Novel and Traditional Risk Factors for Coronary Artery Disease

To all patients and their families affected by Coronary Artery Disease.

To Nadja, Aalyah and Amir.

Örebro Studies in Medicine 215

DEMIR DJEKIC

Novel and Traditional Risk Factors for Coronary Artery Disease:
Role of Coronary Artery Calcium, Lipidomics, Psychosocial Factors and Diet

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Abstract

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Background: The aim of the research reported in this thesis was to determine the association of novel and traditional risk factors with coronary artery calcium (CAC), a marker of subclinical coronary artery disease (CAD) in healthy individuals. In addition, we investigated the effects of a vegetarian, compared to a meat diet, on novel and traditional risk factors in patients with diagnosed CAD.

Methods: Studies I-II evaluated the inter-laboratory reproducibility of liquid chromatography-mass spectrometry (LC-MS) lipid analysis and the association of serum lipidome with CAC in a cohort of 70 patients. Studies III and IV analysed data of 1067 participants in the pilot study of the Swedish CArdioPulmonary bioImage Study to determine associations of psychosocial (residential area, education, housing, and social support) and traditional risk factors with CAC. Cardiac computed tomography was used to obtain a coronary artery calcium score (CACS) (Studies I–IV). Study V employed a crossover design in which 31 patients with CAD were randomly allocated to a four-week vegetarian diet alternating with four weeks of an isocaloric meat diet. Enzyme-linked immunosorbent assay was used to measure oxidised LDL-cholesterol. Plasma metabolome, including choline, trimethylamine N-oxide, L-carnitine, and acetyl-carnitine, as well as plasma lipidome were determined with LC-MS. Gut microbiota and faecal short- and branched-chain fatty acids were analysed with 16S rRNA gene sequencing and gas chromatography-MS, respectively.

Results: In Study I, two laboratories independently identified six lipids in common that differentiated serum of patients with CACS >250 from that of those with CACS=0. Study II, revealed higher levels of phosphatidylcholine(PC)(16:0/20:4) and lower levels of PC(18:2/18:2), PC(36:3) and phosphatidylethanolamine(PE)(20:0/18:2) in patients with CACS >250 than found in those with CACS=0. Study III showed a CACS >0 prevalence of 46.3% and 36.6% in low and high socioeconomic residential areas, respectively, but the traditional risk factor–adjusted odds ratio for CACS >0 was not significantly higher in subjects living in low socioeconomic areas. In Study III, the traditional risk factor–adjusted odds ratio for CACS >100 relative to CACS=0 was significantly higher in women with low education level and living in a rented apartment. Studies III and IV showed traditional risk factor–adjusted odds ratios for CACS >0 to be significantly higher in women with a family history of premature cardiovascular disease and low social support. No relationship of psychosocial factors with CAC was observed in men. The vegetarian diet implemented in Study V significantly lowered mean oxidized LDL-cholesterol (-2.73 U/L), total cholesterol (-0.13 mmol/L), LDL-cholesterol (-0.10 mmol/L), and body mass index (-0.21 kg/m2), as well as the relative abundance of PCs, PEs, and several microbial genera compared with the meat diet. The effect of the vegetarian diet on oxidized LDL-C was associated with higher relative abundance of *Ruminococcaceae* genera and of *Barnesiella* and reduced abundance of *Flavonifractor*. The vegetarian diet lowered the relative abundance of ceramide(d18:1/16:0) and triacylglycerols with saturated fatty acyl chains and raised the relative abundance of triacylglycerols with high carbon and polyunsaturated fatty acyl chains compared with the meat diet.

Conclusions: Novel and traditional cardiovascular risk factors are associated with subclinical CAD. Psychosocial factors are associated with subclinical CAD in women, but not in men. Short-term intervention with a vegetarian diet in individuals with CAD can positively impact novel and traditional factors that have been associated with risk of future cardiovascular events.

Keywords: Novel risk factors, coronary artery calcium, lipidomics, lipidome, psychosocial factors, vegetarian diet, gut microbiota, metabolome.

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V. **Djekic D***, Shi L*, Brolin H, Carlsson F, Särnqvist C, Cao Y, Bäckhed F, Tremaroli V, Landberg R, Frøbert O. Effects of vegetarian diet on cardiometabolic risk factors, gut microbiota and metabolome in patients with ischemic heart disease. A randomized, cross-over study. Under review.

Permission to reproduce the figures from the studies presented in this thesis was obtained from all co-authors and publishers. *Co-first authorship.

SWEDISH SUMMARY

Bakgrund: Föreliggande avhandlingsarbete hade två övergripande mål. Det första var att undersöka sambanden mellan nya och traditionella riskfaktorer på förekomsten av kalk i kranskärlen (CAC) hos friska individer som inte tidigare drabbats av kardiovaskulär sjukdom. CAC-värdet (CACS) är ett mått som kan användas som markör för mängden åderförkalkning i kranskärlen och där högre värden signalerar ökad risk för framtida insjuknande i kranskärlssjukdom (CAD). Det andra huvudmålet var att undersöka effekten av en livsstilsintervention med vegetarisk kost (VD) på nya och traditionella riskfaktorer hos individer med etablerad CAD.

Metod: Delarbete I och II undersökte reproducerbarheten av vätske-kromatografi-masspektrometri (LC-MS) och associationer mellan serum lipider och olika utbredning av CAC i en kohortstudie av 70 patienter.

Delarbete III-IV analyserade data från 1067 individer i pilotstudien av Swedish CArdioPulmonary bioImage Study för att undersöka samband mellan psykosociala faktorer (bostadsområde, utbildningsnivå, boendeform och socialt stöd), traditionella riskfaktorer och CAC. CACS bedömdes med datortomografisk undersökning av kranskärlen i delarbete I-IV.

I delarbete V (cross-over interventionsstudie) randomiserades 31 patienter med CAD till färdigproducerad VD och kost med kött (meat diet - MD). Oxiderat LDL-kolesterol analyserades med enzymkopplad immunads-orberande analys, olika plasma metaboliter och plasma lipider mättes med LC-MS. Mag- och tarmkanalens bakterieflora analyserades med 16S rRNA gensekvensering och fekala fettsyror med korta respektive grenade kedjor med gaskromatografi-MS.

Resultat: Delarbete I identifierade sex olika lipider vars nivåer var olika i serum från patienter med CACS >250 och patienter utan CAC. Analys vid två oberoende laboratorier gav samstämmiga resultat.

Delarbete II påvisade signifikant högre nivåer av lipiden fosfatidylkolin (16:0/20:4) och lägre nivåer av lipiderna fosfatidylkolin(18:2/18:2), fosfatidylkolin(36:3) och fosfatidyletanolamin(20:0/18:2) hos individer med CACS >250 jämfört med de utan CAC.

Delarbete III påvisade en prevalens för CACS >0 på 46.3% och 36.6% för individer från socialt utsatta områden och välbärgade områden. Individer från socialt utsatta områden hade en ökad risk för CACS >0 samt CACS >100, jämfört med individer från välbärgade områden. Den ökade risken kunde dock förklaras av traditionella riskfaktorer för CAD. Kvinnor, men inte män, med låg utbildningsnivå och som bodde i hyreslägenhet hade en ökad risk för CACS >100, jämfört med kvinnor med hög utbildningsnivå eller som bodde i villa. Riskökningen kunde inte förklaras av traditionella riskfaktorer. Kvinnor, men inte män, med ärftlighet för prematur kardiovaskulär sjukdom (delarbete III) samt lågt socialt stöd (delarbete IV) hade en högre risk för CACS >0, risken var oberoende av traditionella riskfaktorer. Kvinnor med låg social integration var ungefär 14 år äldre i kranskärlen jämfört med kvinnor med måttlig eller hög social integration.

Delarbete V visade att en 4 veckors lång intervention med VD, jämfört med MD, lede till signifikant lägre nivåer av oxiderat LDL-kolesterol, LDL-kolesterol, total kolesterol, vikt, lipiderna fosfatidylkolin, fosfatidyletanolamin, ceramid(d18:1/16:0) och triglycerider med mättade fettsyror. VD lede samtidigt till signifikant högre nivåer av triglycerider med fleromättade fettsyror, jämfört med MD. Förekomsten av vissa bakterietyper i mag- och tarmkanalen påverkades under VD, jämfört med MD. Individer med högre nivåer av vissa bakteriearter i mag- och tarmkanalen svarade bättre på VD, med lägre nivåer av oxiderat LDL-kolesterol.

Konklusion: Nya och traditionella riskfaktorer är associerade med subklinisk CAD. Psykosociala riskfaktorer var associerade med subklinisk CAD hos kvinnor men inte män, där traditionella riskfaktorer var mer betydelsefulla. Patienter med etablerad CAD påverkas positivt av VD, med förändringar i nivåerna av både nya och traditionella riskfaktorer, som tidigare associerats med återinsjuknande i CAD.

ABBREVIATIONS

CAC = Coronary artery calcium
CACS = Coronary artery calcium score
CAD = Coronary artery disease
CT = Computed tomography
CVD = Cardiovascular disease
FAC = Fatty acyl chain
HDL-C = High density lipoprotein cholesterol
HNR = Heinz Nixdorf Recall
HU = Hounsfield unit
LC-MS = Liquid chromatography-mass spectrometry
LDL-C = Low-density lipoprotein cholesterol
MD = Meat diet
MESA = Multi-Ethnic Study of Atherosclerosis
PC = Phosphatidylcholine
PCI = Percutaneous coronary intervention
PE = Phosphatidylethanolamine
SCAPIS = Swedish CArdioPulmonary bioImage Study
SCORE = Systematic COronary Risk Evaluation
SES = Socioeconomic status
SM = Sphingomyelin
TC = Total cholesterol
TG = Triacylglycerol
TMAO = Trimethylamine N-oxide
VD = Vegetarian diet
VERDI = VEgetaRian Diet in patients with Ischemic heart disease

INTRODUCTION

Coronary artery disease (CAD) is an important manifestation of cardiovascular disease (CVD) and the leading cause of death globally (1). The relationship of traditional factors such as age, sex, blood pressure, smoking, and cholesterol levels with CAD development has been studied for decades and is currently used by risk assessment systems to estimate CVD risk. Family history of premature CVD, psychosocial factors, obesity, and markers of subclinical disease including coronary artery calcium (CAC), carotid artery plaques, and ankle-brachial index have emerged as risk factors for CVD, independent of traditional risk factors (2). They may serve as risk modifiers, and their presence or absence can be a rationale for reclassification of individuals originally identified as having intermediate risk of a CVD event based on traditional risk factors to either high or low risk.

Identification of certain lipids by liquid chromatography-mass spectrometry (LC-MS) and quantification of oxidised low-density lipoprotein cholesterol (LDL-C) have been shown to improve accuracy of CAD event prediction (3, 4). The gut microbiota and its metabolite trimethylamine N-oxide (TMAO) have been suggested to play a role in CAD development (5).

In observational studies (I–IV), we investigated associations of both novel and traditional risk factors with subclinical CAD, as indicated by CAC assessed by computed tomography (CT), in subjects with no known history of cardiovascular disease. In Studies I and II, we investigated inter-laboratory reproducebility of LC-MS and the association of serum lipidome with subclinical CAD. Studies III and IV, the pilot study of the Swedish CArdioPulmonary bioImage Study (SCAPIS), explored the association of psychosocial factors and traditional risk factors in subjects with subclinical CAD.

An interventional study (Study V), VEgetaRian Diet in patients with Ischemic heart disease (VERDI), compared a four-week vegetarian diet with an isocaloric conventional meat-containing diet to investigate short-term changes in oxidised LDL-C, plasma metabolome, including TMAO, choline, L-carnitine, and acetyl-carnitine, as well as the gut microbiota, and faecal short- and branched-chain fatty acids along with traditional risk factors, in patients with a history of CAD treated with percutaneous coronary intervention (PCI) and receiving standard medical therapy. In this thesis, we also present supplementary findings of the VERDI trial pertaining to the effects of a vegetarian diet compared to a meat diet on the plasma lipidome and the association of plasma lipidome with the atherosclerotic burden in CAD patients.

BACKGROUND

Definition of cardiovascular and coronary artery disease

Cardiovascular disease encompasses disorders of the heart and/or blood vessels. The most common types of CVD include CAD, cerebrovascular disease, and peripheral artery disease. Coronary artery disease is characterized by atherosclerotic plaque formation in the arteries that supply the heart muscle (myocardium) with oxygenated blood. Accumulation of atherosclerotic plaques is initially asymptomatic and may remain stable for decades, a condition referred to as subclinical CAD (6). Eventually, usually not before middle to late adulthood, the build-up of atherosclerotic plaques in the coronary arteries may cause narrowing or complete blockage of blood flow to the myocardium, or an atherosclerotic plaque may rupture, leading to clinically overt CAD (7).

Coronary artery disease may present clinically as stable angina pectoris, acute coronary syndrome (unstable angina, non-ST-elevation myocardial infarction, or ST-elevation myocardial infarction), or sudden cardiac death.

History of coronary artery disease

Leonardo da Vinci (1452–1519) was among the first to describe atherosclerosis (8). Coronary artery disease was traditionally thought to be a disease primarily affecting modern humans; however, a 2013 study of mummies from several ancient cultures revealed presence of atherosclerosis in vascular beds, including the coronary arteries (9).

Epidemiology of coronary artery disease

Coronary artery disease mortality has declined from 1990 to 2015 in high-income countries including Sweden, mainly due to lifestyle changes and improved treatment (1, 10). Fewer people smoke, blood lipids are better regulated with statin drugs, exercise and healthy eating are promoted, there is increased availability of invasive coronary therapy, and myocardial infarction is treated with revascularization and dual-antiplatelet medications (11, 12).

A 2019 study reported that CVD in high-income and some middle-income countries has fallen to the point of causing fewer deaths than cancer (13). However, approximately 7.3 million acute myocardial infarctions and 8.9 million deaths due to CAD occurred globally in 2015 (1). Consequently, CAD remains a serious public health concern.

According to the Swedish National Board of Health and Welfare, in 2018 there were 92 000 deaths in Sweden, with CVD accounting for approximately 23%, followed by cancer at 22%. That same year saw approximately 24 800 cases of acute myocardial infarction resulting in 5800 deaths, equivalent to an incidence of 322/100 000 and mortality of 74/100 000 (14).

Incidence of acute myocardial infarction and mortality resulting from CAD are reported to vary widely with education level. The rate of acute myocardial infarction in Sweden in 2018 was 250/100 000 and 589/100 000 in women and men, respectively, who had completed compulsory school, compared to 113/100 000 and 375/100 000 in those with post-secondary education (14). Mortality following acute myocardial infarction also differs with educational level. Death of individuals 45–74 years in the 28 days following acute myocardial infarction was reported to be approximately 18% vs. 12% in men with compulsory vs. post-secondary education level and 20% vs. 12% in women (14).

Pathophysiology of coronary artery disease

Atherosclerosis is a dynamic, asymmetric, focal process that involves the innermost layer (tunica intima) of large and medium-sized arteries. Early signs of atherosclerosis may appear in childhood with fatty streak formation (15).

Fatty streaks are preceded by an injury to the endothelium and enhanced by cardiovascular risk factors, resulting in endothelial dysfunction, increased permeability, and retention of low-density lipoprotein cholesterol (LDL-C) in the intima (16, 17). Oxidation of LDL-C in the intimal space leads to expression of adhesion molecules on the endothelium. Adherence of white blood cells is followed by their rolling and migration across the endothelium into the intima (18). Scavenger receptors on differentiated macrophages take up oxidized LDL-C particles and are eventually transformed into foam cells composed of droplets of intracellular lipids (19).

During adulthood, fatty streaks may disappear or advance to atheroma (20). Atheromas, or atherosclerotic plaques, appear as necrotic, half-moon shaped lipid cores at the intima-media boundary. Initially, coronary plaques grow toward the media, but, as the atheroma becomes larger than roughly 40% of the artery cross-sectional area, the lesion begins to protrude into the lumen, impacting coronary blood flow (21).

As the lipid core is increasingly covered by fibrous tissue, atheromas may develop into fibroatheromatic plaque. Multiple lipid cores may exist,

surrounded by fibrous layers, while other lesions predominantly calcify or contain a fibrotic layer without a lipid core.

Smooth muscle cells contribute to extracellular connective tissue matrix formation of the fibrous cap by migrating from the media to the site of injury in the intima, where they transform from a contractile to an active synthetic phenotype, producing collagen fibres (22). Anti-inflammatory cytokines such as transforming growth factor-beta, interleukin-10, and platelet-derived growth factor, released by type 2 helper T cells and endothelial cells, stimulate smooth muscle cells to produce collagen fibres, stabilizing the fibrous cap (23, 24). Stimulated type 1 helper T cells may enhance synthesis of interferon-gamma, which further results in release of pro-inflammatory cytokines and inhibits smooth muscle cell proliferation and migration (20, 23, 25). This weakens the fibrous cap, potentially making the plaques more vulnerable to breakage.

Risk factors for coronary artery disease

Conditions predisposing an individual for atherosclerotic CAD can be categorized as nonmodifiable risk factors, including advanced age, male sex, and family history of premature CVD, or as modifiable factors such as hypertension, unfavourable blood lipid levels, diabetes mellitus, abdominal obesity, smoking, negative psychosocial factors, alcohol consumption, absence of regular physical activity, and lack of daily consumption of fruits and vegetables (26–28).

Recently, findings of the Prospective Urban And Rural Epidemiological (PURE) study suggested that approximately 70% of CVD events and deaths could be related to modifiable risk factors (29).

Risk estimate systems and atherosclerotic CVD event risk-modifiers

Because of interaction among risk factors, efforts have been made to develop risk estimation systems incorporating multiple elements. Several CVD risk assessment systems have been devised for use in predicting 10-year probability of a fatal or nonfatal CVD event in healthy individuals without history of CVD (2, 30). Recommended assessments include the Framingham Risk Score, Systematic COronary Risk Evaluation (SCORE), and the Pooled Cohort Equations (30–32). Traditionally recognized risk factors such as older age, male sex, elevated systolic blood pressure, elevated total cholesterol (TC), and smoking are components of most models, with age as a measure of total exposure time to other risk factors.

In adults >40 years of age, unless they are categorised as high or very high risk, use of a system to assess overall CVD risk is recommended by both European and American guidelines (2, 30).

The SCORE risk charts were developed to estimate the 10-year probability of a fatal CVD event in a population from European low and high CVD risk countries. Based on the traditionally quantified factors of age, sex, serum cholesterol, systolic blood pressure, and smoking, SCORE classifies patients into low (0–1%), intermediate (2–4%) and high (>4%) absolute risk for future CVD. In treatment guidelines, drug therapy is indicated for high-risk patients. For intermediate risk patients, the evidence base is less certain, and initiation of drug therapy depends on physician interpretation of data and patient preference.

Cardiovascular disease risk modifiers include psychosocial parameters such as socioeconomic status (SES) and social support, family history of premature CVD, body mass-index, abdominal obesity, CAC, carotid atherosclerotic plaque, and ankle-brachial index (2). Assessment of these factors is critical, since their presence or absence may warrant reclassification in intermediate risk patients. In individuals with an estimated low or extremely high risk based on traditional risk factors, the presence of modifiers is unlikely to change lifestyle recommendations or drug therapy.

Metabolomics and Lipidomics

The fields of metabolomics and lipidomics are defined, respectively, as the study of metabolites and lipids in tissues and body fluids (33). The terms metabolome and lipidome refer to the complete set of molecules (metabolites) and lipids of molecular weight <1500 daltons (34). The most commonly used analytical techniques in metabolomics and lipidomics are mass spectrometry (MS) and nuclear magnetic resonance spectroscopy. A comparison of strengths and weaknesses of the techniques has been summarized (35).

Mass-spectrometry-based lipidomics has been applied in large population-based studies to evaluate the lipidome (3, 36–38), and lipidomics has been used to investigate the serum lipidome of patients with CAC (39). Based on detection of specific lipids, investigators were able to differentiate patients with coronary calcification from those with no calcification. These findings have, however, not been reproduced. This is relevant, since lack of reproducibility has been considered a shortcoming of lipidomic studies (40). In Study I, we evaluated the potential of lipidomics

to reproduce previously obtained results pertaining to the association of serum lipidome and CAC.

Lipids

Table 1 illustrates potential involvement of lipids in CAD. Lipids are structurally complex and can be subdivided into eight main classes: fatty acids, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides. Each lipid class is characterized by a common head group with several possible conjugated fatty acyl chains (FAC) resulting in the formation of >20 000 lipids. Phosphatidylcholines (PC) are classified as glycerophospholipids composed of a choline head group, a glycerol, and two FACs.

The number of carbons and double bonds presented in the FAC may differ. Phosphatidylcholine (16:0/18:2), for example, can possess one FAC of 16 carbons and no double bonds (palmitate) or one FAC of 18 carbons and two double bonds (linoleate). If the exact number of carbons and double bonds in each FAC of a PC cannot be determined, but the total number is known, the PC is designated PC(38:2). Fatty acids may be involved in the formation of CAD through distinct pathophysiological mechanisms (Table 1).

In the clinical setting, levels of cholesterol biomarkers such as TC, LDL-C, and high-density lipoprotein cholesterol (HDL-C) are measured to evaluate an individual's risk for CAD. In addition, cholesterol and apolipoprotein ratios are assessed, with ratio of apolipoprotein B to apolipoprotein A1 suggested to be the best indicator of myocardial infarction risk (41).

Meta-analyses have reported triacylglycerol (TG) levels to be an independent predictor for CAD, findings that are supported by research indicating that individual genetic variation has an impact on serum TG levels (42–44). Clinical measurement of TG levels is expressed as the sum of several TG types with no evidence for the existence of 'bad' and 'good' subtypes as is seen in cholesterol. Plasma lipid measurements (TG, TC, HDL-C, LDL-C) provide only a narrow snapshot of lipid metabolism. Recent advances in MS allow investigation of low-abundance lipids in the micro- and nano-molar range. Establishment of plasma or serum lipidome via lipidomics is of relevance to identification of diagnostic biomarkers and potential generation of hypotheses with respect to mechanisms specific to CAC. Hence, in Study II, we investigated the association of serum lipidome with CAC.

Psychosocial risk factors

The European Society of Cardiology recommends the assessment of socioeconomic status (SES) and social support for use as risk modifiers for potential reclassification of individuals with an estimated intermediate CVD risk into a higher or lower risk group (2). Socioeconomic status can, for example, be determined by an individual's education level, income, assets, possessions, type of residence and occupation (individual-level SES), and by area of residence (area-level SES) (45). Social support may be defined as the number of people in an individual´s social network, frequency of contacts, perceived tangible support, and emotional support. The link of SES or social support with risk of subclinical atherosclerosis and, eventually, clinical events such as acute coronary syndrome and cardiovascular mortality is not well understood. Biological (neuroendocrine, immune, coagulation), environmental (pollution, access to and use of health care, recreational facilities) and behaviour (lifestyle choices, adherence to prescribed medications/lifestyle recommendations) mechanisms are likely to be involved. In Studies III and IV, we used data from the pilot SCAPIS study conducted in Gothenburg in 2012, to investigate the association of psychosocial risk factors with CAC.

Table 1. Lipid classes and their potential contribution to coronary artery disease pathophysiology

Lipid classes	Lipid subclasses	Subtypes	Biological effects
Fatty acids	Fatty acids	n-3 PUFA (EPA[20:5], DHA[22:6], LNA[18:3])	↓ Arrhythmias, ↓ Thrombosis and plaque growth, ↓TGs, ↑ Endothelial function and ↓ inflammation (46, 47)
	Eicosanoids	TxA_2	↑ PLT aggregation, vasoconstriction and inflammation (48)
		PGI_2	↓ PLT aggregation and vasodilation (49)
		PGD_2	↓ PLT aggregation and vasodilation (50)
		PGE_2	↑PLT aggregation and vasodilation (51, 52)
		LTC_4, LTD_4, LTE_4	↑ Coronary vascular resistance and myocardial oxygen extraction, coronary vasospasm and ↑ inflammation (53-55)
		Lipoxins, resolvins, protectins	↓ Inflammation (56)
Glycerolipids	DG TG		↑ Insulin resistance (57) ↑ Dysfunctional HDL and small, dense LDL (58)
Glycerophospholipids	PC PE PS PI		↑ Atherosclerosis (59) ↑ Thrombosis (60) ↑ Thrombosis (61) ↑ Inflammation and atherosclerosis (62)
Sphingolipids	Ceramides Sphingomyelins		↑ ROS, ↑inflammation (63, 64) ↑ Subendothelial LDL aggregation (65, 66)
Sterol lipids	Cholesterol Cholesteryl esters Steroids Bile acids/salts		↑ Inflammation and atherosclerosis (67) ↑ Plaque rupture and Thrombosis (68) ↑ Atherosclerosis (69) ↑ Lipid and energy metabolism (70)

DG = diacylglycerol; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; HDL = high-density lipoprotein; LDL = Low-density lipoprotein; LNA = α-linolenic acid; LTC4 = leukotriene C4; LTD4 = leukotriene D4; LTE4 = leukotriene E4; n-3 PUFA = Omega 3 polyunsaturated fatty acids; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PGD2 = prostaglandin D2; PGE2 = prostaglandin E2; PGI2 = prostaglandin I2; PI = phosphatidylinositol; PLT = platelet; PS = phosphatidylserine; ROS = reactive oxygen species; TG = triacylglycerol; TxA2 = thromboxane A2.

Role of diet in cardiovascular disease

It has been estimated that approximately 11 million premature deaths annually could be prevented by a global shift to a diet characterized by increased intake of plant-based foods and a reduction in consumption of red and processed meat, along with restricted caloric intake (71). Coronary artery disease was reported to be virtually non-existent in a population with dietary habits characterized by low saturated fat and cholesterol intake and plasma TC levels of <3.6 mmol/l (72). In the prospective epidemiological Cornell China Study, nutritional intake, cardiometabolic risk factors, and outcomes were compared in 6500 adults recruited from rural mainland China to those of adults 20–74 years of age in the US (73). In rural China, the total energy intake of fat was 14% compared to 36% in the US, and the total energy intake of animal protein was 1% compared to 10% in US. The total daily fibre intake was 33 grams (11 grams in US), and carbohydrate intake was 71% of the total energy intake (42% in US). Mean serum TC levels were 3.3 mmol/L in rural China compared to 5.2 mmol/L in the US. The age-standardized CAD mortality rate in rural China during the study period of 1973–75 was 3.4/100 000 in women and 4.0/100 000 in men, compared to 18.9/100 000 in women and 66.8/100 000 in men in the US (73). The mortality rate in rural China was inversely associated with consumption of green vegetables and positively associated with plasma apolipoprotein B, which, in turn, was positively associated with animal protein intake and frequency of meat intake.

Recent data of the China Health and Family Planning Commission's Statistical Yearbook shows that CAD mortality has increased in rural areas of China from 28/100 000 in 2002 to 105/100 000 in 2014 (74), a trend possibly explained by changes in dietary habits along with other lifestyle changes. In the past two decades, there has been reduced energy intake from carbohydrates, increased intake of fats and cholesterol, and a decreased intake of fruits and vegetables in China. In 2010, average TC levels in adults were about 4 mmol/L in China, and there was a significant increase of mean TC of approximately 8% from 2003 to 2013 (74).

In a randomized controlled multicentre trial in Spain, 7447 subjects at high CVD risk, defined as the presence of diabetes mellitus or at least three CVD risk factors, were randomly assigned to a Mediterranean diet enriched with extra-virgin olive oil, a Mediterranean diet enriched with mixed nuts, or a control diet with advice to reduce fat consumption (75). After a median follow-up of 4.8 years, the primary end point of any cardiovascular event, defined as myocardial infarction, stroke, or death from cardiovascular

cause, occurred in 3.8%, 3.4%, and 4.8% of subjects allocated to Mediterranean diet with olive oil, Mediterranean diet with nuts, and the control group, respectively (75). Following adjustment for baseline characteristics, subjects allocated to Mediterranean diet with olive oil or with nuts experienced 31% and 28% lower incidence of any cardiovascular event, respectively, compared to the control group (75).

Prospective studies have reported association of a vegetarian diet with a 25% reduction in the incidence of, and mortality related to, CAD and a 41% reduction in all-cause mortality (76, 77). The precise mechanisms by which a meat-free diet may have positive effects on the cardiovascular system and reduce mortality have not been entirely clarified. Randomized controlled dietary trials have shown that a plant-based diet affects oxidised LDL-C, lipid profile, body mass index, inflammatory markers, blood pressure, insulin sensitivity, HbA1c, and fasting glucose levels (78–81). In the Lifestyle Heart Trial, a plant-based diet as part of an intensive lifestyle change in patients with angiographically verified CAD was reported to reverse CAD. Forty-eight patients with moderate to severe CAD not receiving lipid-lowering drugs were randomized to a lifestyle consisting of a low-fat vegetarian diet, aerobic exercise, stress management, smoking cessation, and psychosocial support or to usual care (82). After one year, mean baseline LDL-C level (3.72 mmol/L) was reduced by 1.48 mmol/L, and the frequency of angina episodes was reduced by 91% in the intervention group. The mean percentage diameter stenosis at baseline was decreased by 3.1% in the intervention group after 5 years. In the control group, the percentage diameter stenosis increased by 11.8% in the same time period (83). Data from a small prospective study of 22 participants with angiographically verified severe CAD and a follow-up of 12 years showed similar findings (84). Long-term observational studies comparing vegetarian and meat-containing diets have not been standardized with respect to diet components, leading to diverse findings (85–87). Non-vegetarian participants may consume different quantities of red meat, white meat, fish, eggs, and dairy products, leading to bias (85). In observational studies comparing long-term vegetarians to long-term omnivores, findings may have been influenced by elements other than the diet *per se*, such as total caloric intake, adherence to diet, and differences in traditional risk factors (85, 87).

There is a scarcity of well-designed controlled studies evaluating the impacts of a vegetarian diet on patients with history of CAD who are receiving standard medical treatment. In Study V, we conducted an

interventional trial (VERDI) to evaluate short-term changes in oxidised LDL-C, plasma metabolome including TMAO, choline, L-carnitine, and acetyl-carnitine, as well as the gut microbiota, faecal short- and branched-chain fatty acids and traditional risk factors in subjects with documented CAD. We also present supplementary data obtained from the VERDI trial with respect to effects of a vegetarian diet compared to a meat diet on the plasma lipidome and the association of plasma lipidome with the atherosclerotic burden in CAD patients.

Coronary Artery Calcium

Definition

Coronary artery calcium is defined as calcium deposited in the coronary artery wall (88). In asymptomatic individuals without a history of CVD, CAC can be used as a proxy for subclinical CAD. In 2007, an Expert Consensus Document of the American College of Cardiology created a CAC score (CACS) system based on the CAC relationship with CAD outcomes in observational studies: no calcification (0), mild calcification (1–100), moderate calcification (101–400), severe calcification (401–1000), and extensive calcification (>1000) (88). Other authorities suggest CACS >300 as a cut-off to denote high-risk individuals (89). According to the European Guidelines, CACS ≥300 or ≥75th percentile for age, sex, and ethnicity indicates increased CVD risk (2).

The risk for a coronary event has been demonstrated to increase with higher CACS. For example, the hazard ratio for a coronary event during a median follow-up period of 3.8 years was reported to be 3.6, 7.7, and 9.7 in subjects with CACS =1–100, CACS =101–300, and CACS >300, respectively, compared to those with CACS=0 (89).

Quantification

Computed tomography is the most commonly used and the most sensitive method of detection, localization, and quantification of CAC in the clinical setting, although CAC can be detected by other imaging modalities including chest X-ray, coronary angiography, echocardiography, and cardiac magnetic resonance imaging (90). Coronary artery calcium has traditionally been quantified by the Agatston method (91): An area larger than 1 mm^2 in a coronary artery with an attenuation of greater than 130 Hounsfield units (HU) represents a calcified lesion. The HU is a linear transformation obtained from the original attenuation coefficient

measurement. This results in a scale ranging from -1000 HU for air to ≈2000 HU for very dense bone (cochlea) at standard temperature and pressure. Each calcified lesion is given a density factor of 1, 2, 3, or 4, indicating HU attenuation of 130–199, 200–299, 300–399, or ≥400, respectively.

The density factor is multiplied by the CAC area in square millimetres to obtain the Agatston score. The tomographic slices of the heart are ≈2.5 mm thick, with approximately 50–60 slices generated during a scan. Ultimately the scores of the calcifications in each topographic slice are combined, providing a total CACS.

Mechanism

The precise mechanisms of CAC deposition are not fully understood. In the past, vascular calcification was thought to be an inevitable passive process of aging (90). More recently, it has been suggested to be an active process, similar to ectopic bone formation, regulated by osteoblast differentiation and mineralisation of vascular smooth muscle cells (92). This transformation may be caused by downregulation or upregulation of transcription factors (93). Oxidized LDL-C, hyperlipidaemia, palmitic acid [16:0], glucose, and inflammatory cytokines are likely to induce vascular calcification (94–96).

Population-based bioimage studies

Major population-based prospective bioimage studies involving CAC scoring are summarized in Table 2. In Sweden, the SCAPIS study enrolled 30 000 participants aged 50–64 years from a random population in six university hospitals from 2014 through 2018 (97).

Risk factors for, and prevalence of, coronary artery calcium

Reported risk factors for CAC in individuals without CAD history are similar to those for atherosclerotic CAD and include advanced age, male sex, white race, higher body mass index, unfavourable lipid and glucose levels, family history of CAD, high fibrinogen and c-reactive protein levels, and low creatinine (98). In the Multi-ethnic Study of Atherosclerosis (MESA) study, the prevalence of CACS >0 in 55–64 year-old healthy participants of all included ethnicities was 45% (99), while another study showed the corresponding value in indigenous South American Tsimane to be only 17% (100).

The 75th percentile CACS in a study of 55–64 year-old healthy white, African American, Hispanic, and Chinese men residing in the US was 155, 40, 75, and 67, respectively. Corresponding CACSs for women were 16, 5, 2, and 18 (99). In a population-based study of German subjects aged 45–74 years, the Heinz Nixdorf Recall (HNR) study, CACS >0 was calculated in 82% of men and 55% of women (101).

The incidence of CACS >0 increases with the number of modifiable and/or traditional CVD risk factors; however, data from MESA showed 12% of subjects with CACS >100 to exhibit no traditional CVD risk factors (102). Individuals with a CACS >300 and no traditional CVD risk factors showed a CAD event rate 3.5 times that of subjects with CACS=0 and three or more traditional risk factors (102).

Progression of CAC

Absence of coronary calcification at a single examination does not preclude its appearance later in life. In middle-aged patients undergoing annual CT scanning, a conversion from CACS=0 to CACS >0 was reported in approximately 25% during a five year follow-up period (103). In MESA, progression of CAC was associated with increasing risk of CAD events and all-cause mortality (104, 105).

Association of CAC with CAD and all-cause mortality

The association between CAC and obstructive CAD was investigated in 1851 symptomatic CAD patients who underwent coronary angiography and electron-beam CT (106). The authors reported cross-sectional sensitivity of 95% and specificity of 66% for CAC in identifying obstructive CAD. Symptomatic patients with no CAC exhibited less than 1% likelihood of significant coronary stenosis (107).

The Swedish National Board of Health and Welfare does not recommend use of CACS in patients presenting with acute-onset chest pain in the emergency setting to infer the presence of obstructive CAD (108).

Table 2. Summary of major population-based bioimage studies of coronary artery calcium

Studies	MESA	HNR	RCCS	DHS	CARDIA	SCAPIS
Study period	2000–2002	2000–2003	1997–2000	2002–2004	2000–2001	2014–2018 (pilot 2012)
Age group	45-84	45-74	55-85	30-65	33-45	50-64
Sample size (women %)	6814 (53)	4275 (53)	2013 (54)	2971 (49)	2831 (53)	30 000 (50) (pilot 1111)
Exclusion criteria	History of CVD, pregnancy, treated cancer, weight >300 pounds, cognitive inability, language barrier	Inability to give informed consent, conditions precluding follow-up over 5 years, severe psychiatric disorder and pregnancy	Not stated	Subjects ineligible, incapable, with language barrier or incarcerated were excluded	Not stated	None
Population	Stratified for ethnicity, sample from six US communities	Random sample from a German population	Sample from the Rotterdam Elderly study random sample from a Netherlands population	Stratified for ethnicity, random sample by postal address in Dallas County, Texas, US	Randomly recruited from four US urban areas in Alabama, Illinois, Minnesota, and California	Random sample of a Swedish population

CAC = coronary artery calcification; CARDIA = Coronary Artery Risk Development in Young Adults; CVD = cardiovascular disease; DHS = Dallas Heart Study; HNR = Heinz Nixdorf Recall Study; MESA = Multi-Ethnic Study of Atherosclerosis; RCCS = Rotterdam Coronary Calcification Study; SCAPIS = Swedish CArdioPulmonary bioImage Study.

HYPOTHESES AND AIMS

The overall hypothesis driving the research reported in this thesis is that novel and traditional risk factors are associated with subclinical CAD, as reflected in CAC, and that dietary intervention might modify these associations in patients with CAD. The principal aims, therefore, were to determine associations of novel and traditional risk factors with subclinical CAD and to study the effects of a vegetarian compared to a conventional meat-containing diet on novel and traditional risk factors in patients with history of CAD. We addressed this in five studies:

I. To evaluate the replication of lipidomics studies and reproducibility of previous lipidomic findings in patients without history of coronary artery disease events but with evidence of coronary artery calcium.

II. To identify potential mechanisms and novel diagnostic lipid biomarkers of coronary artery calcium deposition in patients without history of coronary artery disease.

III. To determine association of socioeconomic status and traditional risk factors with coronary artery calcium in a random population-based sample free from history of coronary artery disease.

IV. To investigate the relationship of social support with coronary artery calcium in a random population-based sample free from history of coronary artery disease.

V. To compare effects of a vegetarian diet to those of an isocaloric conventional meat-containing diet by assessing differences in oxidised LDL-C, plasma metabolome including trimethylamine N-oxide, choline, L-carnitine, and acetyl-carnitine as well as the gut microbiota, faecal short- and branched-chain fatty acids, and traditional risk factors in patients with a history of coronary artery disease.

   In addition, we present supplementary data evaluating the effects of a vegetarian compared to a meat-containing diet on plasma lipidome and the association of plasma lipidome with atherosclerotic burden in patients with coronary artery disease.

METHODS

Ethics

All observational studies (I–IV) included in this thesis were approved by the ethics committee of Umeå University, Sweden (2010-228-31M), (2012-307-32M), (08-11M) and the regional radiation safety authority research committees involved. The Regional Ethical Committee, Uppsala, Sweden approved Study V (2016/456), which is registered at ClinicalTrials.gov (NCT02942628).

All studies were carried out in compliance with the Helsinki declaration. All participants gave written informed consent.

Overview of studies and methodology

A summary of methods used in Studies I–V is presented in Table 3. Studies I and II were based on a cohort of seventy patients from the University Hospital of Umeå with exertional angina but no evidence of obstructive CAD revealed on coronary angiography. Studies III and IV were based on data obtained in the pilot SCAPIS study performed at the Sahlgrenska University hospital in Gothenburg with a random sample of 1111 men and women from Gothenburg.

Study V was a randomized controlled trial of thirty-one subjects recruited at the University Hospital, Örebro.

Table 3. Methods employed in the presented research

Studies	Study I	Study II	Study III	Study IV	Study V
Study Design	Prospective cohort (observational studies)		Cross-sectional study (observational studies)		RCT (interventional study)
Study year	2009-2011		2012		2017-2018
Sample size (women %)	70(57)		1067(50)		31(6)
Study age groups	43-83		50-64		45-81
Inclusion criteria	Myocardial ischemia on a cardiac stress test and absence of obstructive CAD		50–64 years of age and residing in a preselected high- or low-SES residential area		>18 years of age, stable CAD, PCI >1 month prior to study inclusion and on SMT
Exclusion criteria	Prior MI, PCI, or CABG, significant valvular disease, chronic heart or renal failure		Inability to understand, speak, or write Swedish		Inability to provide informed consent, already following a vegetarian or a vegan diet, vitamin B deficiency, food allergy, CABG, life expectancy <1 year
Exposures and predictors (method)	Serum lipidome (LC-MS)		SES, FH, obesity, traditional risk factors	Social support (ISSI)	4-week intervention with isocaloric VD and MD
Response variables and outcomes (method)	CAC (Cardiac CT)		CAC (Cardiac CT)		Oxidised LDL (ELISA), traditional risk factors, plasma metabolome and TMAO (LC-MS), gut microbiota (16S rRNA gene sequencing), faecal SCFA and BCFA (GC-MS)
Statistical analysis	PCA, OPLS-DA, unpaired t-tests	PCA, OPLS-DA, unpaired t-tests, FDR correction	Logistic regression	Logistic regression, random forest	Linear mixed model, random forest, FDR correction

BCFA = branched chain fatty acids; CABG = coronary artery bypass grafting; CAC = coronary artery calcium; CAD = coronary artery disease; CT = computed tomography; CVD = cardiovascular disease; ELISA = enzyme-linked immunosorbent assay; FDR = false discovery rate; FH = family history of premature CVD; GC-MS = gas chromatography-mass spectrometry; ISSI = interview Schedule for Social Interaction questionnaires; LC-MS = liquid chromatography-mass spectrometry; LDL = low-density lipoprotein; MD = meat diet; MI = myocardial infarction; OPLS-DA = orthogonal partial least squares – discriminant analysis; PCA = principal components analysis; PCI = percutaneous coronary intervention; RCT = randomized controlled trial; SCFA = short-chain fatty acids; SES = area- and individual-level socioeconomics; SMT = standard medical therapy; TMAO = trimethylamine N-oxide; VD = vegetarian diet.

Study populations and study design

Studies I and II

Seventy patients presenting to the Umeå Heart Centre with chest pain from 2009 through 2011 were prospectively included.

Inclusion criteria:

1) Evidence of myocardial ischemia on cycle exercise stress electrocardiogram
2) Absence of significant obstructive CAD (>50 % stenosis) demonstrated by coronary angiography

Exclusion criteria:

1) History of prior myocardial infarction, PCI, or coronary artery bypass grafting
2) Significant valvular heart disease
3) Chronic left heart failure (left ventricular ejection fraction <45%)
4) Chronic renal failure (creatinine >130 mmol/L)

Approximately 12 months following cardiac stress testing and coronary angiography, patients were invited to participate in the study. Enrolled patients underwent cardiac CT, stress echocardiography, and venous blood sampling. All patients completed questionnaires concerning lifestyle, medical history, and current medications.

Study III and IV

Cross-sectional data from the 2012 pilot SCAPIS study were analysed and used in Studies III and IV. The pilot study included 1111 men and women aged 50–64 years recruited in the city of Gothenburg, Sweden.

Inclusion criteria:

1) Age 50–64 years
2) Residing in pre-selected high- or low-SES residential areas

Exclusion criterion:

1) Inability to understand, speak, or write Swedish

Participants were recruited using Swedish personal identification numbers from three low-SES and three high-SES areas. The areas were selected based on official statistics of average income and education level (97). A recent study from Denmark reported that persons living in low-SES areas were significantly less likely to participate in population-based cohort studies, compared with those living in less deprived areas (109). Therefore, to obtain a balanced number of participants from high- and low-SES areas, a greater number of individuals from low-SES areas were invited (n=2243).

The overall participation rate was 49.5% (39.9% and 67.8% in low- and high-SES areas, respectively). Participants provided detailed information relating to health; lifestyle habits such as smoking status, exercise activity, and alcohol consumption; family history of stroke and myocardial infarction; medication; occupational and environmental exposures; psychosocial well-being; and education.

Study V

Study V (VERDI) was a crossover randomized clinical trial of 31 patients diagnosed with CAD, treated with PCI, and receiving guideline-recommended medical therapy. Participants were assigned to four weeks of a vegetarian diet (VD) alternating with four weeks of a conventional diet including meat (MD), separated by a washout period of 4 weeks. Recruitment took place at the outpatient clinic at the Department of Cardiology, Örebro University Hospital, from September 2017 to March 2018. The study period was completed in June 2018.

Inclusion criteria:

1) >18 years of age
2) Stable CAD
3) PCI more than 30 days prior to the study
4) Treated with standard medical therapy including aspirin and cholesterol lowering drugs

Exclusion criteria:

1) Age <18 years
2) Unstable CAD
3) PCI treatment during the preceding 30 days
4) Inability to provide informed consent
5) Already following a vegetarian or a vegan diet
6) History of vitamin B12 deficiency
7) History of food allergy
8) History of gastric bypass surgery
9) Life expectancy <1 year

All participants were randomly allocated to one of the two intervention sequences (VD-washout-MD or MD-washout-VD) at a 1:1 ratio.

Exposures and predictors

Studies I and II

The exposure of Studies I and II was serum lipidome detected by LC-MS.

Studies III and IV

The primary exposure variable in Study III, area-level SES, was participant residential area (high vs. low). Individual-level SES was evaluated by responses to a questionnaire and measured as level of education and current housing (own house or apartment vs. rental apartment).

In Study IV, a comprehensive questionnaire based on the condensed version of the Interview Schedule for Social Interaction (ISSI), a psychiatric interview questionnaire, was used as a measure of social support (110). The condensed version has been validated and shown to be reliable (110) and, across multiple studies, has been demonstrated to predict cardiovascular outcomes (111–115). The questionnaire yields two subscales, *Social integration* and *Emotional attachment*. Social integration refers to the availability and function of peripheral contacts as assessed by quantitative measures of the social network, including a sense of belonging and accessibility of practical help and support. Emotional attachment describes the availability of emotional support through close family and friends.

Family history of premature CVD was self-reported and defined as having a mother or father with myocardial infarction or stroke before the age of 65 and 55 years, respectively. Obesity was defined as body mass index of >30 kg/m^2.

Blood pressure was measured with an automated electronic sphygmomanometer (Omron M10-IT, Omron Health care Co, Kyoto, Japan) twice in each arm, and the mean was calculated for each arm. The highest mean blood pressure of the two arms was used. Hypertension was defined as blood pressure >140/90 mmHg, self-reported history of hypertension, or use of antihypertensive drugs. Diabetes mellitus was defined as self-reported history of diabetes, a HbA1c level of ≥48 mmol/mol, or a plasma glucose level >7 mmol/L. Dyslipidaemia was defined as self-reported history of hyperlipidaemia or use of statin therapy. Smoking status was classified as non-smoking, current smoking, or former/occasional smoking.

Study V

Participants were provided with ready-made frozen lunches and dinners (Dafgård, Källby, Sweden) based on Swedish recipes. The VD was a lacto-ovo-vegetarian diet allowing consumption of egg and dairy products. We based the MD on the average meat consumption per person in Sweden, corresponding to a daily intake of 145 grams (116). At the first study visit, a research dietitian provided information on strictly following the meal plans, including self-provided side dishes, breakfasts, and two snacks per day in addition to the provided main meals. The meal plan listed five to six options to choose from for breakfast, snacks, and as side dishes to the main courses. During the interventions, all subjects were asked to complete a daily food diary, recording whether they had eaten the main dishes and which options they had chosen for breakfast and snacks, and noting any deviations from the diet plan.

The plan was adapted according to individual energy expenditure and was calorie and macronutrient balanced. Energy requirements for each subject were calculated by multiplying basal metabolic rate by physical activity level. Henry´s energy equation was used to calculate the basal metabolic rate and the physical activity level values according to Nordic nutrition recommendations based on physical activity reported by participants at the first study visit (117, 118). Adherence to the diet was assessed by three-day weighed-food records registered prior to each intervention period and during the least week of each intervention.

Response variables and outcomes

Studies I–IV

Cardiac CT scan was performed in Studies I and II using a 64-slice scanner (Light Speed VCT, XT; GE healthcare, Milwaukee, Wisconsin) and, in III and IV, with a dual-source scanner equipped with a Stellar Detector (Somatom Definition Flash, Siemens Medical Solution, Forchheim, Germany) (Figure 1). In Studies I–IV, an electrocardiogram-gated protocol was used, adapted to subject weight and heart rate. The Agatston method was used to determine the CACS, and participants were classified into groups based on total CACS.

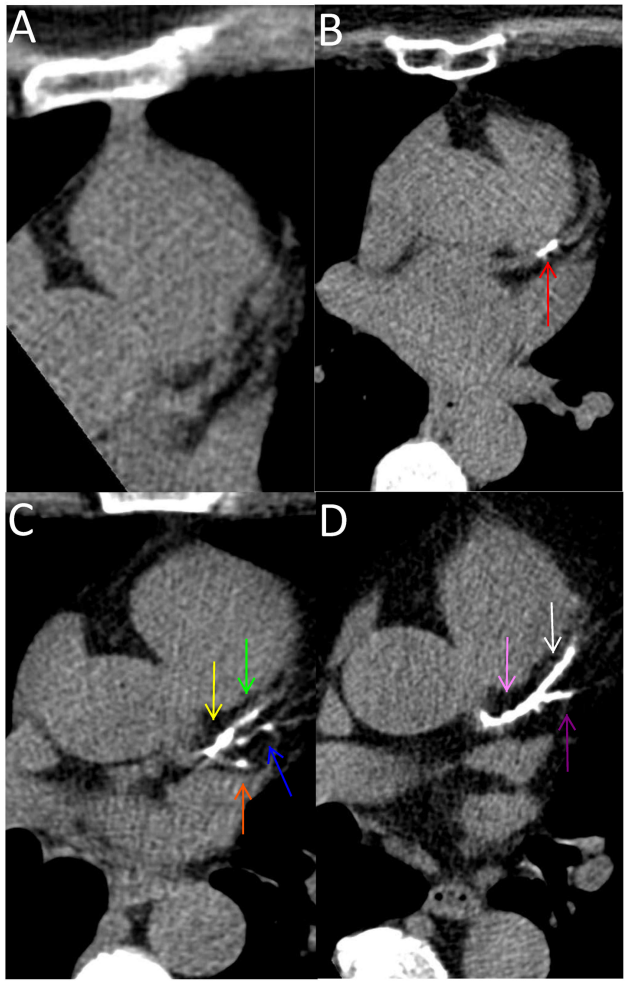

Figure 1. Coronary artery calcium imaged by cardiac computed tomography without contrast in a participant from the pilot SCAPIS study. Cross-sectional view at the level of left main coronary artery and its branches, the left anterior descending artery (LAD) and left circumflex artery (LcX). A: CACS=0; B: CACS =100, red arrow shows calcified plaque in LAD; C: CACS =250, moderate coronary artery calcium, arrows indicate calcified plaques along left main coronary artery and proximal LAD (yellow arrow), mid-LAD (green arrow), LcX (orange arrow), and diagonal artery (DA) (blue arrow); D: CACS =750, severe coronary artery calcium, calcification along the proximal LAD (pink arrow), mid-LAD (white arrow), and DA (purple arrow).

Study V

The primary outcome of Study V was differences between interventions in oxidized LDL-C levels. Secondary outcomes were differences of blood lipids, weight, body mass index, blood pressure, heart rate, HbA1c, quality of life, plasma metabolome including choline, TMAO, L-carnitine, and acetyl-carnitine, as well as the gut microbiota, and faecal short- and branched-chain fatty acids.

Supplementary data

Atherosclerotic burden of subjects in VERDI trial was determined by visual analysis of coronary angiograms performed at the time of hospitalization for coronary events (Figure 2). The coronary arterial circulation was divided into 15 segments according to the definition of the American Heart Association (119). The percent diameter stenosis, number of stenotic lesions, and longitudinal extent of atherosclerosis was estimated in each segment of the coronary circulation. The estimate was conducted before and after PCI, and the smallest branches (<1 millimetre) were excluded from analysis. The degree of stenosis was determined as the percentage of diameter stenosis in the most severely narrowed studied segment.

To determine the percentage of diameter stenosis, a non-narrowed section 5 millimetres proximal or distal to the stenotic lesion was used as reference. The number of significant lesions, defined as stenosis >50%, was calculated for each segment. A stenotic lesion twice the length of the normal lumen diameter was considered two lesions. A maximum number of three stenotic lesions was recorded in each segment. The total number of stenotic lesions was calculated as the sum of stenotic lesions in all segments. The Sullivan extent score was used to define the longitudinal extent of coronary atherosclerosis (120).

Coronary atherosclerosis was defined as irregularity of a vessel wall obstructing >20% of the total lumen and was estimated relative to the total length of the studied segment. A lesion totally occluding any segment was defined as at least 50% longitudinal extent. The lesion percentage of longitudinal extent in each segment was multiplied by a factor representing the surface area of the studied segment relative to the entire coronary circulation. The left main coronary artery accounted for 5%, the left anterior descending artery 35%, the left circumflex artery 30%, and the right coronary artery 30%, according to the Sullivan extent score definition. A Sullivan extent score can be a value from 0 to 100.

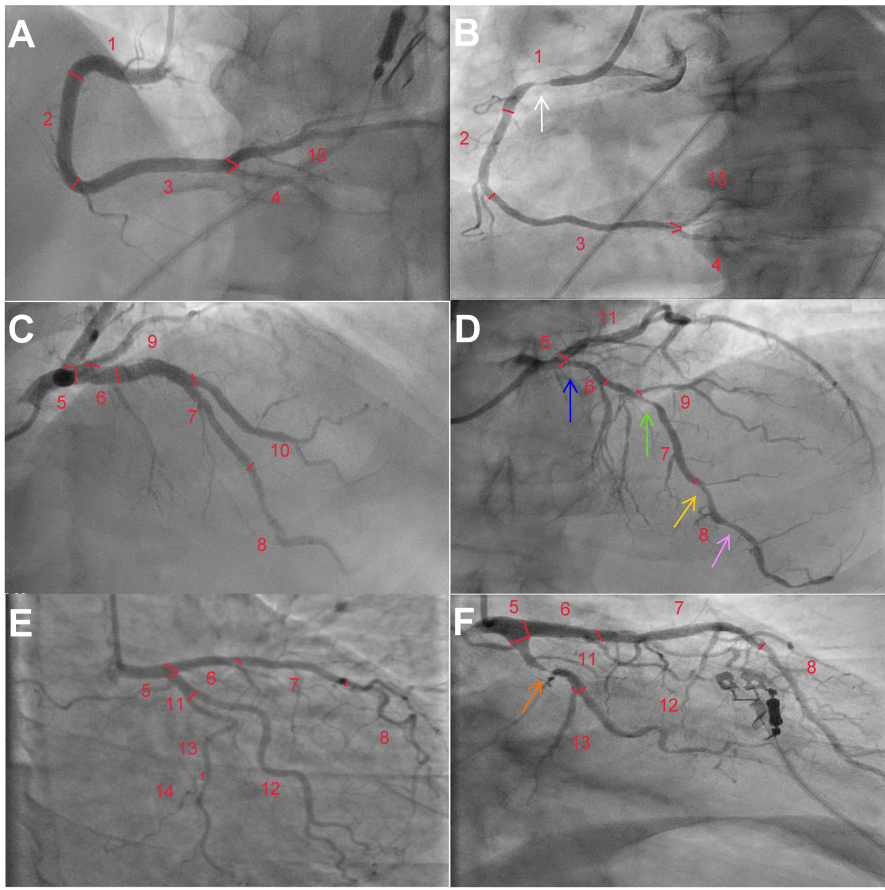

Figure 2. Coronary angiogram, VERDI participants (Study V). A, C, and E show 15 normal segments of the coronary circulation classified according to the AHA. B: significant obstructive coronary artery disease with stenosis ≈90% (white arrow) in segment 1; the longitudinal extent of atherosclerosis was ≈70%. D: Arrows from right to left show ≈40% (blue), ≈60% (green), ≈60% (yellow), and ≈40% (pink) stenosis in segments 6,7, 8, and 8, respectively. Longitudinal extent of atherosclerosis was ≈80% in segment 6, ≈90% in segment 7, and ≈80% in segment 8. F: ≈90% stenosis in segment 11 with estimated ≈50% longitudinal atherosclerosis. A: 1 = Proximal right coronary artery (RCA), 2 = middle RCA, 3 = distal RCA, 4 = right posterior descending artery, and 15 = left posterior descending artery; C: 5 = left main coronary artery, 6 = proximal left anterior descending artery (LAD), 7 = middle LAD, 8 = distal LAD, 9–10 = first and diagonal branches of LAD; E: 11 = proximal left circumflex (LCx), 12 = obtuse marginal branch, 13 = distal LCx, 14 = posterolateral branch.

LABORATORY METHODS

Studies I and II

Venous blood samples were drawn from patients at the time of recruitment and held at 20 °C to clot before serum was separated by centrifugation. Serum samples were stored at -80°C at the Academic Clinical Physiology Department, Umeå until biochemical analysis. Two samples from each patient were used for comparative lipidomics analysis, one of which was analysed at Imperial College in London, and the second at the Swedish Metabolomics Centre in Umeå, Sweden at a later date.

Aliquots of the samples were prepared by mixing 20 μL serum and 110 μL organic solvent (2:1 v/v chloroform:methanol) in an Eppendorf tube. The sample was shaken at 30 Hz for 2 min, stored at 20 °C for 30–60 minutes, and centrifuged at 11 000 x g at 4°C for 3 min. A 50 μL sample of the organic phase was transferred to a microvial and 70 μL internal standard was added. Quality control samples were prepared by pooling 10 μL of each extracted sample and stored at -80°C until analysis.

For sample analysis, we used untargeted lipidomics, LC (separation method) coupled with MS (detection method). The samples were initially analysed with positive electrospray ionization polarity and subsequently re-analysed with negative polarity, using the same instrument. Chromatographic separation was performed on an Agilent 1290 Infinity UHPLC-system (Agilent Technologies, Waldbronn, Germany). A 1 μL sample of the extracted serum was injected into an Acquity UPLC system (Waters Corporation, Milford, MA, USA). The lipid compounds were detected using an Agilent 6550 Q-TOF mass spectrometer equipped with jet stream electrospray ionization (Agilent Technologies, Waldbronn, Germany).

Studies III and IV

Venous blood samples for biochemical analyses were collected from individuals after an overnight fast. Blood serum collected in EDTA tubes was used to measure levels of TG, TC, LDL-C, and HDL-C in mmol/L by enzymatic and calorimetric methods on a Cobas® 8000 modular analyser series instrument (Roche Diagnostics, New York, USA) according to the manufacturer´s recommendations.

Study V

Blood samples were obtained at clinic visits between 07.00 and 10.00 after overnight fasting. Participants were asked to collect a stool sample in a special sealed container on the day before each follow-up visit.

Venous blood samples were collected in evacuated plastic tubes. Samples were centrifuged in a cooling system at 1560 x g for 10 min at -40°C and stored at -80°C in aliquots for analysis at the Clinical Research Laboratory of Örebro University Hospital. Plasma-oxidized LDL-C concentrations were measured with a sandwich enzyme-linked immunosorbent assay kit (Mercodia, Uppsala, Sweden) using a specific murine monoclonal antibody, mAb-4E6, directed against the oxidized antigenic determinants on the oxidized ApoB molecule, as described by Holvoet et al. (121).

Concentrations of TC, LDL-C, HDL-C, TG, high-sensitivity C-reactive protein, apolipoprotein A1 and B, and HbA1c were measured according to standard methods.

Untargeted metabolomics analysis was performed by high-performance LC-quadrupole time-of-flight MS (Agilent Technologies). Reverse-phase chromatography was applied using an ACQUITY UPLC HSS T3 Column (130Å, 1.8 µm, 2.1×100 mm, Waters), with both positive and negative electrospray ionization modes.

Plasma TMAO, choline, L-carnitine, and acetyl-carnitine were analysed by high performance LC-MS on an Exion UHPLC coupled to a QTRAP 6500+ MS/MS system, both from AB Sciex LLC (Framingham, USA).

Gut microbiota was analysed by DNA extracted from stool samples by repeated bead-beating and subjected to 16S rRNA gene sequencing in an Illumina Miseq instrument (2 × 250 bp paired-end reads, V2 kit; Illumina, San Diego, California, USA) after PCR amplification of the V4 region with 515F and 806R primers.

The levels of short-chain fatty acids (SCFA) acetate, propionate and butyrate; branched-chain fatty acids (BCFA) isobutyrate and isovalerate; succinate; and lactate in faecal samples were analysed using gas chromatography-MS (Agilent Technologies).

Supplementary analysis

Untargeted lipidomics analysis was performed by ultra-high-performance LC-quadrupole time-of-flight MS (Agilent Technologies). Reverse-phase chromatography was applied using an ACQUITY UPLC® BEH C18 column (2.1 mm × 100 mm, particle size 1.7 µm) with the samples analysed in positive ionization mode.

STATISTICAL ANALYSIS

Categorical variables are shown as counts and percentages, and continuous variables are presented as mean ± standard deviation when normally distributed and as median and interquartile range in cases of non-normal distribution.

Studies I and II

Univariate unpaired t-tests at the 95% confidence level were performed assuming equal variance, and p-values were calculated.

Principal component analysis, an unsupervised method, was used to detect trends and outliers in Studies I and II (122). Orthogonal partial least squares (supervised method) discriminant analysis was used to correlate variation in the data with calcium score class (Studies I and II) (123). Validation of models in Studies I and II consisted of cross-validation analysis of variance and permutation tests. In Study II, the Benjamini-Hochberg procedure was used to correct for multiple testing, and a logistic regression model was used to test the models adjusted for potential cofounders (122).

In Study I, variables with orthogonal partial least squares-discriminant analysis correlation coefficients of >0.6 and a p-value of t-test <0.05 were considered statistically significant. In Study II, variables with a t-test p-value <0.05, false discovery rate p-value <0.10, and logistic regression model p <0.05 were considered significant. Multivariate analysis was performed in SIMCA 14 (Umetrics AB, Umeå, Sweden) and univariate analysis in MATLAB® (MathWorks®, Natick, USA).

Studies III and IV

Multiple logistic regression models were fitted to the data to assess statistical association between CAC and area-level SES (Study III) and social support (Study IV), independent of cardiovascular risk factors.

Model-estimated odds ratios and 95% confidence intervals are presented. We report the outcome (CAC) as binary in the analysis as opposed to a continuous outcome, due to skewness of the continuous variable.

In Study IV, a robust nonparametric machine-learning method (random forest) that optimizes predictive accuracy by fitting an ensemble of trees was used to stabilize model estimates and to identify the variables having the highest association with CAC (124). The analysis was conducted using R software, v.3.5.0. (R Foundation for Statistical Computing, Vienna, Austria).

Study V

Sample size calculation was based on previous findings in which a vegan diet reduced oxidized LDL-C by 10% as compared to no intervention (78). Considering similar effects in our study and a standard deviation of oxidized LDL-C of 13%, we needed to study 27 patients in a crossover design to allow rejection of the null hypothesis that the experimental and control treatments were identical with a probability (power) of 0.80 and a type I error probability of 0.05. Based on an estimated 10% dropout rate, we included 31 subjects.

The effect of dietary intervention was evaluated using a linear mixed model that included the fixed effects diet, sequence of allocation, and diet duration. Missing values were imputed in an intention-to-treat analysis using the last observation carried forward method for participants who were randomized but did not receive intervention (n=2) and for early dropouts (n=2). A two-sided p-value <0.05 was considered statistically significant.

A random forest model based on multilevel data was applied for pair-wise comparisons to identify differences in individual plasma metabolites and microbial taxa of VD and MD. This method has been successfully used to identify differences inherent in diet in cross-over intervention studies (125). Discriminative metabolites and microbial taxa in VD vs. MD selected by random forest model were subjected to a linear mixed modelling. The significance values were adjusted using the Benjamini-Hochberg false discovery rate, and a value of $p < 0.05$ was considered significant. The analysis was performed using R software, v.3.5.0. (R Foundation for Statistical Computing, Vienna, Austria).

Supplementary data

We applied the same statistical univariate methods for lipidomics data as in Study V for metabolomics data. In addition, plasma lipids were hierarchically clustered using weighted gene correlation network analysis. The resulting lipid modules were identified using the eigenvector of each module, designated the module eigenlipid, which is defined as the first principal component of the standardized expression profiles. Pearson correlation was used to quantify the relationship between module eigenlipids and CVD risk factors. The differences in module eigenlipids were determined using paired t-test.

RESULTS

Study I

Analyses at Imperial College London and the Swedish Metabolomics Centre revealed a number of lipids that discriminated serum samples of patients with CACS >250 from those with CACS=0. Some lipid biomarkers were identified molecularly, while this was not possible with others. Lipid identification was done independently at the centres.

Both centres identified six lipids that significantly differed in subjects with CACS >250 and CACS=0 (Figure 4), detecting higher levels of PC(16:0/20:4) and lower levels of PC(18:2/18:0), PC(18:2/18:2), SM(d18:1/16:0), SM(18:1/22:0), and SM(d18:1/23:0) in sera of patients with CACS >250 compared to CACS=0.

Study II

We identified 102 lipids of four main classes: glycerolipids, glycerophospholipids, sphingolipids, and sterols. After false discovery rate correction, four lipids significantly discriminated serum samples of patients with CACS >250 from those with CACS=0: Higher levels of PC(16:0/20:4) and lower levels of PC(18:2/18:2), PC(36:3) and phosphatidylethanolamine (PE)(20:0/18:2) were found in those with CACS >250 compared to CACS=0. Levels of individual TGs did not significantly differ with CACS category.

Study III

The prevalence of CACS >0 was 46.3% and 36.6% in low and high socioeconomic areas, respectively. In the age- and sex-adjusted logistic regression model, the odds ratios (95% CI) for CACS >0 and CACS >100 were 1.62 (1.17–1.92) and 2.48 (1.63–3.82), respectively, for participants living in low SES areas (reference: high SES area). In the multivariable model, after adjustment for CVD risk factors, the odds ratios for CACS >0 and CACS >100 were not significantly higher in participants from low SES areas.

Associations of CVD risk factors with CACS >0 in the total study population and in men and women are presented in Figures 3, 4, and 5, respectively. In the total study population, both novel and traditional risk factors were independent predictors of CACS >0 (Figure 5). In men, the traditional CVD risk factors age, current smoking, diabetes mellitus, hypertension, and dyslipidaemia were independently associated with CACS >0 (Figure 6). Family history of premature CVD was associated with CACS >0 in women but not in men (Figures 6 and 7). The traditional risk factors age, current smoking, dyslipidaemia, and LDL-C were independently associated with CACS >0 in women.

In the CVD risk-factor-adjusted logistic regression models, the odds ratios for CACS >100 were 4.80 (CI 1.08–26.31) and 4.13 (CI 1.13–13.82) in women who had completed primary school/compulsory education vs. a university degree and in women living in a rented apartment vs. owning a house, respectively. In men, there was no significant association between any SES measure and CACS >0 or CACS >100, after adjustment for CVD risk factors.

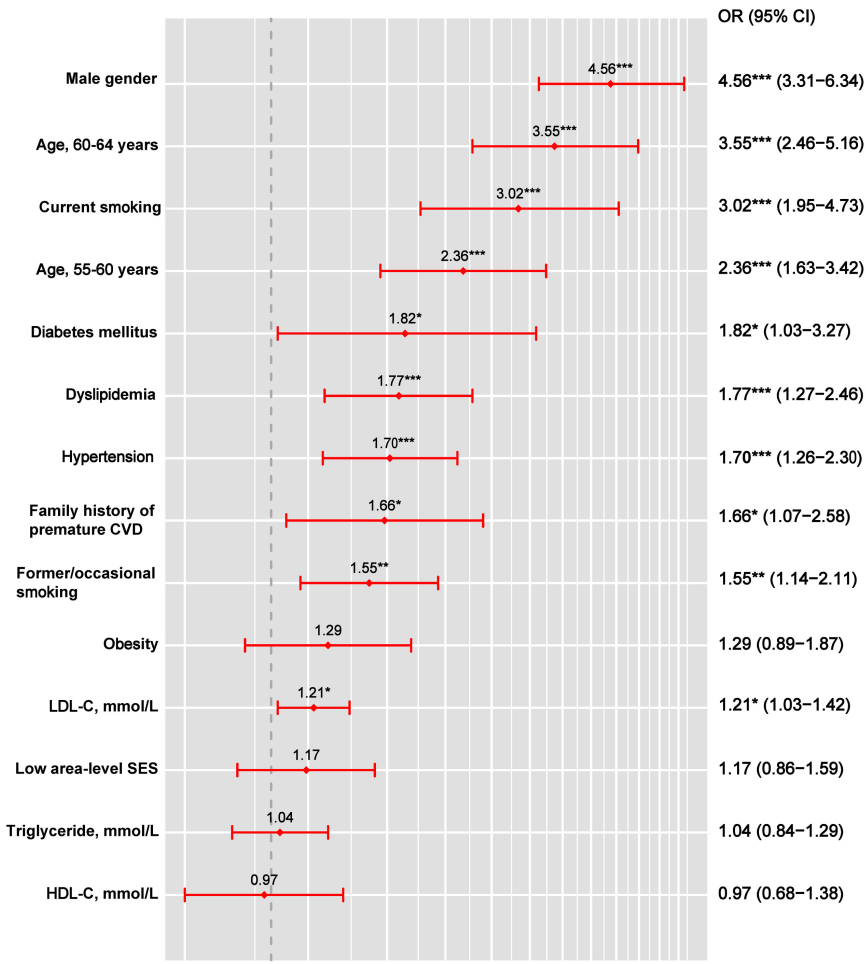

Figure 3. Forest plot showing association of CACS >0 with CVD risk factors in total SCAPIS pilot study population. CI = confidence interval; CVD = cardiovascular disease; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; OR = odds ratio; SES = socioeconomic status. *p <0.05, **p <0.01, ***p <0.001.

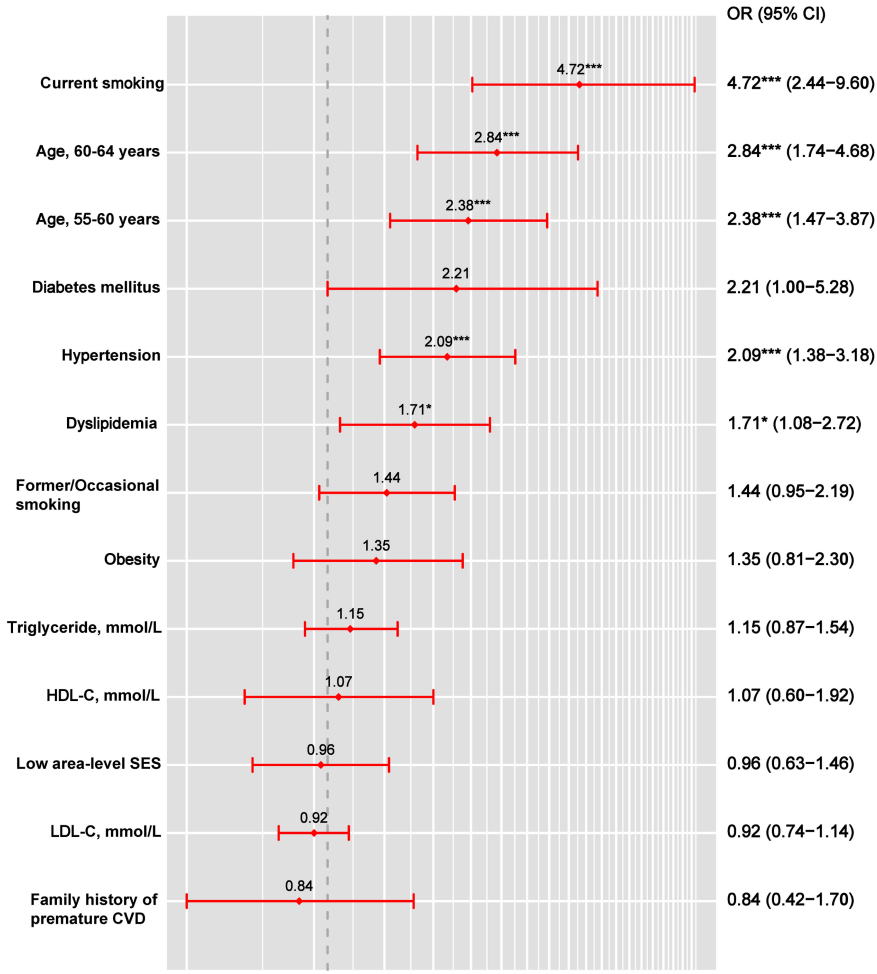

Figure 4. Forest plot showing association of CACS >0 with CVD risk factors in men, pilot SCAPIS study. CI = confidence interval CVD = cardiovascular disease; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; OR = odds ratio; SES = socioeconomic status. *p <0.05, **p <0.01, ***p <0.001.

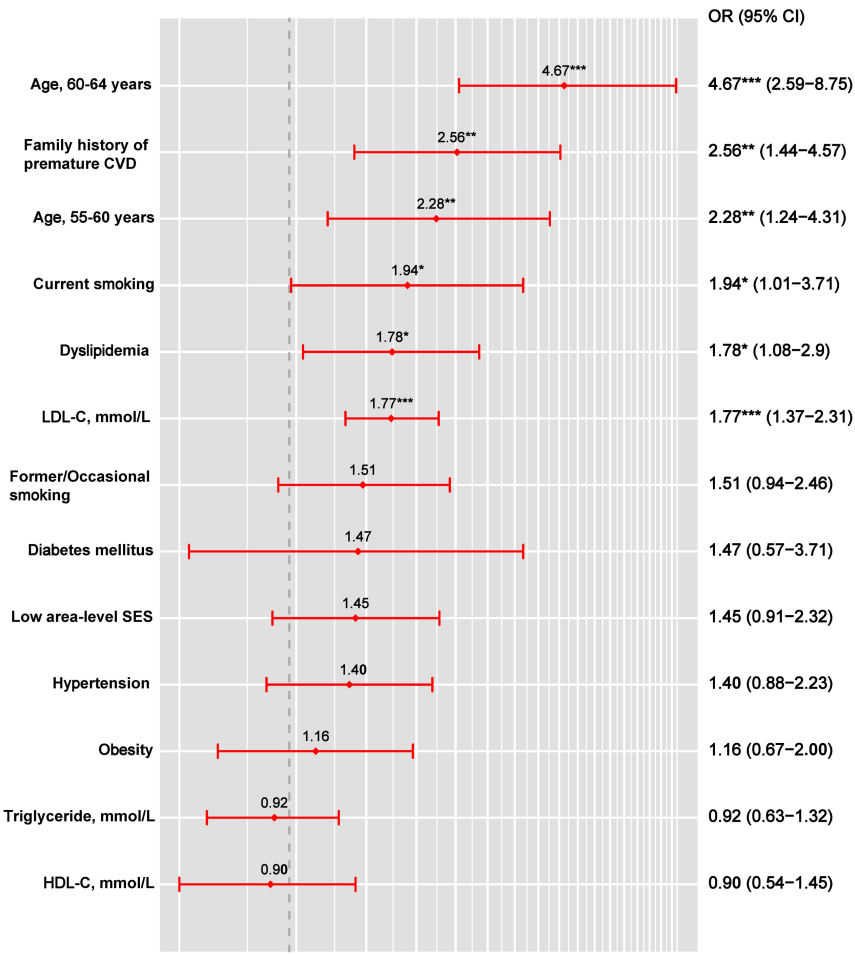

Figure 5. Forest plot showing the association of CACS >0 with CVD risk factors in women, pilot SCAPIS study. CI = confidence interval CVD = cardiovascular disease; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; OR = odds ratio; SES = socioeconomic status. *p <0.05, **p <0.01, ***p <0.001.

Study IV

In men, no measure of social support was significantly associated with prevalence of CACS.

In the CVD risk-factor-adjusted logistic regression models of the total study population, the odds ratios for CACS >0 and CACS >100 were not significantly higher among participants in the lowest category of social integration, emotional attachment, and social support (reference: highest group). In women, after adjusting for traditional risk factors, the odds ratios (95%CI) for CACS >0 were 2.47 (1.23–5.12), 1.87 (0.93–3.59) and 4.28 (1.52–12.28) for subjects in the lowest categories of social integration, emotional attachment, and social support, respectively. With the machine learning analysis (random forest), social integration was the fourth most important predictor of CACS >0 of 12 cardiovascular risk factors assessed in women.

We illustrated the complex interaction of social integration with the most important traditional CVD risk factors in females (Figure 6). Independent of age, LDL-C, and systolic blood pressure, the probability of CACS >0 was higher in women with a social integration score <10. In addition, women aged 50 years with lower social integration levels had approximately the same prevalence of CACS >0 as 64-year-old women with moderate or high social integration levels.

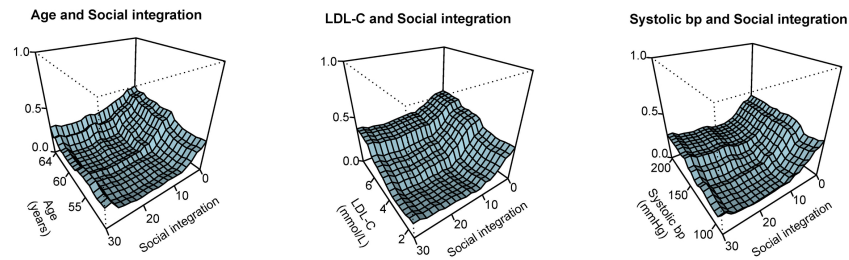

Figure 6. Three-dimensional partial dependence plots generated from the machine learning models in women. We illustrate the most important interactions among social integration and traditional CVD risk factors (X1- and X2-axis) and their impact on CACS >0 (Y-axis) adjusted for 12 cardiovascular risk factors in the random forest models. bp = blood pressure; LDL-C = low-density lipoprotein cholesterol.

Study V

Of 150 individuals eligible for the study, 31 agreed to participate [29 men (93.5%), median age 67 years], and 27 (87%) completed the study. Two dropped out before completion of the first intervention and two before completion of the second intervention.

Of the 31 original participants, five (16%) were diagnosed with stable angina pectoris, three (10%) had unstable angina, twelve (39%) ST elevation myocardial infarction, and twelve (39%) non-ST elevation myocardial infarction. All participants were receiving statin therapy, 29 (94%) were treated with aspirin, and 20 (65%) were on P2Y12 inhibitors.

Significantly lower levels of oxidized LDL-C (-2.73 U/L; p=0.015) were recorded following the VD than after the MD. After the VD, compared with after the MD period, subjects exhibited lower mean levels of TC (-0.13 mmol/L; p=0.01), LDL-C (-0.10 mmol/L; p=0.02), body weight (-0.67 kg, p=0.008), and body mass index (-0.21 kg/m2, p=0.009). No differences between diets were observed for HDL-C, TG, ApoB, ApoA1, ApoB/ApoA1 ratio, HbA1c, high sensitivity C-reactive protein, systolic or diastolic blood pressure, heart rate, quality of life, choline, TMAO, or acetyl-carnitine.

In the random forest model, 46 microbial genera and 33 plasma metabolites significantly differed in VD and MD. In a linear mixed model and after adjustment for false discovery rate, the relative abundance of 12 microbial genera and 28 metabolites differed significantly with intervention. We found significant higher relative abundance of *Shuttleworthia*, *Akkermansia*, and *Fusicatenibacter* genera and lower relative abundance of *Parasutterella*, *Oscillospira*, *Parabacteroides*, *Melainabacteria*, *Clostridium_sensu_stricto*, *Negativibacillus*, *Anaerofilum*, and *Ruminiclostridium* in the faecal samples after the VD than the MD. No significant differences were observed in faecal levels of short- and branched-chain fatty acids.

Significantly higher relative plasma abundance of subaphylline, acoric acid, 3-2-oxopropanoic acid, DG(16:0/20:3) and lignoceric acid and lower relative plasma abundance of 3-methyl-L-histidine, N-acetylanonaine, 4-hydroxy nonenal mercapturic acid, 2-hydroxylauroylcarnitine, dihydroxy-stearic acid, tetracosanedione, and several PEs [(PE(18:1/18:1), PE(20:3/18:1), PE(20:2/18:1), PE(18:1/20:4), PE(18:0/22:5), and lysoPE(22:0)] and PCs [(PC(14:0/O-1:0), PC(18:1/18:1), lysoPC(16:0), lysoPC(16:1), PC(18:1/22:4), and PC(16:1/18:1)] were found after VD compared with MD.

In an exploratory analysis, we found that baseline relative abundance of 14 genera could discriminate responders (VD vs. MD <0) from non-responders: Oxidized LDL-C decreased with the VD in individuals with higher faecal relative abundance of *Ruminococcaceae* genera and *Barnesiella* and with reduced abundance of *Flavonifractor*.

Supplementary data

In the subjects enrolled in VERDI trial, the number of stenotic coronary arteries and longitudinal extent of coronary atherosclerotic burden were strongly correlated (r=0.80, p=<0.001).

We identified 214 plasma lipids in four main lipid classes, glycerolipids, glycerophospholipids, sphingolipids, and sterols.

There was no association between any of the 214 identified lipids and number of stenotic lesions or longitudinal extent of CAD, after false discovery rate correction.

In a generalised linear mixed model with adjustment for false discovery rate, 43 plasma lipids significantly differed with diet. Specifically, compared to MD, VD caused higher relative abundance of the several TGs [TG(52:3), TG(52:4), TG(52:5), TG(54:4), TG(54:5), TG(58:9), TG(14:0/18:2/18:2), TG(16:0/18:2/18:2), TG(16:0/18:2/18:3), TG(18:2/18:1/16:0), and TG(16:0/18:2/22:6)] and lower relative abundance of several TGs [TG(47:0), TG(50:0), TG(50:1), TG(51:1), TG(54:1), TG(55:1), and TG(16:0/18:0/18:1)], ether PCs [PC(O-32:1), PC(O-34:2), PC(O-36:3), PC(O-36:4), PC(O-36:5), PC(O-38:5), PC(O-34:4), PC(O-38:5), and PC(O-38:6)], several PCs [PC(33:0), PC(36:1), PC(18:0/18:1), PC(18:0/22:6), and PC(P-18:0/22:6)], PE(P-38:4), PE(P-18:0/22:6), cholesterol ester(18:0), ceramide(d18:1/16:0), and lyso-PC(16:0). Clustering analysis showed that VD lowered eigenlipid modules dominated by PCs, sphingomyelins, and FACs of TGs with saturated double bonds, and increased module eigenlipid of TGs having high numbers of carbons and polyunsaturated FACs.

DISCUSSION

Principal findings

The major findings of this research are illustrated in Figure 7.

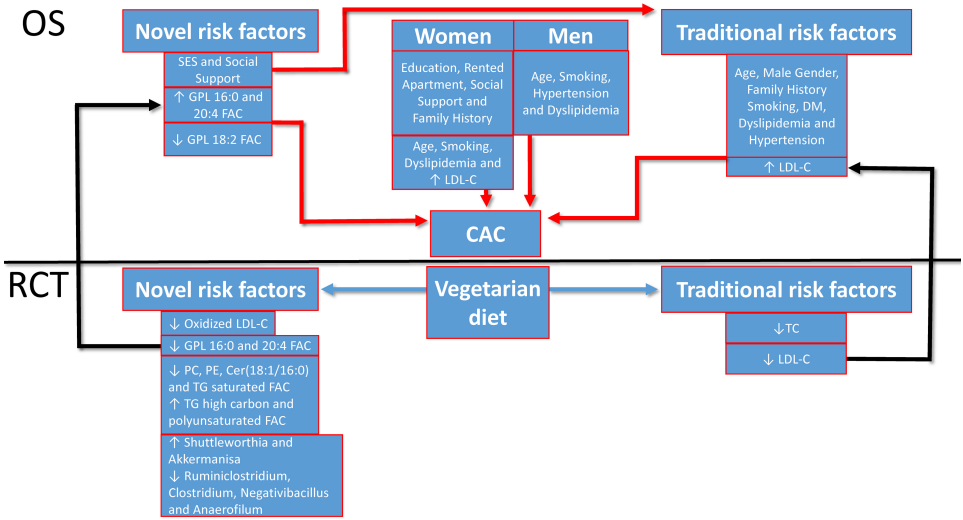

Figure 7. Interrelationships of the main findings of this thesis. Red arrows indicate association, blue arrows indicate causation, and black arrows show risk factors associated with coronary artery calcium that were favourably affected by the vegetarian diet. BMI = body-mass index; CAC = coronary artery calcium; Cer = ceramide; DM = diabetes mellitus; FAC = fatty acyl chain; GPL = glycerophospholipid; LDL-C = low-density lipoprotein; OS = observational studies; PC = phosphatidylcholine; PE = phosphatidylethanolamine; RCT = randomized control trial; SES = socioeconomics (area and individual level); TC = total cholesterol; TG = triglycerides.

- Novel and traditional risk factors were associated with subclinical CAD as indicated by CAC (Studies I–IV).
- Lipidomics results were reproducible in highly specialised laboratories (Study I). We found higher levels of glycerol-phospholipids with 16- and 20-carbon FACs (PC(16:0/20:4) and lower levels with 18-carbon FACs [(PC(18:2/18:2), PC(36:3), and PE(20:0/18:2)] in patients with subclinical CAD compared with those without subclinical CAD (Study II).
- Area and individual level SES and social support were associated with subclinical CAD in SCAPIS participants aged 50–64 years. However, these associations were explained by high levels of traditional risk factors (Studies III–IV). Family history of premature CVD in the total pilot SCAPIS population was associated with subclinical CAD independent of traditional risk factors (Study III). Family history of premature CVD, lower education level, living in a rented apartment, and low levels of social support were, independent of traditional risk factors, associated with subclinical CAD in women but not in men (Studies III–IV). In middle-aged men and women, traditional risk factors were associated with subclinical CAD.
- Using an experimental cross-over design, we observed significant differences in oxidised LDL-C, body-mass index, glycerophospholipids, L-carnitine, individual gut microbial taxa, and the traditional CVD risk factors TC and LDL-C after a four-week VD compared to MD in individuals with a history of CAD on standard medical therapy. The change in oxidized LDL-C with the VD was associated with a specific pattern of gut microbiota at baseline (Study V).
- A vegetarian diet lowered glycerolipids, glycerophospholipids, sphingolipid and sterol lipids, and TGs with saturated FACs and increased TGs with polyunsaturated and high carbon FACs (Supplementary findings).
- Glycerophospholipids (16- and 20-carbon FACs) and LDL-C, which are associated with subclinical CAD independent of traditional risk factors (Studies I–IV), were significantly lower after a VD compared with an MD (Study V).

Reproducibility of lipidomics analyses (Study I)

Inconsistency of results among laboratories may be a barrier to clinical application of findings, but limited effort has been made to evaluate inter-laboratory agreement in lipidomics studies of patients with subclinical CAD.

A nuclear-magnetic-resonance–based lipidomics study identified higher levels of mono-unsaturated fatty acids and lower levels of omega-6 fatty acids and docosahexaenoic acid (22:6) associated with CVD events in three population-based cohorts (total n=13 441, with 1741 events). These findings were confirmed by LC-MS and gas chromatography-MS platforms (126).
Lipid species of FACs with low carbon and double bond content and TG(54:2), CE(16:1), and PE(36:5) have been associated with higher cardiovascular risk and shown to provide more accurate risk stratification in studies of two cohorts (n=2138, with 135 events) (38). These studies highlight the importance of evaluating reproducibility in order to ensure the robustness of results of lipidomic analyses.

The pre-analytic phase and sample-handling are crucial steps in lipidome analysis that may affect reproducibility of results (127). Plastic polymers contained in the anticoagulant lithium-heparin blood sample tube may, for instance, suppress metabolic signals of ion masses. The analysis of serum vs. plasma lipidome is a crucial factor that may affect the reproducibility of results. Blood sample tubes for preparing plasma can be put immediately on ice while those for serum need to be left to clot at room temperature, usually for 30 minutes. Exposure to room temperature and blood clotting may activate platelets to release a variety of lipid metabolites, affecting the serum metabolome analysis (127). Haemolysis resulting from strong aspiration, vigorous shaking of the tube, high centrifugation speed, and high ambient temperature can cause release of enzymes and metabolites from red blood cells, which may account for up to 18% of the variation in the observed metabolome (127).

Methods of sample preparation and LC-MS analysis differed slightly between the two laboratories used in our study. This may have influenced the results and resulted in mutual identification of fewer lipids discriminating subclinical CAD samples from the control group than might have been found with identical procedures. However, despite this, the results were in sufficient agreement to draw conclusions. Our study suggests that lipidomics findings in patients with subclinical CAD are reproducible in highly specialized laboratories with experienced technicians. Demonstrating consistency in results of lipidomics analysis is an important step toward clinical application.

Serum lipidome and subclinical CAD (Study II)

Phospholipase A2 hydrolysis of PC(16:0/20:4), a lipid biomarker identified at higher levels in patients with CAC, releases palmitic(16:0) and arachidonic acid(20:4). A recent report comprising 10 800 participants in a primary and secondary prevention cohort revealed that inclusion of PCs with palmitic acid to a panel of ceramide lipids resulted in improved prediction of CAD events (3). Palmitic acid has been shown related to vascular calcification by formation of reactive oxygen species (128). Arachidonic acid is a major component of eicosanoid production, which is an essential regulator of inflammation (129, 130). Our findings are in agreement with a prospective nested case-control study of 744 cases and 744 controls aged 47–83 years that showed plasma levels of arachidonic acid to be positively associated with acute myocardial infarction (131).

Phosphatidylethanolamines are involved in several biological mechanisms, one of which is autophagy, a process that involves breakdown of cellular components by lysosomes (132). Intracellular PE deficiency has been linked to dysfunction in autophagy and formation of reactive oxygen species (133). We found lower levels of PE(20:0/18:2) in patients with subclinical CAD, an implication that the calcification process might be associated with autophagy dysfunction.

These findings identified potential diagnostic lipid biomarkers, allowing generation of hypotheses concerning their involvement in the pathological processes leading to subclinical CAD.

Socioeconomic status and subclinical CAD (Study III)

The association of neighbourhood-level SES and CAC was investigated in the HNR study (Table 2), and a significant relationship was found in men, but not in women, after adjustment for traditional risk factors (134). The participants in the HNR study were not randomly enrolled stratified by area, and the unemployment rate was used as the marker of area-level SES. In the CARDIA study (Table 2), women 32–50 years from low area-level SES had a higher risk of CAC independent of biological and behavioural factors, but similar associations were not found in men of similar age range (135). Together with the findings of the present study, this might indicate that the influence of living in a low-SES environment on coronary atherosclerosis is mediated through higher levels of traditional CVD risk factors.

A 12-year follow-up of the MESA study (Table 2) showed increased density of health food stores in residential areas to be associated with slower progression of CAC (136). It is likely that multiple factors conducive to a healthy lifestyle differ in low- and high-SES areas, such as the presence and cost of recreational facilities, perceived safety, air pollution, aspects of the built environment, and sociocultural elements, which may contribute to CAC (137).

Findings from the main SCAPIS study will provide more data. In contrast to the HNR and CARDIA studies, age and sex of the randomly selected participants were matched within the residential area categories, a strength of the current study that was also not a feature of the main SCAPIS study. Hence, the design of the current study reduced the likelihood of confounding.

Middle-aged women may to some degree be protected from CAD and tend to develop CAD 7–10 years later than men (138). A potential underlying mechanism for this phenomenon is a beneficial effect of oestrogen on traditional risk factors including blood lipids (higher LDL-C and decreased HDL-C), elevated blood pressure, and high body weight (138). However, oestrogen may fail to counteract mechanisms related to family history of premature CVD, education, or living conditions. These non-standard risk factors may be more important in women, since middle-aged men are more like to exhibit higher levels of traditional risk factors.

Social support and subclinical CAD (Study IV)

Few studies have investigated the relationship of social support with CAC in persons randomly selected from the general population, and findings have been mixed. In the Coronary Artery Risk Development in Young Adults (CARDIA) study (Table 2), low social cohesion was associated with CAC in women aged 32–50, but not in men of similar age, independent of CVD risk factors (135). Being single or widowed was, independent of traditional risk factors, associated with elevated CAC in 783 men and women (mean age 57.4±9.3) (139). In contrast, no association was observed between social support and CAC in a study of 528 asymptomatic men and women aged 53–76 years (140).

Measures of CAC can be used to determine biological age or 'vascular age' (141). In the HNR study, women aged 45–74 years with LDL-C >4.9 mmol/L were reported as 17 years older in vascular age than their counterparts with LDL-C levels of 2.6 mmol/L (142), and current smokers were about 10 years older in vascular age compared to non-smokers (143).

Study IV showed women with low, compared to moderate or high, social integration levels to be about 14 years older in vascular age using a random forest model. In other words, social support appears to be a crucial risk factor and comparable to traditional risk factors. The source of sex discrepancy in the association between social support and subclinical CAD may be that a greater number of women (459 vs. 450) successfully completed the questionnaire quantifying social support, and a higher proportion of men showed manifest CAD, most likely with presence of CAC, resulting in fewer data for men. Secondly, women may be more likely to express their emotions than men (144).

A lack of social support may alter the hypothalamus-pituitary-adrenal axis, resulting in hypocortisolaemia or hypercortisolaemia and white blood cell glucocorticoid receptor resistance, which eventually leads to enhanced pro-inflammatory cytokine production (108, 145). We found that women, but not men, with lower levels of social support had higher white blood cell counts and high sensitivity C-reactive protein levels. Low-grade inflammation may have been a factor in the association of low social support with subclinical CAD in women.

Whether psychosocial factors should be considered a risk factor has been a matter of some debate, however, the current European guidelines, published in 2016, state that it remains unknown whether routine screening for psychosocial risk factors would lead to fewer events (2).

Impact of a vegetarian diet on cardiovascular risk factors, plasma metabolome, plasma lipidome, and gut microbiota and the association of plasma lipidome with atherosclerotic burden (Study V)

The formation of oxidized LDL-C via oxidative modification of LDL-C is a crucial step in the development and progression of fatty streaks and atherosclerotic plaques (146). Oxidized LDL-C level is higher in individuals with a CAD history and is a strong predictor of CAD events, independent of traditional CVD risk factors (4, 121). A significant reduction of oxidized LDL-C was observed in a three-month vegan dietary intervention study in patients with rheumatoid arthritis not receiving cholesterol lowering drugs (78). Monitoring of TC, LDL-C, HDL-C, and TG levels is recommended in patients diagnosed with CAD (2). A meta-analysis of 11 randomized vegetarian diet studies reported significant reductions in TC, LDL-C, and HDL-C in healthy subjects; in patients with diabetes; in obese subjects; and

in patients with rheumatic arthritis (147). In Study V, we observed significantly lower TC and LDL-C with the VD compared to the MD. To the best of our knowledge, no previous studies have investigated the impact of a vegetarian diet on oxidized LDL-C and lipid profile in a homogenous population with a history of CAD receiving standard medical therapy. Our study expands current information and showed significantly lower oxidized LDL-C, TC, and LDL-C after only four weeks of a VD compared to MD.

Subjects with higher C-reactive protein levels have higher risk of recurrent cardiac events following PCI (148). A meta-analysis of 18 studies found no short-term differences in high-sensitivity C-reactive protein levels in vegetarians compared to omnivores (149). However, a vegetarian diet of at least two years duration resulted in high-sensitivity C-reactive protein levels lower than in subjects that ate meat (149). We found no differences in high-sensitivity C-reactive protein levels between the VD and MD in our study period.

A meta-analysis of seven randomized controlled trials reported evidence that both systolic and diastolic blood pressure were significantly lower in subjects consuming a vegetarian diet than with an omnivore diet (mean difference of 4.8 mm Hg systolic and 2.2 mm Hg diastolic) (81). In six of the seven clinical trials, participants were not receiving blood pressure lowering drugs. We found a numeric difference of systolic blood pressure of 2.3 mm Hg in VD and MD, which did not reach significance.

It has been demonstrated that quantification of individual lipids of the plasma lipidome may improve prediction of CVD events, beyond that provided by traditional risk factors, in primary (38) and secondary prevention cohorts (36, 64). Analysis of ceramides has been introduced in clinical practice at the Mayo Clinic in US to determine individual risk of CAD events (150). Recently, large primary and secondary prevention cohort studies reported that a panel of seven glycerophospholipids and sphingolipids predicted CVD events and mortality with greater accuracy than prediction models based on traditional risk factors (3). Specifically, higher levels of ceramide(d18:1/16:0), ceramide(d18:1/18:0), ceramide(d18:1/24:1), ceramide(d18:1/24:0), and PC(16:0/16:0) and lower levels of PC(16:0/22:5) and PC(14:0/22:6) were correlated with recurrent CAD events.

Unpublished data acquired in the VERDI trial showed participants to exhibit lower relative abundance of PCs with palmitic acid (16:0) and ceramide(d18:1/16:0) with consumption of VD compared to MD. It has been suggested that these lipids might contribute to formation of unstable

plaques through vascular immune and inflammatory processes (150). Phosphatidylethanolamines are involved in blood clotting and the formation of thrombin, and lower levels of these lipids after VD vs. MD may partly explain the lower risk of CAD associated with vegetarianism (60).

In a cross-over study of 113 healthy participants (mean age 51 years, 78% female), a low-calorie vegetarian diet resulted in significantly higher TG levels than the low-calorie Mediterranean diet (151). A primary prospective cohort study of 685 healthy subjects found that TGs with a low carbon number and low numbers of double bonds had the strongest predictive value of CVD events (38). Therefore, we sought to investigate the effect of vegetarian diet on TG qualities. Unpublished data acquired in the VERDI trial showed that VD reduced TGs with low double bond content and increase TGs with high double bond and carbon content. These findings suggest that the VD reduced several individual lipids of the plasma lipidome that have been associated with higher risk of recurrent CAD events. We found the relative faecal abundance of genus *Akkermansia* to be higher with VD than with MD. Low *Akkermansia* abundance is associated with an increased risk of developing metabolic disease such as type 2 diabetes and obesity (152). In obese subjects in a six-week interventional trial with caloric restriction, relative abundance of *Akkermansia* was shown linked to beneficial effects on body fat distribution and fasting plasma glucose (153).

The change in oxidized LDL-C seen with the VD was associated with a specific gut microbiota pattern at baseline dominated by several genera of the families Ruminococcaceae and by *Barnesiella*, a gut microbe that might play an important role in clearance of intestinal infections and immunomodulation (154, 155). These findings suggest an interaction of specific gut bacteria and a VD in reduction of oxidized LDL-C.

To link the results obtained from observational studies included in this thesis, we performed a lipidomics analysis and assessed the degree of atherosclerotic burden by visual analysis of coronary angiograms in the subjects of the VERDI trial (supplementary data). We found no association of any component of the plasma lipidome with atherosclerotic burden after correction for false discovery rate, possibly due to the small sample size. However, we observed that the glycerophospholipids with 20:4 and 16:0 FACs and the LDL-C levels that were associated with subclinical CAD in the observational studies were significantly lower in subjects with CAD after the VD than after the MD.

METHODOLOGICAL CONSIDERATIONS

Major differences between observational and interventional studies have been described (156). In brief, in an observational study, researchers are able to detect only associations among exposures and outcomes, but causation cannot be inferred. Strengths of observational studies are that they are typically large and relatively inexpensive, and investigation of relationships of multiple exposures and outcomes is possible. On the other hand, observational studies may be prone to bias, and it is difficult to completely control for residual cofounders in statistical models. The randomized controlled trial in which subjects are randomly allocated to different treatments is considered the gold standard for evidence-based medicine (157). A strength of the research reported in this thesis is that we used a combination of observational and controlled intervention studies to investigate relationships of potential consequence to CAD prevention. Biomarkers can identify individuals affected by disease, evaluate disease progression, and monitor treatment. For atherosclerotic CVD, TC and LDL-C are routinely measured to evaluate risk (2). In addition to exogenous factors such as diet, cholesterol levels are influenced by the complexities of endogenous biosynthesis and conversion to bile acids (158). Single biomarkers are unable to provide sufficient information about the biochemical pathways responsible for CAD. To obtain a broader picture of underlying biochemical mechanisms, we applied lipidomics and metabolomics (Studies I, II, and V).

In Study I, we used a paired t-test to compare the relative levels of specific plasma lipids in various degrees of subclinical CAD. However, when conducting multiple comparisons, there is a chance of obtaining false positive differences in the levels of lipids of tested groups (rejection of null hypothesis) (159). To counteract false positive results, we adjusted for false discovery rate in Study II.

In Study III, we determined the risk of CAC in participants from low area- and individual-level SES using traditional statistical methods. Applying a machine-learning approach in Study III might have generated additional information with respect to the relative importance of risk factors for CAC.

Study IV employed questionnaires to measure social support. A condensed version of the Interview Schedule for Social Interaction was used, which was shown reliable and validated in previous studies (110). These questionnaires have been shown to predict CAD outcomes in multiple studies in Sweden and were therefore used to assess social support in the

SCAPIS study (111–115). The questionnaires use two subscales, *social integration* and *emotional attachment*, each with six questions. We combined the social integration variables to obtain an overall score. The scores were divided into tertiles consisting of a lower quartile, the two intermediate quartiles, and an upper quartile. Another option would have been to divide the scores into quartiles; however, this would not have changed the odds ratio for CAC when comparing lower vs. upper quartiles. Due to the extremely skewed distribution of the Emotional Attachment Scale, with most participants rating their emotional attachment as high, the only option was to separate the scale into three categories: 0–4, 5, and 6.

Subclinical CAD can be measured by several imaging techniques, but CT is the most commonly used technique. In Studies I–IV, the traditionally used Agatston method was applied to measure the extent of coronary artery atherosclerosis. This method has been standardized and unchallenged until recently, with new studies demonstrating that CAC density and volume scores may further improve CVD event prediction (160). However, compared to other markers of subclinical atherosclerotic disease, such as carotid intima-media thickness and carotid plaques (161, 162), thoracic aortic calcification (89, 163), brachial flow-mediated dilation, C-reactive protein, family history of CVD, and ankle-brachial index, CAC measured by the Agatston method has proven to be the best predictor of future CVD (89, 161–164). Use of other imaging techniques in asymptomatic individuals, such as CT angiography to detect coronary stenosis, has not proven superior to CAC in predicting acute coronary syndrome and all-cause mortality (165, 166).

Instead of modelling the risk of an outcome (CAC) with logistic regression, an alternative could have been to use a linear regression (Studies III and IV). The logistic regression model gives odds ratio as a measure of association, while linear regression estimates means. The CAC is measured on a continuous scale, but is not normally distributed. However, others have used log (CAC+1), which also could have been an option in Studies III and IV to evaluate the association of psychosocial factors with CAC. We considered logistic regression models to be optimal, since they provide simpler interpretation of results. Estimating means using the exponent of log-transformed CAC values would generate inaccurate estimates of the true mean of the study population. Log transformation can only be performed on positive CAC values, and therefore it would have been necessary to add a constant (in this case, 1) to CAC values before the log transformation. As the value of the added constant increases, the level of

significance increases, which may impact the results generated relative to the null hypothesis.

Given that HbA1c reflects plasma glucose concentration over the preceding three months, and that our VD and MD interventions covered four weeks, an oral glucose tolerance test revealing insulin resistance could have been performed on the subjects in Study V.

External validity

External validity (generalizability) constitutes the level of confidence at which research findings can be extrapolated to a real-world population and potentially applied to clinical practice.

In Studies I and II, we identified a group of lipids that differentiated serum samples of patients with subclinical CAD from those of controls, findings that may improve risk classification. However, before these findings can be transferred to clinical practice, they need to be validated in a larger and different primary preventive study sample. Secondly, a study design with equal distribution of cardiovascular risk factors among subclinical CAD subjects and controls is needed to minimize the risk of bias from residual cofounding. Thirdly, more precise methods such as targeted lipidomics should be considered in order to reveal details of the absolute concentrations of lipids.

Study III demonstrated that differences in subclinical CAD between individuals living in high and low SES residential areas could be primarily explained by differing levels of other CVD risk factors. While the limited exclusion criteria in Study III increases its generalizability, these findings might not extrapolate to the developing world where area-level SES differences are accompanied by wider differences in living conditions. Still, although there have been, thus far, no studies of SES and subclinical CAD outside high-income countries, recent research shows that low education is a risk factor for CVD across low-, middle-, and high-income countries (167).

Caution must be used in generalizing the results of Study IV with respect to the relationship of social support and CAC to men and women outside the studied age group and population.

Previous studies have shown that a vegetarian diet may improve lipid profile. However, there is a lack of research investigating the impact of vegetarianism on patients with history of CAD who are receiving standard medical therapy. The design of Study V and its measures of relevant outcomes that are routinely monitored in clinical practice in patients with

known CVD ensures high generalizability. The limitations of Study V include small sample size, male-bias, and a short intervention time, not allowing conclusions as to the long-term effects of a vegetarian diet.

Internal validity

Confounding bias

A confounding factor is a variable associated with exposure that is also independently associated with the outcome. Confounding can occur when unmeasured variables are present in both the exposed and an unexposed group. This can result in over- or under-estimating an effect or in obtaining a false positive association.

In Study I, the source of the differences in serum lipidome of subclinical CAD patients and controls may have been in age, sex, or statin treatment, as these factors differed significantly between groups. In attempting to evaluate the interlaboratory reproducibility of previous findings, we applied the same statistical methods as those used at Imperial College in London. In Study II, we tried to control for age, sex, and statin treatment in a logistic regression model. Despite that, the possibility of residual confounding from LDL-C, HDL-C, and TG cannot entirely be ruled out.

In Study III, controlling for cofounders was addressed in the study design by randomly inviting participants from high and low SES-areas and by matching with respect to age and sex. In addition, we adjusted for CVD risk factors in the multivariable logistic regression models, further reducing the likelihood of confounding. However, we did not adjust for the total smoking burden (packs/year), therefore the chance of residual confounding cannot entirely be excluded, although this would not change the main conclusion, since the risk of CAC in low SES-areas was explained by traditional CVD risk factors, and smoking is a positive confounder. In Study IV, we adjusted for the total smoking burden and other covariates in the logistic regression models.

The participants in Study V were initially randomized to either a vegetarian or meat-containing diet, resulting in an equal distribution of confounders between the groups. In addition, each subject received both interventions, therefore, there was no possibility of bias from confounding, provided that the participants kept to their assigned diet. The dietary interventions were not double-blinded, hence, there is a risk of ascertainment bias. Research has suggested that studies not double-blinded

are likely to favour the experimental treatment over the control, and can result in an exaggeration in effect estimates of up to 17% (168).

Selection bias

Selection bias can be introduced when the study population does not represent the target population. Since we investigated patients with chest pain and signs of myocardial ischemia on a cardiac stress test in Studies I and II, selection bias cannot be entirely ruled out.

The difference in the participation rate of low- and high- SES areas in Studies III and IV may have introduced selection bias, as participation was significantly influenced by SES residential area (169). However, according to statistics from registries regarding non-participants, participants and non-participants displayed no significant differences in CVD or traditional CVD risk factors (169).

Subjects in Study V were randomly allocated at a 1:1 ratio to one of the pre-selected interventions, but the risk of selection bias cannot be entirely excluded. From a large population, 150 individuals were asked to participate in a study, and 31, mostly men agreed; hence there is a high risk of selection bias. Also, a potential for bias existed due to inclusion in the analysis of data of subjects who did not complete the study.

Information bias

Information bias can occur during data collection and can be subcategorized as misclassification bias, regression to the mean, and ecological fallacy.

Misclassification can be a consequence of measurement errors in the exposure or outcome, or in confounders, which may eventually result in the exposed/diseased participants being wrongly classified as non-exposed/non-diseased or vice versa.

We have no reason to believe that assessment of CAC was misclassified to the extent of significantly impacting the results in the observational studies (Studies I–IV). In Studies I and II, the between-observer agreement was satisfactory, with a median inter-class correlation coefficient of 0.87 (0.65–0.99), and intra-observer variation was low, with a median intra-class correlation coefficient of 0.88 (0.66–0.99). The reproducibility of calcium scoring was also high in Studies III and IV. With repeated calculation of the CAC from 50 randomly selected subjects, the kappa measure of agreement was 0.91 and 1.00 for identifying participants with a CACS >0 and >100, respectively.

In order to avoid misclassification in Study V, each task was completed by the same research nurse. However, subjective factors in study nurse behaviour could hypothetically have induced misclassification.

Regression to the mean may occur if a random covariate is extreme on the first measurement but closer to the mean on the second measurement. In Studies I–IV, standard procedures were used to account for this, and the study design of Study V eliminated risk of regression to the mean, as the chance of bias was equally distributed between groups with respect to order of diet intervention (VD or MD).

Ecological fallacy is a failure in reasoning that arises when an inference is made about an individual based on the aggregate group. In Studies I and II, the differences in mean relative abundance of individual lipids with and without subclinical CAD does not necessary mean that we can predict the presence of subclinical CAD in individuals. There is a possibility of unequal distribution of the detected lipids among patients with subclinical CAD. Also, regarding the results of Studies III and IV, we cannot draw the conclusion that women with family history of premature CVD, low SES, or social support will have subclinical CAD. Although these findings give rise to questions of the potential underlying mechanisms of the association of novel and non-standard risk factors with subclinical CAD, it should be stressed that the interpretation of the findings of this research should not be made at an individual level.

Random error

Random error is statistical variation in the measured data due to limitations in precision of the measurement device. Random error can be reduced by calculating a mean from a set of measurements or by increasing the study population sample size. A limitation of all studies included in this thesis was the relatively small sample sizes. In Study V, we conducted a sample-size calculation prior to conducting the study in order to enhance precision.

In Study V, the primary outcome variable (oxidized LDL-C) was measured twice, and the intra-assay coefficient of variation was <10% for 95.7% of samples. For secondary outcome variables (plasma metabolome and lipidome, gut microbiota) a cut-of value of 30% was used as intra-assay coefficient of variation.

CONCLUSIONS

I) Results of lipidomics analyses in patients with subclinical CAD are reproducible in highly specialized and experienced laboratories.

II) Identification of the serum lipidome may be of value in assessment of patients with subclinical CAD and in identifying subjects at high risk of CAD events.

III) Low-SES residential area is positively associated with subclinical CAD mediated through higher levels of traditional CVD risk factors.

IV) Lack of social support is associated with subclinical CAD in middle-aged women, but not in men, with of lack of social support exhibiting an association with atherosclerotic burden independent of traditional CVD risk factors.

V) In patients with a history of CAD treated with PCI and on standard medical therapy, a vegetarian diet results in lower oxidized LDL-C and improvement in traditional risk factors and positively affects plasma metabolome and gut microbiota genera.

CLINICAL IMPLICATIONS

- Identifying patients at high risk of CAD may aid in making individual lifestyle recommendations and initiating primary preventive medical treatment. Lipidomics may promote generation of new hypotheses with respect to the potential biochemical mechanisms involved in subclinical CAD.

- The observed presence of higher levels of traditional CVD risk factors in participants from low area-level SES and in those with less social support may warrant expanded patient screening to identify traditional CVD risk factors in the relevant populations.

- Coronary artery disease patients that were willing to adopt a vegetarian diet were shown to adhere to the diet in the short-term. In patients with a history of CAD, vegetarianism may result in reduced plasma low-density lipoprotein cholesterol and lower levels of novel risk factors, both associated with higher risk of coronary events. Gut microbiota assessment in patients with CAD could help to identify individuals with a favourable response to a vegetarian diet.

FUTURE PERSPECTIVES

Lipidomics application to a larger cohort of participants with subclinical CAD may lead to a lipidome signature of subclinical CAD, which could aid in precision of screening. In addition, these studies can generate development of novel medical therapy targeting the involved biochemical mechanisms of subclinical CAD.

Analysis of data from the main SCAPIS study may provide more conclusive information on the relationship of area-level SES and social support with subclinical CAD. If revalidated in the main SCAPIS study, interventional community-based programs may be designed to encourage lifestyle changes to reduce the social inequality in subclinical CAD and the incidence of CAD. Women may have risk factors for CAD that differ from those affecting men, and basing recommendation for coronary angiography solely on traditional factors may expose them to unnecessary hazards for results that are often normal. Conversely, reliance on the presence of traditional risk factors to predict obstructive CAD may result in too many women in whom the condition goes unrecognized. It is possible that screening for family history of premature cardiovascular disease, socioeconomic factors, and social support in women could result in improved cardiovascular care.

A healthy diet in CAD patients may results in a decrease of recurrent CAD events, resulting in more cost-effective secondary prevention. Conducting a larger and longer-term vegetarian diet interventional study might produce more knowledge about adherence to, and impact of, vegetarianism. Since 2016, CAD patients hospitalized at the Cardiology Department at the University Hospital in Örebro receive vegetarian food exclusively, and more solid evidence of the effects of a vegetarian diet in these patients may support this policy.

Ongoing cytokine analysis of the obtained VERDI samples will provide information concerning the mechanisms of the well-known association of a plant-based diet with lower incidence of recurrent CAD.

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