Lipids, Homocysteine and Vitamin A

New hypotheses from patients with cardiovascular disease

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Summary

Lipid parameters such as apolipoproteins B (apoB) and A1 (apoA1), and metabolites related to one-carbon metabolism including plasma total homocysteine (tHcy) have consistently and frequently been related to cardiovascular disease (CVD) risk. However, there is still residual risk present after applying lipid-lowering therapies, and most studies in secondary prevention show that tHcy-lowering therapies are ineffective. Vitamin A denotes a class of essential fat-soluble nutrients with effects in growth, development and vision. The major bioactive form, retinoic acid (RA), has target genes in lipid and homocysteine metabolism and has been shown to induce enzymes associated with homocysteine production and scavenging of atherogenic lipoproteins. Moreover, serum concentrations of the major circulating form of vitamin A, retinol, have been associated with increased risk of the metabolic syndrome and mortality in some observational studies. In this PhD project we aimed to explore whether 1) vitamin A measured as serum retinol interacted with common risk factors including apoB, apoA1 and plasma tHcy with regards to CVD risk and 2) identify factors that are associated with serum retinol in patients with established CVD. Utilizing data from more than 4000 patients hospitalized for suspected coronary artery disease in Western Norway between 2000-2004, we show for the first time that the risk of CVD associated with apolipoprotein B (apoB), apolipoprotein A1 (apoA1) and their ratio (apoB/A1) are modified by circulating concentrations of retinol. Specifically, apoB and apoB/A1 were positively associated, whereas apoA1 was inversely associated with risk of incident acute myocardial infarction (AMI) in patients with elevated concentrations of serum retinol. In a subsequent study in 2205 patients, we showed that the risk association for plasma tHcy with AMI was more pronounced in patients with elevated serum concentrations of retinol. Finally, we show that factors positively associated with serum retinol in the concentrations included markers of metabolic risk such as plasma total cysteine, serum creatinine and uric acid. Factors inversely related to serum retinol included C-reactive protein and plasma serine. Taken together, our results suggest a role for vitamin A in modification of CVD risk, and that serum retinol associates with biomarkers of metabolic risk. However, the results from this project must be replicated in initially healthy cohorts in order to establish clinical relevance, and potential mechanisms and hypotheses presented herein should be elucidated in mechanistic studies.

Acknowledgements

The beginning of this story dates back to 2013 and my master's program in Human Nutrition at the University of Bergen. Back then I was undecided as to what to do once I finished my degree as there seemed to be few options around. However, during the year or so of work with my thesis, it became clear to me that I wanted to pursue a career in research. This hunch was reinforced by working with the preventive cardiology research group led by Professor Ottar K. Nygård. The intellectual curiosity you instilled in me has been absolutely essential for the present work, and I am grateful for your continued guidance through the last four years which has been, and will continue to be, an inspiration in the pursuit of new challenges.

Eventually, I ended up at the University of Oslo in 2015 where I was taken in by my initial main supervisor Professor Helga Refsum and started the work on the present project. Since day one, you have been supportive in your mentoring and have been generous in giving me freedom to explore and grow at my own pace while at the same time providing advice during key times.

In time, main supervision duties were transferred from Helga to Dr. Kathrine J. Vinknes, and when I write this and think back, I realize that I have been somewhat deluded in regarding myself as an independent PhD student. Kathrine, you have been my professional rock the last four years. I am grateful that your door is always open, for your friendly, professional and encouraging advice, for our discussions, for letting me vent loudly in your office, and for trusting me enough to include me in your exciting projects. I look forward to our future collaborations, which I am confident will be fruitful, and I am more than happy to start giving back. Thank you.

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Oslo, May 2019

Thomas Olsen

List of scientific papers

The thesis is based on the following papers published in, or submitted to, international scientific publications.

PAPER I

Olsen T, Vinknes KJ, Svingen GFT, Pedersen ER, Tell GS, Blomhoff R, Drevon CA, Ueland PM, Midttun O, Refsum H, Nygard OK (2017) Cardiovascular disease risk associated with serum apolipoprotein B is modified by serum vitamin A. Atherosclerosis. doi:10.1016/j.atherosclerosis.2017.07.020

PAPER II

Olsen T, Vinknes KJ, Svingen GFT, Pedersen ER, Dhar I, Tell GS, Blomhoff R, Ueland PM, Midttun O, Refsum H, Nygard OK (2018) The risk association of plasma total homocysteine with acute myocardial infarction is modified by serum vitamin A. Eur J Prev Cardiol 25 (15):1612-1620. doi:10.1177/2047487318788587

PAPER III

Olsen T, Vinknes KJ, Blomhoff R, Lysne V, Midttun Ø, Ueland PM, Svingen GFT, Pedersen EKR, Drevon CA, Refsum H, Nygård OK (2019) Amino acids of homocysteine metabolism, inflammatory markers and creatinine are associated
with serum retinol in patients with cardiovascular disease. Submitted as per March 2019

Abbreviations

ABCA1	ATP-binding cassette transporter A1
ABCG2	ATP-binding cassette transporter G2
AMI	Acute myocardial infarction
ApoA1	Apolipoprotein A1
ApoB	Apolipoprotein B
CBS	Cystathionine-β-synthase
CE	Esterified cholesterol
CI	Confidence interval
CPH	Cox proportional hazards
CRABP2	Cellular retinoic acid binding protein-2
CRBP1	Cellular retinol binding protein-1
CRP	C-reactive protein
CVD	Cardiovascular disease
CVDNOR	Cardiovascular Disease in Norway Registry
E %	Energy percent
EDTA	Ethylenediaminetetraacetic acid
eGFR	Estimated glomerular filtration rate
FFQ	Food frequency questionnaire
GAM	Generalized additive models
GNMT	Glycine N-methyltransferase
HDL	High-density lipoprotein
HR	Hazard ratio
HSC	Hepatic Stellate Cells
ICD	International Statistical Classification of Diseases
IQR	Interquartile range
LDL	Low-density lipoprotein
LDL-C	LDL-cholesterol
LRAT	Lecithin:retinol acyltransferase
LXR	Liver X Receptor
oxLDL	Oxidized low-density lipoprotein
R^2_{adj}	Adjusted R2

RA	Retinoic acid
RAE	Retinol activity equivalent
RALDH1	Retinaldehyde dehydrogenase-1
RAR	Retinoic acid receptor
RBP4	Retinol binding protein-4
RE	Retinyl ester
RXR	Retinoic X receptor
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SD	Standard deviation
STRA6	Stimulated by retinoic acid receptor-6
TC	Total cholesterol
TG	Triglycerides
T_h1	Type 1 T helper
tHcy	Total homocysteine
TTR	Transthyretin
VLDL	Very low-density lipoprotein
WECAC	Western Norway Coronary Angiography Cohort
WENBIT	Western Norway B-vitamin Intervention Trial
WENOCARD	Western Norway Cardiovascular Disease Registry
WHO	World Health Organization

Contents

1 Introduction

1.1 Definitions and epidemiology of cardiovascular disease

According to the World Health Organization (WHO), cardiovascular disease (CVD) comprise diseases of the major arteries including coronary artery disease, cerebrovascular disease, pulmonary embolism, peripheral vascular disease, deep vein thrombosis, congenital heart disease, rheumatic heart disease and cardiac arrhythmias [1]. Common to several of these conditions is the stenosis of major arteries as a result of atherosclerotic lesions leading to partial occlusion, ischemia and ultimately total occlusion and necrosis of the surrounding tissues following loss of oxygen. Heart disease resulting from ischemia and occlusion of the epicardial coronary blood vessels is commonly termed ischemic heart disease and includes angina pectoris and acute myocardial infarctions (AMIs).

In 2012, about 17.5 of the 58 million deaths caused by chronic or noncommunicable disease can be attributed to CVD on a global scale [2]. More recently, the Global Burden of Disease 2016 reported that ischemic heart disease accounts for 17.3 % of the total deaths globally and 25.8% in high- and middle-income countries [3]. Additionally, recent reports have shown that the burden of ischemic heart disease is highest in Central Asia, Eastern Europe including the former Soviet Union states as characterized by the highest age-standardized ischemic heart disease mortality rates per 1000 persons [4-6]. In Norway, the number of CVD-related deaths has been receding the last 40-50 years, totaling at 13010 deaths in 2013 [7]. This was reflected in a recently published evaluation of data from the Cardiovasular Disease in Norway (CVDNOR) project showing that the incidence of AMIs, hospitalizations and deaths related to CVD has declined from 2001 to 2014 [8]. However, an apparent increase in AMIs among younger individuals ($\leq$ 45 years) remains a concern [9]. The majority of incident CVD cases do not result in death and numbers from the CVDNOR show that more than 90 % of patients with AMIs survived after 30 days, indicating increased survival.

The importance of lifestyle alterations in the management and prevention of CVD risk were described as early as in the 1960's as reported in Framingham and Seven Countries cohorts [10,11]. In addition, lipid-lowering therapies have been successful in

reducing CVD mortality [12]. However, residual risk still remains after treatment [13] and in light of unfavorable global trends, the WHO have launched several global targets in order to attain 25 % reduction of noncommunicable diseases including ischemic heart disease, by 2025 [2]. Among those targets are the management of modifiable risk factors and metabolic risk markers, that can accelerate the progression of CVD.

1.2 Pathophysiology of ischemic heart disease

The single-most important risk factor for ischemic heart disease is atherosclerosis of the epicardial arteries. Atherosclerosis is a lifelong process involving a number of pathophysiological mechanisms including endothelial damage of the arterial wall and the subsequent immune reaction that leads to accumulation of lipids, immune cells, pro-inflammatory agents, thrombus formation and finally plaque rupture [14]. The resulting thrombus or embolus may result in occlusion of the arteries and reduce blood flow, which can result in an AMI. A brief summary of the pathological mechanisms will be given below.

The endothelial cells coating the intima of arterial walls are usually resistant to adhesion of leukocytes. However, following damage by mechanical or chemical stressors including hypertension and oxidative stress the endothelial layer of the intima becomes compromised [15]. This can in turn cause the endothelial cells to express adhesion molecules, which bind and enable the migration of monocytes into the intima and their differentiation to macrophages [14]. Increasing monocyte and macrophage numbers as well as changes in the composition of the arterial wall may further increase the permeability of the endothelial layer, leading to the influx, modification and retention of low-density lipoprotein (LDL) [16]. Oxidized LDL-particles (oxLDL) are phagocytized by macrophages, which become foam cells that sustain and aggravate the inflammatory response of the atherosclerotic lesion [17]. In addition, smooth muscle cells migrate from the media to the intima to stabilize the lesion, a process to which extracellular proteins such as collagen contribute [18]. The accumulation of smooth muscle cells and extracellular proteins in the intima form a fibrous cap that covers the lipid core of the lesion and contributes to the characteristic bulge of the arterial wall into the arterial lumen [14]. When this bulge results in stenosis that comprises more than 50 % of the lumen, it has traditionally been considered clinically significant [19,20], although this topic has been under some debate [21,22]. Besides occlusion of

the artery, plaque rupture may occur, particularly when plaques contain less smooth muscle cells and collagen but are otherwise rich in macrophages [14]. Upon plaque rupture, pro-coagulant processes are initiated leading to thrombus formation and further occlusion of the artery. The thrombus may also dissociate from the plaque and travel in the circulatory system as an embolus and lodge itself in arteries distant from the rupture site, partly or fully impeding blood flow to respective tissues.

1.3 Biomarkers

Assessment of CVD risk depends heavily on quantification of various circulating biomarkers because of their relation to disease progression [23,24]. For example, subendothelial retention of apolipoprotein B (apoB)-containing LDL particles in the arterial wall occurs relatively early in the atherosclerotic process [25,26] and can lead to cholesterol accumulation in plaques and atherosclerotic progression. In contrast, high-density lipoproteins which contain apoA1 are traditionally inversely associated with risk [12]. Besides apoB, a plethora of circulating compounds are associated with the propagation of this condition and includes inflammatory markers [27,28], coagulation factors [29], other apolipoprotein and non-lipoprotein lipid compounds [30-33], diabetes-related markers [34,35], one-carbon related metabolites [36-40] and markers of kidney function [41]. Thus, biomarkers provide potential predictive evidence for the pathophysiological state of the organism and are applicable to support CVD risk assessment through stratification of patients at high risk. An overview of the biomarkers relevant to the work in this thesis follows below.

1.3.1 Lipoproteins, their metabolism and relation to disease

Lipid compounds such as total cholesterol (TC), LDL, very low-density lipoprotein (VLDL), triglycerides (TG), high-density lipoprotein (HDL), lipoprotein (a), apoB, apolipoprotein A1 (apoA1) and the apoB/apoA1-ratio constitute traditional lipid biomarkers for both primary and secondary cardiovascular events [23,24,42,43]. A simplified overview of lipoprotein metabolism is given in **Figure 1**. Lipoproteins are essentially transporters of lipid-soluble compounds in the organism, and their metabolism is complex and occurs more or less ubiquitously including in the liver, vascular endothelium and circulatory system and other tissues [44]. Each lipoprotein consists of protein components, apolipoproteins, and several lipid components such as

TG, free and esterified cholesterol (CE), phospholipids and to a limited extent, fat-soluble vitamins. Forward lipid transport is the transport of lipids to peripheral tissues from the liver incorporated in VLDL, and from intestines incorporated in chylomicrons. VLDL consists of apoB, CE, which are crucial for apoB folding, and TG. Following hepatic secretion, endothelial lipases hydrolyze VLDL-bound TGs and consequently decrease particle size and increase particle density to yield LDL. LDLs are either absorbed by peripheral tissues or cleared by the liver. Chylomicrons produced in the intestine consist of a truncated version of apoB, TG and CE. Although chylomicron metabolism differs from the metabolism of VLDL, the same lipases are involved in TG hydrolysis prior to chylomicron remnant clearance by the liver. In reverse lipid transport, cholesterol and phospholipids are transported from extrahepatic tissues to the liver for degradation and excretion with HDL. HDL particles consist of up to 4 apoA1 molecules, which, like apoB, are produced and secreted from the liver into the circulation. Molecules of apoA1 recover cholesterol recycled to the cell surface of peripheral tissues by intracellular transporters including the ATP-binding cassette (ABC) transporter A1 (ABCA1) and ABCG2. Cholesterol rich HDLs can then be taken up or cleared by the liver. An alternative route of clearance is that transfer proteins such as cholesteryl ester transfer protein mediate the exchange of cholesterol for triglycerides between HDLs and VLDL in the circulation. The resulting LDL can then be taken up by the liver or other tissues by the LDL receptor or it can be oxidized and scavenged by macrophages in the atherosclerotic process.

ApoB-containing LDL can be retained and accumulate in the arterial wall and consequently lead to atherosclerotic lesions in part due to the deposition of cholesterol [25,26]. In risk management, circulating LDL-cholesterol (LDL-C) is traditionally calculated by the Friedwald formula* [23]. But this approach has been regarded as inaccurate for risk stratification purposes compared with modern approaches such as direct quantification of LDL [45-47]. Direct measurement of the protein constituents of lipoproteins has also been proposed as more accurate compared to LDL-C calculation [48-52]. ApoB is associated with all the lipoprotein particles considered to be proatherogenic, including VLDL, IDL and LDL, and indicates the total number of proatherogenic lipoprotein particles in the circulation. On the other hand, although chylomicrons and VLDL contain small numbers of it, apoA1 is mostly thought to

* (LDL-C = TC – HDL-C – TG/5)

reflect levels of anti-atherogenic HDL particles [53]. Several studies have emphasized the association with, and predictive properties of, apoB in relation to CVD risk [54-60]. ApoA1 on the other hand, has not been demonstrated to improve predictive value compared with conventional parameters [50,61]. The relative amount of apoB as marker of peripheral cholesterol transport to apoA1 as a marker of reverse cholesterol transport has since been considered a superior predictor of risk compared to either apolipoproteins [53]. Large-scale studies such as the INTERHEART study reported that the apoB/apoA1-ratio was associated with ~3-fold increased odds of getting an AMI [42]. Furthermore, patients in the highest tertile of apoB/apoA1-ratio had higher risk of developing major coronary events [57]. Moreover, following 1-year of statin use, only apoB/apoA1-ratio remained a significant predictor of future events. Despite the indication of apoB/apoA1 being a superior risk predictor, clinical trials demonstrating benefits following reduction of this ratio is lacking.

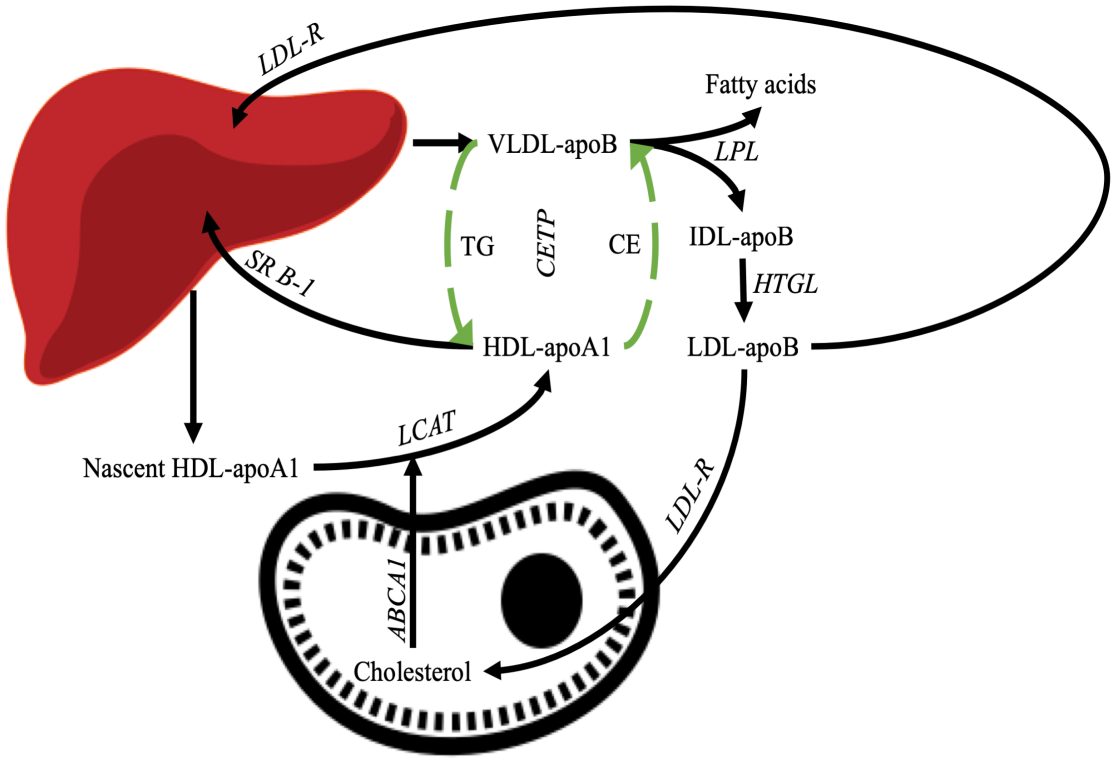

Figure 1: Simplified overview of lipoprotein metabolism. Abbreviations: VLDL, very low-density lipoprotein; apoB; apoA1, apolipoprotein A1; LPL, lipoprotein lipase; IDL, intermediate-density lipoprotein; HTGL, hepatic triglyceride lipase; LDL; low-density lipoprotein; LDL-R; low-density lipoprotein receptor; ABCA1, ATP-binding cassette A1; HDL, high-density lipoprotein; TG, triglycerides; CETP, cholesteryl ester transfer protein; CE, cholesteryl ester; SR B-1, scavenger receptor B1.

1.3.2 Homocysteine, one-carbon metabolites and their relation to disease

Homocysteine is a non-protein sulfur-containing amino acid that resides at the intersection of several metabolic pathways [62]. First, homocysteine is formed via catabolism of the essential amino acid methionine. This degradation occurs via multiple steps and involves 1) adenosylation of methionine to produce the universal methyl-donor S-adenosylmethionine (SAM) by methionine adenosyltransferase [63], 2) demethylation of SAM by SAM-dependent methyltransferases yielding S-adenosylhomocysteine (SAH) in a reaction termed transmethylation [64] and finally 3) hydrolyzation of SAH by SAH hydrolase, producing homocysteine and adenosine [65]. Formation of SAH decreases methylation capacity by reducing the SAM:SAH ratio [66], and it is of general belief that synthesis of creatine and phosphatidylcholine are the two major consumers of SAM and thus considerable sources of homocysteine production [67]. Homocysteine may be remethylated to methionine via the folate/cobalamin-dependent methionine synthase or by the alternative betaine-dependent betaine-homocysteine methyltransferase reaction [68,69]. The third and final pathway involving homocysteine is the transsulfuration pathway [70]. Transsulfuration describes the two-step, irreversible catabolism of homocysteine, and it involves the conversion of homocysteine to cystathionine by cystathionine-β-synthase (CBS) [71] and further to cysteine by cystathionine-γ-lyase [72], both of which are dependent on vitamin B6.

Elevated plasma concentrations of total homocysteine (tHcy) have frequently been linked to CVD. This hypothesis was proposed as early as 1969 by McCully [73], but received little attention until Wilcken and Wilcken showed that cysteine-homocysteine disulphide was significantly higher in patients with coronary artery disease compared with controls after an oral methionine load [74]. This finding indicated that homocysteine metabolism was altered in patients with coronary artery disease, and resulted in a myriad of studies aiming to identify the mechanisms underlying this finding. For example, *in vitro* studies demonstrated that cells treated with 1-10 mmol/L of homocysteine expressed increased tissue factor activity [75] and smooth muscle cell growth [76], which are processes implicated in atherosclerosis. In an epidemiological study, the association between tHcy and risk of CVD was mediated by oxidative stress biomarkers [77]. However, whether the role of homocysteine in

CVD development is causal or not remains to be established. Clinical trials in humans indicate that tHcy is merely a biomarker of poorly understood pathological mechanisms [78] as lowering of tHcy with B-vitamins have not been shown to improve prognosis among patients at risk for coronary events according to meta-analyses [36,79,80]. Notably, these neutral findings have mostly been observed in populations with established disease that are already receiving some sort of treatment. It is thus unlikely that tHcy-lowering therapy will contribute to risk reduction in these patients that are otherwise optimally treated. Indeed, a recent trial in a large population with no prior history, but at risk of CVD, show promising results in terms of stroke prevention with folic acid supplementation in addition to enalapril compared to enalapril alone [81,82]. These findings are supported by results of a recent meta-analysis [83]. Thus, work is still being done in order to unravel and understand the relationship between plasma tHcy and disease affecting the circulatory system in particular.

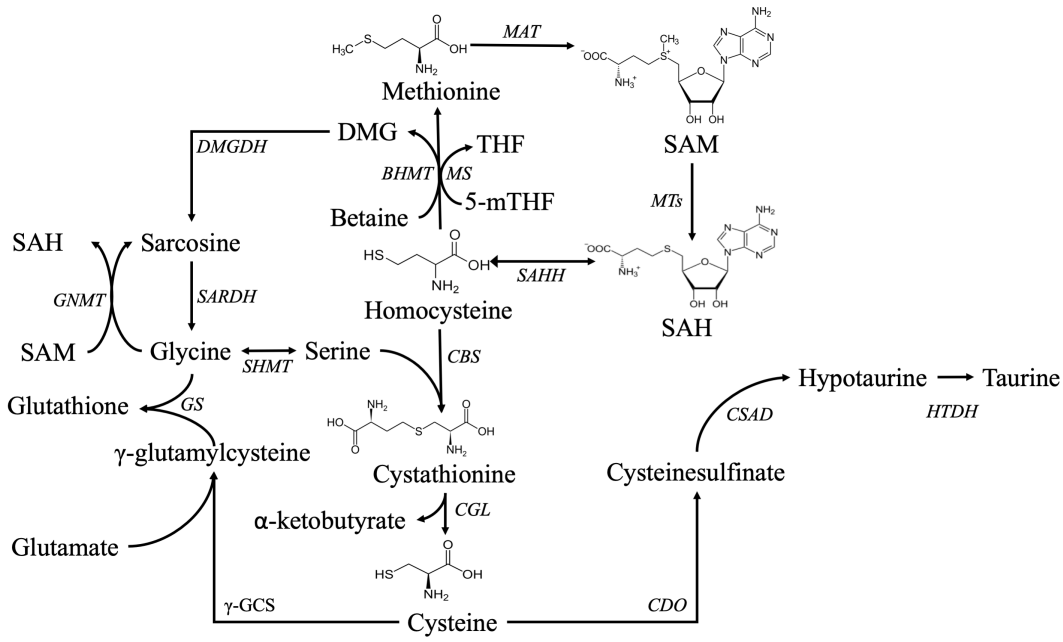

Figure 2: Schematic overview of homocysteine and sulfur metabolism. Abbreviations: MAT, methionine adenosyltransferase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; SAHH, S-adenosylhomocysteine hydrolase; MS, methionine synthase, BHMT, betaine-homoycsteine methyltransferase; 5-mTHF, 5-methyltetrahyrofolate; THF, tetrahydrofolate; DMG, dimethylglycine; DMGDH, dimethylglycine dehydrogenase; SARDH, sarcosine dehydrogenase; SHMT, serine-hydroxy methyltransferase; GNMT, glycine *N*-methyltransferase; CBS, cystathionine-β-synthase; CGL, cysteine-γ-lyase; CDO, cysteine dioxygenase; CSAD, cysteine sulfinate decarboxylase; HTDH, hypotaurine dehydrogenase; γ-GCS, γ-glutamylcysteine synthetase; GS, glutathione synthase

1.4 Vitamin A – a general overview

Vitamin A designates a class of essential lipid-soluble micronutrients with several important biological effects ranging from growth and development to vision [84]. There are several precursors and vitamers of vitamin A including provitamin A carotenoids and retinoids that are present in foodstuffs of both plant and animal origin, respectively. In plants, the major sources are carotenoids such as β-carotene, termed provitamin A, whereas animal sources contain esterified retinol, retinyl esters (RE). β-carotene is absorbed and then cleaved to retinaldehyde and subsequently to retinol in intestinal mucosal cells, whereas REs are cleaved to retinol in the intestinal lumen prior to absorption. In mucosal cells, retinol is re-esterified and transported in chylomicrons via the lymphatic system into the circulation, taken up to some extent by target tissues and ultimately by the liver. Some retinoic acid (RA) is also present in the foods of animal origin but is not considered quantitatively important compared to RE [85]. As a note on terminology, vitamin A will be used in generic terms only (both carotenoids and retinyl esters). I will otherwise refer to specific metabolites.

1.4.1 Metabolism of vitamin A

A simplified overview of the intracellular metabolism of vitamin A is outlined in **Figure 3**. The main storage site of vitamin A is in the liver as REs in lipid droplets of hepatic stellate cells (HSCs) [86,87]. However, REs is also present in the lung, testis, kidney, heart and adipose tissue [88]. The mechanism for metabolism and storage of vitamin A is not fully known, but it is complex and involves a host of transport proteins, enzymes and regulatory mechanisms. A brief overview follows below.

Upon hepatic absorption in dietary adequacy, retinol is transferred from hepatocytes to HSCs where it is bound by cellular retinol binding protein 1 (holo-CRBP1), which in turn serves as a substrate for lecithin:retinol acyltransferase (LRAT) yielding RE and 2-acylglycerophosphocholine as its products [89-91]. The importance of CRBP1 and LRAT in RE formation has been demonstrated in knockout models in which RE depletion of liver stores develop [92] and susceptibility to vitamin A deficiency increases [93]. Liver stores of RE are mobilized when retinol availability is declining, a process catalyzed by RE hydrolases [84] and partly promoted by apo-CRBP1 through inhibition of LRAT-activity [89]. Following mobilization of liver

stores, retinol is either transported to target tissues or metabolized to RA which is a ligand for nuclear transcription factors, retinoic acid receptors (RARs) [84]. The exact mechanism(s) of retinol transport from HSCs to the circulation is/are not known. However, essentially all retinol is transported tightly bound to retinol binding protein-4 (RBP4) [94]. RBP4 seems to also be essential to hepatic retinol secretion considering that *Rbp*$^{-/-}$-mice do not mobilize hepatic RE stores in the liver [94-96]. The retinol-RBP4 complex subsequently binds to transthyretin (TTR) which allows for retention of the retinol-RBP4-TTR complex in the kidneys [97]. Stimulated by retinoic acid receptor-6 is the only known receptor for the retinol-RBP4-TTR complex in target tissues, but is not considered essential for uptake outside the retina [98]. Once inside the cell, retinol dissociates from RBP4 and may be further metabolized after binding to CRBP1 [84]. The reversible and rate-limiting oxidation of all-*trans*-retinol to all-*trans*-retinaldehyde is predominantly catalyzed by a microsomal short-chain dehydrogenase reductase, retinol dehydrogenase, and its importance has been demonstrated in knockouts that develop severe disabilities and malformations [99-101]. Next, all-*trans*-retinaldehyde may either be reduced back to retinol or oxidized to all-*trans*-RA in an irreversible reaction catalyzed by retinaldehyde dehydrogenase 1 (RALDH) [102,103].

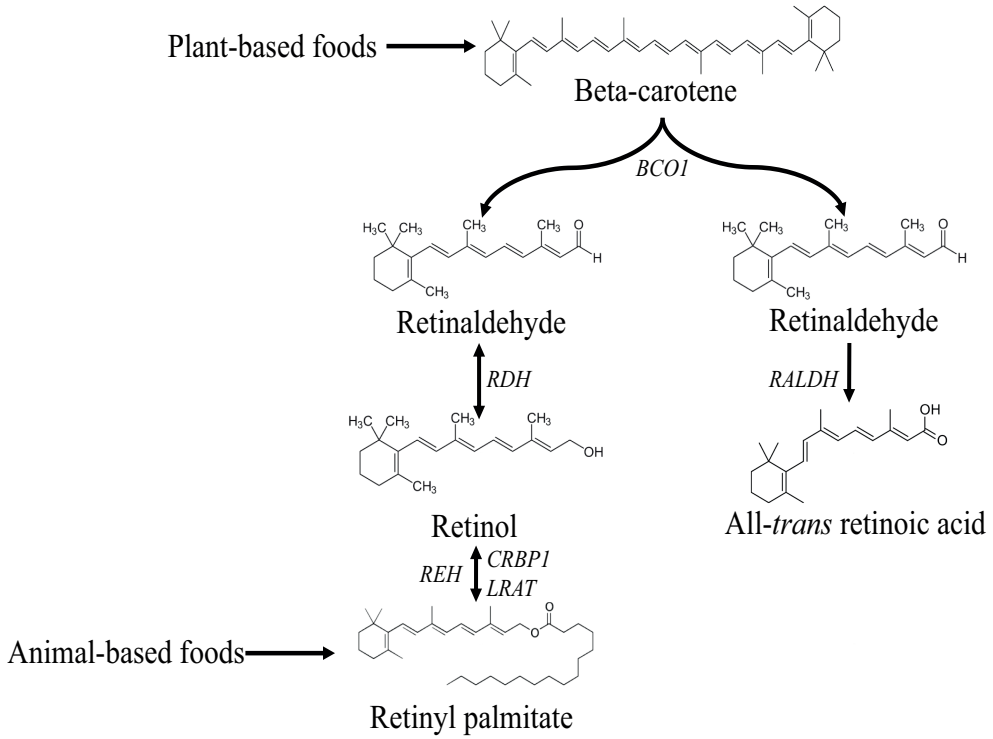

Figure 3: Vitamin A metabolism and function. Abbreviations; BCO, beta-carotene dioxygenase 1; RDH, retinol dehydrogenase; RALDH, retinaldehyde dehydrogenase; REH, retinyl ester hydrolase; CRBP1, cellular retinol binding protein 1; LRAT, lecithin:retinol acyltransferase

All-*trans*-RA is a ligand for nuclear RARs (isoforms α, β, and γ) [84], and its intracellular transport from the cytosol to the nucleus is facilitated by cellular retinoic acid binding protein-2 [104-107]. The RARs have a wide array of target genes that are implicated in growth, development and metabolism of macronutrients [108]. Finally, all-*trans*-RA can be isomerized to 9-cis-RA which is a ligand for nuclear retinoid X receptors (subtypes α, β, and γ), however, the endogenous relevance of this ligand is not known, as evidence of endogenous production is currently lacking [84].

1.4.2 Factors associated with vitamin A in the circulation

In 1984, Olson published results from a partly simulated experiment, postulating that retinol concentrations in plasma are kept under tight homeostatic control [109]. Their data indicated that plasma retinol decreased only when liver stores of vitamin A were nearly depleted (liver RE content 20-30 μg/g liver). Olson also demonstrated that plasma retinol increased when liver stores exceeded 300-500 μg/g of liver. Subsequent studies have supported these findings by showing that participants who administered >2000 retinol activity equivalents (RAE)/day for several years did not exhibit increased circulating retinol compared with participants who supplemented with lower doses or not at all [110,111]. Despite these findings suggesting that dietary or supplemental intake of vitamin A does not affect plasma retinol concentrations [112-114], vitamin A status has been positively associated with certain food groups such as meat intake [115], and the same study showed that energy-adjusted intake of vitamin A predicted serum concentration, but this finding has yet to be replicated. Moreover, a recent observational study found that subjects who were supplemented with multivitamins had somewhat increased circulating concentrations of retinol [116].

Another important factor that positively associates with retinol in plasma is kidney function. A compromised kidney function is often seen in lifestyle diseases such as diabetes type 1 and 2 and CVD [117,118]. Although retinol is not thought to be excreted to a large extent by the kidneys, some degradation products including retinoyl-β-glucuronide, have been detected in the urine [119]. Indeed, patients with compromised kidney function have several-fold higher circulating concentrations of reitnol in the blood [120-125], and kidney function or creatinine is thus a potential confounder when studying potential vitamin A-disease relationships.

Other epidemiological variables associated with circulating retinol include common risk factors of CVD such as TGs and total cholesterol, both of which have been positively associated with circulating retinol in observational studies [126,127]. In addition, markers of the acute phase response to inflammation, such as C-reactive protein (CRP), were inversely related to circulating concentrations of retinol [128]. Acute-phase reaction and systemic inflammation result in lower circulating concentrations of retinol due to increased sequestration/decreased mobilization in/from tissues, and thereby resulted in biased assessment of retinol status in individuals suffering from inflammatory conditions [129].

Thus, although the prevailing view is that retinol is kept under tight homeostatic control and regarded as relatively stable in the circulation, there are several factors as described above that may affect circulating concentrations of retinol.

1.4.3 Vitamin A and traditional risk markers in metabolic disease

The role of vitamin A in development of common lifestyle diseases is somewhat controversial. For instance, supplementation of all-*trans*-RA has been associated with both favorable and unfavorable alterations of lipid metabolism, depending on the experimental model system used [130]. One study showed that supplementation of *all-trans*-RA upregulated the expression of scavenger receptors in macrophages [131], which is a common trait in the pathology of atherosclerotic progression [132]. A similar observation has recently been made for both holo- and apo-RBP4 [133]. Furthermore, one metabolic syndrome mouse model exhibit increased plasma concentrations of retinol and less RA signaling in tissues [134]. This finding was recently corroborated by a human observational study, which showed that circulating retinol concentrations were positively associated with the metabolic syndrome [135]. Moreover, low and high concentrations of serum retinol are associated with all-cause mortality [127], whereas a meta-analysis reported that circulating retinol concentrations tended to be positively associated with all-cause mortality [136]. Supplementation of β-carotene and retinyl esters in patients at risk of lung cancer increased CVD mortality by 30 % [137]. In contrast, one observational study showed that low retinol concentrations increase the risk of cardiovascular disease and mortality [126]. This finding is supported by a cross-sectional study showing that serum retinol was inversely associated with intima-media

thickness [138]. Finally, A recent nested case-control study showed that serum retinol was linearly and inversely associated with first stroke [139].

The relationship of retinol with disease may pertain to the fact that RARs have possible target genes related to metabolism of lipids, lipoproteins, apolipoproteins [108,140,130], protein and carbohydrates [141]. Serum retinol was positively associated with serum TGs and total cholesterol in healthy adults [126,127]. In addition, lipid metabolism seems to be altered following pharmacological treatment with RA in humans, which typically promotes elevated concentrations of LDL-C and TGs and decreased concentrations of HDL cholesterol [142]. Retinoid treatment can involve hepatic VLDL secretion [130]. In rodents, it also induces the enzyme glycine *N*-methyltransferase (GNMT) [143,144], which is associated with hepatic cholesterol trafficking [145]. GNMT is also involved in the production of homocysteine [146], and increased activity may potentially affect circulating concentrations of tHcy, which in turn has been positively associated with serum retinol in a human population [126].

1.5 Knowledge gap and rationale of thesis

Incident CVD remains one of the leading causes of death in the Western world. The WHO has launched a global target for reduction in all non-communicable diseases with a focus on modifiable risk factors including lipids and other disease markers that may be of importance to CVD development. My interpretation of this statement is twofold. First, we should continue to emphasize preventive measures such as smoking cessation, dietary alterations and lipid-lowering treatment that may lower circulating risk factors including markers related to diabetes, inflammation and oxidative stress. However, studies show that even when we prevent CVD events (for example by using lipid-lowering treatment), circulating pro-atherogenic lipids still represent a residual risk among treated patients [13]. This makes sense considering that the pathophysiological mechanisms that ultimately culminate in cardiovascular events are multifactorial and draw upon several aspects of biology and metabolism throughout the life-course. Thus, secondly, I deduce from the WHO statement that we should strive to identify novel markers and effect modifiers of traditional risk factors to potentially improve risk stratification, treatment and prevention if results can be reproduced.

Due to its potency as a ligand for nuclear receptors with a wide array of target genes, retinol and its metabolites are potential effect-modifiers on which traditional risk

markers of CVD may depend. Indeed, bioactive retinoids such as RA are implicated in gene expression related to lipid metabolism and is also involved in the induction of enzymes related to homocysteine metabolism [137,127]. These studies are predominantly experimental, and to our knowledge few studies have described circulating retinol in CVD and none have investigated its potential effect-modifying properties in terms of CVD risk. Hence, there is a need to clarify the relevance of vitamin A and circulating retinol in CVD risk and development. An important point is that the elucidation of vitamin A in CVD risk has placed an exclusive focus on retinol or other metabolites alone, and not whether it interacts with other common risk factors of CVD. Thus, whether these interactions exist with regards to risk assessment, remains an open research question, and a comprehensive investigation of retinol in this context has yet to be done. Finally, factors associated with circulating retinol in plasma of patients with established CVD have not been rigorously described which poses a challenge with regards to potential confounding in the study of retinol and its relation to disease.

Based on the totality of the evidence and the lack thereof, we took the opportunity to study potentially novel interactions on the observational level with regards to traditional risk factors such as lipid parameters, homocysteine and retinol in order to generate hypotheses for further study. In addition, we characterize covariates that are positively and negatively associated with circulating retinol in these patients.

2 Aims

Based on the literature, the relationship of retinol with metabolic disease has yet to be resolved. Thus, to further elucidate this matter, the aim of this thesis was to study the interaction of retinol with traditional risk factors of CVD, including commonly used markers such as lipid parameters and tHcy. Moreover, we will evaluate the factors associated with retinol in the circulation, and possible associations with metabolites related to macronutrient metabolism. The studies will be carried out in a population with established CVD, and a flow-chart of the populations used is given in **Figure 4**. Separate aims of each manuscript are presented below:

1. Evaluate the interaction of serum lipid parameters with serum retinol in relation to incident CVD risk (**Paper I**, published 2017)
2. Evaluate the interaction of plasma tHcy with serum retinol in relation to incident CVD risk (**Paper II**, published 2018)
3. Evaluate factors associated with serum retinol in the circulation (**Paper III,** under review as per May 2019)

3 Materials and methods

3.1 Population

The Western Norway B-vitamin Intervention Trial (WENBIT) (ClinicalTrials.gov identifier: NCT00354081) and the Western Norway Coronary Angiography Cohort (WECAC) served as source populations for the results from the present project (**Figure 4)**. The WENBIT was a clinical trial aiming to study the effects of lowering plasma tHcy with B-vitamins on the morbidity and mortality of CVD [147]. A total of 3090 patients were enrolled and recruited from Haukeland and Stavanger University Hospitals upon admission for suspected angina pectoris, acute coronary syndromes and aortic valve stenosis. Significant coronary artery disease was angiographically verified in the majority of the patients (75 %), and was defined as $\geq$ 50 % narrowing of one or several epicardial arteries. In addition, 2119 patients that underwent coronary angiography at Haukeland and Stavanger University Hospitals were recruited and followed for clinically relevant endpoints, but not enrolled in the WENBIT due to resource limitations. Together, the WENBIT and the additional 2119 patients comprise the WECAC and consist of 5209 patients in total. The overall aim of the WECAC was to identify novel prognostic markers of cardiovascular endpoints [40].

To obtain a fairly homogenous population, we utilized data from the patients with stable angina pectoris only, and excluded patients with acute coronary syndromes (n = 519*)*, valvular stenosis (n = 331) and other indications (n = 195). We further excluded patients with missing data on serum retinol (n = 45*)* yielding a total of 4117 patients available for analysis in **Paper I**. In **Paper III** we further excluded individuals with extremely low or extremely high concentrations of retinol (n = 2) yielding 4115 patients available for analysis. Because plasma tHcy is markedly affected by B-vitamin treatment [40], we excluded patients that received B-vitamin therapy in the WENBIT in **Paper II**. This was done in order to avoid misclassification of participants, considering that baseline tHcy concentrations would no longer be representative of the long-term concentrations and thereby potentially complicate interpretation. The resulting study population in **Paper II** thus consisted of participants in the WENBIT who received placebo treatment and non-WENBIT participants from the WECAC (n = 2205).

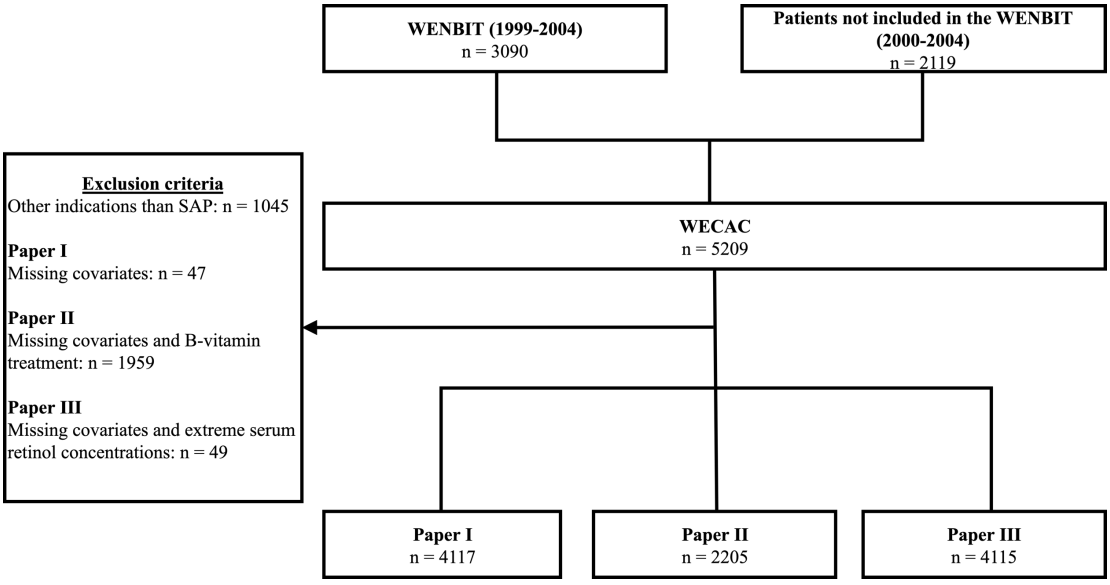

Figure 4: Flowchart of the sub-projects and the source populations.
Abbreviations: WENBIT, Western Norway B-vitamin Intervention Trial; WECAC, Western Norway Coronary Angiography Cohort; SAP, Stable angina pectoris.

3.2 Clinical outcomes

In **Paper I** and **II** the main outcome was incidence of AMI. AMIs were defined according to the revised definitions published in 2001 [148]. In brief, the definition of an AMI includes blood elevation of markers of myocardial necrosis (troponin or the myocardial band-portion of creatine kinase) and at least one ischemic symptom, abnormal electrocardiography or coronary artery intervention as well as pathological findings of an AMI including cardiac wall motion abnormalities or loss of tissue perfusion.

In **Paper I**, follow-up data were collected up to 2006 from the Cause of Death Registry Statistics Norway (www.ssb.no) and Western Norway Cardiovascular Disease Registry (WENOCARD; https://cvdnor.w.uib.no/wenocard). The WENOCARD contains discharge diagnoses from the participating hospitals in this particular project, which were verified against hospital records. In addition to the above definition, AMI outcomes included disease coded according to the International Statistical Classification of Diseases, 10th revision (ICD-10) as I46.1 and R96 corresponding to sudden cardiac death and cardiac death and thus included fatal AMIs. The outcomes were evaluated and verified by an endpoint committee as described in the original WENBIT publication [147].

In **Paper II**, follow-up data were obtained until December 31st, 2009 from the CVDNOR project (CVDNOR; https://cvdnor.w.uib.no/) project [149]. The aim of the CVDNOR project is to study trends in CVD incidence and recurrence and comprise data from 1994 to 2009. The outcome data from the CVDNOR that were collected until 2006 is equivalent to those of the WENOCARD. All outcome data were linked to the 11-digit personal identifier code of the participants.

In **Paper II**, 42 participants were censored for clinical outcomes beyond 2006 because they denied extended follow-up.

3.3 Clinical biochemistry

Blood samples were collected 1-3 days prior to coronary angiography (Haukeland University Hospital) or on the same day as the cardiac procedure (Stavanger University Hospital). Plasma samples were collected into EDTA- or sodium citrate-containing tubes, whereas serum samples were collected into tubes containing a gel separator. After centrifugation, plasma and serum samples were stored at -80 °C until analysis.

Routine laboratory analyses were performed in fresh samples at the Haukeland and Stavanger University Hospitals. Estimated glomerular filtration rate (eGFR), which was included in the statistical models of **Paper I** and **II** was calculated according to the formula published by the Chronic Kidney Disease Epidemiology Collaboration [150]. Several metabolites were quantified using one or more of the platforms at Bevital A/S (www.bevital.no) [151-154]. Briefly, plasma concentrations of methionine, tHcy, cystathionine and total cysteine in plasma were measured using gas chromatography tandem mass spectrometry, whereas plasma neopterin, and serum retinol and creatinine were measured by high performance liquid chromatography tandem mass spectrometry. Serum concentrations of apoA1, apoB, and TGs were measured using immunoassays. Serum concentrations of C-reactive protein (CRP) were measured using ultrasensitive immune-matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Serum uric acid concentrations were included in the routine laboratory analyses.

3.4 Dietary assessment

In **Paper III** we used dietary data obtained from a 169-items food frequency questionnaire (FFQ) to evaluate dietary determinants of serum retinol in the circulation.

We excluded patients with very high (>3585 kcal/d for females, >4180 kJ/d for males) or very low energy intakes (< 720 kcal/d for females, 835 kcal/d for males) and patients who did not complete the FFQ. In total, 1962 patients were eligible for analysis. The FFQ was developed at the Department of Nutrition, University of Oslo (Oslo, Norway) and was designed to collect data on habitual dietary intakes during the past two years in the population. The FFQ and has been validated for total energy intake and several nutrients [155-157]. Depending on the food item, the frequency of consumption was given in times per day, week or month, whereas quantity was given as regular household measures. Values from the FFQ were then converted to nutrient intakes by using a software (Kostberegningssystem 3.2) developed at the Department of Nutrition, University of Oslo. Food groups were given as grams per day. In **Paper III**, relevant food groups such as meats (processed and unprocessed red meats, venison and poultry), eggs, fish and shellfish (processed and unprocessed fish products), total dairy (milk, cheese, yoghurts, cream and ice cream), fruits and berries (including juice), vegetables, and alcohol intake were included.

3.5 Statistical analyses

3.5.1 Descriptive analyses

We applied the log-transformation for data that was not normally distributed in **Paper I-III**. One challenge with this type of transformation is that it complicates the interpretation of the data because it is not immediately clear what the transformed numbers represent. A common method of presenting descriptive non-normal data is by utilizing the median as a summary measure and the interquartile range or full range as the measure of dispersion. Another option is to back-transform (exponentiate) the numbers and present the exponentiated mean (geometric mean) and standard deviation (geometric standard deviation [SD]) of the log-transformed data. In **Paper I**, we presented baseline data as median (interquartile range [IQR]). However, after careful consideration we decided to present geometric means and standard deviations in **Papers II** and **III**. Although this may seem less intuitive, there are several good reasons for doing this. Briefly, when using parametric tests such as linear regression (see below) on log-transformed data, it makes sense to report the central tendency based on the data that was used for analysis rather than the median which in the case of a log-normal distribution may be skewed to the left [158]. Finally, the geometric mean closely

resembles the population median, and is considered more efficient and statistically flexible compared to the median [159].

In order to assess baseline differences in continuous covariates between retinol tertiles, we used quantile regression to assess trends by the median (50^{th} percentile) across the three retinol groups. In short, we quantified whether the median concentrations of the covariates differed between the three groups. As explained previously, we considered log-transformation of non-normally distributed data to be more appropriate than the median for descriptive data. Thus, in **Paper II** and **III** we used ordinary least squares linear regression to assess trends across retinol tertiles in the log-transformed variables. Categorical data were presented as *n* (%) in all papers, and trends across tertiles were assessed by ordinary logistic regression.

3.5.2 Main outcome analyses

Cox proportional hazards model

We used Cox proportional hazards (CPH) models to estimate the hazard ratio (HR) of AMI associated with apoB, apoA1 and apoB/apoA1 (**Paper I**) and tHcy (**Paper II**). The CPH procedure allows for the estimation of HR associated with a unit increase in the predictor per unit of time (days) adjusted for common and potential confounding factors [160]. To be more formal, we aimed to estimate the hazard at a given time interval for a group with a given set of explanatory variables. The HR is obtained by dividing the hazard of one group by that of another. In **Paper I** and **II**, we reported HR per SD increase in the log-transformed predictors. This measure is continuous and reflects the HR associated with one-unit change in the predictor, i.e., the HR per SD increase in the log-transformed predictor, and corresponding 95 % confidence intervals (CI). The CPH procedure is considered semi-parametric for technical reasons beyond the scope of this thesis, but to quote Kleinbaum "it closely approximates a correct parametric model" and is considered more robust and flexible than parametric alternatives [160]. However, the CPH model is not without its assumptions, including independent censoring and the concept of proportional hazards. Independent censoring can be problematic in survival analysis due to the concept of competing risks, which is discussed below.

For both papers, the models were built using a typical epidemiological approach. We first created one simple model adjusted for age and sex, and then one

multivariate model to adjust for confounding factors associated with both the exposures and the outcomes. After identifying potentially relevant confounding factors, we entered each of them to the simple model. If the covariates changed the effect estimate by >10 % we included them in the final multivariate model as proposed previously [161]. In **Paper I**, the multivariate models consisted of age, sex, smoking, C-reactive protein, number of stenotic vessels, left ventricular ejection fraction, hypertension, statin use at discharge from hospital and eGFR. In **Paper II**, the model included age, sex, smoking, statin and aspirin prescription at discharge, fasting status, estimated glomerular filtration rate and apolipoprotein B. To test for interaction between the main predictors (apoB, apoA1 and apoB/apoA1 with retinol in **Paper I** and tHcy with retinol in **Paper II**) and retinol, we included an interaction term (example: log-transformed tHcy × retinol) to both the simple and multivariate models.

Assumptions of the Cox proportional hazards model

The product of the CPH procedure in part relies on the concept of proportional hazards. Inherent in this assumption is the notion that the hazard associated with the explanatory variables are constant over time. For example, the CPH procedure assumes that the hazard explained by a set of variables for individual 1 is proportional to the hazard for individual 2. Visually, this means that the distance between the survival curves for the two individuals is proportional to one another over time. Thus, the HR approximates the average distance or ratio between the curves. The assumption of proportionality can be assessed visually and by formal statistical tests. For each of the predictors in the model, we can calculate residuals for each subject getting the event. The calculation of the residuals is complex and beyond the scope of this thesis, but the main concept is that these residuals should not be correlated with survival time. A test for correlation will give an effect size (Spearman's Rho) and p-value. If the p-value is low, then it indicates a significant correlation between the residuals and time. This can also be illustrated by plotting the residuals against time, in which the regression line should be flat and centered. For illustrative purposes, I have constructed such a plot (**Figure 5**) for our multivariate model from **Paper II** showing that the assumption of proportional hazards holds for this model. A disadvantage of the formal test is that it relies heavily on p-values, which is largely driven by sample size. Thus, the graphical and formal test of correlation should be used in combination, which we specified in the methods sections of both **Paper I** and **II**.

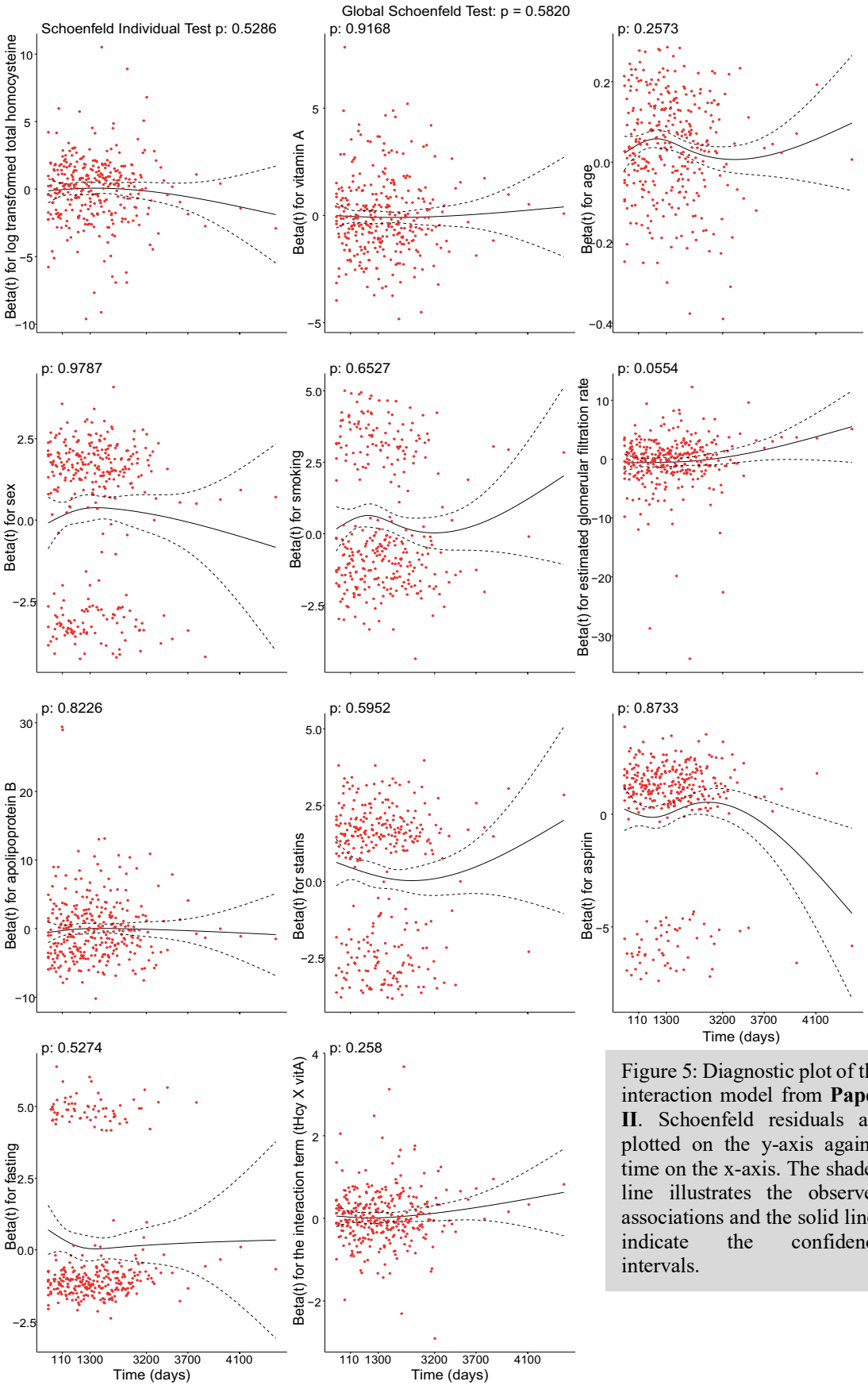

Figure 5: Diagnostic plot of the interaction model from **Paper II**. Schoenfeld residuals are plotted on the y-axis against time on the x-axis. The shaded line illustrates the observed associations and the solid lines indicate the confidence intervals.

Competing risks analysis

The concept of competing risks has gained substantial attention in cardiovascular epidemiology in recent years. In time-to-event analyses, a competing event is an event that precludes and hinders the event of interest [162]. For example, in **Paper II** we treated non-cardiovascular death as a competing event to AMI. The nature of traditional survival analysis treats the patients with a competing event as censored observations even if they are not able to experience the event of interest [160]. This represents a potential violation of an assumption in survival analysis demanding that censored observations are independent. In other words, traditional survival analysis assumes that subjects that are censored for any reason have the same probability of getting the event as those that remained alive. This may be unreasonable in the context of competing events, as subjects that die from non-cardiovascular causes will not get an AMI.

Because censored observations are removed from the risk set, this may lead to overestimation of the observed effect sizes [163-166]. In **Paper II**, we assessed whether the HRs of AMIs were affected by the presence of non-cardiovascular death in the risk set. Based on the assumption of independent censoring, a traditional CPH model would assume that censored subjects due to non-cardiovascular death would have the same probability of getting an AMI had the competing event not occurred, which may bias the results. Thus, we fitted the sub-distribution hazard function proposed by Fine & Gray [167]. The interpretation of the sub-distribution HR is similar to the interpretation of HRs from traditional CPH models.

Ordinary linear regression

For **Paper III** we used ordinary least squares linear regression for our main analyses. Model building and selection were generally performed in a similar manner as for the CPH procedure, but included a number of different covariates. We created several regression models, one for each hypothesized predictor, all of which were adjusted for age and sex unless otherwise specified. Moreover, for the 1962 participants that completed the FFQ we additionally included total energy intake in models including dietary predictors. Energy adjusted analyses are considered essential to minimize bias in models including dietary predictors [168]. We used the nutrient density method to adjust models with macronutrients as the main predictors, and the calculated units are

thus given in energy % (E %). Food groups and vitamin A intakes were given as densities (50g/1000 kcal and 200 RAE/1000 kcal, respectively).

For the sake of comparison, estimates were reported as standardized β, indicating the SD change in the outcome variable per SD increase in the predictor of interest. To increase interpretability, we also reported the unstandardized β and the 95 % CIs for each predictor. For models where both predictors and outcome were log-transformed, the unstandardized βs represent the % change in the outcome per % increase in the predictor. For models where only the outcome variable was log-transformed, the unstandardized βs were back-transformed and thus represent the % change in the outcome per unit increase in the predictor. In order to quantify the explained variance of the models, we calculated and reported the adjusted R^2 (R^2_{adj}). The R^2_{adj} is considered less biased than the unadjusted and often positively biased R^2 [169] and quantifies the explained variance in the outcome explained by the combination of predictor variables in a model. The R^2_{adj} was calculated for all models in **Paper III.**

Multiple comparisons

Multiple statistical tests are commonplace in epidemiological studies such as the individual projects included in this thesis. Briefly, when performing several statistical tests, some may yield significant p-values purely by chance and thus increase type 1 errors, i.e., the false rejection of null hypotheses. Several approaches for the adjustment of multiple testing were used in the manuscripts included in this thesis.

In **Paper I**, we applied the Benjamini-Hochberg procedure to correct for the false discovery rate [170]. This approach is considered less conservative and more powerful than the standard Bonferroni adjustment, and thus keeping the type 2 error rate relatively low. The critical p-value in **Paper I** was set to 0.04.

After careful consideration we elected not to adjust for multiplicity in **Paper II**. This decision was made on the basis of arguments by Rothman [171], that our analyses served with the purpose of generating hypotheses rather than making firm conclusions. This view has also been supported by others [172], and the critical p-value was thus set to < 0.05.

In **Paper III**, the above argument made for **Paper II** would also suffice. However, due to the large sample size and the fact that statistical significance are driven by a large *n* [173], we used the conservative Bonferroni correction in order to filter out

minor or negligible effects. After Bonferroni adjustment for multiplicity, the critical p-value was set to < 0.001.

Figures

All figures illustrating results in this thesis, including the figures from **Paper II** and **III** were made using the "ggplot2" package for R [174,175]. Figures made to assess linearity of associations were based on generalized additive models (GAM). This flexible modelling approach allows for the visualization of non-linear and linear relationships between predictor and outcome variables, and the mathematical basis have been described in detail elsewhere [176]. Moreover, we used the approach published previously by Lamina *et al.* to visualize interactions between predictor and outcome variables in **Paper II** [177].

Statistical software

All statistical analyses were carried out using R versions 3.2.1-3.4.4 [174]. Packages that were used for the main statistical methods and organization included "survival" [178] and the packages included in "tidyverse" [179].

4 Summary of results

4.1 Paper I – Cardiovascular disease risk associated with serum apolipoprotein B is modified by serum vitamin A

We examined the associations of apoB, apoA1 and their ratio (apoB/apoA1) with risk of incident AMI and the possible interaction with serum retinol in patients with stable angina pectoris. In total, 4117 patients were eligible for analysis and consisted of mostly males (72 %) with a median (IQR) age of 62 (55, 70) years. About 32 % were smokers, and 40 % had previously experienced an AMI. Median (IQR) apoA1 concentration in serum was 1.30 (1.13, 1.48) g/L, apoB was 0.87 (0.73, 1.04) g/L, the ratio of apoB to apoA1 was 0.67 (0.54, 0.84) and serum retinol concentrations were 2.82 (2.45, 3.29) μmol/L. In terms of prescription medication, about 72 % of the patients were statin users at baseline, whereas statin prescription was 80 % at discharge from the hospital. Median (IQR) follow-up to the first event was 4.6 (3.6, 5.7) years, and the incidence of AMI in this time-period was 8.2 %. When assessing the risk association of apoB, apoA1 and apoB/apoA1 with AMI in crude models for the total population, the HRs per SD (95 % CI) were 1.11 (1.00, 1.24, p = 0.06) for apoB, 0.85 (0.76, 0.94) for apoA1 and 1.21 (1.08, 1.34) for apoB/apoA1. These associations were attenuated after multivariate adjustment for age, sex, smoking, C-reactive protein, number of stenotic vessels, left ventricular ejection fraction, hypertension, statin use at discharge from hospital and eGFR (apoB, HR per SD 1.09 [0.98, 1.22, p = 0.115], apoA1, 0.95 [0.85, 1.06, p = 0.359], apoB/apoA1, 1.11 [0.99, 1.24], p = 0.070).

We observed significant interactions for apoB and apoB/A1 with serum retinol on incident AMI. When we stratified the population according to serum retinol tertiles (1^{st}: < 2.58 μmol/L, 2^{nd}: 2.58 – 3.09 μmol/L, 3^{rd}: > 3.10 μmol/L), there were trends for more hypertension, higher serum TGs, apoB, apoA1 and total cholesterol whereas the extent of coronary artery disease tended to be lower with increasing retinol concentrations. Overall, we observed no relevant associations between the apolipoproteins and risk of AMI in the 1^{st} and 2^{nd} serum retinol tertiles. In the 3^{rd} serum retinol tertile, we observed significant risk associations of log-transformed apoB (HR per SD 1.42, 95% CI [1.17, 1.73], p < 0.001), apoA1 (0.76 [0.63, 0.91], p = 0.003), and apoB/apoA1 (1.60 [1.31, 1.95], p < 0.001) in simple age- and sex-adjusted models. In the multivariate model the estimates were somewhat attenuated (apoB: 1.35 [1.11,

1.65], p = 0.002, apoA1: 0.87 [0.71, 1.05], p = 0.152, apoB/apoA1: 1.42 [1.16, 1.74], p < 0.001).

Finally, we performed sensitivity analyses from which patients receiving B-vitamin intervention in the WENBIT were excluded. We generally observed similar relationships between the apolipoproteins and AMI, but note that the inverse association between apoA1 and AMI was stronger and significant in the 3rd tertile of serum retinol (HR per SD: 0.61, 95% CI: 0.47, 0.80, p < 0.001).

4.2 Paper II – The risk association of plasma total homocysteine with acute myocardial infarction is modified by serum vitamin A

We explored the potential interaction between plasma tHcy and serum retinol in relation to incident AMI in patients with stable angina pectoris. The total study population consisted of 2205 patients after exclusion of patients receiving B-vitamin treatment in the WENBIT. The total cohort consisted of 64.3 % men, with a geometric mean (geometric SD) age of 62.3 (1.24). The cohort included 31.2 % smokers, and 37.1 % who had experienced a previous AMI. Serum retinol concentrations were 2.93 (1.28) μmol/L. The geometric mean (geometric SD) follow-up time were 7.0 (2.4) years, and the incidence of AMI was 15.1 %. In a simple model adjusted for age and sex (model 1), the HR per SD increase in log-transformed tHcy was 1.22 (95 % CI: 1.10, 1.35, p < 0.01). This relationship was attenuated in model 2 by further adjustment for smoking, estimated glomerular filtration rate, statin prescription, aspirin prescription, fasting and apoB (HR per SD: 1.12, 95 % CI: 0.99, 1.26, p = 0.071).

We observed a significant interaction of tHcy and retinol on incident AMI. We then stratified the cohort into tertiles of circulating retinol (1st tertile: < 2.65 μmol/L, 2nd: 2.65 – 3.20 μmol/L, 3rd: > 3.20 μmol/L). Patients across tertiles were of similar age whereas the upper tertile of serum retinol were more likely to contain men and hypertensive patients. In model 1, we observed a significant association for tHcy and AMI only in the 3rd retinol tertile (HR per SD 1.41, 95 % CI: (1.21, 1.64, p for interaction < 0.001). In the fully adjusted model 2, this association remained significant (HR per SD: 1.25, 95 % CI: 1.04, 1.52, p for interaction = 0.03).

To examine the possibility for competing risk of non-cardiovascular mortality, we carried out a competing risks analysis and calculated the subdistribution HR adjusting for the presence of non-coronary causes of death. Overall, 505 patients died,

of which 274 (54.3 %) from non-coronary causes. The association between tHcy and AMI in the upper tertile of retinol remained unchanged (subdistribution HR per SD: 1.24, 95 % CI: 1.04, 1.49, p for interaction = 0.03) when we adjusted for the presence of non-coronary causes.

Finally, we performed sensitivity analyses in patients with and without significant coronary stenosis. We observed a statistically significant interaction between tHcy and retinol in patients with, but not without, coronary significant coronary stenosis. In the upper tertile of retinol the HR per SD was 1.47 (95 % CI: 1.26, 1.71) in patients with stenosis, and 1.04 (95 % CI: 0.58, 1.56) in patients without stenosis in model 1.

4.3 Paper III – Homocysteine and related amino acids, inflammatory markers and creatinine are associated with serum retinol in patients with cardiovascular disease

We explored associations of CVD risk factors with serum concentrations of retinol in patients with stable angina pectoris. In total, 4115 patients were included in the outcome analyses. The cohort included 72 % males, 31 % smokers, and 40.3 % that had experienced an AMI. The geometric mean (geometric SD) age was 60.8 (1.2) years. Geometric mean (geometric SD) for circulating concentrations of relevant metabolites were: serum retinol concentrations; 2.84 (1.26) μmol/L, serum creatinine; 90.2 (1.22) μmol/L, serum CRP; 3.64 (2.73) mg/mL, serum uric acid; 347 (1.28) μmol/L, plasma neopterin; 8.57 (1.47) nmol/L and plasma tHcy; (1.38) μmol/L. Mean (SD) RAE intake/1000 kcal was 945 (606), whereas meat intake was 54.8 (23.4) g /1000 kcal.

In models adjusted for age and sex (model 1) the strongest predictors of serum retinol included serum creatinine (standardized β: 0.38 [95 % CI: 0.35, 0.42]), serum uric acid (0.30, [0.26, 0.33]), plasma total cysteine (0.26, [0.23, 0.29]) and serum TGs (0.26, [0.23, 0.29]). Plasma serine (-0.15, [-0.18, -0.12]) and serum C-reactive protein (-0.15, [-0.18, -0.12]) were negatively associated with serum retinol. When we additionally adjusted the models for serum creatinine, which is a significant confounder of several of the hypothesized associations, the associations for uric acid (0.20, [0.27, 0.24]) and total cysteine (0.17, [0.13, 0.20]) remained significant. All the observed associations were linear. When we included all variables that were significant after

adjustment for multiple testing into one model, the total fraction of variance explained for serum vitamin A was 30 %.

Of the 4115 patients that were included, 1962 completed the FFQ. A weak association for energy-adjusted meat (standardized β: 0.08, [95 % CI: 0.03, 0.12]) and vegetable (0.04, [0, 0.08]) intake and serum retinol concentrations were observed in an age-, and sex-adjusted model. No associations were observed for other food groups including fruits and berries, eggs, dairy and fish. Dietary RAE intake was not significantly associated with serum retinol concentrations (0.02, [-0.03, 0.06]).

The associations were similar when we stratified the population according to serum retinol tertiles.

5 Discussion

5.1 Methodological considerations

5.1.1 Study design

Prospective and cross-sectional design

The studies in **Paper I** and **II** were of prospective design, relating the exposures (retinol, apoB, apoB/apoA1, tHcy) to an outcome occurring in the future (AMI). For the study in **Paper III** we used a cross-sectional design to evaluate associations between potential predictive factors of retinol in serum. In contrast to a prospective design, establishing the direction of an association is impossible in cross-sectional studies because the exposure and outcome variables are measured at the same time. For example, in **Paper III**, we could not conclude that elevated concentrations of plasma total cysteine lead to elevated retinol concentrations in serum, or vice versa. Some inherent weaknesses pertaining to both cohort and cross-sectional studies should be considered.

Subgroup analyses

One purpose of observational studies is to gain some understanding about relevant exposure-disease relationships. This purpose relies to a certain degree on comparing disease risk in subgroups of exposed and non-exposed individuals in order to generate new hypotheses that can be tested more formally in clinical trials. However, an exaggerated use of data mining to identify subgroups in the population that are at increased risk of the disease can be problematic [180]. Problems may arise for several reasons such as decreased power due to smaller group sizes and the type 1 errors that may occur as a consequence of multiple comparisons (see section 5.1.4). Combined, these potential sources of error can make it difficult to separate signal from noise in the data and may contribute to the so-called reproducibility crisis in science [181]. Guidelines for observational research stress that results reported from subgroup studies in which hypotheses have not been defined *a priori* should be interpreted as purely exploratory and even downplayed in results, discussions and conclusions [180,182,183]. Moreover, tests for interaction, in which one covariate modifies the effect of another on a given outcome, should be clearly specified. Although it can be argued that subgroup analyses should be avoided in its entirety based on the limitations

discussed above, they can be useful for generating hypotheses and identifying groups of the population that may be at excessive risk of disease [184,185]. Such findings can help in designing better trials in the future, in order to optimize treatment and prevention in the present era of personalized cardiovascular medicine [186]. In any case, results from such analyses should be interpreted carefully, and all details of analyses should be provided to ensure reproducibility.

In **Paper I** and **II** we grouped the population according to tertiles of circulating retinol concentrations and assessed lipid- and tHcy-associated risk of incident CVD. Considering that we ignored the initial randomization to B-vitamin treatment, the division of the population by retinol concentrations can lead to problems such as skewed distributions of measured and unmeasured confounding factors (see section 5.1.2). However, we provided thorough details of the analyses in the methods section to enhance reproducibility in future investigations. We also stressed on multiple occasions throughout both manuscripts that the results should be interpreted with care and considered exploratory until future studies can confirm or refute our findings. Finally, the current subgroup analyses were founded in biologically plausible mechanisms that will be discussed in greater detail below.

5.1.2 Bias

Random error, regression dilution and exposure misclassification

In all three papers we only measured the exposure variables and model covariates once. This is problematic because a sole baseline measurement is prone to random error that can lead to exposure misclassification, i.e., participants are allocated to the wrong exposure group [187]. One consequence of exposure misclassification is that the observed values deviate from the true values. This phenomenon manifests itself in statistical analyses as regression dilution, which occurs when the sum of random errors in the baseline data bias the disease-associations towards the null [188]. In **Paper II** that addresses the relationship between tHcy and AMI, we decided to exclude participants that underwent B-vitamin treatment, which would affect plasma tHcy concentrations as we expected exposure classification of the participants to change after study commencement. Although the same arguments can be made against retinol and apolipoproteins, we point out that retinol is normally stable over time [109]. This is also reflected in the intraclass correlation coefficient which was shown to be excellent for

retinol concentrations in serum [152]. As for apolipoproteins, around 80 % of the population was treated with statins upon discharge from the hospital, and it is probable that lipoprotein concentrations would remain relatively low across the entire cohort over time, especially considering that statin treatment at baseline was around 70 %. In summary, random error may have introduced bias in our data, and consequently on the evaluated disease-relationship. However, given that the stability of especially retinol seems to be adequate in serum, the magnitude of regression dilution bias was hopefully low for the main exposure variables. But, imprecision in measurement of confounders may also contribute to bias, as discussed below.

Confounding

A confounding factor is by definition a variable that is associated with the exposure and the outcome of a study, that can introduce bias if it is not accounted for [189]. Ideally, in a prospective cohort study, exposed and unexposed groups are assumed to be exchangeable. In other words, the disease frequency or risk in the exposed group is assumed to be similar to the unexposed group if the exposure had not been present. In contrast to trials where this exchangeability can be achieved by randomization, it is rarely the case for prospective cohort studies. This pose a problem in all three papers included in this thesis but especially in **Paper I** and **II**.

When we assessed AMI risk for apoB, apoA1, and tHcy in **Paper I** and **II**, we subdivided the population into tertiles according to serum retinol. The patient characteristic tables found in the supplement of **Paper I** and in the main text of **Paper II** clearly show that the distribution of common risk factors, including history of CVD and inflammatory markers were not evenly distributed between the groups. The skewed distribution of these factors can lead to confounding and unpredictably biased risk estimates by influencing the interaction of apoB, apoA1, apoB/A1 (**Paper I**) and tHcy (**Paper II**) with retinol in relation to AMI events. To deal with confounding, we adjusted for known risk factors of CVD that were also associated with the predictors in the models.

However, simply adjusting the statistical models for confounding factors will not necessarily eliminate bias, as measurement error in their data collection may impact attempts to adjust for them. For example, imprecise data collection on confounding factors can attenuate the relationship between confounder and outcome [190,191], whereas effects on the confounder-exposure association is less predictable [192]. Thus,

residual confounding that we were unable to adjust for, remains, and we should take care in declaring our results as independent [192-194]. One example from our models is smoking status, which was categorized as never, former and present smoker. Because this measure is categorial, we are unable to adjust for smoking intensity, which may be a source of residual confounding. In addition, although we had data on a multitude of potential confounders, we cannot exclude the possibility of unmeasured confounding by other unknown factors.

Information bias

Information bias arises when we randomly or inaccurately collect data that are imprecise and deviate from their true values. There are several examples of information bias in observational research, including but not limited to outcome validation and recall bias. In this project, the AMI outcome variable of the prospective analyses in **Paper I** and **Paper II** was defined according to the consensus statement published by the Joint European Society of Cardiology and the American College of Cardiology [148]. Outcome information was thus likely to be accurate, and complied with international standards. In **Paper I**, endpoint information was obtained from the WENOCARD that is linked to the 11-digit personal number of each participant. An endpoint committee subsequently validated the outcome [147]. This rigorous practice minimizes the risk of bias in the outcome information in **Paper I**, but we cannot exclude the possibility of inaccurate reporting and erroneous validation by the end-point committee. In **Paper II**, outcome information up to 2009 was derived from the CVDNOR project, and the diagnostic data are thus contributed by hospitals. The data quality has been described in detail elsewhere [149,195], and included both in-hospital and out-of-hospital deaths following AMI [9]. As for **Paper I**, we cannot exclude the possibility of improper reporting of outcomes in **Paper II**. The definition of incident AMI was based on the presumption that no AMIs had occurred <7 years prior to the incident event. We cannot exclude the possibility that an AMI had occurred before this 7-year limit.

Another form of bias that is common to observational research is introduced by the use of questionnaires [196]. For example, inaccuracies in the questionnaire and recall bias in the participants may lead to errors in the data collection that in turn bias results. In **Paper III**, we used data from FFQs to study dietary determinants of serum retinol in the circulation. The main purpose of using FFQs is to record habitual dietary

intakes of the participants. Questionnaires of this type is prone to severe bias including misreporting of dietary intakes, additive bias during conversion into food weights and nutrient intakes, person-specific biases and random error, all of which may lead to significant misclassification of participant exposure [197]. Another issue with the FFQs used in this population is that although they have been validated previously for energy intake and some macronutrients [155-157], the misclassification of dietary vitamin A intakes in the FFQs compared to 24-hour recall was around 60 % [156], which could have introduced significant bias in our results concerning the effect of vitamin A intakes on retinol concentrations in the blood. More or less all of the estimates related to dietary predictors of serum retinol were close to the null, and it should be noted that the misclassification introduced by systematic bias in the FFQs generally biases associations towards the null. Isolated, this can be a problem and lead to type 2 errors. We note however, that retinol in circulation is generally not associated with dietary intakes because of its stringent homeostatic regulation [109].

Selection bias

Selection bias refers to systematic differences between the study sample and the population to which we want to generalize our findings. In this project, the population consisted of patients admitted to the hospital for suspected coronary artery disease with stable angina pectoris that subsequently underwent coronary angiography. Because stable angina pectoris is not necessarily angiographically verified, it can be argued that our population have more severe disease and are at an increased risk compared to stable angina pectoris patients that are not admitted to the hospital.

One aspect of selection that we addressed in **Paper II** was the exclusion of about 50 % of the population because they received B-vitamin treatment. The reason for this was that baseline concentrations of plasma tHcy were likely to change in this group after the commencement of the B-vitamin supplementation [147], and could have complicated the interpretation of our findings. To evaluate whether the excluded portion differed systematically from the included portion of the patient population, we have presented key characteristics in **Table 1**. The non-B-vitamin treated patients had lower prevalence of coronary stenosis at baseline. However, other key risk factors including previous AMIs and the incidence of new AMIs during follow-up were similar in both populations. An argument can also be made that the exclusion was unnecessary considering that the B-vitamin treatment was not associated with the outcome as

reported previously [147]. However, because the ranks of participants were likely to change, we decided to proceed with the exclusions for the sake of interpretability.

Table 1: Key characteristics at baseline for patients receiving no B-vitamin treatment (included in **Paper II**) compared to those who did (excluded from **Paper II**)[1,2]

	No B-vitamin treatment (*n* = 2205)	**B-vitamin treatment (*n* = 1912)**
Retinol, μmol/L	2.93 (1.28)	2.73 (1.24)
Male sex, n (%)	1418 (64.3 %)	1542 (80.6 %)
Age, y	62.3 (1.24)	61 (1.18)
ApoB, g/L	0.89 (1.31)	0.85 (1.3)
ApoA1, g/L	1.33 (1.22)	1.25 (1.23)
Triglycerides, mmol/L	1.51 (1.67)	1.58 (1.67)
tHcy, μmol/L	11.0 (1.40)	10.4 (1.36)
eGFR, mL/min per 1.73 m^2	83.0 (1.34)	88.6 (1.22)
BMI, kg/m^2	26.4 (1.16)	26.6 (1.14)
CRP, mg/mL	3.24 (2.67)	4.18 (2.08)
1-3 stenotic vessels, n (%)	1380 (62.6 %)	1701 (89.0 %)
Smokers, n (%)	689 (31.2 %)	614 (32.1 %)
LVEF < 60, n (%)	1784 (80.9 %)	1456 (76.2 %)
Previous AMI, n (%)	817 (37.1 %)	841 (44.0 %)
Incident AMI during follow-up, n (%)	333 (15.1 %)	298 (15.6 %)

[1]Abbreviations: ApoB, apolipoprotein B; apoA1, apolipoprotein A1; tHcy, total homocysteine; eGFR, estimated glomerular filtration rate; BMI, body mass index; CRP, C-reactive protein; LVEF, left ventricular ejection fraction; AMI, acute myocardial infarction.
[2]Continuous variables are presented as geometric means (geometric standard deviations)

In **Paper III**, we used an FFQ to assess habitual dietary intakes among the participants. Of the study population that was handed the FFQ (only WENBIT participants), about 20 % did not return it. It is possible that the presence of health-conscious individuals, which are generally more compliant to study protocols, may have introduced some selection bias to our results. However, the portion of patients that did not complete the FFQ in **Paper III** did not differ systematically to those that completed the FFQ as

presented in **Table 2**. However, we note that non-completers had slightly higher incidence of AMI during follow-up compared to completers, although the distribution of baseline risk factors were somewhat similar.

Table 2: Key characteristics at baseline for patients that returned FFQs and those that did not[1,2]

	Returned FFQ (n = 1962)	**Did not return FFQ (n = 481)**
Retinol, μmol/L	2.75 (1.24)	2.68 (1.26)
Male sex, n (%)	1569 (80.2 %)	376 (78.2 %)
Age, y	60.9 (1.18)	61.1 (1.2)
ApoB, g/L	0.85 (1.3)	0.87 (1.32)
ApoA1, g/L	1.25 (1.22)	1.23 (1.26)
Triglycerides, mmol/L	1.57 (1.64)	1.6 (1.7)
tHcy, μmol/L	10.4 (1.34)	10.7 (1.43)
eGFR, mL/min per 1.73 m^2	88.3 (1.22)	88.3 (1.25)
BMI, kg/m^2	26.6 (1.14)	26.8 (1.15)
CRP, mg/mL	4.09 (2.1)	4.61 (2.07)
1-3 stenotic vessels, n (%)	1737 (88.8 %)	432 (89.8 %)
Smokers, n (%)	582 (29.8 %)	200 (41.6 %)
LVEF < 60, n (%)	1519 (77.7 %)	341 (70.9 %)
Previous AMI, n (%)	854 (43.7 %)	228 (47.4 %)
Incident AMI during follow-up, n (%)	295 (15.1 %)	89 (18.5 %)

[1]Abbreviations: ApoB, apolipoprotein B; apoA1, apolipoprotein A1; tHcy, total homocysteine; eGFR, estimated glomerular filtration rate; BMI, body mass index; CRP, C-reactive protein; LVEF, left ventricular ejection fraction; AMI, acute myocardial infarction.
[2]Continuous variables are presented as geometric means (geometric standard deviations)

5.1.3 Generalizability

Results from the present population do not necessarily translate to a general population, but rather to a population with established CVD. The present population included mostly Caucasian men, in which more than 80 % were treated with lipid-lowering therapies. In addition, a substantial proportion of the population were discharged with B-vitamin treatment. In other words, it can be argued that our findings only translate to individuals in intensive secondary prevention of major cardiovascular events.

Compared to other cohorts where patients with coronary artery disease were enrolled, such as the Euro Heart Survey on Coronary Revascularization [198], the WECAC cohort generally displayed similar characteristics with respect to age, sex, previous AMIs, coronary artery bypass graft surgery and percutaneous coronary interventions. However, smoking was more prevalent among WECAC patients. In addition, prescription of beta-blockers and aspirin at discharge were similar in another sub-cohort of the Euro Heart Survey on Coronary Revascularization compared to WECAC, although statin prescription in the WECAC was about ~10 % higher at discharge [199]. Based on two papers published by Sulo *et al.* from the CVDNOR project, we can roughly calculate the incidence of AMI to be between around 9 % between 2001 and 2009 in the Norwegian population [8,9], whereas the incidence in our cohort was more than 15 % in the same time-frame. A description of other risk factors in initially healthy and representative populations is given in **Table 3**. WECAC differ in key aspects such as smoking and use of prescription medication, which makes it difficult to generalize our findings to the general population. However, with respect to the presence of cardiovascular risk factors, patients in the WECAC are similar to other, large European cohorts, and the findings can be generalized to patients with established CVD.

Table 3: Key characteristics at baseline for patients in the WECAC and other Norwegian cohorts[1,2]

	WECAC (n = 4115)	**Data from healthy reference populations**
Incidence of AMI 2001-2009	15 %	9 %[3]
Total cholesterol, mmol/L	5.1 (1.2)	5.5 (1.1)[4]
Age, y	61.2 (10.4)	57.3 (11.4)[4]
BMI, kg/m^2	26.8 (4.0)	27.2 (4.4)[5]
Smokers, n (%)	582 (29.8 %)	6383 (12.6 %)[5]
Lipid-lowering, %	80.0 %	28.0 %[6]

[1]Data is given as mean (standard deviation) for continuous variables and count (%) for categorical variables
[2]Abbreviations: AMI, acute myocardial infarction
[3]Data obtained from Sulo *et al* (*n* =1.5 million) [8,9]
[4]Data obtained from Tromsø 7 (*n* = 21083) [200]
[5]Data obtained from HUNT 3 (*n* = 50666) [201,202]
[6]Data obtained from the Norwegian Prescription Database [203]

It is important to note that the serum retinol concentrations observed in this particular population is higher than in persons that reside in the same geographical area [204] and compared to other cohorts consisting of initially healthy populations [205,206]. It is not known whether other cohorts consisting of patients with established CVD present with similar retinol concentrations, and thus whether the results regarding the effect-modifying properties of retinol are transferrable to other similar populations.

5.1.4 Statistical methods

We applied well-known statistical methods in all papers. The main outcome analyses in **Paper I** and **II** included CPH models which are semi-parametric and considered robust to deviations from normality in the data [160]. The assumption of proportional hazards was met in both papers. Because categorization can lead to information loss [207], we tested interaction between retinol and apoB, apoA1 and apoB/apoA1 (**Paper I**), and tHcy (**Paper II)** formally on a continuous scale and then by stratification. In **Paper I**, the population was categorized according to tertiles of serum retinol, which may have led to some loss of information. In **Paper II**, we applied the approach of Lamina *et al*. to initially plot the interactions on a continuous scale [177]. By doing so, we were able to identify a threshold where the risk associated with tHcy appeared to increase, and which corresponded to the third tertile. Thus, the initial interaction plot justified the decision to look more closely at the risk within tertiles.

In **Paper III**, the main outcome analyses included ordinary least squares regression. Because the data were not normally distributed, we log-transformed the data. Although it can be argued that this sort of transformation complicates interpretation, it allows for easy calculation of units and estimates in percentage, which should be familiar to most readers. The log-transformation yielded normally distributed and less heterogenic residuals, which are key assumptions in linear regression.

Because non-linear associations can bias estimates obtained from linear regressions or simple correlational analyses and otherwise obscure true effects, we assessed linearity of associations by creating GAM-curves in **Paper II** and **III**. GAM-curves can aid in the interpretation of findings and the underlying data, as well as provide insight in the potential biological mechanisms at play. In **Paper II**, the GAM-curves revealed variable and inconclusive associations between tHcy and AMI in the 1st and 2nd retinol tertile, whereas this association was linear in the 3rd tertile. In **Paper**

III, the associations between the different covariates and serum retinol were more or less linear.

Model building

There are no specific guidelines for model building and covariate selection for multivariate statistical models [208]. In all papers we built models primarily based on the epidemiological method. To be specific, we identified potential confounding factors and entered them separately into the model. If the covariates changed the effect estimate of the exposure on the outcome by >10 % we included them in the final multivariate model as proposed previously [161]. This approach is data-driven and thus there is some risk of unnecessary overadjustment bias that in turn can induce rather than reduce bias [209]. In hindsight, methods for evaluating the impact of the model covariate selection such as the use of directed acyclic graphs (DAGs) should have been applied [210]. One problem with DAGs is that they are purely qualitative, and thus should be used in combination with considerations of biological plausibility and initial data analysis that determine the strength of associations between the exposure and the outcome.

Multiple comparisons

The literature is torn on the topic of multiple comparisons in observational research. There has been some debate on strategies for adjustment of multiplicity, and whether one should correct for multiplicity at all in exploratory studies [211,171,172]. The issue revolves around the thought that more tests will increase the probability of finding interesting results, and thus unfortunately, false positives [181]. Adjusting the p-value for multiple tests reduce the false positive rate, but can in turn decrease power and increase type 2 errors [171]. In **Paper I** we adjusted the p-values by using the approach of Benjamini-Hochberg, whereas we opted not to adjust for them in **Paper II**. The reason for this is that our tests are purely exploratory and not meant to inform decision-making, but rather generation of new and testable hypotheses [171,172]. As Rothman argues, it is better that new hypotheses are tested and eventually refuted in subsequent studies, rather than inflating type 2 errors and obscuring potentially important results by adjusting p-values. With this in mind, we argue that the p-value adjustments in **Paper I** were unnecessary. Another timely reason for not adjusting for multiple testing is the unjustified focus on the p-value as an indication of an actual effect. In fact, the

American Statistical Association have published several statements in recent years discussing the drawbacks of p-values in science [212] and ultimately urging scientists to abandon them completely [213]. Thus, in this project we have we stress that the effects should be interpreted as they are, and tried not to rely too heavily on p-values.

5.2 Ethical considerations

Long-term follow-up of the population in this study has been approved by the Regional Ethical Committee, and the study protocols adhere to the Declaration of Helsinki. The patients that were enrolled in this cohort were recruited upon hospitalization primarily due to health concerns, and were not subject to unnecessary hazards related to the study.

5.3 Discussion of results

Discussion of the results will be organized in two parts. The first part will discuss the few epidemiological considerations and possible mechanisms underlying the findings from **Paper I** and **II**. **Paper III** will be discussed separately in the next section.

5.3.1 Epidemiological considerations – Paper I and II

The results that reported in **Paper I** and **II** are difficult to put in an epidemiological context because very few or no other studies that we are aware of have assessed the interaction between lipid parameters with retinol, and tHcy with retinol in the context of CVD. Serum retinol has been associated with increasing lipid parameters in serum [126,127], and the retinol transport protein RBP4 has been associated with decreased clearance of VLDL [214] and hypertriglyceridemia [215] but no studies that we know of have explored the potential interaction between lipid parameters and serum retinol. Retinol has in itself been both positively [127,135] and negatively [126,127,139] associated with lifestyle diseases and mortality, but whether or not these effects are mediated through interaction with lipid or homocysteine metabolism, or are independent, have not been assessed. One recently published nested case-control study, reported an interaction between plasma tHcy and retinol on the association with stroke [139]. Notably, in patients with baseline tHcy < 10 μmol/L the inverse association between retinol and first stroke was stronger, whereas this supposed protective effect was not present in subjects with tHcy > 10 μmol/L. Interestingly, the seemingly protective effects of retinol diminished as tHcy increased, and it would be interesting to assess whether tHcy-associated risk of stroke was modified by retinol in this population. The results from this particular study differs somewhat from our own in that tHcy exacerbates the effects of low retinol concentrations on stroke incidence, whereas our focus has been on the effect-modifying properties of retinol on tHcy. Taken together, the findings presented in **Paper I** and **II**, can shed new light on the epidemiological associations between retinol and lifestyle diseases.

5.3.2 Potential mechanisms - preface

The discussion that follow will be based in potential biological mechanisms that may underlie the reported findings. Although the studies in the present project are of observational nature, the results presented are novel, and a comprehensive discussion

of plausible mechanisms are of particular importance in the pursuit of new and testable hypotheses. As outlined in the introduction, retinol is both positively and negatively associated with disease and mortality in observational studies. Also, in experimental studies vitamin A show some ambiguity depending on the retinoid that is under study, and which model system is being used and tissue examined. To exemplify this, one study by Bilbija *et al.* demonstrated that RA signaling is enhanced in the human atherosclerotic plaques of coronary heart disease [216], whereas one study by Trasino *et al.* demonstrate that RA signaling is decreased in the liver of obese mice that otherwise exhibit increased serum concentrations [134]. Moreover, a recent study showed that LRAT, which is crucial for storage of vitamin A as RE in tissues, is downregulated in the metabolic syndrome [217], possibly contributing to increased serum concentrations. The findings from **Paper I** and **II** will thus be discussed based on the assumptions that elevated circulating retinol reflects 1) increased RA availability and signaling (section 5.3.2, *tissue sufficiency*, or 2) decreased RA availability signaling (section 5.3.3, *tissue insufficiency*).

Perspective - Tissue sufficiency

Interaction between apoB and retinol

An overview of the relevant mechanisms in this section is presented in **Figure 6**. In **Paper I**, we present findings showing that the risk of AMI associated with apoB and the ratio of apoB to apoA1 was modified by retinol. The strongest association for apoB and apoB/A1 with AMI was observed among patients with circulating concentrations of serum retinol concentrations exceeding 3.10 μmol/L. This interaction indicates that elevated serum retinol potentiates the pro-atherogenic properties of apoB which is retained in the vascular wall during progression of the atherosclerotic lesion [25,218].

A central process in atherosclerosis is the migration of macrophages to the inner layer of the arterial wall, where they will scavenge oxidized apoB-containing LDL-particles and develop a foam-like exterior [17,219]. One explanation for our finding may thus be the demonstration that all-*trans*-RA can upregulate expression of scavenger receptors in monocytes residing in the endothelial wall [131]. This experiment by Wuttge and colleagues demonstrated that treatment of cells with all-*trans*-RA not only increased the expression of CD36 (a membrane glycoprotein present

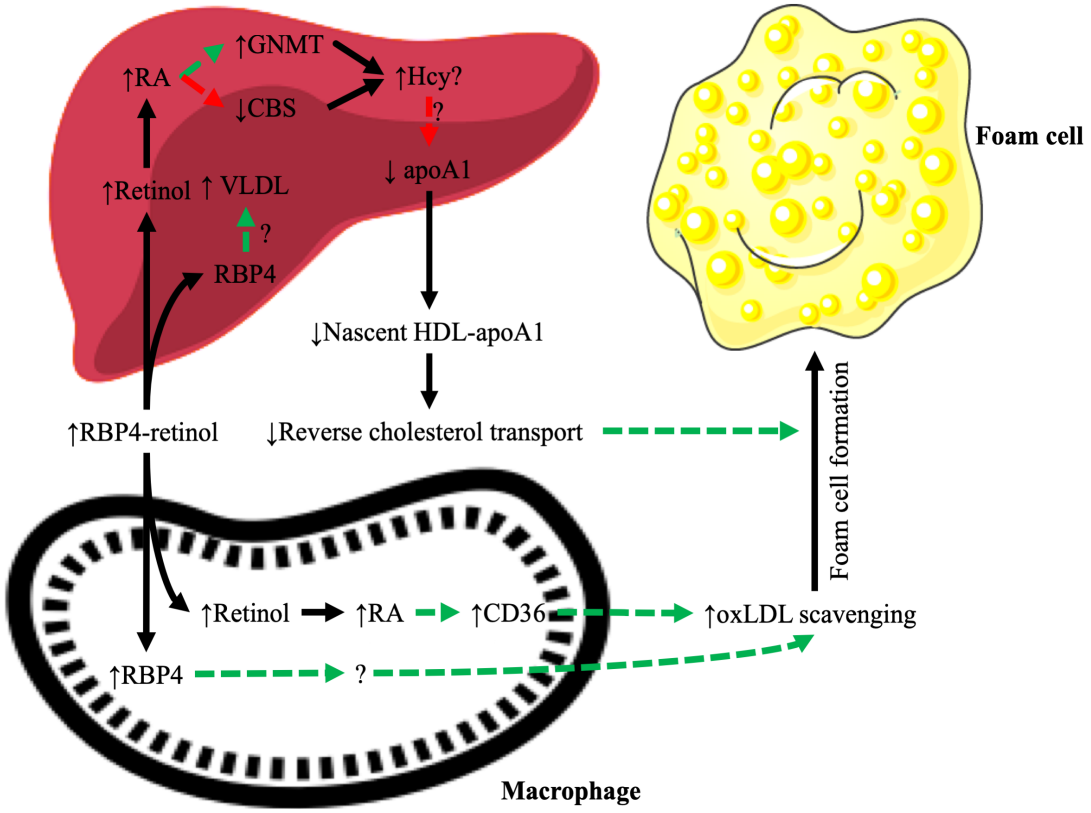

Figure 6: Proposed mechanisms in tissue sufficiency when retinol concentrations in circulation are thought to reflect intracellular concentrations. Increased intracellular retinol can serve as a precursor for retinoic acid synthesis which in turn can affect intrahepatic homocysteine metabolism and consequently apolipoprotein A1. Increased retinoic acid in macrophages can upregulate CD36 and subsequently scavenging of oxidized LDL and lead to foam cell formation. Retinol-binding protein-4 can exacerbate oxidized LDL scavenging in macrophages and disrupt lipid metabolism in the liver. Abbreviations: ApoA1, apolipoprotein A1; GNMT, glycine N-methyltransferase; Hcy, homocysteine; HDL; High-density lipoprotein; RA, retinoic acid; RBP4, retinol-binding protein-4; oxLDL, oxidized low-density lipoprotein; VLDL, very low-density lipoprotein.

on different cells, including macrophages), but also led to increased macrophage scavenging of oxLDL. Importantly, oxLDL can in turn promote continued expression of CD36 and uncontrolled macrophage uptake of oxLDL [220,221,131]. In addition to all-*trans*-RA signaling and activity, a recent publication by Liu *et al.* demonstrated that both apo- and holo-RBP4 can promote cholesterol uptake and macrophage foam cell formation [133]. Whether the delivery of retinol to the tissues and subsequent RA signaling is involved was not reported. Another relevant role for RBP4 includes the apparent inverse association with VLDL clearance from the circulation. One *in vivo* kinetic sub-study study among 221 patients with type 2 diabetes mellitus, showed that RBP4 in plasma was strongly and inversely associated (r = -0.72) with the fractional catabolic rate of VLDL [214]. Moreover, synthetic retinoid administration inhibits lipoprotein lipase activity in the skeletal muscle [222]. The increased exposure of apoB-

containing lipoprotein particles to macrophages that reside in the endothelial wall by decreasing their clearance, may be another explanation for why we observed the interactive effects between apoB and retinol in **Paper I**.

Interaction between plasma total homocysteine and vitamin A

In **Paper II**, we observed a significant interaction between plasma tHcy concentrations and retinol on AMI risk. We have identified some potential mechanisms that may underlie these interactions, but only if retinol in serum correlates to actual RA signaling intracellularly.

Perhaps the most relevant mechanism involves the activity of the GNMT enzyme. GNMT is a methyltransferase that scavenges methyl groups from SAM in times of excess intracellular concentrations, forming the biologically inert sarcosine, and SAH [146,223-225], which is then readily converted to homocysteine [226]. Administration of vitamin A in the form of all-*trans*-RA induced GNMT activity in the liver of mice [143,144,227-230]. The potentially increased GNMT activity induced by retinol/RA can affect homocysteine metabolism in the liver and be a mediator of the interaction we observed in our population. Indeed, all-*trans*-RA administration simultaneously prevents the expression of CBS [229], the rate-limiting enzyme in homocysteine degradation [231]. Thus, retinol may exert intra-hepatic effects on homocysteine metabolism with consequences for lipid metabolism and CVD risk (see below).

Homocysteine, apoA1 and vitamin A – A potential interplay

Based on the findings discussed above, we speculate that patients within the upper retinol tertile may have increased RA signaling in the liver as well as increased GNMT activity consistent with previous findings [143,144,227-230]. Upon GNMT induction and CBS inhibition, homocysteine may increase in the liver, which in turn may confer profound metabolic effects that may explain why tHcy is more closely associated with AMI risk in patients with high serum concentrations of retinol. One intriguing possibility that ties together the results from **Paper I** and **II** in this project, is that homocysteine may disrupt the synthesis of apoA1 in the liver and inhibit reverse cholesterol transport. Indeed, several observational studies have shown that plasma tHcy was inversely associated with apoA1-containing HDL particles in the circulation [232-234]. Animal experiments with $CBS^{-/-}$ models, had hyperhomocysteinemia,

hypercholesterolemia and reduced HDL-cholesterol in the circulation [235]. In patients with coronary artery disease, HDL was inversely associated with plasma tHcy [236] and homocysteine led to reduced apoA1 synthesis in the liver [237]. Thus, the increased risk of AMI associated with plasma tHcy in the upper tertile of serum retinol may be related to increased GNMT activity, decreased CBS activation and hepatic accumulation of homocysteine. This may serve as a potential explanation for the inverse association observed for apoA1 with AMI among patients with high circulating concentrations of retinol. However, the potential relationship with GNMT is complex and may also mediate beneficial effects as discussed in greater detail in the next section.

Perspective - Tissue insufficiency

Another biological mechanism for the observed interactions includes the possibility that elevated retinol in the circulation signifies reduced concentrations in the tissues and dysregulated RA signaling. One experimental mouse model of the metabolic syndrome showed that blood concentrations of retinol were high when tissue concentrations were low and led to reduced RA signaling in the liver [134]. In addition, storage capacity of vitamin A seems to be lower in the metabolic syndrome, as signified by lower LRAT expression in a young population with this condition [217]. This is interesting as metabolic diseases including metabolic syndrome are directly relevant to the development of CVD. The following paragraphs will discuss mechanisms related to reduced intracellular RA signaling.

Plasma apoB and retinol

Several animal studies show beneficial effects of retinoic administration on lipid metabolism [130]. Although human participants treated with synthetic retinoids develop dyslipidemia [238,239], mice treated with all-*trans*-RA for 4 days had lower circulating apoB-containing VLDL concentrations and experienced favorable changes in gene expression related to lipid metabolism [240]. Similar effects have been shown after administration of one oral dose of all-*trans*-RA in mice with vitamin A deficiency [241]. RAR agonism has been proposed as a potent inhibitor of apolipoprotein C-III [242], a positive regulator of plasma TG concentrations. Collectively, these observations suggest that vitamin A is required for normal lipid metabolism in tissues. In a situation where increased serum concentrations of retinol may reflect low

intracellular concentrations; lipid metabolism may be dysregulated as a result and unfavorably affect CVD risk.

Interaction between apoB, apoA1 and retinol

We observed a clear trend for an interaction between retinol and apoA1 in the total population of **Paper I**. In addition, when we excluded patients who received B-vitamin treatment, there was a statistically significant interaction between apoA1 and retinol with respect to CVD risk. Thus, we can assume that patients in the upper retinol tertile with low concentrations of apoA1 are at an increased risk of AMI, which will be the basis of the following discussion.

Although the view of apoA1 as anti-atherogenic has been challenged lately [243], it has traditionally been described in this way due to its effects in peripheral cholesterol metabolism, including the transport of cholesterol from peripheral tissues back to the liver [53]. Efflux of intracellular cholesterol and phospholipids from monocytes and macrophages to apoA1 in the circulation is mediated by a set of transport proteins including ABCA1 and ABCG2 [244]. In 2003, Costet and co-workers demonstrated that mRNA and protein concentrations of ABCA1, and to a moderate extent, ABCG1, were upregulated in macrophages that were treated with all-*trans*-RA [245,246]. The described *in vitro* effects of vitamin A on apoA1 reverse cholesterol transport thus indicate that apoA1 and HDL should be higher in patients with elevated retinol. However, assuming that circulating concentrations of retinol were high when tissue concentrations were low [134,217], inefficient RA signaling in macrophages may have led to lower cholesterol efflux. This may in turn result in cholesterol accumulation in plaques and foam cell formation.

A final, and intriguing hypothesis revolve around the suggested dual role for apoB-containing oxLDL in the pathogenesis of atherosclerosis. Whereas the prevailing view is that macrophage scavenging of oxLDL leads to atherosclerotic progression [17,219], others have reported that it can exert anti-inflammatory effects through the upregulation of relevant, anti-inflammatory genes in immune cells [247-249]. In addition, oxysterols that are present on oxLDL [250] are ligands for the nuclear transcription factor, liver X receptor (LXR) [251], which can upregulate expression of RALDH1 and consequently RA synthesis in the liver [252]. RA signaling is required for a wide specter of regulatory processes, including normal monocyte differentiation to macrophages [253]. Taken together, these demonstrations may be of importance in

considering the total evidence in **Paper I**. Here, we observed that the association between apoB/A1-ratio and AMI in patients with high circulating concentrations of retinol, was likely driven by low concentrations of apoA1. ApoA1-containing HDLs transfer cholesterol in exchange for TGs to apoB-containing LDLs in the circulation in a reaction facilitated by cholesteryl ester transfer protein. In theory, low apoA1/HDL may reflect less cholesterol exchange between HDL and LDL, which in turn may impair LXR-induced synthesis of RA in the liver. Considering that 1) RA promote hepatic GNMT induction [143,144,227-230], and 2) that hepatic GNMT activity has emerged as an important regulator of hepatic cholesterol trafficking with beneficial effects in non-alcoholic fatty liver disease (NAFLD) [145], low RA signaling in the liver may have downstream effects that are detrimental to hepatic immune response and lipid metabolism, which over time potentially can promote NAFLD and increase the risk of CVD [254]. In addition, GNMT ablation in apoE$^{-/-}$ mice exacerbated hyperlipidemia, inflammation and atherosclerotic progression [255]. Thus, future research should explore whether elevated retinol concentrations are actually linked to a protective mechanism that may fail under circumstances with low apoA1 or high tHcy concentrations.

5.3.3 Discussion of Paper III

After revealing new and potentially important interactions between common risk factors of CVD and retinol, we proceeded to evaluate which covariates that most strongly correlated with retinol in the same core population in **Paper III**. Because serum retinol is thought to be kept under tight homeostatic control [109], the interest in identifying potential determinants have been relatively limited. One recent study evaluating serum retinol among Norwegian adolescents showed weak associations for fat mass, albumin level, physical activity and lunch habits with serum retinol in boys, whereas girls showed inverse associations for fat mass, and positive associations for lean mass, serum vitamin D, plasma calcium, serum total cholesterol and contraceptive use with serum retinol [256]. In 1988, one cross-sectional study showed that retinol in the circulation was higher among males and in patients using medication for hypertension [257]. Moreover, data from two additional observational studies reported that factors positively associated with plasma retinol included male sex, plasma TGs,

cholesterol, lipid treatment and alcohol consumption [258,259]. The most important findings from **Paper III** are discussed in an epidemiological context below.

The kidneys and retinol

Serum creatinine exhibited the strongest age- and sex-adjusted association with serum retinol in our population. One previous study in subjects > 65 years showed that subjects with eGFR < 60 mL/min/1.73 m^2 had much higher circulating concentrations of serum retinol [206]. Moreover, elevated serum concentrations of creatinine have been positively associated with serum retinol in a previous study [260]. One report from 1981 demonstrated that 40 patients with uncharacterized diagnosis but undergoing chronic haemodialysis, had significantly increased circulating concentrations of retinol compared with reference values [261]. Similarly, patients with chronic renal failure may have twice or higher serum levels of retinol than healthy controls [120-125], which seems to be consistent through all stages of renal disease [262]. It has been postulated that decreased kidney function contributed to the underestimation of vitamin A deficiency in Sub-Saharan populations [263] due to increased renal retention of retinol and RBP4. Taken together with previous observations, the positive association between serum creatinine and retinol also seems prevalent among patients with established CVD. However, considering that only a minimum amount of retinol is normally excreted in the kidneys [260], confounding factors and unidentified signalling pathways between the kidney and liver may induce this association [264].

Because serum creatinine turned out to be particularly strongly associated with retinol, and renal function can predict elevated concentrations of many metabolites in the circulation, we adjusted regression models accordingly in **Paper III**.

Amino acids of transsulfuration and retinol

One of the strongest positive associations with serum retinol was found for plasma total cysteine, a conditionally essential sulfur amino acid which is produced in the final step of the transsulfuration pathway [231]. Cysteine is closely linked with adiposity and insulin resistance in both human and animal studies [265-270] as well as with weight regain after bariatric surgery [271], but to our knowledge, this is the first observation of an association between cysteine and retinol in a human population. Considering that this association was cross-sectional, it is difficult to determine the direction of causality, i.e., whether cysteine led to high retinol concentrations or vice versa. Interestingly, we

also observed that serine, an amino acid produced in glycolysis [272] important for transsulfuration [231] and glutathione production [273], was inversely related to serum retinol. In light of the findings from **Paper II**, showing that retinol modifies the association between tHcy and AMI, these associations between cysteine and serine, key components of transsulfuration, strengthen the argument for a relationship between homocysteine metabolism and retinol. Future studies are needed in order to elucidate and assess the relevance of these associations.

Inflammatory markers and retinol

The literature describing the relationship between inflammation and retinol is comprehensive. For example, one of the hallmarks of retinol deficiency is respiratory and intestinal infections that contribute to child mortality in developing countries and that retinol supplementation can reverse this [274]. It is now recognized that vitamin A can exert effects that both promote [275] and impede [276] inflammatory responses. Inflammation is also considered a substantial part of atherosclerotic progression, characterized by migration of monocytes to the atherosclerotic plaque and their differentiation into macrophages and subsequently foam cells [14,17,244]. In **Paper III**, we show that serum concentrations of CRP were inversely associated with circulating concentrations of retinol. CRP is elevated during the acute phase response, whereas retinol bound to RBP4 (holo-RBP4) is inversely related to the acute phase response [128]. CRP is further positively associated with CVD and a significant predictor of major cardiovascular events [28]. Tracer studies show that serum retinol was reduced during the acute phase response, but reappears in the circulation when inflammation resolves [277]. It is thus not surprising that CRP was inversely associated to serum retinol in our study.

What is slightly perplexing however, is the finding that other markers of inflammation including neopterin and uric acid were positively associated to retinol in our population. Neopterin is a pteridine that is regarded as a marker of interferon-γ-mediated immune activation and is positively associated with CVD [278]. Specifically, neopterin typically increase when activation of Type 1 T helper (T_h1) cells occur. Vitamin A has generally been considered a suppressor of T_h1-cell activation [279,280], however, administration of physiological doses of RA has been shown to activate T_h1-cells [281], which may affect circulating neopterin concentrations, and possibly reflect the positive association we observed in the present study.

Regarding uric acid, one observational study among 10893 healthy individuals in the National Health and Nutrition Examination Survey reported a linear association between uric acid and retinol [282]. Production of uric acid is catalysed by xanthine oxidase, and the activity of this enzyme has been related to development of endothelial dysfunction [283] which is central to the initiation of atherosclerotic progression [14]. Interestingly, xanthine oxidase can oxidize retinaldehyde to RA in tissues [284]. Thus, the underlying factor for the observed association between uric acid and retinol, may be increased xanthine oxidase activity in these patients.

Diet and retinol

Except for alcohol intake, dietary factors have generally not been regarded as predictive of plasma retinol concentrations [259,285], although one study found that energy-adjusted RAE intake correlated weakly with serum concentrations in 1042 subjects in a community-based cross-sectional study [115]. As part of **Paper III**, we also investigated whether dietary intakes of macronutrients, RAE and major food groups were associated with retinol in the circulation. In general, all of the associations were very weak in terms of effect sizes and proportion of explained variance. The most prominent dietary factor that was associated with retinol in the circulation was meat intake which predicted 3.6 % increase in retinol per 50 g/1000 kcal intake of meat. Positive associations of a smaller magnitude were observed for protein and alcohol intake (E %) as well as vegetable intake (50 g/1000 kcal). No association was observed for RAE intake and retinol in the circulation. The novelty of these findings lies in the fact that dietary intakes of macronutrients and foods in relation to serum retinol have not been described in patients with established CVD. Overall, our findings suggest that dietary intakes are not important for circulating retinol concentrations unless the dietary content becomes deficient or extremely excessive.

5.3.4 Summary of discussion

The discussion regarding findings from **Paper I** and **Paper II** is meant to provide basis for future experimental studies that may further unveil the mechanisms underlying the interactions observed for lipids, tHcy and retinol with regards to incident cardiovascular disease. In summary, we have proposed mechanisms from two viewpoints: In the context of *tissue sufficiency*, elevated retinol concentrations in serum may reflect

increased RA signaling as a result. This may in turn lead to increased expression of scavenger receptors in macrophages, increased uptake of oxLDL and progression of the atherosclerotic lesion. Moreover, induction of GNMT in the liver can lead to accumulation of hepatic homocysteine which in turn may accelerate apoA1 degradation and impair reverse cholesterol transport. In the context of *tissue insufficiency*, possible mechanisms include reduced expression of genes associated with macrophage export of cholesterol. In addition, low apoA1 concentrations may reflect disrupted cholesterol exchange between HDL and LDL that ultimately may lead to reduced RA signaling, with potential ramifications for the normal immune response and atherosclerotic risk.

Much work remains in order to confirm the involvement of these mechanisms in the observed interactions. It should also be noted that the different results by previously published experimental studies may be due to the use of different model systems. Accordingly, considering the pluripotency of RA and its wide array of target genes [108], the evidence as a whole thus suggest that vitamin A may exert different effects at different stages and in different aspects of atherosclerosis, and depend on the model system and potentially the population.

The findings from **Paper III** confirm the relationship between creatinine/kidney function, diet and serum retinol that have been observed in previous observational studies. The positive association between creatinine and serum retinol has been described in several populations, although the causal mechanism for how the kidneys affect serum concentrations of retinol remains somewhat elusive. Moreover, we confirm that dietary intakes of macronutrients and specific food groups largely remain neutral in predicting serum retinol concentrations. Taken together with the novel findings for retinol and amino acids of homocysteine metabolism and inflammatory markers, these epidemiological associations seem to reflect an unfavorable metabolic risk profile in CVD patients with elevated retinol.

5.3.5 Future considerations and a note on interpretation

There is reportedly a reproducibility crisis in science, and the infamous and important article by John P. Ioannidis from 2004 argued that most research findings are false, and cannot be reproduced because of selective reporting and an unhealthy focus on p-values [181]. In addition, Timothy Lash wrote in 2017 about the harm done to reproducibility by null hypothesis testing, including, but not limited to, the failure to account for

systematic bias in epidemiology and an exaggerated focus on p-values instead of estimation in study design and planning [286]. Thus, the first and crucial priority of future studies based on the current project would be to replicate the results presented herein. A natural first step would be for other large cohorts with available data on total homocysteine, lipids, retinol and relevant clinical outcomes to investigate the reported interactions. In particular, these interactions should be assessed in initially healthy populations to improve generalizability and further establish the potential clinical relevance. Although these findings may be clinically meaningful to patients with established CVD, it is not certain whether additional treatment benefits would be uncovered in a population that is already considered optimally treated. We are currently in the process of obtaining data from the Hordaland Health Studies (https://husk-en.w.uib.no), which is a large cohort consisting of initially healthy individuals with data on tHcy, fat-soluble vitamins, dietary intakes and mortality.

Another possibility would be to re-analyze data from clinical trials that reported increased cardiovascular mortality in subjects receiving supplements consisting of β-carotene and retinyl esters. The Beta-Carotene and Retinol Efficacy Trial [137] reported some 30 % increase in cardiovascular mortality in the group that received the supplements compared to the placebo group. It would certainly be interesting to evaluate whether some of these effects were mediated through disrupted lipid metabolism or interactions with homocysteine metabolism.

A final step that lie some years in the future is to design clinical trials to determine the causality of the associations. This particular endeavor poses a challenge because retinol concentrations in serum are not thought to be treatable. As mentioned above, retinol concentrations do not fluctuate much unless vitamin A intake remains excessive or deficient over longer periods of time [109]. Furthermore, inducing vitamin A deficiency by decreasing intake is hardly ethical, considering the harmful effects of vitamin A deficiency [84]. One possibility however, would be to screen subjects and populations at risk, and then enroll patients with elevated retinol concentrations ranging beyond normal reference ranges. One could then randomize these subjects to lipid- or homocysteine-lowering treatment to evaluate whether these patients would benefit more from treatment. Another option is to plan subgroup analyses according to serum retinol concentrations in existing trials to examine whether these patients benefit more from treatment. For example, according to ClinicalTrials.gov at the time of writing, there are currently 14 trials recruiting participants for investigating effects of proprotein

convertase subtilisin/kexin type 9-inhibitors on dyslipidemia and cardiovascular outcomes that in theory could implement the study of planned subgroups according to retinol concentrations. These trials could in theory implement the study of planned subgroups according to retinol concentrations.

The findings for tHcy presented in this thesis should be interpreted with some caution, considering that many studies have been largely unsuccessful in lowering incidence of CVD with B-vitamin treatment [36,79,147,287], and question the causal relationship between tHcy and disease. Although some evidence indicate that tHcy-lowering may work in primary intervention of stroke [82,83], we cannot exclude the strong possibility that tHcy may merely be a marker of metabolic risk rather than causally related to disease. However, we should consider that tHcy-lowering treatment efficiency may depend on baseline concentrations [288], and that other factors related to inflammation may also modify the association of tHcy with disease [77]. Thus, re-analysis of tHcy-lowering trials with information on serum retinol will allow the investigations of whether patients with elevated retinol concentrations had treatment benefits and can yield useful information. Such post-hoc subgroup analyses are prone to considerable bias [289], but may aid in the interpretation of the present results, and, more importantly, lead to the generation of new hypotheses for future studies and trials.

As a final note on interpretation, we cannot exclude the presence of reverse causality, in which the presence of pre-existing disease in these patients are the reason for the associations that we observe. Especially for retinol, not much is known about how established CVD potentially influence its metabolism. Notably, serum retinol in the WECAC at baseline was indeed higher that in some initially healthy populations. For example, in the third National Health and Nutrition Examination Survey (NHANES), serum retinol concentrations ranged from 1.15 and 1.08 (1^{st} percentile) to 3.46 and 3.49 (99^{th} percentile) µmol/L in males and females, respectively, in similar age groups as in our project [205]. Using the same cut-offs in the WECAC show that retinol ranged from 1.66 to 5.05 µmol/L for males (n = 2961), and 1.50 to 4.94 for females (n = 1157). In an initially healthy sub-cohort of the Hordaland Health Studies, the mean serum retinol concentrations were 2.44 µmol/L [204], whereas in our population it was 2.92 µmol/L, and thus about 20 % higher. For this reason, the metabolism of retinol in CVD should be addressed in order to establish whether serum concentrations are affected by metabolic disruptions, or if retinol can act directly on the risk associations observed for lipid parameters and homocysteine.

6 Conclusions

In conclusion, the results of this thesis indicate that the CVD risk associated with lipid parameters such as apoB, apoA1, apoB/A1 and plasma tHcy were modified by serum concentrations of retinol. These associations were only observed among patients with elevated circulating retinol concentrations. We also examined factors associated with markers of unhealth, including creatinine, cysteine and some inflammatory markers. These results may shed light on the rather ambiguous relationship of retinol with cardiometabolic disease.

There are many questions yet to be answered: Firstly, it is imperative to replicate the findings in other populations to establish reproducibility and clinical relevance. If replicated, our results may contribute to improved risk prediction and risk stratification of patients, which in turn can lead to more accurate treatment and prevention of CVD. Secondly, data from existing cohorts and new trials should be designed in order to pinpoint the specific mechanisms by which retinol potentially contributes to the increased atherogeneity associated with lipids and homocysteine. Thirdly, an intriguing question is whether the interaction observed in this study is mediated by the biological actions of RA or RBP4, of which the latter have been more strongly related to metabolic disease, or whether the interactions we observe are products of other, potentially protective mechanisms that fail under certain circumstances. Fourthly, we were able to identify only about 30 % of the total variation in serum retinol, and thus, considerable work remains to identify other factors that contribute to circulating concentrations of retinol including hepatic mobilization and tissue uptakes. On a final note, the presence of systematic biases may have affected our results, underlining the importance of confirming or rejecting the generated hypotheses in future studies. The interactions and associations we observe may simply be due to several metabolic phenomena that arise in a population with lifestyle disease, and unmeasured confounding as a result may have had profound effects on the observed relationships.

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Errata

Page	Original text	Correction	Corrected text	Reason
3	In 2012, about 17.5 (46 %) of the…	Deletion	In 2012, about 17.5 of the…	The number is from a previous draft which I forgot to delete. It is wrong.

Appendix

I

Atherosclerosis 265 (2017) 325–330

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Atherosclerosis

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Cardiovascular disease risk associated with serum apolipoprotein B is modified by serum vitamin A

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ABSTRACT

Background and aims: Apolipoproteins B (apoB) and A1 (apoA1) are major protein constituents of low-density and high-density lipoproteins, respectively, and serum concentrations of these apolipoproteins are associated with risk of atherosclerosis. Vitamin A (VA) has been implicated in lipoprotein metabolism. We evaluated the associations of serum apoB, apoA1 and their ratio (apoBAR) with risk of incident acute myocardial infarction (AMI) and the possible modification by serum VA.
Methods: Risk associations were assessed by Cox regression, and presented as hazard ratios (HRs) per standard deviation (SD) increment in log-transformed values of the lipid parameters, among 4117 patients with suspected stable angina pectoris, located in Western Norway. Interactions with VA were evaluated by including interaction terms in the models. The multivariate model included age, sex, smoking, hypertension, number of stenotic coronary arteries, left ventricular ejection fraction, C-reactive protein, estimated glomerular filtration rate and statin treatment at discharge.
Results: Median (25th, 75th percentile) age of the 4117 patients (72% male) was 62 (55, 70) years. ApoB and apoA1 were higher among patients in the upper *versus* lower tertiles of VA. During a median of 4.6 (3.6, 5.7) years of follow-up, 8.2% of patients experienced an AMI. Overall, we observed no significant associations between lipid parameters and AMI after multivariate adjustment. However, apoB and apoBAR were associated with AMI among patients in the upper tertile of VA (HR per SD 1.35, (95% CI: 1.11–1.65), and 1.42 (1.16–1.74), respectively, p for interactions $\leq$0.003).
Conclusions: The associations of apoB and apoBAR with incident AMI were confined to patients with elevated VA.

1. Introduction

High systemic low-density lipoprotein (LDL) cholesterol and low high-density lipoprotein (HDL) cholesterol are associated with increased risk of atherosclerotic cardiovascular disease (CVD) [1]. The LDL particle is generated from very low density lipoproteins (VLDL) in the circulation and is involved in cholesterol transport from the liver to peripheral tissues, whereas the HDL particle is involved in reverse cholesterol transport from peripheral tissues to the liver [2,3]. The composition of the LDL particle includes one molecule of apolipoprotein B100 (apoB); thus, the number of apoB molecules equals the number of VLDL and VLDL-derived particles in the circulation [4]. Apolipoprotein A1 (apoA1) is the main constituent of HDL, and although each HDL particle may contain as

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much as five apoA1 molecules, systemic apoA1 levels may indicate HDL cholesterol concentrations [5]. However, apoA1 predicts CVD risk also independently of HDL cholesterol [6], and some studies show that apoB, apoA1 and their ratio (apoBAR) are considered more accurate predictors of CVD incidence than LDL and HDL cholesterol in untreated populations [7–10], as well as in patients receiving statin therapy [11,12].

Vitamin A (VA) designates a class of lipid-soluble nutrients collectively referred to as retinoids [13]. From the liver, VA is released as retinol bound to retinol-binding protein 4 (RBP4), which subsequently binds to transthyretin in plasma. In target tissues, retinol is converted to the bioactive retinoic acid (RA), which acts as a ligand for the transcription factors termed RA receptors. These receptors have possible target genes related to lipid and lipoprotein metabolism [14–16], and serum VA has been shown to be positively associated with serum triglycerides and total cholesterol in healthy adults [17,18]. Retinoid administration induces the enzyme glycine *N*-methyltransferase (GNMT) [19,20] in rodents, which may influence hepatic cholesterol trafficking with possible ramifications for VLDL composition [21]. Moreover, retinol is strongly associated with its transport protein in serum [22] and systemic RBP4 concentrations have been linked to cardiovascular outcomes in epidemiological studies [23,24]. Interestingly, both retinol-bound and retinol-free RBP4 promotes expression of scavenger receptors in macrophages [24]. These scavenger receptors bind to oxidized apoB-containing LDL particles, which in turn may lead to foam cell formation and progression of atherosclerosis [25]. Taken together, VA and lipoprotein metabolism seem to be interconnected. However, to the best of our knowledge, interactions between VA and apoB that influence risk of CVD have not been studied in humans.

The aim of this study was to assess the interaction of apoB, apoA1 and apoBAR with VA in relation to risk of AMI, in patients with suspected stable angina pectoris (SAP). More specifically, we conducted explorative interaction analyses to determine whether the risk relationships of apoB, apoA1 and apoBAR with incident AMI are modified by serum VA, and whether serum VA was an independent risk factor for incident AMI.

2. Patients and methods

Eligible subjects for this prospective study included 4164 patients undergoing elective coronary angiography for suspected SAP at Haukeland ($n = 3413$) and Stavanger ($n = 751$) University Hospitals, Norway, in the period 2000–2004. A total of 61.8% ($n = 2573$) of these patients participated in the Western Norway B-Vitamin Intervention Trial (WENBIT) (clinicaltrials.gov: NCT00354081) and received either 1) folic acid, vitamin B_{12} and vitamin B_6, 2) folic acid and vitamin B_{12}, 3) vitamin B_6 or 4) placebo [26]. Subjects with missing values for serum apoB, apoA1 and/or VA ($n = 47$) were excluded, yielding a total of 4117 subjects available for analysis. All participants gave their written consent to participate. The study protocol was in accordance with the principles of the Declaration of Helsinki, and approved by the Regional Committee for Medical and Health Research Ethics, the Norwegian Medicines Agency, and the Norwegian Data Inspectorate.

Endpoint information was collected from the Western Norway Cardiovascular Registry [27] and the Norwegian Cause of Death Registry until December 31st, 2006. Incident AMI was classified according to the revised definition of myocardial infarction [28]. Non-fatal AMI occurring <24 h after baseline coronary angiography, percutaneous coronary intervention (PCI), or coronary artery bypass graft surgery (CABG) were excluded.

Information about medical history and medications at baseline and discharge from hospital were collected from each participant and verified by hospital records [26]. Trained personnel collected blood samples and registered blood pressure and anthropometric data. The majority (69.3%) of the population provided non-fasting samples. Hypertension was classified by preexisting diagnosis, and diabetics as participants previously diagnosed with diabetes (type 1 or 2) or having serum glucose ≥7.0 mmol/L or non-fasting serum glucose ≥11.1 mmol/L. Smoking status was determined by self-reported current smoking or having quit within the last four weeks, and verified by plasma cotinine ≥85 nmol/L measured as previously described [29]. The severity of coronary artery disease (CAD) was angiographically verified and scored between 0 and 3 according to the number of stenotic epicardial vessels with >50% lumen reduction. Fasting was defined as no ingested foods or beverages >6 h prior to blood sampling. Estimated glomerular filtration rate (eGFR) was determined according to the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula [30]. Dietary information was obtained from 2484 participants who completed a 169-item semi-quantitative food frequency questionnaire (FFQ) handed out on the day of inclusion and returned by mail or at the 1-month follow-up visit. The FFQ was developed at the Department of Nutrition, University of Oslo, Norway and was previously validated for several nutrients [31–34].

Serum blood samples were collected at baseline, and angiography was performed. Serum samples were stored at −80 °C until biochemical analyses. Serum concentrations of apoB and apoA1 were measured and determined by the Roche Hitachi 912 and 917 systems (Roche Diagnostics, GmbH, Mannheim, Germany), respectively. VA was measured as serum *all-trans*-retinol at BEVITAL AS (www.bevital.no) using liquid chromatography-tandem mass spectrometry as described previously [35]. Serum concentrations of C-reactive protein (CRP) were determined by an ultrasensitive immunoassay (N Latex CRP Mono, Behring Diagnostics, Marburg, Germany).

Continuous and categorical variables are presented as median (25th, 75th percentile) and percentage (%), respectively. Trends according to VA tertiles for continuous and categorical variables were assessed with unadjusted median linear or logistic regression. Skewed variables were log-transformed before analyses. Partial correlations for log-transformed VA with apoB, apoA1 and apoBAR were assessed with Pearson's correlation coefficient (r) and adjusted for age, sex, smoking, left ventricular ejection fraction and kidney function. Hazard ratios (HRs) per one standard deviation (SD) increment in log-transformed apoB, apoA1 and apoBAR, along with their respective 95% confidence intervals (CI), were calculated using Cox proportional hazards models. Two models were created: Model 1 was adjusted for age and sex, whereas model 2 was adjusted for age, sex, smoking, hypertension, number of stenotic vessels at coronary angiography, left ventricular ejection fraction, CRP, eGFR and statin prescription at discharge from hospital. Adjustment for diabetes, fasting status, and triglycerides did not alter the results materially and were thus not included in the final models. Potential effect modifications by VA were explored *post hoc* by adding interaction terms for apoB, apoA1 or apoBAR with VA (continuous), along with the separate variables in a multivariate model identical to Model 2. To determine whether the B-vitamin intervention affected the results, we carried out sensitivity analyses stratified by B-vitamin intervention in WENBIT. We considered p-values <0.05 significant. In order to adjust for the false discovery rate that may increase with multiple testing, the Benjamini-Hochberg procedure was applied [36]. The corrected significant p-value was set to <0.04.

Statistical analyses were carried out using the "base" and "survival" packages for R 3.0.2 (the R Foundation for Statistical Computing, Vienna, Austria).

3. Results

Baseline characteristics of the total study population are presented in Table 1. The population included 72% of men and 28% of women with a median (25th, 75th percentile) age of 62.0 (55, 70) years.

The baseline characteristics according to serum VA concentrations are shown in Supplemental Table 1. Median (25th, 75th percentile) circulating concentrations of VA were 2.30 (2.10, 2.45), 2.82 (2.69, 2.95) and 3.53 (3.29, 3.90) μmol/L in the 1st to 3rd VA tertiles, respectively. Participants with higher VA were more likely to have hypertension, and higher serum concentrations of triglycerides, apoB, apoA1 and total cholesterol, as well as lower CRP and eGFR. Moreover, patients in the upper VA tertile had less extensive CAD at coronary angiography. There were no trends according to statin, aspirin or beta-blocker prescription at baseline or discharge across VA tertiles. Finally, no trends were observed for dietary VA intake across tertiles of serum VA concentrations (data not shown).

The significant associations for VA with apoB and apoA1 at baseline were still present after multivariate adjustment for age, sex, smoking, left ventricular ejection fraction and eGFR ($r = 0.12$ and 0.19 respectively, both $p < 0.01$).

During median (25th, 75th percentile) 4.6 (3.6, 5.7) years of follow-up, 338 patients (8.2%) suffered an AMI. The risk associations with apoB, apoA1 and apoBAR are presented in Table 2. Overall, a trend of increased risk was found for apoB and apoBAR, whereas apoA1 was associated with a decreased risk of AMI in Model 1. These effects were attenuated by multivariate adjustment in Model 2. Moreover, no significant associations were found for VA and AMI in Model 1 (HR per SD: 1.10, 95% CI: 0.96–1.21, $p = 0.11$) or in Model 2 after multivariate adjustment (HR per SD: 1.07, 95% CI: 0.91–1.23, $p = 0.28$).

We observed a significant interaction between apoB and VA for AMI (p for interaction = 0.003), whereas the interaction between apoA1 and VA was of borderline statistical significance (p for interaction = 0.064). We also observed an interaction between apoBAR and VA in relation to AMI risk (p for interaction<0.001). The interactions between lipid parameters and VA in relation to AMI risk were further explored by stratification according to the VA tertiles as presented in Table 2, and the same statistical models were used as for the total population. In Model 1, serum apoB concentrations and apoBAR were associated with increased risk of AMI only in the upper tertile of VA ($p < 0.001$), which remained significant in Model 2 ($p < 0.002$). Furthermore, apoA1 was inversely associated with AMI in Model 1 in the upper VA tertile ($p = 0.003$). This effect was slightly attenuated in Model 2 after inclusion of left ventricular ejection fraction in the multivariate model ($p = 0.152$).

In general, we observed similar results as in the total population among patients who received B-vitamin treatment in WENBIT (data not shown). However, in patients who received placebo or did not participate in WENBIT ($n = 2237$, No. of events = 181), we observed a trend for an interaction for apoB and VA in relation to AMI (p for interaction = 0.062) whereas the interaction of apoA1 and apoBAR with VA in relation to AMI was significant (p for interaction<0.001), as presented in Supplemental Table 2. After stratification, we found an inverse association between apoA1 and AMI in the upper VA tertile (HR per SD: 0.61, 95% CI: 0.47–0.80, $p < 0.001$) in the multivariate model. Finally, apoBAR was positively associated with AMI in the upper VA tertile (HR per SD: 1.71, 95% CI: 1.30–2.26, $p < 0.001$) in the multivariate model.

Table 1
Baseline characteristics of the participants.[a]

	Total population
	$n = 4117$
Age	62 (55, 70)
Male sex, %	72.0
Hypertension, %	46.8
Diabetes, %	11.0
Current smoker, %	31.6
Previous CVD, %	
Previous AMI	40.3
Previous CBVD	6.9
Previous PVD	9.0
Previous CAD	30.5
Previous CABG	11.5
Previous PCI	19.2
Serum lipids	
LDL, mmol/L	2.90 (2.40, 3.70)
HDL, mmol/L	1.20 (1.00, 1.50)
TG, mmol/L	1.50 (1.08, 2.14)
ApoA1, g/L	1.30 (1.13, 1.48)
ApoB, g/L	0.87 (0.73, 1.04)
ApoB/ApoA1	0.67 (0.54, 0.84)
Total cholesterol, mmol/L	4.90 (4.30, 5.70)
Vitamin A, μmol/L	2.82 (2.45, 3.29)
CRP, mg/mL	1.78 (0.87, 3.67)
eGFR, mL/min per 1.73 m^2	91 (78, 99)
Extent of CAD, %	
No significant stenosis	25.2
One-vessel disease	23.1
Two-vessel disease	22.3
Three-vessel disease	29.4
LVEF <50	9.9
Medication at baseline, %	
Statins	72.4
Aspirin	80.3
Beta-blocker	73.4
Medication at discharge, %	
Statins	80.0
Aspirin	81.5
Beta-blocker	72.5

AMI, acute myocardial infarction; apo, apolipoproteins; apoBAR, apolipoproteinB/A1-ratio; CAD, coronary artery disease; CABG, coronary artery bypass graft surgery; CBVD, cerebrovascular disease; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate, LVEF, left ventricular ejection fraction; PVD, peripheral vascular disease; PCI, percutaneous coronary intervention; TG, triglycerides; VA, vitamin A.

[a] Categorical variables are presented as % and continuous variables as medians (25th, 75th percentile).

4. Discussion

In this large prospective study among 4117 patients with SAP, we show for the first time that the risk relations of lipid-related parameters were modified by serum concentrations of VA. Initially, we did not observe an association for apoB, apoA1 and apoBAR with AMI following multivariate adjustment. However, interaction analyses revealed that serum concentrations of apoB and apoBAR were associated with increased risk of AMI in patients with elevated VA only. We also showed, in a sensitivity analysis, that the interaction between apoA1 and VA reached statistical significance in participants not receiving B-vitamin treatment.

There are several possible biological mechanisms that may explain the statistical interaction we observed in this study. The retinol transport protein, RBP4, has been associated with risk of CVD, and a recently published study showed that both retinol-bound and retinol-free RBP4 promoted CD36 expression and cholesterol uptake in macrophages [24]. Moreover, one *in vitro* study of cells incubated with *all-trans* RA demonstrated a rapid induction of CD36 on both the mRNA and protein level [37]. This scavenger receptor is present on the surface of macrophages and binds to apoB-containing oxidized LDL particles, which are implicated in foam cell formation, platelet activation, inflammation and

Table 2
Associations between apolipoproteins B and A1 and their ratio with acute myocardial infarction according to tertiles of vitamin A.

	Total population		Vitamin A tertiles						*p* for interaction
	HR per SD (95% CI)	*p*	<2.58 μmol/L		2.58–3.09 μmol/L		>3.10 μmol/L		
			HR per SD (95% CI)	*p*	HR per SD (95% CI)	*p*	HR per SD (95% CI)	*p*	
ApoB									0.003
Model 1[a]	1.11 (1.00–1.24)	0.058	0.92 (0.78–1.10)	0.356	1.07 (0.88–1.30)	0.489	1.42 (1.17–1.73)	<0.001	
Model 2[b]	1.09 (0.98–1.22)	0.115	0.91 (0.76–1.09)	0.287	1.08 (0.88–1.32)	0.458	1.35 (1.11–1.65)	0.002	
ApoA1									0.064
Model 1	0.85 (0.76–0.94)	0.003	0.99 (0.82–1.19)	0.904	0.75 (0.61–0.93)	0.007	0.76 (0.63–0.91)	0.003	
Model 2	0.95 (0.85–1.06)	0.359	1.10 (0.91–1.34)	0.313	0.83 (0.66–1.03)	0.084	0.87 (0.71–1.05)	0.152	
ApoB/A1									<0.001
Model 1	1.21 (1.08–1.34)	<0.001	0.94 (0.78–1.13)	<0.001	1.22 (1.01–1.47)	0.040	1.60 (1.31–1.95)	<0.001	
Model 2	1.11 (0.99–1.24)	0.070	0.87 (0.73–1.05)	0.135	1.17 (0.96–1.43)	0.112	1.42 (1.16–1.74)	<0.001	

apoB, apolipoprotein B; apoA1, apolipoprotein A1; apoB/A1, ratio of apolipoprotein B to A1; HR per SD, hazard ratio per standard deviation increase.
[a] Cox proportional hazards model adjusted for age and sex.
[b] Cox proportional hazards model adjusted for age, sex, smoking, C-reactive protein, number of stenotic vessels, left ventricular ejection fraction, hypertension, statin use at discharge from hospital and estimated glomerular filtration rate.

ultimately the progression of atherosclerosis [25]. Finally, a study in vascular smooth muscle cells demonstrated a ~7-fold induction of the lectin-like oxidized LDL receptor by *all-trans* RA [38], which, upon binding of oxidized LDL, may promote endothelial dysfunction, foam cell formation and vascular smooth muscle cell apoptosis in atherosclerotic lesions [25].

The administration of *all-trans*-RA has been associated with enhanced expression of glycine-*N*-methyltransferase (GNMT) in rodents [19,20]. This enzyme has recently been implicated in cholesterol metabolism, as GNMT knock-out mice exhibit hepatic lipid accumulation, suggesting a possible role of GNMT in hepatic VLDL export [39]. In cell cultures, GNMT has been associated with Niemann Pick-C2, a protein essential for cholesterol transport in the liver [21], with potential ramifications for VLDL composition and export. Thus, VA activity in the liver may be important for the synthesis, packaging and secretion of VLDL from the liver to the peripheral tissues, via alterations in GNMT. However, preclinical studies have also suggested that GNMT inhibition in atherosclerotic plaques may impair reverse cholesterol transport (RCT) [39], a process by which apoA1-containing HDL particles play a crucial role. Notably, we observed a trend towards an inverse relationship between apoA1 and incident AMI among patients with high VA, particularly in those that did not receive B-vitamin treatment, including folate, which is a potent inhibitor of GNMT [40].

In this study, we present a significant interaction of apoB and apoBAR with circulating concentrations of VA in relation to AMI. As VA is an abundant nutrient in several affluent societies, a high intake may promote adverse effects in combination with elevated serum apoB and apoBAR. However, VA intake did not correlate with circulating concentrations and did not differ across VA tertiles in our population, and is unlikely to influence the observed interaction. We observed effect modifications of apoB and apoBAR by VA in patients with SAP. If confirmed in other populations, determination of VA in combination with apoB, apoA1 and apoBAR may become useful in CVD risk assessment. Additionally, it may be useful to reanalyze data from larger existing studies to assess whether the CVD risk associated with apoB, apoA1 and apoBAR is modified by VA. Finally, the observed interaction may clarify the observed increased CVD mortality in a population receiving vitamin A supplements [41].

The major strength of this study is its large and well-characterized population with long-term follow-up. Moreover, as the median circulating concentrations of VA in the total population and across VA tertiles were generally higher as compared with other studies [17,42], this population is well-suited for the study of high concentrations of VA and the possible interaction with traditional risk markers.

Our study has several limitations. The majority of blood samples were drawn from non-fasting subjects, which may affect circulating concentrations of serum lipids. However, when we adjusted for fasting status and serum triglycerides in the multivariate model, the effect sizes and interactions remained unchanged. The majority of the population (>80%) received apoB-lowering statin treatment, and the observed effect sizes for apoB and apoBAR may be biased in the total population, as well as the VA tertiles, and not generalizable to untreated populations. However, apoB is associated with CVD also in statin-treated populations [43] and our results may thus hold merit for patients undergoing lipid-lowering therapy. Additionally, in our sensitivity analysis, the B-vitamin treatment seems to mask the interaction of apoA1 with VA, and we cannot exclude further bias introduced by the trial intervention. The interpretation of these results are further complicated by the finding that VA levels in blood seem to be high when levels in tissues are low [44], and the fact that we did not measure RA, which is the major bioactive retinoid. It should be noted that little is known about the regulation of serum VA concentrations, as it is generally thought to be unaffected by dietary intake [45]. However, one recently published study showed that serum VA was slightly higher in supplement users than in non-users [46]. Thus, we cannot exclude the possible influence of unreported long-term high-dose supplementation on circulating concentrations of VA among our participants. Moreover, there may also be other unmeasured and unknown factors that affect serum concentrations of VA that have not been accounted for.

In conclusion, our results suggest that the risk of incident AMI associated with apoB and apoBAR is modified by VA whereas the interaction between apoA1 and VA was only present in patients not receiving B-vitamins in the original trial. Reanalysis of data from existing trials with VA supplements would be useful to determine whether potential risk associated with such treatment is via modification of circulating apolipoprotein concentrations. Finally, the possible mechanisms by which VA potentially increases the atherogeneity of apoB should be elucidated in experimental models.

Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

OKN conceived and designed the study; OKN, GFTS, ERP and PMU conducted the research; TO and KJV conducted the statistical analyses; ØM and PMU were responsible for measuring vitamin A in serum; TO, KJV, GFTS, ERP, GST, RB, CAD, PMU, HR and OKN interpreted the results; TO, KJV, GFTS, ERP, GST, RB, CAD, PMU, ØM, HR and OKN wrote, critically revised and approved the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2017.07.020.

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The risk association of plasma total homocysteine with acute myocardial infarction is modified by serum vitamin A

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Abstract

Background: Plasma total homocysteine (tHcy) has been implicated in the development of cardiovascular disease, but the mechanisms remain unclear. Vitamin A (Vit-A) is involved in homocysteine metabolism and we therefore explored the potential interaction between plasma tHcy and serum Vit-A in relation to incident acute myocardial infarction.

Methods: Cox proportional hazards models were used to assess the prospective relationships between tHcy and acute myocardial infarction in 2205 patients from Western Norway undergoing elective coronary angiography for suspected stable angina pectoris. Results are reported as hazard ratio per standard deviation increase in log-transformed tHcy. An interaction term for tHcy × Vit-A was added to multivariate models including age, sex, smoking, apolipoprotein B fasting, statin and aspirin prescription and estimated glomerular filtration rate.

Results: Geometric mean (geometric standard deviation) age of the participants (64.3% men) was 62.3 (1.24) years. Plasma tHcy was higher among participants in the upper versus lower Vit-A tertile. During 7 (2.4) years of follow-up, 15.1% suffered an AMI. A significant association of plasma tHcy with AMI in the total study population was observed. When we stratified the population according to Vit-A tertiles, plasma tHcy was associated with acute myocardial infarction only in the upper Vit-A tertile (hazard ratio per SD: 1.25, 95% confidence interval: 1.04–1.53, $p_{interaction} = 0.03$).

Conclusions: The risk relationship between plasma tHcy and acute myocardial infarction was modified by serum concentrations of Vit-A in patients with suspected stable angina pectoris. This finding may clarify the relationship between tHcy and cardiovascular disease.

Keywords
Homocysteine, vitamin A, cardiovascular disease

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Introduction

The non-protein, sulphur-containing amino acid homocysteine is produced by intracellular transmethylation reactions where a methyl group is transferred from the universal methyl donor S-adenosylmethionine (SAM) to a methyl acceptor.[1] This reaction produces S-adenosylhomocysteine (SAH), which is readily hydrolysed to adenosine and homocysteine in a reversible reaction. In the circulation, homocysteine undergoes disulphide exchange reactions, forming symmetrical and mixed disulphides, which are collectively referred to as total homocysteine (tHcy).[2] Elevated plasma concentrations

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of tHcy have been linked to increased risk of cardiovascular disease (CVD) in observational studies,[3] findings that were recently suggested to depend on oxidative stress and inflammation.[4] However, tHcy-lowering treatment with B-vitamins did not improve prognosis in secondary prevention trials, and the potential underlying pathological mechanisms for the risk relationship of tHcy with CVD remain unclear.[5]

Vitamin A (Vit-A) designates a class of fat-soluble compounds abundant in Western diets.[6] In target cells, retinol is a precursor for the major bioactive retinoid, retinoic acid (RA), which is a ligand for nuclear RA receptors. The role of Vit-A in CVD development is unclear,[7] but we have shown that serum Vit-A modified the risk association between lipid parameters and acute myocardial infarction in the same core population as the current study.[8] Moreover, expression of genes involved in synthesis of *all-trans*-RA is increased in some inflammatory conditions[9] and is promoted by mediators of oxidative stress and inflammation such as oxysterols.[10] This potentially increased synthesis of *all-trans*-RA may have metabolic ramifications for homocysteine. Circulating tHcy has been positively associated with Vit-A in an observational study,[11] and *all-trans*-RA is a potent activator of glycine *N*-methyltransferase (GNMT),[12–14] which produces SAH and homocysteine under conditions of excess intracellular SAM.[15] GNMT is involved not only in the regulation of intracellular homocysteine concentrations,[16] but also in lipid metabolism[17] and cellular immune activation.[18]

Taken together, the association of plasma tHcy with risk of CVD may be modified by Vit-A through several biological mechanisms. However, evidence is limited, predominantly experimental and little is known about this relationship in humans. The aim of this observational study among more than 2000 patients with suspected stable angina pectoris was to explore whether the relationship of plasma tHcy with risk of AMI was modified by serum concentrations of Vit-A.

Subjects and methods

A total of 4164 patients undergoing elective coronary angiography for suspected stable angina pectoris at Haukeland ($n = 3413$) and Stavanger ($n = 751$) University Hospitals, Norway, in the period 2000–2004 were enrolled in this study. In total, 61.8% ($n = 2573$) participated in the Western Norway B-Vitamin Intervention Trial (WENBIT) (clinicaltrials.gov: NCT00354081) and received either: 1) folic acid, vitamin B_{12} and vitamin B_6, 2) folic acid and vitamin B_{12}, 3) vitamin B_6 or 4) placebo.[19] Subjects with missing data on plasma tHcy or serum Vit-A and those who received B-vitamin treatment ($n = 1959$) in the WENBIT were excluded, yielding a total of 2205 subjects available for the present study. Written informed consent was obtained from all participants and the study protocol was in accordance with the principles of the Declaration of Helsinki, and approved by the Regional Committee for Medical and Health Research Ethics, the Norwegian Medicines Agency, and the Norwegian Data Inspectorate.

Baseline data and biochemical analyses

CVD history and prescription of medications at baseline were self-reported and verified by hospital records.[19] Patients with diabetes were classified by previous diagnosis (type 1 or 2), having fasting or non-fasting serum glucose ≥ 7.0 or ≥ 11.1 mmol/L, respectively, or having baseline glycated haemoglobin $> 6.5\%$. Definitions of smoking and severity of coronary artery disease as well as the calculation of glomerular filtration rate (eGFR) have been described previously.[20]

Blood samples, mostly non-fasting (80%), were collected at baseline. Plasma and serum samples were stored at –80°C until analysis. Plasma concentrations of tHcy were analysed using gas chromatography mass spectrometry,[21] whereas serum Vit-A (as *all-trans* retinol)[22] and neopterin[23] by liquid chromatography tandem mass spectrometry by BEVITAL AS (www.bevital.no). Serum concentrations of apolipoprotein B and C-reactive protein (CRP), and dietary Vit-A intake were measured as described previously.[8,20]

Follow-up and clinical endpoints

The participants were followed from baseline to the date of acute myocardial infarction (AMI) diagnosis or death, or to 31 December 2009 (the end of follow-up), whichever came first. AMI cases were ascertained via record linkage to national hospital discharge diagnoses obtained from the Cardiovascular Disease in Norway project (www.cvdnor.no) to 31 December 2009.[24–26] The primary outcome was hospitalization or death attributed to AMI (coded I21, I22, I46.1, R96, and R98 according to the *International Statistical Classification of Diseases 10th version*). If more than one AMI event occurred in a participant during the follow-up period, only the first event was considered. An 11-digit personal identifier, unique to each Norwegian resident, was used to link baseline variables with study endpoints.

Statistical analysis

Due to non-normality, continuous variables are presented as geometric mean (gM) (geometric standard

deviation (gSD)). Categorical variables are given as percentages (%). Baseline characteristics are presented for the total population and according to serum Vit-A tertiles. Trends across Vit-A tertiles were assessed with unadjusted ordinary least squares or logistic regression. Skewed variables were log-transformed before analyses. Survival analyses were carried out using Cox regression and reported as hazard ratios (95% confidence intervals (CIs)) per 1 SD log-transformed plasma tHcy in one model containing log-transformed tHcy (continuous), age (continuous) and sex (binary), and an additional model including additional adjustment for smoking (binary), statin prescription (binary), aspirin prescription (binary), fasting status (binary), eGFR (continuous) and apolipoprotein B (continuous). To assess the effect-modification by Vit-A, Vit-A (continuous), log-transformed tHcy (continuous) and their interaction term (Vit-A × tHcy) were added to the models. To assess the interaction in the presence of competing risks, we computed subdistribution hazard ratios from identical models based on the approach proposed by Fine and Gray.[27] To explore whether the results differed in patients with and without significant coronary stenosis, we evaluated the Vit-A × tHcy interaction in patients with 1–3 stenotic epicardial vessels as well as patients with no significant stenosis in models adjusted for age and sex. The assumption of proportionality was evaluated visually by log–log plots and quantitatively by calculating Schoenfeld residuals. To assess any non-linear relationships between tHcy and risk of AMI according to Vit-A tertiles, we constructed generalized additive model (GAM) dose–response curves based on the age- and sex-adjusted models in each Vit-A tertile. To visualize the interaction of log-transformed tHcy with Vit-A, we constructed an interaction plot. In brief, we plotted the hazard ratio of log-transformed tHcy against a given range of Vit-A (0–7 μmol/L), and thus illustrated how the association of log-transformed tHcy with AMI changes with increasing concentrations of Vit-A. We considered p-values < 0.05 significant. The statistical basis and R-codes for this procedure have been published.[28] Statistical analyses were carried out using the 'base', 'survival' and 'cmprsk' packages, whereas the GAM plot was created using the 'mgcv' package for R 3.0.2 (the R Foundation for Statistical Computing, Vienna, Austria).

Results

Baseline characteristics

Baseline characteristics of the total study population and according to Vit-A tertiles are presented in Table 1. The total population included a total of 2205 participants of which 64.3% were male with a gM (gSD) age of 62.3 (1.24) years. The gM (gSD) serum concentration of Vit-A was 2.93 (1.28) μmol/L. Cut-offs for serum Vit-A in the tertiles were < 2.65, 2.65–3.20 and > 3.20 μmol/L in the first, second and third tertile, respectively. Participants in the upper Vit-A tertile tended to be men, have hypertension and higher concentrations of low-density lipoprotein cholesterol, apolipoprotein B, triglycerides, apolipoprotein A1, neopterin and tHcy, whereas CRP and eGFR tended to be lower. No trend for Vit-A intake was observed across serum Vit-A tertiles.

Association between plasma tHcy and AMI

During gM (gSD) follow-up time of 7.0 (2.4) years, 333 (15.1%) patients were diagnosed with AMI. As expected, there was a positive risk relationship between plasma tHcy and incident AMI; however, the association was somewhat attenuated in the multivariate model (Table 2).

Interaction with Vit-A

As presented in Table 3, we found a significant interaction between tHcy and Vit-A in relation to AMI in both models. We constructed GAM plots to assess non-linearity between tHcy and AMI in each Vit-A (Figure 1). As can be seen from Figure 1, we found no associations between tHcy and AMI in the first and second Vit-A tertiles; however, a positive association was seen in the third Vit-A tertile. This is further illustrated in Figure 2, which shows that the risk relationship of AMI associated with tHcy increases in a fully adjusted model when Vit-A concentrations exceed ~ 3 μmol/L.

Competing risks analysis

In total, 505 patients died, of which 274 (54.3 %) due to non-coronary causes. Based on this finding, we calculated subdistribution hazard ratios for having an AMI in the presence of non-cardiovascular mortality. The association of tHcy with AMI was generally unchanged in all multivariate models including the upper tertile of Vit-A (subdistribution hazard ratio: 1.24, 95% CI: 1.04–1.49, p for interaction = 0.03).

Interaction with Vit-A in patients with and without significant coronary stenosis

We stratified the population according to the prevalence of significant coronary artery disease to evaluate whether the interaction between tHcy and Vit-A differed in the two groups. In patients with at least one significant coronary stenosis ($n = 1380$), 286 (21%)

Table 1. Baseline characteristics for the total study population ($N = 2205$) with suspected stable angina pectoris.

	Total study population $N = 2205$	First Vit-A tertile < 2.65 μmol/L $n = 728$	Second Vit-A tertile 2.65–3.20 μmol/L $n = 727$	Third Vit-A tertile > 3.20 μmol/L $n = 750$	p for trend[a]
Age, years	62.3 (1.24)	61.4 (1.20)	60.3 (1.21)	60.4 (1.20)	0.229
Male sex, *n* (%)	1418 (64.3)	438 (60.2)	469 (64.5)	511 (68.1)	0.001
Fasting, *n* (%)	418 (19.8)	150 (21.6)	137 (19.6)	131 (18.2)	0.155
Smoking, *n* (%)	689 (31.2)	233 (32.0)	235 (32.3)	221 (29.5)	0.323
1–3 stenotic vessels, *n* (%)	1380 (62.6)	490 (67.3)	454 (62.4)	436 (58.1)	0.005
Diabetes mellitus, *n* (%)	274 (12.4)	106 (14.6)	75 (10.3)	93 (12.4)	0.157
Hypertension, *n* (%)	1030 (46.7)	330 (45.3)	317 (43.6)	383 (51.1)	0.025
LVEF < 60, *n* (%)	1784 (80.9)	593 (81.5)	592 (81.4)	599 (79.9)	0.327
Previous AMI, *n* (%)	817 (37.1)	273 (37.5)	264 (36.3)	280 (37.3)	0.958
ApoB, g/L	0.89 (1.31)	0.86 (1.30)	0.89 (1.30)	0.93 (1.31)	<0.001
ApoA1, g/L	1.33 (1.22)	1.29 (1.23)	1.33 (1.21)	1.37 (1.22)	<0.001
LDL-C, mmol/L	2.97 (1.39)	2.89 (1.40)	2.98 (1.38)	3.03 (1.39)	0.001
HDL-C, mmol/L	1.26 (1.34)	1.25 (1.35)	1.27 (1.33)	1.27 (1.32)	0.703
Triglycerides, mmol/L	1.51 (1.67)	1.3 (1.61)	1.46 (1.62)	1.79 (1.7)	<0.001
CRP, mg/mL	1.97 (2.99)	2.43 (3.27)	1.85 (2.93)	1.71 (2.7)	<0.001
eGFR, ml/min per 1.73 m^2	83.0 (1.34)	88.5 (1.20)	85.7 (1.21)	75.6 (1.51)	<0.001
Total homocysteine, μmol/L	11.0 (1.4)	10.4 (1.36)	10.7 (1.36)	11.8 (1.44)	<0.001
Neopterin, nmol/L	8.94 (1.51)	8.45 (1.44)	8.65 (1.42)	9.76 (1.64)	<0.001
Retinol intake, RAE/day	1576 (1.83)	1548 (1.73)	1584 (1.91)	1618 (1.91)	0.526
Medications at discharge, *n* (%)					
Statins	1605 (72.8)	526 (72.3)	525 (72.2)	554 (73.9)	0.286
Aspirin	1642 (74.5)	542 (74.5)	540 (74.3)	560 (74.7)	0.621
Beta-blockers	1479 (67.1)	490 (67.3)	478 (65.7)	511 (68.1)	0.601

Values are geometric mean (geometric standard deviation) for continuous variables and *n* (%) for categorical variables.
[a]*p*-values for trends across tertiles of vitamin A were calculated by linear regression for continuous variables and logistic regression for categorical variables.
Vit-A: vitamin A; LVEF: left ventricular ejection fraction; AMI: acute myocardial infarction; ApoB: apolipoprotein B; ApoA1: apolipoprotein A1; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; CRP: C-reactive protein; eGFR: estimated glomerular filtration rate; RAE: retinol activity equivalents

Table 2. tHcy-related risk of acute myocardial infarction in the total population.[a]

Total population (no. of events, 333/2205)	HR per SD (95% CI)	p -value
Model 1[b]	1.22 (1.10–1.35)	< 0.01
Model 2[c]	1.12 (0.99-–1.26)	0.07

[a]Risk as HRs (CIs) per 1 SD log-transformed plasma tHcy computed by Cox proportional hazards model.
[b]Adjusted for age and gender.
[c]Adjusted for age, gender, smoking, estimated glomerular filtration rate, statin prescription, aspirin prescription, fasting and apolipoprotein B.
tHcy: total homocysteine; HR: hazard ratio; CI: confidence interval

events occurred, whereas 47 events (5.6%) occurred in patients without significant stenosis ($n = 825$). Risk estimates and CIs are presented in Table 4. There was a significant interaction between tHcy and Vit-A in relation to AMI in patients with, but not without, significant coronary stenosis.

Discussion

Principal findings

In this prospective study among more than 2000 patients with stable angina pectoris, we showed that plasma tHcy was higher in patients with elevated systemic Vit-A concentrations and that the association between tHcy and risk of AMI was primarily confined to patients in the highest tertile of serum Vit-A.

Homocysteine and Vit-A

Our findings are of interest considering our previously published results indicating effect modification by

Table 3. Total homocysteine-related risk of acute myocardial infarction across tertiles of vitamin A.[a]

	Tertiles of serum vitamin A[b]			
	First	Second	Third	*p* for interaction
No. of events/*n*	117/728	108/727	108/750	
Model 1[c]	1.10 (0.90–1.34)	1.11 (0.90–1.38)	1.41 (1.21–1.64)	<0.001
Model 2[d]	1.08 (0.88–1.33)	1.09 (0.86–1.38)	1.25 (1.04–1.52)	0.03

[a]Risk as hazard ratios (95% confidence intervals) per 1 SD log-transformed plasma total homocysteine, computed by Cox proportional hazards model.
[b]Cut-offs for serum vitamin A tertiles were < 2.65, 2.65–3.20 and > 3.20 μmol/L.
[c]Adjusted for age and gender.
[d]Adjusted for gender, smoking, estimated glomerular filtration rate, statin prescription, aspirin prescription, fasting and apolipoprotein B.

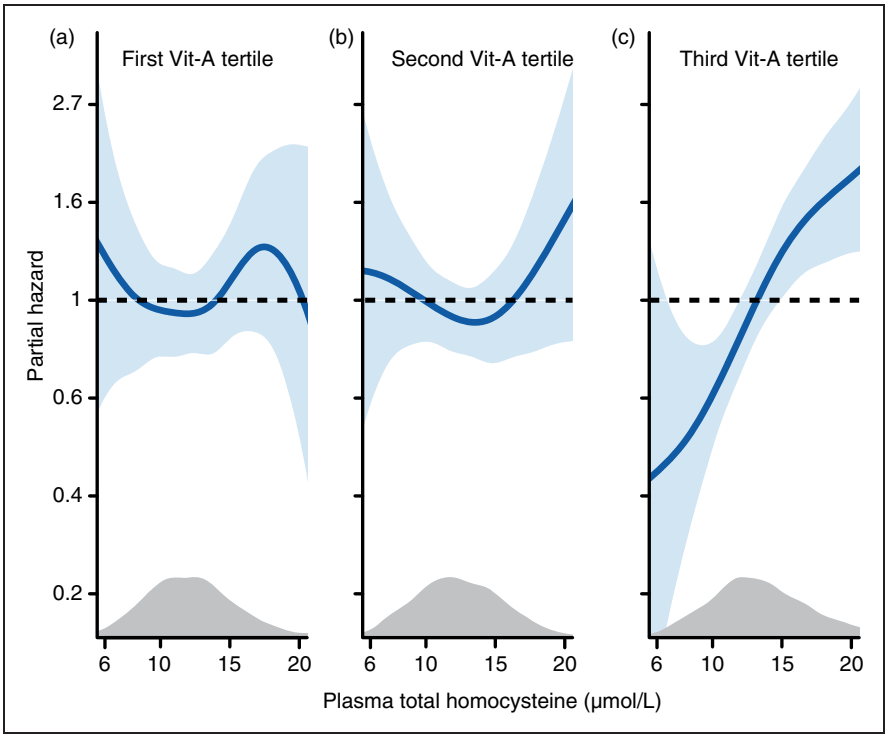

Figure 1. The dose–response relationship between log-transformed total homocysteine and acute myocardial infarctions in the first (a), second (b) and third (c) tertile of vitamin A computed by generalized additive models. Data are adjusted for age and sex. The blue shaded areas around the curve represent the 95% confidence intervals. Kernel distribution plots show the distribution of total homocysteine.
Vit-A: vitamin A

serum Vit-A on the association between traditional lipid parameters and incident CVD.[8] We also demonstrated a positive association between baseline plasma tHcy and serum Vit-A, which is in line with findings among overweight but otherwise healthy individuals.[11] Positive associations for Vit-A,[29] its transport protein retinol-binding protein 4[30] and cardiovascular endpoints has been reported from some prospective cohorts, but no interaction with other common risk factors have been reported. To our knowledge, epidemiological evidence on the interaction between tHcy and Vit-A with respect to incident AMI or CVD in general has not been assessed previously as far as we know.

Potential mechanisms

Experimental data on the interaction between homocysteine and Vit-A are limited, but possible mechanisms may include inflammation and lipid metabolism. Inflammatory conditions such as non-alcoholic fatty liver disease increase hepatic expression of genes

involved in *all-trans*-RA synthesis.[9] Inflammatory markers such as oxysterols stimulate synthesis of *all-trans*-RA in the liver,[10] which in turn may affect homocysteine through induction of GNMT.[13,15,31] Notably, GNMT plays a role in the production of interferon-γ, which drives synthesis of the macrophage-specific inflammatory marker neopterin.[18,32] This pteridine has been related to risk of major cardiovascular events in humans[32] and potentiated tHcy-mediated risk of CVD in the same core population as the present study.[4] Neopterin was higher among patients with elevated Vit-A in the present study, and the tHcy-associated risk we observed in our study may reflect pro-inflammatory conditions that contribute to atherosclerosis. In contrast, CRP, which, unlike neopterin, is produced in the liver as part of the acute phase response[33] and is associated with aspects of CVD risk[34] and complications,[35] tended to be lower among patients with elevated Vit-A. Hence, the interaction between Vit-A and tHcy and the potential link with inflammation seems to relate to specific components of immune activation. This hypothesis needs further exploration in future studies.

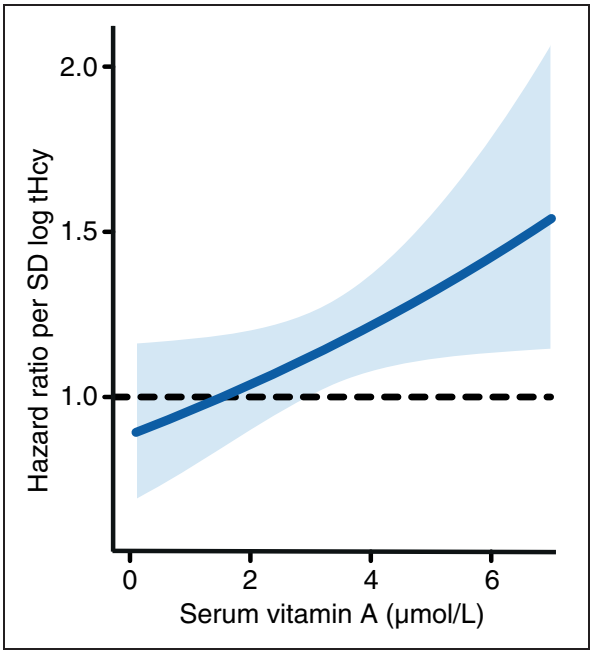

Figure 2. Illustration of the interaction between serum vitamin A and log-transformed total homocysteine on the continuous scale. The blue shaded areas around the curve represent the 95% confidence intervals.
tHcy: total homocysteine

Accumulation of cholesterol is crucial for atherosclerotic plaque progression and leads to foam cell formation from endothelial-derived smooth muscle like cells,[36] and may relate to impaired reverse cholesterol transport to high-density lipoprotein (HDL).[37] We recently showed that low concentrations of apolipoprotein A1 (apoA1), the major protein constituent of HDL, was associated with excess risk of cardiovascular events in patients with elevated Vit-A concentrations.[8] Homocysteine, partially produced by GNMT in the liver, accelerates hepatic clearance of HDL[38] and inhibits synthesis of apoA1 in the liver of mice and men with coronary heart disease.[39] Thus, homocysteine may exert detrimental effects on reverse cholesterol transport from atherosclerotic plaques and the dynamic exchange of cholesterol between HDL and low-density lipoprotein particles in the circulation by limiting HDL availability. However, this proposed mechanism is complicated by the demonstration that GNMT ablation in macrophages impaired reverse cholesterol

Table 4. Total homocysteine-related risk of acute myocardial infarction across tertiles of vitamin A in patients with and without significant coronary stenosis at baseline angiography.[a]

	Tertiles of serum vitamin A[b,c]			
	First	Second	Third	*p* for interaction
Stenosis				
No. of events/*n*	92/461	101/459	93/460	
	1.08 (0.88-1.34)	1.15 (0.93-1.42)	1.47 (1.26–1.71)	0.02
No stenosis				
No. of events/*n*	19/276	13/274	15/275	
	1.04 (0.63–1.71)	0.73 (0.36–1.48)	1.04 (0.58–1.56)	0.56

[a]Risk as hazard ratios (95% confidence intervals) per 1 SD log-transformed plasma total homocysteine, computed by Cox proportional hazards model adjusted for age and sex.
[b]Cut-offs for serum vitamin A tertiles were < 2.62, 2.62–3.15 and > 3.15 μmol/L for patients with coronary stenosis.
[c]Cut-offs for serum vitamin A tertiles were < 2.73, 2.73–3.30 and > 3.30 μmol/L for patients without coronary stenosis.

transport,[37] indicating that the outlined mechanism involving Vit-A and GNMT may be tissue-dependent.

Taken together, we speculate that elevated Vit-A activity modifies the tHcy-associated risk of CVD. Notably, tHcy-associated risk among these patients may reflect immune activation and disrupted lipid metabolism. However, well-defined mechanistic studies are needed to explore this potential biological interaction in greater detail, and possibly with regard to different tissues.

Relevance

Recent dietary intake of Vit-A is not likely to affect the observed relationship as it does not affect serum Vit-A concentrations except in individuals with extreme excess or deficient intake.[40] Thus, the high circulating Vit-A levels may reflect metabolic disturbances, as has been shown in animal models.[41] Adjustment for important confounders such as kidney function, which is a determinant of both serum Vit-A[42] and plasma tHcy,[43] did not attenuate the interaction significantly. Although the interaction between tHcy and Vit-A appeared to be independent of other known risk factors, it is essential to replicate the findings in other studies.

In an attempt to explore whether the interaction differed according to the prevalence of coronary artery disease we stratified our patients based on the presence of significant coronary stenosis. We observed a significant interaction between tHcy and Vit-A in patients with 1–3 stenotic epicardial vessels, but not in patients without significant stenosis. These observations indicate that the interaction was confined to patients with more severe disease; however, the statistical power to detect the interaction in patients without coronary stenosis was weak (47 AMI events). These subgroup observations are exploratory but highlight the necessity of investigating this interaction in large and initially healthy cohorts.

Strengths and limitations

This study included a large and well-characterized population with established CVD. Given the high levels of circulating Vit-A compared with other populations,[29,44] this cohort may have been particularly suitable for the current study objective. In contrast, the results reported in this study may not be generalizable to the general population because the patients were at increased CVD risk upon recruitment.

The majority of samples were drawn from non-fasting subjects, and tHcy has been found to be modestly increased following food intake,[45–47] but adjustment for fasting state in the multivariate models did not alter the risk estimates significantly. The findings and proposed mechanisms are further complicated by the possibility that Vit-A concentrations do not necessarily reflect biological activity in tissues. Finally, as this study is of an observational nature, it does not allow for causal inference due to the possibility of residual confounding such as long-term use of high-dose supplements.[40,48]

Conclusions

The results from the present exploratory study suggest that the association of circulating concentrations of tHcy with incident AMI is confined to patients with elevated serum concentrations of Vit-A. These findings may shed light on the hitherto unclear relationship between tHcy and CVD. We speculate that tHcy-associated risk may reflect disturbed lipid metabolism and thus experimental and mechanistic studies are needed to elucidate this interaction in a biological system.

Author contribution

OKN conceived and designed the study; OKN, GFTS, ERP and PMU conducted the research; TO and KJV conducted the statistical analyses; ØM and PMU were responsible for measuring vitamin A in serum and tHcy in plasma; TO, KJV, GFTS, ERP, GST, RB, ID, PMU, HR and OKN interpreted the results; TO, KJV, GFTS, ERP, GST, RB, ID, PMU, ØM, HR and OKN wrote, critically revised and approved the final version of the manuscript.

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Declaration of conflicting interests

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III

Homocysteine and related amino acids, inflammatory markers and creatinine are associated with serum retinol in patients with cardiovascular disease

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Abstract

Purpose: To evaluate biomarkers and dietary factors associated with retinol in serum of patients with suspected coronary artery disease with focus on lipids, inflammatory markers, intermediates of homocysteine/one-carbon metabolism and kidney function.

Methods: We used cross-sectional data from 4116 patients hospitalised for suspected coronary artery disease from Western Norway. Dietary data were obtained from a subgroup of 1962 patients using a food frequency questionnaire. Potential biomarkers and dietary factors were explored using ordinary least squares regression modelling adjusted for age and sex. Regression coefficients and corresponding confidence intervals are given as % change in serum retinol per unit change in the predictors. Analyses were performed in the total population and in strata of serum retinol tertiles.

Results: In age- and sex-adjusted models, plasma total cysteine were positively associated (standardized β: 0.26, 95% CI [0.23, 0.29], whereas plasma serine were negatively associated (-0.15, [-0.18, -0.12]) with serum retinol. Other positively associated biomarkers included uric acid (0.30, [0.26, 0.33]), neopterin (0.22, [0.18, 0.25]) and serum creatinine (0.38, [0.35, 0.42]). C-reactive protein was inversely associated with serum retinol (-0.15, [-0.18, -0.12]). When we included significant biomarkers in a multivariate model, the model explained 30% of the variability in serum retinol. The results were generally similar in the lower and upper tertiles of serum retinol. Weak or no associations were observed for dietary factors.

Conclusions: The strongest positive associations were observed for serum creatinine, uric acid and plasma total cysteine with retinol. Negative predictors included C-reactive protein and the amino acid serine.

Key words: Retinol; vitamin A; cardiovascular disease; creatinine; cysteine; uric acid

Introduction

Vitamin A is an essential, fat-soluble micronutrient that that refer to all-*trans* retinol and its bioactive metabolites retinaldehyde, retinoic acid (RA) as well as retinyl ester and pro-vitamin A carotenoids [1]. Following dietary intake from foods of plant and/or animal origin, retinol is transported as retinyl esters with triacylglycerol-rich chylomicrons to the liver where it can either be stored in hepatic stellate cells or exported to peripheral tissue bound to retinol-binding protein 4 (RBP4) and transthyretin. Retinol is then converted to RA in target tissues where it functions as a ligand for nuclear RA receptors with several target genes involved in growth and differentiation, metabolism of macronutrients [2], and the immune system [3].

We have recently reported that elevated serum retinol potentiates the risk of incident acute myocardial infarctions associated with traditional risk markers in subjects hospitalised for suspected coronary artery disease, including lipids and total homocysteine [4,5]. The circulating retinol concentrations in these patients exceed those reported from other cohorts [6-9], and retinol concentrations above the clinical reference range have been associated to with the metabolic syndrome [10] and cardiovascular disease (CVD) [11]. In addition, plasma concentrations of RBP4, which circulates with retinol in a nearly 1:1 manner, were elevated in conditions characterized by metabolic dysfunction such as diabetes type 2 [12], obesity [13] and atherosclerosis [14].

In spite of these recent observations, biomarkers associated with circulating retinol concentrations have not been well characterized in populations with CVD. Although early findings suggest that serum retinol is under tight homeostatic control except during conditions of deficiency or extreme excess [15], recent reports suggest that patients with chronic kidney disease have higher [16], whereas inflammation may reduce [17] serum retinol. Thus, the aim of this exploratory cross-sectional study was to identify biomarkers and dietary factors associated with serum retinol in a large cohort of patients with suspected or established CVD. Our focus was primarily on biomarkers relevant for CVD risk prediction including circulating lipid parameters, homocysteine and inflammatory markers, as well as dietary factors and markers of kidney function. Analyses were conducted for the total population and following stratification of patients according to tertiles of serum retinol.

Methods

Study design

The study population has been described extensively elsewhere [18]. Briefly, 4164 patients were initially included in this study. All patients were recruited upon planned angiography for suspected stable angina pectoris at Haukeland (n = 3413) and Stavanger (n = 751) University Hospitals, Norway and 61.8% (n = 2573) were enrolled in the Western Norway B-vitamin Intervention Trial (WENBIT) (clinicaltrials.gov: NCT00354081) [19]. Patients with missing data on serum retinol (n = 46) and with extremely low (< 0.8 μmol/L) (n = 1) or high (> 9.0 μmol/L) (n = 1) concentrations of retinol were excluded from the study, yielding a total of 4116 eligible patients for analysis. Informed consent, ethical approval, and necessary permissions from the Norwegian Medicines Agency and the Norwegian Data Inspectorate were obtained. The study was carried out according to the Declaration of Helsinki.

Baseline data, biochemical analyses and food frequency questionnaire

Acquisition of clinical and diagnostic data including information on the presence and extent of coronary artery stenosis as determined by coronary angiography, body composition and smoking habits have been described in detail previously [18]. In addition, mostly non-fasting blood samples (80%) were collected at baseline and serum samples were stored at -80 degrees Celsius until analysis. Plasma concentrations of methionine, total homocysteine, cystathionine and cysteine were analysed using gas chromatography-mass spectrometry [20,21], whereas serum *all-trans* retinol [22], creatinine [23] and plasma neopterin [24] were measured by high performance liquid chromatography/tandem mass spectrometry by BEVITAL AS (www.bevital.no). Serum concentrations of total cholesterol, apolipoprotein A1 and B, C-reactive protein (CRP), and dietary vitamin A intake were measured as described previously [4,18]. Serum uric acid measurements were part of the routine laboratory analytical panel [18].

Baseline dietary data were obtained from 2068 WENBIT participants from a 169-items food frequency questionnaire (FFQ). After exclusion due to noncompletion of the FFQ, very high (>15000 kJ/d for females, >17500 kJ/d for males) or very low energy intake (< 3000 kJ/d for females, 3500 kJ/d for males), 1962 patients were eligible for analysis. The development of the FFQ has been described elsewhere, and has been validated for total energy intake and several nutrients [25-27]. Frequency of consumption and measures were converted by a conversion system developed by the Department of

Nutrition, University of Oslo (Kostberegningssystem, version 3.2; University of Oslo). Vitamin A intake is given as retinol activity equivalents (RAE), and conversion values are based on the official Norwegian Food Table.

Statistical analysis

The majority of the continuous data were not normally distributed. Log-transformed continuous variables are therefore presented as geometric means (gM) (geometric standard deviations [gSD]). Intake data on macronutrients are presented as proportion of total energy intake (E%), and food items as densities (g/1000 kcal). Vitamin A intake is given as RAE/1000 kcal. Categorical variables are given as *n* (%). Baseline characteristics are given for the total population.

We used ordinary least squares regression to evaluate individual predictors of log-transformed concentrations of serum retinol. All models were adjusted for age (continuous) and sex (categorical) unless otherwise specified, and plasma/serum predictors were log-transformed. Models including dietary intakes were additionally adjusted for energy intake as described in the previous paragraph. No data transformation was applied to the dietary data. Estimates are reported as standardized β which indicate the standard deviation change in serum retinol per standard deviation change in the exposure variable.

For the sake of interpretability, we also report unstandardized βs (95% confidence interval). In models where the exposure and outcomes are log transformed, the βs represent the % change in serum retinol indicated by 1 % increase in the exposure variable, respectively. These were subsequently multiplied by 10, and thus represent the % change in serum retinol indicated by 10 % increase in the exposure variable. For models including dietary data, the β represent the % change in serum retinol indicated by 1 E% (macronutrients), 50 g/1000 kcal (food groups) or 200 RAE/1000 kcal. Additionally, we calculated the adjusted R^2 to obtain variance explained by each model. The linearity of unadjusted associations was visualized using generalized additive model (GAM) plots. We applied the Bonferroni correction for multiple comparisons, and the adjusted p-value was set to 0.001. All statistical analyses were performed using R-studio v.1.1.447 and packages included in the “tidyverse” as well as “sjstats”. All figures were made with the “ggplot2” package.

Results

Baseline characteristics

Baseline characteristics and dietary intake for the total population are presented in **Table 1** and **2**. Serum concentrations of retinol ranged from 1.02 to 7.65 μmol/L, and the gM (gSD) was 2.84 (1.26) μmol/L. The cohort consisted of 71.9% men, and gM (gSD) age was 60.8 (1.19) years, BMI was 26.5 (1.16) kg/m^2 and creatinine was 90.2 (1.22) μmol/L. A total of 25.9% were smokers, whereas 74.8% had significant coronary stenosis at baseline angiography. For the 1962 patients that completed the FFQ, mean (SD) energy intake was 2073 (694) kcal whereas vitamin A intake was 945 (606) RAE/1000 kcal/d.

Biomarkers associated with retinol in the total population

All analyses are presented in **Figure 1.** Regression coefficients and their confidence intervals are given in **Table 3**. The strongest associations were found for serum creatinine, uric acid, triglycerides and plasma total cysteine, all of which were positively associated with serum retinol. Other significant positively associated biomarkers included plasma neopterin, plasma total homocysteine and serum apolipoprotein A1. Negatively associated biomarkers included CRP and plasma serine. Because serum creatinine is used as a marker of kidney function, which may confound associations between biomarkers and serum retinol in the circulation, additional models were created and adjusted for serum creatinine. The results remained significant for the associations of plasma total cysteine (standardized $\beta = 0.17$, 95% CI: 0.13, 0.20, $p < 0.001$), serum uric acid (standardized $\beta = 0.20$, 95% CI: 0.17, 0.24, $p < 0.001$) and plasma serine (standardized $\beta = -0.11$, 95% CI: -0.14, -0.07) with serum retinol, whereas the remaining associations were severely attenuated (data not shown).

We evaluated the linearity of the associations by fitting unadjusted GAM-curves for the associations of serum creatinine, uric acid, triglycerides as well as plasma total homocysteine, cysteine and neopterin with serum retinol as shown in **Figure 2**. All associations appeared to be linear.

To assess the fraction of variance explained by all significantly associated biomarkers, we included age, sex, serum creatinine, total cholesterol, apolipoprotein A1 and B, triglycerides, uric acid and CRP, and plasma total homocysteine, cystathionine, total cysteine, serine and neopterin in a multivariate regression model and calculated the adjusted R^2. The total proportion of variance

explained was 30%, and the predicted vs. observed values of log-transformed retinol based on this model are illustrated in **Figure 3.**

Associations between dietary intakes and serum retinol

In total, 1962 patients completed the FFQ, and associations of dietary factors and retinol in serum are presented in **Table 4.** A weak, positive association was observed for meat and vegetable intake with serum retinol, whereas no significant associations were observed for other dietary variables including energy-adjusted RAE intake.

Factors associated with retinol in patients with low and high concentrations

Because of the substantial range in serum concentrations of retinol (1.02 to 7.65 µmol/L) we explored potential predictors, separately in patients in the lower and higher ranges of serum retinol. Results according to serum retinol tertiles are presented in **Figures 4** and **5.** Results from the 1st and 3rd tertile (retinol < 2.57 and > 3.08 µmol/L, respectively) showed generally the same, but slightly weaker associations as for the total population. Associations for serum creatinine, plasma total cysteine and serum uric acid remained prominent in the 1st and 3rd tertiles. The associations for dietary intakes with serum retinol were essentially similar as for the total population (data not shown).

Discussion

Principal findings

Serum retinol have generally been considered to be under tight homeostatic control [15], and we have previously shown that retinol in serum of patients hospitalised for suspected coronary artery disease may range beyond what has been observed in other cohorts [4-9]. Factors associated with this variation have not been elucidated to a meaningful extent in CVD patients. In this exploratory study, we report observed associations for circulating and dietary factors with serum retinol in a large cohort of patients with suspected coronary artery disease. The most prominent associations were observed for creatinine, triglycerides, uric acid, serine and the sulphur amino acid cysteine. When we adjusted for serum creatinine, several of the associations were attenuated, whereas those of total cysteine and uric acid with serum retinol remained significant. Other positively associated biomarkers included plasma neopterin, but this association was attenuated after adjustment for creatinine. With the exception of meat and vegetable consumption, we found no significant associations between dietary intakes and serum retionl.

Serum creatinine and retinol

We observed a positive association between serum creatinine, a marker of kidney function, and serum retinol. This is in line with previous findings showing that retinol in serum increases in chronic kidney disease [16,28] and that estimated glomerular filtration rate is an important determinant of retinol in an elderly population [29]. One case-control study found that serum retinol was positively associated with hypertension, and the authors speculate that this effect might have been mediated by kidney dysfunction [30]. As illustrated by the GAM plots, the positive association between serum creatinine and serum retinol in our study was presumably linear. Whereas only small amounts of retinol and its degradation products are cleared in the healthy kidney [1], results from patients with kidney disease suggest that the kidneys contributes significantly to the clearance of retinol and its degradation products [16,28]. However, we do not have urinary data on retinol available, which may have been useful in the evaluation of the relationship between kidney function and retinol status [31].

Plasma cysteine, serine and serum retinol

To our knowledge, this is the first study demonstrating associations of plasma total cysteine and serine with serum retinol. In metabolism, serine is produced from glycolysis, serve as a precursor for glycine [32], and may condense with homocysteine to produce cystathionine and ultimately cysteine in transsulfuration [33,34]. In observational studies, serine is inversely related to components of the metabolic syndrome [35,36] whereas positive associations have been observed for cysteine [37,38]. Because there is little literature available on the possible relationship between cysteine and serine with retinol, it is difficult to interpret the direction of the observed associations in the present study, but some evidence from other populations with lifestyle diseases suggest that retinol may affect the metabolism of these amino acids. Notably, circulating concentrations of retinol bound to RBP4 was associated with dysregulated glucose metabolism [12] which may impact serine production from glycolysis and partly explain the observed inverse association between serine and retinol. Furthermore, animal models have demonstrated that enzymes involved in homocysteine and cysteine metabolism can be induced by RA administration [39-41]. Interestingly, both serine – as a glycine precursor – and cysteine, are central to the hepatic formation of glutathione [33,34,42] a major antioxidant of which plasma concentrations can be low in subjects with obesity and CVD [43,44]. Whether the possible effects of retinol on serine and cysteine influence glutathione status is not known, and future studies should thus seek to address whether elevated cysteine and reduced serine in lifestyle disease and CVD 1) reflect reduced glutathione synthesis and 2) whether this effect is mediated by the bioactive RA.

Inflammatory markers and serum retinol

Studies on the relationship between inflammation and vitamin A are extensive [3,17,45]. Markers of the acute phase response, such as CRP, are considered inversely related to retinol [17]. It is generally accepted that systemic inflammation may contribute to increased sequestration of retinol in tissues and subsequently to reduced serum concentrations. In line with this notion, we observed an inverse association for CRP with retinol. In contrast, other inflammatory markers associated with CVD risk, such as uric acid [46], were positively associated with serum retinol. This particular finding reflects those of others, which have shown a positive association between uric acid and retinol in large and healthy cohorts [47,48]. There may be several potential unmeasured factors that affect this association, but interestingly, the enzyme that produce uric acid – xanthine oxidase – has been linked to endothelial dysfunction in atherosclerosis [49] and can also catalyse the formation of RA [50]. Further, neopterin, a

marker of the pro-atherogenic T_h1 cell-mediated monocyte activation that has been linked CVD risk [51,52] was positively associated with retinol in the present investigation. It is not known whether neopterin itself affects retinol concentrations, however, *in vitro* studies show that RA administered in physiological doses activate T_h1 cells [45] which in turn can contribute to proliferation of monocytes into macrophages and increased neopterin concentrations [51]. Our findings indicate a potential interplay between inflammatory processes in atherosclerosis and retinol and should be addressed in future studies. Specifically, the uptake and metabolism of retinol, and activity of RA in immune cells during inflammatory processes specific to atherosclerosis would provide insight in this context.

Diet and serum retinol

The observed associations for the dietary predictors were weak, which is not unusual considering the relatively stable concentrations of circulating retinol [53,54]. Although the existing evidence for the association for meat intake and retinol is somewhat conflicting [55,56] we did observe positive association between meat intake and serum retinol. Meat (in particular processed meat) intake should be limited in the context of CVD prevention [57], and taken together with the other results of the present investigation indicate that high serum concentrations of retinol at least in part are explained by factors related to an unfavourable risk profile in these patients. We cannot exclude the possibility that the bias present in dietary assessment tools obscured the true associations.

Strengths and limitations

The major strength of our study was the large, well-characterized cohort including more than 4000 patients, the majority with angiographically verified coronary artery disease, which provided a solid basis for the evaluation of biomarkers and dietary factors associated with serum retinol. However, our findings cannot be generalized beyond the study population, because serum concentrations of retinol appear to be elevated in patients with CVD compared to a presumably healthy population residing in the same geographical area [58]. Further limitations include the cross-sectional design, which complicates the interpretation of the direction of the associations. The interpretation of the associations between total cysteine and uric acid with retinol were particularly complicated, because it is unclear whether cysteine and uric acid affect retinol or vice versa.

Although not strictly a limitation it should be noted that the proportion of variance explained by the models was generally low. Overall, the adjusted R^2 varied from 4% to ~15%, for the models, whereas a multivariate model including the most strongly associated biomarkers explained about 30 % of the total variation in serum retinol. Although some of the unmeasured variation may be attributed to genetics [59,60], we emphasize that very little is known about factors affecting retinol in the circulation, and that a continued effort should be undertaken to further explore this particular knowledge gap.

Conclusion

Biomarkers associated with retinol in patients with established CVD include metabolites that are linked to metabolic disease, kidney function, and inflammation. Future observational and experimental studies should assess the potential causal direction and the clinical relevance of these associations. Finally, it would be useful to assess these associations in healthy populations, to further uncover the role of retinol in health and disease.

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Conflicts of interest

The authors declare that they have no conflicts of interest

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FIGURE LEGENDS

Fig. 1 Forest plot of predictors of serum retinol. Abbreviations: CRP, C-reactive protein; apo, apolipoprotein; BMI, body mass index, SD; standard deviation

Fig. 2 Generalized additive model plots of the unadjusted linear association between selected predictors and log-transformed serum retinol

Fig. 3 Observed vs. predicted values of log-transformed serum retinol derived from a linear regression model including age, sex, total cysteine, uric acid, creatinine, neopterin, total cholesterol, apolipoprotein A1 and B, triglycerides, total homocysteine and cystathionine

Fig. 4 Forest plot illustrating predictors of serum retinol in the lower retinol tertile. Abbreviations: CRP, C-reactive protein; apo, apolipoprotein; BMI, body mass index, SD; standard deviation

Fig. 5 Forest plot illustrating predictors of serum retinol in the upper retinol tertile. Abbreviations: CRP, C-reactive protein; apo, apolipoprotein; BMI, body mass index, SD; standard deviation

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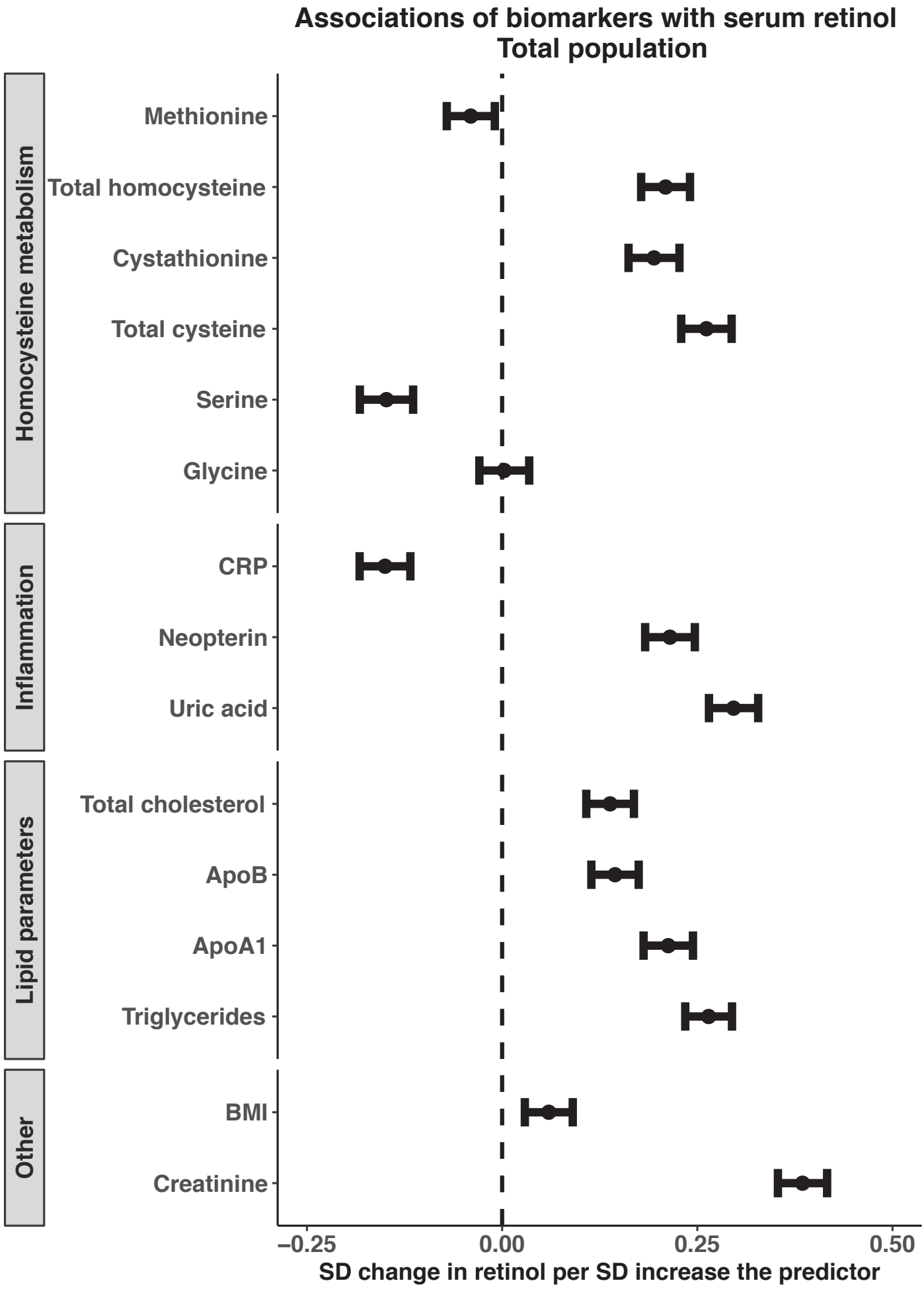
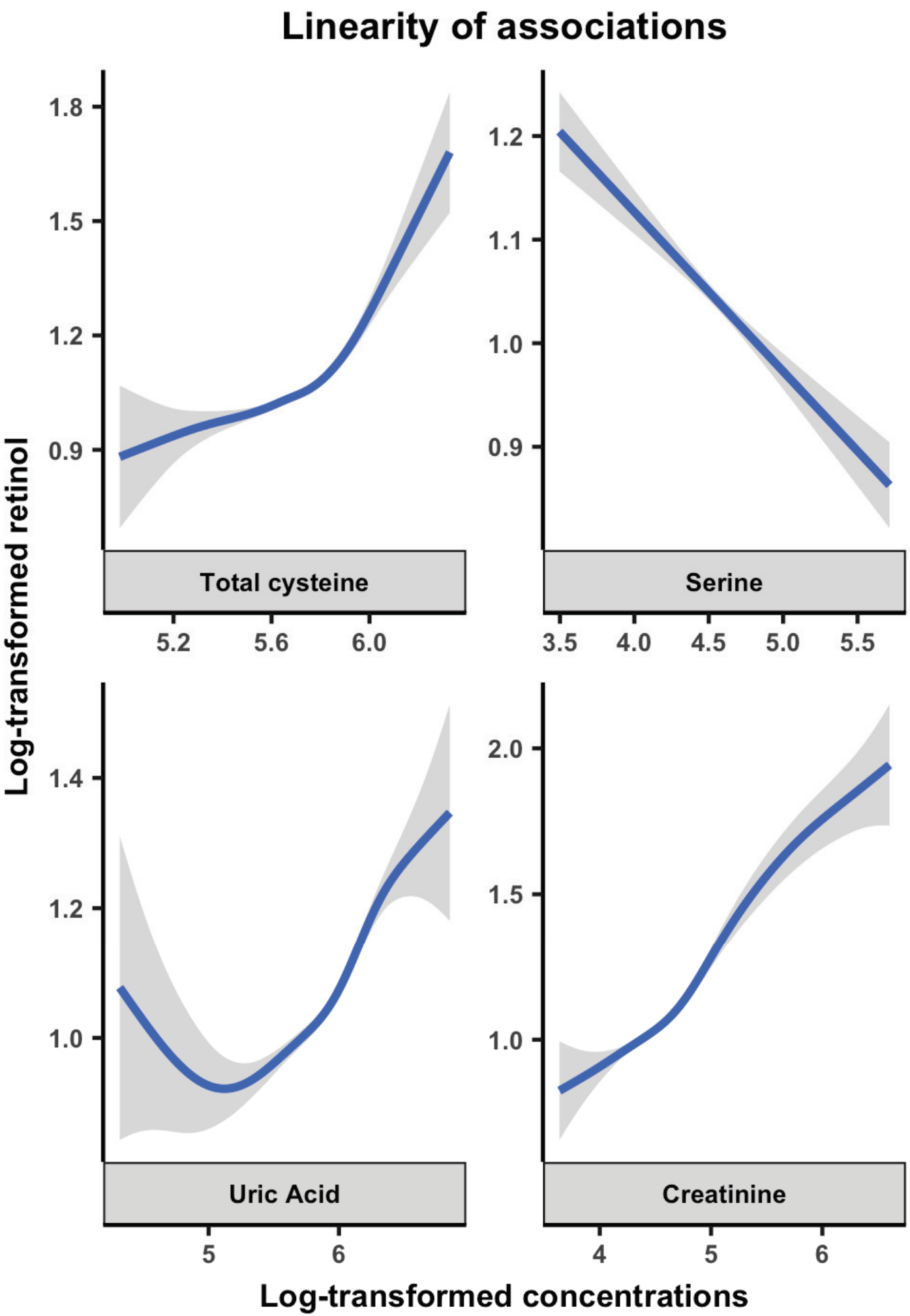
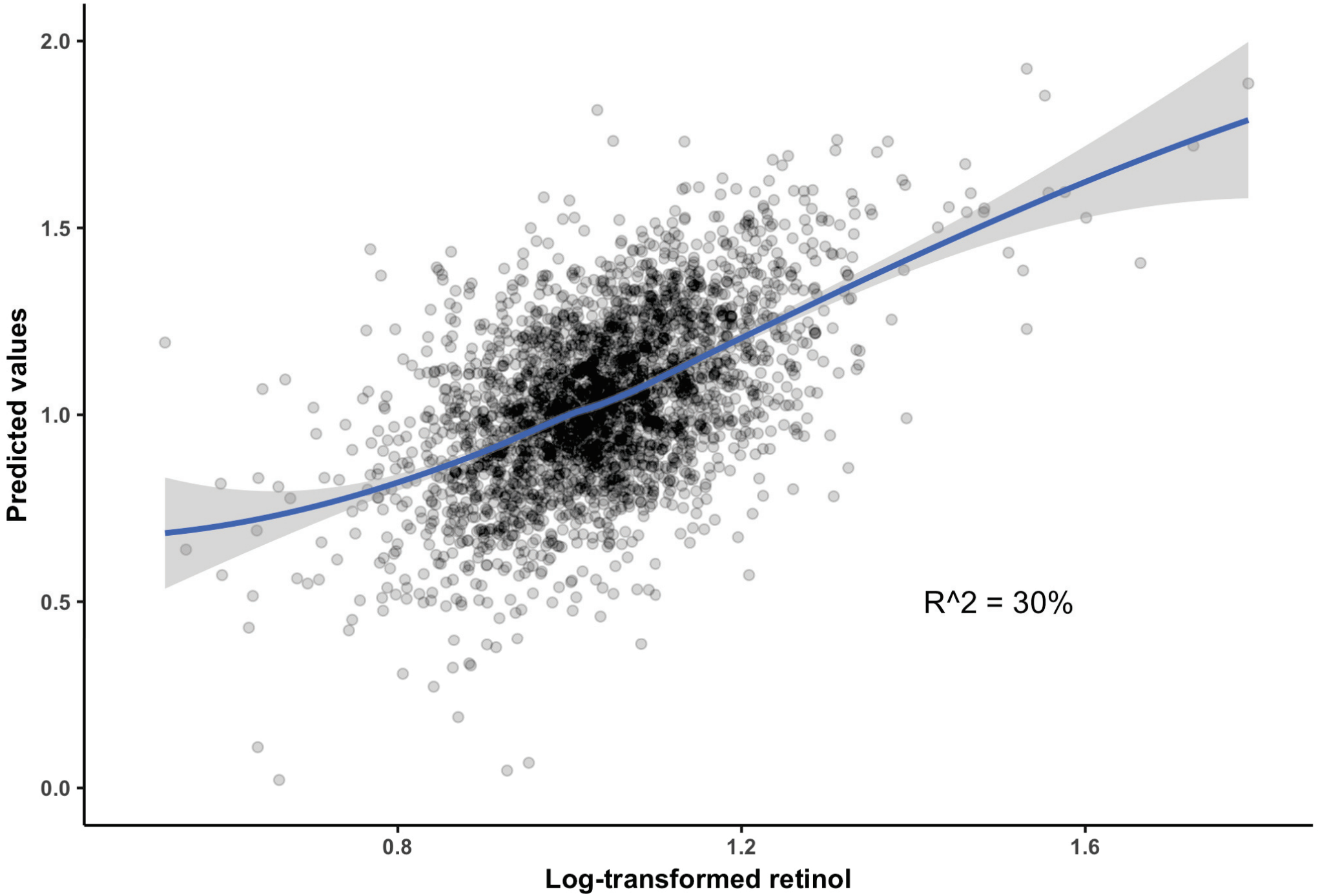
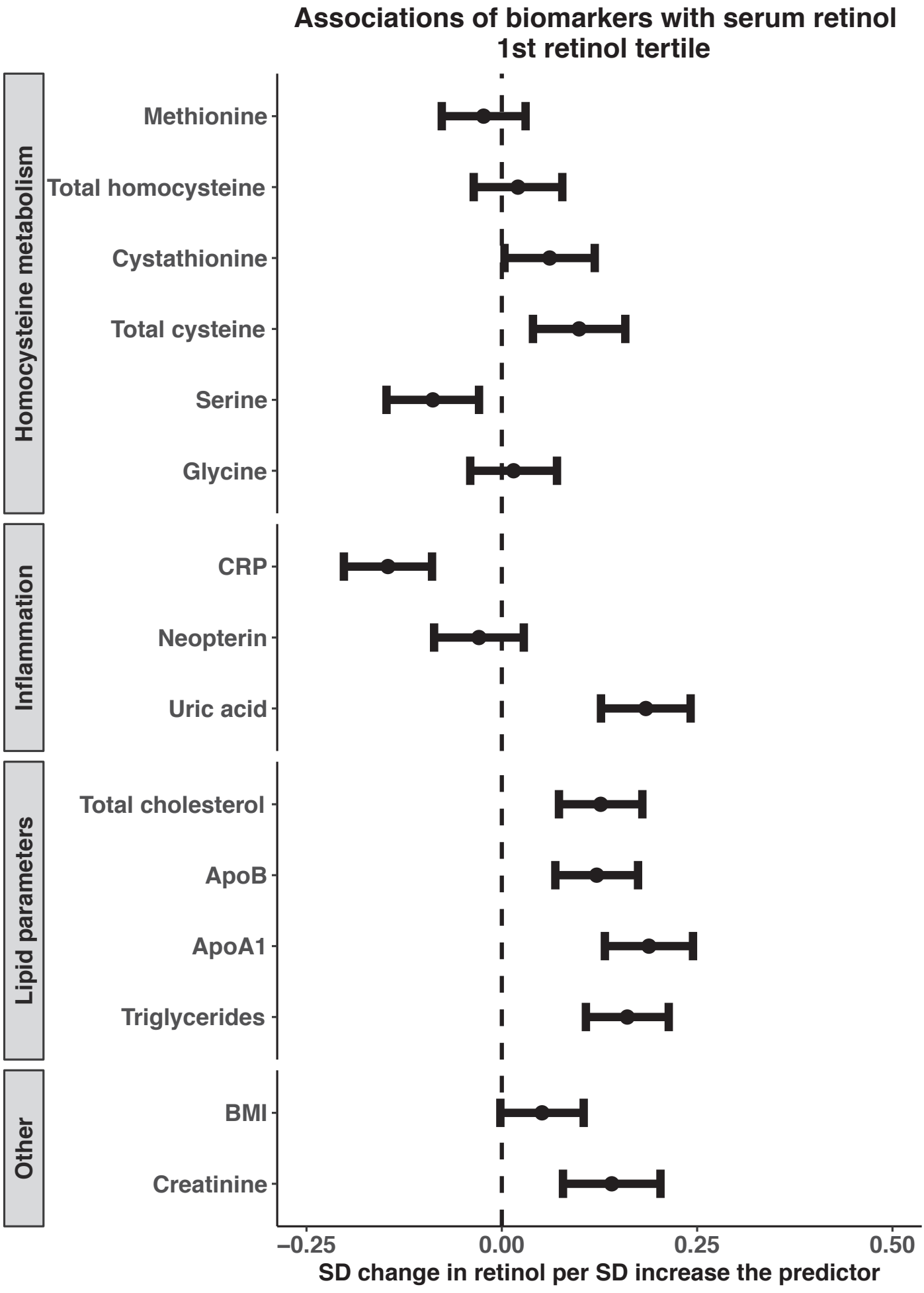

Associations of biomarkers with serum retinol
Total population
Homocysteine metabolism
Methionine
Total homocysteine
Cystathionine
Total cysteine
Serine
Glycine
Inflammation
CRP
Neopterin
Uric acid
Lipid parameters
Total cholesterol
ApoB
ApoA1
Triglycerides
Other
BMI
Creatinine
–0.25
0.00
0.25
0.50
SD change in retinol per SD increase the predictor


Linearity of associations
Log-transformed retinol
Total cysteine
Serine
Uric Acid
Creatinine
Log-transformed concentrations
1.8
1.5
1.2
0.9
5.2
5.6
6.0
1.2
1.1
1.0
0.9
3.5
4.0
4.5
5.0
5.5
1.4
1.2
1.0
5
6
2.0
1.5
1.0
4
5
6


2.0
1.5
1.0
0.5
0.0
Predicted values
0.8
1.2
1.6
Log-transformed retinol
R^2 = 30%


Associations of biomarkers with serum retinol
1st retinol tertile
Homocysteine metabolism
Methionine
Total homocysteine
Cystathionine
Total cysteine
Serine
Glycine
Inflammation
CRP
Neopterin
Uric acid
Lipid parameters
Total cholesterol
ApoB
ApoA1
Triglycerides
Other
BMI
Creatinine
–0.25
0.00
0.25
0.50
SD change in retinol per SD increase the predictor

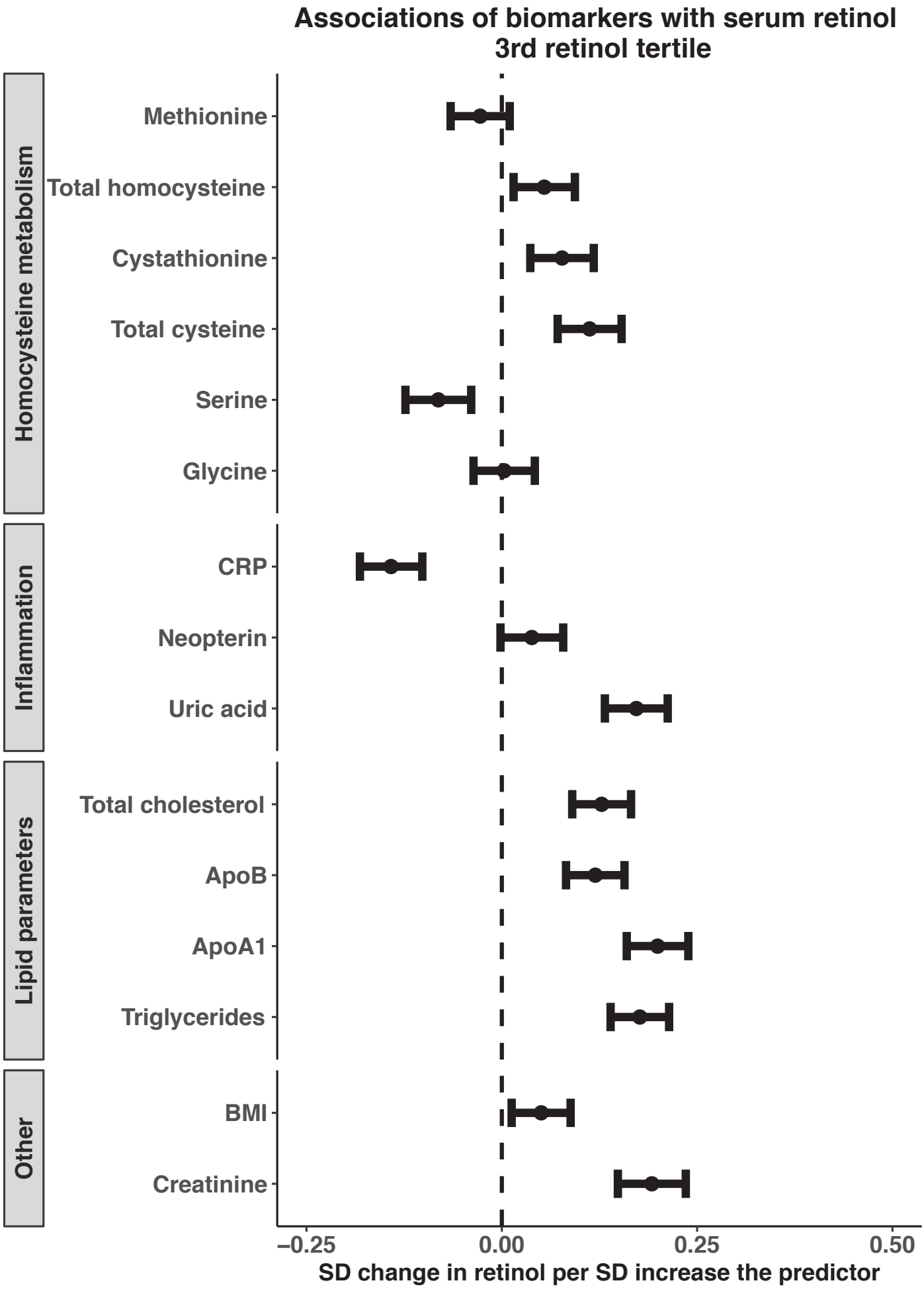

Associations of biomarkers with serum retinol
3rd retinol tertile
Homocysteine metabolism
Methionine
Total homocysteine
Cystathionine
Total cysteine
Serine
Glycine
Inflammation
CRP
Neopterin
Uric acid
Lipid parameters
Total cholesterol
ApoB
ApoA1
Triglycerides
Other
BMI
Creatinine
–0.25
0.00
0.25
0.50
SD change in retinol per SD increase the predictor

Table 1: Baseline characteristics of the total population (n = 4116)[1]

Retinol, µmol/L	2.84	1.26
Age, y	60.81	1.20
Male sex, n (%)	2997	71.9
Smokers, n (%)	1321	31.7
Body mass index, kg/m^2	26.48	1.16
Lipid parameters		
Apolipoprotein B, *g/L*	0.87	1.31
Apolipoprotein A1, *g/L*	1.29	1.23
Triglycerides, *mmol/L*	1.54	1.67
Homocysteine metabolism, µmol/L		
Methionine	26.99	1.30
Total homocysteine	10.70	1.38
Cystathionine	0.28	1.81
Total cysteine	290.04	1.14
Inflammation		
C-reactive protein, *mg/mL*	3.64	2.43
Uric acid, *µmol/L*	347.28	1.28
Neopterin, *nmol/L*	8.57	1.47
Extent of CVD		
1-3 stenotic vessels, n (%)	3120	74.9
Previous acute myocardial infarction, n (%)	1680	40.3

Ejection fraction <60%, n (%)	3277	78.7
Kidney function		
Creatinine, *µmol/L*	90.24	1.22

[1]Baseline characteristics of the total population. Continuous variables are presented as geometric means and geometric standard deviations. Categorical variables are presented as count and per cent.

Table 2: Baseline nutrient and food intake[1]

Nutrients, E%		
Vitamin A, RAE/1000 kcal	945	606
Protein	16.7	2.55
Carbohydrate	48.9	6.46
Total fat	31.9	5.61
PUFA	7.21	1.98
MUFA	10.3	2.03
SFA	11.8	2.65
Alcohol	2.06	3.16
Foods, g/1000 kcal		
Meat	54.8	23.4
Vegetables	105	80.9
Fruits and berries	124	86.1
Eggs	8.38	6.30
Dairy	154	112
Fish	53.7	28.8

[1]Baseline mean and standard deviation intake of nutrients and food groups

Table 3: Regression coefficients and confidence intervals for predictors of vitamin A[1]

Lipid parameters	Standardized β (95% CI)	β per 10% increase (95% CI)	p-value	Adjusted R^2
Total cholesterol	0.14 (0.11, 0.17)	1.47 (1.15, 1.8)	< 0.001	2.17 %
Apolipoprotein B	0.14 (0.11, 0.17)	1.37 (1.04, 1.51)	< 0.001	2.36 %
Apolipoprotein A1	0.21 (0.18, 0.24)	2.44 (2.12, 2.81)	< 0.001	4.36 %
Triglycerides	0.26 (0.23, 0.29)	1.22 (1.10, 1.32)	< 0.001	7.08 %
Homocysteine metabolism				
Methionine	-0.04 (-0.07, -0.01)	-0.36 (-0.62, -0.11)	0.007	0.44 %
Total homocysteine	0.21 (0.18, 0.24)	0.85 (0.62, 1.00)	< 0.001	4.29 %
Cystathionine	0.19 (0.16, 0.23)	0.35 (0.21, 0.52)	< 0.001	3.80 %
Total cysteine	0.26 (0.23, 0.29)	4.81 (4.21, 5.32)	< 0.001	7.11 %
Serine	-0.15 (-0.18, -0.11)	-1.52 (-1.88, -1.17)	< 0.001	2.17 %
Glycine	0.00 (-0.03, 0.30)	0.03 (-0.27, 0.31)	0.823	0.30 %
Inflammation				
C-reactive protein	-0.15 (-0.18, -0.12)	-0.41 (-0.49, -0.32)	< 0.001	2.50 %
Uric acid	0.30 (0.26, 0.33)	2.81 (2.52, 3.23)	< 0.001	9.71 %
Neopterin	0.22 (0.18, 0.25)	1.43 (1.21, 1.62)	< 0.001	4.34 %
Body mass				
Body mass index	0.06 (0.029, 0.09)	0.97 (0.47, 1.47)	0.013	0.60 %
Kidney function				
Creatinine	0.38 (0.35, 0.42)	4.51 (4.19, 4.93)	< 0.001	14.5 %

[1] Regression coefficients for various predictors. All models were adjusted for age and sex. Coefficients represent the percentage change in vitamin A per 10% increase in the predictor and the adjusted R^2 represents the predictive power of the models

Table 4: Regression coefficients and confidence intervals for dietary predictors of vitamin A[1]

Macronutrients	Standardized β (95% CI)	β per E% (95% CI)	p-value	Adjusted R^2
Protein	0.063 (0.02, 0.11)	0.52 (0.16, 0.89)	0.005	1.10 %
Carbohydrate	-0.062 (-0.11, -0.018)	-0.21 (-0.36, -0.07)	0.004	1.18 %
Total fat	0.002 (-0.041, 0.045)	-0.02 (-0.19, 0.14)	0.77	0.70 %
PUFA	-8e-04 (-0.045, 0.043)	-0.11 (-0.58, 0.36)	0.65	0.79 %
MUFA	0.022 (-0.022, 0.065)	0.15 (-0.31, 0.61)	0.53	0.83 %
SFA	-0.0053 (-0.048, 0.038)	-0.12 (-0.47, 0.22)	0.48	0.81 %
Alcohol	0.077 (0.026, 0.13)	0.47 (0.15, 0.79)	0.004	1.01 %
Micronutrient		β per 200 RAE/1000 kcal (95% CI)		
Vitamin A	0.017 (-0.029, 0.062)	0.29 (-0.03, 3.27)	0.09	0.80 %
Foods		β per 50 g/1000 kcal (95% CI)		
Meat	0.076 (0.032, 0.12)	3.60 (1.54, 5.66)	< 0.001	1.30 %
Vegetables	0.04 (-0.0033, 0.084)	0.91 (0.33, 1.50)	0.002	1.15 %
Fruits and berries	0.005 (-0.039, 0.049)	0.13 (-0.41, 0.68)	0.63	0.70 %
Eggs	0.012 (-0.032, 0.056)	0.55 (-7.13, 8.25)	0.89	0.70 %
Dairy	0.048 (0.0048, 0.092)	0.41 (-0.01, 0.82)	0.06	0.91 %
Fish	-0.0068 (-0.051, 0.037)	-0.02 (-1.67, 1.61)	0.97	0.72 %

[1] Regression coefficients for various dietary predictors. All models were adjusted for age, sex and energy intake. Coefficients represent the percentage change in vitamin A per 10% increase in the predictor and the adjusted R^2 represents the predictive power