	0.38	0.54
Butanoic acid, methyl ester	36	55.7 (191.4)	25.6 (99.5)	1	163.75	2.34	0.13
Pentanoic acid, 3-methyl-	33	127 (580.3)	23 (123.8)	1	164.02	2.52	0.11
Hexanoic acid	23	173.5 (898)	114 (549.5)	1	163.94	0.36	0.55
Hexanoic acid, methyl ester	118	235.4 (487.3)	119.3 (359)	1	166.82	3.14	0.08
Octanoic acid, methyl ester	224	2662.3 (4077.2)	1463.1 (2606.2)	1	167.86	4.78	***<0.05***
Octanoic acid	67	340.9 (1157.6)	103.6 (356.7)	1	167.8	2.54	0.11
Decanoic acid, methyl ester	209	1263.3 (1820.2)	601.9 (1069.3)	1	169.46	5.94	***<0.05***
Dodecanoic acid, methyl ester	55	83.9 (243.2)	13.3 (65.9)	1	169.84	4.45	***<0.05***

Compounds significantly different represented by different letters. Significance level taken as $p<0.05$. *Multiple comparison adjusted p-value >0.05 (Bonferroni). *n*= Valid observations.

Supplementary Table 4.4: Relative concentration of volatile compounds in preterm breastmilk by quintiles of New Zealand deprivation index

		Q1	Q2	Q3	Q4	Q5	NZdep effect			
	n	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Num. df**	**Denom. df**	**F**	***p***
Chloromethane	10	1.9 (15)	4 (21.1)	0 (0)	2 (13.9)	1.9 (16.4)	4	157.25	0.31	0.87
Pentane	26	63.5 (301.5)	52.7 (492.3)	74.6 (278.4)	91.6 (334.8)	207.2 (981.3)	4	152.96	1.04	0.39
Thrichloromethane	123	3436 (14005.4)	1004.9 (4522.2)	104.8 (227.6)	2397.8 (10499.1)	439.2 (2244.1)	4	155.33	1.87	0.12
Furan, 2-methoxy-	37	0.9 (7.4)	8.5 (31.1)	2.3 (12.1)	9.4 (39.2)	4 (15.1)	4	152.79	1.61	0.17
Amylene hydrate	62	101.7 (276)	116.3 (315.5)	76.1 (240.1)	103.9 (232.1)	81.7 (242.9)	4	155.32	0.26	0.9
Toluene	36	339.1 (1359)	217.8 (724.7)	0 (0)	529 (3922.8)	10.9 (82.2)	4	154.38	0.98	0.42
2-Ethyl-1-butanol	15	24 (89.3)	18.8 (104.9)	1.9 (12.8)	2.4 (20.6)	8.2 (45.7)	4	152.79	1.38	0.24
Acetone	351	6055.5 (7195.4)	5418.2 (10811.4)	4568.2 (6984.6)	3623.8 (3174.5)	3282.5 (2817.2)	4	155.63	2.14	0.08
1-Hepten-3-one	29	112.2 (356.2)	65.5 (269.1)	63.2 (269.5)	41.7 (262.8)	16 (112.5)	4	156.61	1.34	0.26
Eucalyptol	38	49.5 (322.2)	95.4 (456.2)	3 (19.9)	151.1 (380.1)	184.2 (1189.8)	4	156.91	0.64	0.64
o-Cymene	224	3076.1 (3672.1)^{AB}	2921.5 (4437.3)^{AB}	4062.4 (5853.4)^{A}	1623.1 (2326.3)^{B}	1487.7 (2808.5)^{B}	4	158.1	4.81	***<0.01***
Butanal, 3-methyl-	25	452.1 (1326.3)	145.1 (698.6)	194.4 (751.4)	74.5 (510.9)	164.7 (928.2)	4	155.77	1.61	0.17
Hexanal	126	4212.9 (14904.2)^{AB}	2818 (10213.7)^{A}	5340.9 (11795.9)^{AB}	2337.4 (7197.1)^{A}	9453.9 (18930.2)^{B}	4	153.1	4.92	***<0.001***
Octanal	15	43.7 (149.6)	18.6 (95.8)	42.1 (213.2)	20.9 (182)	7 (50)	4	153.98	0.98	0.42
Butanoic acid, methyl ester	36	76.4 (213.9)	58.8 (205.7)	40.5 (141.4)	44.9 (153.8)	34.3 (156)	4	153.09	0.74	0.57
Pentanoic acid, 3-methyl-	33	199.2 (782)	121 (610.9)	104.4 (354.2)	35.7 (166.4)	77 (449.6)	4	153.08	1.03	0.4
Hexanoic acid	23	126.8 (588.9)^{A}	194.3 (838.3)^{A}	604 (1880.2)^{B}	62.8 (330.3)^{A}	43.7 (387.7)^{A}	4	153.09	4.67	***<0.01***
Hexanoic acid, methyl ester	118	294.2 (592.4)^{A}	270.5 (526.9)^{A}	300 (574)^{A}	140.9 (294.8)*^{B}	123.1 (341)*^{B}	4	156.96	2.75	***<0.05***
Octanoic acid	67	228.1 (518.8)^{A}	409.2 (1220.2)^{AB}	871.4 (2254.2)^{B}	87.7 (273)^{A}	130.2 (474.7)^{A}	4	157.43	4.57	***<0.01***
Octanoic acid, methyl ester	224	3076.1 (3672.1)^{AB}	2921.5 (4437.3)^{AB}	4062.4 (5853.4)^{A}	1623.1 (2326.3)^{B}	1487.7 (2808.5)^{B}	4	158.1	4.81	***<0.01***
Decanoic acid, methyl ester	209	1281.3 (1551.9)^{AB}	1471 (1978.2)^{B}	1840.1 (2594)^{B}	815.8 (1137.5)^{AB}	678.3 (1242.8)^{A}	4	161.04	4.33	***<0.01***
Dodecanoic acid, methyl ester	55	42.3 (129.8) ^{AB}	94.3 (261.5) ^{AB}	171.9 (397.5)* ^{A}	38.7 (134.2) ^{AB}	47.2 (158)* ^{B}	4	161.91	2.61	***<0.05***

Compounds significantly different represented by different letters. Significance level taken as $p<0.05$. *Multiple comparison adjusted p-value >0.05 (Bonferroni). n= Valid observations.

CHAPTER 5

CORTICAL OXYGENATION CHANGES DURING GASTRIC TUBE FEEDING IN MODERATE AND LATE PRETERM BABIES

Chapter published in Nutrients

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5.1 Introduction

Smell and taste are intimately connected to nutrition and behaviour, directly influencing food preferences (Lipchock et al., 2011; Reed and Knaapila, 2010). Sensory cues originating from smell and taste of food can trigger physiological responses facilitating digestion and metabolism of ingested nutrients (Power and Schulkin, 2008). The integration of taste and smell information arising from the peripheral sensory organs is believed to occur in the orbitofrontal cortex (OFC) (Avery et al., 2020; de Araujo et al., 2003; Okamoto and Dan, 2007; Small and Prescott, 2005). The OFC integrates information from the five sensory modalities (visual, auditory, somatosensory, gustatory and olfactory), forming a complex network with other brain areas, such as the insula, amygdala and piriform cortex (Kringelbach, 2005).

Neuronal activity induces an increase in cerebral blood flow to supply the oxygen required to meet cellular metabolic demand (Fox et al., 1988). During sensory stimulation there is a rise in oxygen delivery to the brain cells which leads to an increase in concentration of oxygenated haemoglobin in the cerebral cortex, including the OFC (Kringelbach, 2005; Villringer and Chance, 1997). Changes in haemoglobin oxygen content provide information about oxygen delivery and utilisation by neuronal cells, coupled to cerebral activity (Brazy et al., 1985), and can be measured non-invasively by near infrared spectroscopy (NIRS) (Villringer and Chance, 1997). Oxygenated (O_2Hb) and deoxygenated (HHb) haemoglobin absorb near infrared light at different wavelengths (Dix et al., 2017) which can be detected by NIRS, thereby allowing estimation of changes in the oxygen content of haemoglobin associated with cortical activity (Aslin and Mehler, 2005; Brazy et al., 1985).

Changes in cerebral oxygenation in the OFC have been associated with neuronal activation of the olfactory cortex (Aoyama et al., 2010; Bartocci et al., 2000, 2001; Frie et al., 2018, 2020), and can be detected by NIRS following exposure to a variety of odours in adults (Harada et al., 2006), term and preterm babies (Aoyama et al., 2010; Bartocci et al., 2000, 2001; Frie et al., 2018, 2020), including the odour of the maternal breast (Frie et al., 2020), nosocomial odours (Bartocci et al., 2001; Frie et al., 2018) and odour of breastmilk and formula (Aoyama et al., 2010). Similarly, oxygenation changes in the prefrontal cortex measured by NIRS have been associated with processing of taste information in adults (de Araujo et al., 2003; Okamoto et al., 2009).

Provision of smell of breastmilk in tube-fed preterm infants has been linked to increased sucking behaviour and milk ingestion (Bingham et al., 2007; Raimbault et al., 2007), and possibly may lead to more rapid attainment of full oral feeds and reduced length of hospital stay (Muelbert et al., 2019), although the quality of evidence is very low. Similarly, oral administration of small amounts of colostrum has been associated with faster attainment of full tube feeds, but again evidence is of very low quality (Nasuf et al., 2018).

The studies linking smell to potential activation of the OFC in preterm babies have been conducted in carefully controlled settings and there are no studies investigating cortical activation with taste stimulation in preterm infants. It is, therefore, not clear whether activation of the OFC can be detected in preterm babies in the usual setting of tube-feeds in a newborn nursery, nor whether the administration of milk through a gastric feeding tube itself can lead to increases in oxygenation in the OFC. Thus, our study aimed to describe cerebral oxygenation changes in the OFC measured by NIRS in moderate-late preterm babies

(birth between 32 and 36 weeks' gestation) following exposure to smell and taste of milk and during tube feeds.

5.2 Methods

5.2.1 *Study population*

This is a cohort study nested within a multicentre, factorial, randomised controlled trial, the DIAMOND Trial (Bloomfield et al., 2018) and was approved by the national Health and Disability Ethics Committee (HDEC 16/NTA/90). Written informed consent was obtained from parents or caregivers. Briefly, the DIAMOND trial is investigating the impact of different nutritional approaches on feed tolerance, body composition and neurodevelopment in moderate-late preterm (MLP) babies. The three main factors under investigation are: 1) provision of intravenous nutrition with parenteral nutrition compared to intravenous dextrose; 2) provision of enteral nutrition with exclusive maternal breastmilk compared to milk supplementation with infant formula or donor breastmilk, and 3) exposure to smell and taste of milk before all tube feeds compared to no smell and taste of milk prior to tube feeds. Eligible babies were born between 32^{+0} and 35^{+6} weeks' gestation, admitted to one of five neonatal intensive care units (NICU) in New Zealand, had an intravenous canula sited for clinical reasons, and their mother intended to breastfeed. Babies with congenital abnormality or for whom a particular mode of nutrition was clinically indicated were not eligible.

The current study included a sub-sample of participants in the DIAMOND trial who were born before 35 weeks' gestation, exclusively tube-fed and admitted to the NICU at Auckland

City Hospital (ACH) and whose parents / guardians gave informed consent for this additional NIRS assessment. We did not include babies born ≥35 weeks' gestation as these babies often initiate oral feeding attempts within the first postnatal days. Thus, the sample size was determined by the number of consented babies <35 weeks' gestation at one site enrolling babies into the DIAMOND trial.

5.2.2 ***Assessment procedure***

NIRS assessments were performed on day 5 (±2) days and day 10 (±2) days after birth, during feeds where there was no sucking attempt at the breast prior to the tube feed. The assessment sequence was adapted from previous studies with newborn/preterm babies that provided smell exposure for 30 seconds (Aoyama et al., 2010; Frie et al., 2020). For the intervention group, the assessment consisted of a five minute baseline reading period (P0), a one minute period of exposure to smell (P1), a 2 minute interval, then a one minute period of exposure to taste (P2), another 2 minute interval, then a period of tube-feeding of variable duration (P3). Babies in the control group were monitored for the equivalent time; however, no placebo or sham stimulation was delivered. The total assessment duration for both groups consisted of 11 minutes before the feed plus the duration of the feed, which varied according to the volume of feed (Figure 5.1). As no previous study has investigated cerebral oxygenation changes following exposure to taste in babies, we undertook some pilot assessments which determined that one minute of sensory exposure was feasible and well tolerated by babies. For consistency, equal duration of sensory stimulation was used for both smell and taste exposure.

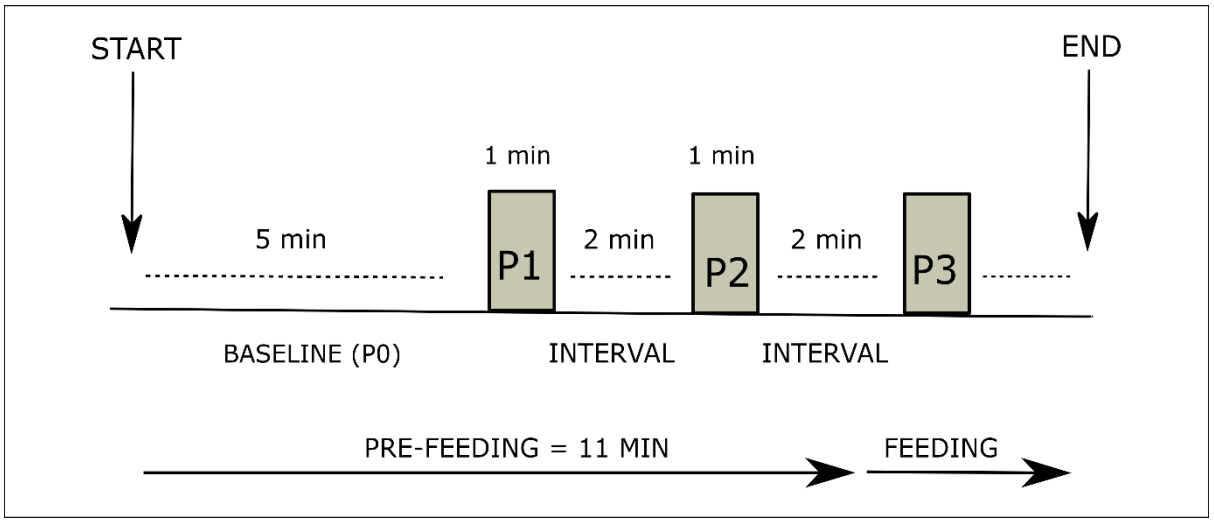

Figure 5.1: Assessment sequence

Pre-feeding consisted of baseline period (P0, five minutes); period 1 (P1, one minute), corresponding to smell exposure in intervention group); period 2 (P2, one minute), corresponding to taste exposure in intervention group; and feeding period (P3, duration according to feeding volume).

5.2.3 ***Sensory stimulation***

Smell and taste stimulation were provided using the milk fed to the infant at the time of the assessment (either breastmilk, fortified breastmilk or infant formula). Smell stimulation was provided during P1 by placing a gauze containing approximately 0.2 mL of milk close to the baby's nose and moving this slowly from one nostril to the other at a distance of approximately 1–2 cm for 1 minute. For taste exposure during P2, a syringe containing 0.2 mL of milk was used to place drops of milk into the baby's mouth for 1 minute. Gastric tube feeding (P3) was administered according to local NICU guidelines.

5.2.4 ***Cerebral oxygenation monitoring***

Cerebral oxygenation levels were monitored at the bedside using a double-channel NIRS device (NIRO-200, Hamamatsu, Japan) at wavelength 775 – 850 nm. Concentration of oxygenated haemoglobin (O_2Hb) were recorded every second and stored in a dedicated personal computer. Onset and completion of P1, P2 and P3 were marked on the NIRS device. Emitter and receiver optodes were placed bilaterally 2 cm above the midpoint of the line connecting the external angle of the eye to the homolateral tragus, reflecting placement anterior to T3/T4 and anterior to F7/F8 in the international 10–20 system for electrode placement (Kabdebon et al., 2014), overlying the orbitofrontal cortex (Figure 5.2). The differential pathlength was set to 3.85 cm and optical pathlength to 11.5 cm. The transmitter and receiver optodes were placed 3 cm apart in a purpose-designed holder attached to the forehead with double-sided adhesive tape, covered by a bandage. Babies were swaddled and incubators covered consistent with standard practice in this nursery. Room light and noise were diminished as much as possible to minimise interference; however, the room environment was kept as close as possible to daily routines to mimic the real-life scenario.

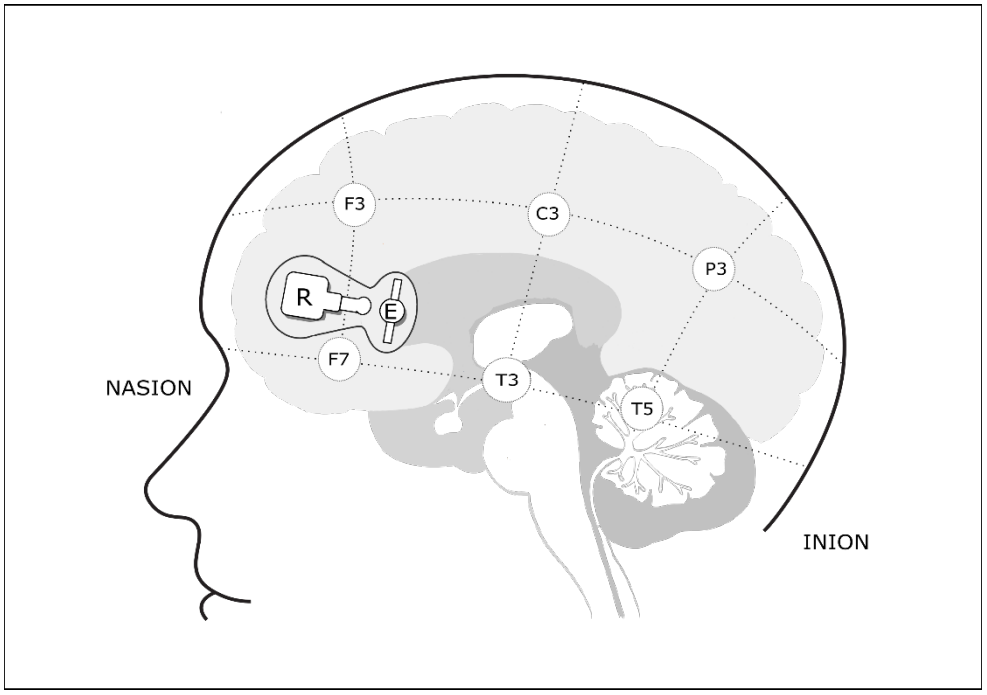

Figure 5.2: Lateral view representing optode position

Optodes were placed bilaterally with emitter (E) positioned anterior to T3/T4 and receiver (R) positioned anterior to F7/F8 according to the international 10–20 system.

5.2.5 ***Data processing and statistical analysis***

In order to minimise slow oscillations and artefacts common to NIRS recordings, data smoothing was performed using a 15 second moving average. Data points that were more than 2 standard deviations from the mean were considered artefacts and were excluded. Baseline O_2Hb concentration for each baby was defined as the mean of a 30 second stable period within P0. The maximum change in O_2Hb concentration (mmol/L) from baseline was then calculated for P1, P2 and P3 separately for left and right hemispheres. To explore the effect of exposure to smell and taste of milk and tube feeding on change in O_2Hb concentration, we used linear mixed model regression analysis with repeat measurements clustered within each baby, and assumed an unstructured covariance matrix between the measurements (P1, P2 and P3). We tested the interaction between O_2Hb changes, group and

optode location (Model 1 – left versus right), postnatal day at assessment (Model 2 – day 5 versus day 10) and sex (Model 3 – boys versus girls). Model-adjusted estimates for each group at each period and assessment were estimated using fixed effects models, adjusted for sex, gestation, and baseline oxygenation. All variables were tested for normality before analysis. Statistical analysis were performed with SAS version 9.4 (SAS Institute Inc., Cary, NC, USA).

5.3 Results

5.3.1 ***Study Population***

Between June 2018 and March 2020, 62 moderate-late preterm babies admitted to NICU at Auckland City Hospital were enrolled in the DIAMOND trial and 50 parents / caregivers consented to NIRS assessments. Thirty-five of these underwent the first assessment at a median postnatal age of 4 (range 3 – 6) days and 18 underwent the second assessment at a median age of 10 (range 8 – 12) days (Figure 5.3). Median gestational age at birth was 33 (range 32 – 34) weeks, 69% of babies were boys and the majority of babies received breastmilk as their feed during the assessments. Baseline characteristics did not differ between control and intervention groups (Table 5.1).

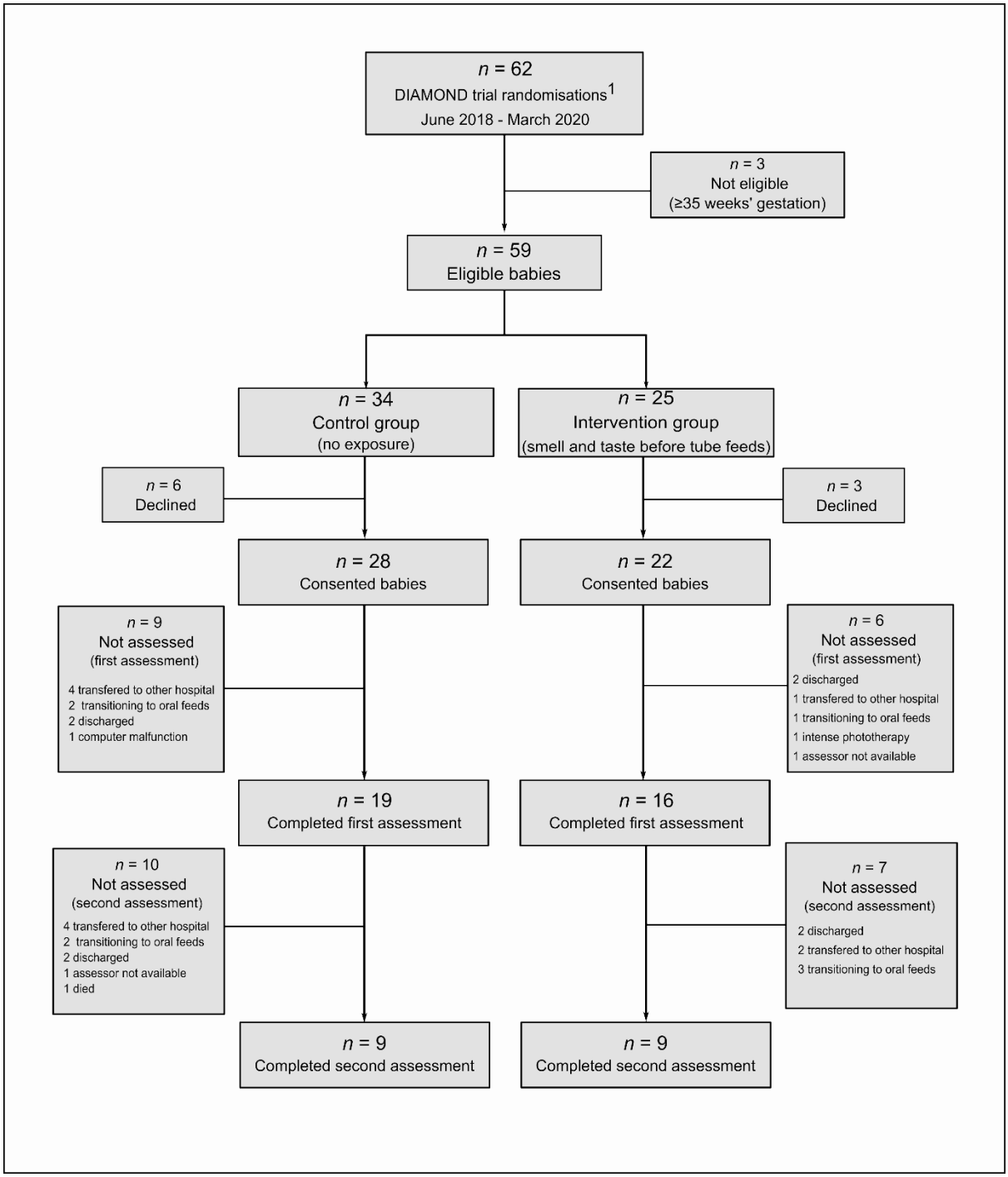

Figure 5.3: Study flowchart

[1]Total recruited at Auckland City Hospital during the study period. n= number of participants.

Table 5.1: Study group characteristics

	Study group		
	Control (*n*= 19)	**Intervention (*n*= 16)**	***p***
Participant characteristics:			
Boys	14 (74)	10 (63)	0.59
Gestational age, weeks	33 (32 - 34)	33 (32 - 34)	0.93
Birth weight, grams	1883 (306)	1909 (393)	0.81
Birth length, cm	43.6 (2.7)	43 (3.1)	0.50
Birth head circumference, cm	30.6 (1.2)	30.4 (2.0)	0.81
Gestational age, weeks	33 (32 - 34)	33 (32 - 34)	0.93
Discharge weight, grams	2557 (299)	2411 (375)	0.16
Duration of NICU stay, days	28 (11 - 51)	24 (10 - 36)	0.30
In-hospital weight gain, g/Kg/day	10.8 (4.1)	9.0 (4.6)	0.28
Maternal age, years	35 (21 - 50)	32 (18 - 45)	0.20
Birth by Caesarean-section	14 (74)	13 (81)	0.71
Antenatal corticosteroid received	18 (95)	13 (81)	0.57
At assessment 1:			
Postnatal age at assessment, days	5 (3 - 6)	4 (3 - 5)	0.15
Volume of tube feed, mL	15 (2 - 43)	18 (4 - 38)	0.61
Duration of assessment, min	20 (14 - 31)	21 (11 - 41)	0.91
Milk fed during assessment			0.61
Breastmilk only	16 (84.2)	12 (75)	
Mixed feeding *	1 (5.3)	3 (18.7)	
Infant formula	2 (10.5)	1 (6.3)	
At assessment 2:			
Total participants	9 (47)	9 (56)	
Boys	8 (89)	8 (89)	
Postnatal age at assessment, days	10 (8 - 11)	10 (8 - 12)	0.86
Volume of tube feed, mL	40 (26 - 50)	29 (16 - 48)	0.30
Duration of assessment, min	27 (24 - 35)	26 (22 - 44)	0.80
Milk fed during assessment			0.38
Breastmilk only	8 (88.9)	7 (77.8)	
Mixed feeding *	0	0	
Infant formula	1 (11.1)	2 (22.2)	

Data are presented as mean (SD), median (range) or *n* (%). NICU: neonatal intensive care unit. *Mixed feeding means the baby received a combination of breastmilk and formula during the feed.

5.3.2 ***Model 1: The effect of laterality***

This model included data from the first assessment only and data from three participants were excluded for signal artefacts that rendered the data of poor quality for analysis, resulting in 32 assessments (18 in the control and 14 in the intervention group) included. There was no difference in lateral cortical activation on either side between babies in the control and intervention groups for any period ($F_{(2,59)}$= 0.88, p=0.4) (Table 5.2). Babies in the intervention group showed significant increase in O_2Hb concentration from baseline for each of the three periods on the right side and for P2 (taste) on the left side of the brain (p<0.05). Babies in the control group showed a significant increase in O_2Hb concentration from baseline on the left side for P1 and bilaterally for P3 (p<0.05). They also showed a significant increase from P2 to in P3 on the left side (p=0.01) (Figure 5.4A).

5.3.3 ***Model 2: The effect of postnatal day***

As there was no difference in O_2Hb concentration between the right and left sides, measurements from both sides were combined in order to explore the effect of postnatal age at the assessment. This model included data from first and second assessments (n= 53) and the excluded data from first assessment from three participants were imputed by maximum likelihood. Overall, there was no significant interaction between period, study group and postnatal day ($F_{(2, 95)}$= 0.41, p= 0.7); however, there was a significant interaction between postnatal day and study period ($F_{(2, 95)}$= 5.29, p= 0.006) (Table 5.2). In both study groups, O_2Hb concentrations measured during P2 were significantly higher in the first than the second assessment (p<0.001). Further, O_2Hb concentration in the control group was significantly

higher during P3 (feeding) than in P1 and P2 (p<0.05) in both assessments. Similarly, in the intervention group O_2Hb concentration in the second assessment was significantly higher during P3 than during P2 (p<0.001) (Figure 5.4B).

5.3.4 ***Model 3: The effect of sex***

This model included the first assessment only (n= 32) as only one girl in each group was assessed twice. There was a significant interaction between period, study group and sex ($F_{(2,59)}$= 4.95, p= 0.01). In girls in the intervention group O_2Hb concentration during P2 and P3 (taste and feed) was higher than baseline and P1 (smell) (Table 5.2). In contrast, in girls in the control group O_2Hb concentration was higher than baseline during P1 (p= 0.01), and also significantly higher than the intervention group (p= 0.03).

Conversely, in boys in both groups O_2Hb concentration was significantly increased from baseline concentration in all periods (p<0.01) but there were no significant differences amongst periods or between groups.

Finally, when comparing boys and girls within groups, we found that only boys in the intervention group had an increase in O_2Hb concentration during P1 (smell of milk), and this was significantly higher than in girls exposed to smell of milk (p= 0.002) (Figure 5.4C).

Table 5.2: Changes in oxygenated haemoglobin concentration from baseline (mixed models estimates)

	Control group			Intervention group		
	Period			Period		
Model estimates	Smell (P1)	Taste (P2)	Feed (P3)	Smell (P1)	Taste (P2)	Feed (P3)
A) Side: $F_{(2, 59)}$= 0.88 p= 0.4						
Left side	3.2* [a b] (0.5 – 6.0)	2.8 [a] (-0.9 – 6.5)	6.4* [b] (2.1 – 10.7)	2.3[a] (-0.8 – 5.8)	5.0* [a] (0.8 – 9.2)	4.3 [a] (-0.6 – 9.2)
Right side	2.2 [a] (-0.2 – 5.3)	2.9 [a] (-0.8 – 6.7)	5.7* [a] (1.4 – 10.0)	3.7* [a] (0.7 – 6.7)	4.4* [a] (0.2 – 8.6)	6.1* [a] (1.3 – 10.9)
B) Postnatal day: $F_{(2, 95)}$= 0.41 p= 0.7						
Assessment #1	2.9* [a] (0.05 – 5.8)	2.9* [a §] (0.01 – 5.9)	6.1* [b] (2.8 – 9.4)	4.0* [a] (0.8 – 7.2)	5.7* [a §] (2.4 – 9.0)	6.2* [a] (2.5 – 9.9)
Assessment #2	2.5 [a] (-1.1 – 6.2)	-0.7 [b §] (-4.4 – 3.0)	5.0* [a] (0.7 – 9.4)	4.0* [a b] (0.2 – 7.8)	1.9 [a §] (-2.0 – 5.8)	7.6* [b] (3.1 – 12.0)
C) Sex: $F_{(2, 59)}$= 4.94 p= 0.01						
Boys	3.7* [a] (1.4 – 6.1)	4.2* [a] (1.0 – 7.4)	8.0* [b] (4.3 – 11.7)	6.6* [a ≈] (3.4 – 9.8)	5.5* [a] (1.4 – 9.5)	7.2* [a] (2.6 – 11.8)
Girls	4.5* [a Δ] (0.8 – 8.1)	3.2 [a] (-1.8 – 8.2)	4.7 [a] (-1.0 – 10.6)	-1.2 [a Δ ≈](-4.8 – 2.3)	5.6* [b] (0.7 – 10.5)	3.8 [b] (-1.9 – 9.5)

Mixed models estimates for three-way interaction between group, period and A) Side; B) Postnatal day; C) Sex. Model adjusted for sex (except C), gestation and baseline oxygenation. Values are expressed in mean *mmol/L* with respective 95% Confidence Interval (95% CI). * Represents significant change from baseline. [a,b] Different letters represent significant period difference. § Represents significant day difference. Δ Represents significant group difference. ≈ Represents significant sex difference.

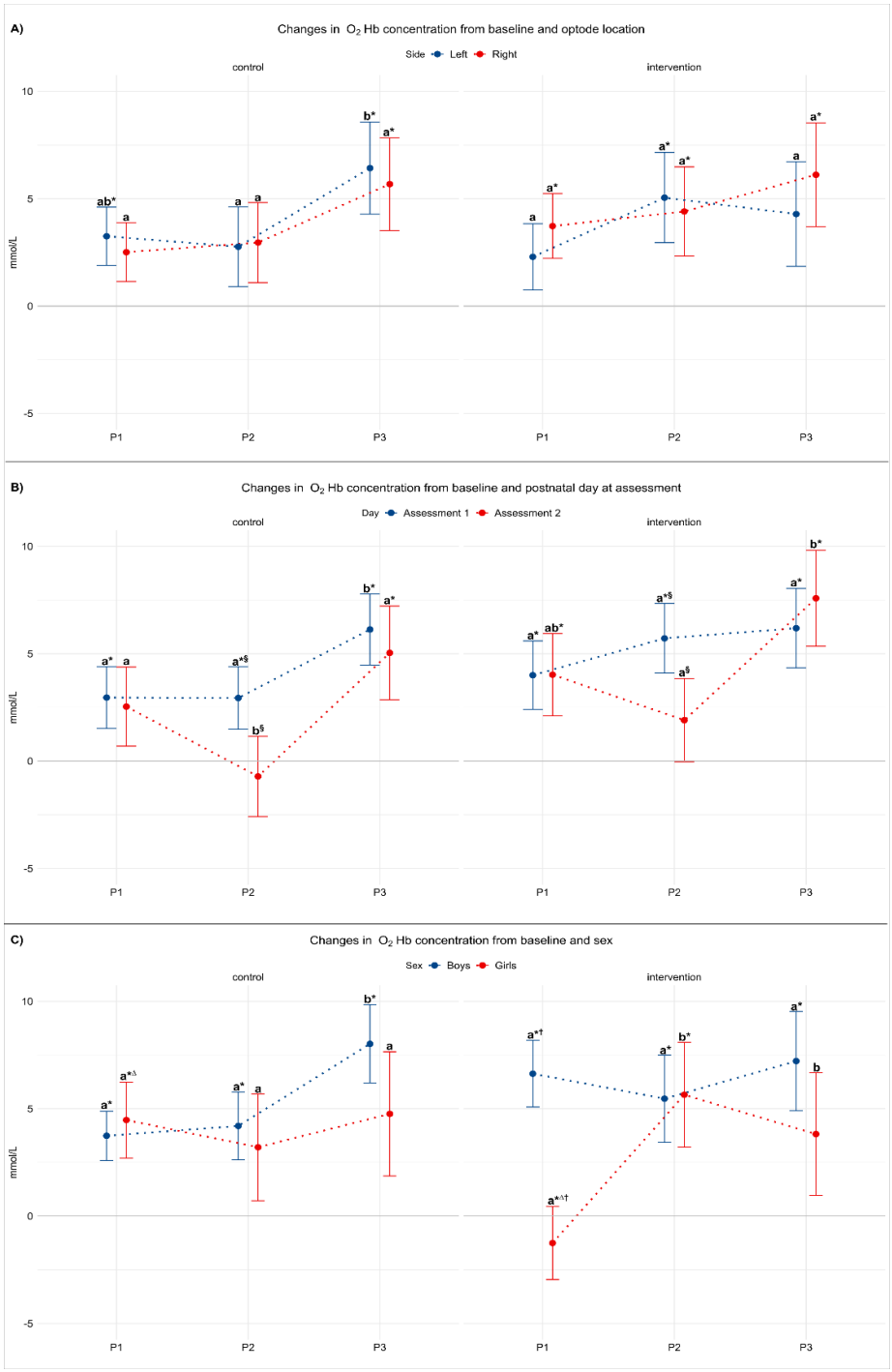

Figure 5.4: Model estimates adjusted for sex, gestation and baseline oxygenation

*Values are mean with 95% confidence intervals. A) Effect of laterality (control n= 18, intervention n= 14). B) Effect of postnatal day (control n= 19, intervention n= 16). C) Effect of sex (control n= 18, intervention n= 14). *Significant change from baseline. [a,b] Different letters represent significant differences amongst periods. § Significant difference between assessment 1 and assessment 2. Δ Significant group difference. †Significant sex difference. Statistical significance taken as p < 0.05 for all comparisons. P1: period 1 (smell exposure in intervention group). P2: period 2 (taste exposure in intervention group). P3: period 3 (feeding period in both groups). O_2Hb= oxygenated haemoglobin.*

5.4 Discussion

5.4.1 ***Main findings***

In contrast to our hypothesis and to previous studies exposing babies to smell in highly controlled settings (Aoyama et al., 2010; Bartocci et al., 2000; Frie et al., 2018, 2020), we did not find that exposure to smell activated the OFC in moderate-late preterm babies. However, changes in O_2Hb concentration during exposure to smell of milk were significantly higher in boys than in girls, whereas girls only showed an increase O_2Hb concentration during exposure to taste. Further, we found that, in the clinical rather than experimental setting, gastric tube feeds resulted in the greatest change in OFC oxygenation.

5.4.2 ***The effect of side***

We found similar O_2Hb concentration changes in both left and right OFC with no clear evidence of laterality of response to sensory stimulation. Previous studies in well-controlled settings (Aoyama et al., 2010; Frie et al., 2018, 2020) have shown that newborn babies are aware of their olfactory environment and are capable of discriminating between different smells, but the evidence for laterality in the response is not consistent. For example, in full-term babies exposed to smell of colostrum and vanilla, increases in oxygenation were detected in the left OFC (Bartocci et al., 2000), whereas a study including both term and preterm babies reported bilateral increases in OFC O_2Hb concentration that were greater with breastmilk than with formula (Aoyama et al., 2010). In contrast, exposure to unpleasant smells (such as detergent and adhesive remover) resulted in decreased OFC oxygenation that was more notable on the right than the left (Bartocci et al., 2001). Conversely, in studies

including both preterm and term babies, bilateral increase in oxygenation in the OFC were observed following exposure to unpleasant smells (hand sanitiser and adhesive remover), with preterm babies showing greater cortical activation at term corrected age compared to the early postnatal period (Frie et al., 2018). There is some evidence that laterality may be affected by maturity, with one study reporting that exposure to the smell of the maternal breast elicited no cortical activation in very preterm babies, only right cortical activation in late preterm babies but bilateral cortical activation in full-term babies, leading the authors to suggest that odour processing at a cortical level may be present in neonates born from 32 weeks' gestation onwards (Frie et al., 2020). As our cohort is limited to moderate- and late-preterm babies, with most falling in the moderate preterm category, we were unable to assess the impact of the degree of prematurity in cortical processing of smell.

5.4.3 ***The effect of postnatal day***

Lower O_2Hb concentration in response to milk placed in the mouth (P2) was observed during the second assessment compared to the first. It is possible that babies developed habituation to taste of milk with increasing exposure through breastfeeding attempts / trials, resulting in weaker activation of the OFC. Habituation is often described in adults as a reduction in physiological and behavioural responses following repeated eating episodes (Epstein et al., 2009); however, it is yet to be determined if this occurs in preterm babies. Decreasing O_2Hb concentration in response to smell of colostrum with increasing postnatal age has been reported previously (Bartocci et al., 2000), potentially indicating that older babies more familiarised with smell of milk through previous feeding experiences became less sensitive to smell of milk as feeding frequency increased (Bartocci et al., 2000). However, a

study investigating whether newborn babies (preterm and term) could discriminate between the smell of breastmilk and formula, in which babies were grouped according to their feeding experience (exclusive breastmilk versus mixed feeding), did not find any interaction between previous feeding experience and OFC oxygenation (Aoyama et al., 2010). In our study, the majority of babies were fed breastmilk (with or without multi-component bovine-based fortifier) during the assessment and thus we were unable to further clarify the effect of milk type on OFC oxygenation.

5.4.4 ***The effect of sex***

We found contrasting patterns of oxygenation in the OFC for boys and girls. Boys showed a significant increase in O_2Hb concentration when exposed to smell of milk and also during the feeding period, whereas girls showed no changes in O_2Hb concentration in response to smell of milk, but a steep increase in O_2Hb concentration in response to taste of milk. This is consistent with a previous report of difference between boys and girls in response to smell of the maternal breast, where boys showed bilateral activation of the olfactory cortex but girls showed activation only of the left olfactory cortex (Frie et al., 2020). In general, women are reported to be more sensitive than men to a variety of odours (Doty and Cameron, 2009), possibly due to differences in circulating neuroendocrine hormones (Doty and Cameron, 2009). The possible differences in OFC oxygenation between preterm girls and boys, and whether this has any clinical relevance, requires further investigation.

5.4.5 ***OFC activation without sensory stimulation***

In contrast to our hypothesis, the control group showed an increase in O_2Hb concentration during P1 and P2 despite receiving no sensory stimulation. Although Frie *et al.* (2020) reported bilateral activation of the frontal cortex when late preterm babies were exposed to a control stimulus, consisting of a clean cloth washed with odourless detergent (Frie et al., 2020), in our study no stimulus at all was provided.

However, in our study the majority of babies were awake during the assessments and it seems possible that infants in the control group were responding to smells or other sensory input originating from their surroundings. The NICU environment provides numerous sensory inputs, including handling, light, noise and exposure to nosocomial odours, all of which may impact the development of sensory systems of the immature brain (Bröring et al., 2017; VandenBerg, 2007). We designed our study to be conducted in the normal NICU environment, as we wished to determine whether exposure to smell and taste has any clinically-relevant impact, so we cannot exclude the possibility that other sensory inputs were inducing cortical activation. Since our focus was on activation of the OFC, the optode pairs were placed only over this region, but the responses observed in the control group could reflect more general cortical activation and not activation of the OFC alone. Future studies are needed to confirm potential cortical activation related to NICU environment stimuli.

5.4.6 ***The effect of feeding***

Research into the potential role of smell exposure in preterm babies is predicated upon the assumption that tube-fed babies miss out on a potentially beneficial sensory stimuli that

may promote feed tolerance and digestion through activation of the cephalic phase response (Muelbert et al., 2019). Our unexpected finding that the greatest increase in O_2Hb concentration in the OFC occurred in response to tube feeds raises the possibility that this mode of feeding does, in fact, activate olfactory and / or gustatory receptors. This may occur through various routes. For example, olfactory receptors have been found throughout the gastrointestinal tract and in organs that assist digestion and nutrient metabolism, such as the pancreas and liver, and possibly may be stimulated during feeding, mediating the secretion of digestive hormones (Braun et al., 2007; Calvo and Egan, 2015; Maßberg and Hatt, 2018). Similarly, taste receptors are expressed throughout the gastrointestinal epithelium and are involved in regulation of food intake, digestion and the secretion of the digestive hormones such as ghrelin, leptin, glucagon and cholecystokinin (Calvo and Egan, 2015). Whether these receptors located outside of the oronasopharynx activate the OFC is unknown. Alternatively, it is possible that small quantities of milk, or the volatile compounds within milk responsible for smell, reflux from the stomach into the upper gastrointestinal tract, activating olfactory and gustatory receptors and the OFC. The presence of a feeding tube passing through the gastro-oesophageal junction increases the likelihood of such reflux by maintaining patency of that junction (Omari et al., 2002). Indeed, gastroesophageal reflux is a normal physiological phenomenon in most healthy babies and can occur in every feed with or without vomiting (Rosen et al., 2018).

5.4.7 ***Strengths and limitations***

We nested this experimental cohort study within the DIAMOND trial to take advantage of the random allocation of babies to experimental and control groups. This allowed us to test

the effect of exposing tube-fed babies to smell and taste of milk before and during feeds compared to no sensory stimulation with tube feeds. We also aimed to assess babies at the bedside in an environment similar as possible to the usual clinical setting, and most babies were awake during the assessment, which may have influenced the baby's level of awareness of the surroundings and affected the measurement of the outcome. In contrast, no previous studies investigated changes in O_2Hb concentration in the context of feeding, and all used a carefully controlled environment and assessed babies while asleep.

Given the study design, it was not possible to blind the assessor to the intervention, and no control stimulation was used, which may be considered limitations. Our study also had a small sample size, limited by the number of trial participants whose parents/caregivers consented for the assessment and the fact that NIRS was available at only one recruiting site, and the unequal distribution of moderate and late preterm infants did not permit us to assess the effect of degree of prematurity on activation of OFC. Similarly, most babies received breastmilk or fortified breastmilk during NIRS assessments and therefore we did not have sufficient numbers to analyse the effect of type of feeding on oxygenation changes in the OFC. Additionally, we used a double-channel NIRS device with only two pairs of emitter-receiver optodes, which may have less spatial resolution and less sensitivity to detect changes in cortical oxygenation (Aslin and Mehler, 2005) compared to multi-channel devices with a larger number of emitter-receiver optodes (Frie et al., 2018, 2020). Nevertheless, our findings are consistent with other studies using same device (Aoyama et al., 2010; Bartocci et al., 2000, 2001).

5.5 Conclusion

We have shown that changes in cerebral oxygenation in the OFC can be detected in moderate and late preterm infants before and during tube feeding. The greatest increase in O_2Hb concentration in the OFC occurred in response to tube feeds, suggesting that gustatory and olfactory receptors may be activated in this feeding mode. Boys but not girls showed activation of the OFC following exposure to smell of milk. Early life sensory exposures are a critical for the development of smell and taste preferences and preterm babies may be capable of processing stimulations coming from their environment. The impact of sensory stimulation prior to tube feeds on clinical outcomes such as feed tolerance, attainment of feeding skills and development of feeding preferences, as well as the impact of feeding experience and NICU environment on cortical activation, remains to be determined.

CHAPTER 6

GENERAL DISCUSSION

6.1 Summary of findings

Nutrition is a key factor in the care of preterm babies. Provision of enteral nutrition can be a major challenge as feeds often are not well tolerated. Feeding intolerance impacts the progression of nutritional support with repercussions for growth and duration of hospital stay (Fanaro, 2013). The importance of sensory cues in the enjoyment and metabolism of food in adults is well recognised, yet little attention has been paid to the potential role of smell, taste and cephalic phase responses (CPR) in preterm infants. Most of these infants will receive some of their initial feeds via a gastric tube, potentially bypassing smell and taste receptors in the oronasopharynx.

6.1.1 *Exposure to smell and taste of milk: Is there any impact on health outcomes of preterm infants?*

The evidence for any effect of exposure to smell and taste of milk to improve feeding in preterm infants currently is limited by the small number of heterogeneous studies of low quality. The evidence from these studies was analysed systematically in this thesis through a Cochrane review assessing whether the effect of sensory stimulation delivered with enteral feeding could improve nutrition of preterm infants. To be included in the systematic review, studies had to be randomised or quasi-randomised trials, provide the intervention before or during tube feeds, and the sensory stimulation should consist of smell and/or taste of milk (either breastmilk or formula).

Only three studies met these inclusion criteria and trials were small, assessed different groups of preterm babies and used different methods for delivery of the intervention (Beker et al., 2017; Davidson et al., 2015; Yildiz et al., 2011). Despite the very low quality of available evidence, the meta-analysis indicated a reduction of up to four days in duration in hospital stay (mean difference -3.9 days, 95% CI -7.0 to -0.7) and a trend towards faster attainment of oral feeds (mean difference -2.6 days, 95% CI -5.1 to 0.02). However, the overall effect of exposure to smell and taste of milk to improve feeding in preterm infants remains unclear and more studies are needed.

It is possible that our review was conducted "too early to smell" the effect, as suggested by Schriever et al. (Schriever et al., 2019). Since completion of our review, new studies have been published (Davidson et al., 2019; Lee, 2019), and studies employing smell stimulation with other substances (anise, cinnamon, vanilla) have been identified (Cao Van et al., 2018; Schriever et al., 2018). Nevertheless, growing interest in smell and taste stimulation in preterm infants, with the potential for inclusion into clinical practice without systematic appraisal of potential benefits and harms (no trial reported adverse effects in a systematic manner), highlights the relevance of conducting this review to guide future studies. For example, an electronic survey of neonatologists and paediatricians in Australia and New Zealand found that 30% (*n*=36) of clinicians reported regular provision of smell or taste of mother's milk prior to tube feeds (Alexander and Bloomfield, 2019), despite the limited reliable evidence of clinical benefit.

Only one of the four additional trials mentioned above would meet the criteria for inclusion in our systematic review. Davidson et al (2019) randomly assigned 36 preterm babies born between 28 and 32 weeks' gestation to receive smell of BM or water (sham

stimulus) before tube feeds once a day until attainment of full oral feeds. Parents, NICU staff and outcome assessor were blinded to study condition. A conference abstract related to this study was identified in our search strategy but the limited data available at that time meant the study did not contribute to the meta-analysis. Full results are now published: there was no difference in time to achieve full oral feeds between the control and intervention groups, but *post hoc* analysis revealed a trend in the subgroup of infants born before 31 weeks' gestation for babies in the intervention group (*n*= 9) to achieve full oral feeds sooner than babies in the control group (*n*= 7) (mean postnatal days 41 versus 49 *p*= 0.06), although no variance estimate was reported (Davidson et al., 2019).

The study by Lee (2019) was an observational study reporting several physiological parameters, including gastric residual volume (measured by syringe aspiration) and time to attain full oral feeds, in two groups of non-contemporaneous preterm babies (28–32 weeks' gestation) who were or were not exposed to smell of BM with tube feeds. Data on the babies who were exposed to smell of BM twice a day three times per week for 15 days were collected prospectively; data on those who did not receive smell stimulation were collected retrospectively from medical charts of gestational age-matched peers (Lee, 2019). There were no differences in gastric residual volume or attainment of full oral feeds between study groups but, in the intervention group, gastric residual volumes were significantly less on days in which the intervention was delivered compared to days on which it was not (Lee, 2019). However, this study is susceptible to serious risk of bias through its study design and does not meet the criteria for inclusion in the systematic review.

The other two studies were RCTs and randomly assigned preterm babies to intervention (smell stimulation) or control (no smell exposure) groups with smell being provided by

artificial compounds rather than breastmilk (Cao Van et al., 2018; Schriever et al., 2018). Neither trial found an effect on duration of hospitalisation or time to full oral nutrition. In both trials, the authors reported post hoc subgroup analyses, that it is not clear were prespecified, with a trend towards earlier discharge home for babies with a birth weight >2000 g (the sample size of this sub-group was not provided) in the intervention group in one trial (Cao Van et al., 2018) and shorter hospitalisation and transition to oral feeds for babies exposed to the intervention for >65% of tube feeds in the other trial (Schriever et al., 2018). Thus, inclusion of these RCTs could introduce further heterogeneity in the assessment of the effect of the intervention.

Our search strategy also identified two ongoing randomised controlled trials: the DIAMOND trial (Bloomfield et al., 2018) and the TASTE trial (Beker et al., 2019), and these should contribute to future systematic reviews. These RCTs will be sufficiently powered to assess the role of smell and taste stimulation with milk during tube feeding on important clinical outcomes such as time required to reach full enteral and oral feeds, growth, length of hospital stay, duration of parenteral nutrition, potential adverse effects associated with delivery of the intervention and others.

Ultimately, these new studies will help determine the overall effectiveness and safety of sensory stimulation with smell and taste on important health outcomes for preterm infants. To assess whether there is an "optimal timing" for effective sensory stimulation associated with the degree of prematurity (Davidson et al., 2019), or a sex-specific response to the intervention (Tottman et al., 2020b), sub-group analyses or individual participant data (IPD) meta-analyses may be warranted. Should there be an effect of exposure to smell and taste of milk on reducing hospital stay in preterm infants, this simple, non-invasive and

inexpensive intervention could be of great value to both families and health care systems by reuniting families sooner and reducing costs of hospitalisation. Furthermore, this intervention can be delivered easily by parents or caregivers, allowing them to engage in the care of their preterm baby, thereby contributing towards a positive NICU experience.

Our Cochrane review adopted stringent inclusion criteria to minimise potential confounding associated with provision of sensory stimulation outside the feeding context, such as the calming effect associated with smell of BM (Alemdar and İnal, 2020; Badiee et al., 2013) or immune modulation related to oropharyngeal administration of colostrum (OAC) (Ma et al., 2021; Nasuf et al., 2018; Panchal et al., 2019; Tao et al., 2020). Of four systematic reviews aimed at determining the effect of OAC in the prevention of infections and death (Ma et al., 2021; Nasuf et al., 2018; Panchal et al., 2019; Tao et al., 2020), three found that infants exposed to OAC (0.2 mL every 2-3 hours initiated within the first 48 hours of life) achieved full enteral feeds approximately three days earlier than infants not exposed to OAC (Ma et al., 2021; Nasuf et al., 2018; Tao et al., 2020). One review also reported that infants in the intervention group were discharged from hospital 10 (95% CI 2-10) days earlier than infants in the control group (Tao et al., 2020), but this effect was not statistically significant in the other reviews (Ma et al., 2021; Nasuf et al., 2018; Panchal et al., 2019). Again, evidence was judged to be of low quality.

The biological mechanism for the outcomes reported in these systematic reviews of OAC remains unclear. One possible mechanism is that immune protection from early OAC leads to fewer nosocomial infections and hence fewer sick babies who require shorter hospitalisation, but only one of the reviews substantiates this hypothesis as OAC was associated with reduction in the risk of necrotising enterocolitis (NEC) and ventilator-

associated pneumonia in very-low birth weight infants (Ma et al., 2021), while the other reviews found no effect of the intervention on the incidence of NEC or sepsis (Nasuf et al., 2018; Panchal et al., 2019; Tao et al., 2020). A second possible mechanism is that the provision of small amounts of colostrum into the oropharyngeal cavity leads to activation of the CPR as smell and taste receptors are expressed in the mouth, nose, oropharynx and throughout the gastrointestinal tract (Calvo and Egan, 2015; Maßberg and Hatt, 2018). This may then lead to secretion of digestive hormones and enzymes, improving tolerance to tube feedings and more rapid progression of enteral feeds resulting in early attainment of oral feeds and faster discharge home. Finally, it is possible that OAC results in improved nutrient and caloric intake, leading to faster growth and ultimately earlier discharge home, although information on actual nutritional intakes and growth outcomes were not assessed (Panchal et al., 2019; Tao et al., 2020) or were limited to a single study of low quality reporting no effect of OAC on weight at discharge (Nasuf et al., 2018).

6.1.2 ***Sensory cues in infant feeds: The story told by volatile compounds***

To identify the compounds that preterm babies are actually exposed to during smell and taste stimulation, we investigated the profile of volatile compounds present in several types of milk commonly fed to preterm infants including, importantly, preterm BM that had been fortified with a bovine-based multicomponent milk fortifier, a common approach to increasing nutritional intakes in preterm and growth-restricted babies; and novel human milk-based products aimed at providing a 100% human milk based diets to preterm infants.

In addition to finding volatile compounds in BM and infant formulas similar to those reported previously in a study of BM from 10 mothers of full term babies and 11 brands of powder and liquid formulas (Hausner et al., 2009), we found two important additional findings. First, BM with added fortifier and stored under refrigerated conditions had more volatile compounds associated with enzymatic lipolysis and lipid autoxidation compared to unfortified BM. This is particularly important for clinical practice, as guidelines recommend that BM can be fortified and stored for up to 24 hours under refrigeration (Steele, 2018). Active lipases naturally present in BM can initiate lipolysis leading to release of free FA which in turn may reduce milk pH, contributing to protein denaturation (Donovan et al., 2017), but pasteurisation can inactivate BM lipoprotein lipase and bile salt dependent lipase and may prevent further lipolysis (Peila et al., 2016).

Our results indicate that changes to the volatile components of BM were already occurring when fortified BM stored for 8 hours or longer was retrieved from dedicated refrigerators in the neonatal care unit, demonstrated by increased products of lipid breakdown (fatty acids, aldehydes, ketones) compared to fresh preterm breastmilk. These findings are consistent with previous reports of increased osmolality of fortified BM within 1-2 hours of fortification (Barbero et al., 2020; Kreins et al., 2018). Nevertheless, a direct comparison of BM samples before and after fortification would be required to confirm such an association.

Taken together, these data raise the possibility that fortification may affect BM properties in a way that could have clinical implications and further research is required. This future research should quantify changes in properties of fortified BM over 24 hours of refrigerated storage by assessing different physicochemical parameters (changes in pH,

osmolality, lipid peroxidation and volatile lipid oxidation products). It would be even more helpful if assessments were performed at regular intervals similar to feeding regimens (for example, every 2–3 hours) and address the impact of BM processing such as freeze-thaw cycles and pasteurisation. This would provide more robust evidence for optimal fortification practices and lead to improved fortification guidelines, especially for units without the support of a human milk bank and where targeted/personalised fortification is not feasible.

The second interesting finding from the analysis of volatile compounds in different milks was the abundance of aromatic hydrocarbons in human milk-based products compared to BM. Human milk-based products recently came onto the market as an alternative to common infant formulas and bovine milk-based fortifiers in an attempt to provide an exclusive human milk-based diet when fortification of BM is required and mother's own milk or donor milk is not available. Even though the concept is promising and there is a growing interest in these products, there is little evidence of benefit over current options (Grace et al., 2021). The presence of aromatic hydrocarbons in BM has been associated with exposure to anthropogenic volatile contaminants (Blount et al., 2010; Kim et al., 2007). Given the lipophilic nature of these compounds, bioaccumulation in fat tissue deposits may occur and further lipid mobilisation for milk synthesis might be the metabolic pathway by which these compounds appear in BM in detectable quantities.

Human milk-based products are produced by for-profit companies in the US and are made from pooled pasteurised BM from multiple donors (up to 250 has been reported) (Thibeau and Ginsberg, 2018). According to information available on the manufacturer's website, donors undergo health screening to confirm eligibility and donated BM is screened for several pathogens (HIV, HBV/HCV, Zika Virus) and illicit substances (Prolacta Bioscience,

2019). Mothers are paid about US$ 1 per ounce of BM (approximately 30 mL), which may add up to US$ 600-800 per month (Thibeau and Ginsberg, 2018). Ethical concerns have been raised about treating breastmilk as a commodity following reports of adulteration of BM purchased over the internet with cow's milk (Keim et al., 2015) and views of selling BM as a legitimate way to make money (O'Sullivan et al., 2018).

There is a paucity of data about sociodemographic characteristics of donors, their motivations for selling BM and whether adequate BM supply is secured for their own infants with only surplus BM being sold (Thibeau and Ginsberg, 2018). In addition, these products are commercialised at a high cost to hospitals (average US$ 200-300 daily cost per infant) and differing health insurance coverage in certain jurisdictions may heighten disparities in health care. Thus, more research is urgently needed to assess clinical, ethical and societal implications, benefits and harms of neonatal exposure to human milk-based products.

In contrast, investing in human milk banks (HMBs) appears to be more appropriate given that they are well-regulated and follow robust and evidence-based protocols (Moro et al., 2019). In addition, HMBs provide human milk to vulnerable and sick infants at low cost to hospitals and in some countries also provide free community breastfeeding advice and support. Nonetheless, the use of pasteurised donor BM (PDBM) is also the subject of debate as BM composition is highly variable between and within individuals and even with fortification PDBM may not meet protein and calorie requirements of some preterm infants (John et al., 2019) and thus may be associated with slow weight gain. Therefore, as currently the only documented benefit of feeding with PDBM compared to formula is the reduced risk of NEC in very low birth weight preterm infants (Brown et al., 2019), more research is needed

to determine the effect of PDBM feeding on other health outcomes and for babies at low risk of NEC.

Furthermore, although donated BM is submitted to thermal treatments to inactivate some bacteria and enveloped viruses this is less effective for non-enveloped viruses and bacterial spores, although new pasteurisation methods, such as high pressure processing and ultraviolet light, are being developed (Hartmann, 2019). Some thermal treatments can inactivate bio-active compounds present in fresh BM such as immunoglobulins, lactoferrin and lysozyme (Buffin et al., 2018; Paulaviciene et al., 2020), but the effect on BM macronutrients is less clear as considerable heterogeneity between studies exists, mostly due to differences in thermal treatment and the method of macronutrient analysis (Paulaviciene et al., 2020; Peila et al., 2016; Piemontese et al., 2019). Combining BM from multiple donors might minimise individual variability in macronutrient composition of PDBM (John et al., 2019), but more research is needed to ensure optimal composition of PDBM and determine potential benefits of its use for nutrition of preterm infants.

This thesis also investigated for the first time longitudinal changes in the volatile compounds in preterm BM, demonstrating that volatile compounds in preterm BM are influenced by lactation stage, maternal ethnicity, socioeconomic status and maternal diabetes. It is well established that BM composition changes with progress of lactation, but the novelty of this study relies on the analysis of a large number of samples of preterm BM and in the identification of changes in specific volatile fatty acids and fatty acids esters over the course of lactation.

Interestingly, our findings showed that medium-chain fatty acids were more abundant in colostrum compared to transitional breastmilk, with a significant increase from postnatal

day 3 to day 5. An association between the concentration of MCFAs in BM and length of gestation has been demonstrated previously (Moltó-Puigmartí et al., 2011; Thakkar et al., 2019). However, contrary to our findings, both of those studies detected higher MCFAs in transitional milk compared to colostrum (Moltó-Puigmartí et al., 2011; Thakkar et al., 2019) even though they used similar definitions for colostrum and transitional milk (<first week and between first and second week postpartum, respectively). In contrast, mature milk was defined as BM collected between 28-32 days postpartum in one study (Moltó-Puigmartí et al., 2011) and 2-16 weeks postpartum in the other (Thakkar et al., 2019). The differences between our findings and those may be explained by the small sample size in those studies or by specific characteristics of the study population such as ethnicity or socioeconomic status, which were not reported, or maternal health characteristics (both studies excluded mothers with gestational diabetes, for example).

We found that the relative concentration of FA and FAe was lower in breastmilk of mothers from low socioeconomic deciles, with diabetes and also in Pasifika mothers. In New Zealand, about 5 – 6% of women develop diabetes in pregnancy (Jowitt, 2016; Lawrence et al., 2019), with Māori, Pasifika and Indian mothers being more prone to develop GDM (Jowitt, 2016). Several studies have suggested that GDM influences the composition of BM, but evidence is conflicting (Peila et al., 2020). Lower socioeconomic status previously has been associated with lower concentrations of decanoic and dodecanoic acids, and of monounsaturated and polyunsaturated FAs (MUFAs and PUFAS), in BM of Iraqi mothers (Al-Tamer and Mahmood, 2006). Diet may also modulate the profile of FAs in BM (Nasser et al., 2010; Yahvah et al., 2015). Taken together, these results highlight the contribution of maternal characteristics to BM composition and future studies should explore further the

potential of dietary interventions during pregnancy and lactation to modulate preterm BM composition.

The clinical relevance of higher MCFA in preterm compared to term BM and in early compared to later stages of lactation remains unclear. It is possible that the immature development of the mammary gland associated with premature parturition could result in temporary synthesis of triacylglycerides with higher MCFA content, which may resolve as lactation progresses (Thakkar et al., 2019). However, another possibility could be related to preferential utilisation of these FA for energy production by preterm infant. As colostrum is produced in small amounts, a preferential oxidation of MCFA for production of energy could occur to preserve long-chain PUFAs for other essential functions such as immune protection, neurodevelopment, cell membrane formation and synthesis of bioactive lipids (Demmelmair and Koletzko, 2018; Moltó-Puigmartí et al., 2011). The endogenous synthesis of essential FA from their precursors linoleic acid and α-linolenic acid is limited and therefore these FAs are mainly sourced from maternal stores. Furthermore, MCFA have antimicrobial properties (Huang et al., 2011) and may be involved in regulation of energy homeostasis (Ichimura et al., 2009). Nevertheless, the biological and nutritional relevance of these findings are yet to be determined.

Diet-induced flavour changes to BM can influence infant feeding behaviour (Debong and Loos, 2020). In a study in which mothers consumed garlic extract capsules (Mennella and Beauchamp, 1991a) and vanilla-flavoured drink (Mennella and Beauchamp, 1996), traces of these flavours in BM could be detected within 1 hour of consumption and infants spent more time breastfeeding, sucked more and consumed more milk (measured by infant body weight before and after consumption) when consuming flavoured BM compared to breastfeeding

trials in which the mother had not consumed these flavoured foods (Mennella and Beauchamp, 1991a, 1996). In contrast, the opposite has been reported when mothers consumed small amounts of ethanol spiked into orange juice (approximately 0.3 g per Kg body weight), with infants consuming significantly less BM compared to trials in which mothers drank orange juice without alcohol (Mennella and Beauchamp, 1991b). These studies suggest that self-regulation of milk intake might be mediated by sensory cues in BM.

Because volatile compounds (xenobiotic and dietary-derived) are mostly small and hydrophobic, they can be absorbed into the maternal circulation by passive diffusion or via carrier-mediated mechanisms and can be transported to organs (liver, kidneys), where odourants undergo biotransformation into secondary metabolites before being excreted through breath, skin, urine, faeces or BM (Debong and Loos, 2020). However, the bioactivity and metabolic fate of odourants and their metabolites in BM are not fully understood and the implications for infant health and development remain to be elucidated.

We also detected anthropogenic volatile contaminants in preterm BM. We opted for an untargeted approach, as a screening method seemed more appropriate given the exploratory nature of the study. Therefore, the relationship between the presence of contaminants in preterm BM and infant outcomes (i.e. nutrition, growth) should be further investigated. Given the growing use of donor BM and human milk-based products, in which we also have detected elevated concentration of anthropogenic volatile compounds, targeted quantification approaches are required to determine the level of exposure and ascertain the potential for harm for preterm infants (Blount et al., 2010; Kim et al., 2007).

The analysis of volatile compounds detected in biological samples, often referred to as the volatilome, has received much attention given the non-invasive nature of sample

collection, relatively robust analytical platforms and the potential to inform health status (de Lacy Costello et al., 2014; Mansurova et al., 2018; Pereira et al., 2020). Different patterns of volatile compounds detected in breath, urine, saliva and faeces have been used as biomarkers to monitor pulmonary, urinary, oral and gastrointestinal tract functions, including infection and different types of cancer (Ahmed et al., 2016; Pereira et al., 2020; Schmidt and Podmore, 2015) and may even assist in the diagnosis of NEC (Wright et al., 2021). Analysis of volatile oxidation products may be used to monitor oxidation stability in infant formulas (Jia et al., 2019), lipid peroxidation and aroma changes in BM (Elisia and Kitts, 2011; Spitzer et al., 2010). However, no study so far has characterised the profile of volatile compounds in BM in the context of maternal health/disease.

6.1.3 *Smell, taste and the cephalic phase response in preterm infants: The unexpected findings*

Contrary to my initial hypothesis, there was no difference in oxygenation in the orbitofrontal cortex measured by near infrared spectroscopy between babies exposed and not exposed to smell and taste of milk before tube-feeding. Intriguingly, gastric tube feeding did increase oxygenation in the OFC, which was not expected and has not been reported previously. To some extent, this finding is reassuring as it seems possible that compounds present in milk (such as volatile compounds, fatty acids and sugars) may act on olfactory and gustatory receptors present in the gastrointestinal tract (Calvo and Egan, 2015; Maßberg and Hatt, 2018), or even in the oropharynx via regurgitation (Omari et al., 2002), leading to activation of the OFC without requiring direct administration of sensory input from smell and

taste. Nonetheless, the underlying mechanism for cerebral activation of the OFC during tube feeds remains to be determined.

We also observed a sex-difference in the pattern of oxygenation in the OFC upon exposure to smell, in agreement with previous studies (Frie et al., 2020), raising the possibility that the development of sensory perception differs between boys and girls. Growing evidence suggest that boys and girls have different health outcomes (Cheng et al., 2016; Galante et al., 2020a; Tottman et al., 2020a, 2020b). Therefore sex-differences should be accounted for in the care preterm babies and also should be considered when conducting research and reporting findings.

We were unable to confirm that provision of smell and taste of milk with tube feedings can trigger CPRs, at least in a way that could be detected by monitoring oxygenation in the OFC with NIRS in a real life setting within the NICU environment. It is possible that the approach used was not ideal for this purpose, at least for assessments performed in a clinical setting. Assessments were intentionally conducted at the cotside in the context of regular feeding of preterm infants and most babies were awake during the assessments. It is possible that other sensory stimulation (light, noise, smells from the local environment) interfered with measurement of the activation of the OFC by smell and taste, given that the OFC integrates information from the five sensory modalities (visual, auditory, somatosensory, gustatory and olfactory) (Kringelbach, 2005). In addition, visible reactions to smell (sniffing and head movements) and taste (lips/tongue movement and sucking) were observed in 10 out of 16 babies in the intervention group, but these were recorded by the investigator performing the assessment and only for babies exposed to smell and taste of milk.

We attempted to assess the potential for smell and taste to elicit a CPR by measuring changes in cortical oxygenation. Considering the endocrine effect of CPR, future studies investigating exposure to smell and taste of milk in preterm infants would benefit from the measurement of key hormones such as insulin, glucagon, GLP1, ghrelin in response to provision of smell and taste compared with sham exposure prior to feeding. Blood sampling is an invasive procedure and should only be considered in research involving babies when alternatives are not available. On the other hand, collection of salivary hormones is non-invasive and seems to be well tolerated by infants (Maron et al., 2015) and, therefore, could be an alternative to blood sampling.

Growing interest in salivary biomarkers as a non-invasive complementary diagnostic tool has been propelled by high-throughput 'omics' technologies permitting global or targeted gene and metabolite analysis in body fluids with low metabolite concentration (Pappa et al., 2019; Yen et al., 2021), including salivary hormones (Goodson et al., 2014; Shi et al., 2015) and RNA (Maron et al., 2015). Whole-transcriptome analysis of salivary biomarkers led to the identification of five key regulatory genes differently expressed amongst preterm babies classified as successful and non-successful oral feeders (Maron et al., 2015), raising the prospect of utilising a genomic approach to the assessment of smell and taste stimulus on the physiology of the preterm infant. However, microRNA analysis yields binary results: either genes are expressed or not, and thus clinical applicability is limited. In contrast, investigation of the salivary proteome with immunoassays allows quantification of targeted proteins at relative low cost (Khanna et al., 2017). For example, proteins involved in hunger signalling and energy homeostasis (neuropeptide Y2 receptor, NPY2R; and adenosine-monophosphate-activated protein kinase, AMPK) were detected and quantified in saliva of

preterm infants and their expression permitted clear differentiation between successful and non-successful oral feeders (Khanna et al., 2017). These findings led to the development of an ongoing multicentre RCT, the NOuRISH trial (Neonatal Oral Feeding-readiness In Salivary High-throughput diagnostics), investigating diagnostic power of these salivary biomarkers to predict acquisition of oral feeding skills in extremely preterm infants (Barlow et al., 2017).

As an emerging field of research, several challenges remain to be addressed for adoption of salivary biomarkers into clinical practice. These include: 1) determination of normative thresholds to distinguish between healthy and sick individuals with validation and normalisation against plasma biomarkers; 2) understanding and accounting for potential influence of nutritional status (prandial state), age, sex and developmental stages on salivary biomarkers; 3) adequate sample collection, stabilisation and processing protocols to ensure standardisation, and 4) volume of sample required, as limited production of saliva occurs in preterm infants (Hassaneen and Maron, 2017; Yen et al., 2021). For example, a systematic review of studies investigating salivary cortisol reactivity to NICU procedures in preterm infants reported a success rate of less than 80% for 6/16 studies, with only four studies reporting a success rate >90% (Mörelius et al., 2016).

The amount of saliva required varies with the methodological approach. A method for measuring salivary cortisol in preterm infants has been validated in samples of 100 µL (Neu et al., 2007) but smaller volumes of 10-25 µL of saliva have been used to quantify metabolic hormones such as insulin, ghrelin and leptin in 11-year old children (Goodson et al., 2014) and salivary mRNA in infants (Maron et al., 2015).

In general, salivary biomarkers are present at lower concentrations compared to plasma and correlations between plasma and saliva concentrations have been reported to vary

between an R^2> 0.6 for glucose (Patel et al., 2015) to an R^2 > 0.9 for IgG profiles in healthy adults (Hettegger et al., 2019), with an R^2> 0.7 reported for levels of 13 drugs, including caffeine, in preterm infants (Hutchinson et al., 2018). Nonetheless, analysis of correlations between salivary and plasma biomarkers in preterm population are scarce and thus clinical applicability as diagnostic tool remains to be explored.

6.2 Limitations

Some limitations of the research reported in this thesis must be acknowledged. Breastmilk samples collected within the DIAMOND trial created the opportunity to obtain a representative number of samples of preterm BM. However, as part of a large RCT, the demographic information collected was restricted to that required for the main trial. Maternal information was limited to sociodemographic and perinatal information (delivery mode, antenatal steroid course and maternal diabetes). This research could have been improved if other factors were known, such as maternal body mass index, diet, and smoking.

The study population was composed solely of MLP preterm babies and their mothers. Therefore, it is unreasonable to extrapolate current findings to other infants, either more preterm or full-term. For example, it is not possible to ascertain if longitudinal changes of volatile FA and FAe in preterm BM also occur in BM produced by mothers of very preterm infants, even though an inverse association between the concentration of MCFAs in BM and length of gestation has been demonstrated previously (Moltó-Puigmartí et al., 2011; Thakkar et al., 2019).

Only one recruiting site had the NIRS device available which meant that participants in this sub-study were limited to babies recruited at Auckland Hospital whose parents consented to additional assessment with NIRS. A larger sample size with a better distribution of moderate and late preterm babies, as well as babies fed with formula and BM, would have enabled us to explore the effect of gestational age and feeding mode on activation of the OFC. Furthermore, the NIRS device used had a limited range of optodes that were not ideal for preterm babies. Newer devices have more optode pairs and head caps designed to fit tiny heads and which secure sensors in the appropriate position, providing better spatial resolution and more accurate assessment of cerebral oxygenation in preterm infants. Furthermore, considering visible reaction to smell and taste displayed by some babies, assessments should have been recorded and infants' reaction assessed by an investigator blinded to the intervention.

6.3 Conclusion

This thesis has expanded our current understanding of sensory exposure to smell and taste of milk in tube-fed premature infants. Currently, it is not possible to recommend the provision of smell and taste of milk to accelerate feeding in preterm infants. However, the systematic review should be updated as findings of new studies are made available. The profiling of odour-active volatile compounds in preterm BM and several milks fed to preterm infants revealed that fatty acids and their esters are the major constituents responsible for the sensory cues in preterm breastmilk, supporting the evidence for discriminatory physiological responses exhibit by infants exposed to different smells. Some maternal factors

known to influence breastmilk composition potentially could be modulated with dietary and life style interventions and should be further explored to improve breastmilk composition for nutrition of preterm infants. Additionally, our findings indicate that urgent further research is needed into the impact of refrigerated storage of fortified breastmilk in accordance with current guidelines to ensure that this is not leading to changes in the physicochemical properties of the milk that could be detrimental. Even though the effect of exposure to smell and taste of milk on improving nutrition of preterm babies remains uncertain, there may be more appropriate approaches to measure CPR in preterm infants and sex differences should be considered in future studies. The development and validation of non-invasive diagnostic tools in preterm population is much needed and in future will provide complementary information about infants' health and metabolism to guide clinical decisions.

Taken together, the comprehensive research presented within this thesis demonstrates that preterm babies are exposed to a variety of sensory stimulation during the postnatal period and are able to respond to stimulations arising from their environment. Boys and girls may establish sensory perception at different developmental stages, reinforcing that the traditional "one size fits all" model of neonatal care is outdated.

APPENDICES

Appendix I: Exposure to the smell and taste of milk to accelerate feeding in preterm infants (Protocol)

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[Intervention Protocol]

Exposure to the smell and taste of milk to accelerate feeding in preterm infants

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ABSTRACT

This is a protocol for a Cochrane Review (Intervention). The objectives are as follows:

To determine whether exposure to the taste or smell (or both) of milk administered with tube feedings can accelerate progress to full sucking feeds without adverse effects in preterm infants. We will also assess these effects in subgroups with different modes of administration, gestational age, birthweight, and type of milk.

BACKGROUND

Description of the condition

Because of immaturity of neurologic and digestive systems, preterm infants (born before 37 weeks' gestation) are often unable to co-ordinate sucking, swallowing, and breathing in order to feed. Initial nutrition is usually provided intravenously and via a tube which goes through the nose or mouthinto the stomach, with a gradual transition to sucking feeds as co-ordination improves (Toce 1987). Usually, enteral feeds (feeds provided via the gut) start at small volumes and are increased slowly until full enteral feeds are tolerated.

Feeding intolerance is defined as the inability to digest enteral feedings in association with increased gastric residuals (fluid remaining in the stomach after tube feeds), abdominal distension or vomiting, or both (Moore 2011). It often leads to a delay in attainment of full enteral feeds and prolonged intravenous nutrition (Fanaro 2013). Prolonged intravenous nutrition can increase the risk of: infection; cholestasis (impaired bile flow) (Gargasz 2012); impaired development of the gut mucosa;necrotising enterocolitis (severe intestinal inflamation) (Fanaro 2013); and increased morbidity and mortality (The SIFT Investigators Group 2013).

Taste and smell are important for the appreciation of food but also have a significant role in nutrition. In response to sensory cues, a sequence of pre-absorptive physiological responses is triggered by the brain, collectively referred to as cephalic phase responses (Smeets 2010).

The cephalic phase response plays an important role in the activation of physiological processes at multiple sites to optimise digestion, including increased salivation, increased peristaltic movements, and increased secretion of digestive enzymes and digestive-related hormones, all of which are active in the newborn (Lipchock 2011; Mattes 1997; Zolotukhin 2013).

The pathways underlining the cephalic phase response to taste and smell stimulation are diverse and stimulate different parts of the

digestive system. First, the increase in salivation starts the process of digestion as a result of the presence of salivary enzymes (such as α-amylase and lingual lipase), salivary insulin, and the moistening of the digestive bolus to assist swallowing. Further down the gastrointestinal tract, the cephalic phase response initiates the release of gastric secretions containing gastrin, gastric acid, trypsin and gut peptides. It also initiates the release of hormones such as ghrelin, glucagon-like peptide-1, leptin and somatostatin, as well as increasing gut motility. Smell and taste also are known to stimulate gastric emptying by increasing contraction of segments of the gastrointestinal tract. Lastly, the release of pancreatic secretions that are rich in digestive enzymes such as lipase, amylase and cholecystokinin assist further digestion of nutrients. In addition to the pancreatic secretions released in the gut, the pancreas also releases insulin and glucagon into the bloodstream in response to sensory stimulation.

All of the responses described above contribute to food digestion and absorption (Mattes 1997; Zolotukhin 2013). However, little is known about the effects of taste and smell stimulation in preterm infants, despite the presence of functional taste receptors from 18 weeks' gestation and flavour perception from around 24 weeks' gestation (Lipchock 2011).

Fetal swallowing of amniotic fluid starts by the end of the first trimester and reaches up to 750 mL/day by 34 weeks' gestation (Dasgupta 2016). Thus, fetal smell and taste receptors are exposed to the components of amniotic fluid for many weeks before birth and at equivalent gestations to those of infants born preterm (Bloomfield 2017), suggesting that the first sensory experiences happen *in utero*.

Distinct olfactory reflexes have been demonstrated in neonates after 32 weeks of gestation, with infants presenting different responses to the smell of substances such amniotic fluid, colostrum or peppermint oil, varying from sucking response alone to a combination of sucking and arousal-withdrawal reflex (Bingham 2003; Marlier 1998; Sarnat 1978). These findings suggest that the olfactory system is fully functional in preterm infants after 32 weeks of gestational age.

Tube feedings bypass the oral and nasal cavities, so tube-fed infants have limited exposure to the taste and smell of their feeds. Therefore, there is little stimulation of the cephalic phase response of digestion, which is important for digestion and tolerance of feeds. The provision of smell and taste exposure to preterm infants receiving tube feedings is currently being applied in the care of some preterm infants based on the assumption that there is biological plausibility for a possible benefit, despite lack of evidence to support this practice. More importantly, potential adverse effects have not been assessed; these could include risks such as aspiration, gagging or choking, bradycardia, desaturations or increase in oxygen requirement.

Description of the intervention

The intervention consists of placing a few drops of the milk with which the infant is being fed on the infant's lips and tongue in order to provide the taste of milk, and placing a cotton bud or gauze soaked with a few drops of milk close to the infant's nostril to provide the smell of milk. The exposure should be done before starting the tube feed in order stimulate the cephalic phase response of digestion.

How the intervention might work

Preterm infants being fed via gastric tube have limited exposure to the taste and smell stimulation which trigger the cephalic phase response of digestion, and this might contribute to feeding intolerance and prolonged intravenous nutrition.

Exposure to the taste and smell of milk before tube feeding may stimulate the cephalic phase response of digestion and assist digestion by increasing salivation, triggering peristaltic movements of gut, secretion of digestive enzymes and release of digestion-related hormones such as ghrelin, leptin, gastrin, insulin and others (Power 2008).

Why it is important to do this review

Prolonged exposure to intravenous nutrition increases the risk of late-onset sepsis, prolonging hospital stay and increasing health costs. In addition, delayed enteral feeding can result in degeneration of gastrointestinal mucosa and increase the risk of necrotising enterocolitis once the tube feedings start, impacting significantly on infant survival and hospital costs (Johnson 2014). Thus any interventions that accelerate transition to enteral feedings, and then to sucking feedings, would be of considerable potential benefit to infants, their families and the healthcare system.

It is increasingly common for staff in neonatal nurseries to include exposure to the smell or taste (or both) of milk in the process for tube feeding preterm infants. This is largely based on the belief that this must be beneficial , which could lead to performance bias when assessing the effects of the intervention. Furthermore, this additional intervention requires staff time (and therefore cost), and there is also the potential for adverse effects such as choking or aspiration. Reliable evidence is required on the benefits as well as possible risks of this intervention.

OBJECTIVES

To determine whether exposure to the taste or smell (or both) of milk administered with tube feedings can accelerate progress to full sucking feeds without adverse effects in preterm infants. We will also assess these effects in subgroups with different modes of administration, gestational age, birthweight, and type of milk.

METHODS

Criteria for considering studies for this review

Types of studies

We will include any published or unpublished randomised or quasi-randomised trials where the unit of randomisation is the infant, or cluster-randomised trials where the neonatal unit or hospital is the unit of randomisation. We will exclude cross-over trials and non-randomised trials such as controlled before-and-after studies.

Types of participants

Preterm infants (born before 37 weeks' gestation) of both sexes and all ethnicities who are receiving any enteral feeds and have not yet reached full sucking feeds.

Types of interventions

We will include studies that report the exposure to the taste or smell (or both) of breast milk or formula milk, immediately before or at the time of tube feedings.

For smell stimulation, we will include in this review interventions that consist of delivering the smell of milk to preterm infants using a gauze with a few drops of milk placed in the cot/incubator close to the infants' nose, or using a cotton bud soaked with milk or other forms of administration of the smell of milk (eg. using an olfactometer adapted to a pacifier).

For taste stimulation, we will include in this review studies that report placing a few drops of milk on the infant's lips or tongue using a syringe, or other forms of oral administration of a small amount of milk (eg. using a pacifier dipped in milk).

We will explore the following comparisons.

1. Exposure to the smell and taste of milk with tube feeds versus no exposure
2. Exposure to the smell of milk with tube feeds versus no exposure
3. Exposure to the taste of milk with tube feeds versus no exposure

Types of outcome measures

Primary outcomes

1. Time to reach full sucking feeds (defined as the removal of the feeding tube), measured in days.
2. Adverse effects related to the intervention, such as aspiration, gagging/choking, bradycardia, desaturations or increase in oxygen requirement during the intervention period.

Secondary outcomes

1. Duration of parenteral nutrition (defined as the removal of intravenous nutrition line), measured in days.
2. Time to reach full enteral feedings (150 ml/Kg/day, or as defined by the trialists), measured in days.
3. Feed intolerance (resulting in cessation or reduction in enteral feeding), during the period of hospitalisation.
4. Necrotising enterocolitis (Bell's stage 2 or more) (Walsh 1986), during the period of hospitalisation.
5. Late infection (bacterial or fungal infection confirmed by presence of blood or cerebrospinal fluid infection with initiation of symptoms beyond 48 hours after birth) (ANZNN 2015), during the period of hospitalisation..
6. Growth from birth to discharge (weight, height/length, head circumference and z-scores; gain in these parameters from birth to 36 weeks' postmenstrual age or to term equivalent age; body composition).
7. Exclusive breastfeeding at time of discharge (WHO 2008).
8. Time to first discharge home, measured in days.

Search methods for identification of studies

We will use the criteria and standard methods of Cochrane and Cochrane Neonatal (see the Cochrane Neonatal search strategy for specialized register). We will search for errata or retractions from included studies published in full-text on PubMed (www.ncbi.nlm.nih.gov/pubmed) and report the date this was done within the review. We will not limit the search to any particular geographical region, language or timing of publication.

Electronic searches

We will conduct a comprehensive search of databases, including: Cochrane Central Register of Controlled Trials (CENTRAL, current issue) in the Cochrane Library; MEDLINE via PubMed (1996 to current); Embase (1980 to current); and CINAHL (1982 to current), using search terms unique to the review topic, plus database-specific limiters for randomised controlled trials (RCTs) and neonates (see Appendix 1 for the full search strategies for each database). We will search clinical trials registries for protocols of ongoing or recently completed trials (clinicaltrials.gov; ICTRP; ANZCTR and the ISRCTN Registry). We will search using both English and American spelling. We will not apply language restrictions.

Searching other resources

Additionally, we will review the reference lists of all identified articles for relevant articles not identified in the primary search. We will also approach well-known researchers in this area to identify any unpublished or ongoing research.

Data collection and analysis

We will follow the standard data collection methods of the Cochrane Neonatal Group.

Selection of studies

Two authors will independently evaluate and appraise the retrieved studies, following the steps below.

1. Screen titles and abstracts to select relevant reports and exclude studies not relevant for this review
2. Access the full text of potentially relevant reports
3. Use a reference software management to combine search results and remove duplicate records of the same report and combine multiple reports of the same study
4. Examine full-text studies for compliance with the eligibility criteria determined for this review
5. Where appropriate, corresponded with study authors in order to elucidate any issue regarding selected studies, such as request for missing results or complementary information
6. Make final decisions on study inclusion and proceed to data collection

We will resolve disagreements by discussion and if necessary, a third review author will mediate for differences in interpretation. We will record the selection process in sufficient detail to complete a PRISMA flow diagram (Moher 2009), and 'Characteristics of excluded studies' table.

Data extraction and management

We will develop a data extraction form to extract data from eligible studies. Two review authors will independently extract data from each eligible study. Information extracted will include, but not be limited to: source details, eligibility assessment, methodological details, characteristics of participants, details of intervention and outcomes reported. Any disagreement will be resolved by discussion and if necessary in discussion with a third review author. We will enter details from the data extraction form into Review Manager 5 (Review Manager 2014). Where review authors are authors of an included trial, we will ensure that those authors are excluded from any decision-making regarding inclusion of the trial in this review, and they will not be involved in data extraction or quality assessment relating to that trial.

Assessment of risk of bias in included studies

Two review authors will independently assess the risk of bias (low, high, or unclear) of all included trials using the Cochrane 'Risk of bias' tool (Higgins 2017). We will assess the risk of bias the following domains.

1. Sequence generation (selection bias)
2. Allocation concealment (selection bias)
3. Blinding of participants and personnel (performance bias)
4. Blinding of outcome assessment (detection bias)
5. Incomplete outcome data (attrition bias)
6. Selective reporting (reporting bias)
7. Any other bias

Any disagreements will be resolved by discussion or by a third assessor. See Appendix 2 for a more detailed description of risk of bias for each domain. We will enter the assessed risk of bias into Review Manager 5 (Review Manager 2014).

Measures of treatment effect

We will use the numbers of events in the control and intervention groups of each study to calculate risk ratios (RRs) for dichotomous data. We will calculate mean differences (MDs) between treatment groups where outcomes are measured in the same way for continuous data. Where outcomes are measured differently we will report data as standardised mean differences (SMDs). We will report risk differences (RDs), and where a significant effect is found we will calculate the numbers needed to treat to benefit (NNTB) or the numbers needed to treat to harm (NNTH). We will report 95% confidence intervals (CIs) for all outcomes.

Unit of analysis issues

The unit of analysis will be the participating infant in individually randomised trials and an infant will be considered only once in the analysis. The participating neonatal unit or section of a neonatal unit or hospital will be the unit of analysis in cluster randomised trials. We will analyse them using an estimate of the intra-cluster correlation coefficient (ICC) derived from the trial (if possible), or from a similar trial or from a study with a similar population as described in the *Cochrane Handbook for Systematic Reviews of Interventions* (Higgins 2011). If we use ICCs from a similar trial or from a study with a similar population we will report this and conduct a sensitivity analysis to investigate the effect of variation in the ICC.

If we identify both cluster-randomised trials and individually randomised trials, we will only combine the results from both if there is little heterogeneity between the study designs, and the interaction between the effect of the intervention and the choice of randomisation unit is considered to be unlikely.

Any possible heterogeneity in the randomisation unit will be acknowledged and a sensitivity analysis will be performed to investigate possible effects of the randomisation unit.

Dealing with missing data

We intend to carry out analysis on an intention-to-treat basis for all outcomes, where feasible. We will analyse all participants in the treatment group to which they were randomised, regardless of the actual treatment received, whenever possible. If important missing data (in the outcomes) or unclear data are identified, we will request the missing data by contacting the original investigators.

We will make explicit the assumptions of any methods used to deal with missing data. We may perform sensitivity analyses to assess how sensitive results are to reasonable changes in the assumptions that are made. We will address the potential impact of missing data on the findings of the review in the 'Discussion' section.

Assessment of heterogeneity

We will observe the clinical and methodological characteristics of the included studies in order to assess if the studies are sufficiently similar for meta-analysis to provide a clinically meaningful summary. We will do this by inspecting the forest plots and assessing statistical heterogeneity using the Chi^2 test and the I^2 statistic, considering the guidelines recommended by the Cochrane Neonatal Group for interpretation of results. We will consider an I^2 value of less than 25% to represent no heterogeneity; 25% to 49% to represent low heterogeneity; 50% to 74% to represent moderate heterogeneity; and more than 75% to represent high heterogeneity.

We will consider an I^2 value greater than 50% and a low P value (less than 0.10) in the Chi^2 test for heterogeneity to indicate substantial heterogeneity (Deeks, 2017). If we detect substantial heterogeneity, we will explore possible explanations in sensitivity/subgroup analyses. We will take statistical heterogeneity into account when interpreting the results, especially if there is any variation in the direction of effect.

Assessment of reporting biases

We intend to conduct a comprehensive search for eligible studies and we will be alert for duplication of data. If 10 or more trials are identified for meta-analysis, we will assess possible publication bias by inspection of a funnel plot. If we uncover reporting bias that could, in the opinion of the authors, introduce serious bias, we plan to conduct a sensitivity analysis to determine the effect of including and excluding these studies in the analysis.

Data synthesis

We will evaluate studies for potential clinical diversity and restrict meta-analysis to situations where clinical consistency is apparent. Where substantial heterogeneity is detected, we will test the potential causes in subgroup and sensitivity analysis. We will use a fixed-effect model to combine data where it is reasonable to assume that studies were estimating the same underlying treatment effect. If there is evidence of clinical heterogeneity we will try to explain this based on the different study characteristics and subgroup analyses.

Quality of evidence

We will use the Grading of Recommendations Assessment, Development and Evaluation (GRADE) approach, as outlined in the GRADE Handbook (Schünemann 2013), to assess the quality of evidence for the following (clinically relevant) outcomes.

1. Time to reach full sucking feeds (defined as the removal of the feeding tube), measured in days
2. Time to reach full enteral feedings (150 ml/Kg/day, or as defined by the trialists), measured in days
3. Feed intolerance (resulting in cessation or reduction in enteral feeding)
4. Duration of parenteral nutrition (defined as the removal of the intravenous nutrition line), measured in days
5. Necrotising enterocolitis (Bell's stage 2 or more) (Walsh 1986)
6. Late infection (bacterial or fungal infection confirmed by presence of blood or cerebrospinal fluid infection with initiation of symptoms beyond 48 hours after birth) (ANZNN 2015)
7. Adverse effects related to intervention such as aspiration, gagging/choking, bradycardia, desaturations or increase in oxygen requirement

Two review authors will independently assess the quality of the evidence for each of the outcomes above. We will consider evidence from randomised controlled trials as high quality, but downgrade the evidence by one level for serious (or two levels for very serious) limitations based upon the following: design (risk of bias), consistency across studies, directness of the evidence, precision of estimates and presence of publication bias. We will use GRADEpro GDT to create a 'Summary of findings' table to report the quality of the evidence for these specified outcomes.

The GRADE approach results in an assessment of the quality of a body of evidence in one of the four following grades.

1. High: we are very confident that the true effect lies close to that of the estimate of the effect.
2. Moderate: we are moderately confident in the effect estimate: the true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different.
3. Low: our confidence in the effect estimate is limited: the true effect may be substantially different from the estimate of the effect.
4. Very low: we have very little confidence in the effect estimate: the true effect is likely to be substantially different from the estimate of effect.

Subgroup analysis and investigation of heterogeneity

Where we identify sufficient data, we plan to carry out the following subgroup analyses using a fixed-effect model.

1. Type of administration of smell exposure (cotton swab or similar soaked with milk placed close to infants' nostril versus placed by the infant's side)
2. Type of administration of taste (cotton swab or similar soaked with milk placed on infant's lips and tongue versus syringe administration of milk directly onto the infant's lips and tongue versus use of pacifier to deliver taste of milk)

3. Type of exposure (provision of smell and taste versus provision of taste only versus provision of smell only)

4. Gestational age (less than 28 weeks' versus 28 to less than 32 weeks' versus 32 to less than 37 weeks' postmenstrual age)

5. Type of diet (exclusively human milk versus formula versus human milk + formula)

6. Intrauterine growth restricted or small for gestational age (less than 10th centile or as defined by the trialists) versus appropriately grown at birth

We will investigate whether the results of subgroup analyses are significantly different by examining the overlap of confidence intervals and performing the test for subgroup differences available in Review Manager 5 software (Review Manager 2014).

Sensitivity analysis

Where we identify substantial heterogeneity, we will conduct sensitivity analysis to determine if the findings are affected by inclusion of only those trials considered of adequate methodology with a low risk of selection and performance bias. We will report results of sensitivity analyses for primary outcomes only.

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* *Indicates the major publication for the study*

APPENDICES

Appendix 1. Search strategies

PubMed:
(((((((((Taste[MeSH] OR Taste Perception[MeSH] OR Smell[Mesh] OR Olfactory Perception[Mesh] OR Odorants[MeSH])) OR ((taste*[tiab] OR tasting[tiab]))) OR gustat*[tiab]) OR ((smell*[tiab] or smelt[tiab]))) OR olfact*[tiab]) OR odor*[tiab])) AND (((((Milk, Human[MeSH] OR Infant Formula[MeSH] OR Colostrum[MeSH])) OR ((milk*[tiab] or breastmilk*[tiab]))) OR formula*[tiab]) OR ((colostrum[tiab] or colostral[tiab])))) AND ((((infant, newborn[MeSH] OR newborn OR neonate OR neonatal OR premature OR low birth weight OR VLBW OR LBW or infan* or neonat*) AND (randomized controlled trial [pt] OR controlled clinical trial [pt] OR randomized [tiab] OR placebo [tiab] OR drug therapy [sh] OR randomly [tiab] OR trial [tiab] OR groups [tiab]) NOT (animals [mh] NOT humans [mh])))
Embase:

1	exp taste/
2	(taste$ or tasting).ti,ab.
3	gustat$.ti,ab.
4	exp odor/
5	exp smelling/
6	(smell$ or smelt).ti,ab.
7	olfact$.ti,ab.
8	odo?r$.ti,ab.
9	1 or 2 or 3 or 4 or 5 or 6 or 7 or 8
10	exp breast milk/
11	(milk$ or breastmilk$).ti,ab.
12	exp artificial milk/
13	formula$.ti,ab.
14	exp colostrum/
15	(colostrum or colostral).ti,ab.
16	10 or 11 or 12 or 13 or 14 or 15
17	(infan* or newborn or neonat* or premature or very low birth weight or low birth weight or VLBW or LBW).mp
18	exp infant/

(*Continued*)

19	17 or 18
20	(human not animal).mp.
21	(randomized controlled trial or controlled clinical trial or randomized or placebo or clinical trials as topic or randomly or trial or clinical trial).mp
22	19 and 20 and 21
23	9 and 16 and 22

CINAHL:

S1	(MH "Taste")
S2	TI (taste* OR tasting) OR AB (taste* OR tasting)
S3	TI gustat* OR AB gustat*
S4	(MH "Smell")
S5	(MH "Odors")
S6	TI (smell* OR smelt OR olfact* OR odor*) OR AB (smell* OR smelt OR olfact* OR odor*)
S7	S1 OR S2 OR S3 OR S4 OR S5 OR S6
S8	(MH "Milk, Human+")
S9	(MH "Infant Formula")
S10	(MH "Colostrum")
S11	TI (milk* OR breastmilk* OR formula* OR colostrum OR colostral) OR AB (milk* OR breastmilk* OR formula* OR colostrum OR colostral)
S12	S8 OR S9 OR S10 OR S11
S13	(infan* OR newborn OR neonat* OR premature OR low birth weight OR VLBW OR LBW) AND (randomized controlled trial OR controlled clinical trial OR randomized OR placebo OR clinical trials as topic OR randomly OR trial OR PT clinical trial)
S14	S7 AND S12 AND S13

CRS Web:

1	MESH DESCRIPTOR Taste EXPLODE ALL AND CENTRAL:TARGET
2	MESH DESCRIPTOR Taste Perception EXPLODE ALL AND CENTRAL:TARGET
3	MESH DESCRIPTOR Smell EXPLODE ALL AND CENTRAL:TARGET
4	MESH DESCRIPTOR Olfactory Perception EXPLODE ALL AND CENTRAL:TARGET
5	MESH DESCRIPTOR Odorants EXPLODE ALL AND CENTRAL:TARGET
6	(taste* or tasting):ti,ab AND CENTRAL:TARGET
7	gustat*:ti,ab AND CENTRAL:TARGET
8	(smell* or smelt):ti,ab AND CENTRAL:TARGET
9	olfact*:.ti,ab AND CENTRAL:TARGET
10	odor*:ti,ab AND CENTRAL:TARGET
11	#1 OR #2 OR #3 OR #4 OR #5 OR #6 OR #7 OR #8 OR #9 OR #10
12	MESH DESCRIPTOR Milk, Human EXPLODE ALL AND CENTRAL:TARGET
13	MESH DESCRIPTOR Infant Formula EXPLODE ALL AND CENTRAL:TARGET
14	MESH DESCRIPTOR Colostrum EXPLODE ALL AND CENTRAL:TARGET
15	(milk* or breastmilk*):ti,ab AND CENTRAL:TARGET
16	formula*:ti,ab AND CENTRAL:TARGET
17	(colostrum or colostral):ti,ab AND CENTRAL:TARGET
18	#12 OR #13 OR #14 OR #15 OR #16 OR #17
19	(infan* or newborn or neonat* or premature or preterm or very low birth weight or low birth weight or VLBW or LBW) AND CENTRAL:TARGET
20	#11 AND #18 AND #19

Appendix 2. Risk of bias tool

We will use the standard methods of Cochrane and Cochrane Neonatal to assess the methodological quality of the trials. For each trial, we will seek information regarding the method of randomisation, blinding and reporting of all outcomes of all the infants enrolled in the trial. We will assess each criterion as being at a low, high, or unclear risk of bias. Two review authors will separately assess each study. We will resolve any disagreement by discussion. We will add this information to the table Characteristics of included studies. We will evaluate the following issues and enter the findings into the risk of bias table:

1. Sequence generation (checking for possible selection bias). Was the allocation sequence adequately generated?

For each included study, we will categorise the method used to generate the allocation sequence as:

- low risk (any truly random process e.g. random number table; computer random number generator);
- high risk (any non-random process e.g. odd or even date of birth; hospital or clinic record number); or
- unclear risk.

2. Allocation concealment (checking for possible selection bias). Was allocation adequately concealed?

For each included study, we will categorise the method used to conceal the allocation sequence as:

- low risk (e.g. telephone or central randomisation; consecutively numbered sealed opaque envelopes);
- high risk (open random allocation; unsealed or non-opaque envelopes, alternation; date of birth); or
- unclear risk

3. Blinding of participants and personnel (checking for possible performance bias). Was knowledge of the allocated intervention adequately prevented during the study?

For each included study, we will categorise the methods used to blind study participants and personnel from knowledge of which intervention a participant received. Blinding will be assessed separately for different outcomes or class of outcomes. We will categorise the methods as:

- low risk, high risk or unclear risk for participants; and
- low risk, high risk or unclear risk for personnel.

4. Blinding of outcome assessment (checking for possible detection bias). Was knowledge of the allocated intervention adequately prevented at the time of outcome assessment?

For each included study, we will categorise the methods used to blind outcome assessment. Blinding will be assessed separately for different outcomes or class of outcomes. We will categorise the methods as:

- low risk for outcome assessors;
- high risk for outcome assessors; or
- unclear risk for outcome assessors.

5. Incomplete outcome data (checking for possible attrition bias through withdrawals, dropouts, protocol deviations). Were incomplete outcome data adequately addressed?

For each included study and for each outcome, we will describe the completeness of data including attrition and exclusions from the analysis. We will note whether attrition and exclusions were reported, the numbers included in the analysis at each stage (compared with the total randomised participants), reasons for attrition or exclusion where reported, and whether missing data were balanced across groups or were related to outcomes. Where sufficient information is reported or supplied by the trial authors, we will re-include missing data in the analyses. We will categorise the methods as:

- low risk (< 20% missing data);
- high risk (≥ 20% missing data); or
- unclear risk.

6. Selective reporting bias. Are reports of the study free of suggestion of selective outcome reporting?

For each included study, we will describe how we investigated the possibility of selective outcome reporting bias and what we found. For studies in which study protocols were published in advance, we will compare prespecified outcomes versus outcomes eventually reported in the published results. If the study protocol was not published in advance, we will contact study authors to gain access to the study protocol. We will assess the methods as:

- low risk (where it is clear that all of the study's prespecified outcomes and all expected outcomes of interest to the review have been reported);
- high risk (where not all the study's prespecified outcomes have been reported; one or more reported primary outcomes were not prespecified outcomes of interest and are reported incompletely and so cannot be used; study fails to include results of a key outcome that would have been expected to have been reported); or

- unclear risk.

7. Other sources of bias. Was the study apparently free of other problems that could put it at a high risk of bias?
For each included study, we will describe any important concerns we had about other possible sources of bias (for example, whether there was a potential source of bias related to the specific study design or whether the trial was stopped early due to some data-dependent process). We will assess whether each study was free of other problems that could put it at risk of bias as:

- low risk;
- high risk;
- unclear risk

If needed, we plan to explore the impact of the level of bias through undertaking sensitivity analyses.

CONTRIBUTIONS OF AUTHORS

Mariana Muelbert wrote the first draft of the protocol and subsequent drafts, with significant editorial assistance by JH. All review authors contributed to subsequent drafts and approved the final version.

DECLARATIONS OF INTEREST

Frank Bloomfield and Jane Harding have designed and are steering committee members of a randomised controlled trial of different nutritional approaches to feeding moderate-to late-preterm infants that includes provision of smell and taste as one intervention. Mariana Muelbert is one of the research team members on this trial (DIAMOND Trial, Australian New Zealand Clinical Trials Registry ACTRN12616001199404).

There is no other conflict and in particular no benefits of any kind have been received by the authors in relation to any element of the proposed review.

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2. Jane Harding: None known.
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Appendix II: Nutritional policies for late preterm and early term infants - can we do better?

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Nutritional policies for late preterm and early term infants – can we do better?

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ABSTRACT

Late preterm (LP) and early term (ET) infants can be considered the "great dissemblers": they resemble healthy full-term infants in appearance, but their immaturity places them at increased risk of poor short- and long-term outcomes. Nutritional requirements are greater than for full-term babies, but there are few good data on the nutritional requirements for LP and ET babies, leading to substantial variation in practice. Recent data indicate that rapid growth may be beneficial for neurocognitive function but not for body composition and later metabolic health. Breastfeeding the LP or ET infant can be challenging, and mothers of these infants may need additional support to breastfeed successfully. Future research should investigate nutritional requirements of LP and ET infants for optimal growth, addressing both short- and long-term outcomes and the potential trade-off between neurocognitive and metabolic benefits.

1. Trends in late preterm and early term birth

Late preterm (LP) and early term (ET) are defined as births at 34^{+0} to 36^{+6} and at 37^{+0} to 38^{+6} weeks gestation, respectively [1]. Whereas the definition of preterm birth as birth before 37 weeks gestation is widely accepted [2], the definition of term birth as birth between 37 and 42 completed weeks gestation has been questioned given that maternal, neonatal and childhood outcomes vary considerably across this range. Recent recommendations are that births between 37^{+0} and 38^{+6} be designated "early term," births between 39^{+0} and 40^{+6} as "full term" and births after 41^{+0} as "late term" births [3]. These definitions take into account the continuum of fetal maturation and that infant mortality rates and adverse health outcomes are lowest for births occurring at full term [4].

LP birth accounts for ~75% of all preterm births [5]. In the USA, there has been an estimated 25% increase in late preterm births between 1990 and 2006 [1]; by 2016, LP births accounted for 7%, and ETB 25%, of all live births, equating to more than 275,000 and one million babies, respectively [6]. Similar trends have been observed in Australia, where between 2001 and 2009 planned births (labour induction or pre-labour caesarean delivery) have increased by 40% at < 37 weeks gestation and by 52.5% at 38 weeks gestation [7].

Many factors have contributed to the increasing incidence of LP and ET birth, including increasing use of assisted reproductive technologies, higher rates of multiple births, and increasing use of obstetric interventions [7].

2. Outcomes following LP and ET birth

Although LP and ET babies are apparently "well" when compared to more preterm babies, they can be thought of as "the great dissemblers:" they often look like full-term babies and so are treated as such, although in fact they have a degree of immaturity that places them at higher risk of many clinical problems, and these outcomes are inversely correlated with gestational age [1,8–10]. Compared to term-born peers, LP and ET infants are at increased risk for requiring special education (adjusted odds ratio (aOR); 95% confidence interval (CI): 1.16; 1.12–1.20; and 1.53; 1.43–1.63, respectively) [10] and LP have twice the risk for neurodevelopmental disability (relative risk (RR): 2.19; 95% CI: 1.27–3.75) [11].

LP infants also are more likely to be obese by 3–5 years of age [12] and, as young adults, are more likely to require prescriptions for hypertension and diabetes [13,14], translating into increased risk of mortality from cardiovascular and endocrine disorders in those both LP and ET [15,16].

Faster growth following LP birth is associated with better childhood [17] and adult neurocognitive functioning [18]; intriguingly, however, there may be a trade-off with greater risk of childhood overweight/obesity [17]. These data suggest that nutrition and growth may be just as important for the LP and ET baby as for the extremely preterm baby.

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3. Challenges for nutrition of LP and ET babies

Providing adequate nutritional support for LP and ET infants presents challenges different from those for the extremely preterm baby. First, location of care in a newborn nursery or postnatal ward varies [19,20], and may impact upon practice and outcome. In the Late And Moderately preterm Birth Study (LAMBS) in the UK, 35% of LP infants received all or part of their neonatal care in a neonatal intensive care unit (NICU) [5]. Of those receiving care in a postnatal ward, almost 84% required a non-routine review, 60% of which were due to unexpected concerns. Of LP neonates admitted to the postnatal ward in the UK, 10% required hospital re-admission from home, suggesting that some of the complications seen in LP and ET infants may not be identified during the short period of admission to postnatal wards [19].

Further, there are significant variations in management of moderate and LP infants, mostly related to respiratory support, fluids, and nutrition [5,20]. A survey of clinicians in Australia and New Zealand found wide variation in approach to the initial nutritional support of LP babies while waiting for mother's own milk (MOM) to meet the baby's needs [21]. In an hypothetical scenario of an LP neonate born with appropriate weight-for-gestational-age, 53% of respondents, while waiting for sufficient MOM to meet the infant's needs, would initiate nutritional support with 10% dextrose, with most of the remainder commencing infant formula. Of those providing 10% dextrose, almost 50% would continue 10% dextrose as the only additional nutritional support for three or more days while waiting for MOM [21]. This variation in practice may reflect the lack of high-quality evidence around optimal nutrition support in the LP population and the long-term outcomes of common complications of LP and ET birth, such as neonatal hypoglycaemia.

4. Hypoglycaemia

Hypoglycaemia, or its perceived risk, is one of the commonest reasons for additional nutritional support in LP infants. At birth, the constant supply of glucose from the maternal circulation ceases abruptly. The fall in blood glucose concentration triggers a reduction in insulin secretion and increased secretion of counter-regulatory hormones such as glucagon, catecholamines, and cortisol, which initiate the endogenous synthesis of glucose from glycogenolysis and gluconeogenesis. This physiological sequence is a normal and transitional adaptation to postnatal life, and blood glucose concentration usually stabilises by 72 h of life, rising steadily to a normal range of > 3.9 mmol/L [22]. Disruption of this pathway leads to hypoglycaemia [23].

Transitional neonatal hypoglycaemia is influenced by many factors such as birth weight, gestational age, body stores, presence of metabolic conditions and maternal health during gestation [24]. It is pertinent to note that 50% of hepatic glycogen stores, a key source of glucose in the immediate newborn period, are deposited between 36 and 40 weeks gestation.

The overall incidence of hypoglycaemia (defined as < 2.5 mmol/L) in a population-based cohort was 19% [25]. However, among LP infants and ET infants at risk of developing hypoglycaemia (e.g. because of maternal gestational diabetes), 50% developed blood glucose concentrations < 2.6°mmol/L in the first 48 h after birth [26]. Infants who are exclusively breastfed tend to have lower blood glucose concentrations during the first days after birth compared with infants fed formula [27].

It is important to note that there is a lack of consensus on the definition and management of hypoglycaemia, again reflecting the limited evidence available to determine the safest approach [23,24]. The American Academy of Pediatrics (AAP) suggests blood glucose thresholds for treatment of asymptomatic hypoglycaemia in infants at risk, including LP infants, of < 1.4 mmol/L in the first 4 h after birth, < 1.9 mmol/L from 4 to 24 h after birth, and a threshold of < 2.2 mmol/L for symptomatic infants, with a target blood glucose concentration of > 2.5 mmol/L for all infants requiring treatment [28]. The British Association of Perinatal Medicine recommend blood glucose thresholds for treatment of asymptomatic term infants of < 1.0 mmol/L, or two measurements < 2.0 mmol/L, or < 2.5 mmol/L in symptomatic infants [29]. By contrast, the World Health Organization recommends maintaining blood glucose concentrations > 2.6 mmol/L in all asymptomatic infants, while the US Paediatric Endocrine Society recommends > 2.8 mmol/L in the first 48 h after birth and > 3.3 mmol/L thereafter [30,31]. Differences in thresholds for diagnosis and treatment will markedly affect the reported incidence of neonatal hypoglycaemia, potentially under- or over-estimating the risk in a population [23], and there is no evidence about whether thresholds or treatments should be different for LP and ET infants from those for full-term infants.

The first line of treatment for hypoglycaemia is usually feeding, preferably with breast milk. Breast milk produced in the first days after birth has a lower carbohydrate content than formula. However, there is also some evidence that breastfeeding may have a more sustained effect on blood glucose concentrations in hypoglycaemic babies than formula feeding [23]. A randomised controlled trial (RCT) has shown that treatment of hypoglycaemia in LP and ET infants with oral dextrose gel is effective in restoring blood glucose concentrations, reducing separation of mother and baby for treatment of hypoglycaemia (RR: 0.54; 0.31–0.93) and reducing the likelihood of formula feeding at 2 weeks of age (RR: 0.34; 0.13–0.90) when compared to treatment with placebo [26]. Thus, dextrose gel and breastfeeding are preferable to formula feeding and provide a safe and non-invasive treatment for hypoglycaemia. If hypoglycaemia is profound or recurrent, then admission to a neonatal unit and provision of intravenous glucose may be required.

Based upon use of dextrose gel for treatment of hypoglycaemia, attention has turned to the potential for use of dextrose gel for preventing neonatal hypoglycaemia in babies at risk, including LP babies. An initial dosage RCT has reported that this approach is promising, with any dose of prophylactic oral dextrose gel reducing the risk of neonatal hypoglycaemia compared with placebo (RR: 0.76; 0.62–0.94) [32]. A larger trial is now in progress to assess effects on NICU admission [33].

5. Hypernatraemia

Neonatal dehydration leading to hypernatraemia can affect healthy term neonates but is more common in preterm infants due to their higher water content, lower fat tissue stores and a more permeable skin. Hypernatraemia is mainly associated with excessive weight loss (> 10% of birth weight) and feeding difficulties [34] and is estimated to occur in two to 58 cases per 100,000 live births each year [34]. It is often asymptomatic; however, apnoea, bradycardia, lethargy or irritability and convulsions can occur, resulting in permanent injury. Management involves rehydration therapy, either enterally with provision of breast milk if available or infant formula, or, in some cases, intravenous infusion of fluids [34]. Prevention centres around monitoring of postnatal weight loss and of breastfeeding efficacy [34].

6. Hyperbilirubinaemia

Exclusively breast-fed infants have higher concentrations of total serum bilirubin (TSB) than formula-fed infants, even when consuming adequate volumes of breast milk [35]. Substances in breast milk – including steroids, fatty acids, cytokines, β-glucuronidase and the epidermal growth factor – result in elevated TSB through increased enterohepatic reabsorption of bilirubin, decreased bilirubin excretion, or through inhibition of uridine diphosphate glucuronosyltransferase 1A1 [36], the sole enzyme responsible for the glucuronidation of bilirubin. This can lead to breast-milk jaundice, which is two to four times more common in LP than in term babies because of hepatic immaturity and feeding difficulties [37]. Poor milk intake can also result in dehydration leading to late onset neonatal jaundice, also referred to as inadequate

breastfeeding jaundice [35]. Promotion and support of successful breastfeeding is a key element for prevention of severe neonatal jaundice [35].

7. Energy and nutrient requirements of LP and ET babies

Nutritional support in LP and ET babies needs to be sufficient to avoid short-term complications of inadequate nutrition, adequate to support optimal brain development, yet not promoting excessive growth that may predispose the infant to increased adiposity even by term-equivalent age [38].

Nutritional support for preterm infants often is targeted at supporting growth equivalent to intrauterine growth trajectories. However, there is inevitable weight loss after birth due to loss of extracellular fluid, and growth charts derived from cross-sectional data from infants born at different gestational ages are unlikely to represent optimal postnatal growth of preterm infants. Furthermore, preterm neonates are, as a population, relatively growth-restricted compared with their gestational-age matched in-utero peers who go on to be born at term [39]. Longitudinal preterm growth charts recently have been published, but the sample size is small [40].

Most nutrition guidelines provide recommendations for more preterm (< 32 weeks) or very low birth weight neonates (< 1500 g) but few provide nutritional recommendations for LP and ET babies [41]. The increased numbers of LP and ET births suggests that new research should focus on best nutrition practices among this growing population.

Guidelines from the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) recommend that total energy intake for preterm infants should be 110–135 kcal/kg/d [42], regardless of gestational age. This recommendation takes into account that resting energy expenditure does not vary with gestational age and is ~45 kcal/kg/d; that requirements for new tissues are ~4.5–4.9 kcal/kg and for fat and protein deposition are 1.55–1.6 and 5.5–7.75 kcal/g, respectively; and that an optimal intrauterine weight gain of 17 g/kg/d will require 76–83 kcal/kg/d of energy intake for preterm babies [42]. However, more recent guidelines based upon fetal growth, fetal accretion rates and intestinal absorption estimate that, for LP and ET babies, fetal growth should be between 13 and 11 g/kg/d respectively; energy intake 127 and 115 kcal/kg/d and protein intake 3.1 and 2.5 g/kg/d respectively [41].

These lower recommendations for LP babies are consistent with the findings of an RCT in five European countries which compared different protein concentrations in term infant formula [43]. Term babies were randomised to receive either low protein formula (1.25 and 2.05 g/100 mL in initial and follow-on formula, respectively) or high protein formula (1.6 and 3.2 g/100 mL in initial and follow-on formula, respectively) during the first year. Breastfed children served as reference group. Babies randomised to higher protein content formula had higher weight at the age of two years [43], increased risk for excessive body fat in the second year that lasted until school age [44], and a greater than two-fold increased risk of obesity by 6 years of age (OR: 2.43; 95% CI: 1.12–5.27) compared to the low protein content group [45]. These findings suggest that excessive protein intake in early life can cause increased adiposity in childhood among term-born children and raise the question of whether this also may explain the increased body fat at term-corrected age in LP infants [38], although it must be emphasised that, to date, there are only observational data in LP babies.

Breast milk may not provide sufficient micronutrients and vitamins for LP infants. The AAP recommends multivitamin and iron supplements for all preterm infants until receiving a diverse complementary diet [46]. Additional phosphate and calcium also may be required [41]. Breast-milk fortifiers can be added to breast milk to provide adequate intake of minerals but are recommended for use only in very low birth weight or very preterm babies with the AAP recommending that fortification of breast milk is only required for babies with birth weights < 1500 g [46].

8. Breastfeeding in LP and ET babies

The Academy of Breastfeeding Medicine suggests that to support breastfeeding of LP infants, there should be early initiation of breastfeeding (within the first hour where possible) and, should mothers and babies be separated, stimulation of milk production through regular expressing and frequent skin-to-skin contact or 'kangaroo cuddles' [47]. This approach also improves mother–infant bonding, exclusive breastfeeding rates, and it reduces costs of care [47]. Breastfeeding LP infants successfully can be challenging as they are less alert, have poorer coordination of sucking–swallowing–breathing reflexes and have delayed maturation of the autonomic system that can predispose to cardiorespiratory instability [48]. Expressed breast milk may need to be given by gavage, cup, bottle, syringes, or finger-feeding [47]. Cup feeding may improve breastfeeding rates up to six months among LP babies when compared to bottle-feeding, but compliance is problematic as it may increase feeding time [47] and require greater attention to possible adverse effects (choking, vomiting) that may be concerning for parents. Retrospective cohort data from the Pregnancy Risk Assessment Monitoring System (PRAMS) in the USA found that LP babies are less likely to be initially breastfed and to be breastfed for 10 weeks or longer compared with full-term infants [49]. Other data suggest that this also is true for ET infants in the USA, who are significantly less likely than full-term infants to be breastfed one month postpartum (OR: 0.77; 95% CI: 0.60–0.99) [50]. These data suggest that improved evidence on how best to support successful breastfeeding in LP and ET babies is needed.

9. Breastfeeding support for mothers of LP and ET babies

Mothers who birth preterm are more likely to experience factors that may impact upon lactation, including separation from their infant, medical conditions that may have contributed to the early birth such as diabetes and pre-eclampsia, multiple births, and birth via caesarean section [47]. Various breastfeeding assessment tools are available, although few have undergone adequate assessment and testing, with the Early Feeding Skills Assessment and the Bristol Breastfeeding Assessment Tool probably the most robust [51]; "lactation technology," such as nipple shields and hospital-grade breast pumps, may also facilitate breastfeeding LP and ET babies [47].

10. Transition from enteral nutrition via tube feeds to oral feeds

For many LP babies, a brief requirement for gastric tube feeding is not uncommon. A retrospective review of 647 moderate-to late-preterm infants in six New Zealand NICUs between 2005 and 2011 reported that gestational age, birth weight, days of parenteral nutrition support, and clinical condition were significantly associated with time to start oral feeds and time required to attain full oral feeding [52]. In this study, on average LP infants had their first oral feed attempt in the first two days after birth, reaching full oral feeds by the eighth day. Local practice impacted upon timing and the authors suggested that the lack of specialised services to support feeding may have contributed to differences in transition time [52].

11. Role of smell and taste stimulation

Late preterm and ET infants receiving tube-feeds may miss out on exposure to olfactory and flavour stimulation because tube feeds bypass the nasal and oral cavities where these sensory perceptions mostly occur [53]. Smell and taste of food initiate a sequence of pre-absorptive physiological responses that are triggered by the brain, preparing the body to digest, absorb and metabolise food before food is ingested. Evidence from a pilot randomised trial in very preterm babies (< 29 weeks gestation) indicated that exposure to taste and smell of milk before each feed may reduce time to full enteral feeds and improve weight gain, but sample size was small [54]. An ongoing RCT is

investigating the role of smell and taste prior to tube feedings on time to full sucking feeds and body composition [55]. Results should shed light on whether this simple intervention to support early nutrition of LP babies is of benefit.

12. Breast-milk substitutes

When MOM is not available, the WHO recommends donor human milk (DHM) as the preferred alternative [56]. There are few data on the benefits of DHM in LP and ET infants, although in more preterm infants DHM has been associated with a positive impact on any breastfeeding on discharge [57] and a lower incidence of necrotising enterocolitis when compared to formula feeding [58]. However, growth is recognised to be slower in DHM-fed infants, and most recommendations are that DHM should be fortified to provide adequate nutrition [58]. MOM remains preferable to DHM and effort should be focused on supporting lactation and breastfeeding in mothers, where this is possible [59].

When neither MOM nor DHM are available, then a variety of artificial formulas are available with differences in energy, protein, and mineral content intended to mimic the nutritional content of human milk. Standard term formulas typically provide 68 kcal/100 mL of energy and 1.4–1.7 g/100 mL of protein in addition to calcium and phosphate, whereas preterm formulas are energy- and protein-enriched to provide typically 80 kcal/100 mL of energy and 2.0–2.4 g/100 mL of protein. In term infants, this protein content results in greater infant weight gain and fat mass from 2 to 6 years of life [43,45]; whether this may also be the case in LP infants is not known.

The fat components of infant formulas are also different from those in human milk. Lipids in human milk are essentially milk fat globules of triglycerides enveloped by a three-layer emulsifier membrane (phospholipids, proteins and cholesterol), whereas infant formulas have lipids with different molecule size and emulsifier membrane architecture. Palmitate in infant formula is low in the sn-2 stereoisomer compared with breast milk, which is high in this isomer. Novel infant formulas addressing fat structure [60] and the proportion of sn-2 palmitate [61] suggest that current infant formulas can be improved substantially, resulting in better tolerance, stool composition and, potentially, other outcomes such as bone health. Future research should address the effect of formulas that reflect more closely the composition of human milk on later growth, body composition, and neurodevelopment in LP and ET formula-fed infants.

13. Post-discharge formulas

For formula-fed infants, nutrient-enriched post-discharge (or “follow-on”) formulas are available which have higher energy density, increased protein concentrations and greater mineral and vitamin content compared with standard term formula. The evidence for the benefit from post-discharge formula in preterm babies is of moderate quality and inconsistent, and currently is insufficient to support their routine use [62,63], although there may be benefit for infants who have been identified as growing poorly on standard formula or who have ongoing mineral or vitamin deficiencies. However, there are no data to support their use in LP or ET infants.

14. Parenteral nutrition

Parenteral nutrition (PN) is usually considered when provision of nutrients via the enteral route is clinically contraindicated or will result in nutrient insufficiency. The composition of PN varies from intravenous infusion of carbohydrates (mostly dextrose) alone, combinations of dextrose and amino acids in addition to minerals and vitamins, and separate infusion of lipids. There is little evidence regarding whether PN is more beneficial than 10% dextrose in LP infants while waiting for maternal milk supply to meet demand and for full enteral feeds to be tolerated [64] but its use in LP infants appears to be rare [21]. Each day of parenteral support in LP infants has been reported to predict an increase in time to achieve full oral feeds of 2 h (hazard ratio: 0.92; 95% CI: 0.89–0.95) [52]. Although it has been reported that 27% of LP infants require intravenous infusions compared to 5% of term babies [65], very few LP and ET babies will receive parenteral nutrition support, with this usually reserved for babies with congenital malformations predicted to lead to delays in reaching full enteral feeds.

14.1. Practice points

- The incidence of LP and ET birth has increased significantly in recent years, mostly due to increased obstetric intervention.
- LP and ET infants are at increased risk of developing short-term and long-term adverse outcomes.
- There is significant variation in nutrition support for the LP and ET infant.
- Nutrition of the LP/ET infant may be related to both short- and long-term outcomes.
- Maternal breast milk remains the optimal feed for LP and ET infants.
- Breastfeeding the LP/ET baby can be challenging, and increased support is needed.

14.2. Research directions

- Optimal nutritional support for LP and ET infants to support neurodevelopment and healthy body composition should be investigated in well-designed RCTs with appropriate sample sizes.
- The potential effect of smell and taste stimulation for tube-fed infants on time to full oral feeds, body composition, and breastfeeding rates should be assessed by RCTs.
- More detailed information on the composition of breast milk in mothers of LP and ET infants may improve understanding of the nutrient requirements of these babies.
- Further research into the potential benefits of novel infant formulas for those LP and ET infants for whom breast milk is unavailable should focus on longer-term outcomes such as development and body composition.

Conflicts of interest

None declared.

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Appendix III: Smell and taste in the preterm infant

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Smell and taste in the preterm infant

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ABSTRACT

Olfaction and gustation are critical for the enjoyment of food but also have important metabolic roles, initiating the cephalic phase response that sets in train secretion of hormones important for metabolism and digestion before any food is actually ingested. Smell and taste receptors are functional in the fetus and there is evidence for antenatal learning of odours. Despite enteral nutrition and metabolism being major issues in the care of very preterm infants, often little consideration is given to the potential role of smell and taste in supporting these processes, or in the role they may have in encoding hypothalamic circuitry in a way that promotes healthy metabolism in the post-neonatal period. This review will discuss the evidence for the role of smell and taste in the newborn infant.

1. Introduction

The importance of the olfactory and gustatory senses for the enjoyment and metabolism of food has been recognised for well over 120 years. Henry Fincks wrote "*To which of our senses are we most indebted for the pleasures of the table? To name the sense of taste in answer to this question would be quite as incorrect as to assert we go to the opera to please our eyes. More incorrect, in fact, because many do attend the opera chiefly on account of the spectacle; whereas, in regard to gastronomic delights it is safe to say that at least two-thirds of our enjoyment is due to the sense of smell.*" [1]. Pavlov's experiments in dogs with exteriorised oesophagi demonstrated that the presence of food in the mouth, without it ever reaching the stomach, stimulated copious gastric secretions; in contrast, if bread was inserted into the stomach via tube without the dog being aware, gastric secretions were substantially less and the bread remained in the stomach undigested for over an hour [2]. The activation of the pathways involved in digestion in anticipation of food is now known as the cephalic phase response. The cephalic phase response increases gut motility and the secretion of enzymes and hormones such as ghrelin, insulin, leptin, glucagon-like peptide-1, cholecystokinin, pancreatic polypeptide and gastrin that result in tighter regulation of blood glucose concentrations and enhanced digestion [3]. Gastro-intestinal motility, digestion and metabolic control are major issues in nutrition of the preterm infant [4], yet the standard mode of enteral feeding before sucking feeds are established is to provide milk via a gastric tube, at intervals and volumes decided by the clinician, usually without any accompanying smell or taste. A recent survey of Australian and New Zealand neonatal units found that, even in moderate-late preterm babies, only approximately 30% of units routinely provided smell or taste before feeds (Alexander and Bloomfield, unpublished data). This brief review will discuss the potential role of smell and taste in the nutritional care of the preterm infant.

2. Ontogeny of olfaction and gustation

The olfactory system is complex and, in most mammals, consists of the main or primary olfactory system, located in the upper part of the nasal cavity, and the vomeronasal organ, located in the nasal septum and of uncertain significance in humans. The primary olfactory receptors are present by the eighth week of gestation and have a mature appearance by the end of the second trimester [5,6]. Olfactory marker protein, considered to correlate with neuroreceptor functionality and connectivity with the main olfactory bulb, is expressed in the olfactory mucosa by 28 weeks' gestation and in the main olfactory bulb by 32–35 weeks' gestation [7]. Taste receptors detect the five tastes and flavours (bitter, sweet, sour, salty and umami) and begin to develop at 7–8 weeks' gestation, maturing by the middle of the second trimester [8,9]. Fetal swallowing of small amounts of amniotic fluid begins at the

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end of the first trimester and reaches up to 750 mL/day by 34 weeks' gestation, when the gastrointestinal tract is beginning to develop mature migrating motor complexes that will result in directional cranial-caudal peristalsis [10]. Thus, fetal smell and taste receptors are exposed to the components of amniotic fluid for many weeks before birth and at comparable gestations to preterm infants.

Odour of amniotic fluid is known to be important for recognition of kin in a variety of animals; for example, newborn lambs and rat pups are preferentially attracted to areas impregnated with their own amniotic fluid rather than to areas impregnated with amniotic fluid from an unrelated female [11]. Healthy, 3-day old term newborns preferentially turn their heads towards a pad impregnated with their own amniotic fluid rather than towards a pad impregnated with unfamiliar amniotic fluid [6]. Interestingly, this occurs similarly in both breast-fed babies (who may be re-exposed to similar volatiles through mothers' milk) and bottle-fed infants [6]. In two-day old term newborns, the duration of head orientation to pads impregnated with amniotic fluid or colostrum is similar and significantly greater than orientation towards a control stimulus of water. However, by four days after birth, babies exhibit a clear preference for mothers' milk over amniotic fluid, although a preference for amniotic fluid over water remains [11]. That these preferences are related to prenatal, rather than postnatal, olfactory learning is demonstrated by a study performed in healthy term babies immediately after birth before the routine use of skin-to-skin at birth [12]. Babies were washed in water containing 0.05% potassium permanganate and placed in a cot until the placenta was delivered. One of mother's nipples/areolae was randomly assigned to be treated with amniotic fluid and the baby placed prone in the midline on the mother's chest. Twenty-three out of thirty babies chose the breast treated with amniotic fluid, 21 of whom were observed to examine both sides of the mother's chest before making a choice. Twenty-two of the thirty babies successfully grasped a nipple and began to suck without help; 17 of these chose the nipple treated with amniotic fluid. These data suggest that the odour of amniotic fluid is a stronger attractant for a newborn baby than simply the mother's odour. Memory of prenatal olfactory learning appears to persist for some time after birth. Babies separated from their mothers immediately after birth and fed formula via bottle for 10–14 days without contact with their mothers, generated greater expression pressure, suck frequency and suck efficiency during a bottle feed at 10–14 days when exposed to filter paper impregnated with expressed mothers' milk rather than with formula or water [13]. Similarly, babies of mothers who drank carrot juice in the last trimester but not during lactation demonstrated fewer negative facial expressions in response to weaning cereals prepared with carrot juice, again presumably indicating memory of flavour of amniotic fluid [14]. Perinatally-acquired odours also generate long-term memory, as babies exposed to a camomile balm used prophylactically to prevent sore nipples exhibited preference for camomile odour over violet odour at both 7 and 21 months of age [15].

Thus, babies born at term demonstrate prenatal functioning of olfactory receptors that retain memory of amniotic fluid volatile constituents into the post-natal period and beyond. Although some of the volatile fingerprints have been identified in breast-milk [16], further research is needed to understand which components generate the greatest response from babies.

3. Olfaction in preterm newborns

More recent evidence indicates that olfaction is also important in preterm newborns. Some innovative approaches have been taken to investigate the effect of stimulation with odour on preterm infants' non-nutritive sucking behaviour. Bingham has developed an ‘olfactometer’ which can be controlled remotely to deliver test or control odours through a programmed schedule (non-contingent) or contingent upon infant sucking [17]. In 7 moderately preterm newborns (33–34 weeks' gestation), stimulation with breast-milk odour during a tube feed resulted in a greater median number of sucks compared with control. In 29 preterm babies with a mean gestational age of 30 weeks, tested at a mean corrected gestational age of 33 weeks, exposure to milk odour via a specially adapted and instrumented pacifier resulted in increased measures of suck pressure and burst activity in those receiving breast milk odour compared with formula odour [18]. Similarly, in a very small non-blinded study, exposure of preterm infants to breast-milk odour once a day for five days, commencing the day before first attempting breast-feeding at 35 weeks' post-conceptional age resulted in longer sucking bouts, increased numbers of long sucking bouts and increased milk intake (assessed by weighing immediately before and after feeds) [19]. Exposed babies also were discharged home significantly earlier. Interpretation of these studies is complicated by the very small number of participants.

Two randomised controlled trials and one observational cohort study have assessed the impact of early colostrum administration in preterm babies on clinical and feeding outcomes [20–22]. These have reported that administration of small amounts of oral colostrum to extremely preterm or extremely low birthweight (ELBW) infants commencing within the first three days after birth is safe. A cohort study reported increased rates of receiving breastmilk as the majority of enteral feeds six weeks later [22] and a randomised trial of sixteen ELBW babies reported a significantly shorter time to full enteral feeds of 150 $mL{\cdot}kg^{-1}{\cdot}day^{-1}$ (14 vs 24 days) [21]. The intervention in this study was 0.2 mL oral colostrum every 2 h for 48 consecutive hours, beginning 48 h after birth. The magnitude of the effect seems extremely large for such a brief intervention and it is not clear that the blinding method described (syringes covered with tape) would have been effective. A larger randomised, placebo-controlled trial of 48 babies born before 28 weeks' gestation who received 0.2 mL oropharyngeal colostrum or water every 3 h for 72 h from 48 to 96 h after birth did not find any difference in time to full enteral feeds or to hospital discharge [20]. However, there was a statistically significant reduction in clinical (but not proven) sepsis (12/24 vs 22/24), although rates in the placebo group were extremely high. Nevertheless, significantly greater urinary levels of secretory immunoglobulin A, and lower levels of salivary transforming growth factor β and interleukin 8, at one and two weeks suggest that there may be an underlying mechanism that would support such an effect that is worthy of further study. A large RCT (n = 622) designed to address the question of whether oropharyngeal mother's milk in extremely low birthweight babies will reduce the incidence of sepsis and necrotising enterocolitis is underway [23].

Longer exposure of tube-fed preterm babies to smell or to smell and taste may shorten time to full enteral feeds, although further research is needed. A trial of 51 infants < 29 weeks' gestation randomised babies to smell and taste of milk before each feed or no olfactory/gustatory exposure to milk until 32 weeks' gestation [24]. Babies in the intervention group tended to reach full enteral feeds earlier (median 13.5 vs 15.5 days). Similarly, a trial of 80 preterm babies between 28 and 34 weeks' gestation reported that administration of breast milk odour during tube feeds three times a day until oral feeds were attained led to full oral feeding 3 days earlier and a four day reduction in hospital stay [25]. Neither of these trials was blinded.

4. Smell, taste and the potential impact on metabolism

In addition to the potential for improving tolerance of enteral feeds through stimulation of the digestive tract, olfaction also may impact upon the central appetite-regulatory pathways. Information on odour is passed from the olfactory bulbs to the primary olfactory cortex via the lateral olfactory tract. Functional MRI studies in adults demonstrate activation of the lateral and anterior orbito-frontal gyri [26]. From there, information passes to the orbito-frontal cortex, where conscious perception of smell occurs, and also to the hypothalamus via the amygdala of the limbic system. Near-infrared spectroscopy (NIRS) studies in term and preterm neonates have demonstrated changes in

cerebral oxygenation in the pre-frontal area in response to odour stimulation [27–29]. This is assumed to reflect changes in blood flow and, therefore, neuronal activity. Term infants demonstrate greater changes in cerebral oxygenation in response to maternal breast milk odour than to formula odour [29] and decreased oxygenation, thought to reflect deactivation, has been demonstrated in preterm infants in response to unpleasant odours associated with neonatal intensive care such as detergent and disinfectant [27]. In newborn rabbit pups, the maternal mammary pheromone upregulates expression of the Fos protein, a regulator of cell proliferation and differentiation, to a greater extent than a control odour in the posterior piriform cortex and the lateral hypothalamus [30]. The posterior piriform cortex is an associative cortex, thought to play a role in encoding responses to odour through learning and anticipation [31]. The lateral hypothalamus is a key area for feeding behaviour, with output neurons to a variety of brain areas including the arcuate nucleus, part of the hypothalamic appetite-regulatory centre [32]. Hypothalamic circuitry largely develops antenatally in humans and primates, with neurogenesis occurring in the first trimester and circuit formation beginning in the late second trimester and extending throughout the third trimester [33,34]. Development of the hypothalamic appetite-regulatory pathways has been demonstrated to be affected by nutritional signals during fetal life, even from very early pregnancy. For example, maternal periconceptional undernutrition in sheep leads to epigenetically-regulated changes in glucocorticoid receptor and pro-opiomelanocortin expression in the ventral hypothalamus, which includes the arcuate nucleus, in the late gestation fetus that persist through to adult life [35,36]. In rats, maternal undernutrition leads to postnatal hyperphagia and increased fasting leptin and insulin concentrations [37]. The postnatal phenotype could be reversed by neonatal leptin administration during the first two weeks after birth [38]. In rats, the hypothalamic circuit development that occurs in utero in humans occurs in the early postnatal period [39], indicating that leptin and insulin may be important hormones in encoding hypothalamic appetite-regulatory and metabolic pathways. Increased levels of insulin during this critical period of hypothalamic differentiation have been associated with altered development of cholecystokinin, galanin and neuropeptide Y (NPY) neurons [40,41], all of which are involved in appetite regulation. The immature regulation of insulin transport across the blood brain barrier during the neonatal period in rats provides a mechanism by which the developing hypothalamus can be exposed to elevated levels of insulin.

It therefore is reasonable to postulate that ordinary fluctuations of metabolic hormones, such as insulin and leptin, in the neonatal period might be important in determining healthy encoding of the hypothalamic pathways. This might particularly be the case in preterm infants who are ex utero at a time when critical development of circuitry is occurring. The cephalic phase response mentioned above is active in the newborn and is important for initiating secretion of these hormones. In addition to systemic secretion, these hormones also are present in saliva, secretion of which, of course, is stimulated by smell and taste [42]. Indeed, salivary concentrations of NPY2 receptor, involved in hunger signalling, have been reported to have a 95% positive predictive power for feeding immaturity (defined as inability to sustain full oral feeds), although the negative predictive power was weak [43]. A randomised controlled trial (sample size 180 extremely preterm infants) to determine whether pulsed orocutaneous intervention simultaneous with feeding will accelerate time to full oral feeds (ClinicalTrials.gov NCT02696343) is underway [44]. A randomised factorial experiment (sample size 530 moderate-late preterm infants) is investigating whether the simpler approach of providing smell and taste before all tube feeds will have a similar effect, as well as mitigating the increased fat mass seen by term-corrected age in preterm infants [45,46] (Australia and New Zealand Clinical Trials Registry ACTRN12616001199404).

5. Conclusion

Smell and taste are powerful sensory inputs that develop during fetal life and are important in the transition to postnatal feeding. In addition to the importance of smell in recognition of the mother, smell and taste initiate metabolic pathways that promote digestion and metabolic control. Feed intolerance in the newborn period is a major problem in the extremely low preterm baby and even moderate-late preterm birth carries increased risk of impaired metabolic health in later life [47,48]. Further research should investigate whether exposing tube-fed preterm infants to smell and taste of mother's milk may improve these outcomes for future extremely preterm babies.

Conflict of interest statement

F H Bloomfield is the Principal Investigator of the randomised factorial trial referred to in the penultimate paragraph (ACTRN12616001199404) which is funded by the Health Research Council of New Zealand. He holds numerous other research grants.

F H Bloomfield is the Abbot Nutrition Lecturer at the 2017 Perinatal Research Society (USA). The lecture is on a topic largely unrelated to this manuscript (the role of nutrition on development of the pancreas).

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