Choline consumption in Norway

Dietary sources, association with one-carbon and lipid metabolism and cardiovascular disease risk

Anthea Van Parys

Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
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Scientific environment

This project was conducted from February 2018 to November 2021 at the Centre for Nutrition, Department of Clinical Science, Faculty of Medicine, University of Bergen. The main supervisor was initially Professor Dr. Med. Ottar Kjell Nygård and from June 2020 Dr. Vegard Lysne. The co-supervisors were Professor Jutta Dierkes, Dr. Jannike Øyen, and from June 2020 Professor Dr. Med. Ottar Kjell Nygård.

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Anthea Van Parys

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Abbreviations

AI	Adequate intake
AMI	Acute myocardial infarction
ApoB	Apolipoprotein B
ApoE	Apoliprotein E
AR	Average requirement
ATP	Adenosine triphosphate
BADH	Betaine aldehyde dehydrogenase
BHMT	Betaine-homocysteine S-methyltransferase
BMI	Body mass index
CBS	Cystathionine-β-synthase
CDP	cytidine 5-diphosphocholine
CGL	Cystathionine-γ-lyase
CHD	Coronary heart disease
CHDH	Choline dehydrogenase
CHT	High-affinity choline transporters
CI	Confidence interval
CK	Choline kinase
CLT1	Choline transporter-like protein 1
CPT	CDP-choline: 1,2-diacylglycerol cholinephosphotransferase
CT	CTP:phosphocholine cytidylyltransferase
CVD	Cardiovascular disease
CVDNOR	Cardiovascular disease in Norway

DAG	Directed acyclic graph
DHA	Docosahexaenoic acid
DMG	Dimethylglycine
DMGDH	Dimethylglycine dehydrogenase
EFSA	European Food Safety Authority
ER	Endoplasmic reticulum
FFQ	Food frequency questionnaire
FMO	Flavin-dependent monooxygenase
γ-BB	γ-butyrobetaine
GPC	Glycerophosphocholine
Hcy	Homocysteine
HDL	High-density lipoprotein
HR	Hazard ratio
HUSK	Hordaland Health Study
LDL	Low-density lipoprotein
LysoPC	Lysophosphatidylcholine
MS	Methionine synthase
mTHF	5-methyltetrahydrofolate
MTHF	5,10-methylenetetrahydrofolate
MTHFD1	Methylenetetrahydrofolate dehydrogenase 1
MTHFR	Methylenetetrahydrofolate reductase
NAFLD	Non-alcoholic fatty liver disease
NAM	National Academies of Medicine

NHS	Nurses' Health Study
NORKOST2	National Dietary Survey among Men and Women aged 16-79 1997
OCT	Low affinity polyspecific organic cation transporters
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEMT	Phosphatidylethanolamine-N-methyltransferase
PI	Prediction interval
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SAP	Stable angina pectoris
SARDH	Sarcosine dehydrogenase
SHMT	Serine hydroxymethyltransferase
SLC44A1	Solute carrier family 44 member 1
SM	Sphingomyelin
SNP	Single nucleotide polymorphism
STROBE	Strengthening the Reporting of Observational studies in Epidemiology
TG	Triglycerides
tHcy	Total homocysteine
THF	Tetrahydrofolate
TMA	Trimethylamine
TMAO	Trimethylamine N-oxide
TML	Trimethyllysine
TPN	Total parenteral nutrition

USDA	US Department of Agriculture
VLDL	Very-low-density lipoprotein
WENBIT	Western Norway B-vitamin Intervention Trial
WHO	World Health Organization

Abstract

Background:

Choline is an essential nutrient involved in a wide variety of physiological functions. Through its metabolite betaine, it is closely connected to the one-carbon metabolism, and phosphatidylcholine (PC) is essential for the formation and secretion of very-low-density-lipoprotein in the liver, connecting choline to lipid metabolism. Choline is found in foods and in the body in different forms. Food products of animal origin contain higher amounts of total choline, and mainly fat-soluble forms, per unit weight compared to plant-derived products. An adequate intake (AI) has been set in the US and Europe, however, not yet in the Nordic countries.

Both the one-carbon and lipid metabolism are closely related to the risk of chronic diseases. However, few studies have investigated the association between dietary choline intake and cardiovascular disease, and findings are contradictory. In **Paper I and II** the association between dietary choline intake and the risk of acute myocardial infarction (AMI) in patients with pre-existing coronary heart disease was addressed. Further, dietary intake of total choline and individual choline forms and their dietary contributors have not been investigated, which was the objective of **Paper III and IV**. Additionally, in **Paper IV**, the association between choline intake and plasma concentrations of one-carbon and lipid metabolites was explored in healthy community-dwelling adults.

Methods:

Western Norway B-vitamin Intervention Trial (WENBIT)

Patients with stable angina pectoris from WENBIT (n=1981 and 1929, for **Paper I** and **Paper II** respectively) were included. Dietary data was derived from a food frequency questionnaire (FFQ) and choline content of food items was quantified using the U.S. Department of Agriculture (USDA) Database for Choline Content of Common Foods, release 2. Cox regression models were used to estimate the association between total choline intake or intake of individual choline forms and risk of AMI. Effect modification was investigated for trimethylamine N-oxide (TMAO)

and trimethyllysine (TML) and mediation analysis was applied considering TMAO as a mediator.

Hordaland Health Study 1997-1999 (HUSK)

HUSK included community-dwelling adults, and 5746 of these were included in the current analysis. Dietary data were derived from the same FFQ used in the WENBIT cohort and choline intake was estimated using the USDA choline database. To explore the relationship between one-carbon and lipid metabolites and total dietary choline intake, choline intake was modeled as a polynomial spline.

Results:

The geometric mean (95% prediction interval) total energy-adjusted choline intake was 287 (182, 437) mg/d and 260 (170, 389) mg/d in the WENBIT and HUSK populations, respectively. PC was the main consumed form in both study populations and eggs contributed most to total choline intake (12.6% and 15.3% in WENBIT and HUSK respectively). Other main dietary contributors were milk, vegetables, potatoes, and lean fish. Most of the individual choline forms were primarily obtained from animal-based food sources, apart from free choline. In patients with SAP, increased intakes of energy-adjusted total choline (Hazard ratio (HR) [95% confidence interval] 1.10 [1.02, 1.19]), PC (1.23 [1.07, 1.41]), and sphingomyelin (1.15 [1.03, 1.30]) were associated with higher AMI risk. We did not observe any effect modification by plasma TMAO and TML, nor any mediation by TMAO. In community-dwelling adults, dietary choline consumption showed clear associations with plasma concentration of one-carbon metabolites and to a lesser extent with serum lipid metabolites.

Conclusion:

Choline was mainly consumed in the form of PC and mostly obtained from animal food sources. The self-reported choline intake was below the established AI for most of the participants. Further, total dietary choline, PC, and sphingomyelin were positively associated with AMI risk. Also, dietary choline was associated with the plasma concentration of metabolites of the one-carbon and lipid metabolism.

Further studies investigating choline intake in Nordic populations are warranted to allow for the establishment of dietary recommendations. To be able to estimate the dietary choline intake more accurately, the choline content of foods should be included in the Norwegian food composition table. The association between dietary choline and the one-carbon and lipid metabolism requires clarification as these are closely related to the risk of chronic diseases.

List of Publications

I. Van Parys A., Lysne V., Svingen G.F.T., Ueland P.M., Dhar I., Øyen J., Dierkes J., Nygård O.K. (2019). Dietary choline is related to increased risk of acute myocardial infarction in patients with stable angina pectoris. *Biochimie*, 173, 68-75. https://doi.org/10.1016/j.biochi.2019.11.001

II. Van Parys A., Lysne V., Øyen J., Dierkes J., Nygård O.K. (2020). No effect of plasma trimethylamine N-oxide (TMAO) and plasma trimethyllysine (TML) on the association between choline intake and acute myocardial infarction risk in patients with stable angina pectoris. *Human Nutrition & Metabolism*, 21. https://doi.org/10.1016/j.hnm.2020.200112

III. Van Parys A., Karlsson T., Vinknes K.J., Olsen T., Øyen J., Dierkes J., Nygård O.K., Lysne V. (2021). Food sources contributing to intake of choline and individual choline forms in a Norwegian cohort of patients with stable angina pectoris. *Frontiers in Nutrition*, 8:676026. https://doi.org/10.3389/fnut.2021.676026

IV. Van Parys A., Brække M.S., Karlsson T., Vinknes K.J., Tell G.S., Haugsgjerd T.R., Ueland P.M., Øyen J., Dierkes J., Nygård O.K., Lysne V. (2021). Assessment of dietary choline intake, contributing food items and associations with one-carbon and lipid metabolites in middle-aged and elderly adults: the Hordaland Health Study. *The Journal of Nutrition,* nxab367. https://doi.org/10.1093/jn/nxab367

Contents

1. Introduction

1.1 Choline

1.1.1 Origin story

The history of choline research started in Paris in 1850 when French chemist and pharmacologist Theodore Gobley isolated a molecule from human brain and carp fish eggs that he called “lecithin”. In 1862, Adolph Strucker investigated the composition of pig and ox bile. He noticed that when boiling the lecithin from this bile a new chemical originated, which he called “choline” from the Greek for bile “chole”. Eventually, it was discovered that lecithin was in fact phosphatidylcholine (PC). How choline is incorporated into PC was described for the first time in 1954 by Eugene Kennedy when he discovered the cytidine 5-diphosphocholine (CDP) pathway. Finally, in 1960 the endogenous pathway for forming PC from phosphatidylethanolamine (PE) via methylation using S-adenosylmethionine (SAM) was identified by Jon Bremer and David Greenberg; the phosphatidylethanolamine-N-methyltransferase (PEMT) pathway [1].

Although first discovered in the 1800s, it took until 1932 before the essentiality of choline was considered when observed that lecithin prevented fatty liver in dogs and rats. However, it was believed that this was only the case in animals and that choline consumption was not required for humans [1]. This view started to change with the observation of decreased plasma choline concentrations in patients receiving total parenteral nutrition (TPN) by Burt *et al.* in 1980 [2]. Later papers demonstrated that these patients also developed fatty liver and liver dysfunction which was attributed to the very low choline concentration in TPN. In 1991, Zeisel *et al.* conducted the first study investigating experimental choline deficiency in healthy adults. Eight healthy volunteers were given a choline-deficient diet for three weeks. Mean plasma choline concentration decreased by 30% and the volunteers developed fatty liver and liver dysfunction. All of this was reversed by reintroducing choline, as choline chloride capsules, into the diet [3]. Finally, in 1998, the Food and Nutrition Board of the US Institute of Medicine (currently known as the National Academies of Medicine

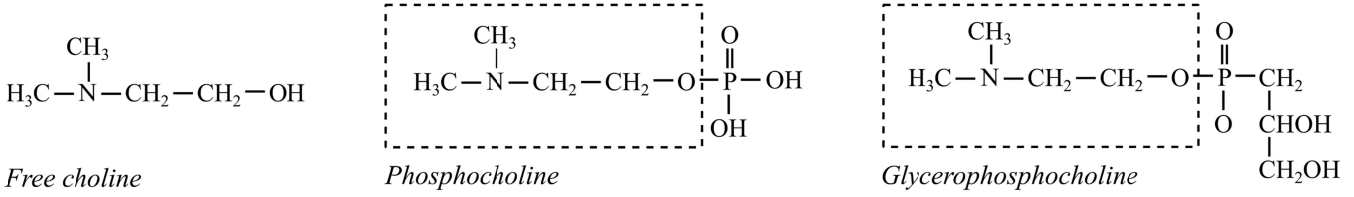
[NAM]) published a recommended Adequate Intake (AI), thereby recognizing choline as an essential nutrient, although based on limited evidence [4]. Eighteen years later, in 2016, the European Food Safety Authority (EFSA) followed and published an AI for choline as well [5]. As per today, no dietary recommendations for choline exist in the Nordic countries [6]. However, it will be included in the next version of the Nordic Nutrition Recommendations 2022.

1.1.2 Absorption and distribution

Intestinal absorption

Choline (molecular weight 104 g/mol) can be found in various forms. In the diet, it is provided as lipid-soluble forms (PC, lysophosphatidylcholine [lysoPC], and sphingomyelin [SM]) and water-soluble forms (free choline, phosphocholine, and glycerophosphocholine [GPC]) [7]. The chemical structures of the choline forms are depicted in **Figure 1**. Typically, lipid-soluble forms are mainly found in animal-derived food items such as eggs, beef, chicken, and milk while water-soluble forms are mainly obtained from plant-based food items [8].

Water-soluble choline forms



Lipid-soluble choline forms

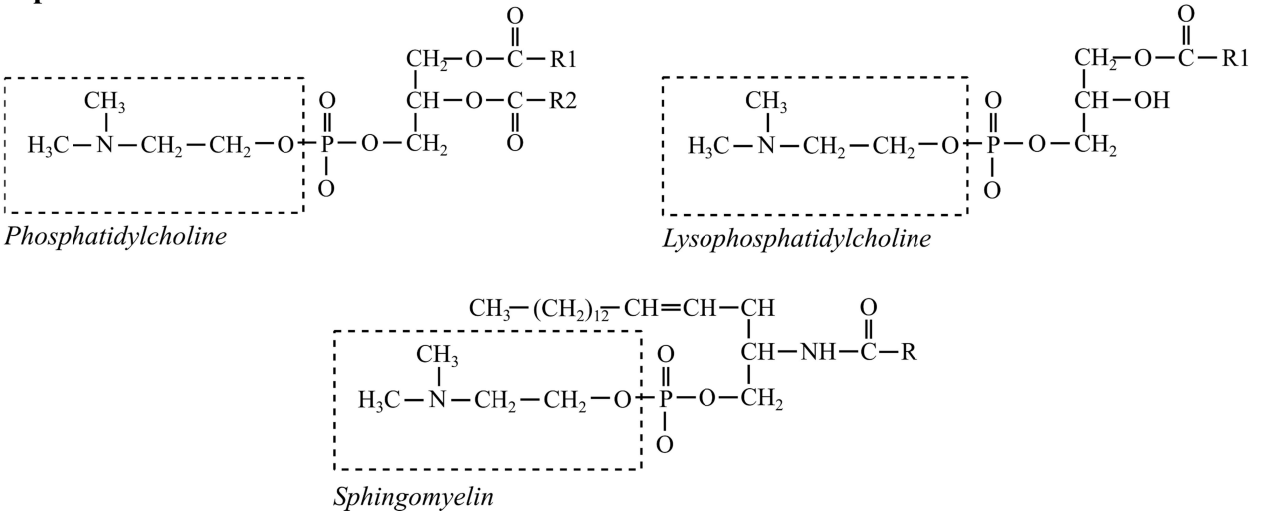


Figure 1: *Chemical structures of water and lipid-soluble choline forms. The dashed box indicates free choline. R indicates a fatty acid chain. (Adapted from Wiedeman et al.* [7]*)*

Dietary choline is absorbed in the jejunum and ileum. However, some dietary choline is metabolized to betaine or methylamines (e.g., trimethylamine [TMA]) by gut bacteria. The digestion and absorption of water and lipid-soluble choline forms differ considerably, and more is known about the latter. Pancreatic and mucosal enzymes digest the water-soluble forms thereby releasing free choline. This free choline enters the enterocyte via choline transporter-like protein 1 (CLT1), also known as solute carrier family 44 member 1 (SLC44A1), a saturable organic cation transporter. Choline transport relies on facilitated diffusion depending on the choline concentration and the electrical potential across the cell membrane. Due to its crucial role, choline absorption is restricted by CLT1 capacity. So far, no data is available on choline bioavailability and percentage of intestinal absorption in humans [5, 9]. In the enterocyte, free choline can be irreversibly oxidized to betaine, or it may enter the portal circulation and be transported to the liver. Free choline enters hepatocytes via the abovementioned transporter. In the hepatocyte, most choline enters the CDP-pathway by phosphorylation and is eventually used to synthesize PC for membranes and lipoproteins [4, 10].

About half of the ingested PC is hydrolyzed in the intestinal lumen by pancreatic phospholipase A2 to lysoPC and free fatty acids. Once in the enterocyte, lysoPC is either degraded to GPC and further to free choline which enters the portal circulation, or reacetylated to PC by lyso-PC-acyl-CoA-acetyltransferase 3. This PC is incorporated in chylomicrons, which enter the lymphatic system, bypass the first-pass metabolism of the liver, and reach other peripheral organs such as adipose and muscle tissue. The other half of ingested PC and about half of the dietary SM remain undigested and enter the lymphatic system in chylomicrons. Once arrived in the liver, PC is crucial for hepatic synthesis of lipoproteins [10–12].

Distribution to tissues

Choline can be distributed in various tissues by three different transporter mechanisms: high-affinity choline transporter 1 (CHT1), polyspecific organic cation transporters (OCT1-3), and choline transporter-like proteins (CTL1-5). Each transporter has specific characteristics regarding choline affinity, sodium dependence, and tissue

distribution [13]. CHT1, also known as solute carrier family 5 member 7, is present in the presynaptic terminals of cholinergic neurons and other non-neuronal cholinergic cells. It has a high affinity for choline and is part of the rate-limiting step in acetylcholine synthesis in cholinergic neurons. CHTs are regulated by neuronal depolarization, second messengers, and acute drug treatment [5, 13, 14]. Low-affinity choline transporters, such as OCT1-3 and CTL1-5 primarily supply choline for the synthesis of PC or other phospholipids [13]. OCT1-3 (or SLC22A1-3) is predominantly expressed in the kidneys and liver while CTL1-5 (or SLC44A1-5) is found in a wide variety of tissues, such as the central nervous system, muscle, and heart [5, 13, 14]. CTL1 and CTL2 are expressed in the blood-brain barrier, and play a vital role in a variety of functions of the central nervous system by facilitation choline transport across the blood-brain barrier. [13].

Plasma choline concentration

Plasma choline concentration, i.e., plasma free choline, has been associated with a range of biological and lifestyle factors including age, sex, gene polymorphisms, smoking, exercise, folate status, kidney function, and body composition. It has been shown that plasma choline levels increase 10-15% after a meal and are particularly responsive to large intakes of dietary choline sources [15]. However, it has previously been reported that long-term dietary choline intake is not associated with plasma choline levels [16–18]. A possible explanation could be that plasma levels are tightly regulated and that endogenous synthesis changes depending on dietary choline intake [19]. Indeed, after a seven-day fasting period, plasma choline concentration only decreased modestly while plasma PC concentration remained unchanged in healthy volunteers [20]. However, the mechanisms determining plasma choline concentration remain to be fully elucidated.

1.1.3 Choline metabolism: four main fates

In general, choline metabolism can be divided into four main pathways involving the synthesis of betaine, phospholipids, trimethylamines, and acetylcholine [7]. Choline is a major methyl group source in the diet and is linked to the folate-mediated one-carbon metabolism via betaine. Choline is also vital for normal cell functioning as phospholipids are essential to cellular structure. Additionally, adequate dietary choline is needed for hepatic lipid homeostasis. In the intestine, TMA can be formed directly from undigested choline and is metabolized in the liver after absorption. Finally, it also affects cholinergic neurotransmission via acetylcholine synthesis [21, 22]. An in-depth overview of these four main pathways is provided in **Figure 2**.

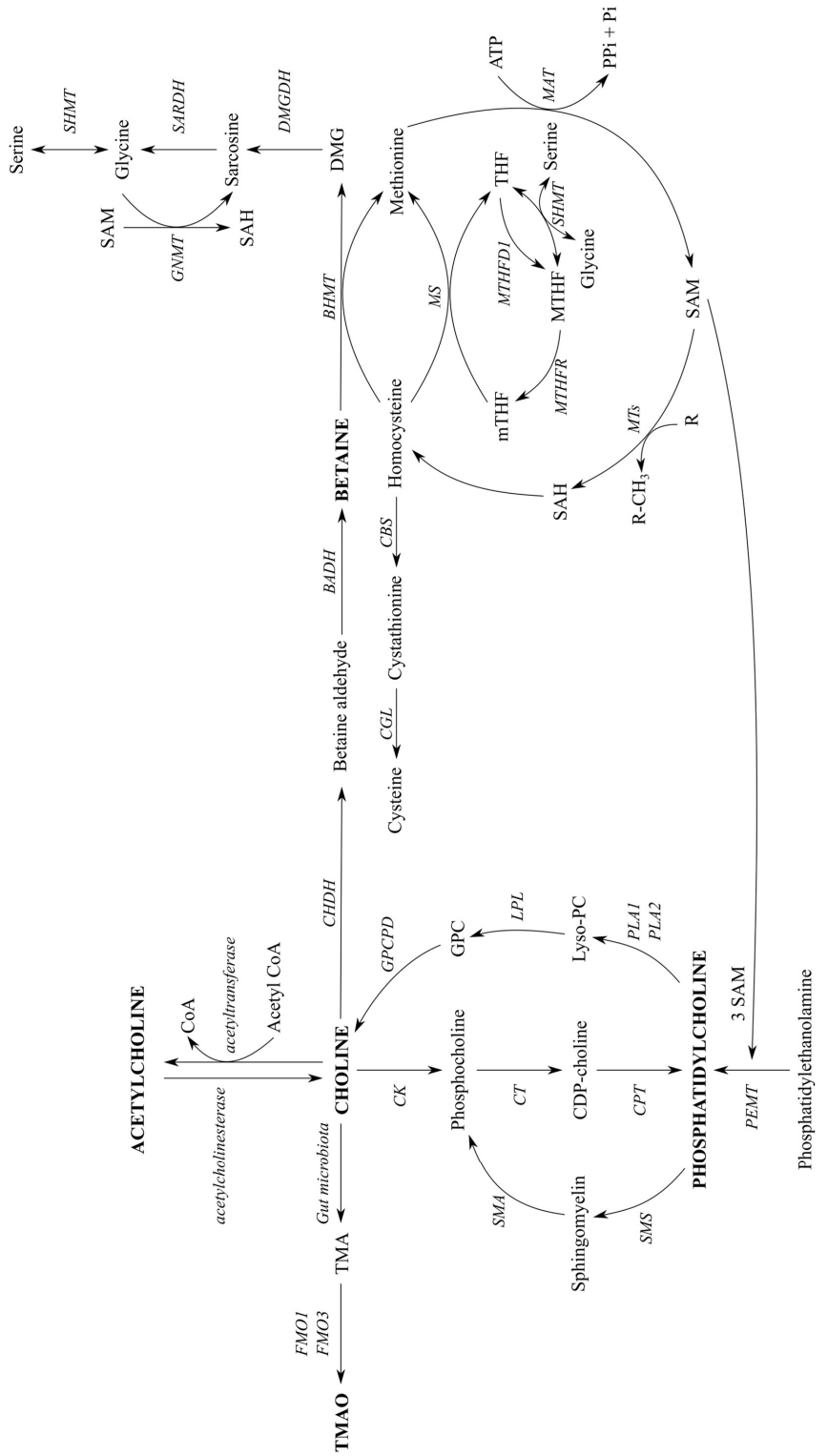

Serine
SHMT
Glycine
SARDH
Sarcosine
DMGDH
DMG
SAM
GNMT
SAH
BHMT
Methionine
ATP
MAT
PPi + Pi
MS
THF
Serine
SHMT
MTHFD1
MTHF
Glycine
mTHF
MTHFR
SAM
ACETYLCHOLINE
CoA
acetyltransferase
Acetyl CoA
acetylcholinesterase
CHOLINE
CHDH
Betaine aldehyde
BADH
BETAINE
CBS
Homocysteine
Cysteine
CGL
Cystathionine
MTs
R
SAH
R-CH3
GPCPD
GPC
LPL
Lyso-PC
PLA1
PLA2
Phosphocholine
CK
CT
CDP-choline
CPT
PHOSPHATIDYLCHOLINE
3 SAM
PEMT
Phosphatidylethanolamine
Gut microbiota
TMA
FMO1
FMO3
TMAO
SMA
SMS
Sphingomyelin

Figure 2: *Overview of the four main pathways in the choline metabolism*

ATP: Adenosine triphosphate; BADH: Betaine aldehyde dehydrogenase; BHMT: Betaine-homocysteine S-methyltransferase; CBS: Cystathionine-β-synthase; CGL: Cystathionine-γ-lyase; CHDH: Choline dehydrogenase; CK: Choline kinase; CPT: CDP-choline: 1,2-diacylglycerol cholinephosphotransferase; CT: CTP:phosphocholine cytidylyltransferase; DMG: Dimethylglycine; DMGDH: Dimethylglycine dehydrogenase; FMO: Flavin-dependent monooxygenase; GNMT: Glycine N-methyltransferase; GPC: Glycerophosphocholine; GPCPD: Glycerophosphocholine-phosphodiesterase; LPL: Lysophospholipase; MAT: Methionine adenosyl transferase; MS: Methionine synthase; mTHF: 5-methyltetrahydrofolate; MTHF: 5,10-methylenetetrahydrofolate; MTHFD1: Methylenetetrahydrofolate dehydrogenase 1; MTHFR: Methylenetetrahydrofolate reductase; MTs: Methyltransferases; PEMT: Phosphatidylethanolamine-N-methyltransferase; PLA1/3: Phospholipase A1/3; SAH: S-adenosylhomocysteine; SAM: S-adenosylmethionine; SARDH: Sarcosine dehydrogenase; SHMT: Serine hydroxymethyltransferase; SMA: Sphingomyelinase; SMS: Sphingomyelin synthase; THF: Tetrahydrofolate; TMA: Trimethylamine; TMAO: Trimethylamine N-oxide

Phospholipid synthesis

The first and main fate of choline is phospholipid synthesis. Both endogenous and exogenous choline molecules are mainly converted to PC, an essential phospholipid in mammalian cells and tissues that accounts for approximately 95% of the total choline pool [23].

The major pathway for PC synthesis from choline, the CDP-pathway, was first discovered in the 1950s by Eugene Kennedy and is often referred to as the “Kennedy pathway” [24]. Upon entry into the cell, choline is phosphorylated to form phosphocholine, a reaction catalyzed by choline kinase (CK) (**Figure 2**) [23]. Even though it is the first step in the CDP-pathway, it is not rate-limiting and does not regulate PC synthesis. The rate-limiting step is the second one, where CDP-choline is

generated from phosphocholine by CTP:phosphocholine cytidylyltransferase (CT) [25]. CT activity is mainly regulated by the reversible movement of CT on and off the endoplasmic reticulum (ER) and/or the nuclear membrane. When bound to either of these, CT is active. Otherwise, it appears as an inactive, soluble form [26, 27]. In the third step, conversion of CDP-choline leads to PC formation enabled by CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT), a membrane protein mainly found on the ER [25]. The supply of CDP-choline and diacylglycerol regulates this final step [19].

A second pathway for PC synthesis is the PEMT pathway which comprises three sequential methylations of PE by PEMT with SAM as a methyl donor [28]. Based on findings from animal studies, it is estimated that this pathway accounts for about 30% of hepatic PC synthesis [29]. The PEMT pathway occurs predominantly in the liver, which is the only mammalian organ where it is quantitatively significant [19].

In the liver, the synthesized PC can be incorporated in very-low-density lipoprotein (VLDL), which regulates the release of this phospholipid from the liver. The PC can afterward be transferred to high-density lipoprotein (HDL) in plasma [12]. Thus, PC is essential for triglyceride and cholesterol export from the liver through assembly and secretion of VLDL. Decreased hepatic PC, either due to a choline-deficient diet or impaired biosynthesis, leads to impaired VLDL secretion from the liver, fat accumulation (steatosis), and eventually non-alcoholic fatty liver disease (NAFLD) [10, 12, 30, 31]. Partial loss of PEMT activity due to a genetic variant in the human PEMT gene is found more frequently in patients with NAFLD, making PEMT activity an important predictor of NAFLD in humans [32]. Further, decreased hepatic PC results in reduced plasma HDL levels through inhibition of hepatic HDL formation and increased HDL cholesterol uptake from the circulation [30]. Finally, the composition of PC generated through the PEMT pathway differs from that generated by the CDP-pathway, as the former is rich in long-chain polyunsaturated fatty acids such as docosahexaenoic acid (DHA) and arachidonic acid [33]. Indeed, PEMT activity is crucial for mobilizing these essential fatty acids from the liver to the plasma thereby delivering them to peripheral tissues [34]. DHA is fundamental for normal

development and function of the brain and low cerebral concentrations have been associated with several adverse neurological outcomes such as Alzheimer's, Parkinson's, schizophrenia, and depression [35]. The current body of evidence suggests that adequate dietary choline and DHA consumption is required to maintain PC-DHA levels in both plasma and brain, as lysoPC is the preferred DHA carrier across the blood-brain barrier [36], and might slow the onset of adverse neurological outcomes [37].

Since PC is the major constituent of mammalian cellular membranes, it is involved in a wide variety of biological functions [30]. Besides being a vital element of lipoproteins, as discussed above, PC can also be incorporated and secreted into bile [38]. This makes PC important for intestinal fatty acid uptake and chylomicron secretion. Additionally, PC is not only linked to lipid metabolism via lipoprotein synthesis and bile but is also a vital component, together with PE, of the surface of lipid droplets [39]. PC is also thought to be involved in *de novo* lipogenesis via inhibition of sterol regulatory element-binding proteins which regulate expression of genes involved in fatty acid, phospholipid, and triacylglycerol synthesis [39]. Apart from lipid metabolism, PC seems to be involved in other metabolic processes. It is for example a major constituent of lung surfactant and the intestinal brush border and the phospholipid content of muscles affects their insulin sensitivity [30, 39]. Interestingly, it is thought that the PC/PE ratio in the mitochondrial membrane can modulate mitochondrial energy production. Mitochondrial phospholipids may play a role in programmed cell death, autophagy, and mitochondrial fusion. As mitochondrial function has been associated with cardiovascular disease (CVD), diabetes mellitus, cancer progression, and neurodegenerative disease, it is not unlikely that PC plays a role in them as well [39].

Betaine and the one-carbon metabolism

The second main fate of choline is irreversible oxidation to betaine by the enzymes choline dehydrogenase (CHDH) and betaine aldehyde dehydrogenase (BADH) in the mitochondria [40, 41]. Betaine can also be obtained directly from the diet from e.g., wheat bran, wheat germ, beets, and spinach [40]. Intracellular betaine is an osmolyte

and regulates cell volume and thus tissue integrity and stabilizes proteins. Most importantly, betaine is a methyl donor and hereby links choline to the one-carbon metabolism [22, 40, 41]. The one-carbon metabolism is a set of biochemical enzymatic reactions where one-carbon groups such as methyl and formyl, are transferred between compounds. Central metabolic pathways include the choline oxidation pathway, the folate cycle, the methionine-homocysteine cycle, and the transsulfuration pathway.

The choline oxidation pathway begins, as mentioned above, with the oxidation of choline to betaine which can act as a methyl donor for homocysteine (Hcy) remethylation to methionine catalyzed by betaine-homocysteine S-methyltransferase (BHMT) (**Figure 2**). This step links the choline oxidation pathway directly to the methionine-homocysteine cycle and occurs primarily in the liver and kidney [40]. As it donates a methyl group, betaine is converted to dimethylglycine (DMG) which is further metabolized in the mitochondrion by dimethylglycine dehydrogenase (DMGDH) to sarcosine and finally by sarcosine dehydrogenase (SARDH) to glycine [42]. Glycine is a precursor for a wide range of biosynthetic pathways including glutathione, purine, creatine, and heme synthesis [43]. When exogenous glycine, i.e. glycine from the diet, is unavailable it may be provided by (reversible) conversion of serine in a reaction catalyzed by serine hydroxymethyltransferase (SHMT) [44]. This makes serine essential when no exogenous glycine is available for purine and glutathione synthesis [45]. Additionally, serine can be synthesized from glucose providing a route from carbohydrates to glycine [43]. Another vital fate of glycine is decarboxylation by the glycine cleavage system in the mitochondria which transfers one carbon unit from glycine to tetrahydrofolate (THF), generating 5,10-methylene THF (MTHF) which can enter the folate cycle in the cytosol [46].

The methionine-homocysteine cycle is another major component of the one-carbon metabolism and links choline to Hcy, methionine, folate, and B-vitamins. Hcy is a nonproteinogenic sulfur-containing amino acid formed from the essential amino acid methionine in a multistep pathway [47]. The first step is the formation of SAM, a universal methyl donor, from methionine and adenosine triphosphate (ATP). After donating its methyl group to acceptor molecules such as DNA, RNA, proteins, amino

acids, etc., S-adenosyl homocysteine (SAH) is formed, a reaction catalyzed by several methyltransferases. Finally, SAH undergoes deadenosylation resulting in the formation of Hcy [48]. The formed Hcy can subsequently be remethylated back to methionine using either a methyl group from 5-methyltetrahydrofolate (mTHF) or betaine. The former is catalyzed by methionine synthase (MS) and requires methylcobalamin (vitamin B12) as a cofactor, while BHMT is the responsible enzyme in the latter [47, 49]. MS is found in all cells, while BHMT is tissue-specific and is found in high concentrations in the liver and kidneys [49]. Alternatively, Hcy can be permanently catabolized by aggregating with serine to form cystathionine, a route known as the transsulfuration pathway, in a reaction catalyzed by cystathionine β-synthase (CBS) [48]. Finally, cystathionine can be further reduced to cysteine by cystathionine-γ-lyase (CGL) [50]. Both enzymes require pyridoxal-5-phosphate (vitamin B6) as a cofactor [49].

The last component of the one-carbon metabolism is the folate cycle, the intracellular metabolism of folate. The active form of folate is THF which can receive a one-carbon molecule from either serine via a reversible reaction catalyzed by SHMT or from formate via methylenetetrahydrofolate dehydrogenase 1 (MTHFD1) [43, 49, 51]. The former results in MTHF directly, while the latter results in 10-formyl THF which can be dehydrogenated by MTHFD1 to form MTHF. This MTHF is reduced to mTHF via MTHF reductase (MTHFR), an enzyme that requires riboflavin (as flavin adenine nucleotide) as a cofactor [49]. Finally, mTHF is used for remethylation of Hcy to methionine as mentioned earlier, resulting in THF.

As a precursor of the universal methyl donor, SAM, choline plays a role in the regulation of gene expression via epigenetic mechanisms such as DNA or histone methylation [41, 52]. In 1984, it became clear that choline could modify DNA methylation when Wilson *et al.* observed that rats fed a diet very low in choline and methionine had decreased hepatic DNA methylation [53]. This was later found to be correlated with changes in the expression of a wide range of hepatic genes [54] and was even linked to the spontaneous development of liver carcinoma [55]. Nutri-epigenomics is an up-and-coming discipline exploring the role of dietary influences on

gene expression. Even though in its infancy, understanding how nutrients such as choline affect epigenetics is important to comprehend both developmental abnormalities and the origin of chronic illnesses [41, 52].

Trimethylamines

The third possible fate of choline is bacterial conversion in the cecum and the colon to TMA [56] (**Figure 3**). Other choline-containing compounds (e.g., PC), betaine, and L-carnitine and the L-carnitine metabolite γ-butyrobetaine (γ-BB) are also known to be TMA precursors in the gut [57, 58]. Gut microbiota plays an essential role in the conversion of precursors as gnotobiotic mice do not produce TMA [56, 59], and treating healthy mice with antibiotics decreases TMA production [56]. Additionally, TMA can be directly obtained from dietary sources such as fish, which is also rich in trimethylamine N-oxide (TMAO) [60]. Finally, γ-BB can be synthesized endogenously from trimethyllysine (TML) and converted to L-carnitine and potentially TMA and TMAO [61].

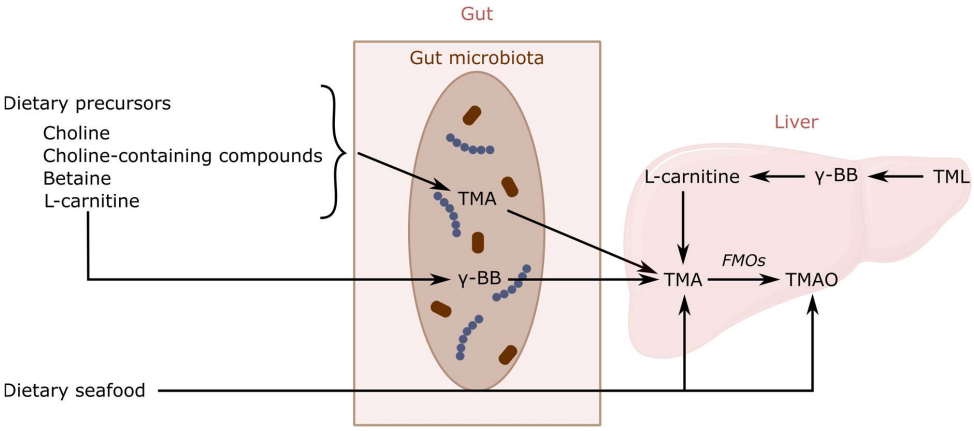


***Figure 3**: Overview of various pathways contributing to trimethylamine N-oxide synthesis. γ-BB: γ-butyrobetaine; FMOs: flavin monooxygenases; TMA: trimethylamine; TMAO: trimethylamine N-oxide; TML: trimethyllysine.*

After absorption from the intestine via passive diffusion into the portal circulation, TMA undergoes oxidation to TMAO in the liver, a reaction modulated by flavin-dependent monooxygenase (FMO) isoforms 1 and 3 [62]. The specific activity of

FMO3 is ten times higher than FMO1 activity in the liver [62] and mutations in FMO3 lead to excretion of TMA in breath and sweat, known as fish-odor syndrome or trimethylaminuria [63]. TMAO is a known osmolyte in tissues, a molecular chaperone for protein stabilization which is crucial for maintaining enzyme activity and regulates various aspects of cholesterol and sterol metabolism [57, 62, 64, 65]. About a decade ago, TMAO was first linked to CVD [57]. This association will be discussed in depth in *Chapter 1.3 Dietary choline and cardiovascular disease*.

Several environmental and host factors, dietary intake, and host genetics are thought to influence circulating TMAO levels, however, there is limited human population-based evidence so far. Recently, fish, red meat, and egg intake have been associated with serum TMAO levels [66]. However, the results of several studies assessing the association between dietary intake and circulating TMAO levels were inconclusive, leaving the major dietary contributors to TMAO open for investigation [67–70]. Also, the specific gut bacteria taxa metabolizing nutrients into TMA remain unidentified in humans as does the potential interaction between dietary factors and gut microbiota and its effect on circulating TMAO levels. Recently, Mei *et al.* identified several gut microbial species that might produce TMA to be positively associated with serum TMAO and they speculated that their observed relationship between red meat intake and TMAO levels might be depending on these species [66].

Acetylcholine

Finally, the fourth fate of choline takes place in neuronal tissues, where choline is, together with acetyl-coenzyme A, essential for the formation of the neurotransmitter acetylcholine. This process, catalyzed by acetyltransferase, takes place in the presynaptic terminal of cholinergic neurons both in the central and peripheral nervous systems. After release in the synaptic cleft, acetylcholine is broken down by acetylcholinesterase to acetate and choline, which is taken up again in the presynaptic nerve ending [71]. Neuronal choline uptake relies on CHT capacity which is the major limiting factor for acetylcholine synthesis [12, 71]. However, acetylcholine formation is also modulated by dietary choline intake [72]. Choline from the circulation crosses the blood-brain barrier via CTL1 and 2 at a rate proportional to blood choline

concentration [13]. This is the only neuronal choline source as *de novo* synthesis does not occur in nervous tissue [73].

1.1.4 Dietary recommendations

In 1998, the first AI for choline was published by the Food and Nutrition Board of the US Institute of Medicine (currently known as NAM) [4]. Their AI was based on the abovementioned depletion-repletion study conducted in eight healthy male volunteers. After three weeks on a diet deficient in choline but with adequate amounts of methionine, folate, and vitamin B12, plasma choline and PC values dropped and serum alanine aminotransferase, a marker of liver damage, increased compared to the control group. Plasma choline and PC increased and ALT values returned to baseline when choline, as choline chloride capsules, was reintroduced in the diet [3]. Additionally, it has been shown that individuals receiving TPN deficient in choline but adequate in methionine and folate developed fatty liver and liver damage defined by increased ALT activity. In some individuals, this was resolved after a source of choline was provided [74–78]. In the depletion-repletion study, the choline dose that prevented ALT abnormalities was 500 mg/d [3], which agrees with findings from the TPN studies [74, 78]. However, this amount still led to decreased plasma choline concentrations due to which the AI was finally set at 550 mg/d for adult males (equals 7 mg/kg/d for an average male of 76 kg). The AI for women was based on the same data and was set to 425 mg/d. However, based on animal data, the choline need for pregnant women was thought to be higher due to the large amounts of choline being transported from the mother to the fetus. Therefore, the AI for pregnant women was set at 450 mg/d. Choline is particularly important during embryogenesis and perinatal development of the fetus e.g., for brain development [52]. Also, high maternal choline intake by itself or together with intake of other one-carbon nutrients is associated with decreased risk of neural tube defects [79–82]. Finally, animal research has also shown long-lasting positive effects of maternal choline intake on cognitive function in offspring, however, this remains debated in humans [37, 52, 83, 84]. The choline requirements for lactating women increased due to the substantial amount of choline in human breast milk. According to available data at the time, human breast milk contained 160-210 mg/L choline [4]. Since human milk is the main choline source for 0–6-month-old infants,

the AI set for this age category reflects the observed mean intake of choline by this group. An overview of AI per age category is presented in **Table 1**. Due to the limited scientific evidence, an average requirement (AR) or population reference intake (PRI) has not been derived in the existing dietary guidelines.

Table 1: Dietary recommendations for choline

	NAM – 1998 [4]			EFSA – 2016 [5]	
Life stage	**Age**	**AI (mg/d)**		**Age**	**AI (mg/d)**
		Males	**Females**		
Infants	0-6 mo	125	125	0-6 mo	120
	7-12 mo	150	150	7-11 mo	160
Children	1-3 y	200	200	1-3 y	140
	4-8 y	250	250	4-6 y	170
	9-13 y	375	375	7-10 y	250
	14-18 y	550	400	11-14 y	340
				15-17 y	400
Adults	≥19 y	550	425	≥18 y	400
Pregnancy	-	-	450	-	480
Lactation	-	-	550	-	520

AI: adequate intake; EFSA: European Food Safety Authority; mo: months; NAM: National Academies of Medicine; y: years

In 2016, EFSA issued an AI for choline based on observed mean intakes in healthy populations from 12 national surveys in nine (former) EU countries (Finland, France, Germany, Ireland, Italy, Latvia, the Netherlands, Sweden, and the United Kingdom) between 2000 and 2011 [85]. Reported mean estimated total choline intakes ranged from 269-468 mg/d in adults with men having slightly higher reported intakes compared to women (332-468 mg/d vs. 269-404 mg/d, respectively) [85]. Data for infants (<1 year) were available from three surveys, for children aged 1-3 years from four surveys, and seven surveys for older children [5]. EFSA set the AI of 400 mg/d based on these studies, supported by one depletion-repletion study which showed that

this amount of choline was adequate to reverse organ dysfunction due to choline deficiency in 70% of the study subjects [86]. In the end, choline recommendations set by EFSA are slightly lower than the AI advised by NAM (**Table 1**), but it must be noted that both AIs are based on very limited scientific evidence which did not allow either institution to publish an AR or PRI. Until today, there are no dietary recommendations for choline in the Nordic countries [6], but an evaluation of choline will be included in the new Nordic Nutrition Recommendations published in 2022.

Factors influencing dietary requirements in humans

Several factors can influence an individual's choline requirement. As mentioned earlier, sex is one of them. Choline requirements are higher for men and postmenopausal women compared to premenopausal women. This is most likely due to the ability of estrogen to increase *de novo* choline synthesis via the PEMT pathway. Indeed, the promoter region for the PEMT gene is estrogen-responsive, making premenopausal women more resistant to developing organ dysfunction when on a low-choline diet [87]. Pregnancy and lactation increase the need for choline due to increased transport from mother to fetus or breast milk [4]. Thirdly, since the choline, folate, methionine, and vitamin B12 metabolisms are so interrelated, disturbances in the availability of one nutrient result in compensatory changes in the others [19]. Further, as betaine is a methyl donor, it has a choline-sparing effect and might therefore influence an individual's choline requirement (see *1.1.3 Choline metabolism: four main fates*) [40, 41]. Finally, genetic variability is known to impact choline requirements. Indeed, several enzymes from the one-carbon metabolism are encoded by genes that have single nucleotide polymorphisms (SNPs) leading to alterations in gene expression or enzyme activity [88]. For example, a genetic variation in the MTHFD1 1958A allele (rs2236225) has been linked to the risk of muscle damage and elevated hepatic fat content. Premenopausal women with this SNP were 15 times more likely to develop signs of choline deficiency on a low-choline diet compared to non-carriers [89].

Current dietary intake

So far, dietary choline intake has been estimated in only some cohorts globally, mainly in European and North American countries. As mentioned earlier, dietary choline intake ranged from 332-468 mg/d in men and from 269-404 mg/d in women in nine European countries [85]. Similar findings have been observed in the US where estimated mean intakes ranged from 258-323 mg/d in women and 302-405 mg/d in men [90–94]. Interestingly, all studies concluded that most individuals did not achieve the AI for choline set by respectively EFSA or NAM. To the best of our knowledge, dietary choline intake has only scarcely been studied in Norway besides the findings reported in our studies [17, 95]. It has to be mentioned that choline is not included in most food composition databases, including the Norwegian one (www.matvaretabellen.no), thereby complicating the estimation of dietary choline intake. Choline content of local foods is often not available, and the choline content of available foods might differ due to geographical differences.

1.1.5 Dietary choline sources

A wide variety of foods contain a significant amount of choline or choline-containing compounds [8]. Eggs, beef, chicken, milk, and certain plant foods such as cruciferous vegetables and several types of legumes are food items high in choline. Consumption of the individual choline forms depends on the dietary pattern as animal-based foods contain mainly lipid-soluble choline forms while plant-derived foods are rich in water-soluble forms. Additionally, animal products contain more total choline per unit weight compared to plant products [96].

Of all the choline forms, PC is the main form in foods, both in the form of PC or in the form of lecithin, a PC-rich fraction originating during commercial purification of phospholipids. Lecithin is a commonly used emulsifying agent often added to foods or can be taken as a supplement [97]. **Figure 4** shows the total choline and PC content in selected food items illustrating the differences between choline sources.

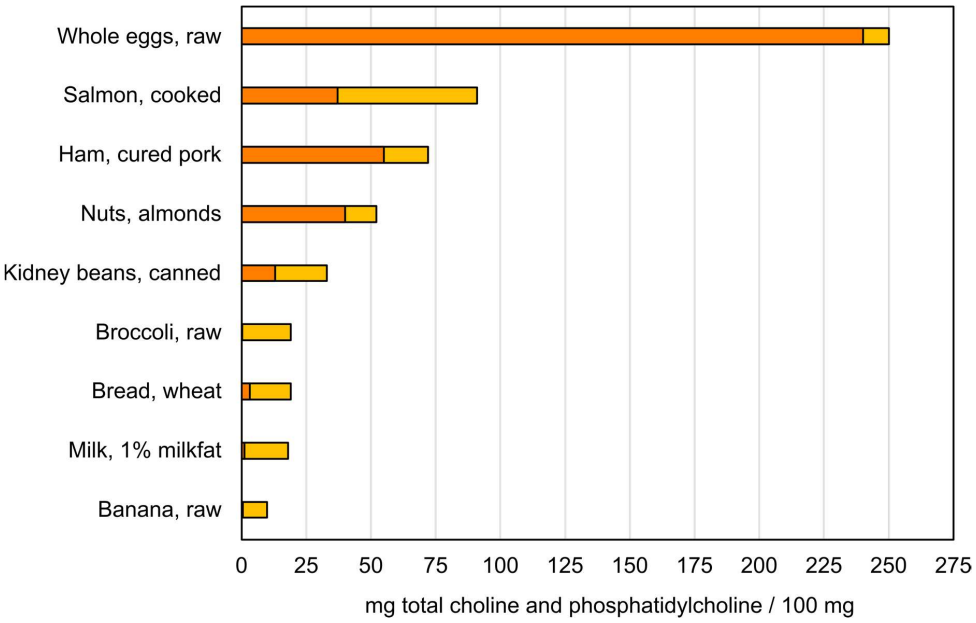


Figure 4: *Total choline and phosphatidylcholine content per 100 mg in a selection of food items. The phosphatidylcholine content is marked orange, while the remaining amount of choline is depicted in yellow. Source: Bachelor thesis Hanna Olberg "Dietary choline and cognitive function and decline in adults". Permission for publication was obtained from the author.*

Moreover, food preparation methods influence the presence of the different choline forms. For example, cooking vegetables and most pulses reduces the concentration of water-soluble choline forms and, for pulses, increases the PC content [97, 98]. Unfortunately, not much is known about the effect of preparation method and duration in other food items but it may affect choline forms and content as well [10]. Finally, multi-component foods such as mixed dishes or fast food can differ substantially in total choline content due to differences in recipes, inconsistency in the formulation used by the manufacturers, or variation in choline content of individual ingredients [8].

Besides choline, foods also contain betaine which cannot be converted to choline but, as it is a methyl donor, has a choline-sparing effect (see *1.1.3 Choline metabolism: four main fates*) [40, 41]. Rich sources of betaine are typically plant-derived foods,

especially grain products [8]. This might also be true for folate and vitamin B12, as a diet deficient in either of these nutrients increases choline demand and is often associated with increased plasma Hcy [88, 99]. Additionally, rats on a choline-deficient diet have a higher folate demand [100]. However, patients receiving TPN without choline but with adequate methionine and folate still develop fatty liver and liver damage [74–77]. Also, healthy humans with normal folate and vitamin B12 status fed a choline-deficient diet still developed liver damage [3]. It seems that these mechanisms are only able to compensate for a certain degree of choline deficiency.

1.2 Cardiovascular disease

CVD is a collective term for conditions affecting the circulatory system and includes coronary heart disease (CHD), cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis, and pulmonary embolism [101]. The most common cause of CVD is atherosclerosis, a process defined by the subendothelial accumulation of lipoproteins, immunocyte infiltration, inflammation of the arterial wall, and endothelial dysfunction [102], leading to the build-up of plaque in the artery walls. Major modifiable risk factors for atherosclerosis include smoking, lack of physical activity, overweight and obesity, insulin resistance, diabetes, high blood pressure, and dyslipidemia, characterized by elevated serum total and low-density lipoprotein (LDL) cholesterol, decreased HDL cholesterol, and changes in the main lipoprotein levels accordingly [103]. Note that many of these risk factors are diet-related. CHD or ischemic heart disease occurs when these plaques are located in the walls of the arteries that supply the heart with oxygen-rich blood (coronary arteries) [104]. Acute myocardial infarction (AMI) is the most severe manifestation of CHD resulting in cardiac ischemia and irreversible damage to the heart muscle [105].

In 2019, ischemic heart disease was the leading cause of mortality globally, responsible for 16% of all deaths which equals 8.9 million people [106]. A report from the American Heart Association from 2017 projected the prevalence and economic cost of CVD in the United States through 2035. In 2015, 41.5% of the US population (102.7

million people) had at least one CVD condition which is thought to increase to 45% or 131.2 million Americans in 2035. This goes hand in hand with a colossal economic burden due to medical and indirect (e.g., loss of productivity) costs summing $555 billion in 2016 and an estimated $1.1 trillion by 2035 [107]. In Norway, 21% of the population had established CVD in 2020 or was at high risk for developing it. Although CVD mortality has drastically decreased since the 1970s due to better screening, medical and technological development, and a decrease in risk factors such as smoking, it is expected that the number of people living with CVD will increase over the coming years. This increase is caused by the growing proportion of elderly people in the population and higher CVD survival rates [108].

According to the World Health Organization (WHO), the most important behavioral risk factors of CVD are unhealthy diet, physical inactivity, tobacco use, and harmful use of alcohol [101]. A healthy diet as defined by the Nordic Nutrition Recommendations is described as rich in vegetables, pulses, fruits and berries, nuts and seeds, whole grains, fish and seafood, vegetable oils, and low-fat dairy products and low in processed and red meat, foods with low nutrient density and high added sugar, fat and salt content [6]. The current dietary recommendations and especially the food-based dietary guidelines are made to reduce the risk of chronic diseases including CVD. However, a comprehensive evaluation of the relationship between diet and CVD is beyond the scope of this thesis.

1.3 Dietary choline and cardiovascular disease

Dietary choline has been linked to CVD risk via several mechanisms and/or metabolites such as TMAO, lipid metabolism, and Hcy. Especially the relationship between dietary choline and TMAO and possibly CVD has gained a lot of interest in the past decade [109]. So far, evidence remains scarce and results contradictory despite the growing interest regarding the association between dietary choline and CVD risk. Indeed, a recent systematic review and meta-analysis of prospective studies conducted by Meyer *et al.* found no association between dietary choline and incident CVD risk.

However, the authors emphasize the scarcity of relevant articles and the heterogeneity of the included studies [110].

TMAO

The role of gut microbiota in the relationship between dietary choline and CVD has been described for the first time in 2011 [57]. Dietary choline, and other choline-containing compounds, were converted to TMA in a gut microbiota-dependent manner, and later to TMAO in the liver by FMOs (see *Chapter 1.1.3*). Further, elevated levels of circulating TMAO were associated with CVD risk possibly due to increased atherosclerosis [111]. In atherosclerosis-prone apolipoprotein E (ApoE)$^{-/-}$ mice, Wang *et al.* observed higher foam cell formation due to an increase in cell surface expression of two proatherogenic scavenger receptors: scavenger receptor A and cluster of differentiation 36, when these mice were fed TMAO or its dietary precursors [57]. Additionally, also in ApoE$^{-/-}$ mice, TMAO reduced reverse cholesterol transport [58]. Other possible mechanisms might be elevated platelet activation [112] or prolongation of the hypertensive effect of angiotensin II [113]. However, Aldana-Hernandez *et al.* did not observe any effect of dietary choline, TMAO, or betaine on atherosclerosis development in either ApoE$^{-/-}$ or Ldlr$^{-/-}$ mice [114]. Additionally, Ldlr$^{-/-}$ male mice fed a diet high in PC showed a decrease in atherosclerotic lesions despite a two-fold increase in plasma TMAO levels compared to mice fed a control diet or a diet high in choline. Moreover, circulating proatherogenic cytokine levels were reduced in the PC group, but not in the control nor the choline group suggesting that dietary PC might even decrease atherosclerotic development [115]. Several recent meta and dose-response analyses reported a positive association between plasma TMAO and CVD risk, however, it remains unclear whether this association is causal [109, 116, 117]. Furthermore, TMAO concentration is influenced by the generation of TMA by the gut microbiota, permeability of the gut-blood barrier, oxidation by FMOs, and excretion through the kidney [118]. Kuhn *et al.* reported a high within-person and between-person variation (coefficient of variation 46.7% and 24.7% respectively) in TMAO levels over time, which may discourage the use of TMAO as a risk marker for CVD [68]. Finally, several dietary sources high in TMAO, such as fish, have favorable health

effects in addition to providing a wide range of essential nutrients, e.g. omega-3 fatty acids, necessary for many biological functions [119]. A diet low in TMAO and its dietary precursors may therefore not be recommended based on the current knowledge.

Lipid metabolism

A second relationship between dietary choline and CVD could be via lipid metabolism, however, evidence is limited. Choline is involved in lipid metabolism through PC which is an essential component of VLDL. It is therefore essential for the transport of cholesterol and triglycerides (TG) from the liver to other organs and tissues as described in *Chapter 1.1.3*. Indeed, in vivo induction of the BHMT enzyme in rats fed a betaine-rich diet led to increased apolipoprotein B (ApoB) mRNA levels and elevated secretion of TG and ApoB-containing lipoproteins from the liver, while also decreasing hepatic TG levels. The underlying mechanism is thought to be the induction of *apob* gene expression by dietary betaine [120]. As choline is a precursor of betaine, it is not unthinkable that it might have similar effects. However, it has also been shown that betaine supplementation can increase plasma LDL and TG concentration [121] while also lowering HDL concentration [122], although the latter was not observed for choline supplementation [122]. Further, a recent cross-over study found that intake of three eggs per day or choline bitartrate for 4 weeks showed an increase in plasma TG, LDL, and LDL/HDL ratio, while plasma HDL levels decreased compared to baseline. Total cholesterol increased only after egg intake. Interestingly, when comparing the two interventions, the plasma concentration of total cholesterol, HDL, LDL, ApoA-I, and ApoE, increased more after egg intake compared to choline bitartrate. No difference was observed for changes in LDL/HDL ratio or ApoB concentration [123]. These findings indicate that the relationship between dietary choline and lipid metabolism might be dependent on the choline form. The current evidence is rather limited, and further research is needed to explore this relationship.

Homocysteine

Finally, choline is linked to Hcy through its precursor betaine which is a methyl donor in the remethylation reaction of Hcy to methionine, as mentioned in *Chapter 1.1.3*. Elevated circulating total Hcy (tHcy) concentrations (hyperhomocysteinemia) have

been associated with over 100 different diseases, syndromes, and health outcomes [124]. However, whether this is a causative factor or merely a biomarker of these conditions remains heavily debated [125]. Hyperhomocysteinemia can be caused by a defective methionine metabolism such as defects in key enzymes or lack of vital cofactors such as folate, vitamin B6, B12, and B2, increased methionine intake, a wide variety of drugs, disease state (e.g., renal disease), pregnancy and lactation and changes in cellular Hcy export. Dietary Hcy is not regarded as a significant contributing factor since the concentration in most food items is negligible [48, 124]. Plasma or serum tHcy is a known biomarker for CVD risk and is thought to cause atherosclerotic plaques based on case studies [126]. Indeed, it was later confirmed in animal models and cell cultures that high levels of Hcy damages cells and tissues of arteries via increased release of inflammation mediators, and elevation of oxidative stress which leads to oxidation of LDL and other atherosclerotic plaque constituents [126, 127]. Several studies have observed a tHcy-lowering effect of dietary choline indicating that dietary choline intake could decrease CVD risk [86, 91, 121, 128, 129]. The tHcy-lowering effect is most likely due to increased betaine-dependent remethylation of Hcy or methionine. Another possible mechanism could be a decreased endogenous PC synthesis via PEMT, which lowers the tHcy production from SAM [129]. Finally, reduced synthesis of Hcy in peripheral cells should also be considered. The tHcy reduction is most likely a combined effect of these possibilities.

However, the implication that dietary choline could decrease CVD risk via lowering tHcy assumes that Hcy is a causative factor of CVD which continues to be a matter of dispute illustrated by findings reported by B vitamin supplement studies. A meta-analysis by Clarke *et al.* included eight randomized trials (37 485 individuals) investigating the effect of B-vitamin supplementation on disease risk. Although the supplementation led to lower plasma tHcy, the meta-analysis could not identify an effect of a 25% reduction in plasma tHcy concentration for at least 5 years on risk for CHD, stroke, cancer, or mortality [130]. However, these trials have some inherent limitations. Most importantly, these were secondary prevention studies performed in patients with confirmed CVD and not primary prevention studies including healthy

participants. Other limitations are fortification of the study population, duration of treatment, concomitant diseases, and B-vitamin dosage. These studies and their limitations have been described in detail previously [131, 132]. Finally, these studies investigated the possible tHcy-lowering effect of B-vitamins and its risk for several CVD outcomes, which does not necessarily translate to the association of decreased tHcy due to choline supplementation with CVD.

2. Knowledge gap and rationale for the thesis

Choline has been recognized as an essential nutrient for 23 years and yet our knowledge regarding its intake in the population and dietary requirements remains poor. Although essential, little is known about the global choline status, as dietary intake is only sparsely investigated and reported. The fact that current dietary reference values are restricted to AI and cannot be elaborated to an AR or PRI due to lack of sufficient data painfully illustrates this point. Until now, dietary choline intake in Norway has only scarcely been investigated and no dietary reference values are in place.

The number of available studies is even lower when focusing on the dietary intake of the individual choline forms, while evidence suggests that this might play a role when investigating the relationship with one-carbon and other metabolic pathways. Additionally, the main dietary contributors to total choline intake and intake of the individual choline forms remain to be clarified. This thesis aimed to explore choline intake and its contributors in two Norwegian cohorts, one consisting of patients with CVD and the second one consisting of community-dwelling adults.

Further, it is well known that metabolites of the one-carbon and lipid metabolism are linked to the risk of several major lifestyle diseases, including CVD. Unfortunately, the current understanding of how dietary choline intake influences these metabolisms is very limited. Investigating the relationship between dietary choline and metabolites involved in these vital pathways is therefore of great interest and one of the aims of this thesis. Finally, we aimed to explore the association between dietary choline, as well as individual choline forms, and AMI risk in CVD patients.

3. Study objectives

The overall objectives of the thesis were to investigate dietary choline intake and contributing food items, and to explore the association of choline intake, including individual choline forms, with circulating levels of metabolites related to the one-carbon and lipid metabolism and AMI risk. This was investigated in two Norwegian cohorts where one cohort consisted of patients with pre-existing CVD (stable angina pectoris [SAP]) and the other one of community-dwelling adults.

Specific study objectives:

- To investigate dietary choline intake, including individual choline forms, and to map food items contributing to their intake in patients with SAP (**Paper III**)
- To investigate dietary choline intake, including individual choline forms, and to map food items contributing to their intake in community-dwelling Norwegian adults. Additionally, to investigate the association between total dietary choline intake and circulating levels of metabolites related to one-carbon and lipid metabolism (**Paper IV**).
- To investigate the association between dietary choline, including individual choline forms, and risk of AMI in patients with SAP (**Paper I**).
- To investigate whether there was any effect modification by plasma TMAO or TML on the association between dietary choline intake and risk of AMI observed in Paper I (**Paper II**).

4. Subjects and methods

4.1 Study population and design

4.1.1 Paper I, II & III: The Western Norway B-vitamin Intervention Trial (WENBIT)

Study cohort

Between 1999 and 2004, 3090 adult patients undergoing elective coronary angiography due to suspected CHD were enrolled in the Western Norway B-vitamin Intervention Trial (WENBIT, ClinicalTrials.gov Identifier: NCT00354081) performed at Haukeland University Hospital, Bergen and Stavanger University Hospital, Stavanger in Norway. The WENBIT study was a randomized, double-blind, placebo-controlled prospective secondary prevention study investigating the effect of vitamin B treatment on mortality and cardiovascular outcomes [133]. For the studies included in this thesis only patients diagnosed with SAP were included (n = 2573). Exclusion criteria were missing dietary data, including choline intake (n = 565), extreme energy intake (<3000 kJ or >15000 kJ for women and <3300 kJ or >17500 kJ for men) (n = 27). In **Paper III** we additionally excluded patients with ≥10 E% from alcohol (n = 52), resulting in 1981 patients eligible for analyses in **Paper I and II** and 1929 patients in **Paper III**. **Figure 5** illustrates the selection process in these papers.

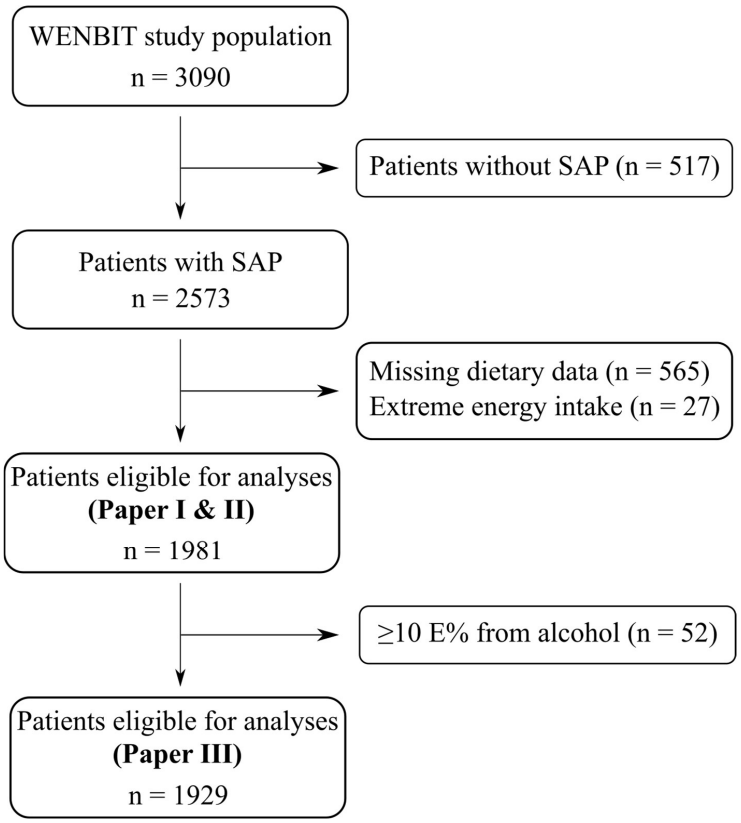


Figure 5: *Flowchart illustrating the selection process in Paper I, II, and III. SAP: stable angina pectoris; WENBIT: Western Norway B-vitamin Intervention Trial*

Baseline data

Clinical information regarding patients' lifestyle and medical history was obtained through self-administered questionnaires or interviews and verified by hospital records. Additionally, blood samples were taken at baseline. Routine biochemical analyses were conducted at the laboratories in the recruiting hospitals, whereas study-specific analyses were conducted by Bevital AS, Bergen, Norway (www.bevital.no). Smoking habits were evaluated based on self-reports and serum cotinine levels >85 nmol/L at baseline. Cotinine is a nicotine metabolite and is commonly used as a biomarker of tobacco smoke exposure as the serum or plasma concentration increases with increased exposure [134]. Diabetes mellitus was defined according to pre-existing diagnosis,

HbA1c >6.5%, fasting blood glucose ≥7 mmol/L, or non-fasting blood glucose ≥11.1 mmol/L in line with the WHO guidelines [135].

Study endpoints

The primary endpoint of **Paper I** was incident AMI, including fatal and nonfatal events, classified according to the revised definition of AMI criteria (ICD-10 codes I21, I22, I46.1, R96, and R98) [136]. Information regarding study outcomes was obtained from the Cardiovascular Disease in Norway (CVDNOR; https://cvdnor.b.uib.no) project, which provided information on patients being discharged with a CVD diagnosis between 1994 and 2009 from 42 Norwegian public hospitals, and the Cause of Death Registry at Statistics Norway (www.ssb.no).

4.1.2 Paper IV: The Hordaland Health Study 1997-1999 (HUSK)

Study cohort

The Hordaland Health Study 1997-1999 (HUSK, ClinicalTrials.gov Identifier: NCT03013725, https://husk.w.uib.no) was conducted during 1997-1999 in western Norway. HUSK is a cooperative project between the University of Bergen, the National Health Screening Service (now the Norwegian Institute of Public Health), and local health services. The main aim of the HUSK project was to quantify the burden of potentially modifiable risk factors in the general population and to pave the way for etiological studies of specific risk factors concerning diseases. The recruitment of this cohort was based on a previous cohort from 1992-1993 where all individuals living in the Hordaland County (currently part of Vestland County) born during 1953-1957 (n = 29 400) were invited. Additionally, 4849 individuals born during 1950-1951 and 4338 individuals born in 1925-1927 who previously participated in the Hordaland Homocysteine Study were invited. The study protocol has been described in detail previously [137]. In the end, data from 7016 HUSK participants was available for the current project. We excluded patients with an incomplete food frequency questionnaire (FFQ) (n = 922), missing plasma choline values (n = 30), extreme energy intake (<3000 kJ or >15000 kJ for women and <3300 kJ or >17500 kJ for men) (n = 198) and with ≥10 E% from alcohol (n = 120). A total of 5746 participants remained eligible for the analyses.

Baseline data

Information regarding lifestyle, health behavior, and medical history was obtained through self-administered questionnaires at baseline. Smoking was defined based on self-reported smoking habits and serum cotinine levels >85 nmol/L. Participants were considered diabetic according to self-reported diagnosis. Hypertension was considered present when the participant reported use of medication for hypertension.

Participants also underwent a brief health examination which included measurement of height and weight and delivered a venous, non-fasting blood sample. Biochemical analyses of plasma concentrations of one-carbon metabolites and cotinine were performed by Bevital AS, Bergen, Norway (www.bevital.no). Serum samples of total cholesterol (TC), HDL-cholesterol, and TG were analyzed within seven days at the Department of Clinical Chemistry, Ullevål University Hospital, Oslo. LDL-cholesterol concentration was calculated using the Friedewald equation [138] if TG <4.5 mmol/L. LDL-cholesterol was not calculated for participants with serum TG >4.5 mmol/L (n = 137) as the Friedewald equation is not applicable in this case.

4.2 Dietary assessment

4.2.1 Dietary intake

Dietary data in both WENBIT and HUSK was obtained from a 169-item FFQ, which is an adaptation of a 180-item FFQ from 1992 developed at the Department of Nutrition, University of Oslo (**Appendix 1**). The FFQ was designed to assess the habitual food intake of Norwegian adults over the past year and has been validated for intake of energy, macronutrients, and a range of micronutrients [139–141]. Notably, the FFQ was not validated for choline intake. The frequency of consumption was given per day, week, month, or never consumed depending on the food item. Portion sizes were given as units (e.g., slices, pieces, etc.) or household measures (e.g., cups, spoons, etc.). In addition to food items, the FFQ included the nine most common single and multivitamin supplements at the time of the study, but no specific questions regarding choline supplementation were included. Daily nutrient intake was calculated using the software system “Kostberegningsystemet” (KBS, version 3.2.) developed at the

Department of Nutrition, University of Oslo, Norway. The nutrient database used is mainly based on the official Norwegian food composition table (www.matvaretabellen.no).

In WENBIT, the FFQ was given to the patients at the first study visit, filled out, and returned at the one-month follow-up visit or returned by mail to the study center. HUSK participants were handed the FFQ on the day of their health examination, filled it out at home, and returned it by mail to the HUSK project center.

4.2.2 Choline composition data

Choline composition data are currently unavailable within the Norwegian food composition database. Choline content of food items was therefore quantified using the U.S. Department of Agriculture (USDA) Database for Choline Content of Common Foods, release 2 [8]. This database contains the choline content of over 630 food items, analyzed using liquid chromatography-electrospray ionization-isotope dilution mass spectrometry [8]. Information regarding total choline content is provided both in the database and in this project as the sum of five choline forms: free choline, GPC, phosphocholine, PC, and SM. The choline content was available for 134 food items included in the 169-item FFQ. For the remaining items, choline content was estimated using nutritionally equivalent foods. For multi-component foods (e.g., ready-meals, dishes), choline content was calculated for each ingredient in the FFQ recipe.

In **Paper III and IV**, the food items were categorized into 10 main food groups (dairy, drinks, eggs, fat, fish, fruit, grain products, meat, vegetables, and other, or sweets and snacks, in **Paper III and IV**, respectively), and subsequently into categories and subcategories based on nutritional similarities. For example, the food group “dairy” is divided into “milk”, “cheese”, and “other dairy”. Whereas the category “other dairy” is divided into the subcategories “yogurt”, “cream”, “ice cream”, and “pudding”.

4.3 Statistical analyses

4.3.1 Paper I

Baseline variables and dietary intake are reported as median (25th, 75th percentile) for continuous variables or counts (percentages) for categorical variables. Patient baseline characteristics and dietary intake across quartiles of energy-adjusted choline intake were compared by median linear or logistic regression for continuous and categorical variables, respectively. The nutrient density method was used for energy-adjustment of macronutrients (E%) and food groups (g/1000 kcal), whereas the residual method was used for choline.

A Kaplan-Meier plot was used to visualize differences in survival across the quartiles of total choline intake, assessed by the log-rank test. Cox regression models were used to estimate the association between total choline intake or intake of individual choline forms and risk of AMI. The hazard ratios (HR) and 95% confidence intervals (CI) were reported per daily increment of 50 mg for total choline and PC and of 5 mg for the remaining choline forms. The first model included adjustment for reported energy intake, the second model was additionally adjusted for age and sex, and the final model for reported energy intake, age, sex, and smoking. Confounders were selected beforehand based on available literature and subject knowledge at the time of writing, using a directed acyclic graph (DAG) (see **Figure 6** for an example of a DAG). Traditional risk factors for CHD, fasting status, statin use at baseline, and study intervention were not included in the model since they were not assumed to be causally linked to choline intake and thus not considered a confounder. Generalized additive models were plotted for the association between total choline intake and intake of individual choline forms and AMI risk to explore non-linear relationships.

Effect modifications were studied according to subgroups of traditional risk factors for CHD such as age, sex, body mass index (BMI), hypertension, smoking, diabetes mellitus, estimated glomerular filtration rate, baseline serum lipid parameters, and statin use at discharge and prior AMI. Continuous variables were dichotomized according to their median value and interactions were formally tested by adding interaction product terms with the continuous variable to the final Cox regression

model. Statistical analyses were performed using R version 3.4.3 (The R Foundation for Statistical Computing, Vienna, Austria), the packages within the "*tidyverse*" [142] and the "*survival*" [143], and "*forestplot*" [144] packages.

4.3.2 Paper II

In **Paper II**, which is a post hoc analysis of **Paper I**, we investigated possible effect modification by TMAO or TML and mediation by TMAO on the association between dietary choline intake and risk of AMI. Effect modification is present when the effect of the exposure (dietary choline intake) on the outcome (risk of AMI) varies across strata of a third variable (TMAO or TML) [145]. Effect modification was investigated by adding TMAO or TML as an interaction term to a Cox regression model adjusted for energy intake, age, sex, and smoking and investigating whether the effect of dietary choline intake varied in different quartiles of TMAO or TML concentration at baseline. Subgroup analyses were performed, stratifying for the original B vitamin intervention. Additionally, we applied a mediation analysis to the effect of dietary choline in AMI risk, considering TMAO as a mediator (**Figure 6**). Mediation analyses quantify the proportion of an exposure-outcome association working via a mediator (a variable on the causal path between exposure and outcome) [146]. DAGs will be discussed in detail in *6.1 Methodological considerations*.

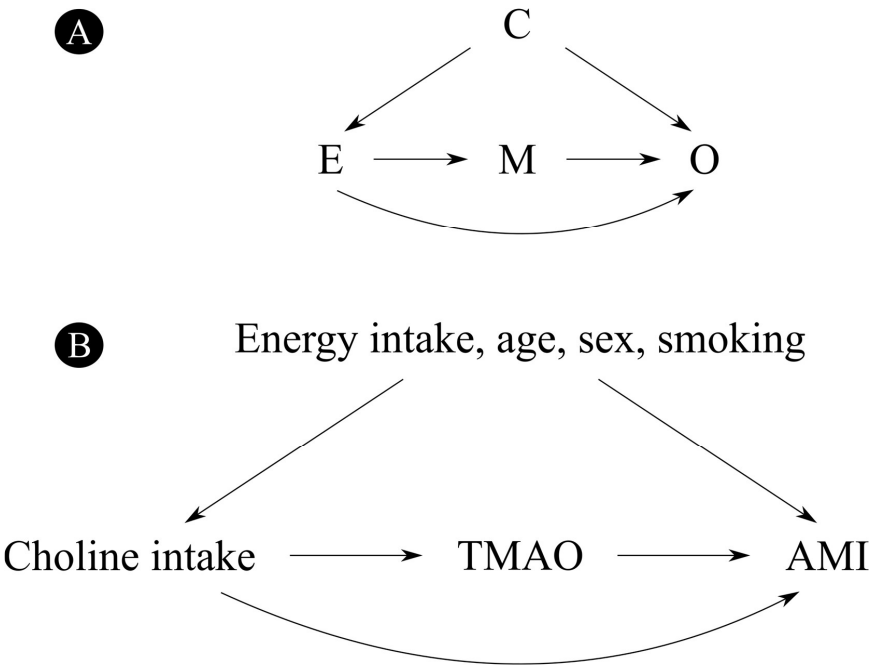


Figure 6*: Directed acyclic graph for mediation analysis in general (A) and the current analysis (B). The variables with arrows pointing towards both choline intake and AMI are considered confounders for the association between choline intake and AMI while TMAO is considered a mediator. AMI: acute myocardial infarction; C: confounder; E: exposure; M: mediator; TMAO: trimethylamine N-oxide; O: outcome.*

Statistical analyses were performed using R version 3.6.1 (The R Foundation for Statistical Computing, Vienna, Austria), the packages within the *"tidyverse"*[142] and the *"survival"* [143], and *"medflex"* [147] package for survival and mediation analyses respectively. The mediation analyses were based on logistic regression analyses because survival models were not implemented in *"medflex"*. The estimates are expected to be similar, however, less powerful [146].

4.3.3 Paper III

Continuous variables were reported as geometric means (95% prediction interval (PI)). The 95% PI renders the limits of the interval as defined by [(geometric mean)/(geometric standard deviation)^2, (geometric mean) x (geometric standard

deviation)^2]. Note that this is different from the method used in **Paper I and II.** Many biological measurements show a relatively skewed distribution, which comes close to a log-normal distribution, instead of a normal distribution. As most observations lie below the mean, the interval provided within ±1 standard deviation from the mean covers more than the expected 68.3% of the distribution and might even contain negative values. Therefore, we choose to report continuous variables as geometric mean (95% PI) instead, as this is most commonly more fitting for biological data [148]. Dietary variables were energy-adjusted using the density method, while choline was adjusted for reported energy intake by the residual method.

The percent contribution of each food (sub)category to total choline intake and intake of individual choline forms was calculated using: [(choline provided by the food (sub)category/total choline from all food (sub)categories)]*100.

In accordance with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) checklist and statement [149], we chose not to report p-values. Given the exploratory and descriptive nature of this study, the main results are presented visually. All statistical analyses were performed using R version 3.6.1 (The R Foundation for Statistical Computing, Vienna, Austria) and the packages within the "*tidyverse*" [142].

4.3.4 Paper IV

As in **Paper III**, continuous variables were reported as geometric mean (95% PI) and categorical variables as counts (percentage). The density method was used for energy adjustment of all dietary variables apart from choline for which the residual method was used. The same formula as in **Paper III** was used to calculate the contribution of each food (sub)category to intake of total choline and the individual choline forms.

To explore the relationship between one-carbon metabolites and dietary choline intake, choline intake was modeled as a polynomial spline in a model with sex as an interaction term and adjusted for age, BMI, and smoking for total choline and the individual choline forms. The same model was used to explore the relationship between serum lipid metabolites and dietary choline intake. Including statin use, diabetes diagnosis,

folate status, or all three factors as an interaction term to the used model did not alter the observed associations substantially (results not shown). Confounders were selected beforehand based on available literature and subject knowledge at the time of writing, using a DAG.

As in **Paper III**, we presented the main results visually and in accordance with the STROBE checklist and statement, we did not report any p-values. All statistical analyses were performed using R version 1.3.959 (The R Foundation for Statistical Computing, Vienna, Austria), the packages within the "*tidyverse*" ("*dplyr*", "*ggplot2*", "*broom*", "*plyr*", "*ggthemes*",) [142] and the "*survival*" [143], "*splines*" [150], and "*interaction*" [151] packages.

4.4 Ethics

Paper I, II, and III: The Western Norway B-vitamin Intervention Trial (WENBIT)

The WENBIT study was carried out in accordance with the Declaration of Helsinki. The study protocol was approved by the Norwegian Data Inspectorate and the Regional Committee for Medical Health Research Ethics (Regional ethical committee number: 2010/1880, later updated in 2013/2324). All participants provided written informed consent.

Paper IV: The Hordaland Health Study 1997-1999 (HUSK)

The HUSK study was carried out in accordance with the Declaration of Helsinki. The study protocol was approved by the Norwegian Data Inspectorate and the Regional Committee for Medical Research Ethics (Regional ethical committee number: 2009/825). All participants provided written informed consent.

5. Results

5.1 Summary of results in Paper I

In total 1981 patients with SAP from the WENBIT trial were considered eligible for analyses in **Paper I** (**Figure 5**). The cohort consisted mainly of males (80%) and the median age of the participants was 62 years. A high percentage of participants suffered from chronic conditions in addition to CVD such as hypertension (47%) and diabetes (31%). The median (25^{th}, 75^{th} percentile) total energy-adjusted choline intake was 288 (255, 326) mg/d. Dietary choline was mainly consumed in the form of PC (43%), followed by free choline (26%), GPC (22%), SM, and phosphocholine (both 5%).

During a median (25^{th}, 75^{th} percentile) follow-up time of 7.5 (6.3, 8.8) years, 16% of the patients experienced an AMI. The risk of experiencing an AMI increased per 50 mg/d increment of total choline (HR 1.11, 95% CI [1.03, 1.20]) and PC (1.24 [1.08, 1.42]) intake and 5 mg/d increment of SM (1.16 [1.02, 1.31]) in a Cox-model adjusted for energy intake only. These associations persisted after additionally adjusting for age and sex (model 2) and age, sex, and smoking (model 3). HRs for incident AMI according to energy-adjusted choline intake (per 50 mg/d increase for total choline and PC and per 5 mg/d increase for the remaining forms) for all models and all choline forms are depicted in **Paper I, Table 3**. The dose-response relationship between energy-adjusted choline intake and AMI was positive and linear for total choline, PC, and SM (see **Figure 2** in **Paper I**). We did not observe any association for free choline, phosphocholine, and GPC (**Paper I, Table 3**).

5.2 summary of results in Paper II

Due to the close link between TMAO, TML, choline metabolism, and possibly CVD, a post hoc analysis was performed to investigate whether the association between total choline intake and AMI found in **Paper I** could be modified by plasma TMAO or TML. The 1981 patients with SAP included in **Paper I** were also investigated in **Paper II**. We did not observe any effect modification according to baseline plasma TMAO and

TML concentrations (**Paper II, Figure 2**). The interaction coefficient was 0.002 (standard error = 0.004, p = 0.567) for TMAO and -0.06 (standard error = 0.08, p = 0.417) for TML. Results from the mediation analysis showed no indication that the effect of choline on risk for AMI was considerably mediated via plasma TMAO.

5.3 Summary of results in Paper III

In **Paper III**, patients with ≥10 E% from alcohol (n = 52) were additionally excluded, resulting in 1929 patients with SAP from the WENBIT cohort included in these analyses. The geometric mean (95% PI) energy-adjusted total choline intake in the population was 287 (182, 427) mg/d. Only 5.5% of the study population reached the AI of 400 mg/d as defined by EFSA. The main choline sources were fish, followed by dairy, vegetables, eggs, and meat which accounted in total for about 75% of the total choline intake. PC was the primary consumed choline form and was mainly obtained from eggs (28%), fish (19%), and meat (18%). Total choline and all individual choline forms, except for free choline, were mainly obtained from animal-based food sources in our study population. The food groups contributing to choline intake differed greatly between the water-soluble and lipid-soluble choline forms (**Paper III, Figure 1**).

5.4 Summary of results in Paper IV

The total study population (n = 5746) existed of both women and men born in either 1925-1927 (hereafter referred to as “elderly) or 1951 (hereafter referred to as “middle-aged”) from HUSK. Key baseline characteristics of the study population are provided in **Paper IV, Table 2**.

Geometric mean (95% PI) energy-adjusted choline intake for the total population was 260 (170, 389) mg/d and was similar in all age and sex groups. Dietary choline was mainly obtained in the form of PC (44%), free choline (26%), and GPC (22%). Phosphocholine and SM each contributed 5% to the total choline intake. The unadjusted choline intake of most participants was below the AI set by EFSA or NAM. Dairy, vegetables, and eggs were the primary contributing food groups to total dietary

choline intake in this population. Main contributors differed largely between the different choline forms with dairy being one of the most important sources of nearly all choline forms.

Dietary energy-adjusted choline intake was associated with changes in plasma one-carbon metabolites (**Paper IV, Figure 3**) and to a lesser extent with changes in serum lipid metabolites in a model with sex as interaction term and adjusted for age, BMI, and smoking. Some associations were found to be sex-dependent. The associations of PC, free, choline, phosphocholine, and SM intake with plasma concentration of one-carbon metabolites were similar to that of total choline intake. Interestingly, this was not observed for GPC, which was not associated with most of the one-carbon metabolites and showed only a weak positive association with cysteine and TMAO. It must be noted that the uncertainty of the associations for the individual choline forms was large, illustrated by the large CIs.

6. Discussion

The main aim of this thesis was to evaluate dietary choline intake, both total choline and the individual choline forms, choline sources in the diet, and the association of choline intake with CVD, and circulating levels of metabolites related to one-carbon and lipid metabolism in both patients with CVD and community-dwelling adults in Norway. In both cohorts the average choline intake was well below the AI suggested by either NAM or EFSA. Only 5.5% of patients with CVD and 7.6% of community-dwelling adults had intakes exceeding the AI set by EFSA. We found that PC was the main consumed choline form and was mainly derived from animal food products such as eggs and meat. Further, dietary sources differed between water- and lipid-soluble choline forms as water-soluble forms were largely obtained from dairy, vegetables, and fish and lipid-soluble forms from eggs, meat, and fish. Total choline intake was associated with changes in the concentration of plasma one-carbon metabolites and to a lesser degree with serum lipid metabolites. Finally, total dietary choline, PC, and SM, but not the other choline forms, were positively associated with risk for AMI. Mediation analyses suggested that this association was not mediated by TMAO or TML.

6.1 Methodological considerations

6.1.1 Study design & population

Although WENBIT is a prospective, randomized controlled trial [133] the data was used for longitudinal observational analyses in **Paper I, II** and cross-sectional observational analyses in **Paper III**. WENBIT data was considered suitable for observational use because the tHcy-lowering effect of the B-vitamin intervention did neither affect the risk of CVD nor mortality compared to the placebo [133]. Also, the B-vitamin intervention was not associated with choline intake, and it was therefore considered independent from both the exposure and the outcome of interest and not included in the statistical models. The HUSK trial used in **Paper IV** is a prospective population-based cohort study of which we used only data from one time point (baseline) making it cross-sectional. Cross-sectional data is useful for identifying

associations and generating hypotheses but does not allow any conclusions regarding causality and the temporal sequence, i.e., from exposure to outcome or vice versa. The issue with temporality is less present, although not fully absent, in longitudinal studies as the exposure is measured before the outcome is present. Notably, although only reported at one time point, the dietary data reflects the regular diet for the past year while the metabolites of interest were only measured at baseline. Further, the observational nature of both WENBIT and HUSK limits the ability to draw conclusions regarding causality [152]. The exploratory nature of these studies makes them suitable for hypothesis generation which may be tested in other cohort or experimental studies.

Some challenges inherent to observational studies should be addressed. The external validity of a study indicates how well the results obtained in the study population reflect true findings among equivalent individuals outside the study. Internal validity is the extent to which the observed results are representative of the true findings in the study population and thus not due to methodological errors [153]. Errors of concern that can compromise internal validity that will be discussed are systematic errors such as selection bias, information bias, and confounding.

Selection bias

Selection bias is "a distortion in the estimate of the effect due to the manner in which subjects are selected for the study". This is a result of systematic differences in characteristics between those who participate in the study and those who do not, and is present when the relationship between exposure and outcome is different between participants and non-participants [153].

The study participants from WENBIT originated from a population of patients in western Norway who had been referred to coronary angiography due to suspected CHD and were diagnosed with SAP. Since coronary angiography is an invasive diagnostic method, it is not commonly used as a primary diagnostic tool for SAP. It is therefore plausible that our study population had more severe underlying disease compared to the general SAP population. On the other hand, agreeing to participate in a study could reflect greater health awareness and generally better health status compared to non-

participants. This is a form of selection or non-response bias known as the *healthy volunteer effect* [154, 155]. Additionally, socioeconomic status and knowledge about the subject have been shown to influence willingness to participate. Individuals with lower socio-economic status and lower education are less likely to participate, thereby impairing generalizability or external validity which will be discussed later [156–158]. Unfortunately, we did not have data on the socioeconomic status of the participants and the non-participants.

Indeed, some of these issues were observed in the HUSK study. Invitations to participate were sent in 1997-1999 to individuals who had participated earlier in a population study in Hordaland County in 1992-1993. The participation rate was 77%. Non-participants in 1997-1999 consisted of a larger proportion of smokers and had less regular physical activity measured in 1992-1993 compared to individuals willing to participate again in 1997-1999. Moreover, average income and the highest degree of education were lower among non-participants compared to participants for both men and women. Finally, fewer participants were on social security compared to non-participants [159]. The *healthy volunteer effect* is a noteworthy form of selection bias as socioeconomic status not only influences the willingness to participate but is also known to be associated with health outcomes and diet.

Selecting patients with SAP in itself might have induced bias in the form of *collider stratification bias,* a form of selection bias [160, 161]. By only including patients diagnosed with SAP, we directly conditioned (i.e., stratified) all the analyses for SAP. It is not unthinkable that SAP is a collider, i.e., a variable that is caused by two or more other variables, for some variables related to the choline-AMI causal pathway. (This concept will be explained in detail in *Confounding*). Conditioning for this variable might cause an association between two otherwise independent variables. A commonly used example of collider stratification bias is the “obesity paradox” [162]. In individuals with CVD, obesity has an apparent protective effect on mortality, while the association is reversed in the general population. However, only including participants with CVD results in adjusting the analyses for CVD and in a false association between obesity and unmeasured factors. Thus, in a study population consisting of individuals

with CVD, obesity appears to be associated with lower mortality risk. As there are always unmeasured factors, we cannot exclude the presence of possible collider stratification bias in our analyses. Luckily, theoretical work has shown that only a small bias in the estimate results from collider stratification [163].

Information bias

Another error that might attenuate internal validity is information bias, which occurs due to flaws in measuring exposure, covariate, or outcome variables. One type of information bias is misclassification, which can be either nondifferential or differential [153]. Nondifferential misclassification occurs when the measurement error in the exposure is not related to the measurement error in the outcome, so the misclassification is the same across groups. This may reduce the observed effect estimate, also known as *regression dilution bias*. In this case, the measurement error in the exposure variable leads to an attenuation of the linear regression slope describing the relationship between the exposure and the outcome variable thereby underestimating the real association [154]. Differential misclassification on the other hand means that misclassification differs according to the value of other study variables and thus is different in the groups to be compared, or across the extent of exposure. This may result in both under- and overestimation of the effect estimate [154, 164].

Adequate assessment of the exposure is essential for all epidemiological studies and is a particular challenge in nutritional epidemiology. A considerable concern regarding information bias in all papers included in this thesis is the collection of self-reported dietary intake data. A well-known source of error is the participants' inability to recall their intakes accurately and fully, also called *recall bias*. FFQ's are especially prone to this type of bias as the participants are asked to report their intake retrospectively over a longer period [154, 165]. Additionally, social desirability, i.e., responding in a manner following perceived social norms, may result in intentional misreporting of certain foods and thereby introduce *reporting bias*. Personal characteristics such as age, gender, overweight, and obesity are also known to affect food intake reporting [165, 166]. For example, it has been shown that women, more than men, tend to underreport

fat intake [166]. Also, a systematic review from Wehling *et al.* found that having a BMI ≥ 30 was associated with lower reporting accuracy of food intake [167].

Confounding

When a variable is associated with both the exposure and the outcome but is not on the causal path between them, it is a confounder (**Figure 7**) [153]. Confounders can be accounted for by the research design (e.g., by matching or randomization) and/or during the analysis by adjusting for them. However, the latter is only possible if the variable is measured properly [154]. If not adjusted for, or wrongly adjusted for, confounders introduce bias and thereby negatively affect the effect estimate. Even though confounders may be accounted for, there will always be *residual or unmeasured confounding,* e.g. due to measurement errors in the confounders*,* which limits the ability of cohort studies to estimate causality [168].

Accurately selecting covariates, i.e., variables included in the statistical model to adjust for confounding is challenging and no specific guidelines exist. Controlling for all pre-exposure measured variables or all common causes of exposure and outcome or including a covariate purely made on statistical grounds is one approach. Yet, this might result in controlling for a variable that introduces bias or not controlling for a variable that could eliminate bias [169].

Especially selecting covariates based on statistical grounds, such as p-value or model-based selection methods, ignore the underlying causal structure of the hypothesis and may therefore not adequately adjust for confounding [169, 170]. Another approach suggests that "the choice of covariates should be primarily guided by empirical evidence or theoretical knowledge of suspected or established confounding factors" [171]. The difference between both approaches is that the first one is primarily data-driven and based on the available study dataset while the second is based on prior knowledge. The causal relationship between variables can be visually presented by DAGs (**Figure 7**) [169, 170].

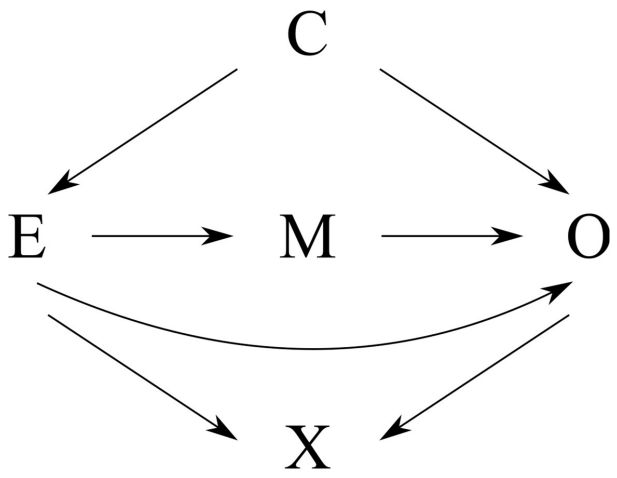


Figure 7*: A directed acyclic graph illustrating a confounder (C), a mediator (M), and a collider (X). E represents the exposure variable and O the outcome variable.*

A DAG is directed since it represents a directed causal "path" along arrows head-to-tail and is acyclic since there is no feedback loop. It represents known causal effects, based on a priori knowledge, with unidirectional arrows. This causal path is the association between the exposure and outcome and consists of a set of arrows between the relevant variables. However, it is also possible to have an alternative, so-called "backdoor" path between the exposure and outcome. Variables that open this path are considered confounders and should be adjusted for so that the alternative path is closed. A variable can also lie on the causal path between the exposure and outcome. In this case, it is a mediator and is of great interest since it represents the causes and mechanisms of the outcome. The path between exposure and outcome including the mediator is called the indirect effect or indirect causal path, while the path without the mediator represents the direct causal effect of the exposure on the outcome. Finally, a variable can have two or more antecedent causes within the pathway of interest. This kind of variable is called a collider and is represented in a DAG as two arrows on one path pointing to one variable. Colliders should not be adjusted for as the path is already closed. However, adjusting for a collider will open the causal path and introduce bias, e.g., collider stratification bias as discussed earlier. DAGs visualize the assumptions

made regarding existing causal effects and aid with the transparency of model building. However, constructing a DAG can be challenging with regards to the selection of variables and directionalities especially when limited a priori knowledge is available [170, 172, 173]. The simplified DAG for **Paper I** is shown in **Figure 8**.

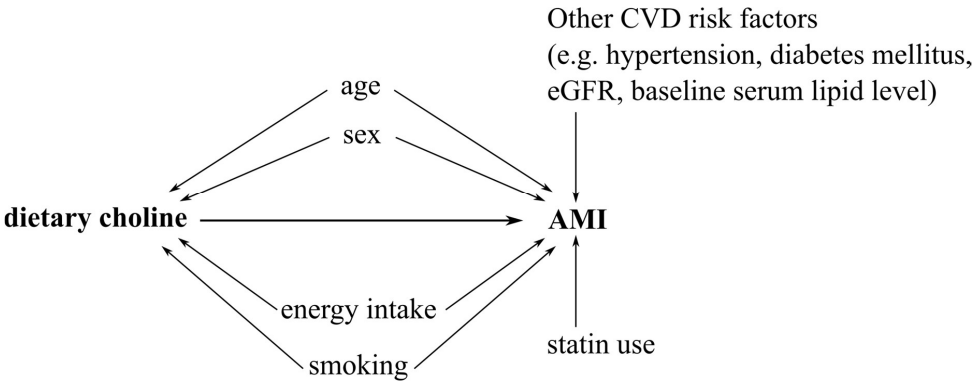


Figure 8*: Simplified directed acyclic graph for the model used in Paper I. AMI: acute myocardial infarction; CVD: cardiovascular disease; eGFR: estimated glomerular filtration rate*

As shown in the DAG, energy intake, sex, age, and smoking were considered confounders and were therefore included as covariates in the Cox regression model in **Paper I**.

External validity

The degree to which the result observed in a study population can be generalized to the target population is referred to as external validity or generalizability. Internal validity is a requirement for external validity [153, 154]. Participants in WENBIT were comparable regarding age, smoking habits, and sex distribution to samples of European patients who underwent invasive evaluation for angina pectoris or acute coronary syndromes and who had verified CHD [133]. The HUSK study population included in **Paper IV** consisted of two groups with each a small age range (46-50 or 70-74 years) and generalizability to other age groups might be limited. Additionally, the study population was homogenous (all Caucasian) which again lowers generalizability to populations of another ethnicity. To investigate the external validity of dietary data to

the overall Norwegian population, the data from HUSK can be compared to data from the National Dietary Survey among Men and Women aged 16-79 1997 (NORKOST2). In 1997-1999, both the recruitment of HUSK patients and the NORKOST2 survey, which gathered data regarding macronutrient intake in the general Norwegian population, were conducted [174]. Mean energy and macronutrient intakes for women and men aged 40-49 and 70-79 in NORKOST2 were similar to what was observed in HUSK (**Table 2**). There were some differences in intake of food groups such as dairy, fish, fruit, meat, and vegetables. However, the differences in intake of food groups between the age groups and sex were similar in both studies. Unfortunately, choline intake was not measured in NORKOST2 and therefore cannot be compared. However, the apparent similarities between NORKOST2 and HUSK support the external validity of the dietary data. Note that the arithmetic mean is reported for HUSK and NORKOST2 in **Table 2** to be able to compare the dietary intake, while in **Paper IV** the geometric mean was reported for HUSK (**Paper IV, Table 3**).

Table 2: Mean dietary intake of energy, macronutrients, and food groups in elderly and middle-aged women and men from HUSK and NORKOST2

	Elderly				Middle-aged			
	Women		Men		Women		Men	
	HUSK	NORKOST2	HUSK	NORKOST2	HUSK	NORKOST2	HUSK	NORKOST2
Age (years)	71-74	70-79	71-74	70-79	47-49	40-49	47-49	40-49
Energy (kcal)	1595	1672	2037	2126	1877	1839	2484	2508
Macronutrients, E%								
CH	52.1	52.9	50.5	51.2	49.5	51.1	48.9	50.5
Protein	16.1	16.5	15.9	16.5	16.1	16.5	15.6	16.0
Fat	30.5	29.7	31.7	30.9	32.4	30.7	32.8	30.9
Alcohol	0.7	0.9	1.5	1.4	1.5	1.6	2.3	2.6
Food groups, g/d								
Dairy	337	388	363	491	289	349	421	533
Drinks	741	740	767	817	989	1064	1122	1212
Eggs	15	16	18	17	16	16	18	19
Fats & oils	21	21	32	30	29	27	42	40
Fish	73	68	107	92	73	62	91	75
Fruit	240	256	235	218	256	222	241	217
Grains	197	197	251	255	225	224	307	318
Meat	63	65	92	96	105	94	142	119
Other	9	9	12	16	14	21	16	26
Vegetables	294	281	347	313	321	256	337	274

CH: Carbohydrates; HUSK: Hordaland Health Study 1997-1999; NORKOST2: National Dietary Survey among Men and Women aged 16-79 1997

6.1.2 Methods of dietary intake assessment

Dietary intake was measured in WENBIT and HUSK with a 169-item semi-quantitative FFQ developed to capture the habitual dietary intake in the Norwegian population. An FFQ is an easy and cost-effective way of assessing dietary intake in large cohorts, is easy to fill out for the participants, and reflects habitual diet. Limitations of an FFQ include non-reporting of food items that are not included in the food list, specificity to the study group, and estimation of portion sizes [175].

Additionally, information bias might be present due to difficulties with recalling food consumption and answering according to social desirability as mentioned previously. Other errors might be introduced via the use of food composition databases. Natural variations in food items or limited information regarding the composition of ready-made or processed products and meals prepared at home are the main causes of these errors [165]. Indeed, a major limitation in the assessment of dietary choline intake conducted in this thesis was the lack of knowledge regarding the choline content of food items included in the FFQ. As per now, choline is not included in the Norwegian food database (www.matvaretabellen.no), therefore the USDA database [8] was used instead. In fact, information regarding the choline content of food items is hardly ever incorporated in food databases. When EFSA investigated the regular choline consumption in several European populations to establish their recommendations in 2015, no European food composition database included choline values [85]. To the best of our knowledge, information regarding choline content in European countries is currently only available in the German food composition database (https://www.sfk.online/#/home), but only for lysoPC and PC. Therefore, differences in consumed food items and composition of foods might have led to substantial measurement error in the analyses [176].

Dietary measurement errors lead to bias in the association between diet and the outcome of interest. Measurement error in cohort studies is usually nondifferential, leading to an attenuation of the relative risks and diminishing statistical power [177]. Importantly, this is true on average when comparing effect estimates of a large number of studies, however, it is not guaranteed that the effect estimate obtained in a single

study is necessarily an underestimation of the true effect. For a single study, it could both be an over or underestimation of the true effect [178]. One way of attempting to deal with this is validating the FFQ via a validation study that evaluates how well the reported intake resembles the true intake in the study population [165]. A validation study provides knowledge regarding the association between the observed and the true value of the variable of interest. This allows the use of regression calibration to adjust the effect estimate for measurement error originating from the used FFQ [177]. Despite being validated for several other factors, the FFQ used in WENBIT and HUSK was not validated for choline which did not allow us to evaluate how well it captured the actual choline intake, nor to adjust for the measurement error. Reported dietary choline intake should therefore be interpreted with caution. However, although not validated for choline, the FFQ still allows ranking of individuals according to their dietary intake level and is therefore suitable to estimate choline-outcome associations as investigated in **Paper I, II,** and **IV**.

Another strategy to mitigate the problems with measurement error in dietary data is energy adjustment. Self-reported energy intake can be used to adjust the measurement error of other self-reported dietary intakes as the error in energy intake correlates with the error in intake of other dietary components [165, 179]. Another reason to adjust for energy intake is to control for confounding which occurs when energy intake is associated with the outcome of interest. The majority of the nutrients are associated with energy intake, either because they provide energy or because individuals consuming a large amount of energy often also consume a higher amount of most nutrients [180].

Further, adjusting for energy intake controls for variation in nutrient intakes caused by extraneous variables (i.e., any variable that can affect the outcome variable but is not measured), for example, physical activity or body composition [180]. Third, estimates of relative risks improve by reducing regression dilution bias and increasing precision with energy adjustment [181, 182].

Dietary nutrient and food intakes were energy-adjusted in all papers. The density method was used for all energy-yielding macronutrients (besides choline) and other food groups, expressing them as energy percentage or intake in grams/1000 kcal, respectively. Dietary choline intake was adjusted for energy intake using the residual method. In this case, the energy-adjusted choline estimate is the residual from the regression model with total energy intake as the independent variable and absolute choline intake as the dependent variable plus the expected nutrient intake for the mean energy intake in the study population (**Figure 9**) [183]. Using this method makes choline intake not only unrelated to energy intake but also directly related to overall variation in food choice and composition.

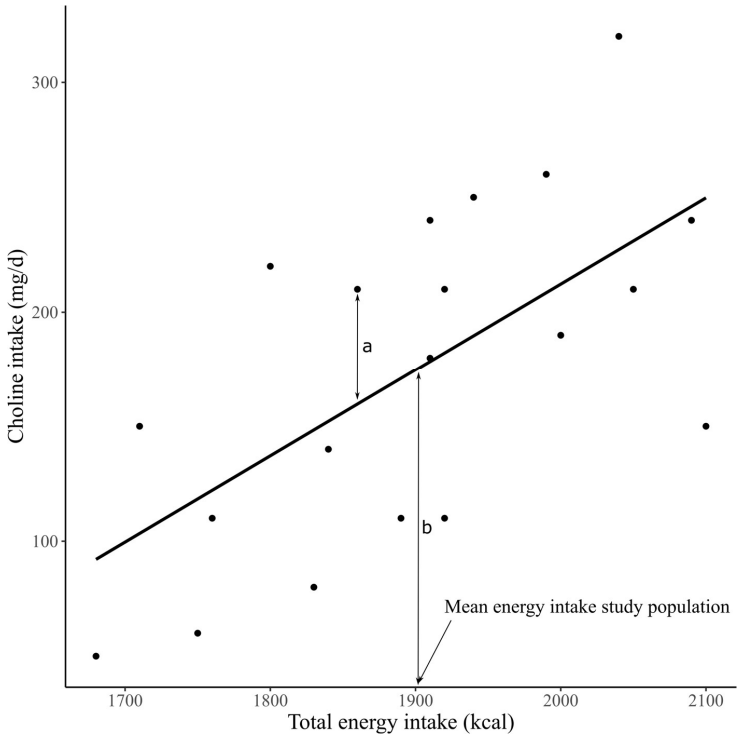


Figure 9: *Visual representation of the residual method for energy adjustment. Energy-adjusted choline intake = a + b. a is the residual for the observation from the regression model with total energy intake as the independent variable and absolute choline intake as the dependent variable. b is the expected nutrient intake for a person with a mean energy intake. (Adapted from Willett et al.* [183]*)*

The effect of energy adjustment on a nutrient regarding data distribution and correlation with variables of interest is shown in **Figures 10** and **11**, respectively. This illustrates the importance of energy adjustment for data analyses and comparison of findings when different adjustment methods are used. The interpretation of energy-adjusted models should be done carefully and is often done incorrectly as discussed in a recent paper by Tomova *et al.* [184].

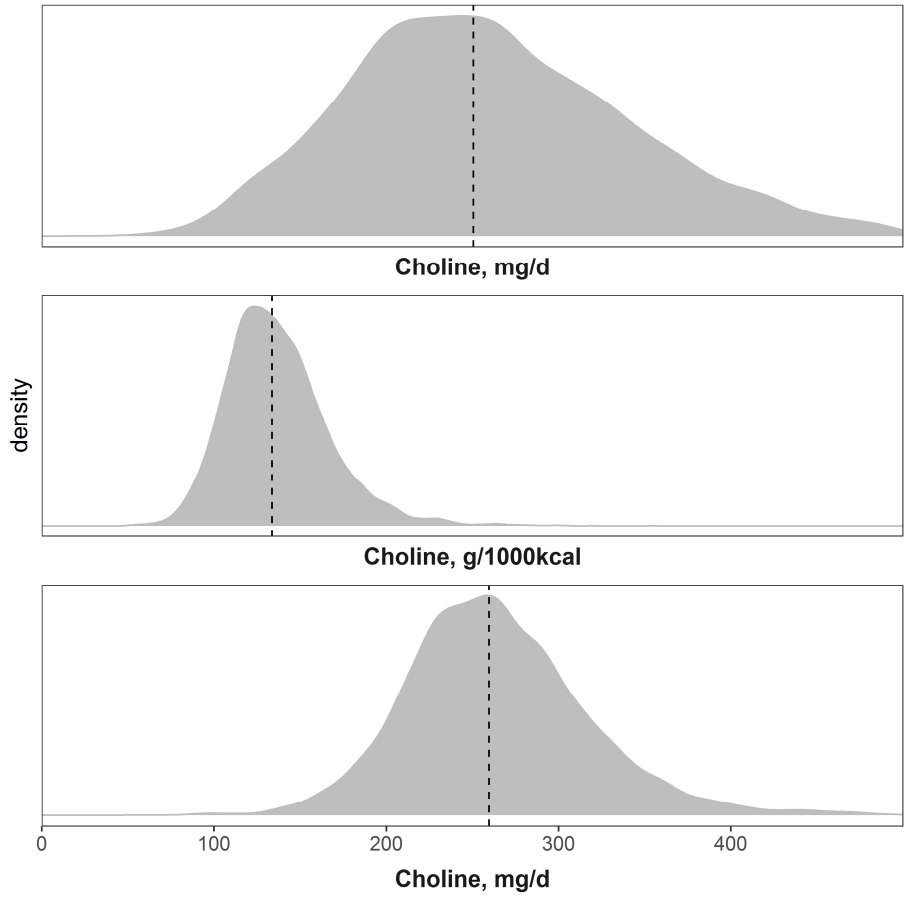


Figure 10: *Distribution of choline intake for participants in* ***Paper IV****. Choline intake was either unadjusted (A), energy-adjusted using the density method (B), or energy-adjusted using the residual method (C). The dotted line indicates the mean intake.*

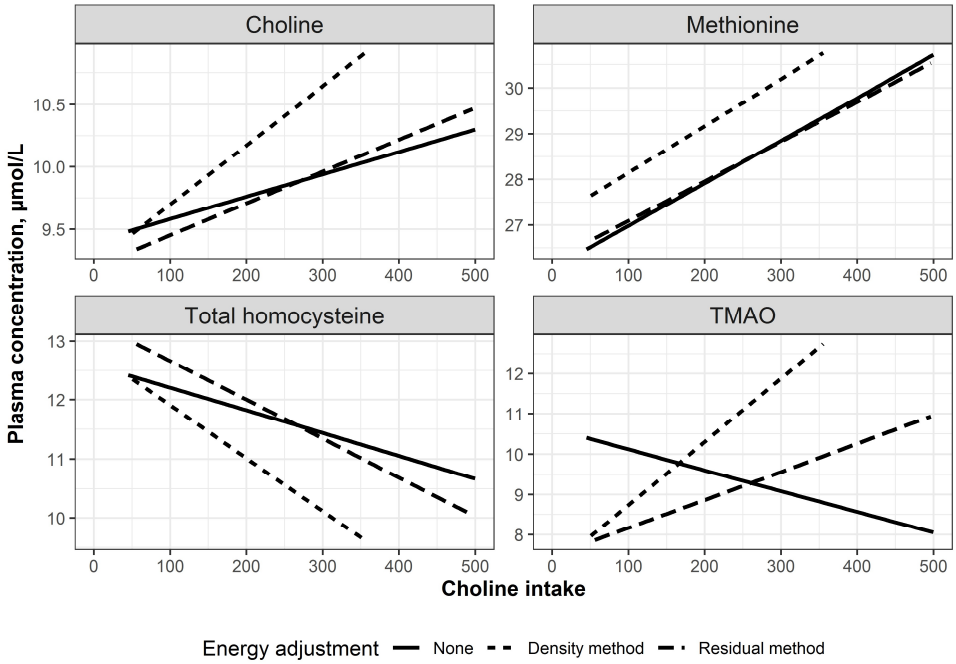


***Figure 11**: Correlation between choline intake and plasma concentrations of choline-related metabolites. Choline intake is expressed in mg/d (unadjusted intake and adjusted intake using the residual method) or in g/1000 kcal (energy-adjusted using the density method). TMAO: trimethylamine-N-oxide.*

6.2 Discussion of main findings

Dietary choline and CVD

In **Paper I**, a positive association was observed between dietary choline intake and the risk of AMI (HR [95%CI] 1.10 [1.02, 1.19] per 50 mg choline increase) in a cohort of patients with SAP. Interestingly, we found only the fat-soluble choline forms, PC and SM, to be associated with AMI risk. Very few studies have investigated this relationship previously and results are contradictory [110, 185, 186]. Meyer *et al.* included four prospective cohort studies in their meta-analysis and did not observe any association between dietary choline intake and incident CVD (relative risk: 1.00 [95% CI: 0.98, 1.02] for an increase of 100 mg choline per day) [110]. Similar findings were observed in a prospective cohort study by Millard *et al.* [186]. However, a positive

association has been reported for CVD mortality suggesting that dietary choline might have more effect on CVD prognosis rather than CVD development [185]. Our observation that only PC and SM were associated with AMI risk is in accordance with findings from the Nurses' Health Study (NHS) and the Health Professionals Follow-Up Study (HPFS) where dietary PC was associated with an increased risk for all-cause and CVD mortality [187]. In a Japanese cohort, however, only SM showed a positive association with CVD mortality risk in men [188]. One explanation for these differences might be residual bias from the diet and other lifestyle factors. In the NHS, HPFS, and WENBIT cohort, PC was largely obtained from eggs and meat, while in the Japanese cohort, a large proportion (15.2% in men and 13.9% in women) of PC was obtained from seafood which is known to contain other compounds which have beneficial effects on cardiovascular health [187, 188]. When investigating the food items contributing to the individual choline forms in **Paper III** and **IV**, it became clear that there were substantial differences in food sources between the lipid and water-soluble forms. Lipid-soluble forms were mainly obtained from animal sources such as eggs, meat, and fish, while vegetables, dairy, and grain products provided most of the water-soluble forms. One could speculate that a high intake of lipid-soluble choline could be a marker of low adherence to dietary guidelines, e.g. high intake of meat and eggs and low intake of vegetables, and is therefore associated with increased AMI risk. It should be taken in mind that these participants were recruited in the late 1990s and that the dietary recommendations at the time were different from the current ones. The recommendations only included macro and micronutrient intake [189], but not food items in contrary to today's Nordic Nutrition Recommendations [6].

Further, findings for a nutrient are not necessarily valid for a food item containing that nutrient, illustrated by the high PC consumption from fish in the abovementioned Japanese cohort. A good example to emphasize this is saturated fatty acid (SFA) intake which should be limited according to current dietary guidelines to prevent CVD. However, it has recently been shown that this statement should be more nuanced as the dietary SFA sources may play an important role. For example, SFA from meat is thought to be inversely associated with health effects while no or a positive association

has been observed for dairy products [190, 191]. Even within dairy products discrepancies were observed as dietary fats from cheese did not have the same effect on blood lipids as from other food matrices [192]. In the middle-aged HUSK participants, SFA from cheese, but not from other food items showed an inverse relationship with risk of incident CHD [193].

The discrepancies between the findings of the current available studies can be attributed to various factors. Although the pathway for choline absorption and transportation is known, the bioavailability of choline and how this is influenced by factors such as food matrix or nutrient-nutrient interactions remains undiscovered. For example, it is thought that PC and SM impair intestinal cholesterol absorption [11, 194], but other possible nutrient-nutrient interactions remain to be investigated. Further, dietary habits vary according to race and/or ethnicity which has been shown to influence choline consumption and dietary sources [92–94]. Additionally, long-term dietary habits have been proven to influence the human gut microbiome which plays an important role in digestion and physiological response to dietary compounds, including choline [195]. For example, it seems that vegetarians have a different gut microbiota composition that has a lower ability to produce TMA compared to omnivores [196]. Indeed, it has been shown that TMAO production in response to choline ingestion is associated with certain microbiota enterotypes, however, studies are scarce and this warrants further investigation [197]. Finally, several SNPs in genes involved in the choline and/or one-carbon metabolism are known to alter dietary choline requirements, such as CHDH, rs12676; MTHFR, rs1801133; MTHFD1, rs2236225; and PEMT, rs12325817 [198, 199]. Additionally, SNPs have been shown to influence the development of liver and muscle damage in case of a low-choline diet [89, 200, 201]. In case of adequate or more than adequate choline intake (respectively 480 and 930 mg/d), common genetic variations in choline metabolizing genes have been shown to alter the metabolic fate of choline, i.e., oxidation to betaine and entering the one-carbon metabolism or catalyzation to phosphocholine and thereby entering the CDP-choline pathway, in women. This might have consequences for disease pathogenesis and prognosis in the long term, but requires further investigation [202].

The biological mechanism by which dietary choline, and more specifically PC and SM, might affect AMI risk remains unexplained. Choline is an essential nutrient and is involved in a wide variety of biological processes. As discussed in *Chapter 1.3*, dietary choline might influence the risk for CVD through several mechanisms given its central role in one-carbon and lipid metabolism, however, these all remain speculative. From epidemiological studies, like ours, we cannot tell the underlying mechanism of an association, despite these often being emphasized when reporting findings. A wide variety of factors might influence not only individual choline requirements but also the phenotype presented at different intake levels complicating both the comparison of reported findings and the search for the underlying mechanism(s). Although explorative and hypothesis-generating, large observational studies are nonetheless useful and warranted to explore the relationship between dietary choline and disease risk further and generate recommendations regarding intake. As worded nicely by Buis & van Roosmalen: "A distinction exists between the scientific and more practical use of epidemiology. The first goal could be defined as to find true causal effects using epidemiological observations and the latter as using these observations to inform health policies to reduce morbidity and mortality."

Dietary choline and TMAO

Due to the recently observed association between TMAO and CVD, we aimed to investigate whether our observed relationship between dietary choline intake and AMI risk in **Paper I** was mediated or modified by TMAO. We did indeed observe an increase in plasma TMAO concentrations with increased dietary choline consumption in the WENBIT study population, however, it did not modify the association between dietary choline and AMI risk (**Paper II**). Several recent systematic reviews and meta-analyses reported a positive association between plasma TMAO concentration and risk of CVD or CVD prognostic factors [109, 116, 117].

The positive association between choline intake and plasma TMAO was also observed in the HUSK study population (**Paper IV**), but only up to a choline intake of ±300 mg/d. Plasma TMAO concentration has been shown to have a large within and between-person variation [68]. Generation of TMAO relies on three main factors: (i)

consumption of dietary precursors (e.g., choline, L-carnitine), (ii) gut microbiota-dependent conversion to TMA, (iii) oxidation of TMA to TMAO by FMOs in the liver. Genome-wide association studies in both mice and humans found that little of the TMAO variation could be explained by FMO variants, indicating that the variation is mainly caused by consumption of dietary precursors and differences in gut microbiota [203]. As mentioned earlier, it has been shown that certain microbiota enterotypes are associated with TMAO production in response to choline consumption [197], although this remains the subject of investigation.

Further, mixed results have been reported regarding the relationship between choline consumption and plasma TMAO concentration. Several studies reported an increase in plasma TMAO concentration after egg consumption [204–206], however other studies did not find these changes [123, 207–210]. It has to be mentioned that all the studies were performed in a small number of subjects and when an increase in plasma TMAO was observed, differences between participants were usually large. Of note, two studies reported that the gut microbiota composition of high versus low TMAO producers differed [206, 211]. Interestingly, also the choline form seemed to be important for the TMAO response. Wilcox *et al.* found an increase in plasma TMAO after an intervention with either choline bitartrate, choline bitartrate and four eggs, or choline bitartrate and four egg whites, while this increase was not observed after consumption of four eggs or capsules containing PC [207]. Similar findings were reported by Cho *et al.* after supplementation with either choline bitartrate or PC [211]. Indeed, the association between choline intake and plasma TMAO differed for the individual choline forms in **Paper IV**, but the uncertainty was substantial, indicated by the large CIs. Dietary choline intake in the HUSK study reflects long-term intake, while plasma one-carbon metabolites were measured at a single timepoint. Since non-fasting blood samples were analyzed, it must be considered that plasma TMAO might have been affected by recent dietary intake. For example, fish intake leads to a substantial increase in plasma TMAO for several hours after consumption [206].

All the above-mentioned findings emphasize the complexity of the relationship between dietary choline intake and plasma TMAO which warrants caution for interpretation of results and the use of TMAO as a biomarker of choline intake.

Dietary choline and one-carbon metabolites

In **Paper IV**, we found that long-term total dietary choline intake, and the intake of the individual choline forms apart from GPC, was associated with plasma one-carbon metabolites. Substantial sex-specific differences were observed, e.g., for betaine, serine, and choline, and uncertainty was considerable for the least consumed choline forms. The inverse association between total choline intake and plasma tHcy observed in the HUSK cohort is in line with findings from previous studies [86, 91, 121, 128, 129]. Unfortunately, hardly any studies have investigated the association between dietary choline and plasma one-carbon metabolites. Cho *et al.* observed a time-dependent increase in plasma free choline, betaine, DMG, and methionine after consumption of animal foods (fish, eggs, or beef) but not fruit [212]. However, these are acute responses to recent choline intake and not to long-term intake. After recording dietary intake and plasma one-carbon metabolites monthly for one year in Gambian women, Dominguez-Salas *et al.* found no relationship between choline intake and plasma choline concentration. However, they did report an increase in plasma tHcy and DMG with increased choline and betaine intake combined. Unfortunately, they did not investigate the relationship between dietary choline intake and other one-carbon metabolites [213].

The metabolisms of choline, folate, methionine, and B-vitamins are closely interrelated and the availability of one nutrient likely results in changes in the utilization and metabolism of the others. The concentration of plasma one-carbon metabolites might therefore not only be associated with choline intake, but also with intake of the other involved nutrients. This was not accounted for in our study, and differences in folate, methionine, or B-vitamin status might have influenced the individual's response to dietary choline intake. Additionally, genetic differences, such as the previously mentioned SNPs, in key enzymes of the choline and one-carbon metabolism, might have further influenced this relationship.

6.3 The way forward

Choline has been officially recognized as an essential nutrient since 1998 and our knowledge and understanding of choline has increased significantly since then. Despite dietary recommendations in the form of an AI being in place in the US and Europe, this is not the case in the Nordic countries. However, choline will be addressed in the new edition of the Nordic Nutrition Recommendations to be published in 2022. Many studies, including ours, found that the choline intake was below the recommended AI. Current knowledge indicates that low choline intake might lead to adverse health effects such as liver and muscle damage in adults and influence cognitive function peri and postnatally. However, an AI, unlike an AR, does not allow to estimate the prevalence of inadequacy or to assess whether an intake below the AI results in suboptimal health status. Therefore, more data is needed to establish an AR and thereby improve dietary recommendations and develop nutritional strategies for choline.

We investigated choline intake in a population with SAP and community-dwelling adults, which is more representative for the general population than the former. However, this was based on dietary data gathered in the 1990s and might not reflect the current intake due to changes in e.g., dietary habits and available foods. More recent data should be used to estimate current choline intake in the Norwegian population. One of the major obstacles is, however, the lack of information regarding the choline content of Norwegian foods. The available USDA database is based on American food items and does not only lack typical Norwegian food items, but the choline content in similar foods might also differ. Data on the choline content of local foods would therefore contribute tremendously to a more accurate estimation of choline consumption.

Choline and its metabolites have a wide variety of physiological functions and are closely related to the one-carbon and lipid metabolism which are associated with the risk of chronic diseases such as CVD. This relationship should be investigated further to unravel choline's role in health and disease development. The complex interplay between choline and dietary components such as folate and methionine but also B-

vitamins should be kept in mind while doing this. Also, as several SNPs have been shown to affect phenotypic response to choline intake, stratifying according to genotype is desirable in further research.

There is a need to increase choline awareness among the public and health professionals alike. Nutrition policies and strategies and dietary recommendations are warranted, especially for vulnerable populations such as pregnant and lactating women. Research institutions, industry, and government should work closely to establish dietary recommendations and support the population to achieve them.

7. Conclusion

One of the aims of this thesis was to investigate the association between dietary choline intake and the risk of AMI in a cohort of patients with SAP. A positive association was observed between dietary intake of total choline, PC, and SM and the risk of AMI during long-term follow-up. This was not observed for free choline, phosphocholine, and GPC. Plasma TMAO and TML did not modify the association. Additionally, the association was not mediated by plasma TMAO.

Further, choline intake and its contributors in a cohort of patients with SAP and community-dwelling adults in Norway were investigated. In both cohorts, choline was mainly consumed in the form of PC and animal food sources were the most important contributors to the intake of all choline forms except free choline. Major dietary choline sources were eggs, milk, fresh vegetables, lean fish, and bread in the WENBIT study population, and eggs, low-fat milk, potatoes, and leafy vegetables in the HUSK study population. Notably, most participants of both study populations did not reach the AI for choline.

Finally, an association between dietary choline intake and plasma concentration of one-carbon metabolites and, to a lesser extent, serum lipids, was found.

Further studies estimating choline intake in Nordic populations are warranted to allow for the establishment of dietary recommendations. Therefore, there is an urgent need for the inclusion of choline in the Norwegian food composition database to estimate the dietary intake of this essential nutrient more accurately. Analyzing food items for total choline and individual choline forms is a vast effort and should preferably be made in a European context. Finally, there is a need to clarify the association between dietary choline, especially the individual choline forms, and one-carbon and lipid metabolites as these are closely related to the risk of chronic diseases.

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Research paper

Dietary choline is related to increased risk of acute myocardial infarction in patients with stable angina pectoris

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ABSTRACT

High plasma choline has been associated with the metabolic syndrome and risk of chronic diseases, including cardiovascular disease. However, dietary choline is not correlated with choline plasma concentrations, and there are few studies and contradictory evidence regarding dietary choline and cardiovascular events. In addition, a recommended dietary allowance for choline has not been established and remains a point of contention.

This study assessed the association between dietary choline, including choline forms, and risk of incident acute myocardial infarction (AMI) in patients with suspected stable angina pectoris (SAP).

In total 1981 patients (80% men, median age 62) from the Western Norway B Vitamin Intervention Trial were included in this analysis. Information on dietary choline was obtained using a 169-item food frequency questionnaire. The Cardiovascular Disease in Norway project provided data on AMI. Risk associations were estimated using Cox-regression analysis using energy-adjusted choline intake.

Median (25th, 75th percentile) total energy-adjusted choline intake was 288 (255, 326) mg/d. During a median (25th, 75th percentile) follow-up of 7.5 (6.3, 8.8) years, 312 (15.7%) patients experienced at least one AMI. Increased intakes of energy-adjusted choline (HR [95% CI] per 50 mg increase 1.11 [1.03, 1.20]), phosphatidylcholine (HR per 50 mg increase 1.24 [1.08, 1.42]) and sphingomyelin (HR per 5 mg increase 1.16 [1.02, 1.31]) were associated with higher AMI risk.

In conclusion, higher dietary intakes of total choline, phosphatidylcholine and sphingomyelin were associated with increased risk of AMI in patients with SAP. Future studies are necessary to explore underlying mechanisms for this observation.

1. Introduction

Choline is an essential nutrient, which to some extent can also be formed endogenously in the liver. Choline appears in both water- and lipid-soluble forms in both body and diet. Water-soluble forms include free choline, phosphocholine and glycerophosphocholine. They enter the liver via the portal circulation after intestinal choline transporter-mediated absorption [1,2]. Lipid-soluble forms include phosphatidylcholine (PC) and sphingomyelin (SM). PC is hydrolyzed by phospholipase A2 to lysoPC prior to absorption in the enterocyte [1,2]. LysoPC can be either reacetylated to PC or further broken down to glycerophosphocholine, and finally free choline [1,4]. PC enters the bloodstream through the lymphatic system incorporated in chylomicrons, thereby being delivered directly to peripheral tissue (muscle and

Abbreviations: AMI, acute myocardial infarction; CAD, coronary artery disease; CRP, C-reactive protein; CVD, cardiovascular disease; DMG, dimethylglycine; FFQ, food frequency questionnaire; MMA, methylmalonic acid; PC, phosphatidylcholine; PLP, pyridoxal phosphate; RCT, reverse cholesterol transport; SAP, stable angina pectoris; SM, sphingomyelin; TMAO, trimethylamine N-oxide; WENBIT, Western Norway B-Vitamin Intervention Trial.

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adipose) before reaching the liver [1]. SM is hydrolyzed by small intestinal brush border enzymes to ceramids and phosphocholine, and the latter is degraded to choline and transported by the portal vein to the liver [4]. Chemical structures of choline forms are provided in Supplementary Fig. 1.

The majority of choline in both diet and body is in the form of PC. Absorbed free choline is mainly used for PC formation via the Kennedy pathway, which is in its turn secreted into bile and very-low density lipoproteins (VLDL) [1,5]. The amount of PC in the bile largely exceeds the dietary supply (respectively 11 g/day vs 1–5 g/day). Approximately 95% of biliary PC is reabsorbed and 40% is returned to the liver, implying an extensive enterohepatic choline cycle [2]. Endogenous PC formation occurs via the phosphatidylethanolamine N-methyltransferase pathway, where PC is formed from phosphatidylethanolamine [1,2,5].

Dietary sources of choline are foods of animal origin like eggs, pork, beef, liver, milk, and plant sources like soybean and wheat germ [7]. Consumption of choline forms is dependent on the individual dietary pattern [1,2,8]. Unfortunately, data on choline content is not included in most food composition databases, including the Norwegian one [9]. There is no consensus so far on dietary choline requirements. While in the US the adequate intake is set to 550 mg/d for men and 425 mg/d for women, based on a few studies involving mostly men [10], values set in the European Union are slightly lower [11]. No reference values have been published specifically for the Nordic countries [12]. Mean choline intake in mainly North American and European countries, was below the dietary recommendation [13]. It is however not possible to conclude on the prevalence of choline intake deficiency since only an adequate intake has been established so far [13]. Additionally, few studies have reported intake of the individual choline forms [1,13], indicating the importance of evaluating choline intake in the population.

Choline is crucial for the synthesis of acetylcholine and major membrane phospholipids. Higher plasma choline has been associated with increased risk of cardiovascular disease (CVD) [5,10,14]. Choline's oxidation product, betaine, links choline to the one-carbon metabolism via its role in the betaine-homocysteine methyltransferase reaction where a methylgroup from betaine is transferred to homocysteine, forming methionine and dimethylglycine (DMG) [10,15]. Elevated plasma total homocysteine (tHcy) has been linked to increased risk of coronary artery disease (CAD) [16] and plasma DMG levels have been associated with risk of future acute myocardial infarction (AMI) [15] in the current population.

Only few and contradictory findings are published on dietary choline intake and CVD risk [14,17]. Additionally, previous studies have reported either no [7] or only marginal [14,18] correlations between plasma choline levels and dietary choline intake. So far, only egg intake has been linked to plasma choline levels in several studies [7,19]. In light of the few existing studies [17], it was the aim of this study to investigate the association between dietary choline, including choline forms, and subsequent risk of AMI in patients with stable angina pectoris (SAP).

2. Patients and methods

2.1. Study cohort

In total 3090 adult patients, undergoing elective coronary angiography due to suspected CAD between 1999 and 2004 at Haukeland University Hospital, Bergen and Stavanger University Hospital, Stavanger in Norway were enrolled in the Western Norway B Vitamin Intervention Trial (WENBIT, NCT00354081). This was a prospective, randomized, double-blind, placebo-controlled secondary prevention study that investigated the effect of vitamin B treatment on mortality and cardiovascular outcomes [20]. The study protocol has been described elsewhere [20]. For the current analysis, we included only patients with suspected SAP (n = 2573). We excluded patients with missing dietary data, including choline intake (n = 565), and those which reported extreme energy intake (i.e. <3000 kJ or >15 000 kJ for women and <3300 kJ or >17 500 kJ for men) (n = 27), which resulted in 1981 patients eligible for analyses.

The study was carried out according to the Declaration of Helsinki and approved by the Norwegian Data Inspectorate and the Regional Committee for Medical Health Research Ethics. All participants provided written informed consent.

2.2. Baseline data

Clinical information on patients' lifestyle and medical history was obtained from self-administered questionnaires or through interviews and verified by hospital records. Participants were defined as smokers based on self-reported smoking habits and serum cotinine levels >85 nmol/L at baseline [15]. Diabetes mellitus was defined according to preexisting diagnosis, HbA1c >6.5%, fasting blood glucose ≥7 mmol/L or non-fasting blood glucose ≥11.1 mmol/L according to the World Health Organization guidelines [21].

2.3. Follow-up and study end points

The primary end point of this study was incident AMI, including fatal and nonfatal events, classified according to the revised definition of AMI criteria (ICD-10 codes I21, I22, I46.1, R96, R98) [22]. Information on study outcomes was obtained from the Cardiovascular Disease in Norway (CVDNOR; https://cvdnor.b.uib.no) project, which reported on patients being discharged with a CVD diagnosis between 1994 and 2009 from 42 Norwegian public hospitals, and from the Cause of Death Registry at Statistics Norway (http://www.ssb.no).

2.4. Dietary assessment

Dietary data was obtained from a food frequency questionnaire (FFQ) given at the first visit and returned by email to the study center or at the one-month follow-up visit. The FFQ was an adaptation of a 180-item FFQ from 1992 developed at the Department of Nutrition, University of Oslo and designed to assess the habitual food intake of Norwegian adults. The adaptation resulted in a 169-food item FFQ designed to obtain information on the usual food intake over the past year. The frequency of consumption was given per day, week, month or never consumed depending on the food item. Portion sizes were given as household measures or units such as slices or pieces. Questions on the use of vitamin or mineral supplements were included, however, there were no questions regarding choline supplementation.

Nutrient intake was calculated using a database and software system developed at the Department of Nutrition, University of Oslo (Kostberegningssystem, version 3.2, University of Oslo, Norway). Intake of choline and individual choline forms was quantified using the U.S. Department of Agriculture (USDA) Database for Choline Content of Common Foods, release 2 [6]. Total dietary choline intake was estimated as the sum of free choline, PC, SM, phosphocholine and glycerophosphocholine. Choline content for food items that did not occur in the USDA database was estimated using nutritionally equivalent foods. For dishes that did not occur in the USDA database, choline content was calculated for each ingredient in the FFQ recipe.

2.5. Biochemical analyses

Routine biochemical analyses were conducted at the laboratories in the recruiting hospitals, whereas study-specific analyses were conducted by Bevital AS, Bergen, Norway (http://www.bevital.no). Choline compounds in plasma were analyzed by liquid chromatography-tandem mass spectrometry [23]. Details on the collection, storage and biochemical analysis of samples have been described previously [15].

2.6. Statistical analyses

Baseline variables and dietary intake are reported as median (25th, 75th percentile) for continuous variables or counts (percentages) for categorical variables. Patient baseline characteristics and dietary intake across quartiles of energy-adjusted choline intake were compared by median linear or logistic regression for continuous and categorical variables respectively. To adjust for reported energy intake the residual method was used for choline intake and nutrient density was calculated for macronutrients, food groups and specific food items [24].

Kaplan-Meier plots were used to visualize differences in survival across the quartiles of total choline intake, assessed by the log-rank test. Cox regression models were used to estimate the association between total choline intake and intake of choline forms and risk of AMI. The hazard ratios (HRs) and 95% confidence intervals (CI) were reported per daily increment of 50 mg for total choline and PC and of 5 mg for the remaining choline forms. The first model included reported energy intake, the second model additionally included age and sex and the final model additionally included smoking status. Traditional risk factors for CAD, fasting status, statin use and study intervention were not included in the model since they were not associated with choline intake and thus not considered being a confounder. Generalized additive models (GAMs) were plotted for the association between intakes of total choline and choline forms as continuous variables with AMI risk to explore non-linear relationships.

Effect modifications were studied according to subgroups of traditional risk factors for CAD, such as age, sex, body mass index, hypertension, smoking, diabetes mellitus, estimated glomerular filtration rate, baseline serum lipid parameters (including low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, apolipoprotein B (apoB) and apoA1) and to statin use at discharge and prior AMI. Continuous variables were dichotomized according to their median value and interactions were tested by adding interaction product terms with the continuous variable to the final cox regression model. Statistical analyses were performed using R version 3.4.3 (The R Foundation for Statistical Computing, Vienna, Austria), and the packages within the "*tidyverse*" *("dplyr", "ggplot2", "tidy", "broom", "purrr", "forcats", "tidyr")* [25], "*survival*" [26] and "*forestplot*" [27] were used for statistical analyses.

3. Results

3.1. Baseline characteristics

Baseline characteristics of the study patients (n = 1981) according to quartiles of energy-adjusted choline intake are presented in Table 1. The cohort consisted of 80% men, and the median (25th, 75th percentile) age was 62 (55, 69) years. In the total population, 28% were current smokers, 47% were diagnosed with hypertension, 31% had diabetes mellitus and 44% had a history of AMI. Moreover, choline intake was slightly inversely associated with plasma betaine, DMG and tHcy, but not associated with plasma concentrations of other one-carbon metabolites. A positive association was shown with plasma trimethylamine N-oxide (TMAO), plasma riboflavin, pyridoxal-5′-phosphate (PLP), and serum cobalamin and folate, whereas no association was observed with plasma methylmalonic acid (MMA).

3.2. Dietary choline intake

Dietary intake of choline and choline species, as well as nutrients and food groups across quartiles of energy-adjusted choline intake is shown in Table 2. The median (25th, 75th percentile) total energy intake was 2036 (1657, 2483) kcal/d and the energy-adjusted total choline intake was 288 (255, 326) mg/d. Total choline intake was mainly derived from PC (123 mg, 43%), followed by free choline (74 mg, 26%), glycerophosphocholine (63 mg, 22%), SM and phosphocholine (both 13 mg, 5%). Higher intake of total choline was inversely associated with intake of carbohydrates and positively associated with intake of fiber and protein. There was a slight increase in total fat, a decrease in saturated fatty acids (SFAs) and no change in intake of monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) with increasing choline intake. Further, positive associations were found with intakes of cholesterol, alcohol, fruits and berries and vegetables. As expected, main dietary choline sources such as meat, fish, eggs and milk were all positively associated with choline intake.

3.3. Dietary choline intake and risk of AMI

During a median (25th, 75th percentile) follow-up time of 7.5 (6.3, 8.8) years, 312 (15.7%) patients experienced an AMI (Table 1). Fig. 1 depicts a Kaplan-Meier plot of event-free survival time across quartiles of energy-adjusted choline intake, showing a difference in event-free survival between the quartiles, with better survival in the first quartile and similar survival in the other three quartiles.

In a cox-model adjusted for energy intake only, we observed increased risk for AMI per 50 mg/d increment of total choline (HR 1.11, 95% CI [1.03, 1.20]) and PC (1.24 [1.08, 1.42]) intakes, and per 5 mg/d increment in SM (1.16 [1.02, 1.31]). These associations persisted after further adjustment for age and sex (model 2) and after additional adjustment for smoking (model 3) (Table 3). Intakes of free choline, phosphocholine or glycerophosphocholine intake were not associated with the risk of later AMI. Subgroup analyses showed no differences in AMI risk with regard to traditional risk factors for CAD (Supplementary Fig. 2).

The dose-response relationship between energy-adjusted choline intake and AMI is shown in Fig. 2. A positive linear relationship was observed for total choline, PC and SM whereas for free choline, phosphocholine and glycerophosphocholine there was no relationship.

4. Discussion

It was the aim of this study to investigate the association of dietary total choline and choline forms on future AMI risk in patients with suspected SAP. In this study, higher dietary choline intake, more specifically total choline, PC and SM, was associated with increased AMI risk during long-term follow-up, and the associations appeared to be linear across the intake ranges. The intakes of free choline, phosphocholine and glycerophosphocholine did not seem to be associated with AMI risk.

4.1. Previous studies on dietary choline and CVD risk

A systematic review and meta-analysis of four prospective studies on dietary choline and risk of incident CVD (defined as CAD, stroke or total CVD) reported no association (risk ratio 1.00)

Table 1
Patient characteristics according to quartiles of energy-adjusted daily choline intake.

Variable	Total cohort	Q1 (≤254)	Q2 (255–288)	Q3 (288–326)	Q4 (>326)	*P* trend
	(n = 1981)	(n = 496)	(n = 495)	(n = 495)	(n = 495)	
Total choline, mg/d	288 (255, 326)	232 (212, 244)	273 (264, 280)	306 (297, 315)	361 (341, 397)	<0.001
Incident AMI, n (%)	312 (15.7)	57 (11.5)	87 (17.6)	78 (15.8)	90 (18.2)	0.007
Age, y	62 (55, 69)	62 (55, 68)	63 (56, 70)	62 (55, 70)	61 (55, 67)	0.036
Male sex, n (%)	1588 (80.2)	429 (86.5)	375 (75.8)	384 (77.6)	400 (80.8)	<0.001
BMI, kg/m^2	26 (24, 28)	25 (23, 28)	26 (24, 28)	26 (24, 28)	26 (25, 29)	<0.001
Cardiovascular risk factors, n (%)						
Smokers	558 (28.2)	125 (25.2)	123 (24.8)	131 (26.5)	179 (36.2)	0.913
Hypertension	938 (47.3)	217 (43.8)	232 (46.9)	237 (47.9)	252 (50.9)	0.324
Diabetes mellitus	613 (30.9)	138 (27.8)	146 (29.5)	145 (29.3)	184 (37.2)	0.504
Cardiovascular history, n (%)						
Prior AMI	864 (43.6)	214 (43.1)	212 (42.8)	207 (41.8)	231 (46.7)	0.92
Prior CABG	285 (14.4)	73 (14.7)	70 (14.1)	65 (13.1)	77 (15.6)	0.796
Prior PCI	450 (22.7)	130 (26.2)	95 (19.2)	110 (22.2)	115 (23.2)	0.009
Medication use, n[a] (%)						
Statins	1769 (89.3)	442 (89.1)	436 (88.1)	445 (89.9)	446 (90.1)	0.609
ACE inhibitors	395 (19.9)	85 (17.1)	96 (19.4)	101 (20.4)	113 (22.8)	0.358
ARB	230 (11.6)	45 (9.1)	58 (11.7)	63 (12.7)	64 (12.9)	0.174
Aspirin	1784 (90.1)	464 (93.5)	446 (90.1)	434 (87.7)	440 (88.9)	0.049
β-Blockers	1533 (77.4)	395 (79.6)	378 (76.4)	383 (77.4)	377 (76.2)	0.214
Diuretics	181 (9.1)	34 (6.9)	48 (9.7)	55 (11.1)	44 (8.9)	0.106
CRP, mg/L	1.7 (0.8, 3.3)	1.7 (0.9, 3.2)	1.5 (0.7, 3.1)	1.5 (0.8, 3.1)	1.8 (0.9, 3.7)	0.977
eGFR, mL/min/1.73m^2	92 (82, 100)	92 (83, 100)	91 (80, 98)	92 (81, 100)	94 (83, 101)	0.059
Plasma levels of one-carbon metabolites, μmol/L						
Choline	9.5 (8.1, 11.2)	9.6 (8.2, 11.2)	9.5 (8.0, 11.2)	9.4 (8.1, 11.2)	9.4 (8.1, 11.4)	0.876
Betaine	39.2 (32.1, 48.0)	40.2 (33.4, 48.9)	39.3 (30.9, 46.8)	39.3 (33.1, 48.4)	38.2 (31.3, 46.7)	0.023
DMG	4.0 (3.3, 4.8)	4.1 (3.4, 4.9)	4.0 (3.2, 4.7)	4.0 (3.3, 5.0)	3.8 (3.2, 4.8)	0.001
Glycine	198 (175, 229)	203 (180, 237)	199 (175, 232)	199 (176, 229)	193 (169, 223)	0.001
Serine	94 (81, 107)	95 (82, 108)	94 (80, 109)	95 (83, 107)	92 (81, 105)	0.354
Methionine	26.6 (22.7, 32.2)	26.6 (22.9, 31.1)	26.2 (22.5, 31.9)	26.5 (22.5, 32.0)	27.3 (22.8, 33.3)	0.177
Total homocysteine	10.2 (8.6, 12.1)	10.4 (8.9, 12.7)	10.2 (8.5, 12.0)	10.2 (8.6, 12.1)	9.9 (8.5, 11.6)	0.004
Cystathionine	0.3 (0.2, 0.4)	0.3 (0.2, 0.4)	0.2 (0.2, 0.4)	0.3 (0.2, 0.4)	0.3 (0.2, 0.4)	0.860
Cysteine	286 (265, 308)	286 (264, 307)	286 (264, 309)	286 (266, 309)	287 (266, 308)	0.681
TMAO, μmol/L	5.7 (3.6, 9.4)	5.3 (3.5, 8.5)	5.6 (3.6, 8.2)	5.8 (3.9, 9.9)	6.1 (3.8, 10.9)	0.004
TML, μmol/L	0.7 (0.5, 0.9)	0.7 (0.5, 0.8)	0.7 (0.5, 0.9)	0.7 (0.5, 0.9)	0.7 (0.5, 0.9)	0.132
Plasma markers of B-vitamin status						
Riboflavin, nmol/L	11.1 (7.6, 17.5)	9.6 (6.6, 15.6)	11.2 (7.8, 17.6)	11.1 (7.8, 16.9)	12.2 (8.3, 19.1)	0.282
PLP, nmol/L	40.8 (29.6, 56.5)	37.9 (27.2, 51.9)	41.6 (29.6, 56.1)	40.4 (29.8, 56.4)	43.4 (32.5, 61.6)	0.001
Cobalamin, pmol/L	340 (260, 428)	317 (239, 399)	338 (259, 433)	344 (259, 435)	365 (286, 449)	0.042
Folate, nmol/L	10.0 (7.3, 14.4)	9.5 (7.0, 12.7)	9.7 (7.0, 13.9)	10.1 (7.6, 15.6)	10.8 (7.8, 15.4)	0.034
MMA, μmol/L	0.2 (0.1, 0.2)	0.2 (0.1, 0.2)	0.2 (0.1, 0.2)	0.2 (0.1, 0.2)	0.2 (0.1, 0.2)	0.158
Serum lipids, mmol/L						
Total cholesterol,	4.9 (4.2, 5.6)	4.8 (4.2, 5.5)	4.9 (4.2, 5.6)	4.8 (4.2, 5.7)	5.0 (4.3, 5.7)	0.496
LDL cholesterol,	2.9 (2.3, 3.6)	2.9 (2.3, 3.6)	2.9 (2.4, 3.5)	2.8 (2.3, 3.6)	2.9 (2.4, 3.7)	0.747
HDL cholesterol,	1.2 (1.0, 1.4)	1.2 (1.0, 1.4)	1.2 (1.0, 1.5)	1.2 (1.0, 1.4)	1.2 (1.0, 1.4)	0.175
TG,	1.54 (1.1, 2.2)	1.6 (1.1, 2.2)	1.5 (1.1, 2.2)	1.5 (1.1, 2.1)	1.5 (1.1, 2.3)	0.529
ApoB, g/L	0.8 (0.7, 1.0)	0.8 (0.7, 1.0)	0.8 (0.7, 1.0)	0.8 (0.7, 1.0)	0.9 (0.7, 1.0)	0.423
ApoA1, g/L	1.3 (1.1, 1.4)	1.3 (1.1, 1.4)	1.3 (1.1, 1.4)	1.2 (1.1, 1.4)	1.3 (1.1, 1.4)	0.837

Continuous variables are presented as medians (25th,75th percentile) and categorical variables are reported as counts (%). Patient baseline characteristics across quartiles are compared by median linear (continuous variables) or logistic (categorical variables) regression. Dietary choline intake is energy-adjusted according to the residual method. ARB indicates angiotensin II receptor blockers; AMI, acute myocardial infarction; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; CABG, coronary artery bypass grafting; CRP, C-reactive protein; DMG, dimethylglycine; eGFR, estimated glomerular filtration rate; MMA, methylmalonic acid; PCI, percutaneous coronary intervention; PLP, pyridoxal phosphate; TG, triglycerides; TMAO, trimethylamine N-oxide; TML, trimethyllysine.

[a] At discharge from hospital.

between dietary total choline and risk of CVD [17]. The studies were, as opposed to the current investigation, performed in initially healthy populations, and no analysis of the individual dietary choline forms was provided [17]. The lack of analyses of choline in food items may be one reason for the low number of studies on choline intake. This calls for action for extended analysis of choline contents in foods.

Indeed, few studies have analyzed the intakes of individual choline forms in relation to CVD. Zheng et al. [28] reported a higher PC intake to be associated with increased risk of all-cause and CVD mortality among healthy American men and women, in particular among diabetic patients. As in our study, PC was the major source of dietary choline. However, the authors did not observe an association between PC intake and incident CVD and suggested that the effects of PC intake may be stronger on CVD prognosis than on CVD development. A Japanese population-based cohort study [29] found a positive association of SM, but not PC or total choline with cardiovascular mortality risk in healthy men. In animal studies, feeding SM to either LDLr KO mice [30] or apoE$^{-/-}$ mice [31] gave contradicting results on atherogenesis. Indeed, our finding of an increase in AMI risk with increasing SM intake is consistent with the findings of Nagata et al. [29], despite differences in choline sources in a typical Japanese diet compared to a Nordic diet [7].

4.2. Possible mechanisms

The underlying mechanisms for potential associations between dietary choline and AMI incidence remain elusive. Digestion of both PC and SM is thought to impair intestinal cholesterol absorption [4,32], thus affecting lipid metabolism. In clinical studies, PC and

Table 2
Daily dietary intake according to quartiles of energy-adjusted choline intake.

	Total cohort	Q1 (≤254)	Q2 (255–288)	Q3 (288–326)	Q4 (>326)	*P* trend
	(n = 1981)	(n = 496)	(n = 495)	(n = 495)	(n = 495)	
Total choline, mg/day	288 (255, 326)	232 (212, 244)	273 (264, 280)	306 (297, 315)	361 (341, 397)	<0.001
Choline forms, mg/d						
Free choline	74 (66, 85)	63 (56, 70)	72 (67, 78)	77 (71, 84)	89 (80, 104)	<0.001
PC	123 (103, 147)	97 (84, 110)	117 (104, 128)	134 (117, 151)	159 (137, 183)	<0.001
SM	13 (11, 15)	10 (9, 12)	12 (11, 14)	14 (12, 15)	16 (14, 19)	<0.001
Phosphocholine	13 (10, 16)	9 (7, 11)	12 (10, 14)	14 (11, 17)	17 (14, 21)	<0.001
Glycerophosphocholine	63 (49, 78)	46 (36, 56)	61 (50, 70)	68 (57, 79)	86 (69, 105)	<0.001
Betaine, mg	135 (105, 169)	144 (116, 180)	127 (99, 159)	133 (102, 166)	135 (108, 169)	<0.001
Energy, kcal	2036 (1657, 2483)	2173 (1809, 2604)	1903 (1547, 2345)	1963 (1581, 2385)	2093 (1692, 2564)	<0.001
Carbohydrates, E%	49.8 (45.5, 54.0)	51.4 (46.9, 55.3)	50.7 (47.4, 54.6)	49.6 (45.2, 53.5)	47.8 (43.3, 52.2)	<0.001
Fiber, g/1000 kcal	11.9 (10.0, 14.0)	11.4 (9.7, 13.0)	12.1 (10.2, 14.3)	11.9 (10.2, 14.2)	12.2 (9.9, 14.8)	<0.001
Protein, E%	16.7 (15.2, 18.4)	15.1 (13.9, 16.6)	16.3 (15.2, 17.8)	17.1 (15.8, 18.6)	18.4 (17.1, 20.0)	<0.001
Total fat, E%	31.2 (27.8, 35.0)	31.9 (28.4, 35.6)	30.7 (27.7, 34.5)	31.2 (27.9, 35.0)	31.1 (27.4, 34.7)	0.035
SFA, E%	11.6 (10.0, 13.3)	12.1 (10.4, 13.8)	11.5 (10.1, 13.0)	11.5 (10.0, 13.2)	11.3 (9.6, 13.1)	<0.001
MUFA, E%	10.2 (9.0, 11.6)	10.2 (9.0, 11.6)	10.1 (8.9, 11.4)	10.4 (8.9, 11.7)	10.3 (9.0, 11.6)	0.208
PUFA, E%	6.9 (5.8, 8.4)	6.9 (5.9, 8.6)	6.6 (5.6, 8.2)	6.9 (5.9, 8.2)	7.1 (5.7, 8.4)	0.016
Cholesterol, mg	278 (216, 359)	257 (195, 324)	255 (197, 327)	285 (217, 356)	338 (261, 420)	<0.001
Alcohol, E%	1.0 (0.0, 2.8)	0.5 (0.0, 2.2)	0.9 (0.0, 2.3)	1.2 (0.1, 2.9)	1.4 (0.2, 3.7)	<0.001
Meat, g/1000 kcal	53.3 (39.0, 68.9)	49.6 (35.9, 64.3)	52.3 (39.2, 67.8)	54.5 (40.2, 69.4)	56.5 (41.5, 73.0)	<0.001
Fish, g/1000 kcal	48.7 (33.6, 68.9)	39.6 (27.0, 58.6)	47.7 (33.8, 65.6)	50.1 (36.4, 67.9)	59.9 (42.0, 81.6)	<0.001
Eggs, g/1000 kcal	7.2 (4.0, 11.2)	4.4 (2.6, 7.0)	6.7 (4.1, 10.0)	8.7 (5.2, 12.6)	10.4 (6.8, 15.3)	<0.001
Milk, g/1000 kcal	117 (41, 193)	70 (21, 125)	112 (33, 179)	138 (68, 214)	170 (84, 249)	<0.001
Fruit and berries, g/1000 kcal	107.2 (66.4, 161.7)	98.3 (61.8, 140.3)	111.8 (70.2, 165.6)	110.4 (69.4, 172.8)	111.5 (63.3, 169.4)	0.005
Vegetables, g/1000 kcal	88.8 (57.5, 133.6)	68.7 (44.4, 98.4)	87.8 (58.4, 122.7)	96.6 (65.2, 146.5)	116.5 (72.5, 177.8)	<0.001
Corn products, g/1000 kcal	106.0 (86.1, 127.2)	116.9 (95.7, 135.8)	110.9 (91.0, 129.2)	104.1 (86.9, 126.2)	94.7 (74.7, 114.6)	<0.001

Continuous variables are presented as medians (25th,75th percentile. Dietary intake across quartiles were compared by median linear regression. Choline and choline forms are energy-adjusted according to the residual method. The nutrient density method was used for other nutrients, food groups and specific food items.
PC indicated phosphatidylcholine; SM, sphingomyelin.

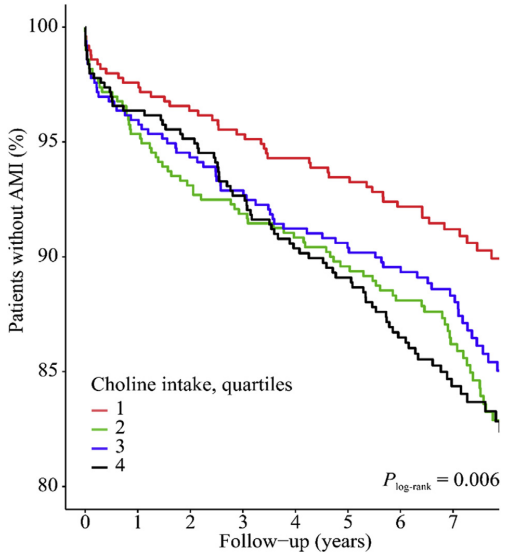

Fig. 1. Kaplan-Meier event-free survival curves for patients with choline intake in quartiles 1 to 4. A non-parametric log-rank test was used to compare survival between quartiles of energy-adjusted choline intake. The *x*-axis is trimmed at 7.5 years.
AMI indicates acute myocardial infarction.

SM from eggs is associated with beneficial changes in biomarkers related to reverse cholesterol transport and high-density lipoprotein characteristics [32,33], suggesting a favorable rather than a negative effect on atherosclerosis. At the same time, dietary choline, and choline-containing compounds such as PC, may exert negative effects through the conversion to trimethylamine by the intestinal microbiota, which is absorbed and transformed in the liver to TMAO by flavin-containing monooxygenase 3 (FMO3) [34]. Several studies, both in animals and humans, reported an association between dietary choline and TMAO formation, as well as a strong positive correlation between plasma TMAO concentration and cardiovascular events [34–37]. Indeed, we also observed increased TMAO levels at higher choline intakes, which may explain the association with increased CVD risk at least partly. Plasma TMAO might advance atherosclerosis by reduction of reverse cholesterol transport, increased macrophage cholesterol accumulation, upregulation of macrophage scavenger receptors and augmented foam cell formation, resulting in increased inflammation and low-density lipoprotein cholesterol oxidation [38,39]. A clear mechanistic link between circulating TMAO and CVD is, however, not yet validated. Additionally, circulating TMAO is not only influenced by diet, but also by the gut microbiome, FMO3 activity and excretion capacity [34,37], factors which were outside the scope of this study. Whether TMAO is a real contributor to atherosclerosis development or merely a marker of underlying pathogenic factors requires further research.

Second, PC is crucial for the formation of VLDL and its secretion from the liver [40]. The major fate of choline is conversion to PC and an estimated 70% of hepatic PC is made via the Kennedy pathway [2], however, to the best of our knowledge, there is no data on the amount of dietary choline incorporated into VLDL phospholipids. PC and other phospholipids produced in the Kennedy pathway may influence lipid metabolism through activation of peroxisome proliferator-activated receptor alpha [41]. In the current study, we found no baseline associations between choline intake and serum lipid parameters, nor did adjusting for such parameters alter the risk association between choline intake and later AMI. However,

Table 3
Hazard ratios for incident AMI according to energy-adjusted choline intake from cox regression analysis.

	Model 1[a]		Model 2[b]		Model 3[c]	
	HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)	P value
Total choline[d]	1.11 (1.03, 1.20)	0.006	1.12 (1.04, 1.21)	0.004	1.10 (1.02, 1.19)	0.015
Free choline[e]	1.02 (0.99, 1.05)	0.280	1.02 (0.99, 1.06)	0.180	1.02 (0.98, 1.05)	0.341
PC[d]	1.24 (1.08, 1.42)	0.002	1.25 (1.09, 1.44)	0.002	1.23 (1.07, 1.41)	0.003
SM[e]	1.16 (1.02, 1.31)	0.019	1.17 (1.04, 1.32)	0.011	1.15 (1.03, 1.30)	0.017
Phosphocholine[e]	1.06 (0.96, 1.18)	0.260	1.07 (0.96, 1.19)	0.203	1.07 (0.96, 1.19)	0.219
Glycerophosphocholine[e]	1.01 (0.99, 1.03)	0.251	1.01 (0.99, 1.03)	0.249	1.01 (0.99, 1.03)	0.249

HR indicates hazard ratio; CI, confidence interval; PC, phosphatidylcholine; SM, sphingomyelin.
[a] Adjusted for energy intake.
[b] Adjusted for energy intake, sex and age.
[c] Adjusted for energy intake, sex, age and smoking.
[d] per 50 mg per day increase.
[e] per 5 mg per day increase.

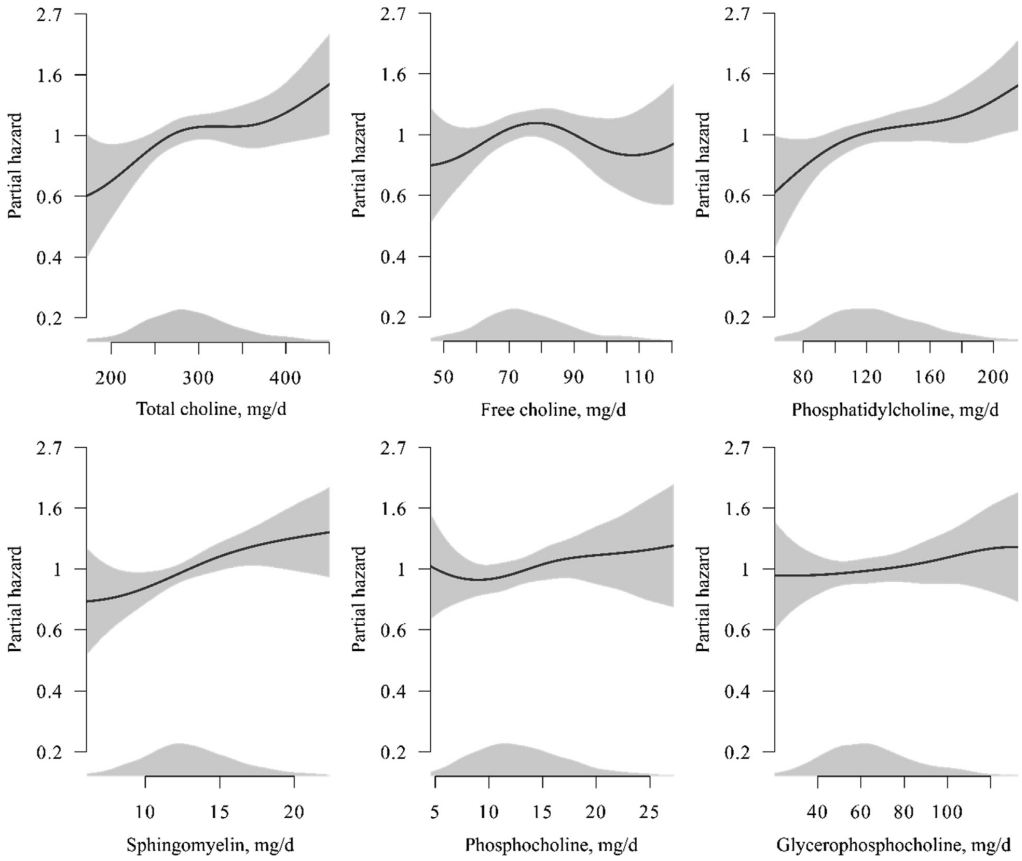

Fig. 2. Association of choline intake with risk of acute myocardial infarction using general additive models adjusted for total energy intake, sex, age and smoking. The solid lines show the observed association and the shaded areas 95% confidence intervals. Density plots indicate the distribution of dietary choline and choline forms.

any association between dietary choline and serum lipid parameters in our cohort might have been masked by high prevalence of statin use, although we did not find any interaction according to statin treatment in subgroup analyses. Nevertheless, our results deem it less likely that the choline-AMI risk relationship is explained by altered lipid levels per se.

Dietary choline intake was inversely associated with plasma tHcy concentrations in our cohort, being in line with several intervention studies showing that choline supplementation lowers plasma tHcy concentrations [42–45]. However, the causality of the association between homocysteine and AMI has been questioned and lowering of tHcy concentrations was not associated with reduced incidence of cardiovascular events in a meta-analysis of 8 randomized trials including 37 485 participants [46].

Also, dietary choline intake has been associated with lower inflammation markers such as CRP, interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) in a cross sectional study involving healthy participants [47]. Pre-clinical studies showed potential anti-inflammatory effects of sphingolipids (with SM as main contributor) but the limited number of human studies and the use of complex phospholipid mixtures instead of single phospholipids, make it difficult to conclude on the effects of sphingolipids on inflammation [48]. In the present study, we did not observe any association between dietary choline and CRP. Il-6, TNF-α and neopterin were not investigated in our study.

The reported median energy-adjusted total choline intake was lower than the recommended intake [5,11]. This is in accordance with observational data from European [49,50], North-American [28,43,51,52] and Norwegian populations [14,18]. Since high choline intake was associated with AMI risk observed in this study and low choline intake has been associated with other adverse health outcomes, such as cancer, neurodegenerative diseases [5,10] and low bone mineral density [18], more research on health effects of choline intake is needed to define an adequate intake. The lack of correlation between dietary and plasma choline and a validated biomarker for choline intake [7,14,18], complicates this determination even more.

Increased PC and SM intake was associated with augmented AMI risk in our population. These fat soluble choline forms are mainly found in products of animal origin like eggs, beef, chicken, fish and milk [5]. Milk, meat and fish consumption increased gradually over the quartiles, while egg consumption in the highest quartile of total choline intake was 2.4 times higher compared to the lowest. Notably, these choline sources tend to have high cholesterol content (especially eggs) [12] and intake should be limited according to current dietary guidelines in context of general and cardiovascular health [12,53]. Additionally, eggs, processed and unprocessed meat are also high in carnitine, a TMAO precursor, and sodium (processed meat) which are associated with CVD risk via different pathways [54]. In contrast, a higher total choline intake was associated with higher vegetable and fibre intake, which are inversely associated with CVD risk [12]. Increased ingestion of plant-based food items result in increased intake of water-soluble choline forms, which were not associated AMI risk in our study. Thus, a high intake of the fat-soluble choline forms may be a marker of an otherwise unhealthy diet and therefore be associated with AMI risk. Importantly, findings for a nutrient are not necessarily valid for a food item containing that nutrient.

Even the association between eggs, which contain a high amount of PC and SM, and CVD remains controversial [54–56]. Further research is needed to explore the underlying mechanisms for the association between fat-soluble choline forms, the food items contributing to their intake and CVD risk.

4.3. Strengths and limitations

Among the strengths of the current study are the large sample size, the prospective design and the long-term follow-up. Detailed clinical and metabolic characterization of the population was available, and dietary intake of all choline forms and plasma concentration of choline were estimated. Additionally, the dietary analyses were adjusted for reported energy intake, which improves the accuracy of the estimates.

To the best of our knowledge, choline data in foods are only presented by the USDA database [6]. There is no data on choline content of Norwegian food items and there are difficulties in replacing local foods with foods included in the database that makes it impossible to exclude discrepancies. Next, the used FFQ was not validated for choline intake [57] and only filled out at baseline which makes it impossible to detect dietary changes over time. Additionally, random measurement error in estimated choline intake may have led to regression dilution bias and attenuated the relationship between choline intake and AMI. The ability to establish causality from this data is limited since it is impossible to exclude residual confounding.

5. Conclusion

In conclusion, increased intakes of total choline and choline forms PC and SM were associated with higher long-term AMI risk in patients with SAP. This is an important finding in light of the lower than recommended average intake in this cohort, and the widespread use of choline supplements. Therefore, future studies are warranted to explore underlying mechanisms for this association, as well as for improving dietary guidelines.

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Declaration of competing interest

No conflict of interest reported by any of the authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biochi.2019.11.001.

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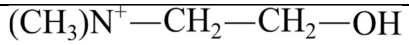
SUPPLEMENTARY MATERIAL FOR ONLINE ONLY

Choline



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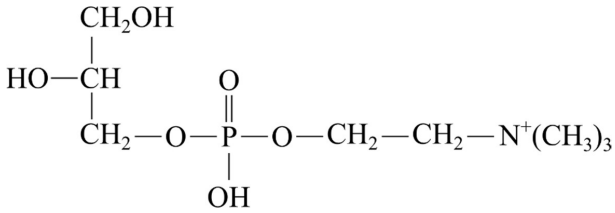

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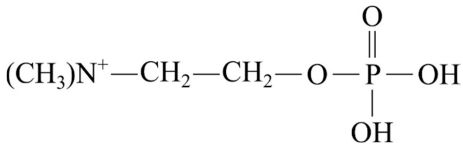

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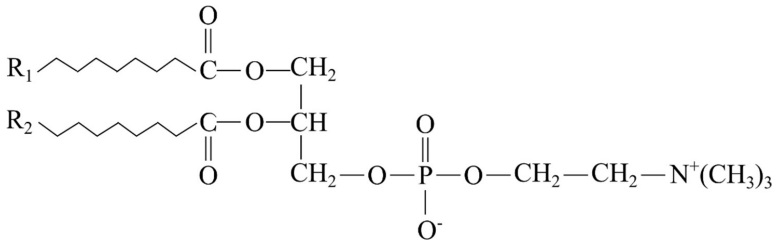

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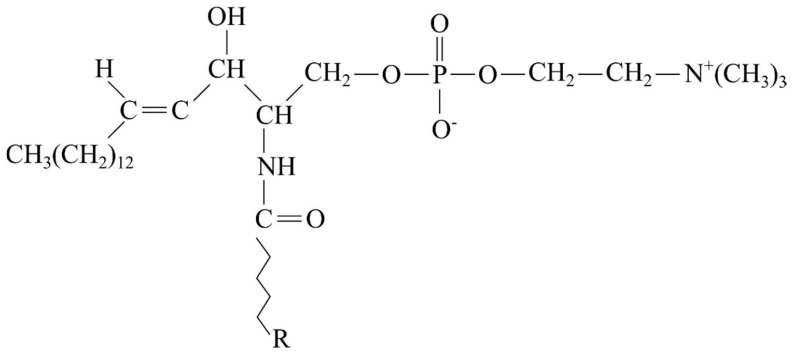
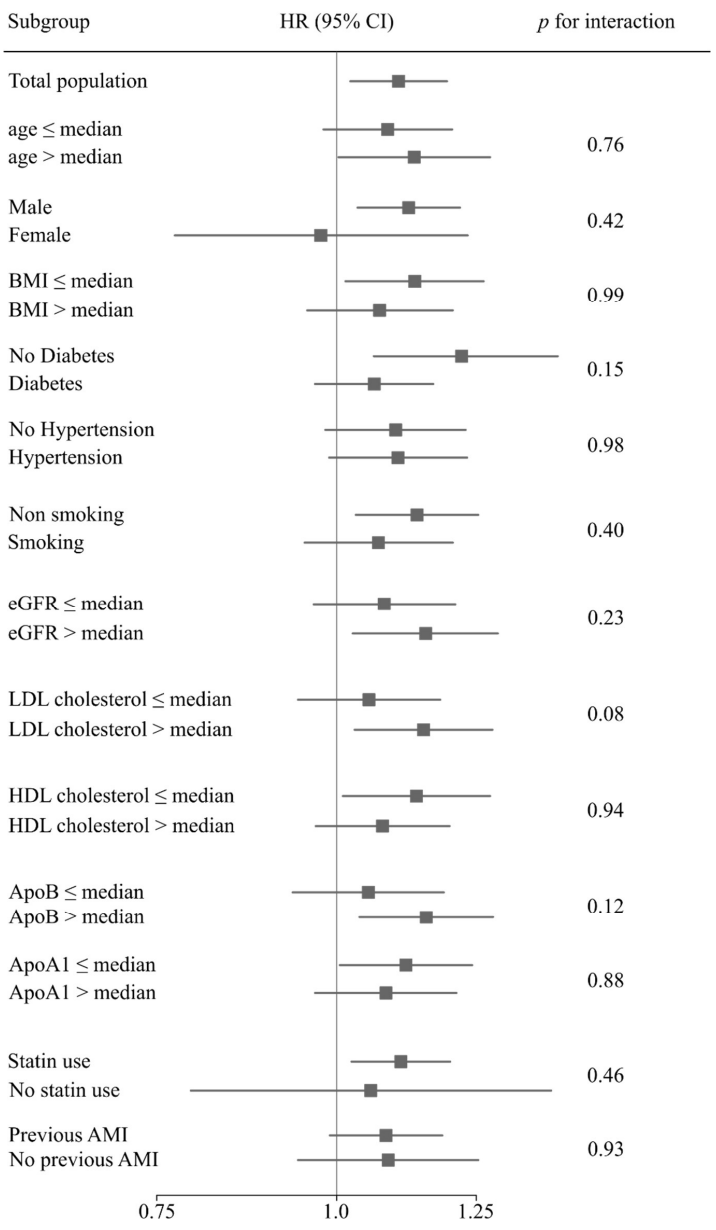


Supplementary figure 1: Chemical structures of choline and choline forms. Adapted from Patterson *et al*. [6].



Supplementary figure 2: Risk estimates per increment of 50 mg/d in total choline for incident AMI according to subgroups based on established risk factors for coronary artery disease based on a cox regression model adjusted for energy intake, sex, age and smoking. Continuous variables were dichotomized according to their median value and interactions

were tested statistically by adding interaction product terms with the non-dichotomized variable to the final cox model adjusted for total energy intake, sex, age and smoking.

AMI indicates acute myocardial infarction; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; CI, confidence interval; eGFR, estimated glomerular filtration rate; HR, hazard ratio.

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No effect of plasma trimethylamine N-Oxide (TMAO) and plasma trimethyllysine (TML) on the association between choline intake and acute myocardial infarction risk in patients with stable angina pectoris

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HIGHLIGHTS

- No effect modification by trimethylamine-N-oxide on the association between choline intake and acute myocardial infarction.
- No effect modification by trimethyllysine on the association between choline intake and acute myocardial infarction.
- No indirect effect of trimethylamine-N-oxide on the association between choline intake and acute myocardial infarction.

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Effect modification

ABSTRACT

Plasma concentrations of trimethylamine N-oxide (TMAO) have been linked to cardiovascular disease (CVD) risk and mortality. TMAO is formed through the bacterial conversion of trimethylamine which is obtained either directly from food, generated from dietary precursors (e.g. choline) or derived from endogenous trimethyllysine (TML) production. In a previous article, we reported an increased risk of acute myocardial infarction with increased total choline intake in patients with stable angina pectoris. Due to the close link between TMAO, TML, choline metabolism and possibly CVD, we investigated whether plasma TMAO and TML modified the effect of total choline intake on acute myocardial infarction (AMI) risk in a post-hoc analysis. We found plasma TMAO and TML do not modify the association between higher dietary choline intake and increased AMI risk. Additionally, this association is not mediated via TMAO.

1. Introduction

Trimethylamine N-oxide (TMAO) is formed through bacterial conversion of trimethylamine (TMA) by flavin-monooxygenases in the liver. TMA is obtained directly from food (e.g. fish, which is also rich in TMAO) or generated from dietary precursors such as choline, choline-containing compounds, betaine and L-carnitine or generated from the L-carnitine metabolite gamma-butyrobetaine (γ-BB) [1,2]. Additionally, γ-BB can be formed endogenously from trimethyllysine (TML) then afterwards converted to carnitine and potentially to TMA and TMAO [3]. An overview of the pathways contributing to TMAO synthesis is provided in Fig. 1. TMAO and its precursors are mainly found in food items of animal origin such as fish, meat, eggs, poultry and milk [1].

In 2011, a link was reported between plasma TMAO and cardiovascular disease (CVD) risk [4]. Since then more evidence has accumulated supporting this association [1]. In animal models, TMAO seems to be proatherogenic [4]; in humans, increased plasma concentrations are associated with elevated risk of CVD and other diseases [1,2]. However, inconsistencies remain and whether or not increased plasma TMAO is causally related to CVD remains unclear [1,2].

We recently reported that higher dietary choline intake is associated with increased risk of incident acute myocardial infarction (AMI) in patients with stable angina pectoris (SAP) [5]. This increase could be attributed only to the lipid-soluble forms PC and sphingomyelin. Additionally, plasma TMAO was positively associated with higher dietary choline intake, while no association between choline intake and plasma

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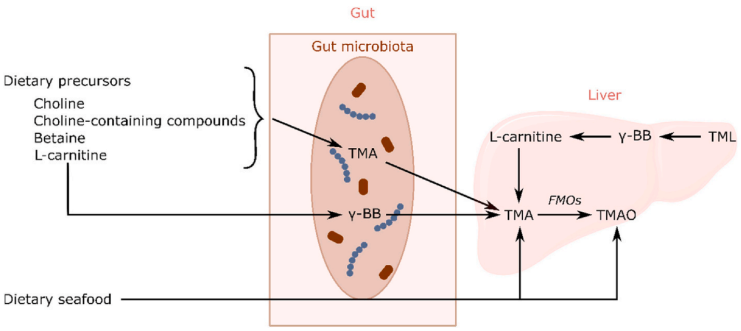

Fig. 1. A general overview of various pathways contributing to trimethylamine N-oxide (TMAO) synthesis. Trimethylamine (TMA) is formed in the intestinal lumen from metabolization of dietary precursors by gut microbiota. Additionally, γ-butyrobetaine (γ -BB) is formed by metabolization of dietary L-carnitine. Both TMA and TMAO can be directly obtained from seafood. After absorption in the liver, TMA is converted to TMAO by flavin monooxygenases (FMOs). Additionally, TMA can be formed endogenously via the formation of γ-BB from trimethyllysine (TML).

TML was observed. In the current post-hoc analysis, we wanted to investigate whether there was any effect modification by plasma TMAO or TML. We based this hypothesis on i) the recently discovered link between plasma TMAO and CVD risk and ii) recent reporting of carnitine synthesis from TML being an endogenous source of TMA, and thus TMAO.

2. Methods

Information on the collection of baseline data, follow-up, and study endpoints can be found in our original article [5].

2.1. Study cohort

In total 3090 adult patients from the Western Norway B-Vitamin Intervention Trial (WENBIT, NCT00354081) were included. This was a prospective, double-blind, placebo-controlled, secondary prevention study were participants were randomized to receive total homocysteine-lowering-B-vitamins. The study protocol has been described in detail elsewhere [6]. Only patients diagnosed with SAP were included (n = 2573). Patients with missing dietary data, including choline intake (n = 565), extreme energy intake (i.e. < 3000 kJ or > 15 000 kJ for women and < 3300 kJ or > 17 500 kJ for men) (n = 27), > 10 E% from alcohol (n = 48) and missing plasma TMAO and TML data (n = 17) were excluded, resulting in 1916 patients eligible for analyses.

The study was carried out according to the Declaration of Helsinki and approved by the Norwegian Data Inspectorate as well as the Regional Committee for Medical Health Research Ethics. All participants provided written informed consent.

2.2. Dietary assessment

A 169-item food frequency questionnaire (FFQ) was used to obtain information on dietary intake. The administered FFQ was an adaptation of an FFQ developed at the Department of Nutrition, University of Oslo designed to obtain information on habitual food intake of the Norwegian population over the past year. The FFQ has not been validated for choline intake and, therefore, we cannot assess how well it captures true choline intake, which is a limitation in the current analysis.

2.3. Biochemical analyses

Plasma TMAO and TML were measured using liquid chromatography-tandem mass spectrometry at Bevital AS (www.bevital.no).

2.4. Statistical analyses

Effect modification by TMAO or TML (continuous scale) was investigated by adding interaction product terms to a Cox regression model which estimated the association between total choline intake and risk of AMI. The model was adjusted for energy intake, age, sex, and smoking. Subgroup analyses were performed, stratifying for the original B vitamin intervention.

Mediation analyses quantify the proportion of an exposure-outcome association working via a mediator (a variable on the causal path between exposure and outcome) [7]. In this study, we applied a mediation analysis on the effect of dietary choline on AMI risk, considering TMAO as a mediator.

Statistical analyses were performed using R version 3.6.1 (The R Foundation for Statistical Computing, Vienna, Austria), the packages within the *tidyverse* and the *survival* and *medflex* package for survival and mediation analyses respectively. The mediation analyses were based on logistic regression analyses because survival models are currently not implemented in *medflex*. The estimates are expected to be similar, however, less powerful [7].

3. Results

The mean total energy-adjusted dietary choline intake was 287 mg/d. Detailed results are available in our original article [5]. Relevant baseline characteristics of the study population included in the current post-hoc analysis are provided in Supplementary Table 1. We previously reported a 10% increased risk of AMI per increment of 50 mg/d in energy-adjusted total choline intake during a median follow-up time (25th, 75th percentile) of 7.5 (6.3, 8.8) years [5]. We did not observe any effect modification according to baseline plasma TMAO and TML concentrations (Fig. 2). The interaction coefficient was 0.002 (standard error = 0.004, p = 0.567) for TMAO and −0.06 (standard error = −0.08, p = 0.417) for TML. Stratifying the analyses for the original B-vitamin intervention yielded generally identical results as in the full population (data not shown).

AMI indicates acute myocardial infarction; TMAO, trimethylamine N-oxide; TML, trimethyllysine.

Results from the mediation analyses show no indication that the choline effect was mediated via plasma TMAO (Table 1).

4. Discussion

In this population, no interaction effect was found between dietary choline and plasma TMAO or plasma TML regarding AMI risk. Further, we did not observe any indication of the choline effect being mediated through plasma TMAO.

Several observational and experimental studies suggested a positive correlation between plasma TMAO and CVD risk and mortality [1]. However, a clear mechanistic link has yet to be proven. Additionally, a recent study [8] failed to reproduce the results from the original paper [4] where TMAO increased atherosclerotic lesion size in mice. Hence,

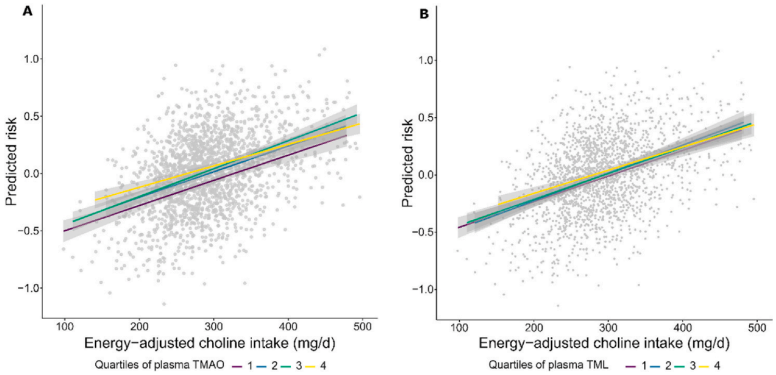

Fig. 2. Predicted AMI risk with increasing energy-adjusted total choline intake across quartiles of plasma TMAO (panel A) and TML (panel B).

Table 1
Summary of mediation analysis via plasma TMAO.

	OR (95% CI)	*p*
Natural direct effect	1.11 (1.01–1.21)	0.027
Natural indirect effect	1.00 (1.00–1.01)	0.608
Total effect	1.11 (1.02–1.21)	0.022

CI indicates confidence interval; OR, odds ratio; TMAO, trimethylamine N-oxide.

the causal relation between plasma TMAO and CVD remains a point of discussion [1,2].

The results of our analyses indicate that the mechanism through which dietary choline is associated with increased AMI risk does not involve TMAO or TML. The hepatic choline metabolism suggests a possible interaction between endogenous choline synthesis and intake from the diet [9]. A choline-deficient diet increases the endogenous synthesis and distribution of choline from other tissues to the liver and the brain [10]. Whether the opposite takes place in the case of a choline excess, to the best of our knowledge, remains unclear. We can therefore only speculate that the balance between dietary choline and endogenous synthesis could be involved in the association between dietary choline and increased AMI risk. Additionally, extensive enterohepatic choline traffic further complicates the determination of the fate of the choline forms after absorption [10]. Thus far, the major part of research on choline and human health has focused on total choline intake [9]. More research is needed on absorption, digestion and metabolism of all choline forms to investigate possible mechanisms by which their intake could increase CVD risk.

5. Conclusion

Plasma TMAO and plasma TML levels do not modify the association between higher dietary choline intake and increased AMI risk. Additionally, this association is not mediated via TMAO.

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Author statement – Van Parys et al.

Anthea Van Parys: Formal analyses, writing – original draft.

Vegard Lysne: Formal analyses.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.hnm.2020.200112.

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SUPPLEMENTAL MATERIAL FOR ONLINE ONLY

Supplementary table 1: Baseline characteristics of patients included in the current post-hoc analysis

	Total population	**Female**	**Male**
n (%)	1916	388 (20)	1528 (80)
Age, years	61 (42.0, 79.0)	63.1 (42.7, 80.0)	60.5 (42.0, 78.0)
BMI, kg/m^2	26.1 (20.0, 34.0)	25.8 (18.0, 37.0)	26.2 (21.0, 34.0)
Smoking[a], n (%)	530 (27.7)	100 (25.8)	430 (28.1)
Hypertension, n (%)	905 (47.2)	199 (51.3)	706 (46.2)
Diabetes[b], n (%)	590 (30.8)	116 (29.9)	474 (31)
AMI, n (%)	311 (16.2)	64 (16.5)	247 (16.2)
Statin use, n (%)	1708 (89.1)	338 (87.1)	1370 (89.7)
Plasma choline, µmol/L	9.6 (5.9, 15.7)	9.1 (5.6, 14.6)	9.7 (6.0, 15.8)
Plasma TMAO, µmol/L	6.2 (1.7, 36.5)	5.6 (1.5, 32.3)	6.4 (1.8, 37.0)
Plasma TML, µmol/L	0.7 (0.4, 1.8)	0.6 (0.3, 1.7)	0.7 (0.4, 1.8)
Choline intake[c], mg/d	287.1 (181.4, 437.6)	293.9 (215.7, 435.2)	285.3 (177.7, 442.9)

Continuous variables are presented as geometric mean (95% prediction interval) and categorical variables as counts (%).
AMI indicates acute myocardial infarction; BMI, body mass index; TMAO, trimethylamine N-oxide; TML, trimethyllysine.
[a] Smoking is defined according to self-report or as plasma cotinine > 85 nmol/L.
[b] Diabetes is defined according to pre-existing diagnosis, HbA1c > 6.5% or a blood glucose measurement > 7mmol/L (fasting) or >11.1 mmol/L (nonfasting).
[c] Choline intake is adjusted for reported energy intake according to the residual method.

III

frontiers in Nutrition

ORIGINAL RESEARCH
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Food Sources Contributing to Intake of Choline and Individual Choline Forms in a Norwegian Cohort of Patients With Stable Angina Pectoris

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Background: Choline is an essential nutrient involved in a wide range of physiological functions. It occurs in water- and lipid-soluble forms in the body and diet. Foods with a known high choline content are eggs, beef, chicken, milk, fish, and selected plant foods. An adequate intake has been set in the US and Europe, however, not yet in the Nordic countries. A higher intake of lipid-soluble choline forms has been associated with increased risk of acute myocardial infarction, highlighting the need for knowledge about food sources of the individual choline forms. In general, little is known about the habitual intake and food sources of choline, and individual choline forms.

Objective: Investigate foods contributing to the intake of total choline and individual choline forms.

Design: The study population consisted of 1,929 patients with stable angina pectoris from the Western Norway B Vitamin Intervention Trial. Dietary intake data was obtained through a 169-item food frequency questionnaire. Intake of total choline and individual choline forms was quantified using the USDA database, release 2.

Results: The geometric mean (95% prediction interval) total choline intake was 287 (182, 437) mg/d. Phosphatidylcholine accounted for 42.5% of total choline intake, followed by free choline (25.8%) and glycerophosphocholine (21.2%). Phosphocholine and sphingomyelin contributed 4.2 and 4.5%, respectively. The main dietary choline sources were eggs, milk, fresh vegetables, lean fish, and bread. In general, animal food sources were the most important contributors to choline intake.

Conclusion: This study is, to the best of our knowledge, the first to assess the intake of all choline forms and their dietary sources in a European population. Most choline was consumed in the form of phosphatidylcholine and animal food sources contributed most to choline intake. There is a need for accurate estimates of the dietary intake of this essential nutrient to issue appropriate dietary recommendations.

Keywords: choline, dietary intake, phosphatidylcholine, FFQ, dietary recommendations

INTRODUCTION

Choline is an essential nutrient with a variety of biological functions. It is a precursor for the synthesis of phospholipids and the neurotransmitter acetylcholine and a source of methyl groups (1, 2). Choline can be synthesized *de novo* via the hepatic phosphatidylethanolamine N-transferase (PEMT) pathway, however, this route is not sufficient to support biological requirements (3).

As choline has important metabolic functions, both dietary intake and circulating concentrations have been associated with several adverse health effects. Dietary deficiency leads to the development of fatty liver disease, liver and muscle damage, and low choline intake has been associated with cancer, neurodegenerative diseases (1, 4), and low bone mineral density (1, 4, 5). On the other hand, elevated plasma choline levels have been associated with an increased risk of cardiovascular disease (CVD) (4, 6). Contradicting findings have been reported concerning the relationship between choline intake and CVD (7). Recent findings from our group suggest an increased risk of acute myocardial infarction with increased dietary choline intake in patients with suspected CVD (8).

There is some uncertainty regarding the required amount of dietary choline. An adequate intake (AI) value for choline was first set by the US Institute of Medicine (currently known as the National Academies of Medicine, NAM) in 1998 (**Table 1**) (2). The European Food Safety Authority (EFSA) published the Dietary Reference Values for Choline in 2016. Similar to the NAM, only an AI was set for choline due to a lack of data to determine an estimated average requirement (**Table 1**) (9). So far, no recommendations have been published for the Nordic countries (10).

TABLE 1 | Adequate choline intake (mg/d) in USA and EU*.

	USA		EU
	Male	**Female**	
Infant (0–6 months)	125	125	120
Infant (7–12 months)	150	150	160
Children (1–14 years)	200–375	200–375	140–340
Adolescents and adults (≥15 years)	550	400–425	400
Pregnancy		450	480
Lactation		550	520

**USA recommendations set by the National Academies of Medicine (2). EU recommendations set by the European Food Safety Authority (9).*

Dietary choline is provided in lipid-soluble forms (phosphatidylcholine or sphingomyelin) or water-soluble forms (free choline, phosphocholine, or glycerophosphocholine). Eggs, beef, chicken, milk and fish, and some plant foods, such as cruciferous vegetables and certain beans, are good sources of choline. Animal products generally contain more choline per weight than plants and contribute most to the intake of the lipid-soluble choline forms (1, 11). Eggs in particular have been shown to make a substantial contribution to total choline intake (12). Further, lecithin (i.e., phosphatidylcholine) is added to many pre-packed foods, which thereby become sources of choline (1).

Choline intake differs between countries as it is dependent on dietary patterns (13) and ethnicity (14). Currently, information on choline intake is mainly available from European and North American countries and has been reviewed by Wiedemann et al. (15) in 2018. Unfortunately, very few studies report on the intake of individual choline forms in addition to total choline intake (15). This could possibly be due to the lack of food composition tables that report on individual choline forms. The USDA database is commonly used for estimation of choline intake (15, 16). Data on dietary choline intake in Norway is scarce (5, 6, 8) and so far, the contribution of different food items to total intake and intake of individual choline forms has not been investigated. Additionally, we were only able to identify one study reporting on contribution of food items to intake of choline forms worldwide (17), emphasizing the knowledge gap regarding this topic.

The aim of this study is to investigate dietary choline intake, including all choline forms, and to map food items contributing to the intake in a Norwegian patient cohort.

PATIENTS AND METHODS

Study Cohort

Between 1999 and 2004, 3,090 adult patients undergoing elective coronary angiography due to suspected coronary artery disease were enrolled in the Western Norway B Vitamin Intervention Trial (WENBIT, NCT00354081) performed at Haukeland University Hospital, Bergen and Stavanger University Hospital, Stavanger in Norway. The WENBIT study was a randomized, double-blind, placebo-controlled prospective secondary prevention study investigating the effect of vitamin B treatment on mortality and cardiovascular outcomes. The study protocol has been described elsewhere (18).

For this study, the source population consisted of the patients from the WENBIT cohort with stable angina pectoris ($n = 2,573$).

Exclusion criteria for the current analyses were missing dietary data, including choline intake ($n = 565$), extreme energy intake (i.e., <3,000 kJ or >15,000 kJ for women and <3,300 kJ or >17,500 kJ for men) ($n = 27$) and >10E% from alcohol ($n = 52$), resulting in 1,929 patients eligible for analyses. Key characteristics of the study population are depicted in **Table 2**.

The study was carried out according to the Declaration of Helsinki and approved by the Regional Committee for Medical Health Research Ethics and the Norwegian Data Inspectorate. All participants provided written informed consent.

Dietary Assessment

A 169-item food frequency questionnaire (FFQ) was given to the patients at the first study visit, filled out by the patients, and returned at the 1-month follow-up visit or returned by mail to the study center. The administered FFQ was an adaptation of an FFQ developed at the Department of Nutrition, University of Oslo designed to obtain information on habitual food intake of the Norwegian population over the past year. Portion sizes were given as units (e.g., slices, pieces, etc.) or household measures. Depending on the food item, the frequency of consumption was given per day, week, month, or never consumed. Questions on vitamin and supplement use were included, however, there were no specific questions regarding choline supplementation. A software system developed at the Department of Nutrition, University of Oslo (Kostberegningssystem, version 3.2, University of Oslo, Norway) was used to calculate energy and nutrient intakes.

Choline Composition Data

Choline composition data are currently not available within the Norwegian food composition database (19). Choline content of food items was therefore quantified using the U.S. Department of Agriculture (USDA) Database for Choline Content of Common Foods, release 2 (11). This database contains the choline content of over 630 food items, analyzed using liquid chromatography-electrospray ionization-isotope dilution mass spectrometry (LC-ESI-MS) (11). Information on total choline content is provided both in the database and in this study as the sum of the five choline forms - free choline, glycerophosphocholine, phosphocholine, phosphatidylcholine, and sphingomyelin. The choline content of food items included in the FFQ but missing in the USDA database was estimated using nutritionally equivalent foods. For multi-component foods (e.g., dishes, fast foods), choline content was calculated for each ingredient in the FFQ recipe.

Food entries were sorted into 41 subcategories based on nutrient similarities. These categories were gathered into 28 main categories. Finally, the main categories were gathered into 10 food groups. A detailed overview is shown in **Supplementary Table 1**.

TABLE 2 | Characteristics of the study population.

	Total population	Female	Male
n (%)	1,929	390 (20)	1,539 (80)
Age, y	61 (42, 79)	63 (43, 80)	60 (42, 78)
BMI, kg/m^2	26 (20, 34)	26 (18, 37)	26 (21, 34)
Smokers[a], *n* (%)	532 (27.6)	100 (25.6)	432 (28.1)
Hypertension, *n* (%)	911 (47.2)	200 (51.3)	711 (46.2)
Diabetes[b], *n* (%)	592 (30.7)	117 (30.0)	475 (30.9)

Continuous variables are reported as geometric mean (95% prediction interval), and categorical variables are reported as counts (%).

[a]Defined according to self-reporting smoking habits and serum cotinine levels >85 nmol/L at baseline.

[b]Defined according to pre-existing diagnosis, HbA1c >6.5%, fasting blood glucose ≥7 mmol/L or non-fasting blood glucose ≥11.1 mmol/L.

Statistical Analyses

Continuous variables are reported as geometric means (95% prediction interval [PI]). The 95% PI renders the limits of the interval as defined by [(geometric mean)/(geometric standard deviation)^2, (geometric mean) × (geometric standard deviation)^2]. The residual method was used to adjust choline intake for reported energy intake. Other dietary variables were energy-adjusted using the density method and are reported as energy % (E%) or g/1,000 kcal.

The percent contribution of each (sub) category to total choline intake and intake of individual choline forms was calculated using the following formula: [(choline provided by the food (sub) category/Total choline from all food (sub) categories)]*100.

In accordance with the Strengthening the Reporting of Observational studies in Epidemiology (STROBE) checklist and statement (20), we chose not to report *p*-values.

All statistical analyses were performed using R version 3.6.1 [The R Foundation for Statistical Computing, Vienna, Austria) and the packages within the *Tidyverse (version 1.3.0)* (21) (*broom (version 0.5.6), dplyr (version 0.8.5), forcats (version 0.5.0), ggplot2 (version 3.3.0), magrittr (version 1.5), purr (version 0.3.4), rlang (version 0.4.5), stringr (version 1.4.0), tidyr (version 1.0.2)*].

RESULTS

The mean (95% PI) age of the participants was 61 (42, 79) years and 80% were men. The participants consumed on average 1996 (982, 3,512) kcal per day of which 49.5 E% came from carbohydrates, 16.8 E% from protein, and 30.9 E% from fat. An overview of the dietary intake in the study population is provided in **Table 3**.

Table 4 shows the energy-adjusted self-reported daily choline intake among the study participants. The geometric mean energy-adjusted total choline intake in the population was 287 (182, 437) mg/d. Women seemed to have a slightly higher choline intake compared to men. Phosphatidylcholine was the major contributor (42.5%) followed by free choline and glycerophosphocholine (respectively 25.8 and 21.2% of total choline intake). Finally, sphingomyelin and phosphocholine contributed to the total intake with 4.5 and 4.2% respectively. Reported energy-adjusted intakes at, or above, the AI of 400 mg/d as defined by EFSA were achieved in only 5.5% of the study population.

In this population, the main choline source was fish, followed by dairy, vegetables, eggs, and meat which accounted in total for about 75% of the total choline intake (**Figure 1**). Phosphatidylcholine was mainly obtained from eggs (28.0%), fish (18.5%), and meat (18.3%). The contribution of these foods combined provided 65% of the total phosphatidylcholine intake. The food category contributing the most to sphingomyelin in the diet was meat (28.5%), followed by dairy (23.3%) and fish (21.7%). Free choline was mainly obtained from vegetables, drinks, grain products, and dairy. Dairy was the main source for both glycerophosphocholine and phosphocholine in our study cohort and accounted for respectively 35.4 and 36.8% of their intake.

Table 5 depicts a more detailed picture of dietary choline sources showing food categories, instead of the larger food groups, contributing to dietary intake of total choline. Eggs contributed most to total choline intake in this population. Additionally, animal-based foods made up seven out of 10 food categories providing most total choline.

The main food categories contributing to intake of the individual choline forms are shown in **Supplementary Tables 2a–e**. Fresh vegetables, bread, coffee, and potatoes supplied half of the dietary free choline intake in our study population. Glycerophosphocholine was primarily obtained from milk and different fish sources, while phosphocholine was mainly acquired through intake of milk, fresh vegetables, and potatoes. The main source of the lipid-soluble phosphatidylcholine was eggs, contributing with 28%. Fresh meat, eggs, milk, and fish products provided half of the ingested sphingomyelin. A full overview of all food groups, categories, and subcategories contributing to choline intake is provided in **Supplementary Table 3**.

Total choline and all individual choline forms, except for free choline, were mainly obtained from animal-based food sources in this study population (**Figure 2**).

TABLE 3 | Dietary intake in the total study population and across genders.

	Total population	Female	Male
n (%)	1,929	390 (20)	1,539 (80)
Energy intake (kcal)	1,996 (983, 3,512)	1,548 (834, 2,861)	2,128 (1,196, 3,594)
Carbohydrates (E%)	48.7 (36.7, 60.8)	49.7 (37.5, 61.3)	48.5 (36.4, 60.4)
Protein (E%)	16.5 (12.1, 22.3)	17.1 (12.8, 23.1)	16.4 (12.0, 22.0)
Fat (E%)	31.5 (21.6, 43.1)	30.7 (21.0, 42.5)	31.7 (21.7, 43.2)
MUFA (E%)	10.1 (6.6, 14.3)	9.8 (6.6, 13.7)	10.2 (6.6, 14.4)
PUFA (E%)	7.0 (4.2, 11.5)	6.5 (4.1, 10.6)	7.1 (4.3, 11.8)
SFA (E%)	11.5 (7.1, 17.7)	11.5 (7.1, 18.5)	11.5 (7.0, 17.4)
Alcohol (E%)	0.2 (0.0, 7.5)	0.0 (0.0, 5.6)	0.3 (0.0, 7.7)
Dairy	126 (17, 413)	135 (26, 445)	124 (16, 403)
Drinks	552 (202, 1,359)	658 (232, 1,562)	529 (197, 1,227)
Eggs	5 (0, 24)	6 (0, 26)	5 (0, 24)
Fats	13 (2, 32)	11 (2, 30)	13 (2, 32)
Fish	45 (10, 119)	46 (10, 124)	45 (11, 119)
Fruit	98 (16, 339)	122 (17, 376)	93 (15, 330)
Grain products	118 (62, 187)	115 (58, 185)	118 (63, 188)
Meat	49 (16, 105)	48 (16, 100)	50 (16, 106)
Other	40 (9, 118)	38 (9, 108)	40 (10, 118)
Vegetables	153 (58, 361)	178 (58, 412)	147 (58, 332)

All dietary intakes are presented as geometric mean (95% prediction interval) and as g/1,000 kcal unless specified otherwise. E% indicates energy percent; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

DISCUSSION

This study aimed to investigate food items contributing to the intake of total choline and individual choline forms. Eggs, milk, fresh vegetables, lean fish, and bread were the main contributors to total choline intake. Choline was mainly consumed in the form of phosphatidylcholine. In general, animal food sources were the most important contributors to choline intake. To our knowledge, this is the first study to assess dietary sources of choline and intake of all choline forms in a European population.

Dietary Sources of All Choline Forms

Eggs contain the highest amount of choline per weight (11) and contribute most to total choline intake in this study. Other good choline sources such as meat, fish, and milk also ranked highest among choline contributors. Similar findings have been reported in other Western cohorts (14, 17, 22–26). Differences in the contribution of food groups between cohorts might be due to

TABLE 4 | Mean energy-adjusted daily reported choline intake in the study population and across sex.

	Total population	% of total choline	Female	Male
n (%)	1,929		390 (20)	1,539 (80)
Total choline, mg/d	287 (182, 436)	-	294 (216, 435)	285 (178, 439)
Free choline, mg/d	74 (49, 114)	25.8	76 (53, 110)	74 (48, 116)
Glycerophosphocholine, mg/d	61 (24, 128)	21.2	62 (31, 109)	61 (23, 132)
Phosphatidylcholine, mg/d	122 (67, 209)	42.5	127 (78, 216)	121 (66, 206)
Phosphocholine, mg/d	13 (5, 26)	4.2	14 (7, 32)	12 (5, 25)
Sphingomyelin, mg/d	13 (7, 22)	4.5	13 (8, 21)	13 (7, 22)

Intakes are reported as geometric means (95% prediction interval).

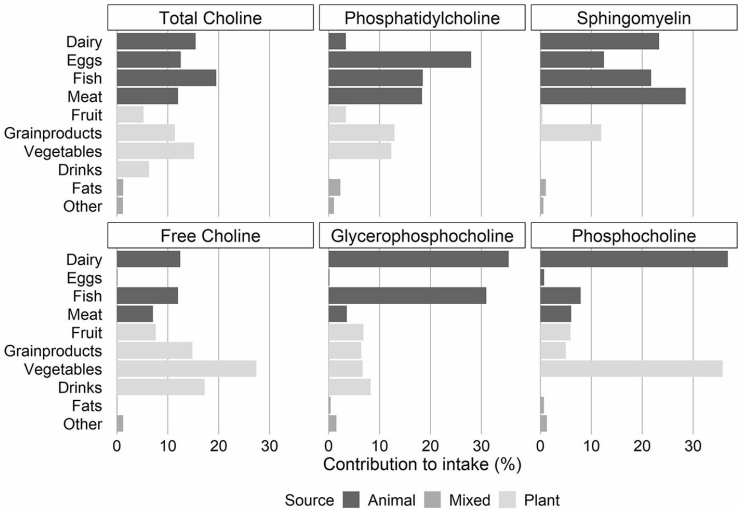

FIGURE 1 | Contribution of food groups to the intake of total choline and individual choline forms.

TABLE 5 | Primary food categories contributing to total choline intake in the study population.

Rank	Food category	Contribution, %	Cumulative contribution
1	Eggs	12.6	12.6
2	Milk	12.1	24.7
3	Fresh vegetables	9.2	33.9
4	Lean fish	8.3	42.2
5	Bread	7.3	49.5
6	Fish products	6.3	55.8
7	Potatoes	5.5	61.3
8	Meat products	5.4	66.7
9	Fresh meat	5.1	71.8
10	Coffee	3.9	75.7

differences in dietary patterns or a different definition of the food groups. However, there was a consensus between the reported studies on the major food groups contributing being eggs, milk, meat, and fish.

A limited amount of studies reports on the intake of individual choline forms. The distribution of the intakes of the individual choline forms observed in this cohort accords with the distribution reported in other Western cohorts (15, 24, 25, 27). The lipid-soluble choline form phosphatidylcholine accounted for around half of the total ingested choline in this cohort. This is not surprising since 60% of total choline was obtained from animal products, which in general contain more choline per unit weight than plants and contain mainly lipid-soluble choline forms (1, 11). We could only identify one study describing the contribution of food items to every individual choline form (17). Similar to our findings, lipid-soluble choline forms were mainly obtained from animal-derived food items, while the contribution of plant foods was larger for the water-soluble forms. Top-ranked contributing food items were also similar for all individual choline forms.

Total Choline Intake

Dietary choline intake has mainly been studied in European and North-American cohorts (15). Based on food consumption data from the EFSA European Comprehensive Food Consumption Database, Vennemann et al. reported a self-reported choline intake ranging from 357 to 468 mg/d for adult men and from 293 to 374 mg/d for adult women in Europe (16). This data was obtained from 10 nationwide surveys in eight different European countries. These values are similar to the dietary choline intake reported in several studies conducted in the USA (14, 17, 22, 23, 26, 28, 29), Canada (25, 30) and New-Zealand (24) where reported intake ranged from 312 to 421 mg/d in adult men and from 258 to 314 mg/d in adult women. The self-reported dietary choline intake in our population was low compared to these values. It is possible that choline intake is lower in Norway compared to other Western countries since choline consumption is dependent on individual dietary patterns. Our population was generally older and our data was collected at an earlier time point compared to the mentioned studies, which might explain this discrepancy. Moreover, it has been shown that race and ethnicity influence choline intake (14, 23), which might explain the discrepancies further. However, the Norwegian dietary habits are quite similar to those of other Western countries and it would, therefore, be unlikely that this causes the lower intake.

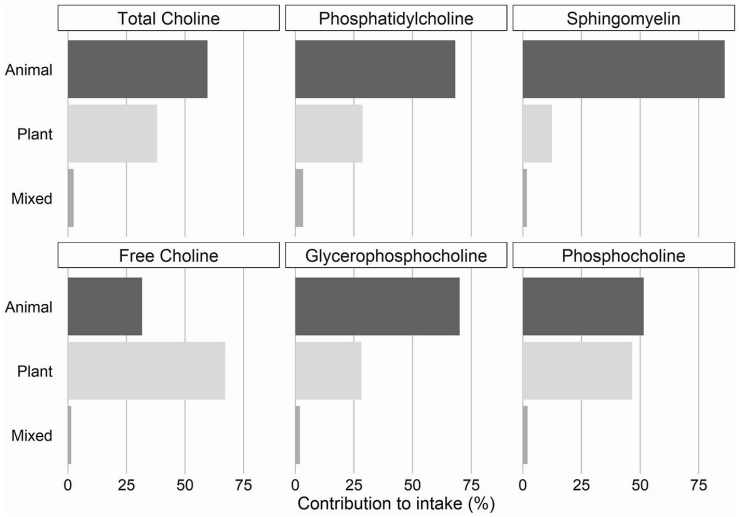

FIGURE 2 | Contribution of animal, plant or mixed food sources to intake of total choline and individual choline forms.

Notably, we used an FFQ that was not validated for choline intake. Therefore, we were unable to evaluate how well it assesses the actual choline intake. Further, studies have used different versions of the USDA database to assess to choline content of foods, which leads to variation in estimates of choline intake. Also, some studies adjusted for energy intake, while others did not. Finally, it has to be taken into account that comparing dietary choline intake between studies must be done with caution due to different methods used to assess dietary habits.

Notably, the total dietary choline intake in our cohort, as in many other studies (15), was below the recommended European and American AI. The self-reported nature of the dietary choline data may have caused an underestimation of actual choline intake due to underreporting. Fischer et al. (28) found that self-reported 3-day weighed food records significantly underestimated daily choline intake compared to the measured choline content in the diet. Additionally, FFQs are subject to social desirability bias meaning that participants tend to overreport food items that are considered "healthy." This could have led to underreporting of "less healthy" food items in this population such as eggs and red meat, which are rich in choline. Egg consumption was discouraged in Norway at the time of data collection which may have lead to underreporting or a true lower egg intake. It also has to be taken into account that the recommended AI of both NAM and EFSA is based on few data. The values set by the NAM for adults are based on a single study performed in males, whereas the values for children were mathematically extrapolated from these adult values (2). EFSA based its estimates on 12 national surveys undertaken in nine European countries (31). Both institutions agree that there is insufficient data to establish average requirements and population reference intakes and therefore only report an AI (2, 31). The lack of data could be attributed to the lack of food composition databases to estimate dietary choline intake. Additionally, folic acid fortification in grains in the US improved folate status in this population. This may reduce choline requirements since folate can be used for remethylation of homocysteine, thereby sparing choline (32) and thus influence dietary requirements set by NAM. Finally, given the definition of an AI, it is not possible to draw any conclusion about the adequacy of choline intake in this study population.

Strengths and Limitations

Several limitations of our study should be acknowledged. First, the Norwegian food composition table does not include values for choline (19). Since choline composition data of European foods is also non-existent, we based our calculation of the choline content of food items on the USDA database (11). The choline content of food items in this database might not always reflect the true choline content of consumed food items in this study population. Especially local foods, which may not be typically consumed in a North American diet needed to be substituted with similar foods with a known choline content. Choline content can also differ due to variations in recipes used by the manufactures or due to differences in choline content of the individual ingredients (16). Additionally, it is not unlikely that the nutritional content of animal food items is influenced by factors such as choline consumption of the animal, season and geographical location, and variation between and within animals, all of which can contribute to discrepancies between estimated and actual dietary choline intake.

Secondly, the administered FFQ was not validated for choline intake, meaning that we were not able to evaluate its ability

to capture actual choline intake. The reported choline intakes should therefore be interpreted with caution. It also did not include information on the food preparation method, which influences the choline content (11, 13). General disadvantages of an FFQ also apply here and include failure to report intake of non-included items due to the fixed-food list and recall bias (33).

Finally, this population of, mainly older, male, cardiovascular patients is not representative of the general population. Additionally, a high percentage of the study population suffered from chronic conditions such as hypertension (almost 50%) and diabetes (30%). Medication use (e.g., statins, aspirin, β-blockers) was also very common in this study cohort (data not shown). The population has been shown to be representative of a general CVD disease population (18), and the results may therefore lack external validity outside such populations. Moreover, their diagnosis might have influenced their true and reported usual dietary intake as these patients may have received dietary advice. Especially consumption of animal food items, which is discouraged in these patients, could have been affected, leading to both reduced intake and underreporting of total choline intake (34).

The strength of this study is that intake of all choline forms, which is heavily understudied, was estimated in a large cohort. Moreover, using an FFQ for dietary assessments avoids day-to-day variations and represents usual long-term intake. This allowed us to collect data on food items that are less frequently consumed. Finally, an FFQ captures usual long-term dietary intake, allowing us to evaluate the main food items contributing to choline intake (33).

CONCLUSION

In conclusion, this study is the first to assess the intake of all choline forms and their dietary contributors in a European population. We found that the main contributors to total choline intake were eggs, milk, fresh vegetables, lean fish, and bread. Most choline was consumed in the form of phosphatidylcholine and animal food sources were the most important contributors to intake off all choline forms except free choline. More research is needed to better understand dietary choline requirements. There is an urgent need for a Norwegian database to more accurately estimate the dietary intake of this essential nutrient. Better understanding of dietary choline intake is essential to improve insight in its association with health outcomes.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: The WENBIT dataset is not publicly available. Requests to access these datasets should be directed to ottar.kjell.nygard@helse-bergen.no.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Regional Committee for Medical Health Research Ethics. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KV and TK calculated the dietary choline intake. AVP analyzed the data and wrote the paper. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.676026/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Material

Supplementary Table 1: Grouping of food items into subcategories, categories and food groups

Food group	Category	Subcategory	Food items
Dairy	Milk	Whole milk	Whole milk
		Low-fat milk	Low-fat milk
			Skimmed milk
			Unspecified milk
	Cheese	White cheese	White cheese >27% fat
			White cheese 27% fat
			White cheese 16% fat
		Brown cheese	Brown cheese
	Other dairy	Yogurt	Plain yogurt
			Fruit yogurt low fat
			God morgen forest fruits
		Cream	Cream 10% fat
			Sour cream
		Ice cream	Ice cream
		Other dairy products	Pudding
Drinks	Coffee		Boiled coffee
			Instant coffee
			Filter coffee
			Decaf
	Tea		Black tea
			Herbal tea
	Soda	Soda sugar	Soda with sugar
			Juice with sugar
		Soda light	Soda light
			Soda with sweetener
	Alcohol	Beer	Alcohol-free beer
			Pilsner beer
		Wine	Wine
		Liquor	Liquor
Eggs			
Fats	Margarine		Soya margarine
			Margarine mixture
			Other margarine
			Light margarine
	Butter		Butter
			Butter-margarine mixture
			Unspecified butter
	Other fats		Mayonnaise
			Remoulade
			Dressing
			Mayonnaise in salads

Fish	Lean fish		Cod
			Pollock
			Lean and half-fatty fish
	Fatty fish		Salmon
			Trout
			Herring
			Mackerel
	Fish products	Minced fish	Mined fish products
		Breaded fish	Breaded fish
			Deep-fried fish
		Fish spread	Mackerel
			Sardines
			Herring
			Caviar
	Shellfish		Shrimp
			Crab
Fruit	Fresh fruit		Citrus fruits
			Apples
			Pears
			Bananas
			Grapes
			Exotic fruits
			Blueberries
			Strawberries
			Cloudberries
			Unspecified fruit
	Canned fruit		Canned apricots
	Other fruit	Juice	Orange juice
			Other juice
			Unfiltered juice
			Nectar
		Jam	Jam
Grain products	Bread	White bread	White bread
			Bread <50% whole wheat
			White rolls
			Rolls <50% whole wheat
		Wholegrain bread	Bread >50% whole wheat
			Rolls >50% whole wheat
			“Birkebeiner” Bread
		Other bread	“Lefse”
			Chapatti
			Taco
			Flatbread
			Crispbread
	Pastries	Buns	Waffles
			“School bun”
			Danish pastry

		Cookies	Cookies
		Other pastries	Cakes with filling
			Cakes made with lard
	Other grains	Rice	Rice
		Pasta	Pasta
		Pizza	Pizza
		Cereal	Cereal
			Oatmeal
Meat	Meat products	Sausages	Wiener sausage
			Cooked sausage
		Liver spread	Liver spread
			Liver spread 13% fat
		Meat spread	Light meat spread
			Salami
			Saveloy
	Fresh meat	Poultry	Poultry
		Venison	Venison
		Other fresh meat	Minced meat dish
			Minced meat products
	Other meat		Unspecified meat
Other	Sugar and sweets	Sugar	Sugar
			Other sweeteners
			Energy-free sweeteners
		Sweet spread	Honey
			Sweet spread
		Chocolate	Chocolate
		Candy	Candy
	Snacks	Chips	Potato chips
		Nuts and seeds	Nuts and seeds
		Other snacks	Other snacks
Vegetables	Potatoes		Fresh potatoes
			French fries
	Fresh vegetables		Carrots
			Kohlrabi
			Cabbage
			Cauliflower
			Broccoli
			Onion
			Leek
			Tomato
			Bell pepper
			Kale
			Spinach
			Mushrooms
			Avocado
			Corn
			Vegetable mix

	Vegetables in stews
	Vegetable spread
Canned vegetables	Canned beans
	Pickled vegetables
	Tomato ketchup
	Other canned vegetables

Supplementary Table 2a: Primary food categories contributing to the intake of free choline

Rank	Food category	Contribution, %	Cumulative contribution
1	Fresh vegetables	16.0	16.0
2	Bread	11.6	27.6
3	Coffee	11.5	39.1
4	Potatoes	10.7	49.8
5	Milk	9.5	59.3
6	Fresh fruit	5.6	64.9
7	Lean fish	5.5	70.4
8	Alcohol	5.0	75.4
9	Meat products	4.8	80.2
10	Fish products	3.8	84.0

Supplementary Table 2b: Primary food categories contributing to the intake of glycerophosphocholine

Rank	Food category	Contribution, %	Cumulative contribution
1	Milk	30.1	30.1
2	Lean fish	13.8	43.9
3	Fish products	8.9	52.8
4	Fatty fish	7.9	60.7
5	Bread	4.8	65.5
6	Potatoes	4.4	69.9
7	Coffee	4.4	74.3
8	Other dairy	4.3	78.6
9	Alcohol	3.5	82.1
10	Fresh fruit	3.5	85.6

Supplementary Table 2c: Primary food categories contributing to the intake of phosphocholine

Rank	Food category	Contribution, %	Cumulative contribution
1	Milk	31.1	31.1
2	Fresh vegetables	27.1	58.2
3	Potatoes	8.3	66.5
4	Other dairy	4.4	70.9
5	Fresh meat	4.0	74.9
6	Fresh fruit	3.6	78.5
7	Fish products	3.5	82.0
8	Lean fish	3.4	85.4
9	Bread	3.1	88.5
10	Other fruit	2.0	90.5

Supplementary Table 2d: Primary food categories contributing to the intake of phosphatidylcholine

Rank	Food category	Contribution, %	Cumulative contribution
1	Eggs	28.0	28.0
2	Fresh meat	8.2	36.2
3	Fresh vegetables	8.1	44.3
4	Lean fish	7.7	52
5	Meat products	7.6	59.6
6	Bread	6.7	66.3
7	Fish products	6.1	72.4
8	Pastries	4.8	77.2
9	Potatoes	3.8	81
10	Shellfish	3.6	84.6

Supplementary Table 2e: Primary food categories contributing to the intake of sphingomyelin

Rank	Food category	Contribution, %	Cumulative contribution
1	Fresh meat	15.7	15.7
2	Eggs	12.5	28.2
3	Milk	12.3	40.5
4	Fish products	10.4	50.9
5	Meat products	9.9	60.8
6	Bread	8.4	69.2
7	Cheese	7.1	76.3
8	Lean fish	4.4	80.7
9	Other dairy	3.9	84.6
10	Fatty fish	3.5	88.1

Supplementary Table 3: Overview of the contribution of various food items to dietary intake of total choline and individual choline forms

Contributing food items (%)	Total choline	Water-soluble forms			Lipid-soluble forms	
		Free choline	Glycerophospho-choline	Phosphocholine	Phosphatidylcholine	Sphingomyelin
Dairy	15.5	12.4	35.4	36.8	3.4	23.3
Milk	12.1	9.4	30.0	31.0	1.7	12.3
Whole milk	1.0	1.0	2.2	3.1	0.1	1.1
Low-fat milk	11.1	8.4	27.8	27.9	1.6	11.2
Cheese	1.4	1.4	1.0	1.4	1.0	7.1
White cheese	1.0	0.9	0.9	1.4	0.7	4.8
Brown cheese	0.4	0.4	0.1	0.0	0.4	2.3
Other dairy	2.1	1.6	4.3	4.4	0.6	3.9
Yogurt	1.1	0.7	2.5	2.9	0.3	1.9
Cream	0.4	0.3	0.7	0.5	0.1	1.0
Ice cream	0.5	0.3	1.0	0.7	0.2	1.0
Other products	0.2	0.3	0.3	0.3	0.0	0.0
Drinks	6.3	17.2	8.2	0.0	0.0	0.0
Coffee	3.9	11.5	4.4	0.0	0.0	0.0
Tea	0.2	0.7	0.0	0.0	0.0	0.0
Soda	0.1	0.0	0.4	0.0	0.0	0.0
Alcohol	2.1	5.0	3.5	0.0	0.0	0.0
Eggs	12.6	0.1	0.1	0.7	28.0	12.5
Fats	1.2	0.1	0.4	0.7	2.3	1.1
Margarine	0.5	0.0	0.2	0.3	1.1	0.0
Butter	0.2	0.0	0.1	0.4	0.2	0.5
Other fats	0.5	0.0	0.2	0.0	1.0	0.6
Fish	19.5	12.0	31.0	7.9	18.5	21.7
Lean fish	8.3	5.5	13.8	3.4	7.7	4.4
Fatty fish	3.1	2.5	7.9	0.6	1.2	3.5
Fish products	6.3	3.8	8.9	3.5	6.1	10.4
Minced fish	1.1	1.0	1.8	0.9	0.8	1.1
Breaded fish	0.4	0.3	0.6	0.2	0.4	0.1

Fish spread	4.8	2.5	6.5	2.5	5.0	9.1
Shellfish	1.9	0.1	0.5	0.4	3.6	3.5
Fruit	5.2	7.6	6.8	5.9	3.4	0.4
Fresh fruit	3.4	5.6	3.5	3.6	2.4	0.2
Canned fruit	0.2	0.0	0.2	0.3	0.2	0.2
Other fruit	1.7	2.0	3.1	2.0	0.9	0.0
Grain products	11.4	14.9	6.4	5.0	13.0	12.0
Bread	7.3	11.6	4.8	3.1	6.7	8.4
White bread	2.5	4.0	1.6	1.3	2.2	2.5
Wholegrain bread	4.4	7.1	2.7	1.6	4.0	5.4
Other bread	0.5	0.6	0.6	0.2	0.4	0.4
Pastries	2.6	1.0	0.7	0.8	4.8	2.2
Buns	2.0	0.7	0.5	0.6	3.7	1.7
Cookies	0.1	0.2	0.0	0.0	0.1	0.0
Other pastries	0.5	0.2	0.1	0.1	1.0	0.5
Other grains	1.5	2.2	0.9	1.1	1.6	1.4
Rice	0.1	0.2	0.3	0.0	0.1	0.0
Pasta	0.2	0.5	0.1	0.0	0.2	0.0
Pizza	0.6	0.9	0.2	0.8	0.5	0.9
Cereal	0.6	0.6	0.3	0.3	0.8	0.5
Meat	12.0	7.1	3.6	6.1	18.3	28.5
Meat products	5.4	4.8	1.5	1.8	7.6	9.9
Sausages	2.2	3.9	0.2	0.9	2.2	4.6
Liver spread	0.9	0.5	0.2	0.3	1.5	1.3
Meat spread	2.2	0.4	1.1	0.5	3.9	3.9
Fresh meat	5.1	2.2	0.9	4.0	8.2	15.7
Poultry	2.4	0.7	0.2	2.6	3.9	10.6
Venison	0.3	0.0	0.1	0.0	0.6	0.6
Other fresh meat	2.4	1.5	0.6	1.4	3.7	4.5
Other meat	1.6	0.1	1.3	0.2	2.5	3.0
Other	1.2	1.2	1.5	1.3	1.0	0.6
Sugar and sweets	0.7	0.7	1.5	1.0	0.4	0.6
Sugar	0.0	0.0	0.0	0.0	0.0	0.0

Sweet spread	0.0	0.0	0.0	0.0	0.0	0.0
Chocolate	0.6	0.4	1.1	0.7	0.3	0.5
Candy	0.2	0.2	0.3	0.4	0.1	0.1
Snacks	0.5	0.6	0.1	0.2	0.6	0.0
Chips	0.0	0.1	0.0	0.0	0.1	0.0
Other snacks	0.1	0.1	0.0	0.0	0.1	0.0
Nuts and seeds	0.4	0.5	0.1	0.2	0.5	0.0
Vegetables	15.2	27.4	6.7	35.8	12.3	0.1
Potatoes	5.5	10.7	4.4	8.3	3.7	0.0
Fresh vegetables	9.2	16.0	2.2	27.1	8.1	0.0
Canned vegetables	0.5	0.7	0.1	0.4	0.5	0.1

IV

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Assessment of Dietary Choline Intake, Contributing Food Items, and Associations with One-Carbon and Lipid Metabolites in Middle-Aged and Elderly Adults: The Hordaland Health Study

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Abstract

Background: Choline is an essential nutrient for humans and is involved in various physiologic functions. Through its metabolite betaine, it is closely connected to the one-carbon metabolism, and the fat-soluble choline form phosphatidylcholine is essential for VLDL synthesis and secretion in the liver connecting choline to the lipid metabolism. Dietary recommendations for choline are not available in the Nordic countries primarily due to data scarcity.

Objectives: The aim of this study was to investigate the dietary intake of total choline and individual choline forms, dietary sources, and the association of total choline intake with circulating one-carbon metabolites and lipids.

Methods: We included 5746 participants in the Hordaland Health Study, a survey including community-dwelling adults born in 1925–1927 (mean age 72 y, 55% women) and 1950–1951 (mean age 48 y, 57% women). Dietary data were obtained using a 169-item FFQ, and choline content was calculated using the USDA Database for Choline Content of Common Foods, release 2. Metabolites of the one-carbon and lipid metabolism were measured in a nonfasting blood sample obtained at baseline, and the association with total choline intake was assessed using polynomial splines.

Results: The geometric mean (95% prediction interval) energy-adjusted total choline intake was 260 (170, 389) mg/d, with phosphatidylcholine being the main form (44%). The major food items providing dietary choline were eggs, low-fat milk, potatoes, and leafy vegetables. Dietary total choline was inversely associated with circulating concentrations of total homocysteine, glycine, and serine and positively associated with choline, methionine, cystathionine, cysteine, trimethyllysine, trimethylamine-N-oxide, and dimethylglycine. A weak association was observed between choline intake and serum lipids.

Conclusions: Phosphatidylcholine was the most consumed choline form in community-dwelling adults in Norway. Our findings suggest that choline intake is associated with the concentration of most metabolites involved in the one-carbon and lipid metabolism. *J Nutr* 2022;152:513–524.

Keywords: choline, dietary intake, one-carbon metabolism, lipid metabolism, phosphatidylcholine

Introduction

Since 1998, choline has been recognized as an essential nutrient, as de novo synthesis was proven to be insufficient (1). It is involved in liver, muscle, and brain functioning and plays a role in diverse processes such as cellular signaling, hepatic lipid metabolism, and methylation-dependent biosynthesis of molecules, including epigenetic regulation and gene expression (2, 3). In addition, phosphorylated choline compounds are elementary structural phospholipids in most cell membranes (4). Phosphatidylcholine (PC) and sphingomyelin are both

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lipid-soluble choline forms, whereas glycerophosphocholine, phosphocholine, and free choline are water soluble. All forms are found in the diet, with PC being the most abundant. Overall, total choline content per unit of weight is higher in animal food sources compared with plant-based sources, which makes eggs, meat, fish, chicken, and dairy good choline sources (5). Nonetheless, plant-based foods such as leafy vegetables, potatoes, grain products, nuts and seeds, and most legumes are also good choline sources, primarily containing water-soluble choline forms.

So far, an adequate intake (AI) for total dietary choline has been set by the National Academy of Medicine (NAM) in the United States (550 mg/d for men and 425 mg/d for women) (1) and by the European Food Safety Authority (EFSA) (400 mg/d for adults) (6). Due to limited scientific evidence, neither of these agencies have published an estimated average requirement. Also, no recommendations have been published as per now for the Nordic countries (7). Choline intake has been investigated in a range of countries worldwide, and even though it has varied largely between investigated countries, the estimated dietary choline intake has mainly been below the recommendations from both NAM and EFSA in most of the studied populations (8–15). So far, the intake of individual choline forms has been investigated in only a few countries (5). Little is known about the consumption and dietary sources of choline and the different forms globally, including in Norway. Because inadequate intake could possibly lead to adverse health effects such as muscle or liver damage (16, 17), choline intake and dietary sources should be investigated to allow for establishment of dietary recommendations.

Choline is closely connected to the one-carbon metabolism, a set of biochemical reactions in which one-carbon groups are being transferred between compounds through its metabolite betaine. Disturbances in metabolites involved in the one-carbon metabolism have been linked to the development of several chronic diseases (18). Other dietary-derived compounds such as folate, betaine, riboflavin, vitamin B-6, cobalamin, and methionine can also generate methyl groups, and their metabolisms are therefore closely related to that of choline (18, 19). For example, choline deficiency may lead to increased de novo choline synthesis via sequential methylation of phosphatidylethanolamine by S-adenosylmethionine, generated from methionine (18). Sex also alters one's choline requirement as estrogen promotes de novo synthesis. Therefore, men and postmenopausal women have a higher choline need compared with premenopausal women (20). Several other factors such as pregnancy, lactation, and gene polymorphisms can also affect one's choline requirement (21). The one-carbon metabolism is complex and characterized by many feedback mechanisms. Even though the association with dietary choline and some one-carbon metabolites has been investigated [e.g., total homocysteine (22) and plasma choline (16)], it remains unclear for several others.

Choline is also involved in the lipid metabolism mainly through PC, which is required for solubilization of bile salts for secretion and, most important, for packaging and export of triglycerides (TGs) from the liver, as a structural part of VLDL (3, 4). Despite the clear association between choline and lipid metabolism, little research has been conducted regarding the relation between choline intake and serum lipids in humans.

The aim of this study was to describe the dietary intake of total choline and the individual choline forms in a community-based population. In addition, the contributions of different dietary choline sources to total choline and individual choline forms were explored. Finally, associations between total dietary choline intake and circulating concentrations of both one-carbon and lipid metabolites were investigated.

Methods

Study population

This study uses data from the community-based Hordaland Health Study (HUSK) (https://husk.w.uib.no/) conducted in western Norway. The recruitment of this cohort in 1997–1999 was based on a previous cohort from 1992–1993 (The Hordaland Homocysteine Study) in which all individuals living in Hordaland county (currently part of Vestland county) born in 1925–1927 or 1950–1952 were invited. In 1997–1999, all living participants born in 1925–1927 or 1950–1951 and residing in the city of Bergen or the neighboring municipalities were reinvited. The main purpose of the HUSK surveys is to gather information for prevention of future disease through *1*) prevention via identification of possible modifiable risk factors for disease and *2*) research via mapping of occurrence to be able to identify the extent of illness, identify causal factors, and be able to better predict future needs for health services. Participants underwent a brief health examination and provided a nonfasting blood sample at baseline. Data from 7016 HUSK participants were available for this study, of whom 6094 completed the FFQ. We excluded 30 participants with missing plasma choline values. Furthermore, participants with extreme energy intakes (<3300 kJ or >17,500 kJ for men and <3000 kJ or >15,000 kJ for women) were excluded ($n = 198$). Finally, we excluded participants with self-reported alcohol intake >10 energy percentage (E%) ($n = 120$). This left 5746 participants eligible for analyses. Excluded participants comprised 306 elderly women, 219 elderly men, 350 middle-aged women, and 395 middle-aged men. All study participants provided written informed consent, and the study protocol was approved by the regional ethics committee for Medical Research Ethics.

Health examination and analytic procedures

Information regarding lifestyle, health behaviors, and medical history was obtained through self-administered questionnaires. Smoking was defined based on self-reported smoking habits and serum cotinine concentrations >85 nmol/L. Participants were classified as having diabetes according to self-report. Hypertension was considered present if the participant reported use of medication for hypertension.

Study procedures for HUSK have been described in detail previously (23). Briefly, participants underwent a brief physical examination, which included measurements of height and weight, and a venous, nonfasting blood sample was taken. Blood samples were collected in evacuated tubes containing EDTA, chilled (at 4–5°C) within 15–30 min, and then centrifuged for 10 min, 4000 × *g* at 10°C within 1–3 h and stored at –80°C until analysis. Metabolites related to one-carbon metabolism and cotinine, as a biomarker of nicotine exposure, were analyzed in 5746 samples at Bevital AS. Betaine, choline, dimethylglycine (DMG), trimethylamine-N-oxide (TMAO), trimethyllysine (TML), and cotinine were measured using LC-MS/MS (24, 25), whereas GC-MS/MS was used for cystathionine, cysteine, glycine, methionine, serine, and total

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Abbreviations used: AI, adequate intake; BHMT, betaine homocysteine methyltransferase; CVD, cardiovascular disease; DMG, dimethylglycine; EFSA, European Food Safety Authority; E%, energy percentage; HUSK, Hordaland Health Study; ICC, intraclass correlation coefficient; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; NAM, National Academy of Medicine; PC, phosphatidylcholine; PEMT, phosphatidylethanolamine-N-methyltransferase; PI, prediction interval; SHMT, serine hydroxymethyltransferase; TC, total cholesterol; TG, triglyceride; TMAO, trimethylamine-N-oxide; TML, trimethyllysine.

TABLE 1 Number of participants with available results per metabolite

		Elderly		Middle-aged	
	Total population	Women	Men	Women	Men
n (%)	5746	1539 (26.8)	1247 (21.7)	1700 (29.6)	1260 (21.9)
One-carbon metabolites, *n*					
Betaine	5570	1501	1199	1669	1201
Choline	5746	1539	1247	1700	1260
Cystathionine	5653	1525	1221	1683	1224
Cysteine	5653	1525	1221	1683	1224
DMG	5746	1539	1247	1700	1260
Glycine	5653	1525	1221	1683	1224
Methionine	5653	1525	1221	1683	1224
Serine	5653	1525	1221	1683	1224
TMAO	5570	1501	1199	1669	1201
TML	5570	1501	1199	1669	1201
Total homocysteine	5653	1525	1221	1683	1224
Serum lipids, *n*					
Total cholesterol	5746	1539	1247	1700	1260
LDL cholesterol	5609	1509	1220	1671	1209
HDL cholesterol	5746	1539	1247	1700	1260
Non-HDL-LDL cholesterol	5746	1539	1247	1700	1260
Triglycerides	5746	1539	1247	1700	1260

DMG indicates dimethylglycine; HDL, high-density-lipoprotein; LDL, low-density-lipoprotein; TMAO, trimethylamine-N-oxide; TML, trimethyllysine.

homocysteine analyses (26). Serum samples of total cholesterol (TC), HDL cholesterol, and TGs were analyzed within 7 d at the Department of Clinical Chemistry, Oslo University Hospital, Ullevål, with reagents from Boehringer Mannheim (Roche) as adapted to a Hitachi 911 analyzer. Cholesterol and TGs were measured by enzymatic methods. HDL cholesterol was measured by a direct, enzymatic inhibition method. LDL cholesterol concentration was calculated using the Friedewald equation: LDL cholesterol (mmol/L) = TC (mmol/L) – HDL cholesterol (mmol/L) – TG (mmol/L) / 2.2 (27). Serum LDL was not calculated for participants with serum TG >4.5 mmol/L as the Friedewald equation is not applicable in this case (n = 137). Non-HDL-LDL cholesterol was used as a surrogate marker for cholesterol in chylomicrons and calculated using the following formula: non-HDL-LDL cholesterol (mmol/L) = TC (mmol/L) – HDL cholesterol (mmol/L) – LDL cholesterol (mmol/L). The number of participants with available results per metabolite is shown in **Table 1**.

Dietary assessment

Information regarding dietary intake was obtained using a 169-item semiquantitative FFQ, which is a slightly modified version of a previously described FFQ (28). The FFQ was handed to the participants on the day of the health examination, filled out at home, and returned by mail to the HUSK project center. The frequency alternatives ranged from once a month to several times a day. In addition, number of units eaten (e.g., slices, pieces) and household measures (e.g., spoons, cups, glasses) were used to capture habitual diet during the past year. In addition to food items, the FFQ included the 9 most common single- and multivitamin supplements at the time of the study, but no specific questions regarding choline supplementation were included. Daily nutrient intake was calculated using the software system "Kostberegningssystemet" (KBS, version 3.2) developed at the Department of Nutrition, University of Oslo, Norway. The nutrient database used is mainly based on the official Norwegian food composition table (https://www.matvaretabellen.no/).

Choline composition data

Total choline was defined as the sum of the 5 individual choline forms: free choline, glycerophosphocholine, phosphocholine, PC, and sphingomyelin. As choline composition data are currently unavailable in the Norwegian food composition table, choline content of foods was quantified using the USDA Database for Choline Content of Common Foods, release 2 (29). From the 169-item FFQ, choline content was available for 134 food items. For the remaining items, choline content was estimated using nutritionally equivalent foods. For multicomponent foods (e.g., dishes, ready-made meals), choline content was calculated for each ingredient in the FFQ recipe. The 134 food items were categorized into 10 main food groups (dairy, drinks, eggs, fats, fish, fruit, grain products, meat, vegetables, and sweets and snacks) and subsequently into categories and subcategories based on nutritional similarities. A full overview of food items and categorizations is provided in **Supplemental Table 1**.

Statistical analyses

Continuous variables are reported as geometric mean [95% prediction interval (PI)] and categorical variables as counts (percentage). The density method was used to adjust dietary variables for self-reported energy intake, and values are reported as E% or g/1000 kcal (30). To adjust dietary choline intake for self-reported energy intake, the residual method was used. The energy-adjusted choline estimate is the residual from the regression model with total energy intake as the independent variable and absolute choline intake as the dependent variable plus the expected nutrient intake for the mean energy intake in the study population (31).

To generate the density plot for total choline intake, we estimated the probability density function using the kernel density estimation (32, 33) implemented via the "geom_density" function of the "ggplot2" package available for R version 1.3.959 (34). The following formula was used to calculate the percent contribution of each (sub)category to both total choline intake and the intake of individual choline forms: [(choline provided by the food (sub)category / total choline from all food (sub)categories)] × 100.

To explore the relation between one-carbon metabolites and dietary choline intake, choline intake was modeled as a polynomial spline in a model with sex as an interaction term and adjusted for age, BMI, and smoking for total choline and the individual choline forms. The same model was used to explore the relation between lipid metabolism metabolites and choline intake. Including statin use, diabetes diagnosis, folate status, or all 3 factors as an interaction term to the used model did not alter the observed associations substantially, and these results are therefore not shown.

TABLE 2 Characteristics of participants in the Hordaland Health Study 1997–1999[1]

Characteristic	Total population	Elderly		Middle-aged	
		Women	Men	Women	Men
n (%)	5746	1539 (26.8)	1247 (21.7)	1700 (29.6)	1260 (21.9)
Age, y	58.6 (47.0, 74.0)	72.4 (71.0, 74.0)	72.4 (71.0, 74.0)	48.0 (47.0, 49.0)	48.0 (47.0, 49.0)
BMI, kg/m^2	25.4 (19.5, 34.6)	25.8 (18.7, 35.5)	25.7 (20.1, 32.5)	24.6 (19.3, 35.1)	25.9 (20.6, 33.9)
Smokers,[2] *n* (%)	1566 (27.3)	243 (15.8)	247 (19.8)	616 (36.2)	460 (36.5)
Diabetes,[3] *n* (%)	201 (3.5)	96 (6.2)	80 (6.4)	7 (0.4)	18 (1.4)
Hypertension,[4] *n* (%)	909 (15.8)	437 (28.4)	342 (27.4)	77 (4.5)	53 (4.2)
Plasma concentration of one-carbon metabolites,[5] μmol/L					
Betaine (*n* = 5570)	36.1 (19.7, 60.5)	34.1 (20.3, 55.2)	41.4 (26.9, 63.6)	31.2 (17.3, 51.6)	41.2 (27.0, 67.6)
Choline	9.6 (6.2, 15.1)	9.6 (6.0, 15.0)	10.8 (7.1, 16.7)	8.9 (5.8, 13.3)	9.6 (6.3, 14.9)
Cystathionine (*n* = 5653)	0.3 (0.1, 0.9)	0.3 (0.1, 1.1)	0.3 (0.1, 1.2)	0.2 (0.09, 0.5)	0.2 (0.1, 0.8)
Cysteine (*n* = 5653)	286 (232, 353)	306 (258, 368)	304 (256, 363)	264 (222, 318)	277 (236, 329)
DMG	4.5 (2.7, 7.7)	4.3 (2.6, 7.5)	4.7 (2.9, 8.1)	4.2 (2.6, 7.3)	4.7 (3.0, 8.1)
Glycine (*n* = 5653)	251 (158, 457)	268 (159, 492)	230 (158, 350)	270 (151, 474)	230 (162, 350)
Methionine (*n* = 5653)	27.5 (17.6, 49.0)	26.2 (17.3, 49.6)	28.8 (18.1, 52.2)	26.6 (16.9, 46.4)	29.2 (19.3, 49.2)
Serine (*n* = 5653)	114 (77, 171)	114 (76, 169)	109 (75, 160)	121 (79, 183)	111 (78, 161)
TMAO (*n* = 5570)	5.6 (1.6, 49.2)	6.6 (2.0, 60.7)	8.2 (2.1, 72.7)	4.0 (1.3, 28.2)	4.9 (1.4, 42.0)
TML (*n* = 5570)	0.6 (0.3, 1.2)	0.6 (0.4, 1.2)	0.7 (0.4, 1.4)	0.5 (0.3, 1.0)	0.6 (0.4, 1.3)
Total homocysteine (*n* = 5653)	11 (6.6, 21.1)	11.7 (7.2, 21.3)	13.2 (8.3, 23.3)	9.2 (5.8, 16.8)	11.0 (7.2, 19.6)
Serum lipids, mmol/L					
Total cholesterol	5.9 (4.1, 8.3)	6.5 (4.5, 8.9)	5.8 (3.9, 8.0)	5.6 (4.1, 7.7)	5.7 (4.1, 8.0)
LDL cholesterol[6] (*n* = 5609)	4.5 (2.8, 6.9)	5.0 (3.1, 7.3)	4.5 (2.7, 6.7)	4.1 (2.6, 6.3)	4.5 (2.9, 6.7)
HDL cholesterol	1.3 (0.7, 2.2)	1.4 (0.9, 2.4)	1.2 (0.7, 2.0)	1.4 (0.9, 2.3)	1.1 (0.7, 1.9)
Non-HDL-LDL cholesterol[7]	0.7 (0.3, 0.2)	0.7 (0.3, 1.9)	0.7 (0.3, 2.0)	0.6 (0.2, 1.8)	0.8 (0.3, 2.4)
Triglycerides	1.5 (0.6, 4.4)	1.6 (0.7, 4.2)	1.6 (0.7, 4.3)	1.3 (0.6, 3.9)	1.8 (0.7, 5.3)

[1] Continuous variables are reported as geometric mean (95% prediction interval), and categorical variables are reported as counts (percentage). DMG, dimethylglycine; TMAO, trimethylamine-N-oxide; TML, trimethyllysine.
[2] Defined according to self-reporting smoking habits and serum cotinine concentrations >85 nmol/L at baseline.
[3] Defined according to self-reported, preexisting diagnosis.
[4] Defined according to self-reported medication use.
[5] *n* = number of participants with available results if different from 5746.
[6] LDL concentration was calculated using the Friedewald equation (27).
[7] Non-HDL-LDL cholesterol was calculated using the following formula: non-HDL-LDL cholesterol (mmol/L) = TC (mmol/L) – HDL cholesterol (mmol/L) – LDL cholesterol (mmol/L).

Given the exploratory and descriptive nature of this study, the main results are presented visually, and in accordance with the STROBE checklist and statement (35), we chose not to report *P* values.

All statistical analyses were performed using R version 1.3.959 (R Foundation for Statistical Computing), including the packages within the "tidyverse" ("dplyr," "ggplot2," "broom," "plyr," "ggthemes") and the "survival," "splines," and "interaction" packages.

Results

Characteristics of the study participants

The total study population (n = 5746) included women and men born in either 1925–1927 or 1950–1951, hereafter referred to as elderly and middle-aged, respectively. There were slightly more women than men among both the elderly and the middle-aged participants (respectively, 26.8% compared with 21.7% and 29.6% compared with 21.9%). Smoking was less common in the elderly participants, whereas diabetes and hypertension prevalence was higher compared with the middle-aged group.

Dietary intake

The dietary intake of the study population is shown in **Table 2**. As expected, energy intake of men was higher compared with women and also higher in middle-aged compared with elderly participants. The macronutrient intake was similar in all groups. Elderly participants consumed relatively more dairy, eggs, fish, fruit, and vegetables compared with the middle-aged participants, whereas meat intake was higher in middle-aged men and women compared with their older counterparts.

Choline intake

Geometric mean (95% PI) energy-adjusted dietary choline intake for the total population was 260 (170,389) mg/d and was similar for all groups (**Table 3**). Dietary choline was mainly obtained in the form of phosphatidylcholine (44%), free choline (26%), and glycerophosphocholine (22%). Phosphocholine and sphingomyelin each contributed ∼5% to the total choline intake.

Among women, 3% of both elderly and middle-aged participants had a non-energy-adjusted choline intake above EFSA's recommendation of 400 mg/d, whereas 7.5% elderly men achieved this AI (**Figure 1**). Around 19% of the middle-aged men achieved this AI. However, this dropped to 2% when using the AI set by NAM (550 mg/d).

Foods contributing to choline intake

Dairy, vegetables, and eggs were the primary contributing food groups to total dietary choline intake in our study population (**Figure 2**). Main contributors differed largely between the different choline forms, with dairy being one of the most important sources of nearly all choline forms.

TABLE 3 Dietary intake among participants in the Hordaland Health Study 1997–1999[1]

Characteristic	Total population	Elderly		Middle-aged	
		Women	Men	Women	Men
n (%)	5746	1539 (26.8)	1247 (21.7)	1700 (29.6)	1260 (21.9)
Energy, kcal/d	1874 (946, 3359)	1527 (839, 2638)	1961 (1091, 3288)	1812 (1020, 2991)	2405 (1357, 3811)
Energy-adjusted choline intake,[2] mg/d					
Total choline	260 (170, 389)	266 (189, 382)	258 (167, 390)	258 (176, 380)	256 (154, 402)
Free choline	68 (45, 102)	69 (49, 100)	67 (45, 102)	67 (46, 101)	68 (41, 108)
Glycerophosphocholine	54 (24, 108)	60 (34, 106)	56 (25, 109)	49 (24, 96)	53 (16, 118)
Phosphatidylcholine	111 (62, 198)	111 (67, 196)	109 (58, 202)	116 (71, 198)	107 (51, 196)
Phosphocholine	12 (5, 24)	13 (7, 24)	11 (5, 23)	12 (6, 25)	11 (3, 23)
Sphingomyelin	11 (6, 19)	12 (7, 18)	11 (6, 19)	12 (7, 18)	11 (5, 20)
Macronutrient intake,[3] E%					
Carbohydrates	49.9 (38.5, 62.4)	51.7 (39.3, 65.2)	50.2 (40.1, 62.4)	49.2 (38.2, 61.8)	48.5 (37.6, 59.4)
Protein	15.8 (11.8, 21.0)	15.9 (11.8, 21.5)	15.7 (11.8, 21.0)	15.9 (11.8, 21.1)	15.4 (11.5, 20.4)
Fat	31.3 (20.6, 42.7)	29.9 (18.4, 42.4)	31.2 (20.9, 42.0)	32.0 (21.9, 43.4)	32.4 (23.1, 42.8)
MUFA	9.8 (6.1, 13.9)	9.2 (5.4, 13.7)	9.7 (6.1, 13.5)	10.1 (6.5, 13.8)	10.4 (7.3, 14.1)
PUFA	6.5 (3.8, 11.4)	5.9 (3.4, 10.2)	6.5 (3.9, 11.1)	6.7 (4.0, 11.9)	7 (4.2, 12.0)
SFA	12.2 (7.4, 18.2)	11.9 (6.7, 18.9)	12.1 (7.5, 18.4)	12.4 (7.9, 17.5)	12.3 (8.4, 17.3)
Alcohol	0.1 (0.0, 6.9)	0.0 (0.0, 5.1)	0.1 (0.0, 8.1)	0.2 (0.0, 6.1)	0.8 (0.0, 7.9)
Intake major food groups,[3] g/1000 kcal					
Dairy	141 (23, 430)	172 (27, 492)	143 (20, 432)	121 (24, 387)	135 (20, 402)
Drinks	392 (122, 1087)	381 (102, 1090)	323 (93, 897)	460 (160, 1203)	398 (124, 933)
Eggs	5 (0, 25)	5 (0.0, 30)	5 (0, 29)	6 (1, 23)	5 (0, 20)
Fats	12 (2, 33)	11 (2, 31)	13 (3, 32)	13 (2, 33)	14 (3, 35)
Fish	35 (8, 104)	35 (8, 110)	44 (14, 115)	32 (9, 93)	31 (8, 87)
Fruit	98 (16, 334)	120 (22, 398)	86 (11, 297)	111 (22, 347)	74 (12, 263)
Grain products	118 (64, 192)	119 (61, 200)	118 (63, 195)	116 (660, 180)	120 (65, 184)
Meat	41 (10, 100)	31 (6, 83)	36 (10, 94)	49 (15, 106)	50 (16, 106)
Vegetables	142 (40, 368)	155 (26, 381)	145 (26, 350)	149 (49, 379)	118 (44, 302)
Sweets and snacks	1 (0, 27)	1 (0, 25)	1 (0, 25)	2 (0, 32)	2 (0, 26)

[1]All dietary intakes are presented as geometric mean (95% prediction interval). E%, energy percentage.
[2]Choline intake was energy-adjusted using the residual method (31).
[3]Intake of macronutrients and major food groups was energy-adjusted using the density method (30).

Besides food groups, we also investigated the food subcategories providing total choline and the individual choline forms. **Table 4** provides a list of the top 10 dietary total choline sources in our study population. Egg (15.3%) and low-fat milk (11.8%) consumption contributed with over one-fourth of the total dietary choline intake. Other, minor choline sources were potatoes, leafy vegetables, and whole-grain bread (respectively, 6.3%, 5.7%, and 5.2%). Contribution of food subcategories was calculated for each individual choline form, and results are presented in **Supplemental Tables 2–6**. Eggs were a major source of the lipid-soluble choline forms, whereas low-fat milk was a top-10 contributor to intake of all choline forms apart from PC.

Contribution of the individual food items to the intake of total choline and the individual choline forms is presented in **Supplemental Table 7**.

One-carbon metabolism

Plasma concentrations of one-carbon metabolites are shown in Table 2. Plasma concentrations of choline, DMG, cystathionine, and TML were similar across all groups. Men in both age groups had higher plasma betaine and methionine concentrations than women. However, the opposite was true for glycine and serine. Middle-aged participants had lower total homocysteine, cysteine, and TMAO plasma concentrations compared with elderly participants.

The relation between total energy–adjusted choline intake and plasma concentrations of one-carbon metabolites is shown in **Figure 3**. In women, plasma concentrations of choline and methionine showed a negative association with total choline intake at low intake levels, and the direction of the association changed at a choline intake of ±200 mg/d. The opposite was true for serine. Total choline intake was negatively associated with glycine and total homocysteine and positively with DMG. Furthermore, at low choline intakes, plasma cysteine and TMAO seemed to increase, whereas plasma concentrations flattened with increasing choline intake and even decreased at high choline intake for TMAO. Finally, no association was observed between dietary choline intake and betaine, cystathionine, or TML in women.

The association between choline intake and plasma concentrations of cysteine, glycine, methionine, TMAO, TML, and total homocysteine in men was similar to that in women. Plasma betaine showed a U-shaped association, whereas a positive association was observed for plasma choline and DMG, except at low intake levels, and for cystathionine until a choline intake of ±300 mg/d. Plasma serine was negatively associated with dietary choline intake, but the curve flattened at an intake of ±200 mg/d.

The associations of PC, free choline, phosphocholine, and sphingomyelin intake with plasma concentration of one-carbon metabolites were similar to that of total choline intake (**Supplemental Figures 1–4**). However, due to the low intake of phosphocholine and sphingomyelin, the 95% CI was rather large for all associations. Interestingly, we did not observe

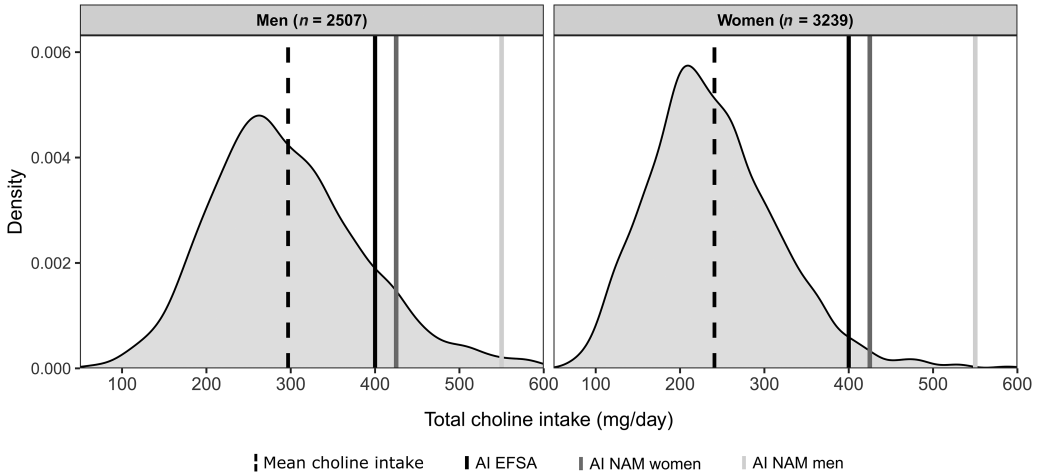

FIGURE 1 Distribution of mean choline intake for participants in the Hordaland Health Study 1997–1999. The solid black line indicates the AI for choline determined by EFSA (400 mg/d). The solid dark grey line indicates the AI for choline determined by NAM for women (425 mg/d), whereas the solid light grey line indicates this for men (550 mg/d). The y-axis shows the density of the observed values calculated using the kernel density estimation (32, 33). AI, adequate intake; EFSA, European Food Safety Authority; NAM, National Academy of Medicine.

an association between free choline and plasma methionine, whereas we noted a positive association for the other choline forms and total choline. Finally, glycerophosphocholine was not associated with plasma concentrations of most of the one-carbon metabolites, but a weak positive association was observed for cysteine and TMAO (**Supplemental Figure 5**).

Lipid metabolism

Plasma TC concentrations were similar in all groups apart from elderly women, who had a higher concentration (6.5 mmol/L) compared with the other groups (5.6–5.8 mmol/L). Both LDL and HDL cholesterol were also higher in elderly women compared with the rest of the population. In general, plasma

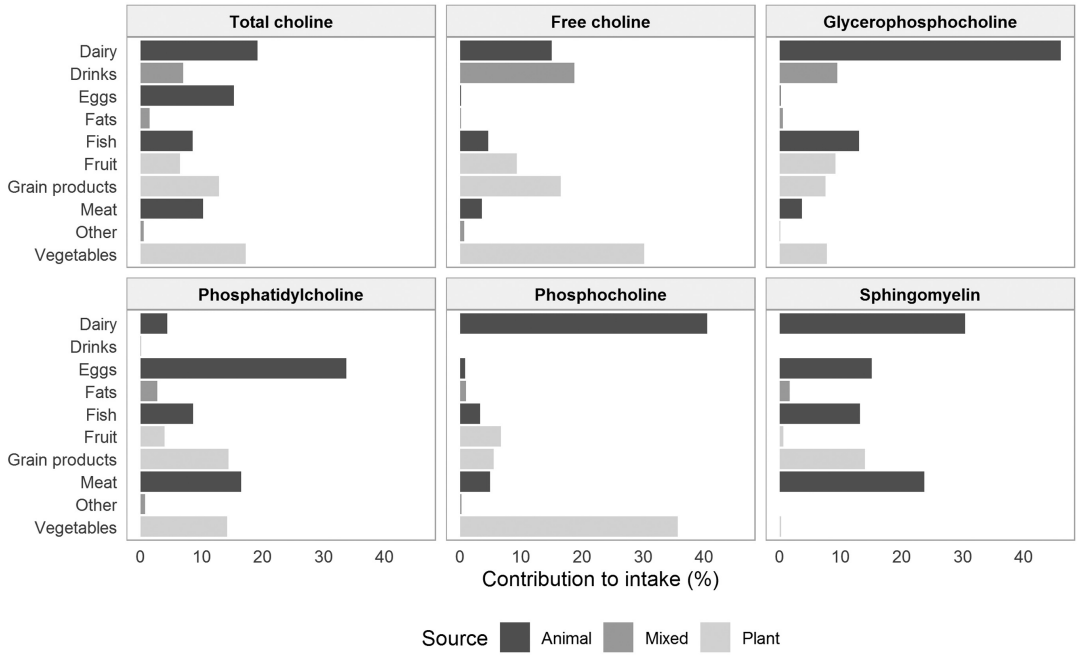

FIGURE 2 Overview of the main food groups from animal, plant, and mixed sources contributing to intake of total choline and individual choline forms for participants in the Hordaland Health Study 1997–1999. Contribution is indicated as percentage of total intake.

TABLE 4 Primary food subcategories contributing to total choline intake among participants in the Hordaland Health Study 1997–1999

Rank	Food item	Contribution, %	Cumulative contribution, %
1	Eggs	15.3	15.3
2	Low-fat milk	11.8	27.1
3	Potatoes	6.3	33.4
4	Leafy vegetables	5.7	39.1
5	Whole-grain bread	5.2	44.3
6	Coffee	4.3	48.6
7	Fresh fruit	4.2	52.8
8	Meat spread	3.7	56.5
9	Other meat products	2.9	59.4
10	White bread	2.8	62.2

cholesterol values were higher in women than men. Plasma TG values were similar in elderly women and men (1.8 mmol/L), whereas they were lower in middle-aged women and highest in middle-aged men (respectively, 1.4 mmol/L and 2.1 mmol/L).

The same model as for one-carbon metabolites was used to investigate the relation between total energy–adjusted choline intake and plasma concentrations of lipid metabolites (**Figure 4**). In women, total, HDL, and LDL cholesterol showed a positive association with choline intake until ±250 mg/d, whereafter the association became negative for TC and LDL cholesterol. Interestingly, this was not observed in men, and for plasma HDL at a low choline intake, a negative relation was found. In both men and women, a negative relation was found between choline intake and plasma TG, and no association was found with non-HDL-LDL cholesterol.

Discussion

This study aimed to investigate the dietary intake of total choline and the individual choline forms, their dietary sources, and their association with one-carbon and lipid metabolites in community-dwelling Norwegian middle-aged and elderly adults. The mean energy-adjusted total choline intake was below the recommended AI set by both EFSA and NAM for women and men in both age groups. Choline was mainly consumed in the form of PC. The main foods contributing to total choline intake were eggs, low-fat milk, potatoes, leafy vegetables, and whole-grain bread. Food sources differed for the individual choline forms. Dietary choline intake was associated with plasma concentrations of one-carbon metabolites and with serum lipids.

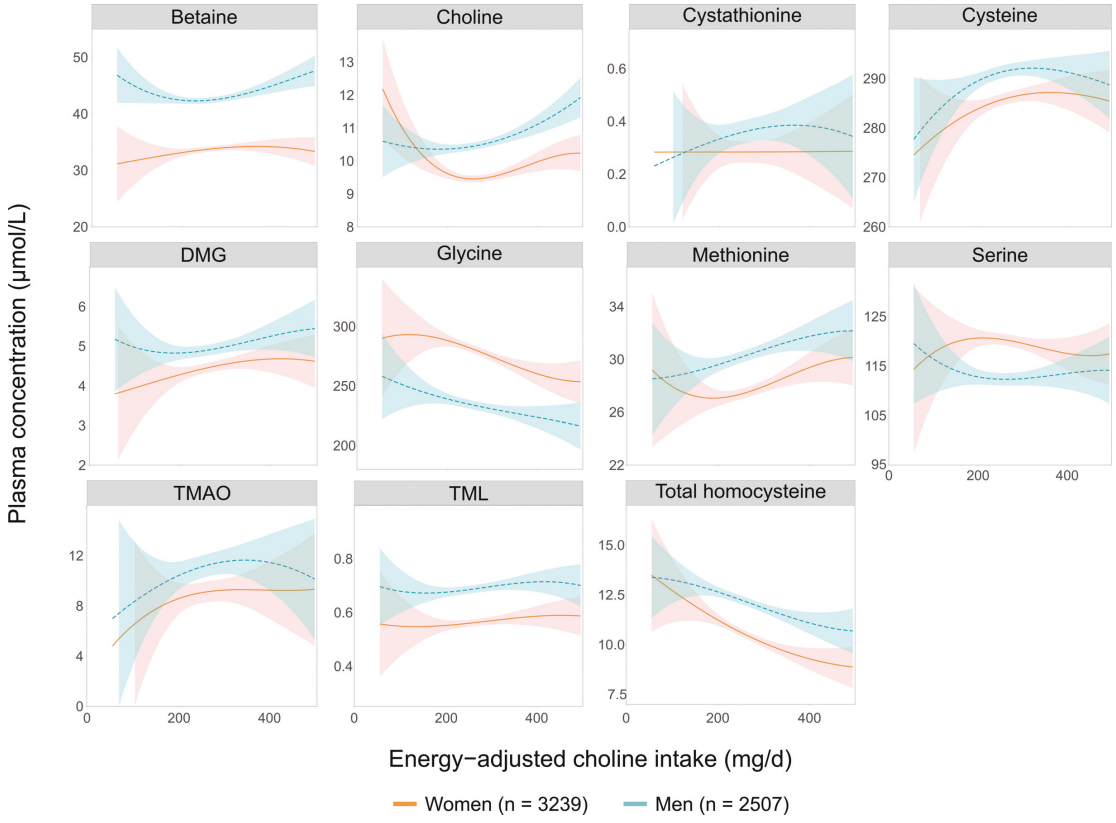

FIGURE 3 Associations between energy-adjusted choline intake and plasma concentration of one-carbon metabolites modeled as a polynomial spline in a model with sex as the interaction term and adjusted for age, BMI, and smoking for participants in the Hordaland Health Study 1997–1999. The dotted red and solid blue lines represent the modeled associations for women and men, respectively, and the colored areas indicate the corresponding 95% CI. DMG, dimethylglycine; TMAO, trimethylamine N-oxide; TML, trimethyllysine.

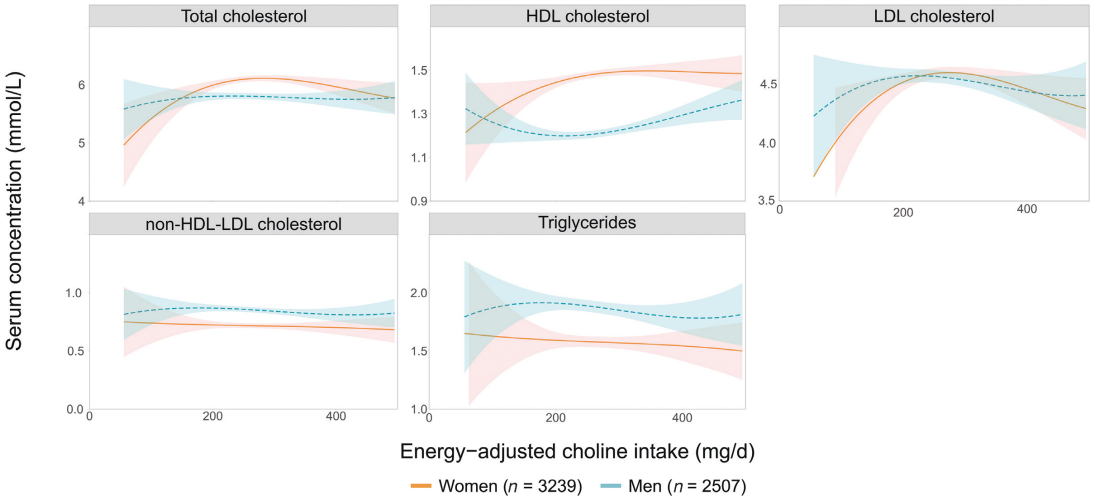

FIGURE 4 The association between energy-adjusted choline intake and serum concentration of lipid metabolites modeled as a polynomial spline in a model with sex as interaction term and adjusted for age, BMI, and smoking for participants in the Hordaland Health Study 1997–1999. The dotted red and solid blue lines represent the modeled associations for women and men, respectively, and the colored areas indicate the corresponding 95% CI.

Dietary choline intake and sources

Dietary choline intake has been estimated in several cohorts globally (9, 11, 12–15, 36–41), and median intake has been estimated previously in our study population (42). In 8 European countries, the non-energy-adjusted estimated choline intakes in adults ranged from 269 to 468 mg/d (15). Similar findings have been observed in the United States, where estimated intakes ranged from 258 to 323 mg/d in women and 302 to 405 mg/d in men (9, 37–40). Interestingly, all studies drew the conclusion that most individuals do not achieve the AI for choline set by EFSA and NAM. The observed values of our cohort were rather low compared with previous findings. However, they are similar to self-reported dietary choline intake in a Norwegian population of patients with cardiovascular disease (43), using the same FFQ as in the current study. This could indicate that dietary choline intake is lower in Norway compared with other Western countries. Interestingly, a Swedish national dietary survey reported an estimated choline intake of 468 mg/d in men and 374 mg/d in women (15), which is higher than our findings. In this survey, dietary choline intake was estimated using a 4-d web-based food record, a method that differs greatly from the FFQ used in this study. The same USDA database was used for estimation of choline consumption. In addition, the reported choline intake was not energy adjusted, and dietary habits in Sweden differ from those in Norway. These factors might explain the observed difference in dietary choline intake. In general, choline intake estimates from various studies should be compared with caution due to substantial differences in method of dietary assessment, study population, and statistical methods.

In our study, the main source of dietary choline was PC, followed by free choline and glycerophosphocholine. The number of studies reporting on intake of individual choline forms is limited, but the available findings agree with ours (5, 14, 36, 44–46). This is not surprising because ~60% of total choline in our study was obtained from animal products in which PC is the predominant form due to its incorporation in mammalian membranes (2). Indeed, several Western cohorts found eggs, meat, fish, and milk to be major contributors to total choline and thus PC intake (14, 36–40, 44). It should be mentioned that our data were gathered in 1997–1999, and available foods and dietary habits might have changed over time. Indeed, when comparing Norwegian consumption data from 1999 with data from 2018, we found a large increase in meat and egg consumption, a large decrease in milk consumption, and a small decrease in fish consumption (in 2018 compared with 2003 for fish consumption) (47). Dietary intake observed in our study population was comparable to the intake observed in the same age groups in the National Dietary Survey among Men and Women from 1997, supporting the external validity of our dietary data (48).

Dietary choline and one-carbon metabolites

Our findings show that dietary choline intake is associated with the concentration of several metabolites involved in the one-carbon metabolism. Because nonfasting blood samples were analyzed, it must be considered that the concentration of metabolites could have been affected by prandial status. For example, it has been shown that plasma choline concentrations increase 10–15% after a meal and are particularly responsive to large intakes of dietary choline sources (16). Also, dietary choline intake in our study reflects the long-term intake, whereas plasma one-carbon metabolites were only measured at a single time point. Nevertheless, we observed that plasma choline was positively associated with self-reported long-term choline intake in men, whereas this association was unclear in women. It has previously been reported that dietary choline intake is not associated with plasma choline concentrations, in contrast with our findings (49, 50). However, it has been shown that plasma choline is associated with egg consumption, which was the main choline contributor in our study population (51). In general, it can be questioned whether plasma choline is a good biomarker of choline intake at all, because it is homeostatically regulated by mechanisms such as de novo synthesis. For example, fasting

plasma choline concentrations did not alter in premenopausal women after a moderate increase in dietary choline intake during 12 wk (52).

We also observed a negative association between choline intake and plasma concentrations of total homocysteine in both men and women. Simultaneously, a positive association with methionine was observed. Indeed, the homocysteine-lowering effect of choline has been previously observed in several studies (17, 22, 36, 40) and is likely to be caused by methionine synthesis and remethylation of homocysteine through choline's metabolite betaine (53). Although plasma betaine concentration was not associated with dietary choline in our study population, a positive association was observed with DMG. A possible explanation could be that betaine is used in the remethylation of homocysteine to methionine and thus forms DMG. In addition, increased dietary choline intake leads to decreased choline de novo synthesis, a process that requires a high amount of methyl groups and therefore methionine (18). Thus, choline intake not only is methionine saving but also actively contributes to methionine synthesis via betaine and the betaine homocysteine methyltransferase (BHMT) pathway (2, 4). Supplementation of betaine has been used to lower homocysteine concentrations in hyperhomocysteinemia (54) and has been shown to decrease homocysteine concentrations by up to 20% in the general population (55). However, high doses of betaine supplementation have been associated with increased LDL cholesterol, whereas this association has not been observed with choline supplementation (22, 56). We did, however, find a positive association between dietary choline intake and serum LDL cholesterol concentrations but only up to an intake of ±250 mg/d.

Choline is a dietary precursor for TMAO, a compound that has been associated with risk of cardiovascular disease (CVD) (57). Indeed, a positive association was observed between dietary choline intake and plasma TMAO concentrations in our study, but only up to 300 mg/d. Afterward, plasma TMAO declined with increasing choline consumption. TMAO can be formed from choline-containing compounds, betaine, and L-carnitine after formation of trimethylamine by gut bacteria (58). Recently, red meat, fish, and egg intakes have been associated with serum TMAO concentrations (59), but results of several studies assessing the association between dietary intake (mainly of choline and egg) and circulating TMAO concentrations were inconclusive (60–63). In addition, the number of studies focusing on TMAO and CVD is increasing, but it remains unclear whether TMAO is a causative factor or a biomarker for CVD (64). In an earlier study, we found that TMAO did not mediate the association between dietary choline intake and risk of acute myocardial infarction in patients with stable angina pectoris (65). Furthermore, a recent review by Thomas and Fernandez (66) investigating the role of diet and TMAO in CVD concluded that further evaluation of how TMAO precursors influence CVD and plasma TMAO concentrations is needed.

Sex differences in the association between choline intake and one-carbon metabolite concentrations have been observed. Indeed, several enzymes are involved in the one-carbon metabolism or up- or downregulated in women compared with men. Sadre-Marandi et al. (67) identified 5 enzymes—BHMT, methionine synthase (MS), methylenetetrahydrofolate reductase (MTHFR), serine hydroxymethyltransferase (SHMT), and phosphatidylethanolamine-N-methyltransferase (PEMT)—with a large difference in expression between women and men. For PEMT, BHMT, and MTHFR, sex hormones are thought to be at the base of the discrepancies in expression and/or activity, whereas the cause remains unclear for MS and SHMT. Subgroup analyses have a high risk of bias, and although their importance in exploratory analyses has been highlighted (68), our results are hypothesis generating and need to be confirmed in further research. In addition, interindividual differences in one-carbon metabolite response to dietary choline should be taken into account. The one-carbon metabolism is regulated by a wide range of enzymes and nutritional factors that may contribute to interindividual differences in the response to choline intake. For example, several of the involved enzymes are encoded by genes containing single-nucleotide polymorphisms, leading to alterations in gene expression or enzyme activity, choline requirement, and possibly metabolite response (21). Finally, the within-person reproducibility of most investigated metabolites is fair to excellent [intraclass correlation coefficient (ICC): >0.75–0.40], except for methionine, choline, and TML (ICC: 0.39–0.15), to allow one-exposure assessment of biomarker status in epidemiologic studies (69).

Dietary choline and lipid metabolites

Effects of dietary choline on serum lipid metabolites remain poorly studied, and findings are inconclusive. Consumption of 3 eggs a day for 4 wk increased total, HDL, and LDL cholesterol in healthy volunteers compared with choline bitartrate supplementation (70). Similar findings were observed in Ldlr$^{-/-}$ male mice after being fed a diet enriched with PC but not free choline (71). However, PC supplementation for 2 wk in 26 healthy volunteers did not alter cholesterol concentration but did increase serum TGs (56). These findings indicate that the choline form might be important when measuring the effect on lipid metabolites. Furthermore, PC was the main consumed choline form, and eggs were its main food source in this study. Because eggs contain a high amount of cholesterol (72), it is not unlikely that the observed short-term increase in serum total and LDL cholesterol might be caused by the composition of the food item providing choline rather than by choline itself. Notably, statin use was not widespread at the time of data collection. When comparing our findings with contemporary populations of the same age, it should thus be kept in mind that reported TC concentrations reflect untreated cholesterol concentrations.

Strengths and limitations

The main advantage of this study is the large sample size of community-dwelling adults. In addition, we have investigated the dietary intake and contributors of individual choline forms, something that is understudied. Furthermore, the FFQ used in this study captured long-term food intake and dietary patterns over time and avoided day-to-day variation in dietary intake (73). It is a method with low cost and low participant burden and is therefore frequently used for large cohort studies (73).

However, several limitations of this study should be acknowledged. Unfortunately, the FFQ used to estimate dietary intake was not validated for choline intake, which did not allow us to evaluate how well it captured the actual choline intake. Inherent limitations of an FFQ, such as systematic measurement errors, also apply to this study and may have led to a systematic underestimation of actual choline intakes (74). Furthermore, the calculation of the choline content of food items was based on the USDA database (29) because the Norwegian food composition table does not include values for choline (www.matvaretabellen.no). As choline content of food items may differ due to season and geographical location and variation between and within choline sources, the choline value obtained from the USDA database might not always reflect the true choline content

of food items (29). This is especially an issue for local foods, which may not typically be consumed in a North American diet. In general, choline intake data from food items are limited, and actual intakes may be underestimated because the choline content of only a small number of foods has been investigated. In addition, choline intakes from food additives such as lecithin cannot be estimated accurately as per now. Considering the increasing consumption of processed foods, it is not unlikely that a significant amount of dietary choline intake is derived from these sources, which leads again to an underestimation of choline consumption in current studies. Finally, the FFQ did not cover possible choline intake from supplements. However, most mainstream multivitamin supplements do not contain choline, especially not at the time of our data collection (1997–1999), and findings from the United States suggest that very few consume choline as a single-nutrient supplement (9). Moreover, it has been suggested that multivitamin or mineral supplement intake does not affect choline intake at the population level (75).

Conclusions

In this study including community-dwelling Norwegian middle-aged and elderly adults, we observed that the self-reported intake of dietary choline was below the established AI for most participants. Furthermore, choline was mainly consumed in the form of PC, and major contributing dietary sources were eggs, low-fat milk, potatoes, and leafy vegetables. Dietary choline was associated with the plasma concentration of one-carbon metabolites and serum lipids.

Further studies that estimate the choline intake in Nordic populations are warranted to allow for the establishment of dietary recommendations and nutrition policy. Also, because this is an exploratory study, there is a need to clarify the association between dietary choline, especially the individual choline forms, and one-carbon and lipid metabolites as these are closely related to risk of chronic diseases.

Acknowledgments

The authors' contributions were as follows—AVP, MSB, and VL: analyzed data and performed statistical analyses; AVP and MSB: wrote the paper; KJV: calculated the dietary choline intake; PMU: analyzed the one-carbon metabolites; and all authors (AVP, MSB, VL, KJV, PMU, TK, GST, TRH, JØ, JD, and ON): read and approved the final manuscript.

Data Availability

Data described in the manuscript, code book, and analytical code will be made available upon request pending application and approval.

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Assessment of dietary choline intake, contributing food items and associations with one-carbon and lipid metabolites in middle-aged and elderly adults: the Hordaland Health Study. Van Parys et al. – Online Supplementary Material

Supplementary Table 1: Grouping of food items into subcategories, categories, and food groups

Food group	Category	Subcategory	Food items
Dairy	Milk	Whole milk	Whole milk
		Low-fat milk	Low-fat milk
			Skimmed milk
		Other milk	Unspecified milk
	Cheese	White cheese	White cheese, full-fat
			White cheese, semi-fat
		Brown cheese	Brown cheese
	Other dairy	Yoghurt	Yoghurt
		Cream	Cream
			Sour cream
		Ice cream	Ice cream
		Other dairy products	Other dairy products
Drinks	Coffee		Coffee
	Tea		Tea
	Soda	Soda sugar	Soda with sugar
			Lemonade with sugar
		Soda light	Soda light
			Lemonade light
	Alcohol	Beer	Beer
			Alcohol-free beer
		Wine	Wine
		Liquor	Liquor

Eggs	Eggs		Eggs
Fats	Margarine		Soya margarine
			Margarine mixture
			Other margarine
			Light margarine
	Butter		Butter
			Butter-margarine mixture
			Unspecified butter
Fish	Fatty fish		Salmon
			Trout
			Other fatty fish
	Fish products	Fish spread	Fish spread
		Other fish product	Other fish product
	Shellfish		Shellfish
Fruit	Fresh fruit		Citrus fruit
			Apple
			Pear
			Unspecified fruit
	Juice		Juice
	Other fruit	Conserved fruit/berries	Jam
			Marmalade
			Canned fruit
Grain products	Bread	White bread	White bread
			Bread <50% whole wheat
		Wholegrain bread	Bread >50% whole wheat
		Other bread	"Lefse"

			Chapatti
			Taco
			Flatbread
			Crispbread
	Pastries	Buns	Waffles
			Yeast baked goods
		Biscuits	Biscuits
		Other pastries	Other pastries
	Other grain products	Pizza	Pizza
		Cereal	Cereal
			Oatmeal
Meat	Fresh/frozen meat	Poultry	Poultry
		Other fresh meat	Unspecified fresh meat
	Meat products	Meat spread	Liver spread
			Other meat spread
		Other meat products	Other meat products
Sweets and snacks	Sugar and sweets	Sugar	Sugar
			Other sweeteners
		Sweet spread	Sweat spread
		Sweets	Chocolate
			Candy
	Snacks	Chips	Potato chips
		Nuts and seeds	Nuts and seeds
		Other snacks	Other snacks
Vegetables	Potatoes		French fries
			Fresh potatoes
	Fresh vegetables	Root vegetables	Carrot

		Turnip
	Leafy vegetables	Cabbage
		Cauliflower
		Broccoli
		Spinach
		Parsley
	Other vegetables	Leek
		Onion
		Tomato
		Bell pepper
		Vegetable mix
		Unspecified vegetables
Canned vegetables		Pickled vegetables
		Other canned vegetables

Supplementary Table 2: Primary food subcategories contributing to free choline intake among participants in the Hordaland Health Study 1997-1999

Rank	Food item	Contribution (%)	Cumulative contribution (%)
1	Coffee	12.1	12.1
2	Potatoes	12.0	24.1
3	Low fat milk	8.7	32.8
4	Wholegrain bread	8.2	41.0
5	Leafy vegetables	8.2	49.2
6	Fresh fruit	6.8	56.0
7	Root vegetables	5.4	61.4
8	Alcohol	5.2	66.6
9	White bread	4.5	71.1
10	Other vegetables	3.8	74.9

Supplementary Table 3: Primary food subcategories contributing to glycerophosphocholine intake among participants in the Hordaland Health Study 1997-1999

Rank	Food item	Contribution (%)	Cumulative contribution (%)
1	Low fat milk	31.7	31.7
2	Fatty fish	5.5	37.2
3	Potatoes	5.4	42.6
4	Coffee	5.0	47.6
5	Fresh fruit	4.9	52.5
6	Alcohol	3.9	56.4
7	Yoghurt	3.7	60.1
8	Whole milk	3.6	63.7
9	Wholegrain bread	3.3	67.0
10	Other milk	3.3	70.3

Supplementary Table 4: Primary food subcategories contributing to phosphatidylcholine intake among participants in the Hordaland Health Study 1997-1999

Rank	Food item	Contribution (%)	Cumulative contribution (%)
1	Eggs	33.8	33.8
2	Meat spread	6.3	40.1
3	Leafy vegetables	6.2	46.3
4	Wholegrain bread	4.7	51.0
5	Other meat products	4.4	55.4
6	Potatoes	4.2	59.6
7	Buns	3.8	63.4
8	Poultry	3.5	66.9
9	Shellfish	3.2	70.1
10	Fresh fruit	2.7	72.8

Supplementary Table 5: Primary food subcategories contributing to phosphocholine intake among participants in the Hordaland Health Study 1997-1999

Rank	Food item	Contribution (%)	Cumulative contribution (%)
1	Low fat milk	26.7	26.7
2	Leafy vegetables	15.1	41.8
3	Potatoes	8.6	50.4
4	Other vegetables	6.3	56.7
5	Root vegetables	5.2	61.9
6	Whole milk	4.2	66.1
7	Fresh fruit	4.0	70.1
8	Yoghurt	3.7	73.8
9	Other milk	2.8	76.6
10	Poultry	2.1	78.7

Supplementary Table 6: Primary food subcategories contributing to sphingomyelin intake among participants in the Hordaland Health Study 1997-1999

Rank	Food item	Contribution (%)	Cumulative contribution (%)
1	Eggs	15.1	15.1
2	Low fat milk	12.2	27.3
3	Poultry	9.7	37.0
4	White cheese	6.4	43.4
5	Wholegrain bread	6.4	49.8
6	Meat spread	6.1	55.9
7	Other meat products	5.4	61.3
8	Brown cheese	3.5	64.8
9	Shellfish	3.1	67.9
10	White bread	2.9	70.8

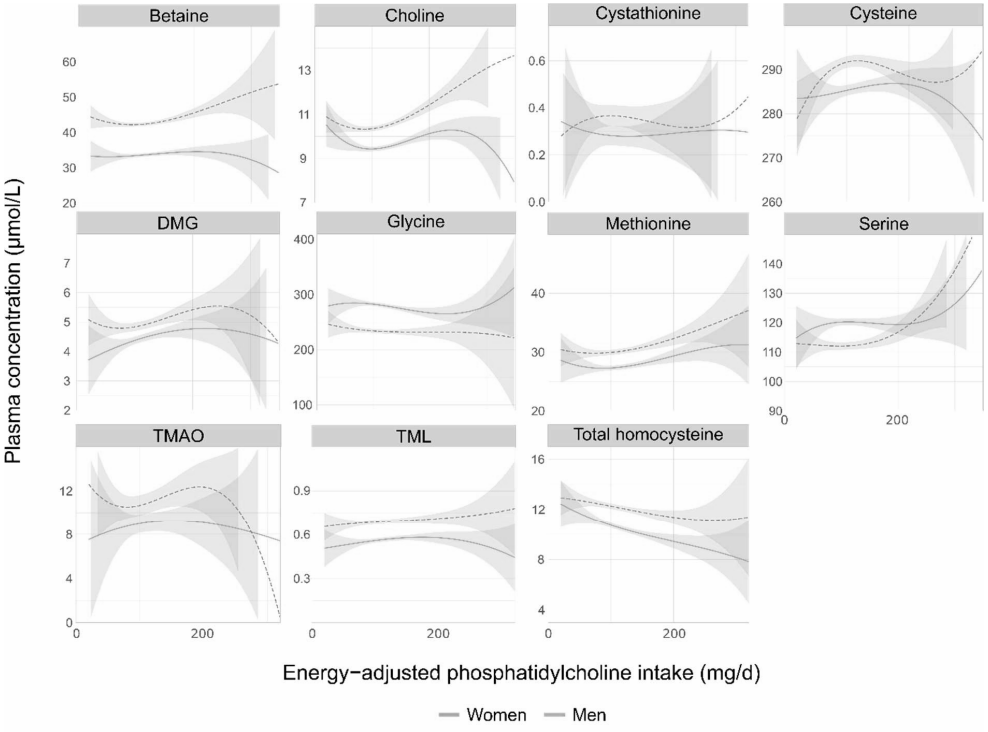
Supplementary Table 7: Main food groups, categories, and subcategories contributing to intake of total choline and individual choline forms among participants in the Hordaland Health Study 1997-1999

		Water-soluble forms			Lipid-soluble forms	
Contributing foods (%)	**Total choline**	**Free choline**	**Glycerophosphocholine**	**Phosphocholine**	**Phosphatidylcholine**	**Sphingomyelin**
Dairy	19.2	15.0	46.1	40.5	4.4	30.4
Milk	14.5	11.1	38.6	33.7	2.1	15.2
Low-fat milk	11.8	8.7	31.7	26.7	1.7	12.2
Whole milk	1.5	1.5	3.6	4.2	0.1	1.7
Other milk	1.2	0.9	3.3	2.8	0.2	1.3
Cheese	1.9	1.8	1.2	1.3	1.5	9.9
White cheese	1.3	1.2	1.1	1.3	0.9	6.4
Brown cheese	0.6	0.7	0.1	0.0	0.5	3.5
Other dairy	2.8	2.1	6.3	5.4	0.8	5.3
Yoghurt	1.5	1.0	3.7	3.7	0.4	2.6
Cream	0.5	0.4	1.0	0.6	0.2	1.4
Ice cream	0.6	0.4	1.3	0.8	0.2	1.3
Other dairy products	0.2	0.3	0.3	0.3	0.0	0.0
Drinks	7.0	18.7	9.5	0.0	0.0	0.0
Coffee	4.3	12.1	5.0	0.0	0.0	0.0
Tea	0.3	1.3	0.0	0.0	0.0	0.0
Soda	0.2	0.1	0.5	0.0	0.1	0.0
Soda sugar	0.2	0.1	0.5	0.0	0.1	0.0
Soda light	0.0	0.0	0.0	0.0	0.0	0.0
Alcohol	2.2	5.2	3.9	0.0	0.0	0.0
Beer	1.8	3.9	3.4	0.0	0.0	0.0
Wine	0.5	1.3	0.5	0.0	0.0	0.0
Liquor	0.0	0.0	0.0	0.0	0.0	0.0
Eggs	15.3	0.2	0.2	0.8	33.8	15.1
Fats	1.5	0.2	0.6	1.0	2.8	1.7

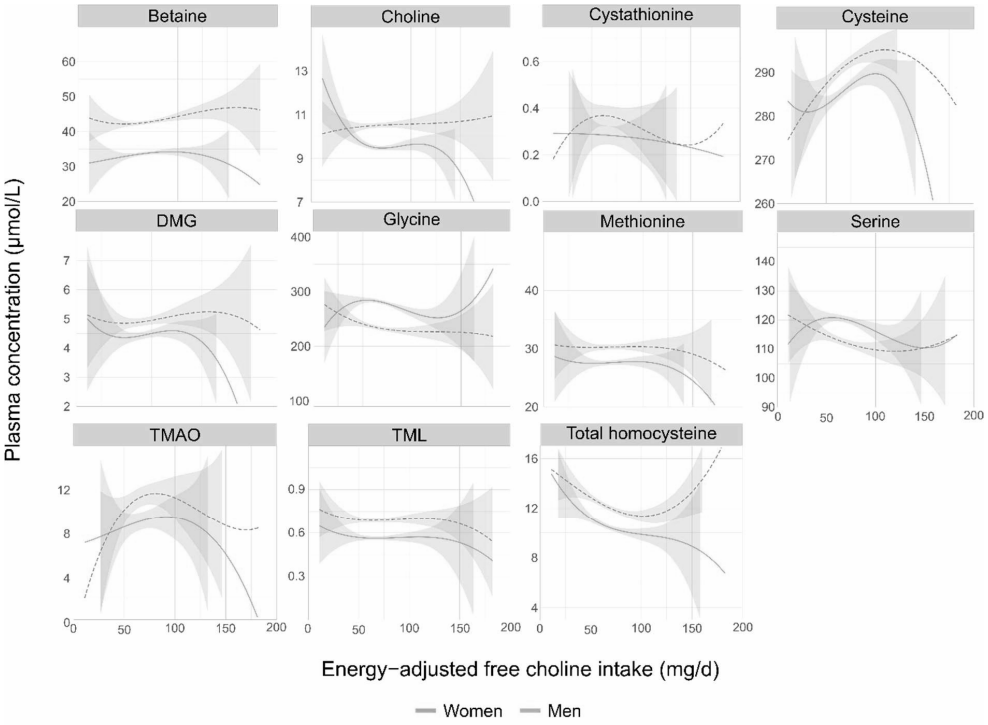
Margarine	0.3	0.0	0.1	0.2	0.7	0.0
Butter	0.2	0.0	0.1	0.2	0.2	0.9
Fish	8.6	4.6	13.0	3.3	8.7	13.2
Fatty fish	2.1	1.7	5.5	0.4	0.8	2.3
Fish products	4.8	3.0	7.0	2.5	4.7	7.8
Fish spread	3.5	2.0	4.7	1.5	3.8	6.4
Other fish products	1.3	1.1	2.3	1.0	0.9	1.3
Shellfish	1.7	0.1	0.5	0.3	3.2	3.1
Fruit	6.5	9.3	9.2	6.7	4.0	0.6
Fresh fruit	4.2	6.8	4.9	4.0	2.7	0.2
Juice	1.3	1.7	1.8	1.6	1.0	0.0
Other fruit	1.0	0.8	2.5	1.1	0.3	0.3
Grain products	12.8	16.5	7.6	5.5	14.4	14.0
Bread	8.5	13.4	6.0	3.3	7.7	9.7
White bread	2.8	4.5	2.0	1.3	2.5	2.9
Wholegrain bread	5.1	8.3	3.3	1.7	4.7	6.4
Other bread	0.5	0.6	0.7	0.2	0.5	0.5
Pastries	2.8	1.2	0.9	0.9	4.9	2.4
Buns	2.1	0.8	0.7	0.7	3.8	1.8
Biscuits	0.1	0.2	0.0	0.0	0.1	0.0
Other pastries	0.6	0.2	0.2	0.1	1.0	0.6
Other grain products	1.6	2.0	0.7	1.3	1.8	1.9
Pizza	0.8	1.2	0.3	0.9	0.7	1.1
Cereal	0.8	0.8	0.4	0.4	1.1	0.7
Meat total	10.2	3.6	3.7	4.9	16.5	23.8
Fresh meat	3.7	0.7	1.4	2.4	5.8	12.3
Poultry	2.2	0.6	0.1	2.1	3.5	9.7
Other fresh meat	1.4	0.1	1.3	0.2	2.3	2.7

Meat products	6.6	2.9	2.3	2.5	10.7	11.4
Other meat products	2.9	1.8	0.7	1.5	4.4	5.4
Meat spread	3.7	1.1	1.6	1.0	6.3	6.1
Sweets and snacks	0.5	0.7	0.1	0.2	0.7	0.0
Sugar and sweets	1.2	1.0	2.5	1.5	0.6	1.0
Sugar	0.0	0.0	0.0	0.0	0.0	0.0
Sweet spread	0.0	0.1	0.0	0.0	0.0	0.0
Sweets	1.2	0.9	2.5	1.5	0.6	1.0
Snacks	0.5	0.7	0.1	0.2	0.7	0.0
Chips	0.1	0.1	0.0	0.0	0.1	0.0
Nuts seeds	0.4	0.5	0.1	0.2	0.5	0.0
Other snacks	0.1	0.1	0.0	0.0	0.1	0.0
Vegetables	17.2	30.2	7.8	35.6	14.2	0.2
Potatoes	6.3	12.1	5.4	8.6	4.2	0.0
Fresh vegetables	10.3	17.4	2.3	26.6	9.2	0.0
Root vegetables	2.0	5.4	0.8	5.2	0.3	0.0
Leafy vegetables	5.7	8.2	0.9	15.1	6.2	0.0
Other vegetables	2.6	3.8	0.6	6.3	2.7	0.0
Canned vegetables	0.6	0.8	0.1	0.4	0.8	0.2

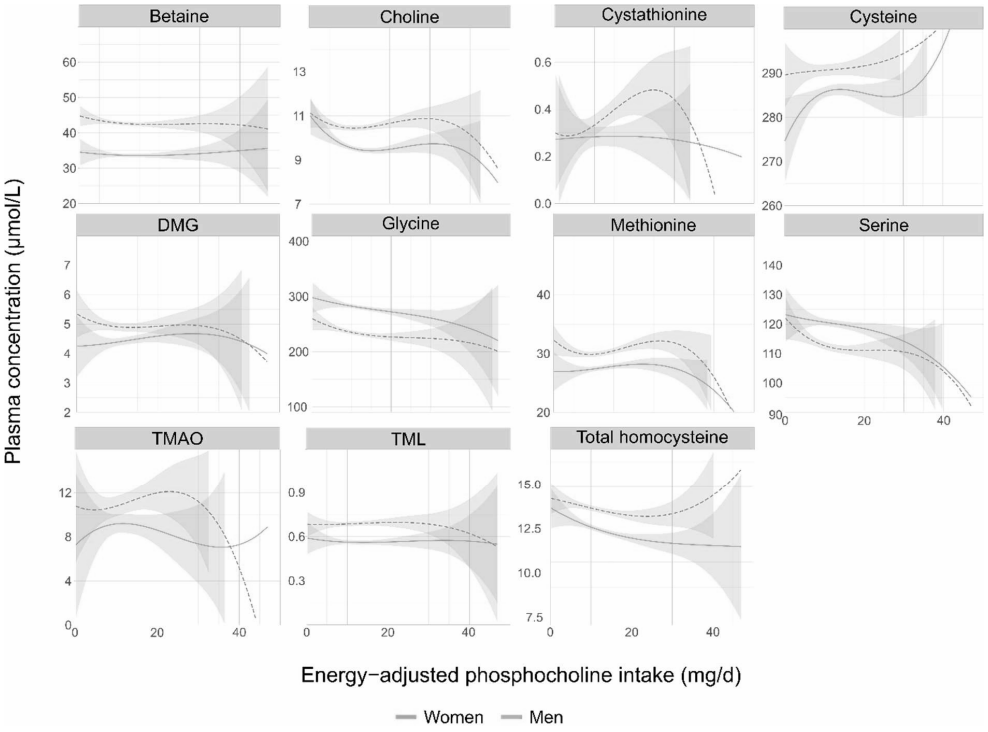
Assessment of dietary choline intake, contributing food items and associations with one-carbon and lipid metabolites in middle-aged and elderly adults: the Hordaland Health Study. Van Parys et al. – Online Supplementary Material



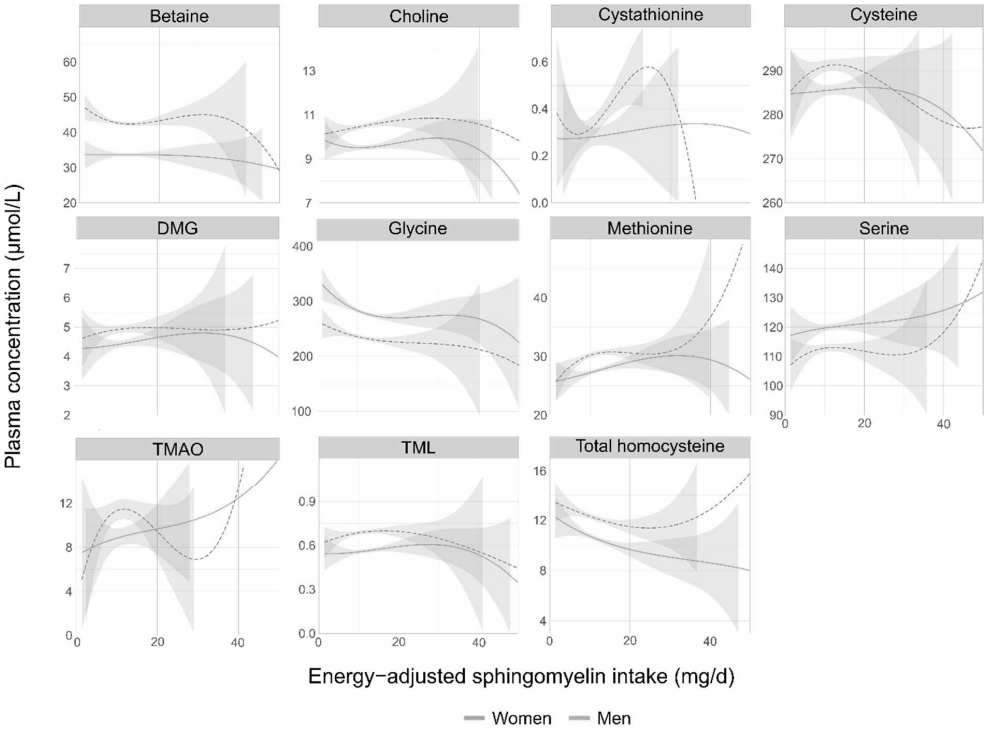
Supplementary Figure 1: Associations between energy-adjusted phosphatidylcholine intake and plasma concentration of one-carbon metabolites modelled as a polynomial spline in a model with sex as interaction term and adjusted for age, BMI, and smoking for participants in the Hordaland Health Study 1997-1999. The dotted red and solid blue lines represent the modelled associations for women and men respectively, and the colored areas indicate the corresponding 95% confidence interval. DMG indicates dimethylglycine; TMAO, trimethylamine N-oxide; TML, trimethyllysine.



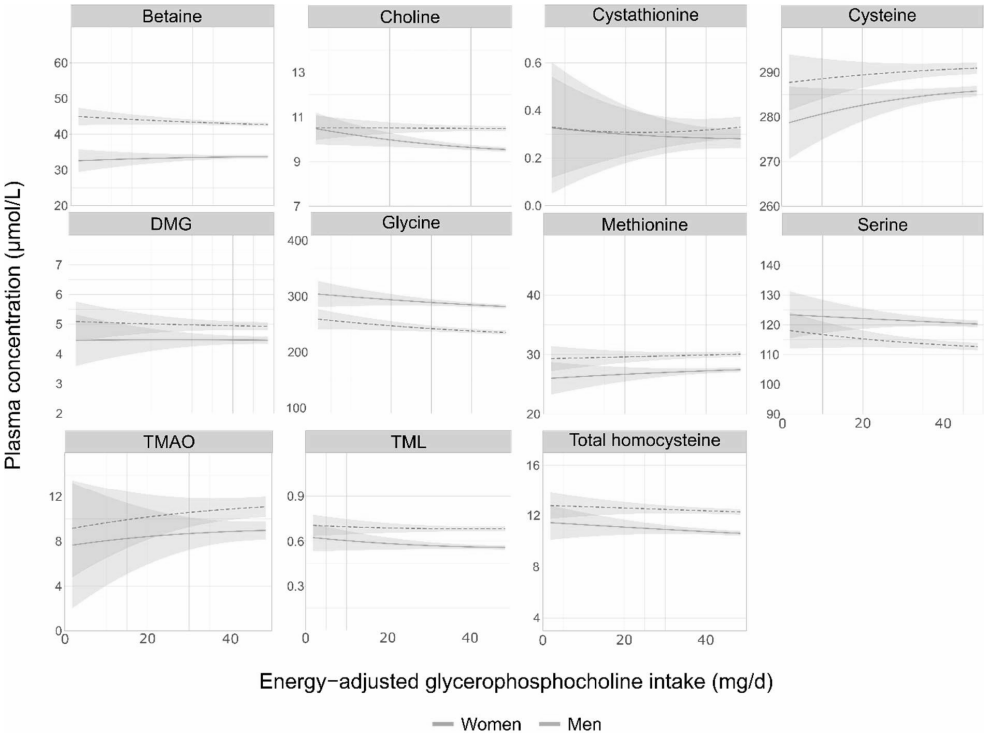
Supplementary Figure 2: Associations between energy-adjusted free choline intake and plasma concentration of one-carbon metabolites modelled as a polynomial spline in a model with sex as interaction term and adjusted for age, BMI, and smoking for participants in the Hordaland Health Study 1997-1999. The dotted red and solid blue lines represent the modelled associations for women and men respectively, and the colored areas indicate the corresponding 95% confidence interval. DMG indicates dimethylglycine; TMAO, trimethylamine N-oxide; TML, trimethyllysine.



Supplementary Figure 3: Associations between energy-adjusted phosphocholine intake and plasma concentration of one-carbon metabolites modelled as a polynomial spline in a model with sex as interaction term and adjusted for age, BMI, and smoking for participants in the Hordaland Health Study 1997-1999. The dotted red and solid blue lines represent the modelled associations for women and men respectively, and the colored areas indicate the corresponding 95% confidence interval. DMG indicates dimethylglycine; TMAO, trimethylamine N-oxide; TML, trimethyllysine.



Supplementary Figure 4: Associations between energy-adjusted sphingomyelin intake and plasma concentration of one-carbon metabolites modelled as a polynomial spline in a model with sex as interaction term and adjusted for age, BMI, and smoking for participants in the Hordaland Health Study 1997-1999. The dotted red and solid blue lines represent the modelled associations for women and men respectively, and the colored areas indicate the corresponding 95% confidence interval. DMG indicates dimethylglycine; TMAO, trimethylamine N-oxide; TML, trimethyllysine.



Supplementary Figure 5: Associations between energy-adjusted glycerophosphocholine intake and plasma concentration of one-carbon metabolites modelled as a polynomial spline in a model with sex as interaction term and adjusted for age, BMI, and smoking for participants in the Hordaland Health Study 1997-1999. The dotted red and solid blue lines represent the modelled associations for women and men respectively, and the colored areas indicate the corresponding 95% confidence interval. DMG indicates dimethylglycine; TMAO, trimethylamine N-oxide; TML, trimethyllysine.

V

HVA SPISER DU?

I dette skjemaet spør vi om dine spisevaner slik de **vanligvis** er. Vi er klar over at kostholdet varierer fra dag til dag. Prøv derfor så godt du kan å gi et **"gjennomsnitt"** av dine spisevaner. Ha det siste året i tankene når du fyller ut skjemaet. Der du er usikker, anslå svaret.

Skjemaet skal leses av en maskin, og derfor er det viktig at du setter et tydelig kryss i avmerket rute.

Riktig markering er slik: ☒

Bruk helst bløt blyant. Feil kan da rettes med viskelær.
Kulepenn og svart tusjpenn kan også brukes.

Av hensyn til den maskinelle lesingen pass på at arkene ikke blir brettet.

Alle svar vil bli behandlet strengt fortrolig.

EKSEMPEL PÅ UTFYLLING AV SPØRSMÅL 1.

Kari Nordmann spiser daglig 5 skiver brød og ett knekkebrød. Hun spiser vanligvis kneippbrød, men i helgene blir det en del loff. I tillegg spiser hun ett knekkebrød hver dag. Hun fyller ut første spørsmål slik:

1. HVOR MYE BRØD PLEIER DU Å SPISE?

Legg sammen det du bruker til alle måltider i løpet av en dag.

(1/2 rundstykke = 1 skive, 1 baguett = 5 skiver, 1 ciabatta = 4 skiver)

	Antall skiver pr. dag													
	0	1/2	1	2	3	4	5	6	7	8	9	10	11	12+
Fint brød (loff, baguetter, fine rundstykker o.l.)	☐	☐	☒	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Mellomgrovt brød (lys helkorn, lys kneipp, lyst hj.bakt o.l.)	☐	☐	☐	☐	☐	☒	☐	☐	☐	☐	☐	☐	☐	☐
Grovt brød (fiberkneipp, mørk kneipp, mørkt hj.bakt o.l.)	☒	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Knekkebrød (kavring, grov skonrok o.l.)	☐	☐	☒	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

Sum skiver pr. dag = 6

Antall skiver pr. uke: 6 x 7 = 42 Tallet brukes i spørsmål 5.

1. HVOR MYE BRØD PLEIER DU Å SPISE?

Legg sammen det du bruker til alle måltider i løpet av en dag.

(1/2 rundstykke = 1 skive, 1 baguett = 5 skiver, 1 ciabatta = 4 skiver)

	Antall skiver pr. dag													
	0	1/2	1	2	3	4	5	6	7	8	9	10	11	12+
Fint brød (loff, baguetter, fine rundstykker o.l.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Mellomgrovt brød (lys helkorn, lys kneipp, lyst hj.bakt o.l.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Grovt brød (fiberkneipp, mørk kneipp, mørkt hj.bakt o.l.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Knekkebrød (kavring, grov skonrok o.l.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

Sum skiver pr. dag = ____

Antall skiver pr. uke: ____ x 7 = ____. Tallet brukes i spørsmål 5.

2. HVA PLEIER DU Å SMØRE PÅ BRØDET?

Merk av både for hverdag og helg, selv om du bruker det samme.

Hverdager		Lørdager, søndager
☐	Bruker ikke	☐
☐	Smør (meierismør)	☐
☐	Bremykt, Smøregod	☐
☐	Brelett	☐
☐	Soft, soyamargarin (pakke, beger)	☐
☐	Solsikke	☐
☐	Oliven	☐
☐	Vita	☐
☐	Olivero	☐
☐	Omega	☐
☐	Soft light	☐
☐	Vita lett	☐
☐	Annen margarin	☐

3. OM DU BRUKER FETT PÅ BRØD, HVOR MYE BRUKER DU?

En porsjonspakning på 12 g rekker til antall skiver

1 ☐

2 ☐

3 ☐

4 ☐

5 ☐

4. MELK SOM DRIKK

(1 glass = 1,5 dl)

	Drikker sjelden/ ikke	Antall glass pr. dag 1/2	1	2	3	4	5	6	7	8+
Helmelk, søt, sur	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Lettmelk, søt, sur	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Lettmelk, ekstra lett	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Skummet melk, søt, sur	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

5. PÅLEGGSSORTER

Bruk sum skiver pr. uke fra spørsmål 1.

Til antall skiver pr. uke

	0	1/2	1	2-3	4-5	6-7	8-14	15-21	22-28	29-35	36+
Brun ost, prim	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Hvit ost, helfet, 27% fett (Jarlsberg, Norvegia o.l., smøreost; eske, tube)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Hvit ost, halvfet, 16% fett (Jarlsberg, Norvegia o.l., smøreost; eske, tube)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Ost med mer enn 27% fett (kremoster, Normanna, Ridderost)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

	0	1/2	1	2-3	4-5	6-7	8-14	15-21	22-28	29-35	36+
Leverpostei, vanlig	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Leverpostei, mager	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Servelat, vanlig	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Lett servelat, kalverull, kokt skinke, okserull o.l.	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Salt pølse, spekepølse (fårepølse, salami o.l.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

	0	1/2	1	2-3	4-5	6-7	8-14	15-21	22-28	29-35	36+
Kaviar	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Makrell i tomat, røkt makrell	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Sardiner, sursild, ansjos o.l.	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Laks, ørret	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Reker, krabbe	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

	0	1/2	1	2-3	4-5	6-7	8-14	15-21	22-28	29-35	36+
Syltetøy, marmelade, frysetøy	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Honning, sirup, sjokolade-, nøttepålegg	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

	0	1/2	1	2-3	4-5	6-7	8-14	15-21	22-28	29-35	36+
Grønnsaker som pålegg (agurk, tomat o.l.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Frukt som pålegg (banan, eple o.l.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Salater med majones	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Majones på smørbrød	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

6. EGG

Antall pr. uke

	0	Mindre enn 1	1	2	3-4	5-6	7	8+
(kokt, stekt, eggerøre, omelett)	☐	☐	☐	☐	☐	☐	☐	☐

7. FROKOSTGRYN, GRØT OG YOGHURT

Svar enten pr. måned **eller** pr. uke. <1 betyr sjeldnere enn 1 gang.

	Gang pr. måned					Gang pr. uke						Mengde pr. gang				
	0	<1	1	2	3	1	2-3	4-5	6-7	8+		1	1 1/2	2	3+
Havregryn, kornblandinger (4-korn, usøtet müsli o.l.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	☐	☐	☐	☐
Cornflakes, puffet ris, havrenøtter o.l.	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	☐	☐	☐	☐
												1-2	3-4	5-6	7+
Havregrøt	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	☐	☐	☐	☐
												1	2	3-4	5+
Sukker til frokostgryn, grøt	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(ts)	☐	☐	☐	☐
												1/2	1	1 1/2	2+
Yoghurt, naturell, frukt	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(beger)	☐	☐	☐	☐
												1/2	1	1 1/2	2+
Lettyoghurt	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(beger)	☐	☐	☐	☐
												1/2	1	1 1/2	2+
Go´morgen yoghurt inkl. müsli	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(beger)	☐	☐	☐	☐
												3/4	1	2	3+
Melk søt, sur på gryn, grøt og dessert	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	☐	☐	☐	☐

8. KAFFE OG TE

(1 kopp kaffe = 1,2 dl 1 kopp te = 2 dl)

	Drikker ikke/ikke daglig	Antall kopper pr. dag: 1/2	1	2	3-4	5-6	7-8	9-10	11+
Kaffe, kokt	☐	☐	☐	☐	☐	☐	☐	☐	☐
Kaffe, traktet, filter	☐	☐	☐	☐	☐	☐	☐	☐	☐
Kaffe, pulver (instant)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Kaffe, koffeinfri	☐	☐	☐	☐	☐	☐	☐	☐	☐
Te	☐	☐	☐	☐	☐	☐	☐	☐	☐
Nypete, urtete	☐	☐	☐	☐	☐	☐	☐	☐	☐

	Antall teskjeer eller biter pr. kopp: 0	1/2	1	2	3	4+
Sukker til kaffe	☐	☐	☐	☐	☐	☐
Sukker til te	☐	☐	☐	☐	☐	☐
Kunstig søtstoff til kaffe eller te	☐	☐	☐	☐	☐	☐
Fløte til kaffe	☐	☐	☐	☐	☐	☐

9. ANDRE DRIKKER?

Svar enten pr. måned **eller** pr. uke. < 1 betyr sjeldnere enn 1 gang.
Merk at porsjonsenhetene er forskjellige. 1/3 liter tilsvarer en halvflaske øl og 2/3 liter tilsvarer en helflaske.

	Gang pr. måned					Gang pr. uke					Mengde pr. gang						
	0	<1	1	2	3	1	2-3	4-5	6-7	8+		1/2	1	2	3	4	5+
Vann	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(glass)	☐	☐	☐	☐	☐	☐
Appelsinjuice	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(glass)	☐	☐	☐	☐	☐	☐
Annen juice, most, nektar	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(glass)	☐	☐	☐	☐	☐	☐
Saft, solbærsirup m. sukker	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(glass)	☐	☐	☐	☐	☐	☐
Saft, kunstig søtet	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(glass)	☐	☐	☐	☐	☐	☐
Brus, Cola, Solo o.l., med sukker	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(liter)	1/4 ☐	1/3 ☐	1/2 ☐	2/3 ☐	1 ☐	11/2+ ☐
Brus, Cola, Solo o.l., kunstig søtet	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(liter)	1/4 ☐	1/3 ☐	1/2 ☐	2/3 ☐	1 ☐	11/2+ ☐
Farris, Selters, Soda o.l.	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(liter)	1/4 ☐	1/3 ☐	1/2 ☐	2/3 ☐	1 ☐	11/2+ ☐
Alkoholfritt øl, vørterøl, lettøl	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(liter)	1/4 ☐	1/3 ☐	1/2 ☐	2/3 ☐	1 ☐	11/2+ ☐
Pilsnerøl	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(liter)	1/4 ☐	1/3 ☐	1/2 ☐	2/3 ☐	1 ☐	11/2+ ☐
Vin	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(glass)	1 ☐	2 ☐	3 ☐	4 ☐	5 ☐	6+ ☐
Brennevin, likør	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(1 dram = 4 cl)	1 ☐	2 ☐	3 ☐	4 ☐	5 ☐	6+ ☐

10. MIDDAGSRETTER

Vi spør både om middagsmåltidene og det du spiser til andre måltider. Tell til slutt sammen antall retter du har merket for og se om summen virker sannsynlig.
En "dl" tilsvarer omtrent mengden i en suppeøse. Med "ss" menes en spiseskje.

	Gang pr. måned										Mengde pr. gang					
	0	<1	1	2	3	4	5-6	7-8	9+		1/2	2/3	1	11/2	2+
Kjøttpølse, medisterpølse	☐	☐	☐	☐	☐	☐	☐	☐	☐	(kjøttpølse)	☐	☐	☐	☐	☐
Hamburger, karbonader o.l.	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐
Grill- og wienerpølse	☐	☐	☐	☐	☐	☐	☐	☐	☐	(pølse)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐
Hamburger-, pølsebrød, lomper	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐
Kjøttkaker, medisterkaker, kjøttpudding	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐
Kjøttdeigretter (saus eller gryte med kjøttdeig, lasagne o.l.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐
Taco (med kjøtt og salat)	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐
Pastaretter	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐

	Gang pr. måned										Mengde pr. gang					
	0	<1	1	2	3	4	5-6	7-8	9+		1/8	1/4	1/2	3/4	1+	
Pizza (500-600 g)	☐	☐	☐	☐	☐	☐	☐	☐	☐	(pizza)	☐	☐	☐	☐	☐	
Biff (alle typer kjøtt)	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1/2	1	1 1/2	2	2 1/2+	
Koteletter (lam, okse, svin)	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1/2	1	1 1/2	2	2 1/2+	
Stek (lam, okse, svin)	☐	☐	☐	☐	☐	☐	☐	☐	☐	(skive)	1-2	3-4	5-6	7-8	9+	
Stek (elg, hjort, reinsdyr o.l.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	(skive)	1-2	3-4	5-6	7-8	9+	
Gryterett med helt kjøtt, frikassé, fårikål o.l.	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1-2	3-4	5-6	7-8	9+	
Lapskaus, suppelapskaus, betasuppe	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1-2	3-4	5-6	7-8	9+	
Bacon, stekt flesk	☐	☐	☐	☐	☐	☐	☐	☐	☐	(skive)	1-2	3-4	5-6	7-8	9+	
Kylling, høne	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1/4	1/3	1/2	3/4	1+	
Leverretter	☐	☐	☐	☐	☐	☐	☐	☐	☐	(skive)	1-2	3-4	5-6	7-8	9+	
	0	<1	1	2	3	4	5-6	7-8	9+		1	2	3	4	5+	
Fiskekaker, fiskepudding, fiskeboller	☐	☐	☐	☐	☐	☐	☐	☐	☐	(kake)	☐	☐	☐	☐	☐	
Fiskepinner	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1-2	3-4	5-6	7-9	10+	
Torsk, sei, hyse (kokt)	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1	2	3	4	5+	
Torsk, sei, hyse (stekt, panert)	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1	2	3	4	5+	
Sild (fersk, speket, røkt)	☐	☐	☐	☐	☐	☐	☐	☐	☐	(filet)	1	2	3	4	5+	
Makrell (fersk, røkt)	☐	☐	☐	☐	☐	☐	☐	☐	☐	(filet)	1/2	1	1 1/2	2	3+	
Laks, ørret (sjø, oppdrett)	☐	☐	☐	☐	☐	☐	☐	☐	☐	(skive)	1	2	3	4	5+	
Fiskegryte, -grateng, suppe med fisk	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1-2	3-4	5-6	7-8	9+	
Reker, krabbe	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl, renset)	1	2	3	4	5+	
	0	<1	1	2	3	4	5-6	7-8	9+							
Risgrøt, annen melkegrøt	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1-2	3-4	5-6	7-8	9+	
Pannekaker	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1-2	3-4	5-6	7-8	9+	
Suppe (tomat, blomkål, ertesuppe o.l.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1-2	3-4	5-6	7-8	9+	
Vegetarrett, vegetarpizza grønnsakgrateng, -pai	☐	☐	☐	☐	☐	☐	☐	☐	☐	(bit/dl)	1-2	3-4	5-6	7-8	9+	
	0	<1	1	2	3	4	5-6	7-8	9+		1/2	1	1 1/2	2	2 1/2+	
Brun/hvit saus	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	☐	☐	☐	☐	☐	
Smeltet margarin, smør til fisk	☐	☐	☐	☐	☐	☐	☐	☐	☐	(ss)	1-2	3-4	5-6	7-8	9+	
Bearnaisesaus o.l.	☐	☐	☐	☐	☐	☐	☐	☐	☐	(ss)	1	2	3	4	5+	
Majones, remulade	☐	☐	☐	☐	☐	☐	☐	☐	☐	(ss)	1	2	3	4	5+	
Ketchup	☐	☐	☐	☐	☐	☐	☐	☐	☐	(ss)	1	2	3	4	5+	

11. POTETER, RIS, SPAGHETTI, GRØNNSAKER

Svar enten pr. måned **eller** pr. uke. <1 betyr sjeldnere enn 1 gang.
Disse spørsmålene dreier seg først og fremst om tilbehør til middagsretter, men spiser du for eksempel en rå gulrot eller salat til lunsj, skal det tas med her.

	Gang pr. måned					Gang pr. uke						Mengde pr. gang				
	0	<1	1	2	3	1	2-3	4-5	6-7	8+		1	2	3	4	5+
Poteter, kokte	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	☐	☐	☐	☐	☐
Pommes frites, stekte poteter	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐
Potetmos, -stuing, gratinerte poteter	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐
Ris	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1-2 ☐	3-4 ☐	5-6 ☐	7-8 ☐	9+ ☐
Spaghetti, makaroni, pasta	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1-2 ☐	3-4 ☐	5-6 ☐	7-8 ☐	9+ ☐
Gulrot	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1/2 ☐	1 ☐	1 1/2 ☐	2 ☐	3+ ☐
Hodekål	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(skalk)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐
Kålrot	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(skive)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐
Blomkål	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(bukett)	1-2 ☐	3-4 ☐	5-6 ☐	7-8 ☐	9+ ☐
Brokkoli	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(bukett)	1-2 ☐	3-4 ☐	5-6 ☐	7-8 ☐	9+ ☐
Rosenkål	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1-2 ☐	3-4 ☐	5-6 ☐	7-8 ☐	9+ ☐
Grønnkål	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐
Løk	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(ss)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐
Spinat, andre bladgrønns.	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐
Sopp	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1-2 ☐	3-4 ☐	5-6 ☐	7-8 ☐	9+ ☐
Avocado	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1/4 ☐	1/2 ☐	3/4 ☐	1 ☐	1 1/4 + ☐
Paprika	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(strimmel)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐
Tomat	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1/2 ☐	1 ☐	1 1/2 ☐	2 ☐	3+ ☐
Tomatbønner, bønner/linser	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐
Mais	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(ss)	1-2 ☐	3-4 ☐	5-6 ☐	7-8 ☐	9+ ☐
Erter, frosne grønnsak-blandinger	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐
Salatblandinger	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1 ☐	2 ☐	3 ☐	4 ☐	5+ ☐
Dressing	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(ss)	1/2 ☐	1 ☐	2 ☐	3 ☐	4+ ☐
Rømme	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(ss)	1/2 ☐	1 ☐	2 ☐	3 ☐	4+ ☐

Hvor mange ganger om dagen spiser du vanligvis grønnsaker utenom grønnsakene du spiser til middag?

0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5+ ☐

12. TYPE FETT TIL MATLAGING

Smør/margarin	Oljer
☐ Smør (meierismør)	☐ Olivenolje
☐ Bremykt	☐ Soyaolje
☐ Melange, Per	☐ Maisolje
☐ Soft-, soyamargarin (pakke, beger)	☐ Solsikkeolje
☐ Solsikke	☐ Valnøttolje
☐ Oliven	☐ Andre oljer
☐ Annen margarin	

13. FRUKT

Svar enten pr. måned **eller** pr. uke. < 1 betyr sjeldnere enn 1 gang.

	Gang pr. måned					Gang pr. uke						Mengde pr. gang			
	0	<1	1	2	3	1	2-3	4-5	6-7	8+		1/2	1	2	3+
Eple	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	☐	☐	☐	☐
Appelsin, mandarin, grapefrukt	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	☐	☐	☐	☐
Banan	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	☐	☐	☐	☐
Druer	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(klase)	☐	☐	☐	☐
Eksotisk frukt (kiwi, mango)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	☐	☐	☐	☐
Annen frukt (fersken, pære m.v.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	☐	☐	☐	☐
Jordbær, bringebær (friske, frosne)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	☐	☐	☐	☐
Blåbær	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	☐	☐	☐	☐
Multer	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	☐	☐	☐	☐

Hvor mange frukter spiser du vanligvis pr. dag?

0	1	2	3	4	5	6	7	8	9+
☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

14. DESSERT, KAKER, GODTERI

Svar enten pr. måned **eller** pr. uke. < 1 betyr sjeldnere enn 1 gang.

	Gang pr. måned					Gang pr. uke						Mengde pr. gang			
	0	<1	1	2	3	1	2-3	4-5	6-7	8+					
Hermetisk frukt, fruktgrøt	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1/2 ☐	1 ☐	2 ☐	3+ ☐
Puddinger (sjokolade, karamell o.l.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1 ☐	2 ☐	3 ☐	4+ ☐
Is (1 dl = 1 pinne = 1 kremmerhus)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1 ☐	2 ☐	3 ☐	4+ ☐
Boller, julekake, kringle	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1 ☐	2 ☐	3 ☐	4+ ☐
Skolebrød, skillingsbolle	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1 ☐	2 ☐	3 ☐	4+ ☐
Wienerbrød, -kringle o.l.	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1 ☐	2 ☐	3 ☐	4+ ☐
Smultring, formkake	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1 ☐	2 ☐	3 ☐	4+ ☐
Vafler	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(plate)	1/2 ☐	1 ☐	2 ☐	3+ ☐
Sjokoladekake, bløtkake, annen fylt kake	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1/2 ☐	1 ☐	2 ☐	3+ ☐
Søt kjeks, kakekjeks (Cookies, Bixit, Hob Nobs)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1-2 ☐	3-4 ☐	5-6 ☐	7+ ☐
Sjokolade (60 g)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(plate)	1/2 ☐	1 ☐	2 ☐	3+ ☐
Drops, lakris, seigmenn o.l.	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(stk)	1-2 ☐	3-4 ☐	5-6 ☐	7+ ☐
Smågodt (1 hg = 100g)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(hg)	1/2 ☐	3/4 ☐	1 ☐	1 1/2+ ☐
Potetgull (1 pose 100g = 7 dl)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1-2 ☐	3-4 ☐	5-6 ☐	7+ ☐
Annen snacks (skruer, crisp, saltstenger, lettsnacks o.l.)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(dl)	1-2 ☐	3-4 ☐	5-6 ☐	7+ ☐
Peanøtter, andre nøtter (1 pose 100g = 4 never)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	(neve)	1 ☐	2 ☐	3 ☐	4+ ☐

15. KOSTTILSKUDD (bs = barneskje, ts = teskje)

	Hele året	Bare vinter-halvåret	Gang pr. uke: 0	<1	1	2-3	4-5	6-7		Mengde pr. gang
Tran	☐	☐	☐	☐	☐	☐	☐	☐		☐ 1 ts ☐ 1 bs ☐ 1 ss
Trankapsler	☐	☐	☐	☐	☐	☐	☐	☐	kapsler	☐ 1 ☐ 2+
Fiskeoljekapsler	☐	☐	☐	☐	☐	☐	☐	☐	kapsler	☐ 1-2 ☐ 3-4 ☐ 5-6 ☐ 7+
Multipreparater										
Sanasol	☐	☐	☐	☐	☐	☐	☐	☐	bs	☐ 1 ☐ 2 ☐ 3 ☐ 4+
Biovit	☐	☐	☐	☐	☐	☐	☐	☐	bs	☐ 1 ☐ 2 ☐ 3 ☐ 4+
Vitaplex	☐	☐	☐	☐	☐	☐	☐	☐	tablett	☐ 1 ☐ 2 ☐ 3 ☐ 4+
Kostpluss	☐	☐	☐	☐	☐	☐	☐	☐	tablett	☐ 1 ☐ 2 ☐ 3 ☐ 4+
Vitamineral	☐	☐	☐	☐	☐	☐	☐	☐	tablett	☐ 1 ☐ 2 ☐ 3 ☐ 4+
Annet	☐	☐	☐	☐	☐	☐	☐	☐	tablett	☐ 1 ☐ 2 ☐ 3 ☐ 4+
Hvis annet, hvilket?										
Jernpreparater										
Ferro C	☐	☐	☐	☐	☐	☐	☐	☐	tablett	☐ 1 ☐ 2 ☐ 3 ☐ 4+
Hemofer	☐	☐	☐	☐	☐	☐	☐	☐	tablett	☐ 1 ☐ 2 ☐ 3 ☐ 4+
Duroferon Duretter	☐	☐	☐	☐	☐	☐	☐	☐	tablett	☐ 1 ☐ 2 ☐ 3 ☐ 4+
Annet	☐	☐	☐	☐	☐	☐	☐	☐	tablett	☐ 1 ☐ 2 ☐ 3 ☐ 4+
Hvis annet, hvilket?										
B-vitaminer	☐	☐	☐	☐	☐	☐	☐	☐	tablett	☐ 1 ☐ 2 ☐ 3 ☐ 4+
C-vitamin	☐	☐	☐	☐	☐	☐	☐	☐	tablett	☐ 1 ☐ 2 ☐ 3 ☐ 4+
D-vitamin	☐	☐	☐	☐	☐	☐	☐	☐	tablett	☐ 1 ☐ 2 ☐ 3 ☐ 4+
E-vitamin	☐	☐	☐	☐	☐	☐	☐	☐	tablett	☐ 1 ☐ 2 ☐ 3 ☐ 4+
Folat (folsyre)	☐	☐	☐	☐	☐	☐	☐	☐	tablett	☐ 1 ☐ 2 ☐ 3 ☐ 4+
Kalktabletter	☐	☐	☐	☐	☐	☐	☐	☐	tablett	☐ 1 ☐ 2 ☐ 3 ☐ 4+
Fluortabletter	☐	☐	☐	☐	☐	☐	☐	☐	tablett	☐ 1 ☐ 2 ☐ 3 ☐ 4+
Annet	☐	☐	☐	☐	☐	☐	☐	☐	tablett	☐ 1 ☐ 2 ☐ 3 ☐ 4+
Hvis annet, hvilket?										

16. NÅR SPISER DU PÅ HVERDAGER?

HOVEDMÅLTIDER som frokost, formiddagsmat, middag, kvelds.

Omtrent klokken

6		8		10		12		14		16		18		20		22		24		2		4
☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

MELLOMMÅLTIDER som kaffe, frukt, godteri, snacks m.v.

Omtrent klokken

6		8		10		12		14		16		18		20		22		24		2		4
☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

17. MENER DU SVARENE I SPØRRESKJEMAET GIR ET BRUKBART BILDE AV KOSTHOLDET DITT?

Ja	Nei
☐	☐

Er det matvarer/produkter du regelmessig bruker, og som ikke er nevnt i skjemaet?

.....

.....

18. ER DU FORNØYD MED KROPPSVEKTEN DIN SLIK DEN ER NÅ?

- ☐ Ja
- ☐ Nei, jeg ønsker å slanke meg
- ☐ Nei, jeg ønsker å legge på meg

19. KJØNN

Mann	Kvinne
☐	☐

Vennligst se etter at du har svart på alle spørsmål.

Takk for innsatsen!

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