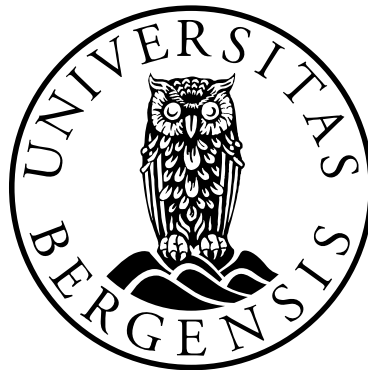# Components of the choline oxidation pathway in relation to acute myocardial infarction, type 2 diabetes and mortality

Prospective observational studies among patients with suspected or verified coronary heart disease in Norway

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LIST OF SELECTED ABBREVIATIONS

ACE – Angiotensin converting enzyme

ACS – Acute coronary syndrome

ACVD– Atherosclerotic cardiovascular disease

AMI – Acute myocardial infarction

Apo – Apolipoprotein

BADH – Betaine aldehyde dehydrogenase

BHMT – Betaine-homocysteine S-methyl transferase

BMI – Body mass index

CABG – Coronary artery bypass graft

CAD – Coronary artery disease

CHDH – Choline dehydrogenase

CBS – Cystathionine-β-synthase

CI – Confidence interval

CoR – Coefficient of reliability

CRP – C-reactive protein

CVD – Cardiovascular disease

DMG – Dimethylglycine

DDH – Dimethylglycine dehydrogenase

GAM – Generalized additive model

GFR – Glomerular filtration rate

GNMT – Glycine-N-methyltransferase

HDL-C – High density lipoprotein cholesterol

ICC – Intraclass correlation coefficient

IDI – Integrated discrimination index

HR – Hazard ratio

LDL-C – Low density lipoprotein cholesterol

LME – Linear mixed effects model

LVEF – Left ventricular ejection fraction

MMA – Methylmalonic acid

MS – Methionine synthase

NAFLD – Non-alcoholic fatty liver disease

NRI – Net reclassification improvement

NORVIT – Norwegian Vitamin Trial

PCI – Percutaneous coronary intervention

PPAR – Peroxisome proliferator-activated receptor

ROC-AUC – Receiver operating characteristics-area under the curve

RCT – Randomized controlled trial

SAH – *S*-adenosylhomocysteine

SAM – *S*-adenosylmethionine

SAP – Stable angina pectoris

SARDH – Sarcosine dehydrogenase

SD – Standard deviation

SSH – Sarcosine dehydrogenase

SMC – Smooth muscle cell

SNP – Single nucleotide polymorphism

STROBE-ME – STrengthening the Reporting of OBservational studies in Epidemiology-Molecular Epidemiology

tHcy – Total homocysteine

TMAO – Trimethylamine *N*-oxide

T2D – Type 2 diabetes

UAP – Unstable angina pectoris

VLDL – Very low density lipoprotein

WECAC – Western Norway Coronary Angiography Cohort

WENBIT – Western Norway B vitamin Intervention Trial

SCIENTIFIC ENVIRONMENT

The current thesis is based upon studies of two Norwegian cohorts, made up of patients with suspected and/or verified coronary heart disease (CHD); The Western Norway Coronary Angiography Cohort (WECAC) and the Norwegian B-vitamin Intervention Trial (NORVIT). Although the NORVIT was a multicentre national intervention study, the scientific environment of the current thesis has mainly been localized to the University of Bergen, with Professor Ottar Kjell Nygård as main supervisor and Professor Per Magne Ueland and PhD Eva Ringdal Pedersen as co-supervisors. However, the work has been a joint collaboration with other local and national research environments, including the Western Norway Cardiovascular Registry (WENOCARD), the Cardiovascular Disease in Norway (CVDNOR) project, the KG Jebsen Center for Diabetes Research, and Bevital AS, as well as scientific co-workers at the Department of Heart Disease, Haukeland University Hospital and Stavanger University Hospital, and the Norwegian University of Science and Technology.

ACKNOWLEDGEMENTS

I first met Dr Ottar Nygård in 2000, when working as an assistant in the Department of Heart Disease at Haukeland University Hospital. Little did I know of his many projects at that time, but soon I found myself in front of a computer plotting WENBIT patient data into the CORDOBA database. At first this meant little more to me than some extra and much appreciated money, but I soon gained more interest into the field of cardiology and continued to work on the database also after graduating in 2003. Upon returning to Bergen in 2006 I felt that everyday clinical practice left little room for academic work. However, my recent hospital internship had ignited an interest in academia, and despite much reluctance from my side, the then appointed professor Nygård convinced me into starting the current research project in 2009, thereby introducing me to scientific work. For that I am extremely grateful. Ottar, your philosophy of supervision is based upon the necessity for your students to work independently, and without you having the need for a detailed control of every aspect of their work. This economic exertion of leadership reflects trust in your employees, and for me this has been essential for being able to carry out the work upon which this thesis rests. Your open-mindedness and truly academic approach, the way you promote your students, and how you unselfishly share your vast knowledge as well as extremely hard earned data is nothing but inspirational.

I am also most grateful to my co-supervisors professor Per Magne Ueland and PhD Eva Ringdal Pedersen for their time and effort spent in both critically reviewing my manuscripts and sharing their expertise within the fields of biochemistry, epidemiology and science in general. To be given the chance to work with international top scientists, I cannot be anything but humble. Per, I know PhD supervision may feel as a slow-going phlebotomy to you, but I hope I have not left you critically anaemic.

Further, I thank all my co-authors for their contributions in all aspects of the scientific work, not least because of their hard effort with the WECAC and NORVIT databases, the latter being chaired by professor Kaare Harald Bønaa at the University of Tromsø. A special tribute goes to professor Grethe Tell at the Department of Global

ABSTRACT

BACKGROUND

The choline oxidation pathway comprises the sequential metabolism of choline into betaine, dimethylglycine (DMG), and sarcosine. In addition, dietary choline and betaine can be turned into trimethylamine *N*-amino oxide (TMAO). Alterations in choline metabolism may relate to cardiovascular disease (CVD) and type 2 diabetes (T2D). Several investigations have focused on systemic concentrations of choline and betaine; however large-scale prospective data are scarce. There is therefore a need for more comprehensive assessments of choline metabolites in relation to incident CVD, T2D and mortality, in addition to investigating any potential benefit in risk prediction from such biomarkers.

AIM

We carried out observational cohort studies of the prospective relationships between plasma DMG and incident acute myocardial infarction (AMI) and mortality, as well as the association between systemic and urinary choline metabolites with the risk of incident T2D. The biomarkers' impact on model discrimination and reclassification of patients at risk was also assessed, as were their test-retest reliabilities and temporal trends according to B-vitamin treatment.

MATERIALS AND METHODS

Analyses on the association between plasma DMG and incident AMI, as well as the relationships between choline metabolites and incident T2D were performed among patients evaluated for suspected stable angina pectoris (SAP). The risk assessment between plasma DMG and mortality included SAP patients and patients with AMI from an independent cohort. Clinical endpoint data were obtained from regional and national health registries. Endpoint analyses on incident AMI and mortality were carried out by Cox regression, whereas analyses on incident T2D were performed by logistic regression. Model discrimination and reclassification were explored by calculating the C-statistics, the integrated discrimination index (IDI), and the

continuous net reclassification improvement (NRI>0), respectively. Mixed linear modelling was used for assessing temporal trends in metabolite concentrations.

RESULTS

Higher plasma DMG was associated with several traditional risk factors for coronary heart disease (CHD). After about four and a half years of follow-up, plasma DMG showed linear relationships with incident AMI among 4154 patients with suspected SAP (age, gender and fasting adjusted hazard ratio (HR) for the fourth vs. first quartile (95% confidence intervals (CI)) 1.95 (1.42-2.68); P<0.001). The relationship between plasma DMG and incident AMI was particularly strong among non-smokers and patients with lower serum apoB and triglyceride levels (P for interaction≤0.03).

Among essentially the same patients, as well as among 3733 patients with AMI who were followed for 7 years, higher plasma DMG was also associated with increased risk of all-cause mortality (age and gender adjusted HRs (95% CIs) for the fourth vs. the first quartile 1.72 (1.21–2.46) and 1.76 (1.42–2.18) among SAP and AMI patients, respectively) and CVD mortality (HRs (95% CIs) 1.94 (1.21–3.11) and 1.97 (1.50–2.59) among SAP and AMI patients, respectively). The associations were only slightly attenuated when adjusting for established CHD risk factors, to which adding plasma DMG also improved risk prediction for both AMI and all-cause mortality. Moreover, plasma DMG showed good to excellent within-person reproducibility throughout repeated measurements among patients not receiving supplementation with folic acid + vitamin B12.

In general, higher plasma choline and lower plasma betaine and serum sarcosine levels were associated with an adverse risk profile of T2D. In urine, most choline metabolites were positively related to an adverse diabetes risk profile. After an average follow-up of 7.5 years, 233 (6.4%) out of 3621 non-diabetic patients were registered with new-onset T2D. Incident T2D was strongly inversely associated with plasma betaine and positively related to urine betaine, DMG and sarcosine (age, gender and fasting adjusted odds ratios (95% CIs) per 1 SD increment 0.72 (0.62-0.83), 1.25 (1.09-1.43), 1.22 (1.06-1.40), and 1.30 (1.13-1.49), respectively). We did not find any relationship between choline or TMAO and incident T2D. The estimates were not

materially altered when adjusting for a range of traditional T2D risk factors and potential confounders, and were similar in sensitivity analyses. Among the choline metabolites associated with new-onset T2D in univariate analyses, plasma betaine and urine sarcosine were most strongly related to incident T2D, and both indices also enhanced risk prediction when added to the multivariate model.

After 1 year, as compared to placebo treatment, supplementation with folic acid + vitamin B12 lowered plasma DMG and sarcosine, but increased plasma betaine and choline. No alterations in plasma TMAO were observed. In urine, we observed similar responses to supplementation as to those seen in blood.

CONCLUSION AND IMPLICATIONS

Among patients with suspected or verified SAP, high plasma DMG was related to increased risk of AMI, as well as all-cause and CVD mortality, the latter endoints being validated among patients with AMI. Moreover, plasma DMG improved risk prediction of both AMI and mortality.

Lower plasma betaine and higher urine betaine, DMG and sarcosine concentrations were related to incident T2D. Plasma betaine and urine sarcosine also improved reclassification of patients at risk. Plasma DMG and betaine, as well as urine sarcosine showed good to excellent within-subject reproducibility among patients not supplemented with folic acid + vitamin B12, justifying one-time assessment of biomarker status.

Our observational findings suggest novel pathophysiological pathways involved in conditions heavily impacting the global burden of disease, warranting more research into the field of one-carbon and choline metabolism in relation to life-style related diseases.

LIST OF PUBLICATIONS

The current thesis is based on the following original articles published in international peer-review journals and referred to by their roman numerals:

PAPER I

Svingen GF, Ueland PM, Pedersen EK, Schartum-Hansen H, Seifert R, Ebbing M, Løland KH, Tell GS, Nygård O. Plasma dimethylglycine and risk of incident acute myocardial infarction in patients with stable angina pectoris. Arterioscler Thromb Vasc Biol. 2013;33(8):2041-8. (Appendix I)

PAPER II

Svingen GFT, Schartum-Hansen H, Ueland PM, Pedersen EP, Seifert R, Ebbing M, Bønaa KH, Mellgren G, Nilsen DWT, Nordrehaug JE, Øyen J, and Nygård O. Elevated plasma dimethylglycine is a risk marker of mortality in patients with coronary heart disease. Eur J Prev Cardiol 2015;22(6):743-52. (Appendix II)

PAPER III

Svingen GFT, Schartum-Hansen H, Pedersen EKR, Ueland PM, Tell GS, Mellgren G, Njølstad PR, Strand E, Karlsson T, Seifert R, Nygård O. The prospective associations of systemic and urinary choline metabolites with incident type 2 diabetes. Submitted, as per January 2016. (Appendix III)

TABLE OF CONTENTS

1   INTRODUCTION

1.1   CARDIOVASCULAR DISEASE

1.1.1   DEFINITION, EPIDEMIOLOGY AND IMPLICATIONS

Cardiovascular disease (CVD) comprises all diseases of the circulatory system (i.e. heart and blood vessels). CVD related mortality has dropped in several countries, including Norway (Figure 1), during the last decades,[1] and this decline has largely been attributed to the identification and improvement of CVD risk factors.[2] Yet, CVD is still the number one cause of mortality globally, accounting for about one third of all deaths,[1] and ischemic heart disease (IHD) is considered the main cause of years lost due to illness and death.[3] In Europe, about 4 out of every 10 deaths before the age of 75 years are due to CVD.[4] Accordingly, in 2012, 13 010 and 4 852 deaths out of totally 41 913 deaths in Norway were attributed to CVD (International Classification of Diseases (ICD) 10 codes I00-I99) and IHD (ICD 10 codes I20-25), respectively, emphasizing CVD as the number one cause of death nationwide.[5]
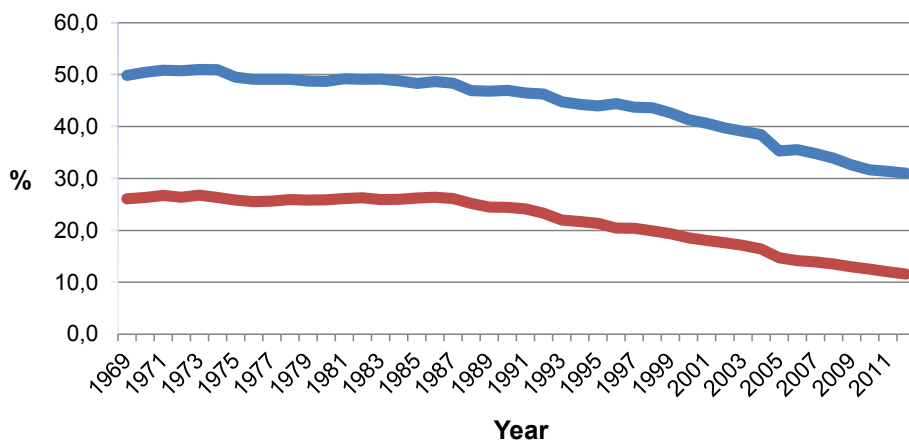
Figure 1. Death from cardiovascular disease (blue line) and ischemic heart disease (red line) as percentages of death from all causes in Norway, during the period 1969-2012.
Data obtained from Statistics Norway.[5]

Most incident CVD events do not immediately cause death; hence CVD has a major impact on overall morbidity. Reliable data on the occurrence of non-lethal CVD

among individuals in Norway has only recently emerged via the Norwegian Cardiovascular Disease Registry (NCDR),[6] contrasting other Scandinavian countries which have had similar national registries for many years.[7, 8] On the other hand, The Western Norway Cardiovascular Registry (WENOCARD; http://cvdnor.b.uib.no/wenocard) has served as a precursor of the NCDR, by recording regional data on CVD hospitalizations in Western Norway public hospitals from 1972 and throughout 2006.[9] Likewise, the Cardiovascular Disease in Norway (CVDNOR; https://cvdnor.b.uib.no/) project collected data on prevalent and incident CVD, as well as diabetes mellitus, from 42 Norwegian public hospitals in the period 1994-2009.

According to the NCDR, approximately 330 000 patients received at least one CVD diagnosis at Norwegian public hospitals in 2013, and almost 60% had incident disease.[6] About 15 000 cases of AMI (ICD-10 I210-I229) were registered and over 23 000 admissions to public hospitals and out-patient clinics were classified as due to angina pectoris (ICD-10 I200-I209). These data correspond to previous reports from regional Norwegian surveys, indicating that the prevalence of non-fatal CVD was approximately 9% among subjects older than 40 years, and mostly related to IHD.[10]

Apart from individual human repercussions, CVD also has a major impact on societal health expenditures and lost productivity. According to a recent report from the European Heart Network and the European Society of Cardiology, the annual overall economic cost from CVD in Europe as per 2009 was estimated to approximately €196 billion. Of these expenses, IHD constitutes about 1/3, with over 60% being related to direct health care expenditures and costs related to lost productivity.[11]

1.1.2 PATHOPHYSIOLOGY AND CLINICAL PRESENTATION OF IHD

Most cases of IHD are caused by atherosclerosis in the coronary arteries (i.e. coronary artery disease (CAD)). The pathophysiological mechanisms involve deposition of apolipoprotein (apo) B and cholesterol containing low-density lipoproteins (LDLs) inside the vessel wall at one or several locations in the coronary artery tree. This is accompanied by low-grade cellular inflammation, smooth muscle-cell proliferation, and fibrosis, forming an atherosclerotic plaque. The plaque may grow both outwards

(positive remodeling) and into the vessel lumen (negative remodeling). An inward growing plaque can potentially obliterate blood flow, giving rise to clinical features of coronary heart disease (CHD) on physical or emotional exertion. Such lesions can be visualized by coronary angiography and quantitatively graded according to luminal obstruction. Symptoms of CHD may vary, but typically include chest pain (i.e. angina pectoris), and result from insufficient blood supply to the distal myocardium to accommodate increased need of oxygen (i.e. ischemia).[12] In patients with stable angina pectoris (SAP) due to reversible ischemia, symptoms will typically arise on a certain level of physical or psychological exertion, and relieved once the precipitating factor is removed or when taking medications that lessen myocardial oxygen expenditure. However, coronary blood flow may be obliterated, either gradually by a growing plaque itself, or abruptly by a thrombus generated on the plaque surface (i.e. atherothrombosis), to such a degree that myocardial cell death occurs distal to the lesion. Myocardial necrosis due to ischemia is the very definition of an acute myocardial infarction (AMI),[13] and puts the patient at risk of both immediate and long-term complications, such as systolic heart failure and cardiac arrest. It is noteworthy, however, that even the presence of non-obstructing coronary atherosclerotic lesions carries a significant risk of atherothrombotic events.[14]

The Framingham Heart Studies have established high age, male gender, dyslipidemia, smoking and hypertension to predict CVD events with an accuracy of approximately 80% among presumably CVD naïve subjects.[15] Although causality between established risk markers and endpoints has been heavily debated, the hypothesis of LDL-cholesterol (LDL-C) deposition in atheromatosis has been strengthened by the favorable results on CVD events and mortality from LDL-C lowering therapy by statins.[16] Statins act primarily by inhibiting the rate-limiting step of the endogenous cholesterol synthesis, thereby enhancing the hepatic clearance of circulating LDLs by increasing the amount of LDL receptors on surface of the hepatocytes.[17] Furthermore, intravascular ultrasound studies have suggested reductions in focal atherosclerotic plaque volume, as well as altered plaque composition by intensive statin treatment,[18] indicating that statin therapy might affect the atherosclerotic plaque more directly. This highlights that atherosclerosis is dynamic,

and that reverse cholesterol transport, as facilitated by high-density lipoproteins (HDLs), might also be influenced by statin treatment. Although some argues against their alleged pleiotropic effects,[19] statins may have beneficial effects on CVD beyond those of lipid modification.[20] On the other hand, statins do not eliminate atherosclerosis, as an incident major cardiovascular event will still occur in a substantial proportion of patients who receive statin therapy.[16] This residual risk may be because of inadequate improvement of lipid status,[21] but also due to the presence of other known[21] and unknown factors influencing risk of atherothrombosis.

Taken together, CVD in general, and CHD in particular, are major determinants of morbidity and death, with huge individual and socioeconomic costs. Intense focus on preventive measures has likely reduced the burden of disease; however, there is still considerable residual risk, warranting research into novel pathophysiological pathways.

1.2   DIABETES MELLITUS

1.2.1 DEFINITION, EPIDEMIOLOGY AND IMPLICATIONS

The term diabetes mellitus comprises diseases characterized by hyperglycemia. Although highly simplified, diabetes can generally be divided into states of either a primary and absolute insulin deficiency (type 1 diabetes), or relative insulin insufficiency due to insulin resistance and pancreatic β-cell dysfunction (type 2 diabetes (T2D)), as well as gestational diabetes and rarer etiologies primarily arising from disorders of the pancreas.[22] The current thesis will mainly deal with T2D.

Diabetes is on the rise worldwide, and the International Diabetes Federation has estimated the global prevalence among adults to 8.3% (382 million people) in 2013, with an expected rise of more than 50% in the next 25 years. Most prevalent and incident cases are due to T2D, and the increase will likely have a particularly strong impact on younger people in developing countries.[23]

The Norwegian Diabetes Register estimates the prevalence of diabetes among adults in Norway to be approximately 4%,[24] which is comparable to our neighboring Scandinavian countries.[25, 26] Accordingly, the Norwegian Prescription Database reported that 170 000 Norwegian citizens used antidiabetic drugs (The Anatomical

Therapeutic Chemical (ATC) classification system and the Defined Daily Dose A10) in 2014,[27] constituting about 3% of the current total Norwegian population. In addition, several patients with T2D do not receive pharmacological antidiabetic treatment, and the International Diabetes Federation states that almost half of all prevalent cases of T2D in the world are likely not diagnosed.[23] Hence, the real prevalence of T2D in Norway is almost certainly much higher than officially claimed.

According to the Global Burden of Disease project, diabetes ranks 14 in terms of causing disability-adjusted life years worldwide,[3] and diabetes was estimated to account for about 11% of total global health care expenditures in 2013.[23] In Norway, a diagnosis of diabetes was registered in 2.5% of the consultations in general practice in 2014,[28] and 2.4% of the admissions to public somatic hospitals and out-patient clinic visits in 2014.[29] In addition, diabetes may be the underlying cause or accompanying condition of a range of other illnesses, and in particular CVD.[23] Diabetes therefore has a huge impact on individuals, public health care and the society as a whole, and motivates research into novel pathophysiological mechanisms in order to prevent disease development and its complications.

1.2.2 PATHOPHYSIOLOGY AND CLINICAL PRESENTATION OF T2D

The pathophysiology of T2D is complex and has yet to be fully delineated; however the main focus has been on insulin resistance, which is defined as the need of supernormal insulin excretion from pancreatic β-cells to keep blood sugar levels within the normal range,[30] and being strongly linked to increasing bodyweight and physical inactivity.[31] However, the β-cells may eventually be unable to compensate for the insulin resistant state, hence hyperglycemia ensues.[31] T2D pathophysiology is also associated with factors other than insulin resistance and β-cell failure per se, such as impaired pancreatic α-cell function, genetic and epigenetic regulation, factors related to the diet and the gastrointestinal system, lipotoxicity, and alterations in the kidneys, adipose tissue and the nervous system.[31, 32]

The clinical presentation of diabetes varies and symptoms may have slow onset. Typical symptoms of hyperglycemia include polydipsia and blurred vision, whereas the inability to utilize glucose as an energy source may lead to fatigue and weight

loss;[33] however, many patients with T2D have little or no symptoms, contributing to the delay of diagnosis.

1.2.3 T2D AND CHD ARE STRONGLY RELATED

The clinical implications of T2D are severe, and excessive mortality is closely related to CVD,[34] and in particular increased risk of CHD.[35] T2D and atherosclerotic CVD (ACVD) seem to act additively in terms of adverse prognosis, and T2D may be regarded as a CHD and stroke equivalent.[36] Conversely, patients diagnosed with CHD are at higher risk of developing T2D, but it is not known whether this association is explained by already impaired glucose homeostasis inherent to CHD in the first place.[37]

T2D is further linked to atherosclerosis by multiple common risk factors, which may be related to insulin resistance and hyperinsulinemia,[31, 38] but could also involve other elements, such as alterations in amino acid metabolism.[39, 40] In addition, several pharmacological treatment options for CVD (i.e. beta-blockers, thiazides, niacin and statins) are associated with adverse glucose control, as well as increased risk of incident T2D,[41] and the use of several glucose lowering drugs (including thiazolidinediones and sulfonylureas) have been associated with higher cardiovascular risk.[42, 43]

The close associations between T2D and CVD suggest that these two non-communicable diseases may not only share some common soil,[44] but also have complementary relationships yet to be resolved. Furthermore, although the diabetes epidemic has been suggested to curtail the steadily declining CHD mortality rates observed during recent decades,[2] there are evidence that increasing rates of obesity and diabetes have not proportionately impacted CVD mortality rates.[45] Although this discrepancy may be influenced by methodological shortcomings,[45] actions to identify specific diabetic phenotypes particularly prone to CHD seem pertinent.

1.3   THE CHOLINE OXIDATION PATHWAY

1.3.1   A BRIEF OVERVIEW

Choline is a quaternary ammonium compound which probably enters the mitochondrion via a specific carrier-mediated transport mechanism.[46] It is metabolized into betaine via a two-step reaction, catalyzed by the enzymes choline dehydrogenase (CHDH) and betaine aldehyde dehydrogenase (BADH), respectively (Figure 2).[47] Betaine diffuses out into the cell cytosol, and is demethylated into dimethylglycine (DMG), as it donates one methyl group to homocysteine (Hcy), which is converted into methionine. This irreversible reaction is catalyzed by the enzyme betaine-homocysteine-*S*-methyl transferase (BHMT).[48] DMG then enters the mitochondrion and is demethylated into sarcosine and glycine via the enzymes dimethylglycine dehydrogenase (DDH) and sarcosine dehydrogenase (SDH), respectively, with tetrahydrofolate (THF) being the methyl group acceptor in both reactions. Whereas DMG can only be produced from betaine, sarcosine may also be obtained by the methylation of glycine in the cell cytosol, catalyzed by glycine-*N*-methyltransferase (GNMT).[49]

1.3.2   CHOLINE AND BETAINE CAN BE PRODUCED ENDOGENOUSLY OR OBTAINED FROM THE DIET

In the human body most choline is present as the phospholipid phosphatidylcholine (PC),[47] which can be converted to free choline by phospholipases.[50] PC production takes place in the liver mainly from choline via the three-step Kennedy (CDP) pathway, or alternatively from phosphatidylethanolamine (PE) by the enzyme phosphatidylethanolamine *N*-methyltransferase (PEMT);[51] however this endogenous production is insufficient for biological choline demands,[47] making choline an essential nutrient. According to the US Institute of Medicine, the average recommended daily choline intake is 550 mg and 425 mg/day for men and women, respectively,[52] but the intake varies extensively, with certain populations probably having insufficient intakes.[53] Nordic authorities have not specifically implemented dietary choline intake in their current nutrition guidelines.[54] Among dietary factors explored in a Norwegian general population sample, only eggs and cholesterol intake

significantly predicted plasma choline, although various other food items are rich in choline, as well.[55]
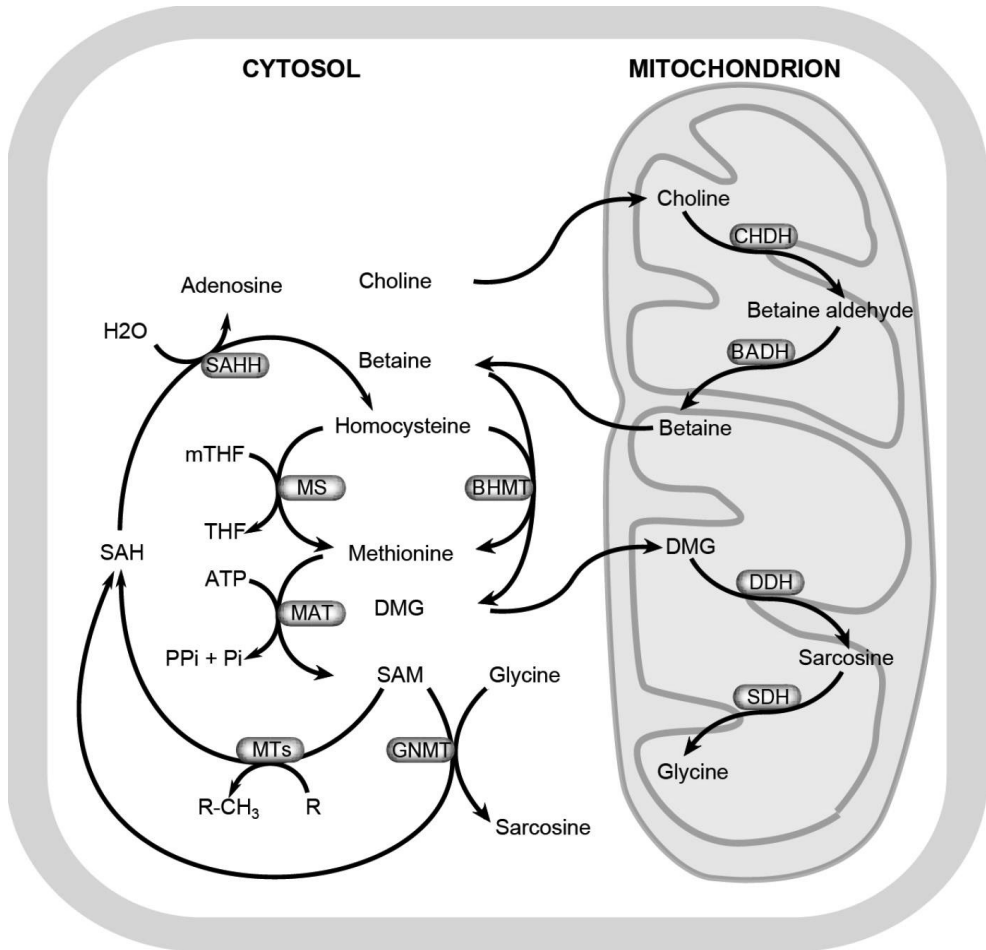
Figure 2. The coinciding choline and methionine cycle pathways.
BADH, betaine aldehyde dehydrogenase; BHMT, betaine-homocysteine-S-methyltransferase; CHDH, choline dehydrogenase; DDH, dimethylglycine dehydrogenase; DMG, dimethylglycine; GNMT, glycine-N-methyltransferase; MAT, methionine adenosyltransferase; MS, methionine synthase; MTs, various methyltransferases; SAH, S-adenosylhomocysteine; SAHH, S-adenosylhomocysteine hydrolase; SAM, S-adenosylmethionine.

Betaine may also be obtained directly through the diet, and plasma betaine has shown strong positive relations with the intake of high-fiber bread,[55] a common food

item in Nordic countries.[56] However, no recommendations on betaine intake currently exist. Besides, no population surveys on either choline or betaine intake have hitherto been carried out in the Nordic countries, thus representative intake and status of choline and related metabolites are largely unknown for subjects immediately comparable to those investigated in this thesis.

1.3.3   RAMIFICATIONS OF CHOLINE METABOLISM

Apart from serving as the source of downstream metabolites, choline is important for the synthesis of neurotransmitters, and PC is crucial in the assembly of cell membranes and lipoproteins,[47] as well as the production of bile.[50] Hence, choline species are found in various tissues, but downstream metabolism of free choline seem most abundant in the liver and the kidney,[47, 50, 57] which also have highest activities of both CHDH[58] and BADH.[59, 60]

CHDH is located to the inner mitochondrial membrane, and in humans the greatest CHDH activity has actually been found in the kidney, followed by the liver.[58] Moreover, CHDH uses flavin adenine dinucleotide (FAD) as a cofactor, and the electron is transferred to the respiratory chain via coenzyme Q10.[58]

BADH is located both in the cell cytosol and inside the mitochondrial matrix.[59] It uses NAD+ (and NADP+) as a cofactor in the irreversible oxidation of betaine aldehyde,[61] and is inhibited by its substrate, betaine aldehyde. BADH is found in several different tissues[47, 62], but betaine is mainly found in the liver and kidney of mammals.[57] This probably reflects the role of betaine not only as a methyl donor, but also as a regulator of cell volume (i.e. as an osmolyte), which is considered of particular importance in the extremely hypertonic environment of the renal cortex. [47, 57]

As expected, the main tissue distributions are similar for betaine as for BHMT, a Zn-containing enzyme which makes up approximately 0.6-1.6% of the total hepatic protein content,[48] the vast amount highlighting the important role for betaine in liver metabolism. The BHMT pathway is involved in regulating levels of methionine and the non-protein amino acid, homocysteine (Hcy). It therefore tightly links betaine to folate metabolism, since methionine also can be formed via folate- and cobalamin-dependent remethylation of Hcy by methionine synthase (MS). Methionine is

important in protein synthesis, but also for the production of the ubiquitous methyl donor *S*-adenosylmethionine (SAM). SAM is necessary for a whole range of transmethylation reactions, including the production of creatine, PC and polyamines, as well as in DNA methylation and epigenetic regulation,[63] the latter referring to the modification of genetic expression without altering the genomic sequence per se.[64] The demethylation of SAM yields *S*-adenosylhomocysteine (SAH), which can be further hydrolyzed into Hcy, hence completing the so-called methionine cycle. Accordingly, the BHMT and MS pathways are complementary to each other. Although the latter pathway traditionally has received the greater attention regarding Hcy remethylation, BHMT might be more important in regulating Hcy levels than previously appreciated.[48,65] The significance of the BHMT pathway in terms of methylation status has been emphasized by in vitro and in vivo studies where impaired BHMT flux leads to lower hepatic SAM levels.[65-67]

The relationship between the MS and the BHMT pathways is further illustrated by several studies suggesting that BHMT flux seems to be inversely related to folate status.[48, 68] BHMT activity is also inhibited by DMG and SAM, and insulin, growth hormone, thyroxin and testosterone, as well as hypertonicity may reduce BHMT activity and/or transcription.[48] Methionine also inhibits BHMT,[69] and it follows that BHMT is up-regulated when dietary intake of methionine is low and intake of labile methyl groups is high.[70, 71] However, dietary methionine excess may also up-regulate BHMT,[72] reflecting dual roles of the BHMT pathway in terms of methionine and Hcy homeostasis.[73] In contrast, increased intakes of choline and betaine up-regulate BHMT flux, as do diabetes,[74, 75] as well as treatment with glucocorticoids.[48]

Hcy is degraded via the vitamin B6-dependent transsulfuration pathway, which is initiated by the condensation of Hcy and serine, catalyzed by cystathionine-β-synthase (CBS). The transsulfuration pathway is essential for the making of cystathionine and cysteine, being pivotal in the production of the intracellular antioxidant glutathione, as well as for taurine and protein synthesis.[69] It follows that inborn errors of CBS lead to Hcy accumulation, and traditionally, betaine has been used in the treatment of such patients.[76] Not only does betaine supplementation increase the remethylation of Hcy into methionine, but increased BHMT flux also

stimulates the transsulfuration pathway,[77] most probably due to increased SAM levels which activate CBS.[69]

DDH and SDH are vitamin B2-dependent enzymes[78], and assumed to have tight connections with the mitochondrial respiratory chain, which utilizes the electrons provided during DMG and sarcosine catabolism.[79] Methyl groups from DMG and sarcosine are transferred to THF, forming 5, 10-methylenetetrahydrofolate. Hence, folate availability may influence the metabolism of DMG and sarcosine, although experimental studies suggest that folate status does not determine the passage of electrons to the electron transfer proteins.[80] Besides generating methyl groups and electrons, DMG has no other known direct metabolic role, although DMG supplementation has been associated with altered immune responses,[81-83] as well as having potentially favorable effects on glucose and lipid homeostasis, oxidative stress, and lung vasculature in poultry.[84] Sarcosine may be a scavenger of excess methyl groups via increased GNMT flux, in particular among patients with impaired SDH.[85] Further, demethylation of sarcosine via SDH yields glycine, which is involved in glucose metabolism, the synthesis of nucleotides, proteins and antioxidants, as well as in cellular signaling.[86]

The concentrations of DMG and sarcosine in tissues other than the liver and the kidneys have been investigated only to a limited degree, as most studies have reported on their blood or urinary levels. DMG was found in rectal cancer cells,[87] and sarcosine has been reported in prostate cancer cells[49] and in the rat cerebral cortex.[88] Nonetheless, studies in animals and/or humans indirectly suggest the presence of DMG and/or sarcosine also in various other tissues, due to their contents of DDH and/or SDH and/or GNMT (Table 1). Since DMG can only be obtained via the BHMT reaction, tissues containing DDH, but not BHMT, may therefore depend upon the import of DMG.

Determinants of circulating DMG and sarcosine concentrations have not been extensively investigated, but DMG levels increase according to higher intake of metabolic precursors and especially betaine,[89, 90] as well as in patients with chronic renal disease.[91] The minor A allele on the single nucleotide polymorphism (SNP)

BHMT 742 G>A (R239Q),[92] is inversely related to plasma DMG,[93] although not associated with plasma total Hcy (tHcy) levels.[92] Polymorphisms of the *sdh* gene influence plasma DMG levels as well,[94] and even genetic traits coding for impaired

Table 1. Tissue specificity of enzymes taking part in downstream choline metabolism

Enzyme		Tissue	Species
CDH	*Protein*	Liver and kidney[58]	Human
BADH	*Protein*	Liver[95]	Rat
		Kidney[96]	Pig
BHMT	*Protein*	Liver, kidney[48]	Human
		Pancreas[48]	Sheep
		Optical lens[48]	Rhesus monkey
DDH	*mRNA*	Liver, kidney, heart, brain, lung[97]	Rat
	Protein	Liver, kidney, lung, heart, spleen, brain[97]	Rat
SDH	*mRNA*	Liver, kidney, lung, thymus, oviduct, prostate, seminal vesicle, heart and brain.[98]	Rat
	Protein	Breast cancer tissue[99]	Human
		Liver, pancreas and adrenal gland.[98]	Rat
GNMT	*Protein*	Liver, kidney, pancreas, salivary gland, jejunum[100]	Various mammals, mainly rats.

BADH, betaine aldehyde dehydrogenase; BHMT, betaine homocysteine *S*-methyltransferase; CHDH, choline dehydrogenase; DDH, dimethylglycine dehydrogeanse; GNMT, glycine *N*-methyltransferase; SDH, sarcosine dehydrogenase.

methylenetetrahydrofolate dehydrogenase (MTHFD1) activity are associated with higher hepatic and plasma DMG concentrations, possibly due to a greater demand for one-carbon units obtained through the choline oxidation pathway.[101]

1.3.4 CHOLINE AND BETAINE METABOLISM IN THE GUT
– THE FORMATION OF TRIMETHYLAMINE-*N*-OXIDE
Dietary choline, and to a lesser extent betaine, may be transformed into trimethylamine (TMA) by bacteria in the gut, and TMA can be further oxidized into trimethylamine *N*-monoxide (TMAO) by hepatic flavine monoxidase 3 (FMO3).[102] Systemic TMAO levels may therefore reflect choline intake, and the gut microbiome might also be regarded as a choline and betaine metabolizing organ.

1.3.5 CHOLINE AND BETAINE IN RELATION TO HEPATIC LIPID METABOLISM

Both choline and betaine are involved in hepatic lipid metabolism, and especially in the export of lipids from the liver. Not only is free choline used in the production of PC, but PEMT uses three molecules of SAM in the alternative production of PC from PE, thus highlighting the need of adequate methylation status for this reaction to occur. The BHMT pathway may also be more directly related to hepatic lipid metabolism as suggested by specific concomitant genetic transcriptions of hepatic *bhmt* and *apob* mRNA.[103] Accordingly, BHMT induction by betaine supplementation resulted in increased production and secretion of VLDL from the liver; however systemic apoB and triglyceride levels were not affected. [103] This was possibly due to increased hepatic clearance via the LDL receptor,[103] suggesting an overall increased transport of apoB containing lipoproteins and cholesterol between the liver and extrahepatic tissues. In humans high-dose betaine supplementation has been associated with a relative increase in serum LDL-cholesterol, and supplementation with both choline and betaine modestly increases serum triglycerides.[104] Accordingly, increased availability of choline and betaine has been inversely related to non-alcoholic fatty liver disease (NAFLD),[47] which is strongly associated with T2D and considered an independent CVD risk factor.[105]

1.3.6 HOMOCYSTEINE AS A CARDIOVASCULAR RISK FACTOR – A LONG STORY MADE SHORT

Since the last half of the 20th century there has been a great focus on the strong relationship between high levels of tHcy (the sum of all Hcy species) in blood and urine, and risk of occlusive CVD (Figure 3). The associations are based upon firm data from numerous epidemiological and experimental studies, suggesting hyperhomocysteinemia to be a causal risk factor for ACVD beyond those identified by the Framingham Heart Studies.[106] Moreover, the solution to the problem seemed straightforward, as well as inexpensive and tolerable, because the administration of folic acid and/or vitamin B6 lowers circulating tHcy by increasing Hcy remethylation or degradation. Hence, several trials with such therapy in order to reduce CVD risk

were launched in the late 1990s and early 2000s; however, despite substantial tHcy lowering effects by the interventions, no improvement in prognosis were observed,[107] nor did later Mendelian randomization studies immediately support elevated tHcy as a causal risk factor for ACVD.[108, 109] In fact, treatment with folic acid and vitamin B12 in the WENBIT was associated with accelerated growth of atherosclerotic plaques.[110] Consequently, the limelight has faded on the "homocysteine theory" (Figure 3), indicating that increased plasma tHcy is merely an epiphenomenon of other pathways responsible for increased CVD risk.
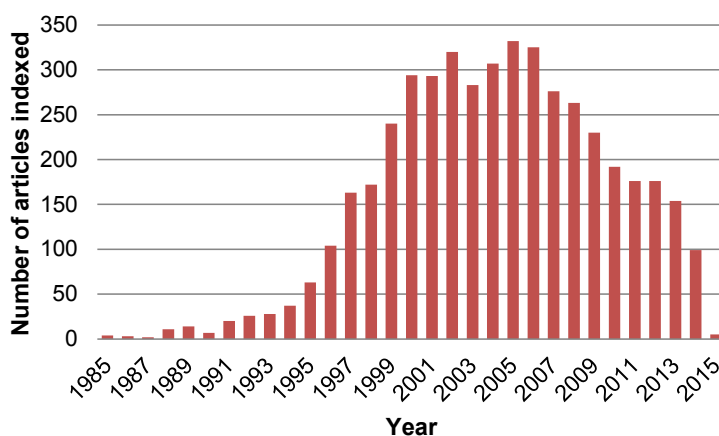
Figure 3. Yearly number of articles indexed in the Pubmed/Medline database by the medical subheadings "homocysteine" + "cardiovascular disease" during the last 30 years. Data obtained from www.pubmed.com

1.3.7   CHOLINE METABOLISM IN RELATION TO CVD AND DIABETES

Small population studies have related higher systemic choline levels to adverse cardiovascular risk, and even suggested that choline in whole blood and serum may yield incremental prognostic information beyond that conferred by conventional risk markers among patients with suspected acute coronary syndrome (ACS; i.e. either unstable angina pectoris or AMI).[111] Accordingly, observational data from a Norwegian general population sample showed that high plasma choline and low betaine were related to an adverse CVD and T2D risk profile, including high BMI,

elevated blood pressure and serum triglycerides, and low serum high-density lipoprotein-cholesterol (HDL-C).[112] On the other hand, a study among about 500 patients with a recent ACS found U-shaped associations between plasma and urine betaine with risk of incident AMI and hospitalizations for heart failure, respectively.[113] Notably, the same study also reported as a secondary finding that increased plasma DMG levels were related to CVD events and mortality.[113] A small study found higher urine DMG, but not betaine, in patients with than without premature vascular disease,[114] and another reported the BHMT 742 G>A minor allele to be inversely related to the extent of angiographic CAD among elderly patients.[92] In summary, these observations indicate that higher circulating choline and DMG levels might reflect adverse CVD risk, whereas the risk relationships with betaine seem a bit more complex. In addition, a recent series of preclinical and human studies has suggested plasma TMAO to play a role in ACVD, and that reducing choline intake might be a means of risk reduction,[102, 115] despite earlier general population based studies reporting no associations between intakes of neither choline nor betaine and CVD risk.[116, 117]

Circulating choline levels according to diabetes status are not well established;[47] however a recent small cross-sectional study from China suggested lower serum choline and betaine concentrations in association with prevalent diabetes and diabetic complications.[118] An inverse association between systemic betaine and existing T2D has been supported by some,[118-120] but not all[57] studies, and experimental investigations have suggested that increased betaine availability may reduce adiposity and improve insulin resistance.[53] A recent Swedish study found robust, but relatively weak, associations between genetic traits coding for lower plasma DMG and increased risk of developing diabetes during long-term follow-up.[94] Among a US male general population lower serum sarcosine levels were observed among those with than without T2D.[121]

Higher dietary intakes of PC were related to increased risk of incident T2D,[122] and higher plasma TMAO levels have been observed among patients with than without T2D.[120, 123] This potentially links choline intake and the gut microbiome to T2D risk,

and the relationship is possibly also modified by the inhibiting actions of insulin on FMO3.[124]

Substantially higher urinary betaine levels have been observed among patients with T2D.[57] Our group showed that urine betaine was highly correlated with urine DMG and sarcosine, and that urine betaine predicted new-onset diabetes within the WENBIT follow-up period;[125] however, no longer-term prospective studies of urinary choline metabolites on incident T2D risk have hitherto been reported.

1.3.8 OPEN RESEARCH QUESTIONS

In summary, there are preclinical and epidemiological evidence of significant alterations in choline metabolism according to both CVD and T2D. However, most prospective epidemiological studies have so far focused on the CVD risk associated with systemic choline and betaine concentrations, in addition to circulating TMAO levels, and relatively little attention has been paid to relationships with DMG and sarcosine. Moreover, with the exception of betaine, any prospective relationships between choline metabolites in the urine and incident disease have yet to be investigated. There is therefore a need for a more comprehensive assessment of several choline metabolites in relation to CVD and T2D in large populations.

2   AIMS OF THE THESIS

The primary goals of the project were to investigate the relationship between plasma DMG levels and risk of incident AMI and mortality, as well as exploring potential associations between circulating and urinary choline metabolites with incident T2D. Secondary aims were to investigate potential improvements in risk prediction and to assess temporal trends of the biomarkers investigated.

SEPARATE PROJECT AIMS

2.1   Paper I: To investigate the association between plasma DMG and incident AMI among patients with suspected SAP.

2.2   Paper II: To explore the relationships between plasma DMG with all-cause, cardiovascular and non-cardiovascular mortality in two independent cohorts of patients with suspected SAP and AMI, respectively.

2.3   Paper III: To study the associations between systemic and urinary choline metabolites with incident T2D among patients with suspected SAP.

3 MATERIALS AND METHODS

3.1 PATIENT POPULATION

3.1.1 THE WENBIT, NORVIT AND WECAC COHORTS

The WENBIT (ClinicalTrials.gov Identifier: NCT00354081) and NORVIT (ClinicalTrials.gov Identifier: NCT00266487) were large, Norwegian, randomized, controlled trials initiated and carried out in the late 1990's and early 2000's to investigate the clinical effect of tHcy lowering therapy with folic acid + vitamin B12 and/or vitamin B6 against placebo in a 2x2 factorial design. The WENBIT[126] enrolled 3090 patients from the Western Norway, the majority (98.6%) having angiographically verified significant CAD. The patients were recruited from Stavanger and Haukeland University Hospitals in Stavanger and Bergen, Norway. During the study enrollment from April 1999 to April 2004, both hospitals carried out percutaneous coronary intervention (PCI) whereas only Haukeland University Hospital carried out open heart surgery. The multicenter NORVIT[127] studied 3749 patients hospitalized for an AMI in all Norwegian health regions, and enrolled patients from December 1998 to March 2002. The patients in the NORVIT were recruited from both local, regional and university hospitals, and did not routinely undergo coronary angiography, as is recommended by current guidelines.[128]

Participants in the NORVIT provided blood specimens at the baseline visit, and study visits after 1 year and at the end of study, whereas participants in the WENBIT provided blood samples at baseline, and at study visits after 1-3 months, 1 year and at the end of study. Most WENBIT study participants additionally provided urine samples at baseline and after 1 year of follow-up.

During the WENBIT study enrollment period, 2119 patients who underwent cardiac catheterization at Haukeland University Hospital, but who were not enrolled in the WENBIT trial, were included in an extended cohort of patients. These patients provided baseline blood and urine samples, and were followed up according to clinical endpoints, but did not attend later study visits. Together with WENBIT participants, these patients totaled 5209 patients, and made up the Western Norway Coronary Angiography Cohort (WECAC) (Figure 3).

In the current work, we chose to focus on WECAC patients with suspected or verified SAP (n=4164), thereby excluding 1045 patients with other indications for angiography (ACS, n=519; valvular disease, n=331; other indications, n=195). Our rationale was to study a rather homogenous group of patients, being less prone to changes in metabolites and biomarkers secondary to the acute phase (e.g. inflammation), as well as having less propensity to adapting life-style changes[129] which could potentially influence risk relationships between baseline values and outcome. Besides, WENBIT patients with ACS had their biosampling done partly before, during, or even after coronary angiography and intervention, without such information being registered in the database, making it possible that such procedures might have influenced biomarker status.

A total of 42 WECAC patients with suspected SAP rejected extended follow-up, and were thus censored in terms of endpoints beyond 31 Dec 2006. We did not have access to follow-up data on patients who moved out of Norway.

3.1.2 FOLLOW-UP AND CLINICAL ENDPOINTS

The WENBIT and the NORVIT cohorts were granted long-term follow-up on incident cardiovascular events, and all-cause, cardiovascular and non-cardiovascular mortality, extending the original trial durations. The rest of the patients making up the WECAC were granted inclusion in the combined WENBIT-NORVIT cohort after approval from the Regional Ethics Committee (Regional Ethics Approval number 2010/1880), which also approved the collection of information on incident diabetes from the CVDNOR project (Regional Ethics Approval number 2013/2324). The Regional Ethics Committee also specifically approved the projects included in the current work; "*DMG og coronarsykdom*" (Regional Ethics Approval number 2010/1881-8) and "*Betaine and choline excretion in urine*" (Regional Ethics Approval number 2010/1747-8).

All endpoints were linked to each patient's unique Norwegian 11-digit person identification number. Incident AMI (paper I) in the WECAC was classified according to American and European guidelines of 2000,[130] but also included patients diagnosed with either "sudden cardiac death" or "sudden death" (ICD-10 codes I46 and R96, respectively),[131] being in line with the definition used in previous Scandinavian

epidemiological surveys.[132] Endpoint information was obtained from the WENOCARD and validated by the WENBIT endpoint committee as previously described.[126] Procedure-related AMI was defined as those occurring within 24 hours after coronary revascularization, and were not included.

Mortality data in both the NORVIT and the WECAC cohorts (paper II) were obtained from the Death Registry at Statistics Norway (http://www.ssb.no). The information on incident AMI and mortality, including time to events, was collected throughout 31 December 2006.

Information on incident T2D was retrieved from the CVDNOR project, assessing public hospital discharge diagnoses ICD E110-E119 throughout 31 December 2009. These latter endpoints were not validated further, nor did we obtain reliable time-to-event data. This was mainly because we had access to detailed hospitalization data for only a limited number of patients. Moreover, for the majority of cases (~90 %), T2D was recorded as a secondary diagnosis and not the main reason for contacting the health care system.

3.1.3 LABORATORY ANALYSES

Baseline study sampling was carried out usually 1-3 days prior to the angiographic procedure among patients enrolled at Haukeland university hospital, whereas patients recruited from Stavanger university hospital usually had their biosampling done at the day of cardiac catheterization. Plasma samples were immediately prepared and stored in tubes either containing ethylenediaminetetraacetic acid (EDTA) or sodium citrate, whereas serum samples were stored in gel-containing tubes. Baseline urine samples were collected by the patients at home on the morning of angiography. Biosampling at later study visits was carried out at as described above, and the patients were not instructed to fast. All biosamples were frozen at -80°C until thawed and analyzed by laboratory staff blinded to the clinical outcomes of the patients. The results from analyses on plasma TMAO first became available during the spring of 2015, and
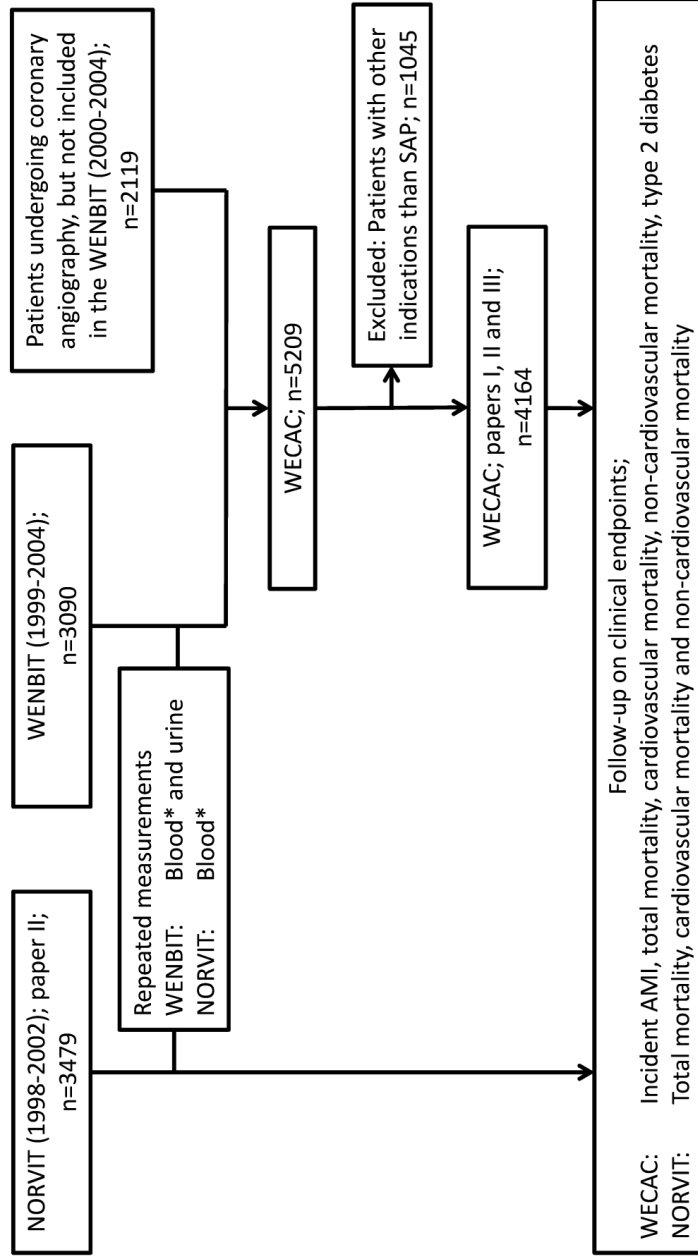
Figure 4. Flowchart depicting the inclusion and exclusion of study patients.
AMI indicates acute myocardial infarction; NORVIT; Norwegian Vitamin Trial; SAP, stable angina pectoris; WECAC, Western Norway Coronary Angiography Cohort; WENBIT; Western Norway B-Vitamin Intervention Trial.
*Plasma and serum samples

therefore we were not able to include this variable in papers I and II.

Routine biochemical analyses were carried out by the local laboratories in each recruiting hospital of the WECAC and NORVIT cohorts. Estimated glomerular filtration rate (eGFR) was calculated according to the Chronic Kidney Disease Epidemiology Collaboration formula.[133] Professor Rolf K. Berge and his research group at the University of Bergen analyzed serum apolipoprotein (apo) A1 and apo B 100 on the Hitachi 917 and 912 systems (Roche Diagnostics, GmbH, Mannheim, Germany), respectively. Study specific blood and urinary analyses were carried out by Bevital As, Bergen, Norway (http://www.bevital.no), using automated methods based on mass spectrometry for investigating large numbers of samples, but requiring small individual sample volumes (Table 2).[134] Urine choline metabolites were measured by a modified liquid chromatography-tandem mass spectrometry (LC-MS/MS) method.[135]

Table 2. Laboratory assays used for analyses of metabolites in plasma/serum and urine

Metabolite	Method
Plasma	
choline	LC-MS/MS[135, 136]
betaine	LC-MS/MS[135, 136]
dimethylglycine	LC-MS/MS[135, 136]
trimethylamine-*N*-monoxide	LC-MS/MS
total homocysteine	GC-MS[137]
riboflavin	LC-MS/MS[138]
5'-pyridoxal phosphate	LC-MS/MS[138]
Serum	
sarcosine	GC-MS[134]
folate	Microbiological assay[139]
cobalamin	Microbiological assay[140]
Urine	
choline	LC-MS/MS[135]
betaine	LC-MS/MS[135]
dimethylglycine	LC-MS/MS[135]
sarcosine	GC-MS/MS[141]

DMG indicates dimethylglycine; GC-MS/MS, gas chromatography mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry.

Serum in citrate tubes had to be used instead of EDTA plasma when assessing sarcosine status in blood, as the EDTA tubes already contained a significant amount of sarcosine.[142] The Bevital laboratory also measured serum C-reactive protein (CRP) by an ultrasensitive immuno- matrix-assisted laser desorption/ionization (MALDI)-MS method, with a detection limit of 0.17 mg/L.[143] Data on serum CRP, lipid fractions, plasma glucose and HbA1c were only available in the WECAC.

The BHMT single nucleotide polymorphism (SNP) BHMT 742 G>A,[144] as well as levels of glycated hemoglobin (HbA1c)[145] were assessed from EDTA whole blood samples, using matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry.

In a subset of 1343 WENBIT participants, of whom the majority were fasting, we also obtained baseline serum insulin and C-peptide from citrate samples, using a solid phase, two-site chemiluminescent immunometric assay (Immulite 2000) from Siemens Healthcare Diagnostics. However, since EDTA plasma is usually used for insulin measurements, and the amount of citrate within each tube slightly varied, we compared EDTA and citrate samples from 40 patients, and found that the insulin concentrations were highly correlated (Spearman's rho 0.95; P<0.0001) (Figure 5); hence, the data obtained from citrate samples was extrapolated into those of EDTA plasma by linear mean regression modelling using a regression coefficient (β) of 1.494. Among fasting, non-diabetic patients with valid insulin and plasma glucose measurements, we calculated the computer-based homeostatic model assessment (HOMA2)[146] of insulin resistance, beta-cell function and insulin sensitivity. The HOMA2 parameters were used in articles II and III.

We also obtained serum levels of high-sensitive cardiac troponin T (hs-cTnT) among 4070 patients with SAP in the WECAC, using a Modular E170 from Roche Diagnostics, with a lower detection limit of 3ng/l. This parameter was not yet available when writing article I, nor was it available among NORVIT patients.
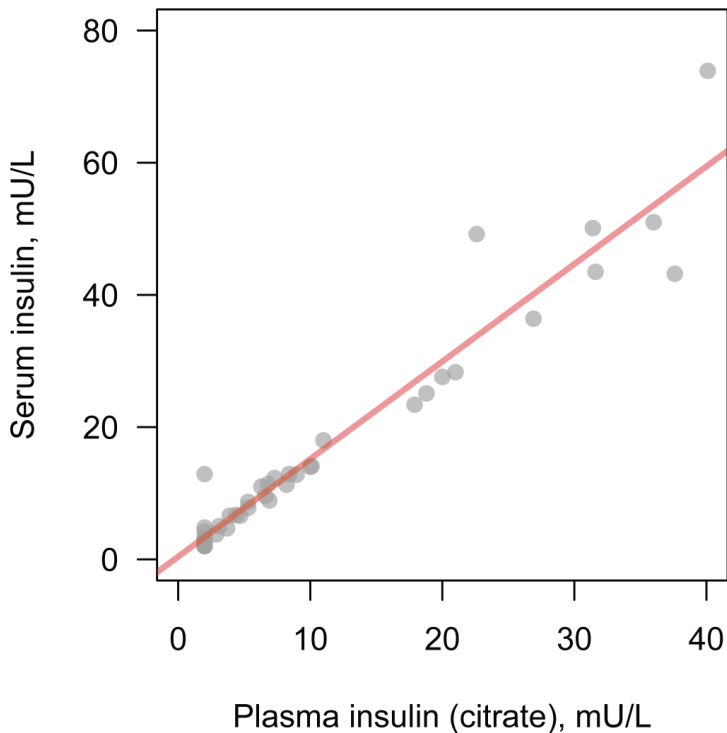
Figure 5. The relationship between insulin in citrate plasma and serum.

3.1.4 DATA ON DIETARY CHOLINE AND BETAINE INTAKE

Among WENBIT participants, 2412 patients provided information on dietary intake of macro- and micronutrients by completing a food frequency questionnaire (FFQ).[147] The daily intakes of total energy, choline and betaine were estimated among 1939 patients,[148] after excluding those with either particularly low (men and women <3300 kJ/day and <3000 kJ/day, respectively) or high (men and women >17500 kJ/day and > 15 000 kJ/day, respectively) estimated energy intake.

3.2   STATISTICAL METHODS

3.2.1 SOFTWARE

For all statistical analyses, we used SPSS for Windows versions 18-21 (SPSS Inc. Released 2009. PASW Statistics for Windows, Version 18.0. Chicago: SPSS Inc.; IBM Corp. Released 2010-2012. IBM SPSS Statistics for Windows, Version 19.0-

21.0. Armonk, NY: IBM Corp.), the free, open-source statistical software R for Windows versions 2.15.0-3.0.2 (The R Foundation for Statistical Computing, Vienna, Austria; packages *nlmee*, *Hmisc*, *ppcor*, *mgcv*, *segmented*, *PredictABEL*, and *ICC*) and SPSS Sample Power Version 2.0 (SPSS, Inc., 2000, Chicago, IL, www.spss.com). Graphics were obtained by the various R packages, and slightly modified according to the standards of each journal by using the free image editing software Inkscape for Windows version 0.48 (www.inkscape.org).

3.2.2 BASELINE DATA

In articles I and II we assessed potential linear relationships between plasma DMG quartiles (i.e. categories according to the 25th, 50th and 75th percentiles) and relevant clinical, anamnestic and biochemical parameters. Traditionally, linear associations between continuous variables have been explored by linear least squares mean regression models and analysis of variance (ANOVA) for continuous and categorical variables, respectively. However, treating the mean as an average measure is problematic, especially in terms of biological data, due to their often extensively right-tailed (non-normal) distribution. In the case of least squares mean linear regression non-normal distribution may also severely influence the regression line.[149] Most commonly, such right-skewed continuous data have been log-transformed before analyses, followed by reporting trends across groups according to their geometric means or back-transformed values. One problem is, however, that such values are quite arbitrary and do not communicate well with the reader. Moreover, regarding linear trends across ordinal categories, ANOVA modeling assumes that data are normally distributed within each group. One way around this problem could be to apply the non-parametric Kruskall-Wallis one-way of variance test. However, this method does not allow for adjusting for covariates. Therefore, we used semi-parametric quantile regression modeling, as provided by the R package *Quantreg*. Quantiles refer to the number of equally distributed parts of a cumulative distribution. In short, quantile regression does not imply assumptions regarding distributions, and allows for covariate adjustment. The method may explore linear trends between any quantile (i.e. percentile) of two continuous variables; however we focused on the

trends according to the median (50 percentile) in all three papers. Quantile regression was also utilized when assessing differences in continuous variables according to two groups (e.g. patients with or without T2D). However, in paper III, we explored the relationships between incident T2D and not only one, but all metabolites downstream the choline oxidation pathway. Thus, it was not feasible to carry out baseline trend analyses according to each one of the independent variables, but rather making an overview over various interrelationships between choline metabolites and other variables of interest (i.e. correlation matrix). Once again, we wanted to adjust the correlations for covariates and we had to overcome the problem of non-normal distributions, the latter violating the assumptions when carrying out Pearson's correlations. We therefore solved this by performing partial ranked Spearman correlations, using the R package *ppcor*.

For categorical and ordinal dependent variables, we performed trend analyses by binary or ordinal logistic regression models, respectively.

3.2.3   ENDPOINT ANALYSES

Time-to-event analyses, censoring and the problem of competing risks. For articles I and II the occurrence of AMI or death was well defined, also in terms of time to events, the latter being of importance when following patients for several years. Hence, we used the well-established method of Cox proportional hazard modeling, where we censored patients who did not experience an event during follow-up, or for whom such information was not available. However, censoring of patients is problematic, because it does not allow us the information on what may have happened to those patients afterwards. In addition, some endpoints may only occur once, for example death. This generates the problem of competing risks, where one endpoint changes the possibility of experiencing another. It has been stated that not properly accounting for competing risk may overestimate the risk association.[150] Thus, in article II, we constructed subdistribution hazard regression models, using the R package *crr*,[151] treating cardiovascular and non-cardiovascular death as competing events.

Cox proportional hazards modeling is quite robust. However, the assumption of proportional hazards is crucial, and was assessed by inspecting log-log plots according

to categorical variables, and calculating and testing Schoenfeld residuals, the latter by using the *cox.phz* function in the R package *survival*.

Endpoint data without information on time to event. Assessing the relationship between baseline and dichotomous endpoint data may also be carried out using binary logistic regression, preferably in analyses of endpoints with a pre-defined follow-up period (for example 30 day in-hospital mortality), or when exploring the relationship in studies without (reliable) information on time to events. We had prospective data on all end-points in our studies, but since T2D is not generally characterized by an abrupt onset of symptoms,[33] we chose to assess risk associations by using binary logistic regression in article III.

Subgroup analyses. The association between an independent and a dependent variable can differ between certain population groups, and may be assessed by performing analyses in each subgroup of interest. Random variation in associations between the independent and the dependent variable may occur across subgroups; therefore the potential effect modification should be tested formally. This is usually performed by including an interaction product term between the subgroup variable (the potential effect modifier) and the independent variable of interest in the model, and should also include the interaction term's lower order components.[152]

    All subgroup analyses were carried out and reported according to current recommendations.[153] Subgroups were obtained from predefined categories (e.g. gender, smoking status or study treatment), or created according to the median value of continuous variables, the latter to make the groups as equally sized as possible. We performed interaction analyses using the same methodology as described above, paying particularly attention to subgroups of established CHD risk factors and baseline status of biological parameters related to the choline oxidation pathway. The former may help identify subgroups in which a novel biomarker may have a more pronounced effect on risk prediction. The latter suggests metabolic mechanisms involved in associations between outcome and metabolite status, as differences in the latter may arise from alterations in the production, catabolism or tissue clearance of the

metabolite, or of their combination.

Model discrimination and reclassification. Model discrimination was assessed by
calculating the Harrells *C*-statistic, which equals the area under the receiver-operating
curve (ROC-AUC; Figure 5). Broadly speaking, the *C*-statistic will provide a
probability score between 0.5 and 1 on how well the model actually predicts patients
with and without the outcome, with the former and latter values corresponding to a
useless coin-toss and a perfect prediction, respectively.
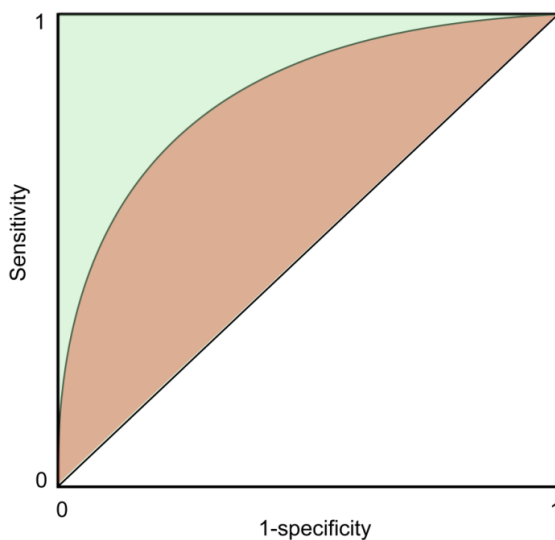
Figure 5. The area under the receiver-operating curve.
The diagonal line illustrates a model with no discriminative value; the brown area illustrates a model with intermediate discriminative predictive value; the green area (also covering the brown area) illustrates an optimal discriminative model.

However, once a few strong and robust predictors are included in the model, the
adding of new variables seldom provides any substantial improvement in the *C*-
statistic.[154] Thus, other, more sensitive, measures of model discrimination and
reclassification have been proposed, with the most notable and utilized being the
integrated discrimination index (IDI) and the net reclassification improvement
(NRI).[154] The IDI can be defined as the difference in discrimination slopes between
models with and without the biomarker(s) of interest; hence it compares the ability of
models to discriminate cases from non-cases. The NRI will provide the percentage of
patients that are being correctly reclassified to new risk categories, penalized by the
percentage of patients that are wrongly reclassified. This estimation is performed both

for patients who do and do not experience an event.

$$NRI_{total} = NRI_{event} + NRI_{non-event}$$

When considering CVD naïve patients, such risk categories for cardiovascular mortality are typically divided according to 0-5%, 5-10%, 10-20% and above 20% of risk of the event within a certain period of time (usually 10 years), as illustrated by the Systematic Coronary Risk Evaluation Project (SCORE) promoted by the European Society of Cardiology.[155] Allocation of patients into a particular risk category will potentially guide the clinician in the decision making of initiating certain preventive interventions. Therefore, the NRI may provide a more clinical useful metric than both the *C*-statistic and the IDI. However, among patients with known (atherosclerotic) CVD, no established risk categories exist, mostly because such patients generally receive quite similar medical treatment (i.e. a statin and aspirin) and life-style advise. In fact, it is advocated against creating new risk categories under conditions were none already exist.[156] Hence, the category free, or continuous, NRI (NRI>0) may serve as a better measure of improvement in reclassification. The NRI>0 equals the net improvement in patients who are either being correctly up- or down-classified on a continuous (category-free) scale from 0 to 100%.[156] Based upon the comparison with Cohen's d, it has been suggested that small, medium and large effect sizes correspond to NRI>0 of <20%, ~40% and >60%, respectively.[157]

3.2.4 REPEATED MEASUREMENTS
Temporal changes in biomarker concentrations. As described earlier, not all WENBIT and NORVIT study patients attended each study visit beyond baseline, and there were also inequalities according to the time spans between sampling points. We could therefore not simply use the well-known and widely used repeated measures analysis of variance (rANOVA), or its derivatives, when analyzing longitudinal biomarker data. Instead, we used the more flexible method of mixed (-effects) linear regression modeling, provided by the R package *nmle*. As opposed to rANOVA, such modeling may include subjects with missing data points, and allows for uneven time-

spans between study visits during follow-up. Moreover, rANOVA modelling requires homogeneous variances in the variables used, whereas in linear mixed modelling, such heterogeneity may be easily adjusted for. Finally, as opposed to the rANOVA method, there is no need for correcting for multiple testing when using the linear mixed modelling approach in the setting of more than one time interval.[158] Data from all study visits in the WENBIT and NORVIT were used for the repeated measures analyses in paper I and II. In paper III we used the baseline and 1-year measurements in WENBIT, since urine sampling was only performed among a few on other study visits. Study treatment effects were explored by including an interaction product term between time and study treatment group in the model.

Within-subject reproducibility. The coefficient of reliability (CoR) is also known as the one-way random effects intraclass coefficient (ICC) and according to Rosner[159] it is defined as

$$\text{CoR} = \text{ICC} = \sigma_\alpha^2 / (\sigma_\alpha^2 + \sigma_\varepsilon^2),$$

where σ_α^2 denotes the between-subject variance and σ_ε^2 the within-subject variance. Hence, a CoR (ICC) approximating 1 will imply very low within-subject variance. CoRs (ICCs) of 0.4-0.75 and ≥ 0.75 are considered fair to good, and excellent, respectively.[159] In paper I and II we used linear mixed modelling when calculating crude CoRs; however in paper III we used the R package #ICC, which also provided a 95% confidence interval together with the ICC estimate.

3.3   THE STROBE GUIDELINES – ENHANCING TRANSPARENCY

The vast majority of epidemiological studies stem from observational data. However, it is imperative that studies originating from observational data, as for any other sources for that matter, have transparent methodology in order to highlight strengths and weaknesses. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement[160] was launched in 2007 as a joint attempt from several authors and medical journals to improve such transparency. The statement

contains a checklist including information to be reported in an observational study, and has been widely endorsed by a number of scientific journals.[161] The STROBE initiative has also been extended, and currently comprises specific guidelines on the reporting on several types of observational studies, including the STROBE: Molecular Epidemiology (STROBE-ME),[162] which was applied in articles I-III.

4 SUMMARY OF THE RESULTS

4.1 PLASMA DIMETHYLGLYCINE AND RISK OF INCIDENT ACUTE MYOCARDIAL INFARCTION IN PATIENTS WITH STABLE ANGINA PECTORIS (PAPER I)

Arterioscler Thromb Vasc Biol. 2013;33:2041-8

Among the 4156 eligible patients with SAP, 72.0% were men and the median (5-95 percentiles) age was 62 (44-78) years. About 1/3 were active smokers, approximately half had hypertension, 40.3% had experienced a previous AMI and 11.8% had self-reported diabetes. Median (5-95 percentiles) plasma DMG was 4.1 (2.6-7.3) µmol/L. Plasma DMG levels were higher among non-fasting patients, and positively related to male gender, current smoking, higher age, hypertension, previous CVD, CRP, the use of beta-blockers and ACEI and/or ARBs, and circulating levels of most one-carbon and choline metabolites, as well as to plasma methylmalonic acid (MMA). Plasma DMG was inversely related to eGFR, serum total cholesterol and apo A1, in addition to the BHMT 742 G>A minor A allele.

During a median (5-95 percentiles) follow-up time of 4.6 (1.6-6.8) years, 343 (8.3%) of the patients experienced an AMI, and the incidence was positively related to increasing plasma DMG quartiles (P for log-rank < 0.001). In crude Cox regression models, the HR (95% CI) for AMI was 2.43 (1.78-3.31; P<0.001) when comparing the 4th vs. the 1st DMG quartiles. Adjusting for age, gender and fasting status somewhat attenuated the risk estimate (HR (95% CI) 1.95 (1.42-2.68); P<0.001), but further adjustment for serum apo A1 and apo B100, diabetes, smoking, and hypertension had only minor influence on the risk associations. Particular strong relationships between plasma DMG and risk of AMI were observed among non-smokers and patients with serum triglycerides or apoB100 < median (P for interaction ≤ 0.03).

Adding plasma DMG to the multivariate model improved model discrimination (Δ*C*-statistic (95%CI) 0.012 (0.001-0.022); P=0.04), and improved reclassification.

In WENBIT patients we observed increasing plasma DMG throughout the study for the group as a whole, but not among patients receiving folic acid + vitamin B12 (with or without vitamin B6). Among patients randomized to placebo treatment, the

CoR for plasma DMG was 0.93 and slightly lower when the variable was log-transformed.

We did not report on the association between plasma DMG and incident AMI in NORVIT; however, when carrying out such analyses in retrospect, we observed a positive association between plasma DMG and AMI also in this cohort, although the relationship was somewhat weaker (Table 3). The association were similar when adjusting for plasma tHcy (HR [95% CI] for the 4th vs. 1st quartile 1.25 [1.00-1.56]; P=0.05), but attenuated when accounting for eGFR, serum total cholesterol, smoking, diabetes and hypertension (HR [95% CI] 1.14 [0.90-1.43]; P=0.28).

Table 3. The relationship between plasma dimethylglycine and incident acute myocardial infarction among patients in the Norwegian Vitamin Trial, n=3733

	Unadjusted		Adjusted for age and gender	
	HR (95% CI)	P	HR (95% CI)	P
Plasma DMG, quartiles				
1	Ref		Ref	
2	1.07 (0.85-1.35)	0.55	1.02 (0.81-1.28)	0.87
3	1.20 (0.96-1.49)	0.11	1.06 (0.85-1.33)	0.61
4	1.61 (1.30-1.98)	<0.001	1.30 (1.04-1.61)	0.02

CI indicates confidence interval; DMG, dimethylglycine; HR, hazard ratio.

4.2 ELEVATED PLASMA DIMETHYLGLYCINE IS A RISK MARKER OF MORTALITY IN PATINTS WITH CORONARY HEART DISEASE (PAPER II)

Eur J Prev Cardiol. 2015;22(6):743-52

The 4150 patients with SAP eligible from the WECAC were generally similar to those included in paper I; hence baseline characteristics and the relationships between baseline plasma DMG and various clinical and biochemical parameters did also not differ to any particular degree from those reported in the previous chapter. In addition, we observed a strong positive relationship between plasma DMG and serum hs-cTnT, and inverse associations between plasma DMG and indices of insulin sensitivity.

The 3733 eligible patients with AMI from the NORVIT cohort had median (5-95 percentiles) age of 63 (44-81) years, and 73.9% were males. As compared to SAP patients, the NORVIT participants were older, more often smokers and had lower eGFR, but had less often preexisting CVD, hypertension or diabetes.

Among patients with AMI baseline plasma DMG was positively related to male gender, age, current smoking, hypertension, serum creatinine, previous CVD, plasma choline and one-carbon metabolites, and the use of beta-blockers and ACEIs and/or ARBs. Plasma DMG was negatively related to eGFR, plasma riboflavin, serum folate and the BHMT 742 G>A minor A allele.

Median (5-95 percentiles) follow-up times were 4.7 (2.8-6.8) and 7.0 (1.1-8.8) years among SAP and AMI patients respectively. Among SAP patients, 308 (7.4%) died, whereas 772 (20.7%) of the patients with AMI died. In both cohorts, the risk of all-cause mortality was positively related to increasing plasma DMG quartiles (P for log-rank<0.001). When comparing the 4th to the 1st DMG quartiles, the crude HRs (95% CI) for all-cause death were 2.52 (1.78-3.57); P<0.001 and 2.80 (2.27-3.44); P<0.001 among patients with SAP and AMI, respectively. Adjusting for age and gender attenuated the risk estimates (1.72 (1.21-2.46) and 1.76 (1.42-2.18)); however, the associations were essentially unaltered in the multivariate models. Plasma DMG was more pronouncedly associated with cardiovascular than non-cardiovascular death, and similar results were obtained when performing competing risk analyses.

Subgroup analyses showed trends towards stronger risk associations between

plasma DMG and all-cause and cardiovascular mortality among SAP patients with serum TG or apoB100 < median.

Adding plasma DMG to the multivariate model improved or trended to improve model discrimination and reclassification for all-cause and cardiovascular mortality, particularly among patients with AMI.

Similar to what was observed in article I, AMI patients who were randomized to either placebo or treatment with vitamin B6 alone experienced a rise in plasma DMG during follow-up; however no changes in plasma DMG were observed for patients receiving folic acid + vitamin B12 (with or without vitamin B6). The CoR for plasma DMG among AMI patients allocated to placebo was 0.72.

4.3   THE PROSPECTIVE ASSOCIATIONS OF SYSTEMIC AND URINARY
CHOLINE METABOLITES WITH INCIDENT TYPE 2 DIABETES (PAPER III)
Submitted, January 2016.

For baseline analyses, 4070 SAP patients were eligible, of whom 71.9% were men, and the median (25-75 percentiles) age was 62 (55-70) years. Patients with T2D (n=449 (11.0%)) had higher plasma choline and TMAO, lower plasma betaine and serum sarcosine, and higher urine choline, betaine, DMG and sarcosine than nondiabetic patients. Higher plasma choline and serum sarcosine, and lower plasma betaine concentrations were correlated with a generally more adverse T2D risk profile. Plasma DMG was positively related to indices of insulin resistance and CRP. Plasma TMAO was positively related to indices of insulin resistance and plasma choline, plasma DMG and serum sarcosine. Most choline metabolites in urine were positively related to an adverse risk profile of T2D.

The levels of all metabolites, except for urine betaine, increased during 1 year of follow-up among WENBIT patients allocated to placebo treatment. Treatment with folic acid + vitamin B12 (with or without vitamin B6) augmented the increase in plasma choline and betaine, whereas plasma and urine DMG and urine sarcosine were lowered, when compared to placebo. Vitamin B6 alone, on the other hand, increased plasma DMG, but dampened the increase in serum sarcosine and urine choline, as compared to placebo treatment. We did not observe any temporal alterations in overall plasma TMAO, also according to WENBIT study treatment.

A total of 3261 patients without registered T2D at baseline were followed for median (25-75 percentiles) 7.5 (6.4, 8.7) years, during which 233 (6.4%) patients were registered with incident T2D. As compared to those who did not develop T2D, patients who did develop T2D had lower baseline plasma betaine, whereas urine betaine and sarcosine were higher. In univariate logistic regression models, plasma betaine was inversely, while urine betaine, DMG and sarcosine levels were positively associated with incident T2D (ORs per 1 SD (95%I) 0.78 (0.69-0.89), 1.27 (1.11-1.45), 1.23 (1.07-1.42), and 1.31 (1.14-1.50), respectively). Adjusting for potential confounders and other T2D risk factors only slightly altered the risk estimates. We also performed a stepwise backwards elimination logistic regression, including all choline metabolites

being associated with incident T2D in univariate analyses. The selection was determined by the Akaikes' information criterion, and left only plasma betaine and urine sarcosine of the choline metabolites in the final model. When including plasma betaine or urine sarcosine, the multivariate model discrimination improved, as did the reclassification of patients according to the NRI>0.

The ICCs (95% CIs) for plasma betaine and urine sarcosine among patients randomized to placebo were 0.62 (0.56-0.66) and 0.69 (0.64-0.74), respectively. Similar ICCs were obtained among patients receiving vitamin B6 only treatment, whereas treatment with folic acid + vitamin B12 (with or without vitamin B6) weakened the test-retest reliability for both metabolites.

5   DISCUSSION

5.1   SUMMARY

The current thesis is based on data from either or both of two independent cohorts of patients with suspected or verified CHD, and the aims were to investigate the associations between plasma DMG and risk of incident AMI and mortality, the relationships between choline metabolites and incident T2D, any improvement in model risk prediction and reclassification for relevant biomarkers, and temporal trends in choline metabolites according to B-vitamin intervention.

The studies show that plasma DMG is a predictor of incident AMI among patients with suspected SAP, and predicts all-cause and cardiovascular mortality among patients with both stable and unstable CHD. We also report that lower plasma betaine and higher urine betaine, DMG and sarcosine are associated with new-onset T2D among patients with SAP. Once adjusted for age, gender and fasting status, all associations were essentially independent of traditional risk factors and potential confounders. Moreover, plasma DMG, as well as plasma betaine and urine sarcosine, may improve risk prediction of the respective endpoints. We also extend previous preclinical studies, by showing that treatment with high-dose B-vitamins influences the concentration of several choline metabolites in blood and urine.

5.2   METHODOLOGICAL CONSIDERATIONS

5.2.1   BIAS

The term "bias" refers to a systematic flaw in study design, and comprises a number of potential errors in obtaining the data on which a study is based.[163]

Selection bias. Selection bias concerns the selection of participants included in the study, thereby influencing the generalizability of the results. Since SAP is often diagnosed and treated without diagnostic angiography, the WECAC population may be regarded as a selected group and could possibly be at different risk of adverse events than SAP patients not admitted for angiography. However, once admitted to tertiary centres for diagnostic evaluation, the vast majority of SAP patients (both with and without angiographically verified CAD) were included in the WECAC during the

recruiting period, thereby strengthening the generalizability of the study results, as compared to only investigating WENBIT participants.

We did not have information on hospitalized AMI patients who were not included in the NORVIT; however, the applicability of the NORVIT data for patients hospitalized with AMI in general is strengthened by the nation-wide multi-centre study design, including primary, secondary and tertiary hospitals.

Most patients in both cohorts had information on systemic levels of choline, betaine and DMG; however, data on serum sarcosine and urine choline metabolites were only available in subpopulations of the WECAC. Thus, a selection bias by excluding patients with missing data was not likely in papers I-II, but may have been present in paper III, although the large study size probably attenuated any such effect. This problem could be assessed by further investigating potential differences between included and excluded patients, and in hindsight I admit that it would have been prudent to do so. On the other hand, when performing multivariate analyses in paper II, we used the statistical technique of multiple imputations to also include patients with one or more missing covariates. This did not lead to any alterations in the results, suggesting that excluding a relatively small number of patients was less likely to confer any serious selection bias.

Repeated measurements were not undertaken in WECAC patients not included in the WENBIT. It is, however, unlikely that WENBIT study treatment would have affected biomarker status otherwise among these patients, as only minor differences in baseline choline metabolites levels were observed among participants and non-participants in WENBIT among the entire cohort for SAP patients in WECAC (Table 4). Yet, we cannot exclude that WENBIT study treatment may have influenced the risk estimates, although such treatment did not statistically significantly alter the results in survival analyses in relatively small groups. In addition, the WENBIT did not randomize patients in strata of clinical presentation; hence the subgroup of SAP patients were most likely not allocated to study treatment in a truly randomized fashion. It would therefore have been more appropriate to investigate not only the temporal trends in choline metabolites according to study treatment, but also to

Table 4. Baseline characteristics among patients in the Western Norwegian Coronary Angiography Cohort according to enrolment into the Western Norwegian B-vitamin Intervention Trial

	WECAC SAP patients		
	Not WENBIT	WENBIT	P*
Male gender, n (%)	946 (59.5)	2050 (79.7)	<0.001
Age, years	62 (55-69)	62 (54-70)	0.59
Current smoking, n (%)	497 (31.2)	823 (32.0)	0.64
Diabetes, n (%)	190 (11.9)	306 (11.9)	1.00
Hypertension, n (%)	731 (45.9)	1215 (47.2)	0.44
Previous CVD, n (%)	755 (47.5)	1648 (64.0)	<0.001
BMI, kg/m^2	26.0 (23.7-28.7)	26.5 (24.5-29.0)	<0.001
HbA1c, %	6.4 (5.8-7.1)	5.8 (5.1-6.6)	<0.001
Plasma glucose, mmol/L	5.6 (5.0-6.6)	5.6 (5.1-6.6)	0.45
eGFR,	88 (74-98)	92 (82-100)	<0.001
CRP, mg/L	1.83 (0.92-4.00)	1.74 (0.85-3.42)	0.002
Lipid parameters			
total cholesterol, mmol/L	5.0 (4.3-5.8)	4.9 (4.2-5.7)	0.04
LDL cholesterol, mmol/L	3.0 (2.4-3.8)	2.9 (2.4-3.6)	0.001
HDL cholesterol, mmol/L	1.30 (1.03-1.60)	1.20 (1.00-1.42)	<0.001
triglycerides, mmol/L	1.42 (1.04-2.04)	1.55 (1.10-2.20)	<0.001
apo A1, mg/dL	1.36 (1.19-1.55)	1.26 (1.10-1.43)	<0.001
apo B100, mg/dL	0.90 (0.75-1.08)	0.85 (0.71-1.02)	<0.001
Extent of CAD at angiography			<0.001
0-vessel disease	764 (48.0)	282 (11.0)	
1-vessel disease	236 (14.8)	730 (28.4)	
2-vessel disease	241 (15.1)	687 (26.7)	
3-vessel disease	350 (22.0)	874 (34.0)	
Systemic one-carbon and choline metabolites, μmol/L			
tHcy	10.8 (8.9-13.2)	10.3 (8.6-12.3)	<0.001
tMet	27.7 (23.5-33.1)	25.9 (21.9-31.2)	<0.001
choline	10.0 (8.5-11.8)	9.5 (8.1-11.2)	<0.001
betaine	39.4 (32.1-48.1)	38.9 (32.0-47.5)	0.32
dimethylglycine	4.4 (3.6-5.4)	4.0 (3.3-4.8)	<0.001
sarcosine	1.5 (1.2-1.8)	1.5 (1.2-1.8)	0.10
Urine choline metabolites, mmol/mol creatinine			
choline	2.30 (1.71-3.17)	1.88 (1.38-2.55)	<0.001
betaine	7.84 (4.99-13.32)	7.28 (4.75-12.73)	0.02
dimethylglycine	3.14 (1.93-5.05)	3.25 (2.13-5.07)	0.04
sarcosine	0.13 (0.08-0.21)	0.14 (0.09-0.22)	0.008
B-vitamers			
riboflavin, nmol/L	11.7 (7.3-19.7)	11.0 (7.6-17.4)	0.07
folate, nmol/L	10.5 (7.8-15.6)	9.9 (7.3-14.5)	<0.001
cobalamin, pmol/L	433 (336-552)	337 (258-426)	<0.001

PLP, nmol/L	43.8 (30.8-66.2)	40.0 (29.1-56.0)	<0.001
Medications prior to the baseline visit, n (%)			
beta-blocker	1102 (69.3)	1955 (76.0)	<0.001
statin	995 (62.6)	2029 (78.9)	<0.001
aspirin	1189 (74.7)	2157 (83.8)	<0.001
ACEI and/or ARB	1102 (69.3)	1955 (76.0)	<0.001
Medications at discharge, n (%)			
beta-blocker	1010 (63.5)	2005 (77.9)	<0.001
statin	1054 (66.2)	2281 (88.7)	<0.001
aspirin	1083 (68.1)	2317 (90.1)	<0.001
ACEI and/or ARB	501 (31.5)	829 (32.2)	0.65
Clinical endpoints, n (%)			
AMI	132 (8.3)	212 (8.2)	0.99
mortality	153 (9.6)	156 (6.1)	<0.001
T2D†	67 (4.8)	168 (7.4)	0.002

ACEI indicates angiotensin converting enzyme inhibitor; AMI, acute myocardial infarction; ARB, angiotensin receptor blocker; BMI, body mass index; CAD, coronary artery disease; CRP, C-reactive protein; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; apo, apolipoprotein; PLP, pyridoxal 5'-phosphate;tHcy, total homocysteine; tMet, total methionine which is the sum of methionine and methionine sulfoxide; T2D, type 2 diabetes.

*Kruskal-Wallis test for continuous and Chi square test for categorical variables

baseline biomarker status. On the other hand, such interaction analyses would certainly decrease statistical power.

Information bias. Overall, only a handful of patients in the WECAC and NORVIT withdrew their consent of long-term follow-up, making the proportion of patients lost to follow-up very low. Since we assessed the risk relationships between baseline choline metabolite status and the clinical endpoints, the primary aims of our studies were not vulnerable to further non-response from the participants. Moreover, the use of mixed linear modelling strengthened the validity of repeated-measures analyses, since data from patients not attending all study visits could be included.

We cannot rule out the possibility that some WECAC patients experienced an incident AMI outside the Western Norway Health region, thereby leading to misclassification of non-cases in paper I. Also, some cases of AMI may have been undiagnosed or not reported properly. On the other hand, it is not likely that risk associations should differ in such subjects as compared to those with endpoints

registered in the WENOCARD, thereby reducing the risk of bias. Incident AMI in paper I was defined according to the revised European Society of Cardiology criteria from 2000,[130] but also included sudden cardiac death (ICD-10 code I461) or other sudden unexplained death outside hospital (ICD-10 code R96)), being in line with definitions used in epidemiological surveys in Sweden and Denmark.[132] Widening the definition of AMI as compared to a more stringent one including only ICD-10 codes I21-22 may thus have increased sensitivity but decreased the specificity of the endpoints in paper I.

Using the nation-wide Cause of Death Registry, the risk association observed between plasma DMG and the hard endpoint all-cause mortality in paper II was not prone to either misclassification or detection bias. We were not able to validate the classification of mortality due to cardiovascular or non-cardiovascular causes, and the reliability of such classifications has indeed been questioned,[164] although the stronger risk association between DMG and cardiovascular mortality strongly agrees with the results in paper I.

The risk relationships between choline metabolites and incident T2D in paper III certainly pose the greatest concerns regarding detection bias in the current thesis. First, many patients with T2D are not diagnosed at all.[23] Second, the diagnosis of incident T2D was dependent on discharge records from most, but not all, Norwegian public hospitals. It is plausible that patients with new-onset T2D, and being hospitalized, may have differed in several aspects from non-hospitalized or non-diagnosed cases. Third, we were not able to validate the T2D diagnoses for each patient. However, when only looking at WENBIT participants during the in-trial follow-up, similar risk estimates between choline metabolites and T2D were obtained as those for the whole study population during long-term follow-up. Since the WENBIT participants were closely monitored by interview, as well as clinical and biochemical assessment, it is not likely that the results in paper III are flawed due to detection bias.

Regression-dilution bias. Risk associations between once-only assessed biomarker status and the clinical endpoint are inclined to be influenced by errors concerning the risk marker assessment per se, including measurement flaws, as well as the short- and long-term variation in biomarker status within each study participant – the latter collectively termed "within-person reproducibility". This error is coined the "regression-dilution bias", because all measurements will tend to regress to less extreme values when performed repeatedly. Consequently one-time assessment biomarker assessment will underestimate (i.e. dilute) the true effect.[165] There have been several suggestions on how to overcome this problem, with perhaps the simplest solution being to directly correct the risk estimates for the ICC in a non-parametric manner (i.e. the corrected association equals the risk estimate/ICC).[166] It follows that when the ICC is relatively low, the real risk estimate will be proportionally higher, whereas a high ICC implies a low degree of within-person variability and a proportionately low chance of regression-dilution bias. From a clinical point of view, however, a biomarker not requiring validation (i.e. having a high ICC) will surely be the more useful.

In the current studies plasma DMG (paper I and II) and betaine (paper III), as well as urine sarcosine (paper III), had ICCs ranging from "good" to "excellent", and comparing to ICCs reported for various other CHD biomarkers, such as serum total cholesterol (0.75) and serum CRP (0.54).[167] Similar ICCs to our findings have been reported for plasma betaine and DMG in other populations, and seem consistent across genders and different ethnical groups.[168] However, a somewhat lower ICC was reported for plasma DMG in the Nurses' Health Study[169] and Lever et al observed ICCs over 8 weeks for plasma betaine and DMG of only 0.43 and 0.15, respectively, when investigating a small population of healthy young men.[170] No studies have hitherto reported on the ICC of urine sarcosine, but high ICCs for the highly correlated urine betaine and DMG have been described.[170]

In all, our findings imply a relatively low risk of regression-dilution bias, favouring the use of the studies metabolites as clinical biomarkers.

5.2.2 CONFOUNDING

For a given risk association *confounding factors* may be defined, quoting Miettinen and Cook, as "extraneous factors that might be explanatory, partially or totally, of the magnitude of the estimate of the effect".[171] Opposite to randomized clinical trials (RCTs), associations from observational studies are prone to confounding; hence, it is not possible to conclude on causal inferences. We can try to minimize this problem by adjusting the statistical models for potential confounders; however residual confounding will essentially always be present, as we are not able to identify or obtain data on all possible confounding variables.

There exist no firm guidelines regarding selection of the most appropriate covariates in statistical models. In general, statistical textbooks advice against solely relying on stepwise inclusion or exclusion procedures based on statistical measures alone, but advocate the inclusion of biological sound covariates, as well. Baseline choline metabolites had statistically significant relationships with several other clinical and laboratory parameters, which could indeed serve as potential confounders. However, we chose not to adjust for all of them due to several reasons: First, including too many variables will inflate the regression model, attenuating power and making the estimate(s) less reliable (i.e. increasing the confidence interval(s)). Second, several parameters may be highly correlated, (for example urinary choline metabolites in paper III), or they may be redundant (for example the inclusion of serum LDL cholesterol, when serum total cholesterol is already in the model), introducing the problems of collinearity and singularity, respectively. A similar issue may arise when including variables that might reflect common biological mechanisms, as discussed with serum creatinine and plasma DMG in paper II, potentially leading to over adjustment.[172] On the other hand, the weakening of a risk estimate when adjusting for a covariate might also suggest pathophysiological mechanisms relevant for the risk associations. We did not, however, assess these issues formally.[173] Third, it is difficult, if not impossible, to compare the performance of models not containing the same covariates. In CHD studies, it is usual to adjust for established Framingham risk factors, although, strictly speaking, these are extensively validated for CHD naïve patients only. Therefore, we chose to primarily adjust for such factors in paper I and II;

accordingly we adjusted for several traditional T2D risk factors and biological plausible confounders in paper III. In papers I and II, we created extended multivariate models, further adjusting for variables with biological plausible (as well as statistical) relationships with DMG, in order to address potential pathophysiological pathways. This was also done regarding the associations between choline metabolites and incident T2D in paper III, although they were not reported due to space limitations.

5.2.3 REVERSE CAUSALITY

This term refers to the situation where the disease itself has influenced biomarker status, potentially impacting the real risk relationship. For example, the estimated daily energy intake among patients with T2D in the WECAC was not different from that of patients without diabetes, although the former had significantly higher BMI. This could be due to under-reporting among T2D patients, but also because these patients had reduced their energy intake as a consequence of their disease. If we were to explore the risk relationship between BMI and T2D, this would surely attenuate the true association.

Reverse causality especially poses a problem in retrospective studies; hence our prospective study design is less prone to this error. Yet, a large proportion of the WECAC and NORVIT patients had established CVD, and we cannot exclude that the disease itself, its complications and/or medical therapy and lifestyle interventions somehow may have influenced plasma DMG levels. This is, however, not immediately supported by studies performed in other populations, reporting similar circulating DMG concentrations as those presented for the WECAC and NORVIT cohorts (Table 5). Also, the risk estimates in paper I were similar when excluding patients without significant CAD at angiography, indicating that reverse causality might not pose a serious limitation. In paper III the risk estimates were similar when excluding patients with possible, yet not diagnosed T2D. Nonetheless, patients identified with higher baseline risk of T2D by conventional measures may also have had life-style or other interventions performed that could influence the status of choline metabolites in blood and urine.

Table 5. Systemic and urine dimethylglycine levels in other populations

Author (year)	Population	N	Specimen	DMG, µmol/L		Method
Holm et al (2003)[135]	Healthy blood donors	60	Plasma			LC-MS/MS
	Fasting			median (25-75 pct)	1.7 (1.3-2.0)	
	Non-fasting			median (25-75 pct)	2.1 (1.6-2.5)	
Fredriksen et al (2007)[93]	General population	10 601	Plasma			LC-MS/MS
	Total			median (5-95 pct)	3.7 (2.4, 5.9)	
	Men			median (5-95 pct)	4.0 (2.7- 6.3)	
	Women			median (5-95 pct)	3.5 (2.3- 5.6)	
Holm et al (2005)[174]	Healthy subjects (fasting)		Plasma			LC-MS/MS
	Total	500		median (10-90 pct)	3.1 (2.0-4.9)	
	Men	208		median (10-90 pct)	3.4 (2.4-5.2)	
	Women	292		median (10-90 pct)	2.8 (1.9-4.6)	
Kirsch et al (2010)[175]	Healthy subjects (fasting)		Plasma			UPLC-MS/MS
	Men	24		median (10-90 pct)	3.1 (2.0-4.3)	
	Women	20		median (10-90 pct)	2.1 (1.5-3.7)	
			Urine*			
	Men	24		median (10-90 pct)	2.1 (0.6-7.0)	
	Women	20		median (10-90 pct)	2.3 (1.3-4.7)	
Melse-Boonstra et al (2005)[176]	Healthy subjects		Plasma			LC-MS/MS
	Men	182		median (10-90 pct)	3.4 (2.4-4.7)	
	Women	126		median (10-90 pct)	3.0 (2.3-4.5)	
Lever et al (2012)[177]	Patients 3 months		Plasma			HPLC

post ACS (fasting)			
Men	383	median (25-75 pct)	3.8 (2.7-5.2)
Women	148	median (25-75 pct)	3.4 (2.2-4.8)
Urine*			
Men	383	median (25-75 pct)	2.9 (1.5-5.9)
Women	148	median (25-75 pct)	2.4 (1.4-4.3)

ACS, acute coronary syndrome; GC-MS, gas chromatography mass spectrometry; HPLC, high-pressure liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; SD, standard deviation; HPLC, high performance liquid chromatography; UPLC-MS/MS, ultra performance liquid chromatography-tandem mass spectrometry;
*Corrected for urine creatinine

5.2.4 MENDELIAN RANDOMIZATION – IMPROVING THE SHORTCOMINGS OF OBSERVATIONAL STUDIES?

One way of omitting the potential pitfalls of residual confounding, reverse causality and certain types of bias is to carry out so-called Mendelian randomization studies.[178] Such studies investigate whether inborn genetic traits affecting biomarker status also are associated with clinical outcomes, thereby bypassing the influence of potential environmental factors. In other words, the subjects investigated are randomized genetically already before they are born. In the era of 'big data' Mendelian randomization studies have been widely embraced, and questioned previously proposed causal relationships for several CHD biomarkers, such as CRP,[179] HDL-cholesterol[180] and tHcy.[108]

As previously mentioned, genetic traits of both BHMT and DDH predict plasma DMG levels, and a proper conducted Mendelian randomization study using the BHMT 742 G>A data from the largely homogenous WECAC and the NORVIT populations could potentially shed more light on any causal relationship between DMG and adverse cardiovascular prognosis. We did, however, carry out logistic regression analyses, which did not indicate any relationships between the BHMT 742 G>A SNP and CVD or mortality risk in both articles I and II (Appendix I, Supplemental Tables IX and X; Appendix II, Supplemental Tables 10-15). This observation strengthens the assumption that elevated plasma DMG is not a causal risk factor for CVD per se.

Mendelian randomization studies are, however, not free of assumptions, and pose several important shortcomings. These include, but are not limited to, genetic pleiotropy, linkage disequilibrium and issues of statistical power, as discussed in the review by Hansen et al:[181] Probably the most important limitation is pleiotropy, meaning that genetic traits may have effects other than those only captured by measuring immediate biomarker levels. Such effects have not, to my knowledge, been investigated for the BHMT 742 G>A SNP; however it has been reported that the SNP does not statistically significantly predict plasma tHcy,[182] nor did we find any differences in either plasma tHcy, betaine or total methionine levels according to this SNP in neither the WECAC or the NORVIT populations (Table 6).

Allele frequencies were assessed according to the Hardy-Weinberg equation. The BHMT 742 G>A SNP was in Hardy-Weinberg equilibrium in the WECAC, but not in the NORVIT cohort. In the WECAC, we observed an approximately 5% decrease of plasma DMG per A allele (Table 4), whereas a decrease in plasma DMG only was observed among homozygotes for the variant allele in the NORVIT.

There was a low minor A frequency, and as we showed in paper I, the impact of the A allele on the endpoint therefore had to be very high if we should have been able to obtain a statistically significant relationship between the genotype and disease risk. This illustrates the problem of insufficient statistical power. In the case of genetic risk associations, this usually necessitates the use of large population samples, as the effects brought about by single genetic traits usually are quite small, being particularly relevant for multifactorial diseases, such as CVD.

5.2.5 GENERALIZABILITY

As discussed in section 5.2.1.1, the current study populations are considered fairly representative of hospital-admitted patients with suspected or verified stable and unstable CHD in present-day Norway. On the other hand, our study subjects were mainly middle-aged to elderly white men, making it not straightforward to extrapolate the results to populations including healthy individuals, as well as those with other age, gender and ethnic characteristics.

5.2.6 MULTIPLE COMPARISONS

The current work includes a large number of statistical analyses. The P-value tells us the probability of falsely rejecting the null-hypothesis on the basis of a proper statistical test (i.e. Type I error). By setting the significance level (α) at 0.05, an average of 1 in 20 comparisons will thus lead to a statistically significant finding just by chance alone. Consequently, the probability of making such a Type I error is high, even when performing a relatively low number of individual tests,[183] and statistical textbooks advice against drawing conclusions without adjusting for multiple comparisons. The most commonly used correction is the one proposed by the Italian mathematician Carl Emilio Bonferroni, where $\alpha = 0.05/m$, and m denotes the number

of hypotheses being tested. On the other hand, the Bonferroni correction is a very conservative adjustment, especially when a large number of correlating parameters are being assessed.[149] Also, some authors emphasize less on formal correction for multiple testing in post-hoc analyses in general, because observational research cannot be confirmative and must be considered hypothesis generating.[183] Although it could have been more prudent to have applied other, less conservative, correction methods,[149] we chose not to correct for multiple testing, which may pose a limitation on the interpretation of our results. When using mixed linear regression models, the problem of multiple comparisons was, at least to some extent, attenuated.

5.2.7   SUBGROUP ANALYSES

Post-hoc subgroup analyses always carry the potential of spurious findings. One of the most well-known examples is probably the one reported for the International Study of Infarct Survival (ISIS)-2 trial, where effects of study treatment were shown to significantly differ according to the astrological assignment of the patients.[184] All post-hoc (subgroup) analyses must therefore be considered hypothesis-generating only. This is particularly stressed in observational studies, where some authors actually have advocated against using formal statistical testing between and within subgroups at all.[185] Nonetheless, although being considered at the lower level of scientific methodological hierarchy, unexpected findings from observational studies have made huge impact on medical sciences, reminding us not to dismiss them on a default basis. For example, hardly anyone would reject the observations of the effect from *Penicillin* fungi on bacterial growth, just because they resulted from untidiness in Sir Alexander Flemming's research laboratory.[186] The crux is that observational findings must be further validated.[187]

Investigating treatment effects according to subgroups may help focusing on patient populations gaining the most benefit from treatment, and could also identify patients who even may be harmed from a particular intervention. Consequently, this has the potential to improve the clinical applicability of results from RCTs, which tend to generalize treatment effects. These issues are also highly relevant for diagnosis and risk assessment in CHD, as has been shown for the use of serum cardiac troponins and

electrocardiograms among patients with suspected ACS,[188] to mention but a few. Notably, in the current growing era of personalized medicine it has been called for an even more advanced profiling of patients in terms of CVD risk, in order to further improve patient care and to cut costs.[189]

Apart from their potentially immediate clinical utility, subgroup analyses in observational biomarker studies may also help elucidate novel pathophysiological mechanisms in an otherwise extremely complex system of biological pathways. This issue will be dealt with in more detail in later sections of the thesis.

In paper I, we found trends towards stronger associations between DMG and adverse prognosis among non-smokers as compared to smokers in WECAC patients; however, there were no signs of any effect modification by smoking in the NORVIT in paper II. Although having relatively similar baseline characteristics, including plasma DMG status, the NORVIT population had a substantially larger proportion of current smokers (paper II). One reason for the lack of any interaction by smoking in the NORVIT could therefore be that more patients may have quitted smoking after having experienced an AMI, thereby diluting any interaction effect. On the other hand, median plasma levels of cotinine, a nicotine metabolite, actually increased throughout follow-up in the NORVIT (data not shown). Although patients might have been less likely to be smoking while being hospitalized, this indicates a low level of smoking cessation, although persistent cotinine levels could also be due to the substitution of combustible tobacco for nicotine replacement products or smokeless tobacco.

The subgroup findings according to lipid parameters will be dealt with in more detail in section 5.4.2.

5.2.8 MODEL DISCRIMINATION AND RECLASSIFICATION

We showed that plasma DMG may improve the risk assessment of AMI and mortality, and that plasma betaine and urine sarcosine may aid in the reclassification of patients at risk of T2D. These findings are, however, not sufficient to bring these biomarkers into everyday clinical use. First, the findings should be extensively validated in other populations, preferably different from the ones studies in papers I-III. Second, the effect sizes of the NRI>0 and IDI were, at best, modest,[157] and not immediately easy

Table 6. The BHMT 742 G>A polymorphism in relation to baseline betaine, total homocysteine, dimethylglycine, and total methionine, incident acute myocardial infarction, mortality, and incident type 2 diabetes

| | N | BHMT 742 G>A | | | P_{trend}* |
		GG	GA	AA	
Plasma, µmol/L					
Betaine					
WENBIT	2427	39.3 (32.4-47.9)	38.3 (31.5-46.9)	40.8 (31.7-49.5)	0.53
NORVIT	3433	32.8 (27.5-39.8)	33.3 (27.2-40.4)	33.0 (26.5-38.5)	0.32
total homocysteine					
WENBIT	2427	10.2 (8.6-12.2)	10.3 (8.7-12.2)	10.3 (8.7-12.8)	0.36
NORVIT	3433	12.1 (10.1-14.7)	11.9 (10.0-14.8)	12.3 (10.3-15.3)	0.93
Dimethylglycine					
WENBIT	2427	4.1 (3.4-4.9)	3.9 (3.2-4.8)	3.7 (3.1-4.5)	<0.00001
NORVIT	3433	4.1 (3.4-5.1)	4.1 (3.3-4.9)	3.6 (3.1-4.5)	<0.0001
total methionine					
WENBIT	2427	26.2 (22.0-31.3)	25.6 (21.9-31.1)	27.0 (21.8-32.4)	0.47
NORVIT	3433	29.5 (25.1-34.1)	29.5 (24.8-34.1)	29.7 (25.3-34.1)	1.00
Urine, mmol/mol creatinine**	2049				
Betaine		7.4 (4.9-12.7)	7.1 (4.7-13.3)	6.4 (4.6-10.3)	0.10
Dimethylglycine		3.4 (2.2-5.2)	3.2 (2.1-5.0)	2.9 (2.0-4.4)	0.02
Incident AMI					
WENBIT	2427	111/1273 (8.7%)	81/993 (8.2%)	15/161 (9.3%)	0.89
NORVIT	3433	372/1899 (19.6%)	218/1258 (17.3%)	47/276 (17.0%)	0.10
All-cause mortality					
WENBIT	2427	86/1273 (6.8%)	58/993 (5.8%)	7/161 (4.3%)	0.18
NORVIT	3433	385/1899 (20.3%)	270/1258 (21.5%)	52/276 (18.8)	0.94
CVD mortality	2427				
WENBIT	2427	47/1273 (3.7%)	32/993 (3.2%)	6/161 (3.7%)	0.71

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NORVIT	3433	237/1899 (12.5%)	155/1258 (12.3%)	32/276 (11.6%)	0.71
Non-CVD mortality					
WENBIT	2427	39/1273 (3.1%)	26/993 (2.6%)	1/161 (0.6%)	0.12
NORVIT	3433	148/1899 (7.8%)	115/1258 (9.1%)	20/276 (7.2%)	0.58
Incident T2D**†	2137	89/1124 (7.9%)	61/868 (7.0%)	11/145 (7.6%)	0.58

AMI indicates acute myocardial infarction; CVD, cardiovascular disease; NORVIT, Norwegian Vitamin Trial; WENBIT, Western-Norway B-vitamin Intervention Trial; T2D, type 2 diabetes.

*Linear trends across groups tested by median linear and logistic regression for continuous and categorical dependent variables, respectively.

**WENBIT participants only

†Patients free of diabetes at baseline.

to communicate, because they do not tell us whether the improvements translate into any significant clinical meaning. This especially concerns the lack of information on patients being reclassified away from the group(s) with intermediate risk, as can be obtained by the category-based NRI. Third, and perhaps most importantly,[190] the current observational studies are insufficient to suggest that patients should be treated differently only on the basis of biomarker status alone. This is especially important among patients with established CHD, who presently receive generally similar treatment, at least in terms of medications and life-style advice.

We used an NRI method based on logistic regression models in all three papers. For papers I and II we did also have information on the time to events, but had to (somewhat arbitrarily) use a cut-off of approximately three years of follow-up, because this was the approximate minimum follow-up for most patients. Therefore, patients who experienced an event after the cut-off period were actually classified as not experiencing an event, which, in addition to not taking into account the time-to-events per se, might have influenced the estimates. Notably, there has recently been launched a method for using Cox regression, instead of logistic regression, models when calculating the NRI, using the R package *SurvIDINRI*. However, this method also makes use of a selected cut-off in follow-up time, similar to our original approach, and consequently it did not alter the results to any particular extent when applied to the current data (data not shown).

There has also recently been raised more profound criticism against the NRI. The NRI per se does not provide us of qualitative measures of the reclassification, i.e. whether unnecessary treatment (or testing) in some patients outweighs the incorrect reclassification of patients who later develop disease. This problem could be addressed by using analyses that take such weighting into account,[191] which obviously necessitates the application of a category-based NRI. Moreover, the NRI may have several other limitations, as outlined by Leening et al,[192] prompting carefulness when applying, reporting and interpreting results based on this methodology.

Moreover, rather than being competitive, the *C*-statistic, the NRI, and the IDI seem to complement each other, and should therefore be reported together when assessing the incremental value of novel biomarkers in risk prediction models.[157] We

did not calculate the IDI in paper I, but the IDI was reported in papers II and III. However, as advocated by others,[192] paper I did report separate NRIs for events and non-events, which, due to space limitations, was not performed in paper II and III.

5.2.9 BIOSAMPLE PROCESSING

Routine laboratory measurements were performed on-site in both the WECAC and the NORVIT. We did not have detailed information on how these analyses were carried out, but it is not likely that established methods deviated considerably according to study sites, or introducing any serious bias according to the risk associations studied.

Among SAP patients in the WECAC, study specific blood and urine samples were usually collected few days prior to or at the day of angiography, and immediately frozen. In the NORVIT, blood samples were collected on-site, and then shipped to the core laboratory at Bevital AS in Bergen before freezing. Thus, in the NORVIT, samples may have been exposed to room temperature for several days, which could have led to overestimating concentrations of plasma free choline, due to formation from PC at room temperature. Long-term storage at $-80\,^{\circ}\mathrm{C}$, however, most likely did not affect any of the choline metabolites.[193]

In the NORVIT, we were able to adjust the DMG-mortality association for time-span from the index AMI until baseline biosampling, but contrary to the WECAC we did not have information on fasting status. However, it is likely that patients presenting with an acute AMI constitute of a mix of fasting and non-fasting patients at the time of biosampling, similar to the SAP patients in the WECAC, and this might be reflected by equal plasma DMG concentrations in both cohorts.

5.2.10 STATISTICAL ANALYSES

We have applied both traditional and more advanced statistical methods in the current thesis, and some have already been more thoroughly discussed in previous sections.

Cox proportional and logistic regression modelling are robust and rely only on a few assumptions, in addition to having being the preferred statistical method in clinical endpoint studies for decades. However, it must be emphasized that relative risk estimates must be considered in light of absolute risk to more fully support their

clinical interpretability. In hindsight, this latter issue should perhaps have received more attention in all three papers.

By using quantile (median) linear regression for baseline analyses, we obtained effect estimates that are easy to communicate with the readers, because we did not have to transform the variables. Non-parametric statistical tests tend to reduce the information conferred by the variables, since their distributions (hence also variances) are not accounted for when using parametric tests. The semiparametric quantile linear regression, on the other hand, not only provides us with a regression coefficient, but also allows us to examine the conditional distribution of a continuous variable according to all percentiles from 0-100. This makes it possible to explore potential differences across the distribution of the independent variable. We did, however, only focus on trend analyses according to the median (50-percentile) in the current work.

Relationships in biological systems are seldom linear, which motivates the use of non-linear statistical approaches. We used GAM when exploring the associations between potential biomarkers and endpoint data, and presented the results as smoothed splines graphics. Whereas non-linear associations were not a major issue in paper I and II, several choline metabolites trended to deviate from linear relationships with incident T2D in paper III. This way of exploring and presenting data may provide more information in terms of potential underlying mechanisms, as well as avoiding the pitfall of concluding with no overall linear association in the case of a U-shaped relationship. We did not use non-linear models when assessing baseline or repeated measures data, which may pose limitations on their interpretability.

5.3   ETHICAL CONSIDERATIONS

5.3.1   LEGAL ASPECTS

All patients included in the current thesis took part on a voluntary, non-commercial basis, and provided signed informed consent. The patient recruitment, diagnostic procedures, sample handling and import of endpoint data were performed according to the Declaration of Helsinki, approval from the Regional Medical Ethics Committee, and Norwegian law on health research.[194] Moreover, all patients in the WECAC

underwent baseline angiography due to clinical indications, and not primarily for study purposes, thereby not exposing the patients to unnecessary hazard.

5.3.2 FINANCIAL ISSUES

The analyses of choline metabolites are presently not implemented in most routine laboratories. Therefore, the hypothetical implementation of choline metabolism biomarkers would likely add considerable cost if applied to large patient populations. Although such issues must be addressed in properly conducted cost-benefit-analyses, a potentially increment in expenditures is another ethical aspect to consider.

5.4   DISCUSSION OF THE MAIN FINDINGS

5.4.1 PUTTING THE FINDINGS INTO AN EPIDEMIOLOGICAL CONTEXT

Papers I and II. The association between plasma DMG and an overall adverse CVD risk profile found in both papers I and II have not previously been reported. However, one study proposed an indirect relationship between plasma DMG and the extent of CAD at angiography according to the BHMT 742 G>A SNP,[92] although a later meta-analysis did not confirm any such relationship,[195] thereby agreeing with our baseline findings in paper I.

The prospective associations between DMG and adverse CVD prognosis are in line with results presented by Lever et al among approximately 500 New Zealand patients with a recent ACS.[113] Although being secondary findings and not reaching statistical significance in models adjusted only for a limited number of potential confounders, the risk estimates in that study were similar to those reported by us in papers I and II. Our validation of the associations between plasma DMG and an adverse CVD prognosis in cohorts that were larger and had a more detailed baseline characterization and longer follow-up time, should be considered a strength. Further, by reporting the potential benefit of adding plasma DMG in risk prediction, and exploring temporal trends according to B-vitamin supplementation, the papers I and II extended current knowledge within the field.

In secondary analyses, we also explored the relationships between other choline metabolites and risk of incident AMI in paper I. Plasma choline was weakly related to

the outcome, but was attenuated once DMG was introduced into the model, suggesting that DMG might have confounded previous reports on positive risk relationships between systemic choline concentrations and CVD events.[111] We did not find any relationship between plasma betaine and incident AMI in paper I, nor was any such overall association evident among WENBIT patients with presumed SAP,[196] thus opposing the findings by Lever et al;[113] however differences in study outcomes may also relate to inequalities between the studied populations.

Paper III. As stated above, previous studies suggested lower systemic betaine[118-120] and higher urine betaine[57] among patients with established T2D as compared to their non-diabetic counterparts. Our prospective findings affirm these observations by also showing that low plasma betaine status, in addition to higher urine DMG and sarcosine predicted incident T2D. The trends were consistent in sensitivity analyses and when adjusting for traditional T2D risk factors, including age, BMI and HbA1c. These results indicate that alterations in choline- and one-carbon metabolism may be present before the onset of clinical disease, and might even predate features traditionally associated with increased T2D risk.

A recent large Mendelian randomization study from Sweden found evidence of higher diabetes risk among patients with lower circulatory DMG concentrations as determined by polymorphisms in the gene coding for SDH.[94] Although the association was weak, the authors concluded that DMG shortage might have a causal role in diabetes development, but did not report on the concomitant levels of other choline metabolites. Notably, the lack of systemic sarcosine data was actually highlighted as a limitation of the study.[94] Indeed, if increased DMG catabolism was responsible for higher relative diabetes risk, one might suspect sarcosine levels to be positively associated with risk, as well. Also, increased downstream DMG metabolism should lead to a higher flux through the BHMT pathway, because of less product inhibition, hence possibly affecting betaine status. Our work reports on the concomitant risk relationships for all choline metabolites. We observed an overall null-effect between plasma DMG and TD2 risk in the multivariate model, and actually a trend towards a higher risk among those with particularly low serum sarcosine, thereby not

immediately supporting the conclusion made by the Swedish authors. It could be, however, that a favourable prognosis related to decreased DMG catabolism among subjects in the Swedish study rather reflected a betaine sparing effect; however a causal relationship between plasma betaine and diabetes was not supported in another Mendelian randomization investigating a specific BHMT polymorphism.[197] Likewise, we did not find a significant difference in the incidence rate of T2D according to the BHMT 742 G>A in the WECAC (Table 6). We have not, however, assessed this relationship in more appropriate statistical models, nor have we investigated it in the NORVIT.

The propensity to having high urine betaine excretion among patients with diabetes has been described in several smaller studies,[57] and a recent report also showed that urine betaine and DMG was highly correlated among young healthy males,[170] hence agreeing with our results in paper III.

5.4.2 POSSIBLE MECHANISMS

A general disclaimer. It must be emphasized that the present thesis primarily represents an epidemiological approach to the relationships between choline metabolism and non-communicable diseases. We have measured metabolites in body fluids, serving as proxies for very complex metabolic processes within subcellular compartments in various tissues. We are therefore not able to draw firm conclusions regarding mechanisms underlying metabolite status or risk relationships. However, previous, as well as the present studies, do suggest interesting, pathophysiological mechanisms in play. Also, these hypotheses may further serve as inspiration for new research projects, both within our own group, but hopefully also for others.

Mitochondrial function, energy metabolism and lipid homeostasis. The transport of choline from the cytosol into the mitochondrion is assumed to be carrier-mediated, and the rate-limiting step in the conversion of choline into betaine.[79] The inverse relationship between plasma choline and betaine with several CVD and T2D risk factors, as observed in our as well as in another[112] study, could therefore relate to impaired choline transport across the mitochondrial membrane. Moreover, *Chdh*

knockout mice had accumulated choline and severely decreased mitochondrial betaine levels in certain tissues, which also exhibited disturbed mitochondrial function and morphology.[198] Also, BADH is dependent on oxidized nicotinamide adenine dinucleotide (NAD+) as a cofactor,[199] and preliminary studies from our group have shown that alterations in the downstream metabolism of tryptophan, essential in the production of NAD+, may relate to incident T2D. Taken together, this suggests that the choline-to-betaine conversion may be impaired among patients more susceptible to CVD and T2D. Plasma choline levels did not relate to prevalent or incident T2D in paper III; however any build-up of choline from decreased choline oxidation might be masked by increased use of choline for other purposes, including augmented hepatic lipid export when energy intake is high, as well as increased choline uptake by the kidneys.[200] This issue could potentially be further explored by molecular tracer studies.

In agreement with the epidemiological data, preclinical studies have also suggested inverse relationships between betaine levels and several indices of insulin sensitivity. For example, rat models of insulin resistance and T2D showed lower hepatic betaine levels, likely due to increased BHMT flux.[201] This might also reflect the positive relationship between the HOMA2-IR and plasma DMG in paper II, although no such association was seen in other, and larger, populations.[94] Moreover, betaine supplementation in insulin resistant rats improved insulin sensitivity in both the liver and in adipose tissue, possibly due to increased adipokine production and lowered endoplasmic reticulum stress.[202]

Most data support an increased BHMT flux in obesity and insulin resistance; however whether this represents a compensatory mechanism, for example due to altered demands for methyl groups, or whether increased BHMT flux is detrimental, remains an open question. Nevertheless, it is interesting that no association between plasma DMG and T2D was observed in paper III, and that betaine status itself does not appear to be causally related to T2D risk, as discussed earlier. This might suggest that for betaine to excess favourable effects, there is a need of both sufficient betaine *and* an adequate BHMT function. On the other hand, BHMT knockout mice actually exhibited increased energy expenditure, reduced body fat and improved glucose metabolism and insulin sensitivity in adipose tissue,[203] similar to choline deprived

mice, further complicating the interpretability in terms of BHMT flux. Professor Steven Zeisel addressed this discrepancy in a recent review.[53] He proposed that different effects might arise from the influence by choline and betaine on PC composition, which again could affect insulin sensitivity via altered regulation of sterol regulatory element-binding protein 1.[53] However, to make matters even more complex, high-dose betaine supplementation in healthy persons did not alter indices of the metabolic syndrome[89] or resting energy consumption,[204] although such therapy increased plasma betaine levels,[89, 204] and thereby likely enhanced BHMT flux, as well.

The link between BHMT and T2D may also relate to the association between statin therapy and increased risk of incident T2D.[205] The BHMT function might actually be attenuated by statins, which were widely used in the WECAC population, because statin therapy has been related to higher plasma betaine in other,[177] as well as in the WECAC cohort (data presented at the EuroPrevent Congress, Amsterdam, The Netherlands, 2014). Notably, simvastatin has been associated with impaired mitochondrial oxidative phosphorylation in skeletal muscle,[206] thereby suggesting a potential relationship with deteriorations in the choline oxidation pathway.

In line with our results, a metabolomics study among almost 900 young and healthy Italian men and women showed an inverse relationship between circulating DMG and LDL-cholesterol, total cholesterol, and triglycerides.[207] We found particularly strong relationships between plasma DMG and CVD risk among those with low levels of (fasting) serum triglycerides and apoB 100. It is thus alluring to speculate whether the increased CVD risk for higher plasma DMG may be related to impaired downstream DMG catabolism. This could potentially decrease BHMT activity by product inhibition, and contribute to decreased VLDL output, as well as impaired oxidation of fatty acids.[203] As treatment with folic acid likely attenuates BHMT flux, it is therefore interesting that we did observe a trend towards a stronger risk relationship between plasma DMG and incident AMI among those who received folic acid intervention in the WENBIT (Appendix 1; Supplemental Tables VI and VIII). A theory of BHMT inhibition is further supported by rat studies showing that peroxisome proliferation-activator alpha (PPARα) agonists, well-known for their

systemic triglyceride reducing abilities, likely impair DMG catabolism via down-regulated expression of DDH and SDH.[208]

The LDL hypothesis regarding ACVD holds firm ground, not least due to the recent results from the IMProved Reduction of Outcomes: Vytorin Efficacy International Trial (IMPROVE-IT)[209] and results from clinical trials investigating treatment with proprotein convertase subtilisin/kexin type 9 antibodies.[210, 211] However, the provoking proposal of increased CVD risk by decreased hepatic VLDL cholesterol output[212] lends support from the fact that non-alcoholic fatty liver disease (NAFLD) is indeed considered an independent CVD risk factor, and is also strongly related to T2D.[213] In addition, studies in apoE knockout mice showed that betaine supplementation led to higher systemic levels of atherogenic lipids, but reduced body weight and atherosclerotic lesions,[214] as well as relieving NAFLD. In line with these data, analyses in the WECAC show that glycine, the immediate sarcosine catabolite, is inversely related to incident AMI, and that the association is stronger when serum apoB levels are low.[215] Notably, diminished GNMT flux has been shown to attenuate hepatic lipid export and induce hyperlipidemia,[216] in addition to impairing reverse cholesterol transport and increasing atherosclerosis.[217] Moreover, low systemic glycine levels have been related to higher risk of T2D.[218] Glycine may be produced from other metabolic pathways than the SDH and GNMT reactions, as well;[86] however our results may indirectly point to a high CVD and T2D risk when the lipid flux between the liver and peripheral tissues is reduced due to curtailed downstream choline oxidation. Hard clinical endpoint data to back up a theory of detrimental effects due to reduced hepatic VLDL export might be obtained from RCTs concerning treatment with mipomersen. This is an antisense oligonucleotide, which impairs hepatic VLDL efflux and lowers systemic LDL levels, but increases the risk of hepatic steatosis.[219]

Further, it is interesting that choline- and one-carbon metabolism may also be related to apo A1 and HDL-cholesterol metabolism. Apo A1 is the major component of the HDL particle, low levels of which have been related to increased risk of both CHD and T2D, albeit Mendelian randomization studies have questioned causal relationships.[220, 221] In line with previous epidemiological data,[112] plasma betaine was negatively associated with both apoA1 and HDL-cholesterol in the WECAC. Also,

studies in mice have suggested that choline deficiency lowers systemic apo A1 synthesis, probably due to reduced *PPARα* transcription,[222] and that supplementation with betaine may improve apo A1 status.[223] These effects could also indirectly relate to BHMT, since PPARα decreases flux through the CBS pathway.[224] In addition, a betaine deficient state may lead to reduced BHMT flux and accumulating Hcy, the latter which may lower apo A1 as well as HDL-cholesterol by several proposed mechanisms, as discussed by Obeid et al.[225]

Ramifications to methyl metabolism, nucleotide production, cell turnover and epigenetic regulation. The interconversion of free folates between the mitochondrial and cytosolic compartments is limited, and one-carbon units therefore move from the mitochondrion to the cytosol mainly as glycine, serine or formate.[79] Formate is used for purine synthesis, as it condenses with THF to form 10-formyl-THF.[64] Furthermore, THF can be methylated by serine via the vitamin B6 dependent enzyme serine-methyltransferase to generate 5, 10-methylene-THF, used for the production of thymidylate, and glycine.[226] Consequently, altered metabolism down the choline oxidation pathway may influence the production of nucleotides necessary for DNA and RNA synthesis.[227] In addition, reduced choline availability might affect genomic stability.[227] These issues may have adverse effects on the cells' regenerative and reparative abilities, and could be linked to cell apoptosis associated with vulnerable atherosclerotic plaques, [228] as well as apoptosis among cardiomyocytes being observed in CVD.[229] The latter could, at least partly, be reflected by the strong positive relationship between plasma DMG and serum hs-cTnT among patients with suspected SAP (paper II), although adjusting for hs-cTnT did not alter the risk associations between DMG and mortality in the WECAC. The mechanisms involved in apoptosis are very complex; however, a propensity towards a catabolic state could potentially be relevant for other organs than the cardiovascular system, as well. Notably, patients with T2D are characterized by reduced mass of pancreatic beta cells, which may be ascribed to a mismatch in the dynamic process of cell replicating and apoptotic cell death.[230]

Choline deficiency[231] and impaired BHMT flux[67, 232] are associated with lower hepatic SAM and increased SAH. The SAM/SAH ratio may reflect the methylation potential,[233] and although partially compensated for by up-regulation of methyltransferases,[64] a lower methylation potential due to impairments along the choline oxidation pathway could influence epigenetic regulation in terms of reduced methylation of cytosine-guanine repeat (CpG) islands in the DNA.[234] However, the extreme complexity of interactions between genes makes it difficult to predict how an overall reduction in methylation capacity may influence phenotype or prognosis. Reflecting this complexity, DNA hypo- and hypermethylation may coexist at specific regions within the genome, yet several studies have associated global DNA hypomethylation with atherosclerosis.[235, 236] Moreover, DNA methylation is inversely associated with age,[237] the latter being far the most important predictor of adverse CVD prognosis in both the WECAC and the NORVIT cohorts (data not shown).

Epigenetic regulation is also important in glucose and energy metabolism,[235] as well as in T2D. The role of methylation was further highlighted in a study among nicotinamide *N*-methyltransferase knocked-out mice, which had a propensity to leanness because of increased energy expenditure. This was explained by increased SAM available for methylation reactions including polyamine synthesis in peripheral adipose tissue, as well as higher flux of oxidized nicotinamide adenine dinucleotide (NAD+).[238] Betaine supplementation was also associated with increased hepatic SAM levels in fat-fed, insulin resistant mice,[239] and a recent study indicated that improved methylation status from betaine might favorably influence several enzymes involved in lipogenesis and fatty acid synthesis, including the fat mass and obesity-associated (FTO) gene.[240]

Choline metabolites in the urine. It is plausible that systemic choline metabolite status primarily reflects altered metabolism in the liver, whereas urinary concentrations of choline metabolites mirror their handling and/or metabolism in the kidney. This is supported by the relative modest correlations between systemic and urinary levels of choline metabolites in the WECAC (paper III), and the low correlation reported between plasma and urinary betaine in previous studies.[57]

However, we observed a negative correlation between baseline plasma and urine betaine among patients with established T2D (paper III), indicating that patients with overt disease actually may have urinary betaine loss contributing to low systemic betaine levels. This agrees with the findings by Lever et al, who reported that some patients with T2D might actively excrete betaine from the kidneys, thereby exceeding 100% fractional clearance for betaine.[241]

Urine betaine excretion has been positively associated with parameters of impaired glucose control in patients with diabetes;[125, 241, 242] however the increase may not be dependent on high systemic glucose levels per se,[243] but a link to hyperosmolarity has been suggested.[57] Proximal tubular dysfunction has been proposed as a likely contributor to increased urinary betaine excretion among patients with diabetes, because betaine is freely filtered in the glomeruli and urine betaine is correlated with urinary concentrations of retinol binding protein, a marker of tubular dysfunction.[242] This corresponds to our findings in paper III, since the predictive ability of urine betaine, as well as DMG and sarcosine, was independent of microalbuminuria which primarily reflects glomerular leakage.[244] Our findings extend previous data, as we also observed that patients who later developed T2D had a 18% and 23% increase in baseline urine betaine and sarcosine concentrations, respectively, as compared to those who did not develop T2D (Article III). A 9.9% increase in urine DMG was also observed; however this difference was not statistically significant. These differences did not seem to be explained by reverse causation due to misclassification of T2D at baseline, as urine choline metabolites levels were similar among patients with possible and patients without T2D. This suggests that altered renal handling and/or metabolism of choline metabolites is present before the onset of overt T2D.

As compared to betaine, relatively little attention has previously been paid to urinary DMG and sarcosine excretion. Betaine ingestion is associated with a transient increase in urine DMG concentration, and a slightly higher fractional excretion has been reported for DMG than for betaine in pigs.[57] Treatment with fibrates, which are PPARα agonists, has also been related to a massive increase in both urine betaine and DMG, and especially so among patients with T2D.[241, 245] Moreover, urine betaine and

DMG correlated strongly with urine choline in another study, which also reported that fenofibrate led to larger increments in urine betaine, than DMG and choline.[245] In paper III, urine choline was not associated with incident T2D; however it is plausible that betaine and DMG, and perhaps also other choline metabolites, may have some common renal handling, also in terms of active excretion from renal epithelial cells.[241, 245]

Almost no sarcosine is excreted in the urine among normal subjects.[246] Higher urinary sarcosine levels have been observed among patients with impaired SDH,[247] probably reflecting the role of sarcosine in scavenging excess methyl.[85] Most sarcosine filtered in the glomeruli is assumed to be absorbed in the tubular system, and sarcosine may compete with glycine for reuptake in tubuli.[247] One might therefore speculate whether increased sarcosine excretion partly results from enhanced renal glycine uptake in a glycine deficient state being associated with T2D. Our data does not support this, however, as urine glycine concentrations did not relate to T2D in the WECAC (data not shown).

Both GNMT[248] and BHMT[48] are abundant in the kidneys, and increased flux over these enzymes, at least locally, could perhaps lead to a spill-over of products from renal cells and into the tubulus system. On the other hand, insulin resistance and T2D may affect GNMT differently in the kidney as compared to the liver. For example, renal GNMT flux might not be up-regulated in prediabetic state, as is the case in the diabetic state. This somewhat contrasts hepatic GNMT flux, which is most likely up-regulated in both conditions.[249] We also observed that treating the patients with B-vitamins altered systemic choline metabolites during follow-up, most probably related to reduced BHMT flux; however less consistent findings were made in the urine. Potential tissue specific alterations in BHMT and GNMT flux brought about by insulin resistance and T2D therefore makes it problematic to draw firm conclusions when only metabolite concentrations in the blood and urine are measured.

Can dietary factors explain our findings? In paper III, we also had data available on estimated daily intake of both choline and betaine in a subpopulation of the WECAC participants, suggesting the daily intake of choline to be far below that recommended

by USDA.[52] We did not, however, find any differences in these measures according to T2D, although plasma TMAO, a proposed marker of choline intake,[102] was about two-fold among those with existing T2D at baseline, the latter agreeing with previous observations.[120, 123, 250] Moreover, recent studies actually found lower plasma TMAO and betaine levels to predict gestational diabetes in women,[251, 252] thereby complicating the relationship between TMAO and diabetes risk further. Higher intakes of PC were recently related to increased T2D risk;[122] however, we did not explore dietary determinants of choline metabolites, nor did we assess the relationships between total choline and betaine intake with the risk of new-onset T2D per se. Nevertheless, including data on choline and betaine intake in the multivariate models in paper III did not affect the risk estimates, nor was plasma TMAO predictive of incident T2D in general.

A recent study actually questioned the alleged causal association between plasma TMAO and ACVD, redirecting the focus to hepatic FMO3 instead.[253] Accordingly, preliminary results from the WECAC also challenge the relationship between plasma TMAO and CVD risk. This agrees with previous epidemiological data reporting no statistically significant associations between intakes of choline and betaine with incident CVD[116, 117, 254]

Relatively weak associations between dietary factors and plasma choline and betaine levels have previously been reported.[55] Although we did not estimate methionine intake, it therefore seems reasonable to conclude that dietary intake of labile methyl groups did not severely impact the overall risk relationships observed in our studies.

6 CONCLUSIONS

The aims of our patient population studies were to investigate prospective risk relationships between plasma DMG with incident AMI and mortality, as well as systemic and urinary choline metabolites in relation to incident T2D. Additionally, we explored temporal changes in systemic and urinary choline metabolites, also according to treatment with B-vitamins crucial for enzymes in the choline oxidation pathway. In conclusion, the current thesis answered the study aims as follows:

6.1 STUDY I

Plasma DMG was positively associated with the risk of incident AMI during about four and a half years of follow-up. The risk associations were independent of traditional CHD risk factors and remained also when adjusting for potential confounders. Adding plasma DMG to a model of traditional CHD risk factors improved model discrimination and suggested enhanced classification of patients at risk. Treatment with folic acid and vitamin B12 lowered plasma DMG levels during repeated measures. Among patients allocated to placebo treatment, plasma DMG showed excellent test-retest reliability.

6.2 STUDY I I

Plasma DMG was positively related to long-term risk of all-cause and CVD mortality in two independent cohorts of patients with suspected SAP and AMI, respectively. The risk associations did not seem to be explained by traditional CVD risk factors, nor by potential confounders, and there were indications of improved risk prediction for plasma DMG beyond traditional CVD risk factors. The attenuation of temporal plasma DMG increments by combined folic acid and B12 treatment was consistent in both cohorts, as was the within-person reproducibility of plasma DMG.

6.3 STUDY III

Low plasma betaine and high urine betaine, DMG and sarcosine were related to increased risk of incident T2D throughout 7 years of follow-up. The risk relationships were not dependent on several T2D risk factors or potential confounders, and adding

either plasma betaine or urine sarcosine suggested improved model discrimination and reclassification of patients at risk. Both metabolites also showed good within-person reproducibility. As compared to placebo, treatment with folic acid with or without vitamin B6 led to relative increases in plasma choline and betaine, but decreases in plasma DMG and serum sarcosine. Similar, although less pronounced alterations were seen for urine choline metabolites.

Our results partly provide important validation on previous results in smaller epidemiological studies, but also add new knowledge into the field of choline metabolism. As described above, the present thesis therefore generates several novel hypotheses relating to diseases like CVD and diabetes, which determinate morbidity and mortality worldwide, thereby warranting further studies within this research field.

7   FUTURE PERSPECTIVES

Our research group will continue to explore the choline oxidation pathway in relation to CVD and T2D, with a particular focus on lipid and energy homeostasis.

We are currently in the process of linking both the WECAC and the NORVIT cohorts (collectively named the Norwegian Study on Coronary Heart Disease; NORCAD) with data from extended follow-up on incident CVD events and mortality throughout 2009, thereby providing us with even more reliable risk estimates; however we must also keep in mind that any potential effect modification by WENBIT and NORVIT intervention might be diluted when analyzing these extended follow-up data. Moreover, the research group is also applying for linkage between the NORCAD and the Norwegian prescription database, which will hopefully allow for more accurate identification of incident cases of diabetes, as well as the ability for exploring risk associations according to pharmacological treatment and patients' metabolic profiles.

In the dawn of the "big data" era it seems imperative that researchers across groups and borders take advantage of each other's data material, in order to optimize publicly funded projects. We therefore also seek to extend our collaboration with other research environments, not least to validate epidemiological findings, but also because experimental research among both animals and humans will provide valuable information on novel pathophysiological pathways. For example, a human dietary intervention trial is about to be launched in our department, partly based upon findings from studies of choline- and one-carbon metabolites in relation to dietary intake patterns in the WENBIT, as well as experiments on mice carried out by the collaborating Lipid Research Group at the University of Bergen.

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Table 1. Baseline characteristics according to type 2 diabetes at baseline

| | Type 2 diabetes at baseline | | | | |
| | No | | Yes | | P^b |
	n^a		n^a		
Age, years	3621	62 (55, 69)	449	65 (58, 72)	<0.000001
Male gender, n (%)	3621	2603 (71.9)	449	325 (72.4)	0.48
Prior CVD, n (%)	3621	2044 (56.4)	449	298 (66.4)	0.001
Current smoking, n (%)	3621	1175 (32.4)	449	113 (25.2)	0.09
Estimated total daily intake	1735		204		
energy, kcal		2052 (1663, 2503)		1912 (1530, 2384)	0.12
choline, mg		242 (193, 302)		240 (182, 299)	0.83
betaine, mg		136 (105, 169)		133 (104, 168)	0.20
BMI, kg/m^2	3618	26.1 (24.1, 28.7)	449	28.1 (25.4, 31.4)	<0.000001
HbA1c, %	3621	6.0 (5.3, 6.6)	449	7.7 (6.7, 8.9)	<0.000001
Plasma glucose, mmol/L	3621	5.5 (5.0, 6.2)	449	10.0 (7.7, 12.5)	<0.000001
HOMA-2	877^c				
β-cell function, %		53 (43, 80)		-	-
insulin sensitivity, %		238 (88, 265)		-	-
insulin resistance		0.40 (0.40, 1.10)		-	-

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	N		N		p
eGFR, mL/min per 1.73m^2	3621	91 (79, 99)	448	90 (74, 99)	<0.001
Plasma/serum					
CRP, mg/L	3620	1.74 (0.85, 3.51)	449	2.15 (1.09, 4.81)	0.009
HDL-C, mmol/L	3620	1.26 (1.01, 1.50)	449	1.10 (0.90, 1.30)	<0.000001
triglycerides, mmol/L	3617	1.47 (1.06, 2.09)	449	1.80 (1.29, 2.63)	<0.000001
ALT, IU/L	3025	28 (20, 38)	378	30 (22, 42)	0.005
total homocysteine, µmol/L	3621	10.4 (8.7, 12.5)	449	10.7 (8.6, 12.9)	0.30
methionine, µmol/L	3621	25.6 (22.5, 31.9)	449	26.7 (22.5, 33.1)	0.31
choline metabolites, µmol/L					
TMAO	3610	5.6 (3.6, 9.3)	446	7.2 (4.3, 12.3)	0.0004
choline	3621	9.6 (8.2, 11.4)	449	10.1 (8.4, 12.2)	0.10
betaine	3621	39.4 (32.5, 48.1)	449	35.6 (28.3, 45.0)	<0.000001
dimethylglycine	3621	4.1 (3.4, 5.1)	449	4.2 (3.2, 5.2)	0.88
sarcosine	3345	1.5 (1.2, 1.8)	422	1.4 (1.1, 1.8)	0.92
B-vitamers					
riboflavin, nmol/L	3605	11.0 (7.4, 18.0)	442	12.9 (8.6, 21.2)	<0.001
folate, nmol/L	3619	10.0 (7.3, 14.6)	449	10.8 (7.9, 15.6)	0.008
cobalamin, pmol/L	3181	362 (275, 466)	400	358 (270, 464)	0.53

PLP, nmol/L	3605	41.5 (29.6, 59.8)	442	39.0 (27.4, 59.4)	0.21
Urine					
choline metabolites, mmol/mol creatinine					
choline	3242	1.98 (1.47-2.69)	399	2.81 (1.85, 4.28)	<0.000001
betaine	3242	6.96 (4.67-42.25)	399	22.27 (10.21, 45.25)	<0.000001
dimethylgycine	3242	3.0 (1.98, 4.64)	399	5.61 (3.50, 8.09)	<0.000001
sarcosine	3242	0.13 (0.09, 0.20)	398	0.25 (0.15, 0.42)	<0.000001
albumin, g/mmol creatinine	2952	0.52 (0.38, 0.86)	375	1.01 (0.55, 3.06)	<0.000001
Medications and supplements, n (%)					
prior to baseline					
statin	3621	2601 (71.8)	449	349 (77.9)	0.009
folic acid	3407	313 (9.2)	408	42 (10.3)	0.39
multivitamins	3407	520 (15.3)	408	46 (11.3)	0.05
at discharge from hospital					
statin	3621	2875 (79.4)	-	-	-
folic acid	3407[d]	226 (6.6)	-	-	-
multivitamin	3407[d]	203 (6.0)	-	-	-

ALT, alanine aminotransferase; BMI, body mass index; CRP, C-reactive protein; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin; HDL-C, high density lipoprotein cholesterol; HOMA2, homeostatic model assessment; PLP, 5'-pyridoxal phosphate; TMAO, trimethylamine *N*-oxide.

[a]Patients with valid measurements

[b]Adjusted for age, gender and fasting status

[c]Fasting patients without established diabetes

[d]Patients included in the Western Norway B-Vitamin Intervention Trial were instructed not to use any additional vitamin supplements

Table 2. Baseline characteristics according to incident type 2 diabetes during follow-up

	Type 2 diabetes during follow-up				P^b
	No		Yes		
	n^a		n^a		
Age, years	3388	62 (54, 69)	233	62 (56, 70)	0.06
Male gender, n (%)	3388	2434 (71.8)	233	169 (72.5)	0.61
Prior CVD, n (%)	3388	1897 (56.0)	233	147 (63.1)	0.06
Current smoking, n (%)	3388	1103 (32.6)	233	72 (30.9)	0.96
Estimated total daily intake	1605		130		
energy, kcal		2053 (1672, 2509)		1997 (1596, 2418)	0.77
choline, mg		242 (193, 302)		242 (182, 301)	0.71
betaine, mg		136 (105, 169)		135 (105, 169)	0.59
BMI, kg/m^2	3385	26.0 (23.9, 28.4)	233	28.9 (26.3, 31.4)	<0.00001
HbA1c, %	3388	5.9 (5.3, 6.6)	233	6.2 (5.5, 7.0)	<0.00001
Plasma glucose, mmol/L	3388	5.5 (5.0, 6.1)	233	6.6 (5.8, 8.3)	<0.00001
HOMA-2	835		42		
β-cell function, %		53 (43, 80)		67 (46, 106)	0.19
insulin sensitivity, %		247 (90, 266)		101 (39, 219)	<0.00001
insulin resistance		0.40 (0.40, 1.10)		1.00 (0.48, 2.55)	<0.00001

	N	Median (IQR)	N	Median (IQR)	p
eGFR, mL/min per 1.73m^2	3388	91 (79, 99)	233	91 (80, 98)	0.13
Plasma/serum					
CRP, mg/L	3387	1.71 (0.84, 3.43)	233	2.34 (1.17, 4.32)	0.09
HDL-C, mmol/L	3387	1.30 (1.08, 1.50)	233	1.10 (0.90, 1.30)	<0.00001
triglycerides, mmol/L	3384	1.43 (1.05, 2.05)	233	1.96 (1.35, 2.69)	<0.00001
ALT, IU/L	2835	27 (20, 38)	190	32 (24, 48)	0.008
total homocysteine, µmol/L	3388	10.4 (8.7, 12.5)	233	10.5 (8.6, 12.9)	0.79
methionine, µmol/L	3388	26.5 (22.5, 31.8)	233	26.9 (22.4, 33.9)	0.08
choline metabolites, µmol/L					
TMAO	3378	5.5 (3.6, 9.3)	232	5.9 (4.2, 9.4)	0.77
choline	3388	9.6 (8.2, 11.4)	233	10.0 (8.2, 11.4)	0.73
betaine	3388	39.5 (32.8, 48.2)	233	36.9 (28.8, 44.6)	0.0009
dimethylglycine	3388	4.1 (3.4, 5.1)	233	4.2 (3.4, 5.2)	0.56
sarcosine	3127	1.5 (1.2, 1.8)	218	1.5 (1.2, 1.8)	0.84
B-vitamers					
riboflavin, nmol/L	3372	11.0 (7.3, 17.8)	233	12.2 (7.7, 20.2)	0.42
folate, nmol/L	3386	10.0 (7.3, 14.6)	233	10.2 (7.8, 15.6)	0.78
cobalamin, pmol/L	2969	363 (275, 467)	212	350 (275, 445)	0.27
PLP, nmol/L	3372	41.5 (29.7, 59.9)	233	41.8 (28.4, 59.8)	0.21

Urine

choline metabolites, mmol/mol creatinine					
choline	3037	1.97 (1.47, 2.69)	205	2.03 (1.45, 2.93)	1.00
betaine	3037	6.91 (4.67, 10.86)	205	8.18 (4.97, 13.60)	<0.00001
dimethylgycine	3037	3.02 (1.96, 4.56)	205	3.32 (2.15, 5.43)	0.61
sarcosine	3037	0.13 (0.08, 0.20)	205	0.16 (0.09, 0.24)	<0.00001
albumin, g/mmol creatinine	2778	0.51 (0.37, 0.84)	192	0.67 (0.46, 1.33)	0.89
Medications and supplements, n (%)					
prior to baseline					
statin	3388	2432 (71.8)	233	169 (72.5)	0.76
folic acid	3184	294 (9.2)	223	19 (8.5)	0.74
multivitamins	3184	488 (15.3)	223	32 (14.3)	0.73
at discharge from hospital					
statin	3388	2678 (79.0)	233	197 (84.5)	0.05
folic acid	3184[d]	215 (6.8)	223	11 (4.9)	0.71
multivitamin	3184[d]	191 (6.0)	223	12 (5.4)	0.76

ALT, alanine aminotransferase; BMI, body mass index; CRP, C-reactive protein; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin; HDL-C, high density lipoprotein cholesterol; HOMA2, homeostatic model assessment; PLP, 5'-pyridoxal phosphate; TMAO, trimethylamine N-oxide.

[a]Patients with valid measurements

[b]Adjusted for age, gender and fasting status

[c]Fasting patients without established diabetes

[d]Patients included in the Western Norway B-Vitamin Intervention Trial were instructed not to use any additional vitamin supplements

Table 3. The associations between systemic and urine choline metabolites with incident type 2 diabetes

	Odds ratio[a] (95% confidence interval)					
	Univariate	P	Adjusted for age, gender and fasting status	P	Multivariate model[b]	P
Plasma/serum						
TMAO	1.05 (0.92, 1.20)	0.46	1.02 (0.88, 1.17)	0.81	1.08 (0.91, 1.27)	0.39
choline	1.06 (0.93, 1.22)	0.37	1.01 (0.87, 1.16)	0.94	0.89 (0.75, 1.06)	0.19
betaine	0.78 (0.69, 0.89)	<0.001	0.72 (0.62, 0.83)	<0.00001	0.74 (0.63, 0.88)	<0.001
dimethylglycine	0.99 (0.86, 1.13)	0.83	0.94 (0.82, 1.09)	0.42	0.92 (0.77, 1.09)	0.33
sarcosine	0.97 (0.84, 1.11)	0.65	0.93 (0.81, 1.08)	0.34	1.07 (0.91, 1.26)	0.43
Urine[c]						
choline	1.09 (0.96, 1.25)	0.20	1.07 (0.92, 1.23)	0.39	1.09 (0.93, 1.29)	0.30
betaine	1.27 (1.11, 1.45)	0.001	1.25 (1.09, 1.43)	0.001	1.23 (1.06, 1.43)	0.006
dimethylglycine	1.23 (1.07, 1.42)	0.003	1.22 (1.06, 1.40)	0.007	1.19 (1.01, 1.39)	0.03
sarcosine	1.31 (1.14, 1.50)	<0.001	1.30 (1.13, 1.49)	<0.001	1.25 (1.07, 1.46)	0.004

TMAO, trimethylamine N-oxide.

[a]Per 1 standard deviation of logarithmically transformed variable

[b]Including age, gender, fasting status, BMI, HbA1c, estimated GFR, CRP, HDL-cholesterol, urine albumin-to-creatinine ratio, and the use of loop diuretics, thiazides, beta blockers, statins and angiotensin converting enzyme

inhibitors and/or angiotensin receptor blockers.

[c]Corrected for urine creatinine.

Table 4. Model discrimination and reclassification

	C-statistic (95% CI)	P	NRI>0 (95% CI)	P	IDI (95% CI)	P
Basic model[a]	0.750 (0.714, 0.786)	-	-	-	-	-
+ plasma betaine	0.751 (0.715, 0.787)	0.79	0.33 (0.19, 0.47)	<0.000001	0.0084 (0.0038, 0.0130)	<0.001
+ urine sarcosine	0.757 (0.721, 0.793)	0.19	0.16 (0.01, 0.31)	0.03	0.0048 (0.0002, 0.0094)	0.04

IDI, integrated discrimination improvement; NRI>0, continuous net reclassification index.

[a]Including age, gender, fasting status, BMI, HbA1c, estimated GFR, CRP, HDL-cholesterol, urine albumin-to-creatinine ratio, and the use of loop diuretics, thiazides, beta blockers, statins and angiotensin converting enzyme inhibitors and/or angiotensin receptor blockers.
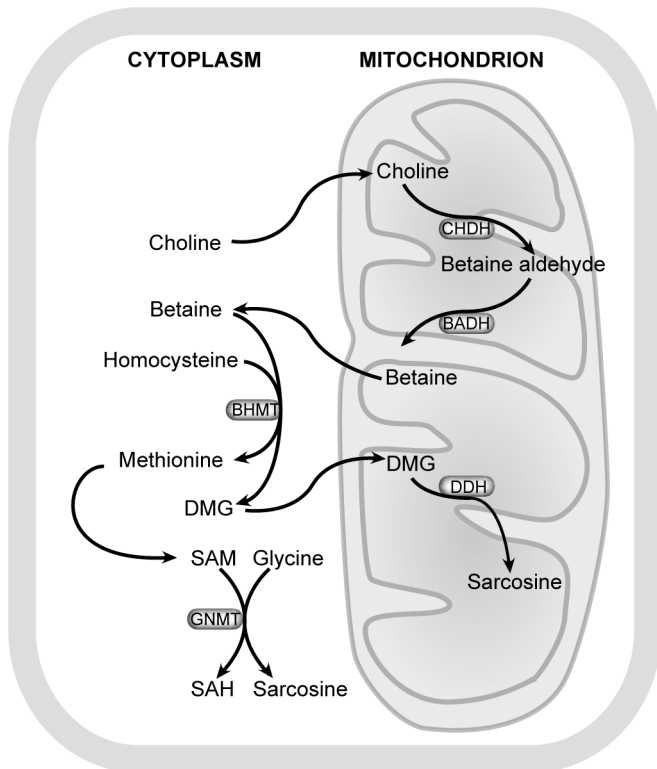
Figure 1. Choline metabolism and its ramifications to homocysteine and methyl group metabolism. BADH, betaine-aldehyde dehydrogenase; BHMT, betaine-homocysteine S-methyl transferase; CHDH, choline dehydrogenase; DDH, dimethylglycine dehydrogenase; DMG, dimethylglycine; GNMT, glycine-*N*-methyltransferase; SAH, *S*-adenosyl-homocysteine; SAM, *S*-adenosylmethionine; SDH, sarcosine dehydrogenase.
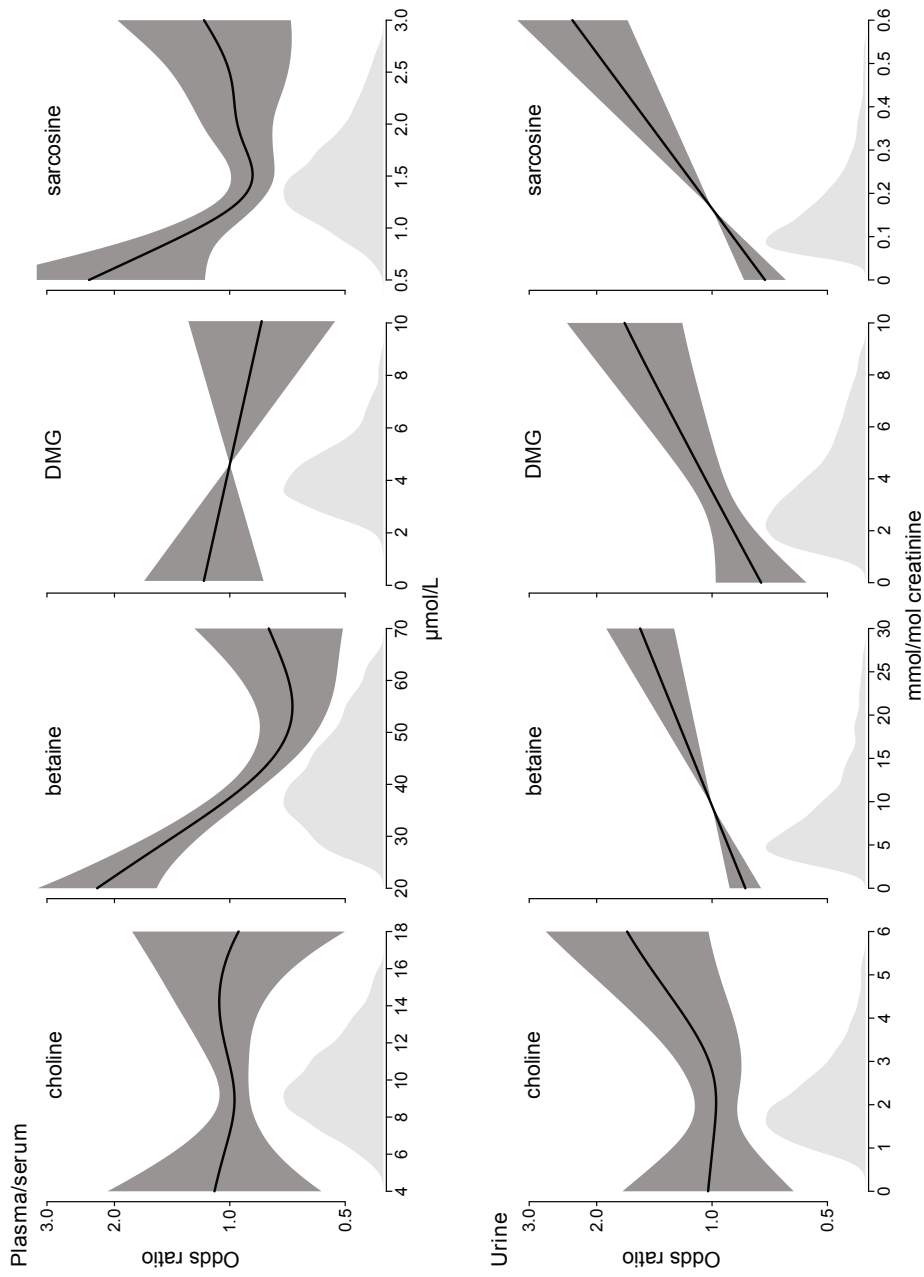
Figure 2. The relationships between systemic and urinary choline related metabolites and incident type 2 diabetes. The solid lines depict the smoothed spline of the generalized additive logistic regressions model, adjusted for age, gender and fasting status. The shaded areas depict 95% confidence intervals. Density plots are aligned along the X-axes.

Supplemental Material

WECAC, n = 4164

Patients with type 1 diabetes, n = 37; patients without data on HbA1c, n = 50; plasma glucose, n = 4; or most circulating choline metabolites, n = 3

Patients eligible for baseline analyses, n = 4070

Patients with type 2 diabetes, n = 449

Patients eligible for follow-up on
- plasma/serum metabolites, n = 3621
- urine metabolites, n = 3242

Patients with missing data on covariates in the multivariate model, n = 672

Patients eligible for multivariable follow-up analyses; n = 2949

Supplementary Figure 1. Flow-chart over patients eligible for the study.
HbA1c, glycated hemoglobin; WECAC, Western Norway Coronary Angiography Cohort.
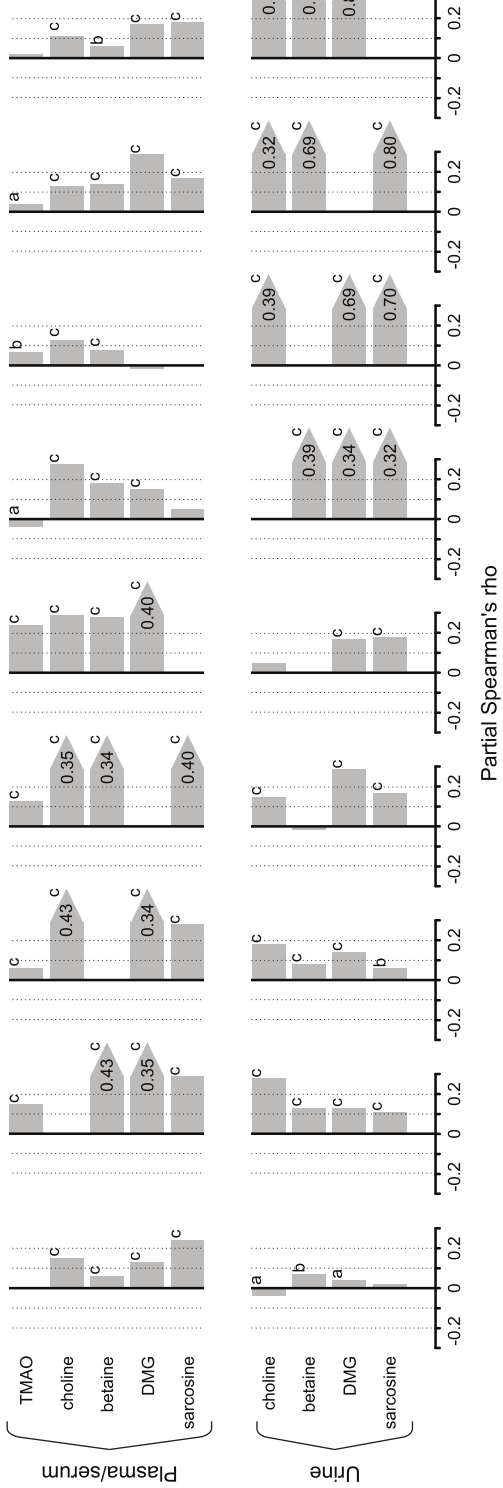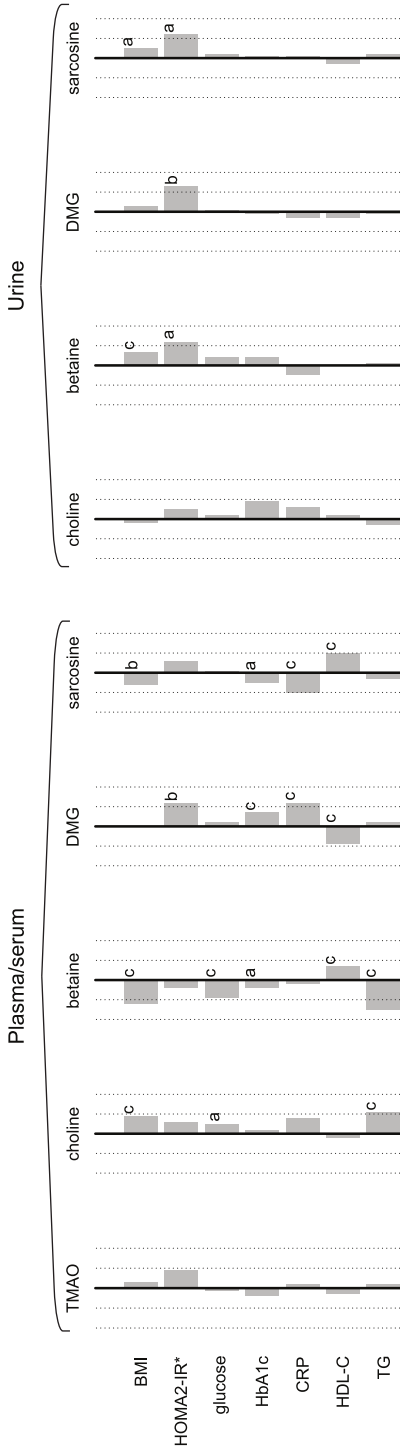
Supplementary Figure 2. Partial correlation plots between systemic and urinary choline metabolites and indices of insulin resistance among patients without type 2 diabetes at baseline.

All correlations are adjusted for age, gender and fasting status. Tapered bars indicate partial Spearman's rho > 0.30, with the actual value superimposed.

BMI, body mass index; CRP, C-reactive protein; DMG, dimethylglycine; HDL-C, high-density lipoprotein cholesterol; HOMA2-IR, homeostatic model assessment of insulin resistance; TG, triglycerides; TMAO, trimethylamine N-oxide.

[a]P<0.05
[b]P<0.01
[c]P<0.001

Supplementary Figure 3. Partial correlation plots between systemic and urinary choline metabolites and indices of insulin resistance among patients with type 2 diabetes at baseline.

All correlations are adjusted for age, gender and fasting status. Tapered bars indicate partial Spearman's rho > 0.30, with the actual value superimposed.

BMI, body mass index; CRP, C-reactive protein; DMG, dimethylglycine; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; TMAO, trimethylamine *N*-oxide.

[a]*P*<0.05
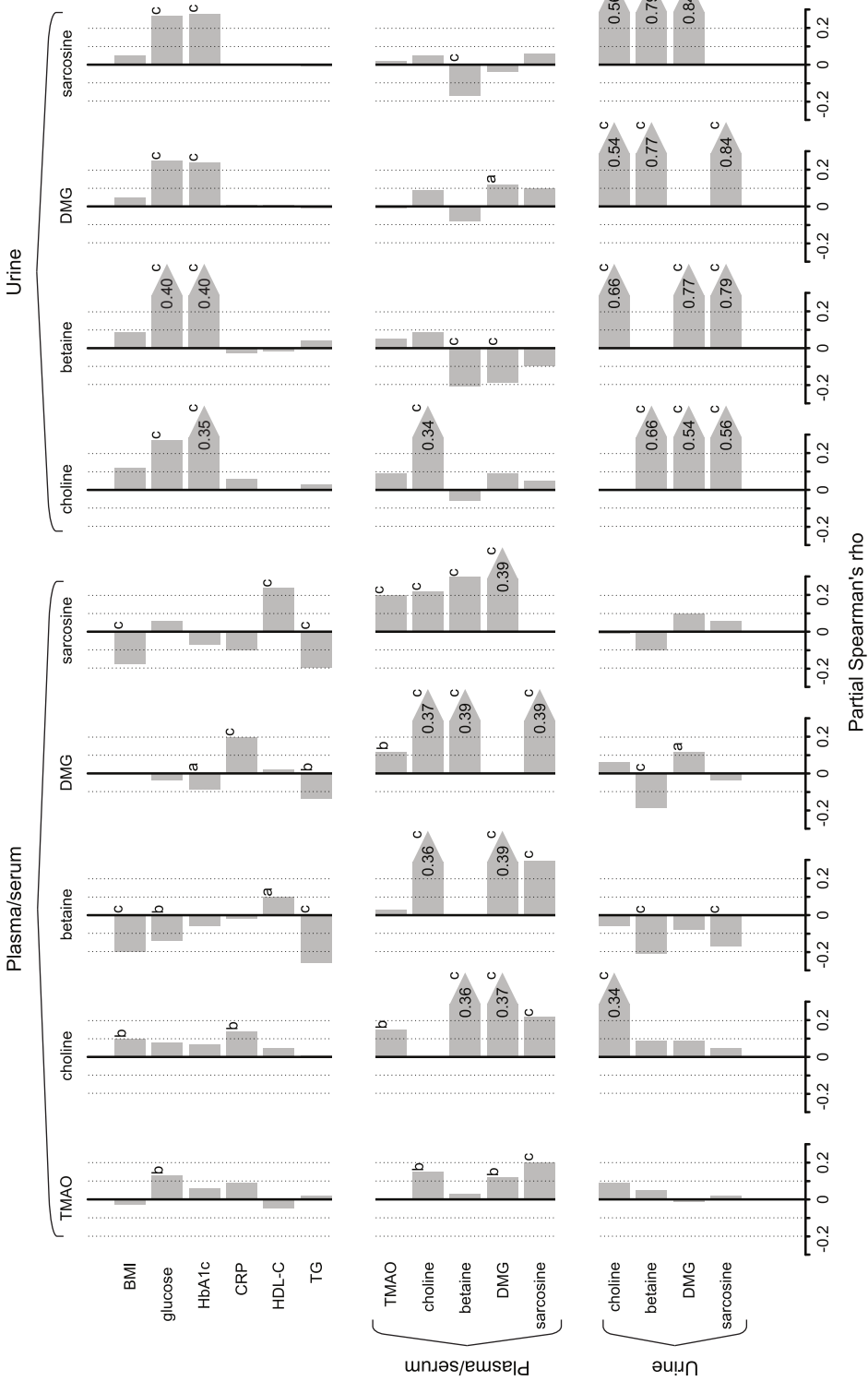[b]*P*<0.01
[c]*P*<0.001

Supplementary table 1. Baseline characteristics according to no, possible and established type 2 diabetes

	Type 2 diabetes at baseline					
	No		Possible		Established	
	n^a		n^a		n^a	
Age, years	2513	62 (54, 69)	1108	62 (55, 70)	449	65 (58, 72)[f]
Male gender, n (%)	2513	1838 (73.1)	1108	765 (69.0)[e]	449	325 (72.4)
Prior CVD, n (%)	2513	1428 (56.8)	1108	616 (55.6)	449	298 (66.4)[e]
Current smoking, n (%)	2513	806 (32.1)	1108	369 (33.3)	449	113 (25.2)
Estimated total daily intake	1343		392		204	
energy, kcal		2038 (1658, 2498)		2091 (1691, 2528)		1912 (1530, 2384)
choline, mg		240 (191, 301)		250 (199, 304)		240 (182, 299)
betaine, mg		135 (104, 169)		138 (106, 169)		133 (104, 168)
BMI, kg/m^2	2511	26.0 (23.9, 28.4)	1107	26.6 (24.4, 29.2)[f]	449	28.1 (25.4, 31.4)[f]
HbA1c, %	2513	5.6 (5.0, 6.0)	1108	7.0 (6.7, 7.5)[f]	449	7.7 (6.7, 8.9)[f]
Plasma glucose, mmol/L	2513	5.4 (5.0, 6.1)	1108	5.7 (5.1, 6.5)[f]	449	10.0 (7.7, 12.5)[f]
HOMA-2	568[b]		309[b]			

	n		n		n	
β-cell function, %		53 (43, 78)		51 (40-84)		-
insulin sensitivity, %		253 (94, 266)		227 (76-263)		-
insulin resistance		0.40 (0.40, 1.10)		0.40 (0.40-1.30)		-
eGFR, mL/min per 1.73m^2	2513	91 (79, 99)	1108	90 (78-99)	448	90 (74-99)
Plasma/serum						
CRP, mg/L	2513	1.69 (0.83, 3.38)	1107	1.86 (0.89, 3.78)	449	2.15 (1.09, 4.81)[f]
HDL-C, mmol/L	2512	1.26 (1.02, 1.50)	1108	1.28 (1.00, 1.50)[d]	449	1.10 (0.90, 1.30)[f]
triglycerides, mmol/L	2510	1.44 (1.06, 2.03)	1107	1.51 (1.08, 2.24)[e]	449	1.80 (1.29, 2.63)[f]
ALT, U/L	2105	27 (20, 38)	920	28 (20, 39)	378	30 (22, 42)[f]
total homocysteine, µmol/L	2513	10.4 (8.7, 12.5)	1108	10.5 (8.7, 12.6)	449	10.7 (8.6, 12.9)
methionine, µmol/L	2513	26.8 (22.6, 32.1)	1108	26.1 (22.1, 31.2)[d]	449	26.7 (22.5, 33.1)
choline metabolites, µmol/L						
TMAO	2504	5.6 (3.6, 9.4)	1106	5.4 (3.4, 9.2)	446	7.2 (4.3, 12.3)[d]
choline	2513	9.6 (8.2, 11.4)	1108	9.7 (8.2, 11.5)	449	10.1 (8.4, 12.2)[d]
betaine	2513	39.7 (33.0, 48.4)	1108	38.5 (31.5, 47.1)	449	35.6 (28.3, 45.0)[f]
dimethylglycine	2513	4.1 (3.4, 5.0)	1108	4.2 (3.4, 5.2)	449	4.2 (3.2, 5.2)

	N		N		N	
sarcosine	2274	1.5 (1.2, 1.9)	1071	1.4 (1.2, 1.8)	422	1.4 (1.1, 1.8)[a]
B-vitamers						
riboflavin, nmol/L	2502	11.0 (7.3, 17.8)	1103	11.2 (7.6, 18.4)	442	12.9 (8.6, 21.2)
folate, nmol/L	2511	10.0 (7.2, 14.9)	1108	10.0 (7.6, 14.1)	449	10.8 (7.9, 15.6)[d]
cobalamin, pmol/L	2215	358 (272, 453)	966	374 (283, 499)	400	358 (270, 464)
PLP, nmol/L	2502	42.1 (29.8, 60.4)	1103	40.6 (29.2, 58.2)	442	39.0 (27.4, 59.4)
Urine						
choline metabolites, mmol/mol creatinine	2258		984		399	
choline		1.95 (1.44, 2.63)		2.07 (1.55, 2.86)[d]		2.81 (1.85, 4.28)[f]
betaine		6.77 (4.59, 10.76)		7.38 (4.78, 11.66)		22.27 (10.21, 45.25)[f]
dimethylglycine		3.00 (2.01, 4.51)		3.15 (1.86, 4.92)		5.61 (3.50, 8.09)[f]
sarcosine		0.13 (0.08, 0.20)		0.13 (0.09, 0.20)		0.25 (0.15, 0.42)[f]
albumin, g/mmol creatinine	2106	0.51 (0.37, 0.82)	864	0.57 (0.39, 0.97)[e]	379	1.01 (0.55, 3.06)[f]
Medications and supplements, n (%)						
Prior to baseline						
statin	2513	1790 (71.2)	1108	811 (73.2)	448	349 (77.9)[e]

folic acid	2391	183 (7.7)	1016	130 (12.8)[f]	408	42 (10.3)
multivitamins	2391	367 (15.3)	1016	153 (15.1)	408	46 (11.3)[d]
At discharge from hospital[c]						
statin	2513	2014 (80.1)	1108	861 (77.7)	-	-
folic acid	2391	116 (4.9)	1016	110 (10.8)[f]	-	-
multivitamins	2391	122 (5.1)	1016	81 (8.0)[f]	-	-

ALT, alanine aminotransferase; BMI, body mass index; CRP, C-reactive protein; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin; HDL-C, high density lipoprotein cholesterol; HOMA2, homeostatic model assessment; PLP, 5'-pyridoxal phosphate; TMAO, trimethylamine N-oxide.

[a]Patients with valid measurements

[b]Fasting patients with stable angina pectoris

[c]Patients were instructed not to use any additional vitamin supplements after inclusion into the Western Norway B-Vitamin Intervention Trial

[d]$P<0.05$; [e]$P<0.01$; [f]$P<0.001$ in mixed linear or logistic regression models, adjusted for age, gender and fasting status. The category "No T2D at baseline" is used as reference.

Supplementary table 2. Choline metabolites at baseline and after 1 year according to WENBIT study treatment

	Baseline	1 year
Plasma/serum, µmol/L		
TMAO		
placebo (ref)	9.0 (8.3, 9.7)	8.5 (7.7, 9.3)
FA+B12+B6	8.2 (7.5, 8.9)	7.6 (6.9, 8.4)
FA+B12	8.6 (7.9, 9.3)	7.9 (7.1, 8.6)
B6	7.7 (6.9, 8.4)	7.6 (6.9, 8.4)
choline		
placebo (ref)	9.8 (9.6, 10.0)	10.4 (10.2, 10.7)[b]
FA+B12+B6	9.7 (9.5, 9.9)	11.0 (10.7, 11.2)[c]
FA+B12	9.7 (9.5, 9.9)	11.0 (10.7, 11.2)[c]
B6	9.9 (9.7, 10.2)	10.5 (10.2, 10.7)
betaine		
placebo (ref)	40.7 (39.6, 41.8)	43.1 (42.0, 44.4)[b]
FA+B12+B6	40.7 (39.6, 41.8)	49.6 (48.4, 50.8)[d]
FA+B12	41.0 (39.9, 42.1)	48.1 (47.0, 49.3)[d]
B6	42.0 (40.8, 43.1)	45.1 (44.0, 46.3)
dimethylglycine		
placebo	4.4 (4.3, 4.6)	4.6 (4.4, 4.7)[a]
FA+B12+B6	4.2 (4.1, 4.4)	4.1 (4.0, 4.3)[c]
FA+B12	4.4 (4.2-4.5)	4.3 (4.2-4.5)[c]
B6	4.2 (4.0-4.3)	4.5 (4.3-4.6)[c]
sarcosine		

placebo	1.63 (1.57-1.68)	1.89 (1.76-2.03)[b]
FA+B12+B6	1.61 (1.56-1.66)	1.55 (1.43-1.68)[d]
FA+B12	1.58 (1.53-1.63)	1.55 (1.41-1.69)[c]
B6	1.59 (1.54-1.64)	1.66 (1.52-1.80)[c]

Urine, mmol/mol creatinine

choline

placebo	1.98 (1.87-2.10)	2.20 (1.09-2.32)[b]
FA+B12+B6	2.13 (2.02-2.24)	2.35 (2.24-2.47)
FA+B12	2.12 (2.01-2.23)	2.29 (2.17-2.40)
B6	2.13 (2.02-2.24)	2.14 (2.03-2.26)[c]

betaine

placebo	10.0 (9.0-10.9)	10.5 (9.6-11.5)
FA+B12+B6	9.3 (8.4-10.2)	11.0 (10.0-11.9)
FA+B12	9.3 (8.4-10.3)	10.0 (9.1-11.0)
B6	9.5 (8.6-10.4)	9.8 (8.9-10.8)

dimethylglycine

placebo	4.1 (3.7-4.4)	4.5 (4.1-4.9)[b]
FA+B12+B6	3.8 (3.4-4.1)	3.6 (3.2-3.9)[d]
FA+B12	3.7 (3.4-4.1)	3.6 (3.2-3.9)[d]
B6	3.6 (3.3-4.0)	3.8 (3.5-4.2)

sarcosine

placebo	0.18 (0.16-0.19)	0.19 (0.18-0.20)[a]
FA+B12+B6	0.16 (0.15, 0.17)	0.13 (0.12, 0.14)[d]
FA+B12	0.17 (0.16, 0.18)	0.13 (0.11, 0.14)[d]
B6	0.16 (0.15, 0.17)	0.18 (0.16, 0.19)

Values are given as mean (95% confidence interval).

B6, vitamin B6; B12, vitamin B12; FA, folic acid; TMAO, trimethylamine *N*-oxide; WENBIT, Western Norway B-Vitamin Intervention Trial.

[a]*P*<0.05 for difference from baseline; [b]*P*<0.001 for difference from baseline; [c]*P*<0.05 for difference from placebo; [d]*P*<0.001 for difference from placebo.

Supplementary table 3. The associations between systemic and urine choline metabolites, and incident type 2 diabetes, excluding patients with indices of possible type 2 diabetes at baseline

	Odds ratio[a] (95% CI)					
	Univariate	P	Adjusted for age, gender and fasting status	P	Multivariate model[b]	P
Plasma/serum						
TMAO	1.18 (1.00, 1.39)	0.05	1.13 (0.95, 1.34)	0.17	1.13 (0.92, 1.40)	0.25
choline	1.08 (0.90, 1.29)	0.40	1.01 (0.84, 1.21)	0.93	0.86 (0.69, 1.08)	0.19
betaine	0.84 (0.71, 1.00)	0.05	0.77 (0.64, 0.93)	0.007	0.83 (0.66, 1.03)	0.09
dimethylglycine	1.05 (0.89, 1.24)	0.57	1.00 (0.83, 1.19)	0.97	1.05 (0.85, 1.30)	0.63
sarcosine	0.94 (0.78, 1.13)	0.50	0.90 (0.75, 1.09)	0.30	1.07 (0.87, 1.33)	0.52
Urine[c]						
choline	1.00 (0.83, 1.21)	0.98	0.97 (0.79, 1.18)	0.73	1.07 (0.86, 1.32)	0.56
betaine	1.19 (0.99, 1.42)	0.06	1.16 (0.96, 1.39)	0.12	1.18 (0.97, 1.44)	0.09
dimethylglycine	1.19 (0.99, 1.43)	0.06	1.16 (0.97, 1.40)	0.11	1.21 (0.99, 1.49)	0.07

| sarcosine | 1.22 (1.02, 1.46) | 0.03 | 1.19 (0.98, 1.43) | 0.07 | 1.22 (1.00, 1.50) | 0.05 |

CI, confidence interval; TMAO, trimethylamine *N*-oxide.

[a]Per 1 standard deviation of logarithmically transformed variable

[b]Including age, gender, fasting status, body mass index, glycated hemoglobin, estimated glomerular filtration rate, C-reactive protein, high-density lipoprotein cholesterol, urine albumin-to-creatinine ratio, and the use of loop diuretics, thiazides, beta blockers, statins and angiotensin converting enzyme inhibitors and/or angiotensin receptor blockers.

[c]Corrected for urine creatinine.

Supplementary table 4. The associations between systemic and urine choline metabolites, and incident type 2 diabetes within the WENBIT study population during the in-trial follow-up period

	Odds ratio[a] (95% CI)					
	Univariate	P	Adjusted for age, gender and fasting status	P	Multivariate model[b]	P
Plasma/serum						
TMAO	1.08 (0.86, 1.35)	0.52	1.04 (0.82, 1.31)	0.75	1.06 (0.81, 1.39)	0.65
choline	1.12 (0.88, 1.42)	0.37	1.06 (0.83, 1.37)	0.64	0.98 (0.75, 1.29)	0.91
betaine	0.88 (0.70, 1.11)	0.28	0.79 (0.62, 1.01)	0.06	0.88 (0.68, 1.15)	0.36
dimethylglycine	0.95 (0.75, 1.21)	0.69	0.92 (0.72, 1.19)	0.53	0.86 (0.65, 1.14)	0.30
sarcosine	1.12 (0.88, 1.43)	0.35	0.99 (0.83, 1.19)	0.95	1.11 (0.89, 1.37)	0.36
Urine[c]						
choline	1.09 (0.87, 1.36)	0.48	1.15 (0.91, 1.45)	0.25	1.14 (0.89, 1.46)	0.32
betaine	1.33 (1.07, 1.64)	0.009	1.36 (1.10, 1.69)	0.005	1.28 (1.03, 1.59)	0.02
dimethylglycine	1.23 (0.98, 1.54)	0.07	1.27 (1.01, 1.60)	0.04	1.26 (0.99, 1.61)	0.06

| sarcosine | 1.32 (1.06, 1.65) | 0.02 | 1.36 (1.09, 1.70) | 0.007 | 1.31 (1.04, 1.65) | 0.02 |

CI, confidence interval; TMAO, trimethylamine *N*-oxide; WENBIT, Western Norway B-Vitamin Intervention Trial

[a]Per 1 standard deviation of logarithmically transformed variable

[b]Including age, gender, fasting status, body mass index, glycated hemoglobin, estimated glomerular filtration rate, C-reactive protein, high-density lipoprotein cholesterol, urine albumin-to-creatinine ratio, and the use of loop diuretics, thiazides, beta blockers, statins and angiotensin converting enzyme inhibitors and/or angiotensin receptor blockers.

[c]Corrected for urine creatinine

Supplementary table 5. The intraclass correlation coefficients for plasma betaine and urine sarcosine over 1 year

	ICC (95% CI)			
	Placebo	FA + B12 + B6	FA + B12	B6
Plasma betaine	0.62 (0.56, 0.66)	0.43 (0.36, 0.50)	0.44 (0.37, 0.51)	0.65 (0.60, 0.70)
Urine sarcosine	0.69 (0.64, 0.74)	0.49 (0.41, 0.56)	0.37 (0.28, 0.46)	0.73 (0.68, 0.77)

B6, vitamin B6; B12, vitamin B12; CI, confidence interval; FA, folic acid; ICC, intraclass correlation coefficient.