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ORIGINAL CONTRIBUTION

TMAO, creatine and 1-methylhistidine in serum and urine are potential biomarkers of cod and salmon intake: a randomised clinical trial in adults with overweight or obesity

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Abstract

Purpose To identify biomarkers to assess participants' compliance in an intervention study with high intake of cod or salmon, compared to a fish-free diet.

Methods In this randomised clinical trial, 62 healthy overweight/obese participants consumed 750 g/week of either cod ($N=21$) or salmon ($N=22$) across 5 weekly dinners, or were instructed to continue their normal eating habits but avoid fish intake (Control group, $N=19$) for 8 weeks.

Results After cod intake, serum concentrations of trimethylamine *N*-oxide (TMAO, $p=0.0043$), creatine ($p=0.024$) and 1-methylhistidine (1-MeHis, $p=0.014$), and urine concentrations (relative to creatinine) of TMAO ($p=2.8\times10^{-5}$), creatine ($p=8.3\times10^{-4}$) and 1-MeHis ($p=0.016$) were increased when compared to Control group. After salmon intake, serum concentrations of 1-MeHis ($p=2.0\times10^{-6}$) and creatine ($p=6.1\times10^{-4}$), and urine concentrations (relative to creatinine) of 1-MeHis ($p=4.2\times10^{-6}$) and creatine ($p=4.0\times10^{-5}$) were increased when compared to Control group. Serum and urine concentrations of TMAO were more increased following cod intake compared to salmon intake ($p=0.028$ and 2.9×10^{-4}, respectively), and serum and urine 1-MeHis concentrations were more increased after salmon intake compared to cod intake ($p=8.7\times10^{-5}$ and 1.2×10^{-4}, respectively). Cod and salmon intake did not affect serum and urine concentrations of 3-methylhistidine, and only marginally affected concentrations of free amino acids and amino acid metabolites.

Conclusion TMAO measured in serum or urine is a potential biomarker of cod intake, and 1-MeHis measured in serum or urine is a potential biomarker of salmon intake.

Keywords Cod · Salmon · TMAO · Creatine · 1-Methylhistidine · 3-Methylhistidine · Amino acids

Introduction

The risk of developing type 2 diabetes and cardiovascular disease have in some studies been shown to be reduced [1–10], whereas others have found no association [11–14], and some have shown that fish intake may increase the risk

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for developing type 2 diabetes [15, 16]. The interpretations of findings in clinical trials including studies on dietary intakes are often undermined by the lack of reliable measures of compliance. For intervention studies on fish intake, the long-chain n-3 PUFAs can be measured in isolated phospholipids from serum or plasma or in isolated red blood cells; however, these methods are time-consuming, expensive, require a relatively large sample volume, and can only be used as biomarkers for intake of fatty fish. Compounds associated with fish muscle proteins measured in blood or urine may be better biomarkers for assessing intake of both lean and fatty fish.

We have recently investigated the use of plasma and urine concentrations of trimethylamine *N*-oxide (TMAO), creatine, 1-methylhistidine (1-MeHis) and 3-methylhistidine (3-MeHis) as biomarkers of fish or fish protein intake in rats, and these results demonstrated that rats fed cod protein or salmon fillet had higher urine concentrations (relative to creatinine) of TMAO, creatine, 1-MeHis and 3-MeHis [17, 18]. In addition, plasma concentrations of creatine and 1-MeHis were also higher in rats fed cod protein or salmon fillet, and rats fed cod protein had higher plasma concentration of 3-MeHis, whereas plasma TMAO concentration was similar in these rats when compared to rats fed diets containing milk proteins as the sole protein source [17, 18]. Thus, our previous findings in rats, and those of others showing increased circulating TMAO concentration after fish/fish protein intake in mice and humans [19, 20], suggest that TMAO, creatine, 1-MeHis and 3-MeHis may be promising biomarkers of fish intake.

The possibility of identifying biomarkers of fish intake is of great value for objectively and reliably assessing compliance in clinical trials investigating the effects of fish consumption on health. The aim of the present study was therefore to identify potential biomarkers of cod and salmon intake. Concentrations of TMAO, creatine, 1-MeHis and 3-MeHis, as well as amino acids and amino acid metabolites, were determined in serum and urine samples from individuals with overweight/obesity who participated in an intervention study investigating potential health effects of high intake of cod or salmon (5 dinners per week for 8 weeks) versus a fish-free diet. Our hypothesis was that TMAO, creatine, 1-MeHis and 3-MeHis could be used as biomarkers of cod and salmon intake.

Methods

Participants, study setting and ethics

The study design, description of study participants, study setting and protocol for study visits have been described in detail previously [21]. In brief, the study was designed as an 8-week randomised, controlled intervention study with a parallel group design, with three intervention arms: Atlantic cod (wild-caught *Gadus morhua*) in weekly doses of 750 g, Atlantic salmon (farmed *Salmo salar*) in weekly doses of 750 g, and a no-fish group as the Control group.

The study population consisted of overweight or obese adults, and all participants were of Norwegian ethnic origin (Caucasian) living in Bergen, Norway. Inclusion criteria were BMI ≥ 27 kg/m^2, fasting blood glucose ≤ 7.0 mmol/l and age 18–69 years. Exclusion criteria were pregnancy, incompatibility with fish consumption (allergies, intolerance and/or dislike), diagnosed diabetes mellitus, heart disease or gastrointestinal disease, use of medications affecting lipid metabolism or glucose homoeostasis, use of anti-inflammatory medications, use of supplements containing n-3 PUFAs, intentional weight loss and large fluctuation in body weight (> 3 kg) during the preceding 2 months. Seventy-six participants were included in the study and were randomly assigned to the Cod group ($N = 27$), Salmon group ($N = 27$) or the Control group ($N = 22$). The participants were randomised into the different groups by the project manager by drawing lots. All examinations were conducted at the Clinical Research Unit at the Haukeland University Hospital, Bergen, Norway.

To enhance compliance the participants were contacted by phone approximately 1 week prior to baseline and end point visits, during which they were informed of the schedule and procedures for the following visit. Also, a text message was sent 1–3 days before the 8 week visit, as a reminder of how to prepare for the upcoming visit. For any inquires during the trial period, members of the research group could be reached by email or telephone. Compliance was monitored through interviews; after 1, 4 and 8 weeks intervention the participants in the fish-eating groups were asked how many dinners with cod/salmon they had not eaten since last contact, instead of asking how well they had complied, to lower the bar for reporting missing intake. As reward for completing the study, participants were offered a dietary consultation with a student dietician at the last visit and all the results of analyses of blood samples.

The study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures were approved by the Regional Committee for Medical and Health Research Ethics of Western Norway (REC no.: 2011/572). Written informed consent was obtained from all participants.

Health professionals performing blood sampling, and personnel conducting the laboratory analyses, were all blinded to participants' group allocation. All data were analysed anonymously. This trial was registered at clinicaltrials.gov as NCT02350595.

Interventions

The fish was provided as frozen, skin-free and boneless fillet portions of 150 ± 10 g (Lerøy Seafood Group ASA), and pallets of fish were chosen at random from Lerøy's warehouse in Bergen, Norway. The fish fillets were supplied free of charge to the participants, and were distributed at the baseline visit or at any time during the study period, if needed. Participants in the Cod group were instructed to eat five dinners containing 150 g of cod fillet per week, and participants in the Salmon group were instructed to eat five dinners containing 150 g of salmon fillet per week. The participants in the fish-eating groups were told not to exceed a total amount of 750 g of fish/week, not to consume any other types of fish or seafood during the study period, and to otherwise maintain their normal eating habits throughout the study period with the exception of the mandatory intake of 750 g fish per week. The Control group was also instructed to continue their normal eating habits, except to completely avoid fish and seafood intake. Participants in all groups were instructed not to change their physical activity level during the 8-week intervention period. The participants' dietary intake (for the 5 days preceding the baseline visit and the 5 days preceding the endpoint visit, including at least 1 weekend-day in each period) and habitual lifestyle (for the preceding 14 days before both baseline and endpoint visits) were recorded using food record charts and a questionnaire for reporting physical activity, as previously described [21]. The reported energy and macronutrient intakes as well as physical activity were not changed within the groups during the study period [21].

Protocol for study visits

The total study period was 8 weeks, with baseline visits between August 22, 2011 and September 19, 2011. Examinations and samplings at baseline and endpoint visits were conducted in the morning after an overnight fast. The participants were instructed not to eat or drink anything except water, and not to use substances containing nicotine after 22.00 hours the previous day, and to avoid physical exercise and alcohol for 24 h before each sampling day.

Participants' height was measured at the baseline visit, using a wall-mounted stadiometer (Seca 222; Seca). Body weight and body composition were measured in a fasted state using a bioelectrical impedance analysis device (InBody 720; Biospace Co. Ltd) at both baseline and endpoint visits. Fasting blood samples were collected at baseline and endpoint visits, in BD Vacutainer SST II Advance gel tubes (Becton, Dickinson and Company) for isolation of serum. The staff complied with a strict protocol for pre-analytical sample handling to ensure high sample quality. Blood samples were centrifuged after 30 min at room temperature, and were immediately aliquoted and frozen at − 80 °C until analyses. Participants provided morning urine at both visits. Urine samples were immediately aliquoted and frozen (− 80 °C) upon participants' arrival to the hospital.

Analyses in serum and urine

Concentrations of potential biomarkers of fish intake, i.e., trimethylamine *N*-oxide (TMAO), 1-methylhistidine (1-MeHis, π-methylhistidine), 3-methylhistidine (3-MeHis, τ-methylhistidine) and creatine, as well as amino acids and related metabolites were measured in serum and urine using liquid chromatography or gas chromatography combined with tandem mass spectrometry, as previously described [22, 23]. TMAO, 1-MeHis and 3-MeHis were measured by adding ion-pairs for the analytes and isotope-labelled internal standards to the existing assays [22]. Arginine was measured in serum, but could not be quantified in urine. Otherwise the same compounds were analysed in serum and urine. Kynurenine [23] and other metabolites in the kynurenine pathway [24] were analysed in serum by published methods. Picolinic acid and quinolinic acid with corresponding isotope-labelled internal standards were added to the previously published assay [24]. The assay precision for the above methods corresponded to within-day CV of 1.4–8% and between-day CV of 1.6–13%, as described in detail elsewhere [22–24]. All metabolites mentioned above were measured at Bevital AS (Bergen, Norway, http://www.bevital.no).

Urine concentrations of albumin and creatinine were analysed on the Cobas c 111 system (Roche Diagnostics GmbH, Mannheim, Germany) using the CREP2 (Creatinine plus ver.2) and ALBT2 (Tina-quant Albumin Urine Gen.2) kits from Roche Diagnostics. The within-day and between-day CVs were < 2% for albumin and creatinine analyses. Reference values for serum creatinine concentration (45–90 µM for women and 60–105 µM for men) and urine albumin:creatinine ratio (0–2.5 mg albumin/mmol creatinine) were set according to the Laboratory of Clinical Biochemistry at Haukeland University Hospital.

Analyses of TMAO, creatine, 1-MeHis, 3-MeHis, anserine and protein in fish fillets

To mimic the participants' preparation of fish prior to consumption, the cod and salmon fillets (15–20 portions of 150 g each) were baked at 180 °C for 20 min, before they were minced, freeze-dried and ground. Fish fillet powders were extracted three times using boiling water as described by Christman [25], and 1-MeHis, 3-MeHis, creatine and TMAO were quantified as described above [22]. Anserine in fish fillets was quantified by HPLC using the Waters Pico-Tag method [26] (Nofima BioLab, Fyllingsdalen, Norway). The protein content (as Nx6.25) in the fish fillets was

analysed by Skretting ARC Laboratory using the Kjeldahl method [27].

Outcome measurements

The primary outcome of the present study was changes in serum and urine concentrations of TMAO, creatine, 1-MeHis and 3-MeHis after a weekly intake of 750 g fillet from either cod or salmon for 8 weeks. Secondary outcomes were changes in amino acids and amino acid metabolites measured in serum and urine.

Sample size

The sample size calculation for this trial was originally conducted with the aim to investigate the effects of high fish intake on postprandial glucose regulation after a standardised breakfast in participants with overweight or obesity [21]. We estimated that it was necessary to include 76 participants divided into three groups to ensure that a minimum of 20 participants in each group completed the trial with satisfactory compliance, with a power of 80% and α of 0.05. Of these, 65 participants were included in statistical analyses [21]. From three of the participants we did not have a sufficient amount of blood serum left for analyses; thus, serum and urine samples from 62 participants were included for laboratory and statistical analyses in the present study.

In the present study we wanted to investigate if TMAO, creatine, 1-MeHis and 3-MeHis measured in serum and urine from this randomised clinical trial may serve as biomarkers for fish intake in future studies. Since this is the first study to compare concentrations of TMAO, creatine, 1-MeHis and 3-MeHis after dietary interventions containing cod, salmon or no fish, measured in both serum and urine, no data were available on effect estimates and for sample size calculation. However, in our previous studies in obese Zucker fa/fa rats we found a complete separation between cod protein group and control group (milk protein-based feed), as well as between rats fed baked salmon fillet and control group (casein-based feed). This separation was observed for urine concentrations of TMAO, creatine, 1-MeHis and 3-MeHis, and for plasma concentrations of creatine, 1-MeHis and 3-MeHis, but groups were overlapping for plasma concentrations of TMAO [17, 18]. Thus, findings in rat studies suggest that even a relatively small study akin to the present work would have the sufficient power for identifying biomarkers of fish intake in humans.

Statistical analyses

Statistical analyses were conducted using SPSS Statistics 25 (SPSS, Inc., IBM Company). Participants who did not complete the study were excluded from the statistical analyses. For analytes in serum and urine, most data were not normally distributed according to the Shapiro–Wilk test, and nonparametric tests (the Wilcoxon's signed-ranks test) were used to investigate changes within groups. For these nonparametric data, the Kruskal–Wallis test was used to compare values between the three groups at baseline. Changes within the groups were compared using the Kruskal–Wallis test, followed by the Mann–Whitney test whenever between-group differences were detected. All statistical testing within and between the groups were unadjusted. Data were not corrected for multiple testing post hoc, in line with the recommendation by Streiner [28] for tests that are not independent of each other. Data are expressed as medians and 25th, 75th percentiles. Categorical data were compared using the Pearson's χ^2 test. All comparisons were two-sided, and $p < 0.05$ was considered statistically significant.

Results

Participant characteristics

In total, 76 participants were included in the study and attended the first study visit, and 68 participants completed the trial. One participant (a woman in the Salmon group) was excluded from statistical analysis because analyses of postprandial blood glucose revealed she had prediabetes, and two participants (one woman in the Cod group and one man in the Salmon group) were withdrawn from analysis because they did not comply with the protocol. From three of the participants (one man in each of the three experimental groups), we did not have a sufficient amount of blood serum for analyses; therefore these three subjects are excluded from all analyses in the present paper. The flow of participants in the study is presented in Fig. 1. For the present investigation, 62 participants [27 men and 35 women; median age 45.5 (25th, 75th percentile 37.1, 53.9) years and median BMI 32.3 (25th, 75th percentile 29.5, 35.9) kg/m^2] were included in the statistical analyses.

Groups were similar at baseline with regard to gender distribution, age, BMI, percentage body fat and percentage muscle mass (Table 1). After 8 weeks, no changes were seen in any of the groups for BMI, percentage body fat or muscle mass (data not presented). Serum creatinine concentration and urine concentration of albumin (relative to creatinine) were measured as markers of kidney function at baseline. All participants had serum creatinine and urine albumin concentrations within normal range (Table 1), and no changes were seen in these markers for kidney function during the intervention period (data not presented). The food diaries showed that most participants consumed dinners that contained either fish or meat (Cod and Salmon groups) or meat (Control group) every day

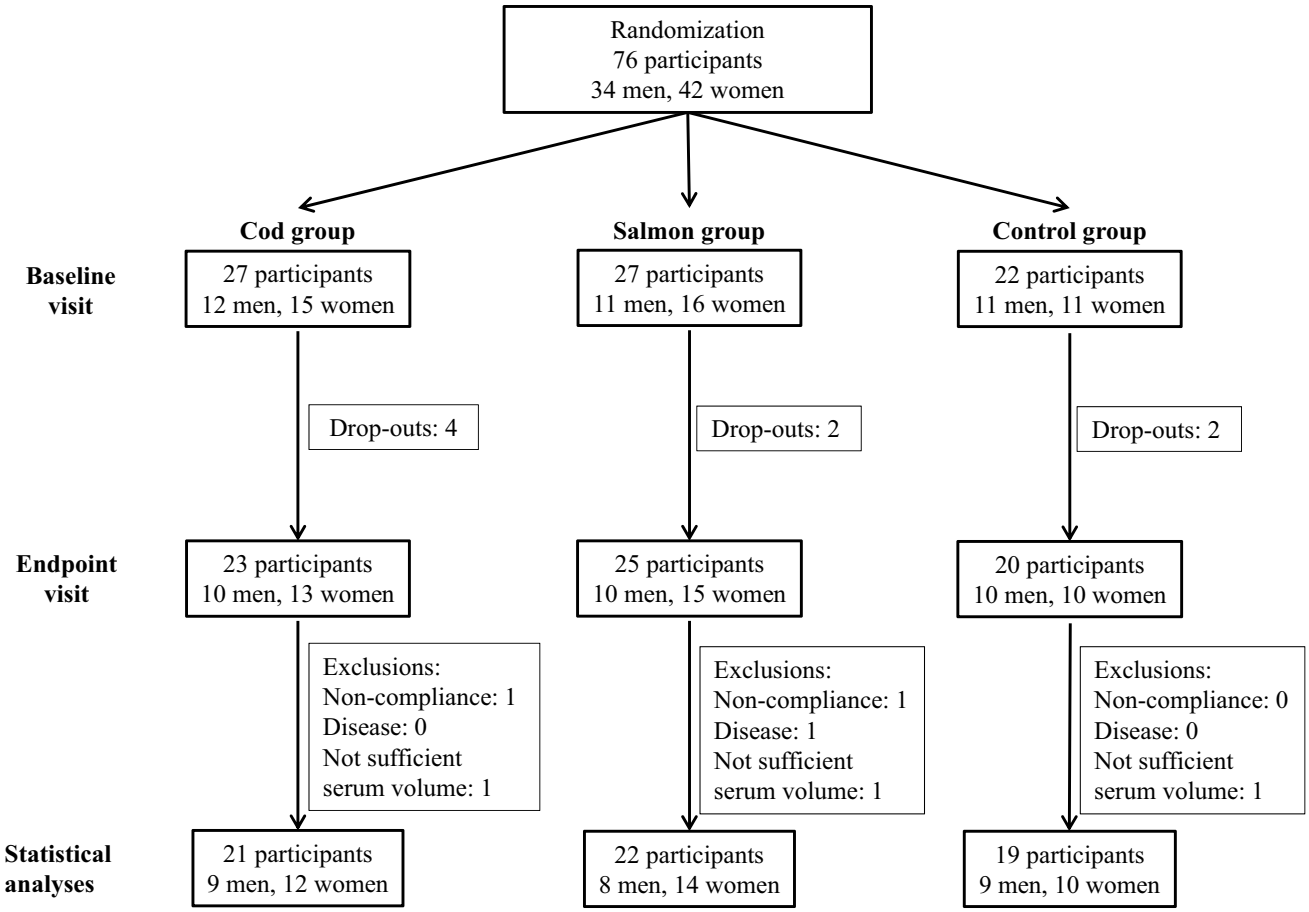

Fig. 1 Flow diagram displaying the progress of participants during the study period. Participants who did not comply with the study protocol were excluded from statistical analysis. Noncompliance was defined as not following the protocol in regard to fish intake, other dietary changes or use of prescription medicine not compatible with the inclusion criteria

Table 1 Participant characteristics at baseline (medians and 25th, 75th percentiles)

	Cod group ($N=21$)		Salmon group ($N=22$)		Control group ($N=19$)		P^a
Men/women	9/12		8/14		9/10		0.77
Age (years)	46.8	37.7,54.2	46.1	43.2,52.6	40.1	31.0,54.4	0.46
BMI (kg/m^2)	30.6	29.1,36.1	32.2	29.9,34.7	33.9	29.1,36.6	0.76
Body fat (%)	39.3	30.6,43.5	40.2	30.1,43.0	39.3	33.1,41.2	0.83
Muscle mass (%)	34.0	32.4,40.9	33.3	31.5,40.0	33.9	32.2,38.2	0.97
Serum creatinine (μmol/L)	71.1	63.6,82.3	77.9	70.8,86.5	73.8	65.5,88.7	0.45
Urine albumin (mg/mmol creatinine)	0.40	0.25,0.75	0.32	0.24,0.62	0.47	0.36,0.58	0.51

[a]Groups were compared at the baseline using the Pearson's χ^2 (categorical data) or the Kruskal–Wallis test (continuous data)

during the 5 days preceding each study visit; only 15 of the 620 reported dinners (2.4%) did not contain meat or fish. None of the participants reported using antibiotics, probiotics or prebiotics at baseline or endpoint, or at any time during the intervention period.

Potential biomarkers of cod and salmon intake in serum and urine

The protein content in cod and salmon fillets were similar; 19.7 (SD 1.0) wt % and 19.5 (SD 1.7) wt %, respectively, as previously published [21]. Table 2 presents the contents of

Table 2 Contents of TMAO, creatine, 1-MeHis + anserine, and 3-MeHis in fillets from cod and salmon

	TMAO	Creatine	1-MeHis + anserine	3-MeHis
Cod fillet (μmol/g protein)	614	248	30	0.19
Salmon fillet (μmol/g protein)	24	357	112	0.11

TMAO trimethylamine *N*-oxide, *1-MeHis* 1-methylhistidine, *3-MeHis* 3-methylhistidine

Means of two measurements; deviations were less than 5% between parallels

TMAO, creatine, 1-MeHis + anserine, and 3-MeHis in cod and salmon fillets (relative to protein content). The TMAO content was much higher in cod compared to salmon, and creatine and 1-MeHis + anserine contents were higher in salmon compared to cod. The content of 3-MeHis was low in both cod and salmon when compared to the other measured compounds, but was higher in cod compared to salmon.

After 8 weeks intervention, concentrations of TMAO in serum and particularly in urine (relative to creatinine) were significantly increased in the Cod group when compared to both the Salmon group and the Control group, but serum and urine TMAO concentrations were not significantly changed in the Salmon group (Tables 3, 4). Serum and urine creatine concentrations were significantly increased in both the Cod group and Salmon group when compared to the Control group, with no differences between Cod and Salmon groups. Concentrations of 1-MeHis in serum and urine were significantly increased in both the Cod group and the Salmon group when compared to the Control group, with a significantly more pronounced increase in the Salmon group compared to the Cod group. Cod and salmon intake did not affect serum and urine concentration of 3-MeHis when compared to the Control group, however, urine 3-MeHis concentration was weakly but significantly increased within the Cod group.

Amino acids and metabolites of tryptophan measured in serum and urine

Cod and salmon intake had little influence on the concentrations of free amino acids in serum and urine after 8 weeks. Serum concentrations of the conditionally essential amino acids arginine and glycine were significantly increased in the Salmon group when compared to

Table 3 Potential markers of fish intake measured in serum at baseline and after 8 weeks (medians and 25th, 75th percentiles)

	Baseline		8 weeks		p^a	p^b	p^c
	Median	25th, 75th percentile	Median	25th, 75th percentile			
TMAO (μmol/L)							
Cod group	3.7	2.8,5.5	6.6	3.9,10.8	0.014	0.010	0.0043^A
Salmon group	6.1	2.5,8.7	6.3	3.7,9.5	0.86		0.35^B
Control group	5.3	3.1,6.7	3.9	2.7,5.1	0.17		0.028^C
Creatine (μmol/L)							
Cod group	48.1	27.1,76.6	53.3	34.1,67.6	0.53	0.0024	0.024^A
Salmon group	39.4	25.0,59.7	57.9	42.7,72.9	0.0035		6.1×10^{-4B}
Control group	47.9	32.6,65.9	42.4	33.4,45.9	0.046		0.25^C
1-MeHis (μmol/L)							
Cod group	3.0	1.6,8.6	6.7	4.2,9.4	0.37	4.5×10^{-7}	0.014^A
Salmon group	3.9	2.1,9.4	20.1	7.8,26.0	8.0×10^{-5}		2.0×10^{-6B}
Control group	4.1	2.0,12.4	2.4	1.3,4.8	0.020		8.7×10^{-5C}
3-MeHis (μmol/L)							
Cod group	4.6	3.4,5.3	4.6	3.8,5.2	0.63	0.40	
Salmon group	4.6	3.9,5.2	4.5	3.8,4.9	0.30		
Control group	4.2	3.7,5.0	4.5	3.6,5.5	0.53		

No differences were seen between the groups at the baseline (Kruskal–Wallis test). Results are presented for 21 participants in the Cod group, 22 participants in the Salmon group and 19 participants in the Control group

TMAO trimethylamine *N*-oxide, *1-MeHis* 1-methylhistidine, *3-MeHis* 3-methylhistidine

[a]Within-group changes are tested using the Wilcoxon's signed-ranks test

[b]Changes within Cod group, Salmon group and Control group are compared using the Kruskal–Wallis test

[c]Changes within the Cod group are compared with the Control group (A), changes within the Salmon group are compared with the Control group (B), changes within the Cod group are compared with the Salmon group (C) using the Mann–Whitney test when the Kruskal–Wallis test showed differences between the groups

Table 4 Potential markers of fish intake measured in urine at baseline and after 8 weeks (medians and 25th, 75th percentiles)

	Baseline		8 weeks		p^a	p^b	p^c
	Median	25th, 75th percentile	Median	25th, 75th percentile			
TMAO (μmol/mmol creatinine)							
Cod group	28.2	20.4,74.1	129.1	70.0,265.2	1.0×10^{-4}	2.6×10^{-5}	2.8×10^{-5A}
Salmon group	63.3	33.5,109.7	80.0	46.9,114.2	0.86		0.31^B
Control group	45.8	29.9,100.0	45.4	26.8,72.5	0.23		2.9×10^{-4C}
Creatine (μmol/mmol creatinine)							
Cod group	6.7	4.5,60.3	12.3	8.2,68.2	0.12	4.2×10^{-5}	8.3×10^{-4A}
Salmon group	9.1	4.7,72.3	25.3	7.9,140.5	5.5×10^{-3}		4.0×10^{-5B}
Control group	10.8	6.5,29.8	7.3	4.3,10.8	4.0×10^{-4}		0.12^C
1-MeHis (μmol/mmol creatinine)							
Cod group	27.3	14.7,82.2	59.8	46.7,82.0	0.30	9.3×10^{-7}	0.016^A
Salmon group	33.4	15.5,102.3	179.2	76.9,222.6	2.3×10^{-4}		4.2×10^{-6B}
Control group	35.5	20.5,108.8	16.7	7.1,49.3	0.024		1.2×10^{-4C}
3-MeHis (μmol/mmol creatinine)							
Cod group	29.4	22.2,34.1	33.0	30.5,36.4	0.033	0.11	
Salmon group	30.6	25.2,37.3	34.7	27.2,40.5	0.10		
Control group	30.0	26.8,38.3	30.7	23.9,32.8	0.72		

No differences were seen between the groups at the baseline (Kruskal–Wallis test). Results are presented for 21 participants in the Cod group, 22 participants in the Salmon group and 19 participants in the Control group

TMAO trimethylamine *N*-oxide, *1-MeHis* 1-methylhistidine, *3-MeHis* 3-methylhistidine

[a]Within-group changes are tested using the Wilcoxon's signed-ranks test

[b]Changes within Cod group, Salmon group and Control group are compared using the Kruskal–Wallis test

[c]Changes within the Cod group are compared with the Control group (A), changes within the Salmon group are compared with the Control group (B), changes within the Cod group are compared with the Salmon group (C) using the Mann–Whitney test when the Kruskal–Wallis test showed differences between the groups

the Control group, but this change was not significantly different from the Cod group (Supplemental Table 1). Serum concentrations of asymmetric dimethylarginine and the indispensable amino acid lysine were significantly increased in the Cod and Salmon groups compared to the Control group, with no differences between the fish-eating groups. In urine, no changes in concentrations of any of the amino acids (relative to creatinine) were observed in any of the groups (Supplemental Table 2).

Serum concentrations of tryptophan and its downstream metabolites in the kynurenine pathway (kynurenine, kynurenic acid, anthranilic acid, 3-hydroxykynurenine, xanthurenic acid, 3-hydroxyanthranilic acid, picolinic acid, quinolinic acid), as well as the kynurenine:tryptophan ratio were not changed in any of the groups (Supplemental Table 1). Urine concentration of kynurenine (relative to creatinine) was significantly increased within the Cod group after 8 weeks, and this change was significant when compared to the Salmon group but not when compared to the Control group (Supplemental Table 2).

Discussion

In the present study, we investigated the effects of high intake of cod and salmon fillets on four potential biomarkers of fish intake, that is, TMAO, creatine, 1-MeHis and 3-MeHis, in serum and urine from adults with overweight/obesity. We also investigated the effects of high intake of cod and salmon fillets on the amounts of free amino acids and amino acid metabolites in serum and urine. To the best of our knowledge, this is the first clinical study to compare the effects of high cod intake, high salmon intake and a fish-free diet on these compounds as possible measures of compliance. The present findings suggest that TMAO measured in serum or urine could be a biomarker of cod intake and 1-MeHis measured in serum or urine could be a biomarker of salmon intake in humans with a varied diet. Our results also demonstrate that a weekly intake of 750 g fillet from either cod or salmon for 8 weeks only marginally affected concentrations of free amino acids and amino acid metabolites in serum and urine in this population.

Reliable biomarkers of dietary intake are valuable for assessing compliance in clinical trials where participants are responsible for administering the intervention themselves.

Fillet from various fish species, including farmed Atlantic salmon and wild Atlantic cod, contain TMAO, creatine, anserine and 3-MeHis [17, 18, 25, 29–35]. More than 95% of orally ingested TMAO is excreted unchanged by the kidneys in humans [36], and creatine is readily absorbed from meat including fish [37]. Around 90% of dietary intake of anserine (a dipeptide of β-alanine and 1-MeHis) is degraded and excreted as 1-MeHis in humans [38], whereas 3-MeHis is formed by methylation of histidine and is released upon breakdown of actin and myosin [39]. These methylhistidines are not reutilised for protein synthesis or metabolised, but are excreted in the urine. It can be anticipated that kidney function will affect the excretion of TMAO, creatine, 1-MeHis and 3-MeHis, and we therefore chose to investigate these biomarkers in a population with healthy kidneys in the present study.

The TMAO content is normally higher in muscle from wild Atlantic cod compared to that of farmed Atlantic salmon. TMAO in cod may be synthesised from trimethylamine (catalysed by trimethylamine oxidase) or obtained from diet (e.g., zooplankton), whereas TMAO in salmon is obtained solely from diet since salmonids lack trimethylamine oxidase [40, 41]. In the present study, we found that the TMAO content (per gramme protein) was markedly higher in cod fillet compared to salmon fillet. In line with this, we found that the increase in TMAO concentrations in serum and particularly in urine were more pronounced after cod intake than after salmon intake. Trimethylamine can be generated by gut microbiota from trimethylglycine (betaine), ergothioneine, dimethylglycine, choline, trimethyllysine and carnitine, amongst others, which are found in common foodstuffs such as seafood, terrestrial meat, egg, dairy products, vegetables, mushrooms, kidneys and liver, before it is transported to liver for oxidation to TMAO [42, 43]. We found no changes in serum and urine concentrations of trimethylglycine, dimethylglycine and trimethyllysine, or in serum choline concentration, from baseline to endpoint within any of the experimental groups, and also no differences between the groups for changes over time. We did not quantify ergothioneine in serum, but since none of the participants reported intake of mushrooms, liver or kidney before or at the end of the intervention period we do not expect any trimethylamine production from ergothioneine. Also, the intake of TMAO from seafood is far higher than the amount of TMAO that can be generated in liver from TMA production from choline or carnitine (or their precursors) by gut bacteria [42, 43]. In some settings, increased serum TMAO concentration has been associated with impaired kidney function due to reduced glomerular filtration, whereas other studies report no causal association of serum TMAO with morbidity or mortality from renal diseases [44]. In the present study, all participants had apparently normal kidney function, based on measurements of serum creatinine and urine albumin, and no changes were seen in these biomarkers during the intervention period. Thus, TMAO seems to be a promising biomarker for cod intake, but not for salmon intake, in this population of adults with overweight/obesity and normal kidney function.

The creatine content was almost 50% higher in fillet from salmon compared to cod, but both serum and urine concentrations of creatine were increased to a similar degree in the Salmon group and in the Cod group when compared to the Control group. Also creatine has the potential to be a biomarker of fish intake, but caution should be exercised since creatine is found in abundant amounts in muscles from both fish and terrestrial animals, and in addition smaller amounts of creatine are found in dairy products [37].

Both 1-MeHis and 3-MeHis are also found in meat of various origins [29–32], and although 1-MeHis is not found in human muscle [25], the use of these compounds as biomarkers of fish intake is challenging in people with a normal varied diet. In the present study, fillets from cod and salmon as 5 weekly dinners each containing 150 g of fish were the major food source of muscle proteins in the fish-eating groups. Based on the food diaries, most of the participants consumed dinners containing either fish or meat (Cod and Salmon groups) or meat (Control group) every day. The Atlantic salmon is a faster swimmer compared to the Atlantic cod, and therefore needs more anserine in skeletal muscle to buffer the higher lactate production [45]. The 1-MeHis + anserine content was almost four times higher in salmon than in cod, and in line with this, the increases in 1-MeHis concentrations in serum and urine were more pronounced in the Salmon group compared to the Cod group. The fourth of the suggested biomarker, 3-MeHis, was found in very low amounts in both cod and salmon fillets (per gramme protein), and neither cod nor salmon intake affected serum and urine concentrations of 3-MeHis in our participants. Based on the present findings, we suggest that 1-MeHis measured in serum or urine could be a biomarker of salmon intake.

In the present study, the concentrations of only a few free amino acids were affected in serum and not at all changed in urine (relative to creatinine), while no tryptophan metabolites were affected in serum after intake of cod or salmon. After 8 weeks intervention the Salmon group had higher serum concentrations of arginine, glycine, lysine and asymmetric dimethylarginine when compared to Control group. Arginine, glycine and lysine are endogenously produced or obtained from the diet. According to the USDA Food Composition Databases [46], the contents of arginine, glycine and lysine are higher in salmon compared to cod, but are lower in these fish species than in red meat. The daily protein intake, the muscle mass (as % of body weight) and the physical activity level were similar in the three experimental groups, and did not change during the course of the

intervention [21]. It is well known the circulating glycine concentration is lower in patients with obesity and/or type 2 diabetes, and the improvement of insulin resistance increases glycine concentration, but the mechanisms behind this are not elucidated [47]. The higher glycine concentration in the Salmon group in the present study fits nicely with the previously reported improved glucose regulation in this group after high salmon intake [21].

The increased serum arginine concentration after salmon intake is in line with findings in obese rats, where the most pronounced difference in amino acids was an almost six times higher concentration of circulating arginine in rats fed salmon compared to rats fed control diet [18], possibly as a consequence of better renal function [48] and thus higher arginine production in proximal tubule compared to controls. Also in these rats, no differences between salmon and control groups were seen for serum or urine concentrations of other amino acids or serum tryptophan metabolites [18]. In contrast to findings in the present study in humans, cod protein feeding in obese rats resulted in higher urine concentrations (relative to creatinine) of all measured amino acids when compared to control group (probably due to better kidney function after cod protein intake), and higher plasma concentration of arginine but similar concentrations of tryptophan metabolites [17].

The present study has some strengths and limitations. The participants had varied diets with a wide range of vegetable and terrestrial animal protein sources combined with high cod or salmon intake or a fish-free diet. Therefore, the findings of higher TMAO, creatine and 1-MeHis after cod or salmon intake are relevant and would strengthen the general applicability of these biomarkers for assessing dietary compliance. On the other hand, a limitation is the generalisability for the present findings, since the fish intake was very high in the Cod and Salmon groups, only two types of fish were used in the intervention diets, and the study population was relatively small and consisted only of adults with overweight/obesity.

To conclude, findings in the present study suggest that in adults with overweight/obesity with normal kidney function, TMAO measured in serum or urine could be a biomarker of cod intake, and that serum and urine 1-MeHis could be a biomarker of salmon intake. Creatine also has the potential to be a biomarker of fish intake, but caution should be exercised since meat from terrestrial animals and dairy products also contain creatine. These biomarkers may be valuable tools for assessing compliance in intervention studies. Further research using additional fish species and a lower weekly intake of fish is needed to evaluate the use of TMAO, creatine and 1-MeHis as biomarkers for assessing compliance for fish intake in general in different populations. Also, more information is needed regarding the kinetics of these biomarkers in humans, i.e., how soon after fish intake can the biomarkers be detected in serum and urine, when do serum concentrations reach a steady-state, how long does it take before these biomarkers have been eliminated from the body after fish intake, and whether other nutrients and physical activity may affect serum and urine concentrations of these biomarkers.

Author contributions HS, GR, GM and OAG formulated the research question and designed the study. IVH, AH, MB and OAG conducted the clinical study. ØM, AM, PMU and OAG analysed the data and performed statistical analyses. OAG drafted the paper and had primary responsibility for the final content. All authors have contributed to the writing and approved the final manuscript. We thank all participants who have contributed to the current study. The kind contribution of fish for the intervention trial by Lerøy Seafood Group ASA (Bergen, Norway) is highly appreciated.

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Compliance with ethical standards

Conflict of interest HS and GR are employed in Skretting Aquaculture Research Centre AS and Lerøy Seafood Group ASA, respectively. Skretting Aquaculture Research Centre AS is a global leader in providing innovative and sustainable nutritional solutions for the aquaculture industry. Lerøy Seafood Group ASA is the leading exporter of seafood from Norway and the world's second largest producer of Atlantic salmon. Skretting Aquaculture Research Centre AS and Lerøy Seafood Group ASA were not involved in on-site data collection. The other authors declare no conflicts of interest.

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