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Higher intake of fish and fat is associated with lower plasma s-adenosylhomocysteine: A cross-sectional study

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Abbreviations:

3G: Gut, Grain and Greens

SAH: s-adenosylhomocysteine

SAM: s-adenosylmethionine

Abstract

Several B-vitamins act as co-factors in one-carbon metabolism, a pathway that plays a central role in several chronic diseases. However, there is a lack of knowledge of how diet affects markers in one-carbon metabolism. The aim of this study was to explore dietary patterns and components associated with one-carbon metabolites. We hypothesized that intake of whole-grains and fish would be associated with lower Hcy, and higher SAM:SAH ratio due to their nutrient content. We assessed dietary information using a four-day dietary record in 118 men and women with features of the metabolic syndrome. In addition we assessed whole-blood fatty acid composition and plasma alkylresorcinols. Plasma *s*-adenosylmethionine (SAM), *s*-adenosylhomocysteine (SAH), homocysteine (Hcy) and vitamin B_{12} was included as one-carbon metabolism markers. We used principal component analysis (PCA) to explore dietary patterns and multiple linear regression models to examine associations between dietary factors and one-carbon metabolites. PCA separated subjects based on prudent and unhealthy dietary patterns, but the dietary pattern score was not related to the one-carbon metabolites. Whole grain intake was found to be inversely associated to plasma Hcy (-4.7% (-9.3; 0.0), p=0.05) and total grain intake tended to be positively associated with SAM and SAH (2.4% (-0.5; 5.5), p=0.08; 5.8% (-0.2; 12.1), p=0.06, respectively, per SD increase in cereal intake). Fish intake was inversely associated with plasma Hcy and SAH concentrations (-5.4% (-9.7; -0.8), p=0.02 & -7.0% (-12.1; -1.5), p=0.01, respectively) and positively associated with the SAM:SAH ratio (6.2% (1.6; 11.0), p=0.008). In conclusion, intake and fish and whole-grain appears to be associated with a beneficial one-carbon metabolism profile. This indicates that dietary components could play a role in regulation of one-carbon metabolism with a potential impact on disease prevention.

Keywords: dietary patterns; homocysteine; long-chain polyunsaturated fatty acids; whole grain; methyl donor metabolism

1. Introduction

One-carbon metabolism and especially high circulating homocysteine (Hcy) levels have been linked to a wide range of diseases such as cardiovascular disease[1], cognitive diseases[2,3], cancer[4,5], metabolic syndrome[6–8] and type 2 diabetes[9–12]. Several mechanisms have been suggested for the associations between one-carbon metabolism and these diseases, many of which, including epigenetic mechanisms, have been linked to the donation of methyl groups for biochemical modification of molecules. One-carbon metabolism donates methyl groups through the global methyl donor *s*-adenosylmethionine (SAM), which is then transformed into *s*-adenosylhomocysteine (SAH)[13,14]. The ratio between SAM and SAH is known as the methylation index and is used as a marker of methylation potential[13,14]. SAH can be further degraded into Hcy, and hyperhomocysteinemia may be a marker of imbalance of the one-carbon metabolism[13,14]. Hcy can be remethylated to methionine which is a precursor for SAM allowing the methyl-donor cycle to continue. The regulation of one-carbon metabolism is complex and understanding this regulation is a first step towards understanding the role of one-carbon metabolism in health and disease[15].

Several nutrients and bioactive components are involved in regulating one-carbon metabolism. Remethylation of Hcy to methionine can occur through folate-mediated methylation by methionine synthase, which is vitamin B_{12} dependent[13,14]. Another remethylation pathway of Hcy involves methyl group donation via betaine, which can be derived from choline. Betaine can convert Hcy through betaine homocysteine S-methyltransferase (BHMT), resulting in formation of methionine and dimethylglycine (DMG) [13,14]. Hcy can also be metabolized through the transsulfuration pathway, which is dependent on vitamin B_6. This pathway is initiated by cystathionine β-synthase (CBS) which converts Hcy to cystathionine, which is further metabolized to cysteine[13,14].

As many components of one-carbon metabolism are derived from diet, including folate, vitamin B_6 and B_{12}, and betaine, uncovering dietary patterns and specific foods that play a role in regulating one-carbon metabolism might be one way of preventing the wide range of diseases associated with elevated plasma Hcy, and one-carbon metabolism dysregulation. Foods such as whole-grains and fish have been proposed to affect one-carbon metabolism due to their nutrient content. Whole-grains are rich in vitamins B_6, folate, betaine and choline[16–19] and intake of whole-grains has been associated with lower Hcy concentrations. Fish are good sources of choline[20] and vitamin B_{12} [21,22] and also the n-3 long-chain polyunsaturated fatty acids (LCPUFA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). A meta-analysis of intervention trials found that fish oil decreased plasma Hcy[23], possibly via upregulation of one-carbon metabolism-related genes involved in the remethylation of Hcy[24,25].

Although the role of diet in regulating plasma Hcy has been studied to some degree[26–28], there is a lack of studies examining the role of diet in the regulation of SAM and SAH levels. We hypothesize that based on their nutrient content, intake of whole-grains and fish as well as biomarkers for the intake of these foods will be associated with lower Hcy, and higher SAM:SAH ratio. In order to examine these hypotheses, dietary patterns and their association with one-carbon metabolites were explored, and associations between intake of whole-grains and fish with one-carbon metabolites determined in adults with features of the metabolic syndrome.

2. Methods and materials

2.1 Study design

This cross-sectional study is based on baseline data from two dietary intervention studies from the 3G Center (http://www.3g-center.dk/about-3g) investigating the effects of gluten and wholegrain on gut microbiota composition and metabolic health[29]. The studies were conducted at the

Department of Nutrition, Exercise and Sports, University of Copenhagen, Denmark in collaboration with Novo Nordisk Foundation Center for Basic Metabolic Research, University of Copenhagen, Denmark. Baseline data from a total of 118 participants were used for cross-sectional analyses of dietary determinants of one-carbon metabolism. The studies were registered at http://www.clinicaltrials.gov (NCT01719913& NCT01731366) and approved by the Regional Ethical Committee of the Capital Region of Denmark in accordance with Helsinki declaration (H-2-2012-064 & H-2-2012-065) and the Data Protection Agency (2012-54-0170 & 2007-54-0269). The study design of the intervention studies has been described in detail by Ibrügger *et al.* [29].

2.2 Participants

The study participants for both studies consisted of men and women aged 20-65 years residing in the Greater Copenhagen area. Participants had to be weight stable and exhibit a metabolic risk profile as the primary aim of the 3G studies were to investigate diet - gut microbiota interactions in people at increased risk of metabolic disorders[29]. The inclusion criteria were a BMI of 25-35 kg/m^2 and/or waist circumference ≥94 cm for men and ≥80 cm for women and furthermore at least one of four of the additional metabolic syndrome criteria; 1) fasting plasma glucose 6.1-6.9 mmol/L, 2) fasting serum HDL-cholesterol ≤1.03 mmol/L for men and ≤1.29 mmol/L for women, 3) fasting plasma triacylglycerol >1.3 mmol/L, and 4) systolic blood pressure >130 mm Hg or medical treatment of hypertension[29]. Pregnant and lactating women were excluded from the study as were individuals with a diagnosed chronic gastrointestinal disorders, diabetes or chronic pancreatitis. Additional exclusion criteria included: pharmacological treatment of dyslipidemia, diabetes, medically prescribed diet, antibiotic treatment (<3 months prior to study start) or intake of pre- or probiotic supplements (<1 month prior to study start), hemoglobin <7.0 mmol/L or blood donation <1 month prior to study start, participation in other biomedical trials (<1 month prior to study start), intense physical activity (>10 hours/week) and alcohol consumption >21 units/week for

men and >14 units/week for women. The studies were registered at www.clinicaltrials.gov (NCT01719913& NCT01731366)

2.3 Dietary registration

Before the baseline examination, participants completed a four-day pre-coded dietary registration, including two weekdays and two weekend days. The dietary registration was developed by the National Food Institute at The Technical University of Denmark. Details about the method and calculation of intake of food and nutrients have been described elsewhere[30]. Briefly, the dietary registration combined choices for the most common food items and dishes consumed in Denmark divided into breakfast, lunch, dinner, and snacks, which in turn were separated into different categories such as bread, spreads, fruit and vegetables, type of meat, and beverages. Further, there was an open answer category for food items not listed in the questionnaire. Portion sizes were given as typical household measures, such as cups and spoons, or were estimated from pictures. Options for cereal products included among others rye bread (with and without sunflower or flax seeds), fine and coarse wheat bread, crisp bread, pasta, bulgur, oatmeal, and other breakfast cereals. In total the pre-coded diary comprised 59 different answering options that may contribute to whole-grain intake[31]. The pre-coded diary had 52 different answering options that could contribute to fish intake, which included options for both fatty fish (salmon, herring, etc.), lean fish (tuna, cod etc.) and fish products (such as traditional Danish fish based mayonnaise spreads) [30,31]. Whole-grain and fish intake was then estimated based on surveys on household use of cereals and cereal products as well as market share on a brand level. Vitamin supplementation was defined as either taken B-vitamin supplement or a multi-vitamin supplement. Fish oil supplementation was any type and amount of fish oil or similar products containing n-3 LCPUFA.

2.4 Laboratory and analytical procedures

All blood samples were drawn by trained laboratory technicians under standardized laboratory conditions in the morning after 8-10 hours overnight fast. Blood samples were taken after the subjects had been reclined for 10 minutes. The blood was drawn via an intravenous cannula in the subjects elbow crease and separated into plasma and cells within 30 min after collection. All blood sample analyses were performed after termination of the study to ensure a lower variation due to analytical batches.

2.4.1 Vitamin B_{12}, total homocysteine, SAM and SAH measurements

EDTA plasma vitamin B_{12} was determined by a chemiluminescent assay (Architect, Abbott Laboratories, Abbott Park, IL, USA). EDTA plasma Hcy was quantified in thawed aliquots by a competitive immunoassay (Architect, Abbott Laboratories, Abbott Park, IL, USA). A tandem mass spectrometry was used to determine s-adenosylmethionine and s-adenosylhomocysteine in plasma, as described in detail previously with only minor differences[32], i.e. a smaller plasma volume (100 µL) and a more sensitive tandem mass spectrometer (API5000 instead of API3000). CV% for quality control samples was between 2-5%.

2.4.2 Fatty acid analysis

Whole-blood fatty acid composition was analyzed at the Department of Kinesiology, University of Waterloo, Canada using a high-throughput gas chromatography method. As described previously, fatty acid methyl esters were prepared from whole-blood samples by direct trans-esterification with convectional heat[33]. Briefly, whole-blood fatty acids were trans-methylated by boron trifluoride in methanol (Pierce Chemicals) in the presence of 2,6-di-tert-butyl-4-methylphenol (butylated hydroxytoluene; Sigma-Aldrich) for 60 min at 90°C. Separation was achieved by the addition of water and hexane, and the fatty acid methyl esters were collected for analysis on a Varian 3900 GC

equipped with a DB-FFAP capillary column (15 m × 0·10 mm i.d. × 0·10 µm film thickness; J&W Scientific from Agilent Technologies). Individual fatty acids were expressed as w/w % of total fatty acids in whole-blood. The inter- and intra-assay CV% were 4.5 and 1.2 % (EPA) and 6.4 and 2.4 % (DHA).

2.4.3 Plasma alkylresorcinols

Alkylresorcinols (AR) are phenolic lipids that reflect whole-grain wheat and rye intake when measured in blood plasma. EDTA plasma total and homologue ratios of AR were measured using a recently published normal-phase liquid chromatography-tandem mass spectrometry (LC-MS/MS) method[34]. Briefly, 100 µL of plasma was extracted using supported liquid extraction (HybridSPE®, Supelco, Sigma Aldrich) with 2 x 800 µL acetone. The resulting extract was evaporated and resuspended in heptane:ethanol solution (95:5v/v). Extracts were run on an LC-MS/MS (LCMS 8030+, Shimadzu Europa GmbH, Duisberg, Germany) and odd and even AR homologues from C17-C26 were measured using multiple reaction monitoring. Intra-batch variation for control samples was 3-15 % while inter-batch variation was 8-18 %, with variation highest around the limit of detection.

2.5 Statistical analyses

Baseline descriptive population data are expressed as means ± SD or medians (25^{th}; 75^{th} percentile). All statistical tests were performed using the R statistical environment (http://cran.r-project.org/, version 3.1.3). Results were considered significantly different at $P<0.05$.

For all statistical analysis dietary intake was corrected for total energy intake and is thus reported as grams/10 MJ and diet as well as baseline phenotype characteristic differences between genders were tested using Student's t-test. Pearson's correlation was used for the correlations between one-

carbon metabolites. Using Spearman's correlation instead did not affect the results so the data from the Pearson's correlation are shown.

For identification of dietary patterns, principal component analysis (PCA) was carried out including 12 food groups. We applied centering and scaling to unit variance (auto-scaling) for preprocessing the data before PCA modelling. The dietary patterns were named based on subjective assessment of the food group loadings in the PCA. The principal component (PC)-scores was extracted for the PC separating the dietary patterns and this was used for univariate analysis for associations with one-carbon metabolites. The linear regression analyses were adjusted for age and gender and for Hcy analysis it was further adjusted for vitamin supplement use. Further adjustment for BMI or total energy intake did not change the results.

As whole-grain intake was determined both using dietary registrations and plasma AR we combined these two measurements using Howe's rank score, which was calculated by ranking the participants according to reported wholegrain intake in the dietary registrations and according to their plasma AR[35]. These two ranks were then added together to give a combined rank. Participants were allowed to have the same score[35]. Plasma AR homologue C17 and C21 ratio was used to differentiate whole-grain wheat and rye[36]. One outlier was removed from further analysis as the AR measurement was >4 SD from the mean.

Multiple linear regression models were also used to examine the association between plasma one-carbon metabolites and dietary intake variables. The models were adjusted for gender and age of participants. Adjusting for dietary supplementation, smoking and alcohol intake did not change the β- or P-values for SAM, SAH and SAM:SAH ratio noticeably, thus, these confounders were left out of the models. The Hcy analyses were adjusted for dietary supplementation use. We also adjusted for total energy intake in the models, but these did not substantially change β- or P-values and thus

the final models are presented without this adjustment. Model validation was performed by visual inspection of residual plots and quantile-quantile plots. Estimates are reported as per standard deviation change in dietary intake. Skewed variables were log transformed and reported as %-change (95%CI), per standard deviation change in dietary intake.

3. Results

3.1 Baseline characteristics

A total of 118 participants had measurements at baseline and were included in the study (See Figure 1 and Table 1). Subjects were on average 49 years old, overweight and had slightly elevated metabolic features (Table 1). In total, 24% used vitamin supplements, equally distributed between men and women, and only 0.8% of our study population smoked. Men had higher plasma concentrations of Hcy (P=0.02) and SAH (P=0.08) and lower SAM:SAH ratio (P=0.002), whereas no gender differences were found for plasma concentrations of vitamin B_{12} and SAM (Table 1).

3.2 Dietary intake

The study population intake of vitamin B_{12}, B_6 and folate were comparable with the average Danish intake levels[37]. Women had a higher folate intake per 10 MJ (p=0.014). Men had a higher meat intake and also a higher vitamin B_{12} intake (p=0.001 & p=0.04, respectively). No gender differences were found for whole-grain and fish intake. Finally, women consumed more vegetables and fruits compared with men (p=0.03 & p=0.008) (Table 2).

3.3 Associations between one-carbon metabolites

We found a negative association between Hcy and SAM:SAH ratio (r=-0.30, p<0.001) and vitamin B_{12} (r=-0.24, p=0.01) (Table 3), but no association between Hcy and SAM (r=0.03, p=0.75), and only a trend between Hcy and SAH (r=0.18, p=0.06). We found a strong positive association

between SAM and SAH (r=0.64, p<0.001) and a negative association between SAM and SAM:SAH ratio (r=-0.22, p=0.02). Yet we found no association between fasting plasma concentrations of vitamin B_{12} and SAM or SAH. SAH concentrations were strongly negatively associated with SAM:SAH ratio (r=-0.79, p<0.001), but were not associated with plasma vitamin B_{12} concentrations (r=0.00, p=0.99). Finally, we found a positive association between the SAM:SAH ratio and plasma vitamin B_{12} concentrations (r=0.18, p=0.05) (Table 3).

3.4 Food patterns and one-carbon metabolites

To examine the relationship between food patterns and one-carbon metabolites we first built a PCA model using relevant available food components. The first PC explained 14.8% of the variation in the data and split the dietary patterns into a more prudent diet (rich in vegetables, fruit, cereals and fish) and a more unhealthy diet (higher in meat, fats, and sugars) pattern (Figure 2a). We found no statistically significant association between this first dietary PC score and Hcy, SAM, SAH and SAM:SAH ratio (Table 4).

Secondly, we included the one-carbon metabolites, SAM, SAH and Hcy, in the PCA model. SAM and SAH grouped together with cereals and Hcy scored opposite to the intake of fish and fruit and vegetables (Figure 2b). The overall dietary patterns remained almost the same.

3.5 Cereals and one-carbon metabolites

To further explore the link between cereals and one-carbon metabolites we used linear regression analyses. These analyses showed that plasma concentrations of SAM and SAH tended to be positively correlated with total cereal intake (2.4 % (-0.5; 5.5), p=0.08; 5.9% (-0.2; 12.1) p=0.06, respectively, per SD increase in cereal intake)(Table 4). On the other hand, the SAM:SAH ratio was not associated with total cereal intake (p=0.17). We further explored if associations were observed for whole-grain intake, but found no statistically significant associations with SAM, SAH or the

SAM:SAH ratio when whole-grain intake was measured by dietary registrations, plasma total ARs, or Howe's rank sum score (p-value between 0.20-0.85). Nor did we find any statistically significant associations between total cereal intake or individual measurements of whole-grain intake or plasma concentrations of Hcy (p-value between 0.33-0.91). However, we found a negative association between Howe's rank sum score for whole-grain intake and Hcy (-4.7% (-9.3; 0.0), p=0.05), suggesting that higher whole-grain intake is associated with marginally lower Hcy concentrations (Table 4). The ratio between AR homologue C17 and C21, which reflects wheat vs rye intake, was not associated with any of the one-carbon metabolites (data not shown). We also performed analyses with total cereal intake adjusted for wholegrain intake as a proxy measure of refined grain and found positive associations between total cereal intake and SAH (12.6% (2.9; 23.2), p=0.01) and a negative association with SAM:SAH ratio(-8.2% (-14.5;-1.7) , p=0.02). We found no associations with plasma Hcy and SAM concentrations.

3.6 Dietary fish intake, fatty acids and circulating concentration of one-carbon metabolites

We found negative associations between fish intake and plasma Hcy and SAH (-5.4% (-9.7; -0.8), p=0.02 & -7.0% (-12.1; -1.5), p=0.01, respectively) and a positive association with the SAM:SAH ratio (6.2% (1.6; 11.0), p=0.008) (Table 4). However, no statistically significant association was found between fish intake and plasma concentrations of SAM (p=0.59). Contrary to the associations with fish intake, we found no statistically significant associations between whole-blood DHA, EPA or EPA+DHA concentrations and one-carbon metabolites (p-values between 0.14-0.88) even though the direction for associations was the same as for fish intake (Table 4). Additionally we found a positive association between fish intake and plasma vitamin B_{12} concentration (8.2% (2.2; 14.5), p=0.007).

3.7 Dietary intake of meat, eggs, fruits, vegetables and circulating concentration of one-carbon metabolites

We also explored the association between eggs, fruits and vegetables which grouped opposite to Hcy, and meat which grouped together with Hcy in the PCA. These analyses showed that higher egg and vegetable intake was negatively correlated with plasma Hcy (-6.5% (-10.8; -2.0), p=0.005; -4.6% (-9.2; 0.2), p=0.06, respectively) and that egg intake was tentatively associated with SAM concentrations (2.7 % (-0.2; 5.7), p=0.07). However, we found no associations between any of the other dietary components and one-carbon metabolites (Table 4).

3.8 Dietary macronutrient energy distribution and circulating concentration of one-carbon metabolites

Fat intake was inversely associated with SAH concentrations (-6.7% (-11.8; -1.2), p=0.02), while being positively associated with the SAM:SAH ratio (5.4% (0.9; 10.1), p=0.02). We found the opposite trend when looking at carbohydrate intake, i.e. a positive association with SAH (9.7% (3.7; 16.1), p=0.002) and a negative association with the SAM:SAH ratio (-8.6% (-12.4; -4.5), p<0.001). However, for protein and fiber intake we found no associations with one-carbon metabolites, and finally no associations between fat and carbohydrate intake and plasma Hcy and SAM concentrations (Table 4).

4. Discussion

In this study we examined the interrelationships between fasting plasma one-carbon metabolites and diet components in overweight Danish adults. Our results showed that one-carbon metabolites did not correlate, as highly as could be expected from metabolic maps of one-carbon metabolism, especially for Hcy and SAM, and Hcy and SAH. Furthermore, we explored food patterns and examined associations between one-carbon metabolites and individual foods. Using a PCA model

to define diet patterns, we did not find any associations, but we did however find links between individual food groups and one-carbon metabolites, especially intake of fish and grains.

The observed inter-correlations between the one-carbon metabolites indicate that there are other components that may have a major effect on fasting concentrations beyond a straightforward feedback based on concentrations. The observed one-carbon metabolite concentrations are comparable to other studies[38–41] and high correlation between SAM and SAH has also been observed earlier[38–40], with only one study not reporting a strong association between the two[42]. Both SAM and SAH were observed to be correlated with the SAM:SAH ratio with SAH being a major determinant, and this too is in line with observations from other studies[38,40]. We found no association between plasma Hcy and SAM, and only a weak association with SAH. This is somewhat in line with the existing literature where most studies find no association between SAM and Hcy[42–44], although one study did show a positive[40] and one a negative association[38]. SAH and Hcy are generally positively correlated[38–40,42–44]. The apparent disconnect between Hcy and SAM and SAH suggest that different dietary components might affect different parts of the one-carbon metabolism. Thus, we examined the role of diet in the regulation of individual one-carbon metabolites.

We used PCA modelling to identify overall dietary patterns and their potential association with the one-carbon metabolites. However, counter to hypothesis, a healthy dietary pattern was not associated with one-carbon metabolites, which is in contrast to observations from other studies that have shown a clear association between a prudent dietary pattern and plasma Hcy concentration[26]. However, we did find that when one-carbon metabolites were included in the PCA model, several food groups appeared to be associated with the one-carbon metabolites.

Cereal intake clustered together with SAM and SAH in the PCA analysis. We hypothesize that this could be due to whole-grains, as intake of whole-grains are frequently associated with several one-carbon metabolites[16,18,19] and are the major dietary source of betaine[18]. There was no association between whole-grain intake and SAM and SAH, although combining estimated intake of whole-grains and whole-grain biomarkers (plasma ARs) showed an overall positive association between SAM and SAH and cereal intake, suggesting that the use of the dietary biomarker improved the estimation of whole-grain intake. The PCA analysis indicated that the relationship between cereals and SAM and SAH was likely driven by refined grain intake rather than whole-grain intake. In our study we lacked specific information on refined grain intake, and to address this we adjusted total cereal intake for wholegrain intake and found positive associations between total cereal intake and SAH and a negative association with SAM:SAH ratio. This could indicate that the association is driven by refined grain intake. This could possibly be accounted for by refined wheat, which although lower in betaine than whole-grain wheat, is still the major source of betaine in most populations[18]. In contrast to our findings, another study, encompassing 610 individuals, found no association between grain intake and plasma SAM, but they did not examine whole-grains and refined grains separately[39]. It is noteworthy that cereal and grain products in Denmark, where the present study was performed, are not fortified with folic acid, which is the case in many other countries, e.g. the US, and this may give rise to differences in the observed association between cereal intake and one-carbon metabolites. Furthermore, the Danish population has a high whole-grain intake[45] and this too could affect the association between intake of cereals and one-carbon metabolites.

While we did not find any correlation between whole-grain and plasma SAM and SAH, we did find that whole-grain intake was inversely correlated with Hcy, which although not as strong as in other studies is in line with previous results [28,46,47]. As the overall results for grain intake and one-

carbon metabolites were weak, the hypothesis of a relationship between grain intake, both whole-grain and refined grain, needs to be further examined in future studies, both in populations where fortification is presents as well as populations, where it is not. Overall, we were able to confirm our hypothesis that whole-grain intake was associated with lower Hcy, but we did not find that it was associated with higher SAM:SAH ratio.

We were able to confirm our hypothesis that fish intake was associated with lower plasma Hcy as well as SAH and therefore a higher SAM:SAH ratio. These results are in line with other studies showing no association between fish intake and plasma SAM[39] and an inverse association between fish intake and Hcy concentration[28]. We speculate that fish intake might be related to one-carbon metabolism as fish is a good source of vitamin B_{12}[48], choline[20], and methionine. However, we found no associations with meat intake, which is ingested at higher levels than fish in this population and is an equally good source of vitamin B_{12}, choline[20], and methionine. This suggests that fish may contribute with other constituents that might affect one-carbon metabolism and the most obvious choice would be the n-3 LCPUFAs. n-3 LCPUFA has been linked to one-carbon metabolism via upregulation of genes and remethylation of Hcy[23–25]. However, no association between whole-blood EPA and DHA concentrations and one-carbon metabolites were detected. Recent studies have found that fish consumption result in a different metabolic profiles compared to meat[49,50], supporting that there could be other compounds from fish aside from n-3 LCPUFA that could be responsible for the association. One limitation of this study was that we did not determine the type of fish eaten, and future studies looking into fatty fish and lean fish separately might elucidate further if the association between fish and one-carbon metabolism is related to their fatty acid content. We analyzed n-3 LCPUFA in whole-blood in the fasting state, which are more variable and dependent on short term intake than e.g. red blood cell fatty acid composition.

Finally, we examined whether overall macronutrient intake was related to the one-carbon metabolites. Total fat intake was found to be inversely associated with plasma SAH and positively associated with the SAM:SAH ratio, while the opposite was found for carbohydrate intake. We did not find any associations between overall macronutrient intake and plasma SAM or Hcy concentrations. These findings are in contrast to Poirier et al. who in a study of 66 participants showed a negative association between fat intake and SAM[51] and another study that found positive associations between fat intake and plasma Hcy and inverse associations between carbohydrate and fiber intake and Hcy[28]. However, our data is in line with another study that did not find any association between protein intake and plasma Hcy and SAM[39]. The associations between circulating one-carbon metabolites, dietary macronutrient and energy intake are complex as has been shown in overfeeding studies[52]. Preservation/stabilization of methionine and SAM concentrations as well as other energy metabolism regulation probably plays a role. Future studies are needed to determine whether energy source matter for one-carbon metabolism or whether this is "just" an overall marker of a certain dietary pattern. Moreover, plausible mechanisms of why energy source should affect one-carbon metabolism should be proposed.

One of the main limitations of this study is its small sample size, especially for a cross-sectional dietary intake study. This may have limited our statistical power to find association between dietary patterns and components and one-carbon metabolites and may also give rise to chance findings. Our findings therefore need to be replicated in future dedicated and statistically powered studies. We have used an explorative approach in the data analysis to avoid overall multiple testing and have focused on foods rather than individual nutrients, which are more often used in studies of associations with one-carbon metabolites. Even though the dietary assessment method has been validated, the method might still be prone to errors such as over- and underreporting and may present a skewed picture of normal eating habits e.g. people tend to eat more healthy during dietary

recording[30]. This could have contributed to an overall attenuation of the true associations, and thus lowered our power to detect associations. A strength of this study is however the inclusion of circulating biomarkers of intake (ARs and DHA and EPA) to complement the dietary intake data, which adds greater validity and statistical power to the analyses[35]. A further limitation of our food registration was that it was conducted over 4 days, which might not be enough to pick up foods that are not consumed daily, in particular fish. Furthermore, as this study has a cross-sectional design it does not allow causal inference which would require that the findings were tested in randomized controlled trials.

In this cross-sectional study of 118 Danish adults we found evidence for associations between fasting plasma concentrations of one-carbon metabolites and overall dietary patterns and macronutrient intake and point towards specific relationships with intake of grains and fish. We also found limited and weaker associations between the one-carbon metabolites than expected, indicating that one-carbon metabolism is governed by complex regulation mechanisms that cannot be easily explained by dietary nutrient intake. As this was a small study these results merely raise questions on what dietary components might be involved in one-carbon metabolism regulation, and more research is needed to establish relationships between diet and one-carbon metabolism and the potential link to lifestyle diseases.

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Table 1 – Baseline characteristics of study participants

	All (n=118)	Men (n=47)	Women (n=71)	p-value
Age (y)	48.8±11.2	49.1±10.7	48.5±11.7	0.775
BMI (kg/m^2)	28.7±3.5	29.0±2.4	28.6±4.1	0.534
Waist circumference (cm)	100±9	104±8	97±9	<0.001
Systolic blood pressure (mmHg)	128±13	134±12	124±12	<0.001
Diastolic blood pressure (mmHg)	81±9	83±9	80±9	0.113
Fasting glucose (mmol/L)	5.7±0.6	5.9±0.5	5.6±0.6	0.008
Fasting triglycerides (mmol/L)	1.33±0.59	1.50±0.72	1.22±0.46	0.013
Fasting HDL cholesterol (mmol/L)	1.30±0.29	1.16±0.27	1.39±0.26	<0.001
SAM (nmol/L)	89.7±15.3	90.9±14.6	88.9±15.7	0.482
SAH (nmol/L)	16.5±6.5	17.7±5.0	15.6±7.2	0.084
SAM:SAH	5.9±1.6	5.4±1.1	6.3±1.8	0.002
Homocysteine (µmol/L)	9.6±2.7	10.3±2.5	9.1±2.7	0.022
Vitamin B_{12} (pmol/L)	306±112	318±132	299±99	0.384

Presented as means ± SD. P-values for gender differences using student's t-test are shown.

Table 2 – Baseline food and dietary intake of study participants

	All (n=118)	Men (n=47)	Women (n=71)	p-value
Energy intake (kJ)	9781±2903	10905.18±3447	9036±2205	<0.001
Protein (g/10 MJ)	85±13	88±14	83±12	0.045
Protein, E%	15±2	15±2	14±2	0.045
Fat (g/10 MJ)	96±13	97±13	96±13	0.807
Fat, E%	36±5	36±5	36±5	0.807
Carbohydrate (g/10 MJ)	262±32	256±32	265±31	0.159
Carbohydrate, E%	44±5	44±5	45±5	0.159
Total fiber per day (g/10 MJ)	26±9	24±7	27±9	0.244
Vitamin supplements, n (%)	28 (24)	11 (23)	17 (24)	1.000
Fish oil supplements, n (%)	14 (12)	5 (11)	9 (13)	0.960
Wholegrain (g/10 MJ)	70±39	71±37	69±42	0.835
Fish and fish product (g/10 MJ)	24±28	24±26	25±29	0.874
Milk and milk product (g/10 MJ)	294±206	277±217	304±200	0.486
Cheese and cheese product (g/10 MJ)	41±29	38±26	42±30	0.473
Cereals and starch product (g/10 MJ)	260±83	271±74	253±87	0.257
Vegetables (g/10 MJ)	213±200	164±88	245±244	0.032
Fruits (g/10 MJ)	191±146	148±118	220±157	0.008
Meat and meat product (g/10 MJ)	104±57	125±57	91±52	0.001
Poultry and poultry products (g/10 MJ)	33±42	36±52	31±34	0.541
Egg and egg product (g/10 MJ)	28±26	27±29	29±23	0.770
Oils and fats (g/10 MJ)	37±15	36±16	37±14	0.931
Sugar and sugar products (g/10 MJ)	51±32	45±35	55±30	0.092
Potatoes (g/10 MJ)	66±67	59±63	70±69	0.392
Vitamin B_{12}, (µg/10 MJ)	5.3±2.4	5.8±2.2	4.9±2.4	0.041
Folate, (µg/10 MJ)	351±134	314±83	375±155	0.014
Vitamin B_6, (mg/10 MJ)	1.5±0.4	1.5±0.4	1.5±0.4	0.714

Values are means ± SD. P-values for gender differences using student's t-test are shown.

Table 3 – Interrelationships between fasting plasma concentrations of one-carbon metabolites in the study participants

	SAM	**SAH**	**SAM:SAH**	**Vitamin B_{12}**
Homocysteine	0.03	0.18^	-0.30**	-0.24*
SAM	-	0.64***	-0.22*	0.07
SAH		-	-0.79***	0.00
SAM:SAH			-	0.18*
Vitamin B_{12}				-

Values are Pearsons *r*. Signs are p-values ^<0.10; *<0.05; **<0.01; ***<0.001

Table 4 – Associations between dietary principal component score, foods, macronutrients, and fasting plasma concentrations of one-carbon metabolites

	Homocysteine	P-value	SAM	P-value	SAH	P-value	SAM:SAH ratio	P-value
Principal component 1	3.1 (-1.9; 8.3)	0.22	-2.1 (-5.0; 0.8)	0.15	0.5 (-5.3; 6.7)	0.85	0.7 (-5.3; 7.0)	0.24
Grains								
Cereal intake (g/10MJ)	0.3 (-4.5;5.3)	0.91	2.4 (-0.5;5.5)	0.08	5.9 (-0.2;12.1)	0.06	-3.1 (-7.4;1.4)	0.17
Wholegrain intake (g/10MJ)	-2.1 (-6.8;2.7)	0.38	1.5 (-1.5;4.5)	0.32	1.0 (-4.8;7.1)	0.75	0.5 (-4.0;5.3)	0.82
Total plasma ARs (nmol/L)	-2.4 (-7.2; 2.5)	0.33	2.0 (-0.9;5.0)	0.17	1.1 (-4.6; 7.3)	0.70	0.9 (-3.7; 5.7)	0.70
Howe's rank sum for wholegrain intake (score)	-4.7 (-9.3;0.1)	0.05	1.7 (-1.3;4.8)	0.26	0.6 (-5.3;6.8)	0.85	1.1 (-3.5;6.0)	0.63
Fish								
Fish intake (g/10MJ)	-5.4 (-9.7;-0.8)	0.02	-1.2 (-4.0;1.7)	0.42	-7.0 (-12.1;-1.5)	0.01	6.2 (1.6;11.0)	0.01
EPA + DHA (%/total fatty acids)	-3.9 (-8.8;1.3)	0.14	0.3 (-3.1;3.8)	0.88	-3.1 (-9.6;3.9)	0.37	3.5 (-1.8;9.0)	0.20
Macronutrients								
Protein intake (g/10MJ)	-1.8 (-6.5;3.1)	0.47	1.7 (-1.3;4.7)	0.27	-1.6 (-7.2;4.4)	0.60	3.3 (-1.3;8.1)	0.16
Fat intake (g/10MJ)	-0.8 (-5.4;4.1)	0.75	-1.6 (-4.4;1.3)	0.27	-6.7 (-11.8;-1.2)	0.02	5.4 (0.9;10.2)	0.02
Carbohydrate intake (g/10 MJ)	3.6 (-1.4;8.8)	0.16	0.3 (-2.6;3.4)	0.83	9.7 (3.7;16.1)	<0.001	-8.6 (-12.4;-4.6)	<0.001
Fiber intake (g/10 MJ)	-3.6 (-8.2;1.2)	0.14	1.7 (-1.3;4.8)	0.27	0.4 (-5.5;6.6)	0.90	1.3 (-3.3;6.1)	0.58
Other foods								
Fruit intake (g/10MJ)	0.6 (-4.3;5.8)	0.81	-0.3 (-3.3;2.8)	0.85	1.4 (-4.6;7.7)	0.66	-1.6 (-6.2;3.1)	0.49
Vegetable intake (g/10MJ)	-4.6 (-9.2;0.2)	0.06	1.8 (-1.2;4.8)	0.23	-1.0 (-6.7;5.1)	0.74	2.8 (-1.8;7.6)	0.23
Meat intake (g/10MJ)	-0.5 (-5.4;4.6)	0.84	1.0 (-2.1;4.1)	0.53	-1.4 (-7.2;4.8)	0.64	2.4 (-2.3;7.4)	0.31
Egg intake (g/10MJ)	-6.5 (-10.8;-2.0)	0.01	2.7 (-0.2;5.7)	0.07	1.3 (-4.4;7.3)	0.66	1.4 (-3.1;6.0)	0.55

Data are presented as β-coefficients in %-change (95%CI) for multiple linear regression models adjusted for age and gender. Homocysteine models were also adjusted for dietary supplementation use. All are reported for 1 SD change (see table 2) in dietary principal component score, foods and macronutrients. Higher principal component values indicate an unhealthier dietary pattern. For Howe's rank sum score higher values is indicating higher wholegrain intake. For the fatty acid analysis n=99. Abbreviations: AR, alkylresorcinols; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; SAH, s-adenosylhomocysteine; SAM, s-adenosylmethionine.

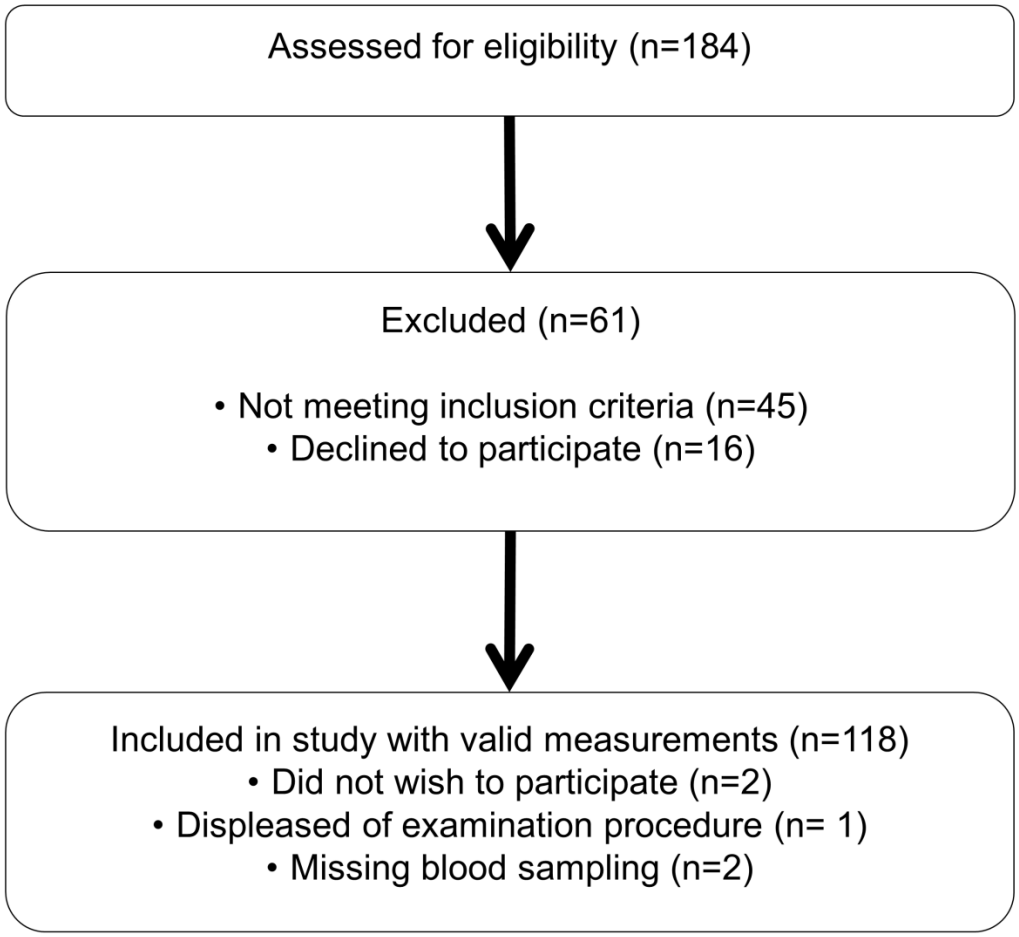

Figure 1 – Flow chart of subjects who were screened, enrolled, and had valid measurements for the study.

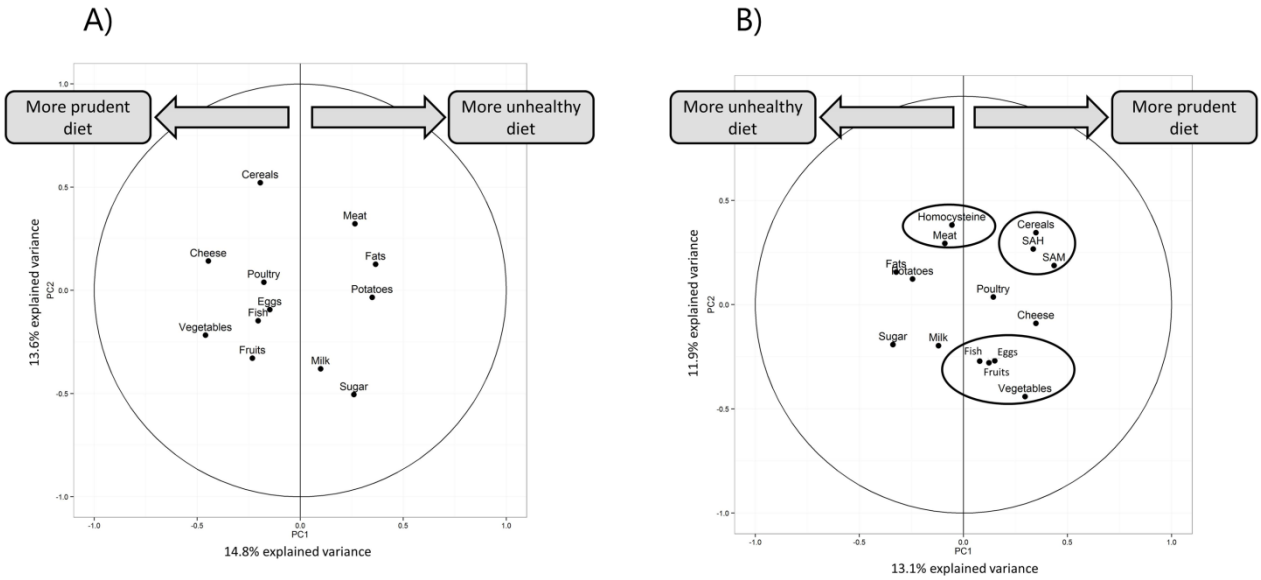

Figure 2 –A) Principal component analysis of dietary patterns – principal component 1, explaining 14.8% of the variation in the data, seems to split the dietary patterns into a prudent against western dietary pattern direction. B) Principal component analysis of dietary components and one-carbon metabolites – S-adenosylhomocysteine (SAH) and S-adenosylmethionine (SAM) seems to group together with total cereal intake (as indicated by the furthers right circle), while homocysteine seems to be opposite of more prudent dietary choices such as vegetables, fruits and fish intake (as indicated by the dark ellipses).