

# Molecular aspects of milk lipid synthesis and secretion in mammary epithelial cells

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## Review

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### Abstract

Milk fat is a high-value component of the U.S. dairy market. It is the major energy component of milk and is responsible for many organoleptic and technological characteristics of milk and dairy products. In addition, milk fat is unquestionably distinctive among all dietary fats that humans consume, as it is not only comprised of several hundred different fatty acids (FAs) but also contains a wide and unique array of bioactive lipids. Milk fat is dispersed in milk primarily in the form of fat globules. These cytoplasmic lipid droplets originate from mammary epithelial cells (MECs) and are secreted into the alveolar lumen surrounded by a membrane. Many advances in our knowledge of specific enzymes involved in milk lipid synthesis, the selectivity of the triacylglyceride (TAG) synthesis enzymes for specific FAs, the molecular mechanisms behind the uptake of long-chain FAs into the cells and the milk lipid secretion process have led to an improved understanding of the biology of milk fat synthesis. However, research to provide deeper insights into the mechanism of lipid synthesis in MECs is warranted and might lead to novel strategies to alter milk fat content and quality to benefit the dairy industry and meet dietary recommendations and consumer demands for foods that positively impact health. In this review, we aimed to provide a general overview of our current knowledge of the molecular aspects of milk lipid synthesis in MECs, from the uptake of blood-derived precursors to the intracellular formation of TAG-rich fat droplets secreted into milk as milk fat globules. We also highlight some current gaps in the knowledge that warrant further exploration. Given the importance of dairy food in the human diet, a better understanding of these processes could help develop novel strategies to alter milk fat composition in ways that benefit both human health and dairy producers.

## Introduction

In the dairy industry, milk fat is an important determinant of milk quality. Bovine milk is composed of 3.5–5% lipids (Fox and McSweeney 2007; Huppertz et al. 2009). Milk fat is the most variable constituent of ruminant milk in terms of both quantity and composition. The makeup of milk fat is an important determinant of both the organoleptic and physical properties of milk, affecting dairy product quality (MacGibbon 1988; Siek et al. 1969; Widder et al. 1991) and therefore the commercial value of milk. The milk value in the United States is mostly determined by the amount of separate components, one of which is anhydrous milk fat. Some specific lipids, such as sphingolipids, are also thought to be highly bioactive and nutritionally functional and therefore may have an impact on human health (Vesper et al. 1999; Anto et al. 2020).

In recent years, many advances have been made in our understanding of milk lipid synthesis, especially in terms of the molecular mechanisms of fatty acid (FA) uptake, the specific enzymes involved in each step of milk lipid synthesis and the milk lipid secretion process. The goals of this review are to (i) review the current knowledge on lipid synthesis in the mammary glands of ruminants, (ii) pinpoint current gaps in knowledge regarding lipid metabolism that could be relevant for a better understanding of the relationship between circulating lipids and milk fat and (iii) provide an overview of the particularities of mammary epithelial cells (MECs) with respect to lipid metabolism. This review will focus mainly on the proteins and enzymes involved in milk fat synthesis in ruminant MECs. A thorough review of the regulation of the genes involved in milk fat synthesis was recently provided by Mu et al. (2021), and we provide only a brief summary here.

## The form, origin and identity of milk lipids

### Milk fat globules

Milk fat is organized into milk fat globules (MFGs), which are structures unique to milk and range in size from 0.1 to more than 10  $\mu\text{m}$ , with an average size between 2.5 and 3.5  $\mu\text{m}$  (Martini et al. 2013). A small portion of milk fat ( $\sim 0.3\%$ ), mainly phospholipids

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and free fatty acids (FFAs), is not associated with MFGs. MFGs originate as lipid droplets in MECs (Chong *et al.* 2011; Fox and McSweeney 2007). Upon excretion into milk, lipid droplets are coated by the apical plasma membrane, which becomes part of the milk fat globule membrane (MFGM) (Chong *et al.* 2011; Fong *et al.* 2007). The MFGM accounts for approximately 2%–6% of the total milk fat and is composed of 25%–70% of lipids, 21%–70% of proteins and 10% carbohydrates, such as mannose and *N*-acetylglucosamine, stemming mostly from the glycosylation of proteins and polar lipids (Keenan and Dylewski 1995; Martini *et al.* 2013; Sun *et al.* 2024). The precise composition of the MFGM has been reported with such disparity due to technical difficulties in separating the membrane from the core of the MFG (Holzmüller and Kulozik 2016). MFGM lipids primarily consist of phospholipids and triacylglycerides (TAGs), with traces of other lipid types (Keenan and Dylewski 1995; Martini *et al.* 2013). MFGs are composed of approximately 98% TAGs, 0.5–1% of phospholipids, 0.2–0.5% sterols (95% of the sterols are cholesterol, 10% of which are esterified) and a small amount of glycolipids, protein and fat-soluble vitamins (Fong *et al.* 2007; Martini *et al.* 2016). The TAGs in MFGs are derived mostly from the lipid droplets of MECs, whereas the phospholipids, glycolipids, proteins and sterols are derived from both lipid droplets and the plasma membrane (Fong *et al.* 2007).

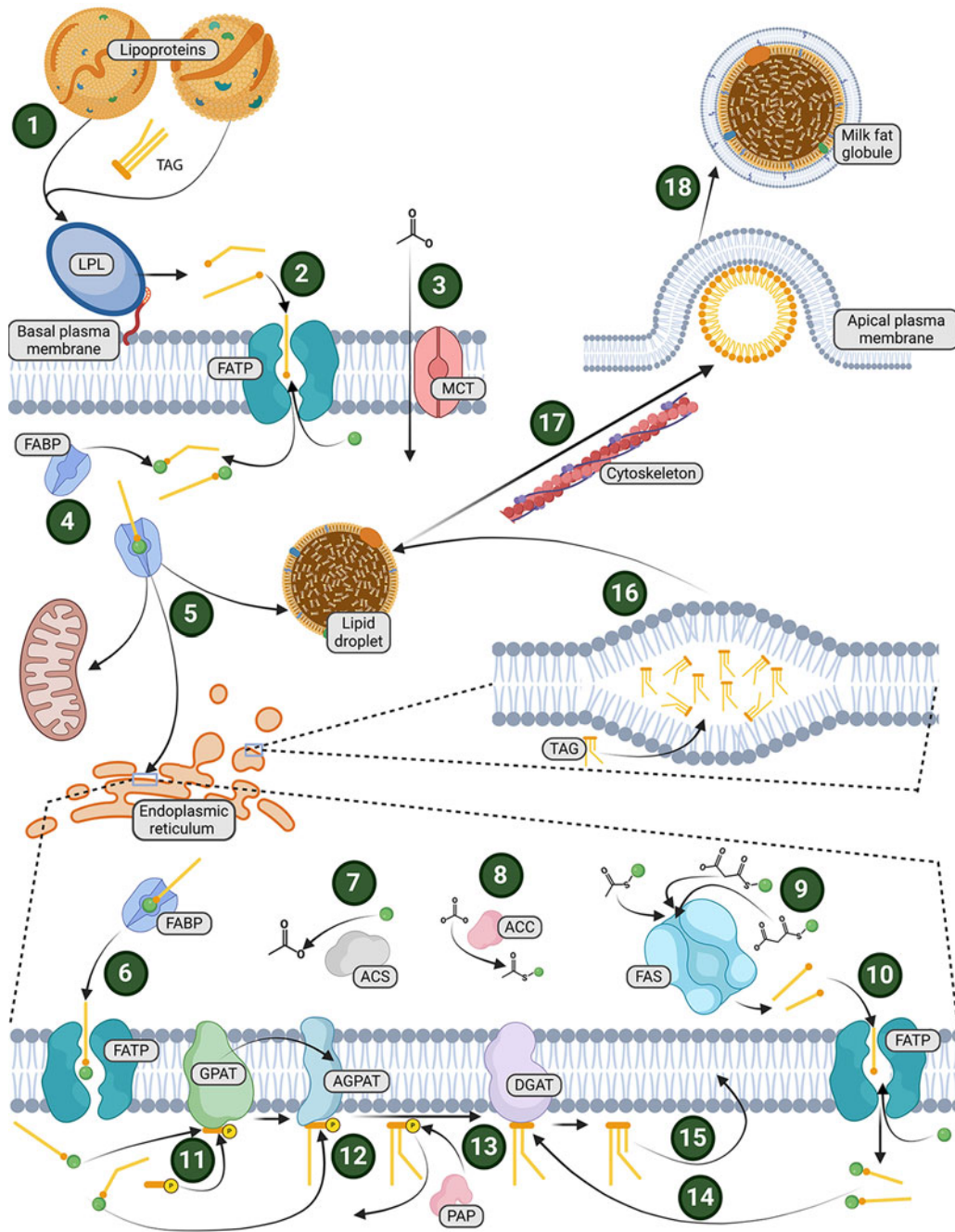
### Lipid droplets in MECs

Lipid droplets are organelles present in most cell types, although they vary greatly in form and physiological roles depending on the cell type and need of the organism (Welte and Gould 2017). In MECs, lipid droplets serve as short-term storage of lipids to be excreted in milk (McManaman 2014). They originate from the endoplasmic reticulum (ER) and consist of a single layer of polar lipids, mostly phospholipids, intertwined with proteins (i.e., glycoproteins) surrounding a core of mostly TAGs with a small amount of free and esterified sterols (Monks *et al.* 2020). The mechanism by which lipid droplets bud from the ER involves first the accumulation of phospholipids in the membrane, which reduces the surface tension between both sides of the membrane and allows both membrane layers to arc outward (Fig. 1). This permits TAG accumulation between the two layers of the membrane until this accumulation leads to the release of the lipid droplet (Thiam and Forêt 2016). Half of the lipid droplet single layer of polar lipids comes from the external layer of the ER membrane, and the other half comes from the internal layer. After budding from the ER, the transport of lipid droplets to the apical membrane is mediated by the cytoskeleton, ensuring that the transport of the lipid droplets is mostly unidirectional and organized (Masedunskas *et al.* 2017). Lipid droplets are metabolically active; TAG synthesis enzymes present in the ER membrane relocate to the lipid droplet's single-layer membrane and continue to produce TAGs to grow the droplet in size as it migrates through the cell (Wilfling *et al.* 2013). The fusion of multiple lipid droplets is also possible and occurs more readily during some pathophysiological conditions, such as clinical and subclinical mastitis. As a result, larger and fewer MFGs are usually associated with lower milk quality (Matsunaga *et al.* 2018). The fusion of lipid droplets in MECs appears to be independent of the droplet's TAG content and is instead mediated by the phospholipid content of the lipid droplet, with the rate of fusion increasing with the phosphatidylethanolamine content in the droplet's membrane (Cohen *et al.* 2017). Lipid droplets are complex organelles that interact with both the mitochondria and Golgi apparatus

(Benador *et al.* 2018), and these interactions support the expansion of lipid droplets. The exchange of lipids between lipid droplets also appears to occur through channels formed between the cell death-inducing DNA fragmentation factor A (DFFA)-like effector A (CIDEA) proteins in the droplets' membranes (Mather *et al.* 2019). MECs deficient in CIDEA produce no droplets larger than 5  $\mu\text{m}$ , suggesting that multiple mechanisms are needed to produce the complete array of droplet sizes (Mather *et al.* 2019). Two other important proteins of MEC lipid droplets are adipophilin (ADPH) and adipose differentiation-related protein (ADRP), which have been shown to regulate lipid droplet size (Chong *et al.* 2011). These two proteins are also essential for the formation of lipid droplets in virtually all cell types. Additionally, volatile fatty acids (VFAs) have been shown to induce the expression of ADRP, along with the expression of most TAG synthesis genes, suggesting that it might play an increased role in ruminants (Sun *et al.* 2016). Insulin signaling has also been shown to be important for the formation and secretion of lipid droplets (Neville *et al.* 2013), and the mammalian target of rapamycin (mTOR)/sterol regulatory element-binding protein (SREBP) 1 axis has been demonstrated to regulate lipid droplet size in the MECs of dairy goats (Zhang *et al.* 2018). However, much of the current knowledge on lipid droplets is based on lipid droplets in either (i) immune cells, where they produce inflammatory mediators such as prostaglandins; (ii) adipocytes, where they act as long-term energy storage; or (iii) MECs from monogastric animals. Owing to the excretory role that lipid droplets play in MECs and the particularity of ruminant animals, there might be important differences in their formation and regulation; and thus, there is currently a need to investigate the mechanisms underlying lipid droplet formation, accumulation and regulation in ruminant MECs in greater depth.

### Milk fat composition

More than 99% of milk fat consist of FA esters. The FA composition of ruminant milk is important for human nutrition and is unique among dietary lipid sources for humans. Over 400 different FAs have been found in ruminant milk (Jensen 2000). Many of these FAs (13–16% of total milk FAs), such as branched-chain FA and *trans*-FAs, are derived from rumen microbial lipid metabolism and are therefore not found in plant- or nonruminant-based fats (Precht and Molkenin 1996; Unger *et al.* 2020). Saturated fatty acids (SFAs) constitute up to 75% of total milk FAs, with palmitic acid (16:0) being the most abundant SFA (25–30% of total milk FAs) (Jensen *et al.* 1991). A 2020 analysis of 194 whole milk samples from 17 different brands in the northeast United States revealed that most commercially available milk contains between 66% and 72% SFAs (Unger *et al.* 2020). Monounsaturated fatty acids (MUFAs) make up approximately 24%–29% of total milk FAs, with *trans*-MUFAs contributing between 2.5% and 4.5% (MacGibbon 2020; Unger *et al.* 2020). Oleic acid (18:1 *c*9) is the main MUFA, accounting for approximately 85% of all MUFAs. Finally, polyunsaturated fatty acids (PUFAs) make up the remainder of milk FAs. Conjugated linoleic acids, of which rumenic acid is the primary isomer (18:2 *c*9,*t*11), constitute up to 1% of total milk FAs (MacGibbon 2020). However, the most abundant PUFA in milk is the *n*-6 FA linoleic acid (18:2 *c*9, *c*12), which typically accounts for more than half of all PUFAs. The most abundant *n*-3 FA in milk is  $\alpha$ -linolenic acid (18:3 *c*9, *c*12, *c*15), which typically accounts for approximately 0.5% of the total milk FA content (Van Valenberg *et al.* 2013). In milk from conventionally raised cattle, the *n*-6/*n*-3 FA ratio is skewed in favor of *n*-6 FA, which averages



**Figure 1.** Diagram depicting the steps of triacylglyceride (TAG) synthesis and milk lipid secretion in mammary epithelial cells. (1) Lipoprotein lipase (LPL) hydrolyzes triacylglycerides in chylomicrons and very-low-density lipoproteins, releasing long-chain fatty acids. (2) The fatty acid transport proteins (FATPs) SLC27A1-6 transfer coenzyme A to long-chain fatty acids while facilitating fatty acid uptake into the cell, potentially with the help of an acyl-CoA synthase long-chain family enzyme. There is the potential involvement of CD36 in an undefined role at this step. (3) Acetate (and other volatile fatty acids) enters the cell either through passive diffusion through the plasma membrane or with the help of monocarboxylate transporters (MCTs). (4) Within the cell, and possibly outside of the cell, seven different fatty acid binding proteins (FABPs) bind the long-chain acyl-CoA. (5) FABPs shuttle long-chain acyl-CoA to different cellular compartments (i.e., mitochondria, the endoplasmic reticulum, and existing lipid droplets), although the preference of each FABP to direct specific fatty acids to which cellular compartment has not yet been established. (6) SLC27A4 facilitates the uptake of long-chain acyl-CoA by the endoplasmic reticulum. (7) The ligase acetyl-CoA synthetase (ACS) adds coenzyme A to acetate. (8) Acetyl-CoA carboxylase (ACC) adds a carboxyl group to acetyl-CoA to synthesize malonyl-CoA. (9) Fatty acid synthase (FAS) utilizes acetyl-CoA and malonyl-CoA to produce coenzyme A and medium- chain fatty acids. (10) An FATP, possibly SLC27A4, takes up short- and medium- chain acyl-CoA into the endoplasmic reticulum. (11) Glycerol-3-phosphate and a long-chain acyl-CoA are combined by glycerol-phosphate acyl transferase (GPAT3 or GPAT4) to form lysophosphatidic acid. (12) Another long-chain acyl-CoA is added to lysophosphatidic acid by one of 5 acyl glycerol-phosphate acyl transferase (AGPAT) enzymes, yielding phosphatidic acid. (13) Phosphatidic acid phosphatase (PAP), also known as lipin, removes phosphate from phosphatidic acid to yield a diacylglycerol. (14) The diacylglycerol acyl transferase enzyme (DGAT) combines diacylglycerol and acyl-CoA, generally either short- or medium- chain acyl-CoA, to form a triacylglyceride. (15) Triacylglycerides accumulate in the endoplasmic reticulum membrane. (16) Accumulation of triacylglycerides in the endoplasmic reticulum leads to budding of a lipid droplet. (17) Lipid droplets travel through actin filaments toward the apical membrane of the cell. They can continue growing by synthesizing more triacylglycerides and fusing together. (18) At the apical plasma membrane, the lipid droplets are released into the lumen of the alveoli, where they becoming milk fat globules.



between 2 and 9 (Unger *et al.* 2020). However, this ratio is highly dependent on feeding practices, and milk from organically raised cows has a much lower *n-6/n-3* FA ratio, typically between 1 and 5 (Unger *et al.* 2020). This variability in the *n-6/n-3* ratio reflects the variability of FAs in different feeds; mammals do not possess the enzymes required to desaturate FAs at positions 3 and 6, meaning that their origin is entirely from the diet (Das 2006). Milk also contains minute quantities of other lipids, such as vitamin A precursors (carotenoids and retinoids) and eicosanoids (e.g., prostaglandins) (Hansel *et al.* 1976; Hulshof *et al.* 2006).

### Origin of the FAs in milk lipids

The FAs used to produce TAGs, phospholipids and other FA esters of the lipid droplets come from two sources. Three different early studies reported that 36%, 46% and 48% of milk fat originates from blood TAGs in ruminants (Gluscock *et al.* 1966, 1983; Palmquist and Mattos 1978). The remainder of milk FAs are synthesized *de novo* in the MECs. This proportion is thought to correspond to the proportion of long-chain fatty acids (LCFAs) in milk. In milk, FAs longer than 16 carbons are typically referred to as LCFAs, which contrasts with the standard definition of LCFAs being FAs with 12–21 carbons. This difference is related to the fact that milk FAs longer than 16 carbons come mostly from the uptake of FAs from the blood, whereas milk FAs shorter than 16 carbons come mostly from *de novo* synthesis in MECs. Milk FAs of exactly 16 carbons can be of both origins, making 16 carbons the pivotal point. Figure 1 shows a diagram depicting the steps of the synthesis of TAGs, lipid droplets and MFGs in MECs.

### Uptake of LCFAs from blood lipoproteins

The TAGs carrying LCFAs that end up in milk travel through the bloodstream mostly in chylomicrons originating from the intestine and in very-low-density lipoproteins originating from the liver. A much smaller proportion of TAGs comes from low-density lipoproteins, which are themselves formed from very-low-density lipoproteins from which a proportion of TAGs has already been released (Ellsworth *et al.* 1988). Milk LCFAs are thought to come both directly from the rumen and from the animal's adipose tissue, the proportion of which appears to be dependent on the energy balance of the animal, with a peak incorporation of FAs from blood TAGs into milk on Day 18 of lactation (Cozma *et al.* 2013; Gluscock *et al.* 1983). In addition to FAs from blood TAGs, the mammary gland can also take up plasma FFAs. While the uptake of plasma FFAs is important in rodents (Del Prado *et al.* 1999) and physiologically possible in ruminants, it has been shown to be significant for the ruminant mammary gland only when there is a very high FFA concentration in the blood, such as during ketosis or immediately *postpartum* (Miller *et al.* 1991). Finally, it has been reported that a small proportion (up to 20%) of C16 FA could be elongated to C18 in caprine MECs (Shi *et al.* 2017), although this conversion has only been reported in dairy cattle when the animals were first fasted for 20 hours (Bishop *et al.* 1969). Importantly, these studies are dated, and given that energy balance appears to play a role in determining the source of the FA taken up by MECs, there is a need to validate that this partition of FA still holds true with modern dairy management practices.

The uptake of LCFAs from circulating TAGs is dependent on lipoprotein lipase (LPL), which hydrolyzes TAGs from lipoproteins to release FAs from the glycerol backbone for uptake. The LPL gene was shown to be upregulated 80-fold in early lactation, with

the expression declining gradually until late lactation to an 8-fold upregulation compared with that in the dry period (Zhao *et al.* 2014). LPL is highly selective for TAGs from chylomicrons and very-low-density lipoproteins because it requires an apolipoprotein for activation (Bengtsson and Olivecrona 1980). The activation of LPL by apolipoprotein C-II of very-low-density lipoproteins and chylomicrons is dependent upon the chain length at position *sn-2* of the phosphatidylcholines for optimal activity (McLean *et al.* 1986). These requirements and the localization of the LPL enzyme, which is anchored to heparin sulfate on the surface of the plasma membranes of endothelial cells (Sivaram *et al.* 1992), may explain why FAs hydrolyzed from lipoprotein TAGs are preferentially selected over the FAs bound in phospholipids or cholesterol esters or from the pool of circulating FFAs bound to serum albumin.

The uptake of LCFAs into the lactating mammary gland is thought to be mostly active (Zhang *et al.* 2021). A strong correlation has been shown between the uptake of FAs and the combined arterial concentration of FFAs and TAGs (Enjalbert *et al.* 1998), but only to a certain extent, after which the uptake reaches saturation (Baldwin *et al.* 1980). The FA translocase CD36, a polyvalent glycoprotein involved in a myriad of different cellular functions, is also crucial to the uptake of LCFAs in various cell types (Coburn *et al.* 2000). A recent study demonstrated that blocking CD36 reversed the increase in TAG synthesis in MECs caused by supplementation with stearic acid (18:0) while also decreasing the phosphorylation of mTOR (Yang *et al.* 2023). In addition to CD36, both FA binding proteins (FABPs) and FA transport proteins (FATPs) are thought to be important for the uptake of LCFA (Schwenk *et al.* 2010). The main mammalian FATP are the 6 solute carrier family 27 proteins (SLC27). All six members of the SLC27 family possess a transmembrane domain and some degree of intrinsic long-chain and very-long-chain acyl-CoA synthetase enzyme activity. Two members, SLC27A1 and SLC27A6, are more highly expressed in the mammary gland than the others, although all six members can be found and their expression levels are dependent upon the stage of lactation (Bionaz and Looor 2008). These proteins are required for the translocation of LCFAs through both the plasma membrane and the ER membrane. The FABPs are a family of small proteins that serve as chaperones for LCFAs and other related molecules, such as eicosanoids and retinol. They act as shuttles for hydrophobic ligands, both intracellularly and extracellularly, where they transport the FAs or their related compounds to their destinations. FABPs associate with the cytosolic tail of the CD36 glycoprotein, which highlights the importance of CD36 in LCFA uptake (Spitsberg *et al.* 1995). Among the nine FABPs, FABP3 is the most highly expressed in the lactating mammary gland, representing more than 90% of the total FABPs at peak lactation (Bionaz and Looor 2008). Because FABP3 is highly expressed during lactation and has been linked with the regulation of FA synthesis and lipid accumulation in the mammary gland (Liang *et al.* 2014), it likely acts as the main carrier of LCFAs from the plasma membrane to either the ER or lipid droplets. During the dry period, FABP4 is the most highly expressed FABP, with both FABP3 and FABP5 being expressed at slightly lower levels (Bionaz and Looor 2008). However, the specificity of each FATP and FABP, their intracellular localizations, and the exact mechanisms for FA translocation have not yet been elucidated.

### *De novo* FA synthesis

The mechanisms of *de novo* FA synthesis in the mammary gland are better understood than those of LCFA uptake. In bovine animals,

acetate and  $\beta$ -hydroxybutyrate are used as carbon sources for FA synthesis (Hillgartner et al. 1995; Miller et al. 1991). Acetate also serves as the main source for NADPH regeneration (Bauman et al. 1974, 1970). Both the plasma concentrations of acetate and  $\beta$ -hydroxybutyrate are significantly higher in ruminants than in nonruminants as microbial carbohydrate fermentation in the rumen yields VFAs. Because VFA uptake in the mammary gland is linked to the arterial concentration, VFAs contribute significantly to milk fat synthesis in ruminants (Urrutia and Harvatine 2017). The remainder of the acetate required for FA synthesis in ruminants comes from glucose via the tricarboxylic acid cycle. While VFAs can diffuse through the plasma membrane, the rate of diffusion is not sufficient to sustain the high requirement of lactating mammary glands. During lactation, the uptake of  $\beta$ -hydroxybutyrate and acetate is dependent on monocarboxylate transporters (MCTs) and sodium-dependent monocarboxylate transporters (SMCTs), also collectively known as solute carrier family 16A (SLC16A), a family of 14 proton-dependent transporters and 2 sodium-dependent transporters (Felmlee et al. 2020). There are nine SLC16A members expressed in the bovine mammary gland, and six of these (i.e., MCT1, 2, 3, 4 and 7 as well as SMCT1) are known to transport  $\beta$ -hydroxybutyrate (Kirat and Kato 2009). Four of these genes (*MCT1*, *MCT3*, *MCT4* and *SMCT1*) are also important for acetate uptake (Aluwong et al. 2010; Halestrap and Meredith 2004). The most highly expressed MCT in the lactating mammary gland is MCT1, which accounts for approximately two-thirds of the total MCT, suggesting that it is the main milk-fat related MCT (Kirat and Kato 2009). The three most important enzyme families for FA synthesis are acetyl-CoA synthetase (ACS), acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), which are highly expressed in the lactating mammary gland. The first step, catalyzed by ACS, is the activation of acetate by linking it to a molecule of coenzyme A, yielding acetyl-CoA. Among the three major isoforms, ACS2 is the most important isoform for milk fat synthesis, since it is located in the cytosol, where *de novo* FA synthesis occurs. Among the two ACC enzymes, ACC1 is the isoform expressed in the mammary gland. Its role is to carboxylate acetyl-CoA to yield malonyl-CoA. A molecule of malonyl-CoA is then condensed with a starting acetyl-CoA by the FAS enzyme. This step is repeated up to seven times, yielding an SFA with up to 16-carbons. Most of the current knowledge on FAS is based on research in rodents performed in the late 20th century (Carey and Dils 1970a, 1970b; Martyn and Hansen 1980). While the same basic principles hold true for ruminants, there are still distinctions to be made. Generally, ruminant FAS has a much greater propensity for incomplete elongation, resulting in the synthesis of a greater proportion of short- and medium-chain FAs. It is thought that propionate can sometimes be used instead of acetate and  $\beta$ -hydroxybutyrate in *de novo* FA synthesis, which results in the addition of three carbons instead of two and typically leads to odd-chain FAs of 15 or 17 carbons in length (Vlaeminck et al. 2006).

## Milk lipid synthesis

### TAG synthesis

The synthesis of TAGs in mammals is a multistep process that has been reviewed in great detail by Coleman and Mashek (2011). However, there are some noticeable differences between TAG synthesis in the mammary gland and in other tissues and even more differences in ruminants. In MECs, TAG synthesis occurs in the

membranes of the ER and lipid droplets (Wilfling et al. 2013). As in other tissues, TAG synthesis requires glycerol-3-phosphate. All tissues, including the mammary gland, can form glycerol-3-phosphate from dihydroxyacetone phosphate, an intermediary in both the glycolysis and gluconeogenesis pathways. However, MECs express glycerol kinase, which can add phosphate at position 3 of glycerol (Rudolph et al. 2007). MECs are thus able to meet their high needs for glycerol-3-phosphate through the combination of intracellular synthesis and the uptake of glycerol from plasma (Chaiyabutr et al. 2002; Waghorn and Baldwin 1984). Acyl-CoA is supplied by one of various acyl-CoA synthetases, either ACSL or SLC27A (Mashek et al. 2007; Soupene and Kuypers 2008), which attaches coenzyme A to an FA. Glycerol-3-phosphate acyltransferase 4 (GPAT4), an internal ER membrane enzyme, combines an activated LCFA and glycerol-3-phosphate to yield lysophosphatidate (LPA) (Bionaz and Loor 2008; Coleman and Mashek 2011; Nagle et al. 2008). In addition to being an intermediary product in TAG synthesis, LPA acts as a signaling molecule with at least five distinct receptors, although its role in the mammary gland outside of glycerolipid synthesis is unknown (Choi et al. 2010). Synthesis of LPA by GPAT is thought to be the rate-limiting step of TAG synthesis, although this has not been confirmed in lactating mammary glands and appears to be both tissue- and isoform-dependent (Coleman and Mashek 2011; Nagle et al. 2008). *GPAT4* gene expression in the mammary gland increases approximately 15-fold during peak lactation but always remains relatively high even during the dry period (Bionaz and Loor 2008). Multiple reasons could explain this. It could be due to the importance of LPA as a signaling molecule (Woclawek-Potocka et al. 2014) and as an essential precursor to phospholipid synthesis or could be linked to the potential regulatory properties of GPAT4, which acts as a transcription factor (Liu et al. 2022). Research on the multiple roles of GPAT4, a relatively novel isoform, in the lactating mammary gland is currently lacking. The FAs incorporated at position 1 of milk TAG are most often C18 in early lactation, which are almost exclusively sourced exogenously to the mammary gland, followed by C16, which can be either of endogenous or exogenous origin, while both C18 and C16 have similar proportions in late lactation (Pacheco-Pappenheim et al. 2022). This is the position most likely to be occupied by LCFAs (Pacheco-Pappenheim et al. 2022). The next step in TAG synthesis is the transfer of a second acyl-CoA to the LPA via the 1-acylglycerol-3-phosphate acyltransferase (AGPAT), yielding a phosphatidate (PA). The AGPAT family contains at least 10 members, but only AGPAT1 to AGPAT5 are known to add an acyl chain to LPA, while AGPAT6 and AGPAT10 have been demonstrated to have GPAT activity instead (Cao et al. 2006; Chen et al. 2008), and AGPAT7, AGPAT8 and AGPAT9 have been shown to be more important for the synthesis of other lipid types, mainly cardiolipins and phospholipids (Agarwal et al. 2006; Cao et al. 2004; Ye et al. 2005). During lactation, AGPAT1 expression in the mammary gland is increased 5-fold, and AGPAT1 is the primary AGPAT isoform, accounting for more than 70% of total AGPAT expression. The expression of AGPAT3 increases twofold during lactation, whereas the expression of AGPAT2, AGPAT4 and AGPAT5 appears to remain relatively constant between lactation and dry periods (Bionaz and Loor 2008). These findings suggest that AGPAT2, AGPAT4 and AGPAT5 might have a constitutive role, whereas AGPAT1, and to a lesser extent AGPAT3, might be the main AGPAT enzyme responsible for milk fat synthesis. Position 2 of TAGs is most often occupied by a C16 FA and most of the C12–C17 FAs present in milk, with a nonnegligible proportion of unsaturated LCFAs, especially half of all linoleic acid, suggesting

that FAs esterified at position 2 are derived from both endogenous and exogenously sourced FAs (Pacheco-Pappenheim *et al.* 2022). For TAG synthesis, PA is then dephosphorylated by a phosphatidate phosphatase (PAP) to yield a diacylglyceride (DAG). There are three PAP isoforms, also known as lipins (LPINs) (He *et al.* 2017), which may play a similar role in lactation, but the precise role of each of these is not yet clear. A specific single-nucleotide polymorphism of LPIN1 at position 406 has been shown to cause a small but significant increase in milk fat percentage in Holstein–Friesian cattle (Du *et al.* 2021), suggesting that LPIN1 may be important for milk fat synthesis, although more research is needed to characterize its role as both an enzyme and a transcription factor in the mammary gland. The existence of multiple spliced variants of LPIN1 with antagonistic effects, with LPIN1- $\alpha$  increasing TAG accumulation and LPIN1- $\beta$  increasing  $\beta$ -oxidation of FA, also needs to be further explored in the mammary gland (Chen *et al.* 2015; Li *et al.* 2014; Reue and Zhang 2008). In the nonlactating mammary gland, the expression of LPIN2 and LPIN3 is 1.5- and 5-fold greater than the expression of LPIN1, respectively (Bionaz and Looor 2008). However, during lactation, LPIN1 expression is increased 20-fold, whereas LPIN2 and LPIN3 expression remain relatively constant (Bionaz and Looor 2008). These findings support the hypothesis that LPIN1 may be a major player in milk fat synthesis. Once dephosphorylated, DAG can serve as the final substrate in the TAG synthesis process. Another ER membrane-bound acyltransferase, diacylglyceride acyl transferase (DGAT), combines a third acyl-CoA with the DAG to form a TAG. There are two known DGATs and each appears to exhibit some preference in selecting a specific FA in the liver. DGAT1 prefers exogenously derived FAs, whereas DGAT2 favors endogenous FAs (Bhatt-Wessel *et al.* 2018; Chitraju *et al.* 2019). In MECs, inhibition of the expression of the main enzymes involved in *de novo* FA synthesis greatly reduces DGAT2 expression (Zhu *et al.* 2015), suggesting that substrate preference holds true in the mammary gland. Approximately 95% of the SCFAs found in milk TAGs are at position 3 on glycerol, indicating that they are incorporated into TAGs mostly by DGAT2 (Marai *et al.* 1969). There is also some evidence indicating that DGAT2 tends to associate with lipid droplets in nonmammary tissue, which could also explain the substrate preference, since FA synthesis occurs in the cytoplasm (Chitraju *et al.* 2019). DGAT1 has been shown to be essential for lactation in mice (Cases *et al.* 2004), which is not known for DGAT2, indicating that DGAT1 might be able to fulfill the same function as DGAT2 but not vice versa. DGAT1 polymorphisms in dairy cattle have also been shown to be important genetic determinants of milk fat composition (Mahmoudi and Rashidi 2023; Schennink *et al.* 2007). Taken together, these studies suggest that both DGAT1 and DGAT2 are responsible for synthesizing milk TAG from DAG, although DGAT2's contribution might be limited to esterifying shorter FAs, whereas DGAT1 appears to have a broader range of potential substrates.

### Polar lipid synthesis

Polar lipids are also a vital part of milk fat. In milk fat, the most abundant polar lipids are glycerophospholipids, mainly phosphatidylethanolamine (31.1–42.0% w/w), phosphatidylcholine (19.2–32.2% w/w), phosphatidylserine (2.8–8.5% w/w) and phosphatidylinositol (4.8–6.2% w/w), followed by sphingolipids, with sphingomyelin (17.9–29.6% w/w) being the most abundant (Bitman and Wood 1990; Fagan and Wijesundera 2004; Rombaut *et al.* 2005). Furthermore, trace amounts of other polar lipid species, such as lysophosphatidylethanolamine,

lysophosphatidylcholine and glycerophosphate ethers, are also found in milk fat (Murgia *et al.* 2003). Glycerophospholipids and sphingolipids represent 0.5–1% of total milk fat and are mostly found in the MFGM, although a fraction is also associated with the membrane residue of skim milk (Simopoulos 2022).

Phospholipid synthesis is crucial for the homeostasis of MECs because the excretion of MFGs results in extensive stress on both the plasma and ER membranes, and the polar lipids lost to the MFGM need to be replaced. Despite this importance, the regulation of phospholipid synthesis, as well as how phospholipid synthesis regulates other aspects of milk fat synthesis, has not yet been well established (Smoczyński 2017).

Phospholipid synthesis shares the first same steps as TAG synthesis, up until one of the AGPATs adds a second FA to an LPA to yield PA. For phospholipid synthesis, PA can be either metabolized to phosphatidylcholine or phosphatidylethanolamine or converted to a DAG by one of the PAPs. DAG can be further converted to different phospholipids, such as phosphatidylinositol. In contrast to TAG synthesis, which occurs mostly on the internal ER membrane, the phospholipid-specific steps are thought to occur mostly on the external ER membrane. A significant proportion of phospholipid synthesis, mainly the conversion of phosphatidylserine to phosphatidylethanolamine, also occurs in the mitochondrial membrane (Vance and Vance 2008). Sphingomyelin synthesis occurs in the Golgi apparatus by transfer of a phosphocholine from a phosphatidylcholine to a ceramide by sphingomyelin synthase (Tafesse *et al.* 2006). Ceramides are themselves produced by condensation of a serine with palmitic acid (16:0) in the ER (Menaldino *et al.* 2003). Phospholipid synthesis is poorly understood in the mammary gland and appears to be involved in the regulation of MFG size (Cohen *et al.* 2017), which is an area of milk fat synthesis that requires further research.

### Regulation of milk fat synthesis

Multiple molecular pathways regulate the expression of genes involved in milk fat synthesis in the mammary gland. The most important transcription factor related to lipid synthesis in the mammary gland may be SREBP (Shimano 2001; Wu *et al.* 2020). The SREBPs are transcription factors with various roles, including promoting the transcription of most lipid synthesis genes, such as *FAS*, *ACC*, *FATP* and *FABP* (Shimano 2001). They are sensitive to both activating signals from the serine/threonine kinase 1 (AKT1) pathway and deactivating signals from the AMP-activated protein kinase (AMPK) pathway. The AMPK pathway is most often activated during periods of negative energy balance (Eastham *et al.* 1988). As such, it tends to inhibit lipid synthesis via two mechanisms. First, it phosphorylates SREBP1, reducing its activity. Second, it inactivates ACC enzymes, preventing the synthesis of FAs (Hardie and Pan 2002). The AKT pathway is more anabolic. In addition to activating SREBP1, it also promotes the formation of lipid droplets, precursors to MFGs (McManaman *et al.* 2004). The other major transcription factor implicated in milk lipid synthesis is peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ). It is activated, among other things, by various FAs, thus increasing the availability of substrate for the regulation of lipid synthesis (Bravo-Ruiz *et al.* 2021). In the context of lactation, PPAR $\gamma$  is also dependent on LPIN 1 for activity (Finck *et al.* 2006). LPIN 1, a key enzyme in TAG synthesis, also acts as a cofactor for PPAR $\gamma$ . The expression of LPIN1 is highly upregulated during lactation, contributing to the high level of lipid synthesis (Bionaz and Looor 2008).



A thorough review of the regulation of the genes involved in milk fat synthesis was recently provided by Mu et al. (2021).

### The role of nonmammary tissue in milk lipid synthesis

In addition to the mammary gland, at least four other organs play important roles in determining the composition of milk fat. The rumen plays two important roles: (i) it supplies the acetate and butyrate required by the mammary gland for *de novo* FA synthesis and (ii) rumen microbes synthesize or alter FAs that eventually become incorporated into milk, such as numerous *trans*-FAs (Precht and Molkentin 1996) or odd-branched chain FAs. Some of these *trans*-FAs are physiologically relevant in that they can induce milk-fat depression (Griinari et al. 1998; Kadegowda et al. 2008; Shingfield and Griinari 2007). The small intestine plays a key role in supplying dietary and ruminal FAs to the mammary gland. The absorption of FAs via enterocytes and their packaging into TAGs and subsequent incorporation into chylomicrons is another important determinant of milk fat composition since chylomicrons are thought to provide approximately half of the LCFAs in milk (Palmquist and Mattos 1978). The liver is another important organ related to milk fat synthesis. It is the site of very-low-density lipoprotein synthesis (Gibbons et al. 2004). Moreover, the liver is an important site of phospholipid synthesis, although the contribution of liver phospholipids to milk fat is currently unknown (Ridgway 2021). It is also the main site of cholesterol synthesis, accounting for approximately 70% of total cholesterol (Kessler et al. 2014). In addition to being an important producer of phospholipids and sterols, the liver is also a site where *de novo* FA synthesis can occur (Bauchart et al. 1996). More importantly, the liver is a major site of synthesis of TAGs that are incorporated into the very-low-density lipoproteins from which half of the LCFAs in milk fat originate (Cozma et al. 2013). Last, adipose tissue is the other important site for supplying FAs for milk fat, although its importance is highly dependent on the energy balance of the animals (Miller et al. 1991).

### Conclusions

In this review, we summarized the biochemistry underlying the synthesis of milk fat, including the individual steps and key proteins (such as transporters and enzymes, Supplemental Table 1) involved in these steps. However, owing to advances in animal husbandry, nutrition and genetics, today's dairy cattle produce a much higher volume of milk than they did half a century ago. Because the partition of FAs in milk from either endogenous or exogenous origins to the mammary gland is dependent on energy balance and animal health, it might be relevant to re-examine their origins to confirm whether findings from half a century ago still hold true in modern practices. The mechanisms underlying the uptake of circulating LCFAs from lipoproteins into cells with the help of FATPs, as well as the intracellular trafficking of the LCFAs with FABPs remain largely unclear, not only for MECs but also for cellular biology in general. The subtlety of lipid droplets in MECs, including their budding in the ER membrane, migration through the cell and exocytosis in milk as MFGs, needs to be further explored, as does the importance of the synthesis of phospholipids in their regulatory roles in lipid droplets and their relationship with ER stress. Last, as many lipid metabolism enzymes come in multiple isoforms, there is still the need to characterize the role of each of these isoforms properly in the context of milk fat synthesis, particularly as some

of these enzymes appear to have dual roles as both enzymes and regulatory proteins.

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