

Plasma Retinol and Cardiovascular Disease Mortality in the BECAC Population

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Preface

This master thesis has been done in association with The Department of Heart Disease and Institute of Clinical Medicine at the Faculty of Medicine and dentistry, University of Bergen. The Department of Heart Disease at Haukeland University Hospital performed the data collection.

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Abbreviations

AMI	Acute myocardial Infraction
BECAC	Bergen Coronary Angiography Cohort
BOND	Biomarkers of Nutrition for development
CAD	Coronary artery disease
CI	Confidence intervals
CRABP	retinoic acid-binding proteins
CRBP	cellular-retinol binding protein
CRP	C-reactive protein
CVD	Cardiovascular disease
DHA	docosahexaeonic acid
eGFR	Estimated glomerular filtration rate
EPA	eicosapentaenoic acid
FFQ	Food frequency Questionnaire
HPLC	high-performance liquid chromatography
HR	Hazard rate
HUS	Haukeland university hospital
LDL	Low-density lipoprotein
LDL-C	Low-density lipoprotein-cholesterol
MCCS	Melburne Colleborate Cohort Study
MS/MS	Tandem mass spectrometry
NCD	Non-communicable disease
NHANES III	The National Health and Nutrition Examination Survey III
OxLDL	Oxidized Low-density lipoprotein
PPAR	peroxisome proliferator-activated receptor
RA	Retinoic Acid
RAR	Retinoic acid receptor
RBP	Retinol binding protein
RXR	Retinoid X receptor
SAP	Stabile angina pectoris
SD	Standard deviation
SMC	Smooth muscle cell
SUS	Stavanger university hospital
UiB	University of Bergen
UiO	University of Oslo
UL	Upper tolerable limit
VAD	Vitamin A deficiency
VSMC	Vascular smooth muscle cell
WENBIT	Western Norway B Vitamin Trial
WHO	World Health Organization

Abstract

Background: Observational studies have suggested a possible link between vitamin A and cardiovascular disease mortality risk. However, randomized supplementation trials have failed to show improvement in survival. There is insufficient evidence on the effects of low and elevated subclinical vitamin A status on mortality risk in populations that show no signs of vitamin A deficiency or toxicity.

Method: We evaluated all-cause, CVD-related and non-CVD-related mortality risk associated with continuous data, quartiles and deciles of plasma retinol concentration, in 3842 patients with established CVD of the Bergen Coronary Angiography Cohort and the Western Norway B Vitamin Intervention Trial.

Results: Over the mean seven years and three months of follow-up, there were 280 deaths, including 149 from CVD and 131 from non-CVD. Patients with high levels of plasma retinol concentration had a higher risk of total and non-CVD mortality than patients with plasma retinol concentration in the 'middle range'. High plasma retinol levels were also associated with increased risk of CVD-related mortality, adjustment for confounders did not change the assumption substantially, but significance was lost. Patients with low plasma retinol also had a higher mortality risk, but the association did not achieve significance.

Conclusion: Our findings suggest that low or high plasma retinol levels does not predict CVD-related mortality singularly but rather predicts increased all-cause mortality.

Impact: The results suggest that further study of association is needed, and for more tailored intervention studies based on participants serum retinol levels, that if possible are adjusted for markers of systemic inflammatory response.

Abstrakt

Bakgrunn: Observasjonsstudier har indikert en mulig sammenheng mellom vitamin A og økt risiko for hjerte- og karsykdomsrelatert dødelighet. Randomiserte intervensjonsstudier har derimot ikke lyktes i å redusere risiko for hjerte- og karsykdomsrelatert død. Det mangler evidens for mulig effekt av høye og lave sub-kliniske nivåer av vitamin A på dødelighet i populasjoner som ikke viser tegn til vitamin A-mangel eller -toksisitet.

Metode: Vi har evaluert total dødelighet, hjerte- og karsykdomsrelatert og ikke hjerte- og karsykdomsrelatert dødelighetsrisiko opp mot kontinuerlige nivå, kvartiler og desiler av plasma retinol, hos 3842 pasienter med etablert hjerte- og karsykdom fra BECAC og WENBIT.

Resultat: Pasientene ble fulgt opp over syv år og tre måneder, hvor 280 døde, 149 av hjerte- og karsykdoms relaterte årsaker og 131 av ikke hjerte- og karsykdoms relaterte årsaker. Pasienter med høye plasma retinol-verdier hadde høyere total dødelighet og ikke hjerte- og karsykdoms-relatert dødelighet sammenliknet med pasienter med middel verdier av plasma retinol. Høye plasma retinol-verdier var også assosiert med økt hjerte- og karsykdoms-relatert dødelighet, justering for kofaktorer endret ikke assosiasjonens størrelse, men endringen i dødelighet var ikke lenger signifikant. Pasienter med lave plasma retinol-verdier hadde også økt dødelighetsrisiko, men assosiasjonen var aldri signifikant.

Konklusjon: Resultatene indikerer at lave og høye plasma retinol-verdier ikke eksklusivt forklarer den økte risikoen for hjerte- og karsykdoms-relatert dødelighet, men heller økt risiko for total dødelighet.

Relevans: Videre utforskning av assosiasjonene analysert i dette studiet er nødvendig. I tillegg kan det virke hensiktsmessig med justering for serum retinol-verdier og markører for inflammatorisk respons i fremtidige intervensjonsstudier.

1 Introduction

1.1 Non-communicable disease

Non-communicable diseases (NCD) are a group of diseases that are defined by the World Health Organization (WHO) as; “a medical condition or disease that is by definition non-infectious and non-transmissible among people” [1]. NCD’s are the leading cause of death globally, responsible for 36 million of the 57 million deaths that occurred globally in 2008. Of the 36 million deaths, the majority were due to cardiovascular disease (CVD), cancer, diabetes and chronic lung disease. Despite the rapid growth of these diseases globally and especially in low- and middle-income populations and countries, a reduction in mortality is seen in other parts of the world. Moreover, the WHO predicts that the number of NCD related deaths are going to increase globally by 15% from 2010 to 2020 [2, 3].

1.2 Cardiovascular disease

As for individual disease prevalence, CVD is the leading cause NCDs and the leading cause of death globally. CVDs are diseases affecting the heart and blood vessels. The underlying pathology is atherosclerosis, which develops over several years and is usually advanced by the time symptoms occur [4].

1.2.1 Definition

WHO defines CVD as; “any disease plaguing the heart and its associated blood vessels. These include a variety of diseases including coronary heart disease, stroke, and peripheral vascular diseases” [5]. WHO divides cardiovascular diseases by their origin, into those due to atherosclerotic origin and those not related to atherosclerosis. The most prevalent diseases related to atherosclerosis are ischemic heart disease or coronary heart disease (e.g. heart attack), cerebrovascular disease (e.g. stroke) and diseases of the aorta and arteries, including hypertension and peripheral vascular disease. The CVDs not directly related to atherosclerosis are congenital heart disease, rheumatic heart disease, cardiomyopathies and cardiac arrhythmias [4, 5].

1.2.2 Prevalence

1.2.2.1 Globally

WHO states in their report from 2010 that CVD is the leading cause of death globally. 17.3 million People died from CVDs in 2008, this is 30% of the total 57 million deaths

globally. Of the 17,3 million people who died from CVDs in 2008 heart attacks caused 7.3 million deaths and stroke 6.2 million deaths [3, 4].

1.2.2.2 In Norway

Similar rates of CVD death were observed in Norway 2012. Of the 41,913 deaths that occurred in Norway 2012, 13,010 (31%) were caused by CVDs according to data published November 2013 by Statistics Norway [6]. The report by Statistics Norway also states that the mortality rate per inhabitant continues to drop as it has done since 1988. So despite the decrease in mortality rate per inhabitant CVDs still causes 30 percent of annual deaths nationally [6, 7].

Side 10 PCD

1.2.3 Risk factors

Risk factors for CVD and especially the atherosclerotic related conditions are known as major contributors to global morbidity and mortality, not only for their promotion of CVD. The risk factors acknowledged by the WHO are divided into groups by origin and listed below [4].

1.2.3.1 Behavioral risk factors

1. Tobacco use
2. Physical inactivity
3. Unhealthy diet (rich in salts, fat and calories)
4. Harmful use of alcohol

1.2.3.2 Metabolic risk factors

5. Raised blood pressure (hypertension)
6. Raised blood sugar (diabetes)
7. Raised blood lipids (e.g. cholesterol)
8. Overweight and obesity

1.2.3.3 Other risk factors

9. Poverty and low educational status
10. Advancing age
11. Gender

12. Inherited (genetic) disposition
13. Physiological factors (e.g. stress, depression)
14. Other risk factors (e.g. excess homocysteine)

1.2.4 Etiology

The main pathological process that leads to heart attacks and stroke is atherosclerosis. The process of atherosclerotic development is the result of numerous risk factors and may start as early as during childhood and adolescence. Atherosclerosis is an inflammatory process affecting the elastic and muscular layers of medium and large-sized arteries throughout the cardiovascular system [4, 8].

As the endothelium of these blood vessels are exposed to above tolerated levels of low-density lipoprotein (LDL) cholesterol, and certain other substances, such as free radicals, damage may occur. The presence of LDL cholesterol and free radicals results in the endothelium becoming permeable to lymphocytes and monocytes. The lymphocytes and monocytes then migrate into the deep layers of the wall of the blood vessel. A series of reactions occur, attracting LDL cholesterol particles to the site. The monocytes engulf the LDL cholesterol particles and then transform into macrophages (foam cells). With the accumulation of foam cells, smooth muscle cells (SMC) migrate to the site from deeper layers of the vessel wall (the media), leading to the formation of a fibrous cap of SMC and collagen. At the same time, the macrophages involved in the original reaction begin to die, resulting in the formation of a necrotic core covered by the fibrous cap. These lesions (atheromatous plaques) enlarge as cells and lipids accumulate [8].

As the accumulation continues, the plaque begins to bulge into the vessel lumen, leading to a thinning of the fibrous cap accompanied by cracking of the endothelial surface of the plaque, which may finally rupture. With the rupture of the plaque, lipid fragments and cellular debris enter the vessel lumen (bloodstream). Thrombotic agents on the endothelial surface react with the intruding particles, resulting in the formation of a thrombus. If the thrombus is large enough, and the blood vessel or cerebral blood vessel is blocked, the result may be a heart attack or stroke [4].

Holligan et al. states that increased plasma low density lipoproteins cholesterols (LDL-C) increase the stress on dysfunctional areas of the arterial wall, this because the small particles

of LDL-C are more susceptible to modification. LDL modification, also known as oxidation of LDL particles, contributes to the activation and accumulation of macrophages and other monocytes. Both intrinsic factors such as antioxidant content, fatty acid composition, and particle size increase the susceptibility of LDL to oxidation and movement through the injured endothelial layer. Extrinsic factors like surrounding pH, local antioxidant concentration, and transitional metal availability contributes to efficiency of oxidation. Oxidized LDL (OxLDL) is atherogenic and is a direct and indirect-chemoattractant for circulating monocytes [8].

Indirect-chemoattractance is a result of OxLDL mediated release of monocyte chemoattractant protein-1 from the endothelial cells in to the lumen. OxLDL inhibits the movement of local macrophages and in turn promotes the differentiation of monocytes into tissue macrophages via the release of macrophage colony-stimulating factor from the endothelial cells. The indirect-chemoattractance process also attracts T-lymphocytes. These actions facilitate the loss of endothelial integrity [8].

Most of the chronic inflammatory diseases are characterized by overproduction of cytokines, chemokines, eicosanoids, and matrix metalloproteinase. These substrates or mediators amplify the inflammation by attracting inflammatory cells to the site and thereby contributing to the tissue destruction. Many of these mediators are positively regulated through nuclear factor kappa-light-chain-enhancer of activated B cells and some are negatively regulated through peroxisome proliferator-activated receptor (PPAR) and liver x receptors [8]. The continued cycle of tissue injury, healing, and repair is the result of cytokines, chemokines and growth factor release. The inflammatory cells and the resident tissue cells produce this release. The process eventually results in the tissue remodeling seen in the vascular lumen during atherosclerosis [9].

1.2.4.1 Heart Attack

A heart attack or myocardial infraction happens when the blood flow to the heart muscle or parts of the heart muscle is cut-off. The restriction of blood supply is often the result of a thrombus in a supplying blood vessel. The reduced blood supply starves the heart muscle of oxygen and nutrients, causing injury, which in turn can result in a heart attack, often referred to as an acute myocardial infraction (AMI). Blockages can cause mild

reduction in blood flow. This may lead to reduced blood supply to the heart (ischemia) and thus chest pain (angina) [4].

1.2.4.2 Stroke

The pathophysiology of strokes is more diverse than that of a heart attack. Ischemic strokes are strokes happening due to thrombus formation in atherosclerotic cerebral blood vessels, or small vessel disease in the brain linked to vascular risk factors. Stroke may also happen because of a haemorrhage (bleeding) event due to the rupture of a blood vessel caused by the presence of an aneurysm, for example, or due to damage from uncontrolled high blood pressure or atherosclerosis (haemorrhage stroke). Additionally, traveling blood clots can cause a stroke if they end up blocking a blood vessel in the brain. For instance if a person has an irregular heartbeat, blood clots may form in the heart and travel through the blood vessels to the brain [4].

1.3 Epidemiological studies

Epidemiology is the science of studying patterns, causes, health, disease and death in a population. Epidemiological studies investigate association and causality between exposure and outcome in populations. The exposure can be a risk factor, a prognostic factor, a diagnostic test, a treatment, or as in this study plasma retinol (vitamin A). While outcome is often measured in prevalence of death or disease. Results of epidemiological studies is usually presented as; risk, rate, prevalence, odds, or frequency of outcome, and comparing outcomes between groups of participants divided according to exposure will yield relevant frequency measures [10].

There are several different ways of performing epidemiological studies. This study will follow a secondary, quantitative, observational, retrospective, cohort design. The dataset used in this study has been assembled prior to the start of this project by another project and consists of quantitative data in form of anthropometric, biochemical and outcome data. The data consist of baseline values as well as outcome data rendering it suitable for a cohort design. The dataset and population is further discussed under the Method section.

The product of epidemiological studies is validated based on the study's design and its transferability to intended population. These two measures are often referred to as internal and external validity, respectively. Internal validity deals with the strength of the study design and its ability to predict causation and association. Contrastingly, external validity deals with whether association or causal relation between treatment and effect seen in the study population is true for of the target population.

Randomized controlled trials (RCT) are considered the gold standard for epidemiological studies, because of their strong internal validity. Observational studies have lower internal validity as exposure is observed rather than introduced. By introducing exposure, RCTs enables the investigator to evaluate causation as the exposure is introduced in one group while no exposure or placebo is introduced in the other group. This allows the investigator to assume that an observed difference in outcome between groups is due to introduced exposure. Contradictory, in observational studies investigators are not able to

assume causation as participants are evaluated based on a pre-existing level of exposure with no controlled manifestation of exposure or outcome.

In addition to the strength provided by introducing exposure, RCTs also gain internal strength over observational studies through randomizing subjects into exposure and control groups. In doing so, the distribution of confounders such as age and gender is expected to be similar between groups (confounders are discussed under limitations). Because the exposure groups are determined by preexisting levels of exposure in observational studies, confounders are not limited through randomization and has to be dealt with in other ways.

The study's applicability to a population is often referred to as the study's external validity. The study population is in most cases only assumed to represent the entire population. Additionally, in some cases such as the Western Norway B-vitamin intervention trial (WENBIT) study and this current study, the study population may be different from the target population. For a study to external valid the differences between study group and target group can not alter the effect of exposure. In a case of high external validity the exposure or outcome are expected to behave identical in target population to what it did in the population under study. In this regard, RCTs do not have structural advantages over observational studies.

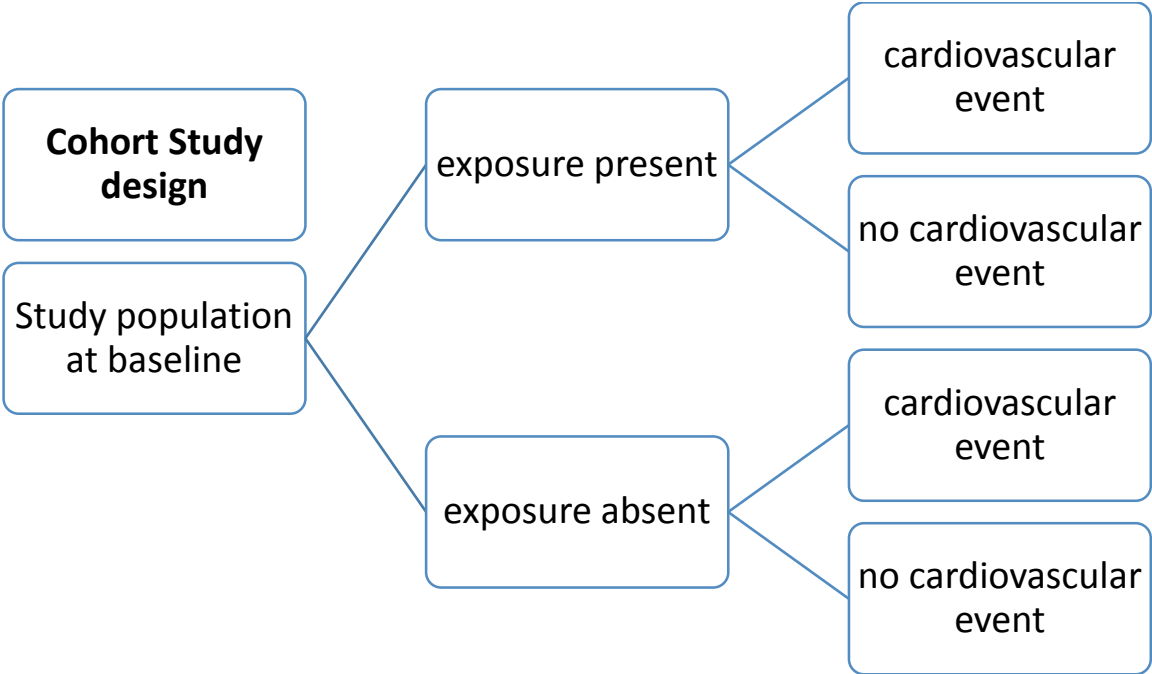
1.3.1 Cohort design

The cohort design is an epidemiological method where a population is followed up over time to evaluate the effect of one or numerous exposures on outcome (Figure 1). The population is divided into groups based on exposure at baseline, in this case plasma retinol level. The population can be divided further based on presence or absence of exposure or may be categorized based on level of exposure and then divided in to two or more groups, for example quartiles. The result of the cohort is gained through factoring the difference in risk of given outcome, in this case CVD death between exposure group. The hazard ratio (HR) is an indicator for the difference in probability of outcome between two or more groups of exposure [10, 11].

The cohort design is similar to random controlled trial (RCT) in that they both evaluate risk of outcome between different exposure groups. Although, in a RCT, the participants are randomly selected into groups, which are then provided with exposure or

placebo. The participants are followed up and outcome is documented, leading to an evaluation of association and causation. In contrast, in a cohort design patients are divided in to groups according to pre-existing exposure. The lack of randomization and control over causation affects the internal validity of the cohort design. The reason randomization is performed in RCTs is to establish groups with identical risk of outcome. In a cohort design, randomization is not possible as exposure controls distribution of patients into groups. Confounding factors may therefore be more or less prevalent in certain groups limiting the power of results. Additionally, when exposure is observed rather than introduced only associations can be made, as there is no evidence of cause-effect [10].

Figure 1, Cohort study design model, modified from Song et al. [11].



1.3.2 Association and causation

Determining the level of association between exposure and outcome is the first step towards understanding a hypothesized link between the two. Association is whether one factor is found more commonly in the presence of another and is defined as different risk in the subsets of the population determined by the subjects’ actual exposure value. If we apply the concept to the cohort model we may ask; is the risk of an outcome higher in the group with exposure? Several epidemiological models and their statistical methods aim to explain

whether two variables are associated, and if so, how strongly, and whether chance or other sources of bias can explain the observed association[12].

Causation is an estimation that goes beyond association. The assumption of causation aims to explain relation between an exposure and following event or outcome. Unlike association measures, effects or causation cannot be directly computed. The lack of data should have explained if a participant's outcome was affected or unaffected by the exposure. For example, if a patient receives a treatment and survives, there is no way to document what had happened if he did not receive the treatment.

The cohort and RCT design can be used to illustrate the problem with causation. As mentioned above, in a cohort study, participants are categorized based on exposure and monitored for outcome (figure 1). However, there is no control over the onset of exposure or the effects of other contributing factors. In a RCT, onset of exposure is controlled by providing one study group with the exposure and another group placebo. Randomization reduces the effect of confounders, as both groups should have equal risk of outcome at baseline. The problem with evidence of causation over association is to prove that a participant would have had a different outcome result had he or she been in the other exposure group, illustrating the need of large study populations and significant numbers of outcomes [12].

1.3.3 Limitations of observational studies

Epidemiological studies following a cohort design have several limitations and factors that has to be considered when constructing the model and evaluating the data. Sources of bias, confounding factors and chance will now be discussed [12].

1.3.3.1 *Bias*

In the context of evaluating clinical studies, bias refers to whether a measure of association between the exposure and the outcome is systematically wrong. In observational epidemiological studies such as our project, two main sources of bias are present, selection bias and information bias.

Selection bias occurs in the design phase of an observational study, and is a potential source of bias although it is poses more of a problem in case-control studies than in cohort

studies. Selection bias occurs if the selection process introduces another, unintended systematic difference between the groups, and this systematic difference is associated with the exposure. In a cohort study, selection bias occurs if the investigator's selection of exposed and reference groups introduces a systematic difference between the groups, other than the exposure, and that the systematic difference is associated with the outcome [10].

In a cohort study, an error in measuring exposure or outcome may cause information bias. Information bias is referred to as either observational bias or misclassification. Observational bias is data recorded that does not accurately represent actual exposure or outcome, intentionally or unintentionally. Misclassification, or Non-differential misclassification as seen in single classification of exposure or outcome. An example of non-differential misclassification is the errors caused by mistyping. In contrast, differential misclassification in a cohort study is the degree or direction of misclassification depending on exposure status, or other variables [10, 11]. For example, the accuracy of serum retinol measurements might be lower for people with higher C-reactive protein (CRP) values.

1.3.3.2 Confounding

Confounding is a concept that aims to characterize the study subjects; patients with a certain characteristics tend to have certain exposures. The aim of an observational study is to examine the effects of the exposure, but sometimes the apparent effect of the exposure is actually the effect of another characteristic that is associated with the exposure and with the outcome. The factor affecting the exposure or outcome is a confounder, provided it is not an intermediate step between the exposure and outcome. An example of this source of bias is CRP, as elevated CRP levels are proven to reduce serum retinol as well as being a risk factor of CVD. CRP then become a source of bias if CRP levels are different across serum retinol groups established based on exposure. There are two principal ways to reduce confounding in observational studies: (1) prevention in the design phase by restricting or matching, and (2) adjustment in the statistical analysis by either stratification or multivariable techniques. These methods require that the confounders are known and measured [10].

As mentioned above, matching is one way of dealing with confounders. This procedure separates the population into different groups that are expected to have the

same value of potential confounders. The groups of the population are then matched based on age or gender. However, with increasing numbers of matching variables, the identification of matched subjects become progressively demanding [10].

Adjusting the statistical analysis through the multivariate modeling is a method that simultaneously control for several variables to estimate the independent effect of each one. Usually, one of the variables in the model describes whether the outcome is observed, and the remaining variables describe the values of potential confounders. A multivariable model will estimate the effect of the exposure on the outcome, given that the exposed patient and reference patient are similar with respect to the confounders in the model. Models such as the Cox proportional hazard model are able to account for confounders, but are treacherous, because there is no limit amount and quality of variables you can enter into the model. All variables entered into such a model affects the outcome and distinguishing between confounding variables and variables creating noise is crucial for limiting of bias [10].

1.3.3.3 Chance

Chance is highly affected by sample size. If the investigator keeps adding to the sample size, the association will eventually become significant. Chance is a measure of accuracy of an estimate. Usually the precision of association between exposure and outcome is expressed as a confidence interval (CI, usually 95% CI). For a given association calculated for a sample population, the confidence interval is a range of values around the association which is believed to contain, within a certain probability (95% CI), the true value of that association. CI can be interpreted as the chance, which, with a 95% certainty, holds the true value of the association if the study is unbiased. Consequently, the wider the confidence interval, the less certain we are that we have estimated the strength of the association precisely [10, 13].

1.4 Vitamin A

Vitamin A is a fat-soluble vitamin that is essential to our diet and readily found in the highest concentrations in food sources such as liver, milk, strong colored vegetables, leafy greens and root vegetables. Absorption, distribution, metabolism, excretion, functions, toxic effects, diagnosis and relation with cardiovascular disease will now be discussed.

1.4.1 Absorption

Oral intake of vitamin A in form of retinol, retinyl esters and provitamin A-carotenoid food sources is essential for maintaining sufficient vitamin A levels in the body, as vitamin A cannot be synthesized *de novo* by humans [14]. Host vitamin A status depends on the sequence of digestion of vitamin A source foods, uptake into the enterocyte, and the handling of these substances inside the enterocyte. Uptake of retinol and provitamin A from the lumen is handled by the enterocytes and dependent on several factors within the lumen. These factors, dietary lipid consumption, bile salts and pancreatic lipase play essential roles as they create appropriate pH milieu and form mixed micells within the lumen to aid absorption [15, 16].

The key vitamin A sources absorbed from the diet; retinol, provitamin A-carotenoids, and retinyl esters (also known as retinol palmitate) have slightly different absorption pathways from lumen and through the enterocyte. Provitamin A-carotenes are either passed intact into the lymphatic circulation, cleaved and converted into a retinoid, or metabolized to an inactive species. As for the esterified and unesterified retinols, absorption from the lumen, through the enterocytes, leads to the lymph or portal system through a series of actions. Retinyl esters are hydrolyzed in the lumen by the pancreatic enzyme pancreatic triglyceride lipase and the intestinal brush-border enzyme phospholipase B. The mechanism of how unesterified retinol is absorbed by the enterocyte is not known. The unesterified retinol inside the enterocyte is paired with cellular retinol binding protein (RBP) type 2, and the complex provides substrate for re-esterification of the retinol thorough the enzyme lecithin retinol acyltransferase. The retinol esters enter chylomicrons together with other dietary lipids, which are released into the bloodstream and transported to the liver. Additionally, some of the unesterified retinol is also absorbed into the portal circulation, the mechanisms of the retinol transport protein facilitating the absorption is not known. Notably, regardless of being absorbed as esterified or unesterified retinol, retinol is at one

point unesterified in the enterocyte before complexed with RBP and further transported or re-esterified [15]. Notably, vitamin A supplementation of retinoids or retinyl esters are absorbed through endothelial cells, in which they joins the normal route of retinol in the enterocyte [15].

1.4.2 Distribution

The majority of total body vitamin A is in some form of storage (90%), with the remaining in active sites in peripheral tissues undertaking vitamin A dependent functions, and in the circulation system (10%). About 80 percent of vitamin A in storage is stored in the liver while the remainder of stored vitamin A is found at peripheral sites [15, 17].

The circulatory system handles the transport of retinol. The chemical actors handling retinol during its distribution between entero- and hepatocyte, and the peripheral tissue are RBP, cellular-RBP (CRBP), cellular retinoic acid binding proteins (CRABP), retinaldehyde dehydrogenase type 2 (RALDH2) and additional enzymes such as retinoic acid receptor's (RAR) and retinoid x receptor's (RXR). The major routes of transport are between enterocytes and lymph system to the liver and its hepatocytes, as well as from the hepatocytes to active sites in the peripheral tissue. Recently absorbed retinol is released from CRBP of the enterocyte into the bloodstream where it joins the retinol-RBP-transthyretin (TTR) complex. When the TTR complex arrives at the hepatocyte, retinol is released from the TTR and binds to CRBPs [15].

Once inside the hepatocyte, the retinol-CRBP accumulates in the endoplasm of the cell until transport becomes available. The retinol-CRBP complex stores of the hepatocyte have two fates. Either retinol rejoins the circulatory system as part of the TTR complex or it is esterified to retinol ester by RALDH2 for storage. Retinol esters are transported within the liver to storage in the fat storing stellate cells. If serum transport complexes (TTR) are available, retinol joins a binding site TTR in serum to maintain the homeostatic level of serum retinol. The remaining CRBP attracts newly absorbed retinol if present, if not esterified retinol is freed from storage.

In states of Vitamin A abundance, the presence of free RBP and synthesis new RBP is modest, and little if any newly absorbed vitamin A moves in to the liver causing further saturation in serum and peripheral tissue. In states of vitamin A deficiency, RBP

concentrations build up in the hepatocyte to bind and conserve available retinol, increasing absorption of serum retinol. Consumption of a vitamin A-rich meal translates into a major release of RBP-bound retinol to feed deprived peripheral tissue [15, 17].

As opposed to retinol and its transport through the portal system, retinol esters are transported from the enterocyte of the digestive system to the hepatocyte of the liver through the lymphatic system. In the enterocyte, newly absorbed retinol esters are packed in chylomicrons before being released into the lymphatic system. The chylomicrons deliver the retinol esters to the hepatocytes, where they are further absorbed by lysosomes. In the lysosomes, the retinol esters are unesterified by acidic hydrolase and retinol is released into the cytoplasm to bind with CRBP for further distribution [15].

The active metabolite of retinol, retinoic acid circulates in blood bound to albumin at a concentration nearly 1,000-fold lower than of retinol. Tissue pools of retinoic acid (RA) are rather short-lived requiring continual replenishment from circulating retinol and RA [18].

1.4.2.1 Vitamin A reference values

Sommer et al. states that there are three cut-off values sufficient to serve a purpose in determining vitamin A deficiency (VAD). VAD can be classified as liver stores below 0.07 $\mu\text{mol/g}$ or serum retinol concentration $< 0.70 \mu\text{mol/L}$. While the cut-off value of adequate vitamin A stores is at serum levels of $> 1.05 \mu\text{mol/L}$ [19]. Brubacher et al. suggest that hypovitaminosis A with anaemia and functional abnormalities in adults, can occur as early as $\leq 1.93 \mu\text{mol/L}$ [20]. The relevance of these values for this study are questionable as the majority of people with western lifestyles have vitamin A levels within the normal range given at serum levels of 500 - 1000 $\mu\text{g/L}$ (2.41 – 4.83 $\mu\text{mol/L}$) [15].

The prevalence of VAD in a population was initially set by WHO as the prevalence of $\geq 5\%$ with a serum retinol concentration $< 0.35 \mu\text{mol/L}$. The original classification was based on its association with the prevalence of eye signs of VAD. Because it was established that adverse effects of VAD occurred before ocular symptoms, the cut-off was reevaluated by the International Vitamin A Consultative group (IVACG), who suggested that in populations with $\geq 15\%$ of population with a serum retinol concentration of $< 0.70 \mu\text{mol/L}$ is defined as significant prevalence of VAD. WHO later implemented the change in cut-off value [19, 21-23]. Sommer et al. argues a wide acceptance for VAD beginning when liver stores of vitamin

A fall below 20 µg/g (0.07 µmol/g) as more accurate, and that serum levels may still be within the homeostatically regulated normal range [19].

1.4.3 Metabolism

The overview of vitamin A metabolism is structured on Solomon et al. chapter on vitamin A in *Present Knowledge in Nutrition* and the review article by Rhee et al. and is illustrated in figure 2

Retinol is the predominant natural circulating retinoid. Retinol and retinyl esters are precursors for active retinoid isoforms, including all-trans, 11-cis, 13-cis, 9,13-di-cis, 9-cis, and 11,13-di-cis, with all-trans retinol being the predominant form. The pathway from absorption to active metabolite will now be discussed [15, 16].

Vitamin A is stored in the liver as retinyl esters or found as retinol in cells or serum, while β-carotene is cleaved in to Vitamin A metabolites based on demand. Retinol undergoes two successive dehydrogenations at its carboxyl alcohol group to generate its active form retinoic acid. Retinol is initially converted to retinaldehyde by Alcohol dehydrogenases (ADH) or short-chain dehydrogenases (SDR). Additionally, retinaldehyde is converted to the active metabolite retinoic acid by retinaldehyde dehydrogenase (RALDH). B-carotene is either symmetrically cleaved into two retinaldehydes or asymmetrically cleaved into one apocarotenal and further converted to retinoic acid. Conversion of retinoic acid back to retinol is not possible [15, 16].

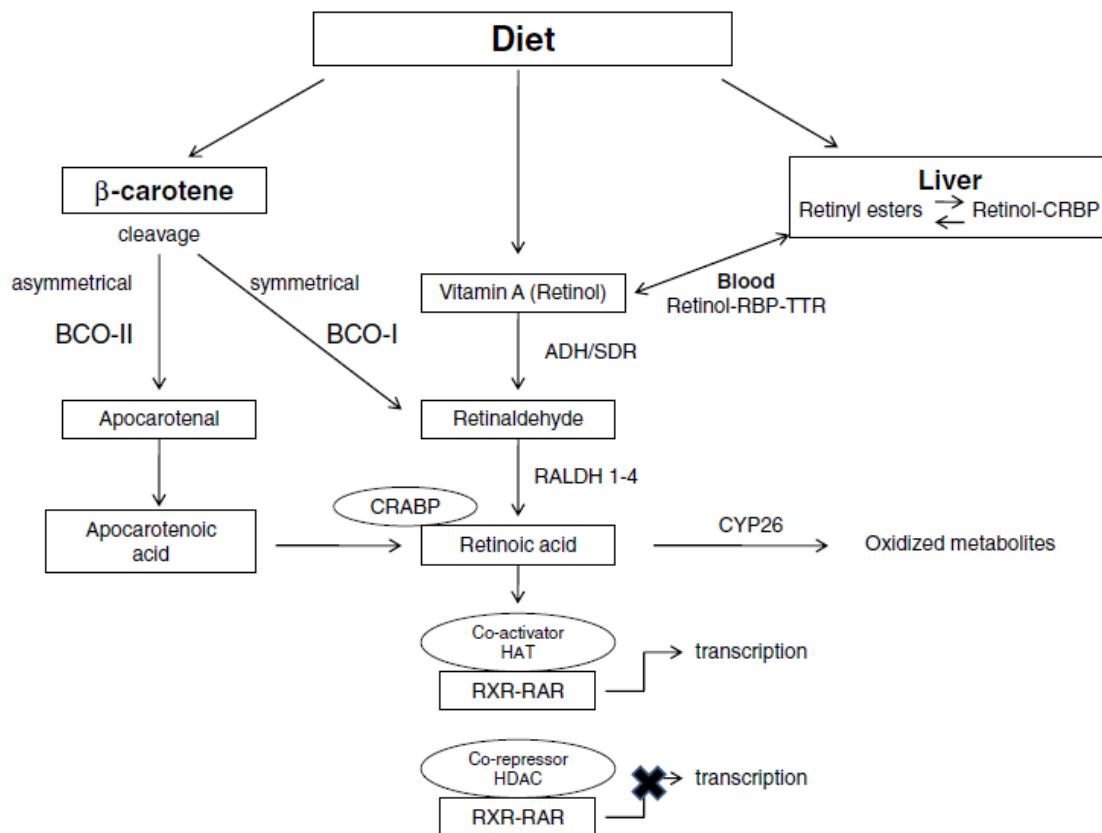
The all-trans retinoic acid is the metabolite product of the retinol metabolism pathway. Retinoic acid exerts the biological effects of retinoids by activating specific members of the steroid hormone nuclear receptor family, thus affecting steroid hormone controlled mechanisms such as metabolism, inflammation, immune function, and development of sexual characteristics. This family includes the RAR and RXR, and their subtypes indicated by the Greek letters α, β, and γ. RARs α, β, and γ bind both all-trans retinoic acid and 9-cis retinoic acid with high affinity [15, 16].

While RXRs α, β, and γ only bind to 9-cis retinoic acid, the affinity of 13-cis retinoic acid is different, as it is not a ligand for retinoid receptors, but can be readily converted to metabolites with agonist properties. The nuclear receptors RARα, RXRα, and RXRβ are

ubiquitously expressed, whereas RAR β , γ , and RXR γ exhibit tissue-restricted patterns of expression. The RAR regulates gene transcription through its participation in dimerization, either as a homodimer or as a heterodimer with partners for various nuclear receptors. Examples of RXRs heterodimeric partners are RARs, PPARs, vitamin D receptor, thyroid hormone receptor, the farnesoid X receptor, and liver X receptor. In the absence of RXRs heterodimeric partners, RXR forms homotetradimers that are transcriptionally inactive but that are rapidly dissociated into active dimers in presence of ligand hormone receptors. The RAR-RXR heterodimer forms on two interfaces, the ligand binding domain, and the DNA binding domain. The complex then interacts with specific response elements in the regulatory region of target genes [15, 16].

Rhee et al. also reviewed studies evaluating the effects of individual RAR and RXRs absence to investigate effects of the individual RARs and RXRs, to further understand the specific effects of individual receptor subtypes. Different mice models were used to evaluate the effect. RAR α deficient mice show features of vitamin A deficiency, indicated by decreased viability, growth defects, and male sterility [16]. Observations from mice that were deficient in single RAR isotype suggested redundancy among RARs, and deficiencies were reversed by retinoic acid treatment. The same effects were observed in RAR β and γ deficient mice. In contrast, RAR double mutants displayed multiple abnormalities and died in utero or shortly after birth. In RXR mutated mouse studies, the result suggests that RXR α has the ability to perform most RXR functions. It also suggests that when RXR deficient mice are born underdeveloped, the cardiovascular and ocular system is especially affected [16].

Figure 2, flowchart of Vitamin A metabolism, provided by Rhee et al. [16]



1.4.4 Excretion

The primary excretion of vitamin A is through excretion of bile, with the net loss dependent on biliary volume. With increased hepatic stores, concentration in bile increases, potentially increasing vitamin A loss. Under normal circumstances, vitamin A loss in urine is fractional. Renal failure, multiple myeloma, febrile infection might lead to increased losses due to excessive urine production. The TTR complex and its ability as a form covalent bonds with other TTRs (homotetramer) is essential in preventing glomular filtration and urinary loss of RBP and retinol [15].

1.4.5 Cardiovascular disease related functions of vitamin A

Vitamin A is a fat-soluble vitamin that is active in numerous functions in the body. Vitamin A and its active metabolites are important in processes like retinol pigmentation for

vision, mucine production, the immune system, reproduction, and intracellular communication. Furthermore, vitamin A metabolites act as ligands in signaling pathways of nuclear transcription, they activate cellular growth and differentiation, and improve bioavailability of iron. They also have antioxidative properties and anti-proliferation properties allowing them to act against proliferative disorders [15, 18, 24, 25]. The metabolites and functions of vitamin A that are deemed relevant for the atherosclerotic process and hence cardiovascular risk will be further discussed in this section.

1.4.5.1 Retinoids and nuclear receptor functions

Most of the functions of vitamin A is through active metabolites, also known as retinoids. The understanding of vitamin A's functions beyond that of vision was improved with the discovery of nuclear receptors in the late 1980s. The family of nuclear receptors comprises of ligand-dependent transcription factors that regulate gene expression by binding to short DNA sequences near target genes. Six different receptors divided into groups were detected [14]. RAR and the isomer RXR each with three subtypes indicated by the Greek letters α , β , and γ , and the metabolic pathway from retinol to retinoic acid and RAR/RXRs has been discussed during evaluation of vitamin A metabolism. In vitro studies have demonstrated that all-trans retinoic acid and 9-cis retinoic acid are highly affinity ligands for RARs, whereas only 9-cis retinoic acid binds with high affinity to RXR.

The retinoid receptors act through dimerization (combining of two molecules) to form homodimers or heterodimers. The binding of these dimers to hormone response elements coordinates the regulation of target genes by RAR ligands. The nuclear RARs function as heterodimers by complexing with one of the RXR, or as a homodimer binding to another RAR. These complexes bind to DNA sequences called RAR elements (RARE) or RXR elements (RXRE) located within the promoter of target genes.

By activating these genes RAR carry out many different transcription functions through the recruitment of a host of positive or negative regulatory proteins, referred to as coactivators or corepressors, respectively. The net effect may either be gene repression, the release of gene repression, gene activation, or gene transrepression. The combination of receptors present in a cell will dictate the eventual outcome of the downstream transcriptome. In addition, the mixture of intracellular ligands will determine whether

RAR/RXR activation results in proliferation, apoptosis, differentiation or survival of cells. Over 500 genes have been suggested to be regulatory targets of retinoic acid. In some cases, the regulation of these genes have been shown to be direct, driven by liganded RAR-RXR heterodimer bound to a DNA response element. While in other cases the regulation appears to be indirect, reflecting the actions of intermediate transcription factors, nonclassical associations of receptors with other proteins, or even more distant mechanisms [14].

Important information about the role of the various RARs and RXRs has been obtained from several studies where one or several of the receptor genes have been deleted in mice. Many, but not all symptoms of vitamin A deficiency syndrome can be recapitulated in such mice. When mutations only affect one receptor subgroup, the mice survive and the abnormalities are minimal, suggesting a functional redundancy between various receptors and isoforms. In double mutant mice lacking either two RAR subtypes or RAR and RXR α or absence of multiple subtypes, the animals do not survive or have severe abnormalities [14, 26]. Transcription regulation on the other hand involves further heterodimer formation between the RXR receptor and either thyroid hormone receptor or the peroxisome proliferator-activated receptor-gamma (PPAR γ) [15].

1.4.5.2 Vitamin A's effect on atherosclerosis

Rhee et al. states that studies testing effects on human cardiovascular events have produced data showing vitamin A involvement in angiogenesis, cellular growth and oxidative balance. However, the results of clinical trials examining the effects of various forms of retinoids on cardiovascular events and mortality, have been conflicting [16].

In vivo evidence suggests that retinoids may influence arterial responses to injury and atherosclerosis. It is important to note that the processes of arterial injury, as might occur during coronary stenting, and that of atherosclerosis, are not the same although some overlap does exist in terms of the processes. Several studies have tested the effect of all-trans retinoic acid on intimal hyperplasia/restenosis after vascular intervention in animal models. Streb et al. found that all-trans retinoic acid treated rats had 35 to 37% higher luminal area in the injured vessel compared with control after induced blood vessel injury [18]. Neuville et al. showed that all-trans retinoic acid inhibited proliferation and increased SMC migration at the site of vascular injury. All-trans retinoic acid also decreased the SMC

markers, consistent with decreased SMC differentiation [27]. These investigators also found that RAR α , but not RXR agonists inhibited SMC proliferation and reduced carotid intestinal hyperplasia.

1.4.5.3 Retinoic acid and atherosclerosis

Some of the effects of vitamin A in the control of atherosclerosis might be assigned to effects of retinoids. Retinoids exert their biological effects on the atherosclerotic process by activating specific members of the steroid hormone nuclear receptor family, including RAR and RXR. RARs α , β and γ bind both all-trans retinoic acid and 9-cis retinoic acid with high affinity, while RXR α , β and γ only bind avidly to 9-cis retinoic acid. The retinoid 13-cis RA is not a ligand for retinoid receptors, but can be converted to one [16].

Most data on nuclear receptors role in development is as mentioned, established through mouse experiments. Kurakula et al. did a review in 2012 on the effects seen in studies evaluating nuclear receptor activity and atherosclerosis [28]. They found that nuclear receptors are proven drugable targets for intervention and even though nuclear receptors may not always affect metabolism beneficially in view of atherosclerosis risk, local nuclear receptor stimulation in the vessel wall through cell-type specific- or nuclear receptor-subtype specific-activation, holds great promise for future treatment of atherosclerosis [28].

Kurakula et al. argues that RAR-mediated atherosclerotic-protection involves decreased SMC proliferation, endothelial cell mediated vasodilation, increased endothelial cell survival and reduced foam cell formation. They also state that RXRs might only heterodimerize with nuclear receptors that have a beneficial role in endothelial cells, SMC, and microphages. Thus specific RXR agonists may also have great direct and indirect cardiovascular-protective properties [28].

1.4.5.4 Retinoids' effect on vascular smooth muscle cells

Additionally vitamin A derived retinoids play important roles in vascular SMC growth, differentiation, and migration during the onset of atherosclerosis as well as under restenosis. During the early stages of vascular injury, medial vascular SMC migrate to the intima where they promote extracellular matrix deposition and reduce lumen size. The switch from a contractile to synthetic SMC phenotype, with decreased expression of SMC differentiation markers, may be central to the arterial response to injury. Recent evidence also

demonstrates that vascular SMC apoptosis may promote intimal hyperplasia, plaque formation and progression of disease. Thus, vascular SMCs are the key players in the normal vessel wall and pathologic response to injury and atherosclerosis [16]. Retinoic acid is believed to have effects on several functions and actions of SMC. It is suggested that retinoic acid alter SMC migration and proliferation, increases phenotype diversity and cell differentiation and regulates cell apoptosis [14, 16, 18, 28]

Retinol has been shown to alter migration patterns of SMC. During the atherosclerotic process, SMC migrates from the media to the intima to further constrict blood flow in the provoked area of the vessel. According to Rhee et al. retinol has been shown to both increase and decrease migration of vascular smooth muscle cells (VSMC). It is reported that retinoids increase the expression of tissue plasminogen activator, a facilitator of cellular migration, and they have been implicated in increasing SMC migration. The findings were not supported by all-studies reviewed [16].

However, retinoid may, according to Rhee et al., control VSMC proliferation, another major factor in vascular response to injury. Still, study results have not been conclusive. Retinoids do in some studies limit VSMC proliferation across study models of different animals. While in other, but similar studies, retinoids are reported to increase VSMC count and initiation of cell division (mitogenesis). The inconsistency in retinoid treatment response might differ depending on whether the cells are dormant or in a growth state. One example of the possible effects is that in rat aortic SMCs, all-trans retinoic acid stimulation alone increases cell number and mitogenesis. However, when the cells were stimulated in the presence of a mitogenic stimulus, cell numbers were reduced and proliferation suppressed [16].

Another suspected mechanism of retinoids role in SMC proliferation was presented by Kurakula et al. They argued that RAR-antagonists inhibit lesion formation. The mechanism responsible was believed to be that RAR α specific synthetic antagonist Am80, decreased SMC proliferation by inhibiting the activity of the transcription factor Krüpel-like factor 5 (KLF5), which is a key regulator in SMC activation. In the absence of ligands, RAR may form a transcriptional complex with KLF5 on the platelet derived growth factor promoter, leading to an increase in SMCs [28].

1.4.5.5 Inflammation

It is established that retinoids and especially retinoic acid also play a role in inflammatory responses in the body [15]. Studies in rats deficient in vitamin A exhibit increased inflammatory response [23]. Vitamin A and all-trans retinoic acid modulate host susceptibility to infection by interfering with both innate and adaptive immune function as well as host inflammatory responses. Retinoic acid effects the inflammatory response by down-regulating B-cell proliferation, T-cell proliferation, pro-inflammatory cytokines and C-reactive proteins [23].

Vitamin A deficient mice have defects in helper T-cell activity. In addition, retinoic acid inhibits T and B-cell proliferation, although evidence of the opposite effects also exist [23]. Retinoic acid may also inhibit B-cell apoptosis through RAR responses, as well as modulating antigen presentation by exerting direct effects on dendritic cells function. For example, retinoic acid enhances dendritic cell maturation and antigen-presenting capacity via RXR receptors, in the presence of inflammatory stimuli such as tumor necrosis factor (TNF) [16].

In addition to the mentioned functions, retinoic acid controlled expression of numerous proinflammatory cytokines. In human monocytes, retinoic acid suppressed the granulocyte-macrophages that produces activin A, a protein that enhances proinflammatory biosynthesis. In contrast, retinoic acid induced interleukin (IL) 1 β production and inhibited IL-1 receptor antagonist expression in activated monocytes. In macrophages, retinoic acid inhibited endotoxin, IFN- γ , and phorbol ester-induced TNF production. Furthermore, in lipopolysaccharide-stimulated macrophages, retinoic acid was found to inhibit IL-12 production. In general, suggest that retinoic acid may limit inflammation by repressing chemokine production [16, 23].

Recent work also implicates retinoids in regulating endothelial adhesion molecule expression, an inflammatory response involved in early stages of atherosclerosis. Retinoid acid suppresses TNF-stimulation of vascular cell adhesion molecule-1 (VCAM1) in endothelial cells, with reduced VCAM1 dependent T-cell binding [16, 29].

CRP is an inflammatory biomarker associated with increased CVD risk. CRP production is regulated primarily by IL-6 in liver, CRP production may also occur on sight in

vascular lesions. Retinoic acid is known to antagonize NF-IL6, a transcription factor involved in regulating IL-6 production. An inverse relationship had been observed between retinol and CRP levels during inflammation and will be further discussed in a later section [16].

1.4.5.6 *Antioxidative properties*

A vital property of vitamin A is its antioxidative action, and Blomhoff et al. reports that Vitamin A deficiency promotes inflammatory reactions [14]. Actions include protection against reactive oxygen species (ROS), ability to quench singlet oxygen, and trapping and neutralizing free radicals. Vitamin A's bind to single oxygen particles and free radicals disallowing them to react with polyunsaturated fatty acids contained in lipids of cell membranes. Thus reducing the preoxidative damage the free radical could have caused cells. At the same time vitamin A suppresses the activity of participating enzymes in the peroxidation of these lipids, and it as prevents oxidative disorders of protein glycosylation in cell membranes [14, 24].

1.4.6 Toxicity

Excessive vitamin A intake may result in toxicity rapidly or over time. Allowing total body vitamin A to exceed the limited storage capacity of the liver, may lead to the supersaturation of the body with vitamin A and the poisoning referred to as hypervitaminosis A. As the hepatic cells fail to absorb available Vitamin A, tissue build up occurs, leading to toxic effects [14, 15].

The upper tolerable limit (UL) for adults is 3000 µg/day of vitamin A [15]. Hypervitaminosis A has been linked to blood levels of vitamin A above 1.0 mg/L (3.5µmol). Toxic effect appears when the liver no longer can absorb excess vitamin A from the blood stream and there is no more serum retinol binding protein (RBP) to bind free retinol molecules. Toxic effects are seen after intake of 150-1200 mg (500 000-4 000 000 IU) of vitamin A over two days by adults, after single dose of 45 mg (150 000 IU) by school-age children, or after intake of about 22 mg (75 000 IU) by small children [24]. There have been reports of children given as much as 500 000 IU by mistake in nyctalopia (night blindness) prevention programs [30], resulting in fatalities, and extreme cases such as polar explorers consuming polar bear liver (Vitamin A content of approximately 10 mg/g). Additionally high doses of vitamin A should be avoided for patients with liver failure and cirrhosis or in-bile

ducts obstruction, because of an increased risk of toxic effects. Pregnant women should avoid high doses of vitamin A, as it may contribute to fetal development anomalies [24]. Patients with hepatic and renal disease and children are especially susceptible to the adverse effects of this vitamin. The supplementation with provitamin A, i.e. β -carotene, could be assumed to be safer, as conversion of β -carotenes to retinol is regulated by serum retinol concentration. However, Bjelakovic et al. performed a meta-analysis of 53 trials with low risk of bias on beta-carotene, vitamin E, and vitamin A supplementation. They concluded that supplementation with beta-carotene in doses higher than the recommended daily allowance (RDA) seems to significantly increase mortality [31].

Hypervitaminosis A can take the form of acute or chronic poisoning.

1.4.6.1 Chronic poisoning

Hypervitaminosis A in the form of chronic poisoning have the following symptoms; chronic fatigue, irritability, double vision, sleep disturbances, renal dysfunction (haematuria), and edemas. Additionally high intake of vitamin A over a period may disrupt the absorption of β -carotene and mineral salts. Excessive intake of vitamin A is also harmful to the osteoarticular system. It may lead to joint and spine pains, as well as decalcification of bones [24]. Studies have indicated although not consistently, an increased risk of fractures in populations taking vitamin A supplements [24, 32-34].

1.4.6.2 Acute poisoning

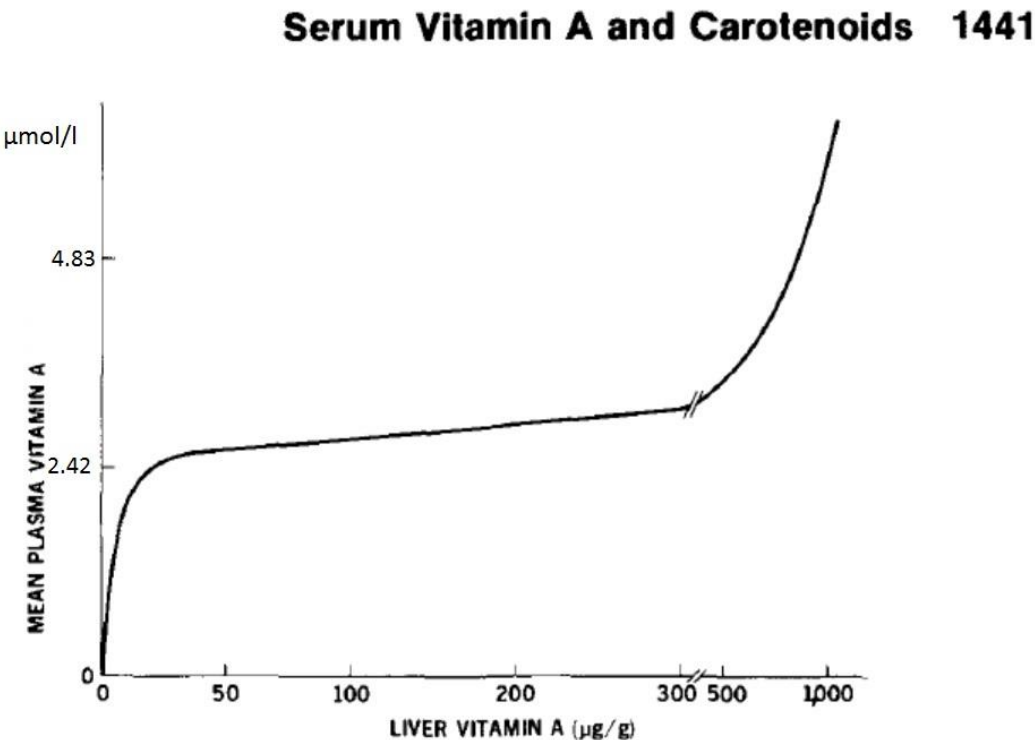
The symptoms of acute poisoning, the other form taken by hypervitaminosis A, are general weakness, dizziness and severe headache, increased intracranial pressure, nausea, vomiting, hepatosplenomegalia (enlargement of liver and spleen), and after a few days skin changes [24].

1.5 Diagnosis of vitamin A status

Liver stores of vitamin A are considered the gold standard for total body vitamin A. Measurements of liver stores are highly invasive and seen as unfeasible in humans. Among several biomarkers, serum retinol levels are frequently used. Serum retinol is regulated by the prevalence of retinol binding protein (RBP) in the hepatocytes [15, 17]. The regulation of RBP in the liver affects serum retinol and creates a plateau stabile serum retinol concentration, while liver stores of vitamin A metabolites may fluctuate. However, there are

multiple factors that affects serum retinol independent of liver stores (zinc deficiency, CRP, estrogen and corticosteroids). Additionally the relation between liver stores of vitamin A and serum retinol is not linear [17]. The historical and current method to measure vitamin A status utilizes its fluorescence properties. The method currently in use is a combination of liquid chromatography and tandem mass spectrometry. During preparation, the samples were protected from heat, light, oxidizing substances to prevent denaturing [35].

Figure 3, Hypothetical relationship between mean plasma vitamin A levels and liver vitamin A concentration, provided by Olsen et. al. [17], original reference values of mean plasma vitamin A axis (Y) have been altered from $\mu\text{g}/\text{dl}$ to $\mu\text{mol}/\text{l}$.



1.5.1 Serum retinol as a biomarker

A biomarker is defined by the Biomarkers of Nutrition for development (BOND) as: “A biological characteristic that can be objectively measured and that serves as an indicator of normal biological processes, pathogenic processes, or responses to therapeutic interventions. Biomarkers can be broadly characterized into three groups: those that measure physical or genetic traits, those that measure chemical or biochemical agents in the biological system (serum retinol, iron, zinc), and those that assess a measurable physical function or future clinical risk” [25]. The limitations and effectiveness of serum retinol as a biomarker will now be discussed.

Serum concentration stays within a narrow range in individuals with adequate liver vitamin A stores. Serum concentration will only fluctuate outside this range under certain conditions. As liver stores become depleted, serum retinol will decrease and when total body vitamin A exceeds the hepatocytes holding capacity serum retinol will increase. As stores become depleted the liver cells absorb available retinol, reducing the serum concentration [15]. The BOND report by Raiten et al. builds on the relation between liver vitamin A stores established by Olson et al. by stating that serum retinol serves as a strong indicator for assessing deficiency and toxicity in individuals and populations with serum levels outside the livers homeostatically controlled range [17, 25]. Although its ability to estimate intake and exposure is weak, as there are large variations in individual responses to doses of retinol [17]. Raiten et al. (2011) also assess the utility of serum retinols for assessing exposure, status and function [25].

Raiten et al. reported that serum retinol reflects long-term intake, but is not sensitive to acute changes. As for vitamin A status serum retinol can be utilized as an indicator of severe and moderate vitamin A deficiency, but is not as widely accepted and used for assessing toxicity. As for serum retinols functionality, low serum concentration is historically used to assess eye function. A concentration of 0.48 $\mu\text{mol/L}$ is elected as a lower cut-off because of its association with xerophthalmia, although no direct cut-off values are established were morbidity/mortality effects begin to occur [25]. No cut-off value for toxicity has been established but as mentioned In the vitamin A toxicity section, Hypervitaminosis A have been linked to blood levels of vitamin A above 1.0 mg/L (3.5 μmol) [24].

Tanumihardjo et al. and the Vitamin A Tracer Task Force have provided an evaluation of the quality of biomarkers of vitamin A status in relation to liver reserve concentrations. The ability of the different indicators and biomarkers to predict total body vitamin A levels is displayed in Figure 4. The bars of the graph in figure 4 indicate to what degree the indicator can predict vitamin A status. Because of the previously discussed homeostatically controlled relation between, serum retinol only reflect deficient and marginal total vitamin A stores [36].

Figure 4, Effectiveness of different biomarkers of vitamin A, provided by: The Vitamin A Status Continuum [36]

Vitamin A STATUS	Deficient	Marginal	Adequate	Sub-toxic	Toxic
LIVER Vitamin A ($\mu\text{mol/g}$)	< 0.07	0.07— 0.1	0.1 — 1.0	> 1.0	?
INDICATOR					
Clinical signs and tests					
Serum retinol					
Breast milk retinol					
Dose response tests					
Isotope dilution					
Liver sample					

The association between vitamin A status, liver reserves, and the indicators currently used. Clinical tests include xerophthalmia, night blindness, and dark adaptometry. Breast milk is specific to lactating woman. Dose response test includes both relative dose response and modified relative dose response. The tracer dilution technique is the only method that provides a quantitative estimate of total body vitamin A pool size. The Figure was originally printed by the Vitamin A tracer task force [36].

1.5.1.1 Acute phase response

An acute phase response to infection, trauma, surgery or other illness is associated with a reduction in serum retinol and other micronutrients. The reduction of serum retinol might be a result of the increased capillary permeability. Hence, the change in the serum retinol seen does not reflect changes in liver or total body vitamin A stores, which are expected to be unaffected [37]. The cross sectional study by Duncan et al. discuss the magnitude of systematic inflammatory response effect on serum micronutrients concentration. They suggest that the increase in permeability allows retinol-binding protein (RBP) to migrate and subsequently reduce serum retinol [38]. Their study indicated that retinol was lower when CRP concentrations were modestly elevated, as indicated by a serum level of CRP between 10-20mg/L. While CRP concentrations of >80mg/L were associated with the largest decrease in vitamin A status with a median decrease in serum levels of 25 - 75 %.The observations by Duncan et al. support previous assumptions concerning the link between systemic inflammation response and serum retinol concentration [38].

The same effect reported by Duncan et al. was documented in the meta-analysis performed by Thurnham et al. (2003). They calculated that during mild infection or early convalescence only 76% of total body vitamin A is represented by plasma levels, giving a underestimation of vitamin stores of 24% [23]. No method of adjustment for this effect has been established, and the reduction of serum retinol in patients with elevated acute phase proteins was acknowledged as a confounder for CVD by the BOND report by Raiten et al.

(2011) as both reduced serum retinol and elevated CVD risk is expected in populations with elevated CRP [25].

1.5.1.2 Obesity

Obese people have lower plasma retinol levels than participants within normal weight range. The origin of this association might be a result of the increased systemic inflammation response seen in obese populations when compared to non-obese [15]. Derosa et al. conducted a study on 363 obese (body mass index (BMI)>30 kg/m²) participants with normal glucose tolerance. Participants with confounding factors of cardiovascular risk were excluded from the study (smokers, diagnosed with CVD within last 12 months, familiar and/or severe dyslipidemia, and stable hypertension under drug treatment). When compared with a non-obese control group Retinol binding protein-4, leptin, IL-6, and CRP were significantly lower in the non-obese group at $p < 0.0001$. Vaspin, resistin, and TNF α were significantly lower in the non-obese group at $p < 0.05$. The data suggest that obese subjects are more likely to have higher levels of inflammation and insulin resistance adipocytokines compared to non-obese [39]. In which indicates that the association between low serum retinol and obesity may be caused entirely or partly through the increased inflammation.

1.5.1.3 Smoking

Another factor is smoking which is found to reduce serum levels of several antioxidant micronutrients, in particular Vitamin C, E, and the pro-vitamin A β -carotene [15]. The review article by Alberg et al. evaluated the influence of cigarette smoking on circulating concentration of antioxidant micronutrients, thereby also vitamin A. The review indicated that smoking significantly lowers circulating micronutrients. Some of the mechanisms among smokers discussed as contributors were; altered diet habits, increased inflammation and increased oxidative stress. They also found that low exposure also reduced serum micronutrients [40]. The decrease seen at low exposure indicating a need to assess smoking exposure beyond being a smoker or not.

Cotinine is an alkaloid found in tobacco and is used as a metabolite of nicotine. Self-reporting is known to misclassify or underestimate the smoking population by 1% to 4.2% [41]. Cotinine is a principal metabolite of nicotine, with a half-life of approximately 20 hours. Inhalation of nicotine among smokers may differ depending on brand of cigarettes and

habits. Wei et al. argues that cotinine represent the nicotine reaching the bloodstream, possibly reducing margin of error [41]. A combination model of self-reported smoking habits and plasma cotinine was used to classify smoking exposure in the WENBIT and BECAC studies [42].

1.6 Previous vitamin A and CVD studies

The association of CVD with the lower end of this range of plasma levels ($< 600 \mu\text{g/L}$ or $2.9 \mu\text{mol/L}$) suggests that a reevaluation of the normal range might be in order [43]. Largely as the effect of subclinical levels of vitamin A is not fully understood, as no efficient method of measuring this range is established [25]. Vitamin A and CVD have been studied in prospective cohort studies, in healthy, elderly and in case control studies. Reviews and studies found relevant are discussed in this section.

The study by Goyal et al. 2013 looked at the hazard ratio of cancer, CVD and all-cause mortality in 16008 participants of the National Health and Nutrition Examination Survey III (NHANES III) population across plasma antioxidants. 4225 died over a median follow-up of 14.2 years (891 cancer-related and 1891 CVD-related deaths). The population was grouped into quintiles based on plasma antioxidant concentrations. The study found a decreased CVD-related HR in the middle quintiles (HR: 0.78 – 0.90) and an increased HR in the upper quartile of plasma retinol concentration (HR: 1.08) with the lower quartile as the referent, none of the assumptions were significant. Adjustment for confounders did not substantially change the assumptions. As for all-cause mortality the HR was lower in all four quartiles (HR: 0.73 – 0.95) with the lower quartile as the referent. All the associations of reduced HR for all-cause mortality were significant before adjustment. After adjustment all none of the associations changed, however, significance was then only 3rd and 4th quartiles [44].

Brazionis et al. reported in 2010 from the Melbourne Collaborate Cohort Study (MCCS), where they reported hazard ratio of CVD across tertiles of plasma retinol. The study included 441 community-dwelling adults from the MCCS population. Their results suggested an increased hazard risk of CVD in the group with plasma retinol levels below $2.24 \mu\text{mol/L}$, when adjusted for age, gender, diagnosed diabetes, self-reported heart condition, plasma cholesterol, plasma triglycerides, plasma homocysteine, plasma CRP and urinary albumin excretion rate [7].

The Prospective epidemiological study of myocardial infraction (PRIME) study was presented by Gey et al. in 2008 and suggested that plasma retinol levels of <601 ug/L (2.90 μmol/L) in a fifth of middle-aged European men placed them at an approximately threefold risk of developing coronary heart disease. The PRIME study included 10600 50- to 59- year old males from Western-European countries. The study compared retinol levels between an exposure group of 150 males that experienced fatal (N=27) or non-fatal (N= 123) coronary heart disease and a control group of 285 randomly selected participants [43].

Reimersma et al. did a case-control study in 1991 on 110 volunteers with stabil angina pectoris and 382 controls. The case-control study aimed to investigat the relation between risk of angina pectoris and plasma concentration of Vitamin A, C and E. No significant relation between plasma levels of vitamin A and risk of angina pectoris was found [45].

Rhee et al. 2012 reviewed studies evaluating atherosclerotic development from a clinical perspective. The studies evaluated were the PRIME study, CARET study, women's health study, the meta-analysis by Bjelakovic et al. also discussed in section 1.6.1 and a study of gene expression mechanism by Kraan et al. [16, 46]. The data presented in the studies led Rhee et al. to conclude that retinoic acid may modulate atherosclerosis in humans by altering gene expression and that retinoids effect on CVD are important [16].

1.6.1 Vitamin A Supplementation reviews

Two independent meta-analysis were performed in 2013 to evaluate the effect of Vitamin A and carotene supplementation. The meta-analysis by Bjelakovic et al. evaluated β-carotene, vitamin A and vitamin E supplementation and its effect on all-cause mortality. The meta-analysis included 53 randomized trials with low risk of bias. These showed that the dose of vitamin A was significantly positively correlated with all-cause mortality. The second meta-analysis, was performed by Myung et al. evaluated vitamin and antioxidant supplementation and CVD risk. The meta-analysis included 50 randomized trials with low risk of bias. Their conclusion was that there is no evidence to support the use of vitamin and antioxidant supplements for prevention of CVD [31, 47, 48].

2 Hypotheses

In this study, we will compare vitamin A status and future cardiovascular mortality risk in patients who previously underwent coronary angiography for suspected Coronary artery disease (CAD) at Haukeland University Hospital, Bergen, Norway (HUS) and Stavanger University Hospital, Stavanger, Norway (SUS).

The objective of the study will be to investigate possible associations between cardiovascular mortality risk and subclinical deficient and toxic plasma retinol concentration.

It is hypothesized that serum retinol concentrations at the extreme ends of the distribution are associated with increased risk for fatal cardiovascular endpoints and for total mortality.

3 Method

3.1 Population under study

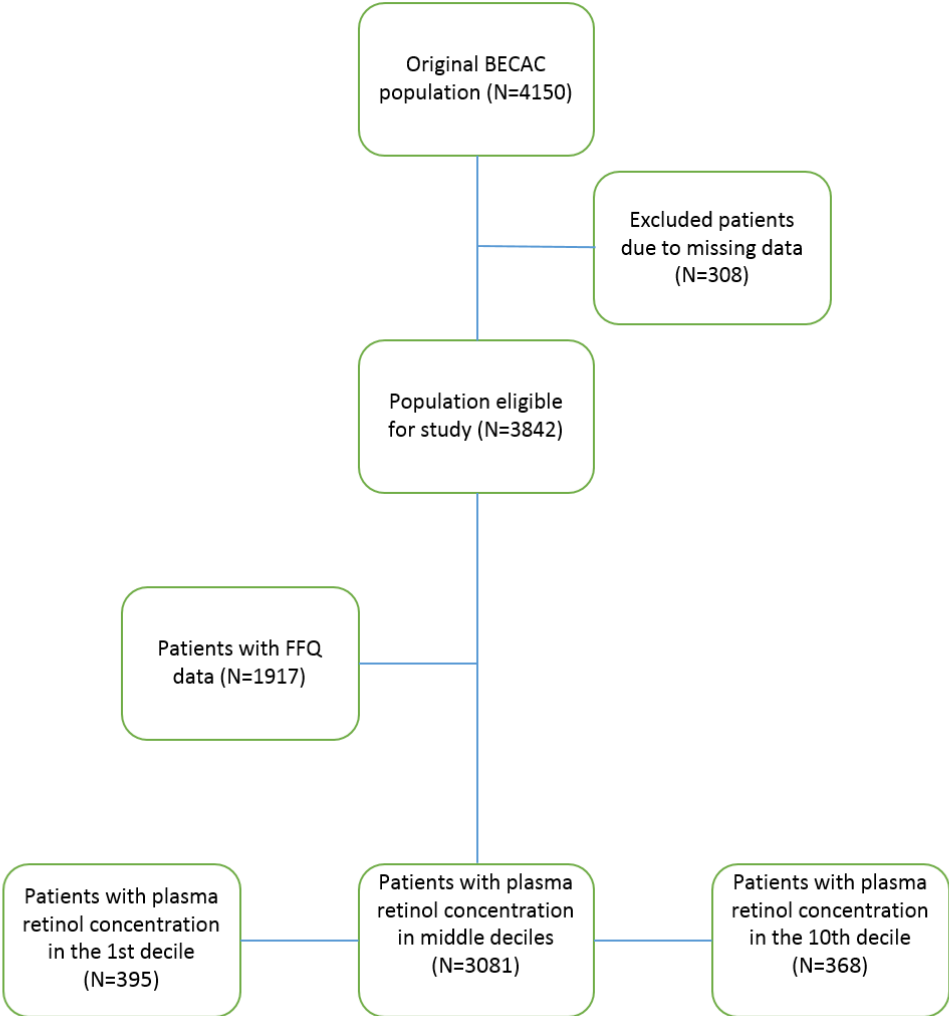
BECAC was established in 1999 to 2004 through the Western Norway B-vitamin intervention trial at Haukeland University Hospital and Stavanger University Hospital. The population available for this study consisted of patients recruited through the Bergen Coronary Angiography Cohort (BECAC) and the Western Norway B Vitamin Intervention Trial (WENBIT; ClinicalTrials.gov number NCT00354081) as explained by Svingen et al [42]. As WENBIT participants were included and part of the BECAC population, the population under study for this project will be referred to as the BECAC population.

The original BECAC population consisted of 4150 adults who underwent coronary angiography at the Department of Heart Disease, HUS, between January 2000 and April 2004. The primary aim of the BECAC study was to study various prognostic markers of cardiovascular end-points and cause specific mortality in patients with suspected heart disease. Furthermore, BECAC established the source population of patients randomized for from WENBIT study at HUS.

The aim of the WENBIT study was to investigate the effect of B vitamin supplementation on mortality and cardiovascular events [49]. Across the population, only those patients admitted due to suspected stable angina pectoris (SAP) (n=3413) were

selected for the BECAC study, of whom 1822 (53.4%) were enrolled in WENBIT. Additionally 751 participants of the WENBIT study with SAP and angiographically verified coronary artery disease (CAD) were recruited to the BECAC population, from SUS. The study population was monitored for the occurrence of acute myocardial infraction (AMI) until December 31st 2006 [42]. Patients with missing baseline covariates specific for this project were also excluded (n=308), resulting in a total population of 3842 as illustrated in flowchart (figure 5).

Figure 5, Flowchart of study population and retinol deciles



3.2 WENBIT supplementation

The patients recruited from BECAC for WENBIT, received folic acid, vitamin B6 or B12 (alone or in combination) or placebo for a median of 38 months [49]. The WENBIT study is a double-blinded study on the clinical effects of homocysteine-lowering therapy in patients

undergoing coronary angiography for suspected CAD. A 2 x 2 factorial design was used in order to assess the effect and combination of folic acid/vitamin B12 and separately vitamin B6 versus no vitamin B6. Baseline characteristics used for the current study was gathered before patients received intervention, additionally the original WENBIT supplementation intervention did not significantly change; mortality, risk of CVD or alter biomarkers of CVD such as IL-6 and CRP [50].

3.3 Baseline Characteristics of participants

Baseline characteristics and information regarding patients' lifestyle and medical history were obtained through self-administered questionnaires and verified by comparing self-reported status with hospital records [42]. Hypertension and diabetes mellitus were defined according to preexisting diagnosis, and patients were classified as diabetic or non-diabetic regardless of having type 1 or type 2. Patients were classified as smokers if they were self-reported as current smokers or having quit within the last four weeks, or if patients had plasma cotinine ≥ 85 nmol/L. The classification "Physical active" included patients self-reporting being physically active twice or more per week. Trained nurses performed blood sampling, blood pressure measurements and assessment of anthropometric data. Left ventricular ejection fraction was obtained either by echocardiography or by ventriculography performed during cardiac catheterization [42].

3.4 Food Frequency Questionnaire

Dietary intake assessment was performed at baseline and only in a sub-group of the WENBIT population, consisting of those recruited from Bergen. Food frequency questionnaires (FFQ) were provided for the patients and returned in hand or through mail. The FFQ used for the assessment of WENBIT patients were developed at the Department of Nutrition, University of Oslo (UiO) [51].

The FFQ included 169 food items that were grouped according to Norwegian meal patterns and was designed to obtain information on usual food intake during the year prior to the study. The frequency of consumption was given per day, week, or month, depending on the food item in question. The portion sizes were given as household measures or units such as slices or pieces. [51].

The FFQ also included questions about supplement intake, including cod liver oil, cod liver oil capsules, and fish oil capsules. Supplement intake was categorized in three groups: whole year or winter use only, number of times per week, and amount per time. Nutrient intake was calculated by using the database and software developed by the Department of Nutrition, UiO (Kostberegningssystemet, version 3.2; UiO, Norway)[51].

The FFQ was returned by 2484 (80.4%) of the patients randomly assigned to the WENBIT. Nineteen questionnaires were excluded because they contained more than one blank page. Patients with very low (<3000 kJ or 717 kcal for woman and <3300 kJ or 789 kcal for men) or very high (>15,000 kJ or 3585 kcal for woman and >17,500 kJ or 4182 kcal for men) estimated daily energy intake were excluded (n=53), leaving FFQ data of 2412 patients to be included in the study [51]. The correlation regression with plasma retinol was performed with 1917 patients as a further 95 was excluded due to missing data.

3.5 Blood Collection and Biochemical Analysis

For BECAC patients undergoing coronary angiography at HUS, venous blood samples were drawn at baseline, usually 1-3 days before the procedure, while BECAC patients undergoing coronary angiography at SUS had samples drawn immediately after the procedure. Blood sampling was carried out around noon in most patients. No relationship between patients time of last meal and blood results was found and blood samples were therefore assumed to be unaffected. Routine laboratory analyses were performed at the hospital laboratories at HUS, or at SUS respectively. Estimated glomerular filtration rate (eGFR) was obtained using the Chronic Kidney Disease Epidemiology Collaborate formula [42]. For study-specific analysis, serum and plasma were immediately prepared and stored in 2mL Vacutainer tubes (Becton, Dickinson and Company, United States) at -80°C until thawed and analyzed by laboratory staff blinded to the clinical outcomes of the patients. Serum cotinine were analyzed using liquid chromatography-tandem mass spectrometry at Bevital AS, Bergen, Norway (<http://www.bevital.no>) [35, 42]. Serum CRP was measured using an ultrasensitive immunoassay, with a detection limit of 0.17 mg/L, applying Behring nephelomer II system (CV 8.1-11.4%; N latex CRP mono, Behring Diagnostics, Marburg, Germany), respectively [42].

3.5.1 Plasma retinol data

Plasma retinol was elected as biomarker for patients' vitamin A status. Status was defined based on blood samples taken at enrolment. Patients' plasma retinol concentration was measured and determined using an automated high-performance liquid chromatography tandem mass spectrometry (HPLC/MS/MS) assay developed by Bevital A/S in collaboration with the Section for Pharmacology, Department of Clinical Medicine at the University of Bergen (UiB) and Laboratory of Clinical Biochemistry, HUS [49]. Midttun et al. 2011 found the assay suitable for analysis of established markers of fat-soluble vitamins A, D and E in 50 µL of human plasma for the BECAC population. The assays are suitable due to the limited amount of plasma available for analysis and due to its precision and repeatability [35].

3.6 Clinical End Points

For the evaluation of plasma retinol levels and CVD risk, all-cause death, coronary heart disease related death and non-coronary heart disease related death, endpoints were used to investigate hazard risk.

According to Svingen et al. endpoint data was obtained from the Cause of Death Registry at Statistics Norway (<http://www.ssb.no>) and the Western Norway Cardiovascular Registry [42, 52, 53]. The latter contains all CVD discharge diagnosis from the patient's administrative systems at Western Norway public hospitals. Medical records were used to verify the registered data. The revised European criteria published in 2000 were applied to classify AMI, including both fatal and non-fatal events, and the study end point was assigned by the WENBT study end-point committee [42, 54]. If death occurred ≤ 28 days after the onset of an event, the event was classified as fatal. Acute myocardial infraction was classified according to the diagnostic criteria of the revised definition of myocardial infraction published in 2000 [51].

Causes of unstable angina pectoris were classified as endpoints if patients were urgently admitted to hospital due to acute attacks of typical ischemic symptoms accompanied by electrocardiographic ST-T findings of myocardial ischemia at rest and/or if coronary angiography during the same hospital stay verified significant progression of their

CAD. Endpoints were recorded in WENBIT and BECAC until December 2005 for this study. Members of the endpoint committee adjusted all events [51].

3.7 Ethics

The WENBIT and BECAC studies followed the guidelines of the Declaration of Helsinki and were approved by The Regional Committee for Health Research Ethics and The Norwegian Data Inspectorate. All Participants provided written informed consent [35, 42, 49].

3.8 Data analysis

3.8.1 Description of variables

The variables that will be used during the statistical analysis are listed in Table 1, with associated abbreviation, units, and description. Values are included due to their role in Cox regression analysis.

Table 1, Description of variables for survival analysis

Variable	Abbreviation	unit	Description
Body mass index	BMI	kg/m ²	
Plasma retinol		µmol/L	
Age		years	
Gender		0/1	Categorical variable, 0=male/ 1=female
C-reactive protein	CRP	mg/L	
Estimated glomerular filtration rate	eGRF	mL/min/1.73m ²	
Apolipoprotein A1	ApoA1	g/L	
Diabetes	DM	0/1	Categorical variable, 0=No DM/ 1 = DM 1 and DM 2
Statin therapy	Statin	0/1	Categorical variable, 0= no treatment/ 1= treatment aimed to reduce cholesterol ≥20%
Physical activity		0/1	Categorical variable, 0= not active/ 1= self-reporting being active twice or more per week
Smoking exposure	Smoking	0/1	Categorical variable, 0= no exposure/ 1= current smokers or having quit within the last four weeks, or if patients had plasma cotinine ≥85 nmol/L
Systolic blood pressure	SBP	mmHg	

3.8.2 Statistical analysis

All statistical analysis was performed using IBM SPSS Statistics for Windows, version 21 (SPSS, Chicago, IL, USA). Because of the large sample size (>30) the sampling distribution is assumed to be normally distributed [13]. Spearman correlation analysis was performed on to evaluate the relation between plasma retinol with confounders and food intake documented by the FFQ. For categorical variables such as smoking exposure, physical activity, gender, statin therapy, and diabetes, independent sample T-tests were used to assess difference in mean plasma retinol concentration between exposure groups of the categorical variable. ANOVA was used to assess the difference in means between plasma retinol deciles. P-values below .05 were regarded to be significant.

3.8.3 Survival analysis

Kaplan-Meier and Cox regression model were used to assess the association of plasma retinol concentrations and the risk of total mortality, cardiovascular mortality and non-cardiovascular mortality. Kaplan-Meier was used to investigate the number of days patients were under study.

Three different models were established: First, plasma retinol was used as a continuous variable. In a second approach, plasma retinol was divided into quartiles, and the third approach used the extreme deciles (1st and 10th deciles) compared to the 2nd to 9th deciles (middle deciles).

In the Cox regression analysis, we used different adjustments: the first model analyzed the effect of plasma retinol in a crude model without adjustments (model 1). Model 2 included adjustment for age (in years) and gender. Model 3 included further adjustments for C-reactive protein, eGFR and apolipoprotein A1. The final model 4 included further adjustments for history of diabetes mellitus (in categories), use of statins (in categories), physical activity (in categories), smoking (in categories), body mass index and systolic blood pressure.

3.8.4 Plasma retinol categories

Because of the initial survival analysis findings already discussed and the objective of evaluating the association between subclinical plasma retinol concentrations and CVD risk, deciles was elected for grouping the population. The 2nd to 9th deciles will be used as the reference category and compared to the 1st and 10th deciles. The 2nd to 9th deciles will from now on be referred to as the middle deciles. Deciles were deemed desirable, as the 1st and 10th deciles is expected to include patients with plasma retinol concentrations outside the homogenically controlled range of plasma retinol previously described. Cut-off values for deciles are presented in Table 2, Cut-off values for plasma retinol deciles

Table 2, Cut-off values for plasma retinol deciles in the present study

	1 st decile	Middle deciles	10 th decile
Number of patients	395	3081	368
Retinol values	≤ 2.15 μmol/L	2.15 to 3.81 μmol/L	≥ 3.81 μmol/L

4 Results

4.1 Population characteristics

Of the 4150 of the original population 3842 patients were included in the data analysis, giving a participation percentage of 92.6%. 308 patients were excluded before data analysis due to missing variables.

The study population is described in Table 3. The average age was (mean \pm SD) 61.7 \pm 10.3 years, and 72.4% were male. On average, the population was overweight with a BMI of 26.8 \pm 3.95 kg/m², 80% received statin therapy, 80% were physically active by self-report, and 11.5% had type 1 or 2 diabetes.

The average plasma retinol concentration was 2.90 \pm .694 μ mol/L with a range from 0.41 μ mol/L to 9.11 μ mol/L. One patient had a plasma concentration indicating vitamin A deficiency and two patients had inadequate levels, while 17.3% had plasma retinol levels of above 3.50 μ mol/L (which according to Rutkowski et al. indicates risk of hypervitaminosis A, reference values are discussed in section 1.4.1 and 1.5.1).

Table 3, Descriptive data for cardiovascular risk factors in BECAC patients, as in the total population and for men and women separately.

Parameters	Total (N=3842)	Male (N=2784)	Female (N=1060)
Plasma retinol	2.90 \pm .694 ^a	2.92 \pm .682	2.86 \pm .725
DM	11.5%	11.8%	10.9%
Physical activity	74.9%	76.0%	71.9%
Smoking	25.8%	27.0%	22.7%
Statin	80.1%	83.8%	27.3%
Age	61.7 \pm 10.3	61.2 \pm 10.2	63.2 \pm 10.5
ApoA1	1.31 \pm .268	1.26 \pm .246	1.45 \pm .274
BMI	26.8 \pm 3.95	26.8 \pm 3.63	26.6 \pm 4.69
CRP	3.57 \pm 6.84	3.52 \pm 7.17	3.79 \pm 5.95
eGFR	88.1 \pm 17.0	89.4 \pm 16.7	84.2 \pm 17.3
SBP	141 \pm 20.7	141 \pm 20.4	14.1% \pm 21.4

ApoA1: Apolipoprotein 1, BMI: body mass index, CRP: C-reactive protein, DM: Diabetes Mellitus, eGRF: estimated glomular filtration rate, SBP: systolic blood pressure.

Data are presented by mean \pm standard deviation for continuous variables, numbers (%) for categorical variables and median (inter quartile range) for biochemical parameters

4.2 Determinants of plasma retinol levels

Table 4 describes the association between plasma retinol and anthropometric and biochemical parameters. Significant associations were observed between plasma retinol and age, body mass index, apolipoprotein A1, eGFR and systolic blood pressure.

In addition, differences in plasma retinol were observed between gender (with males having higher values than females ($\Delta 0.06 \pm 0.04 \mu\text{mol/L}$)) and in smokers (with current smokers having lower concentrations than non-smokers ($\Delta 0.07 \pm 0.03 \mu\text{mol/L}$)), while a history of diabetes, statin use or level of physical activity had no significant effect.

Table 4, Association between plasma retinol and anthropometrics and biochemical parameters in BECAC patients

Parameters	Relationship with retinol	
	Population (N=3842)	
	r	P-value
Age	-.065	.000
ApoA1	.164	.000
BMI	.068	.000
CRP	-.121	.000
eGFR	-.183	.000
SBP	.049	.004

Correlation coefficients are Spearman. Text in bold where $P < 0.05$. BMI: Body Mass Index, SBP: Systolic blood pressure, CRP: C-reactive protein, eGFR: estimated glomerular filtration rate, ApoA1: serum apolipoprotein A1

4.2.1 Plasma retinol and food intake

Table 5 displays the correlation analysis between plasma retinol concentration and food groups documented through the FFQ. Consumption of meat and milk both showed a modest positive correlation with plasma retinol concentration at $r: .092$ ($P: .000$) and $r: .048$ ($p: .036$) respectively.

Table 5, Association between plasma retinol and food groups

Parameters	Relationship with plasma retinol	
	r.	P-value
Meat	.092	.000
Fish	-.012	.600
Egg	.035	.131
Milk	.048	.036
Cheese	-.015	.498
Lean fish	-.018	.422
Oily fish	.006	.789
Processed fish	-.016	.474
Cod liver ^a	.028	.524
Fish oil ^a	.026	.628

Correlation coefficients are Spearman. Text in bold where P for trend <0.05

^aCorrelation between fish oil/cod liver oil and plasma retinol in supplement users

4.2.2 Differences of variables according to plasma retinol deciles

Table 6 presents descriptive data for CVD risk factors at baseline according to retinol deciles. Differences between groups were assessed by ANOVA. Significant differences between plasma retinol deciles was observed for, age Apolipoprotein A1, body mass index, C-reactive protein, Diabetes glomular filtration rate, and systolic blood pressure.

Table 6, characteristics of patients according to plasma retinol deciles, middle, upper and lower deciles of plasma retinol concentration.

Parameters	1 st decile (N=395)	Middle deciles (N=3081)	10 th decile (N=368)	P-trend
Retinol	1.90 ± .223^a	2.86 ± .421	4.34 ± .606	
DM	13.7%	11.0%	13.3%	.160 ^b
Physical activity	72.9%	75.2%	74.2%	.594
Smoking	31.4%	25.7%	20.7%	.003
Statin	78.2%	81.2%	78.8%	.243
Age	63.0 ± 10.7	61.6 ± 10.3	61.9 ± 9.87	.018
ApoA1	1.23 ± .281	1.32 ± .261	1.40 ± .285	.000
BMI	26.1 ± 4.15	26.8 ± 3.89	27.4 ± 4.18	.000
CRP	7.51 ± 14.0	3.17 ± 5.46	2.83 ± 3.62	.000
eGFR	93.2 ± 14.8	88.9 ± 15.5	75.8 ± 24.7	.000
SBP	139 ± 21.9	141 ± 20.3	144 ± 21.6	.003

ApoA1: Apolipoprotein A1, BMI: body mass index, CRP: C-reactive protein, DM: Diabetes Mellitus, GRF: glomular filtration rate, SBP: systolic blood pressure. Text in bold where P < 0.05

^amean ± SD and

^b P values are ANOVA

Data are presented by mean ± standard deviation for continuous variables, categorical data is presented as percentage of exposure

4.3 Survival analysis

The average follow up for total mortality in the 3842 patients was 2659 days (7.3 years, 95% confidence interval 2641-2677 days). In total, 280 patients died during the follow up period (7.9%). Of these, 224 were males and 56 were females. Cardiovascular death occurred in 149 patients (121 males and 28 females) and non-cardiovascular causes of death were noted in 131 patients (103 males and 28 females).

Table 7 presents the distribution between patients who died and patients who were alive at the end of the study by deciles of plasma retinol concentrations. Notably, the number of events in certain subgroups the number of events are relatively low (< 30).

The Kaplan-Meier analysis was used to evaluate the difference in survival between the deciles of retinol in our study. Figure number 6 through 8 and Table 7 provide evidence supporting the assumption that mortality is significantly different between exposure groups among total and male patients, but not among female patients. The mortality rates were highest among patients in the 10th decile and lowest in the middle deciles of plasma retinol. Female patients does not provide sufficient events for further mortality risk analysis.

Table 7, Summary of events in population presented in a cross table of mortality, gender and plasma retinol deciles

Parameters	Total population			1st decile			Middle deciles			10th deciles		
	N	Event	%	N	Event	%	N	Event	%	N	Event	%
All-Cause												
All	3842 ^a	280 ^b	7.86	395	37	9.37	3079	199	6.46	368	44	11.6
Male	2784	224	8.05	261	25	9.60	2249	162	7.20	273	37	13.6
Female	1059	56	5.29	134	12	9.00	830	37	4.46	95	7	7.45
CVD												
All	3842	149	3.88	395	17	4.30	3079	108	3.51	368	24	6.52
Male	2784	121	4.35	261	13	4.98	2249	88	3.91	273	20	7.33
Female	1059	28	2.64	134	4	2.98	830	20	2.41	95	4	4.21
NCVD												
All	3842	131	3.41	395	20	5.06	3079	91	2.96	368	20	5.43
Male	2784	103	3.70	261	12	4.60	2249	74	3.29	273	17	6.23
Female	1059	28	2.64	134	13	9.70	830	17	2.05	95	3	3.16

CVD: cardiovascular death, NCVD: death of non-cardiovascular origin

^a N is given as number of days

^b Number of positive events

Figure 6, Survival function for all-cause mortality according to plasma retinol deciles

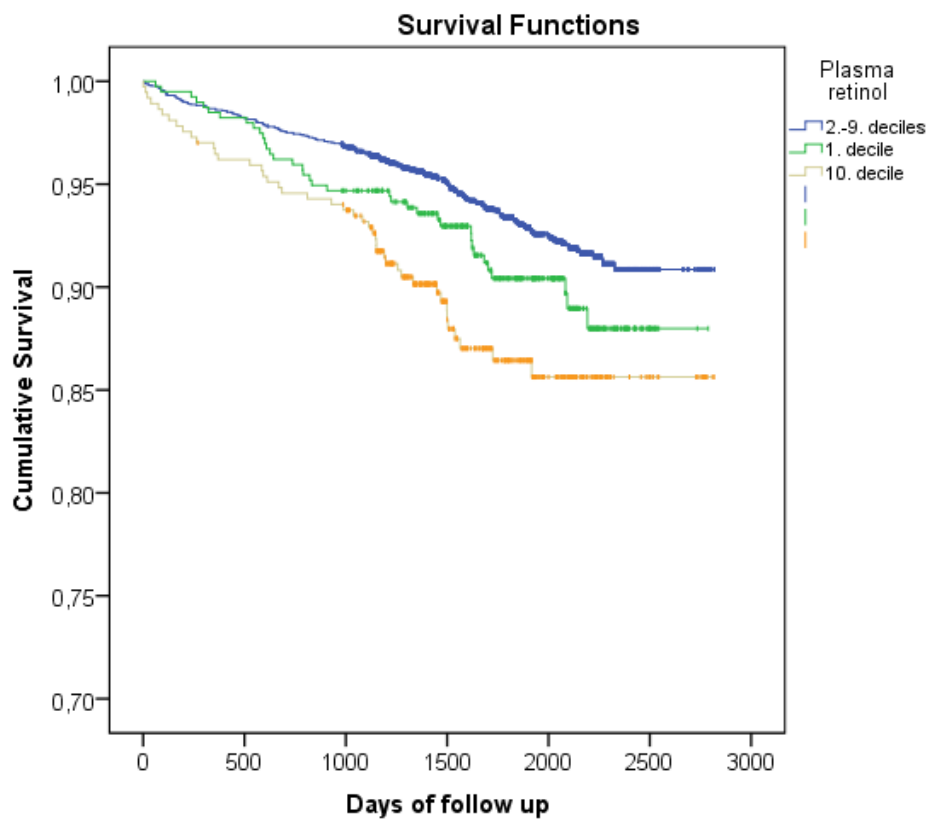


Figure 7, Survival function for CVD-related mortality according to plasma retinol deciles

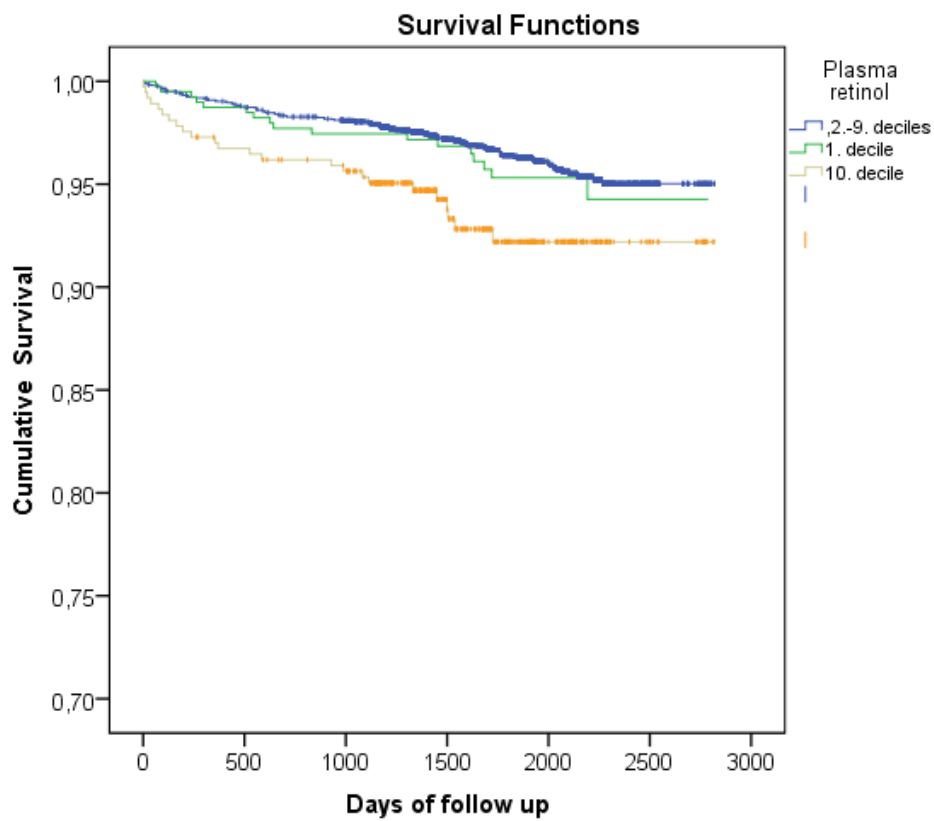
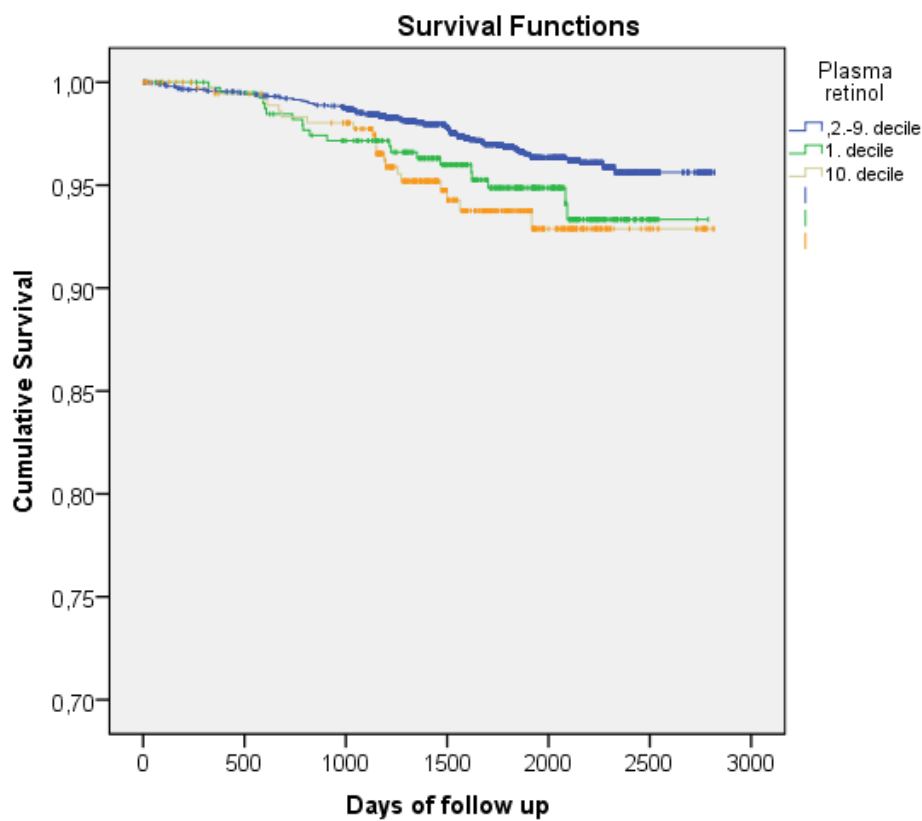


Figure 8, Survival function for non-CVD-related mortality according to plasma retinol deciles



4.4 Cox regression model

4.4.1 Association with total mortality

First, plasma retinol was used as a continuous variable. The crude model without any adjustments showed a risk increase for 1 $\mu\text{mol/L}$ plasma retinol for total mortality of 1.30 with a 95% confidence interval of 1.11, 1.52 (table 8). Adjustment for age and gender did not substantially change the hazard ratio (HR 1.33, 95% CI 1.14, 1.51). Further adjustment also did not change the association (model 3: HR 1.33, 95% CI 1.14, 1.51; model 4: HR 1.33, 95% CI 1.14, 1.51).

When analyzing plasma retinol in quartiles, a significant association of plasma retinol quartiles with total mortality was no longer observed (HR for the 4th quartile, compared to the 1. Quartile was 1.20, 95% CI .87, 1.65). Further adjustment did not change the association substantially (data not shown).

In addition, Cox regression was used to analyze total mortality-risk depending on plasma retinol deciles. When data for the total population were analyzed, all-cause mortality risk was found higher in the 1st (HR 1.38, 95% CI .97, 1.96) and 10th (HR 20.3, 95% CI 1.46, 2.81) decile of retinol in the crude analysis (Model 1, Table 9). The HR was significant, however, only for plasma retinol levels in the upper decile, but not in the lowest decile. Adjustment for age and gender (model 2) did not change the association substantially. Further adjustments also did not change the association (model 3 and 4, table 9).

Table 8, Cox regression models for plasma retinol and all-cause mortality risk in patients of the BECAC study (n=3842) the Hazard ratio was calculated for 1 μ mol/l increase in plasma retinol.

Predictor variable	Hazard Ratio (HR)	95% CI for HR		P-value
		Lower	Upper	
Model 1	1.30	1.11	1.53	.001
Model 2	1.33	1.14	1.51	.000
Model 3	1.30	1.09	1.55	.003
Model 4	1.33	1.12	1.59	.002

ApoA1: apolipoprotein A1, BMI: body mass index, CRP: C-reactive protein, DM: diabetes mellitus, eGRF: estimated glomular filtration rate, SBP: systolic blood pressure, Text in bold where P <0.05

^a P values provided in the Middle deciles row are linear trend across decile groups

^b Hazard ratio represents change in mortality risk

Model 1: crude model

Model 2: adjusted for age (in years) and gender

Model 3: further adjusted for CRP, eGFR and ApoA1

Model 4: Further adjusted for DM, statin, physical activity, smoking, BMI, and SBP

Table 9, Cox regression models for plasma retinol deciles and all-cause mortality risk in patients of the BECAC study (n=3842)

Predictor variable	Hazard Ratio (HR)	95% CI for HR		P-value
		Lower	Upper	
Model 1				
Middle deciles[referent]				.000^a
1 st decile	1.38 ^b	.97	1.96	.072
10 th decile	2.03	1.46	2.81	.000
Model 2				
Middle deciles[referent]				.000
1 st decile	1.27	.89	1.80	.189
10 th decile	2.06	1.49	2.59	.000
Model 3				
Middle deciles[referent]				.006
1 st decile	1.11	.76	1.61	.591
10 th decile	1.80	1.25	2.60	.001
Model 4				
Middle deciles[referent]				.003
1 st decile	1.05	.72	1.53	.802
10 th decile	1.84	1.27	2.65	.001

ApoA1: apolipoprotein A1, BMI: body mass index, CRP: C-reactive protein, DM: diabetes mellitus, eGFR: estimated glomerular filtration rate, SBP: systolic blood pressure, Text in bold where P < 0.05

^a P values provided in the Middle deciles row are linear trend across decile groups

^b Hazard ratio represents change in mortality risk

Model 1: crude model

Model 2: adjusted for age (in years) and gender

Model 3: further adjusted for CRP, eGFR and ApoA1

Model 4: Further adjusted for DM, statin, physical activity, smoking, BMI, and SBP

4.4.2 Association with cardiovascular disease related mortality

First, plasma retinol was used as a continuous variable. The crude model without any adjustments showed a risk increase for 1 $\mu\text{mol/L}$ plasma retinol for CVD-related mortality of 1.39 with a 95% confidence interval of 1.12, 1.71 (table 10). Adjustment for age and gender did not substantially change the hazard ratio (HR 1.39, 95% CI 1.15, 1.71). Further adjustment also did not change the association, but significance was lost when adjusted for model 3 and 4 (model 3: HR 1.18, 95% CI .34, 1.49; model 4: HR 1.22, 95% CI .97, 1.54).

When analyzing plasma retinol in quartiles, a significant association of plasma retinol quartiles with total mortality was no longer observed (HR for the 4th quartile, compared to the 1. Quartile was 1.39, 95% CI .89, 2.18). Further adjustment did not change the association substantially (data not shown).

Cox-regression was also used to analyze CVD-related mortality-risk depending on plasma retinol deciles. When the data for the total population were analyzed, CVD-related mortality risk was found higher in the 1st (HR 1.17, 95% CI .70, 1.95) and 10th (HR 2.01, 95% CI 1.30, 3.16) decile of plasma retinol in the crude analysis (Model 1, Table 11). The HR was significant, however, only for plasma retinol levels in the upper decile, but not in the lowest decile. Adjustment for age and gender (model 2, table 11) did not change the association substantially. After further adjustments significance was lost, HR remained increased for patients in the 10th decile, however, HR indicated decreased risk, although of no significance among patients in the 1st decile (model 3 and 4, table 11).

Table 10, Cox regression models for plasma retinol and CVD-related mortality risk in patients of the BECAC study (n=3842) the Hazard ratio was calculated for 1 μ mol/l increase in plasma retinol.

Predictor variable	Hazard Ratio (HR)	95% CI for HR		P-value
		Lower	Upper	
Model 1	1.39	1.12	1.71	.004
Model 2	1.39	1.15	1.70	.001
Model 3	1.18	.34	1.49	.162
Model 4	1.22	.97	1.54	.096

ApoA1: apolipoprotein A1, BMI: body mass index, CRP: C-reactive protein, DM: diabetes mellitus, eGRF: estimated glomular filtration rate, SBP: systolic blood pressure, Text in bold where P <0.05

^a P values provided in the Middle deciles row are linear trend across decile groups

^b Hazard ratio represents change in mortality risk

Model 1: crude model

Model 2: adjusted for age (in years) and gender

Model 3: further adjusted for CRP, eGFR and ApoA1

Model 4: Further adjusted for DM, statin, physical activity, smoking, BMI, and SBP

Table 11, Cox regression models for plasma retinol deciles and cardiovascular mortality risk in patients of the BECAC study (n=3842)

Predictor variable	Hazard rate	95% CI for HR		P-value
		Lower	Upper	
Model 1				
Middle deciles [referent]				.007
1 st decile	1.17	.70	1.95	.546
10 th decile	2.03	1.30	3.16	.002
Model 2				
Middle deciles [referent]				.006
1 st decile	1.07	.64	1.78	.809
10 th decile	2.06	1.32	3.21	.001
Model 3				
Middle deciles [referent]				.414
1 st decile	.97	.56	1.68	.912
10 th decile	1.41	.85	2.34	.188
Model 4				
Middle deciles [referent]				.359
1 st decile	.89	.52	1.56	.692
10 th decile	1.43	.85	2.40	.173

ApoA1: apolipoprotein A1, BMI: body mass index, CRP: C-reactive protein, DM: diabetes mellitus, eGRF: estimated glomerular filtration rate, SBP: systolic blood pressure, Text in bold where P < 0.05

^a P values provided in the Middle deciles row are linear trend across decile groups

^b Hazard ratio represents change in mortality risk

Model 1: crude model

Model 2: adjusted for age (in years) and gender

Model 3: further adjusted for CRP, eGFR and ApoA1

Model 4: Further adjusted for DM, statin, physical activity, smoking, BMI, and SBP

4.4.3 Association with non-cardiovascular disease related mortality

First, plasma retinol was used as a continuous variable. The crude model without any adjustments showed a risk increase for 1 $\mu\text{mol/L}$ plasma retinol for non-CVD-related mortality of 1.21, however, the increase was not found significant with a 95% confidence interval of .95, 1.54 (table 12). Adjustment for age and gender did not substantially change the hazard ratio (HR 1.24, 95% CI .98, 1.56). Further adjustment also did not change the association, but the adjustments resulted in a gain of significance for model 3 and 4 (model 3: HR 1.44, 95% CI 1.10, 1.88; model 4: HR 1.46, 95% CI 1.12, 1.91).

When analyzing plasma retinol in quartiles, a significant association of plasma retinol quartiles with total mortality was no longer observed (HR for the 4th quartile, compared to the 1. Quartile was 1.02, 95% CI .64, 1.62). Further adjustment did not change the association substantially (data not shown).

Lastly, Cox regression was used to analyze non-CVD-related mortality-risk depending on plasma retinol deciles. When the data for the total population were analyzed, non-CVD-related mortality risk was found higher in the 1st (HR 1.63, 95% CI 1.00, 2.64) and 10th (HR 2.21, 95% CI 1.25, 3.29) decile of retinol in the crude analysis (Model 1, Table 13). The HR was significant in both the lower and upper decile in the crude analysis. Adjustment for age and gender (model 2, table 13) did not change the association substantially, however, the association only remained significant for plasma retinol levels in the upper decile (HR 2.06, 95% CI 1.27, 3.34), but not in the lowest decile (HR 1.51, 95% CI 0.93, 2.45). Further adjustments did not change the association substantially (model 3 and 4, table 13).

Table 12, Cox regression models for plasma retinol and non-CVD-related mortality risk in patients of the BECAC study (n=3842) the Hazard ratio was calculated for 1µmol/l increase in plasma retinol.

Predictor variable	Hazard Ratio (HR)	95% CI for HR		P-value
		Lower	Upper	
Model 1	1.21	.950	1.54	.122
Model 2	1.24	.984	1.56	.069
Model 3	1.44	1.10	1.88	.008
Model 4	1.46	1.12	1.91	.006

ApoA1: apolipoprotein A1, BMI: body mass index, CRP: C-reactive protein, DM: diabetes mellitus, eGRF: estimated glomular filtration rate, SBP: systolic blood pressure, Text in bold where P <0.05

^a P values provided in the Middle deciles row are linear trend across decile groups

^b Hazard ratio represents change in mortality risk

Model 1: crude model

Model 2: adjusted for age (in years) and gender

Model 3: further adjusted for CRP, eGFR and ApoA1

Model 4: Further adjusted for DM, statin, physical activity, smoking, BMI, and SBP

Table 13, Cox regression models for plasma retinol deciles and non-cardiovascular mortality risk in patients of the BECAC study (n=3842)

Predictor variable	Hazard Ratio (HR)	95% CI for HR		P-value
		Lower	Upper	
Model 1				
Middle deciles [referent]				.006
1 st decile	1.63	1.00	2.64	.049
10 th decile	2.21	1.25	3.29	.004
Model 2				
Middle deciles [referent]				.007
1 st decile	1.51	.93	2.45	.096
10 th decile	2.06	1.27	3.34	.003
Model 3				
Middle deciles [referent]				.005
1 st decile	1.25	.75	2.09	.401
10 th decile	2.33	1.38	3.94	.001
Model 4				
Middle deciles [referent]				.005
1 st decile	1.21	.72	2.03	.476
10 th decile	2.38	1.41	4.03	.001

ApoA1: apolipoprotein A1, BMI: body mass index, CRP: C-reactive protein, DM: diabetes mellitus, eGRF: estimated glomerular filtration rate, SBP: systolic blood pressure, Text in bold where P < 0.05

^a P values provided in the Middle deciles row are linear trend across decile groups

^b Hazard ratio represents change in mortality risk

Model 1: crude model

Model 2: adjusted for age (in years) and gender

Model 3: further adjusted for CRP, eGFR and ApoA1

Model 4: Further adjusted for DM, statin, physical activity, smoking, BMI, and SBP

5 Discussion

The discussion section of this paper will focus on explaining the results of our study, with emphasis on comparing our results with the hypothesis and previous studies. In addition, the quality of the study and its model will also be discussed.

5.1 Results

In this study, we evaluated the risk of all-cause, CVD-related and non-CVD-related mortality depending on plasma retinol concentration in a population with established CVD. The main finding of this analysis was that high levels of plasma retinol in patients with coronary heart disease led to a higher risk of total and non-CVD mortality than in patients with plasma retinol levels in the 'middle range'. High plasma retinol levels were also associated with increased risk of CVD-mortality, however, this was no longer significant after adjustment. Patients with low plasma retinol also had a higher mortality risk, but the assumption did not achieve significance.

5.2 Result discussion

5.2.1 Differences in mortality

The survival plots suggested that mortality risk was greatest among patients with elevated plasma retinol, and lowest in the referent levels. The survival analysis supports the assumption that patients with plasma retinol concentration between 2.15 and 3.81 $\mu\text{mol/L}$ have a lower all-cause mortality rate compared to patients with either $< 2.15 \mu\text{mol/L}$ or $> 3.81 \mu\text{mol/L}$.

During the on average 2659 days (7 years and 3 months) of follow-up 7.9 percent of the patients died, of which slightly more were CVD-related compared to non-CVD-related. The number of events allowed the study to show significant association in several models. It also allowed for investigation of the extreme ends to the plasma retinol specter. The original plan was to split the population by gender (matching) and evaluate them separately in addition to the total population analysis to account for differences between sexes. Matching for gender was, however, deemed unsuitable, as numbers of events among women were low and gender was added to adjustment models.

We observed a U-shaped association between plasma retinol and mortality. Patients in the higher and lower deciles having higher mortality than the middle deciles referent creating the U-shape, except for CVD-related mortality after adjustment. The trend is illustrated in figure 9 and 10, which illustrate in unadjusted and adjusted HRs respectively.

Figure 9, HR for all-cause, CVD, and non-CVD-related mortality, HRs are unadjusted

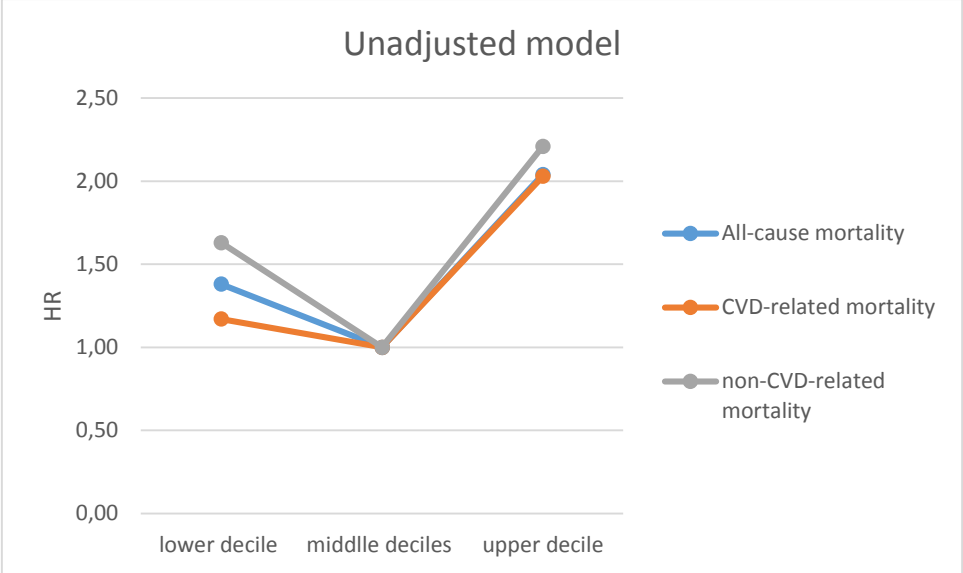
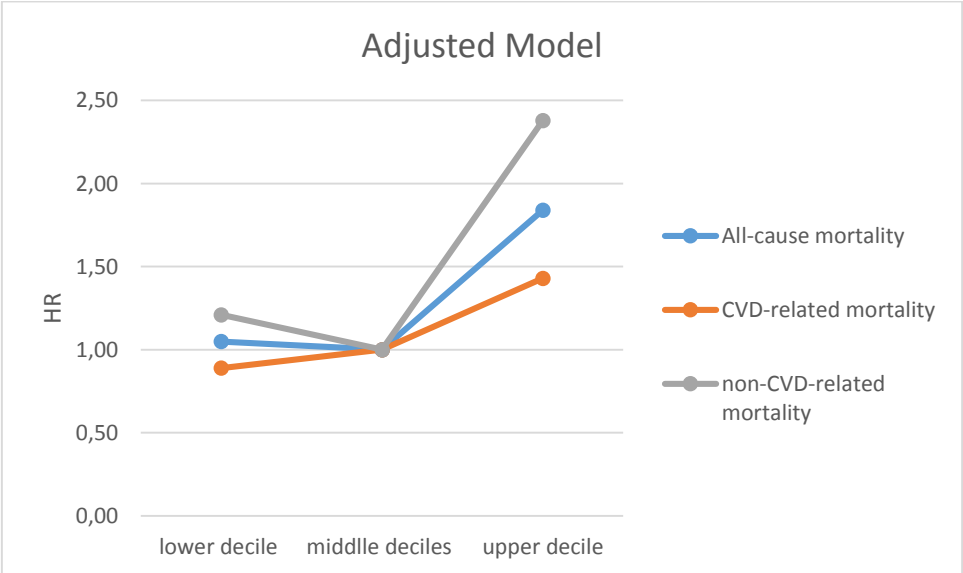


Figure 10, HR for all-cause, CVD, and non-CVD-related mortality, HR are adjusted for variables in Cox regression model 4



5.2.2 Plasma retinol levels and total mortality

The mortality risk analysis revealed that HR increased by 30% per 1 µmol/L plasma retinol and that confounders had little effect on the association. Contradictory, no

association was found when plasma retinol was evaluated in quartiles as done in previous studies. Extreme values of plasma retinol was evaluated to test the hypothesis that upper and lower exposure groups of quartiles included homeostatically controlled plasma retinol levels. The results of the mortality risk analysis for plasma retinol deciles indicated a 38% increased mortality risk for patients in the lowest decile and 106% increased mortality risk for patients in the highest decile, also after adjustments.

5.2.3 Plasma retinol levels and CVD-related mortality

Contradictory to the significance seen in total mortality HR models, increased CVD-related mortality risk was only found significant in the highest decile of plasma retinol before adjustment. The assumptions of increased risk for patients in the highest decile was sustained as the hazard risk stays elevated, however, the hazard risk changes after further adjustments. Among patients in the lower decile of plasma retinol an increase in hazard rate was first indicated, but a lower hazard rate was indicated after adjustment, the association were never of significance.

5.2.3.1 Low plasma retinol concentrations and CVD mortality

The Kaplan-Meier analysis deemed mortality risk for patients with plasma retinol concentration in the lowest decile of distribution to be higher than the middle decile referent and lower than for patients in 10th decile of plasma retinol. Three previous studies have suggested that low plasma retinol concentrations may increase the risk of CVD-related mortality [7, 43, 44]. However, the same results were not seen in the mortality risk analysis. In contrast to previous findings which showed increased risk in participants with concentrations < 2.90 µmol/L, no association of increased or decreased risk in BECAC patients with plasma retinol concentration < 2.15 µmol/L could be made.

5.2.4 Elevated plasma retinol concentration and CVD mortality

BEAC patients with plasma retinol concentration > 3.81 µmol/L were associated with significantly higher mortality risk than patients with < 3.81 µmol/L. After further adjustment for confounders, the mortality risk was 40% higher in patients with elevated plasma retinol concentration, but the difference was no longer significant.

5.2.5 Comparison with previous studies

The models of our study indicated similar assumptions for total mortality and CVD-related mortality. The U-shaped hazard risk distribution is present in previous studies and the current. Although there seems to be a clear trend there are differences in significance of results, size of change and if the highest hazard risk is found at the lower or higher end of the range. Our study stood as CVD-related mortality risk no longer was significant after adjustments and that no assumptions of change for CVD-related mortality risk could be made for patients with low plasma retinol.

5.2.6 Food intake and plasma retinol concentration

Comparing food intake to plasma retinol concentrations provided significant correlations between two out of ten food groups and plasma retinol. Milk and meat had modest positive correlation with plasma retinol. This supports former evidence that plasma retinol stays unaffected by food intake in people with subclinical vitamin A stores [25].

5.3 Method evaluation

In this study, we evaluated the patient's risk of CVD based on the Vitamin A status. The patients were all part of a CVD diagnosis study and some were part of a randomized controlled trial evaluating vitamin B supplementation and CVD risk. The methods utilized in this study and its population will now be further discussed.

5.3.1 Baseline data

The majority of data variables were recorded through robust methods such as blood sampling, and assessment by trained physicians and nurses, or from self-administered questionnaires verified by medical history [42]. The data were originally collected for CVD diagnosis trial and a vitamin B intervention study and thus, some additional data might have been beneficial. Plasma concentrations of other vitamin A metabolites and data on dietary intake of food sources rich in vitamin A could have increased understanding of the population's vitamin A status [42, 49].

In contrast to the more robust data, some data were self-reported. Self-reported smoking habits were combined with cotinine values to make the estimate of exposure more robust, as proposed by Svingen et al. [42], patients' smoking history was not accounted for. The physical activity data was also self-reported as patients were asked to classify

themselves as being active twice a week or not. The data of self-reported physical activity have several limitations. Firstly, patients had to estimate their average physical activity and remember how active they had been the preceding year. Secondly, self-reported physical activity tends to overestimate actual physical activity [55]. Thirdly and lastly, the validity of the activity data is limited by the fact that the cut-off of physical activity twice a week does not reflect the recommendations from the Norwegian Directory of Health, which recommends 20 minutes of activity a day or 150 minutes a week [56].

Additionally, because the significant increased HR of non-CVD-related mortality was indicated in exposure groups of plasma retinol, further data on mortality causes would have been of interest. No such data was available as endpoints were classified as either CVD-related or not CVD-related.

5.3.2 Plasma retinol as a biomarker

The accuracy and repeatability of plasma retinol measuring have been validated and thoroughly discussed in the report by Midttun et al. [35]. While the measurement of plasma retinol is deemed accurate and reliably tested in large studies from small amounts of plasma, questions have been asked regarding its power as a biomarker [15, 35].

The regulation of plasma retinol by the liver and the lack of predictability of the homogenically controlled plasma retinol plateau compromise its effectiveness as a biomarker [17]. Being able to predict cut-off points where plasma retinol no longer is homogenically controlled, could have increased the strength of our assumptions by ensuring that estimations were made on the actual population of participants with subclinical vitamin A deficiency or toxicity. Further, such values would have allowed us to maximize the amount of patients in the exposure groups to gain statistical power. The BOND report by Raiten et al., suggest a need for a separate, complementary biomarker of vitamin A, to account for the homogenically controlled range and as plasma retinol is not found effective in estimating of vitamin A intake or to monitor effects of dietary interventions and food based strategies [25].

A separate problem with plasma retinol as a biomarker is the influence of inflammation and infection. The isolated influence of acute response proteins on plasma retinol in contrast to total body vitamin A is well documented, but the strength and rate of alteration is not

clearly standardized. An effect was found but not proven by Duncan et al. [38], thus a clear standardized approach for adjustment is yet to be established.

5.3.3 Plasma retinol groups

The process of creating the plasma retinol groups for mortality risk estimation provided a conflict of interest. Our first approach was to mimic previous studies by dividing plasma retinol concentration into quartiles. The quartiles were elected to optimize the transferability of our results to the previous studies. As quartiles did not represent trend seen in the mortality risk analysis of continuous plasma retinol data, more extreme groupings were discussed. Two areas of focus were considered when electing the new exposure groups. Firstly, to get sufficient number of patients i.e. events in the exposure groups, and secondly, to avoid including patients with homogenically controlled plasma retinol concentrations. As a result, plasma deciles were elected for the project. This somewhat cautious approach in terms of homogenically controlled range was possible as our population and number of outcomes were large. Even though focus was on having sufficient events in the exposure group and a large population size, the prevalence of events was relatively low, especially when the population was split by gender. A better understanding of changes in serum retinol concentration first explained by Olsen et al. (figure 3) could have improved the number of patients and outcomes in the exposure groups [17].

5.3.4 Dietary intake – FFQ

The advantages of utilizing a FFQ to report dietary intake is that they are cost effective, simple to use, and show higher compliance among participants compared to other methods [51]. Limitations are present as the method is strictly retrospective. Additionally, the method may produce a one-dimensional picture of the participant's food intake and it is relying on the participants' ability to remember what their food intake was like the last year. Underreporting food intake is also a common problem especially among women [57]. The FFQ used is validated, widely used, and is made to fit Norwegian food and meal patterns [51]. However, the FFQ used was developed to investigate eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) fatty acid consumption and did not provide data on vitamin A rich food sources. Data on food groups such as liver, dairy products, dark colored fruits and vegetables as well as vitamin A and β -carotene supplementation was not present in available data set.

5.3.5 Statistical analysis

LDL cholesterol data and other cholesterol related parameters were left out of the regression model due to the extensive use of cholesterol lowering statin treatment (80% of population). In regards to the hypothesis of this study, electing the middle deciles as the referent for the Cox regression analysis was determined the best fit and provided evidence to the assumption of a U-shaped hazard rate distribution. The tested more than one of exposure and our data decision to use several classifications

5.4 Population

There were several benefits of utilizing the WENBIT population for this study. The data-set was easily retrieved from the Department of heart disease at HUS at no cost and without taxing the participants any further as the data already existed.

Performing this study on patients with established CVD poses both benefits and disadvantages. There are several reason why the BECAC population was elected for WENBIT and this study. As the patients had already established CVD, they have an increased risk of new events (exposure). The increased likelihood of events allows the investigator to expect significant results in smaller number of study participants, as opposed to what could be expected when doing the same study with participants from a healthy population. Thus, reducing the amount of resources needed to perform the study, in form of cost, labor, and stress on population.

5.5 Limitations

Strength of introductory information may be questioned as information was primarily gained through reviews and other secondary sources, although, some primary sources where used.

5.5.1 Internal validity

The study design renders assumption of causation invalid. Assumptions of association can be made but structural limitations must be accounted for. Assumptions are limited by low number of events in exposure groups, lack of significance in some models and by alterations assumptions or significance produced by adjusting for confounders. Additionally, we tested multiple hypothesis. Therefore, results must be interpreted with caution.

We do know that the B vitamin intervention given to patients in WENBIT did not affect; mortality, risk of CVD or alter biomarkers of CVD such as IL-6 and CRP [50]. However, we do not have information on whether B-vitamin supplementation will affect plasma retinol concentrations, but we do not have any knowledge on that it does so.

5.5.2 External validity

The cut-off values of the decile groups have little external validity, as they are specific for this study and are not assumed to represent any previously discussed cut-off values for either toxicity or deficiency. The disadvantages of having a population that is predisposed for outcomes cannot be assumed to fully represent the normal population. 53% of deaths were CVD-related in the study. Which is substantially higher than 30% seen in the general population of Norway [6]. The increased prevalence of CVD is assumed to originate from the patients under study's increased risk through previous exposure. The factors increasing rates of CVD in the study population may also have caused exposure and confounding variables to respond differently in the population under study, in relation to how it is expected to react in healthy individuals, reducing validity.

5.5.3 Confounding

The Cox regression model was used to adjust for vascular risk factor exposure, but some potential for unmeasured and residual confounding remains. For example, we did not adjust for dietary vitamin A intake, supplement use or disease history. Gender was added to the model after matching by gender was discarded due to lack of events in exposure groups when divided by gender.

Confounders proved to play a role in for some regression models, as significance was lost or in some test insignificant mortality risks changed from increase to decrease when adjusting for model 2 or model 3. As with any observational study, residual confounding by socioeconomic status, lifestyle variables, and other factors cannot be excluded.

The correlation analysis revealed as expected that CRP was negatively associated with plasma retinol levels. It is established that increased CRP concentrations in plasma reduce plasma retinol without affecting total body vitamin A. The levels of CRP were significantly higher in the 1st decile of plasma retinol at 7.51 mg/L (\pm 14.0 mg/L), which is twice the mean value of the Middle deciles referent and 10th decile (3.17 \pm 5.46 mg/L and 2.83 \pm 3.62,

respectively). The significant higher mean CRP in the 1st decile of plasma retinol may suggest that some of the patients of the 1st decile group may have been false positives.

5.6 Conclusion and suggestions for future work

There are several limitations to our assumptions. However, they follow the same U-shaped trend indicated by Goyal et al. suggesting that the mortality risk is greater in populations with low or elevated plasma retinol [44]. The assumptions also indicated that low or high plasma retinol levels does not predict CVD-related mortality singularly but rather predicts increased all-cause mortality. The accumulation of the increase in HR seen for continuous plasma retinol, the U-shaped HR, and the HR scores throughout the models suggest that mortality risk were higher among patients with elevated plasma retinol concentration.

Several studies have indicated that vitamin A metabolites may have important roles in preventing atherosclerotic CVD development and that vitamin A metabolites may be efficiently affected by supplementation. However, recent randomized supplementation studies have failed to show health benefits from taking vitamin A supplements [47, 48]. The assumptions also indicated increased mortality risk among patients with low vitamin A status, suggesting that sub-groups might benefit from diet intervention or supplementation.

A better understanding of plasma retinol as a biomarker of vitamin A status is needed. Especially when it comes to understanding the relation between vitamin A liver stores, inflammation, and serum retinol. Further investigation of the increased risk seen at the extreme ends of plasma retinol concentration is needed to further support or disprove the assumption and circle in cut-off values for change in mortality risk. The ideal approach to investigating Vitamin A and mortality, would be in a randomized intervention study where supplementation were adjusted for serum retinol and markers of inflammation.

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