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Physiology and Mechanism of Milk Fat Globules Secretion in Dairy Animals: A Review

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ABSTRACT

Milk fat globules (MFGs) are a complex compound secreted in the mammary gland, composed of triglycerides; phosphatidylethanolamine (PE) phosphatidylcholine (PC) with unclear mechanism and the fused lipid droplets enclosed by inner layer derived from the endoplasmic reticulum and outer bilayer is directly derived from the apical plasma membrane as the lipid droplets bud out from the cell. The core components of MFGs, triglycerides are synthesized via the de novo fatty acid pathway and LCFA was directly taken from serum. The de novo fatty acid pathway was significantly dependent on the enzymes acetyl-CoA carboxylase (ACACA), fatty acid synthase (FASN) and acyl-CoA synthetase short-chain family member 2 (ACSS2) to synthesize short and medium-chain fatty acids, whereas the bio-hydrogenation pathway is a principal pathway for the synthesis of long-chain fatty acids. Following this, the milk fat globules coated by the membrane are released from the MEC to the lumen with or without other milk components and the membrane that surrounds the fat globule stabilize the milk fat in its dispersed state, prevent flocculation and globule coalescence, and protects against the deleterious effects of lipases. Even though the secretion mechanism of lipid droplets is controversial hormonal and molecular mechanisms are involved. Moreover, the fat globule size and composition are determined by factors such as stage of lactation, physiological state of the animal, Diacylglycerol Acyltransferase1 (DGAT1) activity, and PC/PE ratio along with breed and animal species. While some studies were conducted on milk synthesis and secretion, the molecular mechanism behind lipid droplet fusion, secretion in the apical membrane and how MFGM is formed and the mechanism of MFG synthesis regulation are undiscovered. Therefore, it needs advanced investigation.

Key words: Fat globule secretion, Fat globule, Fat syntheses, Fatty acid, Fusion, Milk.

Milk is a fluid generated by the mammary glands of female mammals that serve as the newborn's principal source of sustenance. Fat, protein, lactose, vitamins, minerals, and water make up raw milk. Among these milk fat globules (MFG) is one of the components which are difficult to replicate in infant formulas due to its highly complex structure and variable composition. It is composed of a triglyceride core stabilized in an aqueous medium *via* a multilayer lipoprotein membrane called milk fat globules membrane and is generated in mammary epithelial cells via a complicated process (Lee *et al*., 2018).

The MFG's biosynthesis requires a lot of energy and it is the most important economic trait. The precursor for triglyceride synthesis (fatty acid) and then for MGs is derived from the breakdown of blood lipids or from de novo synthesis within the epithelial cell; de novo fatty acid synthesis is catalyzed in the cytoplasm by the enzymes acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). Then the formation of cytoplasmic lipid droplets (CLD) begins with the packing of triacylglycerols (TGs) into micro lipid droplets and from the endoplasmic reticulum of mammary gland alveolar epithelial cells and are surrounded by a phospholipid monolayer. The CLD fuses with the plasma membrane and adds a peripheral bilayer containing a range of bioactive proteins and lipids when it migrates to the epithelial cell's apical pole (Truchet and Honvo-Houéto, 2017). The membrane covered full-fledged MFG (MFGM) is subsequently secreted outside the cell and becomes part of the milk that the newborn consumes (Truchet and Honvo-Houéto, 2017; Smoczyński, 2017).

Although substantial research has been performed over the last two decades in order to better understand the basic mechanisms that enable MFG synthesis and secretion, the intracellular mechanisms remain unknown (Argov *et al*. 2008), and the molecular mechanisms underlying droplet fusion in mammary cells also have received little attention. Therefore, this review is designed to discuss the evidence on the mechanisms of fatty acid synthesis, intracellular mechanisms and molecular control of milk fat globule secretion.

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Overview of milk fat secretion mechanism

Milk fatty acid synthesis

The precursors required for fatty acid synthesis (glycerol and other fatty acid substrates) can be made from scratch or transferred from the blood lipids (Neville and Picciano 1997). Key metabolic features of mammary lipid metabolism, including *de novo* synthesis and FA absorption from the blood, were involved to produce these precursors and synthesize TG; these have been described and quantified in the previous two decades (Bauman *et al*., 2006). While the application of advanced biotechnology has revolutionized our understanding of the molecular mechanism of milk fat synthesis in the mammary gland, the formation of a nascent TG droplet in the rER membrane is poorly understood. Thus, scientists suggest a few hypotheses, including TG production within the rER's eggcup-shaped infolds (Robenek *et al*., 2006), early budding of nascent lipid droplets into the rER lumen (Choudhary *et al*., 2011), and bicelle formation via membrane bilayer scission (Choudhary *et al*., 2011) and bicelle formation through scission of the membrane bilayer (Ploegh, 2007). The schematic diagram below indicates the source of milk fatty acids (Fig 1A) and the process of uptake from the blood vessels (Fig 1B).

According to Palmquist (2006) esterification of the fatty acids at the *sn-1* and *sn-2* sites are carried out to form phosphatidic acid, which is then dephosphorylated to form diacylglycerol. When a third fatty acid is added to the *sn-3* position, a variety of TG is produced, each of which can have various acyl chains at each of the three places. On the other hand, TG synthesis mechanism in cells except mammary gland (intestinal and other tissues) is stepwise esterification of *sn-2*-monoacylglycerol at the *sn-1* and *sn-3* locations. Following this a group of proteins called acyl-CoA synthetase 3, FIT2, seipin, ARF1, COP1/coatomer, plin2 (adipophilin), plin3 (TIP47) and rab18 play a role in lens development and lipid droplet extrusion, as well as helping to consolidate the final structure (Kassan *et al*., 2013; Choudhary *et al*., 2015; Walther *et al*., 2017). Researchers agreed that this fundamental mechanism appears to be conserved and it is the prevailing paradigm for lipid droplet production on the cytoplasmic face of the rER in most cells from yeast to mammals (Szymanski *et al*., 2007; Choudhary *et al*., 2015; Walther *et al*., 2017).

While short-chain and medium-chain length fatty acids, from 4:0 to 14:0 and some 16:0 are synthesized through de novo synthesis, the C18 fatty acids and some 16:0 arise from the plasma lipids or dietary lipids. The bio-hydrogenation pathway is a principal pathway for the synthesis of long-chain fatty acids (Fig 2). These fatty acids are directly derived from the diet and some would be released from adipose tissues. Linoleic acid (9c, 12c-18:2) and linolenic acid (9c, 12c, 15c-18:3), glycolipids, phospholipids and triacylglycerol are among the main lipid and fatty acids originated from dietary lipids. The non-esterified fatty acids are produced through hydrolyzing dietary lipid in the rumen and then subjected to extensive bio-hydrogenation by microorganisms (Jenkins 1993). The process of bio-hydrogenation sequence starts with an isomerization of linoleic acid to produce conjugated linoleic acid (9c, 11t-18:2).

The conjugated linoleic acid is then used to produce vaccenic acid (11t-18:1) *via* a reduction reaction, followed by another reduction to 18:0. Linolenic acid bio-hydrogenation follows a similar pathway (Lee and Jenkins, 2011). The fatty acid mixture produced by bio-hydrogenation is esterified to triacylglycerols, which then circulate in the bloodstream as chylomicrons. The mammary gland absorbs these triacylglycerols and cleaves them to produce non-esterified fatty acids. The mammary gland contains a desaturase system that converts substantial amounts of 18:0

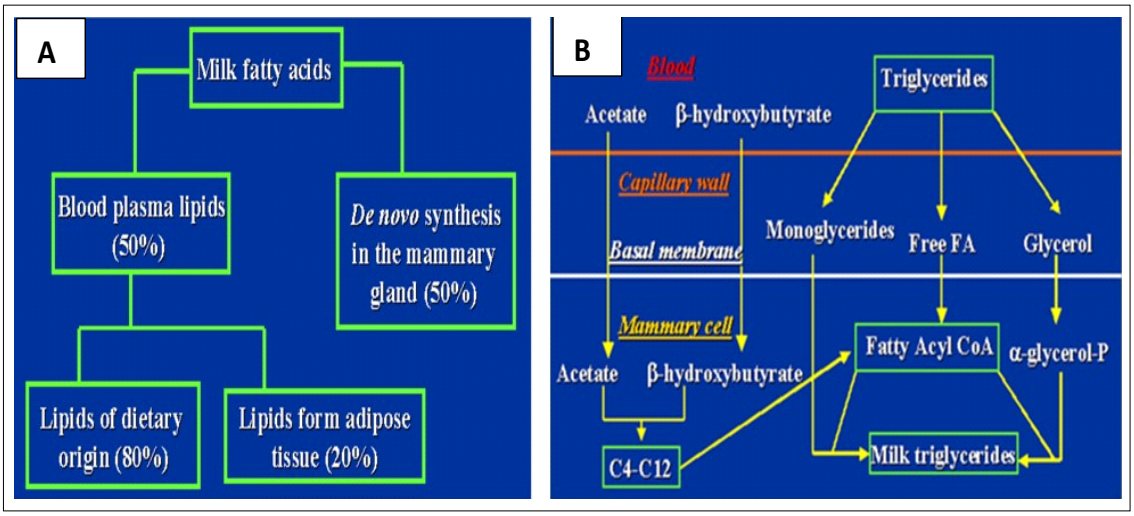

Fig 1: A) Source of milk fatty acids, B) Process of fatty acid uptake form blood.

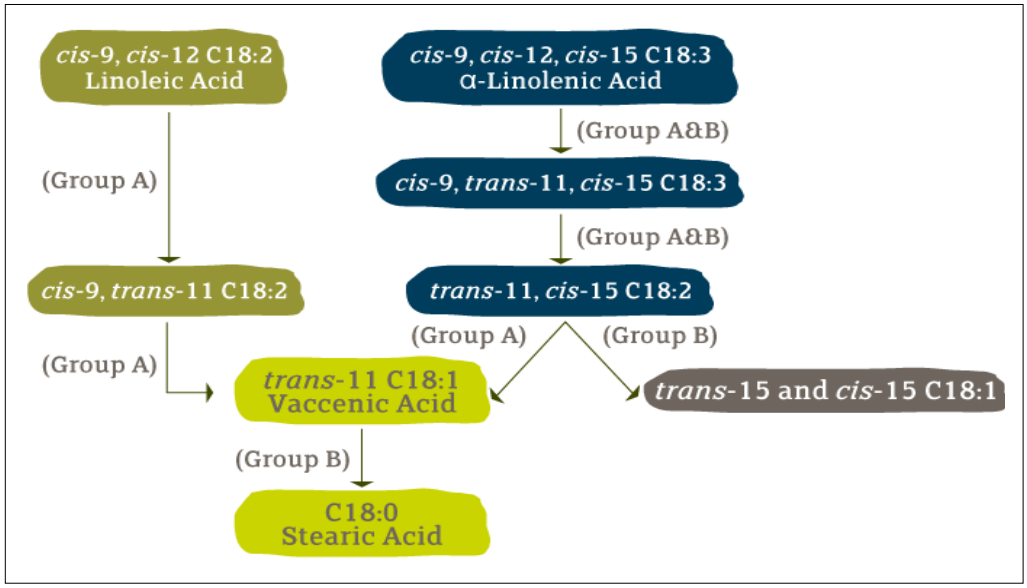

Fig 2: Bio-hydrogenation process.

to oleic acid (9c-18:1) (Lee and Jenkins, 2011). As a result of these processes, the fatty acids in the mammary gland that originate from dietary lipids contain significant amounts of 16:0, 18:0 and oleic acid, small amounts of linoleic and linolenic acids and limited amounts of other monoenoic and dienoic fatty acids such as 11t-18:1 and 9c, 11t-18:2. The process is indicated in the diagram below:

Milk fat synthesis in *de novo* pathway

De novo means "from the beginning" in Latin. Thus, de novo lipogenesis is the process by which fatty acids are synthesized from acetyl-CoA (Diagram 3). This process takes place in the cytoplasm after the Acetyl-CoA exodus from the mitochondria and is converted to malonyl-CoA (3 carbons). The malonyl-CoA is then combined with another acetyl-CoA to form a four-carbon fatty acid (1 carbon is given off as CO_2) (Gropper *et al*., 2008). As a result, short-chain and medium-chain fatty acids are synthesized in the mammary gland (Cowie *et al*., 1951). In FA de novo synthesis, acetic acid (acetate) and β-hydroxybutyric acid (BHBA) penetrate the cell membrane *via* passive diffusion, which is less than the flip-flop and protein-mediated uptake of LCFA (Bionaz and Loor 2008). During lactation, the enzymes acetyl-CoA carboxylase (ACACA), fatty acid synthase (FASN) and acyl-CoA synthetase short-chain family member 2 (ACSS2) were highly up-regulated and the de novo synthesis route of FA was dependent on these three major enzymes (Lee *et al*., 2017). ACSS2 activates acetic acid and BHBA in MECs and converts them to acetyl-CoA by binding to pyruvate. The enzyme ACACA carboxylates acetyl-CoA to create malonyl-CoA and FASN (carried out in the cytosol) synthesizes medium-chain fatty acids (MCFA) or LCFA (Laliotis *et al*., 2010).

Above and beyond, FASN is made up of seven domains: b-ketoacyl synthase (KS), acetyl/malonyl-CoA transferase (MAT), b-hydroxyacyl dehydratase (DH), enoyl reductase (ER), β-ketoacyl reductase (KR), acyl-carrier protein (ACP) and thioesterase I. The core region between the DH and ER domains lacks catalytic activity. Initially, the acyl moiety of acetyl-CoA (initiation substrate) is transferred to the ACP catalyzed by MAT to generate malonyl-CoA (elongation substrate). The acyl moiety is then briefly transferred to KS and MAT catalyzes the transacylation of malonyl-CoA to ACP. Acetoacetyl ACP is then produced *via* decarboxylative condensation catalyzed by KS. This process is catalyzed by KR and DH, which are responsible for the NADPH-dependent reduction of the β-carbon and the dehydration of b-hydroxyacyl-ACP to a β-enoyl, respectively. The ER then catalyzes the formation of a four-carbon acyl chain from the NADPH-dependent reduction of the enoyl. The malonyl-CoA is used as a two-carbon unit in the subsequent elongation cycles. Then, TE I is responsible for the release of a 16-carbon fatty acid from ACP (Asturias, 2006).

Despite the fact, the length of fatty acid chain synthesized in the mammary gland is different among animal species, FASN and TE II is played a significant role in the formation of all types of fatty acids containing C8, C10, C12 and so on (Grunnet and Knudsen, 1978). As a result, animal, they cannot synthesize medium-chain fatty acids *e.g*., rabbit, mouse and pig can synthesize and de novo synthesized medium-chain fatty acids can be incorporated directly into triacylglycerol without the need for an activation step (Knudsen *et al*., 1981).

Moreover, Urrutia and Harvatine (2017), states that acetate and BHBA had multifunctional molecules for ruminants, as primary substrates for the *de novo* synthesis of FA, an energy source for meeting energy requirements (70 per cent) and milk fat synthesis in dairy cows and play an important positive regulatory role in milk fat synthesis through the mTOR/eIF4E signaling pathway in BMECs (Zhao *et al*., 2021). Finally, the TG accumulates and they

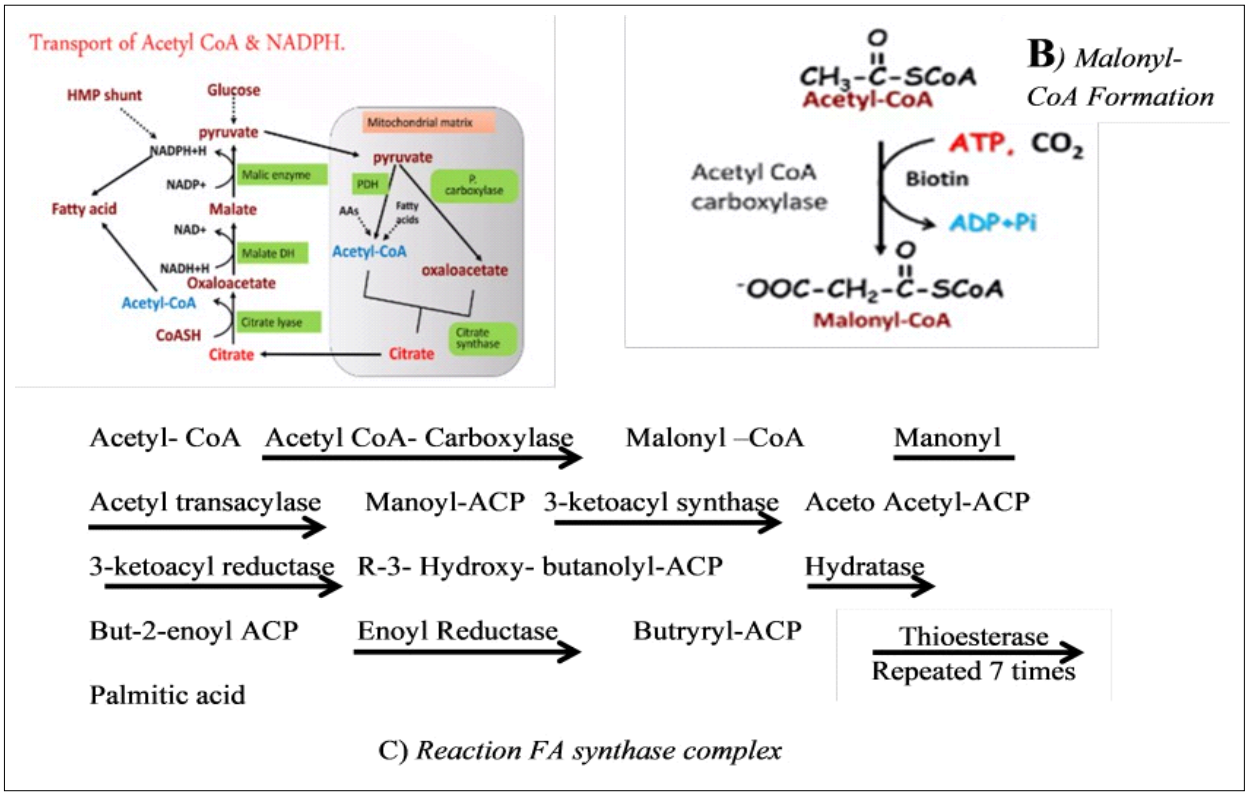

Fig 3: Steps of *De Novo* fatty acid synthesis.

begin to protrude out into the cytoplasm in the form of a micro-lipid droplet, which is coated with a monolayer of phospholipids derived from the exoplasmic leaflet of the rER bilayer (Tauchi-Sato *et al*., 2002).

In general, *De Novo* FA synthesis occurs in three phases (Fig 3, A, B and C), these are transport of acetyl-CoA from the mitochondria to cytosol, carboxylation of acetyl-CoA to malonyl CoA and reaction of FA synthase complex (priming reaction and elongation). These processes are summarized in the diagram below:

Milk fat globule synthesis/assembling

Triacylglycerols, (the intracellular precursors of milk fat globules) the major lipids of milk, are secreted as droplets of variable sizes in the endoplasmic reticulum. The accumulation of neutral lipids (mostly triacylglycerols (TG)) in the ER initiates the synthesis of MFGs within the mammary epithelial cell (Heid and Keenan, 2005, Lopez, 2011). A study conducted on mice revealed the movement of lipid droplets from the basal membrane to the apical membrane with variable speed and then growing by progressive fusions in route, especially at the plasma membrane and in the apical cytoplasm (Mather *et al*., 2019). In addition, lipid droplets are surrounded by a viscous cytoplasm (Luby-Phelps, 2000), which is densely packed with cytoskeletal elements, organelles and many vesicles, some of which may interact with the droplets while in transit.

Moreover, the fused micro-lipid droplets migrate through the cytoplasm of mammary gland epithelial cells from the basal to the apical pole and then the cytoplasmic lipid droplets are engulfed by the apical plasma membrane, resulting in the creation of a unique complex MFGM loaded with diverse bioactive proteins, lipids and other functional molecules (Argov-Argaman, 2019). In the formation of MFGM first, a single monolayer of phospholipids from the ER membrane coats the triacylglyceride core and then the outer bilayer of proteins (Bauman and Griinari, 2003) and polar lipids from the apical plasma membrane of the mammary gland epithelial cells surrounded the MFGs to form the outer bilayer milk fat globule membrane (MFGM). Evidence from different findings reveals that MFGM membrane is made up of the lipids and proteins found in the plasma membrane of epithelial cells, including considerable amounts of cholesterol, phosphatidylcholine and sphingomyelin (Bauman and Griinari, 2003; German and Dillard, 2006). Likewise, the produced triglycerides in the inactive lipid phase are bordered by a monolayer of ER phospholipids and discharged into the cytoplasm as a spherical lipid particle are the main milk fat globule assembling steps in the mammary epithelia (Heid and Keenan, 2005). However, the formation of outer membranes is least understood, what is happens after milk fat globules form and it is unclear whether this process is unequivocally lead by cellular processes or if the globules spontaneously self-assemble based on the composition of the respective surfaces (German *et al*., 2019). Similarly, much speculation has surrounded how the MFGM's outer bilayer membrane becomes permanently

connected with lipid droplets in the cytoplasm (Jeong *et al*., 2013).

In spite, the origin of these outer bilayers are still controversial, researchers believed that the components of the MFGM outer bilayer may come from other membrane structures and organelles within the cytoplasm, such as vesicles with no obvious content in electron micrographs (Wooding, 1971; Wu *et al*., 2000; Mather *et al*., 2001) and secretory vesicles that contain casein micelles and many other skim milk components (Wooding, 1971; Wu *et al*., 2000). The majority of evidence in the literature suggests that the outer membrane layer of MFGs is acquired from cytoplasmic organelles, allowing access to the secretory pathway for subsequent release *via* exocytosis (*e.g*., coronavirus MHV-A59, Tooze *et al*., 1984) or at the cell surface, buds directly from the cell coated with the plasma membrane (Rodriguez Boulan and Sabatini, 1978). The second option is supported by a recent study of mice using intravital images, which found that their growth happens slowly at the apical surface over several hours, even as they bulge out into luminal regions (Masedunskas *et al*., 2017).

The milk fat globules enveloped by the membrane are released from the MEC to the lumen with or without other milk components. Finally, the membrane that surrounds the fat globule stabilizes the milk fat in the dispersed state, prevents flocculation and coalescence of globules, as well as protects against deleterious effects of lipases (Smoczynski, 2017).

Milk fat globule development and size control mechanisms

The intricate orchestration of milk fat globule (MFG) development and the mechanisms that control their size remain a key topic in the dairy industry, as this complex relationship is critical to nutritional and technological considerations. Recent studies have shed light on the dynamic interplay between biological and physicochemical factors that determine MFG formation. These include membrane-associated proteins such as MFGM, which play a multifaceted role in the stabilization and modulating MFG size. Lipid composition and enzymatic activities during mammary gland secretion also contribute to the fine-tuning of MFG size. Although the development and control mechanisms of MFG size in the mammary gland epithelial cell are not yet fully understood, research into these complex relationships provides insights that are essential for improving the nutritional quality and technological applications of dairy products. For instance, Argov-Argaman (2019) believed that the intracellular LD size the regulation is governed by three main mechanisms. Firstly, the concentration of triacylglycerol within a cell is linked to LD size, as demonstrated in Drosophila larval fat bodies (Krahmer *et al*., 2011). This concentration is determined by Tg synthesis, hydrolysis and the availability of phospholipids, which can also modulate LD size. Lipid droplets can grow through Tg incorporation from the endoplasmic reticulum and the proximity and biochemical link between lipid droplets and mitochondria can influence lipid droplet size. The extent of Tg hydrolysis also regulates lipid droplet size (Eynaudi *et al*., 2021). Secondly, the availability of Pl may directly impact LD size, as indicated by genetic interventions that reduced Pl-synthesis genes resulting in enlarged LD in various organisms. Lastly, fusion between LDs is a significant pathway for regulating LD size. This was demonstrated in a study where the percentage of LD engaged in fusion increased with oleic acid treatment compared to palmitic acid treatment (Cohen *et al*., 2015; Cohen *et al*., 2017).

Recent finding by Walter *et al*. (2020) revealed that phosphatidylethanolamine (PE) and phosphatidic acid, which have smaller head groups, are projected to increase LD fusion. The authors suggested that a large amount of DG is diverted towards TG synthesis, Diacylglycerol Acyltransferase 1 (DGAT1) activity increases, diacyl-PL synthesis is reduced, however, the synthesis ePL by CEPT in the ER does not require DG as a precursor and it could provide a viable rescue pathway. As a result, the fusion of LD to form a large MFG (LMFG) phenotype was initiated (Fig 4).

On the contrary, the small MFG (SMFG) phenotype is produced when DG is efficiently channeled towards PL synthesis, either due to lower DGAT1 activity or maybe due to enhanced SM synthase 1 (SMS1) activity in the Golgi apparatus (Deevska *et al*., 2017). Additionally, the SMFG phenotype is produced by phosphatidylethanolamine N-methyltransferase (PEMT) pathway, the precursor of this pathway (PE) produced in the mitochondrion through is decarboxylation phosphatidylserine (PS) by PS decarboxylase (PSD) and the PC/PE ratio increases because PE converts into PC. The amount of PC rises with a high degree of unsaturation and surrounds the LD. The higher ratio of PC/PE inhibits the aggregation of LD; therefore, the SMFG phenotype is synthesized. Likewise, Thiam *et al*. (2013) believed that the cylindrical shape of phosphatidylcholine (PC) prevents LDs fusion and later Cohen *et al*. (2017) proved that polar lipid content of the LD monolayer regulates the LD fusion in mammary epithelial cells, despite the fact that most of the existing information is based on investigations in non-mammary cells. The synthetic and expansion, lipid droplets can grow by coalescing with one another (Boström *et al*., 2005; Walther and Farese, 2012; Barneda *et al*., 2015); intravital data on mice indicate that this is a major mechanism for LD growth in mammary epithelial cells, at least for droplets ≥0.8 nm.

Furthermore, the individual MFGs' final size is regulated by one of two growth methods within the mammary epithelial cell: either local TG production by enzymes in the monolayer surrounding the LD or LD fusion (Wilfling *et al*., 2013; Masedunskas *et al*., 2017). The specific mechanisms behind LD fusion are unknown; however, they may be influenced by the monolayer's PL and fatty acid (FA) composition. Additionally, MFG size varies between

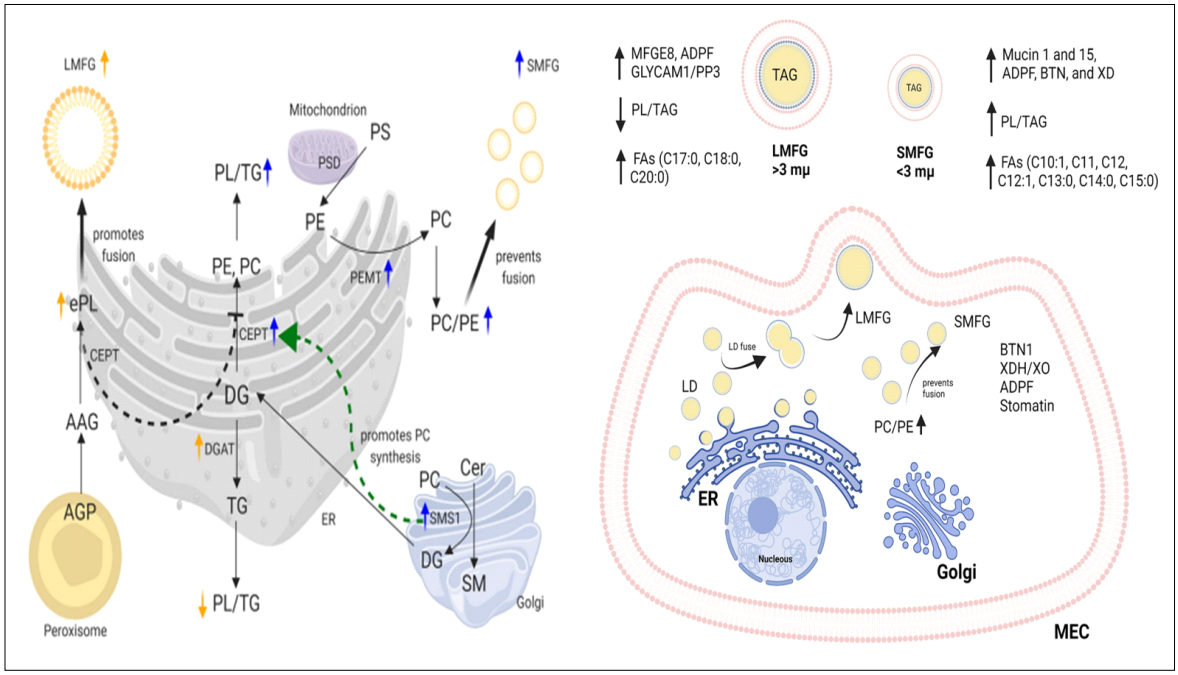

Fig 4: Possible mechanism and metabolic pathways of milk fat size development (Adapted from Walter *et al.,* 2020).

mammalian species and breeds of the same species (Carroll *et al.,* 2006; El-Zeini, 2006), but also between cows of the same breed (Logan *et al*., 2014; Couvreur *et al*., 2007) MFGs in cow's milk range in size from less than 1 m to 15 μm (Michalski *et al*., 2001), with an average size of 2.5-5.7 μm in a herd of Holstein-Friesian cows (Logan *et al*., 2014).

Likewise, the average MFG size varies throughout the lactation cycle based on the number of days in milk since the last calving, as well as throughout the year in response to diet changes (Fleming *et al.,* 2017). Fat content (Wiking *et al*., 2004), fat-to-protein ratio (Couvreur and Hurtaud, 2017) and milking period (Fleming *et al.,* 2017) have also been proposed as influence factors on MFG size. MFG size is also influenced by the animal's physiological state (Fleming *et al.,* 2017; Altenhofer *et al.,* 2015) and the FA composition of milk from cows that produce small or large MFGs can vary (Couvreur *et al*., 2007; Couvreur and Hurtaud, 2017). The magnitude of variation of MFG size due to physiological state of animal's and milk production traits on MFG is less than the overall variation seen between individual animals (Walter *et al.,* 2019).

Generally, it has been widely believed that a variety of metabolic pathways are involved in the development of milk fat globule size (MFGs) in mammary epithelial cells through a variety of processes that influence MFGs. Based on the literature available currently, the following diagram was developed to offer speculative explanations for the observed variations in milk lipidomic profiles between study groups.

Milk fat globule secretion

A lipid droplet (LD), the intracellular precursor of the MFG, is formed and discharged from the ER, enclosed by a single layer of polar lipids and membrane proteins. Lipid droplets are secreted through an outwardly unique mechanism and progressive envelopment of plasma membrane up to the point where the lipid droplet is released from the cell after completely surrounded by plasma membrane (Hans *et al*., 2005). According to the pioneer researchers of this process, Bargmann and Knoop (1959), milk fat droplets are attached to the apical plasma membrane and are gradually enveloped in the plasma membrane until they are dissociated from the cell and completely surrounded by the plasma membrane.

Afterwards researchers believed that the final release of lipid droplets from the cell was a continuous process coordinated with the constitutive release of skim milk components (*e.g.*, water, ions, lactose, casein and other proteins) from secretory vesicles via exocytosis at the apical surface (Mather *et al*., 2019). Findings revealed that major skim milk components accumulate in luminal spaces between milk let-downs, however based on the data obtained from a 2-dimensional histological section of fixed tissue, lipid droplets are also part of this fluid. The Confocal microscopy of 3-dimensional reconstructions of intravital images confirmed that that the majority of lipid droplets observed in2-dimensional scans are linked with cells below or above the plane of section (Mather *et al*., 2019).

In contrast, this lipid droplet secretion process was not observed in a recent study of mice using intravital microscopy and there is no evidence of "free-floating" secreted droplets in luminal spaces (Masedunskas *et al*., 2017). Moreover, the secretion of LD is an intensive process governed by a signal or secretagogue like secretion of proteins and fluid constituents from other exocrine glands (McManaman *et al*., 2006).

Several researchers believed that the contraction of the actin cytoskeleton is responsible for the ejection of lipid droplets. This suggestion is supported by Franke *et al*. (1981) Immunocytochemical studies; he discovered an association of actin with budding lipid droplets. However, Mather *et al*. (2019) reports a different result and conclude that there is no evidence for actin on the cytoplasmic face of budding lipid droplets. Regardless of these ambiguities, the potential role of actin in regulating the final assembly and secretion of lipid droplets at the apical surface requires further investigation using intravital imaging and other techniques.

Moreover, the identities of the cellular components involved in MEC lipid secretion pathways are still unknown and several models are proposed to understand is miracle. Based on the data from tripartite model proposes for MFG secretion from the MEC is facilitated by interactions between three major MFGM proteins: adipophilin (perilipin-2, ADPF), xanthine dehydrogenase/oxidase (XDH/XO) and butyrophilin (BTN). As observed in the model, BTN, XDH/XO and ADPF form a complex on the surface of the fat globule, facilitating bilayer membrane adsorption to the LDs. This complex causes LD membrane deformation and LD budding from the secretory cell (Mather and Keenan, 1998). The BTN/XDH/XO ratio, which is constant across breeds (McManaman *et al*., 2007), varies by species and shows associations with fat content, MFG size and MFG secretion.

Furthermore, Robenek *et al*. (2006) develop a model of lipid secretion from the MEC that challenges the former model. It states that cytoplasmic LD release into milk is exclusively facilitated by BTN homotypic interactions. MEC lipid secretion involves a variety of cellular components. The ADPF C-terminal domain has been shown to bind to the apical plasma membrane (Chong *et al*., 2011). Other models have been discovered in lipid droplets found in adipose (Hörl *et al*., 2011) and liver cells (Stringer *et al*., 2010).

Another complementary model of MFG secretion has been proposed, with casein balancing membrane loss caused by MFG release. Because, Caseins have long been known to be synthesized, transported and released via the secretory pathway via the fusion of casein-containing vesicles with the apical plasma membrane and the soluble N-ethylmaleimide-sensitive fusion attachment protein receptor (SNARE) (Honvo-Houéto *et al*., 2016). While BTN1, PLIN2 and XOR are likely to contribute to MFG budding, attachment to SNARE proteins may connect secretory vesicles not only together, but also with the budding MFG and the apical plasma membrane via the formation of SNARE complexes. As a result, SNARE proteins could promote both casein exocytosis and the connection of the endoplasmic reticulum with the apical plasma membrane, thereby providing a membrane to enwrap the budding MFG (Honvo-Houéto *et al*., 2016) (Fig 5).

The last step of MFG release possibly takes place after the homotypic fusion of the secretory vesicles surrounding the MFG rather than by final scission of the plasma membrane at a budding neck (Honvo-Houéto *et al*., 2016). If this proposed model is correct, the combined release of MFGs and casein micelles from the MEC may contribute to the presence of casein micelles selectively adsorbed onto the native MFGM (Luo *et al*., 2014). Independent of the model, the proteins BTN1, PLIN2, XOR and stomatin appear to play a significant role in MFG secretion, but research on the relationship between protein abundance and MFG size across species is still lacking.

Estimating the amount of lipid associated with the cell surface in groups of GFPcyto mice either immediately euthanized for analysis or after separation from their litters for 4 hours confirmed that lipid droplets accumulate at the apical surface between milk let-downs. Single oxytocin injections removed accumulated lipid from the apical surface. Thus, oxytocin-induced myoepithelium contractions stimulate the release of lipid droplets from the cell as well as previously secreted milk components are ejected from luminal spaces. In several species, including dairy cows, (Lollivier *et al*., 2002), goats (Lollivier *et al*., 2002) and humans (Emery *et al*., 1978), oxytocin-mediated control of lipid droplet secretion may be a common mechanism that explains why the fat content of milk gradually increases as more milk is removed from the gland. Contractions of the myoepithelium induced by oxytocin presumably stimulate mechanical secretion of lipid droplets from the apical surface of epithelial cells, though this may not be the only mechanism, particularly for droplets that are still partially embedded in the cell.

Composition of milk fat globule (MFG)

The milk fat globules hold multifarious configurations and structures which have many properties (Argov *et al*., 2008). MFGM is a thin membrane which encircles around the globules derived from the apical membrane of the lactating cells(Bianchi *et al.,* 2009) and MFGs membrane mass varies from 2-6% of total mass of fat globules (Lopez, 2010). Several research results and literature revealed that proteins, lipids, enzymes, glycoproteins are major components of MFGM and also other minor components were present (Danthine *et al*., 2000).

Recent findings reported that the proteins and lipids; Phospholipids, glycolipids and sphingomyelin (SM) originated from the endoplasmic reticulum and the apical membrane of secreting mammary epithelial cells (MEC). “The surface-active inner monolayer, which surrounds thetriglyceride core, is composed of polar lipids derived from the endoplasmic reticulum” (Thum *et al*., 2022). When investigated via electron micrographs the central layer seems denser and contains proteins. According to Mather and Keenan (1998) the polar lipid outer bilayer membrane originates from secretory regions of the apical plasma membrane of the MEC. Moreover, cholesterol molecules and lightly attached proteins and transmembrane proteins are present in the external layer (Gallier *et al*., 2011).

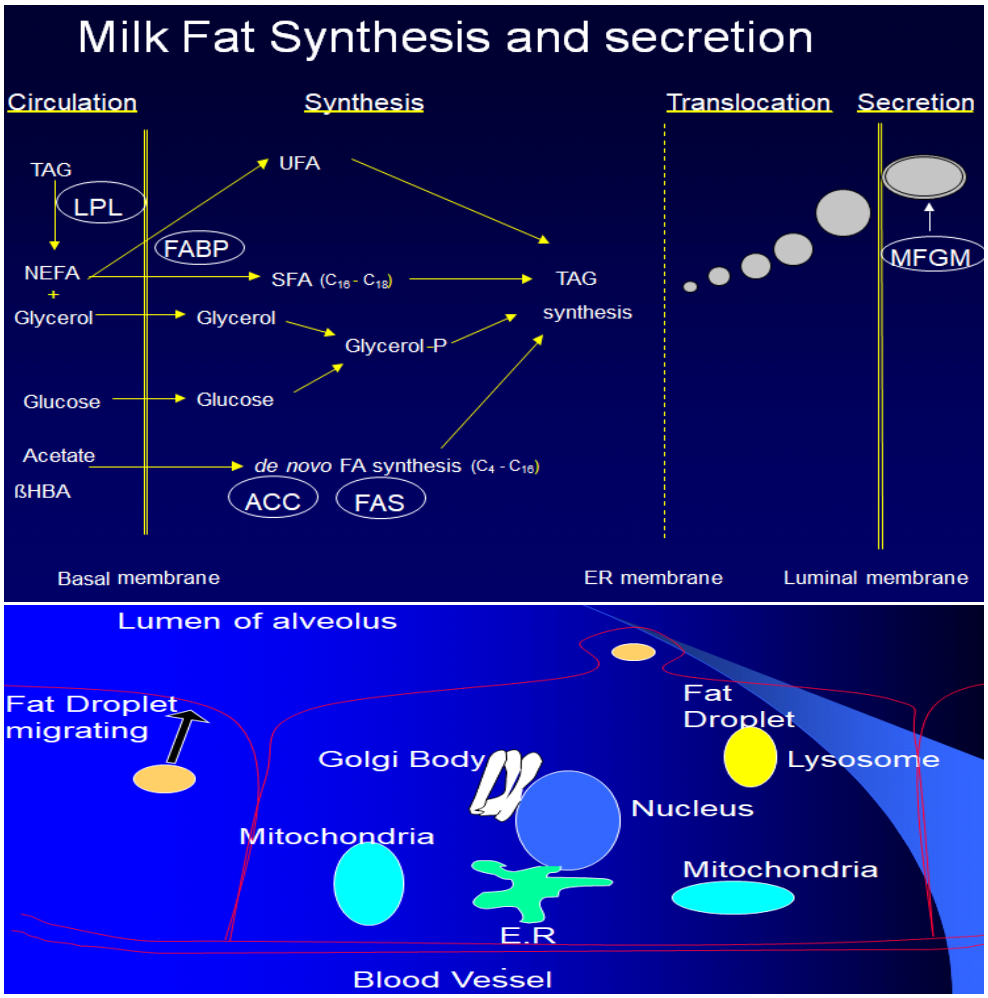

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Fig 5: Milk fat secretion mechanism from the basal and luminal membrane to alveoli.

Carbohydrate domains glycoproteins oriented into the surrounding aqueous phase are also present on the surface (Lopez *et al*., 2010). Unequal distribution of MFGM polar lipids appear because, phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) are concentrated at the inner surface and phosphatidylcholine (PC) and SM are mainly located in the outer layer of the membrane (Thum *et al*., 2023).

Regulation of milk fat synthesis

Nowadays several studies are carried out for better understanding of the molecular mechanisms that can regulate milk fat secretion. However, the regulation of lipid secretion in the mammary alveolar cells is not fully understood yet due to a lack of a suitable model system and potential difficulties distinguishing effects on biosynthesis from effects on secretion. For example, in exocytotic pathway, the importance of glucose in both de novo fatty acid synthesis and lactose synthesis in non-ruminants, hormonal regulation of glucose uptake mechanisms may affect the amounts of both materials available for secretion in humans significantly (McManaman and Neville, 2003). According to Flint and Gardner's, (1994), study on rat different hormones control lactose and lipid secretion, reducing prolactin with bromocriptine significantly reduced lactose concentration while increasing lipid concentration in milk (Flint and Gardner, 1994). The reason for this discrepancy has not been determined, but the findings suggest that the exocytotic and lipid secretion pathways may be under different hormonal control.

According to available resources in literatures, the synthesis and secretion of milk fat globules are regulated by hormonal, molecular and environmental factors. Nutritional regulation of milk fat synthesis is accomplished by either interrelationship between rumen microbial processes and tissue metabolism (milk fat depression) or alterations in rumen bio-hydrogenation of dietary polyunsaturated fatty acids and a specific inhibition of

mammary synthesis of milk fat (Bauman *et al*., 2008). While researchers proposed numerous theories to explain the mechanism of milk fat depression, investigations provide little support for theories because of limitation in the supply of lipogenic precursors. In the later one rumen bio-hydrogenation pathways are altered to produce unique fatty acid intermediates; Trans-10, cis-12 conjugated linoleic acid (CLA) that inhibit milk fat synthesis under certain dietary conditions. Besides findings reveal that pure isomers of trans-10, cis-12 CLA are effective inhibitor of milk fat synthesis and also it is responsible for coordinated reduction in mRNA abundance for key enzymes involved in the biochemical pathways of fat synthesis; diet-induced MFD involves similar mechanism. The findings show that sterol response element-binding protein 1 and Spot 14 play important roles in translational regulation (Bauman *et al*., 2008). Furthermore, Harvatine *et al*. (2009) reported that sterol response element-binding protein 1 (SREBP1) and SREBP-activation proteins are down-regulated during MFD. Importantly; SREBP1 regulates transcription of key lipogenic enzymes.

Similar with nutrition, there are numerous transcriptional regulators that control milk fat synthesis in ruminants. Lipolysis at the MEC membrane, FA uptake from blood, intracellular FA transport, de novo FA synthesis, FA and glycerol activation, FA elongation, FA desaturation, triglyceride synthesis, cholesterol synthesis and lipid droplet formation are all regulated by these regulatory transcriptional or genes. Therefore, ACSS2, FASN, ACACA, CD36, ACSL, SLC27A, FABP3, SCD, GPAM, AGPAT, LPIN, DGAT1, PLIN2, XDH and BTN1A1 are key genes involved in ruminant milk fat synthesis (Mu *et al*., 2021).

CONCLUSION

Milk fat globules originate as small, triacylglycerol rich droplets that are formed on or in endoplasmic reticulum membranes via de novo synthesis or and directly taken from the blood. These droplets are released from endoplasmic reticulum into the cytosol as micro-lipid droplets coated by proteins and polar lipids. The lipid droplets can fuse with each other to form larger cytoplasmic lipid droplets. The lipid droplets can join together to produce larger cytoplasmic lipid droplets and transported to apical plasma membrane, however the mechanism how the lipid droplets transported is still unidentified and are secreted from a cell covered with an outer bilayer. The apical mechanisms, in which lipid droplets are enveloped by the apical plasma membrane and the secretory-vesicle mechanism, in which fat droplets are surrounded by secretory vesicles in the cytoplasm and are released from the surface via exocytosis from intracytoplasmic vacuoles, are two possible mechanisms for lipid secretion. The lipid secretion in the mammary alveolar cells is regulated by coordinated activation mechanisms that determines the availability of fatty acid and glycerol substrates for de novo fatty acid and glycerol synthesis, whereas the molecular level regulation is not explored yet. Therefor extensive study on milk fat synthesis, secretion and formation of fat globule membrane and molecular level regulation of fatty acid synthesis is required.

Conflict of interest

The authors declare that there is no conflict of interest.

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