Association between fatty acid biomarkers of dairy fat intake and insulin sensitivity

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ABSTRACT

Recent epidemiological studies have shown that greater dairy intake is associated with lesser risk of type 2 diabetes (T2D). This inverse association is particularly strong when dairy-specific fatty acids (FA) are used as biomarkers of dairy fat intake, including: pentadecanoic (C15:0), heptadecanoic (C17:0) and *trans*-palmitoleic acid (*trans*-C16:1n-7). One of the defining characteristics of T2D is the reduced sensitivity of tissues to the action of insulin. The objective of this study was to examine the association between biomarkers of dairy fat intake and insulin sensitivity in a cohort of individuals (n = 42) who underwent a 3-hour 40 mU/m^2/min hyperinsulinemic, euglycemic, isoaminoacidemic clamp. Glucose rate of disposal (Rd), glucose rate of appearance (Ra), M/I index, and the Hepatic IR index were taken as measures of insulin sensitivity. Body composition was measured by dual-energy x-ray absorptiometry (DEXA). Total serum FA were measured by gas chromatography. In the entire cohort, fasting serum C15:0, C17:0 and *trans*-C16:1n-7 represented 0.21 ± 0.05%, 0.28 ± 0.06%, 0.45 ± 0.12% of total FA. *Trans*-C16:1n-7 showed a significant inverse association with insulin sensitivity. The cohort was split into normal weight vs overweight/obese T2D participants for analysis. Data were also

analyzed by sex. There were significant differences between serum FA concentrations of *trans*-C16:1n-7 between sexes. Glucose Rd was 57% lower in the overweight/obese T2D group compared to normal weight (9.43 ± 2.39 vs 5.41 ± 1.47 mg/kg LBM/min; $p < 0.001$). Serum *trans*-C16:1n-7 had a significant inverse relationship with insulin sensitivity in the overweight/obese groups. Although C15:0, C17:0, and *trans*-C16:1n-7 are all used as biomarkers of dairy fat intake, the relationships between biomarkers and insulin sensitivity were not consistent. The associations in this cohort were also influenced by BMI and sex, indicating that metabolic status must be considered when interpreting results in large prospective cohorts. Caution is advised when interpreting the relationship between biomarkers of dairy fat intake and insulin sensitivity in heterogeneous cohorts.

RÉSUMÉ

Les études épidémiologiques récentes ont indiqué que la consommation des produits laitiers est associée à un moindre risque du diabète type 2. Cette association inverse est particulièrement forte quand les acides gras uniques aux produits laitiers sont utilisés comme les biomarqueurs de gras laitiers, comprenant: acide pentadécylique (C15:0), acide margarique (C17:0), et acide *trans*-palmitique (*trans*-C16:1n-7). Une des caractéristiques définissant du diabète type 2 est la réduction de sensibilité des tissus à l'action d'insuline. L'objectif de cette étude était d'examiner l'association entre les biomarqueurs de gras laitiers et la sensibilité à l'insuline dans une cohorte des individus (n = 42) qui ont participé en un 40 mU/m^2/min clampe hyperinsulinemique, euglycémique, isoaminoacidemique qui a duré 3 heures. Le taux d'élimination de glucose (Rd), le taux d'apparence de glucose (Ra), l'index M/I, et l'index hépatique IR sont prises comme les mesures de la sensibilité à l'insuline. La composition du corps était mesurée par l'absorptiomètrie à rayons-x en double énergie (DEXA). Les acides gras sériques totaux étaient mesurés par chromatographie en phase gazeuse. Dans la cohorte totale, les acides gras sériques C15:0, C17:0 et *trans*-C16:1n-7 ont représenté 0.21 ± 0.05%, 0.28 ± 0.06%, 0.45 ± 0.12% des acides gras totaux. *Trans*-C16:1n-7 avait une association inverse significative avec les mesures de la sensibilité d'insuline. La cohorte était divisée en un groupe d'individus de poids normaux et un groupe d'individus en surpoids/obèse avec le diabète type 2 pour l'analyse. Les données étaient aussi analysées par le sexe des participants. Il y avait des différences significatives entre les sexes de *trans*-C16:1n-7 sérique. Le taux d'élimination de glucose était 57% inférieur dans le groupe en surpoids/obèse T2D par rapport au groupe de poids normale (9.43 ± 2.39 vs 5.41 ± 1.47 mg/kg LBM/min; $p < 0.001$). Le *trans*-C16:1n-7 sérique avait une association inverse significative avec les mesures de la sensibilité dans le groupe

surpoids/obèse. Même si C15:0, C17:0 et *trans*-C16:1n-7 sont tous utilisés comme des biomarqueurs des acides gras laitiers, les associations entre les biomarqueurs et la sensibilité d'insuline n'étaient pas cohérentes. Les associations dans cette cohorte étaient influencées par IMC et sexe, indiquant que le statu métabolique doit être considéré quand les résultats d'études prospectives des grandes cohortes sont interprétés. La prudence est donc conseillée dans l'interprétation des relations entre les biomarqueurs de gras laitiers et la sensibilité d'insuline des cohortes hétérogènes.

AUTHORS' CONTRIBUTION

Stefanie LaForce is the primary author of this thesis. She was responsible for direct methylation and subsequent gas chromatography of the serum samples used for this study. All statistical analyses were completed by her.

Dr. Errol Marliss provided the serum samples necessary to perform this study and provided essential feedback and critique on the manuscript.

Marie Lamarche was a laboratory technician who took the blood samples necessary to obtain serum during the hyperinsulinemic euglycemic isoaminoacidemic clamp procedure. She also provided participant characteristic data. Marie provided critical feedback on the thesis.

Sherry Agellon was a laboratory technician who trained and assisted Stefanie in the direct methylation of the serum samples as well as the use of the gas chromatograph and was available for consultation concerning the chromatograph output.

Dr. Hope Weiler provided access to the gas chromatograph and relevant software to perform analysis on the serum samples.

Dr. Sergio A. Burgos supervised the work. He designed the study. He assisted in the writing of the literature review for submission as a review article for the Canadian Journal of Animal Science. He provided necessary feedback and critiques of the manuscript. Dr. Burgos reviewed and approved of the thesis.

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1. LITERATURE REVIEW

Milk lipids are important because they confer nutritional, functional, and organoleptic properties to dairy products (MacGibbon and Taylor 2006). Among milk components, milk fat is the most variable in both amount and composition. The composition of lipids in milk varies depending on several factors such as genetics (e.g., breed), environment, the physiological state of the animal, and nutritional management practices (Jenkins and McGuire 2006). Unlike the other major milk components (lactose and milk proteins), milk fat content and composition can be manipulated by altering the cow's diet (Palmquist 2006). There are several reasons to manipulate the composition of dairy products including: the improvement of manufacturing and processing of milk products, the alteration in order to meet governmental dietary guidelines, and the use of milk as a delivery system for specific nutrients (Jenkins and McGuire 2006).

Milk lipids are also an important part of a healthy diet in many countries around the world, including Canada. Consumers and health professionals are increasingly aware of the fatty acid (FA) composition of milk, due to the effects that fat (specifically saturated fat), can have on human health and the potential roles they may play in disease risk. Dairy products such as milk, cream, butter, yogurt, and cheese vary widely in their fat content. However, the primary type of fat found in all of them is saturated fat. This has lead to current dietary guidelines in many countries including Canada to recommend the consumption of low-fat dairy products (Health Canada 2007). However, this recommendation stems purely from the high saturated fat content of dairy products, not from observational evidence of health outcomes in individuals who regularly consume dairy. The fat in dairy products may well have no association with negative health outcomes. Evidence from recent epidemiological studies suggests that dairy fat could possibly be associated with greater insulin sensitivity and protective against the development of

type 2 diabetes (T2D). T2D is a chronic metabolic disease that affects millions of Canadians and is primarily characterized by hyperglycemia (Canadian Diabetes Association 2011). Building on evidence from epidemiological studies, dairy specific FA have become of interest in order to try and determine if there is a causal relationship between dairy fat intake and lower risk of T2D. This potential protective relationship signals the need for further research in this area.

1.1 MILK LIPIDS

1.1.1 Composition of milk lipids

Fat constitutes 3.5 to 4.7% of cows' milk (MacGibbon and Taylor 2006). Lipids are present in milk as globules 0.2 - 15.0 μm in diameter that are emulsified in the aqueous phase enclosed by the milk fat globule membrane (MFGM) (Palmquist 2006). The majority of the lipids found in milk are triglycerides (96-98% of milk lipids) (Jensen 2002). Triglyceride content and composition influences milk characteristics such as the density and melting point of milk fat. Triglycerides are composed of three FA esterified to a glycerol backbone. In bovine milk, these FA are diverse, with over 400 different FA identified to date (Jensen 2002). This diversity is due in part to the variety of FA in the cows' diet, the transformation of dietary unsaturated FA in the rumen and the *de novo* FA synthesis in the bovine mammary gland (Palmquist 2006). The breed, stage of lactation, and diet are among the most important that affect both ruminal and *de novo* synthesis of FA and, as a consequence, the FA composition of bovine milk (Palmquist 2006, Soyeurt et al. 2008, Larsen et al. 2012). Saturated FA (SFA) account for 70-75% of total FA in milk (MacGibbon and Taylor 2006). In addition to SFA, bovine milk also contains monounsaturated FA (MUFA), polyunsaturated FA (PUFA), conjugated FA, and *trans*-FA

(TFA). The major FA in milk are SFA (C4:0 to C18:0) and MUFA (including palmitoleic acid and oleic acid) (MacGibbon and Taylor 2006).

In addition to triglycerides, milk also includes compound lipids such as phospholipids and cholesterol (Shingfield et al. 2008). Phospholipids are polar lipids that comprise about 1% of total milk lipids and can be divided into two main groups: glycerophospholipids and sphingolipids. Glycerophospholipids are composed of a glycerol backbone, a phosphoric acid, a FA, and a hydroxy compound, commonly choline or serine (Contarini and Povolo 2013). Sphingolipids are composed of a sphingoid base, such as sphingosine or phytosphingosine, FA, and either sugar, phosphoric acid, or alcohol (Contarini and Povolo 2013). Dairy products are the most abundant source of sphingomyelin in the diet, with sphingolipids comprising 25% of total phospholipids in milk (Palmquist 2006). Cholesterol is synthesized by the mammary gland and its content in milk is highly associated with total milk fat content because it is present in the MFGM (Clarenburg and Chaikoff 1966). Cholesterol accounts for roughly 0.5% of total milk lipids, with 10% of total cholesterol being its esterified form (Jensen 2002).

1.1.2 Sources of milk lipids

The two main sources of FA in milk are derived from the cow's diet or *de novo* synthesis in the mammary gland (Popják et al. 1951, Palmquist and Conrad 1971, Zhang et al. 2015). Endogenous synthesis of lipids by rumen microbes represent a much smaller proportion of milk lipids but have an important role as biomarkers of dairy intake, as discussed below. Lipids in dairy cattle feed are composed mostly of triglycerides from concentrates (processed grains, supplemental protein and plant-based fats) or glycolipids from forages (Ferlay et al. 2017). The rumen, which accounts for up to 75% of the digestive system of cows, contains microorganisms

such as anaerobic bacteria, archea, cililate protozoa, and anaerobic fungi that produce FA unique to bovine milk (Thivend et al. 1985, Jenkins et al. 2008). Rumen bacteria contain two types of FA synthases, the straight-chain FA synthase and the branched-chain FA synthase (Kaneda 1991). Most long chain FA in milk come from the diet. Indeed, majority of FAs of 18 carbons or more, and about half of FA of 16, in chain length that are taken up by the mammary gland come from triacylglycerol-rich chylomicra and very low-density lipoproteins found in blood plasma (Palmquist 2006).

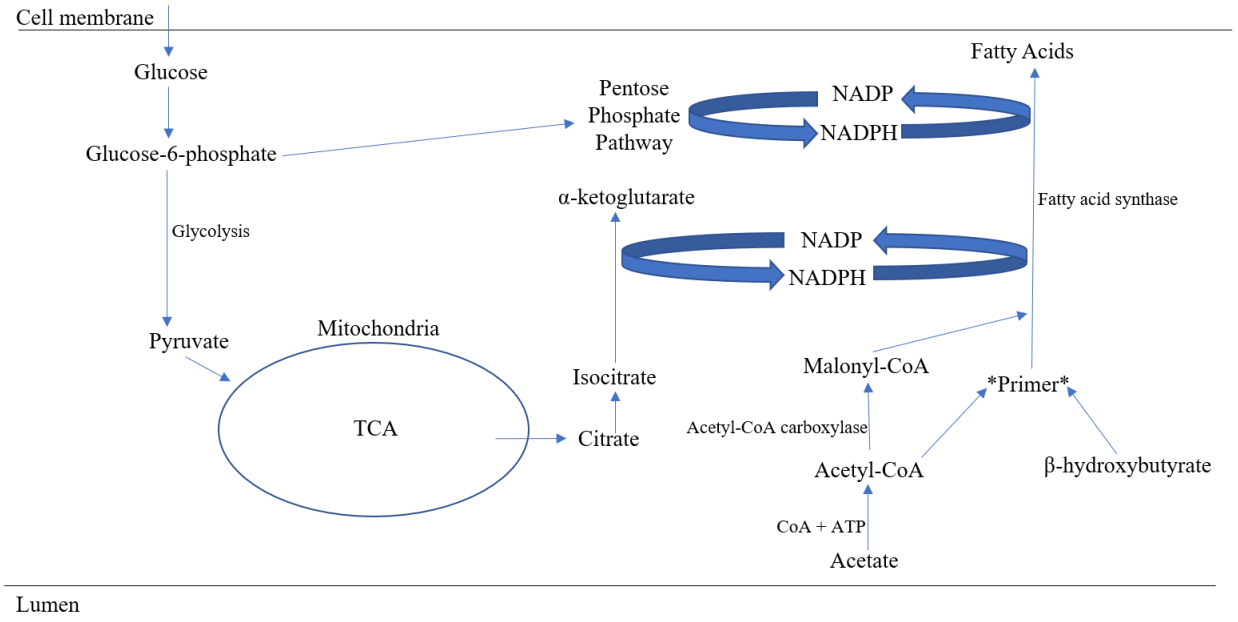

Figure 1-1. Schematic of *de novo* FA synthesis pathway in the ruminant mammary gland. ATP: adenosine triphosphate; NADP/NADPH: nicotinamide adenine dinucleotide phosphate; TCA: tricarboxylic acid cycle. Adapted from (Moore and Christie 1981, Van Soest 1994, Palmquist 2006)

In the mammary gland, FA are formed *de novo* through stepwise elongation via the malonyl-CoA pathway (Figure 1-1). Synthesis of FA in the mammary gland involves the FA synthase which requires a carbon source and the reducing equivalents NADPH and H^+ (Smith et al. 2003). Glucose is oxidized in the pentose phosphate pathway to provide a portion of the NADPH + H^+ necessary for the FA synthase (Palmquist 2006). The carbon sources for ruminants include circulating acetate and β-hydroxybutyrate. These precursors are derived from microbial fermentation of dietary fibre (Lock and Bauman 2004). Acetate is converted to acetyl-CoA by cytosolic acetyl-CoA synthase. The malonyl-CoA pathway involves repeated condensation of malonyl-CoA to an acetyl-CoA primer in a reaction catalyzed by acetyl-CoA carboxylase (Fulco 1983, Vlaeminck et al. 2006). After repeated cycles of adding two-carbons units to acetyl-CoA from malonyl-CoA, a thioesterase specific to a chain length releases the *de novo* synthesized FA (Palmquist 2006). The pathway follows the general form:

$$\text{Acetyl-CoA} + 7\ \text{Malonyl-CoA} + 14\ \text{NADPH} + 14H^+ \rightarrow$$

$$\text{Palmitic acid} + 7\ CO_2 + 8\ \text{CoA} + 14\ NADP^+ + 6H_20$$

Through this pathway, saturated FA from 4 to 14 carbons (C4:0 to C14:0) are synthesized *de novo* in the mammary gland. Although palmitic acid (C16:0) in milk is often derived from dietary intake, it is also synthesized in the mammary gland, especially when dietary fat intake is low (Jenkins et al. 2015).

The mammary gland can also modify milk FA composition by introducing a double bond into saturated FA, a process known as desaturation. The rate limiting enzyme in this process is stearoyl-CoA desaturase. Increasing stearoyl-CoA desaturase activity can be used to increase

beneficial FA in milk such as conjugated linoleic acid (CLA) (Lock and Bauman 2004). In addition, the desaturation of FA also serves to diversify the profile of FA in milk.

Dietary lipids are digested and transformed in the rumen. The first major step in dietary lipids ruminal digestion is hydrolysis by bacterial lipases that hydrolyze ester linkages of triglycerides, phospholipids, and glycolipids to produce free fatty acids (Lock and Bauman 2004). This process is extensive, with approximately 85% of dietary lipids undergoing rumen hydrolysis. Factors that limit the effectiveness of hydrolysis include antibiotic use (Van Nevel and Demeyer 1995) and low ruminal pH (Van Nevel and Demeyer 1996).

The second major transformation of lipids in the rumen is the biohydrogenation of dietary PUFA by the removal of double bonds, converting PUFA to SFA (Figure 1-2). Biohydrogenation serves as a protective measure against the toxic effects that PUFA have on rumen bacteria (Jenkins 1993). A free FA with an exposed carboxyl group is needed to proceed with biohydrogenation, therefore the rate of hydrolysis sets the rate of biohydrogenation (Lock and Bauman 2004). This means that any factor that would affect hydrolysis will also affect biohydrogenation. Two groups of bacteria are involved in biohydrogenation: Group A, which hydrogenate C18 PUFA to *trans*-C18:1 FA isomers, and Group B, which hydrogenate *trans*-C18:1 FA isomers to stearic acid (Harfoot and Hazlewood 1997).

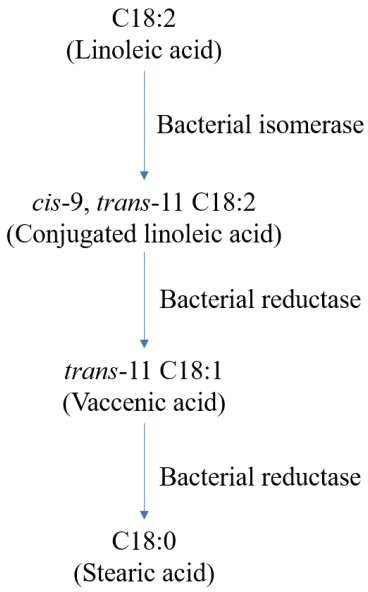

Figure 1-2. Biohydrogenation of a PUFA (linoleic acid) to a TFA (vaccenic acid) and eventually to a SFA (stearic acid). Source: (Shingfield et al. 2010)

1.1.3 Even-chain saturated fatty acids

Even-chain SFA account for approximately 70% of total milk fat (Table 1-1). Palmitic acid constitutes the majority of these, comprising approximately 29% of total milk fat. C14:0 and C18:0 are the next most abundant at over 11% of total FA each. Even-chain SFA are either short, medium, or long-chain FA. As long-chain FA, primarily C18:0 FA, arise from dietary fat intake, the fat content in the bovine diet can cause their concentration in milk to vary widely (Palmquist 2006). Short and medium-chain FA from C4:0 to C14:0 are synthesized *de novo* in the mammary gland, while C16:0 is both synthesized in the mammary gland and derived from dietary fat intake (Popják et al. 1951, Palmquist and Conrad 1971, Zhang et al. 2015). Short-chain FA also lower the melting point of triglycerides which helps maintain milk fat fluidity at physiological temperatures (MacGibbon and Taylor 2006).

Table 1-1. Concentration of major SFA in bovine milk expressed as a percentage of total FA.

FA	% of total FA
C4:0	3.88
C6:0	2.49
C8:0	1.39
C10:0	3.05
C12:0	4.16
C14:0	11.36
C16:0	29.36
C18:0	11.36

Source: Shingfield et al. (2008)

1.1.4 Odd-chain saturated FA

Odd-chain FA (OCFA) are minor FA in milk, typically representing less than 1% of the FA profile of milk (Palmquist 2006). OCFA can be straight-chain or branched-chain FA (BCFA). BCFA are designated as *iso* and *anteiso* forms depending on the fatty acyl Co-A primer from which they are produced. Unlike even-chained FA, OCFA do not arise from acetyl-Co-A, but from odd-numbers carbon primers such as propionyl-CoA and isobutyryl-CoA for straight-chain and branched-chain OCFA, respectively (Figure 1-3) (Ferlay et al. 2017). Thus, unlike mammalian FA synthase, rumen bacteria can accept even (acetyl-CoA) and odd-number carbon molecules as primers for FA chain elongation (Kaneda 1991). Because of their unique source and chemical structure, OCFA and BCFA have garnered interest as biomarkers of dietary intake of dairy products and as potential bioactive molecules.

The proportions in which OCFA are synthesized in the rumen depend on the ruminal bacterial populations, as different species of bacteria contain different FA synthases that can

affect the overall FA profile of milk (Vlaeminck et al. 2006). OCFA made in the rumen can be absorbed in the gut and then transported through the circulation to the mammary gland. Straight-chain OCFA and *anteiso* isomers can also be endogenously synthesized in the mammary gland (Massart-Leën et al. 1983). Evidence for at least partial synthesis of OCFA in the mammary gland is found by comparing the amounts of C15:0 and C17:0 to C18:0 and *cis*-C18:1n-9 in blood plasma versus milk, where the ratio of OCFA to the C18:0 FA is higher in milk (Kay et al. 2005). OCFA that are either made in or taken up by the mammary gland eventually become incorporated into milk.

Table 1-2. Concentration of milk odd- and branched-chain FA concentrations in bovine milk.

FA	% of total FA
***iso* C13:0**	0.040
***iso* C14:0**	0.089
***iso* C15:0**	0.224
***iso* C16:0**	0.209
***iso* C17:0**	0.272
***anteiso* C13:0**	0.083
***anteiso* C15:0**	0.462
***anteiso* C17:0**	0.501
C15:0	1.104
C17:0	0.557
***cis*-9 C17:1**	0.207

Source: Vlaeminck et al. (2006)

As shown in Table 1-2, the proportions of these OCFA and OBCFA in bovine milk are very low compared to the amounts of some SFA and MUFA, such as C14:0, C16:0, and C18:1 (Table 1-1). C15:0 and C17:0 are relatively the most abundant, and therefore the two most commonly

studied OCFA due to their use as internal standards, biomarkers of dairy intake, and biomarkers of certain diseases (Jenkins et al. 2015). The ratio of C15:0 to C17:0 in bovine milk is approximately 2:1. This is in part due to the partial oxidation of abundant C16:0 to C15:0 (Jansen and Wanders 2006).

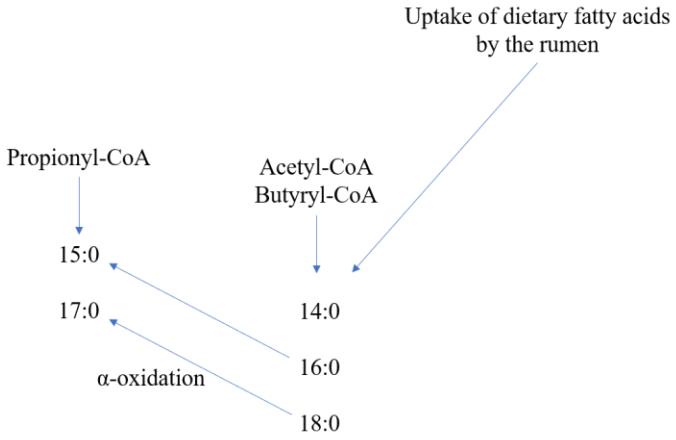

Figure 1-3. Synthesis of OCFA either by step-wise elongation using propionyl-CoA as a precursor or α-oxidation of even-chain FA. Source: (Vlaeminck et al. 2006)

1.1.5 *Trans*-fatty acids

TFA are produced in the rumen of dairy cows through biohydrogenation of MUFA and PUFA. PUFA such as linoleic acid, C18:2, can undergo isomerization and reduction via bacterial populations in the rumen to TFA (Figure 1-2). The final step before reduction of a TFA to a SFA is considered rate-limiting, therefore TFA are able to accumulate and leave the rumen (Shingfield et al. 2010). There are three major TFA in bovine milk, *trans*-C16:1, *trans*-C18:1, and *trans*-C18:2 (Table 1-3). Vaccenic acid (*trans*-C18:1n-11) is the most important *trans*-C18:1 isomer, comprising 30-60% of total *trans*-C18:1 concentration (MacGibbon and Taylor 2006). Aside from hydrogenated vegetable oils, ruminant-derived foods such as dairy products are one

of the main sources of TFA in the diet. A survey that included several European countries concluded that, in those countries, milk and dairy products were the largest source of TFA in the diet (Hulshof et al. 1999).

Table 1-3. Estimations of the major TFA concentrations in bovine milk presented as a percentage of total FA.

FA	% of total FA
***trans*-C16:1**	0.4
***trans*-C18:1**	2.1
***trans*-C18:2**	0.2

Source: Mansson (2008)

1.1.6 Unsaturated fatty acids

In milk, approximately 25% of the FA are monounsaturated, while 2.3% of are polyunsaturated (Table 1-4). The majority of the MUFA in milk derive from C18:1, oleic acid, which constitutes on average 22.8% of total milk FA. The *cis*-bonds present in MUFA and PUFA lower the packing density of triglycerides that contain MUFA and PUFA. Therefore, the quantity of MUFA and PUFA can have a large impact on the melting point of milk fat (MacGibbon and Taylor 2006).

Table 1-4. Estimations of the major MUFA and PUFA concentrations in milk as percent total FA.

FA	% of total FA
C10:1	0.3
C14:1	0.8
C16:1	1.0
C18:1	22.8
C18:2	1.6
C18:3	0.7

Source: Creamer and MacGibbon (1996)

1.1.7 Conjugated fatty acids

Milk also contains geometric and positional isomers of C18:2 that have a double bond termed conjugated FA. These include conjugated linoleic acid (CLA) and conjugated linolenic acid (CLnA). There are two main isomers of CLA, trans-7, cis-9 CLA and cis-9, trans-11 CLA, which represent 0.03 and 0.57 percent of total FA in bovine milk, respectively (Shingfield et al. 2007). Most of the CLA found in milk is synthesized *de novo* in the mammary gland from desaturation of *trans*-11 C18:1, but it can also be produced in the rumen by isomerization of cis-9, 12-cis C18:2 (Bauman 2000). The CLA content in milk is dependent on dietary intake providing the necessary lipid substrates for CLA synthesis in the rumen and factors that affect the rumen environment (i.e., bacterial population distribution) (Bauman and Griinari 2001). In addition, the amount of CLA in milk can be altered by supplementing a cow's diet with fish oils and vegetable oils (Palmquist 2006).

1.1.8 Dietary manipulations of FA in bovine milk

Milk FA composition is, compared to protein and lactose content, the easiest component of milk to modify. There are several reasons why research into modifying milk FA content is done. FA composition affects the characteristics of the resulting dairy products, so modifying FA content can be done to improve the functional properties of dairy products. An additional reason is in order to be more in line with current dietary recommendations for dairy products, which encourage the consumption of dairy products that are low in fat (Health Canada 2007). With the rise of knowledge regarding beneficial micronutrients in foods, producers and researchers are also interested in modifying milk fat composition in order reduce the amount of potentially disadvantageous FA and increase the amount of beneficial FA (i.e. CLA) (Lock and Bauman 2004).

Dairy cattle are fed mainly forages, primarily pasture, and concentrates, which include grains, supplements, and by-products. The most common way of manipulating the FA profile of milk consists of changing the amounts of forages and concentrates that are fed to the dairy cow. This is often seen naturally in seasonal variation of milk fat components caused by the cows' diet. In the summer when cows have access to pasture, C18:0 and C18:1 isomers are found in higher concentrations. During the winter when a higher percentage of the cows' diet is concentrates, the levels of 16:0 and other short to medium chain FA increase (MacGibbon and Taylor 2006). Proportions of both *iso* C14:0 and *iso* C15:0 are also increased when the forage to concentrate ratio is increased, however whether or not this also has an effect on C15:0 and C17:0 remains unclear (Vlaeminck et al. 2006).

Fat supplements are also used to change the FA composition. Sunflower, canola, and linseed oils are all common supplements fed to dairy cattle. Processes such as ruminal degradation, poor

intestinal absorption, or deposition in adipose tissues rather than the mammary glands all contribute to a lower successful transfer rate (Jenkins and McGuire, 2006). Supplements must also be inert or encapsulated in order not to affect rumen metabolism (Jensen 2002). In this vein, considerable interest has been generated in order to attempt to shield dietary FA from biohydrogenation to increase the absorption and delivery of PUFA to the mammary gland. One popular method is to supplement the diet with oilseeds, whose hard outer shells help prevent biohydrogenation (Lock and Bauman 2004). However, when oilseeds are processed for commercial use by methods such as grinding or crushing, their effectiveness decreases. Calcium salts have also been suggested for this purpose, but although calcium salt supplements are available for purchase, their success in preventing biohydrogenation is not fully proven (Jenkins and McGuire 2006).

1.2 DAIRY INTAKE AND TYPE 2 DIABETES

1.2.1 Effect of dairy intake on cardiovascular and metabolic health

The fat content in dairy products is between 70-75% SFA (MacGibbon and Taylor 2006). In general, SFA are viewed as having negative impacts on cardiovascular health. For this reason, current Canadian dietary guidelines recommend the intake of low fat dairy products (Health Canada 2007). The main rationale for these recommendations is that both dietary cholesterol and SFA raise total serum cholesterol concentrations and the low-density lipoprotein (LDL) to high-density lipoprotein (HDL) cholesterol ratio, both of which are risk factors for cardiovascular disease (CVD) (Mensink et al. 2003). High blood pressure, insulin sensitivity, and high LDL cholesterol levels, when present along with obesity and increased blood triglyceride levels, are the conditions that form what is known as metabolic syndrome (Fumeron et al. 2011). The

diagnosis of metabolic syndrome commonly precedes a diagnosis of cardiovascular disease or type 2 diabetes (T2D). High concentrations of dietary SFA are also often associated with other health risks such as smoking, *trans*-fats intake, low-fibre intake, and a sedentary lifestyle (Ericson et al. 2015).

TFA are also associated with increased cardiometabolic health risks, prompting the WHO (2003) to recommend no more than 1% of total energy intake from TFA. These FA increase LDL-cholesterol levels, but also decrease the amount of HDL-cholesterol in the body, which in turn increases the overall total-to-HDL-cholesterol ratio (Mozaffarian 2006). In addition, Mozaffarian et al. (2006) found evidence that TFA are pro-inflammatory, which is a common risk factor of chronic disease.

This blanket statement that fats are universally detrimental to T2D may not be the case. Dairy products have been inversely associated with incident metabolic syndrome, which generally precedes a diagnosis of T2D (Fumeron et al. 2011). Recent studies indicate that OBCFA found in dairy products may have positive effects on insulin sensitivity. This evidence emphasizes the need to account for food sources when establishing associations between saturated fat and disease risk. In this vein, two of the minor OCFA in dairy products, C15:0 and C17:0, are currently of interest in having a potential protective effect against incident type 2 diabetes. Studies regarding this relationship between C15:0, C17:0, and T2D are largely epidemiological in nature and more clinical work is needed to determine what role, if any, these FAs play in disease prevention.

1.2.2 Overview of Type 2 Diabetes

The worldwide prevalence of type 2 diabetes (T2D) is increasing every year, with an estimated number of 624 million cases of incident T2D by the year 2040 (Ogurtsova et al. 2017). In 2015, approximately 3 million Canadians were living with T2D, representing an annual direct health care cost of $3 billion (Canadian Diabetes Association 2015). Although it is possible to control symptoms with diet and pharmaceuticals, T2D can lead to severe complications such as CVD, renal disease, stroke, blindness and lower limb amputations (Canada 2011).

T2D is characterized by the interplay between insulin resistance (IR), defined as a reduced sensitivity of target tissues such as muscle, liver and adipose to the action of insulin, and impaired insulin secretion by pancreatic β-cells. The normal response of β-cells to IR is compensatory insulin hypersecretion to maintain normoglycemia. Over time, β-cells fail to compensate for IR leading to a progressive decline in their function. Consequently, individuals progress over several years from normal glucose tolerance (NGT), to impaired glucose tolerance (IGT), to frank T2D. Prediabetes is a condition characterized by IGT, impaired fasting glucose or elevated glycated hemoglobin (A1c) levels, which places individuals at high risk of developing T2D. As IR can be present for decades before an outright diagnosis of T2D, it is considered the initiating factor in its development.

T2D is thought to be caused by environmental factors acting on genetically susceptible individuals. Lifestyle factors (such as poor diet and a predominantly sedentary lifestyle) are critical environmental factors associated with the T2D (Morio et al. 2016). In addition, a family history of diabetes is a major risk factor for T2D (Hariri et al. 2006, Das et al. 2012). Older individuals are more susceptible to developing T2D, as the body's ability to produce and use insulin naturally decreases with age (Canada 2011). Obesity, especially with excess abdominal

fat, is a primary cause of IR. This naturally leads to the composition of an individual's diet as an important consideration in the development of T2D. Among the environmental exposures that influence diabetes risk, dairy consumption is emerging as a potential protective factor.

1.2.3 Epidemiological Studies

Twelve prospective cohort studies evaluating the relationship between total dairy product intake and T2D risk can be found in Table 1-5. Though many of the studies showed a trend towards total dairy product intake being associated with lower T2D risk, only two studies found a significant association (Choi et al. 2005, Malik et al. 2011). Choi et al. (2005) found that per each serving-per-day increase in total dairy intake, there was a 9% lower risk of T2D (RR = 0.91; 95% CI = 0.85-0.97). Malik et al. (2011) used data from the Nurses' Health Study II and found that in women aged 24-42 years there was a significant association between total dairy intake and T2D risk (RR: 0.75; 95% CI: 0.55, 1.02; *P*-trend = 0.03). In addition, the studies varied greatly in the dairy products included as total dairy products, servings or amounts consumed per day, and adjustment for covariates.

The epidemiological studies discussed here all used forms of self-reported dietary assessment tools, of which food-frequency questionnaires (FFQ) were the most common. FFQs are checklists which include a number of potential food items an individual may consume, including how much of that particular food is consumed in a specified time period. An advantage of FFQs is that they can be validated for specific populations, and once validated, are a more accurate measure of dietary intake compared to other forms of dietary assessment such as food records (Willett 2013). FFQs can also be used to identify dietary patterns and calculate food group intakes as well as for the estimation of disease-risk associations on a per-serving or amount of

food consumed. The main limitations of FFQs are that they rely on participant recall and are therefore predisposed to subjective reporting (Willett 2013). Kratz et al. (2013) have discussed the reporting bias of fat content, which may be of particular concern for dairy products given the cultural stigma against consumption of foods high in SFA in North America.

While these studies provide an overall view of the effects of dairy products on the risk of T2D, this view is limiting in that it does not specifically examine the component of dairy products that is assumed to have negative health outcomes: the fat content. It is therefore of more potential interest to focus on studies that investigated the association between low-fat and high-fat dairy products and T2D risk to truly determine if the fats in dairy products have a negative effect on disease outcome. If a disease association is seen for either high-fat or low-fat dairy products, but not both, then the difference in association may be attributable to the main differences between the products, the dairy fat itself.

Table 1-5. Prospective cohort studies reporting association between total dairy product intake and T2D risk.

Author; country	Study/Cohort; follow up (mean or range)	*n* participants; *n* cases; sex (% men); age (mean or range)	Dietary assessment	Exposure; daily intake	Hazard ratio or relative risk; 95% confidence interval; *P*-trend	Adjustments
Choi et al. (2005); USA	Health Professionals Study; 14 y	41 254; 1243; 100% men; 40–75 y	131-item FFQ	Total dairy products; ≥ 2.9 servings/d	0.75; 0.61–0.93; 0.03	Family history of T2D, hypercholesterol, hypertension, GL,cereal fibre, trans-fat intake, PUFA:SFA ratio
van Dam et al. (2006); USA	Caerphilly Cohort; 8 y	41 186; 1964; 0% men; 21–69 y	68-item FFQ	Total dairy intake (highest vs lowest quintile)	0.93; 0.75–1.15; 0.31	Education, family history of T2D, intake of coffee, sugar, meat, whole grains
Pittas et al. (2006); USA	Nurses' Health Study; 20 y	83 779; 4843; 0% men; 30–55 y	61-166 item FFQ	Total dairy products; 3 vs <1 s/day	0.89; 0.81–0.99; NS	Family history of T2D, intake of coffee, diet, hypertension
Kirii et al. (2009); Japan	Japan Public Health Center–based Study; 5 y	25 877; 634; Men and women; 40–59 y	114-item FFQ	Total dairy products (men); 300 vs <50 g/d	1.18; 0.90–1.56; 0.21	Area, family history of T2D, history of hypertension, coffee, energy-adjusted magnesium intake
				Total dairy products (Women's Health Initiative); 300 vs <50 g/d	0.71; 0.51, 0.98; 0.054	

Montonen et al. (2005); Finland	Finnish Mobile Clinic Health Examination Survey; 23 y	4304; 383; 54% men; 40–69 y	100-item FFQ	Total dairy products; ≥305 vs <39 g/d	0.81; 0.62, 1.08; 0.16	Family history of T2D, geographic area
Malik et al. (2011); USA	Nurses' Health Study II; 8 y	37 038; 550; 0% men; 24–42 y	133-item FFQ	Total dairy products; 2.14 vs 0.62 servings/d	0.75; 0.55, 1.02; 0.03	Family history of T2D, OC use, HRT, PUFA:SFA ratio, GL, cereal fiber, trans fat, processed meat, carbonated soft drinks, fruit drinks, coffee, mutual adjustment for high- and low-fat dairy products
Margolis et al. (2011); USA	Women's Health Initiative; 8 y	82 076; 3946; 0% men; 50–79 y	122-item FFQ	Total dairy products; 3.4 vs 0.5 servings/d	0.93; 0.83, 1.04; 0.15	Race-ethnicity, income, education, family history of T2D, interaction of low-fat dairy × BMI, interaction of yogurt × time
Sluijs et al. (2012); European-wide	EPIC-Interact Study; 16 y	24 475; 10 694; 38% men; 52 y	130-item FFQ	Total dairy products; 628.9 vs 79.7 g/d	1.01; 0.89, 1.13; 0.92	Education, intakes of fruit and vegetables, red meat, processed meat, sugar-sweetened soft drinks, coffee, cereals, cereal products

Struijk et al. (2012); Denmark	The Inter99 Study; 5 y	5953; 214; 48% men; 30–60 y	198-item FFQ	Total dairy products; 204 g/d	0.95; 0.86, 1.06; NS	Intervention group, family history of T2D, education, intake of whole-grain cereal, meat, fish, coffee, tea, fruit, vegetables, change in diet from baseline to 5-y follow-up, waist circumference
Soedamah-Muthu et al. (2013); UK	The Whitehall II Prospective Study; 10 y	4526; 273; 72% men; 35–55 y	114-item FFQ	Total dairy products; 575 vs 246 g/d	1.30; 0.95, 1.77; 0.11	Ethnicity, employment grade, family history of CHD/hypertension, intake fruit and vegetables, bread, meat, fish, coffee, tea
Grantham et al. (2013); Australia	Australian Diabetes Obesity and Lifestyle Study; 5 y	5582; 209; 48% men; 25-88 y	121-item FFQ	Total dairy products; 477 vs 205 g/d	0.71; 0.48, 1.05; NS	Family history of diabetes, education level, triglycerides, HDL cholesterol, systolic blood pressure, waist circumference, and hip circumference
Zong et al. (2014)	Nutrition and Health of Aging Population in China; 6 y	2091; 504; 41% men; 50–70 y	74-item FFQ	Total dairy products; >1 serving/d	0.81; 0.63, 1.05; 0.07	Region, family history of diabetes, and dietary fiber intake, change in waistline

[1]Note: Age, sex, BMI, total energy intake, smoking status, alcohol intake and physical activity were included in all studies.

[2]Abbreviations: BMI, body mass index; CI, confidence interval; CHD, coronary heart disease; EPIC, European Prospective Investigation into Cancer and Nutrition; FFQ, food-frequency questionnaire; GL, glycemic load, HDL, high density lipoprotein; HRT, hormone replacement therapy; OC, oral contraceptive; NS, nonsignificant

Table 1-6. Prospective studies evaluating the association between dairy fat intake and T2D risk.

Authors; country	Study; duration of follow-up (mean or range)	*n* participants; *n* cases; sex (% men); age (mean or range)	Dietary assessment; outcome	Exposure; daily intake (mean or median of highest quantile)	Hazard ratio or relative risk; 95% confidence interval; *P*-trend	Adjustments for covariates[1]
Diaz-Lopez et al. (2016); Spain	Prevención con Dieta Mediterránea; 4 y	3,454; 270; 38%; 67 y	137-item FFQ[2]	Low-fat dairy; 256 g/d	0.65; 0.45-0.94; 0.017	Intervention group, educational level, hypertension, antihypertensive drug use, fasting glucose, HDL-cholesterol, triglycerides concentrations, intake of vegetables, legumes, fruits, meat, fish, olive oil, nuts, alcohol
				High-fat dairy; 20 g/d	0.73; 0.52-1.02; 0.086	
Ericson et al. (2015); Sweden	Malmö Diet and Cancer cohort; 14 y	26,930; 2,860; 31%; 45-74 y	168-item FFQ; 7-d dietary records; diet-history interview	Low-fat dairy; 1.5 portions/d	1.14; 1.01-1.28; 0.01	Diet-method version, season, education level
				High-fat dairy; 3.3 portions/d	0.77; 0.68-0.87; < 0.001	
Hruby et al. (2017); USA	Framingham Heart Study Offspring Cohort; 11 y	2,809; 196; 46%; 54 y	126-item FFQ	Low-fat dairy; 5.4 servings/wk	0.80; 0.46-1.39; 0.88	Parental history of diabetes, dyslipidemia or treatment, hypertension or treatment, consumption of coffee, nuts, fruits, vegetables, meats, alcohol, and fish,
				High-fat dairy; 5.1 servings/wk	0.49; 0.17-1.16; 0.06	

						glycemic index, other dairy intake, weight change, baseline glycemic status
Louie et al. (2013); Australia	Blue Mountains Eye Study; 10 y	1,825; 145; 42%; >49 y	145-item FFQ	Low-fat dairy; 2.1 servings/d	1.09; 0.57-2.09; 0.76	Systolic blood pressure, blood HDL cholesterol, total cholesterol, triglyceride, and calcium
				High-fat dairy; 1.9 servings/d	0.87; 0.48-1.57; 0.60	
O'Connor et al. (2014); UK	EPIC-Norfolk Study; 11 y	4,127; 892; 43%; 40-79 y	7-d food diaries	Low-fat dairy; 325 g/d	0.92; 0.73-1.17; 0.54	Family history of diabetes, social class, education level, consumption of fibre, fruit, vegetables, red meat, processed meat, and coffee
				High-fat dairy; 125 g/d	1.09; 0.87-1.37; 0.08	
Soedamah-Muthu et al. (2013); UK	Whitehall II Study; 11 y	4,255; 273; 72%; 56 y	114-item FFQ	Low-fat dairy; 458 g/d	0.98; 0.73-1.31; 0.88	Race, employment grade, family history of CHD/hypertension, consumption of fruit and vegetables, bread, meat, fish, coffee, and tea
				High-fat dairy; 182 g/d	1.23; 0.91-1.67; 0.17	
Struijk et al. (2013); The Netherlands	Inter99; 5 y	5,232; 214; 48%; 46 y	198-item FFQ	Low-fat dairy; 155 g/d	0.95; 0.85-1.06; NS	Intervention group, diabetes family history, education level, consumption of whole grain cereal, meat, fish, coffee, tea, fruit, vegetables, change in diet, waist circumference
				High-fat dairy; 17 g/d	1.03; 0.77-1.36; NS	

von Ruesten et al. (2013); Germany	EPIC-Potsdam Study; 8 y	23,531; 363; 37%, 35-65 y	148-item FFQ	Low-fat dairy; 100 g/d	1.03; 0.99-1.07; NS	WHR, hypertension, high blood lipids, education level, vitamin supplementation, consumption of other food groups
				High-fat dairy; 100 g/d	1.00; 0.95-1.05; NS	

[1]Note: Age, sex, BMI, total energy intake, smoking status, alcohol intake and physical activity were included in all studies.

[2]Abbreviations: BMI, body mass index; CI, confidence interval; CHD, coronary heart disease; EPIC, European Prospective Investigation into Cancer and Nutrition; FFQ, food-frequency questionnaire; HDL, high density lipoprotein; NS, nonsignificant; WHR, waist-to-hip ratio.

Eight prospective cohort studies have quantified the relationship between high- or low-fat dairy intake and incident T2D (Table 1-6). Of these, two recent studies found that high-fat dairy intake was inversely related to T2D. Ericson et al. (2015) followed 26,930 individuals for 14 y with 2,860 incident T2D cases in the Swedish Malmö Diet and Cancer cohort. High-fat dairy product intake was defined as the sum of butter; regular-fat (≥2.5% fat) of milk, yogurt, and sour milk; cream (>12% fat); and regular-fat cheese (>20% fat). In models adjusted for demographic and lifestyle variables, high-fat dairy products intake was inversely associated with incident T2D (HR = 0.77; 95% CI = 0.68-0.87; *P*-trend < 0.001). Interestingly, higher intake of low-fat dairy products was associated with increased risk, but this association disappeared when high and low-fat dairy were mutually adjusted for other dairy intake. Total dairy fat content was not associated with incident T2D. This study is unique in that dietary assessment combined 7-d dietary records, diet-history interviews, and FFQs, which may have led to a more accurate estimate of dairy intake than other studies. However, dietary data were only collected at baseline, such that potential changes in dietary patterns during the follow-up period could not be ruled out. It is also worth noting that intake of saturated fat (16% of total energy) and the contribution of dairy to total fat intake (30% of total fat) in this cohort was relatively high compared to other populations. Therefore, caution must be exercised when generalizing the results from this study.

In the natural history of the disease, individuals progress from NGT, to IGT, to frank T2D over several years. Prediabetes is a condition characterized by IGT, impaired fasting glucose or elevated glycated hemoglobin A1c levels that places individuals at a higher risk of developing T2D. Using data from the Framingham Heart Study Offspring Cohort, Hruby et al. (2017) showed that baseline glycemic status influenced the association of dairy fat intake with diabetes incidence. In their study, higher intake of high-fat (sum of whole milk, ice cream, cottage and

ricotta cheese, and other cheese) dairy consumption in normoglycemic individuals at baseline was associated with reduced incident prediabetes (HR = 0.68; 95% CI = 0.51-0.92; *P*-trend = 0.03 and HR = 0.75; 95% CI = 0.47-1.17; *P*-trend = 0.03, respectively). Participants with prediabetes at baseline that consumed higher amounts of high-fat dairy had a 70% lower risk of incident T2D (95% CI =0.10-0.92; *P*-trend = 0.03). Therefore, baseline glycemic status may contribute to the heterogeneity observed among studies evaluating the association between dairy fat intake and T2D.

In contrast with Ericson et al. (2015) and Hruby et al. (2017), six studies reported a neutral association between high-fat dairy and T2D incidence. For example, von Ruesten et al. (2013) reported that neither high-fat, nor low-fat, dairy products was associated with T2D risk in the EPIC-Potsdam study. Similar results in fully adjusted models were reported from cohorts in the UK by Soedamah-Muthu et al. (2013) in the Whitehall II study and O'Connor et al. (2014) in the EPIC-Norfolk cohort. One study found that higher low-fat dairy intake was inversely associated with T2D risk (Díaz-López et al. 2016). In this study, the authors studied a cohort of 3,454 elderly participants (mean age 67 y) from Spain. They reported 272 incident cases of T2D during a median follow-up of 4 y. The authors found that total and low-fat dairy products were significantly associated with reduced risk of T2D, but high-fat dairy showed no association.

Several factors may contribute to the inconsistent association between dairy fat intake and T2D. An important factor contributing to study heterogeneity may be variable adjustment for covariates by different investigators. Indeed, while all studies discussed so far controlled for age, sex, body mass index, total energy intake, and smoking status during statistical analyses, the use of additional covariates differed markedly among studies (see Table 1-5 and Table 1-6). Therefore, variable residual confounding may account for the inconsistent results. Furthermore,

study sample size may also help explain some of the inconsistent association among studies. In general, the larger studies tended to yield null or weak associations between dairy fat intake and T2D, whereas smaller studies resulted in stronger associations, often with inconsistent results among dairy fat categories and matrices. In some studies, dairy intake information was collected only at baseline; thus, changes in dietary habits during the follow-up period cannot be ruled out. The differences in median intake between different regions and the fat content of dairy products may also cause issues when evaluating the results, as different regions and cultures may consume vastly different amounts or types of dairy products. In addition, there is no standard categorization of how to separate low-fat from high-fat dairy products, so each study varied in how they classified the dairy products included in their analyses.

1.2.4 Meta-analyses of prospective cohort studies on dairy product intake and T2D risk

There have been seven meta-analyses of prospective cohort studies that have reported associations between total dairy consumption and the incidence of T2D (Pittas et al. 2007, Elwood et al. 2010, Tong et al. 2011, Aune et al. 2013, Gao et al. 2013, Chen et al. 2014, Gijsbers et al. 2016). None of the meta-analyses showed that high dairy intake was associated with an increased risk of T2D, and all but one showed that high dairy intake was associated with a lower risk of T2D. The primary limitation of these meta-analyses is that they rely on self-reported dietary intake, a measurement that is inclined to bias.

The most recent meta-analysis included 579,832 individuals and 43,818 T2D cases from 22 studies, published between 2005 and 2015 (Gijsbers et al. 2016). The authors found that people who consumed greater total amounts of dairy had a 3% lower relative risk (RR) of developing

T2D (RR = 0.97, per 200 g/d increment in dairy consumption; 95% CI = 0.95-1.00; *P* = 0.04) (Gijsbers et al. 2016).

Dairy products differ markedly in fat content and food matrix (i.e., the sum of the nutrients within the specific structure), which may influence their association with diabetes risk. Evidence for an association between total low- or high-fat dairy intake with T2D incidence is inconsistent. For example, the meta-analyses by Gijsbers et al. (2016) reported a trend for an inverse linear association for intake of low-fat dairy intake and T2D risk (RR = 0.96; CI = 0.92-1.00; P = 0.072) per 200-g/d increment. No significant associations were observed for high-fat dairy (Gijsbers et al. 2016). However, this study did not include a recent prospective analysis of the Malmö Diet and Cancer cohort that followed 26,930 individuals for 14 y with 2,860 incident T2D cases. This study found that total intake of high-fat, but not low-fat, dairy products was inversely associated with incident T2D (HR = 0.77; CI = 0.68-0.87; P < 0.001) (Ericson et al. 2015) in a model adjusted for physical activity, smoking, alcohol intake, and education, and body-mass index (BMI). Among dairy food matrices, yogurt intake was consistently associated with reduced risk of T2D (Tong et al. 2011, Aune et al. 2013, Gao et al. 2013, Chen et al. 2014, Gijsbers et al. 2016). A meta-analysis by Pimpin et al. (2016) looked specifically at butter, a high fat dairy product, and found an inverse relationship between butter intake and incident T2D (RR: 0.96; 95% CI: 0.93-0.99; *P*-trend = 0.02). For cheese, most varieties of which are fermented and high-fat, two meta-analyses reported that higher consumption is inversely associated with lower T2D incidence (Aune et al. 2013, Gao et al. 2013), but a third reported no association (Gijsbers et al. 2016). Neither total nor full-fat milk intake was significantly associated with T2D risk (Tong et al. 2011, Aune et al. 2013, Gao et al. 2013, Gijsbers et al. 2016). These results are in line with a Mendelian randomization study which found no association between milk intake and

T2D risk (Bergholdt et al. 2015). For low-fat milk intake, the results are mixed, with studies reporting either inverse or no association with T2D risk. Dose-response meta-analyses reported nonlinear inverse associations for total dairy products (Aune et al. 2013, Gao et al. 2013), low-fat dairy products (Aune et al. 2013, Gao et al. 2013), and yogurt (Aune et al. 2013, Gijsbers et al. 2016), with no further reduction in disease risk at higher intakes (≥ 300-400 g/d of total dairy intake and ≥ 120-140 g/d of yogurt). Taken together, the totality of the evidence from meta-analyses of cohort studies provides strong support for an inverse association between total dairy intake and T2D risk. This association differs among dairy products, with stronger evidence for higher intake of low-fat and fermented dairy products.

While prospective cohort studies offer much in the way of correlation between dairy intake and the incident rate of T2D, they lack the ability to show direct causality between the consumption of dairy products and incident T2D. To this end, randomized controlled trials (RCT) measuring the effect of dairy product intake on T2D risk factors can begin to determine a causal effect of dairy products in prevention of T2D. As reduced sensitivity of tissues to insulin action is one of the primary characteristics of T2D, insulin sensitivity can be used as an unbiased way of evaluating T2D risk.

1.2.5 Determining insulin sensitivity in humans

There are several methods currently used to determine insulin sensitivity in humans in order to predict the risk of developing T2D as well as to evaluate the management of the disease. One of the most common procedures used is the glucose tolerance test (GTT). Glucose tolerance is directly associated with insulin sensitivity. The GTT uses oral, intravenous, or intraperitoneal intake of exogenous glucose to determine the effect on systemic glucose clearance and therefore

measure glucose tolerance (Kim 2009). The main limitation of GTT is that it does not account for endogenous insulin secretion, limiting its use in individuals who have altered or impaired pancreatic function. Insulin tolerance tests (ITT) can also be used to examine systemic glucose clearance through intraperitoneal administration of insulin, as insulin tolerance also correlates with insulin sensitivity (Kim 2009). However, ITT causes severe hypoglycemia which can skew resulting data as systemic processes will try to adjust in response.

The homeostatic method of assessment (Gordon Bell et al.) is a mathematical assessment of the interaction between pancreatic β-cell function and IR in an idealized model which can then be used to compute the steady-state of both insulin and glucose concentrations (Wallace et al. 2004). This relationship between fasting glucose and insulin is an indication of hepatic glucose output and insulin secretion which are maintained via a feedback loop between the liver and β-cells. An additional mathematical assessment is the QUICKI model proposed by Katz et al. (2000). Similar to the HOMA method, this procedure only requires one blood sample from the patient and uses fasting glucose and fasting insulin to calculate insulin sensitivity. Discretion must be used in interpreting HOMA and QUICKI data for individuals with pre-existing conditions that affect insulin sensitivity, as it has been shown to be inaccurate in women with PCOS and prepubertal children (Cutfield et al. 2003, Diamanti-Kandarakis et al. 2004). In addition, these models do not account for systemic changes that occur from a glucose stimulus. However, due to their ease of use and practicality, these methods are used most often for large scale epidemiological studies.

The current gold-standard method for measuring insulin sensitivity is the hyperinsulinemic-euglycemic clamp. This method of addresses the limitations of GTT, ITT, and HOMA. Developed in Andres et al. (1966) and further improved by DeFronzo et al. (1979), it has since

become widely used in clinical and fundamental science research. The hyperinsulinemic-euglycemic clamp allows for the quantification of pancreatic β-cell sensitivity to glucose and the sensitivity of target tissues to insulin (Trout et al. 2007). In the clamp procedure, insulin is administered intravenously at a constant rate to raise and maintain systemic insulin levels (the hyperinsulinemic state). Glucose is then infused at a variable rate until infusion reaches a steady-state that will directly correlate with insulin sensitivity (the euglycemic state). During the last 30 minutes of the clamp procedure, blood samples are taken to measure plasma glucose concentrations, specific activity, and insulin stimulated whole body glucose turnover. In animal models, radiolabeled glucose can be infused in order to measure glucose metabolism in specific organs (Kim 2009). The ability to measure glucose turnover of individual organs in animal models has significantly advanced the understanding the origin of T2D (Kim 2009). Unfortunately, use of the hyperinsulinemic-euglycemic clamp is time-consuming, invasive, and costly compared to other methods, therefore use of this method is generally limited.

1.2.6 Evidence from randomized controlled trials

RCT are needed to provide high-level evidence to guide dietary recommendations regarding dairy intake and T2D risk. Seven RCT have been published on the effects of total dairy product intake on indices of insulin sensitivity and blood glucose/insulin levels in weight-stable adults. Of these, 4 found a positive effect of increased total dairy intake on insulin sensitivity (fasting insulin and HOMA-IR), whereas 3 showed no effect (Table 1-7). Most of these RCTs studied obese and overweight individuals, some selecting for those who met at least 2 criteria of metabolic syndrome (Wennersberg et al. 2009, Stancliffe et al. 2011). None of the studies selected individuals based on prediabetes. The duration of the dairy interventions varied between

4 weeks to 6 months. Studies with less than 12 week interventions failed to detect changes in indices of insulin sensitivity/resistance or glycemic measures (van Meijl and Mensink 2011, Benatar et al. 2014). Studies lasting 12-24 weeks reported a change in at least one glucose-related outcome, suggesting this period is sufficient to detect the effect of dairy intervention on integrated measures of glucose homeostasis. Longer interventions (6-months) led to mixed results and some reported high rates of attrition (>40%) and difficulties in maintaining adherence (Wennersberg et al. 2009, Crichton et al. 2012, Crichton et al. 2012, Rideout et al. 2013). The existing studies also differed in the number of servings, the type and fat content, as well as the accounting for potential confounding variables (e.g. physical activity) that can explain the inconsistent results among studies. Importantly, only one study declared insulin sensitivity as primary outcome. This implies that the sample sizes may be inadequate to properly assess the effect of dairy on this outcome.

One of the largest limitations of RCT studies is the large number of study participants that drop out of the studies due to many of them finding it difficult to comply with the dietary requirements. Quantitatively, the main limitation of these RCT is that they all relied on surrogate measures of insulin sensitivity/resistance. These indices show significant but limited correlation when validated against hyperinsulinemic-euglycemic clamp technique (Otten et al. 2014), which is considered the gold-standard method for quantifying insulin sensitivity (DeFronzo et al. 1979). Importantly, these indices have limited ability to detect changes in insulin sensitivity after an intervention when compared to the clamp (Hays et al. 2006).

Table 1-7. Randomized controlled intervention studies that assessed the impact of altering dairy consumption on insulin sensitivity.

Authors	Study design	*n*; sex (% men); age (mean or range); characteristic	Intervention	Outcome measures	Effects of intervention
Hoppe et al. (2004)	Parallel study of two 1-week interventions	24; 100%; 8 y; healthy	250 g lean red meat/d or 1.5 L skim milk/d	IGF-1, insulin, glucose	↑IGF-1 and fasting insulin in milk group compared to meat.
Hoppe et al. (2009)	Randomised crossover study of two 10-day interventions	11; 100%; 22–29 y; healthy	2.5 L low-fat milk/d or 2.5 L Coca-Cola/d	IGF-1, insulin, glucose	↓IGF-1 after Cola period, no difference between groups for insulin or glucose, or from baseline.
Benatar et al. (2014)	Randomised parallel study of three 1-month interventions	176; 22%; 51 ± 16 y; healthy normal-weight participants	Increase dairy by 2–3 servings/d; maintain usual dairy intake; eliminate dairy	Blood pressure, lipids, CRP, glucose, insulin	No difference between diets.

Rideout et al. (2013)	Randomised crossover study of two 6-month interventions	23; 22%; 18–75 y; BMI 18.5–35 kg/m^2	4 servings dairy/d compared to ≤2 servings dairy/d	Blood pressure, lipids, glucose, insulin	High dairy ↓insulin ($p < 0.05$), improving HOMA-IR. No difference in BP, lipids, weight or body composition.
Crichton et al. (2012)	Randomised crossover study of two 6-month interventions	61; n/a; 18–75 y; BMI >25 kg/m^2	4 servings dairy/d compared to <1 servings dairy/d	Waist circumference, blood pressure, fasting glucose, lipids, CRP	No difference between diets.
Zemel et al. (2005)	Randomised parallel study of two 24-week interventions	34; 32%; 26–55 y; BMI 30–40 kg/m^2	3 servings dairy/d or <1 servings dairy/d	Body composition, blood pressure, insulin, glucose, lipids	↑lean body mass ($p < 0.04$), ↓blood pressure ($p < 0.01$), ↓fasting insulin ($p < 0.04$) in dairy group.
Wennersberg et al. (2009)	Randomised parallel study of two 6-month interventions	113; 67%; 30–65 y; at least 2 components of MetS with habitual dairy intake <2 serves/day	<2 servings dairy/day or 3-5 servings dairy/d	Weight, body composition, lipids, glucose, insulin, HbA1c, C-peptide, inflammatory markers	Total cholesterol ↑ with increased dairy and V-CAM-1 ↓ in women. No change in insulin in milk group but ↑insulin in control resulting in ↑ HOMA-IR ($p = 0.037$).

van Meijl and Mensink (2010)	Randomised crossover study of two 8-week interventions	35; 29%; 18–70 y; BMI >27 kg/m^2 with habitual dairy intake <500 g/day	500 mL low-fat milk and 150 g low-fat yoghurt/d compared to 600 mL fruit juice and 43 g fruit biscuits	Blood pressure, lipids, glucose, insulin, inflammatory markers	↓systolic blood pressure in the dairy period vs control ($p = 0.027$). No difference in glucose, insulin, lipids or inflammatory markers.
Stancliffe et al. (2011)	Randomised parallel study of two 12-week interventions	40; 48%; 37 ± 10 y; BMI 25–39.9 kg/m^2 with >3 components of MetS	>3.5 servings dairy/d compared to <0.5 serves dairy/d	Body composition, blood pressure, glucose, insulin, oxidative and inflammatory biomarkers	Adequate dairy ↓ insulin, improving HOMA-IR vs control ($p < 0.05$). Higher dairy ↓ TNF-α, MCP-1 ($p < 0.05$) and IL-6 ($p < 0.02$).
Pal et al. (2010)	Randomised parallel study of three 12-week interventions	70; 14%; 18–5 y; BMI 25–40 kg/m^2	54 g of whey protein, casein protein or glucose control	Lipids, insulin, glucose, body composition	Whey ↓ insulin vs baseline ($p = 0.012$) and control ($p = 0.049$). Whey ↓ TC and LDL compared to casein and control.

[1]Abbreviations: BMI, body mass index; CRP, C-reactive protein; HbA1c, glycated hemoglobin A1c; LDL, low density lipoprotein.

1.2.7 Biomarkers of dairy intake

To overcome the limitation of self-reported dietary intake, many studies measure the circulating concentrations of the odd-chain FAs (OCFA), pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0), as well as *trans*-palmitoleic acid (*trans*-C16:1n-7) as objective biomarkers of dairy fat (Wolk et al. 1998, Smedman et al. 1999, Wolk et al. 2001, Micha et al. 2010). A biomarker is a measurable nutrient, in this case FA, that serves as an indicator for a specific dietary intake, in this case dairy fat. Humans are thought to be unable to synthesize C15:0 and C17:0 endogenously, such that their concentration in plasma largely reflects dietary intake of ruminant fat sources. As such, circulating serum, plasma, or adipose tissue FA profiles can be compared to disease prevalence to help determine the associated risk. Using biomarkers could reduce heterogeneity from epidemiological studies that do not discriminate between dairy products and where the dairy products themselves have different FA profiles. Previous literature suggests that bodily concentrations of C15:0 can average 0.22% of total FA, while concentrations of *trans*-C16:1n-7 and C17:0 can average 0.27% and 0.4%, respectively (Table 1-8).

The validity of C15:0 and C17:0 as biomarkers has long been of interest (Table 1-9). Wolk et al. (1998) showed that adipose tissue concentrations of C15:0 and C17:0 correlated strongly with their estimated values derived from food records and FFQs. Their work suggested that C15:0 and C17:0 reflected long-term milk intake in populations that naturally consume a large number of dairy products. This was further shown in a follow up study in which they measured C15:0 and C17:0 in adipose tissue, serum cholesterol esters, and serum phospholipids of 114 men aged 40-76 years. Concentrations of C15:0 and C17:0 in all fractions

correlated strongly with weighed food records, with adipose tissue FAs having the strongest association (Wolk et al. 2001).

Table 1-8. Comparison of average known values of C15:0, *trans*-C16:1n-7, and C17:0 in humans (all expressed as percent of total FAs).

Authors	***n*; sex (% men); age (mean or range)**	**Lipid fraction**	**C15:0**	***trans*-C16:1n-7**	**C17:0**
Abdullah et al. (2015)	124; 32%; 39 ± 16 y	Total plasma lipids (control and dairy intervention)	0.22 (control)	0.26	0.39
			0.26	0.25	0.42
Zeleniuch-Jacquotte et al. (2000)	46; 0%; 53 ± 4 y	Serum phospholipids	0.24	--	0.69
Forouhi et al. (2014)	28 557; n/a; >18 y	Plasma phospholipids	0.21 (T2D)	--	0.41
			0.20 (control)	--	0.40
Wolk et al. (1998)	81; 0%; 30 - 77 y	Adipose tissue	0.35	--	0.24
Saadatian-Elahi et al. (2009)	3003; 50%; >18 y	Plasma phospholipids (men and women)	0.14 (men)	--	0.30 (men)
			0.14	--	0.35
Brevik et al. (2005)	110; 100%; 21-55 y	Serum	0.22	--	0.37
Santaren et al. (2014)	659; 45%; 55 ± 9 y	Serum	0.25	0.30	--

Brevik et al. (2005) also found that C15:0 in adipose tissue as well as serum correlated strongly with total dairy fat intake calculated by weighed records. Dietary intervention has also been used to evaluate the effect of dairy intake on plasma concentrations of C15:0 and C17:0. Abdullah et al. (2015) conducted a 4-week intervention study where participants were assigned either a dairy free control diet or a diet in which participants were to consume 3 servings/day of low-fat milk, yogurt, and regular cheese. This resulted in higher plasma concentrations of C15:0 and C17:0, but not *trans*-C16:1n-7, and only minor changes to the rest of the FA profile.

A recent study by Jenkins et al. (2017) found that for a 30% dietary increase in dairy fat, there was a 10% increase in circulating plasma levels of C15:0. Plasma levels of C17:0 did not change after the dietary intervention. The authors performed a further dietary invention with low-fat and high-fat dairy treatment groups. As expected, plasma levels of C15:0 decreased in the low-fat dairy treatment group and increased in the high-fat dairy treatment group. The authors of this study suggest that C15:0 alone is should be used as a biomarker of dairy intake. Further, C17:0 may be incorrectly identified as a biomarker of dairy intake, due to its relationship with the intake of phytol, phytanic acid, and C18:0.

Table 1-9. Associations between dairy fat intake and percent of pentadecanoic acid, trans-palmitoleic acid, and heptadecanoic acid.

Authors	*n*; sex (% men); age (mean or range)	Lipid fraction	Correlation	C15:0	*trans*-C16:1n-7	C17:0
Saadatian-Elahi et al. (2009)	3003; 50%; >18 y	Plasma phospholipid	Spearman	FFQ 0.33 24-HDR 0.15	--	FFQ: -0.08 24-HDR: 0.13
Wolk et al. (1998)	81; 0%; 30-77 y	Adipose tissue	Pearson	Dietary records: 0.63 FFQ: 0.40	--	Dietary records: 0.42 FFQ: 0.23
Santaren et al. (2014)	659; 45%; 55 ± 9 y	Serum	Regression	0.20	--	--
Sun et al. (2007)	327; 0%; 30-55 y	Plasma	Spearman	0.36	0.30	0.21
		Erythrocytes		0.30	0.32	0.16
Micha et al. (2010)	3330; 52%; 72 ± 5 y	Plasma phospholipid	Spearman	--	0.39	--

Seven prospective cohort studies have looked at the association between biomarkers of dairy fat intake and T2D risk (Table 1-10) (Hodge et al. 2007, Krachler et al. 2008, Mozaffarian et al. 2010, Mozaffarian et al. 2013, Forouhi et al. 2014, Santaren et al. 2014, Yakoob et al. 2016). All studies found that higher dairy fat intake was associated with a lower risk of T2D. Three studies reported on *trans*-C16:1n-7; all found substantially lower risk. Confirming these results, a meta-analysis reported an inverse association between *trans*-C16:1n-7 and T2D incidence (RR = 0.58; CI = 0.46, 0.74; $P < 0.001$) (de Souza et al. 2015). Five of the seven studies evaluating C15:0 and two of three studies evaluating C17:0 observed significant inverse associations with incident T2D. Available evidence from dairy biomarkers fat intake studies is consistent in that higher intake of dairy fat is associated with a lower risk of T2D. These results are consistent with some of the results from prospective cohort studies assessing the relationship between diary intake and T2D risk that used FFQ and other dietary records.

Table 1-10. Prospective studies evaluating the association between biomarkers of dairy fat intake and incidence of T2D.

Authors; country	Study; follow-up (mean or range)	*n* participants; *n* cases; sex (% men); age (mean or range)	FA; fraction	Hazard or odds ratio; 95% confidence interval; *P*-trend	Adjustments for covariates[1]
Krachler et al. (2008); Sweden	Vasterbotten Intervention Programme; 5 y	450; 159; 49%; 52 y	C15:0; erythrocyte membrane	0.71; 0.52-0.97; 0.033	HbA1c[2]
			C17:0; erythrocyte membrane	0.54; 0.35-0.83; 0.005	
Forouhi et al. (2014); Europe	EPIC-InterAct cohort; 16 y	27,296; 12,132; 38%; 52 y	C15:0; plasma phospholipid	0.79; 0.73-0.86; < 0.05	Education level, consumption of meat, fruit and vegetables, soft drinks, and total dairy products, baseline HbA1c, incident T2D within 2 y after baseline
			C17:0; plasma phospholipid	0.66; 0.62-0.71; < 0.05	
			C15:0 + 17:0; plasma phospholipid	0.70; 0.66-0.75; < 0.05	
Hodge et al. (2007); Australia	Melbourne Collaborative Cohort Study, 4 y	3391; 346; 47%; 36-72 y	C15:0; plasma phospholipid	0.40; 0.26-0.63; < 0.0001	Country of birth, family history of diabetes, and waist-hip ratio
Mozaffarian et al. (2010); USA	Cardiovascular Health Study;7 y	2,985; 304; 45%; 75 y	*trans*-C16:1n-7; plasma phospholipid	0.38; 0.24–0.62; < 0.001	Race, education level, enrollment site, waist circumference, CHD status, consumption of carbohydrate, protein, whole-fat dairy foods, low-fat dairy foods, and red meat

Mozaffarian et al. (2013); USA	Multi-Ethnic Study of Atherosclerosis; 7 y	2,617; 205; 47%; 62 y	*trans*-C16:1n-7; plasma phospholipid	0.52; 0.32-0.85; 0.02	Race, education level, field center, waist circumference, consumption of whole-fat dairy foods, low-fat dairy foods, and red meat
Santaren et al. (2014); USA	Atherosclerosis Risk in Communities; 5 y	659; 103; 45%; 55 y	C15:0; total serum lipids	0.73; 0.56-0.95; 0.02	Race, center, education level, consumption of fruit and vegetable, red meat, soft drink, and fiber
Yakoob et al. (2016); USA	Nurses' Health Study and the Health Professionals Follow Up Study; 15 y	3,333; 277; 44%; 30-75 y	C15:0; total plasma lipids	0.56; 0.37-0.86; 0.01	Race, family history of diabetes, parental history of myocardial infarction, hypercholesterolemia, hypertension, menopausal status in NHS, postmenopausal hormone use in NHS, consumption of fish, processed meats, unprocessed meats, fruits, vegetables, whole grains, coffee, sugar-sweetened beverages, glycemic load, dietary calcium, polyunsaturated fat, and plasma *t*-C18:1n-7, *t*-C18:2, C16:0, and C18:0
			C17:0; total plasma lipids	0.57; 0.39-0.83; 0.01	
			trans-C16:1n-7; total plasma lipids	0.48; 0.33-0.70; < 0.001	

[1]Note: Age, sex, BMI, total energy intake, smoking status, alcohol intake and physical activity were included as covariates in all studies.

[2]Abbrevations: BMI, body mass index; CI, confidence interval; CHD, coronary heart disease; EPIC, European Prospective Investigation into Cancer and Nutrition; HbA1c, glycated hemoglobin A1c; HDL, high density lipoprotein; HR; hazard ratio; NHS, Nurse's Health Study; WHR, waist-to-hip ratio.

1.2.8 Associations between biomarkers and insulin sensitivity

Evidence from RCT suggests that increased dairy intake is associated with improved insulin sensitivity. To eliminate the difficulties of maintaining compliance with participants in RCT, studies can instead directly assess the relationship between insulin sensitivity and biomarkers of dairy fat. In a study by Kratz et al. (2014), insulin sensitivity was assessed by OGTT and through a hyperinsulinemic-euglycemic clamp procedure in 17 individuals with non-alcoholic fatty liver disease (NAFLD) and 15 age and weight matched controls. They found that phospholipid C17:0, phospholipid *trans*-C16:1n-7, free fatty acid (FFA) C15:0, and FFA C17:0 were all inversely associated with the area under the curve for an OGTT. Phospholipid *trans*-C16:1n-7 was positively associated with both hepatic and systemic insulin sensitivity assessed by the hyperinsulinemic-euglycemic clamp. Nestel et al. (2014) measured phospholipid C17:0 and *trans*-C16:1n-7 in 86 overweight and obese individuals that had been diagnosed with metabolic syndrome. Initially, phospholipid C17:0 was directly associated with insulin sensitivity as measured by HOMA, however, after adjustment for covariates this association was attenuated. No significant association was found between insulin sensitivity and *trans*-C16:1n-7 (Nestel et al. 2014). In a study of 659 healthy adults, C15:0 was positively associated with insulin sensitivity as measured by intravenous glucose tolerance testing (Santaren et al. 2014). *Trans*-C16:1n-7 was negatively associated with insulin sensitivity, but this relationship was attenuated after adjustment for covariates. Of note, in this study *trans*-C16:1n-7 was not significantly associated with total dairy intake but was significantly associated with partially hydrogenated foods (Santaren et al. 2014). In two subsequent studies, *trans*-C16:1n-7 was associated with lower insulin resistance and lower fasting insulin (Mozaffarian et al. 2010, Mozaffarian et al. 2013). However, in the second study, although *trans*-C16:1n-7 correlated positively with dairy

fat intake it was more strongly associated with margarine and french fries (Mozaffarian et al. 2013).

The use of biomarkers has helped clarify the role of dairy fat intake on CVD and T2D risk, but their use is not without limitations. Although serum and adipose tissue FA markers eliminate bias and variability due to misreporting, there are unique caveats that must be considered when interpreting results based on them. First, although they derive primarily from dairy in most prevailing diet patterns, these FA can also reflect the consumption of other forms of ruminant fat, for example that contained in beef or lamb. OCFA are also widely distributed in other food sources including plants and seafood (Abdullah et al. 2015, Riserus and Marklund 2017). Second, serum measurements of dairy-derived FA are not necessarily a more accurate measure of dairy fat intake than FFQs, although their concentration in adipose tissue is a relatively accurate reflection of long-term dairy fat consumption. Importantly, existing biomarkers do not distinguish between dairy matrices even though these differ markedly in their lipid structure and composition (Thorning et al. 2017). Overall dietary intake is not always considered when quantifying the association between FA and CVD risk, which makes it difficult to compare the findings to the type of dairy products being consumed (Matthan et al. 2014). Third and perhaps most problematic, certain serum FA may be altered by metabolic status. For example, one study suggested that the pre-diabetic state itself alters the concentration of linoleic acid in plasma phospholipids (Hodge et al. 2007). While plasma phospholipid linoleic acid was inversely related to diabetes risk, dietary linoleic acid trended in the opposite direction. Both serum and adipose tissue C15:0, C17:0, and *trans*-C16:1n-7 are relatively good biomarkers of dairy fat intake; however, it remains possible that these FAs are influenced by metabolic factors. Contrary to prior assumptions, recent evidence demonstrated they can be endogenously

synthesized by humans from gut-derived propionate (Weitkunat et al. 2017). This would mean that individual genetic differences in metabolism must be considered when doing studies with FAs, as these differences could cause changes to blood FA levels. In addition, the relationship between specific FA biomarkers and cardiometabolic disease risk has not been always consistent between and within studies (see above and (Yakoob et al. 2016)). It may therefore be likely that individual differences in metabolism that may not be considered as confounders would continue to cause variability between studies. Despite these caveats, studies that used FA biomarkers to assess the relationship between dairy fat intake and adiposity or disease end points were broadly consistent with those that assessed dairy fat intake by other means.

1.2.9 Potential Mechanisms of Dairy Products Associated with the Prevention of T2D

The mechanisms by which dairy fat intake may influence diabetes risk are not clear, but there is evidence that dairy intake can influence the levels of metabolites and genes involved in regulation of tissue insulin sensitivity. Bovine milk contains bioactive lipids such as butyrate and medium-chain FAs can act on signaling and transcriptional pathways to modulate gene expression (Shingfield et al. 2008). Low-grade systemic inflammation contributes to the development of IR (Shoelson et al. 2006). Some RCT have showed that a higher intake of dairy products reduced inflammation, as assessed by changes in inflammatory markers (e.g., decreased C-reactive protein (CRP) and interleukin-6 (IL-6), increased adiponectin). In a study by van Meijl and Mensink (2011), after a period of 8 weeks low-fat dairy consumption showed a trend to decrease TNF-α, which is related to obesity, insulin resistance, and the metabolic syndrome. One such study showed that a diet of adequate dairy intake suppressed inflammatory markers as

well as decreasing malonaldehyde and oxidized LDL (Stancliffe et al. 2011). However, a recent systematic review concluded the effect may be neutral (Labonte et al. 2013). Fermented dairy products have been associated with changes in blood metabolites that could be involved in the dairy-induced cardiometabolic effects. For example, a recent metabolomic study identified metabolite signatures of changes in microbial and lipid metabolism in 15 young men who consumed cheese, but not in those that consumed milk, for 14 days (Zheng et al. 2015). Using lipidomic and genomic profiling to identify the metabolites associated with dairy-induced changes in insulin sensitivity may point to potential mechanisms by which dairy products could enhance insulin sensitivity.

2. MANUSCRIPT

Association between fatty acid biomarkers of dairy fat intake and insulin sensitivity

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2.1 ABSTRACT

Background: Epidemiological studies have shown that dairy fat intake, as measured by serum levels of pentadecanoic acid (C15:0), heptadecanoic acid (C17:0), or *trans*-palmitoleic acid (*trans*-C16:1n-7), is inversely associated with incidence of type 2 diabetes (T2D). These biomarkers have been positively associated with surrogate measures of insulin sensitivity, but studies using the hyperinsulinemic-euglycemic clamp in healthy individuals and people with T2D are lacking.

Objective: The primary objective was to investigate the associations between biomarkers of dairy fat intake and measures of insulin sensitivity as determined by hyperinsulinemic-euglycemic clamp in a cohort of normal weight men and women and overweight/obese (ow/ob) men and women with T2D. The secondary objective was to assess the influence of T2D status and sex on serum concentration of dairy biomarkers and their associations with insulin sensitivity.

Methods: Normal weight nondiabetic and type 2 diabetic ow/ob men and women (n = 42) underwent a 3-hour hyperinsulinemic-euglycemic-isoaminoacidemic clamp. Glucose rate of appearance (production, Ra) and disposal (Rd) were measured by 3-[^{3}H]-glucose. The main outcome measures of insulin sensitivity were glucose disposal (M/I index) and the hepatic insulin resistance (IR) index. Fatty acid (FA) profiles in fasting serum samples, including C15:0, C17:0, and *trans*-C16:1n-7, were determined through direct methylation followed by gas chromatography. Pearson and Spearman correlations were used to determine the association between FA biomarkers of dairy intake and insulin sensitivity.

Results: The T2D status group included ow/ob men (n = 14, age = 65.6 ± 8.6 y, BMI = 29.4 ± 2.3 kg/m^2) and women (n = 6, age = 59.3 ± 5.7 y, BMI = 31.2 ± 2.3 kg/m^2). The normal weight group included men (n = 14, age = 35.8 ± 16.3 y, BMI = 22.2 ± 1.6 kg/m^2) and women (n = 8, age = 25.1 ± 5.3 y, BMI = 21.1 ± 1.3 kg/m^2). Participants in the ow/ob T2D group were older, had higher BMI, larger waist circumferences, higher protein intakes, and higher fasting glucose and insulin concentrations. Overall, men had larger waist circumferences, higher protein and energy intakes, higher fasting insulin, as well as higher clamp glucose Ra and Rd. Men had higher concentrations of *trans*-C16:1n-7 (% total FA) than women. Mono- and poly-unsaturated FA differed between groups, the former being lower in the normal group and latter being lower in the ow/ob T2D group. *Trans*-C16:1n-7 was inversely associated with measures of insulin sensitivity in both the normal weight and the ow/ob T2D groups as well as in males. C15:0 showed a trend for a positive association with M/I index in the ow/ob T2D group. C17:0 was not associated with measures of insulin sensitivity but associated inversely with the hepatic IR index in women.

Conclusions: The direction and strength of the association between dairy fat intake and clamped-based indices of insulin sensitivity differed among FA used as biomarkers and between groups. These associations were influenced by both T2D status and sex. More research is needed to discern the precise relationship between dairy fat intake and insulin sensitivity.

Keywords: Biomarkers; dairy fat; insulin sensitivity; serum fatty acids.

2.2 INTRODUCTION

Type 2 diabetes (T2D) is a chronic metabolic disease characterized by chronic hyperglycemia. The prevalence of T2D is increasing worldwide, with an estimated 624 million cases by 2040 (Ogurtsova et al. 2017). If suboptimally controlled, T2D can cause a range of complications including cardiovascular disease (CVD), renal disease, stroke, blindness, and peripheral vascular disease and neuropathy leading to lower limb amputations (IDF 2017). T2D is thought to be caused by environmental factors impinging on genetically susceptible individuals. Diet is a major modifiable lifestyle factor that influences T2D risk.

Dairy products are rich in essential nutrients such as protein, calcium, potassium, as well as vitamins A, D, and B_{12}. However, they also have a high content of saturated fat (Palmquist 2006, O'Donnell-Megaro et al. 2011). Consumption of foods with high saturated fat levels has long been linked to development of the cardiometabolic syndrome, which often precedes T2D and CVD (Wennersberg et al. 2009, Fumeron et al. 2011). This has prompted many countries, including Canada, to recommend the consumption of low-fat dairy products (Health Canada 2007). However, most recent epidemiological studies have revealed that the association between dairy intake and risk of T2D is neutral, with some even reporting a slight inverse relationship (Chen et al. 2014, Ericson et al. 2015, Gijsbers et al. 2016, Hruby et al. 2017).

The relationship between dairy intake and T2D differs depending on the type of dairy products consumed and their fat content. Several meta-analyses have found that milk intake has no association with T2D risk (Tong et al. 2011, Aune et al. 2013, Gao et al. 2013, Bergholdt et al. 2015, Gijsbers et al. 2016). One meta-analysis found no association for cheese intake (Gijsbers et al. 2016), whereas two others found a lower risk (Aune et al. 2013, Gao et al. 2013). One study of butter intake found a lower risk (Pimpin et al. 2016). Yogurt consumption is

consistently associated with lower T2D risk (Tong et al. 2011, Aune et al. 2013, Gao et al. 2013, Chen et al. 2014, Gijsbers et al. 2016). Of eight prospective cohort studies that looked at the effects of low- and high-fat dairy intake, the majority of studies found neutral effects of dairy fat intake on T2D risk (Louie et al. 2013, Soedamah-Muthu 2013, Struijk et al. 2013, O'Connor et al. 2014, Ericson et al. 2015, Díaz-López et al. 2016, Hruby et al. 2017).

Most epidemiological studies evaluating the relationship between dairy intake and T2D have relied on self-reported dietary assessments, which are subject to bias and misreporting. To overcome these limitations, recent studies have measured the concentration of pentadecanoic acid (C15:0), heptadecanoic acid (C17:0), or *trans*-palmitoleic acid (*trans*-C16:1n-7) in serum or plasma lipid fractions as objective biomarkers of dairy fat intake. These fatty acids (FA), which are produced during rumen microbial fermentation, are thought to be derived primarily in the human diet from ruminant food sources (Wolk et al. 1998, Brevik et al. 2005, Micha et al. 2010). Prospective cohort studies using these biomarkers of dairy fat intake have consistently showed lesser risk of developing T2D with higher dairy fat intake, but the strength of the association differed among the specific FA used as dairy fat biomarker, both within and between studies (Hodge et al. 2007, Krachler et al. 2008, Mozaffarian et al. 2010, Mozaffarian et al. 2013, Forouhi et al. 2014, Santaren et al. 2014, Yakoob et al. 2016). However, the physiological mechanisms underlying this association have not been firmly established.

Previous studies that examined the association between dairy fat biomarkers and insulin sensitivity led to inconsistent results. Two studies used surrogate measures of insulin sensitivity or resistance based on fasting glucose and insulin blood measurements, e.g. homeostatic model assessment insulin resistance (HOMA-IR), or glucose tolerance tests (GTT), e.g. Matsuda index (Kratz et al. 2014, Nestel et al. 2014, Santaren et al. 2014). Nestel et al. (2014) found that plasma

phospholipid C17:0, but not *trans*-C16:1n-7, was directly associated with HOMA-IR and Matsuda index. In addition, Santaren et al. (2014) found that while serum C15:0 was associated with intravenous GTT-derived insulin sensitivity index in a subset of the Insulin Resistance Atherosclerosis Study cohort, *trans*-C16:1n-7 was not. Only one study to date has used the gold standard method of determining insulin sensitivity in humans, the hyperinsulinemic-euglycemic clamp. In a cohort of 17 middle aged obese men and women with non-alcoholic fatty liver disease and 15 age- and body mass index (BMI)-matched control individuals, Kratz et al. (2014) found that plasma phospholipid *trans*-C16:1n-7, but not C15:0 or C17:0, was positively associated with both clamp-derived peripheral and hepatic insulin sensitivity in statistical models adjusted for age, BMI and sex. The reason for the inconsistent relationship between specific FA biomarkers of dairy fat intake and insulin sensitivity is not clear but may relate to physiological or metabolic factors that influence FA metabolism and insulin sensitivity.

The primary objective of this study was to examine the relationship between biomarkers of dairy fat intake and insulin sensitivity, as measured by the hyperinsulinemic-euglycemic clamp, in a cohort of men and woman with or without T2D. The secondary objective was to examine the effect of sex and T2D status on the serum concentrations of FA used as dairy fat biomarkers and to evaluate their influence on their relationship with insulin sensitivity.

2.3 MATERIALS AND METHODS

2.3.1 Participants

This study is a secondary analysis of data obtained from 42 participants (28 men, 14 women) previously published by our group (Chevalier et al. 2004, Chevalier et al. 2005,

Chevalier et al. 2006, Pereira et al. 2008, Bassil et al. 2011, Winter et al. 2012, Chevalier et al. 2015, Labonte et al. 2015, Murphy et al. 2015, Burgos et al. 2016). Exclusion criteria for the studies included hepatic, renal, pulmonary, thyroid or cardiovascular disease; any active cancer (except skin); positive serology; pregnancy and breastfeeding in women; smoking and medication; and consumption of diets deviating from standard guidelines. Premenopausal women were studied in the follicular phase. Participants had stable weight (± 3 kg) for at least 6 months preceding the study. They were admitted to the McGill University Health Centre research unit 1-7 d before the clamp, where they consumed an isocaloric, protein-controlled diet for the duration of their stay. All participants gave written informed consent. Ethics approvals were obtained from the hospital's Institutional Review Board.

2.3.2 Hyperinsulinemic, euglycemic, isoaminoacidemic clamp

Participants underwent a 3-h hyperinsulinemic-euglycemic-isoaminoacidemic clamp procedure after fasting overnight. 3-[^{3}H]-glucose was infused throughout to determine the whole-body glucose rate of appearance (Ra) and disposal (Rd). Human insulin (Humulin R; Eli Lilly, Toronto, ON) was infused (40 mU/m^{2}/min) to achieve a physiological postprandial steady state serum concentration. Amino acids were infused to maintain postabsorptive levels of branched-chain amino acids. This elevated concentration of amino acids had no effect on glucose or insulin infusion rates when compared between groups and to previous literature. Body composition was determined by dual energy x-ray absorptiometry (Lunar Prodigy Advance; GE Healthcare, Madison, WI). Fasting serum samples were stored at -80°C until analyzed. Serum insulin was measured by radioimmunoassay (Millipore Corporation, Billerica, MA) or by enzyme-linked immunosorbent assay (Mercodia AB; Upsalla, Sweden) in a subset of participants. Glucose was

measured by the glucose oxidase method. To control individual energy and protein intakes, participants received a controlled diet determined by calculating their resting energy expenditure (Chevalier et al. 2015) multiplied by their activity factor or were instructed to maintain food diaries 2 d prior to admission for the clamp procedure, as described in the publications cited.

2.3.3 Serum FA analysis

Serum FA were methylated using a modification of the direct transesterification procedure described by Lepage and Roy (1986). Serum samples were thawed at room temperature and vortexed well before 190 µL were added to a 12 mL pyrex tube containing 5 mL of 1:10 v/v methanolic hydrogen chloride then vortexed. The tubes were then incubated at 70°C for 1 h in a shaking water bath, then allowed to cool to room temperature. A reference standard mixture of 10 µg/mL methylated C19:0 (CAS No. 1731-94-8; Nu-Chek Prep, Inc. Elysian, MN) in hexane + 0.005% butylated hydroxytoluene was added to each sample followed by 3 mL of 3 M NaCl. After a brief vortex, samples were centrifuged at 2000×*g* for 5 min. The upper hexane layer was then transferred into a gas chromatography glass vial.

The serum analyzed included the 42 study samples, 3 pooled serum samples from healthy donors, a hexane blank, and 3 hexane blanks plus internal standards (methylated C19:0 at 15 µg/mL and C21:0 at 1 µg/mL). A Varian CP-3800 gas chromatograph (Walnut Creek, CA) fitted with a high polarity (cyanopropy)aryl-polysiloxane capillary column (HP88, 60 m x 0.25 mm; Agilent Technologies, Santa Clara, CA) was used to separate FA methyl esters, which were detected by flame ionization. Each sample had 1 µl injected at a split ratio of 1:4. The carrier gas was hydrogen at a constant pressure of 24 psi, with nitrogen as the make-up gas. The temperature cycle of the oven began at 60°C, increased to 120°C at 20°C/min, increased to 180°C at

10°C/min, then to 200°C at 2°C/min and finally to 220°C at 5°C/min where the temperature was held for 6 min to give a total run time of 29 min. The temperature of the injector port and the detector were 270°C and 280°C, respectively. After three serum samples were run, a hexane blank sample was run with an additional increase in temperature to 240°C for a total run time of 41 min to ensure no FA methyl ester remained on the column between samples.

Heneicosylic acid (C19:0; Nu-Chek Prep, Inc. Elysian, MN) was used as the internal standard. Recovery was calculated using the peaks for the internal and reference standards and averaged 104.5%. The amounts of FA were expressed as a percent of total FA. The butylated hydroxytoluene, internal standard, and reference standard peaks were not included when calculating the percent total of FA. Coefficients of variation for C15:0, *trans*-C16:1n-7, and C17:0 peaks from the three pooled samples were 2.3%, 2.4%, and 4.8%, respectively.

2.3.4 Calculations

Glucose turnover was measured during the last 30 min of each phase from the plasma 3-[^{3}H]-glucose specific activity and glucose infusion rate as described by Finegood et al. (1987). The glucose rate of disappearance (Rd), referred to as M value, is a measure of whole-body insulin sensitivity. The insulin sensitivity index (M/I) was calculated by dividing M by the mean insulin concentration during the clamp period. The M/I index represents the amount of glucose metabolized per unit of plasma insulin (DeFronzo et al. 1979). The hepatic IR index was determined by dividing glucose rate of appearance (Ra) by the mean insulin concentration during the clamp period. To account for differences in body composition among participants, the indices were corrected for lean body mass (LBM).

2.3.5 Statistical analyses

All statistical analyses were performed using SAS statistical software (Version 9.4; SAS Institute Inc., Cary, NC). Data were tested for normality using the Shapiro-Wilk test. Non-normally distributed data were log transformed. Where not corrected, non-parametric tests were performed. Homogeneity of variances were assessed using Bartlett's test. To test for differences in baseline characteristics and serum FA concentrations, a two-factor ANOVA adjusted for age was used to analyze parametric data for the main effects of group, sex and the group-by-sex interaction, with *post hoc* Bonferroni test. For non-parametric data, the Mann-Whitney U or Kruskal-Wallis test were used to determine differences between sex and groups, respectively. Pearson or Spearman correlations coefficients, as appropriate, were calculated to examine the relationships between serum concentration of FA biomarkers of dairy fat intake and indices of insulin sensitivity. Partial correlations were then performed to include the following covariates: age, sex, BMI, LBM, protein intake, energy intake, weight, and height. A P-value of < 0.05 was considered significant and $0.05 \leq P \leq 0.10$ was considered a statistical trend.

2.4 RESULTS

2.4.1 Participant Characteristics

The characteristics for the participants in this study (n = 42) are found in Table 2-1. Ow/ob T2D participants were older than their normal weight counterparts ($P < 0.0001$), and *post hoc* Bonferroni testing indicated that within groups, age differed by sex. As intended, BMI was higher ($P < 0.0001$) in the ow/ob T2D group, and *post hoc* Bonferroni testing indicated that within groups, BMI differed by sex. Waist circumference differed by group, with measures being higher in the ow/ob T2D group than in the normal weight group ($P < 0.0001$). LBM was higher

in the ow/ob T2D group ($P < 0.0001$) and in men ($P < 0.0001$). Protein intake also differed by both group and sex, as men consumed more than women ($P < 0.0001$) and those in the ow/ob T2D group consumed more than those in the normal weight group ($P = 0.002$). Energy differed between men and women, with men consuming more kcal/d than women ($P < 0.0001$).

Table 2-1. Participant characteristics.

	Normal weight		Overweight/obese T2D	
	Women	Men	Women	Men
n	8	14	6	14
Age (y)[g]	25.1 ± 5.3[i]	35.8 ± 16.3[j]	59.3 ± 5.7[i]	65.6 ± 8.6[j]
Height (cm)[a]	165.0 ± 7.3	174.6 ± 6.1	163.3 ± 4.1	176.5 ± 7.7
BMI (kg/m^2)[g]	21.1 ± 1.3[i]	22.2 ± 1.6[j]	31.2 ± 2.3[i]	29.4 ± 2.3[j]
LBM (kg)[a,b]	38.3 ± 4.3	52.1 ± 6.0	45.4 ± 5.7	57.9 ± 4.4
Waist circumference (cm)[g]	69.8 ± 4.9	80.2 ± 7.8	100.9 ± 9.2	104.8 ± 7.3
Protein intake (g/d)[a,b]	69.0 ± 13.5	100.3 ± 20.1	90.2 ± 8.3	104.5 ± 12.0
Energy intake (kcal/d)[a]	1737 ± 25.0	2405 ± 48.0	1750 ± 48.5	2448 ± 49.7
Fasting insulin (pmol/L)[g]	35.9 ± 11.2	34.5 ± 22.1	73.5 ± 23.9	115.4 ± 51.2
Fasting glucose (mmol/L)[g]	5.1 ± 0.3	5.3 ± 0.4	7.4 ± 1.7	6.4 ± 1.3
Glycated hemoglobin (%)[g]	5.3 ± 0.5	5.3 ± 0.2	7.3 ± 1.1	6.6 ± 1.1
HOMA-IR[g]	1.4 ± 0.4	1.39 ± 0.9	4.2 ± 1.6	5.5 ± 2.6
Clamp indices				
Glucose Rd (mg/kg LBM/min)[b]	9.5 ± 1.8	9.4 ± 2.4	5.7 ± 0.7	5.3 ± 1.7
Glucose Ra (mg/kg LBM/min)	1.0 ± 0.4	1.0 ± 0.3	0.8 ± 0.2	1.1 ± 0.4
M/I index[g]	15.3 ± 4.1	15.3 ± 8.5	4.8 ± 1.2	3.8 ± 2.9
Hepatic IR index[a,c]	-5.7 ± 1.6[d]	-4.5 ± 1.8[e]	-7.0 ± 1.3[d]	-3.1 ± 1.1[e]

Values are means ± SD.

Abbreviations: BMI, body mass index; HOMA-IR, homeostatic model of assessment insulin resistance; LBM, lean body mass.

M/I index = Δ Glucose Rd (mg/kg LBM/min)/clamp insulin (pmol/L) × 1000.

Hepatic IR index = Δ Glucose Ra (mg/kg LBM/min)/clamp insulin (pmol/L) × 1000.

[a]Sex effect by ANOVA.

[b]Group effect by ANOVA.

[c]Sex-by-group interaction by ANOVA, $P < 0.05$.

[d,e]Within each group, sexes differ by *post hoc* Bonferroni test, $P < 0.05$.

[f]Sex effect by Mann-Whitney U test, $P < 0.05$.

[g]Group effect by Kruskal-Wallis test in both sexes.

[i,j] Within each group, sexes differ by *post hoc* Mann-Whitney U test, $P < 0.05$.

Fasting insulin levels were greater in the ow/ob T2D group compared to the normal weight participants ($P < 0.0001$). Similarly, fasting glucose ($P < 0.0001$), glycated hemoglobin ($P < 0.0001$), and HOMA-IR ($P < 0.0001$) were all higher in the ow/ob T2D group.

Glucose Rd was higher in the normal weight group than in the ow/ob T2D group ($P < 0.0001$). The M/I index was higher in the normal weight group as compared to the ow/ob T2D participants ($P < 0.0001$). The hepatic IR index was higher in men than in women ($P < 0.0001$). There also was a statistically significant interaction on the hepatic IR index between sex and group, indicating that any differences between the groups, while not statistically significant, might be driven by sex ($P = 0.01$).

2.4.2 Serum fatty acid concentration

In the full cohort, 22 FA were reliably identified by gas chromatography in serum samples (Table 2-2). The concentration of serum *trans*-C16:1n-7 differed between sexes, being higher in men than women ($P = 0.046$). Serum C15:0 and C17:0 did not differ significantly between groups or sex. Both serum C16:0 and C18:0 differed between groups at $P = 0.01$, with the serum concentrations being higher in the ow/ob T2D group. There was a statistically significant interaction on C16:0 between sex and group, which could indicate that any sex differences seen, though here were not significant, could be driven by the grouping of these participants. Both C18:1n-9 and C18:2n-6 differed between groups, where C18:1n-9 was higher in the ow/ob T2D group and C18:2n-6 was higher in the normal weight group. C18:2n-6 was the most prevalent FA in the serum samples, followed by C18:1n-9 and C16:0. The concentrations of serum MUFA and PUFA both differed between groups. PUFA were higher in the normal weight group ($P = 0.001$) while MUFA were higher in the ow/ob T2D group ($P < 0.0001$).

Table 2-2. Serum FA concentrations (%of total FA) in the full cohort.

	Normal Weight		Overweight/Obese	
Fatty acid (% of total)	Women	Men	Women	Men
C14:0	1.02 ± 0.21	1.01 ± 0.21	0.96 ± 0.33	0.90 ± 0.24
C15:0	0.23 ± 0.04	0.23 ± 0.05	0.23 ± 0.02	0.18 ± 0.03
C16:0[b,c]	21.71 ± 1.32	21.89 ± 1.81	24.13 ± 1.40	22.14 ± 1.34
***trans*-C16:1n-7[a]**	0.36 ± 0.09	0.45 ± 0.10	0.44 ± 0.12	0.51 ± 0.13
***cis*-C16:1n-7**	1.92 ± 0.74	1.75 ± 1.07	2.42 ± 1.11	1.80 ± 0.73
C17:0	0.25 ± 0.03	0.27 ± 0.06	0.31 ±0.09	0.28 ± 0.05
C18:0[b]	6.85 ± 0.60	6.76 ± 0.89	8.31 ± 6.34	6.46 ± 0.51
C18:1n-11	0.06 ± 0.11	0.21 ± 0.47	1.17 ± 2.64	1.55 ± 5.47
C18:1n-9[e]	18.63 ± 1.47	19.35 ± 5.58	19.97 ± 9.53	23.01 ± 6.59
C18:2n-6[b]	30.84 ± 3.42	31.62 ± 5.08	24.25 ± 2.49	26.30 ± 3.50
C20:0	0.24 ± 0.21	0.26 ± 0.23	0.35 ± 0.29	0.30 ± 0.28
C18:3n-6	0.22 ± 0.19	0.22 ± 0.40	0.14 ± 0.35	0.24 ± 0.28
C18:3n-3	0.36 ± 0.32	0.38 ± 0.32	0.19 ± 0.03	0.38 ± 0.35
***cis*-9, *trans*-11-C18:2n-6**	0.04 ± 0.06	0.04 ± 0.07	0.02 ± 0.04	0.13 ± 0.11
C20:2n-6	0.15 ± 0.13	0.13 ± 0.10	0.20 ± 0.11	0.18 ± 0.11
C22:0	1.14 ± 0.65	1.01 ± 0.65	1.60 ± 0.62	1.07 ± 0.68
C20:4n-6	7.25 ± 1.23	6.66 ± 2.18	8.29 ± 2.90	7.67 ± 2.32
C23:0	0.08 ± 0.09	0.07 ± 0.09	0.04 ± 0.07	0.08 ± 0.09
C20:5n-3	0.61 ± 0.18	0.71 ± 0.35	0.72 ± 0.33	0.70 ± 0.24
C24:0	0.31 ± 0.15	0.39 ± 0.11	0.27 ± 0.08	0.30 ± 0.11
C24:1	0.36 ± 0.29	0.42 ± 0.30	0.27 ± 0.19	0.41 ± 0.33
C22:5n-6	0.11 ± 0.11	0.09 ± 0.12	0.17 ± 0.10	0.06 ± 0.07
C22:5n-3	0.55 ± 0.10	0.93 ± 1.21	0.60 ± 0.18	0.59 ± 0.11
C22:6n-3	1.67 ± 0.31	1.80 ± 0.55	1.85 ± 0.50	1.64 ± 0.49
Total SFA	31.83 ± 7.14	32.07 ± 7.19	33.78 ± 1.96	31.73 ± 1.33
Total MUFA[e]	21.33 ± 8.06	22.19 ± 8.36	26.70 ± 3.76	27.27 ± 4.27
Total PUFA[b]	41.64 ± 9.63	43.21 ± 9.01	36.43 ± 3.57	38.56 ± 4.06

Values are means ± SD.

Abbreviations: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

[a]Sex effect by ANOVA.

[b]Group effect by ANOVA.

[c]Sex-by-group interaction by ANOVA, $P < 0.05$. Within each group, no differences were found between sexes by post hoc Bonferroni test, $P < 0.05$.

[d]Sex effect by Mann-Whitney U test, $P < 0.05$.

[e]Group effect by Kruskal-Wallis test in both sexes. Within each group, no differences were found between sexes by *post hoc* Mann-Whitney U test, $P < 0.05$.

2.4.3 Total cohort FA associations

Serum C15:0 was significantly associated with C17:0 (Pearson $r = 0.50$, $P = 0.001$), consistent with a common dietary source. However, neither C15:0 ($r = 0.02$, $P = 0.90$) nor C17:0 ($r = 0.20$, $P = 0.23$) were associated with *trans*-C16:1n-7.

After adjustment for age and BMI, there was a statistical trend for a weak positive association between C15:0 and glucose Rd ($r = 0.29$, $P = 0.07$) and M/I index (Spearman $\rho = 0.32$, $P = 0.05$) (Figure 2-1), suggesting increasing peripheral insulin sensitivity with higher dairy fat intake. However, a stronger inverse association was found between *trans*-C16:1n-7 and glucose Rd ($r = -0.37$, $P = 0.02$) and M/I index ($\rho = -0.40$, $P = 0.01$) (Figure 2-2). Moreover, *trans*-C16:1n-7 was positively associated with glucose Ra ($\rho = 0.27$, $P = 0.09$) and hepatic IR index ($\rho = 0.28$, $P = 0.08$) (Figure 2-2), both indicative of lower hepatic insulin sensitivity. No associations were found between C17:0 and indices of insulin sensitivity in the total cohort.

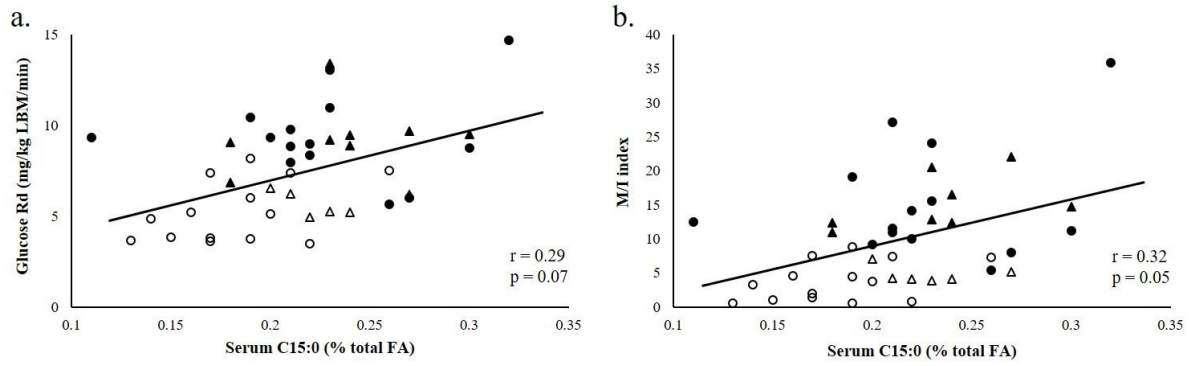

Figure 2-1. Association between serum C15:0 and insulin sensitivity in the total cohort. a) Pearson correlation between C15:0 and glucose Rd and b) Spearman correlation between C15:0 and M/I index in the total cohort (n=42). Normal weight men (●), ow/ob T2D men (○), normal weight women (▲), ow/ob T2D women (Δ).

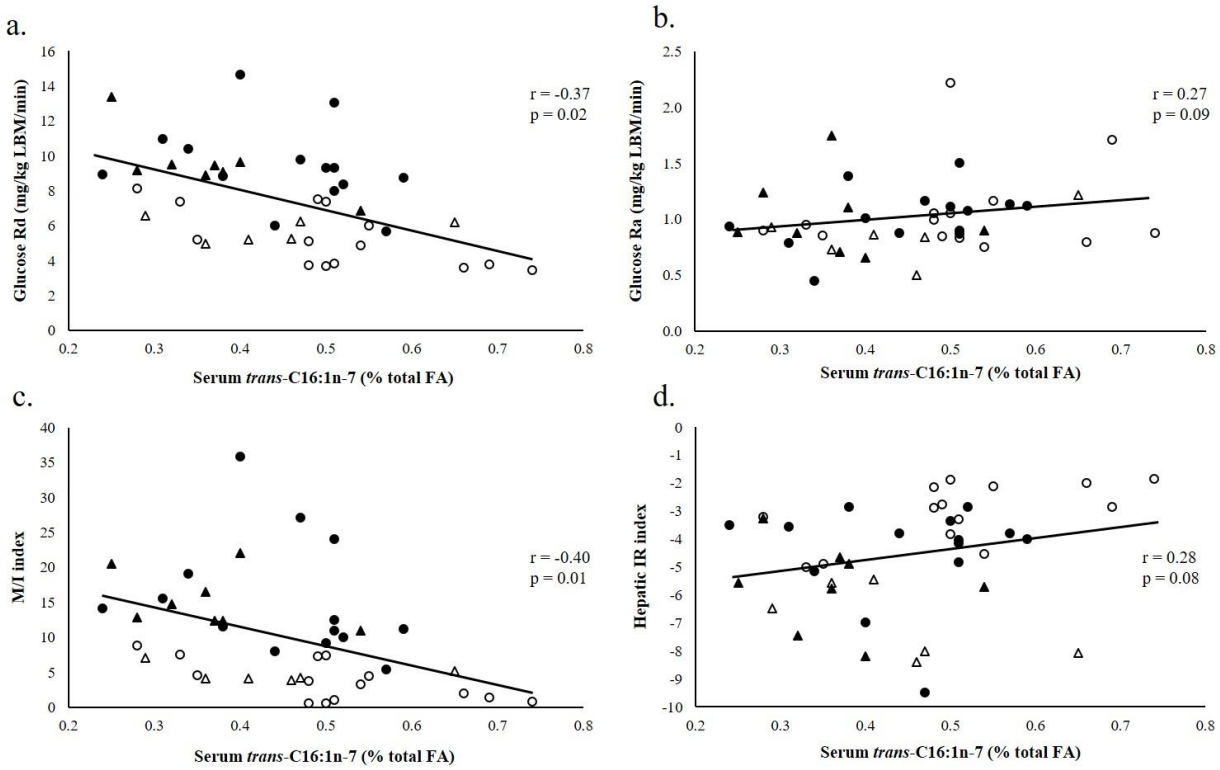

Figure 2-2. **Association between serum trans-C16:1n-7 and insulin sensitivity in the total cohort.** a) Pearson correlation between *trans*-C16:1n-7 and glucose Rd, b) Spearman correlation between *trans*-C16:1n-7 and glucose Ra, c) Spearman correlation between *trans*-C16:1n-7 and M/I index, and d) Spearman correlation between *trans*-C16:1n-7 and hepatic IR index in the total cohort (n = 42). Normal weight men (●), ow/ob T2D men (○), normal weight women (▲), ow/ob T2D women (Δ).

2.4.4 Associations by group

All associations were adjusted for age and BMI. In the normal weight group, serum *trans*-C16:1n-7 showed a statistical trend for an inverse association with the M/I index (ρ = -0.43, P = 0.06; Figure 2-3). No significant correlations were found for either C15:0 or C17:0

with measures of insulin sensitivity, although the directions of their associations were the same as those found for the total cohort.

In contrast, serum C15:0 showed a strong positive correlation with glucose Rd (r = 0.59, P = 0.01) and a trend with M/I index (ρ = 0.43, P = 0.07) in the ow/ob T2D group (Figure 2-4). As in the full cohort, *trans*-C16:1n-7 was inversely associated with glucose Rd (r = -0.49, P = 0.04) and showed a trend for an association with the M/I index (ρ = -046, P = 0.06) (Figure 2-4). In addition, a positive association was found between *trans*-C16:1n-7 and the hepatic IR index (ρ = 0.52, P = 0.03) (Figure 2-4).

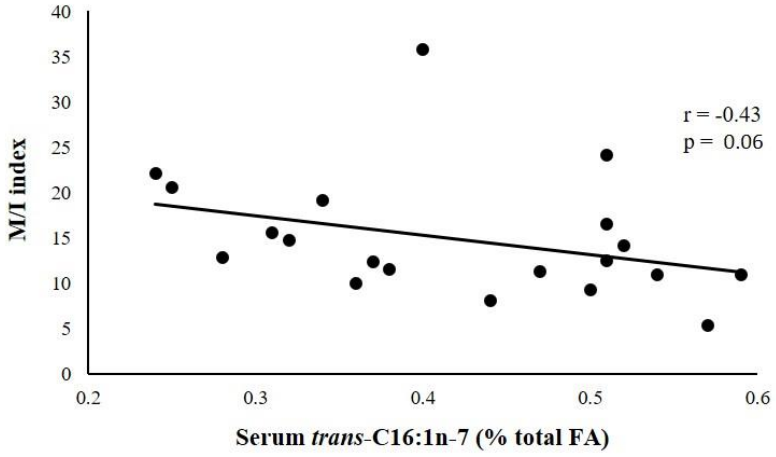

Figure 2-3. Association between serum trans-C16:1n-7 and M/I index in the normal weight group.

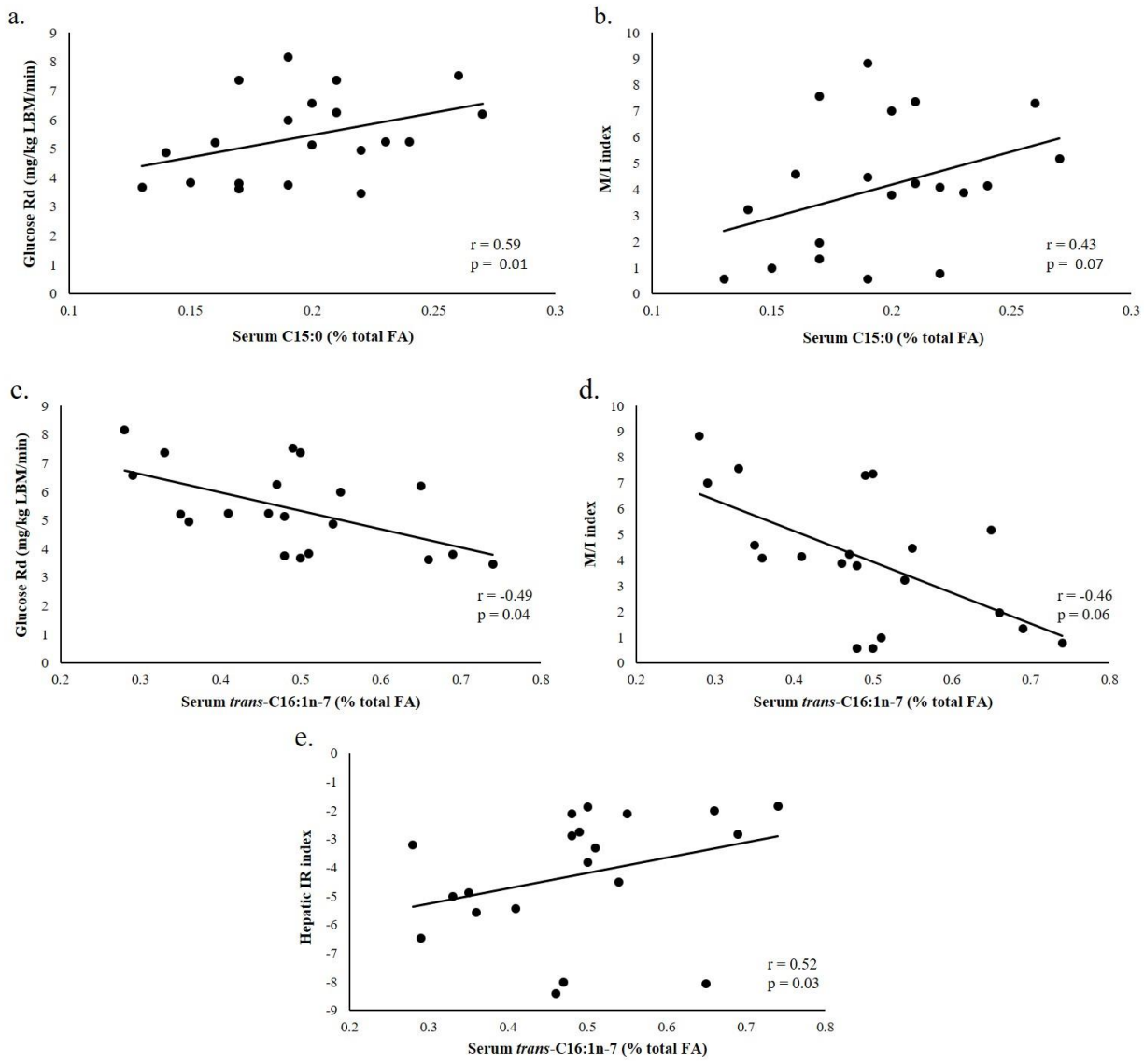

Figure 2-4. **Association between dairy fat biomarkers and insulin sensitivity in the ow/ob T2D group.** a) Pearson correlation between C15:0 and glucose Rd, b) Spearman correlation between C15:0 and M/I index, c) Pearson correlation between *trans*-C16:1n-7 and glucose Rd, d) Spearman correlation between *trans*-C16:1n-7 and M/I index and e) Spearman correlation between *trans*-C16:1n-7 and hepatic IR index and in the ow/ob T2D group (n = 20).

2.4.5 Associations by sex

Because fasting levels of *trans*-C16:1n-7 differed between men and women, we tested for associations by sex. In men, C15:0 was positively associated with hepatic insulin sensitivity (ρ = 0.46, P = 0.02) (Figure 2-5). In addition, *trans*-C16:1n-7 showed a trend for an inverse association with glucose Rd (r = -0.36, P = 0.07) and significant association with M/I index (ρ = -0.50, P = 0.009) (Figure 2-6).

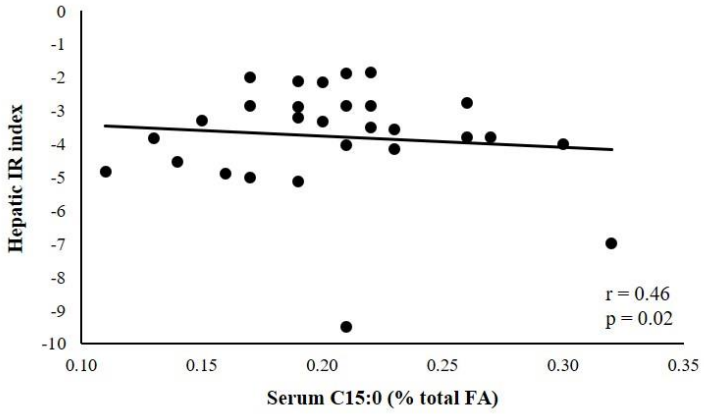

Figure 2-5. Spearman correlation between serum C15:0 and hepatic IR index in men.

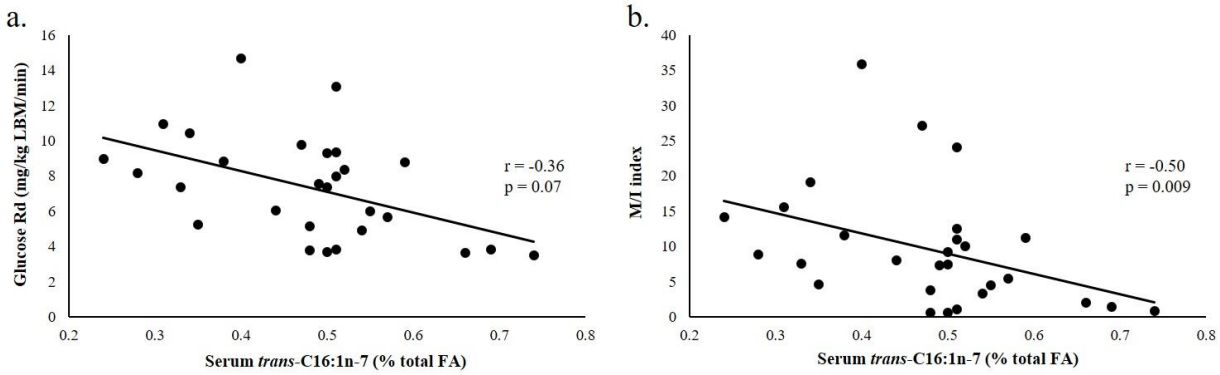

Figure 2-6. **Correlation between serum trans-C16:1n-7 and insulin sensitivity in men.** a) Pearson correlation between serum *trans*-C16:1n-7 and glucose Rd and b) Spearman correlation between serum *trans*-C16:1n-7 and M/I index in men (n = 28).

In women, C15:0 showed a statistical trend to be associated with the M/I index in women (ρ = 0.53, P = 0.08). Spearman correlations also showed inverse associations between both *trans*-C16:1n-7 and C17:0 and the hepatic IR index (ρ = -0.60, P = 0.04 and ρ = -0.88, P = 0.0002, respectively) (Figure 2-7).

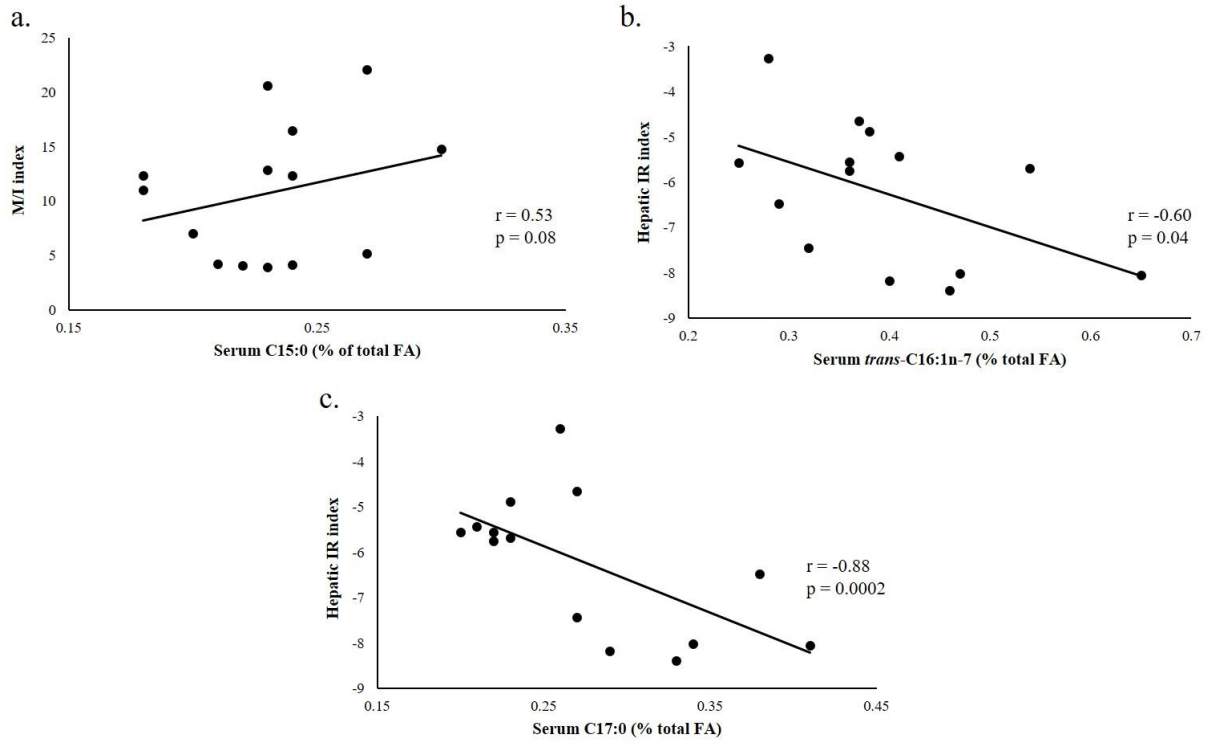

Figure 2-7. Association between serum dairy fat biomarkers and insulin sensitivity in women. a) Spearman correlation between the serum C15:0 and M/I index, b) Spearman correlation coefficient between serum *trans*-C16:1n-7 and hepatic IR index, and c) Spearman correlation coefficient between serum C17:0 and hepatic IR index in women (n = 14).

2.4.6 Associations between biomarkers of fat intake and participant characteristics

Both C15:0 and *trans*-C16:1n-7, but not C17:0, were associated with fasting glycemic and body composition measures in models adjusted for age and BMI in the total cohort. C15:0 showed a trend to be negatively associated with LBM (r = -0.29, P = 0.06) and was significantly associated with waist circumference (ρ = -0.43, P = 0.005) and fasting insulin (ρ = -0.44, P = 0.004). In contrast, *trans*-C16:1n-7 was positively associated with LBM (r = 0.43, P = 0.005), waist circumference (ρ = 0.42, P = 0.005), fasting blood insulin (ρ = 0.42, P = 0.006) and glucose levels (ρ = 0.40, P = 0.009).

2.5 Discussion

To our knowledge, this is the first study that has investigated the relationship between the serum C15:0, C17:0 and *trans*-C16:1n-7 with clamp-based indices of insulin sensitivity in a cohort of healthy normal weight and ow/ob individuals with T2D. Serum FA concentrations were expressed as a percent of total FA, as in previous studies, for comparison. Serum C15:0 levels were comparable to those reported by both Brevik et al. (2005) and Santaren et al. (2014) (0.22 ± 0.05 and 0.30 ± 0.06, respectively). Albani et al. (2016) measured C15:0 in dried bloodspots and found similar concentrations (0.20 ± 0.06), as did Kratz et al. (2014) when C15:0 was measured in phospholipids and free FA (0.18 ± 0.04 and 0.23 ± 0.05, respectively). Abdullah et al. (2015) found similar concentrations in total plasma lipids (0.22 ± 0.01). In contrast, Wolk et al. (1998) found a higher concentration of C15:0 in adipose tissue (0.35 ± 0.07), whereas Saadatian-Elahi et al. (2009) reported a lower concentration in plasma phospholipids (0.14 ± 0.04). Serum concentrations of C17:0 from our cohort fall within the range of previous studies, from 0.24 to 0.69 (Wolk et al. 1998, Wolk et al. 2001, Saadatian-Elahi et al.

2009, Abdullah et al. 2015, Albani et al. 2016), but on average were lower than those found in the study by Kratz et al. (2014) at 0.42 ± 0.07 in total phospholipid FA. Serum *trans*-C16:1n-7 was higher in our study than in three other studies: Kratz et al. (2014), 0.20 ± 0.05; Abdullah et al. (2015), 0.26 ± 0.01; and Santaren et al. (2014), 0.25 ± 0.01.

There are several factors which may account for the differences found among studies. One is the tissue or blood lipid fraction in which the FA were measured. They include adipose tissue (Wolk et al. 1998), erythrocyte membrane (Sun et al. 2007, Krachler et al. 2008), total plasma lipids (Mozaffarian et al. 2013, Yakoob et al. 2016), plasma phospholipid (Saadatian-Elahi et al. 2009, Micha et al. 2010), and total serum lipids (Santaren et al. 2014). This is important, as the FA content in a given tissue or blood lipid fraction is known to reflect different durations of dietary intake. FA in plasma and serum typically represent short term intakes, while FA in erythrocyte membranes and adipose tissue represent medium and long term intakes, respectively (Stanford et al. 1991, Hodson et al. 2008, Burrows et al. 2012, Golley and Hendrie 2014). Therefore, the differences in the concentrations of FA among studies and relationships thereof could reflect different temporal pattern of dairy intake.

In the total cohort, after adjustment for age and BMI, C15:0 showed a trend to be positively associated with measures of systemic insulin sensitivity. A similar trend was found in women, but only for the M/I index. Santaren et al. (2014) similarly found that serum C15:0 was positively associated with insulin sensitivity as measured by intravenous glucose tolerance testing in 659 adults without diabetes. These results are in contrast to the studies by Nestel et al. (2014) and Kratz et al. (2014), who did not find any association between phospholipid and FFA C15:0 and insulin sensitivity.

Serum C17:0 was not significantly associated with measures of insulin sensitivity in the total cohort or within groups but was inversely associated with hepatic insulin sensitivity in women. Results from studies that investigated the association between C17:0 and insulin sensitivity have been inconsistent. Nestel et al. (2014) reported that plasma phospholipid C17:0 was initially associated with insulin sensitivity determined by oral GTT in unadjusted models, but this was attenuated after adjustment for age, sex and BMI. Sun et al. (2007) showed that C17:0 in both plasma and erythrocytes correlated less strongly with dairy fat intake than both C15:0 and *trans*-C16:1n-7. In fact, out of these FA, they concluded that only C15:0 and *trans*-C16:1n-7 could be used as biomarkers of dairy fat intake. Interestingly, C17:0 was more strongly associated with the intake of processed meat and vegetable oils than dairy products in a cross-sectional study of over 3,000 adults (Saadatian-Elahi et al. 2009).

Contrary to our expectation based on associations with T2D risk (Mozaffarian et al. 2010, Mozaffarian et al. 2013, Kratz et al. 2014), serum *trans*-C16:1n-7 was negatively associated with glucose Rd and the M/I index and positively associated with glucose Ra and the hepatic IR index in the total cohort. Santaren et al. (2014) reported that serum *trans*-C16:1n-7 was inversely associated with insulin sensitivity, but this relationship was attenuated after adjustment for covariates, in partial agreement with our findings. However, these results are in contrast to that of Kratz et al. (2014), who found that phospholipid *trans*-C16:1n-7 was positively associated with both hepatic and systemic insulin sensitivity as assessed by the hyperinsulinemic-euglycemic clamp in a cohort that included 17 individuals with non-alcoholic fatty liver disease and 15 age- and BMI-matched controls. The differences in the direction of the association could be explained by differences in the dietary sources of *trans*-C16:1n-7 among studies. Dietary *trans*-C16:1n-7 is not only derived from dairy sources, but can also be consumed from partially

hydrogenated vegetable oils (Jaudszus et al. 2014). Indeed, in the MESA cohort, *trans*-C16:1n-7 was more strongly associated with French fry consumption than any other food, including dairy products (de Oliveira Otto et al. 2013), which in some cases compromise the validity of *trans*-C16:1n-7 as a biomarker of dairy fat intake. Interestingly, in sub-group analysis, the inverse association between serum *trans*-C16:1n-7 and insulin sensitivity was found in the ow/ob T2D group, but not in the normal weight group. These results suggest that alterations in lipid absorption or metabolism in ow/ob individuals with T2D, rather than differences in dietary dairy fat intake, may underlie these associations. Dietary composition calculated by standard techniques would have been useful in distinguishing between these two possibilities. Further research on how BMI and T2D status affects the concentration of these specific FA in serum lipid fractions is needed.

Associations between dairy fat biomarkers and insulin sensitivity were influenced by both sex and T2D status. This implies that the relationships between biomarkers of dairy fat intake and T2D risk may change depending on the metabolic status of the individual. This was further borne out by the associations found between biomarkers and body composition measures. In the ow/ob T2D group, there was in inverse association between C15:0 and LBM. Serum *trans*-C16:1n-7 was positively associated with LBM, and fasting insulin concentrations. In contrast, *trans*-C16:1n-7 was associated with fasting glucose levels, and serum C17:0 concentrations that were associated with LBM in normal weight participants. In summary, the serum concentrations of FA biomarkers of dairy fat intake were influenced by body composition and T2D status.

The main strength of this study was the assessment of insulin sensitivity using the glucose clamp method. Although amino acids were infused to maintain isoaminoacidemia in this clamp, this did not affect glucose infusion rates or measures of insulin sensitivity. In addition, our study

comprised data from a total of 42 individuals carefully phenotyped with anthropomorphic, metabolic and clinical measures. This cohort is larger than the only other study the authors found that also used a hyperinsulinemic-euglycemic clamp procedure (Kratz et al. 2014). Limitations of this study include its cross-sectional design and observational nature, which precludes assessment of causation. Differences in dairy fat biomarker levels among groups might reflect intake of other foods, lifestyle factors or genotype variation. There is no detailed dietary intake information available for this cohort. Therefore, it is not possible ascertain participants' dairy fat intake from dietary records to independently assess the association its association with dairy fat biomarkers (C15:0, *trans*-C16:1n-7, and C17:0) and insulin sensitivity.

2.6 Conclusions

The strength and direction of the association between established biomarkers of dairy fat intake and insulin sensitivity differed markedly between the serum FA used as biomarkers. The strength of the associations differed by sex and between healthy normal weight and ow/ob individuals with T2D. More research is needed to validate the use of these FA as biomarkers of dairy fat intake and to account for dietary, physiological (e.g. sex, BMI) and metabolic (e.g., lipid fractions, T2D status) factors that influence their association with dairy fat intake and insulin sensitivity.

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