

**Folic acid supplementation and biomarkers of
progression of sub-clinical coronary atherosclerosis in
patients with stable angina pectoris**

**A WENBIT sub-study based on coronary angiography and
intravascular ultrasound**

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LIST OF ABBREVIATIONS

- ACS – Acute coronary syndrome
ADMA – Asymmetric dimethylarginine
AMI – Acute myocardial infarction
CABG – Coronary artery bypass graft
CAD – Coronary artery disease
CVD – Cardiovascular disease
CX – Circumflex artery
DS – Diameter stenosis
FA – Folic acid
FCA – Fibrous calcified atheroma
GAM – Generalized additive model
GAMM – Generalized additive mixed model
GFR – Glomerular filtration rate
IVUS – Intravascular ultrasound
IX – Intimal xanthoma
LAD – Left anterior descending artery
LDL – Low density lipoprotein
LME – Linear mixed model
LQMM – Linear quantile mixed model
MCP-1 – Monocyte chemoattractant protein-1
MLD – Minimum lumen diameter
MMP – Matrix metalloproteinase
NORVIT – Norwegian Vitamin Trial
NOS – Nitric oxide synthase
PCI – Percutaneous coronary intervention
PIT – Pathological intimal thickening
QCA – Quantitative coronary angiography
RCA – Right coronary artery
RCT – Randomized controlled trial

SAP – Stable angina pectoris

SMC – Smooth muscle cell

TCFA – Thin cap fibroatheroma

UAP – Unstable angina pectoris

VH-IVUS – Virtual histology intravascular ultrasound

VH-TCFA – Virtual histology thin cap fibroatheroma

WENBIT – Western Norway B vitamin Intervention Trial

SCIENTIFIC ENVIRONMENT

The current thesis is a result of collaboration between several scientific environments. The research was mainly carried out in cooperation between the Department of Heart Disease, Haukeland University Hospital and the Department of Clinical Science, University of Bergen (UiB). The laboratory facilities for analyses related to one-carbon metabolism (vitamins/vitamins, amino acids and metabolites) are organized by the Section for Pharmacology, Department of Clinical Science (UiB), within the frame of the company, Bevital (www.bevital.no). Additional analyses were performed by the group of Asbjørn Svardal at ICS, UiB. IVUS data and VH analyses was provided by Volcano Corp. and the global IVUS registry.

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SUMMARY

Background – Coronary artery disease (CAD) is the leading cause of death in the world. Total plasma homocysteine (tHcy) is an independent risk factor for CAD, and while tHcy is lowered by folic acid (FA)/vitamin B₁₂ (B12) treatment – such therapy has failed to result in clinical benefit.

Aim – Investigate the connection between treatment with FA/ B12 and progression of sub-clinical CAD in patients with stable angina pectoris (SAP) with invasive measurements of CAD progression and associated metabolic changes.

Materials and methods – Patients included in Sub-group studies from the Western Norway B Vitamin Intervention Trial (WENBIT) undergoing percutaneous coronary intervention were randomized to daily treatment with FA/B12 or placebo. Coronary angiograms and quantitative coronary angiography (QCA) were obtained at baseline and follow-up along with virtual histology intravascular ultrasound. Plasma levels of asymmetric dimethylarginine (ADMA), trimethyllysine (TML) and monocyte chemoattractant protein-1 (MCP-1) were collected.

Results –Paper I demonstrated that treatment with FA/B12 was associated with a potential risk of rapid progression of CAD by QCA. Paper II showed that while FA/B12 did not affect levels of ADMA, TML or MCP-1, ADMA and TML predicted progression of CAD by QCA. In paper III MCP-1 was associated with unstable coronary plaques.

Conclusion and implications –Vitamin B treatment showed a potential adverse effect on angiographic progression of CAD. While the methylated amino acids ADMA and TML adversely affected CAD – FA/B12 intervention did not affect either ADMA/TML levels or plaque stability as measured by VH-IVUS.

LIST OF PUBLICATIONS

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

- Paper I** Løland KH, Bleie Ø, Blix AJ, Strand E, Ueland PM, Refsum H, Ebbing M, Nordrehaug JE, Nygård O. Effect of Homocysteine-Lowering B Vitamin Treatment on Angiographic Progression of Coronary Artery Disease: A Western Norway B Vitamin Intervention Trial (WENBIT) Substudy. *The American Journal of Cardiology*. 2010;105:1577-1584.
- Paper II** Løland KH, Bleie O, Borgeraas H, Strand E, Ueland PM, Svardal A, Nordrehaug JE, Nygård O. The Association between Progression of Atherosclerosis and the Methylated Amino Acids Asymmetric Dimethylarginine and Trimethyllysine. *PLoS ONE*. 2013 May 29;8(5):e64774. doi: 10.1371/journal.pone.0064774.
- Paper III** Løland KH, Bleie Ø, Strand E, Ueland PM, Nordrehaug JE, Garcia-Garcia HM, Serruys PW, Nygård O. Effect of Folic Acid Supplementation on Levels of Circulating Monocyte Chemoattractant Protein-1 and the Presence of Intravascular Ultrasound Derived Virtual Histology Thin-Cap Fibroatheromas in Patients with Stable Angina Pectoris. *PLoS ONE* 2013 July 25;8(7): e70101. doi:10.1371/journal.pone.0070101.

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“There is a disorder of the breast, marked with strong and peculiar symptoms, considerable for the kind of danger belonging to it, and not extremely rare, of which I do not recollect any mention among medical authors. The seat of it, and sense of strangling and anxiety with which it is attended, may make it not improperly be called angina pectoris.”

Heberden, W. 1772. Some account of a disorder of the breast. *Medical Transactions*.
The Royal College of Physicians of London. 2: 59-67.

INTRODUCTION

Coronary artery disease (CAD) is a chronic, fibroproliferative inflammatory condition affecting the arterial walls of the epicardial vessels supplying the myocardium.

Typically characterized by decades of clinically silent progressions, it is punctuated by sudden clinical events such as acute myocardial infarctions (AMI), unstable angina pectoris (UAP) or sudden cardiovascular death (SCD) associated with substantial morbidity and mortality¹.

1.1. Clinical entities

The term angina pectoris refers to the subjective chest discomfort or pain experienced during myocardial ischemia². It is often associated with shortness of breath, nausea or diaphoresis. Myocardial ischemia may occur secondary to obstructive CAD, but can also manifest itself due to other perturbations of myocardial oxygen metabolism such as anemia, hypoxemia, hypovolemia as well as other causes.

Stable angina pectoris (SAP) is the clinical entity in which angina pectoris is triggered with exertion in a predictable manner and is relieved by rest or administration of nitroglycerine. When patients present with symptoms of angina which are either new in onset, last >20 minutes, occurs at rest or a previously sustainable level of physical activity or in the wake of a previous AMI, the episode should be considered an acute coronary syndrome (ACS)^{3,4}. Further sub-division into ST- and non-ST-elevation acute coronary syndrome (STEMI and NST-ACS respectively) is based upon the presence or absence of significant ST-elevations in the patient's electrocardiogram³. Depending on whether the myocardial ischemia is sufficient to result in cellular necrosis and release of circulating cardiac markers (e.g. troponin T), the patient is diagnosed with either non-ST-elevation myocardial infarction (NSTEMI) or unstable angina pectoris (UAP)³.

1.2. Cardiovascular epidemiology

In 2008 noncommunicable diseases (NCD) surpassed infectious disease as the leading cause of mortality with 63% of the world's 57 million deaths⁵. Of these 36 million deaths 17.3 million – or 30% of all global deaths – were due to cardiovascular disease (CVD), of which CAD was the most prevalent with 7.3 million⁵. While traditionally considered a disease of industrialized nations and the “Western” world, more than 80% of the world's CVD deaths occur in middle- to low income countries⁵ – exalting a disproportional economic burden of chronic disease management on underdeveloped health care systems.

Risk factors for developing CAD are mostly modifiable and an abnormal lipid profile, smoking, hypertension, diabetes, abdominal obesity, psychosocial factors, consumption of fruits, vegetables, limited amount of alcohol, and regular physical activity account for 90% of the population attributable risk of myocardial infarction (the last four associated with lower risk) worldwide regardless of region and gender⁶. Despite decreasing incidence and mortality of CAD in high-income countries^{7, 8}, the worldwide prevalence continuous to increase^{6, 9}. The prevalence of obesity, diabetes, and hypertension have risen steadily the last decades^{5, 10} reaching epidemic proportions – heralding further rise in CAD incidence and mortality rates.

The reduction in cardiovascular mortality in high-income countries have been attributed to increased risk factor control and advances in disease management such as statin therapy, antithrombotic medication and revascularization procedures such as percutaneous coronary intervention (PCI) and coronary artery bypass grafting (CABG) in patients with clinically unstable CAD¹¹. However, despite these apparent advancements, patients with established CAD have a substantial residual risk for future mortality and morbidity. Of patients ≥ 45 years of age experiencing their first AMI 19% of men and 26% of women will die within a year, increasing to 36% and 46% respectively after five years¹². The rate of recurrence is substantial even in the younger part of the CVD demographic, patients aged 45 to 64 years, 15% of men and 22% of women experience a recurrent AMI or SCD within 5 years¹².

1.3. Pathogenesis of atherosclerosis

Disease processes affecting the arterial wall are termed *arteriosclerosis*, meaning ‘hardening of the arteries’. The term *atherosclerosis* is a distinct form of arteriosclerosis caused by the presence of an atheroma within the arterial lining, originally stemming from the word *atheroma* (Gr. ἀθήρα), meaning gruel. The atheroma is histopathologically an accumulation of soft yellowish material composed of macrophages, extracellular cholesterol deposits and necrotic tissue. Atherosclerosis can affect several parts of the arterial vasculature, including coronary as well as cerebral and peripheral arteries, causing conditions such as AMI, stroke and claudication. CAD is the clinical entity where atherosclerosis affects the coronary arteries, causing symptoms when it compromises the myocardial blood supply¹.

Evidence suggests that coronary atherosclerosis begins as early as the first and second decade of life^{13, 14}. Necropsy studies reveal atherosclerotic lesions in 2% of men 15-19 years of age, rising to 19% in 30-34 year old men¹⁵. Intracoronary ultrasound investigations reveal atherosclerotic coronary lesions in 17% in patients less than 20 years of age undergoing coronary angiography prior to heart transplantation and is related to coronary risk factors¹⁶.

1.4. Histology

Several attempts have been made to characterize the different coronary atherosclerotic lesions, notably the American Heart Association (AHA) lesion classification system¹⁷.¹⁸ The AHA classification was subsequently modified by Virmani et al.¹⁹ and is used in this thesis along with a statement report on terminology by the same authors²⁰.

Table 1. Modified AHA classification based on Morphological description.

Lesion	Abbreviation	Description
<i>Nonatherosclerotic intimal lesions</i>		
Intimal thickening	IT	The normal accumulation of smooth muscle cells (SMCs) in the intima in the absence of lipid or macrophage foam cells.
Intimal xanthoma (previously "fatty streak")	IX	Luminal accumulation of foam cells without a necrotic core or fibrous cap. Based on animal and human data, such lesions usually regress.

Progressive atherosclerotic lesions

Pathological intimal thickening	PIT	SMCs in a proteoglycan-rich matrix with areas of extracellular lipid accumulation without necrosis.
Fibrous cap atheroma	FCA	Well-formed necrotic core with an overlying fibrous cap.
Thin fibrous cap atheroma	TCFA	A thin fibrous cap infiltrated by macrophages and lymphocytes with rare SMCs and an underlying necrotic core.
Calcified nodule	CN	Eruptive nodular calcification with underlying fibrocalcific plaque.
Fibrocalcific plaque	FP	Collagen-rich plaque with significant stenosis usually contains large areas of calcification with few inflammatory cells; a necrotic core.

AHA, American Heart association; SMC, smooth muscle cells. Modified from Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM. Lessons from sudden coronary death : A comprehensive morphological classification scheme for atherosclerotic lesions. *Arteriosclerosis, thrombosis, and vascular biology*. 2000;20:1262-1275.

The modified AHA classification¹⁹ is based on morphological description and includes 7 categories; intimal xanthoma, intimal thickening, pathological intimal thickening, fibrous cap atheroma, thin fibrous cap atheroma, calcified nodule and fibrocalcific plaque as illustrated in table 1. This classification focuses on morphologically recognizable histological patterns and reflects the understanding that atherosclerosis is a dynamic process with multiple stages not necessarily succeeding each other in a linear manner.

1.4.1. Intimal xanthoma and intimal thickening.

Intimal xanthoma (IX), previously named “fatty streaks”, are focal accumulations of lipid-laden macrophages (termed “foam-cells”) occurring at specific branch points throughout the coronary vasculature beginning soon after birth¹⁹. However, current evidence suggests that IXs are not the substrate of progression to more complex coronary lesions, since they both demonstrate the ability to regress and are differently distributed in children than in the adult population^{13, 15, 21}. Intimal thickening (IT) on the other hand occurs at sites associated with atherosclerotic lesions in the adult²¹ and is generally thought to be the precursor of advanced coronary lesions¹⁹. ITs are intimal masses consisting of smooth muscle cells (SMCs), usually clonal²² expansions, and proteoglycan-collagen matrix with little or no infiltrating inflammatory cells.

1.4.2. Pathological intimal thickening.

Pathological intimal thickening (PIT), an intermediate stage between IX/IT and the fibrous cap atheroma, is characterized by the presence of extracellular lipid accumulation typically adjacent to the media with and relative lack of SMCs^{18, 19, 23}. Necrosis is absent, and subluminal macrophage and T lymphocyte infiltration in the intima overlying the lipid pool occurs^{18, 19, 23}.

1.4.3. Fibrous cap atheroma

When a distinct “fibrous cap” of fibrous tissue overlies a lipid core of a certain size, the lesion is characterized as a fibrous cap atheroma (FCA)^{17, 19}. This is generally considered the first of the advanced coronary lesions and is also known as a *fibroatheroma*. The lipid core initially consists of necrotic cellular debris, lipid-laden macrophages and a relative lack of extracellular matrix, while the fibrous cap is constituted by SMCs and collagen fibers and varying presence of macrophages and lymphocytes¹⁹. In more developed FCAs, the necrotic core enlarges, free cholesterol and cholesterol crystals becomes more abundant and separate necrotic cores consolidates as the proteoglycan-collagen matrix is depleted.

1.4.4. Thin fibrous cap atheroma

Further growth of the atheroma and thinning of the overlying fibrous cap is the hallmark of the thin cap fibroatheroma (TCFA) characterized by a fibrous cap of <65 μm thick, typically devoid of SMCs but infiltrated by macrophages secreting matrix metalloproteinases (MMPs)^{19, 20, 24}. TCFA are termed “vulnerable plaques” because they are the anatomical substrates of coronary plaque rupture^{19, 25-27}. The underlying lipid core typically includes necrosis, calcification, neoangiogenesis (*vasa vasorum*) and hemorrhages.

The cut-off at 65 μm is based on previous studies that show that the mean fibrous cap thickness of ruptured coronary plaques were $23 \pm 19 \mu\text{m}$ with 95% of caps <64 μm ²⁴. Since these measurements have been done in *ex vivo* specimens, they are potentially subject to fixation artifacts. *In vivo* measurements with more sensitive optical

coherence tomography (OCT) techniques shows that median (IQR) value of cap thickness in ruptured plaques was 54 (10) μm with 95% of the thinnest cap thickness $<80 \mu\text{m}^2$.

1.5. Progression of coronary atherosclerosis

Progression of coronary atherosclerosis is linked to several factors of which endothelial dysfunction, inflammation, dyslipidemia, plaque rupture, intra-plaque hemorrhage and smoking have been established. Several other, less established mechanisms have been proposed and will be discussed later. The interaction between lipid metabolism and inflammation is shown in figure 1.

1.5.1. Endothelial dysfunction and shear stress

The epithelial monolayer lining the arterial wall is a metabolic active and regulatory important tissue. It is generally accepted that endothelial dysfunction, generally defined as impaired endothelial vasodilator function, is one of the first step in atherogenesis and is due to loss of endogenous nitric oxide production²⁹. Evidence suggests that at least part of this effect is mediated through elevated levels of asymmetric dimethylarginine (ADMA) which is an endogenous nitric oxide synthase (NOS) inhibitor^{30,31}.

Shear stress in vascular biology is defined as the coplanar force vector generated by the movement of blood along the vessel wall. Endothelial cells alter function in response to altered shear stress through mechanoreception³². Turbulent blood flow produces less shear stress than laminar and it is precisely in these regions of the arterial tree – i.e. the inner side of curvatures, near branch points or the outer wall of bifurcations that atherosclerotic lesions tend to amass³³. The role of mechanical, shear, stress in the initiation of the atherosclerotic process is substantial³².

1.5.2. Inflammation

The recognition of the inflammatory response as playing a pivotal role in the formation and progression of atherosclerosis has been one of the leading breakthroughs in the

understanding of the biology of atherosclerosis in recent years. Research has shown relationships with both progression of CAD, plaque instability and rupture, as well as with clinical end-points in numerous studies with different biomarkers. High-sensitivity C-reactive protein (hsCRP) has already earned its mark as an independent biomarker³⁴ of risk and several other inflammatory markers are under investigation.

Activated endothelial cells express surface antigens which facilitates the transmigration of inflammatory cells such as monocytes. Once adherent to the endothelium, monocytes are drawn into the plaque along concentration gradients of chemokines, such as monocyte chemoattractant protein-1 (MCP-1)³⁵. These antigens, commonly known as adhesion molecules, are upregulated in settings of irritative endothelial stimuli such as dyslipidemia, hypertension and hemodynamic alterations¹. In addition, both T-cells (helper and regulatory) as well as B-cells, in a limited number, are present in the intima and probably regulates macrophage activity¹.

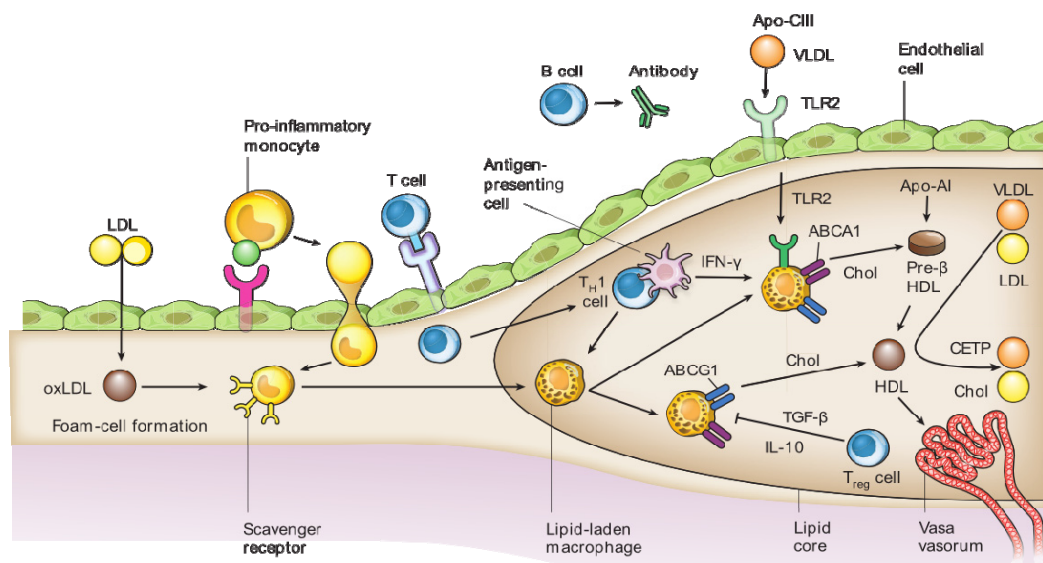


Figure 1. Overview of the intersection between inflammation and lipid metabolism in relation to the development of atherosclerotic lesions.

Oxidized LDL (oxLDL) is engulfed by macrophages by scavenger receptors. Both transforming growth factor beta (TGF-β) and interferon gamma (IFN-γ) is involved. VLDL, very-low density lipoprotein; IL-10, interleukin-10; TLR2, Toll-like receptor 2; CETP, cholesteryl ester transfer protein. Adapted with permission from Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. *Nature*. 2011;473:317-325. Copyright © 2011, Rights Managed by Nature Publishing Group.

1.5.3. Dyslipidemia

Dysregulation of lipid metabolism is paramount to the development of coronary atherosclerosis³⁶. Consistently, elevated LDL cholesterol has shown a strong association with the development of atherosclerotic CVD, while high levels of HDL cholesterol is linked to favorable outcome. Hypertriglyceridemia is also related to increased risk of cardiovascular events, though not as definite as the association between triglycerides and other risk factors^{37,38}.

It is believed that the retention of LDL particles within the intima is a necessary step in atherogenesis. Oxidation of these particles to OxLDL elicits inflammatory stimuli which are thought to be pivotal in the initiation of atherosclerotic progression. OxLDL interacts with scavenger receptors on the surfaces of recruited monocytes¹. Upon OxLDL-phagocytosis, these cells become lipid-laden and termed foam cells due to their characteristic appearance.

1.5.4. Intra-plaque hemorrhage and plaque rupture

It has more recently been appreciated that the expansion of the necrotic core is heavily dependent on both intra-plaque hemorrhage and plaque rupture²⁵. A significant portion of free cholesterol in the necrotic core of the atherosclerotic plaques stems from the cellular membrane of erythrocytes¹. Intra-plaque hemorrhage probably occurs from disruption of weak vasa vasorum which has grown into the plaque from the adventitia as a response to central plaque hypoxia³⁹.

Disruption of the fibrous plaque with subsequent plaque rupture and atherothrombosis does not necessarily result in luminal obstruction and clinical events. In fact, most plaque ruptures are clinically silent^{25,26} and heal without precipitating any symptoms at all. Evidence of atherothrombosis, tissue organization and re-endothelialization are present in most atherosclerotic lesions above 50% diameter stenosis, and only about 11% of plaque ruptures are “virgin” without sign of previous ruptures⁴⁰. While not leading to clinical

events, a silent plaque rupture does lead to significant expansions of the plaque volume, but development of obstructive coronary artery disease following repeat ruptures.

1.5.5. Smoking

Smoking is a major risk factor for cardiovascular mortality and morbidity⁶. It has been associated with both endothelial dysfunction and heightened thrombogenicity⁴¹, possibly mediated through increased inflammation⁴², oxidation of LDL-particles⁴³ and impaired endothelial relaxation⁴⁴.

1.6. The unstable coronary plaque and acute coronary syndromes

James E. Muller et al coined the phrase “the vulnerable plaque” in 1989 when referring to coronary plaques susceptible to rupture⁴⁵. It is now widely acknowledged that such vulnerable plaques is the underlying histopathological feature in most ACS events and most, if not all, atherothrombotic events²⁵. The common causal feature is disruption of the endothelial barrier between the blood and the highly thrombogenic material located within the coronary plaque. These plaques are seldom flow-limiting, angiographically significant stenoses, in fact as many as 75% of vulnerable lesions were identified in coronary segments with <50% diameter stenosis^{26, 46}. Disruption of this endothelial barrier can manifest itself through different mechanism, either a plaque rupture, plaque erosion or a calcified nodule²⁷. In 59 – 75% of ACSs, the event is due to plaque rupture while plaque erosion accounts for 33 – 44%. Calcified nodules are the instigators in only a fraction of the cases, approximately 2-9%^{19, 25}. Depending on the coronary location of the plaque rupture, the blood coagulability at the time of rupture, the size of the thrombus formed and degree of luminal obstruction induced the clinical symptoms range from asymptomatic through unstable angina, non-ST-/ST-elevation infarction and sudden cardiac death²⁵.

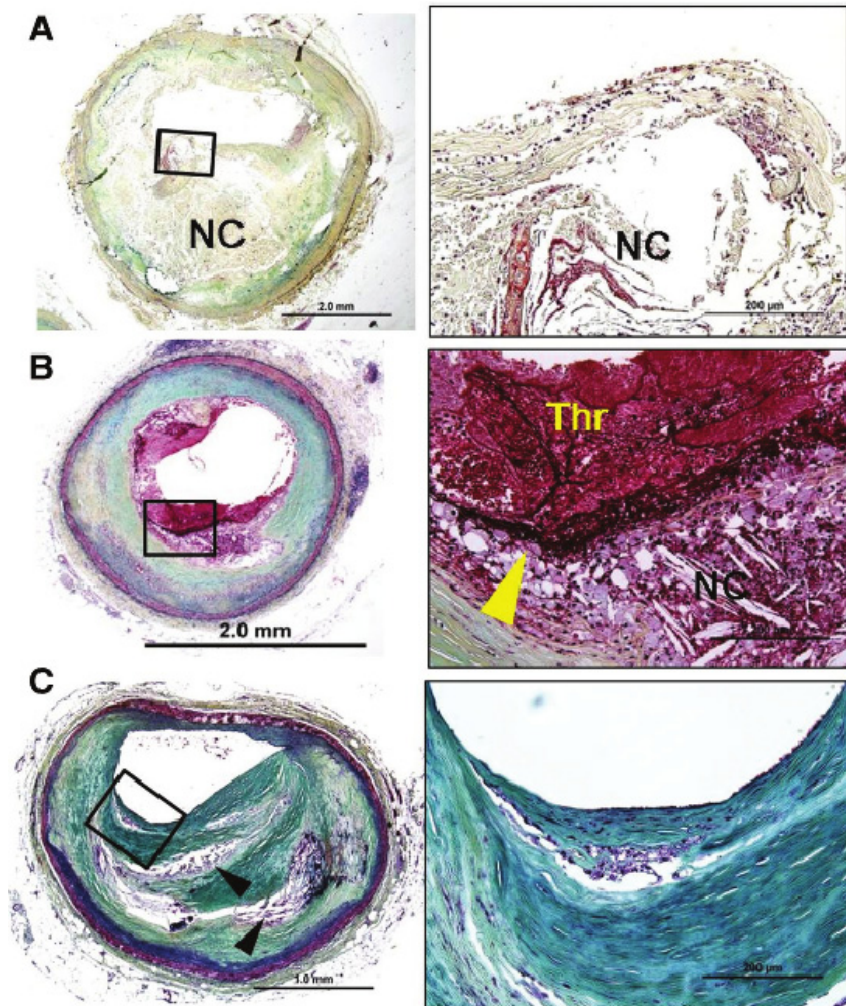


Figure 2. Histopathological images of TCFA lesions, all from patients with sudden cardiac death.

A, example of intact TCFA with necrotic core (NC). **B**, TCFA with plaque rupture and non-obstructive thrombus. Yellow arrow marks site of fibrous cap disruption. **C**, evidence of repeat plaque rupture (black arrow) resulting in 80% luminal occlusion. Adapted with permission from Arbab-Zadeh, A et al. (2012). "Acute Coronary Events." *Circulation* 125(9): 1147-1156. © 2012 Wolters Kluwer Health. All rights reserved.

As previously mentioned, the most prevalent clinical manifestation of plaque rupture is now considered to be nothing, i.e. asymptomatic. This results in negative plaque remodeling and increased luminal obstruction (as seen in figure 2, frame C) with progression of angina as a result, rather than acute clinical events.

In recent years it has been increasingly apparent that such lesions are not satisfactorily diagnosed in clinical practice and there is a growing consensus that early detection and intensive treatment of such lesions are paramount in order to decrease the rate of “unexplained” coronary events in optimally treated patient populations⁴⁷.

Inflammatory activity leads to production of proteolytic enzymes that degrades extracellular matrix and structural integrity of the fibrous cap which is rendered more vulnerable to mechanical stress⁴⁸. These MMPs are collagenases whose expression is induced by inflammatory mediators^{1,48}. Apoptosis of both macrophages and SMCs probably plays an important part in plaque destabilization⁴⁹.

1.7. Biomarkers and risk factors

A biomarker is defined as a “*characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention*”⁵⁰. This in contrast to a risk factor, which is defined as a characteristic, condition or behavior which confers to the individual an increased risk of experiencing disease or injury. In many instances, the difference can be subtle – e.g. whether elevated CRP is a biomarker of atherosclerosis or a risk factor, but most authorities requires a risk factor to have a proven, causal relationship with the disease state⁵¹.

The two biomarkers studied in this thesis both act as an indicator of a pathogenic process, thereby being defined as a biomarker, while evidence also suggest that they are causally related to the development of CAD and represents risk factors.

1.7.1. Monocyte chemoattractant protein-1 (MCP-1)

Monocyte Chemoattractant Protein-1 (MCP-1), also known as Chemokine ligand [C-C motif] 2 (CCL2), is a potent chemokine involved in the recruitment of monocytes, dendritic cells and T_H-cells⁵². MCP-1 is one of the most studied chemokines and is instrumental in the recruitment of monocytes from the blood stream into atherosclerotic plaques⁵². Early evidence showed that mice deficient in either MCP-1,

or the corresponding receptor on circulating monocytes (CCR2) were resistant to the development of atherosclerosis³⁵ implying that it is a *condicio sine qua non* in atherogenesis⁵³.

In basic research, MCP-1 has been linked to macrophage activity in atherosclerotic plaques⁵⁴⁻⁵⁶ and has shown to be associated with coronary risk factors in sub-clinical atherosclerosis⁵⁷. *In vivo* studies reveals that MCP-1 predict restenosis following both balloon-angioplasty⁵⁸ and stent implementation⁵⁹. Clinical studies implies that it is a predictor of CVD mortality in middle-aged obese patients⁶⁰. While some studies have found lack of association⁶¹, this has mainly been in apparently healthy subjects⁶². Genotype-studies are contradictory^{62, 63}.

Most interesting perhaps, is MCP-1s ability to independently predict cardiovascular events in an ACS setting, both in the acute^{64, 65} and chronic phase^{63, 64}. MCP-1 has, as of yet, not been evaluated prospectively in the setting of SAP.

1.7.2. Asymmetric dimethylarginine (ADMA)

Endothelial function is intrinsically connected to nitric oxide (NO) production, and produced by NOS from L-arginine. Asymmetric dimethylarginine (ADMA) is a product of proteolytic degradation of methylated proteins and is a well-known competitive inhibitor of NOS associated with endothelial dysfunction and cardiovascular disease³¹. Plasma levels of ADMA have been associated with all-cause mortality in the general population³⁰, acute coronary events in presumably healthy non-smokers⁶⁶ as well as in patients with established CAD⁶⁷. In addition, ADMA has been shown to be a endogenous inhibitor of endothelial progenitor cells⁶⁸. In-stent restenosis has also been associated with ADMA levels⁶⁹. While ADMA is produced through proteolysis, the regulation and relative importance of this process regarding plasma levels of ADMA has not been studied. Most data on predictors of ADMA levels pertains to mechanisms of degradation⁷⁰.

1.7.3. Trimethyllysine (TML)

Carnitine is necessary for the transport of long-chain fatty-acids into the mitochondria for beta-oxidation and can be obtained either by endogenous biosynthesis or dietary intake. Carnitine deficiency can result from either primary inborn errors of metabolism or from inadequate intake or impaired endogenous production secondary to other causes. The biosynthesis of carnitine starts with 6-N-trimethyllysine (TML). TML is the lysosomal or proteasomal degradation product of proteins containing trimethylated lysine residues such as histones⁷¹.

Like ADMA, TML is produced through post-translational methylation of amino acids in nuclear and cytosolic proteins⁷¹ which is subsequently release through proteolysis; yet unlike for ADMA, associations of TML with CVD have not been previously addressed. In this context, it is interesting to note that both previous speculations⁷² and emerging evidence⁷³⁻⁷⁵ suggest that epigenetic alterations of chromatin is relevant to the development and progression of atherosclerosis. Accordingly we wanted to simultaneously investigate the association of circulation levels of both ADMA and TML with progression of CAD.

1.6. Cardiovascular disease and homocysteine

Hyperhomocysteinemia has been associated with CVD in several prospective studies⁷⁶ and a plethora of explanatory pathomechanisms have been proposed⁷⁷⁻⁷⁹. Interventional treatment with moderate to high doses of B-vitamins, including cobalamin (vitamin B₁₂) and folic acid (vitamin B₉), has been shown to lower total plasma homocysteine (tHcy) by supplying methyl groups for remethylation of homocysteine. While 20 years of intense homocysteine studies have culminated in several large scale clinical trials. The supposed beneficial effect on cardiovascular mortality by homocysteine-lowering folic acid supplementation has repeatedly failed to manifest itself^{78, 80-83}.

Hyperhomocysteinemia has been associated with several risk factors for CVD which has primarily been explained by its putative atherothrombotic effect. Noteworthy, hyperhomocysteinemia results in upregulation of MCP-1⁸⁴ and it has been shown that folic acid supplementation alters the levels of MCP-1 in rats⁸⁵. Interestingly, studies in humans have shown that the presence of obesity alters the direction of the correlation between MCP-1 and tHcy⁸⁶. Hyperhomocysteinemia has several other atherothrombotic attributes such as upregulating interleukin-8 expression, increased SMC proliferation, increased collagen production, inhibition of thrombolytic mechanisms, free radical production, increased platelet reactivity, decreased degradation of ADMA and impaired endothelial function⁸⁷⁻⁹⁰. Even more intriguing, some consider hyperhomocysteinemia as a sign of impaired DNA-methylation, a crucial element in nuclear regulation and that it is through these mechanism that homocysteine is linked to disease^{91, 92}.

1.8. The dark side of folate metabolism

The Western Norway B Vitamin Intervention Trial (WENBIT)⁹³ and the Norwegian Vitamin Trial (NORVIT)⁹⁴ were two large-scale clinical trials conducted in Norway. Neither trial showed any protective effect of intervention with B-vitamins in patients with established coronary heart disease. On the contrary, pooled analyses of these two trials done by our group showed a statistically significant increased risk of cancer incidence and all-cause mortality related to folic acid supplementation⁹⁵. As of now there seems to be no upper limit to the amount of water-soluble vitamins recommended by international guidelines.

A growing body of evidence has shown that both global and site-specific hypo- and hypermethylation of DNA and histones are associated with CVD^{73, 96}. Some studies have shown that low folate intake are linked to DNA hypomethylation⁹⁷, while high doses of FA *induce* aberrant DNA methylation in some⁹⁸ but not all studies^{99, 100}. It has been speculated that the lack of cardiovascular protective effect of homocysteine-lowering FA supplementation is due to a simultaneously increased methylation potential and subsequent epigenetic alterations of gene expression by folate⁷². Histone methylation is

limited to the ϵ -amino groups of amino acid residues in the form of mono-, di- or trimethylation⁷³, and only two amino acids in histones undergo methylation, i.e. arginine and lysine¹⁰¹, which may lead to the production of ADMA and TML, respectively.

Though this area of epigenetic research has gained substantial interest during the last few years, many mechanisms have yet to be elucidated. It is not inherently obvious that excessive supplementation with water-soluble B-vitamins and folic acid to increase nuclear methylation capacity is uniformly positive and data suggest that there might be a “dark side” to folate metabolism. Whether disproportionate folic acid supplementation in certain sub-groups of patients could lead to excessive nuclear DNA methylation and increased risk of cardiovascular disease through alterations in genetic expression is not known, but biochemical inference might suggest so.

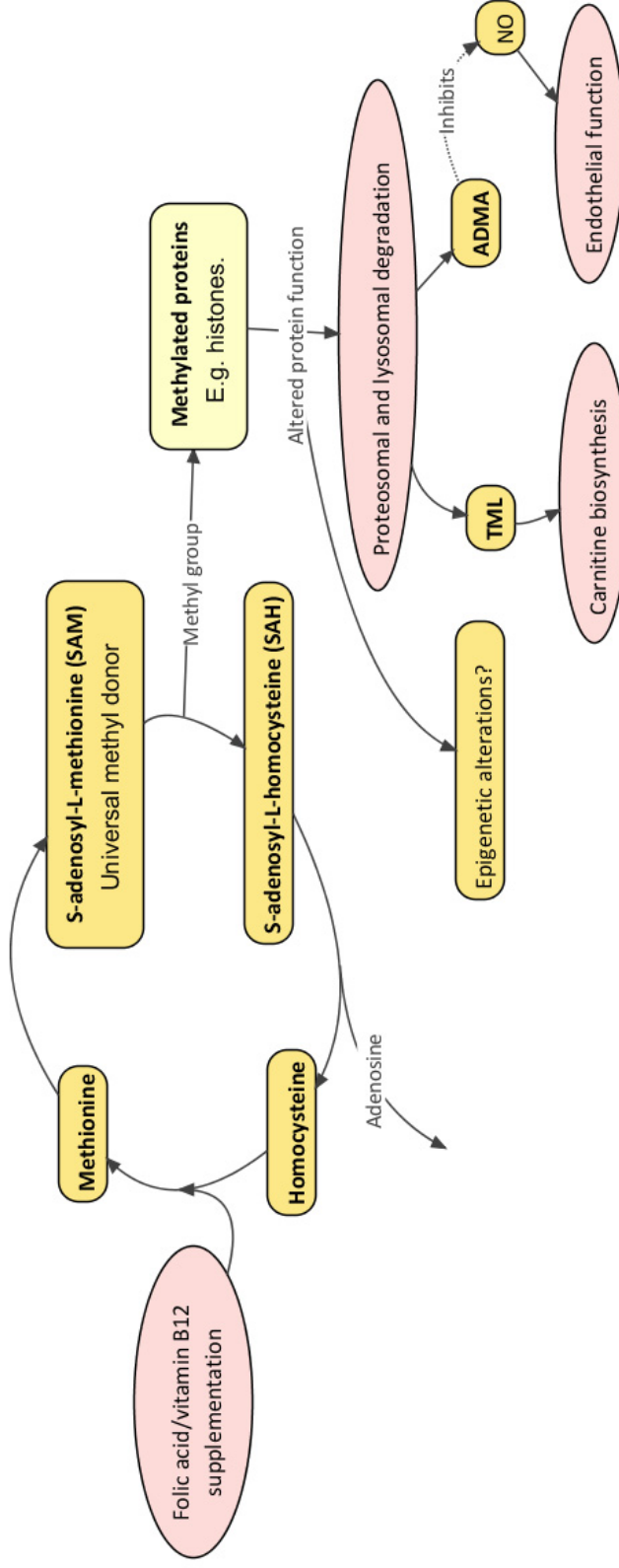


Figure 3. The methylation cycle and its link to formation of asymmetric dimethylarginine and trimethyllysine.

Homocysteine is remethylated to methionine. 5-M-THF is a methyl donor and vitamin B12 a co-factor in this reaction. Methionine is converted to S-adenosyl-L-methionine which is the universal methyl group donor in the cell. Methylation of amino acid residues of proteins alters their function – including epigenetic modification through modification of histones. ADMA and TML are produced through proteolysis of such proteins. Increased methylation status induced by folic acid/vitamin B12 supplementation and raised SAM:SAH-ratio might lead to increased protein methylation and consequently production of methylated amino acids through protein degradation. Abbreviations: 5-M-THF, 5-methyl-tetrahydrofolate; ADMA, asymmetric dimethylarginine; TML, trimethyllysine; SAM, S-adenosyl-L-methionine; SAH, S-adenosyl-L-homocysteine; NO, nitric oxide.

2. AIM AND HYPOTHESIS OF THE THESIS

The main goal of the project was to investigate the connection between treatment with folic acid supplementation/vitamin B12 and progression of sub-clinical CAD in patients with stable angina pectoris (SAP) with invasive measurements of CAD progression and associated metabolic changes.

Separate project aims

- 2.1.** Paper I: Investigate the effect of folic acid supplementation on progression of atherosclerosis in patients with SAP as measured by quantitative coronary angiography (QCA)¹⁰².

- 2.2.** Paper II: Investigate whether the negative effect of folic acid supplementation observed in a sub-group of patients is related to increased plasma levels of the methylated amino acids ADMA and TML as well as assessing their ability to predict progression of coronary atherosclerosis as measured by QCA.

- 2.3.** Paper III: Study the effect of folic acid supplementation on the levels of MCP-1 and consequently on the presence of TCFA as measured by IVUS.

*“There are three kinds of lies:
lies, damned lies, and statistics.”*

Quote attributed to Benjamin Disraeli (1804 – 1881).

3. MATERIALS AND METHODS

3.1. Subjects

The subjects included in the projects for the current thesis participated in the Western Norway B-vitamin intervention trial (WENBIT). This was a double-blinded, placebo-controlled, 2-center trial that included 3,090 adult patients (20.5% women) who had undergone coronary angiography for suspected CAD. Of the 3,090 WENBIT participants, a total of 1,359 (44%) underwent PCI following the baseline angiogram. PCI was performed either as an elective procedure after a previous diagnostic angiography, immediately after the angiography or as a scheduled procedure at the 1 month follow-up. Of these 1,359 patients, 465 (34%) were treated at Stavanger University Hospital, Stavanger, and 894 (66%) at Haukeland University Hospital, Bergen, Norway.

The general WENBIT exclusion criteria were an inability or reluctance to attend long-term follow-up, alcohol abuse, mental illness, and known active malignant disease. For the sub-studies in the current thesis, patients at high risk of procedural complications or who presented with a baseline coronary anatomy of such a nature that repeat angiography would probably prove unsuccessful were also excluded.

All WENBIT participants provided written informed consent, and the patients scheduled for repeat angiography provided additional written informed consent. The Regional Committee for Medical and Health Research Ethics, the Norwegian Medicines Agency, and the Data Inspectorate approved the WENBIT. The Regional Ethics Committee approved the protocol for the present sub-study. The ClinicalTrials.gov identifier was NCT00354081.

3.1.1. WENBIT re-angiography sub-study (WENBIT-RA)

A proportion of the patients undergoing PCI at WENBIT baseline were recruited into a pre-planned sub study (WENBIT re-angiography sub-study, WENBIT-RA) with serial angiography consisting of a planned repeat coronary angiography at approximately 1 year

of follow-up in the main WENBIT-trial for the evaluation of progression (or regression) of coronary atherosclerosis in non-treated segments by QCA. The patients eligible for this sub-study analysis were recruited from the Haukeland University Hospital from October 2001 to May 2004, from a total of 570 patients undergoing PCI at WENBIT-baseline during this time period. The patients asked to participate largely constituted the primary referral population, so as to make scheduled repeat angiography practically possible. The follow-up repeat angiogram was either scheduled to occur approximately 10 months after the initial PCI (to coincide with the 1-year WENBIT control group), or it was performed for clinical indications ≥ 90 days after the PCI. The patients scheduled for repeat angiography who were re-hospitalized for clinical indications within 90 days after the initial PCI were excluded to rule out complications related to baseline PCI (n = 13).

3.1.2. Paper I and II study subjects: quantitative coronary angiography

For paper I and II we used data from repeat coronary angiography to evaluate progression of angiographic CAD. Both baseline and follow-up coronary angiograms were analyzed using quantitative coronary angiography (QCA) in order to identify atherosclerotic lesions in non-intervened vessels and estimate progression during follow-up. In addition to general WENBIT and WENBIT-RA exclusion criteria, we here excluded patients with baseline and/or follow-up coronary angiograms considered unsuitable for quantitative analysis.

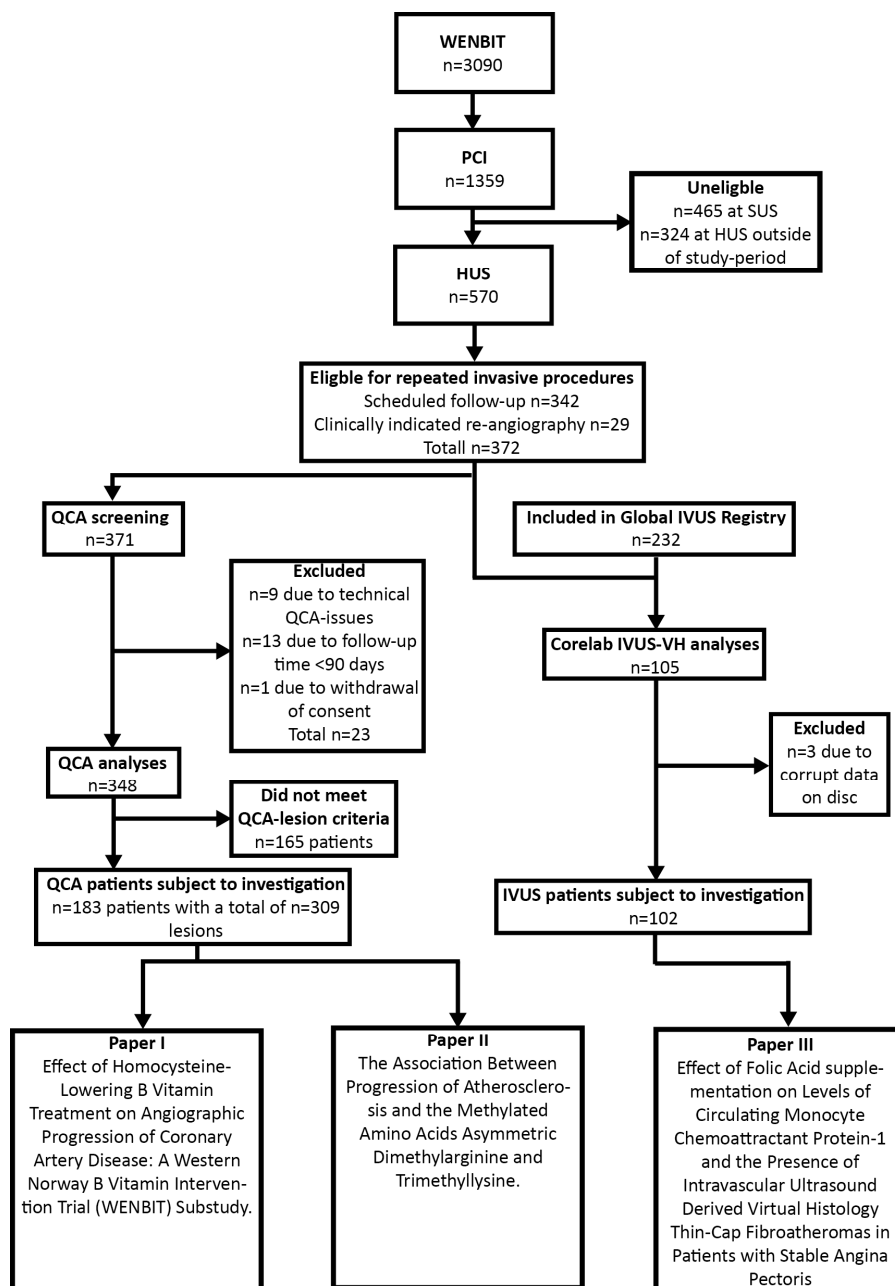


Figure 4. Flow chart of patients from WENBIT, through sub-studies and the corresponding papers in the thesis.

WENBIT, western Norway B-vitamin intervention trial; PCI, percutaneous coronary intervention; HUS, Haukeland University Hospital; SUS, Stavanger University Hospital; QCA, quantitative coronary angiography; IVUS, intravascular ultrasound; VH, virtual histology.

3.1.3. Paper III study subjects: virtual histology

Of the 371 patients undergoing their second coronary angiography in WENBIT-RA, 231 had intravascular ultrasound (IVUS) performed in non-intervened vessels. Of these 231, a cohort 105 patients were also included in the global virtual histology-IVUS (VH-IVUS) registry and IVUS-data was transferred to an external core lab for virtual histology analysis. All study participants were recruited from a subset of patients who after PCI at WENBIT baseline had been scheduled for re-angiography after one year.

3.2. Primary end-points

Analysis of coronary atherosclerosis progression was done using quantitative coronary angiography (QCA) for paper I and II, while presence of vulnerable plaques was assessed using VH-IVUS for paper III.

3.2.1. Quantitative coronary angiography (paper I and II)

The coronary plaque lesions for paper I and II were analyzed using digitalized QCA software (Quantcor QCA, CAAS II, version 5.0, Pie Medical Imaging, Maastricht, The Netherlands). An end-diastolic frame showing the stenosis without foreshortening or vessel overlaps and free of intracoronary guidewires was selected. If the stenosis differed in severity on different projections, the projection demonstrating the most severe stenosis was subject to analysis.

The baseline and follow-up coronary angiograms were analyzed by 2 trained technicians who were unaware of the treatment regimen and who were supervised by an experienced interventional cardiologist. A total of 16 coronary artery segments were evaluated in all patients (i.e., 15 segments according to the American Heart Association standardization

criteria¹⁰³ as shown in figure 5 plus the right atrioventricular branch). Eligible lesions for analysis had a reference diameter of 2 mm, a diameter reduction of 30% at baseline or follow-up, and were adequately visualized at similar projections on both angiograms. The analyzed segment had not been treated with PCI. Cases of disagreement between the observers about the eligibility of a certain lesion were subject to reanalysis by both observers. After all QCA procedures, segments from both observers were compared to ensure equality concerning the accurate numbering of the segments, the correct angiogram analyzed, and the actual stenosis portrayed.

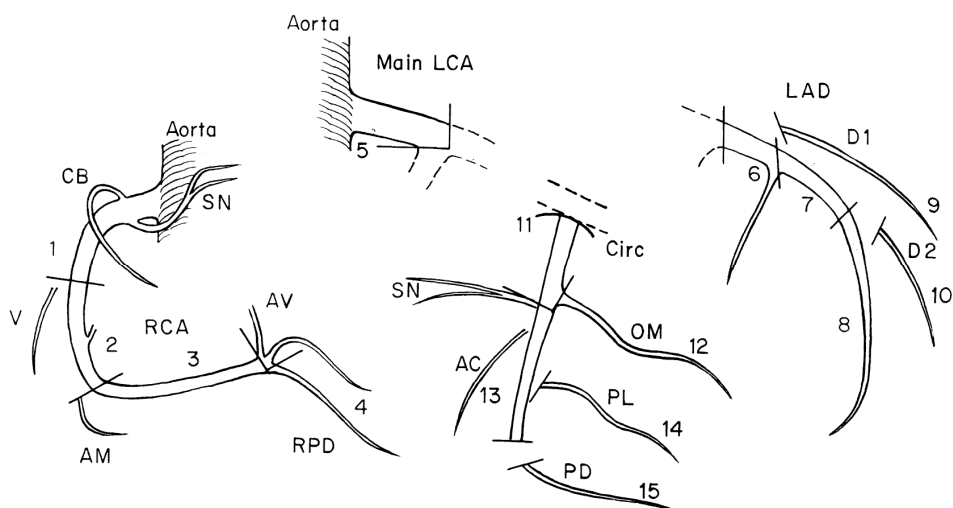


Figure 5. Segments of the coronary arteries as defined by the American Heart Association.

AC, atrial circumflex branch; AM, acute marginal branch; AV, atrio-ventricular node branch; CB, conus branch; Circ, Circumflex artery; D1, first diagonal branch; D2, second diagonal branch; Main LCA, main left coronary artery; OM, obtuse marginal branch; PD, posterior descending artery; PL, posterolateral branch; RCA, right coronary artery; RPD, right posterior descending artery; SN, sinus node branch; V, right ventricular branch. Reproduced from Austen WG, Edwards JE, Frye RL, et al. A reporting system on patients evaluated for coronary artery disease. Report of the Ad Hoc Committee for Grading of Coronary Artery Disease, Council on Cardiovascular Surgery, American Heart Association. *Circulation*. 1975;51:5-40, with permission of the American Heart Association.

The contrast-filled tip of the catheter was used for calibration, and computer-defined obstruction analysis without manual contour correction was used, where applicable. However, ostial stenoses required the use of manually defined obstruction analysis (user-defined reference vessel diameter and stenosis length), and branched artery segment required manual correction of vessel contour.

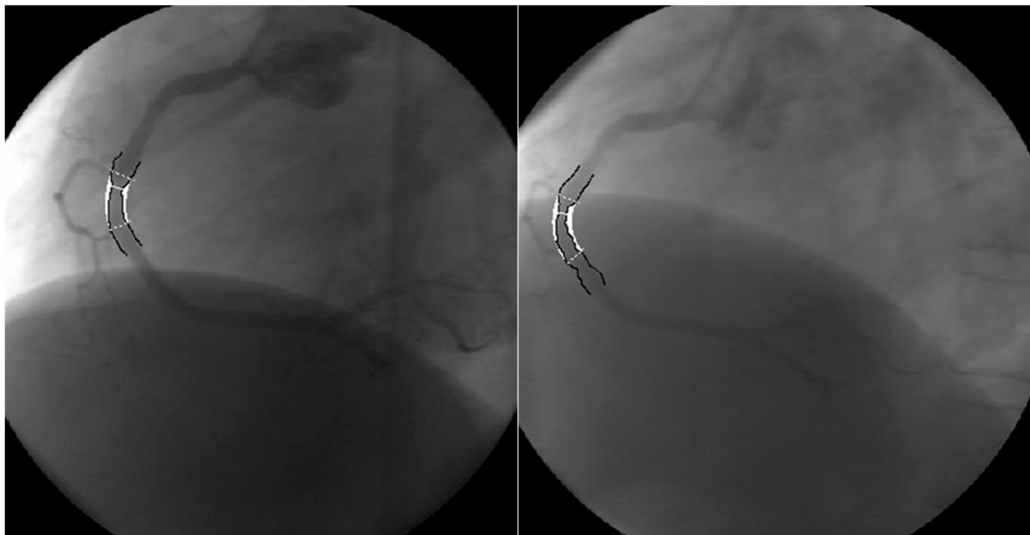


Figure 6. Example of quantitative coronary angiography (QCA).

The left image pane shows a stenosis of the right coronary artery (AHA segment 2) with a minimum lumen diameter (MLD) of 1.93 mm (47% diameter stenosis [DS]). The right image pane shows the stenosis at follow-up (approximately 10 months), now with a MLD of 1.63 mm and a corresponding DS of 55%. Image acquired from Quantcor QCA, CAAS II, version 5.0, Pie Medical Imaging, Maastricht, The Netherlands.

The primary measures for each selected lesion were the minimum lumen diameter (MLD)^{104, 105} and diameter stenosis (DS)^{104, 105} for paper I and DS for paper II. Both parameters were measured as continuous variables, defined as the mean of the values measured separately by each observer.

When all baseline and follow-up lesions had been analyzed by both observers, the interobserver difference in DS was calculated. The 10% of lesions with the largest difference were subject to reanalysis. DS was chosen as the appropriate variable to assess, because it, in contrast to MLD, is a relative measurement, thus reducing any potential calibration errors between the baseline and follow-up angiograms.

For paper I, we additionally defined a post hoc secondary end-point of rapidly progressing lesions as the 25% of all analyzed lesions with the greatest diameter reduction expressed by DS as a categorical yes/no variable.

3.2.2. Intravascular ultrasound and virtual histology (paper III)

One of the most developed technology applicable for *in vivo* identification of TCFA is spectral analysis of IVUS radio frequency data¹⁰⁶ called Virtual Histology-IVUS-(VH-IVUS). With this technique, Virtual Histology Thin-Cap Fibroatheroma (VH-TCFA) is defined as a lipid rich atheromatous lesion with a thin overlying fibrous cap. These lesions have shown to be the most common cause of thromboembolic cardiovascular events^{27, 107, 108}. VH-TCFA also predicts clinical events in a prospective study¹⁰⁹.

IVUS is a technique in which a miniaturized ultrasonic transducer in the 10-40 MHz range is introduced intracoronary in order to visualize the coronary arterial wall. VH-IVUS is based on the spectral analysis of raw backscattered ultrasound data. VH-IVUS renders four different histological tissue components, namely fibrous, fibro-fatty, necrotic core and calcified, all with high correlation with histopathology in *ex vivo* validation studies^{106, 107, 110}. Each of these neointimal components are presented as total volume (mm³) and percentage volume of total neointimal lesional volume.

VH-IVUS data on the 105 patients included in the analyses in paper III were performed by the same interventional cardiologist after intracoronary administration of nitroglycerin 0.2 mg. A 20 MHz, 2.9Fr IVUS catheter (Eagle Eye, Volcano Corp., Rancho Cordova, California) was advanced beyond the selected segment, and a motorized transducer pullback (0.5 mm/s) was performed. VH-IVUS data was lost in three patients due to corrupted data on disc, i.e. 102 patients had complete VH-IVUS data.

All IVUS-data were transferred to the Global VH-IVUS Registry core lab for analyses using the pcVH 2.2 software (Volcano corp., Brussels, Belgium). This is a validated method for visualizing plaque morphology *in vivo*. An IVUS-lesion is defined as being present when the plaque burden (plaque volume / external elastic membrane volume) exceeds 40% over three consecutive frames. A VH-TCFA lesion was present when a necrotic core rich (>10% of cross-sectional area) element was identified in at least 3 consecutive frames as previously described^{109, 111}. While pull-back was done in a per-vessel manner, data from the core lab was provided in a per-lesion form. For the

following analyses we aggregated VH-TCFA to patient-level – i.e. VH-TCFA status was reported per patient.

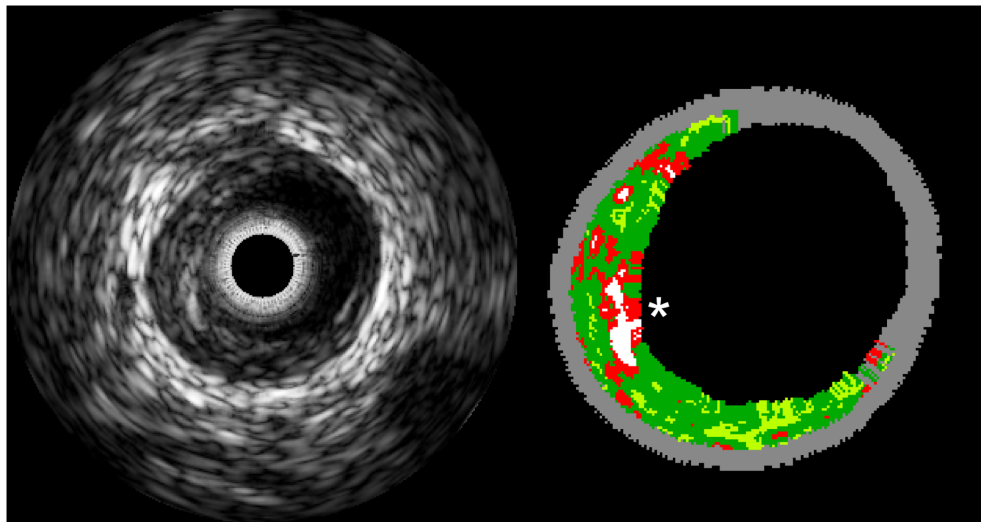


Figure 7. Grayscale IVUS and Virtual Histology of TCFA.

A thin cap fibroatheroma (asterix) in the proximal left anterior descending coronary artery of one of the study patients. The left panel shows an intravascular ultrasound radiofrequency image which is subsequently used to make the virtual histology image in the right panel. Green is fibrous tissue, yellow is fibro-fatty, red is necrotic core and white is calcified tissue.

3.3. Blood samples

For paper I and II blood samples were collected at baseline and follow-up before repeat angiography, corresponding to WENBIT study visits. Routine blood analyses such as hematologic parameters, renal function markers and lipid-related factors were analyzed in fresh samples at the Laboratory of Clinical Biochemistry, Haukeland University Hospital, using standard methods, all blinded to study end-points and randomization. Blood samples for the measurements of total plasma homocysteine (tHcy), ADMA and B-vitamins were analyzed at the laboratory of Bevital AS, Bergen, Norway (www.bevital.no), using previously described methods¹¹². Plasma TML, free carnitine and γ -butyrobetaine were analysed using tandem mass spectrometry as described previously¹¹³ with some modifications of the high-performance liquid chromatography (HPLC) conditions. Estimation of glomerular filtration (eGFR) rate was done using the simplified Modification of Diet in Renal Disease (MDRD)-equation¹¹⁴.

For paper III, additional measurements of MCP-1 were analyzed at the Department of Clinical Science in collaboration with the Lipid Research Group, University of Bergen, Norway, using BioPlex® 200 multiplex array (Bio-Rad Laboratories, Hercules, CA, US).

3.4. Clinical end-points

Subjects analyzed in paper III were followed from the VH-IVUS study inclusion date for approximately 2 years. The follow-up endpoint was defined as a composite of fatal and non-fatal AMI. All events were adjudicated by the WENBIT-endpoint committee, unbeknownst of the MCP-1 serum levels and VH-TCFA status of the patient.

3.5. Statistical methods

3.5.1. General considerations and baseline characteristics

For descriptive data mean (standard deviation [SD]) or median (interquartile range [IQR]) was used. Since most biological parameters are almost invariably skewed, median (IQR) was applied more often for biochemical data.

Dissimilarities in baseline characteristics of continuous variables between subject groups in the different analyses such as TCFA versus non-TCFA in paper III or folic acid/vitamin B12 versus no folic acid/vitamin B₁₂ in paper II were analyzed with either Welch two-sample t-test or chi-square/Fisher's exact test with minimum likelihood where appropriate (i.e. cell number count <5) for categorical data. For some skewed variables logarithmic transformation were in some instances applied in order to approximate normal distribution. Back-transformation was consequently used for data presentation. Alternatively the non-parametric Mann-Whitney U test was applied. In paper I where four intervention groups were compared, differences in continuous variables between groups were investigated using analysis of variance (ANOVA).

A two-sided p-value of < 0.05 was considered statistically significant in all analyses.

3.5.2. The fallacies of linear regression

Linear regression has been the mainstay of medical statistics for several decades.

However, several assumptions underlie the use of simple linear regression which are often violated in its application on biological data¹¹⁵. Simple linear regression fitted using the least squares method generally assumes:

- *Normality of data*. I.e. normality of the residuals at each value of X or Y . More often than not biological or chemical data is skewed, zero-inflated or combinations thereof.
- *Homogeneity*. I.e. the variance of the data should be the same at each value of X . Violation of this is referred to as heterogeneity or heteroscedasticity e.g. if body-mass index has a higher spread in men than females or when serum potassium has a higher variance in patients with lower than higher glomerular filtration rate.
- *Fixed explanatory variables*. Also called weak exogeneity. I.e. that the predictor variable X can be treated as a fixed – as opposed to a random – variable. Essentially assuming that the measured variable X is error-free.
- *Independence*. Violation of error independence is one of the most serious problems and occurs if the Y value at X_i is influenced by other X_i . Both temporal and spatial correlation is more prevalent than one might guess. E.g. patients sampled from the same area may share genetic attributes that influence the way in which a certain biomarker varies between individuals.
- *Linearity*. The response variable is a linear combination of the regression coefficients of the explanatory variables. E.g. the U-shaped relationship between body-mass index and mortality is a violation of this assumption¹¹⁶.

Investigating these assumptions can be exhaustive and several pitfalls exist when one wishes to generalize findings from a sample estimate. In our dataset, several of these assumptions were violated and more advanced techniques were applied accordingly.

3.5.3. Loss of independence and mixed effects modeling

Violating the assumption of independence could potentially severely affect statistical models and renders both p-values and estimates invalid¹¹⁵. Data with a nested or hierarchical structure results in pseudoreplication – e.g. multiple samples from the same individual.

A mixed model is a statistical model which allows for both fixed and random effects. Fixed effects are explanatory variables that consists of non-random quantities – i.e. the levels of the variable included contains all possible levels (or all levels of interest), e.g. gender which would include both male and female. If the levels of a given variable are drawn at random from a population of more possible levels – i.e. study site in a randomized controlled trial. To generalize, if the effect of the levels of a factor could be drawn from a distribution of probability of such effects, the effect is random. While one is interested in the difference of the mean between fixed effects, it is the variance of means across the levels which are of interest regarding random factors. This can be used in a hierarchical structure where random effect is set to an identifier from which multiple samples are drawn – e.g. multiple measurements from the same patient.

In both paper I and paper II repeated measures (“pseudoreplication”) was present. The treatment effect on the primary end-points was associated with up to seven coronary artery segments for each patient. Accordingly, linear mixed effects models fitted by restricted maximum likelihood were applied. The mixed effects model included a random effect term adjusting for the within-patient clustering of coronary artery segments. The models were computed with the software package R (The R Foundation of Statistical Science, Vienna, Austria) using Pinheiro and Bates’ referential implementation¹¹⁷ in the R package *nlme*¹¹⁸.

3.5.4. Non-linear applications

Non-linear relationships between explanatory and response variables are abundant in nature. In the organism the strong preservation of homeostasis in a given system almost

invariably implies that the effect a certain external stressor is altered when one reaches the outer boundaries of such a system. Thus, while the relationship between explanatory variables while be linear in a certain part of the distribution of Y , this is seldom the case when approaching the outer bounds.

While it is possible to model non-linear relationships using a linear regression model with for example a second order polynomial function, one implicitly assumes the form of the relationship. Several solutions to this problem have been proposed, many relating to “smoothing” models pioneered by Hastie and Tibshirani¹¹⁹ with the introduction of generalized additive models (GAM). The additive model fits a smoothing curve through a scatterplot of the data without assuming a given relationship form. Several ways to estimate smoothers have been presented. Further work has been done by Wood¹²⁰⁻¹²² which resulted in the R-package *mgcv*. In our dataset we have used the *mgcv* package for the R software which uses the spline method described by Wood¹²⁰. Generalized additive modelling in the *mgcv* package can be extended to generalized additive mixed effects models (GAMMs) which allow for spatial and temporal correlation in addition to hierarchical structuring of variance, and was used in paper II and III.

GAM was applied in paper III for assessing the effect of folic acid supplementation on MCP-1, VH-TCFA presence and MI. In paper II GAMM was applied when we investigated the association between plasma levels of TML/ADMA and DS and subsequently discarded for a simpler method when a linear relationship was discovered.

3.5.5. Conditional quantile regression

While not an assumption per se, a limitation of linear regression is its (partial) ability to only predict the effect of the explanatory variables on *the mean value* of the outcome. While estimating the mean is often interesting, inference beyond the central tendency is not possible. Quantile regression as pioneered by Koenker and others¹²³⁻¹²⁵ is a method to estimate the conditional quantile of a variable – i.e. to estimate the median value or any other part of the conditional distribution such as the 25th, 75th or

95th percentile. Quantile regression makes no assumption about the distribution at each value of the explanatory variable(s). Thus it is very robust to the presence of outliers in the dataset and needs no variance stabilizing variable transformation.

Recently, the quantile regression method has been extended to allow for both fixed and random effects using a likelihood-based approach to estimate regression quantiles that uses the asymmetric Laplace distribution¹²⁶. Application of this method is possible through the use of the *lqmm* package version 1.02¹²⁷ for the R statistical software.

In paper II a linear quantile mixed model approach using the *lqmm* package was applied to analyze the predictive value of ADMA and TML on progression of sub-clinical atherosclerosis. This was done both to in order to account for outliers in the dataset and to visualize the relationship between the explanatory variables and all parts of the distribution of the outcome variable.

Testing for relationships across the whole distribution in addition to the central tendency seems not to increase the rate of spurious findings. On the contrary, the presentation of several regression lines only helps to distinguish between spurious, asymmetrical and “real” relationships. The *de facto* result is a level of analytical transparency which only helps to expose all relationships in the dataset¹²⁸.

Asymmetrical effects are statistical associations which are more pronounced in a given part of an outcome variables distribution than others. E.g. the relationship between education and cardiovascular risk in women are more pronounced at higher than lower risk levels¹²⁹. While principally different from non-linear modeling which inspects different parts of the explanatory variables distribution – the concept is cognitively translatable. In biological systems with a strong drive for homeostatic control, it would not be unreasonable to expect the effect estimates of a given predictor to differ when looking at the outer boundaries of the outcome parameters – i.e. when any compensatory mechanisms and the redundancy of the system are likely to have been exhausted. Quantile regression allows for this to be modeled.

*“Alle Ding' sind Gift, und nichts ohn' Gift;
allein die Dosis macht, daß ein Ding kein Gift ist
[All things are poison, and nothing is without poison;
only the dose permits something not to be poisonous]”*

Paracelcus (1493 – 1541).

4. RESULTS

4.1. WENBIT study population

All patient material is included from WENBIT which has been extensively described previously⁹³. Briefly, the trial included 3090 patients referred to a diagnostic coronary angiography at either Haukeland or Stavanger University hospital during the time period of 1999-2004. A total of 3096 patients were randomized, six participants withdrew consent immediately after randomization without having started study medication and were excluded from further analyses. Consequently, the study population consisted of 3090 patients, of which 2121 (68.6%) were randomized at Haukeland University Hospital and 969 (31.4%) at Stavanger University Hospital. Clinical indications for referral to baseline angiography were SAP (n=2585 [83.7%]), ACS (n=461 [14.9%]) as well as aortic valve stenosis (n=44 [1.4%]).

4.2. WENBIT-RA study-population (paper I and II)

As previously stated, 348 patients had serial angiographic data available for QCA analysis. Baseline characteristics of these 348 patients are presented in table 2. Briefly, the mean age was 60.0 ± 10.2 years, 17.2% were women, and 31.3% of the patients had a previous history of AMI. The median serum total cholesterol level was 4.8 (IQR 4.2 – 5.6) and serum C-reactive protein was 1.91 mg/L (IQR 0.87 – 5.14). Of the 348 participants, a total of 96% were receiving statin treatment before PCI. No difference was found among the groups regarding dose or type of statin. The median plasma tHcy level was 10.0 $\mu\text{mol/L}$ (IQR 8.1 – 11.0), serum folate was 10.6 nmol/L (IQR 7.7 – 14.2), and 24 of the participants (6.9%) had hyperhomocysteinemia ($>15.0 \mu\text{mol/L}$). In table 4, the baseline characteristics for the 183 patients with angiographic findings who formed the basis of the statistical analyses in paper I and II are shown – i.e. patients with coronary lesions who qualified for QCA analysis according to the inclusion criteria (reference diameter of ≥ 2 mm, DS $\geq 30\%$ at baseline or follow-up, adequately visualized at similar projections on both angiogram, segment untreated with PCI). For this group median (interquartile range [IQR]) age was 60.0 (14.0)

years, 15.8% were women and 27.3% of the patients had a history of prior myocardial infarction. Median (IQR) serum total cholesterol was 5.0 (1.3) mmol/L, serum triglycerides 1.54 (0.87) mmol/L and serum CRP 2.0 (4.9) g/L.

Table 2. Characteristics and Laboratory Findings Among 348 Patients Successfully Studied with Quantitative Coronary Angiography at Baseline.

Characteristics	FA+B ₁₂ +B ₆ (n= 91)	FA+B ₁₂ (n= 87)	B ₆ (n= 87)	Placebo (n= 83)	p - Value
Demographics and clinical characteristics					
Age (years)	59.2 ± 10.9	60.3 ± 10.6	59.7 ± 9.1	60.8 ± 10.1	0.76
Female	14 (15.4%)	16 (18.4%)	16 (18.4%)	14 (16.9%)	0.94
Systolic blood pressure (mm Hg)	138 ± 20	138 ± 19	142 ± 26	144 ± 25	0.19
Body mass index (m ² /kg)	27.1 ± 3.4	26.7 ± 3.3	26.8 ± 2.9	27.4 ± 3.8	0.46
Ejection fraction ^a (%)	62.1 ± 8.7	64.0 ± 8.2	64.1 ± 8.1	65.1 ± 10.0	0.15
Stable angina pectoris	66 (72.5%)	67 (77.0%)	61 (70.1%)	63 (75.9%)	0.72
ACS	25 (27.5%)	20 (23.0%)	26 (29.9%)	20 (24.1%)	0.72
Cardiovascular risk factors					
Extracardial vascular disease ^b	6 (6.6%)	10 (11.5%)	16 (18.4%)	9 (10.8%)	0.11
Prior acute myocardial infarction	25 (27.5%)	29 (33.3%)	31 (35.6%)	24 (28.9%)	0.62
Prior percutaneous coronary intervention	17 (18.7%)	19 (21.8%)	17 (19.5%)	13 (15.7%)	0.78
Prior coronary bypass	6 (6.6%)	2 (2.3%)	2 (2.3%)	3 (3.6%)	0.47
Hypercholesterolemia ^c	47 (51.6%)	51 (58.6%)	48 (55.2%)	52 (62.7%)	0.50
Hypertension ^d	36 (39.6%)	36 (41.4%)	35 (40.2%)	37 (44.6%)	0.91
Diabetes mellitus ^e	6 (6.6%)	9 (10.3%)	9 (10.3%)	9 (10.8%)	0.75
Current smoker	29 (31.9%)	26 (29.9%)	28 (32.2%)	22 (26.5%)	0.84
<i>Extent of coronary artery disease</i>					
One-vessel disease	46 (50.5%)	44 (50.6%)	43 (49.4%)	41 (49.4%)	1.00
Two-vessel disease	32 (35.2%)	27 (31.0%)	32 (36.8%)	32 (38.6%)	0.76
Three-vessel disease	13 (14.3%)	16 (18.4%)	12 (13.8%)	10 (12.0%)	0.69
Medications					
Statins	91 (100%)	86 (98.9%)	84 (96.6%)	80 (96.4%)	0.22
β-adrenergic receptor antagonists	68 (74.7%)	74 (85.1%)	62 (71.3%)	62 (74.7%)	0.16
Calcium antagonists	14 (15.4%)	14 (16.1%)	18 (20.7%)	10 (12.0%)	0.49
ACE-inhibitors or ARB	14 (15.4%)	14 (16.1%)	16 (18.4%)	15 (18.1%)	0.94
Acetylsalicylic acid	88 (96.7%)	86 (98.9%)	86 (98.9%)	83 (100%)	0.41
ADP receptor antagonists	86 (94.5%)	81 (93.1%)	80 (92.0%)	79 (95.2%)	0.82
Laboratory findings					
C-Reactive Protein (mg/L)	1.58 (0.82, 5.32)	2.01 (0.88, 4.91)	1.84 (0.99, 5.05)	2.16 (0.90, 5.53)	0.93
S-LDL-cholesterol (mg/dl) ^f	108.3 (92.8, 131.5)	116.0 (92.8, 143.1)	108.3 (92.8, 131.5)	123.7 (100.5, 146.9)	0.25
S-HDL-cholesterol (mg/dl) ^f	46.4 (38.7, 54.1)	46.4 (38.7, 54.1)	46.4 (38.7, 54.1)	46.4 (38.7, 58.0)	0.97
S-creatinine (μmol/L)	86 (81, 95)	87 (82, 95)	91 (82, 99)	89 (82, 97)	0.46
S-glucose (mmol/L)	5.8 (5.2, 6.8)	5.5 (5.0, 6.5)	5.6 (4.8, 6.6)	5.4 (5.0, 6.5)	0.63

P-total homocysteine (µmol/L)	9.79 (7.96, 11.75)	9.94 (8.72, 11.53)	9.22 (7.42, 11.21)	9.85 (7.73, 11.20)	0.13
S-folate (nmol/L)	9.84 (7.52, 13.62)	9.78 (7.16, 14.7)	10.63 (7.30, 15.23)	10.56 (7.70, 10.56)	0.72

Continuous variables are presented as mean±SD. Analysis of variance (ANOVA) was used to compare the 4 groups. For categorical variables, numbers and percentages are presented and a Chi square test used to compare the 4 groups. Fisher's exact test was used when appropriate. All biochemical parameters are presented as median (IQR). FA, folic acid (0.8 mg); B₁₂, vitamin B₁₂ (0.4 mg); B₆, vitamin B₆ (40 mg); ACE, Angiotensin I converting enzyme; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ACS, composite syndrome consisting of acute coronary syndrome and both ST-elevated and non-ST-elevated myocardial infarction; S, serum; P, plasma. Percentages may not add up due to rounding of numbers.

^aEjection fraction was measured during ventriculography for the majority of the patients. When this was not performed, ultrasonic echocardiography was used.

^bA prior diagnosis of any peripheral or cerebrovascular disease.

^cDefined as a history of untreated total serum cholesterol ≥6.5 mmol/L or familial hypercholesterolemia.

^dDefined as systolic blood pressure > 140 and/or diastolic > 90 mmHg and/or antihypertensive therapy.

^eIncluding diabetes mellitus type I and II.

^fTo convert milligram per deciliter to millimole per liter, multiply by 0.02586.

From the 348 patients undergoing PCI for CAD underwent QCA screening and were subject to the above mentioned QCA screening/inclusion criteria, only approximately half of the patients screened (183 vs. 348) had such lesions. Accordingly, the difference between these two groups is of particular interest, e.g. in regards to investigating selection bias. Table 3 illustrates the differences between those with (n=183) and those without (n=165) coronary lesions which fulfilled the QCA inclusion criteria and were subsequently including in the statistical analysis of the primary endpoints in paper I and II. However, it is important to note that although 165 patients did not have any lesions, they all had CAD and all received at least one coronary stent at baseline – i.e. established CAD. When comparing the two groups, patients with QCA-analyzed stenoses had on average higher systolic blood pressure and had more extensive CAD, while other risk factors and clinical parameters were largely similar. The flow of patients from WENBIT into sub-studies is depicted in figure 4.

Table 3. Baseline Characteristics and Laboratory Findings in Patients eligible for quantitative coronary angiography^a.

Characteristics	Group 1 (n=183)	Group 2 (n=165)	p-value
	QCA-eligible lesions ^a	No QCA-eligible lesions ^a	
Demographic characteristics			
Age - years	60.3 (10.4)	59.6 (10.0)	0.53
Female sex - no. (%)	29 (15.8)	31 (18.8)	0.56
<i>Intervention - no. (%)</i>			
FA/B ₁₂ /B ₆	49 (26.8)	42 (25.5)	0.87
FA/B ₁₂	49 (26.8)	38 (23.0)	0.50
B ₆	40 (21.9)	47 (28.8)	0.19
Placebo	45 (24.6)	45 (27.3)	0.65
Clinical characteristics			
Systolic Blood Pressure (mmHg)	142 (23)	137 (23)	0.03
Body Mass Index (m ² /kg)	27.1 (3.3)	26.9 (3.4)	0.53
Ejection Fraction (% ^b)	63.5 (9.4)	63.6 (8.1)	0.86
Cardiovascular risk factors - no. (%)			
ACS at presentation	57 (31.1)	50 (30.3)	0.86
Prior AMI	50 (27.3)	59 (35.8)	0.09
Prior PCI	34 (18.6)	32 (19.4)	0.85
Prior CABG	6 (3.3)	7 (4.2)	0.64
Hypercholesterolaemia	116 (63.4)	101 (61.2)	0.68
Hypertension	80 (43.7)	64 (38.8)	0.35
Diabetes Mellitus (type I and II)	17 (9.3)	16 (9.7)	0.90
Current smoker	61 (33.3)	44 (26.7)	0.18
<i>Disease severity</i>			
1-vessel disease	75 (41.0)	99 (60.0)	<0.01
2-vessel disease	73 (39.9)	50 (30.3)	0.08
3-vessel disease	35 (19.1)	16 (9.7)	0.02
Medical therapy^c - no. (%)			
Statins	179 (97.8)	162 (98.2)	0.81
β-adrenergic receptor antagonists	139 (76.0)	127 (77.0)	0.82
Calcium antagonists	29 (15.8)	27 (16.4)	0.90
ACE-inhibitors ^d	31 (16.9)	28 (17.0)	0.99
Acetylsalicylic acid	181 (98.9)	162 (98.2)	0.57
ADP receptor antagonists	171 (93.4)	155 (94.0)	0.85
Laboratory findings			
S-C-Reactive Protein (mg/L)	2.0 (4.9)	1.8 (3.2)	0.11
S-LDL cholesterol (mmol/L)	3.0 (1.3)	2.8 (1.1)	0.49

S-HDL cholesterol (mmol/L)	1.2 (0.4)	1.2 (0.4)	0.83
S-creatinine ($\mu\text{mol/L}$)	88 (14)	88 (17)	0.41
S-glucose (mmol/L)	5.6 (1.5)	5.6 (1.5)	0.59
P-total homocysteine ($\mu\text{mol/L}$)	9.8 (3.1)	9.8 (3.5)	0.63
P-folate (nmol/L)	10.3 (6.0)	9.8 (7.3)	0.11

For continuous variables, median and interquartile range within each group is calculated. Mann-Whitney U-test was used to compare the two groups. For categorical variables, number and percentage is presented and a Chi square test was used to compare the four groups. Fisher's exact test was used when appropriate. FA, folic acid (0.8 mg); B₁₂, vitamin B₁₂ (0.4 mg); B₆, vitamin B₆ (40 mg); PCI, percutaneous coronary intervention; CABG, coronary artery bypass graft surgery; ACS, composite syndrome consisting of acute coronary syndrome including both ST-elevated and non-ST-elevated myocardial infarction; AMI, acute myocardial infarction; ACE, Angiotensin I converting enzyme; ADP, adenosine diphosphate; LDL, low-density lipoprotein; HDL, high-density lipoprotein Percentages may not add up due to rounding of numbers.

^aPatients who after QCA screening had coronary lesions which qualified for analysis with reference diameter of 2 mm, a diameter reduction of 30% at baseline or follow-up, and were adequately visualized at similar projections on both angiograms. The analyzed segment had not been treated with PCI.

^bEjection fraction was measured during ventriculography for the majority of the patients. When this was not performed, ultrasonic echocardiography was used.

^cMedication at discharge.

^dIncluding ARB - angiotensin receptor blockers.

4.3. Effect of Homocysteine-Lowering B Vitamin Treatment on Angiographic Progression of Coronary Artery Disease: A Western Norway B Vitamin Intervention Trial (WENBIT) Substudy (Paper I)

The American Journal of Cardiology. 2010;105:1577-1584.

In this sub study of WENBIT, a total of 348 patients, (288 men) with a mean \pm SD age of 60 ± 0.2 years, were available for serial QCA analysis after baseline coronary angiography. A total of 165 patients did not have any detectable lesions by QCA either at baseline or at follow-up. Among the remaining 183 patients, 309 coronary lesions were detected either at baseline or follow-up that fulfilled the angiographically defined inclusion criteria. The patients were followed up for a median of 10.5 months (25th - 75th percentile 9.2 – 11.8) for the assessment of CAD progression using QCA. The baseline tHcy levels were relatively low at $10.0 \pm$ mol/L (25th -75th percentile 8.1 – 11.0), but were still lowered by a mean of 22% in patients receiving folic acid/vitamin B₁₂. At follow-up, we found 309 coronary QCA lesions with a significant decrease from baseline in the minimum lumen diameter of a mean of -0.16 ± 0.4 mm and an increase in the diameter stenosis of $4.4 \pm 0.7\%$. However, while the group effect was luminal narrowing, some coronary segments actually showed regression of atherosclerotic obstruction as shown by an *increase* in MLD. Treatment with folic acid/vitamin B₁₂ or vitamin B₆ was not associated with a change in diameter stenosis or minimum lumen diameter. In a post hoc analysis, folic acid/vitamin B₁₂ treatment was significantly associated with rapid progression (odds ratio 1.84, 95% C.I. 1.07 to 3.18) defined as coronary segments which during follow-up had a change in diameter stenosis belonging to the 4th quartile of Δ DS.

. In conclusion, vitamin B treatment showed no beneficial effect on the angiographic progression of coronary artery disease, and the post hoc analyses suggested that folic acid/vitamin B₁₂ treatment might promote more rapid progression. Patients with such rapid stenosis progression were characterized by having more extensive lesions at baseline.

Table 4. Baseline Characteristics and Laboratory Findings in Patients with Angiographic Coronary Lesions (n=183).

Characteristics	Group 1 (n=98)	Group 2 (n=85)	p-value
	FA, B ₁₂ ±B ₆	Placebo or B ₆	
Demographic characteristics			
Age - years	59.3 (10.5)	61.5 (9.4)	0.14
Female sex - no. (%)	17 (17.3)	12 (14.1)	0.69
Clinical characteristics			
Systolic Blood Pressure (mmHg)	138.3 (20.7)	147.4 (23.9)	0.01
Body Mass Index (m ² /kg)	27.0 (3.2)	27.2 (3.5)	0.79
Ejection Fraction (% ^a)	60.7 (7.4)	61.6 (9.2)	0.45
Cardiovascular risk factors - no. (%)			
Stable angina at baseline angiography	67 (68.4)	59 (69.4)	0.88
ACS at presentation	31 (31.6)	26 (30.6)	0.88
Extracardial vascular disease ^b	7 (7.1)	15 (17.6)	0.03
Prior AMI	24 (24.5)	26 (30.6)	0.36
Prior PCI	22 (22.4)	12 (14.1)	0.15
Prior CABG	5 (5.1)	1 (1.2)	0.14
Hypercholesterolaemia	58 (59.2)	58 (68.2)	0.20
Hypertension	40 (40.8)	40 (57.6)	0.40
Diabetes Mellitus (type I and II)	7 (7.1)	10 (11.8)	0.28
Current smoker	34 (34.7)	27 (31.8)	0.68
<i>Disease severity</i>			0.90
1-vessel disease	36 (36.7)	31 (36.5)	1.00
2-vessel disease	41 (41.8)	38 (44.7)	0.77
3-vessel disease	21 (21.4)	16 (18.8)	0.71
Medical therapy^c - no. (%)			
Statins	98 (100.0)	81 (95.2)	0.03
β-adrenergic receptor antagonists	82 (83.7)	57 (67.0)	0.01
Calcium antagonists	18 (18.4)	11 (12.9)	0.32
ACE-inhibitors ^d	12 (12.2)	19 (22.4)	0.07
Acetylsalicylic acid	96 (98.0)	85 (100.0)	0.19
ADP receptor antagonists	92 (93.9)	79 (92.9)	0.80
Laboratory findings			
S-C-Reactive Protein (mg/L)	1.8 (4.9)	2.5 (4.9)	0.40
S-LDL cholesterol (mmol/L)	3.0 (1.3)	3.1 (1.1)	0.39
S-HDL cholesterol (mmol/L)	1.2 (0.4)	1.2 (0.4)	0.76
S-Apolipoprotein B100 (g/L)	0.87 (0.34)	0.88 (0.28)	0.33
eGFR (mL/min/1.73 m ²)	95 (17)	93 (19)	0.34
S-glucose (mmol/L)	5.5 (1.3)	5.7 (1.7)	0.72

P-total homocysteine (µmol/L)	9.9 (3.1)	9.8 (3.3)	0.27
P-folate (nmol/L)	10.1 (5.8)	11.0 (6.3)	0.20
P-ADMA (µmol/L)	0.50 (0.09)	0.52 (0.12)	0.23
S-Trimethyllysine (µmol/L)	0.85 (0.34)	0.87 (0.40)	0.48
S-Carnitine (µmol/L)	40.5 (7.5)	41.0 (10.1)	0.52
S-γ-Butyrobetaine (µmol/L)	0.98 (0.30)	1.02 (0.28)	0.05

For continuous variables, mean and standard deviation or median and interquartile range within each group is calculated. Student's T-test or Mann-Whitney U-test was used to compare the two groups. For categorical variables, number and percentage is presented and a Chi square test was used to compare the four groups. Fisher's exact test was used when appropriate. All biochemical parameters are presented as median (interquartile range). FA, folic acid (0.8 mg); B₁₂, vitamin B₁₂ (0.4 mg); B₆, vitamin B₆ (40 mg); PCI, percutaneous coronary intervention; CABG, coronary artery bypass graft surgery; ACS, composite syndrome consisting of acute coronary syndrome including both ST-elevated and non-ST-elevated myocardial infarction; AMI, acute myocardial infarction; CHD, coronary heart disease; ACE, Angiotensin I converting enzyme; LDL, low-density lipoprotein; HDL, high-density lipoprotein; eGFR, estimated glomerular filtration rate; ADMA, asymmetric dimethylarginine. Percentages may not add up due to rounding of numbers.

^a Ejection fraction was measured during ventriculography for the majority of the patients. When this was not performed, ultrasonic echocardiography was used.

^b A prior diagnosis of any peripheral or cerebrovascular disease.

^c Medication at discharge.

^d Including ARB - angiotensin receptor blockers.

4.4. The Association Between Progression of Atherosclerosis and the Methylated Amino Acids Asymmetric Dimethylarginine and Trimethyllysine (Paper II).

PLoS ONE. 2013 May 29;8(5):e64774.

A total of 309 coronary artery lesions as well as plasma levels of ADMA and TML were evaluated at both baseline and follow-up. Using novel statistical methods, we showed that at follow-up median (95% CI) DS increased by 18.35 (5.22 – 31.49) percentage points per $\mu\text{mol/L}$ ADMA increase (p-value 0.006) and 2.47 (0.37 – 4.58) percentage points per $\mu\text{mol/L}$ TML increase (p-value 0.021) in multivariate modeling. Treatment with FA/B₁₂ ($\pm\text{B}_6$) was not associated with ADMA or TML levels. ADMA was independently associated with angiographic progression of CAD over a wide distribution of DS using a multivariate model – i.e. the adverse effect of elevated ADMA was similar in respect to both the effect estimate and level of significance across the 10th, 25th, 50th, 75th and 90th percentile of DS. To our knowledge, neither ADMA nor TML has previously been shown to be associated with the progression of de novo atherosclerosis. To conclude, in patients with established CAD, baseline ADMA and TML was associated with angiographic progression of CAD. However, neither ADMA nor TML levels were altered by treatment with FA/B₁₂ ($\pm\text{B}_6$).

4.5. Effect of Folic Acid supplementation on Levels of Circulating Monocyte Chemoattractant Protein-1 and the Presence of Intravascular Ultrasound Derived Virtual Histology Thin-Cap Fibroatheromas in Patients with Stable Angina Pectoris (Paper III).

PLoS ONE. 2013 July 25;8(7):e70101.

In patients with CAD receiving established medical treatment and revascularization with PCI, we found no statistically significant association between treatment with folic acid/vitamin B₁₂ and either levels of MCP-1 or the presence of occult VH-TCFA lesions by IVUS.

Patients treated with folic acid/vitamin B₁₂ had a geometric mean (SD) MCP-1 level of 79.95 (1.49) versus 86.00 (1.43) pg/mL for patients receiving placebo (p-value 0.34), while VH-TCFA lesions were present in 7.8% of patients and did not differ between intervention arms (p-value 0.47). We did however find a strong, statistically significant relationship between VH-TCFA lesions and elevated serum levels of MCP-1. Serum levels of MCP-1 were 1.46 (95% CI 1.12 to 1.92) times higher in patients with VH-TCFA lesions than in those without (p-value 0.005). Additionally, in a post-hoc analysis, MCP-1 levels predicted time to AMI in a Cox proportional hazard model with and without adjustment for established risk factors when followed for median 2.1 years (P-value 0.006).

To conclude, we showed that in patients with SAP receiving established medical treatment, folic acid / vitamin B₁₂ supplementation is not associated with either presence of VH-TCFA or levels of MCP-1. MCP-1 is however associated with VH-TCFA, a finding corroborated by increased risk for future AMI.

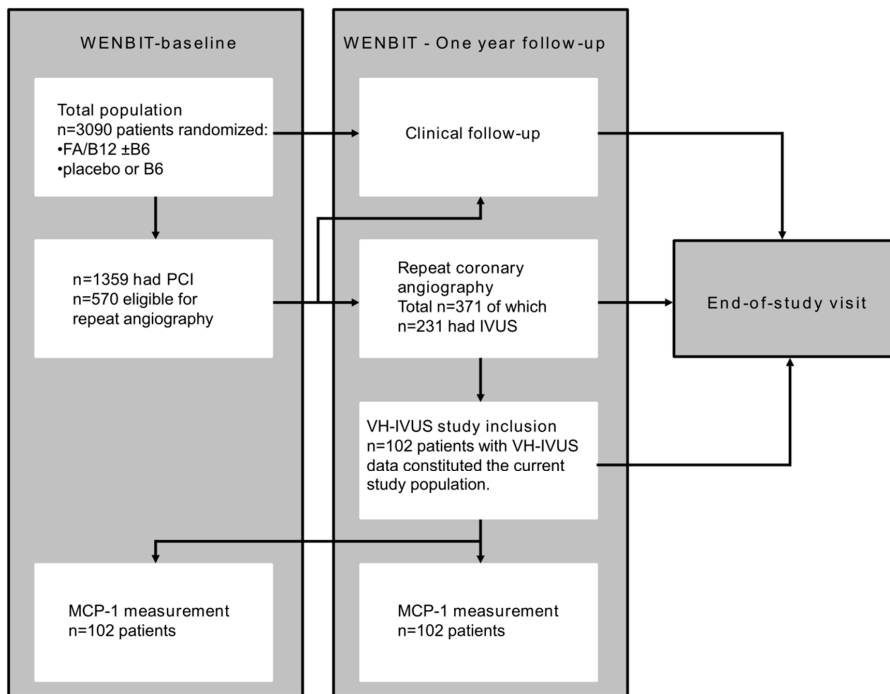


Figure 8. The chart shows the flow of patients from the WENBIT-trial (n=3090) to inclusion in the current IVUS-VH study (n=105).

All patients were randomized to B-vitamin treatment at WENBIT-baseline. Of the 1359 patients who had PCI at WENBIT-baseline, 371 had new, scheduled angiography at the WENBIT-one year follow-up. Of these 371 patients, 231 had received IVUS in a non-intervened vessel. IVUS-VH analysis and MCP-1 measurements were performed on 102 of these 231 patients and constituted our current study population. MCP-1 measurements were performed both at WENBIT-baseline (B-vitamin randomization) and at IVUS-VH study inclusion. Abbreviations are FA, folic acid (0.8 mg); B12, vitamin B₁₂ (0.4 mg); B6, vitamin B6 (40 mg); WENBIT, Western Norway B-vitamin Intervention Trial; IVUS, intravascular ultrasound; IVUS-VH, intravascular ultrasound – virtual histology; PCI, Percutaneous coronary intervention and MCP-1, monocyte chemoattractant protein-1.

*“By science calm’d over the peaceful soul,
Bright with eternal wisdom’s lucid ray,
Peace, meek of eye, extends her soft controul,
And drives the fury passions far away.”*

Sir Humphry Davy (1778 – 1829). Excerpt from “Sons of Genius”. Robert Southey
(ed) Annual Anthology vol.1 (Bristol 1799) pp 93-99.

5. DISCUSSION

5.1. Summary

This thesis is based on sub-studies from the Western Norway B-vitamin Intervention Trial (WENBIT) including patients undergoing both extensive and repeat invasive diagnostic procedures in order to measure rate of progression and (in)stability of CAD in a mostly unselected population of patients with SAP. WENBIT⁹³ was one of several large-scale randomized clinical trials (RCTs) investigating the putative cardioprotective effects of homocysteine-lowering B-vitamin treatment launched during the early 2000s. While universally fruitless, a pattern of a possible detrimental effect in sub-groups started to emerge. The current thesis include studies where we investigated the effect of moderate dose folic acid and vitamin B₁₂ intervention on the progression of coronary atherosclerosis, presence of unstable coronary plaques as well as circulating levels of two methylated amines, ADMA and TML and the inflammatory mediator MCP-1. This thesis adds to the current knowledge of progression of coronary atherosclerosis in patients with SAP. Specifically, that folic acid/vitamin B₁₂ treatment is associated with rapid angiographic progression CAD in a sub-group of patients, but that this is not modulated by either unstable coronary lesions (TCFAs) or circulating levels of either, ADMA, TML or MCP-1. In addition we have shown that the methylated amines ADMA and TML are associated with progression of CAD and that the inflammatory marker and monocyte chemokine, MCP-1, is related to the presence of unstable coronary plaques and possibly future coronary events.

5.2. Methodological discussion

5.2.1. Study design and patient population

Randomized, double-blinded placebo controlled trials (RCTs) are considered the gold standard of clinical investigation designed to minimize the implication of bias and human error¹³⁰. WENBIT is to date the largest clinical RCT ever conducted in the western part of Norway and included patients undergoing coronary angiography for suspected CAD. A total of 3090 patients were randomized in a 2x2 factorial design in order to assess both folic acid/vitamin B₁₂ vs. no folic acid/vitamin B₁₂ and vitamin B₆

vs. no vitamin B₆ on incident cardiovascular events and mortality. Accordingly all sub-studies included patients randomized in the same study design.

Paper I and II conforms to a standard RCT design in which patients are randomized to treatment and followed for a period of time before primary end-points are acquired. Since patients recruited for this sub-study were subject to additional inclusion criteria following randomization, there exists a potential for selection bias which will be discussed further in the following section. In paper I we performed a sub-group analysis of patients with rapidly progressing atherosclerotic lesions which was performed outside of the *a priori* defined analyses. Introduction of such analyses always results in a risk of spurious findings, especially when the amount of baseline variables available for categorization is high, as in registry epidemiology and large-scale RCTs¹³¹. Especially positive (or negative such as paper I in this thesis) trial results in a sub-group from an otherwise negative study should raise suspicion if overstated in the conclusion of a paper. However, within the framework of biological plausibility and supporting evidence they could provide enough evidence to formulate a purely hypothesis generating if interpreted with caution¹³².

When inspecting the baseline data from the patients included in paper I and II, both in tables 2,3 and 4 it is readily apparent that randomization was largely successful and that there was no obvious distortion of covariates introduced by sub-study recruitment of patients.

Paper III has a more complex study design which warrants some consideration. As outlined in figure 8, there are two follow-up time periods. Time period one from baseline and folic acid/B₁₂ randomization with concomitant MCP-1 measurements until coronary angiography and VH-IVUS study inclusion with MCP-1 simultaneous measurements and time period two from VH-IVUS study inclusion until clinical follow-up for incident AMI (median 2.1 years) with no further MCP-1 measurements. The consequence of this is that sub-study recruitment was performed after, for most

patients, about 1 year of treatment with study medication. This in turn, potentially increases the risk of selection bias.

5.2.2. Selection bias

Selection bias is often used to refer to a number of different epidemiological biases, common of which all result in that the association between exposure and outcome differs between the selected patients and those eligible for analysis¹³³. Of these, sample selection bias is probably the most well-known.

While RCTs are considered well suited to minimize the impact of selection bias they are still subject to residual effects. Our patient population consisted of consecutively recruited patient referred to coronary angiography for suspected CAD and thus represents a rather non-selected cohort with mostly SAP. The consecutive approach to patient recruitment and only modest exclusion-criteria mainly pertaining to inability to comply with long-term follow-up is one of our major strengths.

However, sub-studies performed within the WENBIT protocol presented in this thesis raises some concerns regarding sub-trial run-in. In both paper I and II progression of either CAD and/or biomarker activity, is measured in relation to the interventional regimen which in some instances already was started prior to patient inclusion. In WENBIT-RA some patients were recruited for sub-group inclusion (repeat angiography) at the 1 month control and sometimes later. While this allowed for the recruitment of more patients, it also potentially distorts the interpretation of the data. In paper I, where we investigate whether folic acid treatment accelerates CAD progression and did not find anything, we might also observe a “survivor” phenomenon. Those patients, either through genetic or environmental susceptibility, potentially adversely affected by FA might already have been excluded as they had already experienced their clinical event or that a putative “progression” had occurred and reached a new steady-state. This phenomenon has previously been attributed to

conflicting results of the association between hormone replacement therapy and CAD from observational versus randomized data¹³⁴.

For paper III, where sub-study recruitment was mainly conducted at the 1-year clinical follow-up/repeat angiography control, this especially holds true. While we did not detect a significant difference in either MCP-1 levels or VH-TCFA prevalence we cannot exclude a survivor-phenomenon wherein those susceptible to the potentially adverse FA effect would “never reach” the 1 year repeat angiography. With this in mind, one should be careful when concluding from the negative findings of paper III.

5.2.3. Regression dilution bias

Regression dilution bias is the statistical effect observable when a single measurement of a risk factor (e.g. blood pressure) is used instead of the “usual” level of the risk factor during the exposure time-frame. The unadjusted association between a risk factor and outcome is usually weaker than when a measurement over time or repeat measurements are used¹³⁵. Risk estimates based on single measurements tend to underestimate the true effect of the observed relationship due to regression dilution bias. The intraclass correlation coefficient (ICC) of 0.54 for ADMA and 0.37 for TML in paper II are quite modest, as is the ICC of MCP-1 (from n=102) of 0.66. All implying that the “true” adverse effect of these parameters are, most probably, greater than apparent¹³⁵.

Statistical modeling allows for the use of repeat measurements for example through the application of mixed models such as those applied in paper I and II. Had this been applied to investigate risk assessment in paper II, one could potentially further unmask the underlying effect. However, in the setting of a placebo-controlled RCT this becomes difficult as one would have to include the treatment interaction as an interaction term, resulting in massive loss of statistical power. In addition interpretation and presentability of the results diminished with increasing model complexity. Accordingly this was not performed, although in hindsight it might have been prudent to do so.

5.2.4. Confounding

Confounding is the statistical phenomenon wherein an apparent association between two observations is actually explained by a third, unknown factor – the confounder, which both observations are associated with. Numerous examples of such observed relationships that upon close scrutiny have been demonstrated to fall apart riddles the pages of medical history, some having endured for many decades. Several are easily apparent, while others are more subtle.

Confounding is to a certain degree removed by controlling for the confounding factor in the analysis. In all analysis in papers I through III the multivariate analyses are controlled for a substantial number of factors, including not only obvious potential confounders such as age, sex and smoking, but more detailed information about coronary anatomy, ejection fraction and biochemical profiling. On the other end, adjustment for too many potential confounders results in loss of degrees of freedom and subsequently statistical power and model overfitting. In general, analyses in this thesis have been subject to a conservative approach with relatively liberal inclusion of potential confounders. I.e. the multivariate model in paper II includes 16 covariates from 183 patients.

Can residual confounding explain the findings? Theoretically yes, although this would appear unlikely for a number of reasons. Regarding paper II, the fact that adjustment for a wide array of potential confounders resulted in a strengthening, rather than a weakening means that it is more likely a single, instead of multiple, residual confounder is the culprit. Secondly, it implies that the residual confounder would have to be unrelated to any of the included covariates. A completely unknown confounder for progression of CAD without any relationship with current established risk factors seems statistically unlikely. In paper III the relationship between TCFA presence and MCP-1 is strengthened in that MCP-1 (and VH-TCFAs) predicts AMI in the same

cohort – in a way a crude form of internal validation strongly implying that the observed relationship is true.

5.2.5. Generalizability

The concept of generalizability pertains to the ability of the data to make general inferences. All patients were recruited from WENBIT and were accordingly subject to WENBIT inclusion and exclusion criteria as outlined in the methods section. Broad inclusion criteria (patients aged 18 years or older referred to coronary angiography for *suspected* CAD) and few exclusion criteria (inability to comply with long-term follow-up) results in a population pool of patients with established CAD which one could argue does not differ substantially from the “real-life” SAP patients, especially considering it in the framework of a RCT. The level of drop-out from WENBIT were modest, 456 (14.8%) stopped taking study treatment during follow-up of which 46 (1.5%) were due to adverse effects⁹³.

The sub-study recruitment patient flow (figures 4 and 8) in this thesis is complicated. All patients were recruited at, or following, PCI during the sub-study recruitment period and were recruited in a non-selective manner. Meaning that all patients had angiographically verified CAD deemed suitable for invasive treatment. As shown in table 2, there is no major difference between those patients with and those without QCA-analyses performed, i.e. the patient cohort in paper I and II are representative of a mainly SAP cohort of patients with established 2- or the 3-vessel CAD.

5.2.6. Spurious findings and multiple comparisons

When performing multiple statistical analyses and comparisons there is always a risk for false positive findings. A significance level of 5% will result in 1 in 20 false positive findings when there is no real association present. This is a major cause for concern in the age of big data where access to registries with thousands of patients combined with thousands of measured variables results in millions of possible permutations and subsequently spurious findings.

In the post-hoc sub-group analysis of paper I, the fact that the observed relationship between FA/vitamin B₁₂ and rapid progression was statistically significant only when applied to DS and end-points raises the question whether this was just a spurious finding. MLD and DS are both measures of the same phenomenon, luminal narrowing. However, DS is a relative measurement which is not subject to the potential calibration errors of MLDs absolute measurements. However, as a post-hoc finding it should be considered purely a hypothesis-generating finding.

5.2.7. Visualization of plaque progression and stability

QCA has been the standard for coronary visualization for several years and is by far the primary basis for both invasive and surgical treatment choices in CAD. However, inherent shortcomings are readily apparent. QCA is *de facto* a lumenogram, depicting the interior of the epicardial, coronary vessel. As the understanding of CAD progression and ACS broadens, so does our acknowledgement of the disease process within the vessel wall^{4, 25, 48}. QCA has been shown to systematically underestimate the severity of mild stenoses¹³⁶. For the visualization of these aspects of CAD, IVUS and optical coherence tomography (OCT) are much more suited. In addition, diffuse and complex luminal shapes such as thrombosis, dissection and ulcerations are not readily apparent with QCA. However, both IVUS and OCT are time- and cost-consuming in routine clinical practice.

5.2.8. Biochemical considerations

All blood samples were immediately frozen at -80°C by study personnel for storage, which could be considered adequate. Most standard blood analyses were performed by fresh samples at the Laboratory of Clinical Biochemistry, Haukeland University Hospital, which is a high-throughput, high-end institution. B-vitamins and associated compounds were analyzed by Bevitel, using previously described methods¹¹² developed by members of the extended research group and have been extensively validated, and for some compounds the only commercially (or scientifically) available

analysis platform. Plasma TML, free carnitine and γ -butyrobetaine were also analysed using MS/MS as described previously¹¹³, as there were no other commercially available analysis platforms at the time. As paper III is to my knowledge one of the first studies wherein plasma TML have been measured in human subjects we have almost no way of comparing the observed values. It would be very interesting to analyse plasma TML in larger validation cohorts as well as in healthy/younger subjects. As TML is a direct product of proteolysis, it stands to reason that younger and healthier subjects might have higher baseline values due to an increased basal metabolic rate. MCP-1 was measured with established methods and presents no direct problems regarding interpretation.

5.2.9. Statistical analysis

Throughout this thesis we have employed some, at the time, novel and advanced statistical methods most of which have been extensively presented in the methods section. Especially the mixed-effects approach to linear modeling was at the time of publishing (paper I, 2010) quite uncommon in bio-medical literature. Since then however, mixed-model approaches have been applied in an almost exponential manner. The ability to model allow for “variation of variance” at different hierarchical levels is of obvious interest to investigators, but have previously been impossible due to both lack of methods and processing power, as well as availability of applicable software. Mixed effects modeling is now widely applied in meta-analyses, network analyses and observational/epidemiological work.

Quantile regression, although used in the field of econometrics for several decades, is also rather new in biomedical research, also probably in part due to computational and technical requirements. We would argue that not only is it a more thorough and transparent analysis of outcome data, it is also inherently more suitable for the approach to biological systems where a non-parametric method is often the most conservative and reasonable option.

When applying novel, and/or under-utilized statistical methods, one could safely assume that there is an increased risk of software bugs, erroneous application or unintentional misinterpretation of data. This is natural in the biomedical sphere as few researchers have an extensive statistical and/or software development background. This is an inherent problem of “new methods” which we feel is an acceptable risk. It is however important to view such papers as more experimental than those applying more standardized methods.

5.2.10. Inference of causality

Casual inference refer to the process of determining the causality of a sequence of events from statistical data, that A causes B – i.e. concluding that in fact smoking *causes* lung cancer and is not only statistically associated with it. Causality in the setting of observational and epidemiological data is often more difficult to assess than one might intuitively think. Usually, complex multivariable adjusted models might reduce the risk of confounding to an acceptable level – but removing the possibility of reverse causality – that B causes A is within several methodological frameworks impossible. Correlation does not imply causation, as the mantra goes, effectively invalidating observational data as a source of causal inference.

The framework of an RCT is arguably the best suited method for assessing causality in medicine. One could therefore propose that the observed relationship between folic acid supplementation and accelerated progression of CAD is causal – if confounding or spurious findings could have been ruled out. In both paper II and III, reverse causality – i.e. that plasma ADMA/TML and MCP-1 is elevated due to CAD progression or plaque instability respectively and not vice versa cannot be ruled out. Accordingly we have made no claims as to the direction of causality regarding these biomarkers, given the nature of these compounds – we would argue that it seems more probable that they, at least in the context of ADMA/TML are a bystander of disease state predisposing for CAD progression. In paper III, MCP-1 is a well-studied

chemokine intricately linked to the mechanistic understanding of plaque progression, making it difficult to rule out a causal relationship.

More advanced methods of assessing causality in observational data include structural equation modeling (SEM), Mendelian randomization, graphical methods such as direct acyclic graphs (DAGs) and Bayesian networks have recently emerged in the biomedical field¹³⁷. They are however advanced and time- and resource consuming. A SEM approach to paper III was initially explored but abandoned due to its complexity.

5.2.11. Effect modification

Effect modification, the phenomenon that the observed effect of an intervention differs across the strata of a third variable, is not to be confused with confounding¹³⁸.

Technically, effect modification is not necessarily the same as interaction which is defined as the combined/synergetic effect of two interventions – although this is not mutually exclusive at all and is frequently assessed together.

In all papers we have presented extensive baseline tables show only marginal differences between investigated groups. One of the main hypotheses of paper II was to investigate whether folic acid/vitamin B12 supplementation increased levels of ADMA/TML and whether this resulted in altered progression of CAD as assessed by QCA. Since no difference was found, we ruled out an effect modification between folic acid/vitamin B12 intervention and the relationship between ADMA/TML and QCA progression. Had we in fact shown such a relationship, an interaction term might have been added to the linear quantile model in order to test for interaction.

5.2.12. Ethical considerations

As previously stated all patients provided written, informed consent forms for both WENBIT and the WENBIT-RA sub-study. At the time of study conception there was no indication that folic acid/vitamin B12 supplementation would be detrimental – indeed grain fortification was and still is a world-wide, and in many regions

mandatory, phenomenon. The majority of the WENBIT-RA patients underwent an invasive coronary procedure without clinical indication – a potential harmful procedure. This was as previously stated approved by Regional Committee for Medical Research Ethics. The study protocol was in accordance with the principle of the Declarations of Helsinki. The study medication was approved by the Norwegian Medicines Agency and the data registration by the Data Inspectorate.

5.3. Discussion of the main findings

5.3.1. Study I: absence of a group effect and spurious findings

Study I failed to find any effect of folic acid/vitamin B12 supplementation on CAD progression at the population-level¹⁰². In general post-hoc sub-group findings should be considered with extreme caution and only as hypothesis generating findings. However, the sub-group in paper I was not defined by a baseline characteristic, but by the primary end-point itself, DS. When isolating patients who were rapidly progressing – one could argue that it only aids in precipitating an effect which is obfuscated by statistical noise.

A large body of evidence strongly suggests that the net effect of folic acid/vitamin B₁₂ supplementation on CVD is neutral⁸³. However, strong sub-group heterogeneity and differing effects can create an apparent calm sea. In NORVIT, in the group given folic acid/vitamin B12 and vitamin B6 there was observed a trend toward an increased risk (relative risk, 1.22; 95 percent confidence interval, 1.00 to 1.50; $P = 0.05$) for the primary end-point of recurrent myocardial infarction, stroke, and sudden death attributed to CAD. On a similar note, post hoc analyses of WENBIT showed that there was an increased risk of the composite primary end point in the group receiving folic acid/vitamin B₁₂ (HR, 1.34; 95% CI, 1.03-1.75; $P=.03$) compared with placebo. Combined results of NORVIT and WENBIT accordingly showed increased all-cause mortality⁹⁵.

However, paper I showed a potential increase in angiographic progression and not clinical events. Interesting in this regard is a study by Lange et al. where the in-stent restenosis rate after PCI was higher in the folic acid group than in the placebo group (34.5% vs. 26.5%, $P=0.05$), and a higher percentage of patients in the folic acid group required repeated target-vessel revascularization (15.8% vs. 10.6%, $P=0.05$)¹³⁹. While one can argue that the pathomechanisms for in-stent restenosis differs from de novo atherosclerosis, the apparent resemblance is intriguing. Lastly, a recent post-hoc analysis of the SU.FOL.OM3 study (Supplementation en Folates et en Oméga 3) by Blacher et al. looked at the effect of folic acid and omega 3 in a 2x2 factorial designed secondary CVD prevention study with regards to comparing the effect on hard coronary end-points and coronary revascularization¹⁴⁰. In the study, treatment with folic acid or omega 3 did not increase the risk of coronary end-points, but folic acid/vitamin B₁₂ and vitamin B₆ supplementation (560 µg 5-tetrahydrofolate, 3 mg vitamin B₆ and 20 µg vitamin B₁₂) resulted in a statistically significant 52% increase in the rate of coronary revascularization ($p = 0.0008$). The link between angiographic progression and the need for repeat revascularization is obvious. Thus the study by Blacher et al. in many ways validates the findings of paper I and lends credence to the notion of a heterogenic effect of B vitamins. Future studies should more specifically evaluate if there are distinct sub-groups of patients that have clearly benefitted or be harmed by the B vitamin treatment.

5.3.2. Study I: Potential adverse mechanisms

Homocysteine may induce SMC proliferation¹⁴¹ and thusly provide a biologically plausible plaque-stabilizing effect¹⁴². Accordingly, it is not impossible that homocysteine-lowering vitamin B treatment would cause adverse effects in larger, lipid-rich and/or necrotic core lesions by directly leading to thinning of an already marginalized fibrous cap. Repeat plaque rupture is after all the most common cause for rapid plaque progression. Folic acid intervention could also potentially affect both cell proliferation and intracellular methylation status (discussed later). It is also relevant to note that substitution with folic acid does not necessarily lower intra-cellular

homocysteine even though plasma levels are affected. Possibly, folic acid impairs the regulation of methylenetetrahydrofolate reductase by *S*-adenosylmethionine¹⁴³.

5.3.3. Study I and II: Methylation status and folic acid supplementation

The metabolic ramifications of folic acid supplementation are complex and have several potential adverse effects. Firstly, its effect on thymidine synthase promotes cell proliferation directly. This could potentially cause SMC proliferation and both concentric growth of the coronary plaque (causing angiographic progression, SAP and increased revascularization rates) while simultaneously provide plaque stabilization and no increase in clinical event rate. Secondly, the intracellular methylation potential is elevated with folic acid supplementation as the ratio of *S*-adenosylmethionine (SAM) to *S*-adenosylhomocysteine (SAH) increases. The enhanced methylation potential could have a host of adverse effects, including change in the expression of proatherogenic genes and increased production of ADMA^{72, 80}. Both global and site-specific hypo- and hypermethylation of DNA and histones are associated with CVD^{73, 96} and high doses of folic acid induce aberrant DNA methylation in some⁹⁸ studies as has been hypothesized as a possible mechanism in which folic acid might prove deleterious to health^{80, 144}.

5.3.4. Study II: Methylated amines and methylation status

Both ADMA and TML are methylated amino acids produced through proteolysis of methylated proteins. SAM is the global methylation donor for all intracellular methylation reactions. Increased SAM:SAH ratio and thus the intracellular methylation potential is necessarily linked with ADMA and TML – although the regulation of these metabolic steps is not well known.

Research on the pathophysiology of ADMA is based mainly on impaired degradation as a source⁷⁰. TML is produced in the same manner and is considered an obligatory by-product of global protein turnover. However, the degree to which post-translational methylation of amino acid residues is linked with global methylation potential is not

extensively explored¹⁴⁵. It is interesting to note that both previous speculations⁷² and emerging evidence⁷³⁻⁷⁵ suggest that epigenetic alterations, including post-translational methylation of chromatin and histones is relevant to the development and progression of atherosclerosis.

5.3.5. Study II: Lack of effect

We found no effect of folic acid/vitamin B₁₂ on the plasma levels of the circulation amino acids ADMA or TML¹⁴⁶. While some studies have shown that supraphysiological doses of folic acid have led to increased DNA methylation⁹⁸ in vitro and alter the SAM:SAH ratio in humans¹⁰⁰, human studies have failed to demonstrate increased DNA methylation from folic acid supplementation^{99, 100, 147}. DNA methylation and epigenetic regulations are extremely complex and tightly regulated processes^{73, 101, 148} and is probably not rate-limited by availability of methyl donors in an overly simplistic way¹⁴⁸.

However, 183 patients is a modest number and the lack of effect could possibly be explained by a lack of statistical power. A significant increase in the 10th percentile of ADMA in the group receiving folic acid/vitamin B₁₂ does not necessarily reflect a spurious finding. When the statistical power is low and/or the effect modest, one would expect the result of the intervention to be most visible at the extreme ends of the Gaussian distribution – e.g. raising ADMA levels in those patients with the lowest values. That supplying methyl donors would result in a global increase of methylated amino acids in all patients is probably a far too simplistic approach.

5.3.6. Study II: Prediction of CAD progression

The link between ADMA, endothelial NOS inhibition, endothelial dysfunction and increased risk of CVD is well documented^{30, 31, 66, 67} and could fully explain the findings in our data were ADMA predicts progression of QCA¹⁴⁶. While this has not been previously been demonstrated, it fits well with our current understanding of

progression of atherosclerosis in which endothelial dysfunction is pivotal in initiating and sustaining both inflammatory and fibroproliferic activity^{1, 48}.

On the other hand, circulating TML as opposed to ADMA, has not been previously linked with CVD. Since TML is similar to ADMA in that it is a methylated amino acid residue produced through protein turnover – it is tempting to hypothesize that it works in a similar way – that is by inducing vascular dysfunction by inhibiting eNOS. This is however completely unsubstantiated by the current literature, although the list of NOS inhibitors is large¹⁴⁹ and includes such compounds as L-N⁶-(1-Iminoethyl)lysine¹⁵⁰. Testing of this hypothesis is however relatively straight forward and would provide much information.

More intriguing however is TMLs role in carnitine metabolism, where it constitutes the first step human endogenous biosynthesis. Carnitine is involved in fatty acid metabolism^{71, 151, 152} and thus distortion in its biochemistry might represent mitochondrial (dys-)function. Mitochondrial dysfunction is emerging as a possible etiological and pathomechanistic explanation for a wide range of chronic diseases such as cancer¹⁵³, diabetes¹⁵⁴ and CVD^{155, 156}. Carnitine metabolism is dependent upon mitochondrial function and circulating levels of carnitine and acyl-carnitines reflects impairment of fatty acid β -oxidation^{157, 158}. Since TML availability probably is the rate-dependent step in carnitine biosynthesis, or at least TML clearance^{152, 159}, it is possible that mitochondrial dysfunction and impaired fatty acid metabolism results in an increase of TML as conversion to precursor γ -butyrobetaine or carnitine is blocked.

There is substantial co-linearity between TML, γ -butyrobetaine, carnitine and kidney function¹⁴⁶. Hypothetically, TML could act as a confounder for reduced renal function, γ -butyrobetaine or carnitine which all possibly could be related to CVD risk – of which impaired kidney function is by far the best documented¹⁶⁰. Estimated GFR was also significantly associated with progression of CAD in multivariate modeling. However, when we included eGFR in our multivariate models of TMLs effect on CAD

progression it did not substantially alter the effect of TML; suggesting that the observed association between TML and CAD progression is not confounded by kidney function.

However, inclusion of γ -butyrobetaine or carnitine in multivariate modeling did not alter TMLs predictive value and in addition, γ -butyrobetaine or carnitine did not predict CAD progression in a separate analysis when TML was not included. Taken together, this could also suggest that TML has a causal relationship in CAD progression. If elevated TML represents impaired carnitine metabolism as an innocent bystander, one would possibly expect changes in other precursors in carnitine biosynthesis.

5.3.7. Study III: Folic acid supplementation and plaque stability

Repeated rupture and healing of unstable coronary plaques is an important mechanism in which atherosclerotic lesions progress in luminal narrowing – especially rapid progression¹⁶¹. The potentially adverse effect of FA administration could possibly be mediated through plaque destabilization and rapid progression of CAD in such a manner. We found nevertheless no statistical significant effect of folic acid/vitamin B₁₂ on the presence of unstable coronary artery lesions as defined by the presence of VH-TCFA¹⁶².

However, while repeated plaque ruptured is linked to rapid, angiographic progression of CAD it is also strongly associated with clinical events – after all, almost no coronary events happen without plaque rupture or erosion²⁵. Accordingly, since the putative adverse effect of FA on CAD seems rather to result in angiographic progression¹⁰² and repeat vascularization¹⁴⁰ as opposed to clinical event rates⁸² – it would appear plausible that FAs effect is mediated through a different mechanism than plaque destabilization. Consequently, one would perhaps expect the relationship between FA intervention and VH-TCFA presence to be either neutral or inverse.

5.3.8. Study III: MCP-1 and folic acid supplementation

Previous studies have revealed an association between plasma homocysteine and MCP-1^{163, 164}. In addition folic acid supplementation has shown to lower plasma MCP-1^{85, 163, 165}. In paper III we were unable to reproduce these results in patients with SAP¹⁶² which we might have expected. These apparent discrepancies with the literature might reflect different study design and subjects characteristics, including the fact that plasma homocysteine at WENBIT baseline was relatively low (mean tHcy <10 $\mu\text{mol/L}$). Kim et al. reported a correlation of $r = 0.31$ ($p < 0.01$) between plasma homocysteine and MCP-1¹⁶⁴ which was not at all apparent amongst our patients, were the Spearman correlation coefficient between homocysteine and MCP-1 was $r = 0.006$. In addition, the fact that all patients had established CAD reflects a severity, both in time and extent, of the underlying pathological process that might conceivably negate the positive effect. Notably, the fact that most patients were under treatment with statins reflects a non-ignorable degree of anti-inflammatory treatment which could explain the abolished correlation between MCP-1 and homocysteine – and thus a biochemical state in which folic acid might provide an effect. Furthermore, Economou et al. showed that the correlation between homocysteine and MCP-1 is weakened by the presence of obesity and insulin resistance⁸⁶. This however does probably not explain our negative findings, as stratifying according to BMI did not result in significant effects of folic acid/vitamin B₁₂ treatment on MCP-1 levels in our material (data not shown).

5.3.9. Study III: MCP-1 and plaque stability

The primary focus of paper III was to investigate the effect of folic acid/vitamin B₁₂ on plasma levels of MCP-1 and the presence of VH-TCFA. However, we also looked at the relationship between MCP-1 and the presence of VH-TCFA as well as prospective data on time to AMI. In previous studies, MCP-1 has been associated both with the presence of CAD risk factors in sub-clinical atherosclerosis⁵⁷ and CVD mortality in middle-aged, obese patients⁶⁰. Moreover, in several studies by De Lemos et al. MCP-1 is an independent predictor of death or myocardial infarction following an ACS

episode both in the acute setting and long-term follow up^{64,65}. While we had few patients and end-points, we were still able to replicate this relationship in our data set and showed that patients with elevated MCP-1 were significantly higher at risk for experiencing an AMI during 2.1 years of follow-up, both in uni- and multivariate Cox proportional hazard models adjusted for common risk factors.

Regarding VH-TCFAs, we found that MCP-1 levels were linked to the presence of VH-TCFA lesions. Specifically, that patients with VH-TCFA had 46% higher plasma MCP-1. Moreover, 25% of patients in our study with VH-TCFA experienced a coronary event within 2.1 years of follow-up. The last observation is in accordance with the findings of the Providing Regional Observations to Study Predictors of Events in the Coronary Tree (PROSPECT) trial by Stone et al. which validated the predictive value of VH-TCFA lesions on coronary end-points in a prospective manner¹⁰⁹.

Finding from the literature suggest that TCFA-lesions are present in as much as two-thirds of culprit lesions^{19, 166, 167}. A prerequisite for the development of such a lesion is monocyte infiltration, in which MCP-1 plays a pivotal role^{54 55, 56}. MCP-1 is necessary for trans-endothelial migration⁵² and regulation of macrophage activity^{54, 55}, both considered a sine qua non of the destabilization of coronary atherosclerotic plaques^{4, 53}.

Accordingly it is not biologically implausible that the ability of MCP-1 to predict coronary events observed by De Lemos and others is a result of the potential association between MCP-1 and the presence of VH-TCFA lesions which we observed in paper III. Raised circulating levels of MCP-1 might represent increased monocyte recruitment, a necessary step on the ladder towards the formation of VH-TCFA lesions – which in itself are associated with adverse coronary events. To support this hypothesis, recent work by Li et al. showed that MCP-1 levels and mRNA expression is associated with ACS and plaque rupture¹⁶⁸. The strength of the post-hoc findings in paper III is that we are able to demonstrate a statistically significant association between MCP-1, VH-TCFA and future AMI. In a way this is a form of internal

validation of the hypothesis that MCP-1 predicts the presence of high-risk coronary lesions.

6. CONCLUSION

The studies included in the present thesis aimed to investigate the effect of folic acid and vitamin B₁₂ supplementation on progression of sub-clinical CAD in patients with mostly SAP as well as metabolites related to potential mechanisms of progression. In conclusion, the present thesis found the following answers to the study aims:

6.1. Study I: Homocysteine-lowering folic acid/vitamin B₁₂ treatment during median 10 months demonstrated no protective effect on the progression of CAD as expressed by MLD and DS measured by repeat QCA. Moreover, a possible adverse effect by this treatment promoting rapid progression of larger and bulkier lesions was indicated in post-hoc analysis.

6.2. Study II: ADMA and TML were associated with angiographic progression of CAD. However, treatment with folic acid/vitamin B₁₂ did not alter the plasma levels of either ADMA or TML. The possible adverse of folic acid/vitamin B₁₂ on CAD progression is most likely unrelated to the metabolism of methylated amino acids.

6.3. Study III: Folic acid/vitamin B₁₂ supplementation did not alter either MCP-1 levels or the presence of occult VH-TCFA coronary lesions in SAP patients. However, MCP-1 was associated with the presence of VH-TCFA lesions and both were associated with future AMI as well as with each other. The possible adverse of folic acid/vitamin B₁₂ on CAD progression is most likely unrelated to plaque instability and repeated rupture. In addition MCP-1 could be used to select SAP patients for further investigations, including VH-IVUS, to identify patients with VH-TCFA lesions.

In conclusion our findings support the notion that treatment with folic acid/vitamin B₁₂ as secondary prevention in CAD patients is not warranted and may increase progression of CAD in subsets of patients.

FUTURE PERSPECTIVES

We are currently evaluating the effect of the B-vitamin therapy in WENBIT according to selected genotypes within one-carbon metabolism, and preliminary data strongly support that folic acid supplementation is a double-edged sword with detrimental effects according to genotype. Studies replicating our possible findings will be needed, and several such studies are planned. The mechanism in which folic acid might provide progression of angiographic CAD and the need for coronary revascularization without increase in mortality is yet to be elucidated.

On a side note, further investigation should also aim to identify (lifestyle) determinants of plasma TML. Circulating TML levels have a short track-record in the scientific literature and provide a new frontier as a metabolic compound from the nexus of several inter-connecting processes such as methylation, carnitine and fatty acid metabolism and mitochondrial dysfunction. A recent paper by Koeth et al. published in *Nature* showed that plasma *L*-carnitine levels in subjects undergoing cardiac evaluation predicted increased risks for both prevalent CVD and incident major adverse cardiovascular events, but only among subjects with concurrently high trimethylamine-*N*-oxide levels¹⁶⁹. Whether carnitine in this setting is a confounder for the precursor, TML is a very interesting question. A prospective study on TMLs effect on cardiovascular end-points is currently under way.

In regards to MCP-1, it would be interesting to confirm the association between vulnerable plaques and levels of MCP-1 and whether MCP-1 measurement could be used to select SAP patients which are at higher risk for future coronary events and have unstable coronary lesions amendable by PCI.

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