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# Understanding small and large milk fat globule phenotype variation in dairy cows through milk lipidomic characterisation

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## ABSTRACT

Milk fat globules (MFGs) are spherical structures comprising a neutral lipid core that is surrounded by a three-layer membrane. This MFG membrane is of nutritional benefit for infants and adult consumers. Individual variation in the size of MFGs is observed within a herd of the same breed, and this milk production trait, if selected for through breeding programs, could be exploited for a more targeted milk production for specific technological streams. For example, large MFGs are desirable for butter making, whereas small MFGs are preferred for cheese making and direct consumption due to improved sensory properties and increased relative abundance of the beneficial membrane material.

The initial experiment of this work aimed to determine how much the average MFG size is affected by on-farm and animal related factors within a herd subjected to the same diet and environmental conditions. Milk fat globule size of the whole herd was repeatedly measured over a one-year period and the effects of these parameters were estimated using a linear mixed effect model. This analysis showed that stage of lactation, parity and milk yield can affect MFG size, while the impact of fat yield, concentrate intake and number of milkings per day was limited. However, the individual variation within the herd outweighed the effect of individual factors, supporting the possibility of a genetically determined regulation of MFG size.

Based on the data collected for the first experimental chapter, cows were selected for the second and third experiment, which aimed to characterise the small and large MFG phenotypes through an in-depth lipidomics analysis. This analysis included the characterisation of the fatty acid (FA) profile of the MFG core by gas chromatography and the identification of the whole milk lipidome through targeted liquid chromatography tandem mass spectrometry. The analysis of the MFG core FA profile revealed that the

cows with the small MFG (SMFG) phenotype produced milk with higher proportions of unsaturated FAs compared to large MFG (LMFG) cows. This was related to an increased uptake of preformed FAs with a chain length of ≥ 18 carbons, which are sourced directly from the diet or from lipid mobilisation. This characteristic of the SMFG group could potentially lead to the production of milk with an improved nutritional profile.

The results of the third experiment present the most extensive milk lipidomic analysis in the literature to date, with 301 detected lipid species. The results also revealed, for the first time, a potential role for ether phosphatidylethanolamine (ePE) in the regulation of MFG size, showing a higher relative abundance of ePE in the milk from LMFG cows. Ether PEs can reduce the fluidity of biological membranes and are predicted to promote lipid droplet fusion. The milk from SMFG cows, on the other hand, contained higher total phosphatidylcholine (PC) to PE ratios and a higher relative abundance of unsaturated PC species, both attributes that are predicted to prevent lipid droplet fusion. In the final experiment of this thesis an *in vitro* model using cells purified from raw milk and grown on permeable membrane supports was established, which offers the potential to test some of the novel findings of this thesis in future experiments.

## DECLARATIONS

This declaration is to certify that:

i) The thesis comprises only my original work towards the PhD except where indicated in the preface,

ii) Due acknowledgement has been made in the text to all other material used,

iii) The thesis is fewer than 100 000 words in length, exclusive of tables, maps, bibliographies and appendices.

Leonie Walter

28/08/2019

## PREFACE

This thesis comprises chapters that were performed in collaboration with the Commonwealth Scientific and Industrial Research Organisation's Agriculture and Food business unit. I was the lead investigator on all of these chapters. For Chapter 4, gas chromatography was performed by Dr Pushkar Shrestha, CSIRO Black Mountain, Canberra, Australia, who also provided the initial data analysis, including peak identification and calculations of area percentages of the peaks. I conducted the sample collections, subsequent data analysis and wrote the manuscript. For Chapter 5, liquid chromatography tandem mass spectrometry was performed by Dr Vinod Narayana and Dr Dedreia Tull from Metabolomics Australia, who also assisted in the interpretation of the data. Sample collection, peak area selection, data analysis, interpretation and writing of the manuscript were my responsibility.

For all published work in this thesis the respective co-authors have completed The University of Melbourne's co-author authorisation form to certify that the candidate's contribution to the publication was greater than 50%. The candidate's principal supervisor, Professor Brian J. Leury has signed The University of Melbourne's declaration for a thesis with publication form. Manuscripts submitted or intended to be submitted for publication are formatted according to the respective journal's formatting requirements. Supplemental data included in this thesis refers to supplemental files that are intended to be published as part of the research article. Additional material that is not intended for publication but included in this thesis is attached as an appendix at the end of a chapter, where applicable.

This thesis contains the following original manuscripts, where I was the lead author:

| | | |
|---|---|---|
| I. | Full title: | **The effect of physiological state, milk production traits and environmental conditions on milk fat globule size in cow's milk.** |
| | Authors: | Leonie Walter, Sue Finch, Brendan Cullen, Richard Fry, Amy Logan and Brian J. Leury |
| | Status: | Accepted for publication by the Journal of Dairy Research on 01/07/2019 |
| | Candidate's contribution: | 90% |
| II. | Full title: | **Lipid metabolic differences in cows producing small or large milk fat globules: Fatty acid origin and degree of saturation** |
| | Authors: | Leonie Walter, Pushkar Shrestha, Richard Fry, Brian J. Leury and Amy Logan |
| | Status: | In revision following peer review by the Journal of Dairy Science |
| | Candidate's contribution: | 80% |
| III. | Full title: | **Milk fat globule size development in the mammary epithelial cell: a potential role for ether phosphatidylethanolamine** |
| | Authors: | Leonie Walter, Vinod K. Narayana, Richard Fry, Amy Logan, Dedreia Tull and Brian J. Leury |
| | Status: | Submitted for publication to Scientific Reports |
| | Candidate's contribution: | 80% |
| IV. | Full title: | **Investigation on the suitability of milk derived primary bovine mammary epithelial cells grown on permeable membrane supports as an *in vitro* model for lactation** |
| | Authors: | Leonie Walter, Richard Fry, Amy Logan, Brian J. Leury |
| | Status: | Unpublished material not submitted for publication |
| | Candidate's contribution: | 95% |

## LIST OF COMMUNICATIONS AND PRIZES

**Book chapters:**

Logan, A., Walter, L., Watkins, P. (accepted Nov 2019). Chapter 13 – Analytical Methods. In Advanced Dairy Chemistry: Volume 2: Lipids. P.F. Fox and P.L.H. McSweeney, eds. (New York, NY: Springer US).

**Conference presentations and posters:**

Presentation at the International Symposium on Ruminant Physiology, Leipzig, Germany, 2019: Walter, L., Narayana, V.K., Fry, R., Logan, A., Tull, D. and Leury, B.J. Lipidomic profiles of milk from cows with consistently small or large milk fat globule size distributions suggest underlying metabolic differences.

Poster at the International Symposium on Ruminant Physiology, Leipzig, Germany, 2019: Walter, L., Shrestha, P., Fry, R., Leury, B.J. and Logan, A. Towards the identification of milk fat globule size as a potentially beneficial milk production trait: small and large phenotype characterisation.

**Prizes:**

Best pre-confirmation award at the Faculty of Veterinary and Agricultural Sciences' Postgraduate Symposium 2015.

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To Brian, my principal supervisor, thank you for your enthusiasm and encouragement, which always made me leave your office with a lot of motivation, one of the most valuable currencies during a PhD. I would also like to thank you for trusting me to be the right person for the project even though my training was not in animal science.

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And finally, Richard, you and Kerri taught me the basics of cell culture and you were able to put a smile on my face even when I thought cell culture was never going to work for me, thank you!

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To all CSIRO staff based in Werribee, thank you for your support and friendship, you have welcomed me as a master student years ago which contributed to my decision to

stay in Australia for my PhD. I was lucky to be able to do my PhD in collaboration with CSIRO, which provided invaluable access to equipment and expertise, as well as a top-up scholarship from the office of the chief executive. To the University of Melbourne staff in Parkville, thank you for the lunches, chats, co-working and in general for being a good bunch of people. There are too many to name, but I would like to give a special thanks to Kathryn and Priya, as well as Kristy, Vanessa and Darren.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| AAG | Alkylacylglycerol |
| ANOVA | Analysis of variance |
| BSA | Bovine serum albumin |
| BTN | Butyrophilin |
| CEPT | Choline/ethanolamine phosphotransferase |
| CDP | Cytidine diphosphate |
| CE | Cholesteryl ester |
| Cer | Ceramide |
| CI | Confidence interval |
| CN | Carbon number |
| CSN3 | Kappa-casein |
| D[4,3] | Volume-weighted mean diameter |
| D[3,2] | Surface-weighted mean diameter |
| D[*v*, 0.5] | Volume median diameter |
| DG | Diacylglycerol |
| DGAT | Diacylglycerol acyltransferase |
| DIM | Days in milk |
| DMEM/F12 | Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 |
| ECM | Extracellular matrix |
| ER | Endoplasmic reticulum |
| ePC | Ether linked phosphatidylcholine |
| ePE | Ether linked phosphatidylethanolamine |
| ePL | Ether linked phospholipid |
| FA | Fatty acid |
| FAME | Fatty acid methyl ester |
| FBS | Fetal bovine serum |
| FID | Flame ionisation detector |

| | |
|---|---|
| GC | Gas chromatography |
| KRT 8/18 | Keratin 8 or 18 |
| LC-MS/MS | Lipid chromatography tandem mass spectrometry |
| LD | Lipid droplet |
| LMFG | Cows with the large milk fat globule phenotype |
| LPC | Lysophosphatidylcholine |
| LPE | Lysophosphatidylethanolamine |
| LPI | Lysophosphatidylinositol |
| MEC | Mammary epithelial cell |
| MFG | Milk fat globule |
| MFG-E8 | Milk fat globule epidermal growth factor (EGF) factor 8 |
| MFGM | Milk fat globule membrane |
| MUFA | Monounsaturated fatty acid |
| NEFA | Nonesterified fatty acid |
| PBS | Phosphate buffered saline |
| pbMEC | Primary bovine mammary epithelial cell |
| PC | Phosphatidylcholine |
| PDT | Population doubling time |
| PE | Phosphatidylethanolamine |
| PEMT | Phosphatidylethanolamine N-methyltransferase |
| PI | Phosphatidylinositol |
| PL | Glycerophospholipid |
| PLIN2 | Perilipin 2 |
| PUFA | Polyunsaturated fatty acid |
| PS | Phosphatidylserine |
| RM1 | Primary cells from extracted from raw milk and purified by immunomagnetic separation |
| RM2 | Primary cells extracted from raw milk |
| SCD | Stearoyl-coenzyme A desaturase |

| | |
|---|---|
| SCDI | Stearoyl-coenzyme A desaturase index |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SFA | Saturated fatty acid |
| SMFG | Cows with the small milk fat globule phenotype |
| SNARE | Soluble N-ethylmaleimide sensitive factor (NSF) attachment protein (SNAP) receptor |
| SL | Sphingolipid |
| SM | Sphingomyelin |
| SMS | Sphingomyelin synthase |
| TC | Primary cells extracted from bovine mammary tissue |
| TEER | Transepithelial electrical resistance |
| TG/TAG | Triacylglycerol |
| TW | Cells cultured on Transwell permeable membrane supports |
| UFA | Unsaturated fatty acid |
| XOR | Xanthine oxidoreductase |

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# CHAPTER 1

## Introduction

## 1.1 General Introduction

Milk fat globule (MFG) size affects the functional, nutritional and sensory properties of milk and milk products. The native MFG size distribution is variable and depends on a number of endo- and exogenous factors (Martini et al., 2013). Besides its variation between different breeds and species (Carroll et al., 2006), in cow milk it is also affected by physiological factors like stage of lactation (Altenhofer et al., 2015; Evers, 2004) and by the cow's diet (Argov-Argaman et al., 2014; Avramis et al., 2003; Couvreur et al., 2007; Mesilati-Stahy et al., 2015). Furthermore, the natural MFG size distribution can vary between individual cows (Logan et al., 2014a). Throughout this thesis the term MFG size is used to refer to the average MFG diameter, unless otherwise stated.

MFG size as well as casein micelle size influence milk functionality by affecting, for example, rennet gelation properties in cheese. The microstructure of cheese comprises a three-dimensional network of aggregated casein micelles in close association with calcium (Lucey et al., 2003). Aggregation occurs through destabilisation of the casein micelle by enzymatic hydrolysation (rennet gels) of κ-casein which is predominantly found on the surface of the casein micelle or by acidic destabilisation of the casein micelle (acid gels) (Lucey et al., 2003). Enclosed within this network are MFGs, moisture, other substances (e.g. minerals) and starter culture bacteria (Guinee, 2011). Therefore, the rheological properties of cheese are a function of the strength of its protein network and are further influenced by moisture content and the microstructure of the MFGs within the gel network (Guinee, 2011). From a rheological perspective cheese is a viscoelastic material, and the MFGs act as inert fillers, increasing its elasticity when the fat is present in a solid state at low temperatures (Guinee, 2011; Michalski et al., 2002). Rennet gels made from milk with small native MFGs and hence increased surface area exhibit an increased storage module G', a measure for the elasticity of cheese (Michalski et al., 2002). Accordingly, if the native MFGs are larger than the pores formed by the casein

micelles during rennet gelation the gel network is disrupted and softer gels are the result (Logan et al., 2014b; Michalski et al., 2002). Cheese made from milk with smaller MFGs has further been reported to have improved sensory characteristics (Michalski et al., 2007).

It has become increasingly evident that the MFG membrane (MFGM) not only functions to emulsify the neutral lipids of the MFG core, but also provides several health benefits that extend from infants to children after weaning and to adult consumers (Thorning et al., 2016; Timby et al., 2017). Thus, MFG size also influences the nutritional properties of milk and milk products because certain components of the MFGM can have beneficial effects on human health (Martini et al., 2013). For example, infant formula often contains vegetable oils instead of milk derived lipids, leaving the infant deprived of MFGM material and MFGM supplementation to infant formula has resulted in improved cognitive function in randomized control trials (reviewed in Timby et al. (2017)). In healthy adults, dietary MFGM supplementation has been linked to improved muscle strength when combined with regular exercise (Soga et al., 2015) and improved resistance to diarrheagenic *Escherichia Coli* infection (Ten Bruggencate et al., 2016). Moreover, the consumption of buttermilk, which contains the MFGM material, has been shown to reduce plasma cholesterol concentrations in adult men and women (Conway et al., 2013). Another possible health benefit of consuming MFGM material is the potential to prevent obesity. In a mouse model, MFGM-coated lipid droplets (LDs) in the diet of mice in early life resulted in reduced body fat accumulation later in life when mice were exposed to a western style diet challenge (Baars et al., 2016). However, this effect was only found in large LDs with a mode diameter of 2.9 µm, indicating a relationship with the LD macrostructure (Baars et al., 2016). Although the bioactive compound or combination of compounds responsible for the observed effects are often unknown, some more specific roles have also been established. For example, butyrophilin is suggested to

suppress multiple sclerosis and phospholipids (PLs) are believed to prevent colorectal cancer (Spitsberg, 2005).

The concentration of MFGM material in milk depends on the fat content but also on the size distribution of MFGs. At a given fat content, a shift in MFG size towards smaller MFGs leads to higher levels of MFGM material, due to an increased surface area. The size of MFGs dispersed in milk cannot be influenced other than by on-farm cow selection, homogenisation or fractionation methods such as microfiltration or gravity separation (Michalski et al., 2006; O'Mahony et al., 2005). Homogenisation, however, reduces the size of MFGs by disrupting its MFGM and therefore does not result in increased concentrations of MFGM material. Instead, the increased surface area is covered by other milk proteins found in the skim milk phase. Separation technologies such as microfiltration could separate the MFG population in milk into smaller and larger groups for specific technological purposes. Large MFG milk for example could be more suitable for butter production, because large MFGs tend to coalesce, resulting in a more efficient process. However, these technologies would also result in increased processing time and costs. The detailed characterisation of the small and large MFG phenotype in dairy cows, on the other hand, could potentially lead to the identification of genotypes and ultimately genetic selection of cows based on MFG size. Gaining knowledge about the metabolic differences underlying the small and large MFG phenotypes is therefore of major interest for dairy science and potentially the dairy industry.

## 1.2 Objectives and Hypotheses

As outlined in the previous section, MFG size is a potentially desirable milk production trait, due to its distinctive processing properties and the health benefits associated with the MFGM. This may be significant for the dairy industry and the gaps in our fundamental knowledge about

MFG size development in the mammary gland warrant an in-depth characterisation of this trait. Some areas in this field that are currently understudied are outlined in the literature review.

Firstly, although some farm-management, environmental and physiological factors have been shown to influence MFG size, the magnitude of their effect, and in some cases the significance of their effect compared to the individual variability within a herd, remain equivocal. Moreover, the biochemical processes determining the size of intracellular LDs as well as the precise mechanism leading to their secretion as MFGs are not fully understood. Recent advances in this field have provided answers to some of these questions. For example, it has been conclusively shown that LD fusion is involved in MFG size development in the mammary gland (Masedunskas et al., 2017) and that this process depends at least partly on the polar lipid composition of the monolayer surrounding the intracellular LD (Cohen et al., 2017). However, some questions about the relationship between MFG size and the mammary lipid metabolism remain unanswered. For example, it has been suggested that some differences in MFG size are independent from differences in milk production parameters, such as fat content and milk yield (Argov-Argaman, 2019). However, it is unclear how the expression of two distinct phenotypes of small and large MFGs is regulated when other environmental factors and milk production parameters are similar. Likewise, the reported differences in the fatty acid (FA) composition of MFGs with different sizes are inconsistent. It is possible that some of these questions remain unanswered because of the complexity of the milk lipidome, with many lipid species yet to be discovered (Liu et al., 2018). These knowledge gaps present important barriers for the on-farm selection of cows based on their characteristic MFG size. Ultimately, in order to select cows based on MFG size, an understanding of its heritability and the relationship with potential genetic variants of the major lipid synthesising genes will be necessary to achieve the selection of the small and large MFG phenotype through breeding programs. However, to achieve this goal, an in-depth understanding of the fundamental

pathways and key enzymes is required to allow a more targeted search for potential genotypic differences.

Therefore, this thesis aimed to reduce some of these barriers by identifying cows with the small and large MFG phenotype, followed by an in-depth characterisation of the lipid metabolic differences between these animals. This was achieved through three experimental chapters (Chapters 3-5). An additional fourth experimental chapter (Chapter 6) focused on establishing an *in vitro* primary cell culture model for future studies into the regulation of MFG size.

### *1.2.1 First experimental chapter (Chapter 3)*

The goal of the first part of this study was to establish the MFG size distribution within our research herd and estimate the impact of environmental, farm-management and animal-related physiological parameters on MFG size. This was achieved by taking advantage of the extensive daily data collection as part of the automatic milking system used to manage the herd at the University of Melbourne Dairy. The automatic milking system records a range of factors which have previously been shown to affect MFG size (days in milk, fat yield, fat-protein ratio) and some that have not previously been studied (rumination minutes and somatic cell count). Furthermore, the aim was to identify individual animals with the small and large MFG phenotype for the two subsequent experimental chapters that are otherwise similar in major milk production traits and are exposed to the same environmental conditions, including a similar diet. This was important to address the hypothesis that individual variations in MFG size distributions between cows within the same herd outweigh the effect of parameters that could be manipulated through changes in farm management.

### *1.2.2 Second experimental chapter (Chapter 4)*

Based on the identification of cows that consistently produced milk with small or large mean diameters, the second experimental chapter addressed the hypothesis that observed variations

in MFG size distributions between individual cows with the small or large MFG phenotype are related to physiological differences in the lipid metabolism in the mammary gland. The unique lipid metabolism in ruminants allows an estimation of the origin of FAs incorporated into milk from *de novo* synthesis (FAs with less than 16 carbons) or as preformed FAs (more than 16 carbons) from the blood circulation, which can be indicative of the metabolic state of the animal. Differences in energy balance in early lactation are believed to be the reason for the larger average MFG size during the transition period (Argov-Argaman, 2019). This analysis, on the other hand, aimed to establish if the source of FAs also differed between cows that were fed the same diet and were in a stage of their lactation cycle (> 50 days in milk) where negative energy balance is unlikely to be the reason for the differences in MFG size. This chapter focused on the core of the MFG because it contains the majority of all milk lipids. The MFGM was excluded from this analysis to limit the confounding impact of the higher proportion of membrane material needed to coat smaller MFGs. Biological membranes have a distinct FA composition with a high proportion of unsaturated FAs and two thirds of the MFGM are directly derived from the apical plasma membrane, which could have impacted the results.

### *1.2.3 Third experimental chapter (Chapter 5)*

Milk contains an estimated number of around 1000 lipid species (Liu et al., 2018). However, many of these remain unknown and species from minor lipid classes have not been studied in relation to MFG size (Liu et al., 2018). This lack of extensive lipidomic analyses in the literature provides an attractive avenue to study the broader lipid metabolic differences, which could lead to more focused analyses in future studies. Therefore, fresh raw milk samples from the selected animals, were used to compile a comprehensive snapshot of the individual animal's lipid metabolic profile. Because milk fat globules derive their surrounding trilayer membrane entirely from intracellular membranes and the apical plasma membrane, the polar lipid composition of milk, which is mostly derived from the MFGM, can non-invasively provide an

insight into the possible size development prior to secretion. The aim was to capture differences in the major polar lipid classes which are believed to drive the occurrence of LD fusion (Cohen et al., 2017), as well as their metabolic precursors. Due to the high sensitivity of the applied targeted liquid chromatography tandem mass spectrometry (LC-MS/MS) technique, this analysis also included minor lipid classes that have not gained much attention in relation to MFG size, such as lysophospholipids and ceramides. Furthermore, where possible, the FA composition within each of the major lipid classes was also determined.

### *1.2.4 Fourth experimental chapter (Chapter 6)*

A further objective of this thesis was to explore the possibility of establishing an *in vitro* cell culture model to study MFG size development in mammary epithelial cells by isolating primary cell cultures from the raw milk sampled from individual cows (Chapter 6). To achieve this, the aim was to apply a non-invasive technique to collect primary bovine mammary epithelial cells from the milk of individual cows. This method, using an immunomagnetic bead-based technique to purify epithelial cells, has been successfully used in other laboratories to obtain cell material for gene expression analyses (Boutinaud et al., 2008, 2015). Cells derived from the entire cell population in milk have previously been used in cell culture and are reported to produce major milk proteins (Hillreiner et al., 2017). However, it was unknown if purified primary cells from raw milk were functional in terms of their ability to produce LDs, as demonstrated in primary cell cultures derived from mammary tissue (Cohen et al., 2017). The hypothesis was that purified mammary epithelial cells may require different culture conditions than the ones traditionally applied in mixed cultures form mammary tissue, which were also included as controls in this final experimental chapter. Therefore, a 3D-like environment was tested, where mammary epithelial cells extracted from raw milk were cultured on permeable memebrane supports (Transwell inserts). Transwell inserts are frequently used for other

epithelial cell types such as intestinal and retinal epithelial cells (Gao et al., 2017; Sonoda et al., 2009), but have not been used for primary mammary epithelial cells.

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# CHAPTER 2

## Literature Review

## 2.1 Introduction

Milk fat globules (MFGs) supply lipids in a structure that is unique to the mammary gland. Also unique is the wide range of sizes found in cow milk. Previous studies have reported dynamic changes in MFG size distributions based on many factors. However, the physiological reasons for these changes are yet to be identified. This literature review will provide necessary background information on the known mechanisms of lactation physiology and give a detailed account of metabolic pathways involved in MFG formation and secretion. Furthermore, it will summarise the currently available literature on the effect of farm-related factors as well as environmental and physiological parameters on MFG size. Although the focus of this review will be on the lactation physiology of cows and the properties of cow milk, other species and their milk properties will be discussed when appropriate.

## 2.2 The Mammary Gland

### *2.2.1 Evolution of the mammary gland*

The secretion of milk is one of the main characteristics of mammals and the origin of lactation is believed to date back far beyond the appearance of mammals about 160 million years ago (Oftedal, 2012). In fact, lactation may have developed in synapsids, the ancestral lineage to mammals, approximately 310 million years ago (Oftedal, 2012). Oftedal (2002a, 2002b) hypothesised that mammary glands evolved from apocrine skin glands and that milk was secreted in order to protect parchment-shelled eggs in synapsids from drying out. He stated that both types of secretion found in mammary glands, namely exocytosis of secretory vesicles and secretion of cellular components enveloped by the cytoplasmic membrane, are also found in apocrine skin glands.

On the other hand, Vorbach et al. (2006) suggested that the mammary gland originates from the innate immune system and that milk was developed as a secretion for immune protection before its function to nourish the young. This hypothesis was based on the presence of specific peptides, small proteins and enzymes in milk that are also part of the innate immune system. Therefore, they suggested that the mammary gland evolved as a mucous skin gland (Vorbach et al., 2006). Oftedal (2012) supported this hypothesis and stated that the suggested theories are not contradictory, since the evolution of the innate immune system dates back earlier than the development of apocrine-skin glands, suggesting that the apocrine-skin gland can be considered as an intermediate stage of evolution from the innate immune system to the mammary gland.

### *2.2.2 Mammogenesis, lactogenesis and galactopoiesis in the cow*

As opposed to most organs, which are fully developed in the newborn, the mammary gland in mammals changes considerably after birth. Although the processes involved in mammary gland development, lactation and involution are similar for all mammals, this section is focused on the dairy cow. The development of the mammary gland, also known as mammogenesis, starts with the formation of the mammary bud and teat development before birth and continues in the postnatal period with the formation of secondary and tertiary ducts (Hurley and Loor, 2011). Originally consisting of ducts with limited side branching, the mammary gland experiences a first change during puberty (Katz and Streuli, 2007). These changes include elongation of the ducts and the development of additional branches leading to the formation of terminal ductule lobular units (Hurley and Loor, 2011). During pregnancy, further maturation occurs and terminal ductule lobular units develop into clusters of alveoli forming the lobuloalveolar unit (Katz and Streuli, 2007). Complete proliferation and differentiation of mammary epithelial cells, a process commonly referred to as lactogenesis, leads to milk secretion during lactation (Katz and

Streuli, 2007). The ultrastructure of the mammary epithelial cell (MEC) also changes considerably at the onset of lactation, with an increase in cytomembranes, mainly due to a significant growth of the rough endoplasmic reticulum (ER) and Golgi apparatus at the basal side of the MEC (Saacke and Heald, 1974). During galactopoiesis, lactation is maintained, through continuous milk removal and hormonal stimulation (Capuco and Akers, 2011). Finally, the mammary gland again experiences major changes, with involution of the tissue through apoptosis in the three to four weeks after cessation of lactation (Hurley and Loor, 2011).

The regulation of mammogenesis, lactogenesis, galactopoiesis and involution are under the control of several hormones, sometimes through coordinated actions. In brief, estrogen and progesterone are responsible for mammogenesis in the post-pubertal phase and during pregnancy. Progesterone serum concentrations are high throughout pregnancy and estrogen blood concentrations peak towards the end of pregnancy leading to complete proliferation and differentiation of the secretory epithelium (Hurley and Loor, 2011). The onset and maintenance of lactation are stimulated by prolactin and growth hormone, with the latter being directly related to milk yield in cows (Hurley and Loor, 2011). Galactopoiesis is further maintained by insulin-like growth factor 1, glucocorticoids, thyroid hormones and potentially placental lactogen in pregnant cows (Capuco and Akers, 2011)

## 2.3 Milk – The Mammary Secretion

Milk from the bovine mammary gland, like in other species, serves the nutrition of the young but has also become a basic food for human consumption. Alveoli are the smallest functional units of the mammary gland and the site of milk secretion. They contain a monolayer of secretory MECs surrounded by connective tissue and myoepithelial cells

which, upon contraction, are responsible for milk ejection into the alveolar lumen (McManaman and Neville, 2003). Mammary epithelial cells are polarised cells, which take up components from the basal side and secrete products at the apical side. Several alveoli that drain milk into the same duct are called lobules. The milk from the lobules in turn, is drained into the mammary duct and the milk from several mammary ducts is collected in the gland cistern and drained through the teat cistern into the streak canal, where the milk is ejected (Lawrence et al., 2012).

The major milk constituents, although variable in concentrations between mammalian species, are proteins, lipids, lactose, minerals and fat soluble vitamins (Fox and Kelly, 2012). Therefore, milk presents a rich source of a variety of essential nutrients which, due to their different physical properties, are found as dispersed molecules (whey proteins), colloidal particles (casein aggregates with minerals), emulsions (milk lipids) or solubilised in the aqueous continuous phase (lactose, vitamins and mineral salts) (Fox et al., 2015).

### *2.3.1 Milk proteins and lactose*

The protein fraction in bovine milk, as in most dairy species, consists of 20% whey proteins and 80% caseins (Fox and Kelly, 2012). The total protein content varies depending on the influence of several factors, such as breed, diet, health, stage of lactation and individual differences between cows, although to a lesser extent compared to their impact on the fat content (Fox and Kelly, 2012).

Most of the proteins are uniquely found in milk and are synthesised in the mammary secretory cell, whereas other proteins are derived from the blood stream (O'Mahony and Fox, 2013). The site for protein biosynthesis in the mammary gland is the rough ER, where a pool of free amino acids is used to produce the proteins that are subsequently

transported to the Golgi apparatus for packaging. In the Golgi they are incorporated into secretory vesicles and later secreted from the secretory cell by fusion of the secretory vesicle with the apical plasma membrane (Larson, 1979).

The casein fraction of bovine milk comprises $\alpha_{S1}$, $\alpha_{S2}$, β, and κ-casein, with concentrations of 38%, 10%, 36% and 12%, respectively (Fox and Kelly, 2012). Amongst the four caseins a number of genetic variants exist, and they are frequently subject to post-translational modification (Huppertz, 2013). Structurally, caseins are mainly unfolded, due to a high proline content resulting in their low percentage of secondary α-helixes and β-sheets (O'Mahony and Fox, 2013). Furthermore, caseins are phosphorylated which allows them to bind substantial amounts of calcium phosphate through the formation of spherical colloidal particles of approximately 100 nm in size, called casein micelles (Horne, 2011). If caseins were present in the form of individual proteins, the high calcium content in milk would lead to precipitation of the αs1- , αs2- and β-caseins. However, κ-casein on the surface of the micelles, which is soluble at the calcium concentration in milk, protects the other caseins from precipitation (Fox and Brodkorb, 2008). This micellar structure is responsible for many of the technological properties of milk, for example, the scattering of light which is responsible for the white colour of milk (O'Mahony and Fox, 2013). Other important properties of the casein micelle are that it remains in a stable colloidal dispersion upon exposure to heat, dehydration, freezing and homogenization - all important attributes during the initial handling and processing of milk (Fox and Brodkorb, 2008). During cheese making, on the other hand, the casein micelles are destabilised through the hydrolysis of κ-casein using rennet or acidification, leading to coagulation of milk (O'Mahony and Fox, 2013).

Upon acidification at pH 4.6, the casein fraction is separated from the whey protein fraction (Fox and Kelly, 2012). At this pH caseins are insoluble and precipitate, whilst

whey proteins and calcium phosphate stay in solution (O'Mahony and Fox, 2013). The whey fraction in bovine milk contains four major proteins, β-lactoglobulin, α-lactalbumin, immunoglobulins and blood serum albumin, with percentages of 40-50%, 20%, 10% and 10% of the whey protein fraction, respectively (Fox and Kelly, 2012). Colostrum can contain higher proportions of immunoglobulins and the decrease in total protein in milk one week after parturition is mainly attributed to the decrease in immunoglobulins from 10% to 0.1% (Fox and Kelly, 2012). In contrast to the casein fraction, which are exclusively formed in the MEC, some of the whey proteins, blood serum albumin, immunoglobulins and some minor proteins, are derived from the blood (Fox and Kelly, 2012). β-lactoglobulin is the major whey protein in the milk of ruminants, however it is not found in milk from humans, rats and mice (Sawyer, 2013). Compared to the caseins it is a highly structured protein and thus resistant to proteolysis. Therefore, it is believed to be a major determinant of the physico-chemical properties of milk (O'Mahony and Fox, 2013). However, its detailed biological role remains elusive, although it has also been suggested that β-lactoglobulin has nutritional functions, such as a possible role in retinol metabolism (Sawyer, 2013).

α-lactalbumin is a metalloprotein capable of binding calcium (Fox and Kelly, 2012). However, the major function of α-lactalbumin in bovine milk is its involvement in lactose synthesis in the MEC, as part of lactose synthetase, the enzyme responsible for the lactose synthesis from galactose and glucose (Brew, 2013). The final steps of lactose synthesis occur in the Golgi, where it is subsequently packed into secretory vesicles along with the milk proteins (Stelwagen, 2011). Its content in milk partly determines the total milk yield, because it creates osmotic pressure leading to the influx of water into the alveolar lumen (Fox and Kelly, 2012). Other minor proteins in milk are enzymes and proteins of the milk

fat globule membrane (MFGM, 1%). Due to their role in MFG stabilisation and secretion the latter will be discussed in more detail in Section 2.3.2.3.

### *2.3.2 Milk lipids*

The amount of lipids in milk varies considerably between mammalian species, lying within a range from less than 1% in the black rhinoceros to up to 60% in some seals (Oftedal and Iverson, 1995). It is highly influenced by the habitat of the mammal and its suckling young (Oftedal, 2012). The percentage of milk lipids in bovine milk is approximately 3 - 5% (Singh, 2006). Amongst the lipid fraction, the majority are triacylglycerols (TGs) (98%), with the remainder being glycerophospholipids (PLs) (1%), di- and monoacylglycerols, sterols (cholesterol and cholesterol-ester), traces of fat-soluble vitamins and other components (Fox and Kelly, 2012).

Most lipid synthesising tissues produce lipoproteins to make lipids soluble in an aqueous phase, for example in blood (Argov et al., 2008). The mammary gland, however, has developed a unique mechanism to make milk lipids water-soluble: the milk fat globule (Argov et al., 2008; Mulder and Walstra, 1974). The intracellular precursors of MFGs, called cytoplasmic lipid droplets (LDs), in fact have many structural similarities to lipoproteins (McManaman, 2012). Their neutral lipid core contains approximately 95% of TG and they are surrounded by a monolayer comprising polar lipids (PLs and sphingolipids (SLs)) and a number of proteins (McManaman, 2012). Despite these similarities, the mammary gland uses a secretion mechanism vastly different to the mechanisms employed by other lipid synthesising organs (e.g. liver or adipose tissue), leading to major differences in the structure of post-secretory MFGs compared to lipoproteins (McManaman, 2012).

Lipoproteins are released from the cell by fusion of the secretory vesicles with the plasma membrane (Vance and Vance, 1990). The same process (exocytosis) is used in MECs to secrete caseins along with other milk proteins and substances such as water, oligosaccharides and calcium into the alveolar lumen (McManaman and Neville, 2003). The components are integrated in secretory vesicles in the Golgi before being transported to the apical plasma membrane and released into the lumen through fusion of the vesicle with the membrane (McManaman and Neville, 2003).

*2.3.2.1 Lipid droplet formation*

The most commonly accepted theory for the mechanism underlying MFG secretion is that LDs, the intracellular precursors of MFGs, are formed in the ER where they are enveloped by the outer leaflet of the ER, a monolayer of polar lipids and proteins (Heid and Keenan, 2005; Zaczek and Keenan, 1990). Components of the monolayer derived from the cytoplasmic half of the ER membrane, may play a role in MFG secretion by interacting with the plasma membrane (Keenan and Mather, 2006). During transit from the ER towards the apical plasma membrane small LDs can grow in size by fusion, which again is influenced by the components found in the monolayer surrounding the intracellular LD (Keenan and Mather, 2006). Recent studies have provided *in vivo* and *in vitro* time-lapse images of LD fusion events in the MEC and provide compelling evidence that the polar lipid fraction of the monolayer plays a regulatory role (Cohen et al., 2017; Masedunskas et al., 2017) and are discussed in detail in Section 2.5.3.1.

Furthermore, a number of studies by Robenek and co-workers in the last decade have revealed new insights into the potential mechanisms of LD biogenesis and secretion (Robenek et al., 2006b, 2006a, 2009). According to their studies, LDs are potentially formed and most probably grow in size in close proximity to the ER but not within the

ER bilayer membrane (Robenek et al., 2006b). In the proposed process the ER double membrane embraces the LD like an eggcup and egg, allowing the transferal of lipids and LD-related proteins into the droplet (Robenek et al., 2006b).

*2.3.2.2 Milk fat globule secretion*

After transit from the basal to the apical side of the MEC, again a widely unknown process, LDs are secreted by a budding process, where they are enveloped by the bilayer of the plasma membrane (Heid and Keenan, 2005). Hence, the post-secretory structure of MFGs is a lipid core mainly consisting of TGs surrounded by a monolayer derived from the cytoplasmic leaflet of the ER and a bilayer membrane derived from the mammary epithelial cell. This tripartite membrane surrounding the secreted MFG is called the MFGM and was first described by Bargmann and Knoop (1959). The suggested mechanisms for LD formation and secretion are illustrated in Figure 1.

The mechanisms underlying milk lipid secretion on a molecular level still need to be elucidated. A number of proteins are associated with the MFG secretion process: Butyrophilin (BTN), an integral plasma membrane protein; xanthine oxidoreductase (XOR), a protein found in high concentrations in the apical region of MECs during lactation; and perilipin 2 (PLIN2), also known as adipophilin or adipose differentiation-related protein, which is a protein associated with the surface of LDs (McManaman et al., 2002; Robenek et al., 2006a). To date, three different models have been suggested by different researchers, each of which attributes the major role in lipid secretion to one or more of the above-mentioned components.

Mather and Keenan (1998) suggested that these proteins form a tripartite complex, where PLIN2 on the surface of the LD binds to a complex between XOR and the cytoplasmic terminus of BTN, anchoring the LD into the apical membrane of the MEC prior to

secretion. Even though there is scientific evidence supporting this theory (McManaman, 2012), others have challenged the tripartite model (Robenek et al., 2006a). According to Robenek et al. (2006a), BTN plays the major role in MFG secretion through interactions between BTN on the LD monolayer and BTN in the plasma membrane. They localised BTN in high concentrations in the ER, in the monolayer of the LD and the plasma membrane by freeze-fracture replica immunogold labelling electron microscopy and argue that methods used in previous studies do not exhibit the resolution necessary to localise the respective proteins in biological membranes (Robenek et al., 2006a).

Other researchers have reported major roles for XOR in the secretion of MFGs (Vorbach et al., 2002). They showed that mice deficient in XOR produce LDs and transport them to the apical membrane. However, the final secretion step is missing, suggesting a regulatory role of XOR in the secretory process. Lastly, PLIN2 is important for accumulation and growth of LDs in MECs (Russell et al., 2007, 2011) and Chong et al. (2011) later hypothesised that it also involved in milk lipid secretion. They showed that PLIN2 binds directly to the apical plasma membrane, induces its curvature and thus results in a subsequent recruitment of BTN and XOR to the plasma membrane, suggesting a crucial role for PLIN2 in the initiation of the lipid secretion process.

In an attempt to test these previously existing models, Jeong et al. (2013) added a new level of controversy to the matter. They set out to study the location and mobility of BTN, XOR and PLIN2 in LDs and in the plasma membrane of the mammary secretory cell and reported that BTN could not be found on the surface of intracellular LDs but only on LDs located at the plasma membrane. This fundamentally contradicts the model suggested by Robenek et al. (2006a). The same group previously suggested that MFG secretion is coupled with exocytosis of secretory vesicles derived from the Golgi apparatus (Wooding, 1971) and recently provided immunocytochemical evidence for their

hypothesis (Wooding and Sargeant, 2015). They further reported a frequently observed association of Golgi vesicles with LDs, suggesting that Golgi vesicle exocytosis initiates MFG secretion and that part of the MFGM is directly derived from the Golgi vesicle (Wooding and Sargeant, 2015).

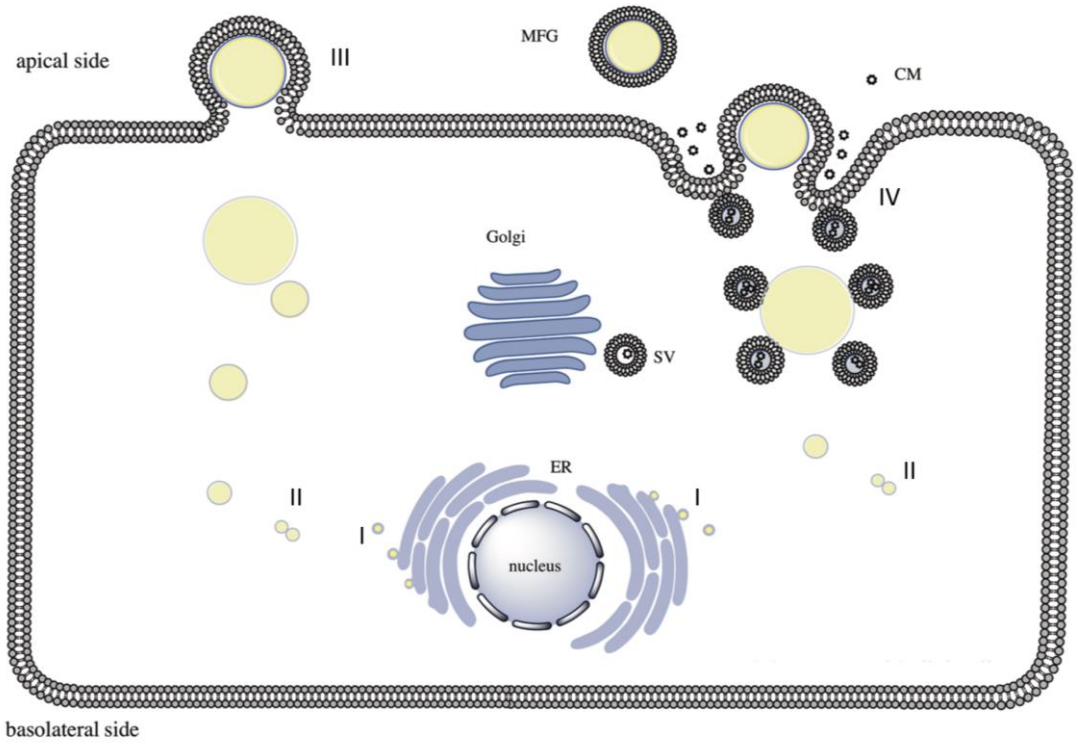


**Figure 1**: Formation of lipid droplets (LDs) and mechanisms leading to the growth, transport and secretion of LDs into milk as milk fat globules (MFGs). Two major pathways for MFG secretion have been suggested both including several steps: I) the process starts with accumulation of LDs in or in close proximity to the endoplasmic reticulum (ER); II) After release from the ER, LDs can grow in size by fusion or growth through local triglyceride and polar lipid synthesis; MFGs are secreted directly through III) the direct envelopment of the LD with a portion of the apical plasma membrane; or IV) the secretion of LDs is combined with the secretion of secretory vesicles (SVs), which contain casein micelles (CMs) and other milk constituents that are released into the apical lumen through exocytosis, where part of the membrane material surrounding the secreted MFG is derived from the bilayer membrane surrounding the SVs.

*2.3.2.3 Structure of the MFG*

Milk fat globules are spherical structures comprising a lipid core surrounded by a trilayer membrane of approximately 10 - 50 nm thickness, as illustrated in Figure 2 (Lopez et al., 2011). The FA composition has been extensively studied, revealing that there are more than 400 different fatty acids, amongst them 12 with a percentage higher than 1%. The MFGM only makes up 2 - 6% of the total weight of the MFG, with an increasing mass in smaller MFGs (Lopez et al., 2011). The lipid core consists of more than 95% TGs (McManaman, 2012). As previously described, the MFGM keeps milk lipids emulsified in the aqueous phase. It also protects the lipid core from lipolysis and prevents aggregation of the lipids and thus prevents the creaming of milk (Deeth, 1997). Besides these crucial functions, the MFGM further provides important factors for the immune system of the neonate, protecting it against bacteria and viruses (Fong et al., 2007).

Due to the MFGM originating from the plasma membrane of the MEC, the composition of the MFGM is similar to the composition of the plasma membrane (German and Dillard, 2006). It primarily contains proteins, glycoproteins, enzymes, neutral lipids (mainly TGs) and polar lipids (mainly PLs and SLs) (Fong et al., 2007). Other components present in smaller or trace amounts are sterols, fat-soluble vitamins and carotenoids (Lopez et al., 2011). The protein content of the MFGM has been reported to be between 25 and 70% of the dry matter, with the remainder being primarily PLs along with some neutral lipids, including cholesterol and TG (Dewettinck et al., 2008; Fong et al., 2007). The quantitative composition of the lipid fraction is also inconsistent in the literature, with some researchers reporting the presence of neutral lipids in the MFGM in relatively high amounts (e. g. 56% of the lipid fraction (Fong et al., 2007)) and others arguing that these

lipids are only present due to contamination during the isolation process of the MFGM from the lipid core (Dewettinck et al., 2008).

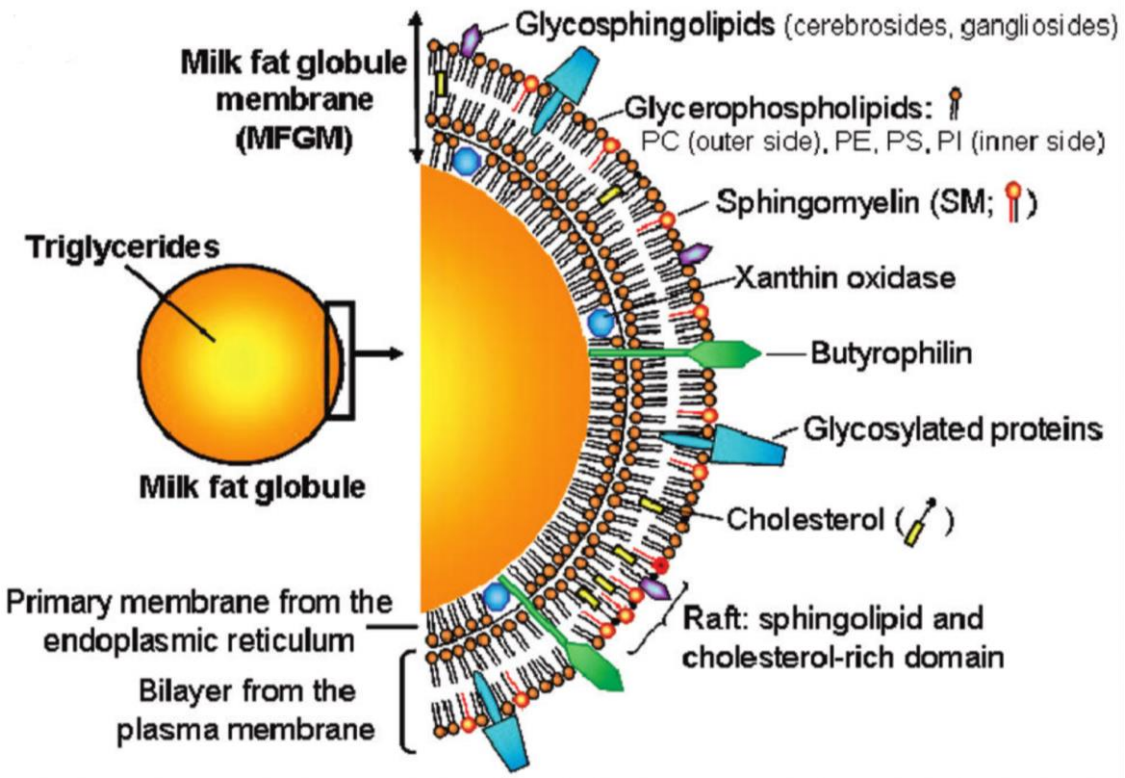


**Figure 2**: Structure of the milk fat globule. A neutal lipid core, consisting mainly of triacylglycerols, is surrounded by a three-layer membrane containing polar lipids and proteins. Sourced from Lopez et al. (2008).

The polar lipids in milk only make up 0.2 to 1% of the total lipids (Lopez et al., 2011). However, they have a major impact on milk characteristics, such as the presence of high contents of protein and lipids in the aqueous continuous phase in milk. This is only possible because of the amphiphilic structure of polar lipids (MacGibbon and Taylor, 2006). Phospholipids contain a glycerol backbone esterified with two FAs (hydrophobic end) and a polar, hydrophilic head (phosphate group and organic residue, e.g. ethanolamine, serine, choline, inositol) (Huppertz et al., 2009; Lopez et al., 2011). Sphingolipids are comprised of a long chain sphingoid base connected with a FA though the amino group, and, in the case of sphingomyelin (SM) which is a phosphosphingolipid,

it is also esterified with a phosphorylcholine group (Lopez et al., 2011). The three major polar lipids in milk are phosphatidylcholine (PC), phosphatidylethanolamine (PE) and SM (Huppertz et al., 2009).

Proteins in the MFGM only make up 1 - 2% of the total protein fraction in milk (Lopez et al., 2011). Herein, the major proteins identified are BTN, XOR, the glycoproteins mucin 1 and mucin 15, PLIN2, periodic acid schiff glycoprotein 6/7, also called lactadherin, cluster of differentiation 36 and fatty acid binding protein (Keenan and Mather, 2006).

## 2.4 Mammary Lipid Metabolism in Ruminants

### *2.4.1 Origin of milk fatty acids*

Fatty acids are the structural subunits of all lipid species of the MFG core and the MFGM and the FA composition of bovine milk has been extensively studied. In bovine milk, FAs are predominantly saturated with a length of four to 18 carbons (C4 to C18), although the monounsaturated FA oleic acid (C18:1) is also found in high proportions (Fox and Kelly, 2012). The FAs with the highest amounts in milk are palmitic acid (C16:0), oleic acid (C18:1), stearic acid (C18:0) and myristic acid (C14:0) (Jensen, 2002). Furthermore, butanoic acid (C4) in ruminant milk is the only FA with four carbons in length found in natural lipids (Fox and Kelly, 2012).

Depending on the length of their carbon chain, FAs in ruminant milk are obtained from two major sources: extracted as preformed FAs from the blood stream (> C16) or *de novo* synthesised in the MEC (C4 to C14) (Huppertz et al., 2009). Some preformed FAs are provided in the form of nonesterified FAs (NEFAs) hydrolysed from TGs stored in adipose tissue. However, predominantly they are supplied in the form of TGs packaged in chylomicrons or very low density lipoproteins from intestinal epithelial cells after

absorption from the diet (Palmquist, 2006). The only FAs that can originate from both *de novo* synthesis and the blood circulation are FAs with 16 carbons in their chain (Huppertz et al., 2009). The proportion of *de novo* synthesised FAs is lower in colostrum but is in balance with the proportion of FAs taken from circulating blood, approximately one week after parturition. In ruminants, the main carbon source for *de novo* FA synthesis is acetate (92%), produced from bacteria in the rumen as an end product of fermentation (Huppertz et al., 2009). For monogastric animals, on the other hand, glucose serves as the main carbon source (Huppertz et al., 2009).

Importantly, in ruminants, the milk FA composition does not directly reflect the FA profile of the dietary lipids (Palmquist, 2006). The reason is that unsaturated FAs (UFAs) are almost completely hydrogenated by rumen bacteria and absorbed predominantly in the form of stearic acid (C18:0). Instead, UFAs in milk are produced by desaturation of saturated FAs (SFAs) in mammary and adipose tissue through the introduction of a cis double bond at the Δ9 position, a reaction catalysed by stearoyl-coenzyme A desaturase (SCD) (Kinsella, 1970). The preferred substrates for SCD are stearoyl- and palmitoyl-CoA and the concentrations of oleic acid and palmitoleic acid, their respective products after desaturation, partly depends on SCD activity (Miyazaki and Ntambi, 2003). Furthermore, oleic acid can be an indicator for increased lipid mobilisation from adipose tissue (Gross et al., 2011) and subclinical ketosis in dairy cows (Van Haelst et al., 2008). Preformed as well as *de novo* synthesised FAs are subsequently incorporated into neutral lipids or polar lipids within the MEC.

### *2.4.2 Biosynthesis of neutral lipids*

The vast majority of lipids secreted into milk are in the form of TGs, comprising about 97% of total lipids in milk (Mather and Keenan, 1998). Structurally, TGs contain three

FAs connected to a glycerol backbone and their synthesis is accomplished mainly in the ER of the MEC. All cell types have the ability to produce TGs, which is crucial for the transport and storage of FAs in a non-toxic form (Coleman and Lee, 2004). The dominant pathway for TG synthesis in most tissues is the glycerol phosphate pathway. An alternative pathway, called the monoacylglycerol pathway, is only relevant in intestinal epithelial cells (Iqbal and Hussain, 2009) and will not be discussed here.

The glycerol phosphate pathway comprises four steps and is shown in Figure 3. The rate limiting step is catalysed by glycerol phosphate acyl transferases, which are responsible for the acylation of glycerol-3-phosphate to form lysophosphatidic acid (Yen et al., 2008). Another important step during TG synthesis is the final step, where diacylglycerol (DG) is acylated to TG. This step is catalysed by diacylglycerol acyl transferases (DGATs) and might be of particular interest for MFG size development because DG is not only the precursor for TG synthesis but also for PL synthesis and therefore represents a possible determinant of LD size (Wilfling et al., 2013).

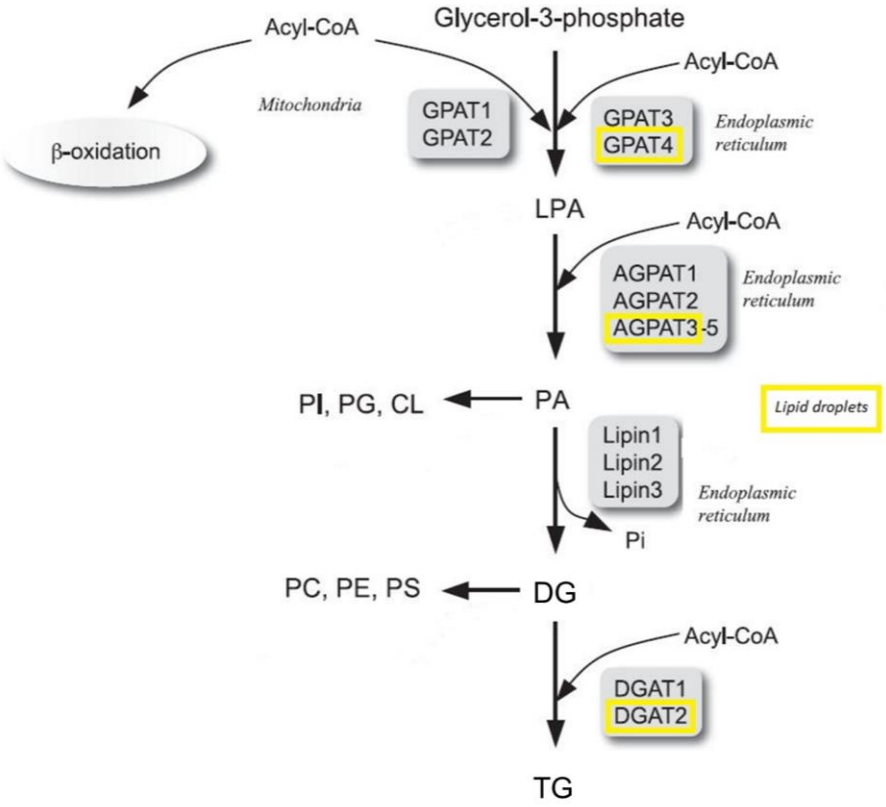


**Figure 3**: Glycerol phosphate pathway for *de novo* triacylglycerol (TG) synthesis. In the endoplasmic reticulum, glycerol-3-phosphate is acylated to form lysophosphatidic acid (LPA) followed by another acylation to form phosphatidic acid (PA). PA is subsequently dephosphorylated to form diacylglycerol (DG), which is the direct precursor for the synthesis of the main glycerophospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE), as well as TGs. Yellow boxes indicate that these enzymes have been identified on the surface of LD in non-mammary cells. AGPAT, acylglycerolphosphate acyltransferase; CL, cardiolipin; DGAT, diacylglycerol acyl transferase; GPAT, glycerol phosphate acyl transferase; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine. Lipins are a family of phosphatidic acid phosphatase enzymes. Modified from Takeuchi and Reue (2009).

### *2.4.3 Biosynthesis of polar lipids*

Polar lipid classes in milk are predominantly PLs (PC, PE, phosphatidylserine (PS), phosphatidylinositol (PI)) and SLs (mainly SM). Pathways for their biosynthesis are interconnected with the TG pathway through their common intermediate DG, which

therefore takes a central role in lipid metabolism. Phospholipids can also be interconverted by headgroup exchange, which can occur in different cellular compartments (Figure 4).

In contrast to PLs, SLs do not contain a glycerol backbone and their synthesis differs from PL synthesis, but is connected to the TG and PL pathways through DG as an intermediate. Sphingolipid biosynthesis starts with the formation of 3-ketosphinganine from its two precursors L-serine and palmitoyl-CoA, which is the rate limiting step catalysed by serine palmitoyltransferase and takes place in the ER (Deevska and Nikolova-Karakashian, 2017). Subsequently, sphinganine is produced from 3-ketosphinganine by a reductase and then acylated to form dihydroceramide. The enzymes responsible for dihydroceramide production are called ceramide synthases and different isomers of these enzymes have a specific affinity for FAs with a certain chain length and saturation (Deevska and Nikolova-Karakashian, 2017). Desaturation of the sphinganine base in dihydroceramide leads to the formation of ceramide. Once transported to the Golgi, ceramide is utilised to produce SM, one of the major membrane lipids (Figure 4). This reaction, where a choline headgroup from PC is transferred to ceramide to form SM catalysed by SM synthase 1 (SMS1), leads to the production of DG and is the point where the pathways for TG, PL and SL synthesis are interconnected (Deevska and Nikolova-Karakashian, 2017). Moreover, it is believed that SL synthesis can regulate the utilisation of DG for PC synthesis instead of TG synthesis. The reason is that SMS1, which requires PC to produce SM from ceramide, can activate PC depletion sensing mechanisms and thus stimulates PC synthesis (Deevska et al., 2017).

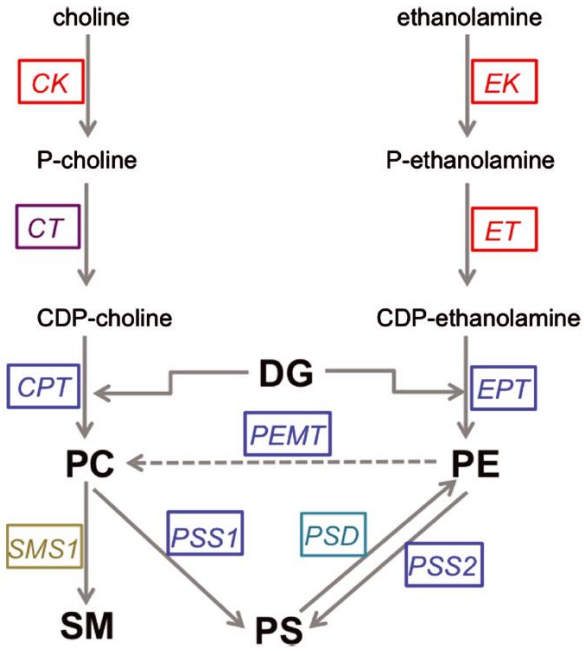


**Figure 4**: Pathways for the synthesis and interconversion of phospholipids in mammalian cells. The pathways shown are cytidine diphosphate (CDP)-choline and CDP-ethanolamine pathways for phosphatidylcholine (PC) and phosphatidylethanolamine (PE) *de novo* synthesis, respectively. Choline or ethanolamine are first phosphorylated by their respective choline kinase (CK) and ethanolamine kinase (EK). This step is followed by the conversion of phosphocholine or phosphoethanolamine to CDP-choline and CDP-ethanolamine by CTP-phosphocholine cytidylyltransferase (CT) or CTP-phosphoethanolamine cytidylyltransferase (ET). CDP-choline and CDP-ethanolamine are then transferred as the respective headgroups to diacylglycerol (DG) by CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT) or ethanolaminetransferase (EPT). PC and ceramides can further be converted to sphingomyelin (SM) by sphingomyelin synthase 1 (SMS1). Phosphatidylserine (PS) is synthesised through PS synthase 1 (PSS1) from PC or by PS synthase 2 (PSS2). PS can also be converted into PE through PS decarboxylase (PSD). Finally, PE can be transformed into PC in an alternative pathway catalysed by the enzyme phosphoethanolamine methyl transferase (PEMT). The dashed line indicates that this reaction is believed to occur mainly in hepatocytes. Colours indicate the cellular compartments in which the respective reactions take place: endoplasmic reticulum (blue), cytosol (red), nucleus (purple), mitochondria (turquoise) and Golgi apparatus (brown). Sourced from Vance (2015).

The only subclass of polar membrane bound lipids that do not rely on DG as a precursor are ether PLs (ePLs). Although the presence of ePLs in milk fat has been reported (Contarini and Povolo, 2013), they are an understudied subclass of PL species and have not been studied in relation to MFG size. This subclass of PLs structurally differs from conventional PLs in the presence of an ether bond (plasmanyl- species) or vinyl ether bond (plasmenyl- or plasmalogen species) instead of an ester bond at the sn-1 position (Lodhi and Semenkovich, 2014). Importantly, this structural difference to their diacyl counterparts requires alkylacylglycerol (AAG) instead of DG as an immediate precursor and ePL synthesis involves peroxisomes (Dean and Lodhi, 2018). In peroxisomes the alkyl-equivalent of lysophosphatidic acid is produced through the acyl dihydroxyacetone phosphate pathway, which can also be a salvage pathway to produce lysophosphatidic acid as a precursor for DG and subsequent TG and PL synthesis (Figure 5).

However, the transferal of the choline or ethanolamine headgroup to AAG is achieved by the same enzymes that catalyse diacyl PC and PE synthesis (collectively called choline/ethanolamine phosphotransferase (CEPT)) and occurs in the ER (Figure 5). Therefore, ePL synthesis competes for the same enzymes in the final steps of synthesis but does not compete for the same DG pool. Ether PLs show different phase behaviour in biological membranes than their diacyl equivalents and are believed to be involved in membrane fusion (Dean and Lodhi, 2018). There is increasing recognition of the physiological importance of ePLs in membrane integrity and function and disorders in peroxisomal function, which result in impaired ePL synthesis, have been linked to diseases such as cancer, neurodegenerative diseases and metabolic disorders (Dean and Lodhi, 2018).

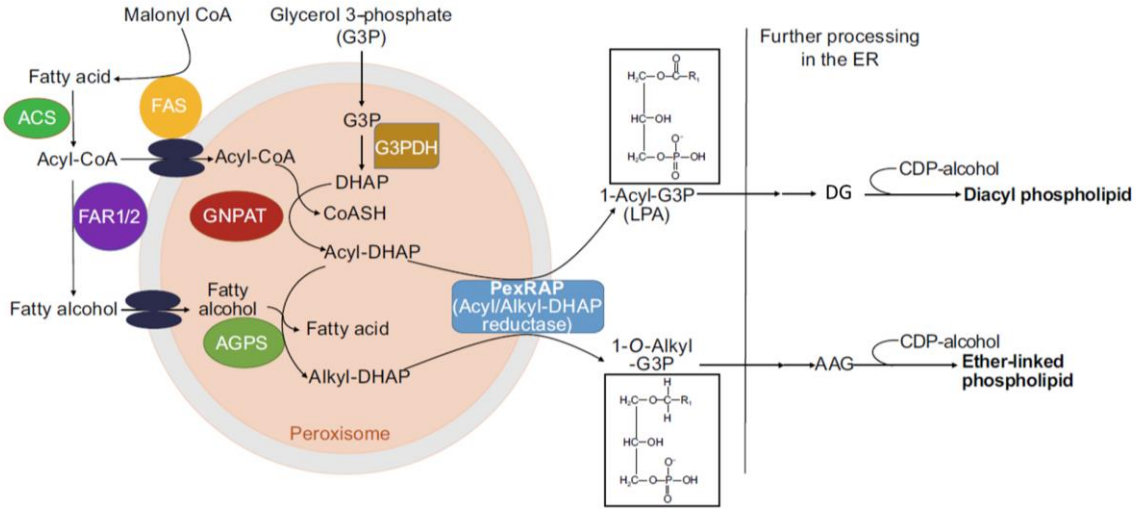


**Figure 5**: Ether phospholipid synthesis through the acyl dihydroxyacetone phosphate (DHAP) pathway. Fatty acids synthesised by fatty acid synthase (FAS) are used in a salvage pathway to produce lysophosphatidic acid (LPA) or are reduced by fatty acyl-CoA reductase (FAR1/2) to produce the fatty alcohol attached to the sn-1 position in ether phospholipids. DHAP is derived from glycerol 3-phosphate (G3P) after dehydrogenation through G3P dehydrogenase (G3PDH). Acyl-CoA is transferred to DHAP by glyceronephosphate O-acyltransferase (GNPAT) to produce acyl-DHAP, which can either directly be reduced to produce LPA or the acyl chain is replaced with an alkyl-chain by alkylglycerone phosphate synthase (AGPS) connected to DHAP via an ether bond (alkyl-DHAP). Reduction of alkyl-DHAP leads to the production of 1-O-alkyl-G3P (AGP), which is the ether equivalent of LPA. After transport of LPA and AGP to the endoplasmic reticulum (ER), they are acylated on the sn-2 position to produce diacylgycerol (DG) or alkylacylglycerol (AAG), the direct precursors of diacyl and ether PLs. In the final step, DG and AAG obtain their respective headgroup through the enzymes of the CDP-choline and CDP-ethanolamine pathways described in Figure 4. Modified from Dean and Lodhi (2018).

## 2.5 MFG Size

The size of individual MFGs varies from under 1 to over 15 μm, with the majority of the fat volume in globules between 1 and 8 μm (Michalski et al., 2001; Walstra, 1969). Furthermore, the average MFG size and its distribution varies between species, breeds

and individual animals (Carroll et al., 2006; Logan et al., 2014; Park et al., 2007). Jersey cows produce milk with larger MFGs than Holstein and Brown Swiss breeds, expressed as the volume mean diameter D[*v*, 0.5] (Carroll et al., 2006). Distinct MFG size distributions between species have also been established, although comparison of MFG sizes between studies are difficult if different methods (microscopy or dynamic light scattering) or measures (surface or volume weighted diameters or spherical diameters of individual particles) are used for particle size analysis. However, a study comparing the MFG sizes of several dairy species based on scanning electron microscopy showed that buffalos produced larger MFGs (average diameter 8.7 µm), whilst cow and sheep milk comprised medium size distributions (both 3.8 µm) and goat and camel milk exhibited the smallest size distributions (3.2 and 3.0 µm) (El-Zeini, 2006). These differences between species are even more apparent, when the percentage of MFGs in different size groups is compared (El-Zeini, 2006). For example, in buffalo milk, only 23% of all MFGs have very small sizes between 0.1 to 4 µm in diameter, whereas in cow, sheep, goat and camel milk 68.4, 55.3, 73.3 and 80.6% fall in this size group, respectively. Furthermore, the percentage of MFGs falling into the group of MFGs larger than 8 µm was 44.1% for buffalo, compared to 5.2% in cow, 1.0% in sheep 9.2% in goat and 0% in camel milk (El-Zeini, 2006). Considering that milk fat content has been identified as one of the major animal factors influencing MFG size, it is important to look at differences in fat content between these species. Table 1 shows minimum and maximum values for the fat content in milk from different species found in the literature compared to their differences in MFG size. In some species (e.g. buffalo, horse) high or low fat content in milk appears to be reflected in large or small MFG size distributions. However, other species that show similar MFG size distributions (e.g. cow and sheep) are remarkably different in their fat content, with sheep milk exhibiting a higher fat content than cow milk. For example,

sheep and buffalo secrete similar amounts of fat per litre of milk, however average MFG size in buffalo milk is more than twice the diameter of average MFG size in sheep milk.

**Table 1**: Fat content and milk fat globule (MFG) size distribution in milk from several species.

| Species | Fat content (g/L) (Claeys et al., 2014) | MFG size[1] (μm) | Reference (For MFG size) |
|---|---|---|---|
| Buffalo | 53 - 90 | 8.7 | (El-Zeini, 2006) |
| Human | 35 - 40 | 5.2 | (Rüegg and Blanc, 1981) |
| | | 4.4 | (Michalski et al., 2005) |
| Cow | 33 - 54 | 3.8 | (El-Zeini, 2006) |
| Sheep | 50 - 90 | 3.8 | (El-Zeini, 2006) |
| Goat | 30 - 72 | 3.2 | (El-Zeini, 2006) |
| Camel | 20 - 60 | 3.0 | (El-Zeini, 2006) |
| Horse | 3 - 42 | 2.8 - 2.9 | (Welsch et al., 1988) |

[1]Note that particle size measurements differ between the studies: El-Zeini (2006) used scanning electron microscopy to measure the spherical mean diameter of individual globules, while for Welsch et al. (1988), Michalski et al. (2005) and Rüegg and Blanc (1981) the volume-weighted mean diameter D[4,3] is shown.

On an individual animal level, several environmental, physiological and management factors have been linked to changes in the average MFG size in cows and other dairy species. However, while some of the estimated effects of these impact factors have been firmly established, others remain equivocal in the literature.

### *2.5.1 Environmental and farm management factors with an impact on MFG size*

Amongst the possible environmental factors, season and feed composition are most frequently reported to affect MFG size. Several studies reported that MFG size is subject to seasonal changes, although the results are often conflicting. While some have suggested that cows produce milk with larger MFGs in winter and autumn compared to summer and spring (Briard et al., 2003; Logan et al., 2014), the opposite has also been

reported, with larger MFGs in spring milk compared to the rest of the year (Fleming et al., 2017a). These differences may be attributed to the seasonal feed compositions depending on the climate and management conditions in different countries. Therefore, the role of season independently from dietary changes and stage of lactation remains unclear. Moreover, in herds practicing seasonal calving, the effect of season cannot be separated from stage of lactation, which represents one of the main impact factors on MFG size and will be discussed later (Section 2.5.2).

In terms of the impact of feed composition, the balance between concentrate and forage in the cow's diet can also impact MFG size (Argov-Argaman et al., 2014; Mesilati-Stahy et al., 2015). In both studies, cows fed a high-concentrate-low-forage diet produced smaller MFGs compared to cows fed a low-concentrate-high-forage diet (Argov-Argaman et al., 2014; Mesilati-Stahy et al., 2015). The authors attributed this to a higher fat content in milk produced by low-concentrate-high-forage cows leading to larger MFGs. However, overall milk yield was higher in the in the high-concentrate-low-forage group. Accordingly, Couvreur et al. (2006) showed that replacing at least 30% of corn silage with fresh grass in the cow's diet led to a decrease in MFG size. However, changes in the ratio of concentrate to forage in the cow's diet can also lead to changes in plasma insulin and NEFA concentrations, which are physiological factors that can also affect MFG size (Argov-Argaman et al., 2014).

Another understudied potential impact factor is the role of the applied milking technology (automatic milking vs. milking parlour) or the frequency and duration of milking (once vs. twice or thrice daily). Some studies reported no effect of milking frequency and technology on MFG size distribution (Abeni et al., 2005; Komara et al., 2009), while others reported an increase in MFG size with increased milking frequency (Wiking et al., 2006). In this context, it also remains unclear if a self-determined milking

frequency by the cows within a herd milked by an automatic milking system affects MFG size.

### *2.5.2 Animal factors with an impact on MFG size*

Stage of lactation is arguably the most studied and best-established animal-related determinant of MFG size. In bovine milk, the average MFG size decreases with ongoing lactation (Altenhofer et al., 2015; Fleming et al., 2017a), while in human milk, MFG size decreases during the first four days of lactation and increases thereafter (Michalski et al., 2005; Rüegg and Blanc, 1981). The reason for the inverse relationship between days in milk and MFG size in bovine milk are not fully understood. However, it has been suggested that it may be a reflection of the metabolic status of the animal, specifically the occurrence of negative energy balance in early lactation, leading to increased plasma NEFA concentrations (Argov-Argaman, 2019). Based on *in vitro* studies, it appears that exposure of MECs to NEFAs instead of lipids in the form of very low density lipoprotein, increases TG content as well as LD number and size (Mesilati-Stahy and Argov-Argaman, 2018). The authors attributed this to increased mitochondrial activity and remodelling of the PL components through the phosphatidylserine decarboxylase pathway, which decreases the PC/PE ratio in these cells through headgroup exchange of PS to PE and takes place in mitochondria. *In vivo*, plasma NEFA levels also appear to play an important role in the regulation of MFG size, but the precise mechanism and effect is still unclear (Argov-Argaman, 2019). This is at least in part due to the correlations between milk yield, fat content, insulin levels and NEFA concentrations which complicate the study of their individual effects on MFG size.

Cows producing milk with a higher fat content often produce larger MFGs compared to those producing milk with a lower fat content (Altenhofer et al., 2015; Couvreur et al.,

2007; Mesilati-Stahy et al., 2015). However, the lower fat content in milk from cows producing smaller MFGs could be due to a dilution effect, because these cows also often exhibit a higher milk yield and the total quantity of milk fat secreted by cows producing either large or small MFGs remained the same (Argov-Argaman et al., 2014; Couvreur et al., 2007). In terms of the impact of the total fat yield on MFG size, Logan et al. (2014a) reported no correlation between the fat yield in milk and MFG size distribution. However, others reported larger MFGs in cows producing milk with higher fat yields and suggested that the production of MFGM material is limited in these cows, leading to a shift in size towards bigger MFGs, requiring less membrane material (Wiking et al., 2004). While the available studies indicate that fat yield and fat content could impact MFG size, a recent combined analysis of several studies revealed that the fat-protein ratio was a better predictor of MFG size than fat content (Couvreur and Hurtaud, 2017). Endogenous factors, such as different genotypes of enzymes involved in lipid synthesis, may also influence fat content and hence MFG size. For example, cows exhibiting the KK genotype of DGAT1, the gene encoding the enzyme catalysing the final step of TG synthesis, produce larger MFGs along with an increased fat content (Argov-Argaman et al., 2013).

In summary, despite several published studies, the impact of environmental and animal factors on MFG size remains unclear. This may be attributed to the fact that it is difficult to study these factors independently from each other. However, it may also indicate that individual differences between cows on a molecular level are major determinants of MFG size (Couvreur and Hurtaud, 2007; Logan et al., 2014). Accordingly, a genetic component in the regulation of MFG size has been suggested with moderate heritability of MFG size as a milk production trait (Fleming et al., 2017b). This, in combination with the possible role of the DGAT1 gene offers the potential to identify and select cows with distinct MFG size distributions (Argov-Argaman et al., 2013).

### *2.5.3 Determinants of MFG size within the mammary epithelial cell*

There are two possible ways for intracellular LDs to grow in size: LD fusion and LD expansion (Wilfling et al., 2013). During LD fusion two LDs are combined into a larger one with the combined volume of the two original LDs. During LD expansion, LDs grow in size through *de novo* synthesis of TGs on the surface of the LD (Wilfling et al., 2013).

#### *2.5.3.1 LD growth through LD fusion*

Recent *in vivo* and *in vitro* studies have conclusively shown that LD fusion contributes to the size development in the MEC (Cohen et al., 2017; Masedunskas et al., 2017). Furthermore, from studies in MECs and other cell types it is known that LD fusion depends on the composition of the monolayer surrounding the intracellular LD, specifically its PL components but also the FA composition of its lipid classes (Arisawa et al., 2016; Cohen et al., 2017). These effects are mainly attributed to the biophysical properties of the monolayer compounds. Arguably the best-established relationship is the impact of PC and PE on the stability of LDs, with PC preventing LD fusion due to its superior surfactant properties compared to PE (Thiam et al., 2013). Briefly, on the PL class level, fusion depends on the area taken up by the head group of the PL and the intrinsic curvature of the molecule in a monolayer. For example, DG, PE and phosphatidic acid have small head groups relative to the FA tail and therefore favor negative curvature, leading to the formation of reverse micelles with the FA chains facing outwards (Thiam et al., 2013). PLs with bigger headgroups relative to the tail ends, such as lysophospholipids and PI, tend to form direct micelles, with the hydrophilic headgroups facing towards the continuous phase. In the third scenario, PLs with a similar area of tail and headgroups, such as PC, have no preferred curvature and are therefore very efficient surfactants (Thiam et al., 2013). Complete fusion of LDs requires the LDs to be in close

proximity and form pores (Thiam et al., 2013). At which distance between the LDs pore formation is achieved depends on the efficiency of the surfactant (Thiam et al., 2013). Furthermore, the pore is stabilised if the curvature of the monolayer matches the bending of the pore, which reduces the line tension and favors fusion. For example, if the monolayer contains PLs with negative curvature, such as PE, and the curvature is negative in the pore, which is the case in oil in water emulsions covered by PLs, then the pore is stabilised and tends to expand, leading to complete fusion (Thiam et al., 2013).

In accordance with this, Cohen et al. (2015) suggested that the PL composition of cellular membranes determine MFG size in MECs. Their study investigated the effect of supplementing different free FAs to the cell culture media on the membrane composition of MECs and its indirect effect on the size of MFGs secreted into the media. The results indicate that palmitic and oleic acid treatments lead to differences in membrane PL composition, which in turn lead to differences in the size distribution of LDs and MFGs. Palmitic acid treatment resulted in smaller LDs and secreted MFGs compared to oleic acid and control treatments. The authors hypothesised that this is due to an increased content of PC stabilising the membrane and thus inhibiting intracellular LD fusion. Oleic acid treatment, on the other hand, increased PE contents in the membrane, inducing membrane fusion and resulting in larger LDs and MFGs (Cohen et al., 2015). In their more recent study, Cohen et al. (2017) manipulated the metabolic pathways for the interconversion of PC, PS and PE and found that independently from total cellular TG synthesis, LD size was modulated by increased PC or PE concentrations. Accordingly, studies in other cell types suggest that LDs with low PC contents are prone to LD fusion and knockout of genes involved in PC biosynthesis results in accumulation of fewer but larger MFGs (Guo et al., 2008; Moessinger et al., 2014).

#### *2.5.3.2 LD growth through LD expansion*

At least one isoenzyme from each group of enzymes catalysing the four steps of TG synthesis have been localised to the surface of growing LDs (Wilfling et al., 2013), as shown in Figure 3. These findings imply that LDs can acquire the capability to synthesise TGs even after being pinched off from the ER, the major site of TG synthesis. Moreover, Wilfling et al. (2013) showed that a subset of LDs missing TG synthesis enzymes on their surface did not grow in size after budding from the ER. In addition to TG synthesis, LD expansion also requires the production of PL material to cover the surface of the growing LD. Indeed, enzymes involved in PL synthesis have also been found on the surface of LDs and have further been connected to differences in LD size (Krahmer et al., 2011; Moessinger et al., 2011; Wilfling et al., 2013). However, LD growth through on-surface TG synthesis has not been demonstrated in MECs and proteomic analyses of MFGM material have not detected all enzymes that would be required for this process (Mather et al., 2019). It is possible that LD growth differs in MECs, which represent a special case compared to other cell types, because LDs are not only energy storage organelles, but are ultimately secreted.

### *2.5.4 Characteristics of small and large MFGs*

#### *2.5.4.1 FA composition of small and large MFGs*

The literature provides evidence that the FA composition of MFGs are related to their size. However, the reported results are conflicting, possibly due to different approaches to obtain MFGs of different sizes. The majority of available studies use bulk or individual milk samples and separate the population of MFGs into groups of different size fractions by gravity separation (Mesilati-Stahy and Argov-Argaman, 2014; Mesilati-Stahy et al., 2011), centrifugation (Lu et al., 2016; Timmen and Patton, 1988) or microfiltration

(Briard et al., 2003; Fauquant et al., 2005; Lopez et al., 2011). Other experimental designs used milk samples from cows that naturally produce MFGs with small or large average sizes (Couvreur et al., 2007) or studied the relationship between average MFG size and FA composition without prior selection based on MFG size (Wiking et al., 2004). Furthermore, the available studies vary in the use of either total lipids for FA analysis (Couvreur et al., 2007; Lopez et al., 2011; Mesilati-Stahy and Argov-Argaman, 2014; Mesilati-Stahy et al., 2011) or prior separation into the MFG core and MFGM materials (Fauquant et al., 2005). Although not consistent across all studies, the results indicate that MFGs with small sizes contain less stearic acid (Briard et al., 2003; Couvreur et al., 2007; Fauquant et al., 2005; Wiking et al., 2004) and total SFAs (Lu et al., 2016), but more polyunsaturated FAs (PUFAs) (Mesilati-Stahy and Argov-Argaman, 2014) and in some studies more monounsaturated FAs (MUFAs) (Couvreur et al., 2007; Lu et al., 2016) than MFGs in the large size groups. Furthermore, Couvreur and Hurtaud (2017) using a large dataset from several studies on MFG size, related small MFGs to lower contents of SFAs and short chain FAs, along with higher contents of MUFAs and PUFAs. Oleic acid as the major UFA in bovine milk, was also related to smaller MFGs (Lu et al., 2016; Timmen and Patton, 1988). However, some studies reported changes in oleic acid depending on lactation stage with cows at 150 days in milk exhibiting higher oleic acid contents in the large MFG fraction (Mesilati-Stahy and Argov-Argaman, 2014). Overall, these results indicate that there are differences in the FA profile of large and small MFGs. However, FA composition also depends on other physiological and environmental factors. While FA profiles of MFGs from different size fractions of the same milk samples were not consistent across the studies, the FA composition of MFGs may be characteristic for the individual animal and reflect its lipid metabolic state (Couvreur et al., 2007).

*2.5.4.2 Composition of the MFGM in small and large MFGs*

The MFGM contains proteins and polar lipids, which are minor milk components in terms of their absolute quantity. However, they are predicted to play a major role in the MFG size development because of their distinct physical properties and role in LD fusion (Thiam et al., 2013). Despite the detailed knowledge of the surfactant properties of polar lipids and their predicted impact on membrane fusion events, reports on MFG size and its relationship to polar lipid concentrations vary greatly.

As seen for the FA composition, a fluctuation in the polar lipid composition depending on the stage of lactation has been reported (Mesilati-Stahy and Argov-Argaman, 2014). The smallest size fraction was characterised by higher SM concentrations at earlier stages of lactation and higher PC and lower PE and PS concentrations at some of the points during the lactation cycles, although this relationship was not consistently observed (Mesilati-Stahy and Argov-Argaman, 2014). In contrast, Lopez et al. (2011) reported decreased PE, PC, PS and PI concentrations with an increase in MFG size. Yet another study reported no significant differences in the proportions of the polar lipid classes and the FA composition within these classes in MFGM material from small or large size fractions obtained by microfiltration (Fauquant et al., 2007). Finally, Lu et al. (2016) reported higher PE and PI concentrations in smaller MFGs, which in the case of PE is in conflict with its proposed destabilising effect in biological membranes predicted to stimulate LD fusion. Across species with different average MFG size distributions, the predicted relationship between the relative proportions of PC and PE and MFG size also does not hold true. For example, albeit a significantly larger average MFG size distribution in buffalo milk compared to cow milk, higher PC proportions were found in the MFGM from buffalo MFGs (Ménard et al., 2010).

The variation in the size dependent polar lipid composition reported in the literature may be attributed to the different separation techniques applied in these studies, for example gravity separation (Mesilati-Stahy and Argov-Argaman, 2014), microfiltration (Fauquant et al., 2007; Lopez et al., 2011) or centrifugation (Lu et al., 2016), which also resulted in substantial differences in the average MFG sizes of the separated fractions.

The MFGM also contains a number of LD associated proteins and transmembrane proteins. Many important functions of these proteins are known, such as the postulated roles in MFG secretion for XOR, BTN and PLIN2 (see Section 2.3.2.2) and the role of perilipins in the regulation of lipolysis particularly in adipocytes (Itabe et al., 2017). However, it remains unknown if the protein components of the MFGM are involved in the size development within the MEC. As for the PL components, some of the proteins found on the surface in other cell types are involved in LD fusion. For example, SNARE (Soluble N-ethylmaleimide sensitive factor (NSF) attachment protein (SNAP) receptor) proteins are believed to be the driving force for membrane fusion in many tissues, for example the release of neurotransmitters from a synaptic vesicle (Truchet et al., 2013). Boström et al. (2007) showed that members of the SNARE family, namely SNAP 23, Syntaxin 5 and vesicle associated membrane protein 4, can be found on the surface of LDs in two non-mammary cell lines and that transfection with small interfering RNA led to a reduction in size of the LDs. Furthermore, the expression of SNARE proteins has been reported in the mammary gland (Chat et al., 2011). In this study certain SNARE proteins were found in close proximity to the secretory vesicles and LDs, suggesting a potential role in casein and milk fat secretion (Chat et al., 2011).

Milk fat globule epidermal growth factor (EGF) factor 8 (MFG-E8), also known as lactadherin, has been the subject of a study by Yasueda et al. (2015) aiming to investigate the role of MFG-E8 in MFG fusion in milk after secretion and hence its impact on MFG

size distribution. The study was conducted with wild-type and MFG-E8 deficient mice. It showed that MFG-E8 can protect MFGs from fusion by binding to PS, which is exposed on the outer surface of the MFGM and is known to be involved in membrane fusion especially in the presence of divalent cations ($Ca^{2+}$). As a result, milk from MFG-E8 deficient mice exhibited a higher proportion of large MFGs (Yasueda et al., 2015).

Few studies have compared the protein components of small and large MFGs. However, in conflict with the suggested function for lactadherin as an inhibitor of post-secretory fusion, Lu et al. (2016) found higher levels of lactadherin in large MFGs. Similarly, SNAP23, one of the SNARE proteins, also showed higher concentrations in the small MFG fraction, which contradicts its suggested role in LD fusion (Lu et al., 2016). However, Lu et al. (2016) also found differences between the small and large MFG fractions in the concentrations of two major MFGM proteins, namely PLIN2 and PLIN3. More specifically, the large MFG fraction contained higher concentrations of PLIN2, while the smaller MFGs were characterised by higher PLIN3 concentrations. Perilipins are a group of LD-associated proteins and contain five members PLIN1-5. While PLIN1 and PLIN4 (also known as S3-12) are exclusively found in white adipose tissue, PLIN2 and PLIN3 (also known as TIP47) are found ubiquitously (Itabe et al., 2017). Furthermore, it has been suggested that during LD biogenesis in adipocytes, tiny LDs are coated by PLIN3, while upon growth PLIN2 coats LDs of intermediate size and large unilocular LDs, which are characteristic for adipocytes, are coated with PLIN1 (Wolins et al., 2006). However, due to the different LD morphology in adipocytes, with large unilocular LDs compared to multiple smaller LDs in other tissues, it is unknown if this dynamic change is also found during LD development in MECs, which do not contain PLIN1.

Overall, the literature is lacking studies on the polar lipid and protein composition of milk and particularly MFGM material from cows that naturally produce MFGs of the small and large phenotype. In contrast to the available studies, which separate individual or bulk milk samples into different fractions, samples from cows with natural differences in MFG size distributions comprise the entire population of MFGs produced in the MEC of the individual animal. This could shed light on the metabolic significance of polar lipid production in relation to MFG size development.

## 2.6 References


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# CHAPTER 3

## The effect of physiological state, milk production traits and environmental conditions on milk fat globule size in cow's milk.

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## 3.1 Introduction

Chapter 3 investigated the impact of on-farm and animal related parameters on milk fat globule size distribution within the herd of Holstein-Friesian cows at the University of Melbourne Dookie Dairy.

This work has been accepted for publication in the Journal of Dairy Research. It has not been published at the time of submission of this thesis. Therefore, the paper is included in this chapter as a manuscript. Supplementary data included in this chapter will be published as a supplementary file as part of the publication.

## 3.2 Manuscript


### SUMMARY

This research paper was carried out to quantify the effects of a range of variables on milk fat globule (**MFG**) size for a herd of Holstein-Friesian cows managed through an automatic milking system with year-round calving. We hypothesised that the overall variation in average MFG size observed between individual animals of the same herd cannot sufficiently be explained by the magnitude of the effects of variables that could be manipulated on-farm. Hence, we aimed to conduct an extensive analysis of possible determinants of MFG size, including physiological characteristics (parity, days in milk, days pregnant, weight, age, rumination minutes, somatic cell count) and milk production traits (number of milkings, milk yield, fat yield, protein and fat content, fat-protein ratio) on the individual animal level; and environmental conditions (diet, weather, season) for the whole herd. Our results show that when analysed in isolation, many of the studied variables have a detectable effect on MFG size. However, analysis of their additive effects identified days in milk, parity and milk yield as the most important variables. In accordance with our hypothesis, the estimated effects of these variables, calculated using a multiple variable linear mixed model, do not sufficiently explain the overall variation between cows, ranging from 2.70 to 5.69 µm in average MFG size. We further show that environmental variables, such as sampling day (across seasons) or the proportion of pasture and silage in the diet, have limited effects on MFG size and that physiological differences outweigh the effects of milk production traits and environmental conditions. This presents further evidence that the selection of individual animals is more important than the adjustment of on-farm variables to control MFG size.



**Key words:** automatic milking system, dairy cow, environmental parameters, milk fat globule diameter, physiological state

Milk fat functions as an important energy source for the neonate and suckling calf. Lipid and protein components of milk also have various nutritional benefits that extend to consumers (Thorning et al. 2016). Milk lipids are enveloped by a membrane made of polar lipids and proteins. This method of lipid packaging and secretion is unique to the lactating mammary gland (McManaman, 2012). The resulting structure is called the milk fat globule (**MFG**) and comprises a neutral lipid core surrounded by a trilayer membrane (Fox and Kelly, 2012). The MFG membrane contains bioactive compounds with noted health benefits (Singh and Gallier, 2017), and small MFG deliver more of this beneficial membrane material due to an increased surface area. A variety of sizes of MFG are found in milk, ranging from < 1 μm to over 10 μm (Michalski et al. 2001). In addition to its variation between different breeds and species (Carroll et al. 2006), MFG size distribution can be affected by stage of lactation (Evers, 2004) and nutrition (Couvreur et al. 2007). Furthermore, MFG size distribution can vary between individual cows (Logan et al. 2014).

Amongst the physiological variables, stage of lactation is negatively correlated to MFG size, with a decrease in size with ongoing lactation in bovine milk (Altenhofer et al. 2015; Fleming et al. 2017). The role of milk production traits is more equivocal in the literature, however recent evidence suggests that fat-protein ratio and fat content are positively related to MFG size (Couvreur and Hurtaud, 2017; Martini et al. 2016). Possible environmental determinants of MFG size are season and diet. For example, rations with a high-concentrate-to-low-forage ratio have been shown to result in smaller average MFG sizes compared to a low-concentrate-to-high-forage diet (Mesilati-Stahy et al. 2015). Several studies also suggested that MFG size is subject to seasonal changes, however the reported effects are often conflicting, mainly because the results are confounded by the association of season with nutrition in pasture fed herds, and with

stage of lactation in herds that practice seasonal calving. Fleming et al. (2017) reported larger average MFG in spring compared to the rest of the year, whereas Logan et al. (2014) showed the opposite with smaller MFG in spring than in autumn. However, in the study by Fleming et al. (2017) management practices and nutrition of the studied herds were unknown and Logan et al. (2014) used a herd practicing seasonal calving. Others have reported no effect of season on MFG size in goats (Salari et al. 2016).

This study examines on-farm and physiological determinants of MFG size, including a broad range of animal and milk production measurements. The MFG size distribution was measured on four sampling days over a one-year period, with one sampling day in each season.

## MATERIALS AND METHODS

### *Animals and Nutritional Management*

The animals used in this study are part of the herd at the University of Melbourne Dookie Dairy in northern Victoria, Australia (latitude 36°25'31.3"S, longitude 145°42'36.6"E). The farm consists of 0.41 $km^2$ of border-check flood irrigated pasture, divided in three grazing areas and a feed pad. Pastures are predominantly perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*). The herd consists of approximately 145 Holstein-Friesian cows, aged between 1.65 and 7.86 years. The cows calve at variable times throughout the year. During the year of this study, 55 of the cows had calved in autumn (32.4%), 55 (32.4%) in winter, 41(24.1%) in spring and 19 (11.1%) in summer. The cows are milked by an automatic milking system (Lely Astronaut; Lely, Maassluis, The Netherlands). They enter the dairy voluntarily for milking and milking is performed with automatic cup attachment trough a laser-based teat detection system as part of the robotic arm. The cows' diet consists of concentrates (cereal grain-based

pellets) and ad libitum pasture, as well as silage and hay when there is insufficient pasture to meet the herd's nutritional requirements. The concentrate (Optimilk Lacta Max, Rivalea Australia - Stockfeeds, Corowa NSW, Australia) is fed during milking in the robot (Lely Astronaut) and cows can finish their individually allocated feed in a feeding station (Lely Cosmix) after milking. Information about the metabolisable energy value of pasture, silage and hay and the calculations used to determine concentrate allowance for each individual cow are provided in the Supplementary File.

### *Milk Sampling*

A total of 274 milk samples from 140 individual cows were taken during the afternoon milking period (13.00 to 18.00) using an automatic sampling system (Lely Shuttle) which samples an aliquot (20 mL) of the entire milking volume. Samples were transported to the laboratory and stored on ice and analysed for MFG size the next day. Sampling was performed once per season over a period of one year, with T1 in winter (n = 51 cows), T2 in spring (n = 54), T3 in summer (n = 95) and T4 in autumn (n = 74). This resulted in sampling frequencies between one and three times for each cow, because not all cows were in milk at all four sampling days or did not enter the dairy for afternoon milking on some sampling days. The year-round calving pattern within our herd allowed us to study the effect of stage of lactation independently from season. Therefore, the effect of nutritional variables such as diet on a whole-herd level and concentrate intake of individual animals were also included to estimate the true effects of seasonal variation on MFG size. The analysis only includes milk samples from cows with a minimum of 10 days in milk (**DIM**).

### *Recording of Animal and Production Traits*

All variables except MFG size (milk yield (kg), DIM, concentrate intake (kg), number of milkings, parity, fat indication (%), protein indication (%), days pregnant, rumination minutes, somatic cell count, weight (kg), age (years) and concentrate per 100 kg of milk) were recorded by the T4C software (Lely), which is the operating system for the Lely Astronaut automatic milking systems. Furthermore, fat yield and fat:protein ratios were calculated based on these variables. This saved data was used to calculate averages for a period of seven days prior to sampling, which was chosen as an appropriate timeframe to allow variables such as a change in diet or temperature to be reflected in MFG size measurements. For cow weight the maximum value recorded during the seven days prior to MFG size analysis was used because cows frequently step outside the balance area, leading to outliers below their actual weight. DIM and age represent the values on the day of sampling. Minimum and maximum temperatures were defined as the lowest and highest recorded temperatures during the seven days prior to sampling. Local weather data was obtained from the University of Melbourne Dookie Campus weather station (latitude E36°23'05.8"S, longitude 145°42'51.2"E) (Anonymous, 2018). Average feed composition was calculated daily for the whole herd and is described in the Supplementary File.

### *MFG Size Analysis*

The distribution of MFG size was measured as previously described (Logan et al. 2014). Briefly, fresh milk samples were diluted 1:1 with 35 mM EDTA, pH 7.0 to dissociate casein micelles and the MFG size distribution was analysed by dynamic light scattering using a Mastersizer 2000 (Malvern Instruments, Malvern, UK). The obscuration rate was kept between 12 to 15% and globule and water refractive indices

were estimated as 1.46 and 1.33, respectively (Michalski et al., 2001). The particle absorption coefficient was 0.001. The volume-weighted mean diameter $D_{4,3}$ was used as the measure for particle size distribution in this study.

### *Statistical Analysis*

Statistical analysis was performed using GenStat statistical analysis software (GenStat for Windows 16th Edition, VSN International, Hemel Hempstead, UK). Linear mixed models were fitted to predict the single variable effects of each measured variable. This analysis identified ten potential explanatory variables that also had a statistically significant effect on MFG size ($p < 0.05$, Tables S1 and S2, Supplementary File). The variables with significant effects on MFG size included five milk production traits (milk yield, fat percentage, fat yield, number of milkings and fat-protein ratio), four physiological variables (DIM, parity, days pregnant, age) and concentrate intake during milking. However, because the fixed effects are highly correlated (see Figure S1 Supplementary File), six variables were selected to study their additive effects on MFG size. A linear mixed model fitted additive effects of six selected variables on MFG size; this is referred to as the multiple variable model. The individual cow's identification numbers and time of sampling were included in the models as random effects. The only exception was the estimation of the effect of sampling day, where only animal number was included as a random effect. The effect of sampling day, as an indicator for seasonal effects, stage of lactation, classified into early (< 100 DIM), mid (100 to 200 DIM) and late (> 200 DIM) and parity were further analysed. Mean differences between factor levels were estimated, and Fisher's LSD method was used to calculate confidence intervals for those differences. Graphs were produced using Minitab 17 Statistical Software (Minitab Inc., State College, PA).

## RESULTS

### *Milk fat globule size*

The average MFG size for individual cows within our herd ranged from 2.70 to 5.69 µm throughout the year of this study, with around 50% of the cows producing MFG sizes between 3.80 and 4.40 µm and 25% producing MFG smaller or larger than 3.80 and 4.40 µm, respectively (Figure 1).

### *Sampling day, diet, environmental temperature and milking frequency*

In our study, the day of sampling did not lead to a statistically significant difference in average MFG size ($F(175.2) = 1.93$, $p = 0.130$. The effect of sampling day was used as an indicator for seasonal effects. Pairwise comparisons between sampling days showed the largest shift in average MFG size between spring and autumn sampling days, with larger globules in milk sampled on the autumn day (4.18 µm) compared to the spring day (4.01 µm). Average MFG size on the summer and autumn sampling days tended to be slightly higher compared to winter and spring sampling days (Figure 1).

Table 1 shows the calculated composition of the cows' diet for each sampling day. While average concentrate consumption for the whole herd was relatively constant across the four sampling days, the proportion of pasture and silage varied. For example, in spring the cows' diet comprised 57% pasture and no silage on the spring sampling day compared to 43% silage and 7% pasture on the winter sampling day. The proportions of silage and pasture were more balanced on the autumn and summer sampling days (Table 1).

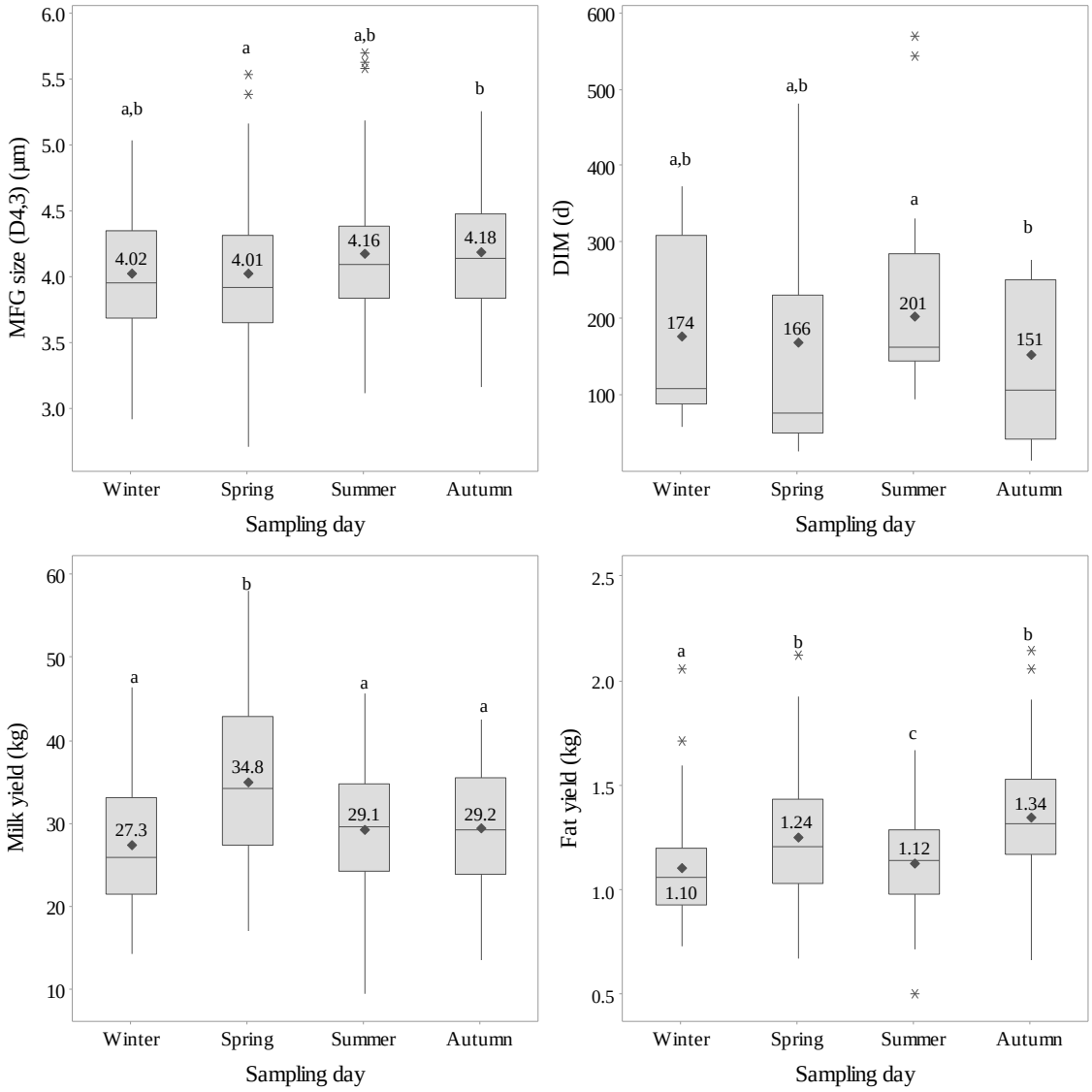


**Figure 1.** Impact of season on the distribution of milk fat globule (MFG) size, days in milk (DIM), milk yield and fat yield in the studied herd. Statistically significant differences ($p < 0.05$, indicated by different letters) between sampling days for each variable were analysed by pairwise comparisons of the sampling days, using the single variable model with cow number as the random effect and the respective variable of interest as fixed effects. Please note, symbols and numbers in the interquartile box show the arithmetic means not the predicted means of the respective variables. Asterisks indicate outliers, which are at least 1.5 times the interquartile range from the edge of the box.

**Table 1.** Environmental temperature and diet composition for the whole herd on the sampling days in winter, spring, summer and autumn

| | Winter (Jun 2016) | Spring (Oct 2016) | Summer (Jan 2017) | Autumn (May 2017) |
|---|---|---|---|---|
| Weather | | | | |
| $T_{ave}$ (°C) | 6.4 | 12.7 | 24.1 | 10.7 |
| $T_{min}$ (°C) | -0.5 | 1.4 | 10.5 | 2.9 |
| $T_{max}$ (°C) | 13.2 | 24.7 | 40.3 | 20.3 |
| Relative humidity (%) | 95.9 | 68 | 55.4 | 82 |
| Rainfall (mm) | 1.8 | 0.5 | 0 | 0.3 |
| Diet | | | | |
| Total ME intake (MJ per cow per day) | 178.5 | 225.9 | 213.9 | 223.1 |
| % Concentrate | 49.6 | 42.7 | 46.1 | 39.4 |
| % Silage | 43.1 | 0.0 | 32.9 | 38.4 |
| % Pasture | 7.2 | 57.3 | 21.0 | 22.3 |

All numbers are averages from the seven days prior to MFG size measurements. $T_{min}$ and $T_{max}$ are the lowest and highest recorded temperatures in the seven days prior to sampling.
ME = metabolisable energy.

***Physiological state and milk production traits***

The cows in the studied herd calve all year round, which is reflected in the wide range of DIM for each sampling day and statistically similar average DIM (Figure 1). Furthermore, the herd comprises cows of different ages and parities (1.65 to 7.93 years and 1 to 6 lactations, respectively) and a variety of milk production traits (Figure 1). The current study identified parity, stage of lactation and milk yield as main effects impacting MFG size (Table 2). MFG size decreased with ongoing lactation by 0.20 µm per 100 DIM ($p < 0.001$, Table 2). A shift towards larger MFG size by 0.16 µm between cows in second and third lactation was also observed ($p = 0.031$, Table 2). Milk yield was negatively related to MFG size with a decrease in size by 0.16 µm for every 10 additional litres of milk per day ($p = 0.047$, Table 2). However, fat yield and the number of milkings per day did not affect MFG size and only a small decrease in MFG size was observed per kg of concentrate in the cows' diet (-0.03 µm, $p = 0.096$, Table 2).

**Table 2.** Effects of selected variables on the average milk fat globule (MFG) size

| Explanatory variable | Mixed effect on MFG size (µm) | 95% Confidence interval | *P* - value |
|---|---|---|---|
| Milk yield (× 10 kg) | -0.16 | -0.32, -0.01 | 0.047 |
| DIM (× 100 d) | -0.20 | -0.26, -0.14 | < 0.001 |
| Concentrate intake (kg) | -0.03 | -0.06, 0.01 | 0.096 |
| Number of milkings | 0.10 | -0.07, 0.27 | 0.240 |
| Fat yield (kg) | 0.30 | -0.06, 0.67 | 0.134 |
| Parity | | | 0.009 |
| Comparison between adjacent factor levels for parity | | | |
| Factor levels | Mean difference for mixed effects (µm) | 95% Confidence interval | *P* - value |
| 2 - 1 | 0.16 | -0.09, 0.41 | 0.207 |
| 3 - 2 | 0.16 | 0.01, 0.31 | 0.031 |
| 4 - 3 | 0.11 | -0.04, 0.25 | 0.149 |
| 5 - 4 | 0.09 | -0.16, 0.34 | 0.481 |

Cow number and sampling day are included as random effects.
DIM = days in milk.

## DISCUSSION

### *Milk fat globule size*

The considerable variation in average MFG size between individual cows is in accordance with previous studies investigating the MFG size distribution within a herd (Logan et al. 2014) or across several herds (Fleming et al. 2017). Logan et al. (2014) measured the MFG size distribution within the herd once in spring and twice in autumn over two consecutive years and the average MFG sizes ranged between 2.5 and 5.7 µm. This considerable variation in MFG size between cows within the one herd of the same breed, exposed to the identical environmental conditions and fed the same diet, which was reported in the study by Logan et al. (2014), led us to hypothesise that physiological differences between individual animals rather than management practice and environmental conditions are in part responsible for their characteristic MFG size. This hypothesis was confirmed by the results of our current study.

***Sampling day, diet, environmental temperature and milking frequency***

Season is often mentioned as a determinant of MFG size because of its known effect on milk fat composition (Briard et al. 2003; Logan et al. 2014). However, there is very little published data about the effect of season on MFG size, partly because it is tightly associated with stage of lactation in herds that practice seasonal calving. The year-round calving pattern in our herd allowed us to estimate the effect of sampling day, across all seasons, more independently from the confounding effect of stage of lactation, which is a limitation of many studies in the literature. The results indicate a limited impact of seasonal effects. However, sampling was performed only once per season, which is a limitation of our study.

Spring sampling stood out as a period with increased pasture intake and milk yield (Figure 1, Table 1). Therefore, changes in milk and fat yield could possibly explain the observed differences between the four days sampled across the seasons. However, average MFG size on the day in winter (4.02 μm) was very similar to the one in spring (4.01 μm), while an increase in milk yield was only observed on the spring day. Accordingly, average fat yields were similar between the autumn and spring days despite the difference in average MFG size (Figure 1).

While daily concentrate intake was recorded for each cow, we did not measure individual pasture and silage intake. However, we calculated an estimated intake of pasture and silage for the whole herd (Table 1). Based on these assumptions we suggest that differences observed between sampling days across the four seasons cannot be attributed to the changes in diet. For example, average MFG size was similar between winter and spring sampling times, when the composition of the diet was markedly different. Pasture intake on the winter day was very low (7.2%) and silage intake was

high, whilst the opposite was the case on the spring day with no silage supplementation and 57% pasture intake. However, this change was not reflected in a change in average MFG size (0.01 µm between the winter and spring sampling days, Figure 1). Therefore, within the perimeters of our study changes in diet on the whole herd level did not impact MFG size.

On the individual level, concentrate intake is subject to changes throughout the lactation cycle because cows are allocated a ration of concentrate during milking, calculated based on their milk production. Even though the cows can finish their allocated concentrate ration in a feeding station immediately after milking, the concentrate intake is correlated with milk production, number of milkings and DIM (see Figure S1 in the Supplementary File). Due to this correlation, the impact of concentrate intake on MFG size distribution should only be looked at in combination with these variables. In our multiple variable model, a small effect of concentrate intake on average MFG size was observed (-0.03 µm per kg of concentrate, $p = 0.096$, Table 2). Intervention studies have shown that MFG size can be influenced for example by increasing the concentrate-to-forage ratio (Argov-Argaman et al. 2014) and through supplementation of the cows' diet with linseed (Lopez et al. 2008), both leading to a small decrease in MFG size of 0.20 ($p = 0.10$) and 0.17 µm ($p < 0.05$), respectively. The observed trend towards smaller MFG size in cows fed a diet with high concentrate-to-forage ratio in the study by Argov-Argaman et al. (2014) is more pronounced than the effect of a voluntarily increased concentrate intake observed in our study (-0.03 µm per kg concentrate, $p = 0.096$). Accordingly, a study in ewes reported no significant effect of increased concentrate-to-forage ratios on average MFG size (Martini et al. 2010). Compared to the variation between individual cows (2.70 to 5.69 µm in our study) the contribution of diet induced

changes in MFG size are limited, supporting our hypothesis that inherent physiological differences may be more important determinants of MFG size.

The average number of milkings per day did not have a statistically significant impact on MFG size (effect per milking 0.10 µm, $p = 0.240$, Table 2). Automatic milking systems such as the one used on our research farm, have been shown to have no effect on MFG size if the number of milkings is the same as in a conventional milking parlour (Abeni et al. 2005). However, milking frequency is often increased in automatic milking compared with conventional milking systems, leading to higher total milk yields, which could in turn impact MFG size. Accordingly, Wiking et al. (2006) reported an increase in average MFG size when milking frequency was doubled from twice to four times daily. This is particularly interesting because the study was performed by milking one udder half twice and the other half four times daily, allowing the authors to look at the effect of increased milking frequency independently from individual cow effects. In our study, the average milking frequency for individual cows on the four sampling days ranged from 1.6 to 3.9 milkings per day. However, the effect of the number of milkings per day on MFG size were not statistically significant (0.10 µm for each additional milking, $p = 0.240$, Table 2). This indicates that the reported effect of the increased milking frequency in our study was in part due to its correlation with other explanatory variables. For example, cows in later stages of lactation tend to come in for milking less frequently (Figure S1 Supplementary File) and have smaller average MFG (see discussion on stage of lactation). Overall, the number of milkings likely does not have a strong impact on MFG size in a system where cows are free to determine their own milking frequency. Although, a small effect might be present as shown in the study by Wiking et al. (2006), where the average MFG size increased by 0.08 µm. Indeed, the estimated effect in our study is of this magnitude.

***Physiological state and milk production traits***

We confirm a major role for stage of lactation in the determination of MFG size and further suggest that parity (the number of lactations) has a significant impact on MFG size (Figure 2, Table 2). Furthermore, we observed an inverse relationship between milk yield and MFG size (-0.16 µm per 10 L of milk produced, p = 0.047, Table 2), whilst fat yield did not significantly impact MFG size (0.30 µm, p = 0.134, Table 2).

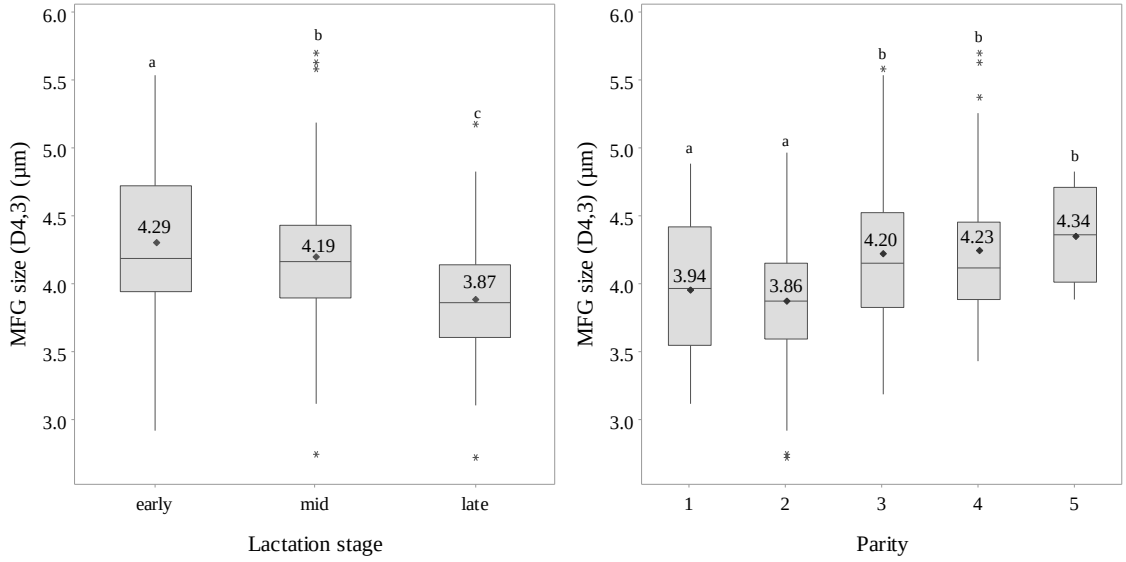


**Figure 2.** Milk fat globule (MFG) size distribution for cows in different stages of lactation and parities. Cows were grouped in early (< 100 DIM), mid (100 – 200 DIM) and late (> 200 DIM) lactation for the purpose of this analysis. Statistically significant differences ($p < 0.05$, indicated by different letters) in MFG size between the groups were analysed by pairwise comparisons, using the single variable model with cow number and sampling day as the random effects and the respective variable of interest as fixed effects. Please note, symbols and numbers in the interquartile box show the arithmetic means not the predicted means of the respective variables. Asterisks indicate outliers, which are at least 1.5 times the interquartile range from the edge of the box.

A higher milk yield in cows producing milk with smaller average MFG size has been reported previously (Couvreur et al. 2007; Mesilati-Stahy et al. 2015). Furthermore, several studies have shown that cows producing milk with a lower fat percentage also

produce smaller MFG (Altenhofer et al. 2015; Mesilati-Stahy et al. 2015). This can be indicative of a higher total milk yield due to a dilution effect in cows secreting the same or increased total fat yield in a larger volume of milk (Couvreur et al. 2007). More specifically, the multiple variable model suggests that when comparing cows in the same stage of lactation the ones producing more milk, and possibly the ones that consume more concentrate, are predicted to exhibit smaller MFG size distributions. Conflicting results about the relationship between MFG size and fat yield have been reported in the literature, with some studies showing no effect of fat yield on MFG size (Couvreur et al. 2007; Logan et al. 2014), while others report an increase in average MFG diameter with increasing fat yields (Wiking et al. 2004).

Stage of lactation, expressed as DIM, had a statistically significant impact on MFG size. This is consistent with previous studies, which also report a decrease in MFG size between early and mid-lactation, however not always between mid and late lactation (Abeni et al. 2005; Altenhofer et al. 2015; Fleming et al. 2017). The inverse relationship between MFG size and DIM can be attributed to a limited availability of membrane material in early and possibly mid lactation, where milk production and fat yield are high (Wiking et al. 2004; Altenhofer et al. 2015). In our study, a steady decrease in milk and fat yield from early-to-mid and from mid-to-late lactation was also observed (data not shown). Therefore, the decrease in fat yield is a possible explanation for the decrease in MFG size with ongoing lactation. However, the effect of fat yield was not statistically significant in our study ($p = 0.134$, Table 2).

We hypothesise that underlying differences on a physiological and possibly molecular level are the true determinants of MFG size and that animal and milk production traits only account for some of the variation seen between individual cows. Overall, the estimated change in MFG size that can be attributed to different stages of lactation is a

decrease of 0.60 µm over a nominal 300-day lactation cycle. This effect therefore does not sufficiently explain the variation between individual animals, ranging from 2.70 to 5.69 µm in average MFG size.

Differences in average MFG size were also observed, when first and second parity cows were compared to cows with three or more lactations. A similar effect of parity on MFG size was previously shown in ewes, with first parity animals producing milk with smaller average MFG size (Martini et al. 2004). Conversely, a recent study by (Fleming et al. 2017) showed no significant impact of parity on MFG size distribution in dairy cows. This extensive study also investigated additive effects of several physiological and environmental variables on MFG size using a linear mixed model. However, 399 individual cows from 44 herds were used as opposed to a single herd in our study. A total of 30 cows from the 140 examined in our study were sampled over two consecutive lactation numbers. It is possible that in the study by Fleming et al. (2017) the effect of parity was relatively small compared to between-herd and between-breed variations, while following the same herd over a one-year period allowed us to capture the independent effect of parity on MFG size.

Overall, the most dominant variables with impact on MFG size identified in our study are physiological parameters. This could indicate that dynamic changes in lipid metabolism throughout the cow's lactation cycle (stage of lactation) and the life cycle (different parities) could drive changes in MFG size. Furthermore, potential differences in the lipid metabolism could also be genetically determined traits and could provide an explanation for the variation in MFG size between individual animals. For example, changes in the origin of fatty acids (FA) incorporated into milk are different throughout the lactation cycle. Previous studies suggest that lipids in milk from cows in early lactation are predominantly derived from body fat stores and circulating FA derived from

the cow's diet (Gross et al. 2011). In mid and late lactation, however, FA are increasingly derived from *de novo* FA synthesis.

## CONCLUSION

We conclude that some of the variation in MFG size seen in dairy cows can be attributed to changes in physiological state of the animal, i.e. stage of lactation and parity; and, in our study, these variables outweighed the effect of seasonal changes in the diet and weather conditions. However, the estimated effects of these variables cannot account for the overall variation in MFG size between individual animals and the span of average MFG size distributions observed in our herd (3 μm) exceeds the effect of stage of lactation, the most dominant variable impacting MFG size in our study, by 5-fold. We suggest that MFG size could be characteristic for each individual animal, possibly based on differences in their lipid metabolism.

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## 3.3 Supplementary data

***Supplementary Methods***

*Metabolisable energy (**ME**) value of the feed*

Silage (9.4 MJ ME per kg dry matter (**DM**), 14.1% crude protein per kg DM, 56% neutral detergent fiber (**NDF**) per kg DM is fed predominately on the feed pad and cows are offered three breaks of pasture each day. Pasture feed quality varies throughout the year, with the lowest quality in summer (January; 9.3 MJ ME per kg DM, 18.8% crude protein per kg DM, 55% NDF per kg DM) and the highest quality in winter (August; ME 11.8 MJ ME per kg DM, 25.3% crude protein per kg DM, 37.9% NDF per kg DM). Feed composition of silage was previously tested by a feed testing laboratory (Feed Quality Service at the Wagga Wagga Agricultural Institute, Wagga Wagga, NSW, Australia) and pasture composition for each month was obtained through the Perennial Pastures Database provided by the Department of Primary Industries Victoria (2018). The concentrate (12.5 MJ ME per kg DM, 16% crude protein; Optimilk Lacta Max, Rivalea Australia - Stockfeeds, Corowa NSW, Australia) is fed during milking in the robot (Lely Astronaut) and cows can finish their individually allocated feed in a feeding station (Lely Cosmix) after milking.

*Calculations for the concentrate allowance of individual animals*

Daily concentrate allowance for the individual animal is calculated based on milk production as follows: animals > 28 days in milk (**DIM**) were fed up to 5 kg concentrate per day for cows producing less than 20 kg of milk; 7 kg for cows producing 20 to 25 kg; 8 kg for cows producing 25 to 30 kg; 9 kg for cows producing 30 to 35 kg; 10 kg for cows producing 35 to 40 kg and 11 kg for cows producing more than 40 kg of milk per day.

Concentrate intake for cows in the first 28 DIM is sequentially increased from 5 kg on the first day, 7 kg per day between day 1 to day 14 and then 9 kg per day up to day 28.

*Calculations for the daily average feed composition of the whole herd*

The cows' average concentrate intake was recorded by the T4C software and silage intake was estimated based on the amount offered in the feed pad and estimated wastage rates. Pasture intake was estimated by a back-calculation technique. The energy from pasture intake was estimated by first calculating the animals' total energy requirements and then subtracting the energy supplied from concentrate and silage. The animals' total energy requirements were calculated according to the procedures of Primary Industries Standing Committee (2007), using daily milk production and milk composition, body weight and gestation.

***Limitations of our study***

Measurements of fat percentage in our study are based on in-line measurements using near infrared spectroscopy, and the data obtained gives a good indication of the fat content in raw milk. However, we observed reduced accuracy for milk with very low-fat percentages (less than 3%), when compared to measurements made using laboratory-based FTIR techniques (data not shown). Therefore, the results from our study only allow us to draw limited conclusions regarding the relationship between MFG size and fat yield.

***Supplementary Results***

**Table S1.** Test statistics for variables included in the linear mixed models for single variable and multiple variable effects

| Explanatory variable | Single variable effects | | | Multiple variable effects | | |
|---|---|---|---|---|---|---|
| | F statistic | df | *P*-value | F statistic | df | *P*-value |
| Primary variables (used in multiple variable model) | | | | | | |
| Milk yield (× 10 kg) | 34.89 | 202.6 | < 0.001 | 4.08 | 81.4 | 0.047 |
| DIM (× 100 d) | 75.31 | 179.8 | < 0.001 | 43.71 | 183.9 | < 0.001 |
| Concentrate intake (kg) | 12.74 | 199.4 | < 0.001 | 2.79 | 211.6 | 0.096 |
| Number of milkings | 19.49 | 202.0 | < 0.001 | 1.39 | 230.1 | 0.240 |
| Fat yield (kg) | 21.23 | 206.4 | < 0.001 | 2.28 | 107.6 | 0.134 |
| Parity | 23.69 | 133.9 | < 0.001 | 3.50 | 223.5 | 0.009 |
| Other variables | | | | | | |
| Fat indication (%) | 21.3 | 196.6 | <0.001 | - | - | - |
| Protein indication (%) | 1.2 | 249.6 | 0.266 | - | - | - |
| Fat:Protein ratio | 21.5 | 214.7 | <0.001 | - | - | - |
| Days pregnant | 32.9 | 170.6 | <0.001 | - | - | - |
| Rumination minutes | 0.5 | 260.4 | 0.480 | - | - | - |
| SCC indication (× $10^3$ cells per mL) | 0.6 | 218.2 | 0.445 | - | - | - |
| Weight (kg) | 0.8 | 205.9 | 0.359 | - | - | - |
| Age | 4.9 | 113.0 | 0.029 | - | - | - |
| Concentrate (kg) per 100 kg milk | 2.0 | 166.2 | 0.156 | - | - | - |

Cow number and sampling day are included as random effects.
SCC = somatic cell count.

**Table S2.** Effects of selected variables on the average milk fat globule (MFG) size fitted using single variable linear mixed models

| Explanatory variable | Single effect on MFG size (µm) | Confidence interval | *P* - value |
|---|---|---|---|
| Milk yield (× 10 kg) | 0.18 | 0.12, 0.24 | < 0.001 |
| DIM (× 100 d) | -0.16 | -0.19, -0.12 | < 0.001 |
| Concentrate intake (kg) | 0.04 | 0.02, 0.06 | < 0.001 |
| Number of milkings | 0.25 | 0.14, 0.35 | < 0.001 |
| Fat yield (kg) | 0.47 | 0.27, 0.67 | < 0.001 |
| Parity | | | < 0.001 |
| Stepwise comparison between factor levels for parity | | | |
| Factor levels (number of lactations) | Mean difference for single effect (µm) | Confidence interval | *P* - value |
| 2 - 1 | 0.05 | -0.22, 0.31 | 0.735 |
| 3 - 2 | 0.30 | 0.15, 0.45 | < 0.001 |
| 4 - 3 | 0.12 | -0.04, 0.28 | 0.144 |
| 5 - 4 | 0.04 | -0.24, 0.31 | 0.801 |
| Other variables | | | |
| Fat indication (%) | -0.18 | -0.26, -0.10 | < 0.001 |
| Protein indication (%) | -0.13 | -0.36, 0.1 | 0.266 |
| Fat:Protein ratio | -0.75 | -1.06, -0.43 | < 0.001 |
| Days pregnant | -0.00 | -0.00, -0.00 | < 0.001 |
| Rumination minutes | 0.00 | 0.00, 0.00 | 0.480 |
| SCC indication (× $10^3$ cells per mL) | 0.00 | 0.00, 0.00 | 0.445 |
| Weight (kg) | 0.00 | -0.00, 0.00 | 0.359 |
| Age | 0.06 | 0.01, 0.11 | 0.029 |
| Concentrate (kg) per 100 kg milk | -0.01 | -0.01, 0.00 | 0.156 |

Cow number and sampling day are included as random effects.

DIM = days in milk.

SCC = somatic cell count.

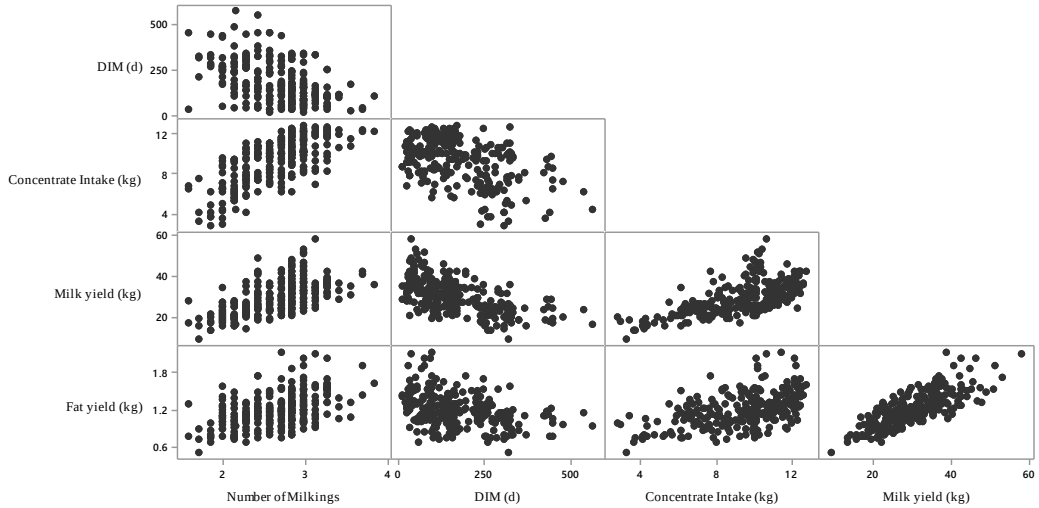


**Figure S1.** Relationship between selected explanatory variables used to fit the multiple variable model. DIM = days in milk. Number of milkings refers to the average number of milking sessions per day, which can vary because the cows enter the milking shed voluntarily for milking.

***References for the Supplementary File***

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# CHAPTER 4

## Lipid metabolic differences in cows producing small or large milk fat globules: Fatty acid origin and degree of saturation

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## 4.1 Introduction

Chapter 4 characterised the fatty acid composition of the milk fat globule core extracted from milk of cows that consistently produced milk with either large or small volume weighted mean diameters in a pre-trial observation period. The milk fat globule membrane separated from the milk fat globule core was used to measure the concentration of the major milk fat globule membrane proteins and data obtained from this analysis is presented in the form of an appendix.

Therefore, Chapter 4 comprises an original manuscript currently under review with the Journal of Dairy Science, as well as addtitional experimental results and discussion in an appendix.

## 4.2 Manuscript


### ABSTRACT

This study compared cows that consistently produce milk with small ($D_{4,3}$ of 2.92-3.83 µm, with an average diameter of 3.29 µm) or large ($D_{4,3}$ of 4.58-5.67 µm, with an average diameter of 4.92 µm) milk fat globule (**MFG**) size distributions in terms of the fatty acid (**FA**) composition of the MFG core. Selected cows fell into the respective size group over at least 3 independent measurements including an observation period prior to the experiment. Further selection criteria were similar milk production traits between cows (milk yield, fat yield, fat-protein ratio) and established lactation (> 50 DIM). However, the selected groups differed in parity (parity 1-3 compared to 3-5 in the small and large MFG groups, respectively) and the small MFG group was an average of 25 DIM later in their lactation period. All cows were under the same nutritional management and environmental conditions. Here we show that cows with the small or large MFG phenotype differ in their lipid metabolism, in terms of the FA composition of the MFG core. Our results indicate that cows with the small MFG phenotype produce milk with higher concentrations of unsaturated FA despite being fed the same diet. We suggest that this characteristic of the small MFG phenotype is the result of an increased uptake of long chain FA from the blood circulation. A relationship between the degree of unsaturation and MFG size was also identified in preliminary studies across other species, namely camels, sheep and goats. These findings show the potential for on-farm selection of cows (and potentially other dairy species) based on MFG size to produce milk with improved nutrient composition. This could lead to purpose-specific separation of milk based on MFG size and FA profile, both known to alter the milk's technological properties.



**Key words:** milk fat globule size, mammalian milk, fatty acid profile

## INTRODUCTION

Milk fat exists as milk fat globules (**MFG**) in the milk of mammalian species. Each globule originates within the mammary epithelial cell (**MEC**) as a cytoplasmic lipid droplet (**LD**) that is enveloped upon secretion with a portion of the plasma membrane of the MEC. The result is the formation of the MFG, a lipid core surrounded by a trilayer membrane. The LD comprises a neutral lipid core containing mainly triacylglycerols (**TAG**) surrounded by a monolayer of phospholipids and proteins. Biogenesis of LD starts either within or in close proximity to the endoplasmic reticulum (Heid and Keenan, 2005; Robenek et al., 2009). Upon release from the endoplasmic reticulum, the LD are transported towards the apical side of the cell and can grow in size by fusion of LD (Masedunskas et al., 2017) or through local TAG and phospholipid synthesis on the surface of the LD (Krahmer et al., 2011; Wilfling et al., 2013). However, the latter process might not be relevant in MEC, because to date, the full set of enzymes required for on-surface TAG synthesis have not been detected in proteomic analyses of the LD envelope (Mather et al., 2019). Lipid droplet fusion, on the other hand, has been demonstrated in the context of mammary LD growth in in vivo and in vitro studies and is believed to depend on the polar lipid and protein composition of the LD envelope (Argov-Argaman, 2019; Mather et al., 2019). After reaching the apical plasma membrane, LD are secreted by a budding process, which involves oxytocin stimulated contraction of the myoepithelial cells surrounding the MEC layer (Masedunskas et al., 2017).

Differences in FA compositions between small and large MFG have been established. For example, milk with smaller MFG tends to contain less stearic acid (C18:0), fewer de novo synthesized FA (< C16), but higher proportions of MUFA and PUFA (Briard et al., 2003; Couvreur and Hurtaud, 2017). Moreover, camel milk, which exhibits the smallest MFG sizes distribution amongst the main dairy species (El-Zeini, 2006), is high in UFA

and contains very little short chain FA (El-Agamy, 2017). Short chain FA, on the other hand, are more abundant in goat milk, which is also amongst the species with a smaller average MFG size compared to cow's milk (Park et al., 2007). The FA incorporated into milk fat in the MEC are obtained from de novo synthesis in the mammary gland (chain length of C4 to C16) or derived from the blood as preformed FA after hydrolysis of TAG from the diet or adipose tissue (chain length of C16 or higher) (Palmquist, 2006). The balance between the two pathways depends on the physiological state of the animal (Palmquist, 2006). Therefore, the FA composition in milk provides an insight into the origin of FA utilized for milk fat production and therefore the lipid metabolism of the individual cow.

Here, we studied the differences between cows that consistently produce milk with small or large MFG size distributions. To identify these animals, the whole herd was tested over 7 sampling days across all seasons (Chapter 3 and some unpublised data). Furthermore, many of the selected animals were sampled additional times over the year prior to this study, resulting in 3 to 9 independent measurements per selected cow (Figure 1). The 2 groups were compared in terms of the FA composition of the MFG core, to gain an insight into the physiological differences between animals that consistently produce small or large MFG but are fed the same diet and are in similar stages of lactation. We hypothesized that differences in the lipid metabolism and specifically the FA origin could be the reason for the observed differences in MFG size between individual cows of the same breed. In this experiment we focused on the FA composition of the LD core, because it contains the vast majority of FA in milk and we wanted to exclude the MFGM from the current analysis to minimize the impact of increased membrane material per unit of fat in the small MFG phenotype. The MFGM comprises polar lipids with higher proportions of UFA (Sánchez-Juanes et al., 2009) and the higher proportion of MFGM material in the

small MFG cows could therefore have impacted the interpretation of our results. A secondary objective was to compare milks from cows with small or large MFG size distribution to milks from sheep, goats and camels. Milks from these species generally differ from bovine milk in their MFG size distribution, as well as their FA composition (Park et al., 2007). We therefore compared the FA composition of the MFG core from several mammalian species to see if these differences are universal.

## MATERIALS AND METHODS

### *Animals and Milk Sampling*

***Cows.*** The cows selected for this study are part of the University of Melbourne Dookie Dairy in northern Victoria, Australia (latitude 36°25'31.3"S, longitude 145°42'36.6"E). The herd consists of approximately 145 Holstein-Friesian cows with a year-round calving pattern and are milked using an automatic milking system (Lely Astronaut; Lely, Maassluis, The Netherlands). The cows are pasture fed all year and are fed concentrate (cereal grain-based pellets) during milking. When there is insufficient pasture to meet the herd's nutritional requirements, the cows' diet is supplemented with silage and hay. The 12 cows selected for this study were selected based on their average MFG size, with 6 cows falling into the group with small MFG and 6 with large MFG. The experiment was performed on 2 cohorts in separate seasons, with the first 6 cows (3 per group) sampled in autumn and the second cohort in winter. Moreover, sampling of each cohort was repeated approximately 2 weeks apart to obtain biological replicates for each cow. The selected cows received the same diet during the time of this study and the concentrate consumption was recorded for each individual cow (Table 1). Another selection criterium was DIM, which was between 184 and 230 DIM for the first and between 57 and 135 DIM for the second cohort of cows. This selection was made based on previous measurements of the whole herd (Chapter 3), throughout the year prior to this study. Pre-

trial measurements to confirm MFG size were performed on 20 mL aliquots collected during afternoon milking (1300 to 1800h) on 7 occasions, where measurements were performed on 50-100 animals. Additional measurements were taken for selected animals during morning milking when milk was collected for other experimental purposes. Particle size analysis was performed the day after milk collection following transport to the laboratory and storage on ice. Raw milk samples (3.5 L per animal) were taken during morning milking (0800 to 1000h) and transported to the laboratory on ice. The fat and protein concentrations were determined using a LactoScope FTIR 20 (Delta Instruments, Melbourne, Australia) for each cow milk sample.

***Camels, Goats and Sheep.*** The camels selected for this study are part of the Camel Milk Co, Kyabram, Victoria, Australia (latitude 36°21'19.7"S, longitude 144°59'53.4"E). The goats and sheep used in this study are part of the Meredith Dairy in Meredith, Victoria, Australia (latitude 37°50'06.9"S, longitude 143°57'51.4"E). Six camels, 6 goats and 6 sheep were randomly selected and sampled once. Camels were wild Australian camels, goats were a mixed breed (approximately 70% Saanen, 20% British Alpine and 10% Toggenburg) and the selected sheep included 1 Awassi, 3 East Frisian and 2 unknown mixed breeds. Raw milk samples (500 mL per animal) were taken during morning milking for camels and during afternoon milking for goats and sheep and were transported to the laboratory on ice. Extraction of the MFG core was performed on the following 2 days. Proximate analysis for fat and protein concentrations in camel, sheep and goat milk were determined according to the Australian Standard 2300 by Dairy Technical Services, North Melbourne, Australia.

### *Milk Fat Globule Size Analysis*

Laser light scattering with a Mastersizer 2000 particle analyzer (Malvern Instruments, Malvern, UK) was used to measure MFG size distributions of the milk samples, according to the procedure described in Logan et al. (2014). Prior to the analysis milk was diluted 1:1 with 35 mM EDTA, pH 7.0 to dissociate casein micelles. The obscuration rate was kept between 12 to 15% and refractive indices for milk fat and water were estimated as 1.46 and 1.33, respectively. The particle absorption coefficient was 0.001. Volume-weighted mean diameter $D_{4,3}$ and surface-weighted mean diameter $D_{3,2}$ were calculated by the software and each sample was measured in duplicate.

***Extraction of the MFG Core***

***Cows.*** Cow milk samples (3.2 L) were first separated into cream and skim milk, and the cream fraction was further separated into MFGM and MFG core fractions. Whole raw milk was centrifuged in 1 L centrifuge bottles (Nalgene, Sigma-Aldrich, Castle Hill, NSW, Australia) at 2500 × g for 30 min at 4°C and the cream fractions were kept at 4 °C overnight. On the next day the cream was washed thrice in 1 L of double distilled water, using gentle agitation with a spoon for 5-10 min followed by centrifugation (2500 × g, 30 min, 4°C). The washed cream was crystallized overnight at 4 °C. On the final day, the washed cream was churned, and the buttermilk and butter phases were separated by filtration through a double layer of cheesecloth. The butter fraction was then heated at 70 °C for 30 min and left to solidify at 4 °C to separate the milk fat from the butter serum. The butter serum was separated from the solid milk fat. The remaining milk fat after separation from the butter serum represents the MFG core. The washed MFG core fraction was kept frozen at - 80°C for FA analysis.

***Camels, goats and sheep***. Extraction of the MFG core fractions from whole milk differed from the process used for cow samples, because churning was not feasible due

to smaller sample volumes and physical properties of camel, goat and sheep milk. Separation of cream from skim milk was performed using 4 × 230 mL centrifuge tubes at 3500 × g for 30 min at 4 °C. Washing was performed by mixing the cream fraction with double distilled water followed by centrifugation (3500 × g, 30 min, 4 °C). Goat and sheep milk samples were washed thrice, while camel milk was only washed twice to reduce losses due to the small MFG size. The washed cream was kept at 4 °C overnight. The cream was mixed with a small volume of double distilled $H_2O$ in a 230 mL centrifuge tube, shaken by hand for 1 min and sonicated for 5 min in a sonication water bath at room temperature. Lastly, the tubes were placed in a water bath at 70 °C for 30 min, followed by 15 min centrifugation at 1500 × g at 4 °C. The milk fat layer was separated from the aqueous phase and kept at -80 °C.

***Fatty Acid Analysis***

The FA composition of milk fats was determined as mentioned in Shen et al. (2001) with some modifications. Briefly, FAME of milk fat were prepared by mixing 10-20 mg fat in a glass vial with 0.3 mL hexane and 0.3 mL 2 M potassium hydroxide in methanol for 5 min at room temperature. After cooling down the mixture, the hexane phase was transferred to a GC vial and was diluted four times with hexane. FAME were analyzed by the Agilent 7890A GC (Agilent Technologies Australia, Mulgrave, VIC, Australia) using a 30 m BPX70 Column (0.25-mm inner diameter, 0.25-µm film thickness, Trajan Scientific and Medical, Ringwood, VIC, Australia). The column temperature was set for 3 min at 40 °C, followed by raising the temperature at a rate of 20 °C/min to 160 °C, which was held for 16 min. The column temperature was further raised to 240 °C at a rate of 20 °C/min and held at this temperature for 3 min. Helium was used as a carrier gas. The injector temperature was programmed at 240 °C with 11.691 psi pressure and 55.58 mL/min flow of helium. The samples were injected in the split mode with a ratio of 50:1

and with a split flow of 51.549 mL/min. The FID detector temperature was 280 °C with a flow of 40 mL /min hydrogen gas and 400 mL/min of air. FAME peaks were identified on the basis of retention time of FAME standards (GLC-607, GLC-674 from NuChek INC., USA and the milk fat reference CRM164 obtained from CSIRO, Agriculture and Food). Peak areas of GLC 607 were used to determine the response factors for individual FAME peaks in the GC and were applied to correct the area percentages of the milk FAME peaks.

### *Statistical Analysis*

Statistical analysis was performed using R version 3.5.1 (R Core Team, 2018). Duplicate measurements of individual cows were averaged and a two-way ANOVA was fitted, with group, DIM and sampling season as fixed effects. The model was fitted using the 'lm' function in combination with the 'tidy' function from the 'broom' package (Robinson and Hayes, 2018). The p-values were adjusted for multiple testing using the procedure by Benjamini and Hochberg (1995), which was applied individually per dataset, namely the milk production data analysis (Table 1), the grouped FA analysis (Table 2) and the individual FA analysis (Table 3). Statistical significance was assumed for p-values $< 0.05$ and p-values $< 0.10$ were defined as trends towards statistical significance. Means and 95% confidence intervals were calculated. Graphs were produced using the 'ggplot2' package (Wickham, 2016).

## RESULTS AND DISCUSSION

### *Milk Fat Globule Size Distribution*

Milk was collected from cows shown to consistently produce milk of either small or large MFG size distributions, selected through an observation period prior to this study (Chapter 3). According to the pre-trial measurements, cows were classified into 2 study

groups with either the small MFG (SMFG) or large MFG (LMFG) phenotype. Figure 1 shows the MFG size distributions of the selected cows during this study and the pre-trial period. This shows the remarkable consistency in average MFG size of individual cows, which in some cows was seen over 2 consecutive lactation cycles (Figure 1).

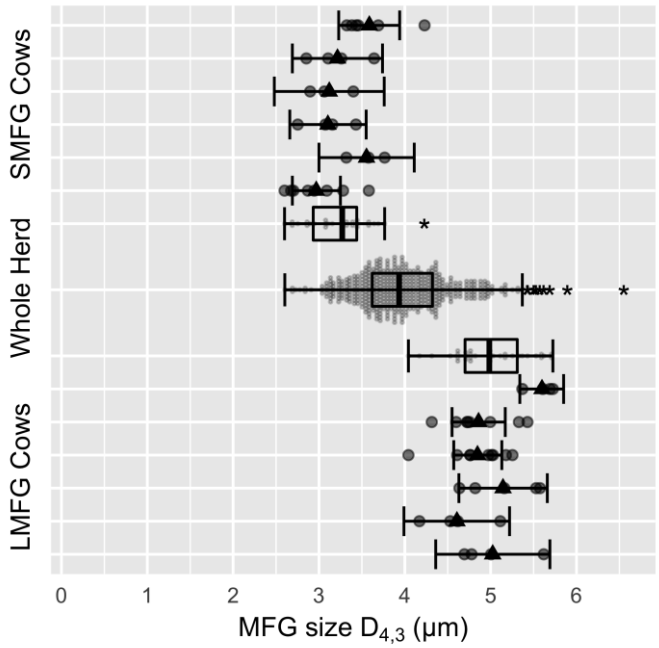


Figure 1: Consistency in volume weighted mean diameter $D_{4,3}$ of milk fat globules (MFG) from selected cows compared to the whole herd. Mean symbols (triangles) and individual values (gray circles) are shown for the 12 selected cows individually, where error bars represent 95% confidence intervals. Boxplots are shown for the SMFG and LMFG group compared to the data collected for the whole herd including the pre-trial period. Asterisks denote outliers 1.5 times the interquartile range above or below the upper or lower quartile, respectively. Each selected cow was measured a minimum of 3 and a maximum of 9 times. SMFG = cows naturally producing small MFG, LMFG = cows naturally producing large MFG.

The average MFG size, milk composition and animal production data of both study groups are shown in Table 1, compared with the same data, where known, for milk

collected from camel, goat and sheep species. The milk from cows in the SMFG and LMFG groups varied significantly in $D_{4,3}$ MFG size ($p = 0.003$, Table 1), with an average diameter of 3.29 µm and 4.92µm, respectively. This confirmed that pre-trial selection indeed led to 2 study groups with significantly different MFG size distributions. Moreover, all cows selected for this study were in established lactation (> 50 DIM), received the same diet and did not differ in milk production traits (milk yield, fat and protein content, fat-protein ratio (Table 1). This was important because it is known that factors such as the fat-protein ratio can influence MFG size (Couvreur and Hurtaud, 2017).

We hypothesized that there may be two distinct phenotypes of cows consistently producing milk with small and large MFG within our herd, thereby allowing for on-farm selection. To test this hypothesis, it was necessary to limit the impact of environmental conditions and milk production traits in order to isolate physiological differences between the SMFG and LMFG groups. While milk production parameters were similar between the studied groups (Table 1), the selected cows differed slightly in DIM (168 in SMFG and 143 in LMFG, $p = 0.199$) and parity (1-3 in SMFG and 3-5 in LMFG), the former being known to impact MFG size (Altenhofer et al., 2015). However, we do not believe this to contribute considerably to the differences observed between the studied groups, because we studied the effect of DIM and parity on MFG size in a previous study using the same herd as the present study (Chapter 3). Based on this analysis, we estimate that 25 additional DIM, observed here for the SMFG group compared to the LMFG group would lead to a 0.05 µm decrease in MFG size. Furthermore, an increase in MFG size by 0.16 µm was observed between parity 2 and 3 (Chapter 3), which in the current study could contribute to the difference between the SMFG (parity 1-3, average 1.8) group and the LMFG group (parity 3-5, average 3.8). However, these results suggest that the

difference in average MFG diameter between the studied groups in the current study (1.63 µm) is considerably greater than the combined effect of the differences in DIM and parity (0.21 µm). Nonetheless, DIM was included in the statistical model as a fixed effect to control for its known impact on MFG size. Parity on the other hand was significantly different between the studied groups ($p = 0.028$, Table 1) and could have contributed to some of the observed differences in FA origin and saturation. This is a limitation of our study as different parities are related to metabolic changes mainly in the transition period, but also later in lactation. For example, increased milk yields in cows in later parities (4 and higher) can lead to a higher loss of body condition during early lactation (Lee and Kim, 2006). Furthermore, in these cows, the recovery from the loss of body condition experienced during early lactation can be delayed and can take up to 4 - 5 months into the lactation cycle (Lee and Kim, 2006). Therefore, although the impact of parity on MFG size remains equivocal in the literature (Martini et al., 2004; Fleming et al., 2017), we cannot separate the observed differences in our study from the potential effect of differences in the lipid metabolism of cows in later parities (here the LMFG group) and earlier parities (the SMFG group).

Table 1: Milk fat globule (MFG) size, milk composition and animal production data in cows producing milk with small or large average MFG size and across several species[1]

| | SMFG Cow[2] | LMFG Cow[3] | p-value[4] | Camel | Goat | Sheep |
|---|---|---|---|---|---|---|
| n | 6[5] | 6[5] | | 6 | 6 | 6 |
| MFG size $D_{3,2}$ (µm)[6] | 2.89 (2.69, 3.10) | 4.04 (3.74, 4.33) | 0.003 | 2.76 (2.62, 2.90) | 3.26 (2.98, 3.53) | 3.53 (3.08, 3.99) |
| MFG size $D_{4,3}$ (µm)[7] | 3.29 (2.96, 3.62) | 4.92 (4.58, 5.25) | 0.003 | 3.33 (3.00, 3.66) | 4.00 (3.50, 4.50) | 4.13 (3.47, 4.78) |
| DIM | 168.3 (117.0, 219.7) | 143.3 (92.0, 194.7) | - | 188.7 (19.0, 396.4) | 96.2 (90.0, 102.3) | 151.2 (56.6, 245.8) |
| Parity | 1.8 (1.1, 2.6) | 3.8 (3.1, 4.6) | 0.028 | 1.3 (0.8, 1.9) | 3.0 (1.9, 4.2) | 2.7 (1.1, 4.3) |
| Concentrate intake (kg) | 8.6 (6.2, 11.1) | 8.4 (5.9, 10.9) | 0.775 | - | - | - |
| Milkings | 2.8 (2.4, 3.2) | 2.8 (2.4, 3.2) | 0.854 | 2.0 | 2.0 | 2.0 |
| Milk yield (kg) | 27.84 (19.41, 36.27) | 29.25 (20.82, 37.68) | 0.854 | 4.87 (2.58, 7.15) | 4.95 (3.71, 6.19) | 1.25 (0.94, 1.56) |
| Fat content (%) | 3.40 (2.56, 4.25) | 3.77 (2.93, 4.62) | 0.729 | 2.15 (1.26, 3.04) | 4.54 (3.60, 5.48) | 5.95 (4.75, 7.16) |
| Fat yield (kg/d) | 0.92 (0.76, 1.07) | 1.01 (0.86, 1.16) | 0.729 | 0.10 (0.05, 0.15) | 0.22 (0.16, 0.29) | 0.07 (0.07, 0.08) |
| Protein content (%) | 2.96 (2.76, 3.16) | 3.00 (2.81, 3.20) | 0.729 | 2.51 (2.02, 3.01) | 2.92 (2.70, 3.15) | 5.14 (4.72, 5.56) |
| Fat/protein ratio | 1.15 (0.92, 1.37) | 1.24 (1.01, 1.47) | 0.729 | 0.85 (0.59, 1.12) | 1.57 (1.21, 1.92) | 1.16 (0.93, 1.39) |

[1]Means (95% CI) are shown.

[2]SMFG = cows naturally producing small milk fat globules.

[3]LMFG = cows naturally producing large milk fat globules.

[4]P-values were calculated using a two-way ANOVA with group (SMFG or LMFG cows), days in milk and sampling season as fixed effects. P-values were adjusted for multiple comparisons using the Benjamini and Hochberg (1995) procedure. Other species are shown for comparison purposes only.

[5]Six cows were sampled in biological replicates on 2 separate days, of which 3 were sampled in autumn and 3 in winter.

[6]MFG size $D_{3,2}$ (µm) = surface-weighted mean diameter.

[7]MFG size $D_{4,3}$ (µm) = volume-weighted mean diameter.

Figure 2 shows the variation in MFG size distribution between the selected SMFG and LMFG groups in comparison to the MFG size distribution of a number of other mammalian species. Interestingly, the average MFG size distribution for camel milk was similar to the SMFG cow group measured in the current study. Moreover, the variation in MFG size between the six randomly selected camels was smaller than in the other species (Table 1) indicating that camel milk may naturally contain MFG of a narrower size range and smaller average MFG size, as previously suggested (El-Agamy, 2017). For sheep and goat milk however, we observed considerable variation in the average MFG size between the six randomly selected animals (Table 1) similar to the range generally found in cow milk (Logan et al., 2014).

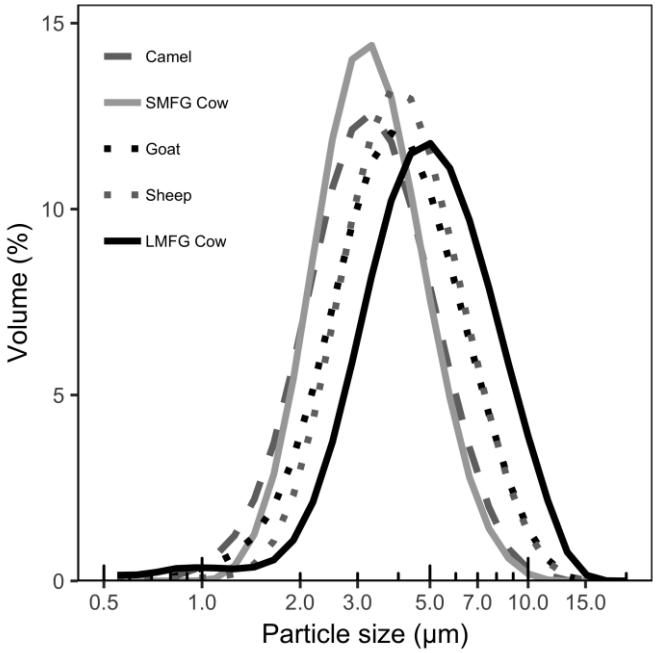


Figure 2: Volume weighted milk fat globule (MFG) size distributions of the selected SMFG[a] and LMFG[b] cows compared to camels[a], sheep[c] and goats[c]. Different superscript letters indicate statistical significance ($p < 0.05$) between the studied species and SMFG and LMFG cows, determined through a pairwise comparison t-test adjusted for multiple comparisons using the Benjamini and Hochberg (1995) procedure. SMFG = cows naturally producing small milk fat globules, LMFG = cows naturally producing large milk fat globules.

### *The MFG Core FA Composition Differs between SMFG and LMFG Cows*

We found clear differences in the FA composition of the MFG core obtained from SMFG and LMFG cows, even though these animals were under the same nutritional management and produced milk of similar composition. The MFG core, comprising predominantly but not exclusively non-polar lipids, contained a higher proportion of total UFA (33.1% of total FAME) in the SMFG group compared to the MFG core form LMFG cows (27.5%, Table 2, Figure 3). This was due to higher concentrations of MUFA and PUFA in the MFG core from SMFG cows (28.4% and 4.8%, respectively) compared to LMFG cows (23.9% and 3.6%, respectively, Figure 3).

Our results are in accordance with a study by Couvreur et al. (2007), but in conflict with another (Mesilati-Stahy and Argov-Argaman, 2014). This could at least in part be due to the difference in study design, with Mesilati-Stahy and Argov-Argaman (2014) using microfiltration to separate milk into groups with different MFG sizes, while in our study and that of Couvreur et al. (2007) the milk of small and large MFG groups was sourced from cows with naturally occurring differences in MFG size. Comparison of these results suggests that, while FA compositions of MFG in a certain size group can vary, the relationship between the overall FA composition in milk and its MFG size is also specific to the individual animal. These results supported our hypothesis that MFG size could reflect individual differences in the cow's lipid metabolism.

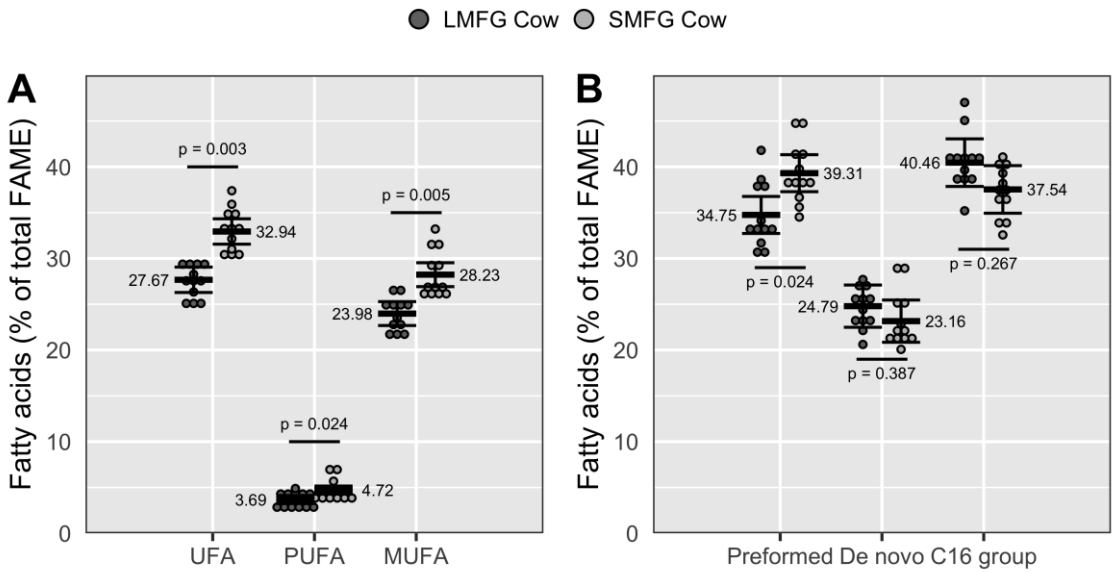


Figure 3: Degree of unsaturation (A) and origin of fatty acids (B) in the small and large milk fat globule phenotypes. Dot plots show individual data points and least square means as thick bars and numbers. Error bars represent the 95% confidence intervals. P-values were calculated using a two-way ANOVA with group (SMFG or LMFG cows), days in milk and sampling season as fixed effects. P-values were adjusted for multiple comparisons using the Benjamini and Hochberg (1995) procedure. De novo = fatty acids derived from de novo synthesis (C4 to C14), C16 group = fatty acids with a chain length of 16 (C16:0 and C16:1), Preformed = fatty acids (≥ C18). SMFG = cows naturally producing small milk fat globules, LMFG = cows naturally producing large milk fat globules.

While the content of UFA can be modified through the cow's diet (Lopez et al., 2008), most UFA are first converted to trans-FA isomers and, if biohydrogenation of the double bonds is complete, they are ultimately converted to the SFA stearic acid in the rumen (Bauman et al., 2011). Hence, the majority of UFA found in milk are produced from the desaturation of SFA by an enzyme called $\Delta^9$ desaturase or stearoyl-CoA desaturase (**SCD**), and this process takes place in mammary and adipose tissue. SCD activity increases with ongoing lactation (Soyeurt et al., 2008) and may change between seasons, where it increases with higher proportions of pasture in the diet (Lock and Garnsworthy, 2003). Furthermore, SCD activity can vary between individual animals and breeds (Lock

and Garnsworthy, 2003; Soyeurt et al., 2008). Indices for SCD (**SCDI**), expressing each product of SCD as a proportion of the sum of product and precursor, can be used as indicators for SCD activity (Garnsworthy et al., 2010). Couvreur et al. (2007) reported a higher C18:1/C18:0 ratio in SMFG cows and the use of SCDI for breeding selection programs has already been suggested (Garnsworthy et al., 2010). In the current study, cows in the SMFG group exhibited a higher SCDI for FA with a chain length of 14 (SCDI14, $p = 0.387$, Table 2), FA of 16 carbons in chain length (SCDI16, $p = 0.387$, Table 2) and FA with 18 carbons (SDCI18, $p = 0.298$). However, this was not statistically significant.

Others have related the K232A polymorphism on the diacylglycerol acyltransferase 1 (DGAT1) gene to changes in milk FA compositions as well as MFG size (Schennink et al., 2007; Argov-Argaman et al., 2013). These studies reported changes in MFG size and FA composition due to genotypic differences on the DGAT1 gene which are comparable to the differences observed between the SMFG and LMFG groups in our study, with cows of the KK genotype producing milk with less UFA, more C16:0 (Schennink et al., 2007) and larger MFG (Argov-Argaman et al., 2013). However, genotypic information on the selected cows was not available in our study and at this stage remains speculative.

Table 2: Fatty acid (FA) origin and degree of unsaturation in the MFG core from cows producing milk with small or large average milk fat globule size[1] and across several species[2]

| | SMFG Cow[3] | LMFG Cow[4] | p-value[5] | Camel | Goat | Sheep |
|---|---|---|---|---|---|---|
| n | 6[6] | 6[6] | | 6 | 6 | 6 |
| Preformed FA (≥ C18) | 39.31 (37.29, 41.33) | 34.75 (32.73, 36.77) | 0.024 | 49.08 (46.32, 51.84) | 43.90 (40.77, 47.03) | 39.49 (31.97, 47.01) |
| De novo FA (C4 to C14) | 23.16 (20.85, 25.46) | 24.79 (22.49, 27.10) | 0.387 | 15.24 (13.1, 17.38) | 26.77 (25.16, 28.38) | 31.34 (25.39, 37.29) |
| SCFA (C4 to C8)[7] | 2.25 (2.03, 2.47) | 2.39 (2.17, 2.62) | 0.387 | 0.11 (0.08, 0.14) | 4.05 (3.72, 4.39) | 4.07 (3.15, 4.99) |
| MCFA (C9 to C14)[8] | 19.13 (16.97, 21.30) | 20.47 (18.30, 22.63) | 0.387 | 12.80 (10.91, 14.69) | 21.33 (19.79, 22.87) | 25.07 (19.69, 30.45) |
| C16 group (C16:0, C16:1c9) | 37.54 (34.94, 40.13) | 40.46 (37.86, 43.05) | 0.267 | 35.68 (32.17, 39.19) | 29.34 (27.46, 31.22) | 29.17 (26.62, 31.72) |
| UFA | 32.94 (31.56, 34.33) | 27.67 (26.29, 29.06) | 0.003 | 42.52 (39.08, 45.96) | 31.78 (29.18, 34.38) | 32.29 (27.81, 36.77) |
| SFA | 67.06 (65.67, 68.44) | 72.33 (70.94, 73.71) | 0.003 | 57.48 (54.04, 60.92) | 68.22 (65.62, 70.82) | 67.71 (63.23, 72.19) |
| MUFA | 28.23 (26.92, 29.53) | 23.98 (22.68, 25.29) | 0.005 | 38.84 (35.42, 42.26) | 28.51 (26.16, 30.86) | 27.52 (23.50, 31.54) |
| PUFA | 4.72 (4.28, 5.16) | 3.69 (3.25, 4.13) | 0.024 | 3.68 (3.24, 4.12) | 3.27 (2.88, 3.66) | 4.77 (3.71, 5.82) |
| SCDI14[9] | 10.50 (8.91, 12.09) | 9.48 (7.88, 11.07) | 0.387 | 10.79 (10.03, 11.55) | 3.26 (2.99, 3.53) | 4.29 (3.05, 5.54) |
| SCDI16[9] | 4.21 (3.42, 5.00) | 3.63 (2.84, 4.43) | 0.387 | 19.74 (17.69, 21.79) | 1.86 (1.50, 2.22) | 4.08 (2.77, 5.38) |
| SCDI18[9] | 69.21 (64.26, 74.16) | 64.23 (59.28, 69.18) | 0.298 | 62.62 (54.8, 70.44) | 66.47 (62.17, 70.77) | 70.85 (63.88, 77.82) |

[1]Least square means (95% CI) are shown for SMFG and LMFG groups.

[2]Means (95% CI) are shown for camel, goat and sheep.

[3]SMFG = cows naturally producing small milk fat globules.

[4]LMFG = cows naturally producing large milk fat globules.

[5]P-values were calculated using a two-way ANOVA with group (SMFG or LMFG cows), days in milk and sampling season as fixed effects. P-values were adjusted for multiple comparisons using the Benjamini and Hochberg (1995) procedure. Other species are shown for comparison purposes only.

[6]Six cows were sampled in biological replicates on 2 separate days.

[7]SCFA = short chain fatty acid.

[8]MCFA = medium chain fatty acid.

[9]SCDI = Stearoyl-CoA desaturase index with SCDI14 = (C14:1c9) / (C14:0 + C14:1c9) × 100, SCDI16 = (C16:1c9) / (C16:0 + C16:1c9) × 100 and SCDI18 = (C18:1c9) / (C18:0 + C18:1c9) × 100.

### *Possible Lipid Metabolic Differences between the SMFG and LMFG Phenotype*

To gain insight into possible lipid metabolic differences between SMFG and LMFG cows we further looked at the origin of the FA in their MFG core. Figure 3 shows the concentrations of de novo synthesized and preformed FA in the studied groups. The results indicate that in SMFG cows the proportion of preformed FA taken up from the blood is higher than in LMFG cows ($p = 0.024$), while the proportion of de novo FA was similar between the groups. Based on this analysis we suggest 2 possible explanations for the observed differences between the SMFG and LMFG phenotypes: 1) long-chain SFA in the SMFG group were utilized to produce polar lipids needed for MFGM production and were therefore reduced in the MFG core; or 2) the observed changes could indicate increased lipid mobilization from adipose tissue in the SMFG group.

Regarding the first suggested mechanism, Mesilati-Stahy et al. (2012) showed that preformed long-chain SFA are preferentially incorporated into the MFGM fraction (polar lipids) rather than the core (TAG) and suggested that this could supply the increased membrane material needed for the formation of smaller MFG. Although we did not determine the FA composition of the MFGM, the increased proportion of blood-derived FA in the MFG core of the SMFG group was due to increased relative proportions of UFA not long-chain SFA (Table 3). This could imply that long chain SFA are channeled towards polar lipid synthesis and could partly explain their reduced proportion in the MFG core of the SMFG group. Moreover, the study by Mesilati-Stahy et al. (2012) suggested that MUFA were preferentially partitioned into the MFG core in cows with higher plasma insulin concentrations, whilst cows with lower insulin levels preferentially partitioned MUFA into the MFGM compartment, suggesting a potential hormonal regulation of this process.

The second possible mechanism is based on the impact of negative energy balance on the milk FA profile. Cows in negative energy balance, i.e. in the early stages of lactation or during feed restriction, exhibit a FA composition similar to the FA profile of the MFG core from SMFG cows in our study, with more oleic acid (C18:1 c9), less palmitic acid (C16:0) and less de novo synthesized FA (Gross et al., 2011). Moreover, oleic acid has been suggested as a potential indicator for subclinical ketosis (Van Haelst et al., 2008). This could indicate increased lipid mobilization from adipose tissue in SMFG cows. However, while dietary FA are derived from TAG packaged in lipoprotein particles and require hydrolysis by lipoprotein lipase, FA from lipid mobilization reach the mammary gland in the form of non-esterified FA (**NEFA**). In vitro evidence suggests that higher NEFA supply to the mammary gland increases LD size (Mesilati-Stahy and Argov-Argaman, 2018) which would be in conflict with the hypothesis that SMFG cows obtain preformed long-chain FA from lipid mobilization. While we did not measure NEFA in the current study to draw a conclusion either way, the concentrate intake, body weight, milk yield and fat-protein measurements provided to us by the milking system suggests that the cows were probably not in negative energy balance and indicate that the first mechanism is more likely to have resulted in the observed differences in the FA profiles of the SMFG and LMFG groups. However, as previously noted the impact of the observed differences in parity between the study groups and its possible effect on body condition recovery could have contributed to some of the observed differences.

Table 3: Fatty acid composition in the small and large milk fat globule phenotype in cows[1] and across species[2]

| Species | SMFG Cow[3] | LMFG Cow[4] | p-value[5] | Camel | Goat | Sheep |
|---|---|---|---|---|---|---|
| C4:0 | 0.71 (0.62, 0.80) | 0.74 (0.65, 0.83) | 0.856 | 0.00 (0.00, 0.00) | 1.00 (0.84, 1.17) | 1.17 (0.90, 1.45) |
| C5:0 | 0.00 (0.00, 0.00) | 0.00 (0.00, 0.01) | 0.668 | 0.00 (0.00, 0.00) | 0.00 (0.00, 0.01) | 0.02 (0.00, 0.03) |
| C6:0 | 0.78 (0.70, 0.86) | 0.83 (0.75, 0.91) | 0.615 | 0.05 (0.03, 0.06) | 1.24 (1.12, 1.36) | 1.20 (0.91, 1.49) |
| C7:0 | 0.01 (0.00, 0.01) | 0.01 (0.01, 0.02) | 0.310 | 0.00 (0.00, 0.00) | 0.02 (0.01, 0.03) | 0.05 (0.02, 0.08) |
| C8:0 | 0.75 (0.66, 0.84) | 0.80 (0.71, 0.90) | 0.659 | 0.07 (0.04, 0.09) | 1.79 (1.68, 1.89) | 1.63 (1.16, 2.10) |
| C9:0 | 0.02 (0.01, 0.03) | 0.03 (0.02, 0.04) | 0.310 | 0.00 (0.00, 0.00) | 0.05 (0.02, 0.09) | 0.12 (0.04, 0.20) |
| C10:0 | 2.31 (1.89, 2.73) | 2.56 (2.14, 2.98) | 0.659 | 0.11 (0.07, 0.14) | 7.11 (6.54, 7.68) | 6.78 (4.72, 8.85) |
| C10:1w1 | 0.22 (0.19, 0.24) | 0.21 (0.19, 0.24) | 0.917 | 0.02 (0.01, 0.02) | 0.16 (0.11, 0.20) | 0.23 (0.15, 0.31) |
| C11:0 | 0.04 (0.02, 0.07) | 0.07 (0.04, 0.10) | 0.310 | 0.01 (0.01, 0.01) | 0.09 (0.01, 0.16) | 0.23 (0.05, 0.40) |
| C12:0 | 3.23 (2.61, 3.85) | 3.53 (2.91, 4.15) | 0.731 | 0.77 (0.58, 0.95) | 3.29 (2.64, 3.94) | 4.78 (3.17, 6.39) |
| C12:1w9 | 0.08 (0.07, 0.10) | 0.08 (0.06, 0.09) | 0.917 | 0.02 (0.02, 0.03) | 0.05 (0.03, 0.07) | 0.08 (0.05, 0.11) |
| C13:0 | 0.07 (0.04, 0.11) | 0.11 (0.08, 0.15) | 0.310 | 0.08 (0.06, 0.09) | 0.09 (0.05, 0.13) | 0.22 (0.08, 0.35) |
| C13:0 isomer | 0.09 (0.07, 0.11) | 0.09 (0.07, 0.11) | 0.954 | 0.02 (0.01, 0.02) | 0.05 (0.03, 0.08) | 0.10 (0.05, 0.15) |
| C14:0 | 11.63 (10.50, 12.77) | 12.39 (11.26, 13.53) | 0.629 | 10.32 (8.79, 11.85) | 10.02 (9.31, 10.73) | 11.92 (10.29, 13.55) |
| C14:0 isomer | 0.09 (0.07, 0.10) | 0.09 (0.07, 0.11) | 0.917 | 0.22 (0.18, 0.26) | 0.08 (0.07, 0.10) | 0.07 (0.04, 0.11) |
| C14:1c9 | 1.36 (1.15, 1.56) | 1.29 (1.09, 1.50) | 0.856 | 1.24 (1.09, 1.40) | 0.34 (0.29, 0.38) | 0.53 (0.36, 0.71) |
| C15:0 | 1.12 (0.87, 1.37) | 1.32 (1.07, 1.57) | 0.579 | 1.38 (1.22, 1.54) | 0.93 (0.69, 1.17) | 1.58 (1.14, 2.03) |
| C15:0 ante-isomer | 0.56 (0.45, 0.66) | 0.54 (0.44, 0.65) | 0.917 | 0.88 (0.74, 1.01) | 0.43 (0.33, 0.52) | 0.58 (0.48, 0.68) |
| C15:0 isomer | 0.10 (0.09, 0.11) | 0.07 (0.06, 0.08) | 0.020 | 0.08 (0.05, 0.11) | 0.03 (0.02, 0.04) | 0.04 (0.03, 0.05) |
| C16:0 | 35.65 (33.22, 38.08) | 38.69 (36.27, 41.12) | 0.310 | 27.74 (25.64, 29.84) | 28.36 (26.43, 30.29) | 27.48 (25.22, 29.74) |
| C16:0 isomer | 0.09 (0.08, 0.11) | 0.09 (0.08, 0.11) | 0.930 | 0.23 (0.14, 0.32) | 0.06 (0.05, 0.08) | 0.12 (0.05, 0.19) |
| C16:1c7 | 0.22 (0.21, 0.23) | 0.20 (0.19, 0.21) | 0.310 | 0.77 (0.61, 0.92) | 0.38 (0.33, 0.42) | 0.37 (0.31, 0.43) |
| C16:1c9 | 1.57 (1.22, 1.93) | 1.47 (1.11, 1.82) | 0.856 | 6.94 (5.53, 8.35) | 0.54 (0.45, 0.62) | 1.19 (0.74, 1.65) |
| C17:0 | 0.63 (0.56, 0.70) | 0.64 (0.57, 0.71) | 0.917 | 0.74 (0.63, 0.85) | 0.56 (0.5, 0.62) | 0.64 (0.52, 0.77) |
| C17:1c9 | 0.31 (0.26, 0.35) | 0.27(0.22, 0.31) | 0.504 | 0.71 (0.61, 0.80) | 0.29 (0.23, 0.35) | 0.37 (0.26, 0.49) |
| C18:0 | 9.10 (7.27, 10.93) | 9.59 (7.76, 11.42) | 0.868 | 14.46 (10.99, 17.93) | 12.69 (10.31, 15.07) | 8.71 (4.75, 12.67) |
| C18:1c9 | 20.54 (19.52, 21.55) | 16.81 (15.79, 17.83) | 0.020 | 24.00 (21.70, 26.30) | 25.04 (23.07, 27.01) | 19.92 (15.45, 24.39) |
| C18:1 isomers | 3.84 (3.46, 4.22) | 3.57 (3.19, 3.95) | 0.615 | 4.85 (4.38, 5.33) | 1.66 (1.3, 2.01) | 4.71 (2.26, 7.17) |
| C18:2 (11t, 15c) | 0.46 (0.43, 0.49) | 0.40 (0.37, 0.43) | 0.201 | 0.31 (0.24, 0.37) | 0.24 (0.19, 0.29) | 0.41 (0.22, 0.61) |
| C18:2 (9t,12t) | 0.40 (0.34, 0.45) | 0.31 (0.25, 0.36) | 0.310 | 0.25 (0.22, 0.28) | 0.22 (0.15, 0.28) | 0.49 (0.32, 0.66) |
| C18:2 | 2.23 (1.78, 2.69) | 1.65 (1.19, 2.10) | 0.310 | 1.69 (1.41, 1.97) | 2.32 (2.15, 2.50) | 2.92 (2.33, 3.51) |
| C18:3 | 0.71 (0.62, 0.80) | 0.59 (0.50, 0.68) | 0.310 | 0.68 (0.53, 0.83) | 0.30 (0.26, 0.35) | 0.42 (0.32, 0.51) |
| CLA C18:2 c9 t11 | 0.92 (0.78, 1.05) | 0.74 (0.61, 0.87) | 0.310 | 0.76 (0.59, 0.93) | 0.19 (0.10, 0.27) | 0.53 (0.27, 0.78) |
| C20:0 | 0.08 (0.04, 0.13) | 0.10 (0.06, 0.15) | 0.786 | 0.35 (0.22, 0.48) | 0.32 (0.30, 0.33) | 0.26 (0.13, 0.39) |
| C20:1d11 | 0.10 (0.09, 0.11) | 0.08 (0.07, 0.09) | 0.310 | 0.30 (0.19, 0.40) | 0.07 (0.06, 0.08) | 0.11 (0.07, 0.15) |

[1]Least square means (95% CI) are shown for SMFG and LMFG groups.

[2]Means (95% CI) are shown for camel, goat and sheep.

[3]SMFG = cows naturally producing small milk fat globules.

[4]LMFG = cows naturally producing large milk fat globules.

[5]P-values were calculated using a two-way ANOVA with group (SMFG or LMFG cows), days in milk and sampling season as fixed effects. P-values were adjusted for multiple comparisons using the Benjamini and Hochberg (1995) procedure. Other species are shown for comparison purposes only.

### *Fatty Acid Composition Across Species in Relation to MFG Size*

The MFG core FA profiles from SMFG and LMFG cows compared to camels, goats and sheep showed general differences between species (Table 3). For example, the MFG core from goat and sheep milk contained higher concentrations of C8:0 and C10:0 and milk fat extracted from camel milk contained very low amounts of short chain FA (Table 3). Most of these differences have been described elsewhere and are not the scope of our study. Here, we aimed to relate certain FA groups, for example UFA and de novo synthesized FA, to differences in the MFG size distributions observed between the camel, goat and sheep milk compared to the milk from SMFG and LMFG cows. Concentrations of UFA seemed to be inversely related to MFG size (Figure 4). Figure 4 shows the concentrations of UFA across the studied species ordered according to increasing average MFG sizes from camels to LMFG cows. Due to the broad distribution of MFG size in goat and sheep milk, the data was analyzed further by grouping the results from all species into small (≤ 3.5 µm) medium (3.5 to 4.5 µm) and large (≥ 4.5 µm) MFG (Figure 4) to reveal a clear relationship between MFG size and UFA across all species (Figure 4).

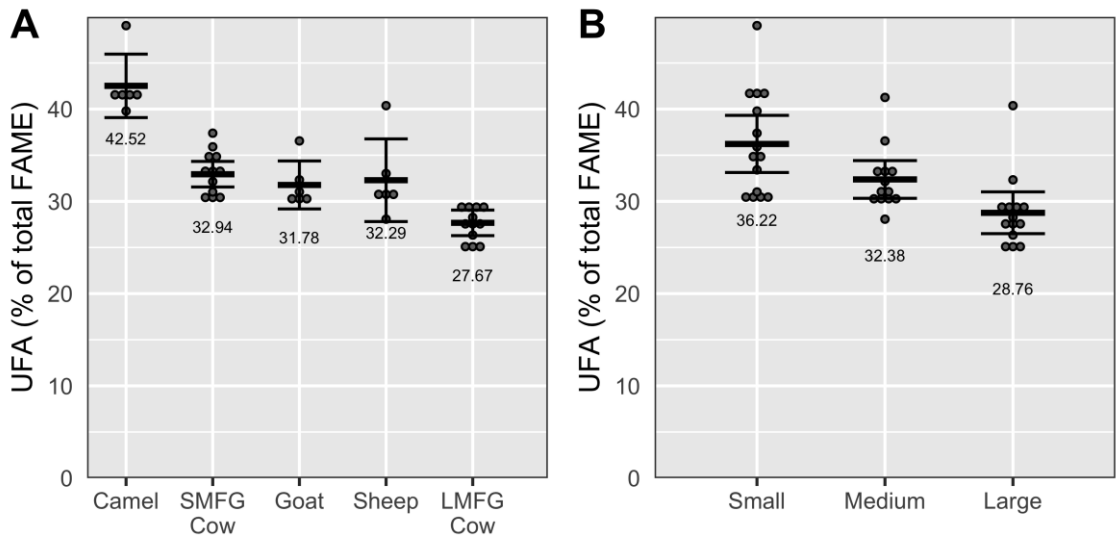


Figure 4: Concentrations of unsaturated fatty acids in the milk fat globule core from the small and large milk fat globule phenotypes as well as camel, sheep and goat milk (A) and grouped into small (≤ 3.5 µm), medium (3.5 to 4.5 µm) and large (≥ 4.5 µm) MFG (B). Dot plots show individual data points and least square means as thick bars and numbers. Error bars represent the 95% confidence intervals. P-values were calculated using a two-way ANOVA with group (SMFG or LMFG cows), days in milk and sampling season as fixed effects. P-values were adjusted for multiple comparisons using the Benjamini and Hochberg (1995) procedure. SMFG = cows naturally producing small milk fat globules, LMFG = cows naturally producing large milk fat globules.

## CONCLUSIONS

The analysis of the FA profile in cows producing milk with either small or large average MFG size distributions revealed a possible link between the origin of FA incorporated into LD, the desaturation activity of the individual animal and its ultimate effect on LD fusion. More specifically, cows with the SMFG phenotype incorporated more FA with a carbon number of 18 or higher into their LD and transformed more of these FA into UFA. In the current study we defined the SMFG and LMFG phenotype as the cows within a single herd that consistently produced MFG of either small or large average size, were fed the same diet and were similar in terms of their milk production traits. Based on this selection we showed that the SMFG phenotype could be exploited to

produce milk with higher nutritional value. However, further studies are needed to test this hypothesis and should focus on the possible links between the DGAT1 gene polymorphisms and MFG size. Further studies should also look at the identification of the small and large MFG phenotypes in goats and sheep and investigate the reason why camels do not seem to show the same variation in MFG size seen in the main dairy species.

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## 4.3 Appendix to Chapter 4

### ADDITIONAL MATERIALS AND METHODS

***Milk fat globule membrane (MFGM) extracts***

For all species, MFGM material was separated from the MFG core as described in the main manuscript of this chapter. The combination of buttermilk and butter serum is the MFGM extract and was frozen at -80 °C followed by freeze-drying and subsequent storage at -20 °C.

***Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) Protein Analysis and Densitometry***

The freeze-dried MFGM extracts were solubilised in 1% SDS (Sigma-Aldrich) and incubated at 30 °C on a rotary mixer (HulaMixer, Invitrogen, Thermo Fisher Scientific Australia Pty Ltd, Scoresby, VIC, Australia). Total protein concentrations were determined for all samples using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Thermo Fisher Scientific Australia Pty Ltd). Samples were standardised to the same protein concentration and 12 µL sample were mixed with 5 µL of LDS sample buffer (Invitrogen) and 1 µL of a standard at varying concentrations (phosphorylase b from rabbit muscle, Sigma-Aldrich). A 2-fold dilution series of the standard was prepared to load a concentration between 0.015 and 1.0 µg and each standard concentration was loaded on 2 separate lanes. The samples were denatured at 95 °C for 5 min and loaded onto Invitrogen NuPAGE 4-12% Bis-Tris Gels (1mm x 15 well, Invitrogen). Electrophoresis was performed at 200 V for 50 min using MOPS running buffer (Invitrogen). Staining was performed using the SimplyBlue SafeStain (Invitrogen) according to the microwave procedure. Gel images were digitised using a GelDoc EZ imager (Bio-Rad Laboratories Pty Ltd, Gladesville, NSW, Australia). The ImageLab 6.0

software (Bio-Rad Laboratories Pty Ltd) was used for densitometry analysis and the main protein bands were quantified by absolute quantification according to the equation obtained by linear regression of the phosphorylase b standard curve.

***Major Protein Band Identification by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)***

Following SDS-PAGE analysis, the main bands of interest were excised from one representative lane for each species to identify the proteins by LC-MS/MS. All chemicals were from Sigma-Aldrich unless otherwise stated. Gel excision was performed using clean sterile scalpel blades and the gel pieces were cut into 1 $mm^3$ cubes. All incubations were performed on an orbital shaker. The gel pieces were de-stained thrice with de-staining solution (50 mM ammonium bicarbonate (AmBic), 50% acetonitrile (ACN)) for 10 min at 37 °C. This step was followed by three incubations in 100% ACN for 10 min at 37 °C. The gel pieces were then subjected to reduction (20 mM DTT in 100 mM AmBic) followed by alkylation in (50 mM iodoacetamide in100 mM AmBic), both at 37 °C for 30 min. After two more washing steps in de-staining solution, the gel pieces were incubated in 100% ACN for 5 to 10 min and then air dried. This was followed by rehydration on ice for 60 min with a trypsin digestion buffer (170 nM trypsin in 50 mM AmBic). The excess solution was removed, and the gel pieces were covered in 50 mM AmBic. Digestion was performed overnight at 37 °C. On the next day, the peptides were extracted during two incubations at 37 °C for 30 min in an extraction buffer (0.1% formic acid in 60% ACN). The extraction buffer containing the peptides was reduced to approximately 10 µL by centrifugal lyophilisation in a vacuum centrifuge.

For peptide sequencing by LC-MS/MS, 3uL aliquots of OPT-302 digests were separated using a two-column chromatography setup comprising a PepMap100 C18 20

mm × 75 μm trap and a PepMap C18 500 mm × 75 μm analytical column (Thermo Scientific). Samples were concentrated onto the trap column at 5 μl/min for 5 min and infused into an Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer (Thermo Scientific) at 300 nl/min running a 120 min gradient from 99% Buffer A (0.1% FA) to 40% Buffer B (99% acetonitrile, 0.1% FA) on a Dionex Ultimate 3000 UPLC system (Thermo Scientific). The Lumos mass spectrometer was operated in data-dependent mode, automatically switching between the acquisition of a single Orbitrap MS scan (resolution, 120,000) every 3 s and the top-20 multiply charged precursors selected for EThcD fragmentation (maximum fill time, 100 ms; AGC of 5 × 104 with a resolution of 30,000 for Orbitrap MS-MS scans).

Mass spectrometry database searches for the identification of milk proteins were performed using the Byonic™ search engine against a custom protein database containing 86 milk protein sequences. High-resolution MS/MS data were searched with non-specific digestion allowing for carbamidomethylation of cysteine (+57Da) set as a fixed modification and oxidation of methionine or tryptophan (+16Da), phosphorylation of serine or threonine (+80Da) acetylation of protein N termini (+42Da) and pyroglutamate formation of N-terminal glutamine or glutamate residues (-17Da/ -18Da) as variable modifications. Total common max was set to 2, total rare max set to 1. The precursor mass tolerance was set to 10 ppm and fragment mass tolerance to 20 ppm with a maximum false discovery rate of 1.0% set for protein and peptide identifications.

***Statistical Analysis***

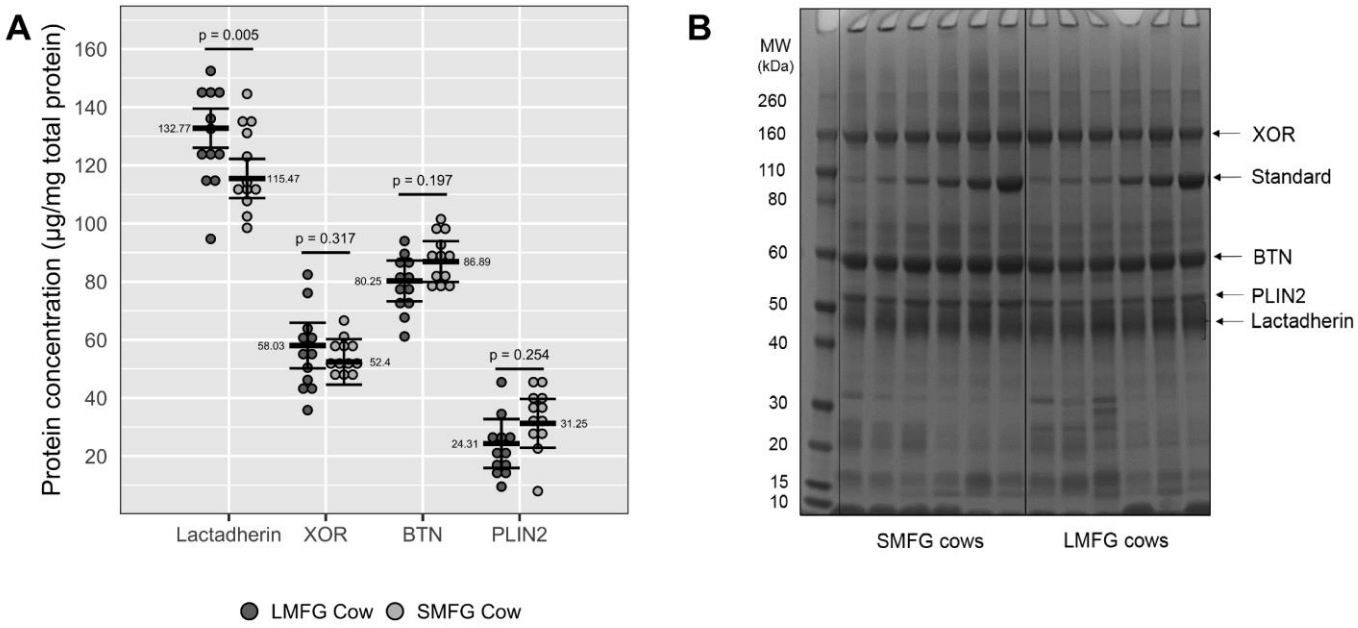
Statistical analysis was performed using a two-way ANOVA with group, days in milk and season as fixed effects to establish differences between cows producing milk with small (SMFG) or large MFGs (LMFG). Results from camels, sheep and goats are

presented as means and 95% confidence intervals and are used for comparative purposes only. Therefore, statistical significance was not determined for the differences between species.

## ADDITIONAL RESULTS AND DISUSSION

### *Concentrations of the Major MFGM Proteins in SMFG and LMFG Cows*

Densitometry analysis of the major bands in SDS-PAGE gels and subsequent identification of these proteins by LC-MS/MS revealed some differences in protein concentrations between the SMFG and LMFG groups, where MFGM extracts from the milk of SMFG cows had lower concentrations of lactadherin ($p = 0.005$, Figure A1). Moreover, MFGM extracts from the milk of SMFG cows had higher concentrations of BTN and PLIN2 compared to LMFG cows, which in turn had higher concentrations of xanthine oxidoreductase (XOR, Figure A1). However, these differences were not statistically significant. We used this analysis to estimate whether MFGM protein concentrations in general could be related to MFG size. While the use of SDS-PAGE and densitometry analysis is not the most sensitive technique and can lead to higher variation, obvious visual differences in the major MFGM protein bands between the groups, where identification was confirmed via LC-MS/MS analysis, indicate that MFGM protein concentrations could possibly be related to the MFG size development in the mammary gland, despite a relatively small sample size.




**Additional Figure A1:** Concentrations of the major milk fat globule membrane proteins in the small and large MFG phenotypes determined by densitometry analysis (A) of SDS-PAGE gels (B). Dot plots show individual data points and predicted means as thick bars and numbers. Error bars represent the 95% confidence intervals. P-values were calculated using 2-way ANOVA with group, days in milk and season as fixed effects and were adjusted for multiple comparisons using the Benjamini and Hochberg (1995) procedure. Proteins were identified by liquid chromatography tandem mass spectrometry analysis. XOR = xanthine oxidoreductase, BTN = butyrophilin, PLIN2 = perilipin 2. SMFG = cows naturally producing small milk fat globules, LMFG = cows naturally producing large milk fat globules. Six cows per group were sampled in biological duplicates on separate days.

The higher concentrations of lactadherin in the MFGM material extracted from LMFG cows compared to SMFG cows ($p = 0.005$, Figure A1) are in agreement with the earlier findings of Lu et al. (2016). Several possible functions of lactadherin have been suggested, including the phagocytosis of apoptotic MEC by adjacent cells and macrophages, as well as the clearance of secreted MFG remaining in the mammary ducts during mammary gland involution (Hanayama and Nagata, 2005). While the role of lactadherin in apoptosis is relatively well established, its physiological role in MEC remains largely unknown, despite being a major component of the MFGM. To date, the

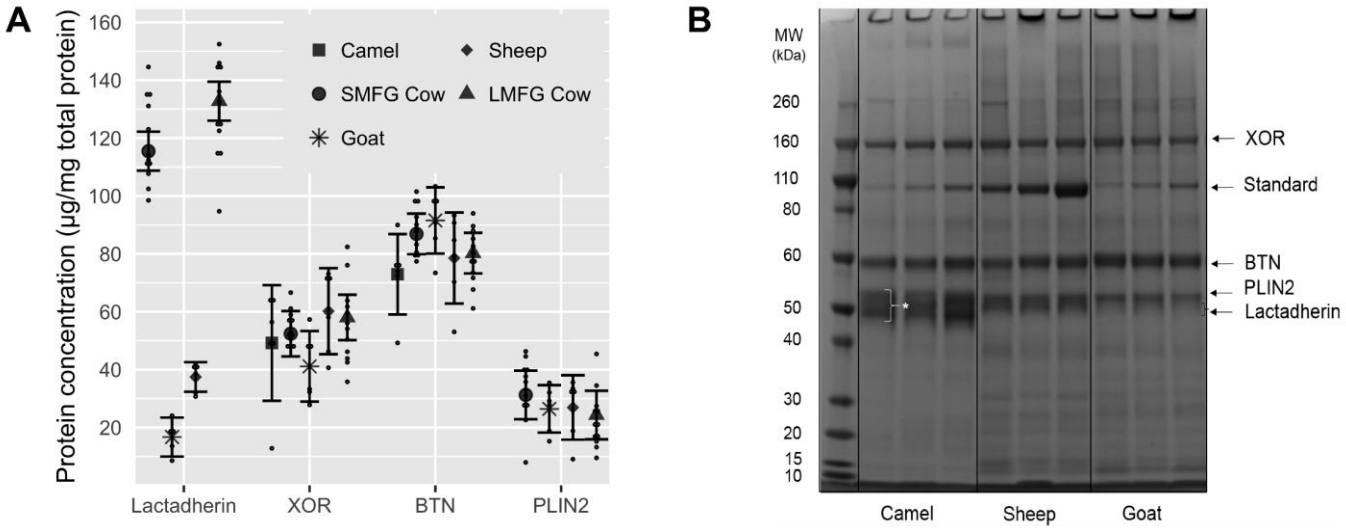
only evidence pointing towards a potential role for lactadherin in the regulation of MFG size is obtained from studies in mice (Nakatani et al., 2013; Yasueda et al., 2015). Yasueda et al. (2015) reported that lactadherin deficient mice produce milk with abnormally large MFG and showed that lactadherin binds to PS exposed on the MFG surface, preventing spontaneous fusion. These results seem at odds with the higher lactadherin concentrations found in MFG from LMFG cows. However, Nakatani et al. (2013) showed that in normal mice, bigger MFG showed a higher staining intensity for lactadherin than smaller globules. They further showed that MFG of similar size from mice in peak lactation had lower staining intensities for lactadherin compared to mice 3 h after forced weaning, suggesting that MFG are coated with more lactadherin during involution, possibly signalling the cells to take up already secreted MFG.

Combined or individual roles for BTN, PLIN2 and XOR in the MFG secretion process have been suggested (Ogg et al., 2004; Robenek et al., 2006a; Chong et al., 2011), although their precise involvement remains unknown. It is possible that higher concentrations of both BTN and PLIN2 could impact the speed of the secretion process, limiting the time for LD growth in SMFG cows. For example, Chong et al. (2011) suggested a major role for PLIN2 in MFG secretion by initiating the secretion process through binding to the apical plasma membrane and subsequent recruitment of BTN and XOR to conclude the budding process. While our results somewhat align with this theory, with higher concentrations of BTN and PLIN2 measured in the MFGM of the SMFG group, overall the differences were not statistically significant and therefore inconclusive. We also did not find increased concentrations of XOR in the MFGM of the SMFG group. Moreover, Robenek et al. (2006a) further showed that BTN can be found in an even distribution between the monolayer of the LD and the opposing bilayer of the plasma membrane, as network forming ridges. These results indicate that BTN alone may be

responsible for LD secretion and that PLIN2, while being involved in LD biogenesis (Robenek et al., 2006b), may not be involved in the final secretion step within the MEC (Robenek et al., 2006a). In line with this, it has been suggested that PLIN2 can act as a FA binding protein and therefore play a role in carrier mediated transport of long-chain FAs, including SFAs, MUFAs and PUFAs, through the plasma membrane of adipocytes (Gao and Serrero, 1999; Serrero et al., 2000). Moreover, in goat MECs, oleic acid supplementation leads to increased colocalisation of PLIN2 with the LD envelope and thus increases the total TAG content, possibly because it can protect TAGs from hydrolysis by adipose triglyceride lipase (Shi et al., 2015). We hypothesise that, in combination with higher SCD activities this could possibly explain the greater relative abundance of UFAs in the MFG core of the SMFG group. However, studies on the role of PLIN2 in LD accumulation in the mammary glands of mice have shown that a drastic decrease in PLIN2 levels in PLIN2 deficient mice prevents the maturation of LD into larger structures, due to the loss of its protective effect against hydrolysis by adipose triglyceride lipase (Russell et al., 2007, 2011). It is important to note that we do not dispute this crucial role for PLIN2 in LD maturation. Here we suggest, that within its physiological range PLIN 2 could further act as a FA binding protein, as shown in adipocytes, and therefore support the incorporation of FA from the bloodstream and at the same time protect LD from hydrolysis by adipose triglyceride lipase, although the results of the current study merely provide preliminary results and will have to be confirmed through more in-depth proteomic analyses.

Across species, the main MFGM protein concentrations varied and are shown in Figure A2. In this analysis, lactadherin content were not consistent with the average MFG size across species. Mean PLIN2 concentrations appeared to be negatively related to MFG size, however the differences between the species were minor possibly due to the diffuse

nature of the bands in the PLIN2 and lactadherin region which could not be clearly separated.




**Additional Figure A2**: Concentrations of the major milk fat globule membrane proteins in camels, sheep and goats compared to SMFG and LMFG cows determined by densitometry analysis (A) of SDS-PAGE gels (B). Dot plots show individual data points, mean symbols as defined in the figure legend and 95% confidence intervals. The species are presented in ascending order of their average milk fat globule size, from smallest (camel) to the largest (LMFG cow) to show possible relationships between the proteins and MFG size. Proteins were identified by liquid chromatography tandem mass spectrometry analysis. XOR = xanthine oxidoreductase, BTN = butyrophilin, PLIN2 = perilipin 2. *identified as lactadherin in camels due to incomplete separation. Therefore, lactadherin and PLIN2 are not shown for camels. SMFG = cows naturally producing small milk fat globules, LMFG = cows naturally producing large milk fat globules.

## ADDITIONAL CONCLUSIONS

Overall, the results obtained through densitometry analysis of MFGM extracts from the selected cows were inconclusive. However, the results indicate that MFGM protein concentrations may differ between the selected cows and could impact the FA composition of the MFG core and could possibly be related to MFG size. Future studies should determine the concentrations of the major MFGM proteins using a more targeted and more sensitive technique to establish their potential roles in MFG size development.

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# CHAPTER 5

## Milk fat globule size development in the mammary epithelial cell: a potential role for ether phosphatidylethanolamine

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## 5.1 Introduction

Chapter 5 characterised the whole milk lipidome of cows producing milk with small or large volume weighted mean diameters across a pre-trial observation period and used samples collected on the same days as the milk used for the experiments in Chapter 4.

This experimental chapter comprises a manuscript submitted to Scientific Reports, as well as supplementary data that is also intended for publication.

## 5.2 Manuscript


### ABSTRACT

Milk fat globule (MFG) size is a milk production trait characteristic to the individual animal and has important effects on the functional and nutritional properties of milk. Although the regulation of MFG size in the mammary epithelial cell is not fully understood, lipid droplet (LD) fusion prior to secretion is believed to play a role. We selected cows that consistently produced milk with predominantly small or large MFGs to compare their lipidomic profiles, with focus on the polar lipid fraction. The polar lipid composition of the monolayer surrounding the LD is believed to either promote or prevent LD fusion. Using a targeted LC-MS/MS approach we studied the relative abundance of 301 detected species and found significant differences between the studied groups. Here we show that the lipidomic profile of milk from small MFG cows is characterised by higher phosphatidylcholine to phosphatidylethanolamine ratios, which is predicted to prevent LD fusion. In contrast, the milk from large MFG cows contained more ether-phosphatidylethanolamine species, which can disrupt biological membranes, reduce fluidity and thus promote LD fusion. This is the first time that a role for ether-phosphatidylethanolamine in MFG size development has been suggested.

## INTRODUCTION

Milk lipids are secreted from the mammary epithelial cell as spherical structures termed milk fat globules (MFGs). They comprise a neutral lipid core surrounded by a membrane derived partially from the endoplasmic reticulum (ER, innermost layer) and the apical plasma membrane (two outer layers) (Heid and Keenan, 2005). The MFG membrane (MFGM) keeps lipids solubilised in milk, but also has several health benefits for the infant and for adult consumers. For example, beneficial effects on cancer, hypercholesterolemia, diabetes and cognitive function have been attributed to the protein, glycerophospholipid (PL) and sphingolipid (SL) components of the MFGM in bovine milk (Spitsberg, 2005). The amount of MFGM material in milk depends on the fat content and MFG size, with smaller globules exhibiting a greater surface area and therefore more surrounding membrane material per unit of fat. Moreover, the processing of milk into butter and cheese is influenced by MFG size. Because larger MFGs are more prone to coalescence, the efficiency of butter manufacturing could be increased by using milk with larger MFGs. Accordingly, cheese manufacturing could benefit from the use of milk with small MFG size distributions. For example, it has been shown that Cheddar cheese made from milk with small MFGs exhibits a reduced springiness and chewiness (Logan et al., 2017) and improved sensory properties of Emmental cheese were reported when it was manufactured from small MFG milk (Michalski et al., 2007). Therefore, the textural properties of cheeses made from milk with different MFG size distributions could be tailored to the desired attributes of the final product.

Variations in MFG size exist between breeds and species of mammals (Carroll et al., 2006; El-Zeini, 2006), but also between animals of the same breed of cows (Logan et al., 2014). Moreover, the average MFG size changes throughout the lactation cycle depending on the days in milk since the last calving and throughout the year in response

to changes in the diet (Fleming et al., 2017). Other suggested impact factors are fat content (Wiking et al., 2004), fat-to-protein ratio (Couvreur and Hurtaud, 2017) and milking period (Fleming et al., 2017). However, these effects are limited compared to the overall variation that is seen between individual animals. The size of MFGs in cow's milk ranges from less than 1 µm to 15 µm (Michalski et al., 2001), with the average size ranging between 2.5 and 5.7 µm within a herd of Holstein-Friesian cows (Logan et al., 2014).

The ultimate size of individual MFGs is determined by two growth mechanisms inside the mammary epithelial cell: either via local triacylglycerol (TG) synthesis by enzymes residing in the monolayer surrounding the intracellular lipid droplet (LD) or by LD fusion (Masedunskas et al., 2017; Wilfling et al., 2013). Membrane fusion events are important for many cellular processes and are believed to involve the action of fusion proteins (Boström et al., 2007) but also depend on the lipid components of the monolayer (Cohen et al., 2017). While most membrane fusion events involve membrane compartments with a typical PL bilayer, LDs only comprise a single layer of PLs and membrane proteins. The MFGM, which includes both the LD monolayer and a portion of the apical plasma membrane, contains several PLs and SLs. Amongst the PLs found in the MFGM, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the major species followed by phosphatidylinositol (PI) and phosphatidylserine (PS) (Dewettinck et al., 2008). Sphingomyelin (SM) is the major SL found in milk and PC (35% of total PL), PE (30%) and SM (25%) are the most abundant species of polar lipids in milk (Dewettinck et al., 2008), although reports differ about whether PC or PE is the most abundant (Contarini and Povolo, 2013).

The exact mechanisms behind LD fusion remain elusive but may depend on the PL and fatty acid (FA) composition of the monolayer. Thiam et al. (2013) comprehensively reviewed the biophysical properties of individual PL and their impact on LD fusion and

the reader is referred to this article for more detail. Briefly, PLs with smaller headgroups, such as PE and phosphatidic acid are predicted to enhance fusion of LDs, while PC has a more cylindrical shape and can protect LDs from fusion. Although most of the available evidence in the literature is based on studies in non-mammary cells, this important role of the polar lipid composition in the LD monolayer in the regulation of LD fusion has also been demonstrated in mammary epithelial cells (Cohen et al., 2017). Moreover, not only the type of polar lipid in the monolayer surrounding the LD determines its size, but also the FA composition of the polar lipids (Arisawa et al., 2016). Apart from SM, which contains predominantly saturated FAs (SFAs), most PLs are rich in unsaturated FAs (UFAs), although PC is less unsaturated compared to PE, PI and PS (Dewettinck et al., 2008). Using a cell line engineered to produce larger LDs, it has been shown that LDs extracted from these cells contained less UFAs in the PE and PC fractions of the LD monolayer compared to control cells with smaller LDs (Arisawa et al., 2016).

Previous studies suggest that MFG size depends on the physiological state of the animal (Altenhofer et al., 2015; Fleming et al., 2017) and that the milk from cows producing small or large MFGs also differs in the FA composition (Couvreur and Hurtaud, 2017; Couvreur et al., 2007). We therefore hypothesised that there are underlying differences in the mammary lipid metabolism between cows consistently producing small or large MFGs. The current study addressed these metabolic differences by focusing on the milk lipidome of the selected cows using triple quadrupole LC-MS/MS analysis. The polar lipid fraction in milk originates predominantly from the MFGM. This MFGM material is partially derived from the ER and the apical plasma membrane and offers a snapshot of the lipid metabolic state of the mammary epithelial cell. Here, we show differences in the polar lipid species and their FA composition between cows producing small or large MFGs. Milk from cows with smaller average MFG size had

higher PC/PE ratios, while cows with larger average MFG size contained relatively more ether-PE (ePE) species. Potential reasons for the observed difference between the studied groups are discussed.

## MATERIALS AND METHODS

### *Animals and nutritional management*

A total of 12 Holstein-Friesian cows were selected from the main herd for this study. Of these, six cows consistently produced milk with small average MFG size (< 3.5 µm, expressed as the volume weighted mean diameter $D_{[4,3]}$), forming the SMFG group. The remaining six cows formed the large MFG size group (LMFG), which produced milk with an average diameter of > 4.5 µm. The cows are part of the herd at the University of Melbourne Dookie Dairy in Victoria, Australia (latitude 36°25'31.3"S, longitude 145°42'36.6"E). The herd comprises approximately 150 cows, which are milked by an automatic milking system (Lely Astronaut; Lely, Maassluis, The Netherlands) and pasture fed and kept outside all year. The cows' diet in the paddock is supplemented with hay and silage if needed and cereal grain-based concentrate is fed during milking. One of the goals of this study was to select cows that consistently produce milk with average MFG sizes on the small or large end of the spectrum observed within a herd but have otherwise similar milk production traits and are in similar stages of lactation. Therefore, cows were surveyed over one year prior to this study and the selected animals were measured between three to nine times.

### *Milk sampling and processing*

Milk from each selected cow was sampled twice on separate days approximately two weeks apart, resulting in a total of 24 samples from 12 cows. The first cohort of six cows, including three per study group, were sampled on two separate days in autumn (March

2016) and the second cohort on two days in winter (June 2016). Milk samples were collected during morning milking (0800 to 1000h). For the lipidomic analysis, whole raw milk samples (30 mL in 5 ml aliquots) were collected and frozen within 1 min from the end of milking in a liquid nitrogen dry shipper and were subsequently kept at -80 °C.

***MFG size measurements and proximate analysis***

Fat and protein concentrations were determined using a LactoScope FTIR 20 (Delta Instruments, Melbourne, Australia). MFG size was measured as previously described (Logan et al., 2014) with a Mastersizer 2000 particle analyser (Malvern Instruments, Malvern, UK). Prior to particle size analysis, milk was mixed 1:1 with 35 mM EDTA, pH 7.0 to dissociate casein micelles and the obscuration rate was kept between 12 to 15%. Refractive indices for milk fat and water were set to 1.46 and 1.33, respectively and the particle absorption coefficient was 0.001. Each sample was measured in duplicate and the volume-weighted mean diameter $D_{4,3}$ was used as the measure for the average MFG size.

***Lipidomic analysis and data curation***

From 24 milk samples, 20 µL of each sample were used for lipid extraction using a modified Folch extraction protocol (Folch et al., 1957). Briefly, 20 µL of milk samples were suspended in 400 µL ice cold chloroform:methanol (2:1, v/v) containing 10 mg/L of each internal standard. The internal standards were PC19:0/19:0, PE-d31, PG17:0/17:0 and TG-d5 19:0/12:0/19:0 (Product # 850367, 8609040, 860374, and 830456, from Avanti Polar Lipids, Alabama, USA). Samples were vortexed and then mixed at 950 rpm for 30 min at 27 ˚C with a Thermomixer C (Eppendorf South Pacific Pty Ltd, Macquarie Park, Australia). Samples were centrifuged at 16,100 × g (Beckman Coulter Microfuge® 22R Refrigerated Microcentrifuge, Beckman Coulter Australia Pty Ltd, Sydney, Australia) for 10 min and the supernatant transferred to fresh LoBind Eppendorf tubes.

Samples were completely dried in a vacuum concentrator (Christ® RVC 2-33, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). The samples were reconstituted with methanol:water-saturated butanol (100 µL, 1:9, v/v). Pooled biological quality control samples (PBQC) were prepared by pooling aliquots of the extracts from each sample and ran after every five samples.

Extracted lipids were processed and detected by Metabolomics Australia (Bio21 Institute, Melbourne, VIC, Australia) as previously described (Huynh et al., 2018, 2019) using an Agilent 1290 liquid chromatography (LC) system and Triple Quadrupole 6490 mass spectrometer (MS, Agilent Technologies Australia, Mulgrave, Australia). Briefly, the lipid material (1 µL) was separated using a Zorbax Eclipse Plus C-18 column (100 mm × 2.1 mm × 1.8 µm, Agilent Technologies Australia, 0.4 mL/min) with a mobile phase solvent system of A: water/acetonitrile/isopropanol (50:30:20, v/v/v) and B: water/acetonitrile/isopropanol (1:9:90, v/v/v), both with 10 mM ammonium formate, using a flow rate of 400 µl/min. After equilibration with 10% B, the gradient elution used was 10%-45% B (2.7 min), 53% B (0.10 min), 65% B (0.20 min), 89% B (0.10 min), 92% B (1.9 min), 100% B (1.9 min), then 10% B (1.1 min) and a total run time of 14 min. The column was preconditioned with 10% B for 30 min before a batch analysis. The lipids were detected in positive ionisation mode with dynamic scheduled multiple reaction monitoring. The MS parameters and multiple reaction monitoring transitions of each lipid class, subclass and individual lipid species have been previously described (Huynh et al., 2018, 2019). Data processing was performed using Agilent's Mass Hunter Quantitative Analysis (QQQ) software (Agilent Technologies Australia).

### *Lipid nomenclature*

Lipid species were named according to the LIPID MAPS nomenclature described in Liebisch et al. (2013). Lipid species identified on a group level were named according to their lipid class followed by the total number of carbons and double bonds (e.g. PC 34:1). Where known, the individual FA composition is denoted in brackets separated by an underscore, e.g. PC (18:0_18:1). The underscore signifies that the stereospecific position number (*sn1* or *sn2*) of the FA on the glycerol backbone is unknown. Furthermore, the O- or P- in the PC and PE lipid classes as well as the lysophospholid species designates the ether or vinyl-ether linkages in plasmanyl-and plasmenyl-species, e.g. PE (O-18:1_18:0).

### *Statistical analysis*

Peak areas of each detected compound were normalised by dividing the respective peak by the aggregate median for all detected compounds (per sample). Ratios of PC/PE and SM/ceramide (Cer) were calculated by calculating the sum of peak areas of the respective lipid class and were not median normalised. The data was transformed using the natural logarithm and the mean of duplicate measurements for each cow were used for statistical analysis. Because sampling was performed across two seasons a two-way ANOVA was used for statistical analysis including the studied groups (LMFG and SMFG cows), days in milk and season as fixed effects using the 'lm' function combined with the 'tidy' function as part of the 'broom' package (Robinson and Hayes, 2018) in R version 3.5.1 (R Core Team, 2018). The procedure by Benjamini and Hochberg (1995) was used to correct false positives for multiple comparisons and only corrected *P*-values are shown. The estimated difference between the means of the two studied groups based on data transformed using the natural logarithm, represents the fold change of SMFG cows

compared to that of the reference group (LMFG cows). If multiplied by 100, the estimated difference between the groups, calculated by the linear model, can be interpreted as a percentage (Cole, 2000). Although absolute abundance of the compounds was not measured, sums of the median-normalised peak areas of individual lipid species within a lipid class were calculated to estimate the difference in relative abundance of each lipid class. The results of this analysis were transformed using the natural logarithm and significance testing was performed using two-way ANOVA, as described for the individual lipid species, including the correction for multiple testing. Graphs were produced using the 'ggplot2' package (Wickham, 2016) in R.

## RESULTS

### *Differences in milk production traits and MFG size between the studied groups*

The average MFG size, expressed as the volume weighted mean diameter $D_{4,3}$, was 1.63 µm larger in the milk from cows in the LMFG group compared to the SMFG group. Other production traits such as milk composition, number of milkings per day and concentrate intake were similar between the studied groups (Table 1). However, there was a small difference in days in milk, representing the number of days since the last calving (25 days difference, Table 1). Furthermore, cows from the SMFG group included primiparous cows (parity 1 to 3), while the LMFG group contained only cows in later parities (parity 3 to 5). Both these parameters can affect MFG size and could be contributing factors to the difference in MFG size. However, we previously estimated the effect of these parameters on MFG size within our herd (Chapter 3). Based on this analysis we estimate that the difference between the groups attributable to the difference in days in milk and parity is in the magnitude of 0.21 µm (0.05 µm decrease due to 25 additional days in milk for SMFG cows and an increase in MFG size of 0.16 µm that was observed between parity 2 and 3). The difference between the selected groups (1.63 µm)

is therefore approximately 8 times the estimated effect of the impact of days in milk and parity. However, days in milk was added to the statistical model as a fixed effect to control for its impact on MFG size.

**Table 1:** Milk fat globule (MFG) size, milk composition and animal production data of the selected cows.

| | SMFG Cow[a] | | LMFG Cow[b] | | *P*-value[c] |
|---|---|---|---|---|---|
| | Mean | 95% CI[d] | Mean | 95% CI[d] | |
| MFG size $D_{4,3}$ (µm)[e] | 3.29 | 2.96, 3.62 | 4.92 | 4.58, 5.25 | 0.003 |
| Days in milk | 168.3 | 117.0, 219.7 | 143.3 | 92.0, 194.7 | - |
| Parity | 1.8 | 1.1, 2.6 | 3.8 | 3.1, 4.6 | 0.028 |
| Concentrate intake (kg) | 8.6 | 6.2, 11.1 | 8.4 | 5.9, 10.9 | 0.775 |
| Milkings | 2.8 | 2.4, 3.2 | 2.8 | 2.4, 3.2 | 0.854 |
| Milk yield (kg) | 27.84 | 19.41, 36.27 | 29.25 | 20.82, 37.68 | 0.854 |
| Fat content (%) | 3.40 | 2.56, 4.25 | 3.77 | 2.93, 4.62 | 0.729 |
| Fat yield (kg/d) | 0.92 | 0.76, 1.07 | 1.01 | 0.86, 1.16 | 0.729 |
| Protein content (%) | 2.96 | 2.76, 3.16 | 3.00 | 2.81, 3.20 | 0.729 |
| Fat/protein ratio | 1.15 | 0.92, 1.37 | 1.24 | 1.01, 1.47 | 0.729 |

[a]SMFG = cows naturally producing small milk fat globules.

[b]LMFG = cows naturally producing large milk fat globules.

[c]*P*-values were calculated using two-way ANOVA with group (SMFG or LMFG cows), days in milk and sampling season as fixed effects and were adjusted for multiple comparisons using the Benjamini and Hochberg (1995) procedure.

[d]CI = confidence interval

[e]$D_{4,3}$ (µm) = volume-weighted mean diameter.

***Lipidomic profile of milk from the studied groups***

Lipidomic analysis was performed on whole raw milk samples which were frozen within minutes of sample collection and thus most accurately represent the metabolic state of the animals. Using a targeted approach, we measured 445 compounds and detected 301 thereof in milk. Although previous reports have reported higher numbers of neutral lipid species (Li et al., 2017), to the best of our knowledge, this study presents the highest number of polar lipids detected in milk to date. These species were from a total of 14 lipid classes, many of which had not been previously the focus of milk lipidomics (Table 2). For example, we found 23 and 19 species of plasmenyl-PE and plasmenyl-PC, respectively. Moreover, we detected 24 Cer species and five and seven additional dihydroceramide (dhCer) and hexosylceramide (HexCer) species. Detected lysophospholipid species included 18 lysophosphatidylcholine (LPC), six lysophosphatidylethanolamine (LPE) and three lysophosphatidylinositol (LPI) species (Table 2). A complete list of the identified lipid species is provided in the supplementary file of this article. Amongst the detected compounds, 27 differed in relative abundance between the SMFG and LMFG groups, of which nine species were significantly different with $P < 0.05$ and 18 species with $P < 0.10$.

To date, about 400 lipid species have been discovered in milk, however the estimated total number is believed to be around 1000 lipid species, with many low abundance compounds yet to be discovered (Liu et al., 2018). The targeted method used in our study allowed us to identify the total carbon number (CN), number of double bonds and the FA composition of PLs which was characterised by collision induced dissociation in positive ionization mode as reported previously (Huynh et al., 2018, 2019). However, cow's milk contains small chain FAs with less than 14 carbons and TG species containing these FAs, resulting in a total CN < 48, will not be detected by this approach. This represents a

limitation of the present study in relation to the TG analysis since a large proportion of TGs have total CNs < 48:1 which was the smallest CN of TG species detected in our study (Liu et al., 2018). Therefore, the differences in the FA composition and origin of FAs between the studied groups were not the focus of the current study. The polar lipid components of milk, on the other hand, contain mostly medium and long chain FAs and the lipidomic approach described here covers the major lipid species in this group. Therefore, the focus of this study was the identification of differences in polar lipid species.

**Table 2:** Lipid classes surveyed and detected in the current study.

| Lipid class | Number of species surveyed | Number of species detected |
|---|---|---|
| PC | 33 | 25 |
| PC-O | 19 | 15 |
| PC-P | 18 | 14 |
| PE | 19 | 17 |
| PE-O | 10 | 9 |
| PE-P | 33 | 23 |
| PI | 16 | 15 |
| PS | 7 | 5 |
| PG | 4 | 3 |
| SM | 35 | 33 |
| CE | 27 | 6 |
| oxCE | 2 | 1 |
| DG | 20 | 18 |
| TG | 44 | 44 |
| TG-O | 3 | 3 |
| Cer | 42 | 24 |
| dhCer | 6 | 5 |
| HexCer | 30 | 7 |
| LPC | 37 | 14 |
| LPC-O | 10 | 3 |
| LPC-P | 4 | 1 |
| LPE | 10 | 5 |
| LPE-O | - | - |
| LPE-P | 4 | 1 |
| LPI | 4 | 3 |
| AC | 12 | 4 |
| Sulfatide | 6 | 1 |
| Ganglioside | 8 | 1 |

AC, acyl carnitine; oxCE, oxidised CE; PG: phosphatidylglycerol; TG-O, can include alkyl-diacylglycerol, dialkyl-acylglycerol or trialkylglycerol.

### *Differences in neutral lipid species*

The milk lipidomic profile contained six TG lipid species that were relatively more abundant in LMFG milk compared to SMFG milk (Figure 1D). However, detailed analysis of the lipid species revealed that the TGs with higher relative abundance in whole milk of LMFG cows mainly contained FAs with one or no double bond ($P = 0.132$, Table 3). Although, the TG classes measured in this study were limited to species with CN ≥ 48, these results align with the results reported by others (Couvreur et al., 2007). Therefore, the results of the milk lipidome in combinations with previously reported results (Couvreur and Hurtaud, 2017; Couvreur et al., 2007), indicate higher saturation in LMFG cows compared to SMFG cows, while fat yield and fat content were statistically similar between the studied groups (Table 1).

### *Differences in polar lipid species*

Comparative analysis of the polar lipid species in in raw milk from SMFG and LMFG cows revealed differences in the two major PL classes, PE and PC (Figure 1B). Milk from SMFG cows contained relatively more of two PC species (PC 36:4 and PC-P 34:0) along with a trend towards higher relative abundance of total PC compared to LMFG cows (16% increase, $P = 0.123$, Figure 2). The PC/PE ratio was also calculated to minimise the impact of intrinsic differences in PL abundance in small compared to large MFGs, due to a higher PL/TG ratio in smaller MFGs. Indeed, in the SMFG group, PC/PE ratios were higher than in LMFG cows (21%, $P = 0.001$). Milk from LMFG cows, on the other hand, differed from SMFG milk in seven PE lipid species (five species with $P < 0.05$ and two species with $P < 0.10$, Figure 1B) Some of the ePE species showed increases in relative abundance of over 45% (PE-P 34:3, PE-P 36:1, PE-P 34:1 and PE-O 34:2, all $P < 0.01$). This led to an increase in the plasmanyl-PE and plasmenyl-PE subclasses of 27% and

33% ($P = 0.104$ and $P < 0.01$, respectively). However, these differences did not lead to a change in the total relative abundance of the total PE lipid class between the groups (Figure 2). These results suggest, for the first time, a possible link between ePE and MFG size.

Ether-PLs (ePLs) contain an ether linked fatty alcohol at the *sn*-1 position, where their diacyl equivalents contain an ester linked FA (Wallner and Schmitz, 2011). Among ePLs, there are two subcategories depending on the nature of the ether bond. The so-called plasmanyl- species contain a 1-O-alkyl ether bond, while plasmenyl- species have a vinyl-ether bond (*cis* double bond at the Δ1 position adjacent to the ether bond) and are also known as plasmalogens (Nagan and Zoeller, 2001). Plasmalogens are mainly found in the form of PE species and their concentrations are reported to reach up to 18% of the total PLs in membranes and can constitute up to 70% of the total PE lipid class depending on the tissue (Nagan and Zoeller, 2001). However, information on the abundance of plasmalogens in the lactating mammary gland is lacking and can only be estimated based on their abundance in milk PLs, which are derived from the plasma membrane of the mammary epithelial cell. These results suggest an abundance of plasmenyl-PE in cow's milk of approximately 4.5% of total PLs, which is less than in camel (6.4%) and human (11%) milk (Garcia et al., 2012; Murgia et al., 2003). The suggested functions of plasmenyl-PE in biological membranes are antioxidative effects (Zoeller et al., 1999), involvement in cholesterol and membrane trafficking (Munn et al., 2003; Thai et al., 2001), signal transduction (Brites et al., 2004) and LD fusion (Glaser, 1994).

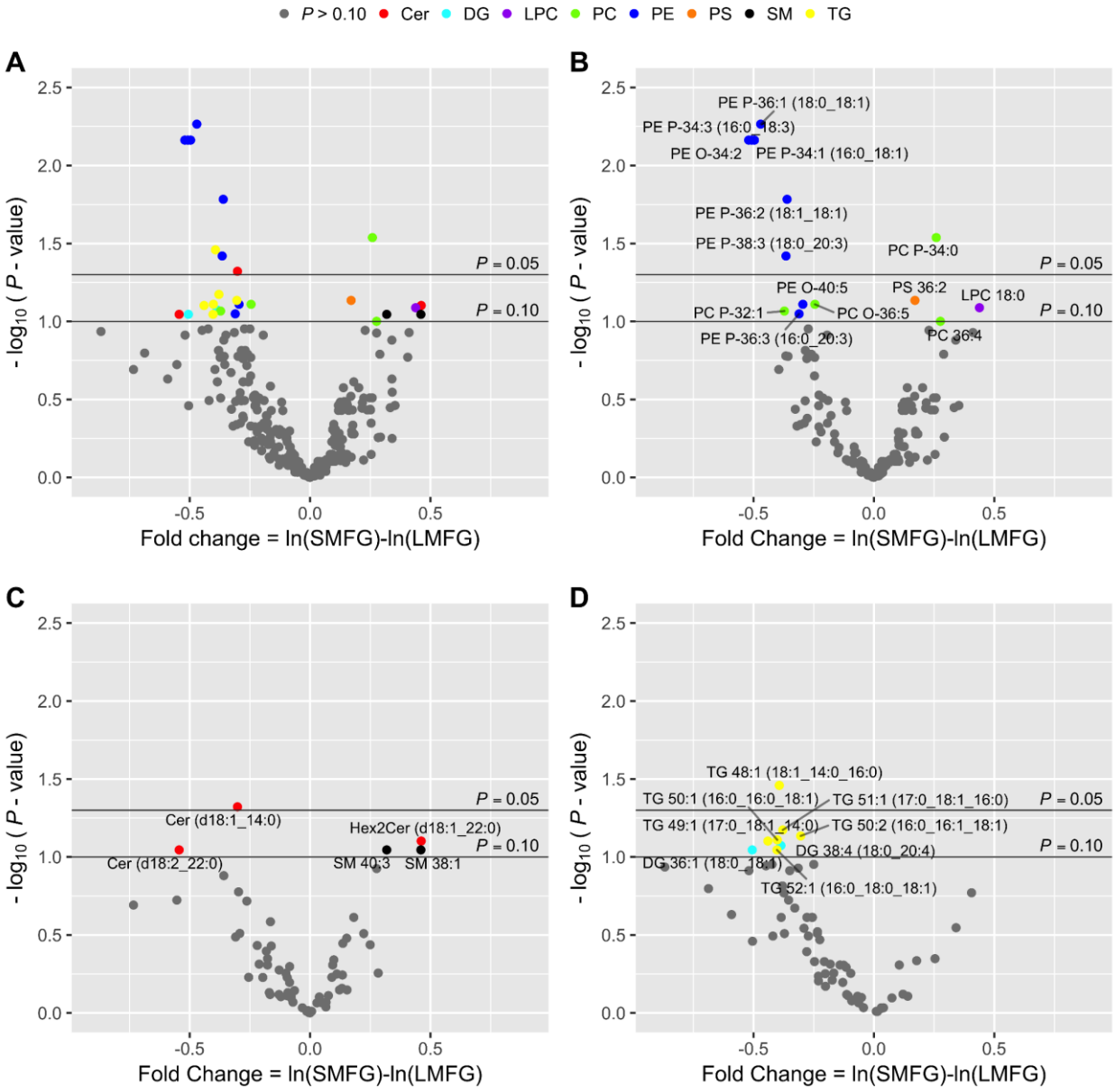


**Figure 1:** Volcano plots showing the milk lipidome of cows with small (SMFG) or large (LMFG) average milk fat globule size. The abundance of lipid species from SMFG relative to LMFG milk are shown as A) total lipid species, B) glycerophospholipid species, C) sphingolipid species and D) neutral lipid species with a total carbon number of 48 and over. The fold change, expressed as ln(SMFG)-ln(LMFG), when multiplied by 100 represents the change in %. Lipid species with positive percentages are more abundant in SMFG milk and lipid species with negative percentages are more abundant in LMFG milk. The horizontal lines denote statistical significance with lipid species above the upper line displaying adjusted *P*-values < 0.05 and above the lower line displaying adjusted *P*-values < 0.10. *P*-values were adjusted according to the Benjamini and Hochberg (1995) procedure.

Some differences were also observed within the Cer and SM classes (Figure 1C). More specifically, the milk of LMFG cows contained more Cer (d18:1_14:0, $P < 0.05$) and Cer (d18:2_22:0, $P < 0.10$) than the milk of SMFG cows, while the relative abundance of total Cer was similar between the studied groups (Figure 2). In contrast, SMFG milk contained a higher relative abundance of Hex2Cer (d18:1_22:0, $P < 0.10$) and two SM species (SM 38:1and SM 40:3, $P < 0.10$) compared to LMFG milk (Figure 1C). Again, this was not related to a difference in total SM abundance (Figure 2). However, SM/Cer ratios were higher in SMFG milk compared to the LMFG group, although this did not reach statistical significance ($P = 0.132$, Figure 2). Cer are SLs, made of a sphingoid base connected to a FA through an amide bond (Bartke and Hannun, 2009). They are also the direct precursors for SM, which is synthesised through the transfer of a choline headgroup from PC to Cer by the enzyme sphingomyelin synthase (SMS) in the Golgi or the plasma membrane, a process that is reversible through the action of sphingomyelinases (Bartke and Hannun, 2009). However, Cer are believed to exert some of their biological functions at low concentrations (Silva et al., 2006), have been linked to insulin resistance (Rico et al., 2015) and may be involved in LD biogenesis (Deevska and Nikolova-Karakashian, 2017).

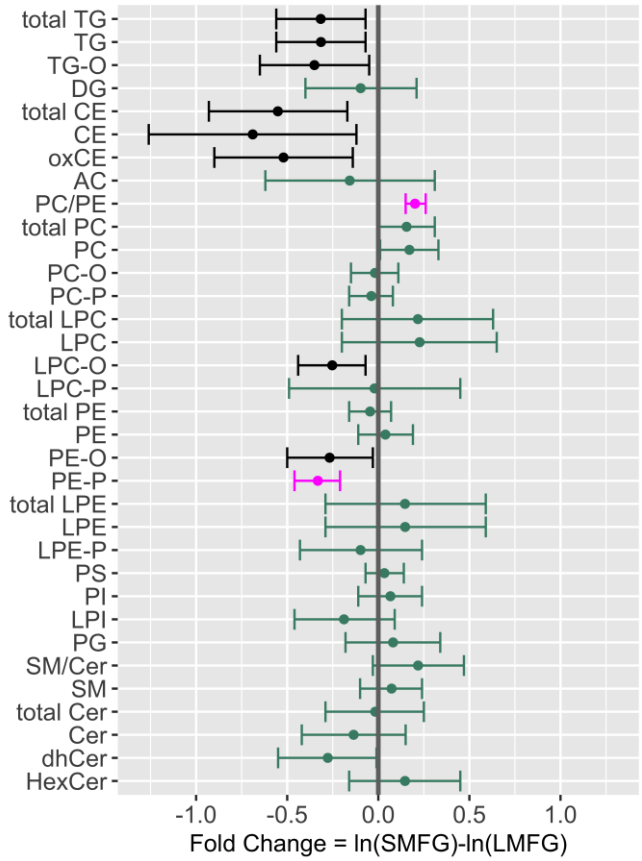


**Figure 2:** The difference in relative abundance of lipid classes and subclasses in milk from cows with small average milk fat globule size (SMFG) compared to cows with large average milk fat globule size (LMFG). Estimated mean difference and 95% confidence intervals are shown. Lipid classes shown in black ($P < 0.10$) or pink ($P < 0.05$) are different between the groups after adjusting the *P*-values according to the Benjamini and Hochberg (1995) procedure.

### *Degrees of saturation within the lipid classes*

Lipid droplet fusion not only depends on the physical properties of the main PLs in its monolayer but is also influenced by the FA composition of the acyl chains and their impact on fluidity and packing density, with UFAs predicted to increase fluidity and prevent LD fusion (Arisawa et al., 2016). Indeed, we found higher unsaturation in some of the polar lipid classes in the milk from SMFG cows compared to the milk from LMFG cows (Table 3), although the results of this analysis did not reach statistical significance after adjustment for multiple comparisons. For this analysis, the data was categorised into

three groups containing no double bonds, a total of one double bond or a total of two or more double bonds in their acyl chains. The milk from SMFG cows contained 24% more PC species and 34% more LPC species with two or more double bonds in their acyl chains (both $P = 0.132$, Table 3), while the abundance of one or no double bonds did not differ between the studied groups. Hence, the increase in relative abundance of total PC species in SMFG milk could be attributed to an increase in the subclass with two or more double bonds. Similar results were observed for the PI lipid class, which tended to contain a 15% higher relative abundance of lipid species with two or more double bonds ($P = 0.132$). Amongst the neutral lipid species, LMFG milk tended to contain higher proportions of lipid species with 2 or more double bonds within the cholesteryl ester (CE) class (57%) and higher proportions of lipid species with at no double bonds (37%) or one double bond (MUFAs, 40%) within the TG class (all $P = 0.132$, Table 3). In contrast to the polar lipids, the neutral lipid classes are found in the LD core and are therefore less likely to affect LD fusion.

**Table 3:** Relative abundance of lipid species with different degrees of saturation in the milk lipidome of cows producing milk with small (SMFG) or large (LMFG) average milk fat globule size. The data is categorised based on the total number of double bonds (DBs) in the lipid species. The relative abundance is expressed as the fold change of ln(SMFG) - ln(LMFG).

| Lipid class | SFA | | | 1 DB | | | ≥ 2 DBs | | |
|---|---|---|---|---|---|---|---|---|---|
| | Fold change[a] | 95% CI[b] | *P*-value[c] | Fold change[a] | 95% CI[b] | *P*-value[c] | Fold change[a] | 95% CI[b] | *P*-value[c] |
| PC | 0.10 | -0.09, 0.30 | 0.533 | 0.07 | -0.08, 0.22 | 0.554 | 0.24 | 0.04, 0.43 | 0.132 |
| PE | 0.17 | -0.17, 0.51 | 0.550 | -0.07 | -0.23, 0.10 | 0.585 | -0.04 | -0.16, 0.08 | 0.592 |
| PI | -0.29 | -0.71, 0.13 | 0.353 | 0.02 | -0.26, 0.30 | 0.903 | 0.15 | 0.03, 0.27 | 0.132 |
| PS | - | - | - | -0.01 | -0.17, 0.14 | 0.903 | 0.06 | -0.02, 0.15 | 0.353 |
| PG | - | - | - | -0.06 | -0.43, 0.31 | 0.823 | 0.15 | -0.10, 0.39 | 0.467 |
| SM | 0.02 | -0.16, 0.20 | 0.866 | 0.07 | -0.12, 0.25 | 0.585 | 0.13 | -0.03, 0.28 | 0.330 |
| Cer[d] | -0.27 | -0.54, 0.00 | 0.230 | 0.01 | -0.26, 0.28 | 0.946 | -0.17 | -0.51, 0.17 | 0.549 |
| LPC | 0.22 | -0.33, 0.77 | 0.585 | 0.18 | -0.07, 0.44 | 0.353 | 0.34 | 0.10, 0.58 | 0.132 |
| LPE | 0.03 | -0.35, 0.40 | 0.903 | 0.11 | -0.38, 0.61 | 0.727 | 0.30 | -0.43, 1.02 | 0.585 |
| LPI | -0.37 | -0.68, -0.06 | 0.132 | -0.17 | -0.53, 0.19 | 0.554 | 0.07 | -0.19, 0.33 | 0.668 |
| AC | -0.16 | -0.62, 0.31 | 0.592 | - | - | - | - | - | - |
| CE | -0.36 | -0.83, 0.11 | 0.353 | -0.47 | -1.15, 0.21 | 0.353 | -0.57 | -0.95, -0.19 | 0.132 |
| DG | -0.14 | -0.50, 0.23 | 0.585 | -0.25 | -0.55, 0.05 | 0.330 | 0.11 | -0.22, 0.44 | 0.592 |
| TG | -0.37 | -0.67, -0.07 | 0.132 | -0.40 | -0.63, 0.16 | 0.132 | -0.22 | -0.48, 0.04 | 0.330 |

[a]If multiplied by 100 the fold change represents the percentage difference of the SMFG compared to the LMFG group.

[b]CI = confidence interval.

[c]*P*-value represents the adjusted *P*-value using the procedure by Benjamini and Hochberg (1995).

[d]Includes the double bond present in the sphingosine base.

## DISCUSSION

Comparative analysis of the lipidomic profiles of milk from the SMFG and LMFG groups showed several important differences, mainly within the PC and PE lipid classes. More specifically, the milk lipidome from LMFG cows was characterised by a higher relative abundance of plasmanyl- and plasmenyl-ethanolamine lipid subclasses, while the milk from SMFG cows showed higher PC/PE ratios and tended to have a higher relative abundance of total PC species (Figure 2). Furthermore, we found that unsaturated species with two or more double bonds within the PC class were relatively more abundant in the milk of SMFG cows compared to LMFG cows. This provides initial evidence that the abundance of ePE within the MFGM could impact MFG size development in the mammary epithelial cell. The biophysical properties of PC compared to PE result in its superior surfactant properties and increased PC/PE ratios in the monolayer surrounding the intracellular LD are predicted to limit LD fusion (Thiam et al., 2013). Moreover, ePL species and particularly plasmenyl-PE have been shown to reduce the fluidity of biological membranes and have been implicated in membrane fusion events (Dean and Lodhi, 2018; Glaser, 1994). Therefore, the milk lipidome from cows presenting small and large phenotypes provides several differences, which could explain their characteristic MFG phenotype.

### *The increased relative abundance ePE in LMFG cows suggests an involvement of peroxisomes in the regulation of MFG size in the mammary gland*

The synthesis of TG from diacylglycerol (DG) occurs in the ER or on the surface of LDs, catalysed by an enzyme called diacylglycerol acyltransferase (DGAT). The two forms of this enzyme are located in the ER (DGAT1) or in LDs (DGAT2) (Wilfling et al., 2013). The pathways for diacyl-PL and ePL synthesis differ in terms of the sites and the organelles involved in their biosynthesis, but the same enzymes are involved in both

pathways (see Figure 3). The dominant metabolic pathway for the synthesis of the major diacyl-PLs, PE and PC is through *de novo* synthesis by the transfer of a cytidine diphosphate (CDP)-choline or CDP-ethanolamine headgroup to DG (Kennedy pathway) (Vance, 2015). In contrast to TG synthesis, where the final step can take place on the surface of the LD, the transfer of the choline or ethanolamine headgroup to DG to form PE and PC takes place in the ER and is catalysed by the enzyme diacylglycerol choline / ethanolamine-phosphotransferase (Fagone and Jackowski, 2009). In ePL synthesis, this enzyme also catalyses the transferal of ethanolamine and choline to alkylacylglycerol, the ether linked equivalent of DG, leading to the formation of plasmanyl-PC and plasmanyl-PE (Nagan and Zoeller, 2001). Plasmanyl-PE is subsequently desaturated to form plasmenyl-PE, while plasmenyl-PC is produced by head group exchange with plasmenyl-PE and not through desaturation of plasmanyl-PC (Braverman and Moser, 2012). Besides these similarities, ePL synthesis requires another organelle, the peroxisome, where the first two steps occur (Nagan and Zoeller, 2001). Peroxisomes are organelles involved in the protection of cells from reactive oxygen species and can perform important functions in the lipid metabolism, such as β-oxidation of very long chain FAs and α-oxidation of branched FAs (Lodhi and Semenkovich, 2014). Close associations between peroxisomes and LDs have been reported and are believed to involve the flux of very long chain FAs and branched FAs from the core of LDs to peroxisomes for oxidation (Binns et al., 2006). Furthermore, very long chain FAs, shortened through peroxisomal oxidation, are subsequently used for ePL synthesis, which directly implicates peroxisomes in the biosynthesis of PLs (Hayashi and Oohashi, 1995). This suggests, for the first time, a possible involvement of peroxisomes in the MFG size development in the mammary epithelial cell.

***Ether PL synthesis as a possible salvage pathway for polar lipid synthesis when DG is preferentially channelled towards TG synthesis instead of diacyl PL synthesis***

Differences in sites of synthesis, however, do not explain why in our study PC synthesis appeared more active in SMFG cows, indicated by higher total PC concentrations than in LMFG cows, while LMFG cows had higher ePE synthesis activity. Since the same enzymes are responsible for the synthesis of ether lipid species and their diacyl equivalents, the final concentration may depend on the relative abundance of their metabolic precursors. Indeed, alkylacylglycerol is predicted to stimulate ePL synthesis, while DG stimulates the synthesis of PE and PC (Honsho et al., 2010). Therefore, increased usage of DG for TG synthesis could lead to reduced DG availability for PC and PE synthesis. Accordingly, MFG size has been related to the DGAT1 K232A polymorphism, with the KK genotype showing lower PL/TG ratios (larger MFG) compared to the AA genotype (Argov-Argaman et al., 2013). This is attributed to a higher activity of the DGAT1 K variant resulting in increased fat content (Grisart et al., 2002) as well as a changed FA composition (Schennink et al., 2007). The results from these studies align with the phenotypical differences seen between the SMFG and LMFG cows in our previous study, where we found less UFAs, but more C16 FAs in the MFG core of LMFG cows (Chapter 4). Accordingly, the K genetic variant is related to an increased content of C16:0 as well as SFAs and reduced contents of unsaturated C18 FAs and C14:0 (Schennink et al., 2007). However, in our study the average fat yield and fat content were similar between the studied groups, which is in contrast to the increased fat content which is usually characteristic for the K variant (Grisart et al., 2004) along with larger MFG size (Argov-Argaman et al., 2013).

In the context of our findings, we speculate that the activity of metabolic pathways that utilise or produce DG could regulate MFG size development in the mammary gland. More

specifically, we suggest that the variation in MFG size between the studied groups could be determined by the metabolic activity of the pathways for TG, PL and possibly SL synthesis. The possible relationship between these pathways and MFG size is illustrated in Figure 3. A common precursor in PL and TG synthesis and a product in SL synthesis is DG. Because PLs are mainly found on the surface of the LD and TGs make up most of the LD core, DG represents an important cross road between PL and TG synthesis and likely impacts the LD core (mainly TGs) to surface (partially PLs) ratio, a characteristic parameter for MFG size. Increased activities of either of the enzymes catalysing TG or PL synthesis from DG, directly affect the availability of DG for the other pathway and therefore determine PL/TG ratios and likely affect LD size. For example, reduced DGAT1 activity in SMFG cows could lead to the accumulation of DG, which promotes the channelling of ethanolamine and choline headgroups towards diacyl-PL synthesis. In the opposite scenario, increased DGAT1 activity in LMFG cows could lead to reduced availability of DG for PL synthesis because it is primarily used for TG synthesis. Consequently, the limited availability of DG as a precursor for diacyl-PL synthesis could lead to a shift towards ePL synthesis in LMFG cows. Therefore, ePL synthesis could represent a possible salvage pathway, when PL synthesis through the conventional pathways is limited, for example when DG is predominantly used for TG synthesis. The fact that plasmenyl-PE is the dominant type of plasmalogens found in most tissues and is the precursor for plasmenyl-PC synthesis explains why mainly ePE species were relatively more abundant in LMFG cows compared to SMFG cows and only two ePC species differed in relative abundance between the groups.

***The increased relative abundance of ePLs and unsaturated PC species in LMFG and SMFG cows, respectively, is predicted to impact LD fusion***

The significance of our results lies in the physical properties of ePLs, particularly plasmenyl-PE, and their potential effect on LD fusion. Plasmenyl-PE with its vinyl-ether bond has been shown to increase the speed of fusion *in vitro* in small unilamellar vesicles (Glaser, 1994). This is attributed to the propensity of plasmenyl-PE to form so called non-bilayer structures at lower temperatures than plasmanyl- and diacyl-PE. Phase transition from the liquid-crystalline to the inverted hexagonal (non-bilayer) state promotes membrane fusion (Nagan and Zoeller, 2001). It is now known that LD fusion contributes to the ultimate MFG size in mammary cells, as shown by *in vitro* (Cohen et al., 2017) and *in vivo* time-lapse imaging analyses (Masedunskas et al., 2017). The increased relative abundance of ePE in LMFG cows could also impact packing density and fluidity of the LD monolayer. Due to the missing carbonyl-oxygen at the *sn*-1 position, ePLs are more lipophilic and this physical property leads to tighter packing of ether lipids in biological membranes compared to their diacyl counterparts, decreasing membrane fluidity (Dean and Lodhi, 2018). Membrane fluidity is also predicted to impact LD fusion (Rahmatyar and Wiking, 2012), with reduced fluidity of the monolayer promoting LD fusion. We therefore suggest that the higher relative abundance of plasmenyl-PE in the milk from LMFG cows is predicted to enhance LD fusion and ultimately influence MFG size. This is the first time that a relationship between the ePE subclass and MFG size has been suggested and the biological role of ePLs in the mammary gland are unknown. However, studies focusing on the pathophysiology of ePL deficiencies have revealed various important biological functions. For example, a possible role of plasmalogens in LD homeostasis has been suggested, characterised by smaller LDs in brown adipose tissue of plasmalogen deficient mice (Brites et al., 2011).

The different physical properties of PC compared to PE, as well as some *in vitro* evidence suggest that PE enhances LD fusion, while PC has a protective effect (Cohen et al., 2017; Guo et al., 2008; Thiam et al., 2013). However, several studies on the PL composition of MFGs with different sizes have reported conflicting results (Lopez et al., 2011; Lu et al., 2016; Mesilati-Stahy et al., 2011) or found no difference in the PL composition between small and large MFGs (Fauquant et al., 2007). These studies separated MFGs from bulk raw milk or individual raw milk samples through gravity separation, microfiltration and centrifugation. We used samples from cows naturally different in MFG size distributions, with the aim to study the lipid metabolic differences between the small and large phenotype. Our results support the notion that increased relative PC abundance and more importantly the significantly increased PC/PE ratio are related to smaller MFG size. In the SMFG group, the milk lipidomic profile showed a trend towards increased relative abundance of total PC and an increase of PC species with two or more double bonds (Table 3). We speculate, that this may be the result of PC synthesis through an alternative pathway to the CDP-choline pathway, namely the phosphatidylethanolamine *N*-methyltransferase (PEMT) pathway (see Figure 3). While the CDP-choline pathway is responsible for the bulk of PC synthesis (about 70%), the PEMT pathway can make up the remaining proportion (McManaman, 2014). Notably, during this process PE is converted into PC, resulting in the production of PC at the expense of PE. The PE used during this reaction is predominantly derived from decarboxylation of PS as opposed to PE derived from the CDP-ethanolamine pathway (Hörl et al., 2011). Moreover, the PC species produced by the conversion of PE into PC contain more long chain and polyunsaturated FAs (PUFAs) compared to the PC species derived through the CDP-choline pathway (DeLong et al., 1999). Although not statistically significant, we found that the elevated PC abundance in SMFG compared to

LMFG milk was partly due to a trend towards higher relative abundance of PC species with two or more double bonds ($P$ = 0.132, Table 3). Interestingly, it has been reported that the concentration of PC derived through the PEMT pathway is higher in the LD monolayer compared to other cellular membrane components (Hörl et al., 2011). Furthermore, the PEMT enzyme has been localised on the surface of LDs or in close proximity to LDs, at sites where they are in close contact with the ER and mitochondria-associated membranes (Hörl et al., 2011). Although, the contribution of this pathway to the total PC content in mammary tissue is unknown, *in vitro* studies on primary bovine mammary epithelial cells have shown that manipulation of the PEMT pathway can affect intracellular LD size (Cohen et al., 2017). Whatever the reason for the increased PC content in milk from SMFG cows, the effect of increased PC/PE ratios, and possibly PC species with higher unsaturation, reduce the propensity of LDs to participate in LD fusion could impact MFG size.

An alternative interpretation of our results is that LD size is determined at a stage during its biogenesis, where LDs are still embedded in the ER. More specifically, it has been reported that the PL composition of the ER membrane determines whether LDs remain embedded in the ER during neutral lipid accumulation or whether budding from the ER occurs (Ben M'barek et al., 2017). This was attributed to the effect of specific PLs on the surface tension of the ER bilayer. For example, PC lowered the surface tension, promoting the release of the LD from the ER, while PE increased the surface tension in the bilayer and kept the LD embedded in the ER (Ben M'barek et al., 2017). The authors further showed that the lipid composition of the LD core affects budding, with the inclusion of 25% of sterol esters into the core of TG-containing LDs preventing budding, even in the presence of PC. However, the presence of either PUFAs or saturated PLs were able to promote the release of LDs containing sterol esters from the ER, further

emphasising the impact of the FA composition of PLs as well as the head group for LD size development (Ben M'barek et al., 2017). Although this has yet to be shown in mammary cells which actively secrete LD, these results present the possibility that large MFGs could not only be the result of LD fusion (Cohen et al., 2017) but could also be the result of differential PL composition in the ER, leading to extended LD growth while attached to the ER. Extended LD growth in the embedded state could explain why milk from LMFG cows contained higher concentrations of ePE, which require the involvement of the ER in their final steps of biosynthesis. The final step of PC synthesis from DG also takes place in the ER, but it has been suggested that PC could alternatively be produced on the surface of or in close proximity to LDs by degradation of PE through the PEMT pathway or by acylation of its precursor LPC through the enzymes of the Lands cycle, which can localise to the surface of LDs (Moessinger et al., 2011).

***Differences in SM and Cer classes between SMFG and LMFG cows***

The lipidomic profile of the milk from the studied cows also revealed differences in some Cer and SM lipid species, which could indicate further metabolic differences between SMFG and LMFG cows. Cer are bioactive lipids with potential roles in insulin signal transduction (Rico et al., 2017). Furthermore, they can change the biophysical properties of the membranes in which they reside, and this has been demonstrated to occur even at low Cer concentrations (Pinto et al., 2011; Silva et al., 2006). Increased plasma concentrations of Cer lipid species have been related to insulin resistance in cows and humans (Kasumov et al., 2015; Rico et al., 2015). In cows, the relationship between Cer lipid species and insulin resistance is apparent when the animals are in negative energy balance due to nutrient restriction or early lactation (Davis et al., 2017; Rico et al., 2017). Cows that are in negative energy balance due to nutrient restriction, have been reported to exhibit increased levels of C22 and C24 Cer in the liver and plasma, along with

increased levels of circulating nonesterified FAs (NEFAs) and insulin resistance (Davis et al., 2017). Accordingly, a reduction of C14 Cer subspecies was related to improved insulin sensitivity in human plasma (Kasumov et al., 2015). Both C22 (Cer d18:2_22:0, $P < 0.10$) and C14 (Cer d18:1_14:0 $P < 0.05$) were relatively more abundant in the milk of LMFG cows in our study (Figure 1C). However, the biological role of Cer in milk are still elusive, because many lipidomic studies on milk did not detect this minor lipid class (Liu et al., 2018). More importantly, it is unknown if increased Cer concentrations in milk reflect increased plasma ceramide concentrations.

In addition to a potential role in insulin signaling, Cer and particularly the balance between SM and Cer may also be involved in LD biogenesis. Some evidence suggests that SL, PL and TG synthesis pathways are interconnected and have important effects on LD composition and size (Deevska and Nikolova-Karakashian, 2017). Within the SL synthesis pathway, SMS1 in the Golgi utilises PC and Cer to produce SM. This leads to further DG production resulting from the removal of the choline headgroup from PC and SM synthesis therefore directly contributes to the available DG pool. Moreover, increased SMS1 activity in hepatocytes has been shown to activate PC sensing mechanisms and increase *de novo* PC synthesis, channelling DGs towards PC synthesis and away from TG synthesis through the DGAT pathway (Deevska et al., 2017). The ratio of SM/Cer was also increased in SMFG compared to LMFG cows (22%, $P = 0.192$). However, this was not statistically significant and may not be the reason for the increased PC/PE ratios observed in the SMFG phenotype in our study. Nonetheless, we speculated that SL synthesis, which involves Cer as the direct precursor of SM, might be intimately connected to PL and TG synthesis, as shown by studies in hepatocytes, and could potentially impact the PL and TG pathways in a similar way in mammary cells (Figure 3).

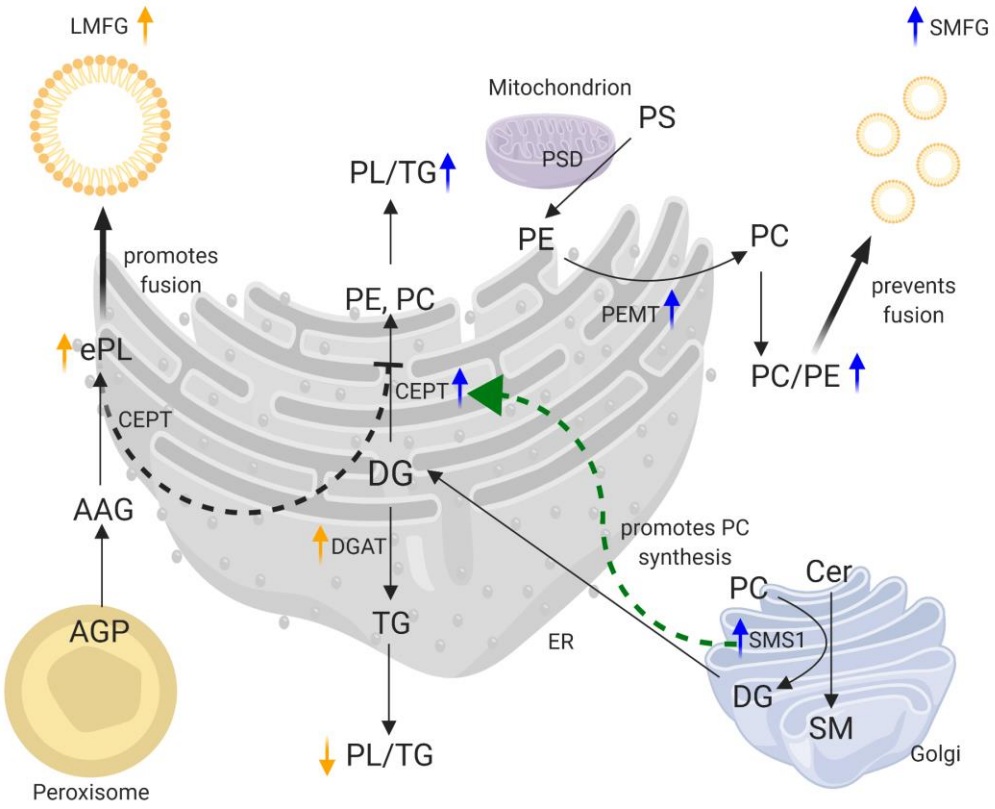


**Figure 3:** Possible mechanisms through which major metabolic pathways contribute to the milk fat globule size development in the mammary epithelial cell. Diacylglycerol (DG) plays a central role in the lipid metabolism in the mammary gland and connects phospholipid (PL), triacylglycerol (TG) and sphingolipid synthesis pathways. DG is utilised to either produce TG or the major PLs, phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylserine (PS). TG synthesis is catalysed by the enzyme diacylglycerol-acyltransferase (DGAT), in the form of two isoenzymes DGAT1 and DGAT2 which are located in the endoplasmic reticulum (ER) or on the surface of lipid droplets (LDs), respectively(Wilfling et al., 2013). The synthesis of PC and PE is catalysed by DG choline/ethanolamine phosphotransferase (CEPT) found in the ER (Fagone and Jackowski, 2009). This enzyme also catalyses the production of ether PLs (ePLs) by transferring a choline or ethanolamine headgroup to alkylacylglycerol (AAG), which is derived from peroxisomal production of 1-O-alkyl-glycerol-3-phosphate (AGP) (Nagan and Zoeller, 2001). We suggest that when DG is more efficiently channelled towards TG synthesis, for example due to increased DGAT1 activity, diacyl-PL synthesis is limited and ePL synthesis through CEPT in the ER could present a possible salvage

pathway that is not reliant on DG as a precursor. This shift towards ePL synthesis promotes LD fusion and thus contributes to the large MFG (LMFG) phenotype. In the small MFG (SMFG) phenotype, DG is channelled more efficiently towards PL synthesis, either due to decreased DGAT1 activity or possibly due to increased SM synthase 1 (SMS1) activity in the Golgi apparatus. SMS1 catalyses the transferal of the choline headgroup from PC to ceramide (Cer) to produce SM and DG, thus contributing to the DG pool. Moreover, increased SMS1 activity can stimulate PC synthesis (Deevska et al., 2017). Another potential pathway contributing to the SMFG phenotype is the phosphatidylethanolamine *N*-methyltransferase (PEMT) pathway, which converts PE into PC and thus increases the PC/PE ratio. The PE used in the PEMT pathway is derived mainly through decarboxylation of PS in the mitochondrion by PS decarboxylase (PSD) and results in PC with a higher degree of unsaturation. Increased unsaturation within the monolayer surrounding the LD and increased PC/PE ratios can prevent LD fusion, resulting in the SMFG phenotype. Figure created with BioRender.com.

## CONCLUSIONS

The results obtained from PL and SL analyses indicated several differences in the lipid metabolism between the studied groups and we have suggested possible explanations for the observed differences. Firstly, milk from SMFG cows contained more PC relative to PE and the PC species were more unsaturated compared to the PC species in LMFG cows. We hypothesise that, in SMFG cows, DG is channelled towards PL synthesis and this process could be directly regulated by reduced DGAT1 activity leading to increased availability of DG. We further hypothesise that the increased PC/PE ratios could be related to the synthesis of PC through the PEMT pathway, which produces PC at the expense of PE and results in PC species comprising more UFAs. However, the main finding of the current study was that the lipidomic profile of milk from the LMFG group was characterised by increased levels of plasmanyl- and plasmenyl-PE. Ether-PL synthesis in these animals could present a salvage pathway for polar lipid synthesis to provide membrane material through a pathway that does not require DG as a precursor.

We speculate that in LMFG cows DG could be channelled towards TG synthesis due to increased DGAT1 activity. Further studies should focus on the precise role of ePL in the mammary gland, their location within the mammary epithelial cell and their absolute abundance, along with the required concentration to exert their suggested biological functions.


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## 5.3 Supplementary data

### Milk fat globule size development in the mammary epithelial cell: a potential role for ether phosphatidylethanolamine

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Supplemental Table S1: List of identified lipid species and putative fatty acid composition, where known, for phospholipid classes in milk

| Phosphatidylcholine (PC) subclasses | | | Phosphatidylethanolamine (PE) subclasses | | |
|---|---|---|---|---|---|
| PC | PC-O | PC-P | PE | PE-O | PE-P[a] |
| PC 30:0 | PC O-32:0 | PC P-30:0 | PE 32:0 | PE O-34:2 | PE P-34:1 (16:0_18:1) |
| PC 31:1 | PC O-32:1 | PC P-32:0 | PE 32:1 | PE O-36:3 | PE P-34:2 (16:0_18:2) |
| PC 32:0 | PC O-32:2 | PC P-32:1 | PE 34:1 | PE O-36:4 | PE P-34:3 (16:0_18:3) |
| PC 32:1 | PC O-34:1 | PC P-34:0 | PE 34:2 | PE O-36:5 | PE P-36:3 (16:0_20:3) |
| PC 32:2 | PC O-34:2 | PC P-34:1 | PE 34:3 | PE O-38:4 | PE P- 36:4 (16:0_20:4) |
| PC 34:0 | PC O-34:4 | PC P-34:2 | PE 36:0 | PE O-38:5 | PE P-36:5 (16:0_20:5) |
| PC 34:1 | PC O-35:4 | PC P-34:3 | PE 36:1 | PE O-38:6 | PE P-38:4 (16:0_22:4) |
| PC 34:2 | PC O-36:0 | PC P-36:2 | PE 36:2 | PE O-40:5 | PE P- 38:5 (16:0_22:5) |
| PC 34:3 | PC O-36:1 | PC P-36:4 | PE 36:3 | PE O-40:7 | PE P-36:1 (18:0_18:1) |
| PC 34:4 | PC O-36:2 | PC P-36:5 | PE 36:4 | | PE P-36:2 (18:0_18:2) |
| PC 34:5 | PC O-36:3 | PC P-38:4 | PE 36:5 | | PE P-36:3 (18:0_18:3) |
| PC 36:0 | PC O-36:5 | PC P-38:5 | PE 38:3 | | PE P-38:3 (18:0_20:3) |
| PC 36:1 | PC O-38:6 | PC P-40:6 | PE 38:4 | | PE P-38:4 (18:0_20:4) |
| PC 36:2 | PC O-40:6 | PC P-40:7 | PE 38:5 | | PE P-38:5 (18:0_20:5) |
| PC 36:3 | PC O-40:7 | | PE 38:6 | | PE P-40:5 (18:0_22:5) |
| PC 36:4 | | | PE 40:4 | | PE P-36:2 (18:1_18:1) |
| PC 36:5 | | | PE 40:6 | | PE P-36:3 (18:1_18:2) |
| PC 37:6 | | | | | PE P-38:4 (18:1_20:3) |
| PC 38:2 | | | | | PE P-38:5 (18:1_20:4) |
| PC 38:3 | | | | | PE P-38:6 (18:1_20:5) |
| PC 38:5 | | | | | PE P-40:5 (18:1_22:4) |
| PC 38:6 | | | | | PE P-40:6 (18:1_22:5) |
| PC 38:7 | | | | | PE P-38:1 (20:0_18:1) |
| PC 40:5 | | | | | |
| PC 40:6 | | | | | |
| PG | PI | | PS | | |
| PG 34:1 | PI 32:0 | PI 38:2 | PS 36:1 | | |
| PG 34:2 | PI 32:1 | PI 38:3 | PS 36:2 | | |
| PG 36:2 | PI 34:0 | PI 38:4 | PS 38:3 | | |
| | PI 34:1 | PI 38:5 | PS 38:5 | | |
| | PI 36:1 | PI 38:6 | PS 40:5 | | |
| | PI 36:2 | PI 40:4 | | | |
| | PI 36:3 | PI 40:5 | | | |
| | PI 36:4 | | | | |

PC-O: plasmanyl-PC, PC-P: plasmenyl-PC (plasmalogen), PE-O: plasmanyl-PE, PE-P: plasmenyl-PE (plasmalogen), PG: phosphatidylglycerol, PI: phosphatidylinositol, PS: phosphatidylserine

[a]Shorthand notation according to Liebisch et al. (2013)

Supplemental Table S2: List of identified lipid species for lysophospholipid classes in milk

| Lysophosphatidylcholine (LPC) subclasses | | | Lysophosphatidylethanolamine (LPE) subclasses | | | LPI |
|---|---|---|---|---|---|---|
| LPC | LPC-O | LPC-P | LPE | LPE-O | LPE-P | |
| LPC 14:0 | LPC O-16:0 | LPC P-16:0 | LPE 16:0 | nd | LPE P-16:0 | LPI 18:0 |
| LPC 15:0 | LPC O-18:0 | | LPE 18:0 | | | LPI 18:1 |
| LPC 16:0 | LPC O-20:0 | | LPE 18:1 | | | LPI 18:2 |
| LPC 16:1 | | | LPE 18:2 | | | |
| LPC 18:2 | | | LPE 20:4 | | | |
| LPC 18:3 | | | | | | |
| LPC 18:0 | | | | | | |
| LPC 18:1 | | | | | | |
| LPC 20:0 | | | | | | |
| LPC 20:1 | | | | | | |
| LPC 20:2 | | | | | | |
| LPC 20:3 | | | | | | |
| LPC 20:4 | | | | | | |
| LPC 20:5 | | | | | | |

LPC-O: O-alkyl-linked LPC, LPC-P: O-alk-1-enyl-linked LPC, LPE-O: O-alkyl-linked LPE, LPE-P: O-alk-1-enyl-linked LPE, LPI: lysophosphatidylinositol

Supplemental Table S3: List of identified lipid groups and putative lipid species, where known, for sphingolipid classes in milk

| Ceramide subclasses | | | | SM |
|---|---|---|---|---|
| Cer | dhCer | HexCer | Ganglioside | |
| Cer (d16:1_16:0) | dhCer 16:0 | HexCer (d16:1_18:0) | GM3 (d18:1_16:0) | SM 30:1 |
| Cer (d16:1_22:0) | dhCer 18:0 | HexCer (d16:1_24:0) | | SM 31:1 |
| Cer (d16:1_24:0) | dhCer 22:0 | HexCer (d18:1_22:0) | | SM 32:0 |
| Cer (d16:1_24:1) | dhCer 24:0 | HexCer (d18:1_24:0) | | SM 32:1 |
| Cer (d17:1_22:0) | dhCer 24:1 | HexCer (d18:1_24:1) | | SM 32:2 |
| Cer (d17:1_24:0) | | Hex2Cer (d18:1_22:0) | | SM 33:1 |
| Cer (d17:1_24:1) | | Hex2Cer (d18:1_24:0) | | SM 34:0 |
| Cer (d18:1_14:0) | | | | SM 34:1 |
| Cer (d18:1_16:0) | | | | SM 34:2 |
| Cer (d18:1_18:0) | | | | SM 34:3 |
| Cer (d18:1_20:0) | | | | SM 35:1 |
| Cer (d18:1_22:0) | | | | SM 35:2 |
| Cer (d18:1_24:0) | | | | SM 36:2 |
| Cer (d18:1_24:1) | | | | SM 36:3 |
| Cer (d18:1_26:0) | | | | SM 37:1 |
| Cer (d18:2_16:0) | | | | SM 37:2 |
| Cer (d18:2_22:0) | | | | SM 38:1 |
| Cer (d18:2_24:0) | | | | SM 38:2 |
| Cer (d19:1_16:0) | | | | SM 38:3 |
| Cer (d19:1_18:0) | | | | SM 39:1 |
| Cer (d19:1_22:0) | | | | SM 40:0 |
| Cer (d19:1_24:0) | | | | SM 40:1 |
| Cer (d19:1_24:1) | | | | SM 40:2 |
| Cer (d20:1_24:0) | | | | SM 40:3 |
| | | | | SM 41:0 |
| | | | | SM 41:1 |
| | | | | SM 41:2 |
| | | | | SM 42:1 |
| | | | | SM 42:2 |
| | | | | SM 43:1 |
| | | | | SM 44:1 |
| | | | | SM 44:2 |
| | | | | SM 44:3 |

Cer: ceramide, dhCer: dihydroceramide, HexCer: hexosylceramide, Hex2Cer: dihexosylceramide, SM: sphingomyelin

Supplemental Table S4: List of identified lipid groups and putative lipid species, where known, for acylcarnitine (AC), cholesteryl ester (CE), diacylglycerol (DG) and triacylglycerol (TG) classes in milk

| AC | CE | DG[a] | Triacylglycerol subclasses[b] | | |
|---|---|---|---|---|---|
| | | | TG | | TG-O[2] |
| Acylcarnitine 14:0 | CE 18:2 | DG 30:0 (14:0_16:0) | TG 48:2 (14:0_16:0_18:2) | TG 50:3 (16:1_16:1_18:1) | TG O-50:1 |
| Acylcarnitine 16:0 | CE 18:3 | DG 32:0 (16:0_16:0) | TG 48:2 (14:0_16:1_18:1) | TG 52:3 (16:1_18:1_18:1) | TG O-52:0 |
| Acylcarnitine 17:0 | CE 20:0 | DG 32:1 (16:0_16:1) | TG 48:3 (14:0_16:1_18:2) | TG 52:4 (16:1_18:1_18:2) | TG O-52:2 |
| Acylcarnitine 18:0 | CE 20:4 | DG 34:1 (16:0_18:1) | TG 50:1 (14:0_18:0_18:1) | TG 49:1 (17:0_16:0_16:1) | |
| | CE 24:0 | DG 34:2 (16:1_18:1) | TG 50:4 (14:0_18:2_18:2) | TG 51:0 (17:0_16:0_18:0) | |
| | CE 24:1 | DG 34:2 (16:0_18:2) | TG 48:2 (14:1_16:0_18:1) | TG 49:1 (17:0_18:1_14:0) | |
| | | DG 36:1 (18:0_18:1) | TG 48:2 (14:1_16:1_18:0) | TG 51:1 (17:0_18:1_16:0) | |
| | oxCE 18:2 +2O NH4 | DG 36:2 (18:1_18:1) | TG 50:3 (14:1_18:0_18:2) | TG 51:2 (17:0_18:1_16:1) | |
| | | DG 36:2 (18:0_18:2) | TG 50:3 (14:1_18:1_18:1) | TG 53:2 (17:0_18:1_18:1) | |
| | | DG 36:3 (18:1_18:2) | TG 49:1 (15:0_18:1_16:0) | TG 51:2 (17:0_18:2_16:0) | |
| | | DG 36:4 (18:1_18:3) | TG 51:2 (15:0_18:1_18:1) | TG 54:0 (18:0_18:0_18:0) | |
| | | DG 36:4 (16:0_20:4) | TG 48:0 (16:0_16:0_16:0) | TG 54:1 (18:0_18:0_18:1) | |
| | | DG 38:4 (18:1_20:3) | TG 50:0 (16:0_16:0_18:0) | TG 54:2 (18:0_18:1_18:1) | |
| | | DG 38:4 (18:0_20:4) | TG 50:1 (16:0_16:0_18:1) | TG 54:4 (18:0_18:2_18:2) | |
| | | DG 38:5 (18:1_20:4) | TG 50:2 (16:0_16:0_18:2) | TG 48:1 (18:1_14:0_16:0) | |
| | | DG 38:5 (16:0_22:5) | TG 50:2 (16:0_16:1_18:1) | TG 54:3 (18:1_18:1_18:1) | |
| | | DG 38:6 (18:2_20:4) | TG 52:1 (16:0_18:0_18:1) | TG 54:4 (18:1_18:1_18:2) | |
| | | DG 38:6 (16:0_22:6) | TG 52:2 (16:0_18:1_18:1) | TG 56:6 (18:1_18:1_20:4) | |
| | | | TG 52:3 (16:0_18:1_18:2) | TG 58:8 (18:1_18:1_22:6) | |
| | | | TG 52:4 (16:0_18:2_18:2) | TG 54:5 (18:1_18:2_18:2) | |
| | | | TG 48:3 (16:1_16:1_16:1) | TG 54:6 (18:2_18:2_18:2) | |
| | | | TG 50:2 (16:1_16:1_18:0) | TG 56:8 (18:2_18:2_20:4) | |

oxCE: oxidised cholesteryl ester

[a]Shorthand notation according to Liebisch et al. (2013)

[b]TG-O: can include alkyl-diacylglycerol, dialkyl-acylglycerol or trialkylglycerol.

**Supplementary References:**

Liebisch, G., J. A. Vizcaíno, H. Köfeler, M. Trötzmüller, W. J. Griffiths, G. Schmitz, F. Spener, and M. J. O. Wakelam. 2013. Shorthand notation for lipid structures derived from mass spectrometry. *J. Lipid Res. 54*: 1523–1530.

# CHAPTER 6

## Investigation on the suitability of milk derived primary bovine mammary epithelial cells grown on permeable membrane supports as an *in vitro* model for lactation

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## 6.1 Introduction

The aim of Chapter 6 was to establish a functional cell culture model using primary bovine mammary epithelial cells extracted from milk and cultured on permeable membrane inserts. The functionality of these cells was assessed in comparison to cells extracted from bovine mammary tissue. Furthermore, culture on permeable membrane inserts was compared to conventional 2D culture and cultivation on an extracellular matrix gel, mimicking the 3D *in vivo* environment.

This chapter comprises a manuscript that has not been submitted to a journal at the time of submission of this thesis.

## 6.2 Manuscript


### ABSTRACT

This study aimed to establish an *in vitro* model for lipid synthesis in primary bovine mammary epithelial cells (pbMECs) extracted from milk and cultured on Transwell permeable supports (TW culture). The suitability of these cells as a functional model for lactation was assessed by measuring κ-casein (CSN3) and diacylglycerol acyl transferase 1 (DGAT1) gene expression, the presence of intracellular lipid droplets and the concentration of triacylglycerol (TG) in the cell lysates. The functionality of the milk derived pbMECs cultured under lactogenic conditions was evaluated by comparing the cells grown on Transwell supports to cells grown on an extracellular matrix (ECM) gel (3D culture) or a simple plastic surface (2D culture) under three different treatment media conditions. Furthermore, the functionality of milk derived cells was compared to pbMECs obtained from bovine mammary tissue. Here we show that in both tissue and milk derived pbMECs, 3D culture offered the most suitable *in vitro* environment and led to increased levels of CSN3 and DGAT1 gene expression along with increased intracellular TG content. The TW culture conditions also resulted in increased DGAT1 gene expression, but not intracellular TG levels and CSN3 gene expression, compared to the 2D conditions. However, milk derived pbMECs cultured on TW inserts showed the highest viability compared to cells grown under 2D or 3D treatments. Interestingly, this was not observed for tissue derived pbMECs, suggesting that TW culture may offer a beneficial environment specifically for milk derived cells. Furthermore, we suggest that extended treatment times under TW conditions could potentially result in increased TG concentrations as indicated by upregulated DGAT1 gene expression and that with further optimisation of the culture conditions, TW culture may present a suitable model for the study of milk lipid synthesis in pbMECs.

## INTRODUCTION

The fully developed lactating mammary gland contains two lineages of epithelial cells: myoepithelial and luminal epithelial cells, with luminal epithelial cells comprising ductal epithelial and alveolar epithelial cells (Rios et al., 2014). Ductal epithelial cells line the ducts of the mature mammary gland and alveolar epithelial cells are the sites of milk secretion during lactation (Van Keymeulen et al., 2011). Myoepithelial cells form a layer of contractile cells surrounding the luminal layer in alveoli and ducts and are vital for the ejection of milk from the milk producing alveolar luminal cells (Masedunskas et al., 2017) and for the movement of secreted milk through the ducts into the teat cistern (Sharma and Jeong, 2013).

The traditional approach to obtain mammary epithelial cells (MECs) for *in vitro* studies, is to isolate cells from mammary tissue obtained after slaughter or from mammary biopsies. One of the major drawbacks of this technique, besides ethical considerations, is the contamination of primary cultures with fibroblasts from connective tissue, as well as adipocytes from the fatty tissue surrounding the mammary gland. An alternative method to obtain MECs is to isolate primary cells from milk (Buehring, 1990; Hillreiner et al., 2017; Sorg et al., 2012). The isolation of cells from raw milk is a non-invasive method and has been applied to study *in vivo* gene expression in lactating mammary tissue (Boutinaud et al., 2015). Milk derived cells from human breast milk as well as bovine milk have also successfully been cultured and used for *in vitro* studies (Buehring, 1990; Danowski et al., 2013; Hillreiner et al., 2017; Sorg et al., 2012). It has been demonstrated that these cells are viable (Boutinaud et al., 2012) and show adequate functionality in terms of their ability to form mammospheres when cultured under 3D conditions and to secrete the major milk proteins (Hillreiner et al., 2017; Thomas et al., 2011). In tissue

culture experiments it has also been reported that MECs can secrete milk fat globules (Cohen et al., 2015).

Animal cell culture is an attractive and often more ethical alternative to *in vivo* studies, especially when using cells that are extracted from raw milk. In addition, the non-invasiveness of this method allows for the repeated sampling of the same animals without affecting the animal's welfare. Hence, although relationships observed in *in vitro* experiments do not easily translate to the *in vivo* environment, their use can be preferred for preliminary studies and cell culture is already extensively used in toxicology and pharmaceutical experiments. Furthermore, primary cell culture has been used in the agricultural sector to study the regulation of lipid droplet accumulation and secretion pathways within the alveolar MEC. For example, using tissue derived primary bovine mammary epithelial cells (pbMECs), Cohen et al. (2017) have shed light on the role of the polar lipid composition of MECs on lipid droplet size. However, the main challenge for animal cell culture is to mimic the *in vivo* environment of the cells as closely as possible. The *in vivo* environment of epithelial cells is characterised by cell-cell and cell-extracellular matrix (ECM) interactions and can be simulated by growing cells in ECM gels (Pampaloni et al., 2007). Moreover, culturing pbMECs obtained from mammary biopsies in ECM gels can induce the expression of genes involved in milk protein and lipid synthesis (Riley et al., 2010). However, this technique requires the separation of the cells from the gel matrix prior to analysis. Alternatively, porous tissue culture treated polycarbonate or polyester membrane inserts can create an environment that also resembles *in vivo* conditions. In this 3D-like technique, cells are grown on a membrane which is suspended in tissue culture wells and creates one compartment above and one below the cells. Thus, the cells are surrounded by cell culture media, with the bottom well representing the basal compartment, where substrates are taken up from the extracellular

environment. The upper well is equivalent to the apical side, where, in the *in vivo* environment, the milk is secreted into the alveolar lumen. Membrane inserts are frequently used to study the gut barrier function and the uptake of drugs or other components in the intestinal epithelial cell line Caco2 (Gao et al., 2017; Pereira et al., 2015). Other applications are the use of membrane inserts to obtain polarised and fully functional monolayers of primary retinal pigment epithelial cells (Sonoda et al., 2009). In MEC culture, permeable membrane inserts have been used with an immortalised cell line, MCF-10A, as well as murine primary MECs (Pai and Horseman, 2008; Stull et al., 2007). However, the use of these inserts to obtain differentiated, polar and functional monolayers of pbMECs extracted from milk, that can be used as an *in vitro* model for lactation physiology in ruminants, is yet to be explored.

Here we intended to establish a non-invasive technique to study lipid droplet accumulation in MECs. Furthermore, we aimed to explore the possibility to collect MECs from cow milk and culture them on permeable membrane inserts. We hypothesised that the use of membrane inserts could present a 3D-like environment which could have the advantage of obtaining polarised cultures with an apical and a basal compartment. This could be exploited to collect and analyse metabolites secreted into the apical well. However, to date it is unknown if this model is suitable for milk derived pbMECs. Therefore, we aimed to test the functionality of milk derived MECs against the use of tissue derived pbMECs and the functionality of both tissue and milk derived MECs cultured on membrane inserts against 3D culture on an ECM gel. In this experiment, the *in vitro* functionality of the three pbMEC cultures was evaluated by measuring the gene expression of κ-casein, a milk protein conserved across all mammalian species and DGAT1, the key enzyme in TG synthesis and a regulator of milk fat content and possibly MFG size (Argov-Argaman et al., 2013). Furthermore, lipid droplet accumulation upon

oleic acid supplementation to induce lipid droplet accumulation was assessed using intracellular lipid droplet staining and quantification of the total TG content in cell lysates. Two concentrations of oleic acid (150 and 300 µM) were chosen to determine the optimal concentration that is able to induce lipid droplet accumulation in the pbMECs without affecting cell viability.

## MATERIALS AND METHODS

### *Experimental Design and Cell Culture Conditions*

This experiment was set up as a 3 x 3 x 3 factorial design. Three primary cell cultures were used, two of which were isolated from raw milk (RM1 and RM2) and one from mammary tissue (TC). Cells were used for the experiment in passage 6, 5 and 4 for RM1, RM2 and TC cultures, respectively. All primary cultures were grown on three different culture matrices (2D culture, 3D culture and Transwell (TW) culture) and within each of these under three different media conditions resulting in a total of nine different treatment conditions per primary cell line (Table 1).

For 2D and 3D culture, 12-well cell culture plates were used to for TG, PCR and viability analyses (Sigma, tissue culture treated, Sigma-Aldrich). The wells used for 3D culture wells were coated with an ECM gel from Engelbreth-Holm-Swarm murine sarcoma (growth factor reduced, without phenol red, Sigma-Aldrich) according to the '3D on-top' method described in the protocol by Lee et al. (2007), using 100 µL of ECM gel per well and media supplemented with 10% ECM gel. TW cultures were performed in 12-well Transwell inserts (Transwell permeable supports, 0.4 µm polyester membrane, 12mm inserts, Costar, Corning Inc., Tewksbury, MA, USA), where the treatment medium was added to the basal well and pure DMEM/F12 to the apical well. Cells for intracellular lipid droplet and immunofluorescence staining were grown on glass coverslips (1.5

thickness, Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) in 6-well plates (Falcon, Coning Inc.). Seeding densities were 100, 000 cells per well in 12- well plates and Transwell inserts. Cells for microscopic analyses were seeded at lower seeding densities with 100,000 cells seeded per well in 6-well plates.

**Table 1:** Experimental design, including three primary bovine mammary epithelial cell cultures extracted from raw milk (RM1 and RM2) or mammary tissue (TC) cultured on three matrices and three culture media conditions.

| Media | Media composition | Matrix | | |
|---|---|---|---|---|
| | | 2D[1] | 3D[2] | TW[3] |
| Ctrl[4] | DMEM/F12<br>+ 5 µg/mL insulin<br>+ 1µg/ml hydrocortisone<br>+5 µg/mL prolactin<br>+1% antibiotic antimycotic solution<br>+0.7% fatty acid-free BSA | RM1<br>RM2<br>TC | RM1<br>RM2<br>TC | RM1<br>RM2<br>TC |
| OA150[5] | Same as Ctrl, with 0.35% fatty acid-free BSA<br>+150 µM oleic acid | RM1<br>RM2<br>TC | RM1<br>RM2<br>TC | RM1<br>RM2<br>TC |
| OA300 | Same as Ctrl<br>+300 µM oleic acid | RM1<br>RM2<br>TC | RM1<br>RM2<br>TC | RM1<br>RM2<br>TC |

[1]Two-dimensional culture on tissue culture wells.

[2]Three-dimensional culture on an extracellular matrix.

[3]Cells cultured on Transwell inserts.

[4]Control cell culture medium, prolactin from sheep pituitary, insulin, hydrocortisone and fatty acid-free bovine serum albumin (BSA) were from Sigma-Aldrich. Antibiotic-antimycotic solution was sourced form Thermo Fisher Scientific.

[5]Treatment medium, 0.35% fatty acid-free bovine serum albumin was used to maintain the oleic acid/BSA ratio consistent with the OA300 treatment medium.

Cells were initially grown to confluence on their respective treatment surface in growth medium made of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS, Bovogen Biologicals Pty Ltd, Keilor East, Australia), 5 µg/mL insulin, 1µg/ml hydrocortisone and 1% antibiotic/antimycotic solution (Anti-Anti 100X, Gibco, Thermo Fisher Scientific). Upon

confluence the media was changed to the three treatment media conditions, described in Table 1. The treatment media were changed daily, over a treatment period of 4 d. All cells were kept in an incubator at 37 ℃ and 5% $CO_2$.

***Isolation of Primary Cells from Raw Milk***

Raw milk (4 L) was obtained from the University of Melbourne Dookie Dairy in Victoria, Australia (latitude 36°25'31.3"S, longitude 145°42'36.6"E), transported to the laboratory at room temperature and was processed on the same day. Milk was defatted by centrifugation (1500 × g, 4 ˚C, 15 min) and the cell pellet was resuspended in phosphate buffered saline (PBS) (without Ca, Mg, Phenol Red; Gibco, Thermo Fisher Scientific Australia Pty Ltd, Scoresby, Australia). The collected cells were washed twice in PBS (1500 × g, 15 min, 4 ˚C), filtered through a 100 µm cell strainer and centrifuged a final time (1500 × g, 15 min, 4 ˚C). For the RM2 cells, the total cell pellet obtained after washing was directly cultured on a T25 cell culture flask. For the RM1 cell line, the cells were resuspended in 2 mL 1% bovine serum albumin (BSA) (Bovogen Biologicals Pty Ltd, Keilor East, VIC, Australia) in PBS, divided in two 2 mL tubes and purified using an immunomagnetic separation technique, as follows.

Magnetic beads (Dynabeads, CELLection Pan Mouse IgG Kit, Invitrogen, Thermo Fisher Scientific) were washed in 0.1% BSA/PBS washing buffer (25 µL Dynabeads per mL cell suspension and 1 mL of washing buffer). The bead suspension was placed in a magnet (DynaMag – 2 Magnet, Thermo Fisher Scientific), the supernatant was discarded and the beads were resuspended in the same volume as the initial volume of Dynabeads (25 µL). The cell pellet was incubated with 50 µL of mouse monoclonal anti-keratin 18 antibody (sc32329, Santa Cruz Biotechnology Inc., Santa Cruz CA, USA) per 1 mL cell suspension and incubated at 4 ˚C for 10 min on a rotary mixer (HulaMixer Sample Mixer,

Thermo Fisher Scientific). The cells were washed with 2 mL 1% BSA/PBS and centrifuged at 350 × g for 8 min to remove unbound antibodies. The supernatant was discarded and the cells were resuspended in 1 mL 1% BSA/PBS. Washed Dynabeads (25 µL per mL cell suspension) were added and incubated at 4 ˚C for 20 min on a rotary mixer. The tube containing the bread bound cells was placed in the magnet for 2 min and the supernatant was removed. The bead bound cells were washed three times by adding 1 mL 1% BSA/PBS, pipetting 2-3 times and placing in the magnet for 2 min. In the final step the cells were resuspended in 200 µL warm complete DMEM containing DMEM/F12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 with L-glutamine and phenol red; Gibco, Thermo Fisher Scientific) supplemented with 1% fetal calf serum (FCS) (New-Zealand sourced, HyClone, GE Healthcare Life Sciences, Utah, USA) and 5 mM $MgCl_2$ (Sigma-Aldrich). The bead-bound cells were detached from the beads by adding 4 µL release buffer (DNase 1) to the 200 µL cell suspension and were incubated at room temperature for 15 min in a rotary mixer tilting at 90 ˚. The tube was placed in the magnet for 2 min and the supernatant containing the cells was transferred to a T25 cell culture dish.

***Isolation of Primary Cells from Mammary Tissue***

Mammary tissue from a Murray Grey heifer was obtained from a local abattoir and transferred to the laboratory in washing buffer (2% Penicillin/Steptomycin (Sigma-Aldrich) in PBS) immediately after slaughter. The tissue was cut into smaller pieces which were washed twice in washing buffer and transferred to a sterile petri dish containing 2 mL growth media (DMEM/F12 supplemented with 10% FBS (Hyclone), 100 U/mL penicillin, 100 µg/mL streptomycin, 1 µg/mL insulin (insulin from bovine pancreas, Sigma-Aldrich) and 0.5 µg/mL hydrocortisone (Sigma-Aldrich). The washed tissue was finely diced with two surgical blades and transferred into a 50 mL conical tube

containing 30 mL digestion media (growth media supplemented with 2 mg/mL collagenase type 3 (Worthington Biochemical Corporation, Lakewood, New Jersey, USA), 0.1 mg/mL hyaluronidase (Sigma-Aldrich) and 0.04% DNase 1 (Sigma-Aldrich). Tissue digestion was performed by placing the tube in a rotary mixer at 37 ˚C for 3-4 h with intermediate dissociation of bigger tissue fragments using a 25 mL pipette. After incubation, the cell suspension was filtered through a 100 µm cell strainer and centrifuged at 350 × g for 5 min. The supernatant was discarded, and the cell pellet was treated with 0.25% trypsin/EDTA (Gibco, Thermo Fisher Scientific). The cell suspension was once again filtered through a 100 µm cell strainer and washed with growth medium before being transferred to the cell culture flask.

***Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)***

Total RNA was extracted from the cultured cells using a protocol combining TRIzol Reagent (Invitrogen, Thermo Fisher Scientific) with the RNeasy mini kit (Qiagen Pty Ltd, Chadstone, Australia). Briefly, cells from pooled duplicate wells in 2D and TW cultures were lysed directly in 1 mL, and 400 µL TRIzol, respectively. Cells grown under 3D conditions were harvested with TrypLE, washed with PBS (1500 × g, 5 min, room temperature) and the cell pellet was lysed in 600 µL TRIzol reagent. Lysed cells were stored at -80 °C. Cell lysates were thawed on ice and homogenised by vortexing for 30 s. The lysate was then transferred to Phasemaker tubes (Thermo Fisher Scientific), 0.2 volumes of chloroform were added per 1 volume of TRIzol and the tubes were mixed by inversion for 30 s. The chloroform-TRIzol mixture was incubated at room temperature for 10 min and centrifuged at 24,400 × g for 10 min at 4 °C. The upper aqueous phase was transferred to a new tube and mixed with 1 volume of 70% ethanol. This mixture was transferred onto a RNeasy spin column and the procedure was performed according to the manufacturer's instructions, omitting the initial cell lysis step. The concentrations of

total RNA and RNA integrity were determined using an Experion gel electrophoresis station and the StdSens analysis kit (Bio-Rad Laboratories Pty Ltd, Gladesville, Australia). All samples in this study had an RNA quality indicator (RQI) of 9.9 or over, except for one RM2 sample cultured under 3D culture and control media conditions, which had some phenol contamination as determined by Nanodrop (Thermo Fisher Scientific) analysis.

Reverse transcription into cDNA was performed using the iScript cDNA synthesis kit (Bio-Rad Laboratories Pty Ltd) with 1 µg of RNA for RM1 and TC cultures and 250 ng for RM2 cultures due to limited RNA concentrations obtained from the latter samples. The applied reverse transcription protocol was 5 min at 25 °C, 20 min at 46°C and 1 min at 95°C and was performed on a T100 Thermal Cycler (Bio-Rad Laboratories Pty Ltd). No reverse transcription controls were also included, and all samples were stored at - 20 °C. The cDNA produced from the RM1 and TC cultures was diluted 1 in 4 with sterile dd$H_2O$. Finally, RT-qPCR was performed using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories Pty Ltd) containing 300 nM of forward and reverse primers (Table 2) in a 20 µL reaction volume containing 2 µL cDNA template for the RM1 and TC samples, and a 10 µL reaction containing 1 µL cDNA in the RM2 samples. A CFX 96 Touch real-time PCR system ((Bio-Rad Laboratories Pty Ltd) was used and cycling conditions were as follows: polymerase activation and DNA denaturation at 95 °C for 30 s followed by 40 cycles of amplification each including 15 s denaturation at 95 °C and 30 s annealing at 60 °C. A melt curve analysis was also performed. Primers were produced by Invitrogen (Thermo-Fisher Scientific) according to published primer sequences shown in Table 2.

The data was processed for each pbMEC culture individually, where each gene of interest was run on the same plate as the reference gene, which was chosen from three

reference genes that were tested in a preliminary experiment using the RM1 and TC pbMECs and four different treatment conditions. The efficiencies of each gene of interest and the reference gene were determined by running a pooled sample standard curve for each primer pair on each plate along with the nine samples from each pbMEC culture condition for the reference gene and the gene of interest. The mean efficiency corrected cycle of quantification ($mCq_E$) from triplicate wells per sample was obtained through the CFX manager software and is calculated as follows using three replicate wells:

$mCq_E = ((Cq_E \text{ rep } 1) + (Cq_E \text{ rep } 2) + (Cq_E \text{ rep } 3))/n$

where $Cq_E = Cq^* (\log(E) / \log (2))$ and E is the efficiency for the respective primer pair.

This data was used to calculate the ΔCq as follows:

$\Delta Cq = mCq_{E(\text{reference gene})} - mCq_{E(\text{gene of interest})}$

Delta Cq values were used to calculate the relative normalised differences ΔΔCq of the sample compared to the respective control sample, which differed for the analysis of the ‘Matrix’ or ‘Media’ effects and is described under ‘Statistical Analysis’.

**Table 2:** Primer sequences, product length, accession numbers and source for the genes measured in the current study.

| Gene name | NCBI accession number[1] | Primer sequence 5' to 3'<br>Forward<br>Reverse | Product length (bp) | Reference |
|---|---|---|---|---|
| Cyclophilin | NM<br>178320.2 | GGATTTATGTGCCAGGGTGGTGA<br>CAAGATGCCAGGACCTGTATG | 120 | Boutinaud et al. (2008) |
| DGAT1[2] | NM<br>174693 | CCACTGGGACCTGAGGTGTC<br>GCATCACCACACACCAATTCA | 101 | Bionaz and Loor (2008) |
| CSN3[3] | NM<br>174294.1 | TGCAATGATGAAGAGTTTTTTCCTAG<br>GATTGGGATATATTTGGCTATTTTGT | 150 | Sigl et al. (2012) |
| KRT8[4] | NM<br>001033610.1 | GCTACATTAACAACCTCCGTC<br>TCTCATCAGTCAGCCCTTCC | 237 | Sigl et al. (2012) |

[1]NCBI: National center for biotechnology information.

[2]DGAT1: Diacylgycerol-acyltransferase 1.

[3]CSN3: κ-casein.

[4]KRT8: Keratin 8.

### *Immunofluorescence Staining*

For immunofluorescence staining the cells were grown to 70 – 80% confluence on glass coverslips in 6-well plates with the control medium. The cells were washed three times with PBS and fixed with ice-cold (-20 ˚C) methanol for 10 min. Fixed cells were again washed three times with cold PBS and blocked in a blocking solution of 1% BSA (Sigma-Aldrich) in PBS for 1 h at room temperature. This was followed by a permeabilisation step in PBS supplemented with 0.05% Triton-X-100 for 15 min at room temperature. Permeabilisation was followed by an incubation with the primary antibody (mouse monoclonal anti-cytokeratin 18 antibody RGE53 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in a 1/50 dilution in PBS overnight at 4 ˚C. After incubation with the primary antibody the cells were washed three times for 10 min in PBS to remove excess antibodies. The cells were incubated with a secondary antibody (goat anti-mouse IgG H&L Alexa Fluor 488 (Abcam, Melbourne, Australia) in a 1/200 dilution in PBS for 1 h at room temperature in the dark. After three more washing steps with PBS the cells were counterstained in a 300 nM DAPI solution (Santa Cruz Inc.) in PBS for 30 min at room temperature in the dark. The stained cells were then mounted on a glass slide with Fluoroshield mounting media (F6182, Sigma-Aldrich) and visualised with a Nikon Eclipse Ti inverted microscope (Nikon Australia Pty Ltd, Rhodes, Australia).

### *Intracellular Lipid Droplet Staining*

Staining of intracellular lipid droplets was performed on cells grown on glass coverslips under all three cell culture media conditions. The cells were fixed in a 3.6% formaldehyde solution (Sigma-Aldrich) in PBS at room temperature for 20 min. Fixed cells were washed four times with PBS and stained with Nile Red (Sigma-Aldrich) in PBS for 15 min, which was initially dissolved in acetone (1mg/mL) and then diluted in

PBS to obtain a final concentration of 200 nM. The cells were washed three times in PBS and stained with DAPI (300 nM) for 5 min before being mounted on a glass slide with Fluoroshield mounting medium (Sigma-Aldrich). Micrographs were captured using the Nikon Eclipse Ti inverted microscope.

***Growth Curve Analysis and Viability***

The growth characteristics of each primary cell line were determined by capturing duplicate images on triplicate T75 flasks daily during the cell propagation phase prior to this experiment. The cells on each image were counted using the 'multi-point' tool in imageJ (Version 1.50i, (Schneider et al., 2012)) and the $\log_{10}$ of the cell number was plotted against the hours of growth from seeding until the cells reached 80-90% confluence. The population doubling time during the exponential growth phase was calculated according to Bryant et al. (1958) as follows:

$$PDT = ((t_2 - t_1) / 3.32 \times (\log_{10}(n2)-\log_{10}(n1)),$$

where PDT = population doubling time, $t_2 - t_1$ = the time period in h between the two time points in the exponential growth phase, $n_2$ = the number of cells counted at the second time point, $n_1$ = the number of cells counted at the first time point.

Viability was determined using the Trypan Blue exclusion procedure. Briefly, 5 µL of the cells suspended in PBS for TG analysis, were mixed with 5 µL of a 0.4% Trypan Blue solution (Sigma-Aldrich) and the total and stained cells were counted using a hemocytometer and a Leica DM500 microscope (Leica Microsystems Pty Ltd, Macquarie Park, Australia).

### *Transepithelial Electrical Resistance (TEER)*

To determine the TEER, quadruplicate measurements were taken for each primary cell line under each media treatment condition on the Transwell culture inserts, prior to TG and RNA extraction after 4 d of treatment. Four blank measurements were taken using Transwell inserts filled with the control media and the mean of the blank measurements were deducted from each sample measurement to control for the resistance created by the membrane insert. Transepithelial electrical resistance was measured at room temperature using a Millicell ERS-2 Epithelial Volt-Ohm Meter fitted with an ERS STX01 probe (Merck Pty Ltd, Bayswater, Australia). The TEER was calculate by multiplying the normalised resistance (Ω) with the effective growth area of the Transwell insert (1.1 $cm^2$) to obtain the TEER in $\Omega \times cm^2$.

### *Intracellular TG Content*

Total TGs were determined using the Picoprobe Triglyceride Quantification Assay Kit (Fluorometric, Abcam). This analysis was performed on duplicate wells and was expressed as TG in µg per million cells, where cell numbers were determined for one of the duplicate wells prior to TG extraction or in µg per µg total protein. The cells from each of the three cell lines grown under the nine treatment conditions were detached using TrypLE Express (Gibco, Thermo Fisher Scientific), washed in PBS, centrifuged at 1500 × g for 5 min at 4 °C and lysed in the triglyceride lysis buffer provided with the kit. The cell lysates were snap frozen in liquid nitrogen and kept at -80 °C. Upon thawing, the cell lysates were homogenised by pipetting, incubated on ice for 10 min and centrifuged for 5 min at 24,400 × g and 4 °C. The supernatant was collected and diluted 10-fold in triglyceride buffer. The samples and standards (50 µL) were incubated with 2 µL lipase for 20 min at 37 °C. Background control wells were incubated with 2 µL of triglyceride

buffer instead. All sample, standard and background wells were mixed with 50 µL reaction mix according to the manufacturers protocol and incubated at 37 °C for 30 min. Fluorescence was measured using a microplate reader (Varioskan Lux Multimode Microplate Reader, Thermo Fisher Scientific) with an excitation and emission wavelength of 535 and 587 nm, respectively. Total protein concentrations were determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Thermo Fisher Scientific Australia Pty Ltd) according to the manufacturers protocol after 10-fold dilution of the cells lysed in triglyceride lysis buffer in double distilled water.

***Statistical Analysis***

Data obtained from TG quantification of duplicate samples was averaged and transformed using the natural logarithm before statistical analysis was performed. The data was expressed as the fold change (FC) normalised to a control sample prior to statistical analysis (i.e. ln(FC) = ln(sample)-ln(control)). Delta Cq values obtained from RT-qPCR analysis were also used to calculate the ΔΔCq values normalised to the respective controls, which differed depending on the analysis of 'Matrix' or 'Media' effects, as follows:

For the analysis of the 'Matrix' effect, the control sample was the sample cultured with the same treatment medium under 2D culture conditions. For the analysis of the 'Media' effect, the control sample was the sample cultured with the control treatment medium on the same matrix as the respective sample of interest. Statistical analysis was subsequently performed by fitting two linear models, one with 'Matrix' and 'Cell line' and one with 'Media' and 'Cell line' as fixed effects and with ΔΔCq values or ln(FC) as the response variables for PCR and TG quantification data, respectively. The linear models were fitted using the 'lm' function in R version 3.5.1 (R Core Team, 2018) in combination with the

'tidy' function as part of the 'broom' package (Robinson and Hayes, 2018). Least square means and pairwise comparisons between factor levels were calculated using the 'emmeans' package in R (Lenth et al., 2019). All data is presented as mean $\log_2$ (FC) for PCR data and mean ln(FC) for TG quantification compared to the control condition ('2D' for 'Matrix', 'ctrl' for 'Media' and 'RM1' for 'Cell line') and its 95% confidence interval. The data were presented on the logarithmic scale so that positive and negative fold changes are on a proportional scale, where a fold change of 2 is as far from the x-axis as a fold change of -2, making the visualisation of the results clearer.

Transepithelial electrical resistance measurements of cells grown on TW inserts, where analysed individually for each cell line using a linear model with 'Media' as the only fixed effect. Viability measurements were also analysed for each cell line individually, however, in this case 'Media' and 'Matrix' were both included in the linear model as fixed effects. Graphs are presented with individual values, means and 95% confidence intervals and were produced using the 'ggplot2' package in R (Wickham, 2016).

## RESULTS

### *Viability and Growth Characteristics of Milk and Tissue Derived pbMECs*

The morphology and growth characteristics differed between pbMECs extracted from raw milk or tissue and viability changed depending on the growth media and matrix conditions. Milk derived cells had a longer population doubling time compared to tissue derived cells, with 32.06 h and 31.07 h for RM1 and RM2, respectively compared to 21.19 h for the TC pbMECs. This could indicate the presence of non-epithelial cells, such as fibroblasts in the TC cells, with shorter population doubling times compared to pure epithelial cultures. However, morphological differences were also observed between the

two milk derived cultures. Notably, RM1 cells were morphologically similar to RM2 cells in sub-confluent cultures, displaying the typical epithelial cobblestone morphology (data not shown). However, RM1 cells grew more densely when they reached confluency and resembled the TC cells, whereas RM2 cells maintained the cobblestone morphology even at confluency (Figure 1, '2D'). In the 3D culture condition on an ECM gel, all three pbMEC cultures formed mammospheres (Figure 1, '3D'). When grown on TW inserts, on the other hand, both milk derived pbMEC cultures grew in monolayers, while TC cells formed domes and duct like structures (Figure 1, 'TW').

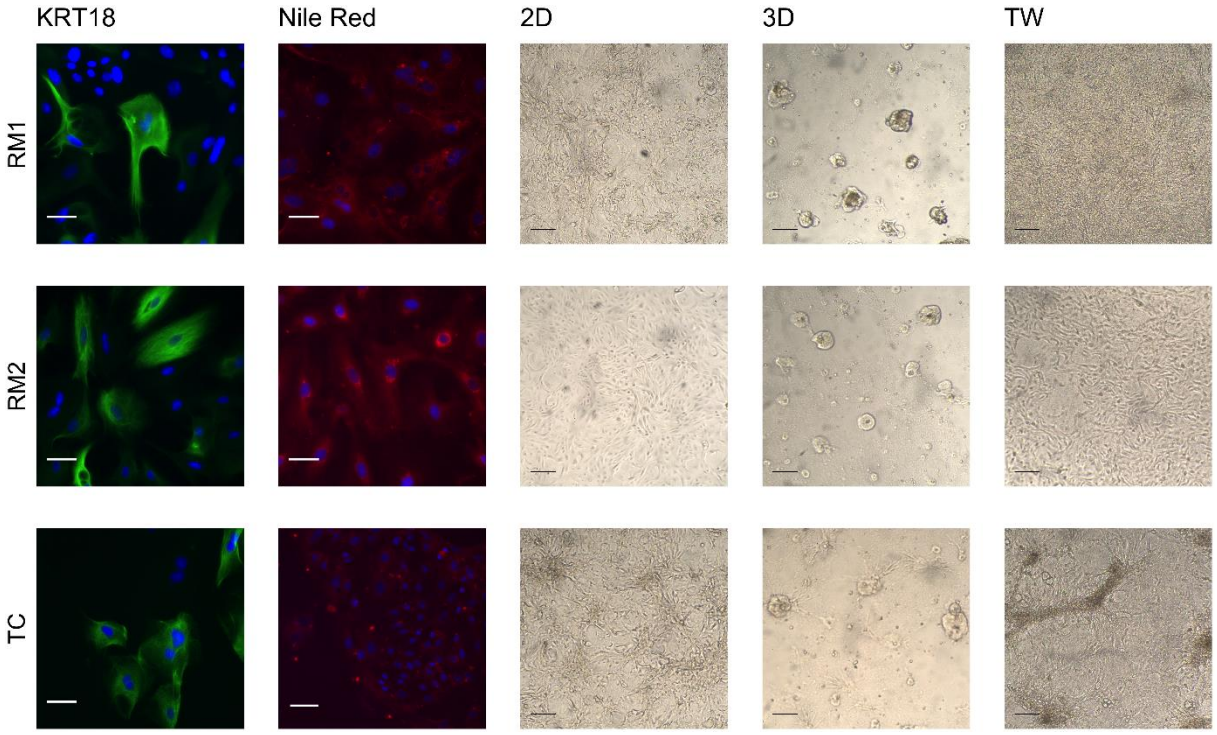


**Figure 1:** Micrographs of primary bovine mammary epithelial cells extracted from raw milk (RM1 and RM2) or mammary tissue (TC). Fluorescence images show cells stained with antibodies against Keratin 18 (KRT18) and intracellular lipid droplets stained with Nile Red. Images were taken using a Nikon Ti Eclipse inverted microscope. Light micrographs show cell morphologies on two-dimensional culture on plastic tissue culture wells (2D), three-dimensional culture on an extracellular matrix (3D) and Transwell culture on inserts with 0.4 μm pore size (TW). Scale bars represent 50 μm for fluorescent images and 200 μm for light microscopy images.

The viability of the pbMEC cultures was also differentially affected by the media and matrix conditions applied in this study. The viability of milk derived pbMECs was increased when they were cultured on TW inserts compared to the 2D cultures, with an estimated effect of an around 17% increase in viability ($P = 0.032$ and $P = 0.036$, for RM1 and RM2, respectively, Table 3). For these cells, 3D culture conditions did not increase the viability compared to 2D treatments. In the tissue derived pbMECs, no significant effect of the culture matrix on cell viability was observed and only the cells grown with OA150 medium showed reduced viability (-15.12%, $P = 0.044$, Table 3), although higher oleic acid concentrations in the OA300 medium did not negatively affect cell viability ($P = 0.298$). A reduction in viability with the highest oleic acid concentration was observed for the RM2 cells (-18.03%, $P = 0.035$), while RM1 cells were not affected by oleic acid in the cell culture medium (Table 3). The observed increase in viability in TW culture compared to 2D culture in the milk derived pbMECs could indicate that this environment more closely resembles the *in vivo* environment, where luminal MECs form a monolayer as a barrier between the alveolar lumen on the apical side and the myoepithelial cells on the basal side. A similar effect of a more *in vivo* like environment on cell viability would also have been expected under the 3D culture conditions but was not observed in the current experiment. This could possibly be related to dead cells being trapped in the ECM gel. Moreover, cells grown on 2D and TW conditions detached more easily which may have preserved their viability compared to a longer time needed for the detachment of cells from the ECM matrix.

**Table 3:** Predicted mean viability (%) of primary bovine mammary epithelial cells extracted from raw milk (RM1 and RM2) or mammary tissue (TC) cultured on three matrices and three culture media conditions. Least square means, standard error (SE), estimated effects and *P*-values are shown.

| | | | | | | Estimated effects[1] | | | | *P*-values[2] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Means (%) | | | SE | Matrix | | Media | | Matrix | | Media | |
| Cells | | 2D[3] | 3D[4] | TW[5] | | 3D | TW | OA150 | OA300 | 3D | TW | OA150 | OA300 |
| | Ctrl[6] | 76.2 | 76.5 | 93.4 | | | | | | | | | |
| RM1 | OA150[7] | 72.2 | 72.5 | 89.4 | 3.57 | 0.30 | 17.15 | -4.01 | -3.24 | 0.938 | 0.032 | 0.406 | 0.479 |
| | OA300[8] | 73.0 | 73.3 | 90.2 | | | | | | | | | |
| RM2 | Ctrl | 81.4 | 86.2 | 98.3 | | | | | | | | | |
| | OA150 | 78.1 | 82.9 | 95.0 | 4.10 | 4.80 | 16.91 | -3.34 | -18.03 | 0.406 | 0.036 | 0.493 | 0.035 |
| | OA300 | 63.4 | 68.2 | 80.3 | | | | | | | | | |
| TC | Ctrl | 90.2 | 83.9 | 83.5 | | | | | | | | | |
| | OA150 | 75.1 | 68.8 | 68.4 | 4.06 | -6.36 | -6.73 | -15.12 | -6.25 | 0.298 | 0.298 | 0.044 | 0.298 |
| | OA300 | 84.0 | 77.6 | 77.3 | | | | | | | | | |

[1]Estimated effects were calculated for each cell line individually using a linear model with 'Media' and 'Matrix' as fixed effects and represent the change in viability of the treatment compared to the respective control condition.

[2] *P*-values were adjusted for multiple testing using the procedure by Benjamini-Hochberg (1995).

[3]Two-dimensional culture on tissue culture wells.

[4]Three-dimensional culture on an extracellular matrix.

[5]Cells cultured on Transwell inserts.

[6]Control cell culture medium, supplemented with prolactin, insulin, hydrocortisone and bovine serum albumin.

[7]Treatment medium, comprising control medium supplements and 150 μM oleic acid.

[8]Treatment medium, comprising control medium supplements and 300 μM oleic acid.

***Polarity of Cells Grown on TW Inserts Assessed Using TEER Measurements.***

Transepithelial electrical resistance was reduced by the addition of 300 µM oleic acid to the cell culture medium for all three pbMEC cultures (Table 4). For the RM2 and TC cells, a substantial decrease in TEER was also observed with lower concentrations of oleic acid at 150 µM (-413.84 and -303.80 $\Omega \times cm^2$, for RM2 ($P = 0.008$) and TC ($P < 0.001$), respectively, Table 4), while RM1 cells were unaffected by the lower oleic acid concentrations. However, under control treatment conditions ('Ctrl', Table 4) all pbMECs showed TEER > 400 $\Omega \times cm^2$, suggesting polarisation of the cells. Moreover, RM1 cultures showed high TEER across all treatments. Reduced TEER in the TC cultures were likely impacted by the formation of domes and duct-like structures (Figure 1, 'TW'). The formation of domes on TW culture can result in gaps in the monolayer around the forming dome and could lead to reduced TEER. The less densely packed morphology observed in RM2 cultures can also affect their ability to form a continuous barrier and reduced TEER in these cells upon oleic acid supplementation could indicate that tight junctions may not have been closed between these cells. Figure 1 shows an example for the formation of domes in TC cultures and the gaps observed in loosely packed RM2 cultures.

**Table 4:** Transepithelial electrical resistance (TEER) of primary bovine mammary epithelial cells extracted from raw milk (RM1 and RM2) or mammary tissue (TC) cultured on Transwell inserts and three cell culture media conditions.

| | Means ($\Omega \times cm^2$) | | | | Estimated effect[1] | | *P*-values | |
|---|---|---|---|---|---|---|---|---|
| Cells | Ctrl[2] | OA150[3] | OA300[4] | SE | OA150 | OA300 | OA150 | OA300 |
| RM1 | 1695.12 | 1722.28 | 1117.76 | 253.67 | 27.16 | -577.36 | 0.917 | 0.055 |
| RM2 | 603.4 | 189.56 | 110.04 | 115.70 | -413.84 | -493.36 | 0.008 | 0.003 |
| Tissue | 414.12 | 110.32 | 76.73 | 37.59 | -303.80 | -337.40 | < 0.001 | < 0.001 |

[1]The estimated effect of different cell culture media conditions was calculated for each cell line by fitting a linear model with 'Media' as a fixed effect and represent the change in TEER of the treatment compared to the respective control condition.

[2] *P*-values were adjusted for multiple testing using the procedure by Benjamini and Hochberg (1995).

[3]Control cell culture medium, supplemented with prolactin, insulin, hydrocortisone and bovine serum albumin.

[4]Treatment medium, comprising control medium supplements and 150 µM oleic acid.

[5]Treatment medium, comprising control medium supplements and 300 µM oleic acid.

### *Functionality of Milk and Tissue Derived pbMECs*

The extracted pbMECs all expressed KRT8 (Figure 2) and stained positively for KRT18 (Figure 1, 'KRT18'), which are markers for the luminal epithelial cell lineage. The gene expression of KRT8 was not significantly influenced by the addition of oleic acid to the cell culture media. However, cells grown on 3D cultures expressed slightly lower levels of KRT8 compared to TW and 2D cultures ($P = 0.100$, Figure 2). A trend towards a reduction in keratin 8 and 18 protein expression in primary cultures of human hepatocytes grown on extracellular matrix compared to the 2D culture environment has previously been reported (Page et al., 2007). In our study, a trend towards reduced KRT8 gene expression was also observed for cells cultured with 150 µM oleic acid ($P = 0.110$, Figure 2). However, overall our results show that independently from the origin and

extraction method the obtained pbMECs were indeed MECs and suitable for the intended experiment.

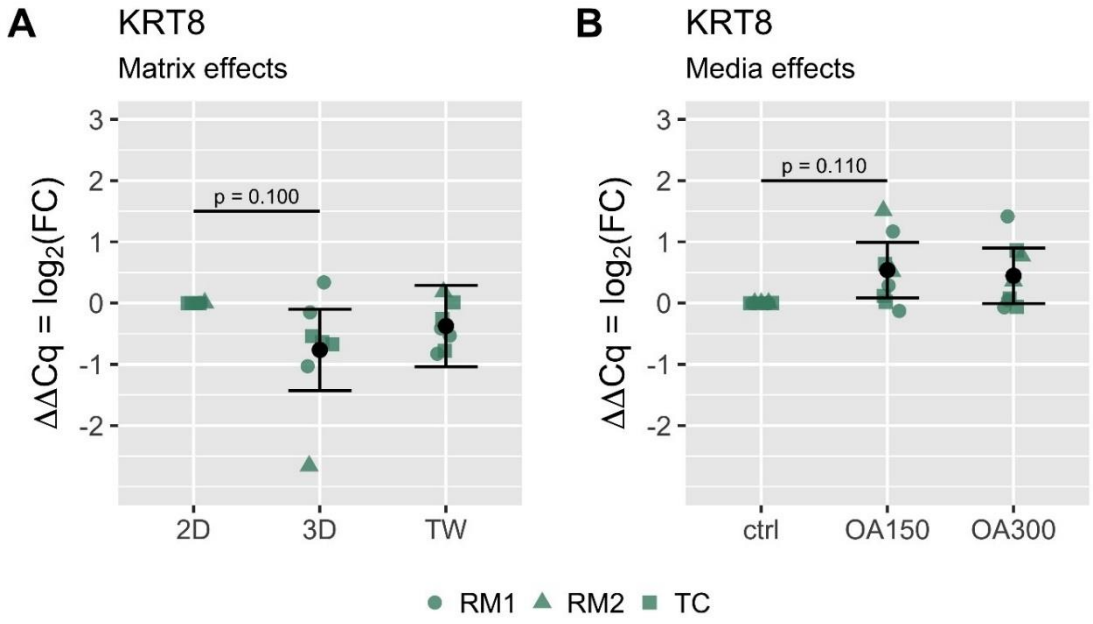


**Figure 2:** Keratin 8 (KRT8) gene expression in primary bovine mammary epithelial cells extracted from raw milk (RM1 and RM2) or mammary tissue (TC), cultured on three matrices (A) and three cell culture media conditions (B). Cells were cultured on tissue culture treated plastic wells (2D), on an extracellular matrix (3D) or on Transwell inserts (TW). The control medium (ctrl) contained lactogenic hormones (prolactin, hydrocortisone and insulin) and bovine serum albumin, whereas the treatment media contained the control medium supplemented with 150 μM (OA150) or 300 μM (OA300) oleic acid. The data is expressed as ΔΔCq values, representing the relative normalised gene expression relative to the respective control condition i.e. '2D' for 'Matrix' and 'ctrl' for 'Media'. *P*-values were adjusted for multiple testing using the procedure by Benjamini and Hochberg (1995).

All pbMEC cultures also expressed genes for κ-casein and TG synthesis (DGAT1) and contained intracellular lipid droplets. For both DGAT1 and CSN3 gene expression, a clear effect of the treatment medium was observed, with cultures treated with 300 µM oleic acid showing a significant increase in these transcripts ($P = 0.019$, Figure 3). Furthermore, cells cultured under 3D conditions expressed higher levels of both DGAT1 and CSN3 compared to the 2D controls ($P = 0.041$ and $P < 0.001$ for DGAT1 and CSN3, respectively, Figure 3). In contrast, TW culture did not lead to a significant upregulation of the CSN3 gene expression compared to 2D controls. However, DGAT1 gene expression in TW cultures was comparable to the 3D treatments and significantly upregulated compared to the 2D control treatment ($P = 0.006$, Figure 3). Intracellular Nile Red staining showed lipid droplets were present under all culture conditions (Figure 1, 'Nile Red') and this was confirmed by quantifying the TG content of the cell lysates (Figure 4). However, fluorometric quantification of the intracellular TG content only partially confirmed the suggested effects from qRT-PCR analysis. The effect of supplementing the cell culture media with oleic acid was in line with the observed upregulation of the DGAT1 gene, with an increase in TG content per total protein, when 300 µM oleic acid was supplemented ($P = 0.047$, Figure 4). However, the observed matrix effect for TGs quantified through the fluorometric technique only reflected the results obtained from DGAT1 gene expression analysis in the cells grown under 3D conditions, where the cells showed increased levels of TGs in the 3D treatment compared to the 2D control ($P < 0.001$, Figure 4C). For cells grown on TW inserts, the observed increase in DGAT1 gene expression was not reflected in the intracellular TG content (Figure 4C).

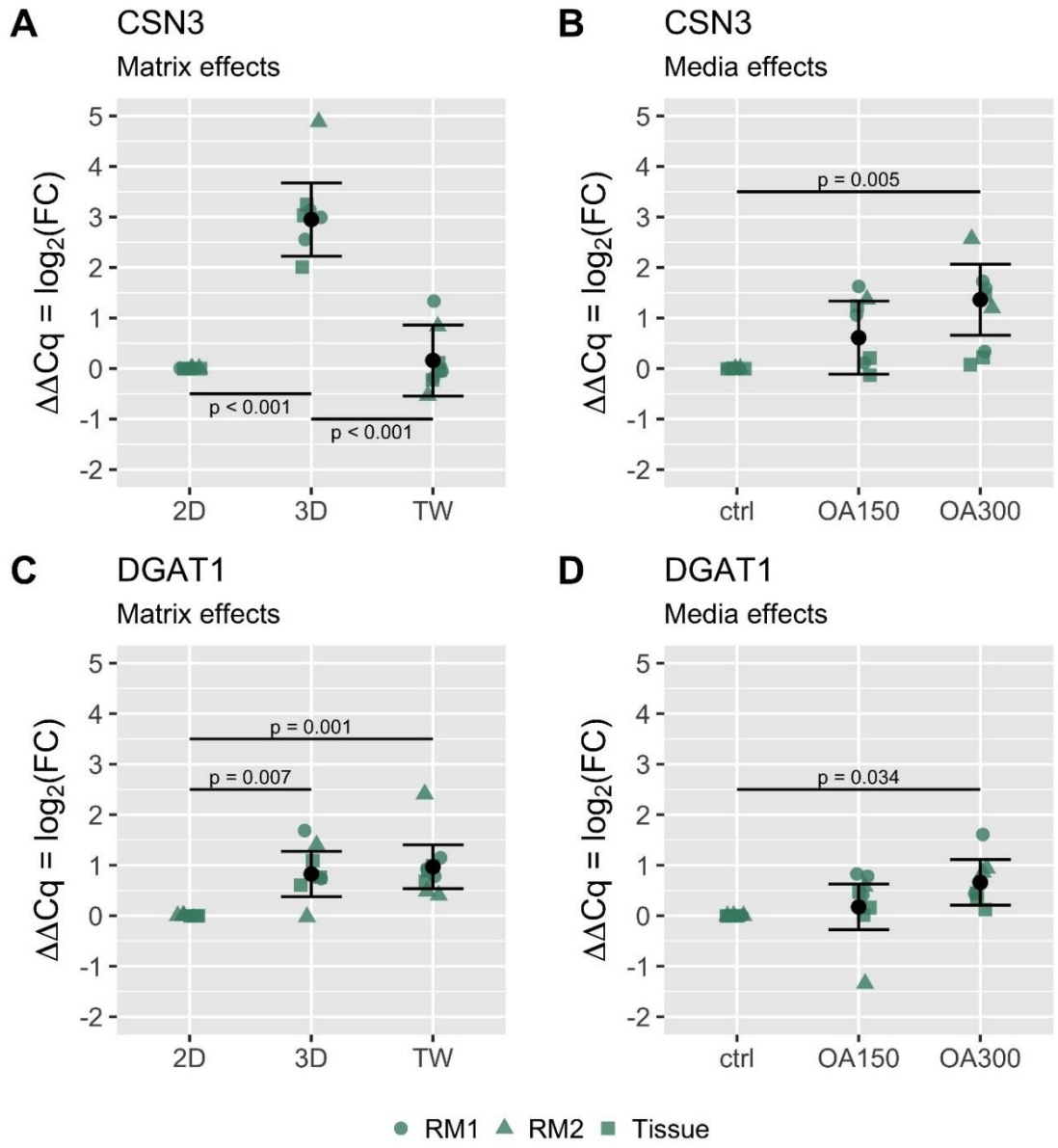


**Figure 3:** Kappa-casein (CSN3) and diacylgycerol-acyltransferase 1 (DGAT1) gene expression in primary bovine mammary epithelial cells extracted from raw milk (RM1 and RM2) or mammary tissue (TC), cultured on three matrices (A and C) and three cell culture media conditions (B and D). Cells were cultured on conventional tissue culture treated plastic wells (2D), on an extracellular matrix (3D) or on Transwell inserts (TW). The control medium (ctrl) contained lactogenic hormones (prolactin, hydrocortisone and insulin) and bovine serum albumin, whereas the treatment media contained the control medium supplemented with 150 μM (OA150) or 300 μM (OA300) oleic acid. The data is expressed as ΔΔCq values, representing the relative normalised gene expression relative to the respective control condition, i.e. '2D' for 'Matrix' and 'ctrl' for 'Media'. *P*-values were adjusted for multiple testing using the procedure by Benjamini and Hochberg (1995).

## DISCUSSION

### *Suitability of Milk Derived Cells for the In Vitro Study of Milk Lipid Synthesis*

This study confirms the suitability of milk derived cells for primary cell culture experiments, as previously demonstrated (Danowski et al., 2013; Hillreiner et al., 2017; Sorg et al., 2012) and is the first to use Transwell inserts to create a 3D-like environment for pbMECs. The extracted cells expressed κ-casein and produced TGs and lipid droplets under all applied media and matrix conditions. However, the morphology of the two milk derived pbMEC cultures, RM1 and RM2, differed in terms of their density at 100% confluence and TEER of the cells, which was higher in RM1 under all treatment conditions. The reported range of TEER in the literature varies depending on the cell type and TEER data has not been reported for pbMECs. For example, intestinal epithelial cells (Caco-2 cell line) have reported TEER values of > 1000 $\Omega \times cm^2$ (Gao et al., 2017), while kidney cells (MDCK cell line) only reach a final TEER of around 120-130 $\Omega \times cm^2$ (Butor and Davoust, 1992). In mammary cell culture, TEER plateaus at around 800 $\Omega \times cm^2$ for mouse primary MECs and > 3000 for the immortalised MCF-10A cell line (Stull et al., 2007). More importantly, Stull et al. (2007) observed a lag-phase of several days in the development of TEER in both cell lines. This could indicate that, in our study, TC and RM2 cells had not yet reached the same level of TEER as the RM1 cells after 3 d of proliferation on growth media and 4 d under treatment conditions. In fact, RM1 cells reached a TEER of > 1700 $\Omega \times cm^2$, which was almost three and four times the observed TEER in RM2 and TC cells, respectively (Table 4). Based on the variation of TEER between the three pbMECs, we suggest that the suitability of milk derived cells for *in vitro* studies could be tested based on TEER measurements. Moreover, treatment times and starting points for future experiments could be optimised based on TEER measurements.

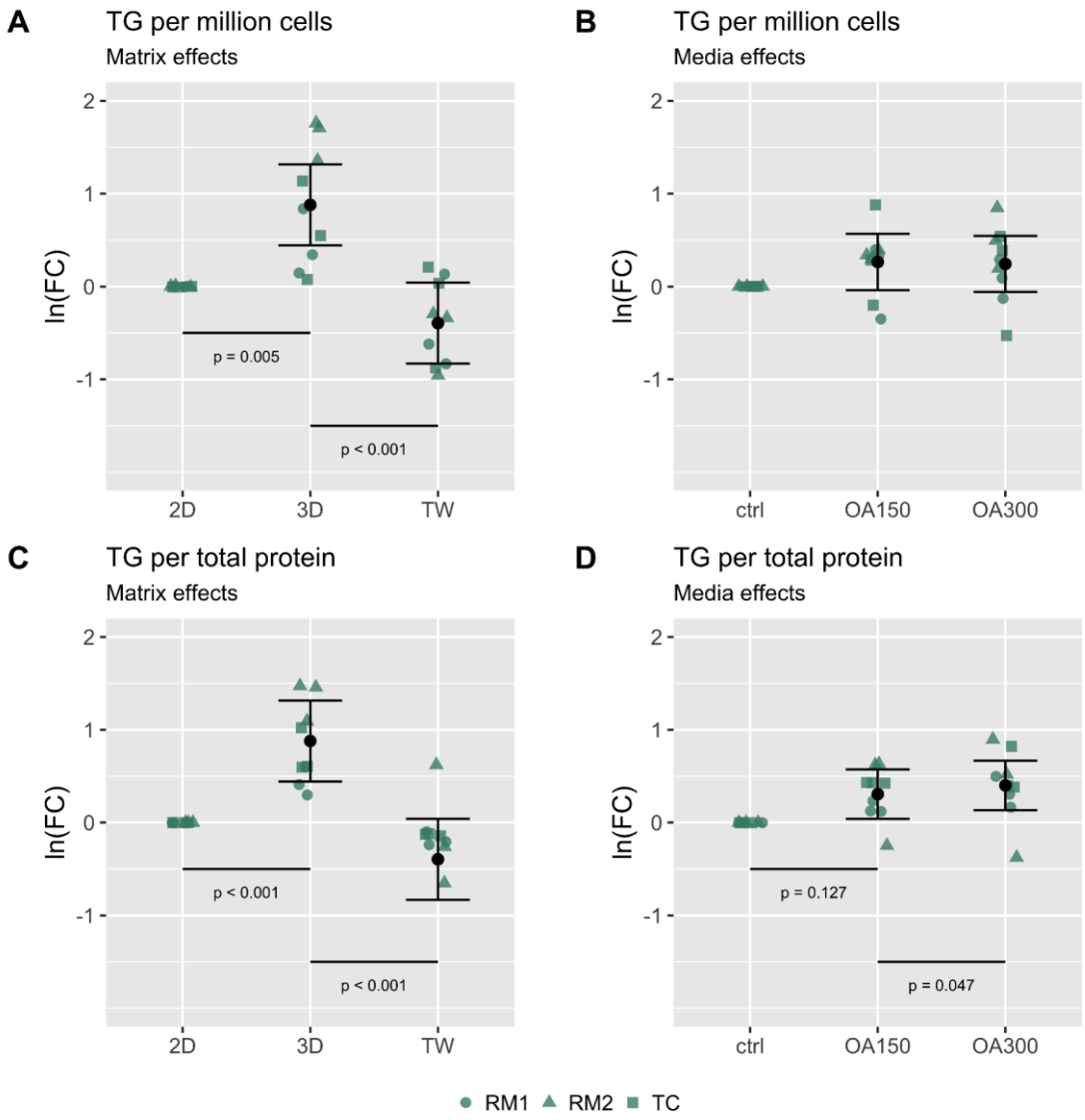


**Figure 4:** Triacylglycerol (TG) content in cell lysates from primary bovine mammary epithelial cells extracted from raw milk (RM1 and RM2) or mammary tissue (TC), cultured on three matrices (A and C) and three cell culture media conditions (B and D). The control medium (ctrl) contained lactogenic hormones (prolactin, hydrocortisone and insulin) and bovine serum albumin, whereas the treatment media contained the control medium supplemented with 150 µM (OA150) or 300 µM (OA300) oleic acid. Cells were cultured on conventional tissue culture treated plastic wells (2D), on an extracellular matrix (3D) or on Transwell inserts (TW). The TG concentration was normalised to the number of cells expressed in µg per million cells (A and B) or to the total protein content of the cell lysates expressed as µg per µg total protein (C and D). The data is expressed as the ln of the fold change compared to the respective control condition, i.e. '2D' for 'Matrix' and 'ctrl' for 'Media'. Absolute TG concentrations were between 9.7 and118.9 ug/Mio cells and 114.9 and 1200.5 ug/ug total protein. *P*-values were adjusted for multiple testing using the procedure by Benjamini and Hochberg (1995).

The cell morphology under 2D culture conditions also differed between the two milk derived pbMECs. In contrast to RM2 cells, the morphology of RM1 cells, which were extracted as purified cultures applying an immunomagnetic separation technique with antibodies against KRT18, resembled the morphology of TC cells on 2D culture (Figure 1, '2D'). Furthermore, immunofluorescence staining against KRT18 showed that some cells in RM1 cultures did not express KRT18, which was also observed in the TC cultures (Figure 1, 'KRT18'). This could indicate the presence of cells from the myoepithelial as well as the luminal epithelial lineage. This was expected in the TC cultures but was unexpected in RM1 cultures which were extracted from milk and purified prior to cell culture. Studies in mice have suggested that KRT18 expressing MECs contain stem cells that are committed towards the luminal lineage, however the authors suggested that these stem cells were rarely able to differentiate into the myoepithelial lineage (Van Keymeulen et al., 2011). However, others have suggested that multipotent stem cells, which are able to differentiate into both myoepithelial and luminal epithelial cells, exist within the mammary gland (Rios et al., 2014). The contamination of pure, KRT18 expressing cultures with only a small number of multipotent stem or progenitor cells could result in mixed cultures of both lineages. However, in the current study, we did not analyse myoepithelial markers and therefore cannot conclusively determine the purity of the milk and tissue derived cultures. Moreover, this study reports results from a single experiment with cells from 3 individual cows that were isolated using different techniques and future work is needed to confirm the reproducibility of these results.

***Suitability of TW Culture as an Alternative to 3D Culture on ECM gels***

Differences in TEER and viability on TW culture were also observed between the three primary cell cultures. Notably, milk derived cells showed higher viability on TW culture compared to 2D and 3D culture conditions. However, this was not the case for TC

cultures. This could again be related to the purity of the cultures, where cultures comprising predominantly epithelial cells could prefer the environment offered by TW inserts. In contrast to 2D cultures, where cells are forced to take up nutrients and secrete compounds on the same side, cells on permeable membrane inserts are in contact with the cell culture medium on the apical and basal side of the cell. This environment more closely resembles the *in vivo* orientation of MECs and could promote the formation of a polarised monolayer. This could present a possible advantage of TW culture for pbMECs extracted from milk.

However, with respect to their overall functionality, cells cultured under 3D conditions exhibited the highest levels of CSN3 gene expression and intracellular TG content, while TW cultures did not differ from cells grown on conventional 2D plastic surfaces in relation to these parameters (Figures 3 and 4). Notably, DGAT1 gene expression was increased in the cells cultured under TW and 3D conditions compared to the 2D treatment and showed a dose response to increased levels of oleic acid in the medium (Figure 3). The conflicting results obtained from DGAT1 gene expression analysis compared to the results obtained from intracellular TG quantification, demonstrate that increased gene expression does not always translate to increased levels of the respective metabolite. However, the fluorometric analysis of TG content is heavily reliant on appropriate normalisation and standardisation. Therefore, we applied two strategies for TG content normalisation, by expression the results in relation to total cell number or total protein and obtained similar results with both strategies (Figure 4). On the other hand, these results could indicate that TW culture had led to increased DGAT1 expression but was not yet reflected in TG levels. In combination, with the possibility that 4 d of treatment in our study had not yet led to the development of the desired level of TEER in RM2 and TC cells, longer treatment times may also be required for TG synthesis in pbMECs on

TW inserts. Moreover, some protocols suggest coating Transwell inserts with an ECM gel or other basement-membrane constituents, like fibronectin or collagen (Sonoda et al., 2009). We decided to test the functionality of the pbMEC on uncoated Transwell inserts to explore the effectiveness of the permeable membrane without the confounding effect of the ECM coating, allowing the direct comparison between 3D culture on an ECM gel and TW culture. Furthermore, uncoated Transwell inserts are successfully used with other epithelial cell types, such as intestinal epithelial cells (Gao et al., 2017). The current study demonstrates that this method could be further developed into a suitable *in vitro* system to study the biochemical pathways behind lipid droplet synthesis in pbMECs. However, at this stage, the 3D culture condition offered a more suitable environment for MEC functionality.

## CONCLUSIONS

We suggest, based on these preliminary results, that TW culture is a promising method, particularly for the study of lipid droplet accumulation. The milk derived RM1 cultures showed high TEER under all treatment media conditions and both milk derived pbMECs showed the highest viability when cultured on TW inserts, suggesting that the permeable membrane supports provided a suitable environment for pbMECs extracted from milk and can induce the formation a polarised monolayer. However, the comparative analysis with 2D and 3D cultures on an ECM gel also showed that at least in terms of CSN3 gene expression and intracellular TG content, the TW cultures were not superior to 2D cultures and were inferior to 3D cultures, which at this stage remain the gold standard for *in vitro* studies.

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# CHAPTER 7

## General Discussion

## 7.1 Introduction

Milk fat globule (MFG) size is important for the technological processing properties and the nutritional value of milk. However, the knowledge gaps associated with the underlying differences in cows producing milk with different average MFG size, prevent its use as a desirable milk production trait that could be selected for in breeding programs. The literature provides evidence for a genetic component in the determination of MFG size in dairy cows, showing moderate heritability of this trait (Fleming et al., 2017a). Furthermore, it has been suggested that the diacylglycerol acyltransferase 1 (DGAT1) K232A gene polymorphism is related to the phospholipid (PL) to triacylglycerol (TG) ratio, a proxy for MFG size (Argov-Argaman et al., 2013). The discovery of a genotype associated with the small and large MFG phenotype would considerably advance the potential of selective breeding based on this trait. In order to break through this next frontier, a detailed characterisation of the small and large MFG size phenotype is needed and farm-management and environmental factors that lead to changes in MFG size must be established to provide management recommendations.

This study addressed some of the existing knowledge gaps. The first experimental chapter dealt with the on-farm factors and estimated their effect on MFG size (Chapter 3) to test the hypothesis that the natural variation in MFG size exceeds the combined effects of environmental, physiological and milk production parameters. The two subsequent chapters (Chapter 4 and 5) provided an overview of the differences in the mammary lipid metabolism of cows presenting the small or large MFG phenotype. These chapters aimed to address the hypothesis that differences in the mammary lipid metabolism between individual cows could be responsible for the distinct small and large MFG phenotypes. Furthermore, a fourth experimental chapter (Chapter 6) was conducted to test the suitability of primary bovine mammary epithelial cell culture as an *in vitro* model which

could be employed to test some of the suggested mechanisms in future studies. This experimental chapter was designed to test the use of Transwell permeable membrane inserts to provide mammary epithelial cells extracted from milk with a 3D-like environment. Culture on Transwell inserts was hypothesised to create an environment resembling the *in vivo* conditions because it promotes growth in a monolayer, which could be more suitable for purified milk derived mammary epithelial cultures. The TW culture conditions were therefore compared to cell culture using an extracellular matrix gel (3D culture) and to 2D culture conditions on conventional tissue culture plates.

## 7.2 On-farm factors to consider when selecting cows based on MFG size

The data presented in Chapter 3 established the most important parameters that impact MFG size for the herd used in this work. The results suggest that the physiological state of the cows had the most significant effect on MFG size. A decrease in size with ongoing lactation led to a shift in MFG size of -0.60 µm over a 300-day lactation cycle. A small increase in MFG size of 0.16 µm was also observed when cows were in later parity (≥ 3) compared to animals in their first two lactations. However, cows in later parity reportedly produce more milk and tend to channel more energy towards milk production rather than replenishing fat stores (Lee and Kim, 2006), which, based on the current estimation, would lead to a concurrent decrease in MFG size, with an additional 10 L in milk produced resulting in a -0.16 µm reduction in MFG size. Another outcome from the investigation on the major environmental factors was that season did not significantly impact MFG size in the herd used for this research. This is in conflict with another study who reported seasonal changes in MFG size (Fleming et al., 2017b). The reason could be that in the herd studied in the current work, season is less confounded by stage of lactation compared to herds practicing seasonal calving and some of the reported effects of season in other herds could be related to the well-established effect of stage of lactation on MFG

size. Another confounding factor related to season is the cows' diet. Dietary changes, such as an altered concentrate to forage ratio, have been established as factors with impact on MFG size and were not the scope of the current research (Couvreur et al., 2007; Mesilati-Stahy et al., 2015). However, the composition of the diet for the studied herd was estimated on a whole herd level and dietary changes that occoured throughout the year of the initial experiment did not seem to alter MFG size. Nonetheless, potential dietary changes might be more relevant in other countries and will have to be considered when selecting and managing cows as distinct subpopulations to produce milk with small and large average MFG size.

Overall, the effects of the parameters studied in the current work were minor compared to the considerable variation in MFG size observed between individual animals (2.7 – 5.7 μm). However, these parameters should be considered when separating cows within a herd based on MFG size. It would be feasible to include the estimated effects of these parameters in a predictive model for selecting animals based on MFG size. This, in combination with the possibility of monitoring MFG size in-line by mid-infrared spectroscopy (Fleming et al., 2017a), provides the potential to combine the selection of individual animals based on MFG size with a more dynamic approach, where animals could further be selected based on their lactation stage and possibly parity. The fact that the most important impact factors for the herd used in this work were related to the physiological state of the cows, confirmed one of the primary hypotheses, which was that distinct differences in MFG size between animals are related to the individual cow's mammary lipid metabolism.

## 7.3 Lipidomic profiles of the small and large milk fat globule phenotype

The main hypothesis arising from the detailed lipidomic analysis reported in Chapters 4 and 5 of this work is that cows with the small or large MFG (SMFG or LMFG) phenotype are characterised by a differential utilisation of diacylglycerol (DG), possibly dependent on the activity of key enzymes in the TG and PL pathways or the availability of their precursors.

The results discussed in Chapter 4 suggest that DG takes a crucial role in the lipid metabolism of TGs, PLs and sphingolipids and that its utilisation for either PL or TG synthesis may regulate MFG size. It is well known that the balance between TG and PL synthesis from their common precursor DG determines the PL/TG ratio, which is frequently used as an indicator for MFG size. Previous work has also demonstrated that the reason for the channelling of DG to TG instead of PL in cows with larger MFG sizes depends on the DGAT1 genotype, which in turn determines the activity of this key enzyme in TG synthesis (Argov-Argaman et al., 2013). Cows with the DGAT1 KK genotype exhibit increased DGAT1 activity resulting in the production of milk with smaller PL/TG ratios and thus larger MFG size (Argov-Argaman et al., 2013). Although genotyping was not performed for the SMFG and LMFG cows in the present work, the fatty acid (FA) profile of the LMFG cows in Chapter 4 aligned with the FA profile reported for the DGAT1 K variant, showing reduced proportions of unsaturated FAs (UFAs) (Schennink et al., 2007). It has been reported that 53% of the variation in C18 UFAs could be explained by the DGAT1 K232A polymorphism (Schennink et al., 2007). In combination with the finding that LMFG cows exhibited a higher relative abundance of ether PE (ePE) (Chapter 5), the possibility of higher DGAT1 activity in these cows could lead to a reduced availability of DG for PL synthesis. This in turn could result in the production of ether PLs (ePLs) through a salvage pathway for PL synthesis. Increased

relative abundance of ePE is predicted to enhance LD fusion, thus resulting in the large MFG phenotype (Glaser, 1994). This suggested mechanism could also explain the larger average MFG size observed in cows in early lactation in Chapter 3. It has been suggested that during early lactation membrane material is the limiting factor for MFG envelopment, leading to the formation of larger MFGs (Wiking et al., 2004). In line with this, ePL synthesis could be a physiological response to this limitation, with the added effect that increased LD fusion, due to ePE instead of diacyl PE synthesis, can concomitantly reduce the required proportion of membrane material to cover the LD. Thus, increasing membrane PL synthesis in the form of ePE could alleviate the animals' high demand for membrane material during early lactation. Cows in the first 10 days in milk were excluded from the analysis in Chapter 3. However, the few cows that were measured in the first 10 days in milk contained the largest MFGs of all observed animals (data not shown). Future studies should look into the abundance of ePE in cows immediately after parturition and address their potential role in the nutrition of the calf.

The SMFG phenotype, on the other hand, was characterised by higher PC/PE ratios, increased proportions of UFAs in the PC, PI and lyso PC (LPC) subclasses and higher proportions of UFAs in the MFG core compared to the LMFG cows. Moreover, an inverse relationship between MFG size and the proportion of UFAs in the MFG core was also observed across other dairy species, namely camels, sheep and goats. This, in combination with the naturally higher PL/TG ratios in smaller MFGs, suggests that in these cows the balance between PL and TG synthesis is shifted towards a higher proportion of PLs compared to the LMFG cows. Moreover, the milk from SMFG cows showed a trend towards higher SM/ceramide (Cer) ratios. It has been suggested that increased sphingomyelin synthase 1 (SMS1) activity in hepatocytes can stimulate *de novo* PC synthesis, because a headgroup transfer from PC to Cer is needed to produce SM

(Deevska et al., 2017). During this reaction DG is produced, adding to the available DG pool for PL and TG synthesis. An increase in SM synthesis in the SMFG phenotype could also explain the observed increase in the proportion of UFAs in the MFG core, because increased SMS1 activity was further related to an increase in UFAs in the TG fraction (Deevska et al., 2017).

## 7.4 Recommended future research

### *7.4.1 Negative energy balance and nutrient partitioning*

A reoccurring interpretation of the results obtained in the current study was that MFG size could be related to differences in the energy balance between cows with the small or large MFG phenotype. It is a known phenomenon that average MFG size is larger in early lactation, where cows may be in negative energy balance and this was also observed in the herd studied in Chapter 3. A recent study showed that intracellular LD size was increased when FAs were delivered to primary bovine mammary epithelial cells in the form of non-esterified FAs (NEFAs), which is the form in which FAs mobilised from fat stores are presented to the mammary gland during negative energy balance (Mesilati-Stahy and Argov-Argaman, 2018). Accordingly, the finding that some Cer species were increased and more importantly the SM/Cer ratio was reduced in LMFG cows, could also suggest a difference in energy balance between the studied groups, because increased concentrations of some Cer species in the liver and plasma have been related to negative energy balance in dairy cows (Davis et al., 2017). However, it is unknown if Cer concentrations in milk can also serve as potential markers for negative energy balance. Moreover, the hypothesis that increased relative abundance of some Cer species could be related to negative energy balance in the LMFG group is in conflict with the FA profile in the MFG core presented in Chapter 4 of this work, where the observed increased

proportion of oleic acid in the core of MFGs from SMFG cows could also indicate lipid mobilisation in these cows (Gross et al., 2011). Furthermore, the diet was similar between the studied groups and all cows were in established lactation (> 50 days in milk), suggesting that negative energy balance is likely not the cause of the differences in the FA profiles reported in the current study. Known markers for negative energy balance in dairy cows, such as increased plasma NEFA levels were not determined in this study and a potential difference in energy balance between the studied groups therefore remains speculative. A possible relationship with the cows' energy balance will need to be the subject of further studies aiming to characterise the small and large MFG phenotype.

Another possible explanation for the differences seen in MFG size between individual animals is that they may differ in their genetically determined ability to channel nutrients towards either milk production or body fat stores. This trait, referred to as nutrient partitioning, seems to be genetically and environmentally driven (Veerkamp et al., 2003). Although it is somewhat selected for when breeding high genetic merit cows for milk yield, Friggens et al. (2007) showed that the heritability of body energy mobilisation is low and natural variation between individual cows exists. Increased nutrient partitioning towards the mammary gland and away from adipose tissue makes the animal more susceptible to negative energy balance, which can lead to reduced fertility and animal health (Friggens and Newbold, 2007). Whilst negative energy balance can occur as a response to environmental changes for example when there is an undersupply of energy through the feed or reduced intake due to heat stress, the notion that this is a partly genetically determined trait also implies that it cannot be prevented through nutritional management (Friggens et al., 2007). Therefore, negative energy balance due to a genetic predisposition of cows to prioritise partitioning of nutrients towards the mammary gland can occur, even when feed availability is not limited (Veerkamp et al., 2003).

Interestingly, nutrient partitioning also changes during the lactation cycle and is influenced by parity and breed (Friggens et al., 2007). The investigation on the origin of FAs incorporated into the MFG core reported in Chapter 4 suggested a higher proportion of preformed FAs in the milk from cows with the small MFG phenotype, which could indicate increased nutrient partitioning towards the mammary gland. Therefore, it is possible that the differences in the ability to partition nutrients, as a genotype which varies between individual cows, could explain the wide range in MFG size distributions between individual animals. To date, no link between MFG size and genetic merit for milk yield has been reported and this possible relationship remains to be investigated.

### *7.4.2 Stearoyl desaturase activity*

The finding that SMFG cows were able to incorporate more preformed FAs into their milk could also be related to increased stearoyl-coenzyme A desaturation (SCD) activity. Desaturation of FAs in the mammary gland is important for membrane and LD fluidity in the mammary cell (Timmen and Patton, 1988). At body temperature, only TGs containing adequate proportions of SFAs with chain lengths of C4 to C11, branched FAs or UFAs are present in liquid form (Knothe and Dunn, 2009). The fluidity of the LD is crucial for unimpaired physiological processes such as LD secretion, and the requirement to maintain LD fluidity limits the ability of the mammary gland to take up long chain SFAs from the diet. Unless the animal is in negative energy balance, long chain FAs taken up by the mammary gland are predominantly derived directly from the diet by hydrolysis of TGs from intestinal lipoproteins, while only small proportions are derived from adipose tissue (Palmquist, 2006). To counteract adverse effects of FAs with higher melting points, the animal can either limit uptake of these FAs and at the same time increase *de novo* synthesis of short chain FAs or reduce the melting points of long chain FAs by inserting a double bond through SCD activity.

The SMFG and LMFG groups in this work did not differ in the proportion of *de novo* synthesised FAs and the main difference was an increased proportion of UFAs and specifically oleic acid in the SMFG group. Although the results were not statistically significant and therefore inconclusive, the data presented in Chapter 4 suggested increased SCD indices for FAs with a chain length of 14 (SCDI14), FAs of 16 carbons in chain length (SCDI16) and FAs with 18 carbons (SDCI18) in the SMFG group. Both, SCDI14 and SCDI18, but not SCDI16, have moderate heritability estimates ($h^2$ = 0.38 for SCDI14 and $h^2$ = 0.19 for SCDI18) (Garnsworthy et al., 2010), suggesting that some variation in SCDI14 and SCDI18 within a population can be attributed to genetic differences. An especially important indicator of mammary gland specific SCD activity is SCDI14 because the desaturation of FAs with a chain length of 14 carbons, i.e. C14:0 to C14:1c9, occurs predominantly in the mammary gland (Mosley and McGuire, 2007). This provides a potential for selection through breeding programs, if it could be shown that the cow's SCD activity is associated with her characteristic MFG size. Our results support a possible connection between desaturation activity and MFG size as suggested by Couvreur et al. (2007), who reported a higher C18:1/C18:0 ratio in SMFG cows and the use of SCDI for breeding selection programs has already been suggested (Garnsworthy et al., 2010).

### *7.4.3 Potential identification of genotypes and genomic selection for MFG size*

Moreover, the hypothesis that SCD activity can influence MFG size offers the potential to identify small and large MFG genotypes based on the reported polymorphisms on the SCD gene (Mele et al., 2007). The polymorphism on the SCD gene with two alleles A and V has a relative frequency of 27% for the AA genetic variant, 60% for VA and 13% for VV (Mele et al., 2007). Cows with the AA genetic variant have higher MUFA concentrations and higher C14:1/C14:0 ratios compared to the VV cows

(Mele et al., 2007). The repeated measurements of the whole herd as part of this work (Chapter 3) revealed that out of the approximately 150 cows, 50% produced milk with MFG sizes between 3.8 and 4.4 µm and 25% produced milk with average MFG sizes smaller or larger than 3.8 and 4.4 µm, respectively. Further examination of the top and bottom quartiles throughout the whole sampling year showed that 49 and 50 individual cows fell into the top and bottom quartile, respectively. Out of these cows, 16 appeared repeatedly in the top quartile and 15 in the bottom quartile, representing about 10% each of the total herd each, which could indicate the presence of a genetic variant.

Another gene polymorphism with impact on MFG size is the DGAT1 K232A gene polymorphism. It has previously been suggested that this gene polymorphism could be related to MFG size (Argov-Argaman et al., 2013) and that it can impact the FA composition in milk (Schennink et al., 2007). Importantly, the characteristics of the small and large MFG phenotypes, described in Chapter 4, aligned with the predicted differences between the AA and KK genotypes and thus support the suggested relationship of the DGAT1 polymorphism with MFG size. Furthermore, the results discussed in Chapter 5 led to the hypothesis that the increased relative ePE abundance in the LMFG group could be related to an increased DGAT1 activity, one of the characteristics of the KK genotype (Argov-Argaman et al., 2013). The suggested reason was that the reduced availability of DG for PL synthesis, due to increased DGAT1 acivity, could lead to ePL synthesis as a salvage pathway. Overall, the DGAT1 and SCD gene polymorphisms are the most promising candidates based on the comparison of the milk lipidome from the SMFG and LMFG phenotypes with the lipid metabolic characteristics described for these genotypes in the literature. Future studies should further investigate their relationship to MFG size.

In addition, genomic selection based on the identification of genome wide DNA markers has been successfully applied for other traits, for example for the selection of

heat tolerant dairy cows (Nguyen et al., 2016). Future studies should aim to apply genomic selection to the selection of cows with distinct MFG phenotypes, because MFG size may be a more complex trait that does not depend on a single gene polymorphism. However, the identification of genome wide markers would require a more regular measurement of MFG size on-farm, for example using mid-infrared spectroscopy as suggested by Fleming et al. (2017b), to be able to relate MFG size to genomic data of the respective cows.

### *7.4.4 The impact of parity on FA composition and lipidomic profiles in milk*

The studied groups in this work differed in parity, with the SMFG group comprising cows in earlier parities (1-3) compared to the LMFG group (3-5). This is a limitation of this study because it is difficult to isolate a potential effect of parity from the effect of the small and large MFG phenotype. However, the estimated effect of parity within the studied herd was minor (0.16 µm between parity 2 and 3) (Chapter 3) compared to the individual variation (3 µm) between cows. Parity could potentially affect nutrient partitioning, although the differences in energy balance between parities are predicted to be less relevant in later stages of lactation (Friggens et al., 2007). Nonetheless, some of the results of this work could at least partly originate in the difference in parity between the studied groups. It also emphasises that MFG size is most definitely a reflection of the metabolic state of the cow. Therefore, future studies should look at the lipidomic differences between animals in earlier and later parities.

## 7.5 Recommended future research using primary cell culture

The potential role for ePE in the determination of intracellular LD size could be further investigated using the *in vitro* model described in Chapter 6 of this thesis. Due to the novelty of this finding, first preliminary studies in primary bovine mammary epithelial

cell culture could shed some light on the potential role of the ePE subclass in LD accumulation. Considerable advancements in the study of MFG size regulation have been made using primary cell culture with cells extracted from bovine mammary tissue (Cohen et al., 2015, 2017; Mesilati-Stahy and Argov-Argaman, 2018). Cell culture experiments using mammary tissue or cells extracted from raw milk could equally be used to study the relationship between LD size and ePE. For example, the effect of the supplementation of different FAs on intracellular LD size and ePL abundance could be measured by fluorescence microscopy and lipid chromatography tandem mass spectrometry (LC-MS/MS). However, although visualisation of ether lipids has been achieved in cell culture by supplementing the culture medium with polyene lyso-etherlipids which are subsequently incorporated into ePLs (Kuerschner et al., 2012), imaging of lipids on a class level remains challenging because available probes to visualise phospholipids in cells are limited (Maekawa and Fairn, 2014). Moreover, the established *in vitro* model in the current work will need to be further optimised in order to minimise the reduction in viability due to FA supplementation.

## 7.6 Conclusions

The experimental chapters within this thesis investigated the extrinsic and intrinsic parameters that determine MFG size in dairy cows. The main goal was to identify the differences in the mammary lipid metabolism between cows that consistently produce milk with small or large MFG size distributions in order to characterise the small and large MFG phenotype. An in-depth characterisation of MFG size as a milk production trait that could potentially be selected for through breeding programs, could lead to a more targeted use of milk for specific processing streams or could lead to improved nutritional value of milk and milk products.

The combined analysis of the FA profile in the MFG core and the analysis of the whole milk lipidome in the selected animals revealed important differences between the small and large MFG phenotypes. The small MFG phenotype was characterised by higher proportions of UFAs in the MFG core, which was related to an improved ability to take up preformed FAs from the bloodstream, possibly due to increased SCD activity or preferential uptake of already unsaturated FAs from lipid mobilisation. The milk from cows with the small MFG phenotype also had higher PC/PE ratios and contained more unsaturated lipid species within the PC, LPC and PI lipid classes. Crucially, the biophysical properties of monolayers with higher PC/PE ratios and higher degree of unsaturation within the polar lipid classes are predicted to prevent LD fusion (Arisawa et al., 2016). Overall, the milk lipidome of SMFG cows identified as part of this work is therefore in good agreement with the small MFG phenotype.

The milk from cows with the large MFG phenotype, on the other hand, contained a higher relative abundance of ePE and a tendency towards smaller SM/Cer ratios. Ether PE species compared to diacyl PE species have been shown to enhance LD fusion (Glaser, 1994). This is in agreement with the large MFG phenotype and is the first time that a possible involvement of ePE species in the regulation of MFG size has been suggested. The novelty of this finding warrants future investigations into the role of ePE species within the mammary epithelial cell and their possible role in the lipid droplet accumulation in other cell types. Furthermore, this finding should encourage investigations into other potential roles of ePL species in lactation physiology, for example a potential role during the transition phase in dairy cows.

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