

Article

# The Dietary Total-Fat Content Affects the *In Vivo* Circulating C15:0 and C17:0 Fatty Acid Concentrations Independently to the Dietary Fatty Acid Compositions; Highlighting Dietary Routes that may Attenuate the Development of Metabolic Disease

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**Abstract:** Pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) have been described as dietary biomarkers of dairy-fat consumption with varying degrees of reliability between studies. It remains unclear how the total amount of dietary fat; one of the main confounding factors in these biomarker investigations, affects C15:0 and C17:0 circulating compositions independently to their relative intake. Additionally, it is unknown how changes in the dietary total-fat affects other fatty acids in circulation. Through two dietary studies with different total-fat levels but maintaining individual fatty acid compositions we were able to see how the dietary total-fat affects the fatty acids in circulation. We saw that there was a significant, proportionate, and robust decrease in the endogenous C15:0 levels with an increase in dietary total-fat. However, there was an increase in the circulating C17:0 compositions as the total-fat increased. To conclude, the dietary total-fat content and fat-type have a very complex influence on the relative compositions of circulating fatty acids, which are independent to the actual dietary fatty acid composition. Knowing how to manipulate circulating C15:0 and C17:0 composition is far-reaching in nutritional/pathological research as they highlight a dietary route to attenuate the development of metabolic disease (both by reducing risk and improving prognosis).

**Keywords:** Biomarkers; dietary total fat; pentadecanoic acid; 15:0; heptadecanoic acid; 17:0; odd chain fatty acids.

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## 1. Introduction

Odd chain fatty acids, specifically pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) have been described as biomarkers of dairy consumption [1]. However, there are varying degrees of reliability for each fatty acid [2-5]. Within large observational human studies, both of these fatty

acids correlate moderately with their individual intake; which are usually attributed to the intake of dairy fat containing products, such as milk and butter [6]. However, within strictly controlled animal models, there are significant differences in the correlation for the individual odd chain fatty acid between dietary intake and their circulating levels. Within animal models, C15:0 behaves as a direct concentration biomarker of intake, with no notable variation caused by metabolism or endogenous lipogenesis. In contrast, there is convincing evidence showing that C17:0 does not directly relate to dietary intake. Instead, C17:0 endogenous biosynthesis has a significant influence on its circulating levels [2, 7]. Furthermore, the literature suggests that C17:0 may have a significant pathophysiological relevance and could be subject to *in vivo* homeostasis. Whether or not these fatty acids relate to dairy intake, their circulating levels show a significant association with a reduced risk of cardiometabolic diseases [8-9].

Investigating biomarkers within human populations is difficult due to many confounding factors. One typical dietary factor, such as the participant's total-fat intake, can vary hugely across a study population [10]; it remains unclear if this independently affects the circulating fatty acid levels. For instance, the effect of the total-fat intake in relation to the total energy intake on circulating odd chain fatty acids (C15:0 and C17:0) biomarkers has not been investigated.

The aim of this study was to investigate the influence of the total-fat intake (relative to total energy intake (% total-fat)) on the circulating C15:0 and C17:0 composition, independent of the dietary C15:0 and C17:0 composition. The current thought is that both C15:0 and C17:0 are homologous dietary biomarkers (supposed to be independent of endogenous metabolic processes). Therefore, both C15:0 and C17:0 *in vivo* should reliably correlate with their dietary compositions irrespective of any other dietary factors, such as the dietary total-fat content.

For this project we decided to make use of samples from existing studies that could provide evidence to achieve the aim of this work. In our opinion this is a more ethical approach than to start new a study demanding the use of more animals. The two studies we selected, included a three-tier-diet dose-response-type study [11]; this will investigate whether there is a linear effect on C15:0 and C17:0 by changing the total-fat content, and a two-tier-diet with different fat sources [12]; this will investigate if any change in C15:0 or C17:0 due to the total-fat levels is consistent across different fat sources (i.e. are there sources of fat that may promote optimal C15:0 or C17:0 levels). The fact that these studies use different lines of rats, different diets formulations, and different time points contributes to the robustness of any reproduced results.

## 2. Materials and Methods

**STUDY ONE – THREE-TIER-DIET DOSE-RESPONSE** - The setup of this study has been described in detail previously [11]. In short, male Sprague-Dawley rats (n = 7-9 per group) were housed under standard conditions. Rats had an intragastric cannula surgically inserted seven days before being separated into three groups, each group receiving an experimental diet with either 5%, 35% or 70% total-fat content (% energy); fat was isocalorically substituted for carbohydrate calories, additionally, protein, vitamin and mineral contents were identical in all diets. The animals had *ad libitum* access to water throughout the study. Blood was collected and processed into serum after 21 days on the experimental diets. All samples were stored at -80°C until analysed. Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved animal facility. Animal maintenance and experimental treatments were conducted in accordance with the ethical guidelines for animal research and were approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences.

**STUDY TWO – TWO-TIER-DIET AND DIETARY FAT SOURCE** - The setup of this study has been described in detail previously [12]. In short, six week old male Wistar rats (n = 6-8 per group) were housed under standard conditions. The rats were separated into six groups and fed one of six experimental diet made up of three different fatty acid compositions (described as basal, lard and fish oil based diets) at two different total-fat levels (11% and 51%; % energy). The rats had *ad libitum* access to demineralised water and food. After twelve weeks the animals were euthanized with

pentobarbital, blood was collected by puncturing the abdominal vein with a heparinised syringe, then centrifuged at 1000 g for 10 minutes at 4°C to obtain plasma. All samples were stored at -80°C until analysed. The INRA institutional guidelines for the care and use of laboratory animals were followed, and all experimental procedures were approved by the local ethics committee in Montpellier, France (reference CEEALR-11 009).

**ANALYTICAL MATERIALS** - Chemicals were obtained from Sigma-Aldrich (Sigma-Aldrich Company Ltd, Dorset, UK). All solvents were of HPLC grade.

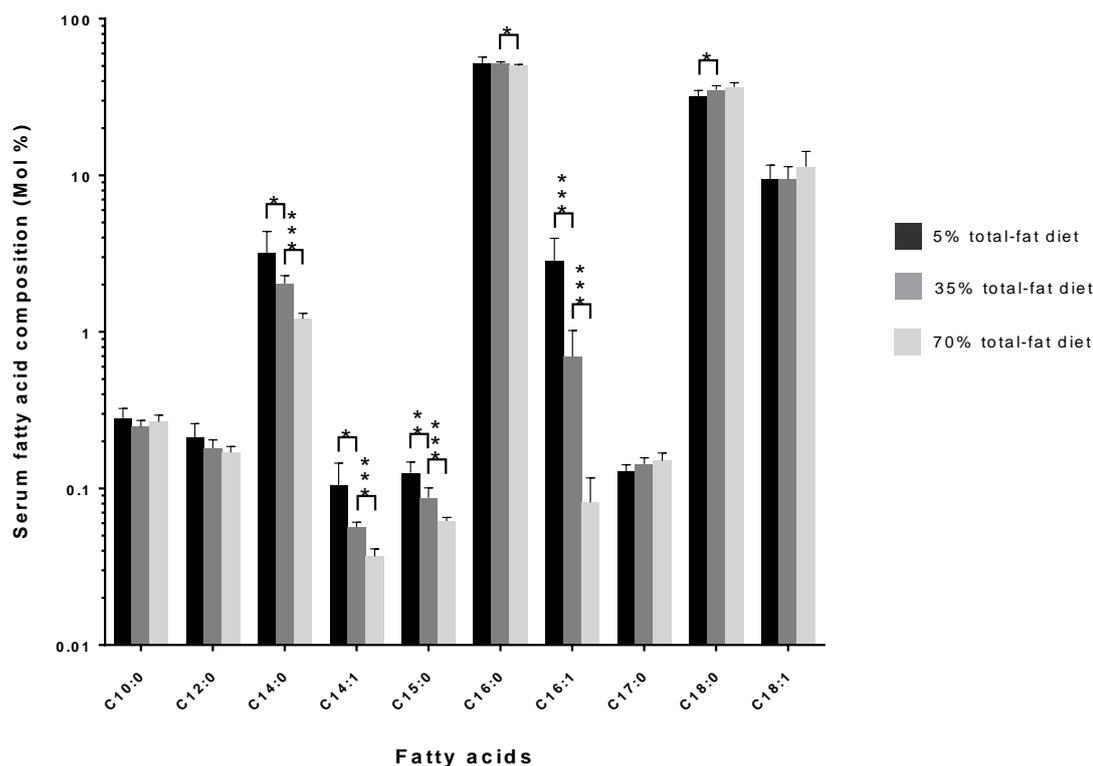
**FATTY ACID METHYL ESTER PREPARATION** - Samples were extracted using the chloroform: methanol: water extraction as previously described by Folch et al [13]. Briefly, chloroform: methanol solution (2:1, 1 mL) was added to 100 µL of plasma/serum. The samples were then vortexed and sonicated for 15 minutes. Water (400 µL) was added to each sample, followed by further vortexing then sonication (15 min), and additional vortexing to ensure complete recovery. Samples were centrifuged (~20,000 rpm, 5 min) and the resulting aqueous and organic layers were separated and dried under a gentle stream of nitrogen. For the analysis of total fatty acids, the samples were derivatised as described previously [2]. To the dried organic extracts; boron trifluoride in methanol (14%, 125 µL), chloroform: methanol (1:1, 100 µL) and internal standard tridecanoic acid-d25 in chloroform (100 µL, 200 µMol) were added. The mixture was thoroughly vortexed, sonicated (30 min) and vortexed a further time to ensure there was no undissolved material. Samples were heated to 80°C for 90 minutes to commence the derivatisation process. After the samples had cooled, water (300 µL) and hexane (600 µL) were added. The samples were vortexed and the organic layer separated into glass vials, blown down to dryness under nitrogen and finally reconstituted in 200 µL of hexane, ready for gas chromatography separation with mass spectrometry detection (GC-MS) analysis.

**GAS CHROMATOGRAPHY MASS SPECTROMETRY PARAMETERS** - Gas chromatography separation was achieved using a 6890N / 5973 Agilent GC-MS system (Agilent Technologies, California, USA) with a HP-88, 30 meter capillary column, 0.25 mm internal diameter and a 0.2 µm film thickness (Agilent 112-8837). Inlet temperature of 250°C. A total oven gradient over 26 minutes from 120°C to 210°C (initial temperature 120°C hold of 1 minute, temperature increase of 10°C per minute to 170°C followed by a hold of 6 minutes, then temperature increase of 3°C per minute to 210°C followed by a hold of 1 minute) with a 5:1 split. Full scan mass spectrometry detection starting after a delay of 2 minutes (mass range from 60-400 Da, transfer line temperature of 280°C, MS-source temperature of 230°C, MS-quadrupole temperature of 150°C). Peaks were integrated using GC/MSD ChemStation (Agilent Technologies, California, USA) and signal intensities were normalized to the internal standard.

**STATISTICAL ANALYSIS** - Homoscedastic t-tests were used between two unrelated equal variance groups to identify any significance/non-significance between them two groups. For the homoscedastic t-tests, a value of  $p \leq 0.05$  was considered statistically significant.

### 3. Results

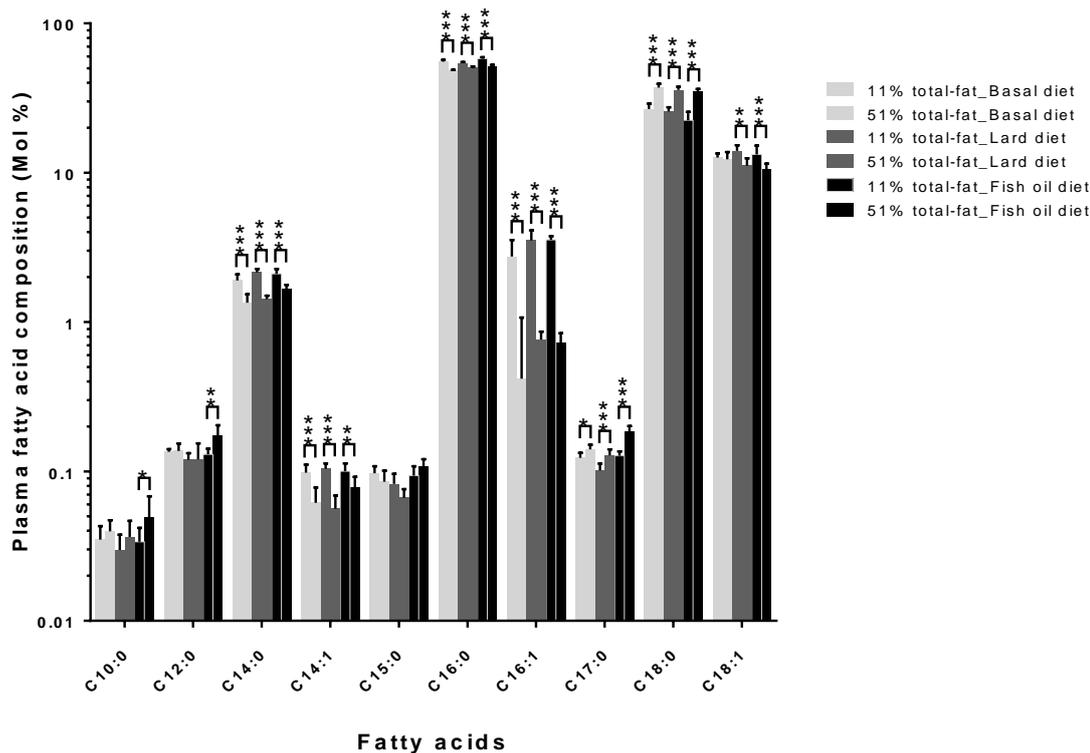
The pathophysiological effects of the three-tier-diet dose-response study has been previously described in detail [11]. However, the effect of changing the dietary total-fat content from 5% to 35% to 70% on the circulating C15:0 and C17:0 compositions are shown in Figure 1.



**Figure 1.** The effect of changing the amount of total-fat within the diet from 5% to 35% to 70% (% energy) on the serum fatty acid composition (Mol %) whilst maintaining identical dietary fatty acid compositions across the three diets ( $n = 7-9$  per group). This is to see if the amount of total-fat within the diet influences the serum fatty acid composition independently to the actual dietary fatty acid composition. The serum samples were analysed by gas chromatography separation with mass spectrometry detection. The significance of the difference between each group is shown by the  $p$ -value star system; where  $p \leq 0.05$  was considered statistically significant ( $p < 0.05 = *$ ,  $p < 0.01 = **$ ,  $p < 0.001 = ***$ ). Error bars represent  $\pm$  standard error of the mean.

As shown in the figure above (see Figure 1), the composition of C15:0 decreases as the dietary total-fat content increases ( $p < 0.01$ ); a ~30% increase in dietary total-fat resulted in a ~30% decrease in circulating C15:0 composition. The C17:0 composition appears to increase, although, statistically non-significantly.

The pathophysiological results of the two-tier-diet and dietary fat source study has been previously described in detail [12]. The effects of changing the diet's total-fat content across the three different formulations of fat are shown in Figure 2.



**Figure 2.** The effect of changing the amount of total-fat within the diet (% energy) from 11% to 51% on the plasma fatty acid composition (Mol %) across three different formulations of dietary fat (Basal, Lard and Fish oil) ( $n = 6-8$  per group). This is to see if the amount of total-fat within the diet influences the plasma fatty acid composition independently to the dietary fatty acid composition. The plasma samples were analysed by gas chromatography separation with mass spectrometry detection. The significance of the difference between each group is shown by the  $p$ -value star system; where  $p \leq 0.05$  was considered significant ( $p < 0.05 = *$ ,  $p < 0.01 = **$ ,  $p < 0.001 = ***$ ). Error bars represent  $\pm$  standard error of the mean.

The amount of dietary total-fat (as a percentage of total energy) has a significant effect on the relative concentrations of circulating fatty acids. This correlation in study one (see Figure 1) was confirmed by study two (see Figure 2) for two-of-the-three diets, where there was a ~40% increase in dietary total-fat content which resulted in a ~25% decrease in the circulating C15:0 (in the basal and lard diets). On the contrary there was a statistically significant increase in the circulating C17:0 composition ( $p < 0.05$ ); this agrees with study one (see Figure 1). Interestingly, there was an increase seen in the circulating C15:0 in the fish oil diet group ( $p > 0.05$ ) within study two (see Figure 2) as the dietary total-fat content increased from 11% to 51%.

The dietary fatty acid compositions were identical between each experimental diet and therefore, any change in the fatty acid compositions *in vivo* were due to the change in the dietary total-fat content.

Interestingly, these results confirm previous studies where increasing the dietary total-fat content decreases *de novo* lipogenesis and lipid desaturation but not fatty acid elongation [14].

#### 4. Discussion

To our knowledge, this is the first report that shows the effects of varying the dietary total-fat (as a percentage of total energy) on the circulating C15:0 and C17:0 fatty acid compositions whilst maintaining the actual dietary C15:0 and C17:0 fatty acid composition (Mol %).

As shown in study one (see Figure 1) there was a statistically significant ( $p < 0.01$ ), proportionate, and robust decrease in the endogenous C15:0 levels with an increase in dietary total-fat content from 5% to 35% to 70% (% energy). However, there was a continuing increase in the

endogenous C17:0 compositions; although not statistically significant. The inverse correlation seen in C15:0 suggests there could be a decrease in the efficiency of intestinal absorption as the dietary total-fat content increases. The correlations with the dietary total-fat content show that C15:0 and C17:0 are not homologous with each other and that they must be discriminated from one another when being studied; either because they originate from different sources or because they could be biologically compartmentalised. These results are further expanded in the supplementary material where the absolute concentration ( $\mu\text{Mol}$ ) of each fatty acid are shown (instead of relative compositions; Mol %) across the three experimental diets and agree with the relative fatty acid compositions (Mol %) (see Figure S1: The circulating fatty acid absolute concentration ( $\mu\text{Mol}$ ) change in response to the three experimental diets). These absolute concentration relationships show that C15:0 and C17:0 are changing with varying dietary total-fat contents and are not artificially created due to changes in endogenous lipogenesis.

In the literature it is shown that C15:0 and C17:0 may originate from different sources; C15:0 comes directly from the diet, while C17:0 is also significantly endogenously biosynthesized [2, 7]. The results presented here support a dietary origin for C15:0, since the *in vivo* C15:0 compositions decrease with an increase in dietary total-fat. The decrease in the *in vivo* C15:0 may be due to a reduced absorption efficiency in the higher fat diets. This absorption efficiency hypothesis is supported by our second study comparing circulating fatty acids across two total-fat levels (11% and 51%) and three different diet formulations (basal fat, lard fat and fish oil based) (see Figure 2). This study showed that a fish oil based diet actually saw an increase in C15:0 ( $p > 0.05$ ) when increasing the dietary total-fat content from 11% to 51%, possibly because the fish oil drives an overall increase in intestinal lumen absorption efficiency [15]. For C17:0, each diet resulted in an increase in the *in vivo* C17:0 composition with an increase in the dietary total-fat content across the three dietary fat formulations. Additionally, the fish oil based diet resulted in a dramatic increase in the *in vivo* C17:0 compositions, which further suggests endogenous biosynthesis since it has been shown that poly-unsaturated fatty acids decrease endogenous fatty acid desaturation [16, 17], possibly increasing endogenous alpha oxidation biosynthesis of C17:0 via increased C18:0 production [2]. This also provides further evidence of a diet high in fish oil being pathophysiologically beneficial.

Many epidemiological studies have shown that there is an inverse association between the relative compositions of odd chain fatty acids and metabolic disease risk [8, 9]. From work in animal models, we showed that this relationship could be causal, at least for C17:0; a reduction in C17:0 could be significant in the development of pathology [2]. It is therefore essential to understand how diet will affect the relative compositions of these two odd chain fatty acids; C15:0 and C17:0. The findings from these studies highlight the need for strictly controlled confirmation of biomarkers, especially when they originate from large human studies due to the inherent complexity of the data sets involved. The effects of the dietary total-fat can be further complicated by processes like *de novo* lipogenesis [14] (influenced by non-fat dietary components, such as carbohydrates or ethanol [18] (see Table S2: The circulating fatty acid compositions (Mol %) changes between an ethanol treated group and their associated control group)).

## 5. Conclusions

From our results, we conclude that the dietary total-fat content and fat-type composition have a very complex influence on the relative concentration of odd chain fatty acid in the circulation, which behaves profoundly different between C15:0 and C17:0. Where an increase in dietary total-fat independently reduces the circulating levels of C15:0 but increases C17:0. Furthermore, a fish-oil based diet proves to be noteworthy as it significantly increases both C15:0 and, to the greatest extent, C17:0. The importance of these findings are far-reaching in nutrition and pathophysiology research as they highlight a possible dietary/nutritional route to attenuate the development of metabolic disease.

**Supplementary Materials:** The following are available online at [www.mdpi.com/xxx/s1](http://www.mdpi.com/xxx/s1), Figure S1: The circulating fatty acid absolute concentration ( $\mu\text{Mol}$ ) change in response to the three experimental diets, Table S2: The circulating fatty acid compositions (Mol %) changes between an ethanol treated group and their associated control group.

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