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The relationship between fish intake and urinary trimethylamine-N-oxide

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Keywords: metabolomics, fish intake, dietary biomarkers, trimethylamine-N-oxide

Abbreviations: AUC, area under the curve; CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid; DHA, docosahexaenoic acid; D_2O deuterium oxide; EPA, eicosapentaenoic acid; ^{1}H NMR , ^{1}H nuclear magnetic resonance; HOMA-IR, Homeostatic model assessment-insulin resistance; NANS, National Adult Nutrition Survey; OPLS-DA, orthogonal partial least-squares discriminant analysis; PUFAs, polyunsaturated fatty acids; ppm, parts per million; PCA, principal components analysis; PLS-DA, partial least-squares discriminant analysis; QUICKI, quantitative sensitivity check index; ROC, receiver operating

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characteristic; TMAO, trimethylamine N-oxide; TSP, sodium trimethylsilyl [2,2,3,3-$^{2}H_4$] proprionate.

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Abstract

Scope: Fish intake has been reported to associate with certain health benefits; however accurate assessment of fish intake is still problematic. The objective of this study was to identify fish intake biomarkers and examine relationships with health parameters in a free-living population.

Methods and results: In the NutriTech study, 10 participants were randomised into the fish group and consumed increasing quantities of fish for 3 days/week during 3 weeks. Urine was analyzed by NMR-spectroscopy. Trimethylamine-N-oxide (TMAO), dimethylamine and dimethyl sulfone were identified and displayed significant dose response with intake (P < 0.05). Fish consumption yielded a greater increase in urinary TMAO compared to red meat. Biomarker derived fish intake was calculated in the National Adult Nutrition Survey (NANS) cross sectional study. However, the correlation between fish intake and TMAO (r=0.148, P < 0.01) and between fish intake and calculated fish intake (r=0.142, P < 0.01) were poor. In addition, TMAO showed significantly positive correlation with serum insulin and insulin resistance in males and the relationship was more pronounced for males with high dietary fat intake.

Conclusion: Urinary TMAO displayed strong dose-response relationship with fish intake; however, use of TMAO alone is insufficient to determine fish intake in a free-living population.

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1 Introduction

Fish is important dietary protein source and oily fish is rich in long-chain omega-3 polyunsaturated fatty acids (PUFAs) [1, 2]. In epidemiological studies, greater intake of fish was reported to have associations with decreased risk of diseases such as cancer [3, 4], cerebrovascular diseases [5, 6], heart diseases [7, 8] and diabetes [9, 10]. However, there are also some inconsistent findings in the literature [11-14]. For example, data from Rhee et al. (2017) suggested that tuna and dark fish intake and long-chain omega-3 PUFAs were not associated with risk of major cardiovascular disease in a large prospective cohort study of women [15]. Engeset et al. (2015) reported no association between fish consumption and overall or cause-specific mortality in an European cohort [16]. One of the reasons contributing to the inconsistent results is the difficulty in obtaining accurate dietary exposure data. Traditional dietary assessment methods, for example, FFQs, 24 h dietary recalls, and food records, are based on self-reporting and can be subject to measurement issues including underreporting or recall errors [17-19]. Usually, the presence of dietary measurement errors attenuates the estimate of disease relative risk when analyzing single exposure variables, and also reduces the statistical power of the corresponding significance test, which can result in an important relationship between diet and disease being obscured [18]. To further investigate and strengthen the evidence for the associations between specific food intake and disease risk, there is an increased interest in developing new approaches for improving the accuracy of dietary intake measurement. One such approach is the application of food intake biomarkers. These biomarkers can be used in conjunction with traditional dietary assessment methods therefore offering a more objective measure of dietary intake [17].

Metabolomic approaches have become an important tool in the identification of novel food intake biomarkers. A number of studies previously examined fish intake biomarkers using metabolomics. One of the biomarkers identified in earlier studies is TMAO, and many studies

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reported high levels of TMAO in urine following fish intake [20-22]. However, TMAO excretion was also reported to increase following red meat consumption [23, 24]. Stella et al. (2006) reported that consumption of a diet high in red meat for 15 days increased urinary TMAO in healthy men [24]. It was reported that dietary L-carnitine, a trimethylamine abundant in red meat could lead to formation of the TMAO by intestinal microbiota metabolism [25]. Furthermore, TMAO levels have been linked to cardiometabolic risk factors in some studies and not in other population groups [26-29]. Other metabolites were also suggested as fish intake biomarkers such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [30, 31]. However, it should be noted that DHA and EPA mostly reflect the intake of fatty fish, and n-3 PUFA supplement intake may affect their association with fish intake. 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid (CMPF) was previously identified as a biomarker of fish intake in a controlled trial using LC-MS non-targeted metabolomics approach [32]. In a recent study carnosine, acetylcarnitine, propionylcarnitine, and 2-methylbutyrylcarnitine showed an increase in urine for all types of meat and fish intake, but they appeared to be generic markers of intake for animal foods [33]. Therefore, there is a need to identify biomarkers which demonstrate a clear contrast with other protein-rich foods such as red meat or poultry. The objective of this study was to identify potential biomarkers of fish intake and examine relationships with health parameters in a free-living population. Examination of the biomarkers ability to estimate intake in the free living population and the relationships with health parameters distinguishes this study from previous work.

2 Materials and methods

2.1 Biomarker discovery; the NutriTech food intake study design

The biomarkers of fish intake were discovered using data from the NutriTech food intake study, reported previously [34]. Ethical approval was received from London Brent Ethics Committee (reference number: 12/LO/0139) and the study was registered (NCT01684917).

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In brief, fifty participants were randomly assigned to one of five different treatment groups equally and consumed different sources of protein: steak (red meat group), chicken breast (chicken group), ham (processed meat group), haddock (fish group), and quorn (vegetarian group) for 3 days/week during 3 weeks.

2.2 Dosage information

A standardized breakfast was provided at 8 am and treatment meals were provided at midday (12 pm) and evening (7 pm) for 3 consecutive days per week over 3 weeks. All meals were designed to provide similar intakes of dietary energy and fibre but macronutrient composition varied over the intervention weeks with carbohydrate decreasing from week 1 to week 3 and protein and fat intake increased from week 1 to week 3. Leftovers were measured and recorded where appropriate. Fasting and postprandial urine and blood samples were collected and further analyzed. The present study focused on the fish group, and the total amount of fish (haddock) provided in the lunch and dinner meals increased from week 1 to week 3. The fish meals were designed for females to receive 90, 180, and 370 g/day of haddock respectively and males to receive 87, 250, and 445 g/day respectively. The average actual fish intake was 88, 222 and 412 g/day in week 1, 2 and 3, respectively (**Table 1**).

2.3 Biomarker confirmation; the NANS cross sectional study

Data from the National Adult Nutrition Survey (NANS) cross sectional study was used to confirm fish intake biomarkers. In this study, 1500 participants were asked to record detailed information on the amount and type of all foods, drinks and nutritional supplements consumed over four consecutive days using a 4-d semi-weighed food record. A detailed description of the data collection has been previously published [35]. Ethical approval was obtained from the University College Cork Clinical Research Ethics Committee of the Cork Teaching Hospitals, and recruitment began in May 2008. Anthropometric measurements were taken by the researcher in the participants' homes, including weight, height, waist and hip circumference and measures of body composition. Fasting urine and blood samples,

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from 565 NANS participants, randomly selected from the main NANS database ensuring equal numbers of men and women across the age range, were collected. The biochemical profile analysis including serum triacylglycerol, total cholesterol glucose, insulin and C-peptide has been described elsewhere [36]. For the current study, the food group for fish & fish products was selected and used to confirm fish intake biomarkers, and 565 participants' fasting urinary spectra were used to identify and quantify fish intake biomarkers. Furthermore, the correlations between biomarkers and metabolic health parameters were investigated.

2.4 Urine analysis by ^{1}H NMR spectroscopy

Urine samples from the NutriTech food intake study and the NANS cross sectional study were analyzed by ^{1}H NMR spectroscopy. Samples were prepared by the addition of 250 µL phosphate buffer (0.2 mol KH_2PO_4/L, 0.8 mol K_2HPO_4/L) to 500 µL urine. After centrifugation at 5360 x *g* for 5 min at 4 °C, 10 µL sodium trimethylsilyl [2,2,3,3-$^{2}H_4$] proprionate (TSP) and 50 µL deuterium oxide (D_2O) were added to 540 µL supernatant. Urine spectra were acquired on a 600-MHz Varian NMR spectrometer by using the first increment of a nuclear overhauser enhancement spectroscopy pulse sequence at 25 °C. Spectra were acquired with 16,384 data points and 128 scans. Water suppression was achieved during the relaxation delay (2.5 s) and the mixing time (100 ms). All ^{1}H NMR urine spectra were referenced to TSP at 0.0 parts per million (ppm) and processed manually with the Chenomx NMR Suite (version 7.5, Inc.; Edmonton, Canada) by using a line broadening of 0.2 Hz, followed by phase correction and baseline correction. Data were normalized to the sum of the spectral integral. Spectral regions of varying bin widths were exported, and the region 4.0-6.0 ppm was excluded. Urine metabolites were identified and quantified by Chenomx NMR Suite software. Metabolites were quantified using the Chenomx NMR suite [37].

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2.5 Statistical analysis

Data are presented as mean ± SEM and were analyzed using IBM SPSS Statistics 20.0. Repeated measure ANOVA was performed to identify metabolites exhibiting significant differences across 3 weeks. Independent samples t-test was applied to identify differences in metabolite concentrations between fish consumers and non-consumers. A P value ≤ 0.05 was considered to indicate significance. The correlation analysis between TMAO excretion and fish intake or metabolic health parameters were performed using SPSS.

Multivariate statistical analysis of the sample dataset was carried out using SIMCA-P software (version 13.0.3; Umetrics) to discover fish intake biomarkers. NMR spectral bins were also exported into SIMCA. All datasets were scaled using Pareto scaling. Principal component analysis (PCA) was applied to explore any trends and outliers in the data. Data were further explored using partial least square discriminant analysis (PLS-DA). Subsequently, orthogonal PLS-DA (OPLS-DA) was performed to analyse NMR data, and the S-line plot was used to identify features that discriminated between intervention weeks.

A receiver operating characteristic (ROC) curve was performed using an internet tool - ROC Curve Explorer & Tester (ROCCET) at http://www.metaboanalyst.ca. ROC curve analysis is performed based on 100 cross validation performance and support vector machines multivariate algorithm. It was used to determine whether the biomarkers can discriminate between fish consumers and non-consumers. The classification performance of biomarkers was assessed by the area under the curve (AUC). A rough guide for assessing the utility of a biomarker based on its AUC is as follows: 0.9-1.0 = excellent; 0.8-0.9 = good; 0.7-0.8 = fair; 0.6-0.7 = poor; 0.5-0.6 = fail [38]. Cross classification analysis was performed to assess the agreement between reported and calculated fish intake. Fish intake was cross-classified to estimate the percentage of participants classified by these two quantification measurements into tertiles of ‘exact agreement’, ‘exact agreement + adjacent’, and ‘disagreement’.

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3 Results

3.1 Identification of potential fish intake biomarkers using ^{1}H NMR spectroscopy

In total, 6 men and 4 women with a mean age (± SEM) of 62 ± 1 y and a mean BMI (± SEM) of 29.19 ± 0.6 kg/m^2 were randomly assigned to the fish group and consumed increasing amounts of fish from week 1 to 3. Characteristics of participants are described in **Table 1**. Fasting urine samples for intervention week 1 and 3 were analyzed using multivariate data analysis. The initial PCA model of ^{1}H NMR urine samples showed no outliers and revealed good separation when comparing high fish intake in week 3 with low fish intake in week 1, (R2X: 0.53 and Q2: 0.17). (**Figure 1**). Further discriminating information between week 1 and week 3 was extracted from PLS-DA model and S-line plot generated from OPLS-DA model. The S-line plot showed the urinary metabolite profiles and revealed differences in metabolite levels between low fish intake in week 1 and high fish intake in week 3 (**Supporting Information Figure S1**). Further examination revealed the discriminatory spectral regions corresponded to the following metabolites: TMAO, dimethylamine, dimethyl sulfone, methylsuccinate, 3-hydroxyisovalerate, guanidoacetate, and N-phenylacetylglycine.

These seven metabolites were quantified in fasting urine samples, and only TMAO, dimethylamine, and dimethyl sulfone significantly increased from week 1 to week 3 ($P \leq 0.05$) (**Table 2**). Urinary excretion kinetics of TMAO, dimethylamine and dimethyl sulfone were also investigated (**Figure 2**). The three metabolites-TMAO, dimethylamine and dimethyl sulfone indicated a dose-response association with increasing fish intake, and TMAO displayed a much higher response in terms of concentration compared to dimethylamine and dimethyl sulfone. Considering both the dose-response data and the acute response TMAO was deemed as the most interesting potential biomarker of fish intake.

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To confirm the specificity of biomarkers indicative of fish intake and to investigate response to different animal foods, TMAO, dimethylamine, and dimethyl sulfone were also quantified in the red meat group. **Figure 3** shows the comparison of TMAO, dimethylamine, and dimethyl sulfone concentrations in fasting urine samples in these two protein groups. TMAO concentrations increased following increasing intake of both fish and red meat. However, fasting urinary TMAO excretions following fish consumption were 4.72 and 6.38 times higher compared to red meat consumption in week 2 and 3, respectively. Dimethyl sulfone excretion showed a similar trend as TMAO, but no significant differences across the weeks were found in the red meat group. There was a weak dose response for dimethylamine in the fish group only. Furthermore, the comparison indicated the large difference in concentrations of these three metabolites between fish and red meat groups and the difference was more pronounced for TMAO.

3.3 Examination of TMAO in the NANS cross sectional study.

To examine the suitability of TMAO as a fish intake biomarker, TMAO was quantified in fasting urine samples from the NANS cross sectional study. Characteristics of the participants are described in **Supporting Information Table S1**. Fifty participants with the highest fish intake (58-258 g/d) were selected and classified as fish consumers, and another fifty participants who had no reported fish intake (0 g/d) were classified as non-consumers. Examination of TMAO levels in fasting urine samples demonstrated that fish consumers (1.03 ± 0.19 mmol/L) had significantly higher concentrations compared to non-consumers (0.36 ± 0.04 mmol/L) ($P < 0.05$). ROC analysis was performed to evaluate the classification performance of TMAO between fish consumers and non-consumers. The AUC value was 0.81 and the specificity and sensitivity were 0.70 and 0.82, respectively, which indicated that TMAO displayed good classification performance for the extremes of fish consumption (**Supporting Information Figure S2**). The association between TMAO and fish intake was further assessed using Spearman's correlation coefficient (**Table 3**). The spearman correlation was 0.148 ($P < 0.01$) between TMAO and fish intake and 0.158 ($P < 0.01$)

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between TMAO and total fish and red meat intake. In female participants, TMAO had a higher correlation with fish intake (r=0.191, $P < 0.01$) compared to males (r=0.128, $P < 0.05$) despite similar levels of fish intake. Furthermore, the relationship between TMAO and different types of fish was examined with the highest observed with the *Gadidae* family (r=0.265, $P < 0.01$) (**Table 3**). Using the data from the intervention study a linear calibration curve was built to relate urinary TMAO concentrations and fish intake, (**Supporting Information Figure S3**). This calibration curve was used to calculate fish intake for 565 NANS participants using their urinary TMAO levels. A correlation of 0.142 ($P < 0.05$) was found between reported fish intake and calculated fish intake.

In cross classification analysis, reported and calculated fish intakes for 565 participants were divided into tertiles. The 'exact agreement', exact agreement +adjacent', and 'disagreement' were calculated. A total of 30.08% participants displayed 'exact agreement'; considering consumers only this increased to 37.50% (**Table 4**).

3.4 TMAO correlates with metabolic health parameters

Examining the relationship between TMAO and metabolic health parameters revealed a number of interesting correlations (**Table 5** and **6**). In male participants, TMAO was significantly correlated with serum insulin, serum C-peptide, and HOMA-IR. To further investigate these correlations, male participants were classified into tertiles based on dietary fat intake. Interestingly, in the background of high fat intake stronger correlations were found: TMAO was positively correlated with insulin (r=0.358, $P < 0.01$), c-peptide (r=0.423, $P < 0.01$) and HOMA-IR (r=0.357, $P < 0.01$).

4 Discussion

In the present study, TMAO, dimethylamine and dimethyl sulfone were identified as metabolites indicative of fish intake. Further examination of the potential biomarkers

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revealed that TMAO displayed a stronger dose response and a calibration equation was developed capturing the relationship between urinary concentration and fish intake . Although urinary TMAO levels were able to distinguish high fish consumers from non-consumers in a free-living population, they performed poorly in estimating actual intake. Our data clearly demonstrates the importance of considering fish intake when assessing TMAO levels; however, it also supports the role of multiple factors contributing to TMAO levels. At the same time the strong correlation between TMAO and metabolic health parameters related to insulin resistance indicate the potential importance of TMAO levels on health. Furthermore, our data highlight the importance of considering sex as a biological variable: the relationships with health parameters were only present in male participants.

A significant increase in urinary TMAO concentration was observed following fish intake, and the present results confirm those of previous dietary intervention or observational studies where high TMAO excretion in urine was associated with fish intake [20, 22, 33, 39]. For example, in an early study by Svensson et al. (1994), urinary TMAO was used as an indicator of dietary fish exposure, and its concentration was positively correlated with weekly fish intake [39]. In the present study TMAO concentration increased with the increase in fish intake allowing a linear relationship to be established. This relationship was used to estimate intake in the free living population. High urinary TMAO concentrations were also observed after the consumption of different fish species including lean and fatty fish [40]. Furthermore, high levels of TMAO were found in various fish species [41-43], supporting the contribution of fish intake to TMAO urinary levels. It is important to acknowledge that there are other dietary factors that can contribute to TMAO levels. For example TMAO can be formed from other nutrients including choline (abundant in eggs) and carnitine (abundant in beef) [44], which may explain the TMAO excretions after red meat (beef) intake. The substrates choline and L-carnitine produce trimethylamine (TMA) by gut microbiota in the intestine and the gut microbiota-derived TMA is subsequently converted into TMAO in the liver [6, 45]. In our study, fish consumption resulted in much higher TMAO concentrations compared to red

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meat consumption, which is consistent with previous results by Cho et al. (2017). In a crossover feeding trial in healthy young men (n = 40) meals containing TMAO (fish), its dietary precursors, choline (eggs) and carnitine (beef), and a fruit control were consumed. Fish yielded higher circulating and urinary concentrations of TMAO (46–62 times; $P < 0.0001$), compared to eggs, beef, or the fruit control [44]. One potential reason for the higher response may be due to the fact that TMAO is actually present in fish. Furthermore, the precursors of TMAO present in animal products are poorly accessible for conversion to TMAO [40].

In the NANS cross sectional study, urinary TMAO was capable of classifying individuals as high fish consumers and non-consumers. However, the overall correlations between TMAO and fish intake were low. These results question the suitability of using only ROC analysis for assessment of food intake biomarkers. A number of factors could influence the TMAO levels including enhanced intestinal production of TMAO and contribution of different foods to TMAO levels. Although TMAO levels have been reported in fish it is important to acknowledge that the levels vary across species with much lower quantities reported in the tissue of freshwater fish [43, 46]. Generally, fish belonging to the *Gadidae* family such as cod and haddock contain high amounts of TMAO. Pelagic fish like sardines, tunas and mackerels have a lower TMAO content [47]. Factors such as fish storage conditions, fish dimension, feeding quality and fishing zone could also influence the endogenous TMAO contents in fish products [41-42], and subsequently contribute to the variation in TMAO excretion after fish intake. This variability in TMAO content across fish species and fish preparations will contribute to a lower correlation to total fish intake. The literature also supports that red meat intake could also contribute to the TMAO levels and consequently the disagreement between biomarker derived and reported fish. In a randomized crossover study, the effect of chronic ingestion of red meat, white meat, or non-meat protein on TMAO metabolism was examined, and red meat but not white meat or non-meat intake, significantly increased plasma and urine TMAO levels [48]. Another potential factor that needs to be

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taken into account is the gut microbiota with studies reporting that high-TMAO producers were characterized by enriched ratios of Firmicutes to Bacteroidetes compared to low-TMAO producers [44]. Furthermore, a study of human intestinal isolates demonstrated that certain bacterial species were capable of production of TMA, the TMAO precursor [49]. Overall, while the results from our acute intervention indicate that TMAO increases with increasing fish consumption caution is needed when interpreting TMAO levels. The results from our cross-sectional data indicate that multiple factors are likely to influence the urinary levels. Overall, we conclude that TMAO alone had a poor ability to estimate fish intake in a free-living population despite showing significant dose-response relationships in a controlled intervention study.

The literature surrounding the metabolic effects of TMAO is controversial. Several studies report TMAO to be associated with health outcomes such as cardiovascular disease and diabetes, and high TMAO levels were found to be a strong marker of all cardiovascular events [50, 51]. However, other studies have found no relationships between TMAO and cardiovascular events, for example, in the PREDIMED (Prevention With Mediterranean Diet) Study no positive significant associations were found between plasma concentrations of TMAO and the risk of cardiovascular disease in individuals at high cardiovascular risk after 4.8 years of follow-up [52]. It is worth noting that recent studies have demonstrated strong associations between systemic TMAO levels and diabetes risk factors. For example, diabetic mice had significantly increased insulin resistance and TMAO concentrations in comparison to non-diabetic controls in an animal study [51]. Furthermore, in a high-fat-diet mouse model, male mice were randomly assigned to the control, high fat, and high fat with TMAO groups. After 3 weeks intervention, dietary TMAO significantly increased fasting insulin levels and HOMA-IR in mice fed the high fat diet. The results suggested that dietary TMAO can exacerbate impaired glucose tolerance, obstruct the hepatic insulin signalling pathway, and cause adipose tissue inflammation [53]. In line with these findings, in our cross-sectional study, a strong correlation between insulin resistance and TMAO was observed in males; the

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results were even stronger for males with habitual high fat intake. However, the mechanism for the sex-specific nature of this relationship remains unclear. It is also worth noting that there are some studies where TMAO levels were associated with positive health parameters indicating the complexity involved in interpreting a single biomarker. A recent study reported that TMAO improved insulin secretion and restored glucose tolerance in an isogenic mouse population model during a high fat diet intervention [54]. Interpreting the data from the present study in conjunction with the literature indicates the need to consider multiple factors when interpreting TMAO levels; in the current context participants with higher habitual fat intake had a stronger relationship between TMAO and markers of insulin resistance.

Other biomarkers of interest were identified in the acute intervention study, however further examination of these revealed poor dose response or lack of specificity. Despite this, it is worth considering these further as they may play a role in future panels of biomarkers related to fish intake. Dimethylamine, which is the most abundant of the short-chain aliphatic amines found in human and animal urine [55], can also be found in fish products [43], and the increment of dimethylamine content follows the demethylation of TMAO, which may occur during frozen storage of fish [56]. In a randomized crossover intervention study where participants were randomly assigned to lean-seafood or non-seafood diet groups, urinary TMAO and dimethylamine concentrations significantly increased after lean-seafood intake [57]. Dimethyl sulfone was also identified as an interesting metabolite of fish intake with increasing dimethyl sulfone observed with increasing fish intake. Dimethyl sulfone is a naturally occurring organic sulfur compound commonly found in variety of fruits, vegetables, grains, milk and cooked beef [58-60]. Other studies have identified some metabolites associated with fish consumption. For instance, the concentrations of long-chain n-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in plasma or serum phospholipid were significantly higher after fish intake [30, 31]. They are mostly used to reflect the intake of fatty fish; furthermore, their association with fish intake can be weakened by the widespread use of n-3 PUFA supplements. Other metabolites including

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creatine, proline, arsenobetaine, 1- and 3-methylhistidines, 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid, phenyalanine, taurine, and docosahexaenoic acid were also found an increment in plasma after cod ingestion [61]. However, these biomarkers were not specific to fish intake. A separate study identified plasma furan fatty acid [3-carboxy-4-methyl-5-propyl-2-furanpropionic acid (CMPF)] as a specific biomarker of fatty fish intake based on LC-MS non-targeted metabolomics approach [32]. For the advancement of quantifying fish intake, it may be possible to combine these potential plasma/urinary biomarkers, for example, combining EPA/DHA to capture oily fish intake with TMAO . Future work should examine this possibility.

The present study has a number of strengths. The detailed analysis of TMAO in different study settings allowed us decipher it had a dose response but also had certain limitations. The combination of an acute feeding study and a cross-sectional study allowed us to examine TMAO levels under different conditions. The ability to stratify according to sex allowed us to identify sex specific relationships. The use of one type of fish in the intervention study(rather than a variety of fish) may have limited our ability to estimate overall fish intake in the free-living population. Lack of microbiome data meant that we could not examine the links between the microbiome and TMAO. Considering the poor agreement with self-reported we have to acknowledge that fish is consumed episodically and there is a chance that both the biomarker data and the self-reported data overestimate intake. However, by assessing intake over 4 days we mitigate this to a certain extent.

In conclusion, the identification of biomarkers in an acute scenario is feasible and gives good potential candidates. However, confirmation of these biomarkers in free-living populations is not trivial. The present example of TMAO indicates that many factors influence the urinary levels making it difficult to use it alone as a marker for fish intake. Likewise, the use of TMAO as a marker for cardiovascular risk should be cautioned; inclusion of dietary contributions including fish intake are essential in any study using TMAO as a marker. In the present population, TMAO had significant positive correlations with insulin resistance parameters for

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males; the relationships were strengthened in the background of a high fat diet indicating the complexity surrounding the interpretation of TMAO levels. Further work is necessary to examine the potential of TMAO in combination with other biomarkers as signatures for fish intake, and importantly, to investigate the association between TMAO and health outcomes.

Author contributions

X.Y., H.G. and LB conducted the research and analyzed NMR data; X.Y. and L.B. analyzed data and wrote the manuscript. M.R. and G.F. provided essential materials in the NutriTech project; B.A.M., A.P.N., J.W., A.F. provided essential materials in the NANS cross sectional study. All authors read and approved the final manuscript.

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Conflict of interest statement

The authors have declared no conflict of interest.

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Table 1 Demographic characteristics of participants and intakes of fish and red meat in the NutriTech Food Intake Study[1].

	Intervention group	
	Fish group	**Red meat group**
n	10	10
Sex, n	4 (F), 6 (M)	5 (F), 5 (M)
Age, y	62 ± 1	58 ± 1
BMI, kg/m^2	29.19 ± 0.6	30.95 ± 1.0
Food intake (g/day)		
Week 1	88 ± 0.5	80 ± 4.1
Week 2	222 ± 11.5	158 ± 11.1
Week 3	412 ± 13.2	283 ± 14.6

[1]Values are presented as mean ± SEM.

Table 2 Metabolites in fasting urine samples across three weeks[1].

mmol/L	**Week 1**	**Week 2**	**Week 3**	***P* value[2]**
Methylsuccinate	0.10 ± 0.02	0.09 ± 0.01	0.11 ± 0.02	0.804
3-Hydroxyisovalerate	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.450
Dimethylamine	0.56 ± 0.09	0.85 ± 0.09	0.90 ± 0.12	0.039
Dimethyl sulfone	0.06 ± 0.01	0.08 ± 0.01	0.10 ± 0.01	0.040
TMAO	1.12 ±0.12	2.50 ± 0.23	3.80 ± 0.33	<0.001
Guanidoacetate	0.44 ± 0.08	0.68 ± 0.15	0.74 ± 0.21	0.152
N-phenylacetylglycine	0.40 ± 0.07	0.45 ± 0.05	0.46 ± 0.07	0.701

[1]Values are presented as mean ± SEM.
[2]Based on repeated measures ANOVA and $P < 0.05$ means urinary metabolites significantly increased from week 1 to week 3 in fish group.

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Table 3 Spearman's correlation between TMAO concentrations (mmol/L) and fish intake/total fish and red meat intake (g/day).

	Spearman's correlation coefficient (r)
Fish intake[1]	0.148**
Fish intake (females)[2]	0.191**
Fish intake (males)[3]	0.128*
Total fish and red meat intake[4]	0.158**
Fish intake only for fish consumers[5]	0.166**
Gadidae family fish intake[6]	0.265**
Other fish intake[7]	0.113

[1] Fish intake for all participants based on food record (n=565)
[2] Fish intake (22.23 ± 1.69 g/day) for all females based on food record (n=281), and average TMAO concentration of 0.53 ± 0.03 mmol/L.
[3] Fish intake (25.56 ± 2.29 g/day) for all males based on food record (n=284), and average TMAO concertation of 0.70 ± 0.04 mmol/L.
[4] Total fish and red meat intake for all NANS participants (n=565)
[5] Fish intake only for consumers (n=312)
[6] *Gadidae* family fish intake which included cod, haddock or hake intake (n=139), and the average intake was 15.50 g/day.
[7] Other fish intake which mainly included salmon and tuna, and also included sardine, herring, trout, or mackerel intake (n=183), and the average intake was 24.68 g/day.
** Correlation is significant at the 0.01 level (2-tailed).
*Correlation is significant at the 0.05 level (2-tailed).

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Table 4 Cross classification by tertiles of reported and calculated fish intakes in the NANS cross sectional study.

	Exact agreement (%)[1]	Exact agreement + adjacent (%)[2]	Disagreement (%)[3]
All participants (n=565)	30.08	80.35	19.65
Fish consumers (n=312)	37.50	83.65	16.35

[1] % of participants cross-classified into the same tertile of intake.
[2] % of participants cross-classified into the same or adjacent tertile of intake.
[3] % of participants cross-classified into 1 tertile apart.

Table 5 Pearson correlation between urinary TMAO levels and metabolic health parameters for male and female participants in the NANS cross sectional study.

Metabolic Health Parameters	Males (n=284)	Females (n=281)
Serum glucose (mmol/l)	0.081	0.022
Serum insulin(μIU/ml)	0.140*	-0.015
Serum C-peptide (ng/ml)	0.193**	-0.019
QUICKI	-0.151*	0.027
HOMA-IR	0.122*	0.005
Serum triglyceride(mmol/l)	0.052	-0.012
Serum total cholesterol (mmol/l)	0.016	0.089
Systolic blood pressure	0.004	0.110
Diastolic blood pressure	0.075	0.022

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).
HOMA-IR: Homeostatic model assessment-insulin resistance; QUICK: Quantitative sensitivity check index.

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Table 6 Pearson correlation between urinary TMAO levels and metabolic health parameters for male participants.

Metabolic Health Parameter	Low Fat intake[1] (n=95)	High Fat intake[2] (n=94)
Serum glucose (mmol/l)	-0.078	0.188
Serum insulin(μIU/ml)	-0.031	0.358**
Serum C-Peptide (ng/ml)	-0.043	0.423**
QUICKI	-0.026	-0.288**
HOMA-IR	-0.008	0.357**
Serum triglyceride(mmol/l)	0.017	0.031
Serum total cholesterol (mmol/l)	0.067	0.040
Systolic blood pressure	0.053	0.016
Diastolic blood pressure	0.123	0.055

[1] Low fat intake: contribution to daily energy intake lower than <30.85%.
[2] High fat intake: contribution to daily energy intake higher than >35.59%.
** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).
HOMA-IR: Homeostatic model assessment-insulin resistance; QUICK: Quantitative sensitivity check index.

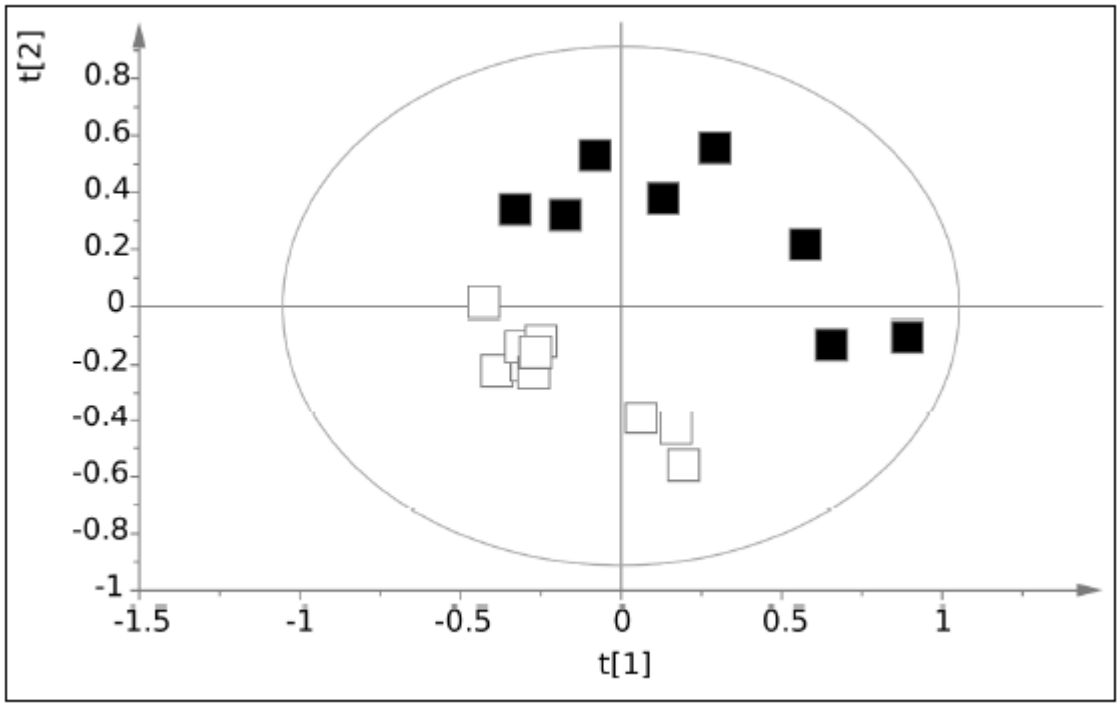
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Figure captions

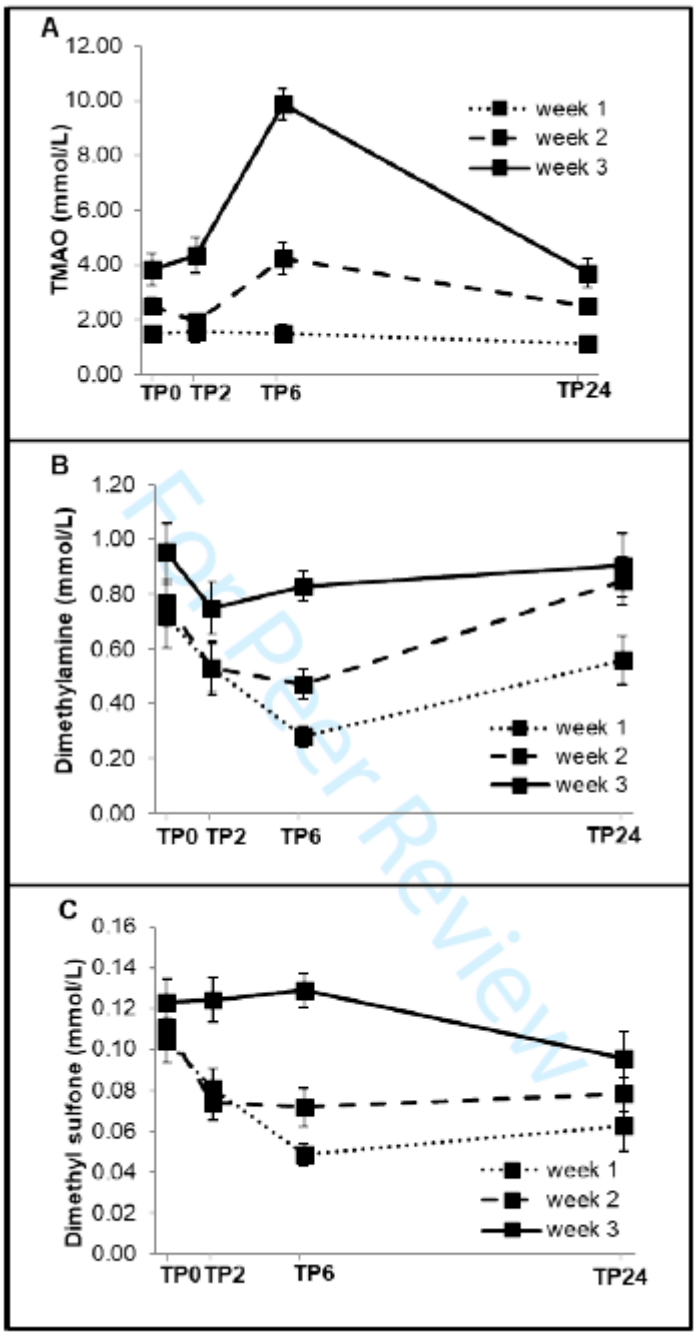
Figure 1 PCA model of fasting urine samples in week 1 (□) and week 3 (■) in the fish group. Week 1, low fish intake (88 g/day); Week 3, high fish intake (412 g/day). R2X=0.53 Q2=0.17. t[1], principal component 1; t[2], principal component 2.



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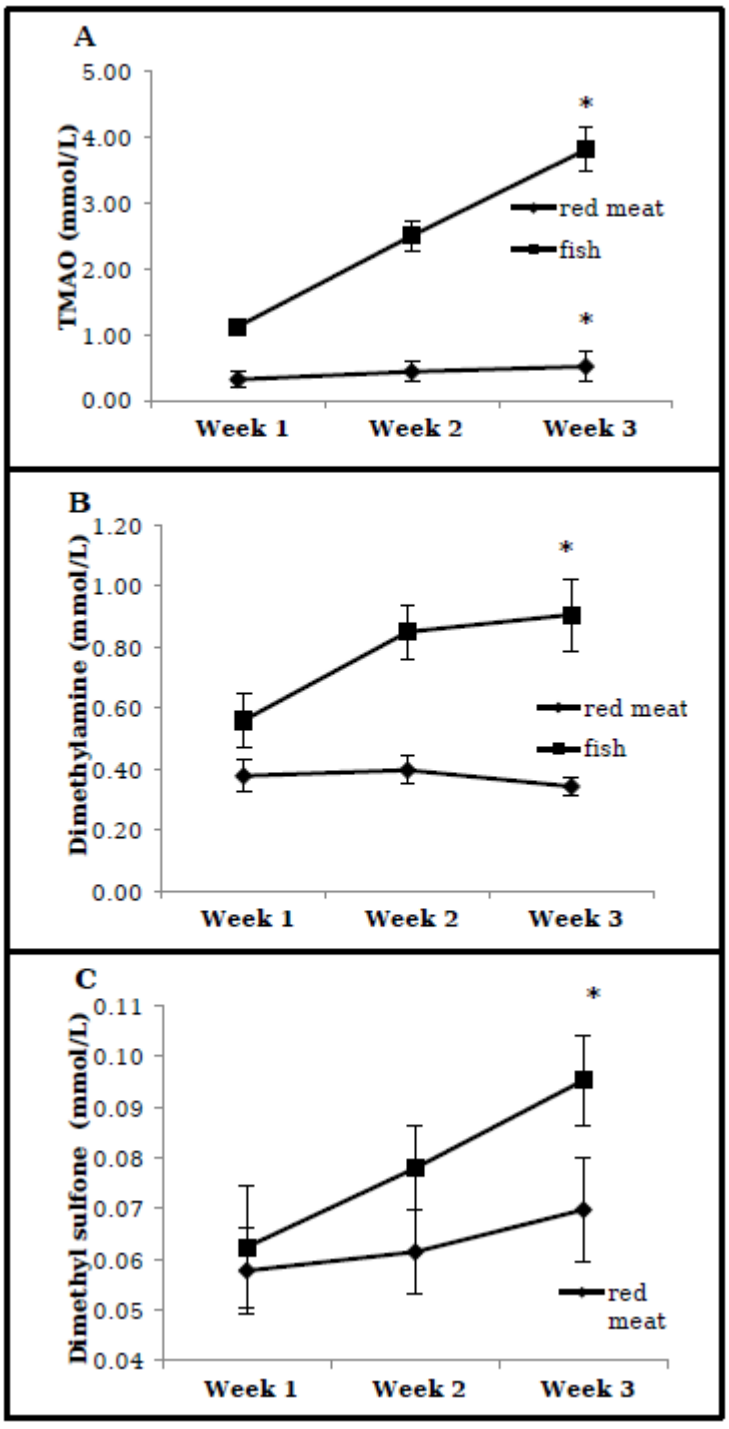
Figure 2 Urinary excretion kinetics of TMAO, dimethylamine and dimethyl sulfone. A: TMAO; B: Dimethylamine, C: Dimethyl sulfone. TP 0 h (void immediately before the midday meal at 11.55 am on day 3 of week1, 2, and 3), TP2 h (spot sample 2 hours after the midday meal on day 3 of week1, 2, and 3), TP6 h (spot sample 6 hours after the midday meal on day 3 of week1, 2, and 3), and TP24 h (day 4 fasting sample in week1, 2, and 3). TP, time point.



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Figure 3 Changes in fasting urinary metabolites in the fish and red meat groups. A: TMAO; B: Dimethylamine; C: Dimethyl sulfone.



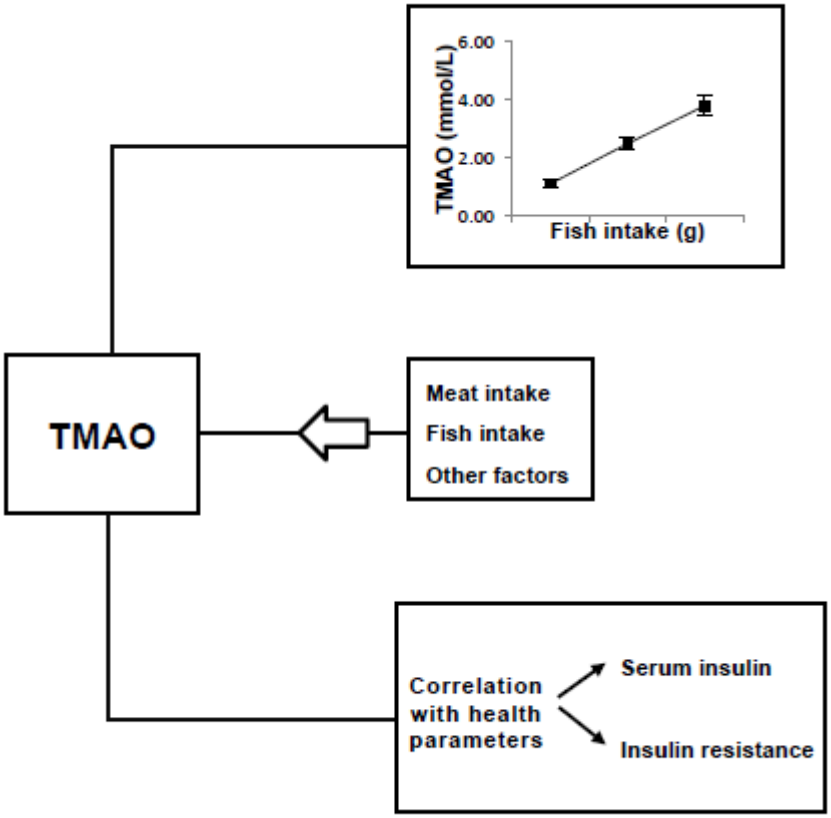
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Graphical Abstract

In a controlled intervention study we demonstrate a linear relationship between fish intake and urinary TMAO levels. However, in a cross-sectional study our results indicate that other factors also contribute to TMAO levels. Caution is needed when interpreting TMAO levels and contribution of dietary fish needs to be considered.



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