UNIVERSITY OF OSLO

Faculty of Medicine

Microbial translocation and cardiovascular disease states

Emphasis on chronic heart failure, diabetes and the metabolic syndrome

Thesis for the degree of Philosophiae Doctor (PhD)

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Oslo 2020

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*Series of dissertations submitted to the
Faculty of Medicine, University of Oslo*

ISBN 978-82-8377-785-7

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Cover: Hanne Baadsgaard Utigard.
Print production: Reprosentralen, University of Oslo.

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Acknowledgement

First, I owe my deepest gratitude to the Center for Clinical Heart Research. This work as well as my PhD education, would not have been possible without the fundamental support from an exceptional research center. A center that has with the unwavering leadership of professor Ingebjørg Seljeflot and Harald Arnesen over the past 20 years, thrived as an excellent center for translational research and a school for PhD students.

Professor Seljeflot, has also been my main supervisor. Her guidance, help and support, has been vital for my successful entry into the exiting world of science. I will always be grateful for her bold decision to enter the untested field of the gut microbiome and cardiovascular disease. Her door is always open, despite of an immense workload.

I would like to thank Sissel, Jeanette, Trine and Charlotte, integral parts of CCHR. They have been central for most of the laboratory work in my thesis, but also supportive in other areas of my work.

I would also like to thank my co-supervisors Marius Trøseid and Svein Solheim. Their expertise in the field of the gut microbiota and cardiovascular disease, respectively, have been indispensable to my work. Marius' enthusiasm, ambition and effectiveness is truly contagious and admirably. Svein' clinical skills have been vital in the presentation and conduction of my works.

Our cooperation with Cristiane Mayerhofer, Kaspar Broch and Lars Gullestad from Rikshospitalet have both been seamless and fruitful. Their work with the design of the GutHeart study, allowed me to enter a well-design study which became the basis for papers III and IV in my thesis.

I would also like to thank Sigrun Halvorsen for facilitating my research but also research in general at the cardiology department Ullevål.

My fellow PhD students Christian, Joanna, Miriam, Hani, Are, Vibeke and Susanne have truly been sources of motivation and help throughout the entire period.

I would furthermore like to thank all other coauthors and personnel at Rikshospitalet, Bodø, and Rio de Janeiro for their help in the GutHeart study.

Finally yet importantly, I would like to thank my wife Kristine, my two children Emma and Benjamin, my sisters and parents for their support in everyday life and their help and sacrifice for me to proceed and complete my PhD education.

Selected abbreviations

CAD	Coronary artery disease
CVD	Cardiovascular disease
DAMP	Damage associated molecular patterns
DOIT	Diet and omega-3 fatty acid intervention trial
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
FMT	Fecal microbiota transfer
HDL-c	High density lipoprotein cholesterol
HF	Heart failure
HFpEF	Heart failure with preserved ejection fraction
HFrEF	Heart failure with reduced ejection fraction
hsCRP	High sensitive C-reactive protein
I-FABP	Intestinal fatty acid binding protein
IL-1	Interleukin-1
IL-6	Interleukin-6
LBP	Lipopolysaccharide binding protein
LDL-c	Low density lipoprotein cholesterol
LPS	Lipopolysaccharide
mCD14	Membrane-bound Cluster of differentiation 14
MetS	Metabolic Syndrome
NF- κ B	Nuclear factor kappa B
NT-proBNP	N-terminal pro-Brain natriuretic peptide
PAMP	Pathogen associated molecular patterns
PUFA	Polyunsaturated fatty acids
sCD14	Soluble Cluster of differentiation 14
SCFA	Short chained fatty acids
TLR	Toll-like receptor
TMAO	Trimethylamine N-oxide
TNF α	Tumor necrosis factor alpha

List of papers

Paper I

Awoyemi A, Trøseid M, Arnesen H, Solheim S, Seljeflot I: **Markers of metabolic endotoxemia as related to metabolic syndrome in an elderly male population at high cardiovascular risk: a cross-sectional study.** *Diabetol Metab Syndr* 2018, 10:59

Paper II

Awoyemi A, Trøseid M, Arnesen H, Solheim S, Seljeflot I: **Effects of dietary intervention and n-3 PUFA supplementation on markers of gut-related inflammation and their association with cardiovascular events in a high-risk population.** *Atherosclerosis* 2019, 286:53-59

Paper III

Awoyemi A*, Mayerhofer C*, Felix AS, Hov JR, Moscovitch SD, Lappegård KT, Hovland A, Halvorsen S, Halvorsen B, Gregersen I, Svardal A, Berge RK, Hansen SH, Götz A, Aukrust P, Åkra S, Seljeflot I, Solheim S, Lorenzo A, Gullestad L, Trøseid M*, Broch K*. **Microbiota-Directed Therapy in Heart Failure with Reduced Ejection Fraction: Results From the Randomized GutHeart Trial.** *Submitted* * Equally contributed

Paper IV

Awoyemi A, Mayerhofer C, Broch K, Hov JR, Moscovitch SD, Lappegård KT, Hovland A, Halvorsen B, Aukrust P, Gullestad L, Solheim S, Trøseid M, Seljeflot I. **Effect of Probiotics and Antibiotics on markers of Gut Leakage in Heart Failure Patients with Reduced Ejection Fraction - Results from the randomized controlled GutHeart trial.** *In manuscript*

Introduction

Cardiovascular disease and risk factors

In 2016, non-communicable diseases were responsible for 71% of all deaths worldwide.

Of these, 44% were caused by cardiovascular diseases (CVD), thus being the leading cause of death especially in low to middle-income countries (1). CVD includes coronary artery disease (CAD), cerebrovascular disease, heart failure, aortic disease, abnormal heart rhythms, valvular heart disease, cardiomyopathies, peripheral artery disease, rheumatic heart disease, congenital heart disease, venous thromboembolic diseases.

Traditionally, risk factors of CVD are divided into modifiable and non-modifiable. The main true non-modifiable risk factors are advanced age, male sex, African or Asian ancestry and family history/genetics. The modifiable risk factors are more abundant. The classical ones are dyslipidemia, diabetes mellitus, hypertension, obesity and metabolic syndrome (MetS). However, from a preventive point of view, the most important ones are the modifiable behavioral risk factors, such as high alcohol intake, tobacco use, unhealthy diet and physical inactivity (2). Of these, an unhealthy diet including a shift towards intake of energy-dense foods high in saturated fats, salt and sugars is probably the main reason for a quadrupling of diabetes, tripling of obesity and doubling the prevalence of hypertension the past 30-40 years (1). Importantly, CVD is preventable by addressing these risk factors, both before the appearance of CVD, but also after, the occurrence of clinical disease.

Surprisingly, despite aggressive treatment of hyperlipidemia, hyperglycemia and hypertension, a considerable portion of patients will still have a high risk of new CV events. Chronic low-grade inflammation has been proposed to be a player in this residual risk. High sensitivity C-reactive protein (hsCRP), a marker of systemic inflammation, but also cytokines such as tumor necrosis factor α (TNF α) has demonstrated strong

association with prevalent and incident CVD (3, 4). Further elaboration on the role of inflammation in CVD follows in the next subchapters.

Strategies targeting central inflammatory pathways had failed to demonstrate any reduction in CV risk compared to conventional treatment when the present work was started out, and there were no available treatment strategies for reducing inflammation in CVD.

Atherosclerosis

The notion that atherosclerosis, being an important underlying process in CVD, is caused by deposits of cholesterol in large to medium-sized vessels, is over a century old (5). Furthermore, the established link between dietary intake of fat and atherosclerotic disease, is arguably one of the most important medical discoveries of the 20th century. However, we now know that the etiology behind is much more complex and multifactorial than initially believed. An interplay between different cell types such as immune cells, endothelial cells and smooth muscle cells undoubtedly contributes to disease initiation and progression (6).

Vascular intimal retention of cholesterol-rich lipoproteins, especially apolipoprotein B (apoB)-containing lipoproteins, is believed to be the principal driver of atherosclerosis. Thus, efforts directed towards decreasing the atherosclerotic burden, would naturally be directed at reducing apoB-containing lipoproteins, especially low-density lipoprotein cholesterol (LDL-c). In 1994, the Scandinavian 4S study, paved the way for targeting LDL-c with statins in coronary heart disease. The study demonstrated a 34% risk reduction in coronary deaths and non-fatal myocardial infarctions with a LDL-c goal which is today considered conservative (7). Further studies with more potent LDL-c lowering strategies, have cemented the role of LDL-c in CVD. However, despite

aggressive lowering of LDL-c and addressing other known risk factors of CVD such as smoking, hyperglycemia and hypertension, atherosclerosis still occurs.

The pathophysiological processes behind atherosclerosis are very complex and involve many biological systems (Figure 1). Here I will only attempt to give a short overview of the main processes investigated in my thesis.

Figure 1. Innate immune responses in atherosclerosis. Reused with permission from Anton Gisterå et al.(8)

As mentioned, several immune cells such as monocytes, monocyte-derived macrophages, mast cells and T-cells also contribute to the atherosclerotic process. Upon endothelial activation, monocytes migrate into the intima and differentiate into type 1 macrophages (9). Activated macrophages express both scavenger receptors and toll like receptors (TLRs). The scavenger receptors internalize modified, especially oxidized LDL-c and ultimately forms foam cells, a key component of the atherosclerotic core. The TLRs binds damage associated molecular patterns (DAMPs) such as cholesterol crystals and oxidized LDL-c and pathogen associated molecular patterns (PAMPs) such as bacterial toxins.

Both pathways eventually lead to an increased production of pro-inflammatory cytokines and chemokines such as interleukin-1 and -6 (IL-1 and IL-6), TNF α and monocyte chemoattractant molecule-1 (MCP-1), the latter attracting more monocytes and other immune cells to the inflamed intima. As the disease progresses, a core of cells, lipids and connective tissue, forms the atheroma or the atherosclerotic plaque (10, 11). The entity becomes symptomatic either due to simple expansion of the atheroma, erosion or rupture of the overlying fibrous cap. A subsequent narrowing of the vessel lumen restricts the blood flow, and when occurring in coronary arteries, the silent atherosclerotic plaque may transform into ischemic heart disease.

Metabolic syndrome

Metabolic syndrome (MetS) describes a consortium of conditions that translates into an increased risk of CVD and all-cause mortality (12). From the 1800 century, specific anthropomorphic phenotypes have been associated with symptomatic coronary artery disease (13). Although the concept of MetS stems from the 1920s, the term was first used in 1981 to describe the coincidence of obesity, hyperlipoproteinemia, diabetes, gout and hypertension (14). Today, almost 40 years later, there is still no universal definition of the syndrome. However, the central components of adiposity, dyslipidemia, insulin resistance/hyperglycemia and hypertension, are now widely accepted as the central features of the syndrome. In 1998, the World Health Organization (WHO) defined MetS as the presence of insulin resistance, impaired glucose tolerance or diabetes mellitus type 2, together with at least two of the following: hypertension, dyslipidemia, increased waist/hip ratio or increased body mass index and microalbuminuria (15). The European Group for the study of Insulin Resistance (EGIR) redefined some of the cutoff values, excluded microalbuminuria and defined hyperinsulinemia as a central part of the

syndrome. In the present work we used the “Third Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (NCEP ATP III)” from 2001 for the definition of MetS (16). This definition includes at least three of the following five components: abdominal obesity (waist circumference > 88 cm in females and > 102 cm in males), elevated triglycerides (>1.69 mmol/L), reduced HDL cholesterol (< 1.03 in females and < 1.29 mmol/L in males, high blood pressure (>130/85 mmHg) or elevated fasting glucose (> 6.1 mg/mL). Importantly, none of the criteria were a prerequisite. The definition has later been modified to include patients on drug treatment for dyslipidemia, for elevated blood pressure and for hyperglycemia, however, this is not taken into account in the present work (17).

International Diabetes Federation (IDF), the American Association of Clinical Endocrinology (AACE) and the American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI) have all also published their revised definition of MetS. IDF and AHA/NHLBI used abdominal obesity as a necessity for the diagnosis of MetS. IDF has also suggested different cutoffs according to ethnicity. Similar to the WHO definition, AACE are focused on impaired fasting glucose or impaired glucose tolerance as the indispensable criterion (15, 17). Although not being a part of any MetS definitions, it has been discussed whether chronic low-grade inflammation should be added as well as hypercoagulability through increased plasma levels of tissue plasminogen activator inhibitor-1 (PAI-1), fibrinogen (figure 2), and coagulation factors VII and VIII (18-20) . The CV-risk associated with MetS is higher than can be accounted for by the combined single components alone (21). It is associated with increased levels of markers of systemic inflammation such as CRP, TNF α and IL-6. Moreover, the more components

present, the higher the markers of systemic inflammation, and the higher the risk of new CV events (22).

The source of this chronic inflammation in MetS is not completely clear. One hypothesis is that it originates from the adipose tissue, more specifically adipocytes and adipose tissue macrophages. However, populations of B and T-cells, neutrophils, dendritic cells and mast cells have also been proposed to contribute (23).

Figure 2. Pathophysiology of the metabolic syndrome. Superimposed and contributory to the insulin resistance produced by excessive FFA is the paracrine and endocrine effect of the pro-inflammatory state. Reused and modified with the permission from Eckel et al. (24)

Upon excess energy intake, an unhealthy expansion of visceral adipose tissue leads to lipolysis and release of free fatty acids, which in turn binds to TLRs in the adipose tissue with subsequent pro-inflammatory signaling through nuclear factor kappa B (NF-κB) (25). Conversely, inhibiting NF-κB has been shown to suppress cytokine production of the adipose tissue ex-vivo (26). It is suggested that MetS not only contributes to increased inflammation, but the effect of inflammation on target tissues such as the liver and skeletal muscle, also contributes to insulin resistance and obesity, arguably the key

components of MetS (19). However, the direction of the potential causality is not known. Interestingly, chronic low-grade inflammation seems to induce MetS-like features in mice through activation of TLRs and NF- κ B (27).

To this date, pharmacological treatment of metabolic syndrome is only focused on addressing the individual components of the syndrome.

Heart failure

Heart failure (HF) is defined by a set of clinical symptoms and signs (dyspnea, ankle edema, elevated jugular venous pressure pulmonary crepitation and peripheral edema) due to a cardiac abnormality. It is a clinical syndrome caused by reduced cardiac output and elevated intracardiac pressures (28).

An aging population and the successful treatment of CAD have resulted in the rise of heart failure as a global pandemic. The prevalence is well above 26 million globally (29). This corresponds to 1-2% of the adult population in the western world. Despite improved management of heart failure over the last two decades, mortality remains high with a 1-year mortality rate of about 20% in Europe (30).

Heart failure with reduced ejection fraction

There are two main subgroups of heart failure characterized by the rather arbitrary level of left ventricular ejection fraction (LVEF) $>$ or $<$ 40%, namely heart failure with preserved and reduced ejection fraction (HFpEF and HFrEF, respectively). A mid-range subgroup has also recently been introduced (28). Although HFpEF and HFrEF share the same clinical symptoms and signs such as congestion, shortness of breath and fatigue, they vary considerably in prevalence, incidence, pathophysiology and treatment. While the incidence of HFrEF shows encouraging signs of declining, HFpEF is increasing in

both incidence and prevalence (29, 31). In the GutHeart study, a part of the thesis, we only included patients with HFrEF.

The major risk factors of HFrEF in the western world are age, male sex, smoking, hypertension, myocardial infarction, valvular heart disease, obesity and diabetes. In developing countries, especially sub-Saharan Africa and Latin America, infective cardiomyopathies also contributes considerably (32). Both treatment and prevention of HFrEF are directed towards addressing these risk factors. In contrast to HFpEF, well-documented pharmacological treatment options proven to reduce morbidity and mortality exist (28). These treatment modalities reduce the workload of the heart, but also block some of the neurohormonal maladaptive processes in HF, such as chronic activation of the renin-angiotensin system, the adrenergic nervous system and the natriuretic peptide system. Although pharmacological treatment has improved the survival in HFrEF, mortality remains as poor as certain disseminated cancers (30).

Heart failure with reduced ejection fraction and inflammation

Subjects with HFrEF are characterized by increased circulating markers of systemic inflammation (33). The suggested role of innate immunity in heart failure started with the observation of acute heart failure as a feature of gram-negative septic shock (34). Around 1990, increased levels of TNF α was recognized in subjects with heart failure (4, 33). Later, other cytokines such as IL-1 β , IL-6 and IL-18 have been found increased in subjects with heart failure. Cardiac inflammation can be initiated from sources distal to the heart, but ultimately exerts its deleterious effects mainly through activation of pattern recognition receptors (PRR) such as the toll-like or/and nod-like receptors. These receptors can be activated by bacterial wall products such as lipopolysaccharide (LPS) and peptidoglycans, or intracellular content producing sterile inflammation (35). Inability

to resolve an on-going inflammation may lead to a low-grade chronic inflammatory state. TNF α , IL-1 β and IL-6 have all demonstrated negative inotropic effects, while sustained cytokine production has been shown to contribute to adverse remodeling (36). However, clinical trials targeting inflammation in HF have largely been disappointing. Some trials have shown an increase in LVEF, nonetheless, none have demonstrated any effects on CV mortality (37).

Gut microbiota and dysbiosis

The term gut microbiota denotes the collective community of microorganisms in the gut, including bacteria, viruses, fungi, archaea and protists. The present work has focused mainly on the bacterial community in fecal samples, which serves as a surrogate for the community in the large intestines.

The microbiota is slowly modified from birth by our surroundings, where diet probably plays a major role. From birth, the bacterial community is less diverse, consisting of bacteria specialized in utilizing milk mono and disaccharides. After introduction to solid food, the microbiota increases in diversity and reaches an adult-like composition by the age of three (38). The composition of an individual's microbiota is as diverse as a fingerprint. However, the ratio of the main phyla is more or less consistent. According to some studies, we share about 30 percent of phylotypes, while the rest differs substantially (39). Bacteroidetes and firmicutes are the main phyla in the gut. They are anaerobic gram-negative and gram-positive bacteria, respectively. They are the most abundant phyla, while others such as actinobacteria, verrucomicrobia and proteobacteria are also present (figure 3) (40).

Figure 3. **The structure of the human intestinal microbiota across the life cycle.**

Adapted from Kostic et al. (41) This figure was made available by Attribution-NonCommercial 4.0 International (CC BY-NC 4.0).

The gut microbiota serves as a barrier of growth for pathogenic bacteria. Furthermore, it contributes to vitamin production, immunomodulation, regulating colonic health, glucometabolism and muscle energy expenditure. It exerts its effect either directly or by production of microbial metabolites such as short chain fatty acid (SCFA) and secondary bile acids (42). These bind to different types of G-coupled, olfactory and farnesoid receptors within and distal to the gut, thus functioning as an endocrine organ (43).

A healthy gut is typically associated with high bacterial diversity. Conversely, the main characteristics of dysbiosis often includes low diversity (44). GI-related diseases such as inflammatory bowel disease (IBD) are associated with a large degree of gut dysbiosis. Studies typically show low microbial diversity, decrease in firmicutes and an increase in both bacteroidetes and proteobacteria. This leads to a decrease in firmicutes/bacteroidetes ratio (F/B), which is an enduring measure of dysbiosis in some disease state, but also a feature of aging (45, 46). However, also specific species such as *Faecalibacterium praunsnitzii* has been associated with favorable diagnosis while *Mycobacterium paratuberculosis* have been associated with adverse effects in IBD (47, 48). However, a causal relationship is yet to be established. In systemic diseases such as diabetes mellitus, obesity, hypertension and metabolic syndrome the results are even more diverse and

associative (49-52). A few seminal animal and human studies 10-15 years ago, demonstrated the transferability of metabolic features such as bodyweight and insulin sensitivity between subjects by use of fecal microbiota transfer (FMT) (53, 54). The role of gut microbiota was further emphasized by a subsequent study demonstrating that germ-free mice do not increase body weight upon a high fat, high sugar diet (55).

Dysbiosis in cardio-metabolic diseases are in some studies associated with an increase in the phyla firmicutes and a decrease in bacteroidetes, thereby increasing the F/B ratio, however these findings are not consistent (52). Further down the phylogenic tree, the results between studies are even more diverse. Nevertheless, some bacterial species such as *Akkermansia muciniphila* and *Faecalibacterium prausnitzii*, two known SCFA producers, have been shown to be inversely correlated to glucose intolerance, diabetes mellitus and body weight (43). Furthermore, *Enterobacter cloacae* B29, a gram-negative pathobiont, was demonstrated to be increased in the morbid obese, and was associated with hyperinsulinemia, hyperglycemia and hypertension (56). The prevalence of the bacteria decreased significantly during weight loss and improved the metabolic profile. *E.cloacae* produces LPS, a known activator of innate immunity (vide infra) that is hypothesized to contribute to the pro-inflammatory phenotype observed in obesity (56).

In CAD, an increase in firmicutes/bacteroidetes ratio have been observed (57). Others have demonstrated a reduction in bacteria important for SCFA synthesis as well as an increase in species within the phylum proteobacteria (58, 59). Furthermore, bacteria originating from the gut microbiota have been identified in atherosclerotic plaques, however their role are uncertain (60). In heart failure, the degree of dysbiosis has been associated with the degree of congestion, and thus the severity of the disease (61).

Although results vary, many of these studies report on a reduction in the *Ruminococcacea*

and *Lachnospiracea* families, both in the phylum firmicutes, and both known to be butyrate producers (62-64).

Innate immunity

Innate immunity or natural immunity ensures a swift, but unspecific defense against invading pathogens, but also clearance of damaged endogenous material and cells.

Importantly, the innate immune system does not have a memory, thus it will elicit the same response regardless of type and repetition of the insult.

A limited number of microbial and non-microbial structures stimulate the innate immune system. These are referred to as DAMPs or PAMPs (vide supra). Both DAMPs and PAMPs, bind to PRRs. In the field of microbiota-induced inflammation, the extracellular TLR and the cytosolic NOD-like receptors (NLR) are the most studied. Upon activation, both receptors cause an increased expression of inflammatory cytokines and adhesion molecules through NF- κ B and interferon regulatory factor (IRFs). There are many cells involved in the innate immune reaction, and many express TLRs and NLRs, however, leukocytes and monocytes/macrophage are believed to play the most important role in the ensuing inflammatory response (figure 4).

In the gut, the first line of defense is the gut epithelia and the inter-epithelial tight junctions, which regulate the passage of molecules between the gut and systemic circulation. The tight junctions are regulated by the gut microbiota, but also by its metabolites such as SCFA (66). The paneth and goblet cells in the epithelial layer produce antimicrobial peptides and mucus providing further protection to the gut epithelia. Furthermore, the cells of the gut epithelium, also express a variety of extra and intracellular PRR.

Figure 4. Major host immune cells, pattern recognition receptors and cellular functions involved in innate immune defence. Adapted from Cui Hua Liu et al. (65)

Upon leakage or translocation of bacteria or bacterial wall products from the gut lumen to systemic blood, an interaction with their specific TLRs ensures a pro-inflammatory response.

The LPS-TLR4 signaling is the most studied pathway. LPS seems to be unable to interact with TLR4 directly, but requires binding to several co-receptors. In the classical pathway involving cells which express membrane bound CD14 (mCD14), LPS first binds to LPS-binding protein (LBP). LBP shuttles LPS to mCD14 and to the complex TLR4/MD-2, which leads to dimerization of the complex. Downstream this interaction leads to increased transcription of pro-inflammatory cytokines (figure 5).

Cells and tissues that do not possess mCD14, rely on the soluble form of CD14 for their response to PAMPs.

Figure 5. Schematic view of the TLR4 signaling pathway. The figure is used with the permission of Diamond et al. and publisher: Dove Medical Press Limited. (67)

Markers of gut leakage/gut related inflammation

Lipopolysaccharide (LPS)

LPS is a surface molecule located on the outer membrane of all gram-negative bacteria. It consists of a hydrophobic lipid A region attached to a core oligosaccharide and an O antigen (Figure 6). It is a highly potent activator of the innate immune response through binding to LBP, membrane-bound or soluble CD14 and subsequently TLR4/MD-2 complex (vide supra) (68).

Figure 6. Structure and immunogenicity of lipopolysaccharide from Gram-negative bacteria. Lin et al. (69). This figure was made available by Attribution-NonCommercial 4.0 International (CC BY-NC 4.0).

High levels of circulating LPS is observed in a gram-negative septicemia, usually released upon lysis of bacteria. However, circulating levels of LPS is also detectable in presumable healthy subjects, devoid of a gram-negative bacteremia (70). It is reasonable to expect that the source of free circulating LPS is likely to be derived from the gastrointestinal tract due to the high density of gram-negative bacteria in the intestines. The amount of circulating LPS is believed to depend on the amount of LPS in the intestinal lumen and the integrity of the intestinal wall barrier. However, increased levels of LPS postprandially have also been observed without evidence of a dysfunctional barrier (71). Thus, two main mechanisms of LPS leakage have been proposed: i) paracellular leakage due to dysfunctional tight junctions in the large intestines ii) intracellular active uptake with dietary fat in the small intestines and transport with chylomicrons extracellularly (figure 7) (72, 73).

Figure 7. Modes of LPS transport across the intestinal barrier. Reused with permission from Stephanie Thomas, PhD. (74)

Not all LPS-molecules have agonistic effects on TLR4. The lipid A region determines the potency of LPS. In detail, the number and phosphorylation of the attached acyl groups seem to affect the immune response. The classical pathogenic bacteria from the phylum proteobacteria have six acyl groups attach to a disaccharide backbone (figure 6), while commensals from the phylum bacteroidetes usually have five or less (75). Studies indicate that LPS from bacteroidetes, which accounts for 80-90% of the total microbiota LPS in healthy adults, are immunosilent or even immunoinhibitory, while the immunopotent LPS from proteobacteria, in particular from the family enterobacteriaceae, accounts for 5-15% (76).

Experimental studies over a decade ago demonstrated that chronic exposure to LPS causes weight gain, hyperglycemia and hyperinsulinemia in rodents (27). In humans, high levels have been shown associated with prevalent metabolic syndrome, diabetes mellitus (77, 78), triglycerides and inversely with HDL-cholesterol.

The role of LPS in the process of atherosclerosis is still not fully elucidated. E-coli LPS has been found in human atherosclerotic plaques co-localized with antibodies to LPS and TLR4, indicating an active role (79). Leakage of LPS through intestinal mucosal injury

has been shown to correlate significantly with post-infarction systemic inflammation after ST elevation myocardial infarction, and furthermore to predict adverse cardiovascular events (80). In HF, LPS has been shown to be increased in patients with signs of edema, compared to patients without, and decreased upon diuretic treatment (70).

Cluster of differentiation 14 (CD14)

Cluster of differentiation 14 (CD14) is a 55kDa large protein, existing in a membrane-bound form with a glycosylphosphatidylinositol (GPI) anchor (mCD14) and in a soluble form (sCD14). mCD14 is expressed on a variety of immune cells, predominantly by monocytes, macrophages and to a lesser degree on neutrophils (81). Moreover, it is also expressed in other cells such as in skin and intestinal epithelium (82). mCD14 was originally described as a PRR due to its recognition and binding of LPS (83). It may however also bind to other bacterial wall products such as lipoteichoic acid and peptidoglycans of gram-positive bacteria. Even endogenous proteins such as heat shock protein 60 have been described to activate mononuclear immune cells through CD14-binding (84).

sCD14 originate mainly from a protease-dependent cleavage of the membrane-bound form upon monocyte/macrophage activation, but can also be secreted within vesicles (85). Furthermore, there is substantial evidence that hepatic production of sCD14 also contributes to the circulating levels, and both IL-6 and TNF α have been shown to induce hepatic production of sCD14, thus it can also be characterized as an acute phase protein (82).

Despite a high affinity for LPS, at low concentrations, sCD14 requires the interaction of LPS-binding protein (LBP) (*vide infra*) to accelerate binding (86). After binding to the dimeric LPS-LBP complex, sCD14 might transfer LPS either to mCD14 or directly to the

TLR4/ Lymphocyte antigen 96 complex (TLR4/MD-2) (87). The latter is particularly important in cells that do not express mCD14, such as vascular smooth muscles or endothelial cells, thus may play an important role in the process of atherosclerosis (88). Increased levels of sCD14 have in some studies been shown to independently predict future cardiovascular risk and all-cause mortality, especially in subjects with chronic kidney failure, human immunodeficiency virus (HIV) and liver disease (89-91). However, in the general population, the results are somewhat conflicting. One study showed that sCD14 could predict coronary artery stenosis >50 % in patients eligible to undergo coronary CT and increased levels have been reported in patients with stable and unstable angina pectoris compared to healthy controls and in chronic heart failure patients (90, 92-94). However, other studies have shown no association with coronary artery disease (CAD) (95).

In experimental studies, sCD14 have been shown to decrease insulin action in response to a high fat diet in mice, and knockout of CD14 have been shown to improve their gluco-metabolic profile and decrease the mesenteric adipose tissue inflammatory gene expression (96, 97).

Lipopolysaccharide binding protein (LBP)

Lipopolysaccharide binding protein (LBP) is a 60kDa serum glycoprotein with high affinity for LPS (98). LBP is mainly produced by hepatocytes, but also extrahepatic tissues such as the lung, the intestine and the gingiva (99, 100). It is synthesized upon stimulation by LPS, but also cytokines such as IL-1, IL-6 and TNF α , thus it is also characterized as an acute phase protein.

Small amount of LBP is detectable in physiological conditions, but increases upon stimulation (101). The protein is highly dynamic. It binds free LPS or aggregates of LPS.

It also acts as an opsonin by binding LPS attached to the surface of bacteria (102). The presence of LBP greatly enhances the sensitivity and the binding capacity of both mCD14 and sCD14 to microscopic amounts of LPS. Thus, at low LPS concentrations, LBP is regarded to be essential for immune cell activation (103). Its main function is to recognize and bind LPS. LPS is then transferred and presented to CD14 after catalyzing LPS micelles into recognizable monomers (104). LBP has also been suggested to transfer LPS to intracellular PRRs independent of CD14 (105).

LBP may also attenuate the immunostimulatory effects of LPS by regulating its binding to CD14. LBP can bind and internalize LPS aggregates through mCD14 and it can also dislodge LPS that have already been bound to mCD14, and transfer LPS to lipoproteins. The latter is believed to be protective against LPS-induced inflammation (106) in a concentration-dependent manner (107, 108).

In the physiological state, leakage of microbiota components is believed to contribute to the circulating levels of LPS and consequently to LBP, and levels of LBP are therefore discussed in association with gut leakage. Circulating levels of LBP have also been shown to correlate to the degree of LPS biosynthesis in the microbiota, and furthermore, the presence of butyrate producers in the microbiota seems to associate with low levels of LBP (109).

In CVD, high levels of LBP have been associated with subclinical and clinical atherosclerosis, and with increased risk of cardiovascular mortality and all-cause mortality after an ischemic stroke (110-113). Very limited data is available on the role of LBP in HF and most of the current knowledge is extrapolated from studies on LPS in HF (70, 114).

Intestinal fatty acid binding protein (I-FABP)

Intestinal fatty acid binding protein is a cytoplasmic protein located in the villi of intestinal epithelial cells. It is most prevalent in the small intestines. Its function varies, including transport of long chained fatty acids into the endoplasmic reticulum and intracellular buffering (115). After transport of fatty acids to the endoplasmic reticulum, triglycerides and ultimately chylomicrons are formed, which can transfer dietary fatty acid into systemic blood (116).

Upon intestinal mucosal injury or intestinal diseases, I-FABP leaks extracellularly and is measurable in blood indicating intestinal epithelial cell damage (117). Thus, it has also been suggested as a marker of gut leakage (118), although there are some discrepancies. In studies where participants are expected to have a large extent of epithelial damage, I-FABP correlates well with the degree of endotoxemia (119, 120), whereas in other diseases associated with gut leakage, but no evident damage to the intestinal epithelium, I-FABP seems not to be a suitable marker of gut leakage (121, 122). Nevertheless, I-FABP has been reported increased in diabetics and in obesity, but the cause of the increase is not clear (122, 123).

Zonulin

Zonulin is the only known physiological regulator of intercellular tight junctions. Enteric infection of the small intestine have been shown to increase expression of Zonulin and subsequently intestinal permeability (124). Zonulin has been associated with body mass index, fasting insulin, triglycerides and IL-6 (125). However, this has not been studied in the present thesis.

Microbial metabolites

There are >2000 microbial metabolites known today, several of which are biologically active and have an impact on our health as well as disease (126). The two metabolites which have gained the most attention in the field of CVD are SCFAs and trimethylamine-N-oxide (TMAO). Both metabolites were studied in the GutHeart trial, as part of the thesis.

SCFA – Butyrate

Short chained fatty acids (SCFA) are byproducts after bacterial fermentation. The three main SCFA are acetate, propionate and butyrate. The main source of substrate for SCFA production is dietary fibers. Several bacteria are capable of acetate production, however only a limited number of bacteria can produce propionate and butyrate. Butyrate is produced through two distinct microbial pathways, through the phosphotransbutyrylase and butyrate kinase, and the butyrate CoA-transferase (butyryl-CoA/acetate CoA-transferase) pathways, the latter being the most important. Most butyrate producers belong to the firmicutes phylum, more specifically the order of clostridia, however the order of bifidobacteriales from the phylum actinobacteria, also contributes (127).

There are many proposed effects of SCFA. They act as the main energy substrate for colonocytes and regulate the assembly of tight junction proteins, thereby maintaining the gut wall barrier integrity. They also inhibit pathogen proliferation by lowering pH (66, 128).

SCFAs bind to several receptors of which the most abundant are the free fatty acids receptors (FFAR). In the colon, they may trigger glucagon-like peptide (GLP) 1, associated with increased glucose uptake. However, FFARs are also present in

leukocytes, pancreatic β -cells, adipocytes and even neurons, thus affecting gluco-metabolism and innate immunity (129). SCFAs also bind to other G-coupled receptors exerting potential effects on the cardiovascular system (130).

Bacteria capable of SCFA production, especially butyrate, have gained much attention both in cardio-metabolic and CV diseases. A reduction in butyrate producing bacteria has been associated with diabetes mellitus and CVD (58, 62, 131). Furthermore, butyrate and propionate have been shown to protect against diet-induced obesity (132).

TMAO

Upon dietary intake of choline, phosphatidylcholine, and l-carnitine in meats, dairy products, eggs and some fish, trimethylamine (TMA) is cleaved by microbial TMA lyase (133). TMA is then oxidized by the hepatic flavin monooxygenase 3 (FMO3) resulting in trimethylamine N-oxide (TMAO) (134). The production of TMAO is dependent on a functional microbiota, thus germ-free mice essentially have no TMAO, and vegans and vegetarians have lower levels. (135). TMAO has been demonstrated to increase the formation of foam cells by increased expression of macrophage scavenger receptors and to adversely affect lipid metabolism (133). It also has prothrombotic effects through increased platelet responsiveness to ADP and thrombin and increased platelet adhesion through increased Ca^{2+} release from platelets (136).

TMAO have in experimental HF studies been associated with adverse ventricular remodeling, dilation, wall thinning and increased fibrosis (137).

Increased levels of TMAO has been demonstrated to predict incident CVD in atherosclerotic disease independent of traditional risk factors (138). Increased levels have also been associated with poor prognosis both in acute and chronic heart failure (139, 140).

Although studies indicate that reduced TMA precursor intake may reduce circulating TMAO, the effect of TMAO interventions on CV outcome remains unclear.

Treatment modalities

Treatment of CVD can be divided into primary and secondary prevention. In primary prevention, that is, before evidence of clinical disease, the degree and intensity of treatment are tailored after a person's individual risk. The risk of CV death can be estimated using widely available risk scores such as SCORE and NORRISK, however the use of such risk scores are recommended for individuals that already are at increased risk of CV disease. (141). From a population point-of-view, several life-style changes and modifications are in general cost-effective, and save lives. Most life-style changes work by reducing risk factors of CVD.

With the evidence of high blood glucose, high cholesterol and/or high blood pressure, reducing the levels is vital for CVD prevention. In some patients, life-style changes may be sufficient, however, concomitant pharmacological treatment is needed in many cases.

Diet

According to the ESC 2016 guidelines on CVD prevention, a healthy diet should be the cornerstone of CVD prevention (2). A reduction of saturated and trans unsaturated fatty acids, reduction of salt, alcohol and sugar-sweetened soft drinks and an increased intake of fibers, fruits, nuts, legumes and fish are all recommended. The reduction in CV-risk is believed to partially be mediated through a reduction in risk factors such as hypertension, obesity, metabolic syndrome and diabetes (2).

The Mediterranean diet as a concept, offers the best studied diet that confers a reduced risk of CVD (142). The Lyon Diet Heart Study successfully demonstrated a 50-70%

lower risk of CVD with Mediterranean diet in secondary prevention compared to control after 46 months (143). Furthermore, these changes were independent of traditional risk factors such as cholesterol and blood pressure. In primary prevention, the PREDIMED study demonstrated a 30% risk reduction in major CV events with Mediterranean Diet supplemented with nuts or olive oil compared to a low-fat control diet, however, it has recently been retracted due to inconsistencies in the randomization process (144).

The role of dietary fibers in CV-risk and health is not completely elucidated. In a systematic analysis from Threapleton et al from 2013, an increase in dietary fibers of 7 g/day, yielded a 9 percent risk reduction in CVD (145). High fiber diets may reduce LDL-c and increase HDL-c (146). However, which type of fibers and the mechanisms behind are not clear. Nevertheless, an increased intake is encouraged due to low cost and potential benefits beyond the CV system.

Omega-3 fatty acids

When considering the main macronutrients in foods and their association to CV-risk, disease and health, substituting saturated fat with polyunsaturated fatty acids (PUFAs) consistently lowers triglycerides and LDL-C, and reduces CV-risk (147).

Monounsaturated fatty acids have demonstrated beneficial effects on lipid profiles, however not on CV-risk. The marine n-3 PUFA, i.e. eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have significant effects in lowering triglycerides, without affecting LDL-C or HDL-C to any significant degree. Several studies have shown benefits of marine n-3 PUFAs on CV-risk, however it is still debated (148, 149). Also intake of fish at least once a week, has been associated with reduction in coronary artery disease (150).

The mechanisms behind a potential reduction of CVD by marine n-3 PUFA are unclear. There are many pathways and systems that have been suggested to be impacted by n-3 PUFA (151). There is evidence that n-3 PUFA supplementation reduces heart rate and blood pressure, and has favorable effects on vascular endothelium (152, 153). It has been reported to enhance nitric oxide production in experimental studies and to reduce markers of endothelial activation (154, 155). Importantly, it has also been suggested to have anti-thrombotic effects as well as anti-inflammatory effects, the latter potentially related to resolution of inflammation by resolvins (151). It has also been suggested that n-3 PUFA may impact CVD by reducing gut leakage and thus contributing to systemic anti-inflammatory effects (156).

Saccharomyces boulardii

S.boulardii CNCM I-745 is a probiotic yeast which do not occur naturally in the normal human microbiota. In clinical studies, it has been demonstrated effective in reducing symptoms and restoration of the normal microbiota in subjects with gastrointestinal (GI) diseases associated with diarrhea (157). Furthermore, it has been demonstrated to have a preventive effect against potential dysbiosis (158). Thus, in clinical medicine, it is used as prophylaxis against antibiotic-associated diarrhea and other conditions associated with diarrhea.

There are several purposed modes of action. Cell wall components of the yeast such as β -glucans, serves as substrates for the microbial short chained fatty acid (SCFA) – fermenters. Thus, administration of *S.boulardii* is associated with an increase in fecal butyrate, a SCFA vital for colonic health (vide supra) (159). *S.boulardii* can synthesize polyamines, an organic compound involved in enterocytes proliferation and differentiation, and they may also bind pathogenic bacteria, thus inhibiting their growth

(160, 161). It can also neutralize bacterial toxins such as *Clostridium Difficile* but also lipopolysaccharide from *E-coli* (162, 163). Excitingly, *S. boulardii* have been suggested to interfere with NF- κ B signaling pathways, that may translate to immune-inhibitory properties (164). *S. boulardii* seems however, to have little or no effect on specific taxa or total microbiota composition in presumable healthy individuals without symptomatic GI disease (158).

Rifaximin

Rifaximin is broad-spectrum antibiotic with bactericidal and bacteriostatic effects. Due to a systemic absorption of merely 0.4 %, it is considered a locally acting antibiotic. It has been shown to have effects against gram-positive and negative aerobic and anaerobic bacteria. Rifaximin has demonstrated its effectiveness as treatment for infective GI diseases as well as treatment of hepatic encephalopathy (165). In non-infective GI diseases such as inflammatory bowel disease and irritable bowel syndrome, the results are highly diverse (166, 167). Besides a direct antimicrobial effect, it has also been shown to inhibit NF- κ B, thus exerting anti-inflammatory effects (168).

The antibiotic has also been suggested to have a eubiotic effect on the microbiota. Most studies demonstrate beneficial effects of specific taxa, such an increase in the beneficial taxa ruminococcaceae, lactobacillaceae and bifidobacteriaceae and a decrease of the pathogenic enterobacteraceae (169). Although a decrease in the bacterial load is often seen, most studies show no change in the overall composition of the microbiota (169).

Hypothesis of the thesis

We hypothesize that leakage of bacteria from the gut is an important mechanism in patients with cardio-metabolic diseases, especially metabolic syndrome. We believe that gut leakage contributes to the different component of the syndrome and drives the increased cardiovascular risk through chronic low-grade systemic inflammation.

In heart failure, we hypothesize that the microbiota through gut leakage or microbial metabolite-signaling, leads to adverse effects on the heart and contributes to the poor prognosis in chronic heart failure. Importantly, we also believe these effects are preventable by modifying the gut microbiota.

Aims of the thesis

Overall aims

The overall aim of the present work was to explore and elucidate the role of the gut microbiota in cardio-metabolic diseases, symptomatic atherosclerotic disease and in heart failure. We focused on the role of the gut leakage, circulating microbiota biomarkers, systemic inflammation and their relations to different intervention strategies.

Specific aims

- i) To explore any difference in markers of gut leakage in subjects with and without metabolic syndrome, and whether such markers associated with systemic inflammation (Paper I)
- ii) To investigate the effect of n-3 PUFA and/or diet intervention on markers of gut leakage in a high CV-risk population (Paper II)

- iii) To explore any association between leakage markers and future cardiovascular events in the same CV-risk population (Paper II).
- iv) To explore whether potential modulation of the gut microbiota with antibiotics or probiotics in patients with heart failure would improve cardiac function and circulating microbiota biomarkers (Paper III).
- v) To explore whether intervention with antibiotics or probiotics in patients with heart failure would impact gut leakage markers and systemic inflammation (Paper IV)
- vi) Moreover, to explore any associations between gut leakage markers, microbiota alpha diversity, microbial biomarkers and biomarkers of systemic inflammation (Paper IV)

Methods

Study population & Design

My doctoral thesis is based on two different study populations namely the DOIT population and the GutHeart population. Both will be presented separately below.

The DOIT study (Papers I & II)

The DOIT (Diet and omega-3 fatty acid intervention trial) population are survivors of the Oslo study which was a randomized controlled trial designed to explore the effect of diet intervention on coronary heart disease (170). In total, 1232 men in the age span 40-49 years with high risk of coronary heart disease were included. The Oslo study was conducted between 1972-77 and the main results were published in 1981.

The survivors were invited to a follow-up study in 1997. Of the 910 survivors, 563 men aged between 65-75 years were willing to attend the DOIT study (155). The intervention principles are described below. All participants were followed for three years for intervention effects and for CV endpoints registration.

The first two papers in the thesis utilized the DOIT population. Due to lack of samples from some patients, we analyzed blood from 482 of the original 563 participants.

Intervention

The participants were randomized to intervention with n-3 PUFA supplementation or placebo, or diet counselling vs no diet counselling. They were randomized in a 2 x 2 factorial design. One group with both n-3 PUFA supplementation and diet intervention, one group with n-3 PUFA supplementation only, one group with placebo capsules and diet intervention, and the last group with placebo capsules and no diet intervention.

Participants randomized to n3-PUFA supplementation received a total of 2.4g of marine n-3 PUFA (35% EPA and 20 % DHA). The placebo capsules consisted of corn oil.

The patients who were assigned to dietary intervention received advice on a healthy diet consisting of increased intake of fat from plant sources, vegetables, fruit and fish. They were advised to decrease intake of meat and fat from animal sources. Overweight individuals were encouraged to lose weight. They received diet counseling for 30 min at baseline and after three months. They were later followed with phone calls every six months by a nutritionist.

Laboratory analyses

Blood samples were collected in fasting state between 08:00 and 10:00 am. Routine analyses were performed by conventional methods. Serum and plasma samples were prepared as described in the papers, and kept frozen in a biobank at -80°C until analyses. Commercially available enzyme-linked immunosorbent assays were used to determine LBP and sCD14, as detailed described in the papers. We decided not to measure LPS in the DOIT-population due to contamination concerns as the blood samples were close to 20 years old.

The GutHeart Study (Papers III & IV)

Screening patients for the GutHeart (Targeting Gut microbiota to treat Heart failure) trial was started in 2016. Patients were enrolled from outpatient clinics at Oslo University Hospital Rikshospitalet (Oslo, Norway), Oslo University Hospital Ullevål (Oslo, Norway), Nordlandssykehuset (Bodø, Norway), and Instituto Nacional de Cardiologia (Rio de Janeiro, Brazil).

Patients with heart failure with left ventricular ejection fraction (LVEF) < 40% at the time of randomization were included. Importantly, they had to have an acceptable acoustic window on echocardiography for assessment of LVEF. Furthermore, only symptomatic patients in New York Heart Association Functional class II and III were included. The patients also had to be on optimal guideline recommended therapy for HF at least three months prior to randomization. As of 2016, optimal pharmacological treatment included maximal up-titrated doses of beta blockers and angiotensin converting enzyme inhibitors or angiotensin receptor blocker. Mineralocorticoid receptor antagonists were added if patients remain symptomatic. Angiotensin receptor neprilysin inhibitor (ARNI) was recently introduced for patients with LVEF below 35% in despite of the abovementioned treatments (28). In the GutHeart population, 14 % used ARNI.

We excluded patients on probiotics or antibiotics during the past three months, patients that received cardiac resynchronization therapy during the past six months, patients with acute coronary syndrome within 12 weeks, patients with gastrointestinal tract diseases and patients with other significant co-morbidities including polypharmacy.

Intervention

Patients were randomized to three months' intervention with the non-absorbable antibiotic rifaximin 550 mg tablets, one tablet twice a day or the probiotic yeast *S.boulardii* 250 mg capsules, 2 capsules twice a day, both on top of optimal medical treatment, or optimal medical treatment only.

Echocardiography

All study participants underwent an echocardiography at baseline before randomization and at the end of the intervention after three months. Exams were performed after a

standardized protocol in concordance with the European society of echocardiography and performed at each individual study center (171). All images were analyzed offline at Oslo University hospital. We used the modified Simpson's method for calculation of LVEF.

Laboratory analyses

Blood sampling

All samples were drawn in a fasting condition at baseline, at one month for safety purposes and at the end of study (three months). We allowed intake of small amounts of water prior to sampling. Routine analyses were measured by conventional methods. Serum and plasma samples were prepared as described in the papers and kept frozen at -80°C until analyzed. EDTA plasma was used to measure LPS, assessed by the Kinetic Chromogenic LAL Assay, Lonza BioScience, Basel, Switzerland. We used commercially available ELISAs to measure LBP, I-FABP, sCD14, CRP, IL-6 and IL-10, as detailed described in the papers. For NT-proBNP electrochemiluminisence immunoassay (ECLIA) was used. TMAO was determined in serum by stable isotope dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS). More details are given in the manuscripts.

Stool samples

Samples were collected before start of intervention and after three months. Patients received a home-sampling kit for stool collection. Careful instructions were given. Collection was performed in tubes containing a deoxyribonucleic acid (DNA) stabilizing solution (PSP Spin Stool DNA kit, Stratec Molecular GMBH, Berlin, Germany). Time for sampling was registered by the patients. The samples were either delivered personally or sent by postal mail.

To define microbiota diversity we utilized the V3–V4 region of the 16S ribosomal ribonucleic acid (rRNA) gene for metagenomic sequencing. Libraries were sequenced on the Illumina MiSeq platform (San Diego, California, USA) and at Norwegian Sequencing Centre (Oslo, Norway). The butyrate producing capacity of the microbiota was determined by predicting the abundance of the gene encoding the rate-limiting enzyme Butyrate-acetoacetate CoA transferase. PICRUST2 was used (172).

Statistical analysis

We used non-parametric and parametric statistics in the papers, however, most analyzed variables were skewed. For simple correlations, bivariate Spearman's correlation or Pearson were used as appropriate. To explore between group differences, we used Mann-Whitney U-test, Kruskal-Wallis test or Pearson chi-square. Mantel-Haenszel test was used for trend analysis across quartiles.

In paper I, odds ratio by logistic regression was used to demine risk. In paper II we used a cox regression model to analyze the risk of experiencing an endpoint. Conventional CV risk factors were used as covariates, but also confounders that associated to both the CV outcome and the marker in question with a p-value less than 0.2. We also used area under the curve (AUC) to determine the discriminative ability of our suggested biomarkers.

The GutHeart study was powered to detect a 5-percentage point increase in LVEF in either intervention group compared with the control arm with a power of 80% and α of 5%. The 5-percentage point increase was based on a pilot study from Costanza et.al, exploring the effect of *S. boulardii* on LVEF (173). With a presumed standard deviation of LVEF of 7.5 percentage point, 37 patients would be needed in each group.

For the interventional part of paper II, III and IV, paired sample t-tests or Wilcoxon signed-rank tests were used to explore within-group treatment effects as appropriate. One-

way ANCOVA was used to assess treatment effect between groups. Baseline values were used as the covariate in these analyzes. Variables that did not conform to the assumptions of the test were logarithmic transformed. IBM SPSS statistics version 24.0 and 25.0 were used for all statistical analyses.

Summary of results

Paper I

Markers of metabolic endotoxemia as related to metabolic syndrome in an elderly male population at high cardiovascular risk: a cross-sectional study

Markers of gut leakage (LBP and sCD14) were not significantly different in the group with metabolic syndrome (n= 182) compared to the group without (n=300). We did however find that the higher the quartiles of LBP, the higher the risk of having MetS (p for trend = 0.05). Quartile 4 had a close to two-fold increased risk of having MetS compared to quartile 1. This was not seen for sCD14 (Figure 1, paper I).

Figure1. Paper I. Quartiles of LBP (A) and sCD14 (B) levels as related to the prevalence of metabolic syndrome in the total population.

Paper II

Effects of dietary intervention and n-3 PUFA supplementation on markers of gut-related inflammation and their association with cardiovascular events in a high-risk population

LBP levels increased significantly from baseline to 36 months in all groups, whereas sCD14 did not change significantly. There were however, no significant between-group difference in change for the n-3 PUFA group or the diet intervention group compared to their respective control (Figure 1. Paper II).

Figure 1. Paper II. Plasma levels of both markers at baseline and after 36-month intervention according to the factorial design. (A and B) for LBP and (C and D) for sCD14.

A total of 53 patients experienced a CV endpoint after three years. When dichotomizing LBP levels at median, subjects with the highest LBP had a two-fold increased risk of experiencing an endpoint. This association remained significant even after correcting for

conventional risk factors, real confounders and CRP in a multivariate cox regression model (HR 2.00, 95% CI 1.11-3.58; $p=0.02$). For sCD14, there was a tendency towards an increased risk when having levels above median ($p= 0.06$) (Figure 2. Paper II).

Figure 2. Paper II. Kaplan-Meier curves for event free CV survival, comparing levels of LBP (A) and sCD14 (B) above and below median values.

Paper III

Microbiota-directed therapy with rifaximin or *Saccharomyces boulardii* in heart failure with reduced ejection fraction: Results from the randomized GutHeart trial

We could not demonstrate any significant effect of rifaximin or *S Boulardii* intervention on LVEF. For rifaximin vs control, LVEF increased 1.2 percentage points (95% CI -0.7 - 3.2, $P=0.22$) and for *S boulardii* vs control, 0.2 percentage points increase (95% CI -1.9 - 2.2, $P=0.87$). Within groups, we observed an increase in LVEF for rifaximin ($p<0.05$) (Figure 3b, Paper III).

Figure 3b. Paper III. Within-group changes in LVEF. Left panel: Change in LVEF from baseline to three months for all participants. Right panel: The bars indicate mean levels at baseline and three months for all groups.

Moreover, there were no significant between-group difference for Shannon Index, butyrate-producing capacity, TMAO, CRP or NT-proBNP (Figures 4a and 4b, paper III).

Figure 4a. Paper III. Baseline-adjusted mean microbiota diversity and butyrate-producing capacity

Figure 4b. Paper III. Baseline-adjusted mean circulating biomarkers.

Paper IV

Effect of Probiotics and Antibiotics on markers of Gut Leakage in Heart Failure Patients with Reduced Ejection Fraction - Results from the randomized controlled GutHeart trial

We found no between-group difference in levels of LBP, I-FABP, LPS or sCD14 for *S. boulardii* or rifaximin compared to control (Figure 1, Paper IV). Furthermore, there were no within-group changes for any of the groups. No interventional effect was also not observed for IL-6, IL-10 or CRP (all $p > 0.05$).

Figure 1. Paper IV. Within-group and baseline-adjusted levels of sCD14, LPS, LBP and I-FABP for intervention versus control.

Baseline-levels of LBP and I-FABP were significantly correlated to NT-proBNP ($\rho=0.2$, $p=0.03$ and $\rho=0.3$, $p<0.01$, respectively). High levels of LBP and I-FABP were associated with an increased likelihood of having NT-ProBNP above median. ROC curve analyses revealed an AUC of 0.70, outperforming either variable individually (AUC LBP 0.63 and 0.64 for IFABP) in predicting high vs low NT-proBNP levels.

I-FABP was associated with Shannon Index ($\rho=-0.24$, $p=0.016$), TMAO ($\rho=0.39$, $p<0.001$) and the butyrate-producing capacity of the microbiota ($\rho=0.20$, $p=0.04$). sCD14 were associated with TMAO ($\rho=0.25$, $p=0.003$).

LBP was associated with IL-6, IL-10 and CRP ($\rho=0.25-0.40$, $p<0.01$ for all). sCD14 correlated to IL6 and CRP ($\rho=0.27$, 0.31 , $p<0.01$ for all). IFABP correlated to IL-6 ($\rho=0.20$, $p=0.02$). LPS did not correlate to any of the measured inflammatory markers.

Ethical considerations

In papers I and II, all initial approvals from regulatory governments were present, and all data were anonymized according to the plan (2012), before the present investigations were performed. The interventions were regarded as safe and without discomfort for the participants.

In paper III and IV, all patients were thoroughly informed in writing, but also orally before they gave their written consent. The study investigator had no prior role as a physician for the patients enrolled. All patients were monitored closely, and all were equipped with a telephone number they could contact the investigator in the case of side effects. This was done due to the limited experience with long-term treatment with *S.boulardii* and rifaximin. All adverse events were registered consequently, and an external safety committee evaluated all serious adverse events. Furthermore, we cooperated with the patients' primary physicians as well as hospitals in case of any form of disease or heart failure worsening.

Both studies were performed in compliance with the Helsinki Declaration. It was approved by the Regional Ethics Committee (REK) and registered at ClinicalTrials.gov NCT00764010 (Papers I and II) and NCT02637167 (Papers III and IV). All subjects gave their written informed consent.

Discussion

Methodological considerations

Study population & design

Paper I and II were based on the DOIT population, which again was based on the survivors from the Oslo study from 1972. As a result, our study displays a number of limitations that impacts the generalizability of our results. The most obvious limitation is that all the study participants were at relatively high age (65-75 years) and all of male sex. There are unquestionable biological differences between males and females. The manifestations of cardio-metabolic diseases are different between sexes as are CV risk (174). Furthermore, females seems to have higher TLR expression, possibly through the impact of sex hormones, higher firmicutes/bacteroidetes ratio and higher proportions of the phylum proteobacteria (174). Thus, we cannot exclude that our result would be different in a more sex-balanced population.

These study participants were actually survivors despite a high-risk CV profile over a period of 30 years. About one fourth of the population had died within these years. Of the survivors, only two thirds joined the DOIT-study, and we can therefore not dismiss the possibility that the healthiest of the survivors were included. Of the 563 included, we had sufficient blood samples from 482 participants. Thus, we subjected both papers to a substantial selection bias. We know that treatment of cardio-metabolic and CV diseases have changed significantly during the past 20 years. We also know that different pharmaceuticals affects microbiota composition (175), which may influence the gut-leakage markers. Thus, a study population on more contemporary CV drugs might have a different outcome.

The sample size in the GutHeart study was based on a pilot study by Costanza et al (173) in which 20 HF patients were randomized to three months' treatment with *S.boulardii* or placebo. The *S.boulardii* arm experienced a 6.6 percentage point increase in LVEF compared to the placebo arm. Based on these numbers we hypothesized a 5 percentage point increase with a standard deviation of 7.5% based on an expected inter-observer variability of $\pm 7\%$ as previously reported (176). Thus, we calculated that we needed 37 patients in each arm. When calculating sample size from a small study with only 10 individuals in each arm, it is easy to overemphasize the effect size and consequently the sample size. Possibly, a larger study would give different results, however that is unlikely given the consistent neutral effects across all endpoints in the study.

Only patients with HFrEF were included, thus our results cannot be extrapolated to include the growing amount of patients having HFpEF. Furthermore, we only included patients in NYHA class II and III, and not class IV. This will be discussed thoroughly later in the discussion section.

Both the DOIT study and GutHeart study were randomized controlled trials, which is a strength of the studies. The interventions in the DOIT study were marine n-3 PUFA which was placebo-controlled and diet intervention. Importantly, corn oil was used as placebo, which is thought to be inert. The recent landmark study REDUCE-IT demonstrated a beneficial effect of EPA on CV endpoints (177). However, the use of mineral oil as a placebo has received much attention. The participants in the placebo-group, increased their CRP and had more gastrointestinal symptoms such as diarrhea, compared to the EPA group. From a microbiota point-of-view, a compound that increases gastrointestinal transit time cannot be considered inert. Furthermore, a major strength of the DOIT study was the measurement of the serum fatty acids profile as a measure of compliance.

The GutHeart study was designed as an open label study with a control group, but without placebo for the active treatments due to problems with creating a double dummy for two interventional arms. However, despite of the open label design, the laboratory assessments were performed in a blinded fashion.

Outcome assessment

Echocardiography

In the GutHeart study we used 2D-echocardiography (2DE) to measure the primary endpoint, LVEF. LVEF is used as a surrogate marker for left ventricular systolic function and in clinical HF trials it confers prognostic information (178). According to the recommendations from the European Association of Echocardiography, 2DE is considered acceptable as proof-of-concept of treatment in phase II trials as the GutHeart trial essentially is, but is controversial as measure of primary endpoint in phase III studies (171).

The 2DE exams were performed at each individual study center, but as an attempt to reduce the inter-observer variability, the endpoint measurements were mostly done at Oslo University Echocardiography Core Lab. We also included a good image quality as an inclusion criterion in the study.

2DE is a feasible, cheap and fast way to assess LVEF. In the GutHeart population, more than half of the patients had some type of implantable cardiac device. The use of cardiac magnetic resonance imaging (CMR), considered to be gold standard in LVEF determination, would have complicated the conduction of the study. Thus, we cannot rule out that more subtle changes in LVEF could have been detected by usage of CMR.

Lastly, more patients could have been eligible for the study with imaging techniques that could enhance the endocardial border making it easier to trace, such as 3D-echocardiography and usage of intravenous contrast.

Microbiota analysis

In the GutHeart study, we examined the fecal samples by using 16S ribosomal RNA amplicon sequencing. Which region to sequence is widely discussed, however we utilized the V3-V4 region (179). The method is not free from potentials errors; especially errors in sample preparation and sequencing are common. To minimize errors, all samples were prepared and analyzed at the Oslo University Hospital, Rikshospitalet by experienced bioengineers.

Metagenomic shotgun sequencing allows for better species resolution than 16s amplicon sequencing (180), however, for comparisons of differences in paired samples for such as alpha diversity, 16s is arguably the most cost-effective method.

We only analyzed microbial richness and diversity represented by Shannon Diversity Index.-Thus, we cannot refute that our interventions might have had significant effects on some specific taxa of the microbiota. However, no such change could be translated into increased LVEF, changes in gut microbiota metabolites, potential butyrate producing capacity or gut leakage markers, which was our objective.

General discussion

Gut leakage as a marker of cardiovascular risk factors

Prior studies have demonstrated that chronic stimulation with LPS contributes to hyperglycemia, hypertriglyceridemia and obesity (27). Furthermore, antibiotic treatment could attenuate the effect of high fat diets on circulating LPS, intestinal permeability,

adipose tissue, glucose tolerance and systemic inflammation (181). A central hypothesis is that gut leakage through the actions of LPS causes systemic inflammation, which can lead to or amplify metabolic disorders (figure 8).

Figure 8. Hypothesis for bacteria-induced metabolic disease. From reference (181), with permission

In the DOIT-population of high-risk elderly men, we failed to demonstrate that participants with MetS had higher levels of gut leakage markers compared to participants without (Paper I). High LBP levels, nevertheless, increased the risk of having MetS. Our results were somewhat disappointing, as a previous study had demonstrated an association between high levels of LBP and MetS (182). However, this was a Chinese cohort with modified NCEP-ATP III criteria for MetS, which may make the results incomparable to a northern European cohort. Also, more recently, a study with patients on hemodialysis, demonstrated an association between LBP and MetS (183). As uremia is an extreme non-physiological condition, the studies cannot be compared.

The finding that LBP was higher in individuals with high waist circumference (>102 cm) compared to those with low, but not associated with BMI, is in line with a recent study, which demonstrated no association between increasing BMI and either LBP and LPS (184). As discussed in paper I, waist circumference is associated with the amount of intraabdominal fat, while BMI is a more crude and unspecific measure. The amount of intra-abdominal fat correlates with circulating LPS in the obese (185). Furthermore, after weight loss, LBP has been shown to decrease significantly along with circulating levels of LPS (186).

LBP has been shown to associate with cardio-metabolic risk factors in several studies and to be increased in diabetics compared to non-diabetics (187). Hyperglycemia per se is hypothesized to contribute to increased intestinal permeability leading to translocation of bacterial products and systemic inflammation (188). The suggested mechanism is increased paracellular leakage through dysfunctional tight junction (125). A recent systematic review involving 2424 subjects with diabetes, showed that the presence and the severity of diabetes was associated with increased levels of LBP (189). We explored this mechanism by dividing the population according to their fasting glucose, however no significant association with the measured leakage markers was found. We did, however, find a weak correlation between serum insulin and LBP, a finding that harmonizes with prior studies demonstrating an association between LBP and measures of glycemic control (187).

Dyslipidemia, especially low HDL-c, reduces clearance of LPS, thus ensuring a low-grade chronic inflammation (190). The LPS/HDL ratio has also been suggested to be a marker of LPS activity, and has been associated with components of MetS (78). As we did not measure LPS, no such ratio could be judged. There was however, no association between levels of LBP or sCD14 and HDL-c.

Gut leakage as a marker of cardiovascular disease

At the time of our investigation there were limited number of reports that addressed the potential association between gut leakage markers and CAD. In one study LBP was found to be higher in patients with angiographically confirmed CAD (110), and LBP was shown to independently predict total and cardiovascular mortality (111). In paper II, high levels of LBP, independent of conventional risk factors, predicted a twofold increased risk of CVD over a three-year period, even after correction for CRP. This finding has later been confirmed in a Japanese population (191).

We found that sCD14 was associated with a tendency towards increased risk of CVD, but this tendency was lost after correcting for confounders. Previous studies on CVD and sCD14 have been more diverse. The C-260T substitution in the promoter region has been associated with increase prevalence among subjects with CAD through differential expression of mCD14 and sCD14 in retrospective studies (192). However, large prospective studies have failed to show any importance of this polymorphism for incident cardiovascular events (95, 193). Nevertheless, a recent GWAS study has identified important alleles associated with lower sCD14 expression and with incident CV events (194).

The role of gut leakage in CVD is unclear. Although gut bacteria has been identified in the atherosclerotic plaque, there is little evidence that they have an active role and are not innocent bystanders. Carnevale et al did, however, demonstrate the presence of antibodies against E-coli LPS co-localized with TLR4 in an atherosclerotic plaque, suggesting a more active role (79).

The seminal Cantos study, demonstrated the feasibility of targeting IL-1 β in CAD (195).

We hypothesize that gut leakage is an upstream process that in part contributes to the

immune activation and the production of pro-inflammatory cytokines such as IL-1 β .

Bacterial wall products may also contribute to the thrombotic process following a plaque rupture by increasing platelet aggregation through overproductions of eicosanoids and reduced synthesis of von Willebrand factor (196, 197).

The latest studies have focused more on bacterial metabolites such as TMAO, changes in the microbiota and specific taxa with beneficial or potentially detrimental effects in CAD. Unfortunately, there is a lack of studies investigating both gut leakage markers and microbial metabolites. Thus, we do not know if gut leakage and increased TMAO are dysbiotic features that tend to coexist. Interestingly, we found an association between TMAO and both sCD14 and I-FABP. The role and the importance of that association, remains to be elucidated.

The two gram-negative bacteria *Bacteroides vulgatus* and *Bacteroides dorei* were found to be relatively depleted in CAD compared to healthy controls (198). Infusion of the same bacteria in apolipoprotein E-deficient mice, inhibited atherosclerosis formation.

Furthermore, treatment with *Bacteroides* decreased both plasma levels of LPS and circulating pro-inflammatory cytokines. The study did not measure any other gut microbiota leakage markers or metabolites, nevertheless it cemented the possible role of gut leakage in CAD.

Gut microbiota in heart failure

There are accumulating evidence that the gut microbiota differs in patients with HF compared to controls. Moreover, microbial metabolites such as TMAO confer prognostic information in HF. However, there were no clinical human studies that had demonstrated any effect of microbiota modulation on prognosis in HF when we started the GutHeart study. It was therefore designed as a proof-of-concept study where we attempted to

demonstrate a potential causal effect of gut microbiota modulation in HF. In papers III and IV, we could not demonstrate any significant effect of our interventions on LVEF, NT-proBNP and markers of gut leakage and selected markers of systemic inflammation. Importantly, the study failed to significantly modulate the microbiota diversity, thus we are unable to refute our null hypotheses, that gut microbiota intervention may influence cardiac function.

A population with HF failure is possibly an ideal population to study the effects of dysbiosis as the degree of dysbiosis and intestinal permeability seems to increase with increasing NYHA-class and the degree of congestion (61, 199). A subsequent bloom of the pathobionts *Shigella* and *E-coli*, and a reduction of butyrate-producing bacteria, may lead to increased fecal and circulating LPS and increased levels of TMAO (200, 201). Conversely, in well regulated and compensated HF, levels of LPS, LBP and sCD14 have been shown to be comparable to healthy controls. LPS can also be reduced with antidiuretic therapy (70). In the GutHeart study, most of the baseline characteristics in our study population were comparable to those observed in contemporary HF trials such as DAPA-HF and PARDIGM-HF (202, 203). As discussed in paper III, we observed a median NT-ProBNP level of 964 pg/ml, as opposed to 1428 and 1594 pg/ml in these other trials. Furthermore, 72 % of the subjects in our population were in NYHA class II at randomization, and the lack of congestion may be a cause of the failure of our interventions. This is in line with a previous study showing no difference in alpha diversity between HF subjects and healthy control despite changes on genus and species level (200).

In paper IV, we found that high levels of I-FABP were significantly associated with high levels of NT-proBNP, but not with LVEF. Combining LBP and I-FABP in the same regression model predicted high NT-proBNP levels better than either marker alone. We

can thus hypothesize that impaired cardiac function may lead to intestinal epithelial cell destruction, subsequent impairment of intestinal wall barrier and leakage of LPS.

Interestingly, high levels of I-FABP have been associated with poor clinical outcome in acute decompensated HF (204). The current knowledge leads us to speculate whether gut leakage and subsequent activation of innate immunity, contributes to the poor survival following an acute decompensated HF (205).

Intervention of the gut microbiota

In paper II, intervention with diet or n-3 PUFA, had no effect on circulating LBP or sCD14, thus our hypothesis failed. Diet is a key modulator of the gut microbiota and an unhealthy diet is an important behavioral risk factor for CVD. Increased intake of simple carbohydrates such as glucose and sucrose and intake of saturated fat are associated with unfavorable changes in the gut microbiota. This essentially transforms the gut into a pro-inflammatory phenotype characterized by TLR activation (206). In contrast, the Mediterranean diet which is rich in polyunsaturated fatty acids, low glycemic carbohydrates such as fiber and more proteins of vegetable sources is believed to exert beneficial effects on the microbiota (207). Effects such as increased SCFA, lower urinary TMAO and lower plasma LPS have also been reported (207, 208).

An association between gut leakage markers and diet has previously been made (72, 209). However, similar to our paper II, prior studies have failed to reduce the markers with diet intervention (210). It is likely that our three-year intervention had a modulatory effect on the microbiota *per se*, but, unfortunately, we did not have samples to investigate the microbiota or its metabolites. However, these potential modulations did not lead to decreased levels of LBP or sCD14.

For the n-3 PUFA intervention, data on the potential effects on the microbiota is disperse. From a gut leakage perspective, which was our objective, the most important suggested effect is enhancement of the gut intestinal wall barrier either directly or through promotion of SCFA producing bacteria (156). This may lead to less leakage of LPS, and thus decreased hepatic and immune cell production of LBP and sCD14. However, again, no interventional effects were observed.

There are several potential causes of our neutral result. First, our population was an older population. There are important temporal changes in the microbiota with increasing age, and increasing age is associated with a more pro-inflammatory microbiota (211). What drives the change, and whether it is modifiable by diet, is unknown. Secondly, many of the study participants used medications for comorbidities. Both the comorbidities and the medications may impact the gut microbiota and the leakage markers.

Furthermore, the degree of dysbiosis in the participants at baseline is unknown. We know that the microbiota of patients with symptomatic CAD differs from healthy control (59). However, the difference between subjects at high risk compared with healthy controls, are not necessarily large (212). With low degree of dysbiosis, it is likely that LPS-leakage occurs by transcellular transport rather than paracellular leakage. Diet intervention and n-3 PUFA are less likely to affect that mode of transport.

In paper III and IV, we used *S boulardii* and rifaximin as interventional drugs. Both drugs have clinically proved their utility as microbiota-restoring drugs in the setting of dysbiosis (157, 169). They are also believed to have anti-inflammatory properties primarily due to their modulatory effects on the gut microbiota, as described. Reversal of dysbiosis requires that the gut microbiota is adversely affected in the first place. In our subjects, the median Shannon diversity index was 5.3. Due to methodological differences, measures of diversity and dysbiosis can rarely be compared between studies. Still, in a different cohort

of patients with HF, Luedde and coworkers reported a median Shannon diversity index of 3.6 (62). This may suggest that the gut bacterial diversity in our patients was higher, and the degree of dysbiosis lower in our patients, which could be the reason for the neutral interventional effect in the GutHeart study.

In Trøseid et al. paper on TMAO-levels in chronic heart failure (139), the levels of TMAO ranged between from mean 7.9 $\mu\text{mol/L}$ in the healthy controls to 12.1 $\mu\text{mol/L}$ in patients with HF and CAD. In our GutHeart population, median TMAO was close to 6 $\mu\text{mol/L}$, this may again indicate a lower degree of dysbiosis

The utility of gut leakage markers

In the present thesis, we have used LPS, LBP, sCD14 and I-FABP as markers of gut leakage. These are the most used markers in clinical research. Of the four chosen markers, only LPS is a direct product of leakage. LPS has however many limitations as a marker of gut leakage. LPS only represent leakage of gram-negative bacteria and not the pool of gram-positive bacteria that most likely leaks conjoined. It is removed from circulation in a matter of a few minutes by the liver (213), thus is arguable a poor marker of chronic endotoxemia. Most of the LPS in the gut, depending on the number of acyl-groups, are unable to elicit an immune response (214). Consequently, measured levels of circulating LPS may not represent its pro-inflammatory potential. Finally, the most known method of LPS measurement, the limulus amebocytes lysate assay, also used in our work, have several limitation (215).

In disease states with a high degree of gut leakage such as in decompensated HF, LPS may be a marker worth measuring. However, with low-grade chronic leakage, such as we hypothesize in GutHeart study, LPS is probably a poor marker. This point is further

highlighted by the fact that LPS did not correlate with any marker of systemic inflammation in paper IV.

In contrast to LPS, LBP and to a certain degree sCD14 are indirect markers of gut leakage. Both are essentially acute phase proteins and a part of the innate immune response to an infection. Despite of the acute phase characteristics of LBP, it rises slowly, peaking after 24-48 hours (101). LBP also reflects the immunological potential of LPS, and not necessarily the total amount of LPS, thus in septicemia, there are not necessarily a correlation between LPS and LBP (216). Therefore, it can be argued that LBP is a more suitable marker of low-grade endotoxemia than LPS.

In our papers, LBP correlated significantly to markers of systemic inflammation and high levels associated with increased risk of having MetS, incident cardiovascular disease and high NT-proBNP levels. A major limitation of LBP as a gut leakage marker is that IL-1, IL-6 and TNF α also can induce the transcription of LBP (101). Therefore, in the cohort of older subjects with a high degree of comorbidities (Papers I and II), it is difficult to pinpoint the source of LBP induction.

sCD14 is increased in a variety of inflammatory disease states, suggesting a role beyond simple LPS signalling (82). Although the hepatic production is regulated as an acute-phase protein, essentially all monocyte activators are able to increase levels of sCD14 (217). As expected, sCD14, similar to LBP, correlated well with several markers of systemic inflammation. However, high levels of sCD14 only demonstrated a tendency towards increased risk of CVD in paper II. When analysing for total mortality, sCD14 above median was associated with an unadjusted 3.5 fold increased risk of dying compared to those under median levels (OR 3.5, 95% CI 1.39-8.95, $p=0.005$). These results are in line with other studies in other disease states linking increased levels of sCD14 with decreased survival (89, 91, 194).

I-FABP is principally a marker of intestinal epithelial cell damage as discussed. Any potential damage to the intestinal wall barrier will most likely facilitate leakage of bacterial wall products into systemic circulation. I-FABP was measured only in the GutHeart population, as it has been suggested to be a possible marker of gut leakage in HF, as discussed. Interestingly, chronic hyperglycemia also causes increased I-FABP levels possibly due to increased intestinal cell turnover (122), and it could therefore have been of interest also in relation to the presence of MetS (Paper I).

Conclusions

- I. Gut leakage markers were not significantly increased in study participants with MetS compared to those without. However, LBP above median levels were associated with the increased risk of having MetS (Paper I). Both sCD14 and LBP correlated moderately with markers of systemic inflammation. We conclude that LBP might have a role in MetS. High prevalence of comorbidities and usage of medication can have affected our result. With a true control group, the result would likely be more convincing.
- II. Intervention with n-3 PUFA and diet intervention did not affect any marker of gut leakage (Paper II). We conclude that a more comprehensive or directed approach is probably necessary to affect markers of gut leakage.
- III. LBP but not sCD14 were independently associated with incident cardiovascular events, even after correction for CRP (Paper II). We conclude that LBP is a promising biomarker and/or a treatment target in CVD.
- IV. Intervention with *S.boulardii* and rifaximin did not improve LVEF or cardiac function. Moreover, microbial diversity and the metabolites were not affected (Paper III). We conclude that as we were not able to significantly change the microbiota diversity, we cannot say whether or not microbiota directed therapy may affect the heart. Other strategies should be attempted in the future.
- V. Intervention with *S.boulardii* and rifaximin did not significantly affect any marker of gut leakage or any of the markers of systemic inflammation (Paper IV). Again, we conclude that as we were not able to significantly change the microbiota diversity, we cannot say whether or not microbiota directed therapy may affect gut leakage or the associated systemic inflammation.

VI. LBP and I-FABP were correlated to NT-proBNP and high levels of both markers, predicted high levels of NT-proBNP. I-FABP correlated significantly to microbiota diversity, butyrate producing capacity of the microbiota and TMAO, and sCD14 correlated to TMAO. LBP, I-FABP and sCD14 were all significantly correlated to the markers of systemic inflammation, whereas LPS was not.

We conclude that LBP and I-FABP may be markers of cardiac function and intestinal congestion in HF. Furthermore, in compensated HF, LPS might be a poor marker of gut leakage.

Future perspectives

Our results have strengthened LBP as a candidate biomarker in CVD, however a potential causal relationship is yet to be established.

In patients with a hypothetical large degree of dysbiosis and gut leakage such as in inflammatory bowel disease (IBD), interventions with Mediterranean diet, n-3 PUFA, *S.boulardii* or rifaximin could be feasible. Intriguingly, IBD is associated both with increased levels of LBP and increased risk of CVD (218, 219). However, based on our studies, it is unlikely that such interventions would affect the levels of LBP or sCD14 in the general population. Possibly a more directed form of therapy is warranted.

Drugs such as orlistat, a pancreatic and gastric lipase inhibitor that reduces dietary fat absorption by 30%, have been shown to reduce levels of LPS in obese individuals, possibly by reducing its co-transport with dietary triglycerides (220). If the effect is independent of weight loss, could be achieved in normal weight individuals, or leads to a reduction in LBP, is unknown. Also monoclonal antibodies to LBP and sCD14 have been used in experimental studies (221, 222). On the other hand, both of these proteins play important roles in LPS recognition and signalling, thus the feasibility in human studies, are unknown.

Fecal microbial transplant (FMT) demonstrated a decrease in both IL-6 and LBP in cirrhotic patients (223). As proof-of-concept, a trial with FMT, could be interesting in a CVD trial, however there are several safety concerns regarding the use (224).

Regarding other metabolites such as TMAO and butyrate, the problems are similar.

Although different strategies to reduce TMAO and increase butyrate exist and more are being developed, none have demonstrated an effect on CVD (225).

The need for RCTs is evident in the field of the gut microbiota. The abovementioned CANTOS study, elegantly demonstrated how a field could progress from association to

causation. The GutHeart study have taught us that we cannot easily extrapolate a potential effect of an intervention on the microbiota from one population to another. Even geographical differences might matter. As suggested in paper III, future studies of the gut-axis should demonstrate an effect of an intervention on the gut microbiota composition or metabolites in question before testing the intervention in a RCT.

Synopsis in Norwegian

Da PhD-prosjektet startet våren 2016, ønsket vi å studere forholdet mellom tarmflora, kardiovaskulære risikofaktorer og kardiovaskulær sykdom. Våre hypoteser var at personer med metabolsk syndrom, diabetes, koronarsykdom og hjertesvikt har en endret tarmflora og en endret tarmbarriere. Denne endringen fører til endringer i mikrobielle metabolitter og/eller lekkasje av bakterier fra tarm som fører til sykdomsprogresjon ved metabolsk syndrom, diabetes, koronarsykdom og hjertesvikt. Denne progresjonen kan være forårsaket av flere mekanismer, men man mener at den i stor grad skyldes kronisk lavgradig aktivering av det medfødte immunforsvaret. Behandling av tarmflora ved hjelp av kostholdsendringer, omega-3 fettsyrer, probiotika eller perorale antibiotika med lite systemisk absorpsjon, kan modifisere sykdomsforløpene. På det aktuelle tidspunktet var det få studier som hadde sett på disse sammenhengene.

Studie I og II var basert på DOIT studien (Diet and Omega-3 fatty acid Intervention Trial) fra 1997. Totalt 563 eldre menn med økt kardiovaskulær risiko ble inkludert i den opprinnelige studien. De ble randomisert til intervensjon med middelhavsdiett, eller ikke middelhavsdiett, omega-3 fettsyrer eller placebo i en 2 x 2 faktoriell design og fulgt i 3 år. Kardiovaskulære endepunkter ble registrert i samme periode. Av de 563 personene ble 482 personer inkludert i studie I og II i min PhD grunnet mangel på blodprøvemateriale.

I studie I undersøkte vi om markører for tarmlekkasje (LBP og sCD14) var økt hos pasienter med metabolsk syndrom samt om disse markørene var assosiert med systemisk inflammasjon. Dette var en tverrsnittsstudie basert på baseline data. I alt 40% av populasjonen hadde metabolsk syndrom. Vi fant at pasienter med metabolsk syndrom ikke hadde økte sirkulerende verdier av tarmlekkasje-markører sammenliknet med pasienter uten metabolsk syndrom. Imidlertid var økende verdier av LBP assosiert med

økende risiko for å ha metabolsk syndrom. Pasienter i høyeste kvartil av LBP hadde også dobbelt så høy risiko for å ha metabolsk syndrom sammenliknet i laveste kvartil.

Markører for systemisk inflammasjon, henholdsvis, CRP, IL-6, TNF α og IL-18, var signifikant assosiert med både sCD14 og LBP.

Studie II var basert på det randomiserte prinsippet hvor vi ønsket å undersøke om markører for tarmlekkasje (LBP og sCD14) kunne modifiseres med intervensjon med middelhavsdiet eller omega-3 fettsyrer samt å undersøke hvorvidt de samme markørene kunne predikere fremtidige kardiovaskulære hendelser over en 3 års periode. Til sammen 53 pasienter gjennomgikk et endepunkt i løpet av oppfølgingsperioden. Vi fant at ingen av intervensjonene endret verdiene av LBP eller sCD14 sammenliknet med deres respektive kontrollgrupper. LBP over median verdi var imidlertid, uavhengig av andre risikofaktorer, assosiert med en doblett risiko for å gjennomgå et endepunkt i løpet av 3 år.

I studie III og IV (GutHeart) ønsket vi å undersøke hvorvidt gjærsoppen *Saccharomyces boulardii* (*S.boulardii*) eller rifaximin, et antibiotikum som virker lokalt i tarm, over en 3 måneders intervensjonsperiode kunne i) bedre hjertefunksjon målt ved venstre ventrikkels ejeksjonsfraksjon ii) bedre tarmflora diversitet iii) påvirke mikrobielle metabolitter iv) påvirke markører for tarmlekkasje og v) redusere systemisk inflammasjon hos pasienter med hjertesvikt med redusert ejeksjonsfraksjon.

Vi fant at venstre ventrikkels ejeksjonsfraksjon ikke var signifikant høyere etter intervensjon i verken rifaximin-armen eller i *S.boulardii* armen sammenliknet med kontrollgruppen. For øvrig fant vi ingen effekt på tarmflora diversitetsmålet Shannon Index, metabolitten trimetylamin-N-oksidi, den butyrat-produserende kapasiteten til tarmfloraen, NT-proBNP, lekkasjemarkørene LBP, sCD14, I-FABP, LPS eller CRP, IL-6 og IL-10. Vi fant imidlertid at høye nivåer av LBP og I-FABP var assosiert med høye

nivåer av NT-proBNP. Vi fant også at høye verdier av begge markører kan predikere hvilke pasienter som har høye NT-proBNP verdier.

Vi konkluderer med at lekkasje av bakterielle produkter fra tarm sannsynligvis spiller en rolle i metabolsk syndrom samt koronarsykdom via aktivering av det medfødte immunforsvaret. Spesielt LBP fremheves som en spennende biomarkør og et potensielt behandlingsmål. Det er imidlertid vanskelig å vite om økte LBP verdier virkelig er relatert til tarmlekkasje, eller om det skyldes andre inflammatoriske prosesser i kroppen. LBP er et akutfase protein og syntesen kan derfor påvirkes på lik linje med for eksempel CRP. Det er for øvrig uklart om LBP har en rolle i patogenesen ved kardiovaskulær sykdom.

Ved kronisk hjertesvikt er det igjen LBP og I-FABP som utmerker seg, her som markør for høye NT-proBNP verdier. Vi tror de økte verdiene skyldes tarmveggødem og tarmepitel skade. Uavhengig av mekanismen, er begge markører assosiert med systemisk inflammasjon og økte verdier kan derfor potensielt ha negative virkninger på hjertet.

Før vi kan bevise kausalitet, vil det være helt avgjørende å finne intervensjonsmodaliteter som påvirker både tarmflora diversitet, metabolitter og tarmlekkasje markører. Vi klarte ikke å påvirke verken LBP, sCD14, LPS, I-FABP med kosthold, omega-3 fettsyrer, probiotika eller antibiotika i våre populasjoner. Vi klarte heller ikke å påvirke tarmdiversiteten eller de mikrobielle metabolittene. I DOIT studien var deltakerne eldre, hadde høy kardiovaskulær risiko og var mye medisinert. Dette kan ha influert resultatene. I GutHeart var pasientene velbehandlet målt ved NT-proBNP. Vi tror også at tarmdysbiosen i populasjonen var lite uttalt, hvilket også kan forklare den manglende effekten. Denne forklaringen støttes av at diversitetsmålene i vår populasjon var høyere enn i sammenliknbare studiepopulasjoner. For øvrig var også TMAO verdiene lavere i vår studie enn i andre studier.

En mer omfattende strategi slik som FMT eller en mer direkte metode kreves sannsynligvis for å påvirke tarmfloraen og dermed også våre lekkasje-markører. Det vil være viktig å vurdere nøye type intervensjon opp imot populasjonen som skal studeres.

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RESEARCH

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Markers of metabolic endotoxemia as related to metabolic syndrome in an elderly male population at high cardiovascular risk: a cross-sectional study

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Abstract

Background: Metabolic syndrome (MetS) is a cluster of conditions that conjoined represents a 1.5–2.5 fold increased risk of developing cardiovascular disease (CVD). Recent studies have reported that gut dysbiosis and leakage of bacterial components, may contribute to the metabolic disturbances and systemic inflammation observed in subjects with MetS. Chronic exposure to lipopolysaccharide (LPS) has been shown to induce features of MetS in experimental studies. LPS interacts with the innate immune system, facilitated through LPS-binding protein (LBP) and the co-receptor CD14, both regarded as markers of gut leakage.

Purpose: We investigated whether circulating levels of LBP and sCD14 are associated with the presence of MetS and its components, and further any association with systemic inflammation.

Methods: We examined 482 men, aged between 65 and 75 years, all at high CVD risk. MetS criteria's according to the US National Cholesterol Education Program Adult Treatment Panel III were met in 182 subjects (38%).

Results: Levels of LBP and sCD14 did not differ between individuals with and without MetS. However, a trend towards increased risk of MetS through quartiles of LBP was observed ($p = 0.05$). Individuals in the highest quartile (Q4), had an increased risk of MetS (OR = 1.76, 95% CI (1.04–3.00), compared to the lowest quartile (Q1) ($p = 0.04$). With regard to the separate constituents of MetS, patients who met the waist circumference criterion had significant higher concentration of LBP compared to those who did not ($p = 0.04$). We also found a weak, but significant correlation between LBP and waist circumference ($r = 0.10$, $p = 0.03$). Moderate, yet significant correlations were observed between both LBP and sCD14 and several markers of systemic inflammation ($r = 0.1–0.23$; $p < 0.001–0.04$).

Conclusion: The trend for increased prevalence of MetS observed with increasing quartiles of LBP seems to be mainly driven by central obesity in our male cohort. The associations between LBP, sCD14 and systemic inflammation, indicate a potential role of the innate immune system in MetS.

Trial registration CLINICALTRIALS.GOV, NCT00764010. Registered 01 October 2008—retrospectively registered, <https://clinicaltrials.gov/ct2/show/NCT00764010?term=NCT00764010&rank=1>

Keywords: Metabolic syndrome, Gut microbiota, Lipopolysaccharide binding protein, CD14, Innate immunity, Chronic inflammation, Central obesity

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Background

The prevalence of metabolic syndrome (MetS) is rapidly increasing in the western world. Its health consequences includes a twofold increased risk of developing cardiovascular disease (CVD), a sevenfold increased risk of developing diabetes mellitus type 2 (T2DM) and a 1.5-fold increased risk of all-cause mortality [1, 2].

There is no uniform definition of MetS, which essentially is a testimony of its complexity. MetS is a cluster of clinical and biochemical conditions which are often found to coexist, thus indicating a common pathophysiological background. The common denominators are central obesity, insulin resistance/glucose intolerance, dyslipidemia and hypertension [3], also known as Norman Kaplan's "deadly quartet" [4].

The underlying pathophysiology remains unclear and is widely debated. There is, however, compelling evidence that MetS is associated with chronic low-grade inflammation, typically demonstrated by increased levels of C-reactive protein (CRP) and pro-inflammatory cytokines like interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF α) compared to subjects without MetS [5, 6]. Abdominal adipose tissue seems to be an important source of this inflammatory response, and the size and composition of this compartment have been shown to correlate well with the amount of circulating pro-inflammatory cytokines [7].

Translocation of parts of the gut microbiome, and in particular endotoxins or lipopolysaccharides (LPS) to the systemic circulation, has been proposed to be an early trigger of inflammation and subsequent cardiovascular risk [8]. An increase in plasma LPS can occur in healthy individuals after a high fat meal, partly due to co-transportation over the gut wall together with dietary fat by incorporation in triglyceride-rich chylomicrons [9, 10]. Leakage through dysfunctional tight-junctions have also been suggested [11]. Obese individuals tend to have higher levels of circulating LPS, both in fasting conditions and in the postprandial phase [10], and circulating levels of LPS have been reported to correlate with abdominal obesity and glycemic control [12]. In experimental human studies, chronic LPS exposure has been shown to promote systemic insulin resistance and adipose tissue related inflammation [13].

To communicate with the innate immune system, LPS binds to the LPS-binding protein (LBP), which is pivotal for the binding of CD14 and transfer to the Toll like receptor (TLR) 4 complex [14]. Further activation of NF- κ B and interferon regulatory factors induces transcription of pro-inflammatory mediators. Blockage of LBP or CD14-binding seems to attenuate the inflammatory effect of LPS in animal studies [15, 16]. Circulating

levels of LBP have also been reported to correlate with abdominal obesity and glycemic control [12, 17].

We hypothesize that microbial translocation may contribute to the inflammatory state associated with MetS. We therefore explored any association between circulating levels of LBP and soluble CD14 (sCD14) and the presence of MetS, its components and hyperglycemia. We also explored any association to systemic inflammation.

Methods

Study population

The study participants were enrolled in the Diet and Omega-3 Intervention Trial on Atherosclerosis (DOIT) initiated in 1997. The study was designed as a prospective randomized trial [18]. The subjects were all men, aged between 65 and 75 years, deemed at high cardiovascular risk. They were essentially survivors from the Oslo study cohort, conducted in 1972–1977 [19]. The present investigation is a cross-sectional study on baseline data obtained at inclusion. A total of 563 subjects were included in the study. Blood samples from 482 subjects were available for the present investigation.

MetS was classified using the Adult Treatment Panel III (NCEP) definition [20]. The classification requires three or more of the following risk factors: Abdominal obesity defined by a waist circumference > 102 cm in men, triglyceride levels > 1.7 mmol/L, HDL cholesterol < 1.04 mmol/L in men, hypertension defined by blood pressure $\geq 130/\geq 85$ and fasting glucose ≥ 5.6 mmol/L. The presence of previously diagnosed hypertension outside the definition of MetS, was defined as systolic blood pressure > 140 and/or diastolic blood pressure > 90 mmHg and diabetes as manifest diabetes and/or fasting glucose > 7 mmol/L. We divided fasting glucose according to the American Diabetes Association definition of normal levels, impaired fasting glucose and diabetes mellitus (≤ 5.5 , 5.6–6.9 and ≥ 7.0 mmol/L, respectively). Smokers were defined as current smokers.

Laboratory methods

Blood samples were obtained at inclusion in fasting condition (> 10 h) by standard venipuncture before daily intake of medication between 08:00 and 10:00 a.m. EDTA blood was separated by centrifugation within 1 h at 2500 $\times g$ for 10 min and plasma was kept stored at -80°C until analyses. Serum lipids were determined by conventional enzymatic methods. LBP and sCD14 were analyzed by commercial ELISAs (Hycult Biotech, Uden, the Netherlands and R & D Systems Europe, Abingdon, Oxon, UK, respectively). The inter-assay coefficients of variation were for LBP 8.2% and for sCD14 8.9%. Methods for CRP, IL-6, IL-18 and TNF α have previously been described [21].

Statistics

All statistics were performed using IBM SPSS statistics version 24.0. Demographic data are given as numbers with proportions or medians with 25, 75 percentiles. As most data were not normally distributed, non-parametric statistics were used. For continuous variables, bivariate Spearman's correlations were used. To identify differences between MetS vs no MetS and between the different components of MetS, Mann–Whitney U-tests were used. Kruskal–Wallis test was used to examine differences between groups of fasting glucose. Furthermore, we explored the relationship between categorical variables using Pearson Chi square. For trend analysis, we used Mantel–Haenszel test and risk was expressed as Odds Ratio by logistic regression. $p < 0.05$ was considered statistically significant.

Results

Baseline characteristics and laboratory data of the total study population are shown in Table 1. As displayed, 38% met the criteria of MetS. Within the criteria of MetS, 88% fulfilled the hypertension criterion, 29% the waist criterion, 40% the hypertriglyceridemia criterion, 10% the low HDL criterion and 56% the impaired fasting glucose criterion. Within the individuals who did not fulfill the definition of MetS ($n = 300$), 56% had two criteria fulfilled, corresponding to 35% of the total population. Only 6% of these, corresponding to 4% of the total population, had no criteria. Furthermore, there were 16% diabetics, 33% current smokers, 29% with previously diagnosed cardiovascular disease (CVD) and 31% were treated for hypertension (Table 1).

LBP and sCD14 as related to MetS and its components

Neither LBP nor sCD14 levels differed significantly between individuals with MetS compared to those without, although numerically higher levels of LBP in the MetS group was observed ($p = 0.11$) (Table 2). When dividing LBP and sCD14 into quartiles, we observed a significant trend towards increased prevalence of MetS with ascending quartiles of LBP ($p = 0.05$) (Fig. 1a). Furthermore, when using the lowest quartile (Q1) as the reference group, subjects in Q4 had an increased risk of having MetS (OR = 1.76, 95% CI (1.04–3.00), $p = 0.04$). No such trend was observed across quartiles of sCD14 (Fig. 1b).

Analyzing the separate constituents of the syndrome, concentration of LBP was found to be significantly higher in patients with waist circumference above 102 cm ($p = 0.04$). This was also observed in the ≤ 5.5 mmol/L glucose subgroup ($p = 0.002$), but not in other subgroups of plasma glucose. No other

Table 1 Baseline characteristics of the total study population ($n = 482$)

Age	70 (67.5, 72.6)
Metabolic syndrome	182 (38)
Number of criteria fulfilled	
0	19 (4)
1	113 (23)
2	168 (35)
3	111 (23)
4	60 (12)
5	11 (2)
Body mass index (kg/m^2)	26.5 (24.3, 28.6)
Waist circumference (cm)	98 (92, 103)
Systolic blood pressure (mmHg)	148 (135, 160)
Diastolic blood pressure (mmHg)	84 (91, 77)
Previous hypertension	150 (31)
Previous diabetes mellitus	79 (16)
Previous myocardial infarction	89 (18)
Current smokers	160 (33)
Aspirin	131 (27)
Statins	135 (28)
Antidiabetics	21 (4)
HbA1c (%)	5.6 (5.3, 5.9)
Insulin (pmol/L)	118 (94, 154)
HOMA (units)	4.2 (3.3, 5.7)
CRP (mg/L)	3.27 (1.7, 5.8)
IL-6 (pg/mL)	1.53 (1.00, 2.45)
TNFA (pg/mL)	1.10 (0.78, 1.89)
IL-18 (pg/mL)	274 (212, 350)
LBP ($\mu\text{g}/\text{mL}$)	12.9 (10.4, 15.2)
sCD14 (ng/mL)	1293 (1052, 1515)

Median values (25, 75 percentiles) or numbers (proportions) are given. For abbreviations, see text

differences in LBP levels were observed among the other features of the syndrome (Table 2). sCD14 levels did not differ between the different constituents of MetS.

No trend towards higher levels of LBP nor sCD14 as related to the number of MetS criteria fulfilled was observed in the total population (Data not shown).

When looking at the separate constituents as continuous variables in the whole population, we found a weak, but significant correlation between LBP and waist circumference ($r = 0.11$, $p = 0.02$) (Table 3). Furthermore, sCD14 correlated with diastolic blood pressure ($r = 0.12$, $p = 0.01$), but not with systolic blood pressure. No significant correlations were observed with regard to levels of fasting glucose, triglycerides or HDL, also when excluding statin users (data not shown).

Insulin resistance measured by Homeostasis Model Assessment (HOMA) and Triglyceride/HDL ratio (TG/HDL ratio), did not show any correlations with LBP or

Table 2 Serum concentrations of LBP and sCD14 as related to metabolic syndrome and its separate constituents

	LBP (µg/mL)	p	sCD14 (ng/mL)	p
MetS				
+	13.1 (11.2, 15.5)	0.11	1285 (1057, 1483)	0.71
–	12.7 (10.3, 14.9)		1306 (1053, 1538)	
Waist circumference				
> 102 cm	13.2 (11.2, 15.7)	0.04	1299 (1078, 1494)	0.41
≤ 102 cm	12.6 (10.2, 14.9)		1294 (1033, 1535)	
Blood pressure				
> 130/85 mmHg	12.9 (10.5, 15.2)	0.87	1294 (1051, 1499)	0.36
≤ 130/85 mmHg	13.0 (10.9, 15.0)		1309 (1053, 1627)	
Fasting glucose				
≥ 5.6 mmol/L	12.9 (10.5, 15.3)	0.99	1299 (1065, 1494)	0.95
< 5.6 mmol/L	12.9 (10.6, 15.0)		1293 (1004, 1566)	
HDL-cholesterol				
< 1.04 mmol/L	13.8 (11.1, 15.9)	0.15	1309 (1039, 1594)	0.72
≥ 1.04 mmol/L	12.8 (10.5, 15.1)		1295 (1052, 1518)	
Triglycerides				
> 1.7 mmol/L	13.0 (10.8, 15.4)	0.69	1284 (1061, 1495)	0.87
≤ 1.7 mmol/L	12.7 (10.4, 15.0)		1297 (1030, 1530)	
Fasting glucose				
≤ 5.5	12.9 (10.6, 15.0)	0.99*	1289 (1008, 1558)	0.71*
5.6–6.9	12.8 (10.6, 15.1)		1297 (1072, 1467)	
≥ 7.0	13.1 (10.5, 15.3)		1317 (1056, 1589)	

Median values are given (25, 75 percentiles). P-values refers to differences between groups. * Kruskal–Wallis test. Italic text indicates p-values below < 0.05

sCD14. A weak correlation between LBP and serum insulin was observed ($r = 0.10$, $p = 0.04$) (Table 3).

Among subjects with MetS, both TG/HDL ratio and triglyceride levels, correlated weakly to sCD14 ($r = 0.15$, $p = 0.04$ for both) (Table 3).

LBP and sCD14 as related to systemic inflammation

Moderate, yet significant correlations were observed in patients with MetS as well as the total population between both LBP and sCD14 and several markers of systemic inflammation, represented by CRP, IL-6, IL-18 and TNF- α as shown in Table 3.

In patients with MetS, CRP, IL-6, IL-18 and TNF- α levels were significantly higher compared to those without in the total population, as previously reported [21].

Discussion and conclusion

In the present study, concentrations of LBP and sCD14 did not differ significantly in the subjects with MetS vs no MetS although a numerically higher level of LBP was found in the MetS group. We observed, however, a trend towards increasing risk of MetS through quartiles of LBP, in line with previous reports that have shown significant associations between MetS and LBP [22, 23]. The

a Quartiles of LBP

b Quartiles of sCD14

Fig. 1 Prevalence of MetS as related to quartiles of LBP (a) and sCD14 (b) in the total population. P for trend using Mantel Haenszel test. Comparing quartile 4 to 1 of LBP levels, gives an unadjusted Odds ratio of 1.76, $p = 0.04$ by logistic regression

prevalence of comorbidities and the frequent use of medications in both the MetS and the no MetS group, could explain the limited differences in our study, hence the no MetS group cannot be looked upon as a true control group as they may share some of the same metabolic disturbances as seen in MetS. This is further emphasized by the fact that only 6% of the individuals without MetS, did not fulfill any of the MetS criteria and more than 50% had two criteria fulfilled. There are also limited knowledge about how medications affect leakage of LPS from the gut and the expression of sCD14 and LBP. Previous studies have excluded patients on antidiabetics and statins, or use of such medications have not been properly reported [23, 24].

Looking at anthropometric measures in our study population, none of the markers correlated with BMI, however, a significant association was observed between LBP and waist circumference. Central obesity is thought to play a vital role and even accelerate the metabolic and hormonal disturbances observed in this syndrome. The

Table 3 Correlations between LBP and sCD14 and metabolic and inflammatory variables in the total population and in subjects with MetS

	In the total population (n = 482)				In subjects with MetS (n = 182)			
	LBP		sCD14		LBP		sCD14	
	r	p	r	p	r	p	r	p
BMI	0.05	0.33	−0.04	0.34	−0.06	0.04	0.03	0.70
Waist circumference	0.11	<i>0.02</i>	−0.01	0.89	0.01	0.95	0.13	0.10
Triglycerides	−0.03	0.56	0.01	0.84	−0.01	0.86	0.15	<i>0.04</i>
HDL cholesterol	−0.04	0.37	0.02	0.62	−0.08	0.31	−0.11	0.15
Systolic blood pressure	0.01	0.78	0.01	0.87	−0.05	0.51	0.02	0.80
Diastolic blood pressure	0.04	0.36	0.12	<i>0.01</i>	−0.02	0.79	0.07	0.37
Fasting glucose	0.001	0.96	0.01	0.80	−0.01	0.93	0.10	0.18
Insulin	0.10	<i>0.04</i>	0.02	0.74	0.13	0.08	0.02	0.75
HOMA	0.08	0.11	0.03	0.59	0.10	0.18	0.07	0.39
TG/HDL ratio	−0.01	0.82	0.01	0.86	0.02	0.80	0.15	<i>0.04</i>
CRP	0.22	<i><0.001</i>	0.14	<i>0.003</i>	0.17	<i>0.03</i>	0.09	<i>0.23</i>
IL-6	0.23	<i><0.001</i>	0.16	<i><0.001</i>	0.2	<i>0.001</i>	0.17	<i>0.02</i>
IL-18	0.15	<i>0.001</i>	0.18	<i><0.001</i>	0.17	<i>0.02</i>	0.26	<i><0.001</i>
TNFα	0.15	<i>0.001</i>	0.10	<i>0.04</i>	0.24	<i>0.001</i>	0.06	<i>0.39</i>

For abbreviations, see text. Italic text indicates p-values below <0.05. Subjects with MetS, refers to subjects with 3 out of 5 or more MetS criteria according to the NCEP ATP III definition

amount of intraabdominal fat has been shown to correlate strongly with serum concentration of LPS as well as bacterial DNA found in intraabdominal fat samples [12]. Thus, although correlations were weak, our finding supports an association and potential link between central obesity and metabolic endotoxemia.

Dyslipidemia in MetS is typically characterized with low levels of HDL and high triglyceride levels. LPS has been shown to be inversely correlated with HDL, thus one could expect an equal relationship between LBP and HDL [25]. In our study, LBP was however, not significantly correlated with HDL or with any of the other lipoproteins in the population as a whole, also when excluding patients on statins, which is known to influence inflammation [26]. In the group with MetS alone, triglycerides were significantly correlated to sCD14.

Glucometabolic parameters are also central in MetS. Hyperglycemia has independently been associated with increased leakage of gut microbial content [27]. We did however, not find any significant differences in LBP nor sCD14 levels between the different ranges of glucose.

Of the glucometabolic parameters, only serum insulin showed a weak positive correlation with LBP in the whole population, while TG/HDL ratio correlated weakly, but significantly to sCD14 in the MetS group. We did not observe any correlations between our markers of gut leakage and HOMA, which is probably due to lack of correlation to fasting glucose. Experimental studies with chronic LPS exposure, typically show decreased insulin

sensitivity [13, 28], and several studies have shown correlation between insulin resistance and LBP in humans [17, 29]. Antidiabetics such as sulfonylureas and metformin, whose central mechanisms of action are to stimulate pancreatic beta cells production of insulin and increase glucose sensitivity, respectively, could mask a potential association. However, in our study, the frequency of such drugs was low.

We could also show that the proposed markers of gut leakage, LBP and sCD14, correlate well with downstream mediators of systemic inflammation in the total cohort. For the group with MetS, the correlations were somewhat weaker, which may be due to the reduction in sample size. We have previously shown that IL-6, IL-18, CRP and TNFα, all were significantly higher in the group with MetS compared to those without [21].

LBP is essentially an acute phase protein secreted primarily by hepatocytes and the transcription of LBP is mainly stimulated by IL-1, IL-6 and LPS [30, 31]. Free or membrane-bound LPS is the main ligand of LBP, although it can also bind to other lipopeptides such as Lipoteichoic acid [32], thus not being LPS exclusive. sCD14 can also interact with other pathogen associated molecular patterns (PAMPs) and TLRs, thus not being specific for the LPS–LBP complex [33].

Our population is rather homogenous in terms of being only males and with a narrow age span between 65 and 75 years. Consequently, we were not able to examine differences between sexes and different age groups.

Concentrations of LBP are known to increase with increasing age [23].

We have used LBP and sCD14 as surrogate markers of gut related endotoxemia. As stated, both LBP and sCD14 do not exclusively bind to LPS. Conversely, other PAMPs are able to activate the immune system independent of LBP and sCD14. As a result, we are only able to highlight parts of the gut-related activation of the innate immune system.

Furthermore, we did not register daily alcohol intake. Excessive alcohol intake is associated with increased levels of LPS and LBP in serum [34]. Endotoxemia has also been suggested as an important mechanism behind alcohol induced fatty liver. Thus, this may be an important confounder that we are not able to adjust for.

In our study, multiple analyses were conducted using two dependent variables, thereby adding to the risk of Type I errors. However, we decided not to perform Bonferroni correction, because we look upon this study as a hypothesize-generating study, and believe that a correction would be too strict in this case.

To conclude, our study cohort of elderly men cannot confirm that levels of LBP or sCD14 contribute significantly to the low grade inflammation in subjects with MetS. Nevertheless, there was a trend for increased prevalence of MetS with increasing quartiles of LBP which seems to be mainly driven by central obesity. Furthermore, we reaffirm that both LBP and sCD14 are associated with systemic inflammation, indicating a role of the innate immune system in MetS.

Abbreviations

MetS: metabolic syndrome; CVD: cardiovascular disease; LPS: lipopolysaccharide; LBP: lipopolysaccharide binding protein; T2DM: diabetes mellitus type 2; IL-6: interleukin 6; TNF α : tumor necrosis factor alpha; CRP: C-reactive protein; TLR: toll like receptor; sCD14: soluble cluster of differentiation 14; DOIT: Diet and Omega-3 Intervention Trial on Atherosclerosis; HOMA: Homeostasis Model Assessment; PAMPs: pathogen associated molecular patterns; TG/HDL ratio: Triglycerides/high density lipoprotein ratio.

Authors' contributions

All authors made substantial contributions to conception, design, drafting and critically revising the manuscript. MT, IS and HA have also been involved in acquisition of data. All authors read and approved the final manuscript.

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Acknowledgements

The authors would like to acknowledge Vibeke Norheim Kjær for excellent work in the laboratory and Elsa Hjerkin for her efforts in performing the DOIT study.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets generated and analyzed during the current study are not publicly available due to risk of compromising privacy, but are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The DOIT study was conducted in compliance with the Helsinki Declaration, approved by the Regional Ethics Committee and registered at ClinicalTrials.gov (NCT00764010). Subjects gave their written informed consent for participation.

Funding

This work was supported by the Stein Erik Hagen Foundation for Clinical Heart Research, Oslo, Norway. The funding source had no involvement regarding the conduct or preparation of the study.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 24 April 2018 Accepted: 16 July 2018

Published online: 21 July 2018

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Effects of dietary intervention and n-3 PUFA supplementation on markers of gut-related inflammation and their association with cardiovascular events in a high-risk population

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HIGHLIGHTS

- Markers of gut-related inflammation are not affected by diet intervention.
- LBP and sCD14 are associated with increased risk of cardiovascular disease.
- LBP predicts increased risk of cardiovascular disease independent of CRP.

ARTICLE INFO

Keywords:

Innate immunity
Cardiovascular disease
N-3 PUFA
Diet intervention
LPS-Binding protein
Soluble CD14
CRP

ABSTRACT

Background & aims: Dysbiosis of the gut microbiota is associated with increased levels of circulating lipopolysaccharide (LPS) and subsequent activation of systemic inflammation. Diet is an important modulator of the gut microbiome.

We aimed to investigate whether circulating markers of gut-related inflammation, LPS binding protein (LBP) and soluble CD14 (sCD14) can be modulated by n-3 PUFA supplementation and/or diet counselling, and whether these markers are related to cardiovascular (CV) outcome.

Methods: 484 men aged 65–75 years, at high CV-risk, were included and randomized in a 2 × 2 factorial design to 36-month intervention with dietary counselling, n-3 PUFA supplementation, or both. N-3 PUFA supplementation was placebo-controlled. ELISAs were used for determination of the biomarkers measured at baseline and study-end. A composite endpoint was defined as new CV-events and CV-mortality after 36 months.

Results: There were no significant differences in changes of either LBP or sCD14 in the intervention groups compared to their respective controls (n-3 PUFA vs. placebo: $p = 0.58$, $p = 0.15$, diet vs. no-diet: $p = 0.53$, $p = 0.59$, respectively). The group with LBP levels above median had about 2-fold unadjusted risk of suffering an endpoint compared to the group below (HR 2.22, 95% CI 1.25–3.96; $p = 0.01$). A similar tendency was seen for sCD14 (HR 1.72, 95% CI 0.97–3.03; $p = 0.06$). After adjusting for covariates, LBP remained significantly associated with a two-fold CV-risk, whereas sCD14 gained statistical significance, however, lost when hsCRP was added to the model.

Conclusions: In our population, markers of gut-related inflammation associated with 36-month CV outcome. However, neither n-3 PUFA nor diet intervention had an effect on these markers.

1. Introduction

Inflammation is believed to be a major contributor to the

development of cardiovascular disease (CVD). It is implicated in all phases of atherosclerotic plaque development from initiation to the potentially devastating event of atherothrombosis [1–3]. Targeting

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<https://doi.org/10.1016/j.atherosclerosis.2019.05.004>

Received 25 January 2019; Received in revised form 16 April 2019; Accepted 3 May 2019

Available online 04 May 2019

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inflammation is now looked upon as a feasible method for addressing the substantial residual risk observed after managing conventional risk factors. Studies like IMPROVE-IT have shown a convincing effect on secondary CV events when both LDL-cholesterol and high sensitive C-reactive protein (hsCRP) were lowered by statins and Ezetimibe [4]. The recent hallmark study CANTOS, where hsCRP was lowered independently of LDL-cholesterol, further provides evidence of the causal role of inflammation in CVD [5].

The innate immune system stands at the front line of vascular inflammation. Monocyte-derived macrophages present in the vascular wall initiates inflammation through identification of danger signals [6]. These danger signals may serve as ligands for Toll-like-receptors (TLRs) or primers/activators of the inflammasome. Downstream, they induce transcription of pro-inflammatory cytokines such as interleukin 6 (IL-6) and interleukin 1 β (IL-1 β), which are highly associated with cardiovascular disease [7,8]. Pathogens associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) are also known potent activators of innate immunity through essentially the same signaling receptor family [9].

Antibodies against LPS have been found to co-localize with antibodies to TLR4 and CD68 in atherosclerotic plaques, suggesting an active role in the atherosclerotic process [10]. Furthermore, LPS and other gut derived PAMPs have been suggested to play a role in atherothrombosis [11,12].

The source of circulating LPS in healthy individuals is thought to be derived from the gut. Unfavorable changes in the bacterial composition of the gut microbiome, namely dysbiosis, is suggested to increase the amount of LPS in the gut lumen as well as in systemic blood through translocation of whole bacteria or bacterial components [13].

The effect of circulating LPS is mainly facilitated by LPS binding protein (LBP). LBP is essentially an acute phase protein, mainly produced by the liver and stimulated by LPS, but also IL-1, IL-6 and tumor necrosis factor α (TNF α) have been shown to increase expression of LBP [14,15]. In the circulation, LPS binds to LBP with high affinity. This step is suggested to be a rate-limiting step facilitating further binding to membrane bound cluster of differentiation 14 (CD14), which promotes atherogenic and proinflammatory properties through TLR4, which is a transmembrane receptor on a large variety of cells, including mononuclear immune cells and vascular cells [16].

Dysbiosis as well as several commensal gut bacteria has been associated with increased risk of CVD [17–19]. Diet is the foremost long-term modulator of the gut microbiota. High fat and high sugar diets have been associated with driving the microbiome into a pro-inflammatory phenotype associated with increased circulating levels of LPS [20,21]. Conversely, interaction between the gut microbiota and a low fat, high fiber diet, has been suggested to have protective roles in the development of atherosclerosis, when compared to western diet [22].

We therefore aimed to explore whether diet counselling and n-3 PUFA intervention would reduce markers of gut leakage, namely LBP and soluble CD14 (sCD14), thus reducing the activation of the innate immune system.

Furthermore, we investigated whether baseline levels of these markers were predictive of cardiovascular events in a high CVD risk population.

2. Materials and methods

2.1. Study population

In total 563 males, all aged between 65 and 75 years, deemed at high cardiovascular risk, were enrolled in the Diet and Omega-3 Intervention Trial on Atherosclerosis (DOIT), a prospective randomized trial initiated in 1997. The participants were randomized in a 2 $\times$ 2 factorial design with 36-month intervention with either dietary counselling, n-3 PUFA supplementation, or both. N-3 PUFA supplementation was placebo controlled with encapsulated corn oil [23].

Blood samples from 484 subjects were available for the present investigation. Of these, 241 subjects received 2.4 g n-3 PUFA daily, whereas 243 subjects received placebo. The n-3 PUFA capsules contained 35% eicosapentaenoic acid (EPA), 20% docosahexaenoic acid (DHA), and 3.5 mg tocopherols/g for prevention of fatty acid peroxidation. The n-3 PUFA capsules consisted of an ethyl esterified triglyceride formula.

According to randomization, 237 subjects underwent strict personal dietary counselling, guided by a clinical nutritionist. All of these subjects were advised to reduce intake of saturated fat and meat from animal sources and increase intake of vegetable oils, fruit, fish and vegetables. Calorie-restriction was advised for subjects deemed as overweight. At randomization, 30–45 min of counselling was given, and repeated after three months. They were followed either by telephone consultations or at the outpatient clinic every six months. Compliance of n-3 PUFA and diet intervention was obtained by measurement of serum fatty acids and comparison of food questionnaires obtained at baseline and at the end of the study [23]. Methods for randomization and intervention have previously been published [23].

All participants were followed for 36 months. Endpoints were registered as a composite of new CV events and CV mortality. New CV events were defined as acute myocardial infarction, need for revascularization, aortic aneurysm, peripheral arterial occlusive disease, cerebrovascular events and death due to cardiovascular causes.

The DOIT study was approved by the Regional Ethics Committee, and all subjects gave their informed written consent to participate.

The trial has been registered at clinicaltrials.gov (NCT00764010, www.clinicaltrials.gov).

2.2. Laboratory methods

Blood samples were obtained at inclusion in fasting condition (> 10 h) by standard venipuncture before daily intake of medication between 08:00 and 10:00 a.m. EDTA blood was separated by centrifugation at 4 °C within 1 h at 2500 $\times$ g for 10 min and plasma was kept stored at –80 °C until analyses. Serum lipids were determined by conventional enzymatic methods. LBP and sCD14 were analyzed by commercial ELISAs (Hycult Biotech, Uden, the Netherlands and R & D Systems Europe, Abingdon, Oxon, UK, respectively). The inter-assay coefficients of variation were for LBP 8.2% and for sCD14 8.9%. Method for high sensitivity (hs)CRP analysis has previously been described [24].

2.3. Statistics

As most data were not normally distributed, non-parametric statistics were used. Demographic data are provided as numbers with proportions or medians with 25, 75 percentiles. Pearson Chi-squared test was used to explore baseline differences in categorical variables, whereas Mann-Whitney u-test was used to explore differences between groups in continuous variables. Mantel-Haenszel chi-square statistics was used for trends across quartiles.

Within the treatment groups, Wilcoxon signed-rank test was used to compare baseline levels with levels after the 36-month intervention. Interventional effect of n-3 PUFA or dietary counselling between the groups was explored according to the factorial design with one-way ANCOVA adjusting for baseline levels.

A Cox regression model was used to analyze the risk of having an endpoint during the 3-year follow-up period when dichotomizing LBP and sCD14 levels at median. Levels below and above median was chosen as cut-off values after first dividing both markers into quartiles as related to the incidence of an endpoint. We then visually found a natural cut-off at median level (Supplementary Fig. 2). Both markers are presented with three proposed models. In the first model, covariates which are associated with both CV outcome and the marker in question with a p-value less than 0.2, were included. In the second model, conventional risk factors were included. In the third model, hsCRP was

Table 1

Baseline characteristics of the total study population (n = 484) and according to having and endpoint or not. Median values (25, 75 percentiles) or numbers (proportions) are given.

	Total	Endpoint (–)	Endpoint (+)	p
Age (years)	70.1 (67.5–72.7)	69.88 (67.4–72.7)	71.22 (67.9–72.7)	0.32
BMI (kg/m ²)	26.5 (24.2–28.6)	26.4 (24.1–28.4)	27.4 (24.8–29.4)	0.14
Triglycerides (mmol/L)	1.50 (1.12–1.98)	1.50 (1.12–1.97)	1.63 (1.12–2.13)	0.62
LDL (mmol/L)	4.02 (3.46–4.60)	3.99 (3.38–4.59)	4.05 (3.66–4.60)	0.24
HDL (mmol/L)	1.37 (1.16–1.62)	1.38 (1.17–1.63)	1.34 (1.14–1.55)	0.66
Fasting glucose (mmol/L)	5.6 (5.3–6.2)	5.6 (5.3–6.2)	5.8 (5.3–7.5)	0.07
HbA1C (%)	5.6 (5.3–5.9)	5.6 (5.3–5.9)	5.6 (5.3–6.3)	0.20
Systolic BP (mmHg)	147 (136–160)	147 (135–160)	153 (143–168)	0.02
Current smokers	160 (33)	135 (32)	23 (43)	0.09
History of diabetes mellitus	79 (16)	64 (15)	15 (28)	0.01
History of MetS	182 (38)	159 (37)	23 (43)	0.37
History of hypertension	150 (31)	133 (31)	17 (32)	0.87
Previous CVD	139 (29)	118 (28)	21 (40)	0.07
hsCRP (mg/L)	3.28 (1.78–5.89)	3.08 (1.67–5.80)	4.88 (3.10–6.69)	< 0.001
Statin use	134 (28)	122 (29)	13 (25)	0.54
Antidiabetic use	21 (4)	16 (4)	5 (9)	0.06
Diuretic use	23 (5)	20 (5)	3 (6)	0.75
ACE-inhibitor use	78 (16)	66 (16)	13 (25)	0.09
Aspirin use	131 (27)	107 (25)	24 (45)	0.002
Betablocker use	79 (17)	64 (16)	15 (21)	0.22
Calcium blocker use	82 (17)	74 (17)	8 (15)	0.68
Nitrate use	43 (9)	32 (8)	11 (21)	0.001

Statistical significant differences ($p < 0.05$) are outlined in bold.

Fig. 1. Plasma levels of both markers at baseline and after 36-month intervention according to the factorial design.

(A and B) LBP and (C and D) sCD14. * Indicates statistically significant changes within groups with p -values of < 0.001 . The p -values given refer to difference in changes between n-3 PUFA and placebo (A and C) and diet intervention vs no diet intervention (B and D) (One-way Ancova).

added. Risk is given as Hazard ratio (HR). Kaplan-Meier method was used for time-to-event analysis.

To analyze the discriminative ability of our markers, receiver operating characteristics (ROC) curves were used. We also compared incremental contributions of each marker and the combination of both markers when added to hsCRP. Area under the curves were calculated.

IBM SPSS statistics version 25.0 was used for all statistical analyses.

3. Results

3.1. Baseline characteristics

Of the 484 subjects, a total of 53 patients suffered the composite endpoint of a new CV event and CV mortality.

Baseline characteristics of the patients that experienced an endpoint

compared to those who did not are given in Table 1. Levels of hsCRP and systolic blood pressure were higher in the endpoint group and proportionally there were more diabetics in the endpoint group, as was also the use of aspirin and nitrates (Table 1).

Baseline characteristics according to the randomized groups were well matched (Supplementary Table 1). The diet intervention group was slightly older than controls (median 70.8 vs. 69.7 years, $p = 0.05$). There were borderline significantly more diuretic users in the n-3 PUFA intervention group (7% vs. 3%, $p = 0.05$). Antidiabetics and nitrates were more frequent in the diet control group compared to the diet counselling group ((7% vs. 2%), $p = 0.02$ and (12% vs. 6%), $p = 0.02$, respectively).

3.2. Interventional effects

The effects of 36 months with diet counselling or n-3 PUFA supplementation with regard to serum lipids and lipoproteins have previously been described in detail. In short, both diet and n-3 PUFA intervention significantly decreased serum triglyceride levels [23].

We observed a significant increase of LBP levels in all groups regardless of the intervention modality ($p < 0.001$). There was, however, no significant difference in changes in levels of LBP between groups receiving n-3 PUFA or diet counselling vs. their respective controls ($p = 0.58$ and $p = 0.53$, respectively), (Fig. 1, upper panel).

For the levels of sCD14, the group receiving n-3 PUFA had a non-significant increase over the 36-month intervention period ($p = 0.06$), while the group receiving placebo capsules had a non-significant reduction over the 36-month intervention period ($p = 0.34$). The difference in changes was not statistically significant ($p = 0.15$) (Fig. 1, lower panel). As for diet counselling, we observed no change in sCD14 within the intervention group ($p = 0.81$) and a non-significant increase in controls ($p = 0.28$). However, the difference in changes between the groups was not significant ($p = 0.59$) (Fig. 1, lower panel).

3.3. Clinical endpoint analyses

The concentration of LBP was found to be significantly higher in patients who suffered an endpoint compared to individuals that did not (median 14.09 vs. 12.68 $\mu\text{g/mL}$; $p = 0.01$). A trend towards increased concentration of sCD14 in the endpoint group was observed, however, not statistically significant (median 1354 vs. 1285 ng/mL , respectively; $p = 0.1$). Levels of CRP were significantly higher in subjects experiencing an endpoint, as previously reported [24].

When dividing LBP into quartiles, we observed a positive trend of increased prevalence of an endpoint across ascending quartiles ($p = 0.01$). This was not observed with sCD14 ($p = 0.10$).

When dichotomizing levels at median, there were 36 vs. 17 endpoints in the group above vs. below median LBP (12.88 $\mu\text{g/mL}$) and 32 vs. 19 for sCD14 (1294 ng/mL). In an univariate Cox regression model, the group above the median LBP had significantly higher risk of suffering an endpoint during the 36-month follow up (HR 2.22, 95% CI 1.25–3.96; $p = 0.01$), whereas we observed only a tendency towards higher risk with levels above median sCD14 (HR 1.72, 95% CI 0.97–3.03; $p = 0.06$) (Fig. 2). After adjusting for confounders in a multivariate model, LBP remained significantly associated with CV outcome (HR 2.00, 95% CI 1.11–3.58; $p = 0.02$) and sCD14 achieved statistical significance after adjustment (HR 1.82, 95% CI 1.02–3.23; $p = 0.04$). After adding traditional CV risk factors, including hsCRP in the model, sCD14 no longer remained significantly associated, whereas LBP prevailed (Tables 2 and 3).

We further investigated subjects with combined LBP and sCD14 above the median ($n = 124$). These subjects had no real added risk compared to LBP only when analyzed by univariate Cox regression (HR 2.25, 95% CI 1.29–3.91; $p = 0.004$).

ROC curve analyses revealed a similar AUC for hsCRP alone, LBP alone and LBP + CRP combined (0.63, 0.61 and 0.62). Thus, no

incremental improvement of AUC was obtained when hsCRP was added (Supplementary Fig. 1).

4. Discussion

In our study of high CV risk individuals, neither n-3 PUFA nor diet counselling had any modifiable effects on levels of LBP or sCD14 after a 36-month intervention.

For all groups, levels of LBP increased from baseline during the study period, however, with no differences in changes between the groups. For sCD14, there was no significant change in the diet intervention group and an increase in the n-3 PUFA group, however, not significantly different from their respective controls.

The effect of dietary intervention on prevention of CVD is thought to be through modification of known CV risk factors, however, interactions with the gut microbiota have been suggested to play an important role [25–27].

Diet-induced dysbiosis is characterized by increased LPS-containing luminal bacteria and increased leakage of bacteria, bacterial components or products through a dysfunctional intestinal barrier [21,28,29]. Overall, these changes facilitate leakage of LPS from the gut lumen and subsequent production of both sCD14 and LBP [30]. Conversely, we hypothesized that dietary intervention could counteract these changes. However, no signs of such counteractions were shown in our study.

The effect of n-3 PUFA on the human microbiome is largely unknown. There are experimental and clinical studies showing favorable changes and decreased intestinal wall leakage by supplementation, thus indicating reduced gut-related inflammation [31,32]. Recently, the landmark study REDUCE-IT showed remarkable effects of icosapent ethyl (Vascepa) on incident cardiovascular events [33]. These findings are in contrast to the contemporary meta-analysis by Aung et al., showing no effect of n-3 PUFA on cardiovascular endpoints [34]. In REDUCE-IT, a daily dose of 4 g of icosapent ethyl was used, whereas in the meta-analysis, the dosing displayed great heterogeneity, and in our study, a dose of 840 mg EPA daily was used. Thus, the lack of effect might be a dosing issue.

In line with our results, an intervention study using the Mediterranean diet showed a non-significant increase in LBP over a given time period [35].

We found that levels of LBP were significantly higher in individuals that experienced a CV event. Subjects with levels above median had a close to 2-fold increased risk of experiencing an endpoint during the 36-month follow up, also after correcting for known confounders, traditional risk factors and hsCRP. For sCD14, there was a tendency towards higher levels in the endpoint group and the group above median had a non-significant increased risk of experiencing an endpoint; however, gained significance after correcting for confounders. Statistical significance was, nonetheless, lost after adding other risk factors, including CRP. When analyzing the Kaplan-Meier survival curves for both markers, it is quite evident that the two curves diverge early for LBP, indicating a consistent reduction of event-free survival for the group with high levels. For sCD14, the two curves co-localize approximately until day 650. At this point, the rate of decline in event-free survival for the group with the lowest sCD14 levels is somewhat reduced. Prior studies have shown that high levels of sCD14 correlate well with both short and long-term mortality, more so than non-fatal cardiovascular events [36–38]. Thus, we may hypothesize that the decline in event-free survival is mainly driven by increased mortality; however, the current study was not powered to study mortality *per se*.

As for diagnostic accuracy, LBP did not outperform CRP or added incremental accuracy in predicting CV outcome, as judged by similar AUC levels in ROC curves.

Prior studies have identified differences in the microbial communities of individuals with CVD vs. individuals without, by metagenomic sequencing [18,39,40]. Recently, *Bacteroides vulgatus* and *Bacteroides dorei* were found to be depleted in patients with CAD. Supplementation

Fig. 2. Kaplan-Meier curves for event free CV survival, comparing levels of LBP (A) and sCD14 (B) above and below median values.

Table 2

Multivariate Cox regression model of LBP dichotomized at median for the prognosis of CV outcome.

	Model 1 ^a		Model 2 ^b		Model 3 ^c	
	HR (95% CI)	<i>p</i>	HR (95% CI)	<i>p</i>	HR (95% CI)	<i>p</i>
LBP - below median	Ref.		Ref.		Ref.	
LBP - above median	2.00 (1.11–3.58)	0.02	1.98 (1.10–3.55)	0.02	1.90 (1.05–3.42)	0.03
Aspirin use	2.76 (1.56–4.88)	< 0.001	2.33 (1.25–4.32)	0.01	2.34 (1.26–4.35)	0.01
Current smokers	1.56 (0.90–2.70)	0.11	1.64 (0.94–2.87)	0.08	1.61 (0.92–2.83)	0.1
Total cholesterol	1.35 (1.01–1.79)	0.04	1.43 (1.06–1.91)	0.02	1.41 (1.05–1.90)	0.02
Age			1.06 (0.96–1.16)	0.24	1.05 (0.96–1.15)	0.32
History of diabetes			2.27 (1.24–4.16)	0.01	2.26 (1.24–4.14)	0.01
History of hypertension			0.91 (0.49–1.67)	0.75	0.86 (0.46–1.60)	0.63
Prior CVD			1.59 (0.84–3.00)	0.16	1.60 (0.84–3.04)	0.15
hsCRP					1.04 (0.98–1.11)	0.22

Statistical significance ($p < 0.05$) are outlined in bold.

^a Model 1: Including confounders only.

^b Model 2: Confounders and traditional risk factors added.

^c Model: Confounders, traditional risk factors and hsCRP added.

of the aforementioned bacteria in rodents had anti-inflammatory effects by reducing fecal and blood levels of LPS, however, levels of LBP were not measured [17].

Zhou and colleagues reported increased bacterial richness measured in blood in patients after ST-elevation infarction (STEMI) compared to

controls. Circulating LPS was also increased and associated with adverse cardiovascular events. The same study showed that experimental treatment of STEMI mice with polymyxin B, an antibiotic with LPS-inhibitory properties, reduced infarct size [41]. Conceptually, inhibition of LBP could also be an interesting way to inhibit the effects of

Table 3

Multivariate Cox Regression Model of sCD14 dichotomized at median level for the prognosis of CV outcome.

	Model 1		Model 2		Model 3	
	HR (95% CI)	<i>p</i>	HR (95% CI)	<i>p</i>	HR (95% CI)	<i>p</i>
sCD14 - below median	Ref.		Ref.		Ref.	
sCD14 - above median	1.82 (1.02–3.23)	0.04	1.68 (0.95–2.99)	0.08	1.62 (0.91–2.89)	0.10
Aspirin use	2.10 (1.16–3.81)	0.01	1.98 (1.04–3.77)	0.04	2.02 (1.06–3.84)	0.03
Nitrate use	2.60 (1.22–5.55)	0.01	2.55 (1.18–5.54)	0.02	2.55 (1.18–5.52)	0.02
Current smokers	1.93 (1.09–3.41)	0.02	2.09 (1.17–3.74)	0.01	2.03 (1.13–3.65)	0.02
Age			1.06 (0.96–1.16)	0.24	1.05 (0.96–1.15)	0.29
History of diabetes			2.33 (1.26–4.31)	0.01	2.35 (1.27–4.35)	0.01
History of hypertension			0.72 (0.39–1.35)	0.31	0.68 (0.36–1.29)	0.24
Prior CVD			1.18 (0.62–2.26)	0.62	1.21 (0.63–2.31)	0.57
hsCRP					1.05 (0.98–1.12)	0.18

^a Model 1: Including confounders only.^b Model 2: Confounders and traditional risk factors added.^c Model: Confounders, traditional risk factors and hsCRP added.Statistical significance ($p < 0.05$) are outlined in bold.

circulating LPS.

Associations between LBP and CVD have previously been studied in the Ludwigshafen Risk and Cardiovascular Health Study (LURIC) [42], in which 2959 subjects who were scheduled for coronary angiography were followed for 8 years. They found that LBP was associated with severity of coronary artery disease (CAD), prevalent CAD and both total and CV mortality. However, the predictive value of LBP was lost after controlling for levels of CRP, in contrast to our study in which LBP remained significantly associated with new CV events independently of CRP.

There are many factors that may explain the differences between the studies. Most importantly, we studied an older group of individuals with higher degree of inflammatory burden measured by CRP, and we observed specifically new CV events and CV mortality.

Our study has shown that none of the suggested markers of gut related inflammation was influenced by diet or n-3 PUFA supplementation. However, both markers seem to play a predictive role in CVD. In the context of the utility as biomarkers for clinically evident CVD, both markers show relatively low predictive value. However, as both LBP and sCD14 play important roles in the LPS-LBP-CD14-TLR4 signaling pathway [43], they may represent novel biomarkers of atherosclerosis or even potential treatment targets in CVD. Further studies will be needed to clarify the role of LBP and sCD14 as either having a causal role in cardiovascular inflammation or as innocent bystanders associated with CVD.

Financial support

This work was supported by the Stein Erik Hagen Foundation for Clinical Heart Research, Oslo, Norway. The funding source had no involvement regarding the conduct or preparation of the study.

Author contributions

All authors made substantial contributions to conception, design, drafting and critically revising the manuscript. Dr. Marius Trøseid, Prof. Ingebjørg Seljeflot and Prof. Harald Arnesen have also been involved in acquisition of data. All authors have approved the final manuscript.

Conflicts of interest

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

Acknowledgements

The authors would like to acknowledge Vibeke Norheim Kjær for excellent work in the laboratory and Elsa Hjerkin for her efforts in conducting the DOIT study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.atherosclerosis.2019.05.004>.

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