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Original Research

Baked cod consumption delayed the development of kidney and liver dysfunction and affected plasma amino acid concentrations, but did not affect blood pressure, blood glucose or liver triacylglycerol concentrations in obese fa/fa Zucker rats.☆

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ABSTRACT

Obesity is associated with changes in amino acid metabolism, and studies show that ingestion of fish proteins influence amino acid composition in plasma and urine, in addition to affecting risk factors for metabolic syndrome. Since the majority of fish proteins consumed by humans are as fish fillet, it is of interest to investigate if cod fillet intake affects amino acid composition and metabolic disorders. We hypothesized that a modified AIN-93G diet containing cod fillet would affect amino acid compositions in plasma and urine in obese rats, and also affect risk factors for metabolic syndrome when compared to rats fed a regular AIN-93G diet with casein as the protein source. Obese Zucker fa/fa rats, a rat model of metabolic syndrome, received diets containing 25% protein from lyophilized baked cod fillet and 75% protein from casein (Baked cod diet), or a Control diet with casein for four weeks. The Baked cod diet affected the amino acid composition in plasma, with e.g., lower glycine, histidine, homoarginine, homocysteine, methionine, proline and tyrosine concentrations, but did not affect amino acid concentrations in urine. The concentrations of markers for kidney and liver dysfunction were lower in the Baked cod group, however blood pressure development,

☆ *Abbreviations:* ELISA,enzyme-linked immunosorbent assay; HDL, high density lipoprotein; LDL,low density lipoprotein; TIM-1, T cell Immunoglobulin Mucin-1; TMAO, trimethylamine N-oxide

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fasting and postprandial glucose, and hepatic triacylglycerol concentrations were similar to the Control group. To conclude, substituting 25% of dietary protein with baked cod fillet affected concentrations of some amino acids in plasma and delayed development of kidney and liver dysfunction, but did not affect blood pressure, glucose concentration or fatty liver.

1. Introduction

The prevalence of obesity continues to rise globally, and this is of major concern since obesity is strongly associated with comorbidities such as hypertension, hypercholesterolemia, hyperlipidemia, fatty liver, type 2 diabetes, renal disease, and cardiovascular diseases [1-4]. The criteria for metabolic syndrome include abdominal adiposity, reduced high density lipoprotein (HDL) cholesterol, elevated blood pressure and elevated fasting blood glucose. In addition, several factors such as atherogenic dyslipidemia (elevated low density lipoprotein (LDL) cholesterol, reduced HDL cholesterol), elevated liver fat, fasting insulin, glucose after oral glucose tolerance test, and chronic renal disease are listed as important targets for future research on metabolic syndrome [5]. Fish consumption has been associated with reduced risk of coronary heart disease and heart failure [6-9] and prevalence of type 2 diabetes and metabolic syndrome are low in populations with high fish intake [10-13]. Of these, only Karlsson *et al.* [13] distinguished between the effects of intake of lean and fatty fish. Thus, more research is needed to identify metabolic effects of consumption of lean and fatty fish separately.

The kidneys and the liver are central organs in the regulation of amino acid metabolism, and dysfunctions in these organs may affect the concentrations of amino acids in circulation and in urine. Circulating concentrations of amino acids have been shown to be different in patients with obesity, metabolic syndrome and/or insulin resistance when compared to healthy persons. Specifically, elevated concentrations of branched-chain amino acids (isoleucine, leucine, valine), and reduced glycine (aliphatic) concentration are markers for prediabetes, insulin resistance, and future type 2 diabetes in humans [14,15]. Associations of branched chain amino acids and aromatic amino acids (phenylalanine, tryptophan, tyrosine) with cardiovascular disease and obesity have also been demonstrated [15], and evidence suggests that the kynurenine pathway of tryptophan degradation is upregulated in obesity [16].

The effects of fish intake on amino acids concentrations have not been extensively explored, however a few clinical studies have shown that intake of fish may affect the concentrations of amino acid and their metabolites in circulation and in urine. Fish intake was not a strong determinant of the plasma concentrations of tryptophan metabolites in the kynurenine pathway in patients with coronary artery disease, however lean fish intake had some influence on the kynurenine pathway in a subgroup of patients that also presented diabetes [17]. In non-diabetic adults with overweight or obesity, serum concentrations of asymmetric dimethylarginine and lysine were significantly increased after cod intake [18], but did not affect serum concentrations of kynurenines, however urine concentration of kynurenine (relative to creatinine) was increased [18]. The cytokine interferon-γ stimulate both the conversion of tryptophan to kynurenines and the biosynthesis of neopterin, and the lower neopterin concentrations observed in humans after intake of lean fish [17,19] suggests that the kynurenine metabolism pathway may be affected by consumption of lean fish such as cod.

The obese Zucker fa/fa rat is a widely used model of genetic obesity and presents visible obesity already 3-4 weeks after birth [20]. This rat spontaneously develops abnormalities resembling human metabolic syndrome such as dyslipidemia, insulin resistance, mild glucose intolerance and hyperinsulinemia [20]. In addition, the obese Zucker rat develops proteinuria [21] and high blood pressure [22,23] before the age of 10 weeks, with indications of decreased renal function from around 12 weeks of age [24].

We recently presented findings that inclusion of baked fillet from Atlantic cod (*Gadus morhua*) as part of a regular diet resulted in lower serum cholesterol concentrations in obese Zucker fa/fa rats, most likely through down-regulation of endogenous hepatic cholesterol synthesis [25]. Little is known about the effects of cod fillet consumption on amino acid composition, kidney function, vitamin status, blood pressure, and concentrations of blood glucose and liver triacylglycerol. The main objective of the present study was to investigate the effects of baked cod fillet intake on concentrations of amino acids and related metabolites in plasma and urine, with the secondary aims to investigate effects on kidney and liver function, vitamin status, the development of high blood pressure, blood glucose and liver triacylglycerol concentrations in obese Zucker fa/fa rats. We hypothesized that cod fillet intake would affect amino acid concentrations in plasma and urine in this obese rat model. To test this hypothesis, obese Zucker fa/fa rats were fed modified AIN-93G diets with 25% of proteins from baked cod fillet and 75% of protein from casein (the Baked cod group), or with casein as the sole protein source (the Control group) for 4 weeks. Amino acids were quantified in plasma and urine, and kidney and liver function markers, blood pressure and concentrations of blood glucose and liver triacylglycerols were assessed.

2. Methods and materials

2.1. Animals and diets

Twenty male Zucker fa/fa rats (HsdHlr:ZUCKER-Leprfa, from Harlan Laboratories, Indianapolis, IN, USA) were housed in

Table 1 – Composition of the experimental diets

g/kg diet	Baked cod diet	Control diet
Casein[1]	162.16	216.22
Freeze dried baked cod[2]	62.50	-
Corn starch	503.22	511.67
Sucrose	90.00	90.00
Cellulose	50.00	50.00
Soybean Oil	70.00	70.00
t-Butylhydroquinone	0.014	0.014
Mineral Mix (AIN-93-MX)	35.00	35.00
Vitamin Mix (AIN-93-VX)	10.00	10.00
L-Methionine	1.60	1.60
L-Cystine	3.00	3.00
Choline Bitartrate[3]	2.50	2.50
Growth and Maintenance Supplement[4]	10.00	10.00

[1] Contains 92.5 % crude protein
[2] Contains 80 % crude protein
[3] Contains 41 % choline
[4] Contains vitamin B12 (40 mg/kg) and vitamin K1 (25 mg/kg) mixed with sucrose (995 g/kg) and dextrose (5 g/kg)

Table 2 – Amino acids in the diets

g/kg diet	Baked cod diet	Control diet
Alanine	6.4	5.2
Arginine	7.1	6.4
Aspartic acid + asparagine	14	12
Glutamic acid + glutamine	36	38
Glycine	4.2	3.6
Histidine	4.8	5.1
Isoleucine	8.9	9.2
Leucine	16	16
Lysine	14	14
Methionine	6.8	6.4
Phenylalanine	8.8	9.1
Proline	17	19
Serine	9.6	9.9
Taurine	ND	0.3
Threonine	7.3	7.2
Tryptophan	2.1	2.5
Tyrosine	7.4	7.8
Valine	12	12

Values are means of two measurements, deviation <5% between parallels
ND; not detected

pairs in Makrolon IV cages, and were kept at a 12 h light/dark cycle at 20–23°C. After acclimatization under these conditions for a minimum of seven days, the rats were randomly assigned the two experimental groups with comparable mean body weight. The intervention started when the rats' bodyweight were 355 ± 10 g (mean ± SD), i.e. approximately 8–9 weeks old. The rats were fed modified experimental diets in accordance with the American Institute of Nutrition's recommendation for growing laboratory rodents (AIN-93G) [26], added 1.6 g methionine/kg diet and 1 wt% Growth and maintenance supplement containing vitamin B12 and vitamin K1 as recommended by Reeves [27]. The diets differed only in their protein sources (Table 1). Since the rats would be in the growth phase throughout the intervention period (based on growth charts for Zucker rats from Harlan Laboratories, https://www.envigo.com), and since obese Zucker rats have an impaired protein metabolism leading to poorer protein utilization and requires a greater protein intake to maintain a maximal rate of protein gain during growth [28], we used the AIN-93G diet with 20% (w/w) wt% protein instead of the AIN-93 M diet for maintenance containing 15 wt% protein. The Baked cod diet contained 5 wt% proteins from cod fillet (Atlantic cod (*Gadus morhua*) provided by Lerøy Seafood Group, Hordaland, Norway) and 15 wt% proteins from casein. The addition of 25% of dietary proteins from baked cod fillet is higher than the US and Norwegian recommendations of 300-450g fish/week [29,30], amounting to 9%-13% of the total protein intake based on a daily average total protein intake of 91-96g [31,32], but corresponds well with a previous clinical intervention study where adults had a median intake of 22% of protein intake as fish proteins [33]. Skin free cod fillets were baked in oven (180°C for 20 min) with no added fat. Baked cod fillets were minced using a hand blender, and were thereafter freeze dried and ground. The Control diet contained 20 wt% proteins from casein. Diets were frozen immediately after preparation. Casein was purchased from Sigma-Aldrich (Munich, Germany), the other feed ingredients were purchased from Dyets Inc.

The contents of amino acids and taurine in the diets were analyzed by Nofima BioLab (Hordaland, Norway), and are presented in Table 2.

2.2. *Ethical approval*

The National Animal Research Authority (Norway) approved the study protocol in accordance with the Animal Welfare Act and the Regulation of animal experiments (approval no 2014/6979). All applicable international, national and institutional guidelines for the care and use of animals were followed.

2.3. *Design*

Rats had free access to the experimental diets and tap water for 4 weeks. Feed was given as a powder formula and was contained in ceramic bowls that were too heavy for the rats to knock over, and rats were provided with newly thawed feed every day except Sundays (rats were given double portions on Saturdays). Rats always had access to wooden blocks for chewing and plastic cage inserts for housing. The rats were weighed every seventh day during the intervention period.

The rats were housed separately in cages with grids for 24 h on day 18 of the intervention period for measurements of water intake and collection of urine, without fasting in advance. Urine was stored at −80°C until analysis.

Systolic and diastolic blood pressures were measured in conscious rats at baseline (Day 0, before rats were introduced to the experimental diets) and 3 days before end point (Day 26) using the tail-cuff method (CODA-6, Kent Scientific Corporation, Torrington, CT, USA). Rats were pre-warmed in a heating cabinet for 30 min at 32°C before blood pressure measurements.

A meal tolerance test with a standardized high-sucrose meal was conducted on day 22 of the intervention period, with

the following ingredients per kilo diet: 400 g sucrose, 217 g casein, 70 g of soybean oil, 390 g of sucrose, 210 g of maize starch, 50 g of cellulose, 35 g of mineral mix, 10 g of vitamin mix, 3 g of L-cystine, 1.6 g of L-methionine, 2.5 g of choline bitartrate, 10 g of growth and maintenance supplement, and 0.014g of tert-butylhydroquinone. The rats were housed separately under fasting conditions from 20.00 to 08.00 hours with free access to tap water, before they received a meal corresponding to 2 g of sucrose/kg body weight. All rats were received the same diet for the meal tolerance test, and they were allowed a maximum of 15 min to finish the meal. The dorsal tail vein was punctuated and blood glucose was measured using the Contour blood glucose measuring device (Bayer Consumer Care AG) in the fasting condition and 60 and 120 min after the rats had finished eating the meal.

The rats were euthanized after four weeks intervention while under anesthesia with isoflurane (Isoba vet, Intervet, Schering-Plough Animal Health, Boxmeer, The Netherlands) mixed with oxygen and nitrous oxide, after a 12 h fast with free access to tap water. Blood was drawn from the heart and was collected in Vacuette Z Serum Clot Activator Tubes (Greiner Bio-one) and Vacuette K2EDTA (Greiner Bio-One) for isolation of serum and plasma, respectively. The heart was removed immediately after collection of blood. Liver, kidneys and epididymal white adipose tissue were dissected and weighed. Serum, plasma and organs were frozen in liquid nitrogen and were stored at −80°C until analysis.

Assessors responsible for feeding, weighing, general daily animal care, euthanasia and analyses of samples were blinded to diet groups.

2.4. *Analyses of serum, plasma, urine and liver*

Serum concentrations of creatinine, total protein, carbamide and uric acid were analyzed by accredited methods using the Cobas c702 system (Roche Diagnostics GmbH, Mannheim, Germany) by the Department of Medical Biochemistry and Pharmacology, Haukeland University Hospital (Bergen, Norway). Serum alanine transaminase and aspartate transaminase (measured with pyridoxal phosphate activation), plasma ammonium, and urine concentrations of creatinine, total protein, carbamide and uric acid were analyzed on the Cobas c111 system (Roche Diagnostics) using the ALTL (Alanine aminotransferase acc. IFCC), ASTL (Aspartate aminotransferase), NH3L (Ammonia), CREP2 (Creatinine plus ver.2), TP2 (Total Protein Gen.2 monochromatic), UREAL (Urea/BUN), and UA2 (Uric Acid ver.2) kits from Roche Diagnostics. Lipids were extracted from liver by the method of Bligh and Dyer [34], evaporated under nitrogen and re-dissolved in isopropanol before triacylglycerol was quantified using the TRIGL kit on Cobas c111 (Roche Diagnostics). These analytes were analyzed spectrophotometrically, according to the following test principles: creatinine, enzymatic colorimetric method; total protein, colorimetric assay; carbamide, kinetic test with urease and glutamate dehydrogenase; uric acid, enzymatic colorimetric method; the catalytic activities of alanine transaminase and aspartame transaminase were determined by measurements of the rate of oxidation of nicotinamide adenine dinucleotide; ammonium, enzymatic method with glutamate dehydrogenase; and triacylglycerol, enzymatic colorimetric method.

Urine concentration of T cell immunoglobulin mucin-1 (TIM-1) was quantified using the Rat TIM-1/KIM-1/HAVCR Quantikine enzyme-linked immunosorbent assay (ELISA) kit (RKM100) from R&D Systems, Bio-Techne, Minneapolis, MN, US.

Serum vitamin D was quantified using the 25-OH Vitamin D Total (Rat) ELISA kit (EIA-553; DRG Instruments GmbH).

Plasma for glutathione measurements was added ice-cold 5 % metaphosphoric acid (1:4), mixed and kept on ice for 15 min before centrifugation, and the supernatant was stored at −80°C until analysis. Total glutathione was analyzed using the glutathione detection kit (ADI-900-160) from Enzo Life Sciences AG.

Amino acids, related metabolites and potential biomarkers of fish intake (trimethylamine *N*-oxide (TMAO), 1-methylhistidine (π-methylhistidine), 3-methylhistidine (τ-methylhistidine) and creatinine) were measured in plasma and urine by Bevital AS (http://www.bevital.no) using gas chromatography and high-performance liquid chromatography with tandem mass spectrometry, as previously described [35,36]. Arginine, aspartic acid and glutamine were measured in plasma but could not be quantified in urine. Since sarcosine is present in the Vacuette K2EDTA tubes, sarcosine could not be measured in EDTA-plasma but was quantified in urine. Otherwise, the same compounds were analyzed in plasma and urine. Thiamine (vitamin B1), riboflavin (vitamin B2), nicotinamide (vitamin B3), pyridoxal 5'-phosphate (vitamin B6) and respective vitamers as well as kynurenine [36] and other metabolites in the kynurenine pathway [37] were analyzed in plasma by Bevital AS.

2.5. *Analyses of mRNA gene expression in liver, kidney and adipose tissue*

We purified total RNA from liver, kidney and epididymal white adipose tissue with the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The QIAxpert System (Qiagen, Hilden, Germany) was used to measure RNA concentration and quality. cDNA from 150 ng total RNA per sample was synthesized by using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA), and was diluted with PCR-grade water (1:5) before qPCR was performed (in triplicate) using the LightCycler480 rapid thermal cycler system (Roche Diagnostics GmbH, Basel, Switzerland) with the LightCycler 480 SYBR Green I Master (Roche, Basel, Switzerland). We used the following primer pairs: *Angiotensinogen*; forward primer 3' agcacgacttcctgacttgga'5 and reverse primer 3'ttgtaggatccccgaatttcc'5, *Renin*; forward primer 3' caaaggtttcctcagccaagat'5 and reverse primer 3' ctcggtgacctctccaaagg'5 (Sigma-Aldrich). We tested three primer pairs as reference genes: *60S ribosomal protein L32 (RPL32)*; forward primer 3'gtggctgccatctgttttg'5 and reverse primer 3'ttcttggtcctctttttgacg'5 (Sigma-Aldrich), *60S acidic ribosomal protein P0 (RPLP0)*; forward primer 3'gatgcccagggaagacag'5 and reverse primer 3'gaagcattttgggtagtcatcc'5 (Sigma-Aldrich), and *18S ribosomal RNA (18S)*; forward primer 3'agtccctgccctttgtac'5 and reverse primer 3'gatccgagggcctca'5 (Eurogentec, Seraing, Belgium). Of these, *18S* had least variation in readings and was least affected by the intervention diets in liver samples in triplets, whereas *RPL32* had

least variation in readings and was least affected by the intervention diets of kidney and adipose tissue samples in triplets. Therefore, hepatic mRNA concentrations are calculated relative to *18S* rRNA, and kidney and adipose tissue mRNA concentrations are calculated relative to *RPL32*.

2.6. Outcomes

The primary outcome of this study was to assess the effect of dietary intake of cod fillet on concentrations of amino acids and related metabolites in plasma and urine in obese Zucker fa/fa rats. The secondary outcomes were effects of cod fillet intake on markers of kidney and liver function, the development of high blood pressure, vitamin status, blood glucose and liver triacylglycerol concentrations.

2.7. Sample size

This is the first study to investigate the effects of baked cod fillet as part of a normal diet for growing rodents (AIN-93G) on concentrations of amino acids and related metabolites in plasma and urine in obese Zucker fa/fa rats, therefore the present study is considered to be a pilot study. We included 10 rats in the Control group and 10 rats in the Baked cod group, based on previous studies were we used salmon fillet as part of the AIN-93G diet with 6 rats in each group [38] and recent clinical trials showing that cod fillet in affected biochemical parameters to a lesser extent when compared to salmon fillet [18,39-41].

2.8. Statistical analysis

The experimental groups were compared using Independent Samples T Test since the data were mainly normally distributed (Shapiro–Wilk test). Changes in blood pressure from baseline to end point within each group were tested using the paired sampled T-test. The cut-off level for statistical significance was taken at a probability of 0.05. SPSS Statistics version 25 (SPSS, Inc., IBM Company) were used for all statistical analyses. Rats fed a casein-based diet served as controls. Data are presented as mean ± standard deviation. One rat in the Control group had to be euthanized due to a wound that did not heal and is not included in the results, therefore we analyzed n=10 in the Baked cod group and n=9 in the Control group.

3. Results

3.1. Body weight and growth

The starting weight was similar in the Baked cod group and the Control group, and body weight measured after 1, 2, 3 and 4 weeks were similar between the groups (Fig. 1, *P* values 0.80, 0.87, 0.20, 0.55 and 0.17, respectively).

3.2. Amino acids and vitamins in plasma and urine

Plasma concentrations of the essential amino acids methionine and histidine, the non-essential amino acids alanine, asparagine, aspartic acid, glycine, proline and tyrosine, and of

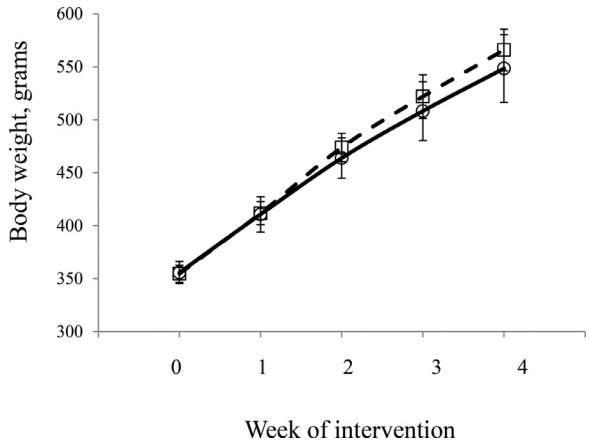

Fig. 1 – Body weight measured at baseline (week 0), and after 1, 2, 3 and 4 weeks of intervention for rats fed Baked cod diet (□ with dotted line, n = 10) or Control diet (o with solid line, n 9). Values are mean and standard deviations. *P* <0.05 were considered statistically significant. No differences were observed between the groups at any time during the intervention period. Groups are compared using Independent Samples T Test assuming equal variances

homoarginine, total homocysteine, α-ketoglutaric acid, nicotinamide and methylmalonic acid were lower in the Baked cod group compared to the Control group (Table 3). The plasma concentrations of the potential biomarkers of fish intake, i.e., 1-methylhistidine, 3-methylhistidine, creatine and TMAO [18], were higher in the Baked cod group compared to the Control group. The groups were similar with regard to circulating concentrations of all measured fat- and water-soluble vitamins (Table 4).

In urine, the concentrations (relative to creatinine) of 1-methylhistidine, 3-methylhistidine, creatine and TMAO were higher in the Baked cod group compared to the Control group, but otherwise no differences were seen between the groups for concentrations of amino acids and related metabolites in urine (Table 5).

3.3. Organ weights and water intake

We have previously published that Baked cod group and the Control group were similar with regard to body weight-to-square body length and weights of selected white adipose tissue depots, liver and thigh muscle relative to body weight at end point, and that the average daily energy intake was similar in the two groups [25]. Now we show that also kidney weight and water intake relative to body weight were similar between the groups (Table 6).

3.4. Blood pressure, blood glucose and liver triacylglycerol

Systolic and diastolic blood pressures were similar between the groups at baseline (Table 6). Blood pressures increased in both groups from baseline to 4 weeks; *P* values for increase in systolic blood pressure were 0.0062 and 0.015 in

Table 3 – Plasma concentrations of amino acids and related metabolites at end point

µmol/l	Baked cod group	Control group	*p*
Alanine	320 ± 43	389 ± 82	0.032
Arginine	21.8 ± 25.2	17.2 ± 21.1	0.68
Asparagine	50.8 ± 3.3	60.5 ± 8.8	0.0046
Aspartic acid	16.1 ± 3.9	22.5 ± 7.1	0.026
Betaine	80.5 ± 10.9	72.1 ± 7.3	0.070
Choline	17.6 ± 2.4	18.0 ± 3.9	0.78
Creatine	192 ± 39	116 ± 47	0.0012
Cystathionine	0.37 ± 0.03	0.42 ± 0.11	0.19
Cysteine (total)	201 ± 27	188 ± 22	0.26
Asymmetric dimethylarginine	0.69 ± 0.06	0.65 ± 0.05	0.11
Symmetric dimethylarginine	0.37 ± 0.04	0.36 ± 0.03	0.53
Dimethylglycine	9.10 ± 1.17	8.38 ± 1.95	0.34
Glutamine	488 ± 48	555 ± 102	0.078
Glutamic acid	99.5 ± 13.9	119.1 ± 36.9	0.14
α-Ketoglutaric acid	37.6 ± 6.1	45.2 ± 8.9	0.042
Glutathione (total)	34.7 ± 11.5	42.7 ± 17.0	0.24
Glycine	108 ± 10	127 ± 17	0.011
Histidine	53.6 ± 4.6	61.6 ± 5.1	0.0024
1-Methylhistidine	15.2 ± 4.3	5.6 ± 0.8	5.2×10^{-6}
3-Methylhistidine	4.09 ± 0.47	2.76 ± 0.17	3.9×10^{-7}
Homoarginine	0.90 ± 0.27	1.18 ± 0.20	0.019
Homocysteine (total)	2.13 ± 0.21	2.56 ± 0.38	0.0065
3-Hydroxyisobutyrate	20.2 ± 4.8	23.2 ± 5.2	0.22
Isoleucine	83.2 ± 11.1	87.2 ± 11.0	0.44
Leucine	134 ± 20	142 ± 22	0.44
Lysine	290 ± 42	302 ± 30	0.50
Methionine	41.8 ± 5.8	48.5 ± 5.9	0.024
Methionine sulfoxide	1.18 ± 0.54	1.58 ± 0.58	0.14
Methylmalonic acid	0.29 ± 0.04	0.36 ± 0.05	0.0061
Ornithine	125 ± 30	142 ± 28	0.24
Phenylalanine	67.2 ± 4.2	71.8 ± 9.0	0.16
Proline	105 ± 18	130 ± 27	0.029
Sarcosine	ND	ND	(-)
Serine	181 ± 12	191 ± 20	0.19
Threonine	183 ± 26	206 ± 29	0.090
TMAO	13.23 ± 16.24	1.56 ± 0.44	0.00017
Trimethyllysine	1.18 ± 0.09	1.15 ± 0.12	0.61
Tryptophan	94.4 ± 12.7	100.5 ± 11.1	0.28
Tyrosine	56.3 ± 8.3	83.9 ± 17.2	0.00029
Valine	192 ± 34	208 ± 28	0.28

Data are presented as means ± standard deviation; n = 10 rats in Baked Cod Group and n = 9 rats in Control Group. *P* <0.05 were considered significant. Groups are compared using Independent Samples T Test assuming equal variances. TMAO; Trimethylamine N-oxide

Table 4 – Circulating concentrations of fat-soluble vitamins, B vitamins and kynurenine pathway metabolites at end point

	Baked cod group	Control group	*P*
Fat soluble vitamins			
all-trans Retinol, µmol/l	1.68 ± 0.30	1.91 ± 0.27	0.10
25-OH Vitamin D (total), ng/mL	24.0 ± 6.4	26.1 ± 5.7	0.47
α-Tocopherol, µmol/l	94.0 ± 10.1	93.3 ± 9.7	0.88
gamma-Tocopherol, µmol/l	3.58 ± 1.36	3.80 ± 0.94	0.69
B vitamins			
Thiamine, nmol/l	165 ± 22	192 ± 52	0.15
Thiamine monophosphate, nmol/l	677 ± 146	672 ± 98	0.94
Riboflavin, nmol/l	77.4 ± 13.2	92.4 ± 21.0	0.077
Flavin mononucleotide, nmol/l	56.6 ± 13.6	53.5 ± 11.6	0.60
Nicotinic acid, nmol/l	<LOD	<LOD	
Nicotinamide, nmol/l	3743 ± 463	4416 ± 851	0.044
N^1-Methylnicotinamide, nmol/l	383 ± 119	417 ± 215	0.67
Pyridoxal 5′-phosphate, nmol/l	1222 ± 115	1090 ± 268	0.17
Pyridoxal, nmol/l	437 ± 79	434 ± 76	0.94
4-Pyridoxic acid, nmol/l	66.5 ± 15.7	67.8 ± 14.7	0.86
Pyridoxine, nmol/l	<LOD	<LOD	
Kynurenine pathway metabolites			
Kynurenine, µmol/l	1.72 ± 0.24	1.64 ± 0.39	0.59
Kynurenic acid, nmol/l	82.9 ± 13.5	84.9 ± 18.5	0.79
Anthranilic acid, nmol/l	84.3 ± 22.0	99.8 ± 23.2	0.15
3-Hydroxykynurenine, nmol/l	5.42 ± 2.98	4.95 ± 1.92	0.69
Xanthurenic acid, nmol/l	14.9 ± 3.3	13.9 ± 2.6	0.47
3-Hydroxyanthranilic acid, nmol/l	3.83 ± 0.95	4.55 ± 0.97	0.12
Picolinic acid, nmol/l	113 ± 26	110 ± 15	0.74
Quinolinic acid, nmol/l	279 ± 79	302 ± 119	0.62

Data are presented as means ± standard deviation; n = 10 rats in Baked Cod Group and n = 9 rats in Control Group. *P* <0.05 were considered significant. Groups are compared using Independent Samples T Test assuming equal variances.
LOD, level of detection (LODs were 1 nmol/L for pyridoxine and 20 nmol/L for nicotinic acid)

the Baked cod group and Control group, respectively, and *P* values for increase in diastolic blood pressure were 0.0046 and 0.0060 in the Baked cod group and Control group, respectively (data not presented). The within-groups changes in systolic and diastolic blood pressures from baseline to end point were not significantly different when the groups were compared (*P* values 0.91 and 0.93, respectively, data not presented), and no differences were seen between groups for systolic and diastolic blood pressures at end point (Table 6).

Gene expressions of *angiotensinogen* in liver and of *renin* in kidney and epididymal white adipose tissue were similar between the dietary groups (*P* values = 0.19, 0.37 and 0.75, respectively, data not presented). Since mRNA expression of angiotensinogen is reduced by fasting in white adipose tissue (but not in liver) [42], *angiotensinogen* mRNA expression in adipose tissue was not measured in our rats as they were fasted overnight.

After 22 days, the fasting blood glucose concentration was similar between the groups (Table 6). The postprandial blood glucose concentrations after intake of a high-sucrose feed were also similar between the groups, measured after 60 and 120 minutes (*P* values = 0.35 and 0.74, respectively). Liver triacylglycerol concentration was similar in the Baked cod group and the Control group.

Table 5 – Urine concentrations of amino acids and related metabolites at end point

µmol per mmol creatinine	Baked cod group	Control group	P
Alanine	56.5 ± 14.9	63.3 ± 44.9	0.66
Asparagine	8.13 ± 5.13	9.81 ± 3.59	0.43
Betaine	92.7 ± 27.0	83.1 ± 48.5	0.60
Choline	46.8 ± 36.2	31.8 ± 18.6	0.28
Creatine	1560 ± 652	31.5 ± 33.5	2.1×10^{-6}
Cystathionine	0.33 ± 0.11	0.50 ± 0.40	0.22
Cysteine (total)	65.5 ± 30.3	55.4 ± 35.4	0.51
Asymmetric dimethylarginine	0.12 ± 0.10	0.23 ± 0.49	0.50
Symmetric dimethylarginine	4.28 ± 0.97	4.30 ± 3.11	0.99
Dimethylglycine	67.1 ± 22.7	64.1 ± 37.8	0.83
Glutamic acid	26.4 ± 10.9	30.2 ± 19.0	0.60
α-Ketoglutaric acid	487 ± 176	354 ± 337	0.29
Glycine	57.5 ± 11.1	87.7 ± 96.8	0.34
Histidine	16.4 ± 4.0	19.2 ± 10.0	0.43
1-Methylhistidine	57.02 ± 38.13	7.64 ± 7.48	1.4×10^{-3}
3-Methylhistidine	36.60 ± 16.63	9.97 ± 8.62	4.8×10^{-4}
Homoarginine	22.9 ± 7.2	33.3 ± 19.0	0.13
Homocysteine (total)	2.74 ± 1.16	2.79 ± 2.70	0.96
3-Hydroxyisobutyrate	7.3 ± 2.6	8.8 ± 8.9	0.62
Isoleucine	8.44 ± 5.39	8.27 ± 3.25	0.94
Kynurenine	0.09 ± 0.04	0.23 ± 0.36	0.26
Leucine	14.9 ± 7.6	19.3 ± 6.8	0.20
Lysine	41.3 ± 8.5	44.2 ± 12.0	0.55
Methionine	25.0 ± 25.4	16.8 ± 8.2	0.37
Methionine sulfoxide	2.43 ± 1.41	2.90 ± 1.73	0.52
Methylmalonic acid	10.97 ± 2.34	13.44 ± 8.37	0.38
Ornithine	5.30 ± 1.72	7.15 ± 5.07	0.29
Phenylalanine	11.2 ± 4.3	15.9 ± 13.2	0.30
Proline	33.1 ± 10.0	48.6 ± 55.1	0.39
Sarcosine	3.81 ± 1.38	3.39 ± 1.90	0.59
Serine	19.9 ± 11.1	21.9 ± 10.3	0.68
Threonine	48.4 ± 55.1	45.3 ± 17.6	0.65
TMAO	2534 ± 1106	69.8 ± 52.6	4.0×10^{-6}
Trimethyllysine	8.23 ± 3.47	6.61 ± 9.09	0.61
Tryptophan	2.89 ± 1.86	4.66 ± 4.80	0.29
Tyrosine	10.9 ± 3.8	18.8 ± 19.0	0.22
Valine	20.3 ± 7.0	23.9 ± 9.5	0.36

Data are presented as means ± standard deviation; n = 10 rats in Baked Cod Group and n = 9 rats in Control Group. p <0.05 were considered significant. Groups are compared using Independent Samples T Test assuming equal variances. TMAO; Trimethylamine N-oxide

3.5. *Markers of organ damage and kidney function, and nitrogen-containing compounds*

Rats fed baked cod had significantly lower circulating concentrations of alanine transaminase, aspartate transaminase creatinine, carbamide, uric acid and ammonium when compared to the Control group (Table 7). The urine concentration (relative to creatinine) of TIM-1 was significantly lower in the Baked cod group, whereas the urine creatinine concentration and concentrations (relative to creatinine) of total protein, carbamide, uric acid and total amount of proteinogenic amino acids (arginine was not measured) were similar between the two groups.

Table 6 – Kidney weight, water intake, blood glucose, liver triacylglycerol and blood pressure

	Baked cod group	Control group	P
Baseline measurements			
Systolic blood pressure, mm Hg	131 ± 14	136 ± 15	0.49
Diastolic blood pressure, mm Hg	92 ± 14	97 ± 12	0.45
End point measurements			
Kidneys, g/kg bodyweight	4.7 ± 0.5	5.1 ± 0.6	0.21
Water intake, g/kg bodyweight/24h	47.2 ± 13.5	42.6 ± 13.1	0.46
Fasting blood glucose, mmol/l	4.8 ± 0.7	4.9 ± 1.3	0.82
Liver triacylglycerols, µmol /g liver	162 ± 39	170 ± 55	0.70
Systolic blood pressure, mm Hg	149 ± 9	150 ± 12	0.75
Diastolic blood pressure, mm Hg	108 ± 5	110 ± 10	0.55

Data are presented as means ± standard deviation; n = 10 rats in Baked Cod Group and n = 9 rats in Control Group. p <0.05 were considered significant. Groups are compared using Independent Samples T Test assuming equal variances.

4. Discussion

Here we show for the first time that feeding rats a diet containing baked cod fillet resulted in lower concentrations of several amino acids and related metabolites in plasma when compared to a control group fed a fish-free diet, but did not affect amino acid composition in urine. The concentrations of 1-methylhistidine, 3-methylhistidine, creatine and TMAO, which are proposed as potential biomarkers of fish intake [18], were higher in both plasma and urine from rats fed baked cod. The Baked cod diet contained more (that is, >1 g/kg diet difference) alanine and aspartic acid + asparagine, and less glutamic acid + glutamine and proline, all of which are non-essential amino acids, when compared with the Control diet, with no differences between the diets for essential amino acids. Differences in the amino acid composition of the Baked cod diet and the Control diet were, in general, not reflected in the plasma and urine concentrations of amino acids in rats fed these diets. Concentrations of markers of kidney dysfunction and liver damage measured in serum or urine suggest that the function of both organs were better in the Baked cod group. Vitamin status is affected by kidney status [43], but no differences were seen between the groups for circulating concentrations of water- and fat-soluble vitamins. Groups were similar with regard to growth, blood pressure development, fasting and postprandial glucose concentrations, and liver triacylglycerol concentration.

Patients with chronic kidney failure are advised to consume a diet with modest protein restriction in order to limit the development of toxic nitrogenous metabolites, uremic symptoms and other metabolic complications [44,45]. However, information is lacking in regard to whether different dietary proteins may have dissimilar impact on kidney func-

Table 7 – Markers of organ damage and kidney function, and nitrogen-containing compounds measured in serum, plasma or urine at end point

	Baked cod group	Control group	P
Serum creatinine, µmol/l	12.3 ± 0.7	13.8 ± 1.2	0.0038
Serum alanine transaminase, U/l	99 ± 29	147 ± 59	0.034
Serum aspartate transaminase, U/l	185 ± 54	301 ± 149	0.034
Serum protein, g/l	60.1 ± 2.6	61.8 ± 3.3	0.23
Serum carbamide, mmol/l	5.9 ± 1.3	7.3 ± 1.5	0.047
Serum uric acid, µmol/l	73.2 ± 25.6	106.6 ± 26.9	0.013
Plasma ammonium µmol/l	89.7 ± 21.5	114.8 ± 16.0	0.011
Urine creatinine, mmol/l	5.4 ± 2.7	5.7 ± 2.7	0.83
Urine TIM-1, ng/mmol creatinine	192 ± 24	266 ± 72	0.0066
Urine total protein, g/mmol creatinine	1.9 ± 0.6	1.8 ± 1.4	0.90
Urine carbamide, mmol/mmol creatinine	378 ± 126	446 ± 56	0.15
Urine uric acid, µmol/mmol creatinine	368 ± 110	335 ± 116	0.54
Urine total proteinogenic amino acids, µmol/mmol creatinine	467 ± 127	533 ± 295	0.52

Data are presented as means ± standard deviation; n = 10 rats in Baked Cod Group and n = 9 rats in Control Group. $P < 0.05$ were considered significant. Groups are compared using Independent Samples T Test assuming equal variances. TIM-1; T cell immunoglobulin mucin-1

tion, and it is of interest that intake of fish has been associated with reduced risk of developing kidney disease [46,47]. We used obese Zucker fa/fa rats aged 8–9 weeks at the start of the intervention (rats were euthanized at age 12-13 weeks), since they develop high blood pressure and proteinuria before or around 10 weeks of age [21-24]. The occurrence of proteins and amino acids in urine are among the earliest indicators of renal dysfunction in both humans and animals [48,49]. Proteins and free proteinogenic amino acids were observed in the urine of all rats, with no difference in concentrations between the Baked cod group and the Control group, thus indicating renal dysfunction in both groups. The relative urine protein concentration in the present study was around three times higher than previously observed in young healthy male Wistar rats [50]. A better urine marker for tubular injury is TIM-1, which is expressed on the proximal tubule apical membrane in response to renal injury but is not detectable in urine when kidneys are healthy [51]. The presence of TIM-1 in urine from all rats also indicates the occurrence of tubular injury in both experimental groups; however, the lower TIM-1 concentration in urine from rats in the Baked cod group suggest that consumption of cod fillet may somehow protect against or delay the development of kidney injury in obese Zucker fa/fa rats. Other findings support this assumption; the lower serum concentrations of creatinine and carbamide in the Baked cod group are suggestive of a less impaired kidney function [51,52] compared to the Control group. This is in line with our previous report on the effects of cod protein intake on urinary markers of renal dysfunction in obese Zucker fa/fa rats [53], indicating a protective effect of cod proteins on kidney function.

Rats fed the Baked cod diet had lower glycine plasma concentration when compared to the Control group, however no differences were seen between the groups for plasma concentrations of branched chain amino acids and kynurenines, and of the aromatic amino acids only tyrosine concentration was lower when compared to the Control group. The lower plasma concentrations of glycine and tyrosine are of interest as these are associated with prediabetes, insulin resistance, cardiovascular disease and future type 2 diabetes in humans [14,15]. Plasma concentrations of the non-essential amino acids alanine, asparagine, aspartic acid and proline, and of α-ketoglutaric acid (the ketone derivate of glutaric acid) were lower in rats fed the Baked cod diet. Alanine transaminase and aspartate transaminase catalyze the conversion between α-ketoglutaric acid and either alanine or aspartic acid, respectively, and glutamic acid and either pyruvate or oxaloacetate, and the lower concentrations of alanine, aspartic acid and α-ketoglutaric acid in the Baked cod group may be a consequence of different activity rates of these transaminases between the groups. The lower histidine and proline concentrations may be caused by increased flux into the tricarboxylic cycle, and in addition the lower proline concentration may suggest that its formation from glutamate is downregulated. In this context it is of interest that fatty liver disease is associated with higher plasma concentrations of several amino acids including alanine, histidine, methionine, tyrosine and proline [54].

The lower total homocysteine concentration in the Baked cod group may be beneficial since elevated homocysteine concentration is associated with increased risk for developing cardiovascular disease in humans [55]. In addition, elevated homocysteine concentration is prevalent in patients with acute and chronic renal disease, underscoring the key role of the kidneys in removing homocysteine from the circulation [56]. The lower total homocysteine concentration together with the lower methionine concentration in plasma may indicate that one-carbon metabolism was affected by the Baked cod diet, as plasma concentrations of homocysteine and methionine are shown to be positively correlated [57]. Plasma concentrations of other metabolites related to the one-carbon metabolism, such as the methyl donors betaine, choline and dimethylglycine, were not different between the dietary groups. Thus, the lower total homocysteine concentration may be a consequence of lower plasma concentration of the precursor methionine, despite similar methionine content in the Baked cod diet and in the Control diet.

The plasma concentrations of arginine, asymmetric dimethylarginine, symmetric dimethylarginine and homoarginine are of interest in the context of blood pressure regulation. Nitric oxide is a potent vasodilator, and is synthesized from L-arginine by endothelial nitric oxide synthase. Asymmetric dimethylarginine (produced by methylation of arginine residues of the intracellular proteins) is an endogenous competitive inhibitor of nitric oxide synthase [58], symmetric dimethylarginine inhibits the transport of arginine [59] and homoarginine is a competitive nitric oxide synthase substrate [60], thus all of these can act to decrease nitric oxide production from L-arginine. Of these four compounds, only

homoarginine concentration was affected by Baked cod diet, with a lower plasma concentration when compared to the Control group. However, blood pressure is also influenced by the renin-angiotensin system where angiotensinogen (mainly produced by the liver) is cleaved by renin (primarily produced by kidneys) to the biologically inactive angiotensin I, which is further converted to the vasoconstrictor angiotensin II by the angiotensin-converting enzyme [61], with renin as the rate determining enzyme [62]. Adipose tissues in humans and rodents contain all component of the renin-angiotensin system, and the activity of this system is increased in obesity [61]. The renin-angiotensin system is important for the blood pressure increase in obese Zucker fa/fa rats, possibly through increased sensitivity towards angiotensin II [63]. We observed no differences between the Baked cod group and the Control group with regard to blood pressure development or gene expressions of *angiotensinogen* in liver or of *renin* in kidney and in adipose tissue, and the kidney weight was similar between the groups. These results are in coherence with our recent findings where a diet containing cod fillet did not affect blood pressure development or nitrite+nitrate concentration in serum from obese Zucker fa/fa rats [64]. From this we conclude that baked cod fillet did not have the potential to prevent or delay the typical blood pressure increase that is expected in obese Zucker fa/fa rats.

Methylhistidines from dietary anserine and cod muscle proteins are not re-utilized for protein synthesis or metabolized but are excreted in the urine, and the concentrations of methylhistidines in urine have been proposed as a useful biomarker of meat intake [65]. Also, the majority of TMAO from diet is excreted unchanged by the kidneys [66]. The higher concentrations of 1-methylhistidine, 3-methylhistidine, creatine and TMAO in plasma and urine of rats fed the Baked cod diet were expected since cod muscle contains relatively high levels of these four compounds [18], and is in line with our recent report showing high urine concentrations of these compounds in obese Zucker fa/fa rats fed cod protein [53].

Consumption of baked cod did not affect the concentrations of individual amino acids and the total amount of proteinogenic amino acids in urine. This was somewhat surprising, since we previously have shown that Zucker fa/fa rats fed cod protein had a much lower urine concentration of almost all measured amino acids and a lower total amount of proteinogenic amino acids when compared to those fed a control diet devoid of fish protein [53]. In addition, circulating and urine markers indicated a better kidney function in rats fed cod protein [53], thus the lower urine secretion in those rats may be a consequence of better kidney function. Results from the present study suggest that although the Baked cod diet seems to offer some protection of the kidneys, this was not sufficient to affect the amount of amino acids that was excreted in urine.

The nutritional advice for non-alcoholic fatty liver disease patients is to achieve weight loss through a hypocaloric diet but does not explicitly address protein intake [67], and effect of fish intake on fatty liver has not been described. Alanine transaminase is a circulating marker for liver function, whereas aspartate transaminase is regarded as a more general marker for organ damage. The lower concentration of serum alanine transaminase in rats fed baked cod indicates that the liver function was better compared to the Control group, although the hepatic triacylglycerol content was similar between the groups. Also, the lower serum aspartate transaminase concentration may support the lower concentrations of markers for kidney dysfunction in the Baked cod group. These findings indicates a protective effect of cod fillet on liver function, and are in line with the observation that obese Zucker fa/fa rats fed salmon fillet had lower serum concentrations of both transaminases when compared to rats fed a fish-free diet but did not affect hepatic triacylglycerol concentration [68].

The rats in the Baked cod group were fed diets containing 25% of proteins from baked cod fillet and 75% of proteins from casein. This intake of fish proteins is higher than the recommendations from the American Heart Association and the Norwegian government, who recommend a fish intake of 300-450g/week (2-3 dinner meals per week) for the general public [29,30]. The daily average total protein intake for adults in the US and in Norway is estimated to be 91-96g [31,32], thus these recommendations corresponds to approximately 60-90g of fish proteins weekly, amounting to 9-13% of the total protein intake. The cod protein intake in the present study corresponds well with a previous clinical study where adults with overweight/obesity consumed 750g per week of cod fillet or salmon fillet; participants had an estimated median intake of 96 g total protein/day with 22% of protein intake as fish proteins [33]. By using 75% of proteins from casein in the Baked cod diet, and 100% of proteins from casein in the Control diet, with extra added methionine and cystine (1.6 and 3.0 g/kg diet, respectively), we ensure that the intake of essential amino acids is adequate and avoid that a potential lack of essential amino acids influence the effects of the diets on biochemical and physiological parameters.

The present study has some methodological strengths and limitations. Strengths include simultaneous measurements of many amino acids and related metabolites. Samples were treated according to a strict protocol for pre-analytical sample handling, and samples were thawed for the first time for these analyses. The measurements of blood pressure were conducted in conscious (unanesthetized) rats using the tail-cuff method (volume-pressure recording), which is a non-invasive and inexpensive method that does not require surgery. Rats were hand-tame and trained to be in the constrainer before the start of the intervention. Limitations to the study includes the rat model used; although the obese Zucker fa/fa rat is a relevant model of human obesity that spontaneously develops comorbidities of obesity [20], the translation of the present findings to humans must be further investigated since there are still important differences between humans and rats, and the findings in the current study could be specific for obese Zucker fa/fa rat.

We hypothesized that intake of baked cod fillet would affect amino acid concentrations in plasma and urine in obese Zucker fa/fa rats. Partially in line with our hypothesis, we found that rats in the Baked cod group had lower plasma concentrations of total homocysteine, homoarginine, the essential amino acids methionine and histidine, the non-essential amino acids alanine, asparagine, aspartic acid, glycine, proline and tyrosine. Regarding parameters associated with the metabolic syndrome, we found that markers of renal and

liver function were less impaired in rats fed baked cod, while we found no differences between the groups for blood pressure development, fasting and postprandial glucose and liver triacylglycerol concentrations. To conclude, substituting 25% of dietary proteins with lyophilized baked cod fillet affected plasma concentrations of several amino acids and delayed the development of kidney dysfunction and liver damage, but did not affect urine amino acid composition, blood pressure development, vitamin status, blood glucose and liver triacylglycerol concentrations when compared to a diet with 100% of proteins from casein.

Author statement

LAV, AD, GM and OAG formulated the research question and designed the study. LAV, AD, MTB and OAG conducted the animal study. LAV, AD, ØM, AM, MHA, PMU and OAG analyzed the data. OAG performed statistical analyses, drafted the paper and had primary responsibility for the final content. All authors have contributed to the writing and approved the final manuscript.

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Author Declarations

The authors declare no conflicts of interest.

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