MTHFD1 and relevant biomarkers in cardiovascular disease

Observational studies in patients with suspected stable angina pectoris in Norway

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Scientific environment

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Abbreviations

ACBA1	ATP–binding cassette transporter A1
ACBG1	ATP–binding cassette transporter G1
ACS	Acute coronary syndrome
apo	Apolipoprotein
BECAC	Bergen Coronary Angiography Cohort
CAD	Coronary artery disease
CVD	Cardiovascular disease
CVDNOR	Cardiovascular Disease in Norway
FFA	Free fatty acid
GNMT	Glycine-N-methyltransferase
HMG-CoA	3-hydroxy-3-methylglutaryl CoA
HWE	Hardy-Weinberg equilibrium
IQR	Interquartile range
MAF	Minor allele frequency
MTHFD1	Methylenetetrahydrofolate dehydrogenase
NAFLD	Nonalcoholic fatty liver disease
NEFA	Non-esterified fatty acids
NPC	Niemann-Pick C disease
OCM	One-carbon metabolism
RCT	Randomized clinical trial
SAM	S-adenosylmethionine
SAP	Stable angina pectoris
SHMT	Hydroxymethyltransferase
SMC	Smooth muscle cell
SRB1	Scavenger receptor type B1
WENBIT	Western Norway B Vitamin Intervention Trial

Abstract

Background: As the leading cause of death globally, cardiovascular disease (CVD) is a major public health burden. Epidemiological and experimental studies consistently demonstrate an association between elevated total plasma homocysteine and increased risk of CVD, but intervention studies with B vitamin treatments to lower homocysteine levels have failed to result in major clinical benefits. The highly interconnected network of one-carbon metabolism (OCM) makes it difficult to delineate pathways associated with pathogenesis and the responsible factors with a simple biomarker. On the other hand, genetic variations with implications on certain metabolic pathway may serve as a more robust proxy for life-time susceptibility to CVD outcomes, which may allow evaluation of causal inference.

Objectives: We investigated a trifunctional gene in OCM, methylenetetrahydrofolate dehydrogenase 1 (MTHFD1), in relation to the risk of acute myocardial infarction (AMI) in patients with suspected stable angina pectoris (SAP) and potential effect modifications from B vitamin treatments. Furthermore, since MTHFD1 and the interconversion between serine and glycine are the major sources of methyl groups for OCM, interactions of MTHFD1 with serine and glycine levels, as well as their independent risk associations, were also studied.

Methods: The study is based on the Western Norway B Vitamin Intervention Trial (WENBIT) and Bergen Coronary Angiography Cohort (BECAC), including 4164 patients with suspected SAP recruited during 2000-2004. All participants underwent blood sampling immediately before or after elective coronary angiography and were subsequently followed for clinical endpoints throughout the year 2006 (Paper I and III) or 2009 (Paper II). Specifically, for paper I and III, the study population was restricted to patients in WENBIT since genetic information was only

available in this cohort (Paper I, n=2381; Paper III, n=2571, respectively) while Paper II incorporated the whole population (n=4164).

Results: Paper I identified that a common and functional MTHFD1 polymorphism, *rs1076991*, was associated with increased risk of AMI. Subgroup analysis revealed that the risk association was likely introduced by the combined treatment with folic acid+B12 and vitamin B6. In paper II, we observed an inverse dose-response relationship between plasma glycine and risk of AMI, primarily in patients with elevated serum apolipoprotein B, LDL cholesterol and apolipoprotein A1. Paper III demonstrated that the risk associations of both plasma serine and glycine on AMI occurrence were modified by two common polymorphisms in the MTHFD1 gene: *rs2236225* and *rs1076991*.

Conclusion: The current project links a key gene in OCM to acute atherosclerotic complications, possibly by interacting with serine, glycine and lipid metabolism as well as with B vitamin treatment.

List of publications

I. Ding YP, Pedersen ER, Johansson S, Gregory JF, Ueland PM, Svingen GF, Helgeland Ø, Meyer K, Fredriksen Å, Nygård OK. B vitamin treatments modify the risk of myocardial infarction associated with a MTHFD1 polymorphism in patients with stable angina pectoris. *Nutr Metab Cardiovasc Dis* 2016;26(6):495-501.

II. Ding Y, Svingen GF, Pedersen ER, Gregory JF, Ueland PM, Tell GS, Nygård OK. Plasma glycine and risk of acute myocardial infarction in patients with suspected stable angina pectoris. *J Am Heart Assoc* 2015;5(1):e002621.

III. Ding YP, Pedersen ER, Svingen GF, Helgeland Ø, Gregory JF, Løland KH, Meyer K, Tell GS, Ueland PM, Nygård OK. MTHFD1 polymorphisms modify the associations of plasma glycine and serine with risk of AMI in patients with stable angina pectoris in WENBIT. *Circ Cardiovasc Genet.* 2016 Nov 21. pii: CIRCGENETICS.116.001483. [Epub ahead of print].

"*Everything we hear is an opinion, not a fact. Everything we see is a perspective, not the truth*."

-- Marcus Aurelius

Contents

1. Introduction

1.1 Cardiovascular disease

Cardiovascular diseases (CVDs) are a group of disorders of the heart and blood vessels, which includes atherosclerosis-related coronary heart disease (CHD), cerebrovascular disease and peripheral arterial disease, as well as non-atherosclerotic diseases like congenital heart disease, rheumatic heart disease, other diseases of the myocardium and valves, deep vein thrombosis and pulmonary embolism [1]. According to the report from World Health Organization, CVDs are responsible for over 17.5 million deaths in 2012, representing 31% of all global death [2]. Although over three quarters of CVD deaths take place in low- and middle-income countries [3], CVDs remains the leading cause of death in Norway, accounting for 33% of total deaths in 2014 [4].

1.2 Overview of atherosclerosis

Among global CVDs deaths in 2012, approximately 7.4 million (42%) were due to CHD and 6.7 million (38%) were due to stroke [2]. Thus, a majority of CVD deaths were caused by atherosclerosis related CVDs. Atherosclerosis is a complex and long-term pathological process has been reported to begin in childhood [5]. Although the detailed mechanism has not been fully understood, evidence suggests that atherogenesis begins with the formation of plaques, which is mainly made of fatty substances, cellular waste products, calcium, fibrin and cholesterol.

1.2.1 Cholesterol synthesis

Cholesterol is a crucial constituent for cell membranes and a precursor for the synthesis of sterol and non-sterol products. It can be derived

from diet or synthesized within the body. While approximately 300-500 mg of cholesterol is provided by dietary sources, human body synthesizes 700-900 mg of cholesterol per day [6].

As the major source of fat from the diet [7], triglyceride is broken down by intestinal and pancreatic lipases and is primarily absorbed as free fatty acids and 2-monoglycerides in the intestine [8]. Once across the intestinal barrier, triglyceride is resynthesized and packaged with apolipoprotein (apo) B-48 to form chylomicrons [9]. The main function of chylomicrons is to transport triglycerides from the intestine to the lung, liver, adipose and muscle tissue [10]. When chylomicrons enter the circulation, they interact with lipoprotein lipase, which is attached to the capillary endothelium of the tissues [11]. The lipoprotein lipase hydrolyses the triglyceride of chylomicrons, resulting in cholesterol-rich chylomicron remnants, free fatty acids (FFA) and monoglycerides [12]. While the released FFA and monoglycerides are picked up locally by body cells for use as energy sources, the cholesterol-rich chylomicron remnants are taken up by the liver cells, through a process which requires apo E for the recognition of the remnant particles [13].

The main site for cholesterol biosynthesis is the liver, followed by the intestine, adrenal glands and reproductive organs [14].Synthesis of cholesterol begins from the condensation of acetyl-CoA to form 3-hydroxy-3-methylglutaryl CoA (HMG-CoA), which is further reduced to mevalonate by HMG-CoA reductase with the involvement of NADPH as the rate-limiting agency [15]. After several energy-consuming steps, lanosterol is formed from mevalonate and can be further converted to cholesterol through a 19-step process [16]. This process is under negative feedback regulation: increased cholesterol intake from the diet suppresses hepatic HMG-CoA reductase and thus decrease cholesterol biosynthesis [17]. Notably, statins are competitive inhibitors of HMG-CoA

reductase. Treatment with statins has been shown to decrease blood cholesterol and diminish atherosclerotic CVD risks [18].

1.2.2 Apolipoproteins and lipoproteins

Apo B is an integral apoprotein found in two forms in humans, apo B-48 and apo B-100. Apo B-48 is synthesized exclusively by the small intestine and is essential for the assembly and secretion of chylomicrons to the blood [19]. Apo B-100, on the other hand, is synthesized in the liver and is essential for the initial lipidation of the nascent VLDL particle [20]. The main function of VLDL is to export cholesterol, phospholipids and triglycerides from the liver to the circulation. In the circulation, triglyceride is progressively broken down into FFA and glycerol thereby delivering energy source to tissues [21]. The resulting remnants can either be cleared from the circulation by the liver or undergoes hydrolysis by hepatic lipase to form LDL [22]. The LDL-particle is small enough to cross the vascular endothelium and supply tissues with cholesterol [23].

HDL, on the other hand, is crucial for the regulation of cholesterol content in cellular membranes, the transport of cholesterol, phospholipids and triglycerides from peripheral tissues back to the liver and in inter-organ exchange of these constituents [24]. HDL is synthesized in the liver and intestine as a lipid-poor particle comprised of apo A1 and phospholipid [25]. The free cholesterol can be subsequently taken up by the apoA1 or HDL binding to scavenger receptor type B1 (SRB1) involving the ATP–binding cassette transporter A1 (ABCA1) [26] and ATP–binding cassette transporter G1 (ABCG1) [27]. Cholesterol esters within HDL can directly be transferred to hepatocytes or other tissues expressing SRB1 [28], or taken up by endocytosis.

Disruptions of these receptors may cause cholesterol accumulation in peripheral tissues and macrophages [29].

1.2.3 Intracellular cholesterol trafficking

Intracellular cholesterol distribution is controlled by a combination of vesicle-mediated transport and protein-mediated monomeric transfer through the aqueous cytoplasm [30]. Visceral cholesterol and lipid trafficking is impaired in Niemann-Pick C (NPC) disease cells, in which exogenously-derived cholesterol and lipids are accumulated in the endosomal and lysosomal compartments [31]. Although the majority of NPC disease is caused by mutation of NPC1 gene [32], approximately 5% of the NPC disease is accounted by NPC2 gene mutation [33]. Current evidence suggests that NPC2 binds cholesterol and transfers it to the membranes of endosomal and lysosomal compartments rapidly [34]. Deficiency of NPC2 in mice has been associated with hepatic cholesterol accumulation [35].

1.2.4 Hepatic lipid accumulation

Patients with nonalcoholic fatty liver disease (NAFLD) are shown to be at increased risk of CVD [36]. NAFLD patients often have dyslipidemia along with other phenotypes of metabolic syndrome. Although the underlying mechanisms for the atherogenic dyslipidemia are not fully clear, it is seemingly derived from the impairment of hepatic lipid metabolism [37]. The main source of excess lipids entering the liver is non-esterified fatty acids (NEFAs) that are released by adipose tissue. These NEFAs possibly mediate insulin resistance [38]. Further, the hepatic lipid accumulation has been shown to promote the formation of apo B-100 and the secretion of VLDL particles with triglycerides [39]

leading to the rise of VLDL remnants and LDL particles and is therefore considered an independent CAD risk factor [40].

1.2.5 Development and progression of atherosclerosis

It has been suggested that the atheromatous plaques are initially developed in the monolayer of endothelial cells that lines the inner arterial surface [41]. Abnormal stimuli, such as dyslipidemia, hypertension or pro-inflammation mediators, may result in adhesion of circulating leukocytes on the surface of arterial endothelial cells [42]. The corresponding changes in endothelial permeability promote lipoproteins to enter the artery wall, where the lipoproteins may be further oxidized and subsequently taken up by monocyte-derived macrophages or endothelial derived smooth muscle cells (SMCs), leading to intracellular cholesterol accumulation and the formation of foam cells [43]. The activated endothelial cells and foam cells can secrete growth factors that stimulate proliferation and migration of arterial SMCs from tunica media (middle coat) into tunica intima (inner coat), forming a fibrous cap with collagen and elastin that covers the atherosclerotic lesion [44]. The ruptured plaques typically have thin, collagen-poor fibrous caps with few SMCs but abundant macrophages [45].

Numerous reports propose a key role of inflammation in the pathophysiology of atherosclerosis [46]. The inflammatory cells may interact with the SMCs and endothelial cells to promote plaque disruption and thrombosis of lipid cores [47]. Inflammation biomarkers, such as C-reactive protein (CRP) and interferon gamma are independent risk markers for cardiovascular disease [48, 49]. Notably, however, the associations of inflammatory biomarkers with risk of CAD do not necessarily reflect causality [50], and the dilemma of “which came first, the chicken or the egg?" remains to be solved.

1.3 Risk factors for CVDs

Large amounts of population-based studies have demonstrated associations between CVD and certain risk markers, which are usually the presence of combinations, such as advancing age, male gender, smoking, unhealthy diet, physical inactivity, obesity, hypertension, diabetes mellitus and hyperlipidemia [51]. Recent evidence has showed that 66% of the decline in total CHD events can be accounted by changes in established coronary risk factors [52]. However, searching for new biological markers can improve the accuracy for disease susceptibility evaluation, as well as to understand more of disease pathophysiology and potential therapeutic implications.

1.4 One-carbon metabolism and cardiovascular disease

One-carbon metabolism (OCM) is a metabolic network that integrates amino acids, glucose and vitamins for supporting the biosynthesis of nucleotides, lipids and proteins, as well as the maintenance of redox status and methylation reactions [53]. This network is compartmentalized in the mitochondria, nucleus and cytoplasm [54]. Mitochondrial OCM is required for the biosynthesis of formylated methionyl-tRNA; the catabolism of choline, purines, and histidine; and the interconversion of serine and glycine. Nucleic OCM is recently suggested to be involved in *de novo* thymidylate biosynthesis [55, 56]. Cytoplasmic OCM is providing the substrates for purine and thymidylate biosynthesis and remethylation of homocysteine to methionine [57].

Experimental and observational studies strongly suggest a role of hyperhomocysteinemia, resulting from OCM disruption, in the development of CVDs [58]. However, randomized clinical trials (RCTs)

aimed for secondary prevention have failed to reduce CVD risks by homocysteine-lowering vitamin B treatments [59]. The highly interconnected OCM network makes it difficult to delineate pathogenic pathways using classical observational approaches. On the contrary, genetic variation with implications on certain metabolic pathways may serve as a more robust proxy for life-time susceptibility to a certain disorder, which may provide more information on causal inference [60]. Indeed, associations between CVD risk factors and several OCM genetic variants have been widely researched [61-63]. However, few studies have focused on the cytosolic methylenetetrahydrofolate dehydrogenase (MTHFD1).

1.5 MTHFD1

1.5.1 MTHFD1 biochemistry

C1-tetrahydrofolate synthase (C1-THF) was first described by Hum *et al.* in 1988 [64]. It catalyzes three sequential reactions in the interconversion of one-carbon derivatives of THF for DNA syntheses and homocysteine remethylation. Two forms of C1-THF synthases are currently known, one in the mitochondrial matrix while the other one in the cytosol [65]. The cytosolic C1-THF is encoded by the MTHFD1 gene [66]. The C-terminal domain of MTHFD1 encodes 10-formylTHF synthetase activity while the N-terminal domain possesses 5,10-methenylTHF cyclohydrolase (C) and dehydrogenase (D) activities. MTHFD1 synthesis activity is responsible for the conversion of formate and THF to 10-formylTHF for *de novo* purine synthesis. The cyclohydrolase activity of MTHFD1 catalyzes the interconversion of 10-formylTHF and 5,10-methenylTHF, which can be further reduced to 5,10-methyleneTHF by the dehydrogenase activity (Figure 1). The 5,10-methyleneTHF reside at a branch point that is required for *de novo* thymidylate synthesis or can be alternatively reduced to 5-methylTHF for homocysteine remethylation [54].

1.5.2 MTHFD1 -1958G>A polymorphism

A common single nucleotide polymorphism (SNP, *rs2236225*), which exists in approximately 20% of European populations [67], has been widely studied. This SNP locates at nucleotide 1,958 of the gene, with a substitution of glutamate to arginine at amino acid position 653 in the synthetase domain of the enzyme [68].

An experimental study using MTHFD1 knockout cell showed that this polymorphism altered protein thermostability of MTHFD1 and reduced 36% of the half-life of the enzyme [69]. The same study also linked this mutation to a 25% decrease in purine biosynthesis rate. In addition, at the metabolic level, this SNP showed borderline significant effect on serum folate levels, but was not associated with plasma homocysteine levels in the same population [70]. Evidence also suggests that individuals carrying this polymorphism have increased susceptibility to choline deficiency under a restricted folate diet [71].

The SNP has been associated with a number of folate related pathologies. It has been associated with neural tube defects [68, 72], pregnancy loss [73], abruptio placentae [74], and congenital heart disease [69]. Moreover, this SNP is associated with higher risk of acute lymphoblastic leukemia [75], intestinal carcinogenesis [76] and gastric cancer [77].

1.5.3 MTHFD1 -105C>T polymorphism

MTHFD1 -105C>T (*rs1076991*) is another mutation located in the promoter region of MTHFD1 gene with a reported minor allele frequency (MAF) of 49% [78].

The minor T-allele has been associated with an approximately 62.5% drop in transcription rate of the MTHFD1 enzyme due to decreased promoter activity [79], which may result in lower amounts of available enzymes and reduce the MTHFD1 flux. Current evidence suggests no associations of this SNP with red cell folate or plasma homocysteine levels [79].

This SNP has been associated with acute lymphoblastic leukemia [80] and higher risk of neural tube defects when combining with *rs2236225* polymorphism [79]. Despite the increasing number of studies on genetic variants of MTHFD1, few studies have focused on associations with CVDs.

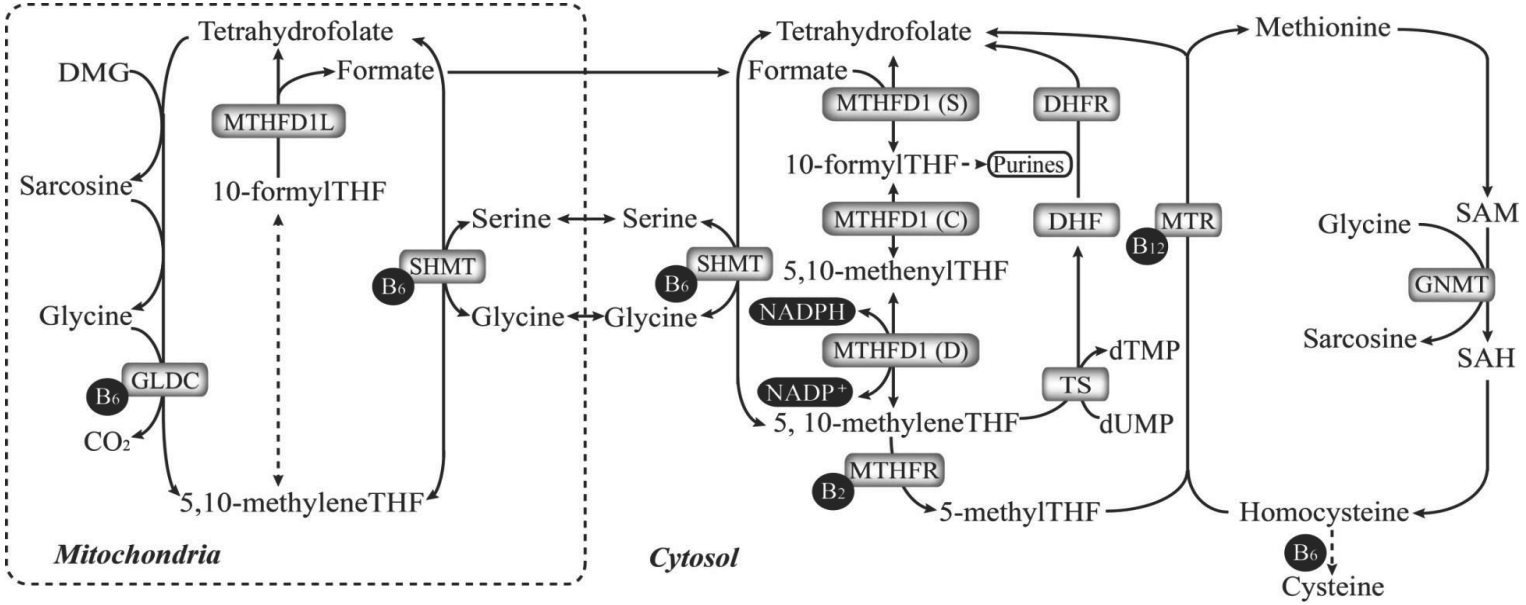

Figure 1. The role of MTHFD1 in the folate-mediated one carbon metabolism (OCM). MTHFD1 encodes a protein with three distinct activities, formate–tetrahydrofolate ligase (S), methenyltetrahydrofolate cyclohydrolase (C) and methylenetetrahydrofolate dehydrogenase (D). Those tetrahydrofolate derivatives catalyzed by MTHFD1 are closely involved in DNA synthesis and homocysteine remethylation. B vitamins (B2, B6, folic acid and B12) are crucial sources of coenzymes that maintain and modulate the normal functionalities of one carbon metabolism. Abbreviations: DHF, dihydrofolate; DHFR, dihydrofolate reductase; DMG, dimethylglycine; GLDC, glycine decarboxylase; GNMT, glycine N-methyltransferase; MTHFD1, methylenetetrahydrofolate dehydrogenase 1; MTHFR, methylenetetrahydrofolate reductase; MTR, 5-methyltetrahydrofolate-homocysteine methyltransferase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SHMT, serine hydroxymethyltransferase.

1.6 Serine and glycine metabolism

1.6.1 Serine metabolism and disease

Serine is a non-essential amino acid that can be synthesized endogenously from 3-phosphoglycerate, or converted from glycine by hydroxymethyltransferase (SHMT). It can also be imported from the extracellular environment by amino acid transporters.

In OCM, serine is a major methyl donor for cytosolic thymidylate and methionine synthesis [81]. Besides, serine can be metabolized directly through the transsulfuration pathway, potentially resulting in the generation of glutathione [53]. Serine may exert a crucial role in the central nervous system, as serine and its enantiomer d-serine have been suggested as novel biomarkers for Alzheimer's disease, schizophrenia and epilepsy [82, 83]. Serine has also been suggested as a potential treatment for amyotrophic lateral sclerosis [84]. However, serine metabolism has not previously been related to CVDs.

1.6.2 Glycine metabolism and diseases

Similar to serine, glycine is also a non-essential amino acid which can be obtained from the diet, or synthesized endogenously from threonine, choline, glyoxylate or serine [85].

In OCM, glycine levels have been shown to influence serine metabolism and methyl flux. The glycine-to-serine conversion rate were elevated in the hepatocytes of rats fed a glycine supplemented diet [86]. Additionally, cellular 5.10-methyleneTHF levels for homocysteine remethylation were decreased, whereas no significant impact on thymidylate synthesis was observed [81]. Increased plasma glycine levels is also associated with decreased s-adenosylmethionine (SAM) levels, possibly by the depletion of available 5-methylTHF, and/or by

stimulating the glycine N-methyltransferase (GNMT) pathway [81]. Notably, plasma and tissue glycine concentrations are regulated by the mitochondrial glycine cleavage system in a vitamin B6-depedent manner, which may therefore reflect vitamin B6 status [87].

Glycine is a predominant constituent of collagen and is utilized in the synthesis of several biologically important compounds, including glutathione, creatine, purines and glucose [88, 89]. It has been inversely associated with several CVD risk factors. Population-based studies have demonstrated a positive association of plasma glycine with estimated glucose disposal rate [90] and a negative association with HbA1c [91]. Plasma glycine has also been inversely associated with obesity [92, 93], hypertension [94] and diabetes mellitus [95, 96]. In addition, glycine intake was shown to decrease plasma free fatty acids, cholesterol and triglycerides levels in animal models [97, 98]. Although mounting evidence has linked glycine status with CVD risk factors, the relationships between circulating glycine levels and clinical cardiovascular endpoints have previously not been evaluated in large-scale observational studies.

1.6.3 Serine, glycine metabolism and MTHFD1

One-carbon units required for the synthesis of thymidylate and methionine are obtained both from formate through MTHFD1 and from the conversion of serine to glycine by SHMT. Therefore, 5,10-methyleneTHF represents a metabolic cross-point of these two sources of one-carbon units. Since both one-carbon sources may associate with CVD risks, it is of interest to explore if these two pathways may interact in association of atherosclerotic CVD outcomes.

2. Objectives

The main purpose of the present project was to investigate the role of the trifunctional MTHFD1 gene in relation to atherosclerotic disease risk in patients with suspected stable angina pectoris (SAP). Since the MTHFD1 function is intercorrelated with serine and glycine metabolism, their potential interactions as well as the independent risk association of plasma serine and glycine were also studied.

Specific aims:

Paper I: Evaluate the effect of a common and functional polymorphism in MTHFD1 on the risk of AMI and the potential effect modifications by randomized B vitamin treatments among 2381 patients with SAP from Western Norway B Vitamin Intervention Trial (WENBIT).

Paper II: Evaluate the risk association of plasma glycine with AMI and the potential effect modifications by lipid profile parameters among 4164 patients with suspected SAP from WENBIT and the Bergen Coronary Angiography Cohort (BECAC).

Paper III: Evaluate plasma serine and glycine levels in relation to AMI risk, as well as potential effect modifications by two common MTHFD1 polymorphisms among SAP patients in WENBIT (n=2571).

3. Material and Methods

3.1 Study design and population

BECAC includes 4164 cardiac patients who underwent elective coronary angiography for SAP, acute coronary syndromes (ACS) or aortic valve stenosis at Haukeland University Hospital, Bergen, Norway (n=3413) or Stavanger University Hospital, Stavanger, Norway (n=751) from 2000 to 2004. Due to capacity reasons, approximately two-thirds were included in WENBIT (ClinicalTrials.gov Identifier: NCT00354081).

WENBIT was a randomized, double-blind and placebo-controlled clinical trial designed to investigate the effect of B vitamin treatments on the risk of CVD events and mortality [59]. In specific, WENBIT had a 2x2 factorial design in which participants with angiographically verified coronary artery disease (CAD) were randomized to receive a daily capsule containing one of the following: combined treatment of folic acid 0.8 mg + vitamin B12 (cyanocobalamin) 0.4 mg + vitamin B6 (pyridoxine hydrochloride) 40 mg; folic acid + vitamin B12; vitamin B6; placebo. In Paper I and Paper III, we included SAP patients with complete genotype data from WENBIT, leading to 2381 subjects in Paper I and 2571 subjects in Paper III, respectively. On the other hand, Paper II includes 4164 patients with suspected SAP from WENBIT and the BECAC.

The study protocol was in accordance with the Declaration of Helsinki, and was approved by the Regional Committee for Medical and Health Research Ethics, Western Norway, the Norwegian Medicines Agency and the Norwegian Data Inspectorate. Besides, all subjects were informed and agreed to participate in extended follow-up including genetic studies (2010/2895/REK vest).

3.2 Baseline data

Clinical information was collected by self-administered questionnaire, which included medical history, CVD risk factors and medications. Smoking status was estimated according to both self-reports and serum cotinine levels. Patients initially claiming to be non-smokers but with serum cotinine concentrations ≥ 85 nmol/L [99] were reclassified as smokers since subjects basically tend to underestimate their actual tobacco exposure [100]. Obesity was defined as body mass index (BMI) ≥ 30 kg/m^2. In paper I, diabetes mellitus was identified by self-reports or by glucose criteria (fasting plasma glucose ≥ 7.0 mmol/L or non-fasting plasma glucose ≥ 11.1 mmol/L). Paper II and Paper III revised the classification of diabetes mellitus by adding HbA1c (≥ 6.5%) into the criteria according to the American Diabetes Association guidelines [101]. The extent of CAD at angiography was scored 0-3 according to the number of significantly stenotic coronary arteries.

3.3 Genotyping and biochemical analyses

Blood samples were obtained at baseline before or immediately after coronary angiography and were stored at -80°C. Routine biochemical analyses were performed at the hospital laboratories of Haukeland University Hospital or Stavanger University Hospital, respectively. For study-specific analyses, plasma homocysteine was measured by gas chromatography coupled with mass spectrometry procedure while plasma pyridoxal 5'-phosphate, serine and glycine levels were measured by liquid chromatography-tandem mass spectrometry [102] at Bevital A/S, Norway (http://www.bevital.no). Serum folate were separately measured by a microbiological assay [103]. The MTHFD1 *rs2236225* and *rs1076991* polymorphisms were genotyped with matrix-assisted laser desorption/ionization mass spectrometry [104].

3.4 Follow-up and clinical endpoints

The primary outcome for the project was AMI (fatal or non-fatal), which was classified according to the International statistical Classification of Disease Tenth Revision (ICD-10) codes I21 and I22, respectively. Study subjects in Paper I and Paper III were followed from the enrollment until the onset of AMI, or until the end of 2006. Information on clinical events in these two papers was collected from the Western Norway Cardiovascular Registry. In Paper II, participants were followed from enrollment until the onset of AMI, or until the end of 2009. Information on the extended follow-up of paper was collected from the Cardiovascular Disease in Norway project (CVDNOR; https://cvdnor.b.uib.no), which reports on patients being discharged with a CVD diagnosis from any of 42 Norwegian public hospitals from 1994 and throughout 2009 [105, 106].

3.5 Statistical analyses

Plasma or serum metabolite concentrations were log-transformed prior to statistical analysis due to their right-skewed distributions. Hardy-Weinberg equilibrium (HWE) and MAF of MTHFD1 polymorphisms were calculated before the analyses. Baseline categorical variables were summarized as percentages while continuous variables were presented as medians with interquartile ranges (IQRs).

Trend differences in baseline characteristics were tested by un-adjusted linear regression for continuous variables (for all three papers) and Kruskal-Wallis rank sum test for categorical variables (Paper I), or logistic regression for categorical variables (Paper II and III). In addition, we adopted additive genetic model in analyses of Paper I and III, in which we assumed a linear risk association among MTHFD1 minor allele carriers.

Hazard ratios for endpoints were calculated by Cox regression. Risk associations of the interested parameter with AMI in all three papers were tested in two models: a simple model adjusted for age (continuous) and gender (male/female); a multivariate model with further adjustment for established CAD risk factors, including smoking status (yes/no), obesity (yes/no), hypertension (yes/no) and diabetes mellitus (yes/no). Paper II and III additionally incorporated extent of CAD at angiography (ordinal), estimated glomerular filtration rate (eGFR, continuous) and statin treatment (yes/no) into the multivariate models. For study-specific reasons, we further included apo A1 (continuous) and apo B (continuous) in the multivariate model of Paper II, and vitamin B6 (yes/no) and folic acid+B12 treatment (yes/no) in Paper III. Proportional hazards assumptions were examined using the Schoenfeld and scaled Schoenfeld residuals.

In Paper I, potential effect modifications of vitamin B treatments on the MTHFD1 *rs1076991* polymorphism were evaluated by adding interaction product terms in the multivariate Cox models. In Paper II, interactions of plasma glycine with pre-specified lipid parameters (apo B, LDL-cholesterol, apo A1 and HDL-cholesterol) were explored according to their median values and tested by including interaction terms in Cox models adjusted for age, gender and the use of statins. In Paper III, potential effect modifications of two MTHFD1 polymorphisms (continuous) on plasma serine or glycine (continuous) were explored separately by including an interaction product in Cox model, which yielded four comparisons under simple adjustment and four under multivariate adjustment. As such, the four interaction tests under each adjustment were examined separately at a significant false discovery rate (FDR) ≤ 0.05 to address multiple hypothesis testing.

Unadjusted cumulative survivals according to MTHFD1 *rs1076991* polymorphism was illustrated by Kaplan-Meier curves (Paper

I). In Paper II and III, we visualized the potential non-linear dose-response relationships of plasma serine and glycine with risk of incident AMI in varied populations by generalized additive model (GAM) plots.

All reported *P* values were two-sided, and $P<0.05$ was considered statistically significant. The statistical analyses were performed in R (R Core Team, Vienna, Austria; version 3.1.0 to 3.2.2) using packages "coin", "genetics", "Hmisc", "lm.beta", "MASS", "mgcv", "stats", "survival" and "visreg" [107].

4. Results

4.1 Study population

4.1.1 WENBIT (Paper I and III)

In total, 2584 patients who underwent coronary angiography for suspected SAP were included in WENBIT, the median (IQR) age was 62 (14) years and 2048 (79.7%) were men. Baseline characteristics did not differ significantly across MTHFD1 polymorphisms (*P*≥0.07, Table 1). However, patients carrying MTHFD1 *rs1076991* polymorphism tended to have higher plasma glycine levels (*P*=0.09) and lower prevalence of diabetes mellitus (*P*=0.08). In addition, the *rs2236225* polymorphism showed borderline significant association with plasma homocysteine levels (*P*=0.07). The observed MAF of the MTHFD1 *rs2236225* and *rs1076991* were 0.48 and 0.43 and did not deviate from HWE (P=0.71 and P=0.31, respectively). We did not observe linkage disequilibrium between the two SNPs (r^2=0.03, D'=0.22).

4.1.2 BECAC (Paper II)

For the 4109 patients in BECAC study, the median (IQR) age was 62 (15) years at baseline and 2960 (72.0%) were males. As outlined in Table 2, patients in the upper glycine quartiles were more likely to be older, females and smokers as compared to patients in the lower quartiles. However, the association between plasma glycine and smoking status was not present after the adjustment for BMI (*P*=0.11). Besides, patients in upper quartiles of plasma glycine also had higher apo A1 (*P*<0.001) and HDL-cholesterol levels (*P*<0.001) and lower serum apo B (*P*<0.001), and tended to have lower LDL-cholesterol (*P*=0.064), which was significant after the adjustment for statin treatment (*P*<0.001).

Table 1. Baseline characteristics according to MTHFD1 polymorphisms in SAP patients in WENBIT

	rs2236225			P_{trend}	***rs1076991***			P_{trend}
	GG (n=652)	GA (n=1202)	AA (n=572)		CC (n=794)	CT (n=1141)	TT (n=446)	
Age, years	62 (13)	62 (14)	62 (15)	0.67	62 (14)	62 (14)	62 (15)	0.77
Male gender, n (%)	520 (79.8)	942 (78.4)	465 (81.3)	0.55	626 (78.8)	909 (79.7)	353 (79.1)	0.83
Current smoking, n (%)	215 (32.8)	387 (32.2)	181 (31.6)	0.62	231 (29.1)	391 (34.3)	147 (33.0)	0.08
Obesity, n (%)	122 (18.7)	231 (19.2)	97 (17.0)	0.46	167 (21.0)	198 (17.4)	80 (17.9)	0.10
Hypertension, n (%)	296 (45.4)	569 (47.3)	278 (48.6)	0.26	390 (49.1)	533 (46.7)	203 (45.5)	0.19
Diabetes mellitus, n (%)	229 (35.1)	437 (36.4)	201 (35.1)	0.98	302 (38.0)	403 (35.3)	145 (32.5)	0.06
eGFR, ml/min per 1.73m^2	92 (17)	92 (18)	92 (19)	0.32	92 (17)	92 (19)	92 (20)	0.33
Serum CRP, mg/L	1.69 (2.37)	1.79 (2.88)	1.78 (2.53)	0.19	1.79 (2.91)	1.70 (2.51)	1.93 (2.65)	0.74
Previous MI, n (%)	285 (43.7)	540 (44.9)	253 (44.2)	0.84	346 (43.6)	521 (45.7)	194 (43.5)	0.86
Statins at discharge, n (%)	574 (88.0)	1595 (80.5)	500 (87.4)	0.77	794 (88.9)	1009 (88.4)	392 (87.9)	0.58
Serum lipid parameters								
Apo A1, mg/dL	1.27 (0.33)	1.26 (0.34)	1.26 (0.33)	0.99	1.27 (0.34)	1.25 (0.32)	1.27 (0.36)	0.40
Apo B, mg/dL	0.85 (0.29)	0.85 (0.32)	0.83 (0.30)	0.14	0.83 (0.30)	0.85 (0.31)	0.85 (0.27)	0.99
LDL-cholesterol, mmol/L	2.90 (1.20)	2.90 (1.21)	2.80 (1.30)	0.26	2.86 (1.22)	2.90 (1.35)	2.90 (1.20)	0.74
HDL-cholesterol, mmol/L	1.20 (0.42)	1.20 (0.40)	1.20 (0.50)	0.79	1.20 (0.49)	1.20 (0.40)	1.20 (0.40)	0.17
Plasma OCM parameters								
Homocysteine, µmol/L	10.1 (3.5)	10.2 (3/7)	10.6 (3.7)	0.07	10.2 (3.5)	10.2 (3.7)	10.4 (3.6)	0.76
Serine, µmol/L	95.1 (26.7)	92.9 (25.9)	92.0 (29.0)	0.13	92.3 (24.7)	93.3 (27.0)	93.2 (26.4)	0.59
Glycine, µmol/L	197.3 (53.6)	198.8 (59.0)	197.2 (56.3)	0.78	196.6 (54.2)	198.6 (58.8)	199.8 (58.7)	0.09

Variables are given in medians (IQR) or counts (percentages).

Abbreviations: MI, myocardial infarction; OCM, one-carbon metabolism.

Table 2. Baseline characteristics according to quartiles of plasma glycine among suspected SAP patients in WENBIT and BECAC

	Plasma glycine quartiles (μmol/L)				P_{trend}
	1st (<178)	2nd (178-205)	3rd (205-243)	4th (>243)	
Age, years	61 (14)	62 (14)	61 (15)	63 (16)	0.037
Male gender, n (%)	817 (79.5)	818 (79.7)	804 (78.2)	521 (50.7)	<0.001
Current smoking, n (%)	299 (29.1)	292 (28.5)	375 (36.5)	337 (32.8)	0.003
Obesity, n (%)	321 (31.2)	183 (17.8)	135 (13.1)	113 (11.0)	<0.001
Hypertension, n (%)	561 (54.6)	483 (47.1)	451 (43.9)	434 (42.3)	<0.001
Diabetes mellitus, n (%)	513 (49.9)	413 (40.2)	393 (38.2)	393 (38.3)	<0.001
HbA1c (%)	6.23 (1.77)	6.02 (1.56)	6.05 (1.33)	6.08 (1.42)	<0.001
Serum					
Apolipoprotein A1, mg/dL	1.24 (0.32)	1.28 (0.34)	1.31 (0.34)	1.37 (0.37)	<0.001
Apolipoprotein B, mg/dL	0.90 (0.31)	0.88 (0.30)	0.85 (0.30)	0.85 (0.33)	<0.001
HDL-cholesterol, mmol/L	1.10 (0.30)	1.20 (0.40)	1.30 (0.42)	1.40 (0.50)	<0.001
LDL-cholesterol, mmol/L	3.00 (1.30)	2.99 (1.30)	2.90 (1.33)	2.90 (1.40)	0.064
eGFR, mL/min/1.73 m^2	94 (18)	92 (19)	91 (20)	86 (24)	<0.001
Serum CRP, mg/L	2.48 (3.73)	1.84 (2.80)	1.58 (2.36)	1.35 (2.09)	<0.001
Troponin T, ng/L	5 (8)	4 (6)	5 (7)	4 (6)	0.088
Prior MI, n (%)	438 (42.6)	436 (42.5)	437 (42.5)	347 (33.8)	<0.001
LVEF (%)	65 (10)	65 (10)	65 (10)	65 (10)	0.092
Angiographic evidence of CAD, n (%)					
No significant stenosis	189 (18.4)	214 (20.8)	260 (25.3)	374 (36.4)	<0.001
Single-vessel disease	255 (24.8)	240 (23.4)	245 (23.8)	210 (20.4)	0.032
Double-vessel disease	250 (24.3)	249 (24.2)	228 (22.2)	188 (18.3)	<0.001
Triple-vessel disease	334 (32.5)	323 (31.6)	295 (28.7)	255 (24.9)	<0.001
Medications at discharge, n (%)					
Aspirin	854 (83.1)	871 (84.9)	849 (82.6)	781 (76.0)	<0.001
Statins	850 (82.7)	855 (83.3)	824 (80.2)	763 (74.3)	<0.001
Beta blockers	774 (75.3)	764 (74.5)	749 (72.9)	694 (67.6)	<0.001
ACEIs	237 (23.1)	233 (21.7)	202 (19.6)	178 (17.3)	<0.001
Loop diuretics	141 (13.7)	102 (10.0)	88 (8.4)	119 (11.5)	0.075

Variables are given in medians (IQR) or counts (percentages).

Abbreviations: ACEI, angiotensin-converting-enzyme inhibitor; CABG, coronary artery bypass graft surgery; CAD, coronary artery disease; GFR, glomerular filtration rate; HbA1c, glycated hemoglobin; MI, myocardial infarction; PCI, Percutaneous coronary intervention.

4.2 Synopsis of results for individual papers

4.2.1 Paper I

B vitamin treatments modify the risk of myocardial infarction associated with a MTHFD1 polymorphism in patients with stable angina pectoris

Nutrition, Metabolism and Cardiovascular Diseases 2016;26(6):495-501.

In this study, we explored the association between MTHFD1 *rs1076991* polymorphism and incident AMI, and the effect modifications by folic acid+B12 and/or vitamin B6 treatment among SAP patients in WENBIT. During a median follow-up time of 4.9 years, 204 participants (8.6%) suffered an AMI. After adjusting for established CVD risk factors, the MTHFD1 polymorphism was significantly associated with AMI (HR: 1.49; 95% CI, 1.23-1.81). A similar association was observed among patients allocated to treatment with vitamin B6 alone (HR: 1.53; 95% CI, 1.01-2.31), and an even stronger relationship was seen in patients treated with both vitamin B6 and folic acid+B12 (HR: 2.35; 95% CI, 1.55-3.57). However, no risk association between the MTHFD1 polymorphism and AMI was seen in patients treated with placebo (HR: 1.29; 95% CI, 0.86-1.93) or folic acid+B12 (1.17; 95% CI, 0.83-1.65).

In conclusion, we demonstrate that B vitamin treatment may introduce a strong association between a common and functional MTHFD1 promoter polymorphism and AMI risk in patients with stable angina pectoris.

4.2.2 Paper II

Plasma glycine and risk of acute myocardial infarction in patients with suspected stable angina pectoris

Journal of American Heart Association 2016;5:e002621.

In this study, we assessed the association between plasma glycine and incident AMI among SAP patients in WENBIT and BECAC. Plasma glycine levels were higher in women than in men and was associated with a more favorable baseline lipid profile and lower prevalence of obesity, hypertension, and diabetes mellitus (all P<0.001). During a median follow-up of 7.4 years, 616 patients (15.0%) experienced an AMI. After adjusting for age and gender, higher plasma glycine was associated with a decreased risk of AMI (HR: 0.88; 95% CI, 0.80-0.95). The association was essentially similar in the multivariate model (HR 0.89; 95% CI, 0.82-0.98). The inverse association was generally stronger in those with apo B, low-density lipoprotein cholesterol, or apo A1 above the median (all P_{int}≤0.037).

In conclusion, plasma glycine was associated with decreased risk of AMI in patients with suspected SAP. This association was particularly strong in those with apo B, LDL-cholesterol or apo A1 levels above the median.

4.2.3 Paper III

MTHFD1 polymorphisms modify the associations of plasma glycine and serine with risk of AMI in patients with stable angina pectoris in WENBIT

Circ Cardiovasc Genet. 2016 Nov 21. pii: CIRCGENETICS.116.001483.

In this study we assessed if plasma serine and glycine may affect the risk of AMI in response to genetic polymorphisms in MTHFD1. During a median follow-up of 4.7 years, 212 patients (8.2%) experienced an AMI. In age- and gender-adjusted analyses, plasma glycine (P<0.01), but not serine (P=0.52) showed an overall association with AMI. However, interactions of MTHFD1 *rs2236225* polymorphism with both plasma serine and glycine were observed ($P_{interaction}$=0.03 for both). Low plasma

serine and glycine were associated with an increased risk of AMI among patients carrying the *rs2236225* minor A-allele. Similarly, low plasma glycine showed stronger risk relationship with AMI in the *rs1076991* CC genotype carriers but weaker associations in patients carrying the minor T-allele ($P_{interaction}$=0.02).

In conclusion, we demonstrate that in patients with SAP, the risk associations for plasma serine and glycine with AMI were modified by the *rs2236225* and *rs1076991* polymorphisms in the MTHFD1 gene.

5. Discussion

5.1 Summary

The current observational studies were based on patients with suspected SAP either recruited to the WENBIT or followed in the BECAC cohort. The aims were to investigate the role of MTHFD1 polymorphisms in relation to AMI risk and their potential interaction with B vitamins treatment. Besides, considering the functional crosstalk between MTHFD1 and the interconversion between serine and glycine, we also explored the independent risk associations of plasma serine and glycine with AMI, as well as their effect modifications with MTHFD1 polymorphisms.

We identified that a common and functional MTHFD1 polymorphism, *rs1076991*, was associated with increased risk of AMI, and the risk association was more likely to be introduced by the combined treatment with folic acid+B12 and vitamin B6. Besides, we observed an inverse dose-response relationship between plasma glycine and risk of AMI, primarily in patients with elevated serum apolipoprotein B, LDL cholesterol and apolipoprotein A1. Furthermore, we demonstrated that the risk associations of both plasma serine and glycine on AMI occurrence were modified by two common polymorphisms in MTHFD1 gene, *rs2236225* and *rs1076991*.

5.2 Study design

The relatively large sample size and the high MAFs of MTHFD1 polymorphisms ensure sufficient power to detect underlying risk associations with CVD outcomes. Besides, prospective studies are suggested as an appropriate approach to explore gene-environment

interactions as they are not prone to biases due to modifications in environmental exposure induced by disease onset [108].

Risk factors may take several years to fully manifest their effect on the development and progression of CVD. Relatively short duration of cohort studies may only identify a part of their effect, while a potential larger effect can be uncovered in extended studies, which is known as the “lag effect” [109]. Therefore, the long follow-up time of the current study should also be considered as strength.

In the current project, we included patients with suspected SAP only. Previous studies have shown that ACS is associated with a strong inflammatory response [110], which affects vitamin B6 status [111] and may accordingly influence plasma glycine levels [87]. Hence, the exclusion of ACS patients was more likely to be able to avoid the influence of acute inflammation on gene-nutrient interactions. Nevertheless, our findings in the current project are primarily relevant for patients with SAP and should preferably be evaluated in patients with different clinical characteristics.

5.3 Regression dilution bias

In population studies, single (as opposed to multiple) measurement may have a large variance and lead to an attenuation in regression coefficient and substantially underestimate of the strength of association [112], which is known as “regression dilution bias”. Notably, a previous study from a subgroup of the current study population exhibited excellent within person reproducibilities of plasma serine and glycine (intraclass correlation coefficients > 0.75) [113], allowing one-exposure assessment for biomarker status of serine and glycine and therefore reducing the risk of regression dilution bias [114].

5.4 Choice of genetic models

Genetic models need to be specified before testing associations between polymorphisms and outcomes. We can assume dominance of one of the alleles by treating the heterozygote and one of the homozygote genotypes as a single category [115]. For example, if *G* is the mutant allele with a high risk, a dominant model refers to a comparison between *CC* genotype and *CG+GG* genotypes, which forces heterozygotes to have the same risk estimates as the homozygotes. Likewise, a genetic model can be assumed as recessive when comparing *CC+CG* genotype to *GG* genotype. Alternatively, a genetic model can be also assumed as additive in which each additional copy of the minor allele increases the response by the same amount. The additive model is the most commonly assumed model and its statistical significance is assessed based on the Cochrane-Armitage test for trend [115, 116]. In the current study, we adopted additive model in all the statistical analyses to demonstrate the differences of log hazard ratios among MTHFD1 genotypes.

5.5 Covariates and confounding

A covariate refers to a variable (ex. age, gender, ethnicity, etc.) that may or may not be associated to the outcome being studied. If the covariate is related to both the interested exposure and the clinical outcome, then this covariate becomes a confounder [117]. Population-based studies primarily use multivariate adjustment approaches to control the effect of covariates and confounders.

In Paper II, we included both the traditional CVD risk factors and lipid parameters in the multivariate model. Although higher plasma

glycine was associated with a generally more favorable cardiovascular disease risk factor profile, the risk estimates of plasma glycine on AMI occurrence remained significant after multivariate adjustment. However, additional adjustment for plasma CRP slightly attenuated the risk associations, possibly reflecting a role of glycine in inflammation. Indeed, plasma glycine has been correlated to CRP levels [118, 119] by regulating the production of pro-inflammatory cytokines [120, 121] and has been suggested as a modulator of the pro-inflammatory state [122].

Comparing to classic epidemiology studies, candidate SNP association studies, on the other hand, are unlikely to be influenced by behavioral and environmental factors since those factors usually do not influence genotype. However, adjustment for those factors that affect the outcome independently may increase estimate precision [115].

In Paper I, we found strong risk association between MTHFD1 *rs1076991* polymorphism and AMI in the age- and gender-adjusted model, which was barely influenced by the multivariate adjustment, indicating an independent role of MTHFD1 in association with atherosclerotic CVD.

In Paper III, the statistical adjustment seemed to have negligible influence on risk associations and effect modifications between serine, glycine and MTHFD1 polymorphisms on AMI occurrence, supporting the hypothesis that their associations to AMI reflect different pathogenic pathways from that of traditional CVD risk factors.

The unidentified confounders are usually referred as “residual confounders”. In genetic studies, population stratification refers to a systematic difference in allele frequencies between cases and controls possibly due to different ancestry rather than association of genes with disease [123], and is recognized as a crucial confounder [124]. However, this might not be the case in the current study, since more than 99% of the

participants in this population were Caucasians [125], which is also supported by the HWE results in Paper I and III. On the other hand, however, serine and glycine are involved in several metabolic pathways and may be influenced by corresponding genetic and metabolic traits, as well as dietary habits, which were not evaluated in the current project. We did not evaluate the lifestyle or dietary determinants of plasma serine and glycine, therefore the possibility of residual confounding cannot be excluded.

5.6 Main findings and possible mechanisms

Among the 2381 SAP subjects included in Paper I, the common and functional MTHFD1 *rs1076991* polymorphism was associated with increased risk of AMI during follow-up. This association was particularly strong in patients allocated to the combined vitamin B6 plus folic acid+B12 treatment.

MTHFD1 mediated one-carbon transfer from formate is one of the major methyl sources for homocysteine remethylation [126]. Experimental studies revealed higher hepatic adenosylmethionine (SAM) levels in MTHFD1 knock-down mice [76]. Recent evidence also linked both folate intake [127] and vitamin B6 intake [128] with elevated SAM levels. Accumulation of SAM has been shown to cause hepatic lipid accumulation [129]. These findings may be indicative of a potential mechanism in which SAM accumulation is involved in the development and progression of atherosclerosis.

In OCM, SAM catabolism is closely associated with betaine-homocysteine methyltransferase (BHMT) and glycine-N-methyltransferase (GNMT) activity. First, elevated SAM has been shown to inhibit BHMT activity [130], which may impair the transcription of both apo B [131] as well as peroxisome proliferator-activated receptors α [132], a nuclear transcription factor involved in the metabolism of

macronutrients (carbohydrate, protein and fat) as well as lipid metabolism [133]. Second, SAM can be catabolized by GNMT in a glycine-dependent manner. Therefore, sufficient glycine availability may be important to scavenge excess SAM and to prevent atherosclerotic CVD outcomes.

We accordingly evaluated the association between plasma glycine levels and risk of AMI in Paper II, and observed decreased AMI risks in patients with higher glycine levels. As mentioned before, glycine can be methylated into sarcosine via GNMT. Impairment of GNMT flux has been associated with hepatic cholesterol accumulation and overt dyslipidemia by downregulation of NPC2 protein [134]. In addition, impaired GNMT flux was suggested to interrupt reverse cholesterol transport by downregulating the expression of SRB1, ACBA1 and ACBG1 [135]. In the same study, GNMT flux was also shown to affect composition of atherosclerotic plaques and regulate inflammation response within atherosclerotic lesions.

On the other hand, glycine can be recognized as the end product of choline oxidation in the mitochondria, and low glycine may therefore be related also to a low BHMT activity. Thus, the excess AMI risk observed among patients with low glycine levels can be induced by impaired apo B transcription and/or incorporation of cholesterol into VLDL as well as reduced activity of reverse cholesterol transport. Notably, all these features are classical components of metabolic syndrome [136] characterized by the secretion of large, buoyant VLDL particles enriched with triglycerides but with low cholesterol, and which ends up the small, dense atherogenic LDL particles [137].

Results from Paper III revealed significant effect modifications of plasma serine and glycine with MTHFD1polymorphisms on the risk of AMI. The underlying mechanisms of these interactions are yet to be elucidated. However, our findings may be related to the severity of

MTHFD1 deficiency, the availability of serine and glycine for activated one-carbon units, as well as the metabolic crosstalk of these two interconnected pathways. Notably, serine and glycine status has been shown to influence the direction of MTHFD1-mediated one-carbon flux [81], which may in turn affect the production [138] and consumption [139] of NADPH, a crucial reductant in fatty acid and cholesterol synthesis, Approximately 80% of the NADPH produced by the MTHFD1 is used for fatty acid synthesis [140], but there are no data linking this flux directly to cholesterol synthesis. In the current study, adjustment for apolipoprotein B, LDL cholesterol and triglycerides had negligible influence on the risk estimates of plasma serine and glycine, suggesting that cholesterol-related atherogenesis may not be relevant for the observed effect modifications by the MTHFD1 polymorphisms.

5.7 Effect modification

In common diseases, the effects of susceptible polymorphisms can be seen as triggering factors while behavior or environmental factors reveal or facilitate the phenotypic expression of such susceptibility genes, which is known as “effect modification” [141]. The susceptible genetic variants are more likely to be response modifiers to exogenous factors (such as age, gender, dietary habits and medical history) rather than a primary etiological role in the predisposition for multifactorial disease [142]. Therefore, a better characterization of interactions between genetic polymorphisms and behavior/environmental factors contributes a vital part in the understanding of pathogenesis of common diseases.

In Paper I, we observed a significant interaction between combined treatment of folic acid+B12 and vitamin B6 with the MTHFD1 *rs1076991* polymorphism on risk of AMI. These findings suggest that intake of B vitamins may influence the risk of atherosclerosis depending

upon the MTHFD1 genetic variants. Since B-vitamin status may be correlated to other lifestyle factors like obesity or the intake of macronutrients, further studies are warranted to examine if the MTHFD1 genotype may influence the associations between such lifestyle factors and atherogenesis, and if such associations may be mediated at least partly by B vitamin status.

Paper II revealed similar trends on the risk estimates of glycine with risk of AMI according to several lipid parameters. Plasma glycine had stronger associations with AMI in patients with higher apo B, LDL-cholesterol and apo A1 levels. These significant interactions suggest that an adequate plasma glycine level may be important to prevent dyslipidemia and atherosclerosis. Studies on life-style and genetic determinants of plasma glycine are therefore encouraged.

Our data from Paper III revealed opposite interaction estimates between plasma serine, glycine and two MTHFD1 polymorphisms. These results possibly reflect complex activities of the tri-functional MTHFD1 gene in the synthetase and dehydrogenase-cyclohydrolase domains. Notably, elevated plasma glycine was associated with a particular beneficial effect against AMI in patients carrying the MTHFD1 *rs2236225* AA genotype, which may be associated with lower transcriptional capacity. Further studies should evaluate if patients carrying this genotype require higher glycine levels to scavenge potentially redundant 10-formyl-tetrahydrofolate production. These findings may be also indicative of a potential role of MTHFD1 in balancing serine and glycine metabolism associated with atherosclerotic progression.

6. Conclusions

The present project aimed to investigate the role of two polymorphisms of MTHFD1 in relation to risk of AMI and the potential interactions by B vitamins treatments. Besides, considering the functional crosstalk between MTHFD1 and the interconversion between serine and glycine, we also explored the independent risk associations of plasma serine and glycine with AMI, as well as their effect modifications with MTHFD1 polymorphisms. Those questions were addressed as follows:

Paper I: We observed an association between the common and functional MTHFD1 polymorphism *rs1076991* and increased risk of AMI in SAP patients. The risk association was confined to patients treated by combined folic acid+B12 and vitamin B6 treatment, whereas no association was observed among those treated with placebo.

Paper II: Elevated plasma glycine was associated with a favorable CVD risk factor profile and decreased risk of AMI in patients with suspected SAP. This association was particularly strong in those with elevated apo B, LDL-cholesterol or apo A1 levels above the median.

Paper III: We demonstrated significant effect modifications of two MTHFD1 polymorphisms (*rs2236225* and *rs1076991*) on the associations between plasma serine and glycine with risk of AMI in SAP patients.

In conclusion, our findings highlight the importance of MTHFD1 in the development and progression of atherosclerosis. We also demonstrated that the association between MTHFD1 polymorphism and risk of AMI was significantly modified by B vitamin treatment. Meanwhile, two polymorphisms in MTHFD1 were observed to modify the risk estimates of plasma serine and glycine on AMI occurrence. Because the risk association of plasma glycine is also modified by lipid levels,

further studies on the functional crosstalk of MTHFD1 in relation to serine, glycine and lipid metabolism should be encouraged.

7. Future Perspectives

In genetic association studies, results validation means to obtain similar findings under modified influencing factors such as ethnicity, phenotype and sampling strategy [143]. It plays an important role in identifying potential genetic variants associated with complex diseases. Therefore, the risk association of MTHFD1 *rs1076991* polymorphism with AMI is warranted to be validated in other populations. Further studies should also evaluate if this genotype may modify the risk of other chronic diseases affected by dietary or lifestyle factors.

Amino acids and vitamins enter the metabolic pathway by donating carbon units or acting as enzyme cofactors in OCM. In this context, OCM functions as a metabolic integrator of nutrient status [53]. Results from the current project strongly suggest the existence of effect modifications between genetic polymorphisms and OCM biomarkers, as well as B vitamin status in association with atherosclerotic CVD. However, the detailed mechanisms have not been fully elucidated. Our findings motivate further studies to unravel the complex roles of OCM in the development of chronic diseases.

We for the first time observed significant inverse association of plasma glycine with risk of AMI. Previously, considerable data has linked low plasma glycine levels with CVD risk factors, such as obesity, hypertension, diabetes mellitus and lipid profile [93, 95, 96, 98]. Therefore, associations of dietary determinants of glycine with CVD outcomes may be of interest to explore in future prospective studies. In addition, extensive experimental data strongly indicates a role of GNMT in the development and progression of atherosclerosis [135, 144]. However, observational studies are scarce. Hence, further population-based studies of GNMT polymorphisms on CVD outcomes should be motivated.

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B vitamin treatments modify the risk of myocardial infarction associated with a MTHFD1 polymorphism in patients with stable angina pectoris

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Abstract *Background:* Methylenetetrahydrofolate dehydrogenase (MTHFD1) catalyzes three sequential reactions that metabolize derivatives of tetrahydrofolate (THF) in folate-dependent one-carbon metabolism. Impaired MTHFD1 flux has been linked to disturbed lipid metabolism and oxidative stress. However, limited information is available on its relation to the development of atherothrombotic cardiovascular disease.

Methods and results: We explored the association between a MTHFD1 polymorphism (*rs1076991* $C > T$) and acute myocardial infarction (AMI), and potential effect modifications by folic acid/B12 and/or vitamin B6 treatment in suspected stable angina pectoris patients ($n = 2381$) participating in the randomized Western Norway B Vitamin Intervention Trial (WENBIT). During the median follow-up of 4.9 years 204 participants (8.6%) suffered an AMI. After adjusting for established CVD risk factors, the MTHFD1 polymorphism was significantly associated with AMI (HR: 1.49; 95% CI, 1.23–1.81). A similar association was observed among patients allocated to treatment with vitamin B6 alone (HR: 1.53; 95% CI, 1.01–2.31), and an even stronger relationship was seen in patients treated with both vitamin B6 and folic acid/B12 (HR: 2.35; 95% CI, 1.55–3.57). However, no risk association between the MTHFD1 polymorphism and AMI was seen in patients treated with placebo (HR: 1.29; 95% CI, 0.86–1.93) or folic acid/B12 (1.17; 95% CI, 0.83–1.65).

Conclusion: A common and functional MTHFD1 polymorphism is associated with increased risk of AMI, although the risk seems to be dependent on specific B vitamin treatment. Further studies are warranted to elucidate the possible mechanisms, also in order to explore potential effect modifications by nutritional factors.

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Introduction

The role of one-carbon metabolism (OCM) in the pathogenesis of atherothrombotic cardiovascular disease (CVD) is not fully understood. Observational studies have shown that elevated plasma homocysteine is associated with increased CVD risk [1], whereas plasma pyridoxal 5′-phosphate (PLP) is inversely related with CVD independent from plasma homocysteine [2]. Although folic acid therapy has been suggested to significantly reduce the risk of first stroke in adults with hypertension [3], several randomized clinical trials aimed for secondary prevention have failed to reduce CVD risk by treatment with homocysteine-lowering folic acid/B12 or by vitamin B6 [4,5]. Thus, the underlying mechanisms for the observed associations in prospective studies are largely unknown. The highly interconnected network of one-carbon metabolism makes it difficult using classical observational approaches to delineate pathways associated with pathogenesis and the responsible factors. However, genetic variation with implications on certain metabolic pathways may serve as a more robust proxy for life-time susceptibility to a certain disorder which may allow causal inference [6].

Associations between CVD risk factors and several genetic variants of OCM have been widely researched [7], but few studies have focused on relationships with the cytosolic methylenetetrahydrofolate dehydrogenase (MTHFD1). MTHFD1 catalyzes three sequential reactions involved in the interconversion of one-carbon derivatives of tetrahydrofolate (THF, Fig. 1) [8]. A common single nucleotide polymorphism (SNP) MTHFD1 −105C > T (*rs1076991*) is located in its promoter region, with a reported minor allele frequency (MAF) of 49% [9]. The minor T-allele has been associated with an approximately 62.5% drop in transcription rate of the MTHFD1 enzyme due to decreased promoter activity [10]. Therefore, this SNP may adequately represent MTHFD1 deficiency. Impaired MTHFD1 flux has been suggested to disturb lipid metabolism in mice by alternating hepatic choline, betaine and dimethylglycine (DMG) concentrations [11] as well as interrupting intercellular NADPH production [12]. Genome-wide association studies (GWAS) additionally found variations in MTHFD1L, the mono-functional counterpart to MTHFD1 in the mitochondria, to be associated with risk of early-onset acute myocardial infarction (AMI) [13]. Hence, genetic variation in MTHFD1 may potentially be associated with CVD risk.

B vitamin status can influence OCM, but it likely depends on the genetic background among individuals [14], reflecting gene−environment interactions. Among participants of the Western Norway B Vitamin Intervention Trial (WENBIT), we therefore performed a candidate gene analysis of MTHFD1 *rs1076991* for its association with AMI, and further focusing on potential interactions with allocation into B vitamin treatments.

Methods

Study design and population

The WENBIT (ClinicalTrials.gov number NCT00354081) was carried out to investigate the effect of B vitamin

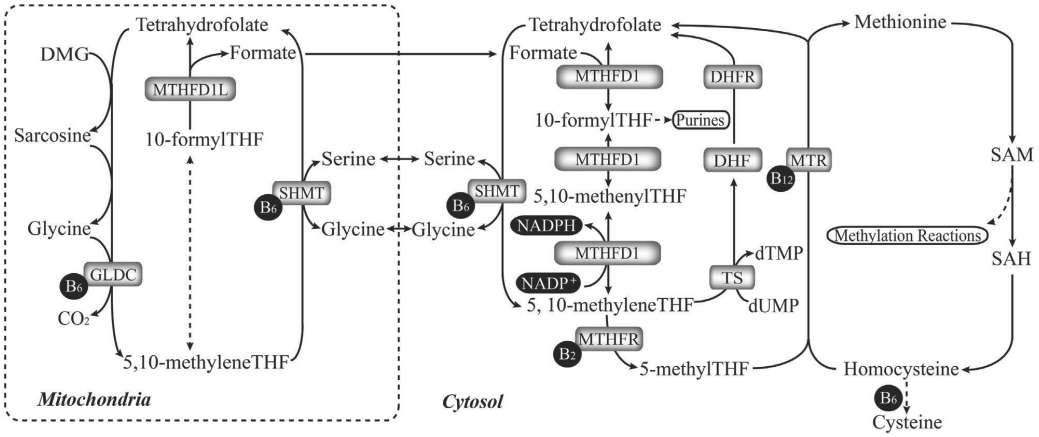


Figure 1 The roles of MTHFD1 enzyme in the folate-mediated one carbon metabolism in cytoplasm. MTHFD1 is a trifunctional C1 tetrahydrofolate (C1-THF) metabolizing enzyme that plays an important role in the folate metabolism. Those THF derivatives catalyzed by MTHFD1 are closely involved in DNA synthesis, as well as homocysteine remethylation. B vitamins (B2, B6, folic acid and B12) are crucial sources of coenzymes that maintain and modulate the normal functionalities of one carbon metabolism. Abbreviations: DHF, dihydrofolate; DHFR, dihydrofolate reductase; DMG, dimethylglycine; MTHFD1, methylenetetrahydrofolate dehydrogenase 1; MTHFR, methylenetetrahydrofolate reductase; MTR, 5-methyltetrahydrofolate-homocysteine methyltransferase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; Sarc, sarcosine; SHMT, serine hydroxymethyltransferase.

treatments on the risk for serious cardiovascular events and mortality [4]. In specific, WENBIT had a 2×2 factorial design in which participants with suspected or verified coronary artery disease (CAD), or aortic stenosis, were randomized to receive a daily capsule containing one of the following: 1) folic acid 0.8 mg plus vitamin B12 (cyanocobalamin) 0.4 mg plus vitamin B6 (pyridoxine hydrochloride) 40 mg; 2) folic acid plus vitamin B12; 3) vitamin B6; 4) placebo. In total, there were 3090 patients enrolled in WENBIT study. Among them, 2584 patients underwent coronary angiography for stable angina pectoris (SAP), 461 for acute coronary syndromes (ACS) and 45 for aortic valve stenosis at Haukeland University Hospital, Bergen, Norway or Stavanger University Hospital, Stavanger, Norway. The current study included patients with SAP only. Participants without successful genotyping ($n = 203$) were excluded, leaving a total of 2381 participants for the final analyses.

The study protocol was in accordance with the principles of the Declaration of Helsinki, and was approved by the Regional Committees for Medical and Health Research Ethics, the Norwegian Medicines Agency and the Norwegian Data Inspectorate. All subjects were informed and agreed to participate in extended follow-up including genetic studies.

Baseline data

Smoking status and the extent of CAD at angiography were defined as previously described [15]. Obesity was defined as body mass index (BMI) $\geq$ 30 kg/m^2. Diabetes mellitus was classified by self-reports or by glucose criteria (fasting plasma glucose $\geq$ 7.0 mmol/L; or random plasma glucose $\geq$ 11.1 mmol/L measured at baseline of the study).

Follow-up and clinical endpoints

The primary outcome for the present study was fatal or non-fatal AMI. Study subjects were followed from enrollment until the onset of AMI, or until the end of 2006. Details on the collection and classification of clinical endpoints have been described previously [16].

Genotyping and biochemical analysis

Clinical information and blood samples were obtained at baseline before or immediately after coronary angiography and the blood samples were stored at -80 °C. Earlier reports have described the biochemical analyses for relevant clinical indices [15,16]. Besides, genotyping of MTHFD1 *rs1076991* polymorphism was performed by MALDI-TOF mass spectrometry [17].

Statistical analysis

Baseline categorical variables were summarized as percentages and continuous variables were presented as medians (interquartile range (IQR)). Hardy-Weinberg equilibrium (HWE) and MAF of MTHFD1 *rs1076991* polymorphism were also calculated. Baseline variables across MTHFD1 *rs1076991* genotypes (CC, CT and TT) were assessed by un-adjusted median linear regression for continuous and logistic regression or Kruskal–Wallis rank sum test for categorical variables.

The risk association between the MTHFD1 polymorphism and AMI was tested in two Cox regression models: A simple model was adjusted for age (continuous) and gender (male/female); a multivariate model was further adjusted for established CAD risk factors including smoking status (yes/no), obesity (yes/no), hypertension (yes/no) and diabetes mellitus (yes/no). Additional adjustments for baseline serum apoA-1, apoB, and angiographic signs of coronary artery disease (CAD) had minor influences on the estimates and were therefore not included in the models. An additive genetic model was used in all survival analyses, in which we assumed a linear risk relationship among MTHFD1 *rs1076991* CC, CT, TT genotypes. This is equivalent to a comparison of the T-allele versus the C-allele. Proportional hazards assumptions were examined using the Schoenfeld and scaled Schoenfeld residuals. Interactions between vitamin B treatments and the MTHFD1 polymorphism on the outcome were evaluated by adding interaction product terms in the multivariate Cox models.

The statistical analyses were performed in R (R version: 3.1.0) using packages “genetics” and “survival” [18]. All reported P values were two-sided, and $P < 0.05$ was considered statistically significant.

Results

Baseline characteristics

Of the 2381 SAP participants included, 1888 (79.3%) were males. The median (IQR) age of the population was 62 (48–76) years. Most baseline characteristics in the study did not differ according to the MTHFD1 *rs1076991* genotypes ($P \geq 0.08$, Table 1). However, diabetes mellitus was inversely associated with the number of T-allele ($P = 0.02$). We did not observe any association between biomarkers for OCM and the MTHFD1 polymorphism at baseline ($P \geq 0.25$). The observed MAF of the MTHFD1 polymorphism was 42.7% and did not deviate from Hardy-Weinberg equilibrium ($P = 0.31$).

The MTHFD1 polymorphism and AMI risk

During a median (IQR) follow-up time of 4.9 (2.8–7.0) years, 204 participants (8.6%) suffered an AMI. Kaplan–Meier curves showed a significant association between the MTHFD1 polymorphism and AMI occurrence ($P_{\text{log-rank}} = 3.5 \times 10^{-4}$) (Fig. 2). After adjusting for age and gender, the minor T-allele was linearly associated with AMI (HR: 1.46; 95% CI, 1.20–1.76), which remained significant after multivariate adjustment (HR: 1.49; 95% CI, 1.23–1.81). Notably, similar results were also observed in the complete WENBIT population (data not shown).

Table 1 Baseline characteristics of the WENBIT cohort according to the MTHFD1 promoter polymorphism.

	CC ($N = 794$)	CT ($N = 1141$)	TT ($N = 446$)	Total ($N = 2381$)	*P* for trend
General characteristics					
Age (year)	62 ± 14	62 ± 14	62 ± 15	62 ± 14	0.71
Gender (male, %)	626 (79)	909 (80)	353 (79)	1888 (79)	0.83
Body mass index (kg/m^2)	26.6 ± 4.6	26.4 ± 4.6	26.4 ± 4.6	26.5 ± 4.6	0.16
Cardiovascular history and risk factors, n (%)					
Prior MI	346 (43.6)	521 (45.7)	194 (43.5)	1061 (44.5)	0.86
Prior PCI	167 (21.0)	255 (22.3)	99 (22.2)	521 (21.9)	0.57
Prior CABG	105 (13.2)	165 (14.5)	60 (13.5)	330 (13.9)	0.79
Hypertension	390 (49.1)	533 (46.7)	203 (45.6)	1126 (47.3)	0.19
Diabetes mellitus	132 (16.6)	162 (14.2)	52 (11.7)	346 (14.5)	0.02
Current smoking	209 (26.3)	357 (31.3)	133 (29.8)	699 (29.4)	0.08
Angiographic evidence of CAD					0.80
No significant CAD	87 (11.0)	133 (11.7)	48 (10.8)	268 (11.3)	
Single vessel disease	234 (29.5)	309 (27.1)	127 (28.5)	670 (28.1)	
Double vessel disease	210 (26.4)	297 (26.0)	127 (28.5)	634 (26.6)	
Triple vessel disease	263 (33.1)	402 (35.2)	144 (32.2)	263 (34.0)	
Biochemistry parameters					
ApoB/ApoA1 ratio	0.7 (0.3)	0.7 (0.3)	0.7 (0.3)	0.7 (0.3)	0.60
Estimated GFR (mL/min/1.73 m^2)	92 (17)	92 (19)	92 (20)	92 (18)	0.42
Serum CRP (mg/L)	1.8 (2.9)	1.7 (2.5)	1.9 (2.7)	1.8 (2.7)	0.74
Serum Creatinine (μmol/L)	90 (16)	91 (17)	90 (17)	90 (17)	0.18
Treatment following baseline coronary angiography, n (%)					0.62
No or medications only	275 (34.6)	378 (33.1)	163 (36.5)	816 (34.3)	
Percutaneous coronary intervention	300 (37.8)	462 (40.5)	169 (37.9)	931 (39.1)	
Coronary artery bypass grafting	193 (24.3)	265 (23.2)	101 (22.6)	559 (23.5)	
Medication at discharge, n (%)					
Aspirin	713 (89.8)	1024 (89.7)	407 (91.3)	2144 (90.0)	0.48
Statins	706 (89.0)	1009 (88.4)	392 (87.9)	2107 (88.5)	0.58
Beta blockers	614 (77.3)	884 (77.5)	358 (80.2)	1856 (78.0)	0.64
ACEIs	178 (22.4)	224 (19.6)	96 (21.5)	498 (20.9)	0.51
Loop diuretics	87 (11.0)	110 (9.6)	40 (9.0)	233 (9.8)	0.23
Biomarkers for OCM					
Plasma homocysteine (μmol/L)	10.2 (3.5)	10.2 (3.7)	10.4 (3.6)	10.2 (3.6)	0.76
Plasma PLP (nmol//L)	40.4 (27.2)	39.0 (26.1)	40.5 (27.5)	40.0 (26.9)	0.89
Serum folate (nmol//L)	9.8 (7.4)	9.9 (7.0)	10.0 (7.4)	9.9 (7.2)	0.24
Serum cobalamin (μmol/L)	331 (160)	341 (173)	333 (172)	337 (170)	0.79
Serum MMA (μmol/L)	0.16 (0.06)	0.16 (0.07)	0.16 (0.07)	0.16 (0.07)	0.25

Abbreviations: ACEI, angiotensin-converting-enzyme inhibitor; Apo, apolipoprotein; CABG, coronary artery bypass grafting; CAD, coronary artery disease; CRP, C-reactive protein; GFR, glomerular filtration rate; MMA, methylmalonic acid; OCM, one carbon metabolism; PCI, percutaneous coronary intervention; PLP, pyridoxal phosphate.

The effect modifications of B vitamin treatment

Table 2 describes the risk associations between the MTHFD1 *rs1076991* and AMI according to WENBIT treatment allocation. Among patients treated with placebo or folic acid/vitamin B12, we found similar trends towards positive associations between the number of T-allele and AMI risk in the multivariate models (HR: 1.29; 95% CI, 0.86−1.93 and 1.17; 95% CI, 0.83−1.65, respectively; $P_{int} = 0.76$). A significant association was observed among patients allocated to vitamin B6 treatment (HR: 1.53; 95% CI, 1.01−2.31), which, however, was not statistically different from placebo treatment ($P_{int} = 0.59$). Notably, we observed a more profound association between the polymorphism and AMI among patients allocated to the combined vitamin B6 and folic acid/B12 treatment (HR: 2.35; 95% CI, 1.55−3.57; $P_{int} = 0.047$ vs. placebo). This interaction seemed to be introduced by a shift from a lower to higher risk of the combined B vitamin treatment according to the number of T-allele (HR among the CC homozygotes: 0.38; 95% CI, 0.12−1.23; CT heterozygotes: 1.14; 95% CI, 0.68−1.93; and TT homozygotes: 1.98; 95% CI, 0.86−4.55).

Discussion

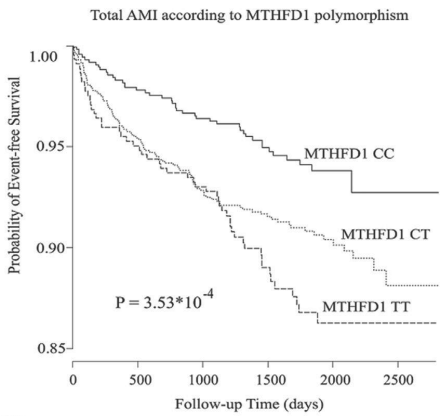
Main findings

A common and functional MTHFD1 polymorphism is associated with increased risk of AMI among the SAP patients in WENBIT. We furthermore observed a significant interaction between combined treatment of folic acid/vitamin B12 and vitamin B6 with the MTHFD1 polymorphism on risk of AMI occurrence.

Strengths and limitations

The MTHFD1 *rs1076991* is a common polymorphism, which ensures sufficient power to detect underlying gene–disease associations. The large sample size and long-term follow-up are strengths of the current study. To decrease the probability of type I errors by multiple testing, we did not assess the existence of other genetic variants besides the prime candidate SNP in the region of linkage disequilibrium (LD) at the MTHFD1 locus. Based on HapMap data, several SNPs in this region are in strong LD with MTHFD1 *rs1076991* and may therefore represent the underlying causes of the observed association (Supplementary table 1 and figure 1). Also, our results should be validated in independent populations, preferably with other clinical and baseline characteristics than the present cohort.

In the current investigation, we excluded patients with an acute coronary syndrome to avoid the influence of acute inflammation on SNP–treatment interactions. Notably, several previous studies have shown that acute coronary syndrome is associated with inflammatory response [19], which affects biomarkers of B vitamin status [20]. Thus, the current results are primarily relevant for patients with SAP only.



No. at Risk						
MTHFD1 CC	794	774	745	498	252	39
MTHFD1 CT	1141	1081	1032	727	362	63
MTHFD1 TT	446	420	403	273	138	26

Figure 2 Kaplan–Meier estimates of AMI according to the MTHFD1 polymorphism.

The cytosolic MTHFD1, diseases and biomarkers

Production of cytosolic C1-THF synthase (10-formyl-THF, 5,10-methenyl-THF and 5,10 methylene-THF) are involved in *de novo* purine and thymidylate synthesis, as well as in homocysteine remethylation. However, we did not observe any association of the MTHFD1 *rs1076991* polymorphism with plasma homocysteine levels, which concurs with results from another study [21]. Population studies have shown associations between MTHFD1 polymorphisms and neural tube defects [22] and gastric cancer [23]. One study showed strong association between MTHFD1 *rs1076991* and B-cell acute lymphoblastic leukemia [24]. To the best of our knowledge, the current study is among the first to focus on the promoter polymorphism of MTHFD1 with AMI occurrence in SAP patients.

Effects of B vitamin treatments on the association of MTHFD1 with AMI

We found a particularly strong association between the MTHFD1 polymorphism and AMI in patients allocated to combined vitamin B6 and folic acid/B12 treatment. Concomitant high levels of folate and B6 resulted in a non-significant beneficial effect against AMI in the MTHFD1 CC homozygous, and showed a more adverse tendency in patients with MTHFD1 T-allele. Vitamin B6 intake is shown to be inversely related to hepatic and plasma glycine levels [25], and positively associated with adenosylmethionine (SAM) [26] due to decreased SAM consumption by GNMT. Accumulated SAM has been shown to cause hepatic lipid accumulation and other atherothrombotic changes, including overt dyslipidemia [27] and oxLDL-induced foam cell formation [28]. On the other hand, adequate folate status is crucial for balancing the transmethylation flux. Folate deficiency has been associated with elevated circulating formate levels, impaired nucleotide synthesis, DNA hypomethylation and hyperhomocysteinemia [29]. Recent evidence linked folate intake with elevated hepatic SAM [30], which is a known inhibitor to betaine–homocysteine methlytransferase [31], which is suggested to regulate liver lipids and to induce apoB expression [32]. Indeed, excess SAM has been associated with hepatic apoB mRNA expression and VLDL assembly [33]. Coupled with the evidence that MTHFD1 deficiency has also been associated with accumulated intercellular SAM [34], these findings may be indicative of a potential mechanism in which lipid accumulation is exacerbated by the combined treatment in those with MTHFD1 deficiency, which may further promote atherosclerosis. Nevertheless, the interaction of the MTHFD1 genotype with glycine, lipid and B6 metabolism should be further explored.

Genetic analysis may provide more insights to the underlying pathophysiology of atherosclerosis. The

Table 2 HRs of AMI by MTHFD1 *rs1076991* polymorphism in WENBIT population and different treatment arms.

	Events/total	Simple model[a]			Multivariate model[b]			P_{int}[c]
		HR	95% CI	*P* value	HR	95% CI	*P* value	
WENBIT	204/2381	1.46	1.20–1.76	1.3×10^{-4}	1.49	1.23–1.81	5.5×10^{-5}	
Placebo	48/601	1.27	0.85–1.90	0.24	1.29	0.86–1.93	0.22	Ref.
Vitamin B6	46/593	1.44	0.95–2.16	0.08	1.53	1.01–2.31	0.04	0.59
Folic acid/B12	61/598	1.19	0.85–1.67	0.31	1.17	0.83–1.65	0.38	0.76
B6 + Folic acid/B12	49/589	2.28	1.51–3.44	9.0×10^{-5}	2.35	1.55–3.57	6.3×10^{-5}	0.047

[a] Simple model was adjusted for age and gender.
[b] Multivariate model was adjusted further for smoking status, obesity, hypertension and diabetes mellitus.
[c] P_{int} refers to the gene-treatment interaction in multivariate models which compared SNP association between placebo group and designated treatment groups.

modulation of AMI risk conferred by MTHFD1 polymorphism through allocation to vitamin B6 and folic acid/B12 in the current study may be interpreted in the context of personalized medicine. Since B-vitamin status may be correlated to other lifestyle factors like obesity or the intake of macronutrients, further studies are warranted to examine if the MTHFD1 genotype may influence the association between such lifestyle factors and atherogenesis, and if such association may be mediated partly by vitamin status.

Conclusion

We demonstrate that B vitamin treatment may introduce a strong association between a common and functional MTHFD1 promoter polymorphism and AMI risk in patients with SAP. Our results may potentially provide insight into the conflicting results of randomized B vitamin intervention trials on cardiovascular disease. Further studies should evaluate if this genotype may modify the risk of chronic diseases affected by other dietary or lifestyle factors.

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Disclosures

None.

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Appendix A. Supplementary material

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.numecd.2015.12.009.

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Plasma Glycine and Risk of Acute Myocardial Infarction in Patients With Suspected Stable Angina Pectoris

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ORIGINAL RESEARCH

Plasma Glycine and Risk of Acute Myocardial Infarction in Patients With Suspected Stable Angina Pectoris

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Background—Glycine is an amino acid involved in antioxidative reactions, purine synthesis, and collagen formation. Several studies demonstrate inverse associations of glycine with obesity, hypertension, and diabetes mellitus. Recently, glycine-dependent reactions have also been linked to lipid metabolism and cholesterol transport. However, little evidence is available on the association between glycine and coronary heart disease. Therefore, we assessed the association between plasma glycine and acute myocardial infarction (AMI).

Methods and Results—A total of 4109 participants undergoing coronary angiography for suspected stable angina pectoris were studied. Cox regression was used to estimate the association between plasma glycine and AMI, obtained via linkage to the CVDNOR project. During a median follow-up of 7.4 years, 616 patients (15.0%) experienced an AMI. Plasma glycine was higher in women than in men and was associated with a more favorable baseline lipid profile and lower prevalence of obesity, hypertension, and diabetes mellitus (all $P<0.001$). After multivariate adjustment for traditional coronary heart disease risk factors, plasma glycine was inversely associated with risk of AMI (hazard ratio per SD: 0.89; 95% CI, 0.82–0.98; $P=0.017$). The inverse association was generally stronger in those with apolipoprotein B, low-density lipoprotein cholesterol, or apolipoprotein A-1 above the median (all $P_{interaction}\leq 0.037$).

Conclusions—Plasma glycine was inversely associated with risk of AMI in patients with suspected stable angina pectoris. The associations were stronger in patients with apolipoprotein B, low-density lipoprotein cholesterol, or apolipoprotein A-1 levels above the median. These results motivate further studies to elucidate the relationship between glycine and lipid metabolism, in particular in relation to cholesterol transport and atherosclerosis.

Clinical Trial Registration—URL: https://www.clinicaltrials.gov. Unique identifier: NCT00354081. **(*J Am Heart Assoc*. 2016;5: e002621 doi: 10.1161/JAHA.115.002621)**

Key Words: amino acids • apolipoprotein • atherosclerosis • glycine • lipids and lipoprotein metabolism • myocardial infarction

Glycine is a nonessential amino acid that can be obtained either via the diet, or synthesized endogenously from serine, threonine, choline, or glyoxylate in the liver and kidney.[1] It is a predominant constituent of collagen and is utilized in the synthesis of several biologically important compounds, including glutathione, creatine, purines, and glucose.[2,3] Glycine exerts anti-inflammatory and antioxidative effects[4,5] and has been inversely associated with several traditional cardiovascular risk factors, including obesity,[6] hypertension,[7,8] and diabetes mellitus.[9]

Increasing evidence suggests that glycine-dependent reactions are associated with lipid homeostasis and cholesterol transport. More specifically, glycine is utilized to catabolize excess S-adenosylmethionine by its remethylation into sarcosine via the enzyme glycine-*N*-methyltransferase (GNMT),[10] and excess hepatic S-adenosylmethionine concentrations have been linked to the regulation of apolipoprotein (apo) B mRNA expression and very low-density lipoprotein formation.[11–13] Disturbances in these reactions have been associated with lipid accumulation both in the liver and in macrophages, which further promotes oxidized low-density lipoprotein (LDL)-induced foam cell formation in the artery wall.[12] Therefore, glycine availability may affect lipid metabolism and thereby further modulate the risk of coronary artery

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disease (CAD). However, data on glycine and the risk of atherosclerotic cardiovascular disease in observational studies are sparse.

We investigated the associations between plasma glycine and incident acute myocardial infarction (AMI) in a large cohort of patients with suspected stable angina pectoris, with a particular focus on potential effect modifications by lipid parameters. The results are reported according to the STrengthening the Reporting of OBservational studies in Epidemiology—Molecular Epidemiology (STROBE-ME) guidelines.[14]

Materials and Methods

Study Population

The study population has been described previously.[15] In brief, a total of 4164 subjects underwent elective coronary angiography for suspected stable angina pectoris during 2000–2004 at Haukeland (n=3413) or Stavanger (n=751) University Hospitals in Western Norway. Among these patients, 85 were taking folic acid supplementation while 583 were taking multivitamins before the study. In addition, 2573 (61.8%) were enrolled in the Western Norway B Vitamin Intervention Trial (WENBIT; ClinicalTrials.gov Identifier: NCT00354081). Patients with missing baseline data on plasma glycine (n=9), lipid parameters (n=1), or glycated hemoglobin (n=45) were excluded, leaving a total of 4109 patients eligible for the current analyses.

The study protocol was in accordance with the Declaration of Helsinki, and was approved by the Regional Committee for Medical and Health Research Ethics, Western Norway, the Norwegian Medicines Agency, and the Norwegian Data Inspectorate. All patients provided written informed consent.

Baseline Data

Clinical information and blood samples were obtained at baseline before coronary angiography. Smoking status was defined according to self-reports and serum cotinine levels (≥85 nmol/L) as previously described.[16] Obesity was defined as body mass index (BMI) ≥30 kg/m^2. Diabetes mellitus was classified by self-reports, glucose measurements (fasting plasma glucose ≥7.0 mmol/L or nonfasting plasma glucose ≥11.1 mmol/L), or by single measurement of glycated hemoglobin ≥6.5% according to the American Diabetes Association guidelines.[17] The extent of CAD at angiography was scored 0 to 3 according to the number of significantly stenotic coronary arteries. Left ventricular ejection fraction was determined by echocardiography or ventriculography.

Clinical End Points

Study subjects were followed from enrollment until the onset of AMI, or until the end of 2009. Information on clinical events was collected from the Cardiovascular Disease in Norway (CVDNOR; https://cvdnor.b.uib.no/) project, reporting on patients being discharged with a cardiovascular disease diagnosis from any of 42 Norwegian public hospitals from 1994 and throughout 2009.[18,19] AMI (including fatal and nonfatal AMI) as the primary end point was classified according to the International Statistical Classification of Disease Tenth Revision (ICD-10) codes I21 and I22, respectively.

Biochemical Analyses

Earlier reports have described the collection and storage of blood samples and the biochemical analyses for relevant clinical indices.[15,16] In addition, plasma glycine was analyzed by gas chromatography–tandem mass spectrometry[20] at Bevital A/S, Norway (www.bevital.no).

Statistical Analyses

Baseline categorical variables are reported as frequencies and percentages, while continuous variables are presented as medians with interquartile ranges. Plasma or serum metabolite concentrations were log-transformed before statistical analysis due to their right-skewed distributions. Baseline variables across quartiles of plasma glycine of the whole population were assessed by unadjusted median linear or logistic regression for continuous and categorical variables, respectively.

Cox regression analysis was used to estimate the association between plasma glycine and risk of AMI. The risk estimates were reported as fifth versus first plasma glycine quintiles, trends across quintiles, and per 1 SD increment in log-transformed plasma glycine. A simple survival model (model I) was adjusted for age (continuous) and sex (male/female). Covariates in the multivariate model (model II) included age (continuous), sex (male/female), smoking (yes/no), obesity (yes/no), hypertension (yes/no), diabetes mellitus (yes/no), extent of CAD at angiography (ordinal), statin treatment (yes/no), and estimated glomerular filtration rate, apolipoprotein A1 (apoA-1) and apoB (all continuous). Since results from experimental studies suggest that glycine may regulate pro-inflammatory cytokines,[21,22] we additionally included C-reactive protein (CRP) in an extended model. Fasting status and baseline revascularization procedures had negligible impact on the risk estimates and were excluded in the final model (data not shown). The assumption of proportionality was examined by the Schoenfeld and scaled

Schoenfeld residuals. Potential nonlinear dose–response relationships between plasma glycine and risk of incident AMI were visualized by generalized additive model plots for both simple and multivariate models.

Potential effect modifications by prespecified lipid parameters (including serum apoB, LDL cholesterol, apoA1, and high-density lipoprotein-cholesterol) were explored according to their median values and tested by including an interaction product term in Cox models adjusted for age, sex, and the use of statins.

The statistical analyses were performed in R (R Core Team, Vienna, Austria; version 3.1.1 & 3.1.2; packages "coin," "Hmisc," "survival," "MASS," and "mgcv"). All reported *P* values were 2-sided, and $P<0.05$ was considered significant.

Table 1. Baseline Characteristics According to Quartiles of Plasma Glycine

	Plasma Glycine Quartiles (μmol/L)				
	1st (<178)	2nd (178–205)	3rd (205–243)	4th (>243)	*P* for Trend
Age, y	61 (14)	62 (14)	61 (15)	63 (16)	0.037
Male sex, n (%)	817 (79.5)	818 (79.7)	804 (78.2)	521 (50.7)	<0.001
Current smoking, n (%)	299 (29.1)	292 (28.5)	375 (36.5)	337 (32.8)	0.003
Obesity, n (%)	321 (31.2)	183 (17.8)	135 (13.1)	113 (11.0)	<0.001
Hypertension, n (%)	561 (54.6)	483 (47.1)	451 (43.9)	434 (42.3)	<0.001
Diabetes mellitus, n (%)	513 (49.9)	413 (40.2)	393 (38.2)	393 (38.3)	<0.001
HbA1c (%)	6.23 (1.77)	6.02 (1.56)	6.05 (1.33)	6.08 (1.42)	<0.001
Serum					
Apolipoprotein A1, mg/dL	1.24 (0.32)	1.28 (0.34)	1.31 (0.34)	1.37 (0.37)	<0.001
Apolipoprotein B, mg/dL	0.90 (0.31)	0.88 (0.30)	0.85 (0.30)	0.85 (0.33)	<0.001
HDL cholesterol, mmol/L	1.10 (0.30)	1.20 (0.40)	1.30 (0.42)	1.40 (0.50)	<0.001
LDL cholesterol, mmol/L	3.00 (1.30)	2.99 (1.30)	2.90 (1.33)	2.90 (1.40)	0.064
eGFR, mL/min per 1.73 m^2	94 (18)	92 (19)	91 (20)	86 (24)	<0.001
Serum CRP, mg/L	2.48 (3.73)	1.84 (2.80)	1.58 (2.36)	1.35 (2.09)	<0.001
Troponin T, ng/L	5 (8)	4 (6)	5 (7)	4 (6)	0.088
Prior MI, n (%)	438 (42.6)	436 (42.5)	437 (42.5)	347 (33.8)	<0.001
LVEF (%)	65 (10)	65 (10)	65 (10)	65 (10)	0.092
Angiographic evidence of CAD, n (%)					
No significant stenosis	189 (18.4)	214 (20.8)	260 (25.3)	374 (36.4)	<0.001
Single-vessel disease	255 (24.8)	240 (23.4)	245 (23.8)	210 (20.4)	0.032
Double-vessel disease	250 (24.3)	249 (24.2)	228 (22.2)	188 (18.3)	<0.001
Triple-vessel disease	334 (32.5)	323 (31.6)	295 (28.7)	255 (24.9)	<0.001
Treatment following baseline coronary angiography, n (%)					
No or medications only	413 (40.2)	424 (41.3)	468 (45.5)	565 (55.0)	<0.001
PCI	374 (36.4)	357 (34.8)	337 (32.8)	283 (27.6)	<0.001
CABG	229 (22.3)	230 (22.4)	209 (20.3)	157 (15.3)	<0.001
Medications at discharge, n (%)					
Aspirin	854 (83.1)	871 (84.9)	849 (82.6)	781 (76.0)	<0.001
Statins	850 (82.7)	855 (83.3)	824 (80.2)	763 (74.3)	<0.001
Beta blockers	774 (75.3)	764 (74.5)	749 (72.9)	694 (67.6)	<0.001
ACEIs	237 (23.1)	233 (21.7)	202 (19.6)	178 (17.3)	<0.001
Loop diuretics	141 (13.7)	102 (10.0)	88 (8.4)	119 (11.5)	0.075

Variables are given in medians (interquartile ranges) or counts (percentages). ACEIs, angiotensin-converting-enzyme inhibitors; CABG, coronary artery bypass graft surgery; CAD, coronary artery disease; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; low-density lipoprotein; LVEF, left ventricular ejection fraction; MI, myocardial infarction; PCI, percutaneous coronary intervention.

Results

Patient Characteristics and Plasma Glycine Levels at Baseline

The median (interquartile range) age for the 4109 patients at baseline was 62 (15) years and 72.0% were males. As outlined in Table 1, persons in the upper glycine quartiles were more likely to be older, females, and smokers as compared to persons in the lower quartiles. However, the association between plasma glycine and smoking status was not significant after the adjustment for BMI (P=0.11). In addition, persons in upper quartiles of plasma glycine also had higher apoA-1 (P<0.001) and high-density lipoprotein-cholesterol levels (P<0.001) and lower serum apoB (P<0.001) and glycine tended to be associated with LDL cholesterol (P=0.064). Notably, after adjusting for statin treatment, plasma glycine was inversely associated with LDL cholesterol (P<0.001).

Plasma glycine was negatively associated with obesity, hypertension, diabetes mellitus, serum CRP, and prior AMI. We observed no association of plasma glycine with left ventricular ejection fraction and cardiac high-sensitive troponin T, but a strong inverse association with the extent of angiographic CAD, the latter also reflected by more use of coronary heart disease medications among patients in the lower plasma glycine quartiles.

Plasma Glycine and Risk of AMI

During a median (interquartile range) follow-up of 7.4 (2.4) years, 616 patients (15.0%) experienced an AMI. After adjusting for age and sex, higher plasma glycine was associated with a decreased risk of AMI (hazard ratio per SD: 0.88; 95% CI, 0.80–0.95; P=0.003). The association was essentially similar after the multivariate adjustment (hazard ratio per SD: 0.89; 95% CI, 0.82–0.98; P=0.017) (Table 2, Figure 1). However, additional adjustment for plasma CRP slightly attenuated the association between plasma glycine and incident AMI (hazard ratio per SD: 0.92; 95% CI, 0.84–1.01; P=0.085). Hazard ratios for AMI across all quintiles of plasma glycine gave similar results and are given in Table 3.

Notably, nearly 62% of the patients were enrolled in the WENBIT and randomly received treatments with folic acid plus vitamin B12, vitamin B6, or placebo. The risk estimates of plasma glycine in WENBIT were not modified by any intervention treatments (all $P_{interaction}$≥0.16, Table 4).

Subgroup Analyses

Figure 2 demonstrates the risk estimates between plasma glycine and AMI occurrence according to several lipid parameters. We observed a stronger negative association between plasma glycine and incident AMI among patients with high as compared to low serum apoB and LDL cholesterol levels ($P_{interaction}$=0.037 and 0.012, respectively). Additionally, we observed a stronger risk estimate of plasma glycine in patients with high as compared to low apoA-1 levels ($P_{interaction}$=0.027) and a similar trend was also seen for high-density lipoprotein cholesterol ($P_{interaction}$=0.16). The estimates were essentially similar after multivariate adjustment (Table 5).

Table 2. Association Between Plasma Glycine and Acute Myocardial Infarction

	Hazard Ratio	95% CI	*P* Value
Model I*			
Per SD increment	0.88	0.80 to 0.95	0.003
Q5 vs Q1	0.68	0.52 to 0.88	0.004
Model II†			
Per SD increment	0.89	0.82 to 0.98	0.017
Q5 vs Q1	0.71	0.54 to 0.94	0.016

*Adjusted for age and sex.
†Adjusted for age, sex, smoking, obesity, hypertension, diabetes mellitus angiographic extent of coronary artery disease, estimated glomerular filtration rate, apolipoprotein A-1, apolipoprotein B, and statin treatment.

Discussion

Principal Findings

In a large cohort of patients undergoing elective coronary angiography for suspected stable angina pectoris, higher plasma glycine was associated with a generally more favorable cardiovascular disease risk factor profile and with a decreased risk of AMI during follow-up, independent of traditional CAD risk factors. Furthermore, the inverse associations between glycine and AMI were stronger among patients with serum apoB, LDL cholesterol, or apoA-1 levels above the median.

Plasma Glycine and Cardiovascular Disease

Consistent with our study, glycine has previously been inversely associated with several coronary heart disease risk factors, and in particular those related to insulin sensitivity, glucose homeostasis, and the metabolic syndrome. Population-based studies have demonstrated a positive association of plasma glycine with estimated glucose disposal rate[23] and a negative association with glycated hemoglobin.[24] Accordingly, plasma glycine has been inversely associated with obesity,[6,25] hypertension,[8] and diabetes mellitus.[9,26] Glycine intake has also been associated with low plasma free fatty acids, cholesterol and triglycerides levels in animal models.[7,27]

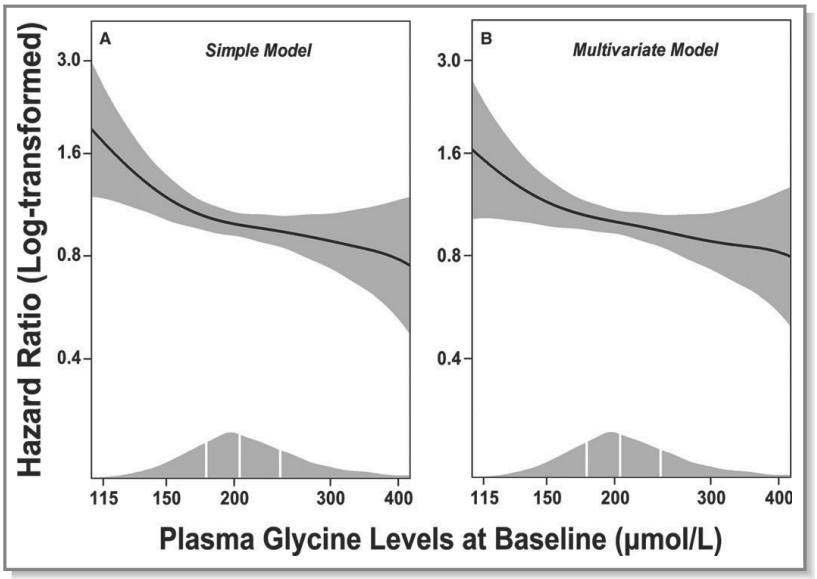

Figure 1. Dose–response associations between (log-transformed) plasma glycine and risk of acute myocardial infarction. Generalized additive regressions are used with the adjustment for age and sex in the simple model (A), and additional adjustment for smoking, obesity, hypertension, diabetes mellitus, angiographic extent of coronary artery disease (ordinal), estimated glomerular filtration rate, apolipoprotein A-1, apolipoprotein B, and statin treatment in the multivariate model (B). The solid lines and the shaded areas represent hazard ratios of plasma glycine and their 95% CI, respectively. The areas under the curve along the X-axes represent the distributions of the plasma glycine concentrations (μmol/L) in the total population. The vertical white lines denote the 25th, 50th, and 75th percentiles of plasma glycine, respectively.

Although high plasma glycine was related to an overall favorable coronary heart disease risk profile, we somewhat unexpectedly observed a positive association between plasma glycine and smoking status. However, this relationship may be explained by the negative correlation between BMI and smoking behavior,[28] as adjusting for BMI rendered the association nonsignificant. The relationships between glycine status and clinical cardiovascular end points have, to the best of our knowledge, not been evaluated previously in large-scale observational studies; hence the current study extends previous knowledge on cardiovascular prognosis according to glycine status.

Table 3. Hazard Ratios (HR) of AMI According to Quintiles of Plasma Glycine Levels

	Model I*		Model II†	
	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
Quintiles				
First	Reference		Reference	
Second	0.89 (0.71–1.13)	0.34	0.93 (0.73–1.18)	0.54
Third	0.76 (0.59–0.97)	0.025	0.81 (0.63–1.04)	0.10
Fourth	0.80 (0.63–1.01)	0.062	0.83 (0.64–1.06)	0.13
Fifth	0.68 (0.52–0.88)	0.004	0.71 (0.54–0.94)	0.016
Trend	0.92 (0.87–0.97)	0.004	0.92 (0.87–0.98)	0.012

*Adjusted for age and sex.
†Adjusted for age, sex, smoking, obesity, hypertension, diabetes mellitus angiographic extent of coronary artery disease, estimated glomerular filtration rate, apolipoprotein A-1, apolipoprotein B, and statin treatment.

Possible Mechanisms

The negative association between plasma glycine and LDL cholesterol in the current study was probably veiled by the intake of statins, since a greater proportion of patients in lower plasma glycine quartiles were prescribed statins. Accordingly, we observed a significant inverse trend between plasma glycine and LDL cholesterol after adjusting for statins, in line with the inverse association with apoB, but positive relationships with apoA-1and high-density lipoprotein cholesterol. This suggests an important role of glycine in lipid metabolism.

Table 4. Association Between Plasma Glycine and Acute Myocardial Infarction in Different Treatment Arms in WENBIT

	Total		Folic Acid+B12		Folic Acid+B12+B6		B6		Placebo	
	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
Model I*										
Per SD increment	0.88 (0.79–0.97)	0.012	0.89 (0.72–1.11)	0.31	0.94 (0.77–1.16)	0.56	0.87 (0.70–1.08)	0.20	0.80 (0.65–0.97)	0.025
Q5 vs Q1	0.71 (0.52–0.96)	0.027	0.73 (0.39–1.38)	0.33	1.01 (0.55–1.83)	0.98	0.74 (0.39–1.40)	0.36	0.45 (0.24–0.84)	0.012
Model II†										
Per SD increment	0.89 (0.80–0.99)	0.046	0.94 (0.74–1.19)	0.61	0.97 (0.78–1.21)	0.80	0.88 (0.70–1.11)	0.29	0.82 (0.66–1.02)	0.07
Q5 vs Q1	0.74 (0.53–1.03)	0.077	0.86 (0.44–1.70)	0.67	1.09 (0.57–2.07)	0.79	0.76 (0.37–1.58)	0.47	0.48 (0.25–0.94)	0.032

HR indicates hazard ratio; WENBIT, Western Norway B Vitamin Intervention Trial.

*Adjusted for age and sex.

†Adjusted for age, sex, smoking, obesity, hypertension, diabetes mellitus angiographic extent of coronary artery disease, estimated glomerular filtration rate, apolipoprotein A-1, apolipoprotein B, and statin treatment.

Indeed, considerable evidence suggests that glycine availability may be important in lipid metabolism and atherosclerosis. First, glycine can be methylated into sarcosine via GNMT, which is mainly confined to the liver and kidney[29,30]; however, rodent studies have shown that the GNMT is also localized to aortic endothelial cells.[12] Impaired GNMT flux was shown to exacerbate lipid accumulation in both the liver and in macrophages, which can further promote oxidized LDL-induced foam cell formation in the artery wall.[12] Furthermore, GNMT flux has also been shown to affect composition of atherosclerotic plaques and regulate inflammation response within atherosclerotic lesions.[12] We observed stronger associations of glycine with AMI among those with higher apoB and LDL cholesterol levels in subgroup analyses. This finding may support the hypothesis that GNMT flux plays a role in plaques formation and the progression of atherosclerosis.

In addition, impaired GNMT flux was suggested to interrupt reverse cholesterol transport by downregulating the expression of scavenger receptors class B member 1 and ATP-binding cassette transporters-A1 and G1.[12] GNMT deficiency has also been associated with hepatic cholesterol accumulation and overt dyslipidemia by downregulation of Niemann-Pick type C2 protein,[31] a regulator of intracellular cholesterol trafficking and homeostasis. In the same study, enhanced GNMT flux was shown to promote cholesterol export from the cells by upregulating Niemann-Pick type C2, a process requiring the involvement of lipid-poor apolipoproteins (apoA-1 and apoE), which may explain the stronger beneficial effect of plasma glycine among patients with higher apoA-1 levels.

Moreover, reduced flux over the GNMT pathway may cause the accumulation of excess S-adenosylmethionine,[10] which is shown to interrupt hepatic apoB mRNA expression and very low-density lipoprotein assembly.[12,13] Accordingly, glycine infusion normalized hepatic triglyceride-rich very low-density lipoprotein secretion in rats fed a high-fat diet,[32] suggesting the necessity of adequate glycine status in avoiding hepatic lipid accumulation, which is considered an independent coronary heart disease risk factor.[33]

Plasma and tissue glycine concentrations are regulated by the B6-dependent glycine cleavage system. Therefore, glycine elevation may reflect B6 deficiency.[34] Interestingly, low B6 status is suggested as a risk marker for CAD.[35,36] However, in part of our population who received B vitamin treatments, we did not observe any significant interaction between plasma glycine and vitamin B6 on AMI occurrence. This finding may indicate that the glycine-related atherogenesis may not be solely dependent on pathways requiring vitamin B6.

Nevertheless, glycine has wide metabolic ramifications, which therefore makes it difficult to make conclusions on any particular pathomechanism involved in the current study. For instance, the negative correlation between plasma glycine and CRP is in line with other studies,[37,38] implying a role of glycine

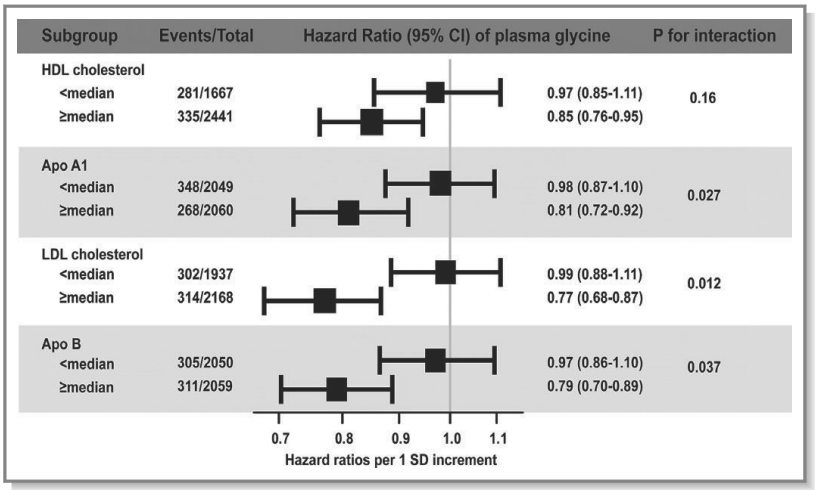

Figure 2. Risk associations between plasma glycine and AMI according to the median values of prespecified lipid parameters. The black squares represent the hazard ratios and their areas are proportional to the subgroup sizes. Horizontal lines represent the 95% CI. AMI indicates acute myocardial infarction; ApoA-1, apolipoprotein A-1; ApoB, apolipoprotein B; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

in inflammation. Indeed, glycine has been shown to directly regulate the production of pro-inflammatory cytokines[22,39] and has been suggested as a modulator of the pro-inflammatory state.[21] These findings may indicate the involvement of glycine-related inflammation in atherogenesis and can at least partly explain the attenuation of the risk estimate of glycine when including serum CRP in the extended Cox model.

Table 5. Risk Associations Between Plasma Glycine and AMI According to the Median Values of Prespecified Lipid Parameters

	HR (95% CI)*	*P* Value	P_{int}
HDL			
Above median	0.86 (0.76–0.97)	0.017	0.22
Below median	0.96 (0.84–1.10)	0.58	
ApoA-1			
Above median	0.81 (0.71–0.93)	0.003	0.039
Below median	0.99 (0.87–1.12)	0.84	
LDL			
Above median	0.80 (0.70–0.91)	<0.001	0.013
Below median	0.99 (0.87–1.13)	0.92	
ApoB			
Above median	0.81 (0.71–0.92)	0.002	0.032
Below median	0.99 (0.87–1.12)	0.84	

AMI indicates acute myocardial infarction; HDL, high-density lipoprotein; HR, hazard ratio; LDL, low-density lipoprotein.
*Adjusted for age, sex, smoking, obesity, hypertension, diabetes mellitus angiographic extent of coronary artery disease, estimated glomerular filtration rate, apolipoprotein A-1, apolipoprotein B, and statin treatment.

Strengths and Limitations

The strengths of the study include the large sample size, detailed baseline clinical characteristics, and its long-term prospective design. Notably, a prior study from a subsample of the current cohort showed that plasma glycine has an excellent within-person reproducibility over time (intraclass correlation coefficient: 0.77 [95% CI: 0.74–0.79]),[40] allowing 1-exposure assessment of biomarker status, as well as low risk of regression-dilution bias.[41]

Several metabolic pathways contribute to glycine formation, and their relative quantitative contributions are not fully elucidated. Glycine concentrations among individuals may therefore be influenced by genetic and metabolic traits, as well as dietary habits, which were not evaluated in the current study. Hence, the possibility of residual confounding cannot be excluded.

Conclusions

Plasma glycine was associated with decreased risk of AMI in patients with suspected stable angina pectoris. This association was particularly strong in those with apoB, LDL cholesterol, or apoA-1 levels above the median. Our findings

motivate further studies to elucidate the role of glycine in regulating lipid metabolism and cholesterol transport in patients with atherosclerosis.

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Disclosures

None.

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