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Research Article

Consumption of whey in combination with dairy medium-chain fatty acids (MCFAs) may reduce lipid storage due to urinary loss of tricarboxylic acid cycle intermediates and increased rates of MCFAs oxidation

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25 **Abbreviations:** **CH**, casein + high amount of medium-chain fatty acids; **CL**, casein +
26 low amount of medium-chain fatty acids; **LCSFAs**, long-chain saturated fatty acids;
27 **MCFAs**, medium-chain fatty acids; **MeS**, metabolic syndrome; **OPLS-DA**, orthogonal
28 partial least squares discriminant analysis; **PCA**, principal component analysis; **TCA**,
29 tricarboxylic acid; **TGs**, triglycerides; **WH**, whey + high amount of medium-chain fatty
30 acids; **WL**, whey + low amount of medium-chain fatty acids

Abstract

Scope: We aimed to investigate whether changes in the metabolome could explain observed changes in body composition in overweight adults after consumption of butter with high level of medium-chain fatty acids (MCFAs) in combination with casein or whey.

Methods and Results: With GC-ToF and LC-Q/MS, we analyzed metabolites in plasma and urine from a 12-weeks randomized double-blinded human intervention including 52-abdominally overweight adults. The participants consumed 63 g/day of milk fat (high or low in MCFAs) and 60 g/day of protein (whey or casein). Urinary loss of the tricarboxylic acid cycle metabolites and a concomitantly increase of glycerol in blood were observed in the whey + high-MCFAs group, indicating potential lower anabolic processes, such as lipogenesis, by draining substrates. High intake of MCFAs resulted in elevated level of urinary adipic (independently of protein type) and plasma sebamic acid (with whey), indicating a potential increase in oxidation of MCFAs, which might lead to energy loss.

Conclusion: The type of protein showed highest effect on the overall metabolic profiles, but ω -oxidation of MCFAs in the liver seemed to be the main reason for the observed reduction in body fat mass after consumption of high MCFAs, independent of type of protein.

1 Introduction

The Metabolic Syndrome (MeS) is defined as abdominal obesity in combination with insulin resistance, dyslipidemia and/or elevated blood pressure. The prevalence of the MeS is globally increasing and is associated with a doubling of risk of cardiovascular diseases (CVD) and a five-fold increased risk of developing type 2 diabetes (T2D) [1].

Dairy products have occasionally been condemned due to the high content of saturated fatty acids (FAs), which has been linked to increased risk of CVD [2]. However, observational studies suggest that dairy food consumption may prevent the development of MeS and its related disorders [3]. The effect of dairy intake on body weight may be ascribed to milk proteins, as both whey and casein stimulate insulin secretion and possess the potential to improve tissue glucose uptake and suppress postprandial blood glucose [4].

Despite the fact that dairy fat has a high energy density, the association between the consumption of dairy products and body weight remains uncertain. Overall, studies have shown a neutral relationship between body weight and the intake of dairy products (regardless of fat content) [5, 6]. Several studies show an association between dairy fat consumption and markers of improved metabolic health and thereby challenge the hypothesis that dairy fat causes metabolic dysfunction [2, 7].

Beside the high content of long-chain saturated FAs (LCSFAs), milk fat also holds a high levels of both short- and medium-chain FAs [8, 9]. Lately, focus has been on MCFAs, which are hydrolyzed and absorbed faster in the gastrointestinal tract than LCSFAs. While LCSFAs are incorporated into chylomicrons for further digestion or storage as triglycerides, MCFAs are transported directly via the portal vein to the liver and shunted into mitochondria for β - and ω -oxidation [10, 11].

Recently, we demonstrated that high level of dairy MCFAs in the diet increased lean body mass and reduced body fat accretion independently on the type of milk proteins administrated in the diet [12]. Dairy products are complex diets that make it important to study their metabolic effects in a diligent way. For such a challenge, metabolomics has the potential to provide a thoroughly, comprehensive and in-depth understanding of the influence of dietary factors on specific conditions such as the MeS by the analysis of low-molecular-weight compounds present in human biofluids collected from an intervention study.

In the present study, we applied untargeted GC- and LC-MS metabolomics to elucidate the influence of milk lipids with high or low content of MCFAs, and milk proteins (whey vs. casein) consumption on the blood and urine metabolic profiles of 52 subjects with abdominal obesity [13]. The objective was to explore, which metabolic changes were induced by long-term consumption of high or low content of MCFAs in combination with casein or whey, to elucidate the previously observed changes in body composition after an intervention diet rich in MCFAs.

2 Materials and Methods

2.1. Chemicals

Methanol and heptane were purchased from Rathburn Chemicals Ltd., Walkerburn Scotland, UK. Methoxamine hydrochloride brought from Alfa Aesar GmbH, Karlsruhe, Germany. Pyridine, sodium acetate, acetonitrile, acetic acid and trimethylsilyl cyanide (TMSCN) from Sigma-Aldrich CHEMIE GmbH, Steinheim, Germany. Myo-inositol-d6 (98%), citric-2,2,4,4-d4 acid (98%) and lauric acid d-23 (98%) internal standards were obtained from Cambridge Isotope Laboratories, Inc. Andover, MA, USA. Milli-Q water was obtained from a SG ultra-pure water system, Barsbüttel, Germany. Finally, sodium trimethylsilyl propanoate-d4 (TSP) and D₂O from Sigma-Aldrich, Brøndby, Denmark.

2.2. Human intervention study

The current study was part of the Dairy Health Study, a 12-weeks, randomized, double-blinded parallel diet intervention study on 52 abdominally overweight participants studying the effects of MCFAs from milk and two different types of milk proteins on postprandial lipaemia [13].

The study has been previously described in detail [13]. In short, participants were randomized to one of four groups according to the intervention diet; Whey + low amount of MCFAs (L-MCFAs) (WL), Whey + high amount of MCFAs (H-MCFAs) (WH), Casein + L-MCFAs (CL), Casein + H-MCFAs (CH).

The main inclusion criteria were as follows: Age $\geq$ 18 y, waist circumference $\geq$ 80 cm for women or $\geq$ 94 cm for men and weight stability. The exclusion criteria were: diabetes, pregnancy or history of severe endocrine, cardiovascular or gastrointestinal disease. At baseline, participants were stratified for the metabolic syndrome to homogenize the four groups. 74 subjects were screened, 63 were randomized and 52 subjects completed the study.

The test products to be consumed daily were 63 g of milk fat/day (high or low in MCFAs) (given as 1 cake, 2 rolls and 25 g of butter) and two protein-shakes (a total of 60 g/day of casein or whey protein). The differences in MCFAs content were achieved naturally by either low-fat feeding (to increase the MCFAs content) or fat supplementation (to decrease the MCFAs content) according to Bohl et al. [13].

During the study, the participants visited the clinic regularly (every other week at start and every 4th week towards the end of the intervention) to ensure compliance and to collect the test products. Dietary guidance was performed to instruct the participants on how to incorporate the test products into their habitual diet and remain weight stable. Three-day weighted dietary registrations were made before and after the intervention period. Based on the dietary registrations, a clinical dietitian calculated the participants energy intake, macro- and micronutrient content by using Master Dietist System, version 1.235 (2007), based on the Danish National Food administration database (as previously published) [13].

139

140 Plasma and urine samples (morning fasted samples) were collected before and after the
141 intervention. A total of 104 plasma and 104 urine samples were collected as fasted samples
142 before (baseline) and after the 12-weeks human intervention with the four different diets WL,
143 WH, CL, and CH [13]. The samples were analyzed by GC-MS (94 plasma and 90 urine
144 samples) and LC-MS (91 plasma and 98 urine samples). Not all samples were successfully
145 analyzed due to two reasons: i) not enough blood (4 samples) and urine (5 samples), and ii)
146 analytical faults; not successful derivatization and sample injection faults for the rest of missing
147 samples.

148

149 The trial was approved by the Central Denmark Region Committees on Health Research Ethics
150 and registered at clinicaltrials.gov as NCT01472666.

151

152 2.3. GC-MS

153 2.3.1. Sample preparation

154 Samples were treated according to Nielsen and Amer et al. [14]. Please consult Supplementary
155 Materials for a detailed description of sample preparations.

156

157 2.3.2. GC-MS analysis

158 The samples were injected in a 1:40 split mode into an Agilent Technologies 7890B GC system
159 coupled to an Agilent Technologies 7200 Accurate-Mass QToF/MS (Agilent Technologies,
160 Germany) according to Nielsen and Amer et al. [14]. Detailed analysis parameters are shown in
161 Supplementary Materials.

162

163 2.4. LC-MS

164 2.4.1. Sample preparation

Sample preparation was performed according to Bruce et al. [15]. Please consult Supplementary Materials for a detailed description.

2.4.2. LC-MS analysis

The LC-MS work was done on a MicroTOF-QMS (Bruker Daltonics, Bremen, Germany) coupled with an Agilent 1200 series capillary HPLC (Agilent, CA, USA), following the method by Zhang et al. [16]. Detailed analysis parameters are shown in Supplementary Materials.

2.6. Data analysis

2.6.1. GC-MS data analysis

Raw GC-ToF/MS data (plasma and urine) were deconvoluted using Mass-Hunter Unknown Analysis software (Quantitative Analysis, Version B.07.00/Build 7.0.457.0, Agilent Technologies), with extracted-ion chromatogram (EIC) peak threshold = 3, EIC signal-to-noise ratio (SNR) threshold = 5, and resolution retention time (R.T) windows size factor = 200. Detected components from all samples were transferred into Mass Profiler Professional software (Version 12.6.4-Build 196252, Agilent Technologies) for retention time alignment (R.T tolerance (min) = 0.05, match factor = 0.3, and delta MZ (low resolution) = 0.2), peaks filtration (abundance filtering by minimum absolute abundance 5000 counts) and metabolites identification using NIST11 library. Detected components were transferred into Mass Profiler Professional software. Data sets were normalized by dividing features over the sum of all meaningful features of each observation (total area normalization), after removing peaks obtained from urea, derivatization reagents and GC column.

After processing, integrated peak areas for the detected features were imported to SIMCA 14 (Umetrics, Sweden) to perform multivariate data analysis. Before the statistical analysis, data were visually explored for outliers by principal component analysis (PCA) [17]. Samples with a Hotelling's T2 value outside the 99% confidence limit were considered as outliers and removed

from the models. Both PCA and orthogonal partial least squares discriminant analysis (OPLS-DA) [18] were applied to develop compliance models based on the most discriminative features detected in plasma and urine, and models were constructed for the whole data set and for different subsets of the samples according to dietary group and time point (before and after intervention). Unit variance scaling of data was performed and the dietary treatments were given as class information in the OPLS-DA models. OPLS-DA models were cross-validated to evaluate the performance of the models. To eliminate noise and non-important metabolites from the interpretation of the results, filtering based on variable importance for the projection (VIP) values > 1 that summarizes the overall contribution of each X-variable to the PLS model [19], was used to detect important metabolites.

Univariate statistical data analysis (2-way mixed ANOVA) was carried out on identified potential metabolites selected from OPLS-DA models (VIP > 1) under SAS Enterprise Guide 5.1, internal standards peaks were used to normalize the GC-ToF/MS data before the univariate data analysis for higher accuracy [15].

2.6.2. LC-MS data analysis

Raw LC-Q/MS data were automatically calibrated in Data Analysis software (Bruker Daltonics, Bremen, Germany) and converted to MZxml files using CompassXport software (Bruker Daltonics, Bremen, Germany). Then data were analyzed under R environment (v.3.0.2; <http://www.r-project.org/>) using XCMS package (v.1.38.0; <http://www.bioconductor.org/>) to obtain a list of integrated peak areas for detected features, characterized by m/z and retention time. CAMERA software (v.1.18.0; <http://www.bioconductor.org/>) was used for the annotation of isotopes and adducts in LC-Q/MS data.

Data were pre-processed and pre-treated before multivariate analysis in Excel software. First, the nearest blank in the analysis sequence was subtracted from each sample; this ensured

subtracting any artificial peaks or noise from any source in the chromatograms taking into account the time drift reduction. Negative values were obtained for some features, therefore, negative values were replaced with blank value. Data sets were normalized by dividing features over the sum of all features of each observation (total area normalization) after blank deduction, which represent the meaningful area of each chromatogram. After processing, integrated peak areas for the detected features were imported to SIMCA 14 (Umetrics, Sweden) to perform multivariate data analysis. Same multivariate data analysis procedure was followed as described for GC-MS data analysis. Univariate data analysis (ANOVA) was performed on selected LC-MS features from OPLS-DA ($VIP > 1$) models, total area normalized data was used for ANOVA analysis as no internal standards were used for the LC-MS analysis. Bruker Daltonik Data Analysis software and the Human Metabolome Database (HMDB) Version 3.6 was used to identify detected metabolites, which contribute to the separation in metabolic profiles.

3 Results and Discussion

The present metabolomics study derived from a 12-weeks clinical trial already published [13]. There was a similar unintended weight gain of 1.4 kg (95% CI: 1.0 – 1.9; $P = 0.677$) across groups after the 12-weeks intervention. However, the lean body mass increased by 981 g (95% CI: 248 – 1713; $P = 0.010$) after consumption of high MCFAs compared with low MCFAs supplementation. Concomitantly, total body-fat percentage increased by 0.70% points (95% CI: 0.10 – 1.31; $P = 0.024$) after intake of low MCFAs butter compared with intake of high MCFAs butter. Both changes were independent of protein type ($P = 0.96$ and $P = 0.99$, respectively). No differences in homoeostatic model assessment of insulin resistance (HOMA-IR), Matsuda index, and diurnal blood pressure or plasma cholesterol concentrations were observed related to MCFAs content or protein type [12]. From the dietary registrations, we observed no significant differences in dietary variables between groups at baseline. All groups significantly increased energy intake, with the casein-supplemented groups increasing energy intake more than the whey-supplemented groups independently of content of MCFAs with

a mean of 1556 kJ/d (95% CI: 384 – 2728; $P = 0.010$). There were no significant changes in macro- and micronutrients between groups after intervention [13].

Unsupervised PCA was performed on GC- and LC-MS results obtained from fasted plasma and urine samples collected after the intervention (Figure 1). Few biological outliers were detected after performing the multivariate data analysis on GC-MS data. Thus, one outlier was detected in the PCA model of fasted urine samples before intervention, and one outlier was detected in the PCA of urine samples from WL group (before vs. after), while three outliers were detected in the LC-MS data in both PCA models of plasma sample before and after intervention. From 140 entries detected in the GC-MS plasma data, an effect of the type of protein (casein versus whey) was separated along PC1 explaining 13% of the variation in data (Figure 1A). The level of MCFAs separated the metabolic profile when whey was the dietary protein (WH versus WL) and a separation was also observed when plotting PC1 versus PC3, explaining 7% (Figure S1) whereas no separation was obtained with respect to level of MCFAs with casein as dietary protein. PCA analysis of the plasma LC-MS data with 362 entries did not show any separation along PC1 but showed separation according to type of protein (casein versus whey) in the diets along PC2, even though this separation was only explained by 6% (Figure 1B). OPLS-DA models were employed to determine metabolites that are responsible for the discrimination and separation between groups (Figure S2, Supplementary Materials). As expected, OPLS-DA models of GC-MS data have higher Q^2 values (0.92 – 0.97) than LC-MS (-0.01 – 0.56) data. This could be explained by differences in which metabolites are being detected by the two techniques, but also by differences in peaks deconvolution and data pre-processing, which include different thresholds for the selection of features between the two data sets.

PCA of the urine GC-MS data showed separation along PC1 (17% explanation of variation) with respect to type of dietary protein (whey vs. casein) (Figure 1C). Supervised OPLS-DA revealed metabolites that could discriminate between the dietary groups according to type of

protein (Figure S3A-B), and levels of MCFAs (Figure S3C-D). LC-MS data of urine samples did not show any changes in the metabolic profiles after the intervention when analyzed by PCA, Figure 1D.

The OPLS-DA models ($VIP > 1$) helped us identifying the metabolites that differed significantly ($P < 0.05$, ANOVA) between the groups after 12-weeks of intervention. The metabolites are summarized in Table 1 (GC-MS, plasma), Table 2 (LC-MS, plasma), and Table 3 (GC-MS, urine). Furthermore, PCA and OPLS-DA models were used to determine metabolites (in plasma and urine) that were significantly changed as a result of the 12-weeks diet in all groups using GC-MS (Figure S4 and Figure S5, respectively). The change in metabolic profiles as a result of the 12-weeks intervention (before vs. after intervention) was less pronounced, indicated by no valid models for results obtained on plasma samples that analyzed by LC-MS (data not shown).

Leucine, isoleucine and valine are branched-chain amino acids (BCAAs) that are known for their action in satiety signaling [20], stimulation of muscle protein synthesis [3], with resultant increased lean body mass [21], and they also enhance insulin secretion and regulate plasma glucose levels [22]. The postprandial levels of BCAAs has previously been shown to increase in blood after whey consumption [23]. In the present study, whey also gives a long-term change in the blood concentrations of the BCAAs, which was not observed in the casein groups (Table 1 and 2). A high level of BCAAs has been associated with low level of aromatic amino acids after direct infusion of BCAAs [24]. However, this was not the case in the present study as the plasma level of tyrosine was higher in the WH group compared to CH groups after the intervention. Recent studies have shown a positive correlation between high levels of BCAAs and obesity [25], and between high levels of both BCAAs and aromatic amino acids and insulin resistance [26]. This was not the case in our study, as no increase in insulin resistance or body weight was observed in WH or WL group [12]. Two intermediates or catabolic by-products of

phenylalanine metabolism, phenylpyruvic acid and 2-phenylacetamide [27] were higher in plasma of WH compared to CH group and the levels of phenylalanine, cinnamic acid and 4-hydroxyphenylacetic acid (the latter a tyrosine metabolite [28]) were higher in WL group compared to CH group (Table 2). This does not agree with the fact that casein have higher amount of phenylalanine and tyrosine than whey protein [29], but this may rather indicate an effect of MCFAs on casein digestibility. Thus, it is recommended to study the postprandial levels of amino acids in blood samples of similar dietary studies that based on a combination of dairy lipids and proteins. The two amino acids derivatives sarcosine and pyroglutamic acid were increased in plasma after WH intervention. Pyroglutamic acid is an amino acid derivative in which the free amino group of glutamic acid cyclizes to form a lactam, which is formed non-enzymatically from glutamate, glutamine, and gamma-glutamylated peptides, but it can also be produced by the action of gamma-glutamylcyclotransferase on an l-amino acid [30]. Sarcosine was higher in plasma of the WH group after intervention compared to the WL, CH and CL groups. Sarcosine is generated from glycine [31], which was higher in the WH and WL compared to CH and CL. The plasma level of threonic acid was increased in the WH and WL groups as a result of the intervention but decreased in the CH and CL group. Contrary, threonic acid was higher in urine of CH compared to WH after the intervention. Threonic acid is a product of ascorbic acid catabolism, but may also be derived from glycated proteins [32]. In summary, the metabolomics analyses revealed differences between the casein and whey protein groups in relation to endogenous protein and amino acid metabolism, but the exact impact of these changes remain unknown. Eventhough the general impression of the metabolic profiles was that the type of proteins had higher impact than level of MCFAs, mostly due to many amino acids and their metabolites being major compounds in the PCA, quiet a lot of lipid metabolites originate from from lipolysis or oxidative metabolism were important as well.

Plasma glycerol level was elevated after intervention for the WH group (Table 1), thus being higher than the levels in plasma from the other three groups. Plasma glycerol has been reported to increase in response to resistance exercise as result of higher lipolysis rates [33].

Interestingly, the plasma levels of lauric acid was higher in the WH group compared to the CH and CL groups and myristic acid was also higher in the WH group compared to all other groups after intervention (Table 1). These results suggest higher triglyceride lipolysis in WH group or a faster clearance of lauric and myristic acids in the CH group (Figure 2). A potential higher lipolysis in the WH group is in contrast with that we previously observed an up-regulation of the LPL-gene only in the CH group of the same clinical study [13] suggesting a faster clearance, but it still remains unclear why it was in the WH group rather than in the CH group that we identified the highest glycerol level. However, Zheng et al. (2015) have reported a higher fecal excretion of glycerol after the consumption of milk- but not cheese-based diet [34]. Milk contains higher levels of whey protein compared to cheese, and this might indicate a higher fat lipolysis in the gut of WH group in the present study. Thus, higher absorption of glycerol, which might be due to changes in microbiota after WH consumption, a hypothesis that needs to be confirmed in future studies. Unfortunately, feces samples were not collected in the clinical study, so it leave us to speculate and put up this new hypothesis. Thus, analysis of the gut microbiota of a dairy-based human intervention, and correlating the data with metabolomics data is recommended for deeper understanding of the effect of milk components on gut microbiota and the connection between microbiota and metabolic profiles.

In plasma, the dicarboxylic acid, sebacic acid, was increased as a result of the WH intervention (Table 1). Moreover, changes in the levels of urinary adipic acid (Table 3) were related to intake of MCFAs showing increased levels in the CH and WH groups compared to the low level MCFAs groups (CL and WL). Medium-chain dicarboxylic acids are produced from direct ω -oxidation or ω -oxidation following β -oxidation of longer-chain dicarboxylic acids (Figure S6)

[11, 35]. But, more studies (including *in vitro* studies) are required to thoroughly understand the exact mechanism of MCFAs oxidation under different conditions, including the dietary matrix. Adipic acid has, previously, been described as a ketosis product in urine indicating use of fat instead of glucose as energy source [36]. One of the possibilities for the endogenous origin of adipic acid is from the ω -oxidation of FAs, which is one of the metabolic pathways of MCFAs (Figure S6). High intake of MCFAs may increase the urinary excretion of corresponding chain length dicarboxylic acids and 2 or 4 carbon atoms less, which was previously observed in acute and short-term studies [37]. Here we report similar finding on a long-term basis indicating enhanced fat oxidation over a long period for diets with high level of MCFAs, independent on the type of protein. Despite results that suggest only short-term increase in energy expenditure after feeding women with medium-chain triglycerides but not long-term effect [38], higher levels of the dicarboxylic acids, adipic and sebacic acids, after the consumption of milk MCFAs may predict a potential high energy expenditure as a result of higher MCFAs oxidation rates [39, 40] (Figure 2). These observations might provide a potential explanation of the clinical observation on increased lean body mass in the two diet groups consuming high levels of MCFAs [12]. Furthermore, levels of the urinary dicarboxylic fatty acids, 3-hydroxyadipic acid and suberic acid, were increased after the consumption of milk-based diet in an animal (pigs) study compared to before the intervention and also as compared to the control diet at the end of the intervention as shown in Nielsen and Amer et al. [14], which also correlates with our findings. Adipic acid concentration was higher in CH urine compared to WH at the end of the intervention, suggesting a synergetic effect of caseins on the ω -oxidation of MCFAs. Higher water solubility of adipic acid compared to sebacic acids may lead to higher excretion of adipic acid in urine but not the longer-chain dicarboxylic acid, sebacic acids. The recognition of the effects of MCSFAs intake on adipic acid and sebacic acid provides insight into metabolic effects. Furthermore, we propose further studies to elucidate the potential of use of adipic and sebacic acids as markers for compliance in future studies with diets including MCFAs, and to study the energy expenditure rates after the long-term consumption of dairy MCFAs.

A reduction in 2-hydroxybutyric acid (α -hydroxybutyric acid) in blood was observed after 12-weeks of CH and CL interventions (Table 1). α -Hydroxybutyric acid was reported as an early marker in blood for both insulin resistance and impaired glucose regulation as a result of increased lipid oxidation and oxidative stress [41] and a good marker for early stage T2D [42]. Furthermore, the level of myo-inositol in blood was reduced after 12-weeks of CH intervention, but increased in the urine of the CL group as a result of the intervention (Table 1 and 3). The interpretation of myo-inositol results is difficult as the CH-group showed higher levels of myo-inositol than the other groups before the intervention (data not shown). 3-Hydroxybutyric acid (β -hydroxybutyric acid) was also reduced in plasma after 12-weeks of CH intervention (Table 1). Previously, β -hydroxybutyric acid was shown to increase in subjects after an 8-weeks intervention with an acidified milk drink [43]. However, together with acetoacetate and acetone, β -hydroxybutyrate is one of the major ketone bodies in diabetic ketoacidosis. The ketone bodies are formed after β -oxidation of FAs into acetyl-CoA, but here casein reduced the level of β -hydroxybutyrate indicating a conversion into first acetoacetate then 3-hydroxy-3-methylglutaryl-CoA, followed by acetoacetyl-CoA and then into acetyl-CoA that through oxaloacetate can enter the tricarboxylic acid (TCA) cycle [44, 45]. The ketone bodies may also be derived from the decarboxylation of leucine [46], and as whey protein has a high content of leucine, this pathway may contribute to the higher level of β -hydroxybutyrate observed in the two whey groups compared to the casein groups. However, it does not explain the reduction in both α -hydroxybutyric and β -hydroxybutyric acids for the CH group, which rather suggests an impact of the combination CH than with the WH diet.

A lower level of urinary 2,4-dihydroxybutyric and 3,4-dihydroxybutyric acids was observed in the WH group compared to the CH group (Table 3). 2,4-Dihydroxybutyric acid, which is an intermediate in the β -oxidation of 4-hydroxybutyric acid, and 3,4-dihydroxybutyric acid were reported in urine of various cases of succinic semi-aldehyde dehydrogenase deficiency [47]. The

level of 3,4-dihydroxybutyric acid was reduced in plasma of the CH group and was higher in the CH group compared to the WH group after intervention, and this might be a result of higher urinary excretion of this metabolite in the CH group but not in the WH group.

An increase in urinary fumaric and succinic acids levels was observed after the 12-weeks only in the WH intervention, whereas no increase was observed in the CH group (Table 3). Succinic acid level was decreased in plasma of WH group after intervention (Table 1), which might be explained by an increase in the urinary excretion of this TCA cycle intermediates in the WH group. Fumaric acid is a precursor to l-malate in the TCA cycle and it is formed by the oxidation of succinic acid. Fumaric acid possesses interesting biological effects including anti-psoriasis, anti-inflammatory, neuroprotective and chemo-preventive activity [48]. Urinary citric acid followed the same pattern as fumaric and succinic acids in this study (Figure 2). TCA intermediates and citrate, in particular, have been shown to stimulate fatty acids *de novo* synthesis [49]. In addition, cytosolic oxaloacetate is metabolized to malate, which by the action of malic enzyme can be further metabolized to pyruvate, a reaction that generates NADPH, the reducing equivalent needed for fatty acids synthesis [50, 51]. Different modulating factors of urinary citrate excretion were previously reported, including protein intake [52]. Another explorative analysis of mice urine demonstrated higher urinary excretion of the TCA cycle intermediate citric acid after intake of a high fat diet combined with whey protein relative to high fat diet combined with casein protein. Thus, this might indicate that increased urinary loss of TCA cycle metabolites drain available substrates for anabolic processes, such as lipogenesis, which led to reduction in lipid storage in whey-fed mice relative to casein-fed mice [53]. The present human study showed the same pattern for urinary citric acid (Figure 2). Moreover, we found that the increase in urinary excretion of TCA cycle metabolites was only associated to the intake of a combination of whey and high level of MCFAs, as the WL intervention and the two casein groups did not show this effect on urinary citric acid. Thus, beside to observed changes in the levels of dicarboxylic fatty acids that were discussed above, these observations might

provide another potential explanation of the clinical observation on increased in lean body mass in the group consuming high levels of MCFAs + whey [12]. Therefore, a PCA analysis was performed in order to test the correlation between clinical and metabolomics observations, by including the clinical data (fat mass, weight, waist, lean body mass, levels of cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), HDL/LDL, and TGs) to the PCA models of GC-MS metabolomics at the end of the intervention. Loading plots show a negative correlation between urinary adipic acid, and TCA cycle metabolites (citric, succinic and fumaric acids) and lean body mass, fatt mass, waist and body weight (Figure 3B). Moreover, both sebacic acid and glycerol in blood show a negative correlation with fat mass, waist and body weight (Figure 3D), which supports our new hypothesis in this study.

In general, the observed discrimination between plasma metabolic profiles of WH vs. WL, but not CH compared to CL, is due to more differences in the observed metabolites between WH and WL than CH and CL and might indicate a higher synergistic effect of whey protein or its degradation products and MCFAs on some metabolic pathways, e.g. fat lipolysis and fat oxidation. Finally, the differences/changes in metabolic profils of this study could be a direct effect of diets intake and/or a diet-effect on the metabolism. An example of changes as an effect of consumption could be the higher levels of BCAAs (valine and leucine/isoleucine) in WH compared to CH after intervention as whey contains higher amounts of BCAAs than casein. On the other hand, the changes in MCFAs oxidation endogenous metabolites, urinary adipic acid and TCA cycle metabolites as well as plasma sebacic acid, in WH and CH groups are examples of diet-effect on the metabolism. The present study emphasizes the importance of implementing more than one analytical technique for the aim of untargeted metabolomics investigation, where they can complement each other.

In conclusion, metabolomics analysis in the human intervention study disclosed significant effects of dairy MCFAs and proteins intake on the blood and urine metabolome. Most

differences in metabolic profile were on the type of proteins, which was not reflected in the phenotypic response in body composition. Drain of available substrates for anabolic processes such as lipogenesis may reduce fat storage in the WH group, while early markers of T2DM were reduced in the casein groups. Most importantly, the metabolic pathway of MCFAs oxidation, ω -oxidation and/or β -oxidation followed by ω -oxidation, may result in reduction in body fat accretion reflecting previous observation on increase in lean body mass, independently on the type of milk protein.

Author contributions: M.K.L. was responsible for the production of milk with high levels of MCFAs, M.B. carried out the human study supervised by S.G and K.H, B.A. carried out GC-MS and LC-MS metabolomics work, M.RC and H.Z. contributed to LC-MS metabolomics work, C.N. contributed to GC-MS analytical work, T.S. contributed to data analysis work, T.K.D. and H.C.B., supervised the study, B.A wrote the manuscript with the contribution of all authors.

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Tables

Table 1. Changes in metabolite levels detected by GC-MS in plasma after 12-weeks intervention with the four different diets, low amount of MCFAs (L-MCFAs) + Whey (WL), high amount of MCFAs (H-MCFAs) + Whey (WH), L-MCFAs + Casein (CL), H-MCFAs + Casein (CH). Spectra match with pure standards. TMS: trimethylsilyl derivative. All changes are significant (* $P < 0.05$, ** $P < 0.001$, 2-way ANOVA).

Metabolite	Main peaks (m/z)	R.T (min)	NIST Match factor (%)	Effect of intervention	Differences between diets after intervention
α -Hydroxybutyric acid	73.0825/147.1206/131.0858/ 148.1228/66.0516	4.60	96	CH-decreased** CL-decreased*	WH>CH** WH>CL** WL>CL** WL>CH*
β -Hydroxybutyric acid	147.1206/73.0825/191.1828/ 117.0838/148.1228	4.79	97	CH-decreased**	WH>CH** WL>CH*
3,4-Dihydroxybutyric acid ^a	73.0825/147.1206/189.0872/ 233.1999/231.1842	6.15	88	CH-decreased**	WH>CH*
Sebacic acid	75.0604/331.1540/129.1227/ 149.1166/117.037	7.91	77	WH-increased**	WH>CH** WH>WL* WH>CL**
Succinic acid	147.1220/73.0824/148.1224/ 149.1166/247.3190	5.58	83	WH-decreased*	
Glycerol	147.1207/73.0827/205.1772/ 117.0833/148.1226	5.41	93	WH-increased**	WH>CH** WH>WL** WH>CL**
Lauric acid	117.0833/257.181/129.1220/ 131.0845/258.1773	7.02	70		WH>CH* WH>CL** WL>CL* WL>CH*
Myristic acid	117.0833/285.1238/75.0613/ 129.1220/286.2129	7.74	80	WL-decreased*	WH>CH** WH>WL** WH>CL**

Valine	144.1739/73.0827/147.1207/ 218.1810/145.1551	5.11	96	WH-increased*	WH>CH* WH>WL* WH>CL*
Glycine (2TMS)	102.1152/73.0827/147.1207/ 103.1003/204.1698	4.55	92		WH>CH*
Glycine (3TMS)	174.1011/248.3599/175.0673 /86.0813/176.1541	5.58	78		WH>CH* WH>CL* WL>CL* WL>CH*
Pyroglutamic acid	156.1410/73.0827/147.1207/ 157.1261/258.1773	6.55	97	WH-increased* CH-decreased**	WH>CH** WH>CL**
Sarcosine	73.0827/116.1062/147.1207/ 160.137/190.1365	5.97	72	WH-increased** CL-increased**	WH>CH** WH>WL* WH>CL* CL>CH*
Threonic acid	73.0827/147.1207/292.2188/ 220.1547/217.1799	6.72	95	WH-increased** CH-decreased** WL-increased* CL-decreased*	WH>CH** WH>CL** WL>CL* WL>CH** CL>CH**
Isoleucine	158.1910/73.0827/218.1810/ 149.1165/159.1737	5.51	88		WH>CH*
Threonine	73.0827/219.1778/218.1810/ 147.1207/117.0833	5.96	94	CH-decreased*	WH>CH*
Alanine	116.1062/73.0827/147.1207/ 75.0613/117.0833	4.45	87	CL-increased*	
Myo-Inositol	305.2304/73.0825/217.1802/ 147.1206/318.2400	8.66	94	CH-decreased**	WL>WH* WL>CH*

^ano available pure standard.

Table 2. Changes in metabolite levels in plasma detected by LC-MS after 12-weeks intervention with the four different diets, low amount of MCFAs (L-MCFAs) + Whey (WL), high amount of MCFAs (H-MCFAs) + Whey (WH), L-MCFAs + Casein (CL), H-MCFAs + Casein (CH). All changes are significant (* $P < 0.05$, ANOVA).

Metabolite	Detected peak (m/z)	R.T (min)	Differences between diets after intervention	Adduct	Adduct MW (DA)	Metabolite MW (DA)	Calculated molecular formula
Valine	118.086	5.15	WH>CH*	M+H	118.0862	117.0789	C ₅ H ₁₂ NO ₂
Leucine/isoLeucine	132.102	6.07	WH>CH*	M+H	132.1019	131.0946	C ₆ H ₁₄ NO ₂
Leucine/isoLeucine	154.083	6.07	WH>CH* WL>CH*	M+Na	154.0838	131.0946	C ₆ H ₁₃ NNaO ₂
Tyrosine	182.081	6.14	WH>CH*	M+H	182.0811	181.0738	C ₉ H ₁₂ NO ₃
Tyrosine	204.062	6.15	WH>CH*	M+Na	204.0631	181.0738	C ₉ H ₁₁ NNaO ₃
Phenylpyruvic acid	165.054	6.15	WH>CH*	M+H	165.0546	164.0473	C ₉ H ₉ O ₃
2-Phenylacetamide	136.075	6.14	WH>CH*	M+H	136.0756	135.0684	C ₈ H ₁₀ NO
3-Hydroxyundecanoic acid	247.128	9.22	WH>CH* WL>CH*	M+2Na-H	247.1280	202.1568	C ₁₃ H ₂₀ Na ₂ O ₃
Pivaloylcarnitine	246.170	11.45	WH>CH* WL>CH*	M+H	246.1699	245.1627	C ₁₂ H ₂₄ NO ₄
Phenylalanine	166.086	9.40	WL>CH*	M+H	166.0862	165.0789	C ₉ H ₁₂ NO ₂
Phenylalanine	188.068	9.40	WL>CH*	M+Na	188.0681	165.0789	C ₉ H ₁₁ NNaO ₂
Cinnamic acid	149.060	9.41	WL>CH*	M+H	149.0597	148.0524	C ₉ H ₉ O ₂
4-Hydroxyphenylacetic acid	175.030	11.76	WL>CH*	M+Na	175.0365	152.0473	C ₈ H ₈ NaO ₃

Table 3. Changes in metabolite levels detected by GC-MS in urine after 12-weeks intervention with the four different diets, low amount of MCFAs (L-MCFAs) + Whey (WL), high amount of MCFAs (H-MCFAs) + Whey (WH), L-MCFAs + Casein (CL), H-MCFAs + Casein (CH). Spectra match with pure standards. TMS: trimethylsilyl derivative. All changes are significant (* P <0.05, ** P <0.001, 2-way ANOVA).

Metabolite	Main peaks (m/z)	R.T (min)	NIST Match factor (%)	Effect of intervention	Differences between diets after intervention
Fumaric acid	245.1434/147.1201/73.0807/ 246.1445/246.1445/75.0599	5.83	95	WH-increased**	WH>CH* WH>CL** WH>WL*
Citric acid	273.1823/347.2169/375.2174 /274.1836/183.1102	7.71	81	WH-increased**	WH>CH*
Succinic acid	147.1201/75.0599/148.1200/ 149.1150/247.3517	5.58	82	WH-increased**	WH>CH* WH>CL** WH>WL**
2,4-Dihydroxybutyric acid ^a	73.0807/103.0994/147.1201/ 219.1740/129.1202	6.06	92		CH>WH*
3,4-Dihydroxybutyric acid ^a	73.0807/147.1201/117.0945/ 292.2209/203.1576	5.81	95		CH>WH*
Adipic acid	73.0807/75.0599/141.1247/ 111.06882/147.1201	6.44	93	WH-increased* CH-increased**	CH>WH* CH>CL** WH>WL*
Threonine	73.0807/218.1788/219.1763/ 147.1199/117.0947	5.96	96	WH-decreased*	
Pyroglutamic acid	156.1407/73.0807/147.1199/ 157.1236/258.1766	6.55	96	CL-increased*	CH>WH*
Threonic acid	73.0807/147.1201/292.2209/ 220.1519/205.1743	6.65	95		CH>WH*
Myo-Inositol	73.0807/305.2305/217.1775/ 147.1201/318.2419	8.66	91	CL-increased*	CL>WH* CL>WL*

^ano available pure standard.

Figure legends

Figure 1. PCA score plots of data obtained from fasted plasma and urine samples after 12-weeks of intervention of the four diets, (▼) low amount of MCFAs (L-MCFAs) + whey (WL), (●) high amount of MCFAs (H-MCFAs) + whey (WH), (Δ) L-MCFAs + casein (CL), (○) H-MCFAs + casein (CH) using GC-MS and LC-MS. A) GC-MS, plasma, B) LC-MS, plasma, C) GC-MS, urine, D) LC-MS, urine.

Figure 2. Illustration of some metabolic changes related to medium-chain fatty acids (MCFAs) oxidation observed in plasma and urine after consumption of milk MCFAs and proteins, WH: whey + high level of MCFAs, and CH: casein + high level of MCFAs. Texts in black are reported mechanisms from literature. Bar plots show level of metabolites in urine and plasma (before and after stand for before and after intervention, respectively). Star annotations represent significant differences between groups for each metabolite.

Figure 3. PCA score and loading plots of clinical data, and GC-MS metabolomics data obtained from fasted urine and plasma samples after 12-weeks of intervention of the four diets, (▼) low amount of MCFAs (L-MCFAs) + whey (WL), (●) high amount of MCFAs (H-MCFAs) + whey (WH), (Δ) L-MCFAs + casein (CL), (○) H-MCFAs + casein (CH). A and B) scores and loadings, respectively, of urine PCA model, C and D) scores and loadings, respectively, of plasma PCA model.