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SUBCLINICAL INFLAMMATION AND THE OBESITY-TYPE 2 DIABETES-CARDIOVASCULAR DISEASE TRIFECTA

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DOCTORAL THESIS

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ABSTRACT

Obesity, type 2 diabetes and cardiovascular disease are major causes of morbidity and mortality in the world. They are known to be linked by an unhealthy diet and low levels of physical activity. Furthermore, obesity is a risk factor for type 2 diabetes, and both increase a person's cardiovascular risk. Subclinical inflammation has been associated with each of the conditions, but there have been limited attempts to establish whether it is another major link between them.

To test our hypothesis subclinical inflammation being an important biological link between the obesity-type 2 diabetes-cardiovascular disease trifecta, we analysed whether subclinical inflammation precedes and predicts, obesity, type 2 diabetes, cardiovascular disease and all-cause death.

Three large, population-based prospective Finnish cohorts and one North American cohort were used for the analyses of the present study, namely FINRISK 1992, FINRISK 2002, DILGOM 2007 and the FHS Offspring Study. The Finnish cohorts were further linked to nation-wide registers, providing information on deaths, disease diagnoses and drug purchases.

We explored links of subclinical inflammation with obesity in both cross-sectional and longitudinal settings using the DILGOM 2007 cohort. Inflammation markers were associated with obesity measures at baseline, as well as with weight gain, increasing waist circumference, increasing body fat percentage, or increasing body-mass index (BMI) during a 7-year follow-up. However, after adjusting for baseline BMI, longitudinal associations were no longer significant for any of the outcomes. This suggests that subclinical inflammation does not precede obesity, and may be its consequence rather than the cause.

With the help of advanced metabolomics, we identified 545 eicosanoids and related oxylipins in the FINRISK 2002, DILGOM 2007 and FHS Offspring Study cohorts. Using stepwise Cox regression analysis, we determined that a three-eicosanoid risk score was associated with incident type 2 diabetes in three independent cohorts. Our findings imply that lipid-derived mediators of inflammation may play a role in the prediction of incident type 2 diabetes.

Using a case-cohort design based on the follow-up of the FINRISK 1992 cohort, we found inflammation markers to be independent predictors of cardiovascular disease and all-cause death, especially in men. IgG class antibodies to periodontal pathogens as well as endotoxin were also associated with incident cardiovascular events, although endotoxin's association was not independent of cholesterol levels.

Further epidemiological studies and randomised clinical trials exploring links between inflammation markers and cardiovascular disease have confirmed the role of subclinical inflammation in the development of atherosclerosis. The findings of this thesis suggest that subclinical inflammation is associated with both metabolic and atherothrombotic aspects of common public health problems. It seems to be at least one of the links between obesity, type 2 diabetes, cardiovascular disease and death.

The findings, together with current literature, suggest opportunities for early detection, estimation of disease risk and prevention as well as therapeutic targeting of subclinical inflammation to reduce the risk of cardiovascular disease. Once we understand better the biological processes underlying subclinical inflammation and the obesity-type 2 diabetes-cardiovascular disease trifecta, we may be able to develop safe and cost-effective therapies addressing metabolic and atherothrombotic characteristics of each of these diseases.

TIIVISTELMÄ

Lihavuus, tyypin 2 diabetes sekä sydän- ja verisuonitaudit aiheuttavat suuren osan maailman tautitaakasta. Epäterveellinen ruokavalio ja liikunnan vähäisyys yhdistävät näitä tauteja toisiinsa. Lihavuus on myös riskitekijä tyypin 2 diabetekselle ja molemmat lisäävät yksilön sydän- ja verisuonitautiriskiä. Matala-asteisen tulehduksen tiedetään olevan yhteydessä kuhunkin näistä taudeista.

Tutkimuksemme tavoitteena oli selvittää, edeltääkö ja ennustaako matala-asteinen tulehdus lihavuutta, tyypin 2 diabetesta, sydän- ja verisuonitauteja sekä kokonaiskuolleisuutta. Käytimme kolmea laajaa, väestöpohjaista suomalaista (FINRISKI 1992, FINRISKI 2002, DILGOM 2007) ja yhtä pohjoisamerikkalaista (FHS Offspring Study) kohorttia. Suomalaisten kohorttien seurantaa varten haimme diagnoosi-, lääkeosto- ja kuolinsyytietoja kansallisista rekistereistä.

DILGOM 2007 -kohortissa tulehdusmerkkiaineet olivat yhteydessä lihavuusmuuttujiin kuten painoon, vyötärönympärykseen, rasvaprosenttiin ja painoindeksiin lähtötilanteessa sekä niiden kasvuun seitsemän vuoden seurannan aikana. Lähtötason painoindeksillä vakioituna tulokset eivät kuitenkaan enää olleet merkitseviä lihavuusmuuttujien kasvulle. Matala-asteinen tulehdus ei siis näytä edeltävän lihavuutta ja niinpä se voisi olla lihavuuden syyn sijaan sen seuraus.

FINRISKI 2002 -, DILGOM 2007 - ja FHS Offspring Study -kohorteissa kolmen eikosanoidin riskipistearvo liittyi lisääntyneeseen riskiin sairastua tyypin 2 diabetekseen. Eikosanoidit saattavat siis olla osallisia tyypin 2 diabeteksen kehittymisessä.

FINRISKI 1992-kohortissa kohonneet tulehdusmerkkiaineet ennustivat itsenäisesti sydän- ja verisuonitauteja sekä kuolleisuutta, etenkin miehillä. Hampaan tukikudostulehdusta aiheuttavat bakteerit olivat myös yhteydessä suurentuneeseen sydän- ja verisuonitapahtumien riskiin, vaikka yhteys selittyi osittain kolesterolitasoilla.

Myöhemmät tulehdusmerkkiaineiden ja sydän-ja verisuonitautien yhteyttä tarkastelevat väestöpohjaiset tutkimukset ja satunnaistetut vertailukokeet ovat vahvistaneet, että matala-asteinen tulehdus vaikuttaa valtimonkovettumataudin kehittymiseen. Matala-asteinen tulehdus yhdistää tutkimiemme yleisten kansantautien metabolisia ja aterotromboottisia piirteitä. Se näyttäisi olevan ainakin yksi lihavuutta, tyypin 2 diabetesta, sydän- ja verisuonitauteja sekä kuolleisuutta yhdistävä tekijä.

Aikainen havaitseminen, tautiriskinarvio, tautien ehkäisy ja matala-asteisen tulehduksen ehkäisy ja hoito sydäntautiriskin vähentämiseksi on mahdollista. Tulehduksen ja tutkimamme tautikolmikon taustalla olevien biologisten mekanismien parempi ymmärtäminen auttaisi kehittämään turvallisia ja kustannusvaikuttavia ehkäisykeinoja ja hoitoja.

To mama
and isi

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Without resilience, challenges such as doing PhDs, may never be overcome. I should therefore not overlook the remarkable examples of my iron grandmothers and aunties of steel, and the impact they have had on both my personal and professional lives. Babcia and mummu, Pelagia, Illon mummu, Anne, Maija, Krysia: your strength and survival instincts have been a major inspiration to me. I owe the resilience I have built up over the years in large part to you. Many thanks to my whole extended family in Finland, Poland and Ghana for your love and support.

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The area of study of this thesis, public health, is a social science, and I would like to express my deepest gratitude to my mum Renata for grounding me in the social and my dad Harri for doing the same with science. I wouldn't be the person I am today without these two exceptional people, who loved and encouraged me unconditionally every step of the way. And I certainly would never have been able to finish this thesis without their amazing support or editing will and skill. Mama and isi, this work is dedicated to the two of you.

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London, October 2021

Karolina Tuomisto

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Tuomisto K, Jousilahti P, Havulinna AS, Borodulin K, Männistö S, Salomaa V. Role of inflammation markers in the prediction of weight gain and development of obesity in adults - A prospective study. Metabol Open. 2019 Aug 27;3:100016. doi: 10.1016/j.metop.2019.100016. PMID: 32812925; PMCID: PMC7424817.

II Tuomisto K, Palmu J, Long T, Watrous JD, Mercader K, Lagerborg KA, Andres A, Salmi M, Jalkanen S, Vasan RS, Inouye M, Havulinna AS, Jousilahti P, Niiranen TJ, Cheng S, Jain M, Salomaa V. A plasma metabolite score of three eicosanoids predicts incident type 2 diabetes – a prospective study in three independent cohorts. *(submitted)*

III Tuomisto K, Jousilahti P, Sundvall J, Pajunen P, Salomaa V. C-reactive protein, interleukin-6 and tumor necrosis factor alpha as predictors of incident coronary and cardiovascular events and total mortality. A population-based, prospective study. Thromb Haemost. 2006 Mar;95(3):511-8. doi: 10.1160/TH05-08-0571. PMID: 16525580.

IV Pussinen PJ, Tuomisto K, Jousilahti P, Havulinna AS, Sundvall J, Salomaa V. Endotoxemia, immune response to periodontal pathogens, and systemic inflammation associate with incident cardiovascular disease events. Arterioscler Thromb Vasc Biol. 2007 Jun;27(6):1433-9. doi: 10.1161/ATVBAHA.106.138743. Epub 2007 Mar 15. PMID: 17363692.

The publications are referred to in the text by their Roman numerals.

ABBREVIATIONS

12-HHTrE	12-Hydroxy-5,8,10-heptadecatrienoic acid
8-iso-PGA1	8-iso-Prostaglandin A1
95% CI	95% confidence interval
BMI	Body-mass index
CHD	Coronary heart disease
COVID-19	Coronavirus Diseases 2019
COX	Cyclooxygenase
CVD	Cardiovascular Disease
CYP-450	Cytochrome P450
DALY	Disability adjusted life year
DILGOM	Dietary, Lifestyle and Genetic Determinants of Obesity and Metabolic Syndrome Study
FFQ	Food Frequency Questionnaire
FDR	False Discovery Rate
FHS	Framingham Heart Study
FINRISK	Finnish Cardiovascular Risk Study
HDL	High-density lipoprotein
HOMA-B	Homeostasis Model Assessment Beta-Cell Dysfunction
HOMA-IR	Homeostasis Model Assessment of Insulin Resistance
HMW	High molecular weight Adiponectin
HR	Hazard ratio
hs-CRP	High-sensitivity C-reactive protein
ICD	International Classification of Diseases
IL-1Ra	Interleukin 1 Receptor antagonist
IL-6	Interleukin 6
IQR	Interquartile range
ISCED	International Standard Classification of Education
LC-MS	Liquid chromatography – mass spectrometry
LDL	Low-density lipoprotein
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LTPA	Leisure-time physical activity
MI	Myocardial infarction
MONICA	Monitoring Trends and Determinants in Cardiovascular Disease
OECD	Organisation for Economic Co-operation andDevelopment
PUFA	Polyunsaturated fatty acid
SD	Standard deviation
THL	Finnish Institute for Health and Welfare
OGTT	Oral glucose tolerance test
TNF-alpha	Tumour necrosis factor alpha
WHO	World Health Organization
WHR	Waist-to-hip ratio

1 INTRODUCTION

Non-communicable diseases account for over 70% of yearly deaths globally. Obesity, type 2 diabetes and cardiovascular disease (CVD) is a trio of non-communicable conditions linked to some extent by an unhealthy diet and low level of physical activity. Heredity and genetics play a part in them as well. Although the obesity-type 2 diabetes-CVD trifecta is non-communicable in its nature, it has also been associated with issues relating to the immune response, namely a chronic, low-grade, system-level inflammation.

Systemic inflammation can be detected by analysing a number of molecules from human plasma. These substances, known as inflammation markers, can be quantified from simple blood samples and they can help to establish the level of inflammation in the body. It is these markers of inflammation that have been associated with obesity, type 2 diabetes and CVD. Could inflammation be another factor to inextricably link the trifecta together, and perhaps contribute to the development of these three devastating diseases?

This lethal trio has deservedly received a lot of attention in terms of their prevention in the past half a century, not least for its macroeconomic effects. A notable scheme in Finland was the North Karelia project, which aimed at reducing premature mortality and incidence of cardiovascular diseases and develop nation-wide approaches to prevention. Later the World Health Organization (WHO) launched an international initiative called the Comprehensive Cardiovascular Community Control project. Ventures such as these in the 1970s brought to light the possibility of lifestyle changes in preventing chronic disease and premature death through community-based disease prevention programmes.

As much as in clinical medicine the aim is to especially treat patients and diseases, in public health the primary focus leans more on the population and prevention of diseases. The past few decades have seen major advances in the treatment of obesity, type 2 diabetes and CVD. However, the declining incidence of CVD has shown that a lot can be achieved through focussing on prevention as well.

In this thesis, I explore the role of chronic, low-grade, systemic inflammation in the development of obesity, type 2 diabetes and CVD, discuss the implications of our findings and review their public health relevance.

2 LITERATURE REVIEW

2.1 EPIDEMIOLOGY

Cardiovascular diseases are a major cause of morbidity and mortality in the world, and the upheaval of obesity and type 2 diabetes seem to be turning around some of the significant work done with preventing coronary heart disease (CHD) and premature deaths in the past half a century.

In this section on epidemiology, I give examples from the high-income countries of Finland and the USA, as the population cohorts used in this thesis are from these two countries. Where possible, I have included examples from lower and middle-income countries, to provide an idea of how they compare with Finland and the USA.

2.1.1 ALL-CAUSE MORTALITY

The average life expectancy has been steadily growing over the past century. In Finland, life expectancy of men has increased by 14 years since 1960 and that of women by 12 years (World Bank 2019). The decline in cardiovascular mortality is likely to be responsible for a considerable part of improved life expectancy (Salomaa et al 2016). Following similar trends, 93.1% of women and 85.6% of men in Finland now live at least to the age of 65, whereas in 1960 the percentages were 79.3% and 60.6%, respectively. Global trends are similar, although there still exist vast differences in life expectancy for example between OECD countries and Sub-Saharan Africa.

In 2019, that is before the global COVID-19 pandemic, there were 55.4 million deaths worldwide (WHO 2020). The patterns of causes of death have also shifted dramatically over the last century, with communicable diseases such as tuberculosis and infant conditions all but disappearing from the Finnish cause of death statistics (Official Statistics of Finland 2010). Worldwide a similar pattern of a switch from communicable diseases to non-communicable diseases has been observed.

2.1.2 CARDIOVASCULAR DISEASES

Although there has been major improvement in prevention, management and rehabilitation from the consequences of myocardial infarction (MI) and stroke, they still represent a lion's share of the CVDs that have occupied the

first places in the global causes of death statistics for at least 20 years. In 2019, nearly 15 million people died of MI or stroke (WHO 2020). Furthermore, according to the Global Burden of Disease Study, ischaemic heart disease and stroke constituted for nearly 13% of disability adjusted life years (DALYs) at all ages globally (GBD 2020).

Myocardial infarctions are usually caused by coronary heart disease (CHD), which is a manifestation of atherosclerosis in the arteries of the heart muscle. Strokes may be either ischaemic, which is more common, or haemorrhagic. The most common underlying cause for both is atherosclerosis present in the arteries supplying brain tissue. Because both MIs and strokes can be lethal even long after the initial acute management, secondary prevention is of vital importance. Those who survive may face a long period of rehabilitation and for working-age adults, a prolonged absence or even retirement from active work. For these reasons, a strong emphasis on primary prevention prevails.

CHD and stroke are rare amongst adults younger than 50. According to the FinHealth 2017 study, CHD prevalence in men over 50 years of age is 14.3% and 7.1% in women of the same age (Koponen et al 2018). The prevalence of stroke in men and women over 50 are more equally distributed at 6.6% and 6.1%, respectively. In 2019, there were 8630 deaths caused by coronary heart disease and 3991 deaths caused by cerebrovascular diseases (Official Statistics of Finland 2019), which corresponds to 23.4% of all deaths in Finland in 2019. The decline in cardiovascular mortality in Finland has been largely attributed to primary prevention, however declined case fatality suggests that improved treatment has played an important role as well (Salomaa et al 2016).

In the USA, the same percentage of 23.4% of all deaths are caused by coronary heart disease or stroke (Center for Disease Control and Prevention 2020a). In Ghana, CVDs caused 19% of all deaths in 2016, whereas in India CVDs constituted as much as 27% of all-cause mortality in that same year (WHO 2018).

2.1.3 TYPE 2 DIABETES

Type 2 diabetes, formerly known as “non-insulin dependent diabetes” or “adult-onset diabetes”, accounts for more than 90-95% of diabetes cases in the world. The American Diabetes Association defines type 2 diabetes as diabetes in “individuals who have insulin resistance and usually relative (rather than absolute) insulin deficiency.” (American Diabetes Association 2014) Most people with type 2 diabetes do not require insulin treatment in the beginning, and many not even during their lifetime.

There is an early stage to the disease known as pre-diabetes. It is defined as impaired fasting glucose and/or impaired glucose tolerance. At early stages

type 2 diabetes and its complications can be delayed and treated. Both the early stages and the disease itself are most often associated with obesity or increased body fat distribution around the waist. Diabetes on the other hand is a major risk factor for CVD.

Prevalence of diabetes, and by extension of type 2 diabetes that makes up most of the cases of diabetes in the world, has increased substantially in the past decades: from 108 million people living with diabetes in 1980, to 463 million in 2019 (NCD Risk Factor Collaboration 2016; International Diabetes Federation 2019). It has been estimated that 1.5 million deaths were caused directly by diabetes in 2019 (WHO 2021a). Moreover, according to the Global Burden of Disease Study, diabetes accounted for nearly 3% of DALY's at all ages globally (Global Burden of Disease Study 2020).

In Finland, it has been estimated that about 15% of adult men and 10% of adult women have type 2 diabetes, either diagnosed or undiagnosed (Koponen et al 2017). In the USA, the statistics are similar to Finland: 14% of adult men were estimated to have been diagnosed or have undiagnosed type 2 diabetes and 12% of adult women (CDC 2020b). More disturbingly however, one in three American adults were estimated to be living with pre-diabetes.

In contrast in Ghana, a systematic review of literature estimated the type 2 diabetes prevalence level in Ghanaian adults to be almost 6.5% (Asamoah-Boaheng 2019). The trend of increasing prevalence in the past decades has also been observed in Ghana, and it is similar to prevalence rates in Sub-Saharan Africa as a whole. The Indian subcontinent suffers from a heavy burden of type 2 diabetes and available data suggest that the Asian Indian population has an increased propensity to develop type 2 diabetes (Unnikrishnan et al 2016). The overall prevalence of type 2 diabetes in 15 of the 28 states of India was 7.3% during the study years 2008-2015, with rates between states varying from 4.3% to 10% (Anjana et al 2017).

2.1.4 OBESITY AND OVERWEIGHT

Obesity is defined by the WHO as a body-mass index (BMI) greater than or equal to 30kg/m^2 (Table 1). Since 1975, the prevalence of obesity has tripled globally (WHO 2021b). Despite some of the world being on track to eliminate undernutrition as per the Sustainable Development Goals (Unicef 2019), many countries are now facing a double burden of diseases associated with both under and overnutrition.

Table 1. *Definitions of adult weight by Body-mass index (BMI)*

Body-mass index	Definitions of adult body weight status
< 18.5	Underweight
18.5-24.9	“Healthy” weight
25-29.9	Overweight
≥ 30	Obese

In 2016, nearly two billion adults were overweight, translating into more than a third of the world’s adult population. Altogether 13% of adults were obese. Figure 1 is a map highlighting the prevalence of obesity in different countries in the world.

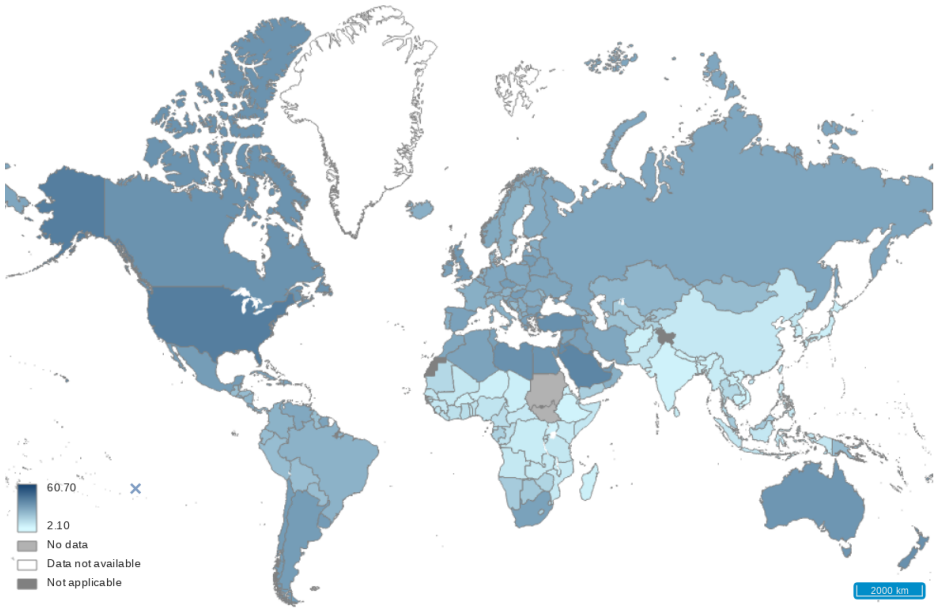

Figure 1 Prevalence (%) of obesity (BMI ≧ 30) among adults. Latest data (2016) and graphic from the Global Health Observatory, World Health Organization 2021. Prevalence of obesity varies between 2.10% (lightest shade of blue) and 60.70% (darkest shade of blue).

In Finland, a quarter of the adult population over 30 years of age is obese and nearly three quarters are overweight (FinHealth 2017). In the USA, the prevalence of adult obesity is 42.5%, whereas the prevalence of overweight adults is similar to Finland (Center of Disease Control 2021).

In contrast in Ghana, a West African country facing the above-mentioned double burden, a quarter of the population is overweight and the obesity prevalence has been estimated at 10-17%, with a higher prevalence in women than in men (Ofori-Asenso 2016, WHO country profile 2016). And although making progress on some of the global nutrition targets, malnutrition of children under 5 is still common in Ghana: according to the Unicef State of Children Report (2019), 19% were stunted (low height-for-age or 'chronic malnutrition'), and 5% were wasted (low weight-for-height, or 'acute malnutrition') (Unicef 2019).

Unlike Ghana or similar African countries, the Asian Indian population has a higher prevalence of obesity overall. In 4 of the 28 states of India, it varied between 12-31% (Pradeepa et al 2015). Furthermore, malnutrition is also major issue: overall 38% of children under 5 were stunted, and 8% were wasted, with a significant variation between the richest 20% and poorest 20% (22-51% for stunting and 2-21% for wasting) (Unicef 2019).

Equally alarmingly, childhood obesity is becoming a serious global issue. In 2016, over 340 million children over 5 years old and adolescents up to 19 years of age were either overweight or obese, and in 2019, a further 38 million children under the age of 5 (WHO 2021b). Obesity is a preventable disorder that can also be overturned to some extent. However, as it is strongly linked with an unhealthy lifestyle, including poor diet and physical inactivity, its management is a big challenge. Apart from lifestyle change, treatment options such as bariatric surgery and weight-loss medicines exist (Carlsson et al 2020; Davies et al 2021).

2.1.5 RISK FACTORS OF OBESITY, TYPE 2 DIABETES AND CARDIOVASCULAR DISEASE

What are the major risk factors for the obesity-type 2 diabetes-CVD trifecta? As economies and societies develop, their risk factor profiles change. Traditional risk factors, for infectious diseases, such as inadequate nutrition, indoor air pollution and unsafe water and sanitation shift to modern risks, for non-communicable diseases, such as smoking, physical inactivity, overweight and urban air quality. A variety of the modern risk factors affect the development of obesity, type 2 diabetes and CVD, and ultimately death.

Obesity is a known risk factor for type 2 diabetes, and both obesity and type 2 diabetes for CVD. Other known risk factors in the obesity-type 2 diabetes-CVD-trifecta include the age and sex of an individual, their socioeconomic status, hyperlipidaemias, hypertension, smoking, physical inactivity, an unhealthy diet, alcohol consumption and poor oral health, as well as the genetic makeup of an individual. Figure 2 shows the estimated global burden

of disease for some of these risk factors in the age groups 25-49 and 50-74 years (GBD 2020).

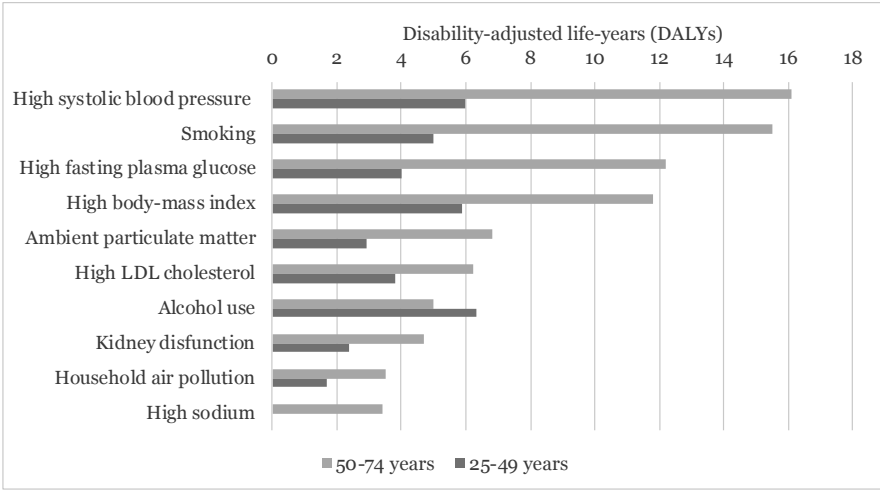

Figure 2 Global burden of disease (in DALYs) of the top 10 risk factors in the age group 50-74 years, comparing to the age group of 25-49 years. Estimate data obtained from the Lancet Global Health Metrics article by GBD 2019 Risk Factors Collaborators (GBD 2020).

As depicted in Figure 3, the interplay between these risk factors and outcomes is complex and it underlines the fact that an individual's health behaviour plays an important role in these connections.

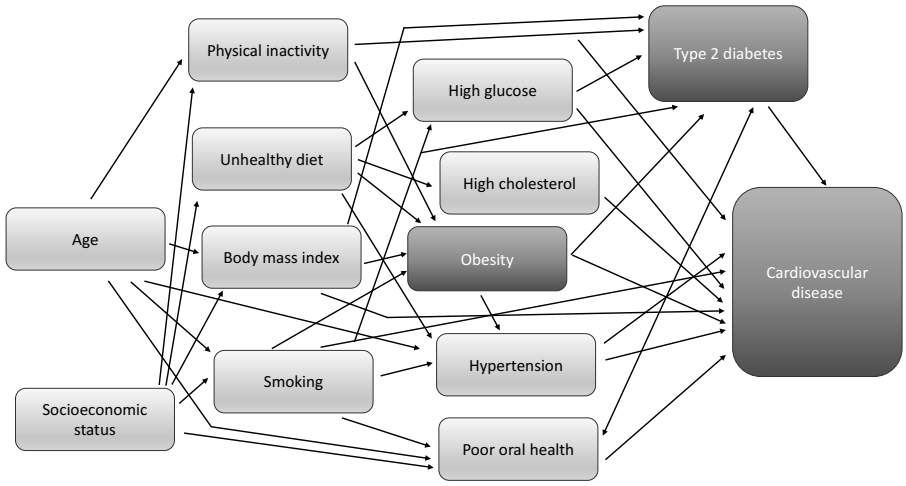

Figure 3 Interactions of some of the risk factors for obesity, type 2 diabetes and cardiovascular disease

Changes in lifestyle have been shown to directly impact on a person's risk of obesity, type 2 diabetes and CVD. For example, the historic North Karelia project in Finland conclusively demonstrated how well-planned and determined multidisciplinary community-based prevention programmes can have a major impact on changes in lifestyle and by extension on the cardiovascular disease burden (Puska & Jaini 2020). Furthermore, type 2 diabetes can be prevented or its onset postponed by changes in lifestyle as well as with medical treatment of risk factors (Tuomilehto et al 2001; Knowler et al 2002).

2.1.6 ECONOMIC IMPACT OF THE OBESITY-TYPE 2 DIABETES-CVD TRIFECTA

In addition to the global burden of morbidity and mortality, the interplay of CVD, type 2 diabetes and obesity, carries a heavy macroeconomic burden.

Across 32 European countries in 2017, stroke alone was estimated to have cost 60 billion euros, 45% of which were health care system costs. This represented 1.65% of the health budgets of these countries. Furthermore, five billion euros were costs to the social care system, 16 billion euros for informal health costs and 13 billion euros in production losses due to morbidity or premature mortality (Luengo-Fernandez et al 2020).

In the USA in 2013, costs of CVD, including CHD and stroke but excluding hyperlipidaemia and hypertension, amounted to 231.1 billion US dollars. Diabetes incurred costs of 101.4 billion dollars in that same year. It was also estimated that spending on risk factors such as treatment of hypertension, hyperlipidaemia, obesity and tobacco cessation were increasing the fastest (Dieleman et al 2016). A systematic review from 2011 assessing the worldwide costs of obesity found that 0.7-2.8% of a country's total health expenditure was spent on direct costs associated with obesity (Withrow et al 2011).

Considering direct costs as well as loss of productivity due to absence from work or early death, Bommer et al (Bommer et al 2018) estimated the global costs of diabetes to be 1310 billion US dollars in 2015. They also projected that the costs will continue to rise by 2030, even if globally set goals in addressing the disease are met. In Finland, it has been estimated that three quarters of the total costs of diabetes between years 2002-2011 incurred from loss of productivity (Koski et al 2018).

A study on the cost of type 2 diabetes in the UK estimated that 44.2% of the cost of its complications were due to CVD, and 35.3% of the overall cost of type 2 diabetes (Hex et al 2012). According to a systematic review of similar studies, treatment of CVD accounted for 20-49% of the overall cost of treating type 2 diabetes (Einarson et al 2018).

2.2 INFLAMMATION

Inflammation was first defined by the Roman medical writer Celsus (1st century AD) as *rubor et tumor cum calore et dolore* (redness, swelling, warmth, pain). Later the Greek physician Galen (129 to 216 AD) added loss of function (*functio laesa*) into the definition that still remains in use today (Scott et al 2004). Pathologically speaking, inflammation can be described as a local response to cellular injury. Vascularised tissues react to tissue damage or infections and invite cells and molecules from circulation to the site in need (Kumar et al 2018; Netea et al 2017). Based on the origins of the word inflammation, its name is thus appropriate: *inflammare* in latin means to ignite, to excite or to set on fire.

Inflammatory stimulus triggers this combined vascular and cellular response and a large number of chemical factors derived from cells or plasma mediate the response (Kumar et al 2018). Only some of these mediators of inflammation are known while a bulk of them are yet to be identified and studied. The mediators may be pro- or anti-inflammatory with local and systemic interplay. These intricate processes take place at molecular, immunological and physiological levels and therefore it is difficult to define inflammation comprehensively.

For the purpose of this thesis, subclinical inflammation will be defined first by generally defining inflammation, then by describing inflammation as the driving process of atherosclerosis, its role in metabolic disorders such as obesity and type 2 diabetes, its links with periodontal disease and finally by detailing different markers of inflammation and examining some of the risk factors for subclinical inflammation.

2.2.1 SUBCLINICAL INFLAMMATION

2.2.1.1 Definition

Inflammation may be acute or chronic. Acute inflammation is, more often than not, a protective response to an invading pathogen and/or tissue damage, that aims to eliminate the cause of the injury. Chronic inflammation may either follow unresolved acute inflammation, such as an oral infection by periodontal pathogens, or develop in a chronic nature from the beginning, such as atherosclerosis.

Chronic inflammation can be thought of as having three stages: an adaptive inflammatory response takes place after an initial trigger such as a stressor. Then a long-term poorly adaptive phase takes place, which further leads to complications (Reilly & Saltiel 2017). Chronic inflammation is characterised

by a prolonged response (weeks or months) with subtle, sometimes concealed and usually harmful effects.

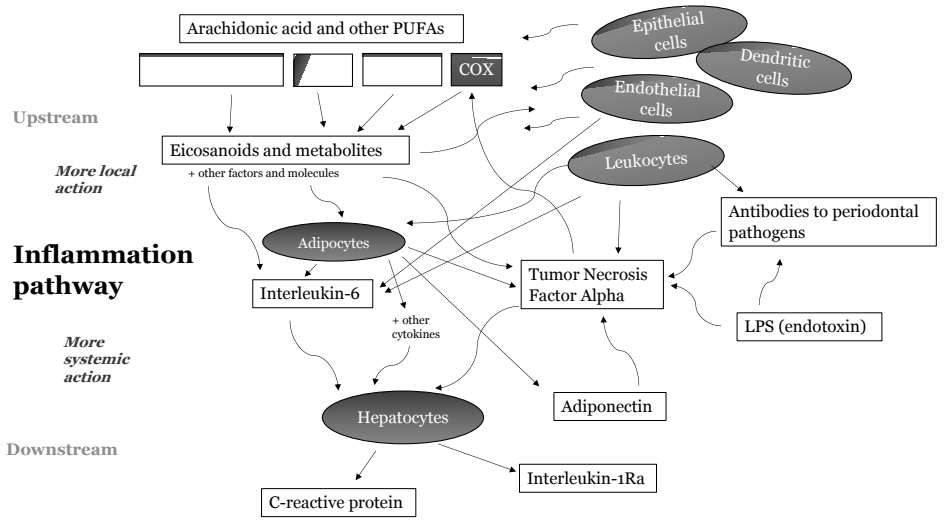

Figure 4 A simplified image showing some of the molecular pathways of inflammation. Straight arrows signify known production/metabolism and curved arrows known modulation. PUFA= polyunsaturated fatty acid; LOX= lipoxygenase; CYP450= cytochrome P 450; COX= cyclooxygenase; LPS= lipopolysaccharide; Interleukin-1Ra= interleukin-1 Receptor antagonist

In chronic inflammation, the cells invited to the damage site are mostly macrophages and lymphocytes (Kumar et al 2018). They produce peptides known as cytokines to mediate inflammation. When chronic inflammation prevails or progresses insidiously, it is often referred to as subclinical inflammation. In this case the classic symptoms described by Celsus are usually not visible to the naked nor the experienced eye, and may only be detectable via laboratory measurements of circulating markers of inflammation, such as cytokines. Cytokines and other markers of inflammation are described in more detail in sections 2.2.2 and 2.2.3.

Inflammation pathways include a complex set of molecules, cells and events. Mediators of inflammation can be categorised into peptide or lipid derived, or upstream and downstream. A simplified image of some of these pathways, including the molecules of interest to this thesis, is presented in Figure 4.

For the purpose of this thesis, the term subclinical inflammation will be used to describe a state of systemic, low-grade, chronic inflammation.

2.2.1.2 Atherosclerosis

Atherosclerosis is a thickening of arterial walls and their loss of elasticity. It is characterised by atherosclerotic plaques that reduce the size of the arterial lumen and that may rupture and locally occlude the artery (Kumar et al 2018). Its manifestations include CHD, stroke and peripheral artery disease.

The development of atherosclerosis is currently explained with the response-to-injury theory, in which endothelial injury leads to a chronic inflammatory response in the arterial wall. In 1999, New England Journal of Medicine published an influential review by Ross defining atherosclerosis as an inflammatory disease (Ross 1999).

According to the hypothesis, excessive production and tissue deposition of endogenous cholesterol and other lipids impair endothelial cell function, allowing for accumulation of lipoproteins within the intima layer of the arterial wall, generating oxidized low-density lipoprotein (LDL) and cholesterol crystals. These in turn activate an inflammatory response that may progress into chronic inflammation. The hypothesis has become more sophisticated over the years (Raggi et al 2018), however the core of it remains the same.

Atherosclerosis involves a state of subclinical inflammation. Yet the pathophysiology of atherosclerosis is intricate and although the contribution of inflammation to it is becoming clearer, it is not yet fully understood (Raggi et al 2018). It is known however that metabolic disorders such obesity and diabetes, and according to increasing evidence periodontal disease, are independent risk factors of atherosclerosis.

2.2.1.3 Metabolic disorders

Metabolic disorders such as the metabolic syndrome, characterised by central obesity, abnormal lipid levels, hypertension and raised fasting glucose, and type 2 diabetes have not been traditionally thought of as inflammatory diseases. However, currently it is being suggested that subclinical inflammation plays an important role in their pathogenesis.

In recent years, a rising paradigm termed ‘metaflammation’ has gained ground in the discussions on the pathogenesis of many chronic diseases, especially metabolic ones. In a review published in Nature 15 years ago, Hotamisligil proposed several evolutionary foundations for why metabolic diseases may be linked to inflammatory processes (Hotamisligil 2006). He suggested the term ‘metaflammation’, characterised by low-grade inflammation combined with high nutrient intake, to capture the crosstalk between immune and metabolic pathways that have been preserved from insects to mammals (Hotamisligil 2017).

The 'fat body' in the fruit fly is in charge of functions of nutrient storage and immune defence. This organ is proposed to have been developed into adipose tissue, the liver and cells of the immune and circulation systems in humans (Hotamisligil 2017), and adipose tissue is what is thought to be at the core of metaflammation (Franceschi et al 2018).

Obesity is strongly associated with metaflammation and it has also been established that metaflammation contributes to the onset of insulin resistance (Gregor & Hotamisligil 2011). The interplay between LDL cholesterol and the inflammatory response in atherosclerosis could also be considered as metaflammation as it relates at least partially to an individual's high intake of LDL cholesterol.

Current knowledge on the role of inflammation as a risk factor for obesity, type 2 diabetes, CVD and death from any cause is summarised in section 2.3.

2.2.1.4 Periodontitis

Periodontitis is an oral disease of the gums. It is a bacterial infection that has developed into a state of chronic inflammation, and it causes destruction of the tooth-supporting apparatus and may eventually lead to tooth loss. Two mechanisms have been proposed as to how periodontitis could contribute to systemic disease. In the direct mechanism, bacteria or parts of them may have access to systemic circulation through the inflamed periodontal tissues. The indirect mechanism involves chronic periodontitis as a source of chronic inflammation that may contribute to the pathogenesis of other inflammatory diseases (Winning & Linden 2015).

2.2.1.5 Other risk factors of subclinical inflammation

It has been established that smoking increases systemic inflammation (Messner et Bernhard 2014). Smoking cessation on the other hand leads to reduced levels of inflammation markers (McElroy et al 2019; Reichert et al 2009), suggesting that the state of subclinical inflammation increased by smoking is reversible in the early stages and possible to reduce. Exposure to other xenobiotics such as air pollutants, hazardous waste or industrial chemicals also play a role in the development of subclinical inflammation (Furman et al 2019).

The diet-physical inactivity-obesity trio affects inflammation in many ways. LDL cholesterol and excessive adipose tissue are established drivers of inflammation as described earlier in this section. High-density lipoprotein (HDL) on the other hand has been identified as an anti-inflammatory agent in the process of inflammation, although it is also known to have pro-

inflammatory effects (Barter et al 2007; Ajala et al 2020). Increasing physical activity and losing weight have also been linked with reducing inflammation (Pedersen 2017; Herder et al 2009).

Arguably the increased incidences of type 2 diabetes and CVD reflect the globally ageing population. Subclinical inflammation progresses with ageing, thus contributing to the pathogenesis of age-related diseases. This phenomenon has recently been referred to as 'inflammageing' (Franceschi et al 2018), a concept closely linked with metaflammation, described above.

Inflammageing has been hypothesised to be driven amongst others by gut microbiota (the latest 'organ' identified in the human body) and by chronic conditions caused by microbes, such as periodontitis (Ebersole et al 2010, Ferrucci & Fabbri 2018). Other risk factors for subclinical inflammation, which are not necessarily linked with age, include chronic stress and poor sleep (de Punder & Pruimboom 2015; Furman et al 2019).

Inflammation may also have developmental origins, being affected by an individual's childhood 'exposome'. This term assumes that a person's total health, which is defined as an individual's genetic factors combined with interaction with the environment, is affected by exposures starting already in the perinatal period. Childhood circumstances, such as early-life stress and childhood obesity, may lead to the development of the individual into a pro-inflammatory phenotype (Furman et al 2019), whereas greater microbial exposure in infancy may be associated with reduced risk of chronic inflammation in adulthood.

Finally, there is no denying that an individual's genotype plays perhaps the ultimate role in the development of inflammation. Many studies have demonstrated that gene polymorphisms of a variety of inflammation markers affect levels of inflammation (Walston et al 2007; Hage & Szalai 2009; Luotola et al 2010).

2.2.1.6 Inflammation markers

Inflammation markers can be thought of as molecular biomarkers found in fresh or frozen blood samples. Their levels give information on the inflammation status of an individual at the time the sample was drawn. Inflammation markers are produced by a variety of cells participating in the inflammation processes such as leukocytes (including macrophages, dendritic cells), epithelial cells (including endothelial cells), adipocytes and hepatocytes.

Inflammation markers may be categorised into peptide-derived and lipid-derived markers of inflammation. They can also be characterised as predominantly pro-inflammatory or anti-inflammatory, although elevated

levels of an anti-inflammatory marker may be a sign of subclinical inflammation as much as those of pro-inflammatory markers.

2.2.2 PEPTIDE MARKERS OF INFLAMMATION

Peptide markers of inflammation include amongst others acute-phase proteins, such as C-reactive protein (CRP) and interleukin-1 receptor antagonist (IL-1Ra), and cytokines such as interleukin-6 (IL-6), tumour necrosis factor alpha (TNF-alpha) and adiponectin, as well as antibodies to pathogens.

A coacting cascade of cytokines and leukocytes initiates and later sustains chronic inflammation. At the local level, cytokines such as IL-1, IL-6 and TNF-alpha act together to activate leukocytes, and their role is especially important in the acute-phase reactions, which are not specific to acute inflammation. The anti-inflammatory adiponectin inhibits inflammation and cell death. Antibodies to pathogens may act as triggers to the local and systemic immune response.

For the purpose of this thesis, only a selection of the well-known markers of inflammation such as cytokines is presented.

2.2.2.1 C-reactive protein

First identified in the early 1930s, C-reactive protein is an acute-phase protein synthesised in the liver. Although it may have beneficial effects during acute inflammation, its prolonged production in chronic inflammatory states has traditionally been thought of as pro-inflammatory. It is already in routine use as a biomarker for acute infections and in recent years high-sensitivity CRP (hs-CRP) tests have also become available to assist clinicians in assessing an individual's risk for CVD, or other chronic diseases. For the purpose of this thesis, the shorter-term CRP will be used to signify hs-CRP, unless the more accurate term (hs-CRP) is warranted for specifying the actual laboratory test.

CRP is situated downstream in the inflammatory pathways and its production is stimulated by IL-6. It has classically been thought of as a pro-inflammatory substance, however it is known to also have anti-inflammatory effects, such as stimulating the production of the anti-inflammatory IL-1Ra (Del Giudice et al 2018). Recently it has been discovered that CRP exists in two isoforms, which may explain the ambiguous role it has been found to play in acute and chronic inflammation (Eisenhardt 2009).

Links between CRP and an array of systemic diseases have been uncovered in the past few decades. CRP is known to be associated with the metabolic

syndrome, type 2 diabetes as well as atherosclerosis and its complications (Ross 1999; Rutter et al 2004), although its role has been increasingly defined to be non-causal (Brunner et al 2008; CCGC 2011). Other diseases to be associated with CRP include rheumatoid arthritis, age-related macular degeneration and a number of neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (Luan & Yao 2018).

2.2.2.2 Cytokines

Cytokines are triggers of both the vascular and cellular responses in inflammation. Apart from leukocytes, they can be secreted by a number of cell types such as epithelial, endothelial and connective tissue cells. Some of the cytokines are involved only in acute inflammation, however most cytokines acting in acute inflammation are also present in chronic inflammation (Kumar et al 2018). Such are for example interleukins 1 and 6 as well as TNF-alpha). For the purpose of this thesis, we will examine more closely IL-1Ra, which is one of the members of the IL-1 superfamily.

Strictly speaking, IL-1Ra is not a cytokine, but an acute-phase protein synthesized in the liver. It was first identified in the 1980s, and is considered a member of the IL-1 family of cytokines (Arend 1998). It was initially called IL-1 inhibitor, as it is a natural regulator of biological activity of IL-1, master regulator of inflammation. Although IL-1Ra is predominantly an anti-inflammatory protein, its elevated levels are usually considered as a sign of on-going inflammation, and it seems to play at least a dual role in inflammation (Dinarello 2017). Similarly to CRP, it has been identified to have more than one isoform, suggesting it is a family of proteins rather than just a single molecule (Arend 1998).

Interleukin-6 is a cytokine, which was also first identified in the 1980s. It is produced mostly by leukocytes such as macrophages and monocytes, but also by adipocytes and endothelial cells. Its major effect is to stimulate production of CRP and other acute phase proteins and it also induces changes in body temperature. It is classically categorised as a pro-inflammatory cytokine and is a commonly used biomarker of inflammation. However, its role in inflammation is no longer considered unambiguous. It has been found that IL-6 inhibits the production of IL-1 and TNF-alpha, meaning that elevated levels of IL-6 may act as a negative feedback loop, contributing to the end of the inflammatory response (Del Giudice 2018).

TNF-alpha is a cytokine that was first identified in 1975. It is predominantly secreted by leukocytes such as macrophages and lymphocytes as well as adipocytes. Its role in inflammation includes induction of the expression of cyclo-oxygenase and lipoxygenase enzymes and of the production of IL-6. It is

considered a pro-inflammatory cytokine, although it has been suggested to have anti-inflammatory effects as well (Zakharova et al 2005).

IL-1 (and by extension IL-1Ra), IL-6 and TNF-alpha have all been linked with a variety of chronic inflammatory diseases, including atherosclerotic diseases, rheumatoid arthritis, and the metabolic syndrome (Ross 1999; Gregor & Hotamisligil 2011; Clarke et al 2018). These cytokines have also been associated with glucose metabolism abnormalities, such as impaired glucose tolerance, insulin resistance and impaired beta cell function (Shi et al 2019).

2.2.2.3 Adiponectin

Adiponectin is an adipokine first identified in 1995. It is secreted predominantly by adipocytes, although other cells express low levels of adiponectin. Its regulators include IL-6, TNF-alpha and other proinflammatory factors (Fantuzzi 2008). Adiponectin is one of the best-known anti-inflammatory peptides and it also protects cells from apoptosis. As for the cytokines described in the previous section, adiponectin plays a role in glucose metabolism by strongly suppressing hepatic gluconeogenesis and locally promoting insulin sensitisation. Elevated levels of adiponectin have been associated with rheumatoid arthritis and type 1 diabetes, whereas low levels of adiponectin have been linked with obesity, type 2 diabetes, atherosclerosis and CVD (Wang & Scherer 2016, Fantuzzi 2008)

2.2.2.4 Antibodies to Gram-negative periodontal pathogens

The most widely studied periodontal pathogens in relation to atherosclerosis are *Actinobacillus actinomycetemcomitans* (nowadays known as *Aggregatibacter actinomycetemcomitans*) and *Porphyromonas gingivalis*, both of which are Gram-negative bacteria. They are serologically heterogeneous as species and they have both been identified as members of the 'periodontopathic microbiota'.

A actinomycetemcomitans and *P gingivalis* provoke and to some extent modulate a local immune response (Lamont et al 2018). Their virulence characteristics include endotoxin activity, leukotoxin production and adherence and invasion of host cells (Fives-Taylor 2000; Holt et al 1999). In periodontitis, bacteria or their parts may be able to enter into the systemic circulation where bacterial components can induce and promote systemic inflammation and proatherogenic responses. These two bacteria and their components trigger a local and systemic antibody response, namely through production of IgA- and IgG- class antibodies. Plasma (and saliva) antibody measurements are used for diagnostics, classification and prognosis of

periodontitis as well as to estimate its activity and success of treatment (Kinane et al 1999).

The DNA of both A Actinomycetemcomitans and P gingivalis as well as viable pathogens have been identified in human atherosclerotic plaques (Haraszthy et al 2000; Kozarov et al 2005). Moreover, antibodies to periodontal pathogens have been linked with obesity, type 2 diabetes and CVD (Larvin et al 2020; Demmer et al 2012; Arboleda et al 2019) as well as with rheumatoid arthritis and systemic lupus erythematosus (Graves et al 2019).

2.2.3 LIPID-DERIVED MARKERS OF INFLAMMATION

Eicosanoids and their metabolites are derived from lipids such as arachidonic acid. For the purpose of a simplified categorisation lipopolysaccharide (LPS), a glycolipid, is here presented under lipid-derived markers.

2.2.3.1 Lipoplysaccharide or endotoxin

Lipopolysaccharide (LPS) is a unique glycolipid found in the outer membrane of Gram-negative bacteria, such as previously described *A actinomycetemcomitans* and *P gingivalis*. The term endotoxin is widely used synonymously to mean LPS. Endotoxin is a bacterial product that can be found in the plasma of healthy individuals (Goto et al 1994), as Gram-negative bacteria may colonise the gastrointestinal, respiratory and genitourinary tracts. Endotoxin is proinflammatory in nature, and it may be produced during acute infections or chronic inflammatory conditions such as periodontitis. Endotoxin has been linked with atherosclerosis by activating leukocytes, increasing oxidative stress and modifying lipoprotein metabolism (Stoll et al 2004).

2.2.3.2 Eicosanoids and related oxylipins

Eicosanoids, and their metabolites, are a group of bioactive lipids that are derived from 20-carbon polyunsaturated fatty acids (PUFAs), most notably from arachidonic acid. The number of carbons in their precursors has given eicosanoids their name (*eicosa*, twenty in Ancient Greek). The main enzymes involved in their production are cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP450), however eicosanoids may be produced through non-enzymatic mechanisms as well. They are categorised as prostaglandins, thromboxanes, leukotrienes and lipoxins.

Nobel prizes in medicine and chemistry have been given to investigators of eicosanoids after discoveries in the 1930s (identification of prostaglandin,

Nobel in medicine 1970) and in the 1960s (derivation of classical eicosanoids from arachidonic acid, Nobel prize in medicine 1982; synthetisation of prostaglandins, Nobel prize in chemistry 1990). In this light, it is interesting to note that still so little is known on eicosanoids and their metabolites. It was not until recently that great leaps in laboratory and computer methodologies suitable for mass detection and identification of eicosanoids were taken, giving rise to advanced metabolomics.

Eicosanoids are important mediators of inflammation especially at local level, for example by modulating cytokines and other inflammatory mediators (Ricciotti & FitzGerald 2011; Dennis & Norris 2015). Through similar functions, they play a role also on the systemic level. Cytokines such as IL-1 and TNF-alpha also act as back-regulators of eicosanoids through activation of the COX pathways (Dennis & Norris 2015).

2.3 CURRENT KNOWLEDGE ON THE LINKS BETWEEN INFLAMMATION AND THE OBESITY-TYPE 2 DIABETES-CARDIOVASCULAR DISEASE TRIFECTA

Obesity, type 2 diabetes and CVD are known to be linked. They also share a variety of risk factors such as an unhealthy diet and physical inactivity. All of them have been associated with subclinical inflammation, but could subclinical inflammation be what links them inextricably together (Figure 5)?

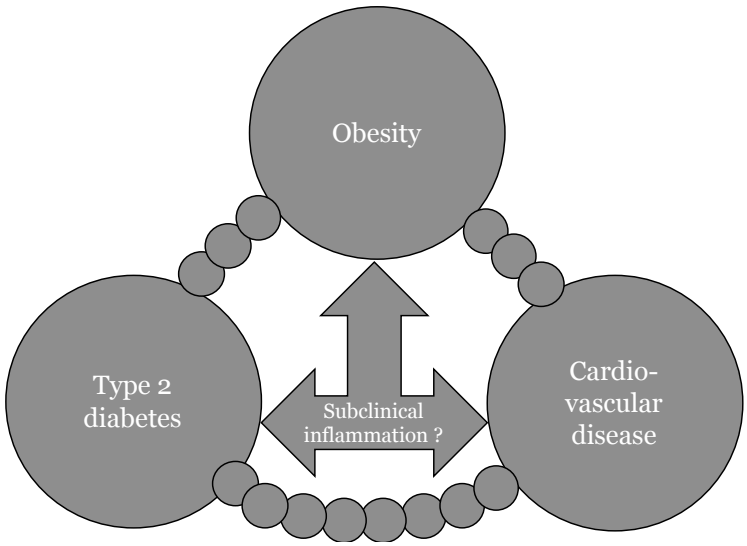

Figure 5 Associations between obesity, type 2 diabetes, cardiovascular disease and inflammation.

2.3.1 INFLAMMATION AND OBESITY

Overweight, obesity and weight gain have been associated with subclinical inflammation (Hotamisligil 2006; Fogarty et al 2008). Adipose tissue has been identified as a site of production of inflammatory markers and of leukocyte accumulation (Hotamisligil et al 1993; Weisberg et al 2003) and to play a role in 'metaflammation' (Hotamisligil 2017). Adipose tissue seems to respond to overnutrition with an immune response, however the initial trigger remains unknown (Reilly & Saltiel 2017).

Based on this, it has been suggested that adipose tissue inflammation is an attempt to adapt to obesity (Reilly & Saltiel 2017). According to animal studies, inflammation in other tissues and the liver is also activated in obesity (Mayoral Monibas et al 2016). However, these are proposed to be reversible states, whereas adipose tissue inflammation seems to continue despite weight loss, at least in mice (Schmitz et al 2016). In human studies of obese individuals who have undergone bariatric surgery, overall inflammation may resolve (Askarpour 2019). However, there is also evidence that adipose tissue inflammation does not resolve in everyone (Schmitz et al 2016).

There has also been research suggesting that inflammation may play a role in the process of weight gain. The Atherosclerosis Risk in Communities (ARIC) study and the Malmö Preventive Studies found that inflammation-sensitive plasma proteins such as fibrinogen, and other putative markers of inflammation, predicted weight gain in middle-aged adults (Duncan et al 2000, Engström et al 2003). The MONICA/KORA study in middle-aged adults found an independent association between elevated levels of inflammatory markers, including CRP, and weight gain (Holz et al 2012). In a study in Brazil, it was found that CRP predicted changes in the BMI-for-age z scores of children under 10 years of age (Lourenço et al 2014).

No study however has presented definitive results as to whether subclinical inflammation is a cause or consequence of obesity. Furthermore, there is no clear understanding on the role of different inflammation markers on weight gain.

2.3.2 INFLAMMATION AND TYPE 2 DIABETES

The role of inflammation in the development of type 2 diabetes has been hypothesised and some data have been presented to support this hypothesis (Pickup & Crook 1998; Kolb & Mandrup-Poulsen 2005; Wang et al 2013). A meta-analysis including 10 prospective studies showed an association of elevated levels of CRP and IL-6 with an increased risk of type 2 diabetes, demonstrating that subclinical inflammation precedes the development of type 2 diabetes (Wang et al 2013). Type 2 diabetes is also closely linked to

obesity and CVD, which are both known to be associated with subclinical inflammation.

TNF-alpha produced by the adipose tissue has been shown to induce insulin resistance (Hotamisligil 1993) and has been suggested to be one of the links between obesity and type 2 diabetes (Shoelson et al 2006). An experimental study infusing TNF-alpha in healthy humans showed that it inhibits whole-body glucose uptake, supporting the role of TNF-alpha in insulin resistance (Plomgaard et al 2005).

Results from the Relationship between Insulin Sensitivity and Cardiovascular Disease (RISC) Cohort study are in line with a strong association between insulin resistance and inflammation (de Rooij et al 2009). The Rotterdam study found associations between a number of novel inflammatory markers with different stages from normoglycemia to type 2 diabetes, suggesting associations of inflammation with incidence of prediabetes, need for insulin therapy and type 2 diabetes (Brahimaj et al 2017).

The MONICA/KORA Augsburg study using a case-cohort design found an association between a group of cytokines and incident type 2 diabetes, which was not affected by adjustments for IL-6 and CRP (Herder et al 2006). A Finnish study using FINRISK97 as the discovery cohort and Health 2000 as a replication cohort identified a number of novel biomarkers as well as adiponectin, CRP and IL-1Ra, associated with incident type 2 diabetes (Salomaa et al 2010).

The Whitehall II study examining IL-1Ra trajectories before the diagnosis of a type 2 diabetes found that IL-1Ra levels showed an accelerated increase in the last years preceding diagnosis, suggesting a presence of an anti-inflammatory response in the years leading up to type 2 diabetes (Carstensen et al 2010). The Diabetes Prevention Program in the USA showed associations between inflammation markers including adiponectin, and incident type 2 diabetes, and furthermore that the studied diabetes interventions (intensive lifestyle modification and metformin) modified these associations (Goldberg 2019).

In summary, inflammation, as portrayed by elevated levels of inflammation markers, seems to precede development of type 2 diabetes, and there is evidence suggesting that this association also exists with the development of prediabetes, insulin resistance and start of insulin therapy. Early interventions by lifestyle change and metformin may modify these associations.

2.3.3 INFLAMMATION AND CARDIOVASCULAR DISEASE

In addition to hypercholesterolemia, defined as increased plasma total and LDL cholesterol, biological factors, such as age and the male sex are known

independent risk factors of CVD. Furthermore, behavioural factors such tobacco smoking, lack of physical activity and harmful use of alcohol increase the risk of CVD. And finally, conditions involved in and associated with the metabolic syndrome, namely decreased levels of HDL cholesterol, elevated blood pressure, high triglyceride levels and obesity, especially abdominal obesity, as well as diabetes, are also major risk factors for CVD. They are all linked with inflammation, as described in previous sections, which underlines the complexity of the links that exist between inflammation and CVD.

A significant and defining role in the formation of atherosclerotic plaques is played by LDL cholesterol (Goldstein & Brown 2015). However, since the definition by Ross in 1999 of atherosclerosis as an inflammatory disease (Ross 1999), it has been all but established that inflammation plays a mediatory, or at the very least a contributory role in the development and progression of atherosclerosis (Ridker et al 2008; Ridker et al 2017; Bäck et al 2019).

The two main complications of atherosclerosis are MI and stroke. Research associating inflammation and the risk of these two CVD events in the past 20 years has been ample and several meta-analyses have been published confirming the link between inflammation and incident CVD (Emerging Risk Factors Collaboration 2010; Kaptoge et al 2014; Piccardi et al 2017).

By far the most investigated of the inflammation markers in relation to CVD is CRP, with evidence supporting its association with incident CVD from cohort studies such as the MONICA Augsburg Survey, the Health ABC study, the Framingham Heart Study, the Helsinki Heart Study, the British Regional Practice study, the Multi-Ethnic Study of Atherosclerosis as well as a large individual participant meta-analysis (Koenig et al 1999; Ridker 2001; Cesari et al 2003; Wilson et al 2005; Emerging Risk Factors Collaboration 2010; Yeboah et al 2012).

CRP has been shown to consistently predict CVD, but the strength of the prediction is still under ongoing debate and many argue that its role should be reviewed (Hermans et al 2019; Avan et al 2018; Yousuf et al 2013). Although CRP has been associated with atherosclerotic plaque presence and plaque area in cross-sectional analyses, results from the Tromsø study suggested that its effect is through mechanisms other than promotion of atherosclerotic plaque formation and progression (Eltoft et al 2017). And finally, Mendelian Randomization studies from the past decade or so have challenged its potential as a causal factor, virtually ruling causality between CRP and CVD out (Nordestgaard & Zacho 2009; CCGC 2011; Zhuang et al 2019).

Cytokines such as IL-6 and TNF-alpha have also been identified as predictors of CVD, however their roles are yet to be clarified and established (Ridker et al 2000; Cesari et al 2003; Luc et al 2003; Pai et al 2004; Danesh et al 2008; Zhang et al 2018). TNF-alpha has been suggested to play a direct role in the

development of atherosclerosis (McKellar et al 2009), whereas there is evidence from a study in mice pointing at IL-6 deficiency enhancing atherosclerotic plaque formation (Schieffer 2004).

A number of clinical trials exploring the usefulness of anti-inflammatory therapy for atherosclerosis have been conducted, namely Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin (JUPITER), Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) and Cardiovascular Inflammation Reduction Trial (CIRT) trials (Ridker et al 2008; Ridker et al 2017; Ridker et al 2019). These clinical trials have strengthened understanding of the connection between subclinical inflammation and CVD, while also raising a number of further questions such as the appropriate target cytokine or function in the inflammation pathways (Ridker 2019).

In short, inflammation has been established to play a contributory if not mediatory role in the development of atherosclerosis, and by extension, its thrombo-occlusive complications. However, the complexity of the different roles played by inflammation markers in subclinical inflammation and the development of CVD is yet to be untangled.

2.3.4 INFLAMMATION, PERIODONTITIS AND CARDIOVASCULAR DISEASE

Current understanding of periodontitis is based on an impaired innate host defence and promotion of inflammation driven by colonisation of keystone pathogens such as P gingivalis and aided by accessory pathogens. In other words, inflammation and dysbiosis develop at the same time and reinforce each other to drive chronic periodontitis in susceptible individuals (Lamont et al 2018). The chronic, inflammatory nature of periodontitis is central to its effects on the systemic level.

Links between periodontitis, inflammation and systemic diseases are not yet clear and for example associations between periodontitis and type 2 diabetes are considered to be bidirectional (Polak & Shapira 2017). On the whole, establishing causality between a number of conditions and periodontitis has been a challenge, and confounding is a further challenge in the interpretation of associations (Winning & Linden 2017). For periodontitis, one of these confounding factors is socioeconomic status, which is difficult to control for meaningfully and entirely.

Patients with periodontitis have elevated levels of inflammation markers both locally in gingival fluids as well as in circulation (Zekeridou et al 2019; Paraskevas et al 2008; Bretz al 2005). Furthermore, periodontal treatment seems to lower amongst others plasma levels of CRP, IL-6 and TNF-alpha,

affect the cardiovascular risk profile of periodontitis patients and improve metabolic control of those with diabetes (Teeuw et al 2014; Artese et al 2015).

There is growing consensus on periodontal disease being at least modestly associated with CHD and CVD, although not all studies have confirmed these associations (Larvin et al 2020; Dietrich et al 2013; de Boer et al 2014). Moreover, there are indications that genetic variations underlying irregular inflammatory reactivity may partially explain the links between periodontitis and CVD (Sanz et al 2020; Loos & van Dyke 2020).

In summary, periodontitis is a chronic inflammatory disease leading to elevated systemic levels of inflammation markers. Although there clearly is a link between periodontal and CVD, the driving elements of the association as well as the fundamental matter of causality remain an open question.

2.3.5 INFLAMMATION AND TOTAL (ALL-CAUSE) MORTALITY

Inflammation, including its acute forms, is a well-known enemy of critically ill patients and medical personnel of intensive care unit. However, systemic subclinical inflammation has also been linked with total (all-cause) mortality, and implicated for example in poor recovery and prognosis in patients discharged from intensive care units (Griffith et al 2016). Inflammation markers IL-6 and CRP have been most commonly associated with all-cause mortality.

Results from the MONICA/KORA Augsburg study and the European Prospective Investigation into Cancer (EPIC) Norfolk studies, amongst others, suggested that elevated levels of CRP are associated with cardiovascular and all-cause mortality (Koenig et al 2008, Ahmadi-Abhari et al 2013). However, another study in a Japanese population showed that CRP did not seem to predict mortality in women (Nisa et al 2016). A later meta-analysis confirmed that elevated serum levels of CRP can predict the risk of all-cause death and proposed that inconsistent results in the past could be attributed to ethnic, age or gender differences in study populations (Li et al 2017).

While the role of CRP in all-cause mortality remains somewhat under debate, the link between IL-6 and total mortality seems established. For example, results from the Rancho Bernardo study suggested associations of elevated IL-6 levels with increased risk of all-cause deaths, reduced survival and decreased lifespan in older adults (Lee et al 2012; Wassel et al 2010). The Memory and Morbidity in Augsburg Elderly Study (MEMO), a follow-up of the MONICA Augsburg S2 study, proposed elevated levels of a number of cytokines, including IL-6 and TNF-alpha, to be associated with all-cause mortality, although the latter's association did not remain significant after adjusting for confounders in a Cox Proportional Hazards model (Baune et al 2011). The

Whitehall II study has later confirmed IL-6 to be a good prognostic marker for all-cause and cardiovascular mortality, in both short and long terms (Singh-Manoux et al 2017).

Inflammation has been shown to be linked with total mortality and IL-6 seems so far to be the most promising prognostic marker. The role of other inflammation markers in survival remains unclear.

3 AIMS OF THE STUDY

The aim of this PhD study is to analyse whether subclinical inflammation precedes and predicts obesity, type 2 diabetes, CVD and deaths from any cause.

The specific objectives are:

1) To analyse whether increased concentrations of inflammation markers (hs-CRP, IL-1Ra, IL-6, TNF-alpha, high-molecular weight [HMW] adiponectin) precede weight gain, increasing waist circumference and increasing body fat percentage (% body fat) and whether these inflammation markers can predict the development of obesity in a longitudinal setting

2) To analyse the association of plasma eicosanoid profiles with the risk of incident type 2 diabetes

3) To analyse whether inflammation markers (hs-CRP, IL-6, TNF-alpha) predict CVD and all-cause (total) mortality

4) To analyse the associations of serum endotoxin, antibodies to periodontal pathogens (IgA and IgG for *P gingivalis* and *A actinomycetemcomitans*) and inflammation markers (hs-CRP, IL-6 and TNF-alpha) with the risk of incident CVD

4 METHODS

4.1 Study cohorts and ethics

The study cohorts for this thesis include those from the FINRISK 1992 and 2007 studies, DILGOM 2007 study and the FHS Offspring Study. Table 2 includes summary information on the cohorts used for each paper. Below the table is a more elaborate description of each study cohort with information on data collection and ethical review of each respective study plan.

Table 2. *Summary of cohorts used*

	FINRISK 1992	FINRISK 2002	DILGOM 2007 and 2014	FHS Offspring
Ages	25-64 years	25-74 years	25-74 years	6-70 years
Sexes	Both	Both	Both	Both
Geographical areas	4	6	5	NA
Follow-up time	10 years (III, IV)	15 years (II)	7 years (I) 10 years (II)	NA
Outcomes of interest/ incident cases (paper)	CVD: n=205 (III), n=185 (IV) CHD: n=151 (III) Death: n=183 (III)	Type 2 diabetes: n=586 (II)	Changes in weight (I) % body fat (I) waist-to-hip ratio (I) BMI (I) Type II diabetes: n=248 (II)	Type 2 diabetes: n=236 (II)
Explanatory variables of interest (paper)	hs-CRP (III, IV) TNF-alpha (III, IV IL-6 (III, IV) IgA and IgG antibodies to periodontal pathogens (IV) endotoxin (IV)	Eicosanoids (II)	hs-CRP (I) IL-1Ra (I) IL-6 (I) TNF-alpha (I) HMW Adiponectin (I) Eicosanoids (II)	Eicosanoids (II)
Study design	Case-cohort	Cohort	Cohort	Cohort
Sample size	7,927	13,498	6,258	5,124
Participants (response rate %)	6,051 (76%)	8,798 (65.2%)	5,024 (80%) Follow-up in 2014: 3735 (82%)	2,886
Final study population (paper)	836 (III) 783 (IV)	6,548 (II)	3,369 (I) 3,905 (II)	2,886 (II)
Sub-populations	N/A	3,092 (with OGTT) 2951 (with cytokines)	1,312 (with body fat % and hs-CRP at follow-up)	N/A

4.1.1 FINRISK Cohorts

The National FINRISK Study was launched in 1972, originally as an evaluation of the North Karelia Project. Those population-based health examination surveys formed a base for the cohort study that today is known as the FINRISK study. First the study concentrated on monitoring CVD risk factors, but more recently the study has considered major non-communicable diseases with an objective to inform policy making in Finland. The Finnish Institute for Health and Welfare (THL, formerly known as National Institute for Health and Welfare, and previously as the National Public Health Institute KTL) has been the coordinator of the project from the start.

The FINRISK cohorts are large population-based random samples of adults aged 25 to 74 years who have lived in Finland since at least one year, who have a personal identification code and who are from selected geographical areas. Each survey year has added a new cohort to the FINRISK study as each of them draws a new independent sample of individuals from the Finnish Population Information system. The surveys have included questionnaires, health examination by trained nurses and blood samples.

Follow-up of the FINRISK cohorts is conducted using national health care registers, which are described in more detail in chapter 4.2 Finnish national registers. These registers are regulated by a solid legal framework and they cover the entire country. Only persons who permanently move out of the country and give up their Finnish nationality are lost to follow-up.

4.1.1.1 FINRISK 1992

In 1992, the stratified random sample included men and women aged 25 to 64 years, drawn from four geographical areas according to the WHO MONICA survey protocol (Vartiainen et al 1994, WHO MONICA 1988). Out of the total sample of 7,927 individuals, 76% participated in the study (n=6,051). A self-administered questionnaire, physical examination and a venous blood sampling formed the baseline survey protocol. The questionnaires were sent to the participants in advance. Participants then attended a health examination by nurses trained for that specific purpose.

The nurses used standardised methods to measure blood pressure, waist and hip circumferences as well as height and weight. Participants had been advised to fast at least for four hours and avoid heavy meals before sampling, because after the examination, venous blood samples were drawn, and a central laboratory conducted the investigations. The venous blood samples were then stored at -20°C to allow for further investigations in the future.

The FINRISK 1992 study population was used for papers III and IV. Using linkages to national health care registers (explained in more detail in chapter 4.2 Finnish national registers), we identified incident CHD events, CVD events and death by any cause (numbers summarised in table 2), and a stratified subcohort (n=400) for the case-cohort study design, explained in more detail in chapter 4.4 Statistical methods. The sample included 999 individuals. However, usable serum samples were no longer available for 161 participants (paper III) and 216 (paper IV). The final study population for analyses for paper III was 836 participants after further excluding two individuals with missing information on anthropometric measurements, and 783 participants for paper IV.

The FINRISK 1992 study plan was reviewed and accepted by the Ethical Committee of the National Public Health Institute on 22 January 1997.

4.1.1.2 FINRISK 2002

In 2002, the stratified random sample included men and women aged 25 to 74 years, drawn from six geographical areas. Out of the invited 13,498 individuals, 8,708 (65.2%) participated. Similarly to 1992, the baseline survey protocol included self-administered questionnaires, physical examination by trained nurses and drawing of blood samples. In addition to giving the venous blood samples, 3,767 individuals 45 years and older, participated in a 2-hour oral glucose tolerance test (2-hour OGTT), which was carried out in a separate visit after an overnight (10-hour) fast. A panel of cytokines was quantified from blood samples of 2,951 individuals, and plasma samples from 8,292 individuals were successfully analysed with directed non-targeted liquid chromatography – mass spectrometry (LC-MS) (Watrous et al 2019, Lagerborg et al 2019).

To obtain the final study population, we used baseline data and register-based linkages (explained in more detail under a later section on national registers) to exclude individuals with prevalent or incident type 1 diabetes (n=79), cancer (n=1,085, excepting non-melanoma skin cancer) or on-going pregnancy (n=44), as explained in more detail in chapter 4.2 National registers and case definitions. Participants with missing values of relevant variables were also excluded (n=546). The final study population consisted of 6,548 individuals.

The FINRISK 2002 study plan was reviewed and accepted by the Ethical Committee for Epidemiology and Public Health of the Helsinki University Hospital District on 19 December 2001.

4.1.2 DILGOM 2007 AND 2014

In April-May of 2007, the 6,258 participants of the FINRISK 2007 cohort were invited to participate in the Dietary, Lifestyle and Genetic determinants of Obesity and Metabolic syndrome (DILGOM) study. Altogether 5,024 (80%) of the invitees participated in the DILGOM 2007 baseline study. The baseline study protocol included questionnaires, physical examination, blood sampling and 2-hour OGTTs.

In 2014, all living participants of the DILGOM 2007 baseline study were invited for follow-up. Altogether 3,735 (82%) of the invitees participated in the DILGOM 2014 follow-up study by returning the survey questionnaire. A total of 1,312 participants from two geographical areas, namely South-Western Finland and the capital metropolitan area, attended the health examinations. Before blood sampling, nurses measured the participants' weight, height and waist circumference following standardised protocols. They also measured the participants' body fat percentage (% body fat). The detailed protocols of the DILGOM study have been published previously (Borodulin et al 2018, Konttinen et al 2018).

The 2,423 participants who were not invited for the health examination in 2014, were sent instructions to measure and report their weight, height and waist circumference and a measuring tape. The validity of these self-reported measurements against the anthropometric measurements performed by trained nurses was confirmed in a previous study (Kanerva et al 2018).

For paper I, individuals with prevalent or incident (during 7-year follow-up) weight-loss causing diseases, such as cancer (except non-melanoma skin cancer), hyperthyroidism, human immunodeficiency virus (HIV) and tuberculosis were excluded from the analyses. Pregnancy at either baseline or follow-up was also used as an exclusion criterion. Visual inspection of the outcome measure distributions further led to exclusion of three individuals that appeared as extreme outliers. Finally, 366 individuals were excluded from the final study population resulting in 3,369 participants being included in the analyses.

For paper II, plasma samples from 4,903 participants of the DILGOM 2007 study were analysed with LC-MS. Similarly to the FINRISK 2002 cohort, we used linkages with registry information to identify cases and to exclude participants with prevalent type 1 diabetes, pregnancy and cancer (except non-melanoma skin cancer). Individuals with missing values of relevant covariates were also excluded and the final study population used as a replication cohort in paper II consisted of 3,905 individuals.

The DILGOM 2007 and 2014 study plans were reviewed and accepted by the Coordinating Ethical Committee of the Helsinki and Uusimaa Hospital

District (decision number 229/E0/2006) on 3 April 2007 and (decision number 332/12/00/2013) on 14 January 2014, respectively.

4.1.3 FHS OFFSPRING

The original cohort of the Framingham Heart Study's (FHS), including 5,209 men and women aged 28-62 years (first generation), was recruited between 1948 and 1952 as a random sample of two third of the adult population of Framingham, Massachusetts, USA. The children and spouses of the original cohort participants joined the FHS Offspring study cohort (n=5,124, second generation) in 1971. The participants from the second generation have been re-examined every 4-8 years thereafter.

Plasma samples of participants of the FHS Offspring study's eighth examination cycle, which took place between years 2005-2008, were analysed with LC-MS. The definition used for diabetes in the FHS Offspring cohort was either fasting plasma glucose ≥126 mg/dL (7.0 mmol/l), or non-fasting plasma glucose ≥200 mg/dL (11.0 mmol/l), or treatment with insulin or an oral hypoglycaemic agent (based on medical history of FHS examinations). A total of 771 participants were excluded due to baseline diabetes or missing data on relevant covariates. The final study population used as a replication cohort in paper II, consisted of 2,886 individuals.

The FHS Offspring study is ethically reviewed annually by the Boston University Medical Center's Institutional Review Board and other relevant institutional boards each time there are changes or additions to the research protocols.

4.2 FINNISH NATIONAL REGISTERS AND CASE DEFINITIONS

Finland hosts a variety of register data. The Act on National Personal Data Registers Kept under the Health Care System (556/1989) and subsequent decrees and related Acts, form the regulatory framework for the upkeep and use of register-based data. The personal identity number that every resident in Finland possesses, allows for wide linkages between registers to be used for research purposes, if appropriate permission is granted by the register controller.

4.2.1 FINNISH HEALTH CARE REGISTERS

4.2.1.1 National Hospital Discharge Register or Register for Health Care

The Care Register for Health Care (since 1994), formerly known and still often referred to as the National Hospital Discharge Register (1968-1993), is a register set up for the purpose of statistics, research and health planning. Data for the register are collected from health centres, hospitals and other institutions providing inpatient care on people who are treated either in these facilities or at home. The original National Hospital Discharge Register contained data on patients discharged from inpatient care at hospitals, the Care Register also includes discharge data from other health facilities, specialized ambulatory care and surgery as well as patient counts of inpatients in health facilities on the last day of the year. It also includes a special addition known as Invasive Cardiac Procedures Register, which was used to link data in papers III and IV, in addition to the Care Register for Health Care. The latter's description is available at: https://thl.fi/en/web/thlfi-en/statistics/information-on-statistics/register-descriptions/care-register-for-health-care

4.2.1.2 Causes of Death Register

The Causes of Death Register has been maintained by Statistics Finland since 1936. The Register used death certificates written by doctors and they include causes of death of persons residing permanently in Finland. The data in the Causes of Death Register are verified against data on deaths from the Population Information System of the Digital and Population Data Services Agency (in short The Finnish Digital Agency), and supplemented with further data available from the Finnish Digital Agency as well as other Statistics Finland data. The description of the Causes of Death Register is available at: https://tilastokeskus.fi/til/ksyyt/index_en.html

4.2.1.3 Drug Reimbursement and Drug Purchase Registers

The Drug Reimbursement and Data Purchase Registers are maintained by the Social Insurance Institution of Finland (Kela) since 1995 (for basic rate of reimbursement, since 1964 for special rate of reimbursement) and are available from a statistical database known as Kelasto. This information is updated on quarterly and annual basis. The medicines administered in the hospital are not reimbursable, so the information includes medicines purchased outside of hospitals. It is important to note that there are recurring amendments to eligibility conditions, which is why year-to-year comparisons

are not always appropriate. The registers descriptions are available at: https://www.kela.fi/web/en/drug-reimbursement-statistics

4.2.1.4 FINAMI and FINSTROKE registers

The FINAMI and FINSTROKE registers are population-based registers to which data were recorded on MIs and strokes, respectively, in selected areas of Finland and during the FINMONICA (1983-1992), FINAMI (1993-2002) and FINSTROKE (1992-97) studies (Salomaa et al 2005, Sivenius et al 2004). The FINAMI study was a continuation of the FINMONICA MI register aimed to record every clinically symptomatic MI and CHD death occurring in adults at least 25 years in four geographical areas during the 10-year period of 1993–2002. Trained nurses collected data from a variety of hospital and medico-legal documents using standardised data collection forms. The FINSTROKE study, on the other hand, was a continuation of the FINMONICA stroke register aiming to record all strokes occurring in adults aged 25 to 74 years in specific geographic areas of Finland during the 5-year period of 1992-97.

4.2.2 VALIDITY OF CARDIOVASCULAR DIAGNOSES IN ADMINISTRATIVE REGISTERS

In general, the quality of data in the Finnish health care registers is good (Sund 2012). Several validation studies of the CVD diagnoses have been previously published with regards to the FINMONICA, FINAMI and FINSTROKE registers (Pajunen et al 2005; Mähönen et al 1999; Leppälä et al 1999). These studies have invariably found that the cardiovascular diagnoses are reliable.

4.2.3 USE OF REGISTER DATA IN PAPERS I TO IV

To identify cases of CVD (papers III and IV) and those of type 2 diabetes (paper II), as well as for excluding participants with specific conditions (papers I and II), different register linkages were used. International Classification of Diseases (ICD) 9 diagnosis codes were in use in Finland until the end of 1995, after which ICD-10 codes have been used.

In paper I, using linkages with the Care Register for Health Care and the Causes of Death Register, we excluded DILGOM study participants with established weight-loss causing diseases prevalent at baseline or incident during the 7-year follow-up, such as cancer (excluding ICD-10 category C44), hyperthyroidism, HIV and tuberculosis. Cancer (excluding ICD-10 category C44) was also used as an exclusion criterion of FINRISK 2002 participants in paper II.

Furthermore, in paper II, we combined information from the Care Register for Health Care, the Causes of Death Register, and the Drug Reimbursement and Drug Purchase Registers using the Finnish personal identification number. For the first two, we used diagnostic codes to extract information on diabetes (E10-E14 for ICD-10 and 250*B for ICD-9). For Drug Reimbursement and Drug Purchase Registers, we identified cases of diabetes using Diabetes medication purchase ATC code A10 or the special reimbursement code for diabetes medications. In cases where a medicine purchase was the only fulfilled criterion, we required at least 3 purchases as well as exclusion of gestational diabetes using ICD codes. The date of first purchase of hypoglycaemic drugs or the onset of special reimbursement was taken as the date of onset of type 2 diabetes. We determined the types of diabetes by using a proxy variable: if a person was under 30 years old and was on insulin only, or insulin and metformin only, or if a person was 30-40 years old when insulin only was started, they were categorised as having type 1 diabetes. All other persons with diabetes, as described above, were considered to have type 2 diabetes.

In papers III and IV, in which we used fatal and non-fatal CHD events, CVD events (CHD + ischemic stroke), and deaths from any cause (paper IV only) as end points, participant data were linked with FINAMI and FINSTROKE registers, as well as the Care Register for Health Care and the Causes of Death Register. We used MI and unstable angina pectoris as main or additional diagnoses in the Care Register for Health (ICD-9: 410 or 4110; ICD-10: I12-I22 or I20.0) for non-fatal CHD events. We also used the FINAMI register and the Invasive Cardiac Procedures Register to include revascularizations (coronary artery by-pass grafting or percutaneous transluminal angioplasty) as CHD events. Diagnoses for fatal CHD events included deaths with CHD or sudden death as the underlying or immediate cause of death (ICD-9: 410-414 and 798; ICD-10: I20-I25, I46, R96, R98), or MI as the contributing cause of death (ICD-9: 410, ICD-10: I21-I22). For fatal and non-fatal stroke events we used ICD-9 diagnosis codes 433, 434 and 436, and the ICD-10 diagnosis codes I63 and I64. Data on all-cause deaths were obtained from the Care Register for Health Care.

4.3 LABORATORY METHODS

4.3.1 PEPTIDE MARKERS OF INFLAMMATION

Quantification of peptide markers of inflammation was conducted using assays described in table 3. For FINRISK 1992 and DILGOM, peptide markers of inflammation were quantified from frozen serum samples. For FINRISK

2002, hs-CRP was quantified from fresh serum samples, whereas the other markers were quantified from previously unthawed heparin plasma samples.

Table 3. *Quantification methods for peptide markers of inflammation*

<table>
<tr><th></th><th>FINRISK 1992 (frozen serum stored at -20°C)</th><th>FINRISK 2002 (fresh serum; frozen plasma stored at -70°C)</th><th>DILGOM 2007 & 2014 (frozen serum stored at -70°C)</th></tr>
<tr><td>hs-CRP</td><td>Solid-phase chemiluminescent immunometric assay (Immulite®, Diagnostic Products Corporation)</td><td>Architect ci8200 Chemistry Analyser (Abbott) using an immunoturbidometric method (CRP Vario, High Sensitivity Method)</td><td>Latex immunoassay (Sentinel diagnostics, Milan, Italy) on Architect® c8000 analyzer (Abbott Laboratories)</td></tr>
<tr><td>IL-1Ra</td><td>N/A</td><td rowspan="3">Bio-Rad's premixed Bio-Plex Pro Human Cytokine 27-plex Assay and 21-plex Assay, and Bio-Plex 200 reader with Bio-Plex V.6.0 software (Bio-Rad Laboratories)</td><td>Enzyme linked immunosorbent assay Human IL-1ra/IL-1F3 Quantikine ELISA Kit, R&D Systems, Inc., Minneapolis</td></tr>
<tr><td>IL-6</td><td rowspan="2">Solid-phase chemiluminescent immunometric assay (Immulite®, Diagnostic Products Corporation)</td><td rowspan="2">Multiplex sandwich immunoassays Milliplex® High Sensitivity Human Cytokine kit, Millipore</td></tr>
<tr><td>TNF-alpha</td></tr>
<tr><td>HMW Adiponectin</td><td>N/A</td><td>N/A</td><td>Enzyme linked immunosorbent assay Human HMW Adiponectin ELISA kit, Millipore</td></tr>
</table>

4.3.2 LIPID MARKERS OF INFLAMMATION

For participants in the FINRISK 2002, DILGOM 2007 and FHS Offspring studies, we used a directed non-targeted LC-MS approach in conjunction with computational chemical networking of spectral fragmentation patterns, to identify eicosanoids and related oxylipins. The methods, which have been described in detail in literature (Watrous et al 2019; Lagerborg et al 2019),

allowed to identify known as well as putative and previously unknown eicosanoids and related oxylipins.

4.3.3 MARKERS OF PERIODONTITIS

In paper 4, for the FINRISK 1992 cohort, we used multiserotype-ELISA to determined serum IgA and IgG antibodies to periodontal pathogens *A actinomycetemcomitans* and *P gingivalis* from frozen sera stored at -20°C (Pussinen et al 2002). We analysed two dilutions of each serum in duplicate with ELISA Units. From the mean absorbance results we calculated continuous variables. In order to decrease inter-assay variation, we calculated a correction coefficient from the mean of the reference serum value. After all the laboratory analyses, the ELISA results data of each plate were normalized using the correction coefficient.

We also determined a combined antibody response by summing up *A actinomycetemcomitans* and *P gingivalis* IgG levels. Finally, we used the Limulus Amebosyte Lysate (LAL) test kit with a chromogenic substrate (HyCult Biotechnology b.v) on diluted samples to analyse serum endotoxin concentrations.

4.3.4 MARKERS OF GLUCOSE AND INSULIN METABOLISM

Members of a subcohort in the FINRISK 2002 study underwent a 2-hour OGTT. Measurements from the 2-hour OGTT were quantified and indices of Homeostatic Model Assessment of Insulin Resistance (HOMA) were calculated using WHO recommendations, as described in detail (Saaristo et al 2005; Matthews et al 1985), respectively.

4.3.5 MARKERS OF LIPID METABOLISM

In the FINRISK 1992 and 2002 studies, total and HDL cholesterol and triglycerides were analysed from fresh serum samples (Sundvall et al 2007; Sundvall et al 2008; Leiviskä et al 2013). In 1992, routine enzymatic methods were used (CHOD-PAP, Boehringer) and the Olli-C analyzer (Thermo Electron Corporation). In 2002, they were analysed using Optima 909 (Thermo Electron Corporation). In DILGOM 2007, analyses of these lipids were done on the Architect c8000 (Abbott Laboratories). For the FHS Offspring study (8th examination cycle), serum total and HDL cholesterol and triglycerides, were analysed using the Hitachi 911 (Roche) and Roche reagents.

4.3.6 MEASUREMENTS OF BODY SIZE, BODY COMPOSITION AND BLOOD PRESSURE

Waist, hip, height and weight of participants were measured by trained nurses in FINRISK 1992 and 2002 studies, however in DILGOM 2007 only one third of the participants were measured by trained nurses and the rest self-measured and self-reported their results (Kanerva et al 2018). Nurses also took the blood pressure measurements in the FINRISK studies using the mercury sphygmomanometer: further details on the measurement equipment and technique have been published previously (Borodulin et al 2018). The systolic blood pressure value used for FINRISK 1992 (papers III and IV) was a mean of the first and second measurement and for FINRISK 2002 (paper II), a mean of the second and third measurement.

In the DILGOM study, body fat % was determined by nurses using a bioelectrical impedance instrument (TANITA TBF-300MA, Tanita Corporation of America, Inc.) (Männistö et al 2014).

4.3.7 LIFESTYLE FACTORS: DIET, PHYSICAL ACTIVITY AND SMOKING

In DILGOM, total energy intake was measured from the answers of participants to a standardised and validated food frequency questionnaire (FFQ) designed to capture the habitual diet over the previous 12 months. Further details of this methodology have been previously published (Kanerva et al 2018). Alcohol intake was based on the participants' responses on their alcohol consumption over the past week. The variable used in our analyses, weekly alcohol intake, thus reflected the absolute amount of ethanol (g/l) ingested over the course of a week.

Leisure-time physical activity (LTPA) was measured based on the questionnaires in all the Finnish cohorts. The question and response options have remained the same over the years in FINRISK and further on in DILGOM. The question used was "How much do you exercise and stress yourself physically in your leisure time? If it varies much according to the different seasons, mark the alternative which best describes the average situation". The response options included four categories, the first being 'low level or no exercise', second 'light exercise, at least 4h per week, third 'aerobic exercise, at least 3h per week' and the last category 'regular exercise at competitive level'. Detailed response options have been previously published (Borodulin et al 2016). For the purpose of analyses in each of the four papers in this thesis, categories three and four were combined.

A standard set of questions was used to assess smoking history in the FINRISK and DILGOM studies. The self-reported smoking status was used across

analyses for all papers (I, II, III and IV). For the purpose of the analyses, participants were classified into 3 categories never smokers, former smokers and current smokers. Those who according to the answers had quit smoking less than 6 months ago were classified as current smokers.

The variable descriptions for each Finnish cohort are available to view (in English) on the internet: https://thl.fi/en/web/thl-biobank/for-researchers/sample-collections/the-national-finrisk-study-1992-2012

4.3.8 EDUCATION

Socioeconomic status was captured in all analyses using the Finnish cohorts with the education variable. The question includes three response options that are specific for different birth cohorts. The birth-cohort specific responses were categorised to reflect the UNESCO International Standard Classification of Education (ISCED) levels: low level (ISCED levels 0-2) referring to a maximum of 9 years of formal education, middle level (ISCED levels 3-4) referring to 10-12 years of formal education and high level (ISCED levels 5-6) referring to 13 years or more for formal education (Harald et al 2007).

4.4 STATISTICAL METHODS

This section describes the rationale and use of statistical methods relevant for each study design as well as the software used for the analyses. To summarise baseline characteristics, we calculated mean values or medians, and either standard deviations (SD) or interquartile ranges (IQR), or where relevant due to skewed distributions geometric means and either anti-logs of SDs or IQRs, for continuous variables, and frequencies for categorical variables. In papers where we compared means and frequencies, we used the t-test (paper III) or the Wilcoxon Ranks Sum test (paper IV) and Chi-square tests (paper III, IV) to compare between participants with CVD or no CVD at baseline, or the Welch Two Sample test (paper I) to compare between baseline and follow-up values.

4.4.1 PAPER I: COHORT DESIGN (LINEAR REGRESSION)

All statistical analyses for paper I were conducted using R (version 3.4.1) and RStudio (version 1.0.153).

We chose CRP, IL-1Ra, IL-6, TNF-alpha and HMW adiponectin as our variables of interest and changes in weight, BMI, waist circumference and % body fat as our outcome variables. The single cohort was examined and their blood samples analysed at baseline and again seven years later. At follow-up

only CRP concentrations were available and only for a subgroup of the cohort. We chose to use linear regression for the statistical analyses.

We determined the appropriateness of a linear regression model for the variables of interest using a residual analysis and as a result, opted to log-transform for the linear regression analyses. For each analysis on a given inflammation marker, we excluded outliers with a difference of more than three standard deviations from the mean of the inflammation marker level. Furthermore, to ensure fluent comparison between results for the different markers, we expressed the associations per one standard deviation (SD) difference in the log-transformed concentrations.

We examined the associations between the explanatory variables (inflammation markers) and the obesity indicators in both cross-sectional (baseline values) and longitudinal settings (change in the values during follow-up) using generalized linear regression models. The models were adjusted for conventional risk factors for weight change and other relevant baseline characteristics including continuous variables such as age, energy intake, alcohol consumption and BMI at baseline and categorical variables including sex, educational status, smoking, and physical activity at leisure time.

Using the whole cohort, we also ran two different logistic regression analyses for serum CRP: with a BMI cut-off point of less than 30kg/m^2 and at least 30kg/m^2, and at least 10% increase in body weight and less than 10% increase in body weight during 7-year follow-up as outcomes. We also conducted a subgroup analysis performing linear regression using only never smokers, with CRP as the explanatory variable and changes in obesity indicators as outcome variables.

Furthermore, we ran another subgroup analysis to establish whether individuals who gained weight during the follow-up also experienced an increase in their inflammation status. The latter was represented by either an increased or a decreased level of CRP at follow-up and we used linear regression models adjusted for age, sex and baseline BMI.

Regression analysis results are presented for both sexes combined, as there were no notable differences in results between women and men.

4.4.2 PAPER II: COHORT DESIGN (TIME-TO-EVENT, SURVIVAL ANALYSIS)

All statistical analyses for paper II were conducted using R (version 3.6.1). Source code for all analyses is available at https://doi.org/10.5281/zenodo.3968712

Eicosanoids, represented by mass spectrometry peaks identified from the laboratory analyses, were the explanatory variables of interest. In the data set, we replaced any missing values with the minimum value for each of the eicosanoid abundances. The data were then normalised by subtracting plate medians from each feature and by dividing the median absolute deviations.

The outcome variable of interest was incident type 2 diabetes, as defined earlier in chapter 4.2. As follow-up time varied and time-to-event data were available, we chose to use Cox proportional hazards regression and a nested modelling approach for our analysis.

We excluded 167 participants with prevalent type 2 diabetes from the FINRISK 2002 study population which allowed us to include 6381 individuals for the Cox regressoin analyses, including 586 participants with incident type 2 diabetes. We tested associations of plasma eicosanoids with incident type 2 diabetes using the above-mentioned methodology, adjusting for well-established risk factors and other confounding factors in three different models: 1) adjusted for age, sex, region of residence, and mass spectrometry plate; 2) further adjusted for BMI, physical activity at leisure time, parental history of diabetes, prevalent CVD, systolic blood pressure, use of antihypertensive medication, triglycerides and use of lipid lowering medication; 3) further adjusted for CRP.

We used Schoenfeld residuals to test for the proportional hazards assumption [Grambsch & Therneau 1994). To control for type I errors in multiple comparisons, we opted to use the false discovery rate (FDR) correction method (Benjamini & Hochberg 1995). We analysed and visualised the statistically significant correlations of plasma eicosanoids and serum CRP with incident type 2 diabetes producing a correlation heatmap. The eicosanoids were ordered using complete linkage clustering.

After forcing the confounding variables into the model, we proceeded to a stepwise Cox regression analysis with forward selection, including the eicosanoids that had statistically significant with incident type 2 diabetes in any of the models 1,2 and 3 described above. We applied the Bonferroni-corrected inclusion threshold $p<0.05/545=0.00009$ (Etymologia 2005), where 545 is the number of eicosanoids validated by the laboratory in the FINRISK 2002 discovery sample.

Using the three eicosanoids that remained after the stepwise regression analysis, we developed an eicosanoid risk score. Each eicosanoid was weighted by their respective regression coefficients in our prediction model.

We determined quartiles for the eicosanoid risk score to examine type 2 diabetes-free survival. Cox regression models and Kaplan-Meier curves were used to demonstrate survival and a log rank test to compare the survival

distributions. Furthermore, we calculated the risk of type 2 diabetes per SD change in the eicosanoid risk score using Cox regression.

We performed replication analyses in the FHS Offspring and DILGOM 2007 cohorts for the three eicosanoids which exceeded the Bonferroni-corrected inclusion threshold ($p<0.05/545 = 0.00009$) in the FINRISK cohort. In the FHS Offspring cohort, Cox regression models were adjusted for age, sex, BMI, systolic blood pressure, use of antihypertensives, and plasma triglycerides. We excluded 301 individuals with diabetes at baseline or missing values in the covariates. This resulted in 236 incident diabetes cases out of a total of 2,115 individuals included in the analysis. In the DILGOM 2007 cohort, Cox regression models were adjusted for the same covariates as in the FINRISK cohort. After excluding 144 participants with prevalent type 2 diabetes, 3,761 individuals remained for the replication analysis, including 248 individuals with incident type 2 diabetes. Finally, we performed inverse variance weighted random-effects meta-analysis of the eicosanoid risk scores in all three cohorts using the metaphor package in R.

To explore possible pathogenetic mechanisms of the eicosanoid effect on the risk of incident type 2 diabetes, we examined whether the three eicosanoids or the eicosanoid risk score were associated with the measures of glucose and insulin metabolism. For each of the quartiles of the eicosanoid risk score, we calculated medians of fasting plasma glucose (fP-Gluc), fasting plasma insulin (fP-Ins), HOMA-IR and HOMA-B. The trends of these measures of glucose and insulin metabolism across the quartiles of the eicosanoid risk score were tested adjusting for age, sex and BMI. We also used Spearman's correlation coeffiecients to explore the associations of the three eicosanoids included in the risk score with the measures of glucose and insulin metabolism.

In order to determine the associations of peptide markers of inflammation with eicosanoids, we also calculated correlations of serum CRP, plasma IL-1Ra, IL-6, TNF alpha and with the three eicosanoids from the risk score. In addition, we ran Cox regression models for the continuous eicosanoid risk score as well as the quartiles of the eicosanoid risk score adjusted for the above-mentioned peptide markers of inflammation in the FINRISK cohort.

4.4.3 PAPERS III AND IV: CASE-COHORT DESIGN (TIME TO EVENT, SURVIVAL ANALYSIS)

We conducted statistical analyses for papers III and IV using SAS (version 8). In both papers, we used a case-cohort sampling design, as it allows for the selection of a sub-cohort that may be used for several different endpoints (Kulathinal et al 2007). Our design used Barlow's time-dependent weighting, where weights for sub-cohort members are defined as ratio of cohort members at risk to sub-cohort members at risk (Barlow 1994), and robust variance

estimators suitable for a Cox regression analysis of case-cohort data (Barlow et al 1999). The case-cohort sampling method has been described in more detail by colleagues (Kulathinal et al 2007) and is characterised by stratification and randomness.

In both papers, we examined the associations of the inflammation markers with traditional risk factors for CVD and with each other (paper III) and of serum antibodies to periodontal pathogens and endotoxin, and the inflammation markers with traditional risk factors for CVD (paper IV) in a cross-sectional setting using Spearman's rank correlations.

We computed hazard ratios (HRs) and their 95% confidence intervals (95%CIs) for CHD (paper III), CVD (papers III and IV) and deaths from any cause (paper III), described in detail in chapter 4.2, using the above-described modified Cox proportional hazards models. The variables of interest described in more detail in chapter 4.3 were:

- markers of periodontitis (serum antibodies to periodontal pathogens and endotoxin) in paper IV
- markers of inflammation (serum CRP, IL-6 and TNF alpha) in paper III and IV

Initially in paper III, we examined event-free survival by dividing the participants with no CVD at baseline to sex-specific quartiles of serum concentrations of inflammation markers. We drew Kaplan-Meier curves to visualise event-free survival for each quartile.

We then ranked the markers of inflammation (paper III) and periodontitis (paper IV) into quartiles for each sex (paper III) and both sexes combined (papers III, IV) for the modified Cox regression analyses. In paper III, the lowest quartile of each inflammation marker was used as a reference category while the other quartiles were included as dummy variables in the models. In paper IV, the three lowest quartiles of each marker of periodontitis were used as a reference category. We also tested for linear trends. Analyses for both sexes combined were used, as sex by cytokine-interaction tests were not significant ($p<0.05$).

In the first Cox regression model, we adjusted for age, and sex when relevant. In the second model, we further adjusted for serum total to HDL cholesterol ratio, systolic blood pressure, smoking, waist-to-hip circumference ratio and diabetes (papers III and IV), and for years of education (paper IV only).

In paper III, we endeavoured to examine whether the effects of inflammation markers were independent of each other, and thus models of CRP were further adjusted for TNF-alpha, and vice versa. Among women, we performed an

additional analysis by further adjusting for hormone replacement therapy (paper III only).

In paper IV, we proceeded to test the joint effect of inflammation and the immune response. We divided the combined antibody response, serum endotoxin concentration and inflammation markers into categories "high" and "low" using the highest quartile limit in the sub-cohort as the cut-off point. For the Cox proportional hazards regression models, we constructed four categories from the immune response and inflammation level:

1) low antibody response or low LPS, + low level of inflammation (reference category)

2) high antibody response or high LPS, + low level of inflammation

3) low antibody response or low LPS, + high level of inflammation

4) high antibody response or high LPS, + high level of inflammation

5 RESULTS

5.1 INFLAMMATION AND CHANGES IN OBESITY INDICATORS (PAPER I)

We followed-up and included in the analysis a total of 3,369 individuals (54.5% women) from the DILGOM 2007 cohort. Participants gained on average 0.70kg of weight during the 7-year follow-up: the most weight lost was 31.5kg and the most weight gained 31.9kg. A larger proportion of women (12.2%) than men (8.3%) gained 10% or more of weight during follow-up. The increase in waist circumference during follow-up (86.1cm to 88.1cm for women and 95.8cm to 98.0cm for men) was statistically significant. Further analyses were run for women and men combined.

We examined cross-sectional associations between inflammation markers, baseline obesity measures and other related factors using linear regression adjusted for age and sex. All the inflammation markers, notably serum CRP, IL-1Ra, IL-6, TNF-alpha and HMW adiponectin, were associated with baseline obesity measures ($p<0.0001$). The inflammation markers were also associated with each other, except HMW adiponectin, which was not associated with IL-6 and TNF-alpha.

In the longitudinal analyses using linear regression adjusted for known risk factors and other possible confounding factors at baseline (Table 4), HMW adiponectin at baseline had a modest and statistically significant association with increases in all of the obesity measures during the 7-year follow-up ($p<0.001$ for all but % body fat, for which $p=0.014$). After further adjusting for baseline BMI, HMW adiponectin remained statistically significantly associated with changes in body weight ($p=0.008$), BMI ($p=0.005$) and waist circumference ($p=0.002$), but not with changes in % body fat.

CRP and IL-1Ra at baseline had modest inverse associations with changes in all of the obesity measures adjusted for known risk factors and other confounding factors at baseline ($p<0.001$). However, after further adjustment for baseline BMI, these associations disappeared. As for IL-6 and TNF-alpha, all associations with the obesity measures were statistically non-significant.

Logistic regression models for CRP, with a cut-off point for BMI at 30kg/m^2 and +10% for change in weight produced similar, statistically non-significant results, as did further subgroup analyses for current smokers, ex-smokers and never smokers with models using CRP. A subcohort analysis in 1,158 participants for whom serum CRP was analysed both at baseline and at follow-up was performed to examine whether there was a change in inflammation status in the weight increase group, however no association was found.

Table 4. *Associations of inflammation markers at baseline with changes in weight (kg), in BMI (kg/m^2), in waist circumference (cm) and in % body fat during the 7-year follow-up*

	n	Model 2	p value for model 2	n	Model 3	p value for model 3
High-sensitivity C-reactive protein, mg/l						
Change in weight	3 158	-0.334	<0.001	3 157	-0.0167	0.877
Change in BMI	3 158	-0.1236	<0.001	3 157	-0.0091	0.812
Change in waist circumference	3 072	-0.5377	<0.001	3 071	-0.1424	0.274
Change in % body fat	1 057	-0.3702	<0.001	1 056	-0.1245	0.264
Interleukin-1Ra, pg/ml						
Change in weight	3 136	-0.3722	<0.001	3 135	-0.0294	0.792
Change in BMI	3 136	-0.1333	<0.001	3 135	-0.0076	0.847
Change in waist circumference	3051	-0.6323	<0.001	3 050	-0.1965	0.149
Change in % body fat	1 055	-0.4005	<0.001	1 054	-0.0619	0.597
Interleukin-6, ng/l						
Change in weight	3 103	-0.1166	0.24	3 102	-0.0173	0.862
Change in BMI	3 103	-0.0471	0.182	3 102	-0.1105	0.753
Change in waist circumference	3 017	-0.1316	0.274	3 016	-0.0051	0.966
Change in % body fat	1 043	0.2152	0.043	1 042	-0.1366	0.192
Tumour necrosis factor alpha, ng/l						
Change in weight	3 143	-0.1873	0.061	3 142	-0.1176	0.236
Change in BMI	3 143	-0.0655	0.065	3 142	-0.0402	0.255
Change in waist circumference	3 057	-0.077	0.525	3 056	0.0063	0.956
Change in % body fat	1 053	-0.0888	0.405	1 052	-0.0154	0.883
HMW adiponectin, ng/ml						
Change in weight	3 163	0.4565	<0.001	3 162	0.2895	0.008
Change in BMI	3 163	0.1652	<0.001	3 162	0.1046	0.007
Change in waist circumference	3 077	0.6377	<0.001	3 076	0.4125	0.002
Change in % body fat	1 060	0.2768	0.014	1 059	0.1401	0.214

The values are regression coefficients per one standard deviation increase in inflammation markers and their p-values from linear regression models adjusted for age, sex, education status, smoking, weekly alcohol intake, daily total energy intake, leisure time physical activity at baseline (model 2) and after further adjustment for baseline BMI (model 3). Inflammation markers were log-transformed for the analysis.

5.2 INFLAMMATION AND INCIDENT TYPE 2 DIABETES (PAPER II)

We analysed whether plasma eicosanoids at baseline were associated with incident type 2 diabetes in three cohorts, namely in a discovery cohort FINRISK 2002 among 6,548 individuals (52.3% women) and two replication cohorts DILGOM 2007 among 3,905 individuals (52.7% women) and FHS Offspring among 2,886 individuals (54.4% women). Further statistical analyses were run for men and women combined.

In the discovery cohort FINRISK 2002, a total of 76 eicosanoids were associated individually with incident type 2 diabetes, after adjusting for age, sex, region of residence, mass spectrometry plate, BMI, family history of diabetes, systolic blood pressure, use of antihypertensive medication, triglycerides, leisure-time physical activity, prevalent CVD and use of lipid treatment. We found that the eicosanoids also correlated with each other.

The 76 eicosanoids were included in the stepwise Cox regression analysis: three eicosanoids remained in the model (Table 5). Two eicosanoids, 8-iso-Prostaglandin A1 (8-iso-PGA1) and 12-Hydroxy-5,8,10-heptadecatrienoic acid (12-HHTrE), were positively associated with the risk of incident type 2 diabetes, while an unknown eicosanoid had an inverse association with it.

Table 5. *Hazard ratios (95% confidence intervals) for incident type 2 diabetes for the three plasma eicosanoids at baseline in the FINRISK, DILGOM and FHS Offspring cohorts.*

	8-iso-PGA1 HR (95%CI)	**Unknown eicosanoid** HR (95%CI)	**12-HHTrE** HR (95%CI)
FINRISK	1.24 (1.25-1.32)	0.71 (0.59-0.83)	1.20 (1.11-1.28)
DILGOM	1.23 (0.74-1.73)	0.96 (0.80-1.12)	1.07 (1.01-1.12)
FHS Offspring	1.20 (1.08-1.34)	0.93 (0.80-1.08)	1.12 (0.99-1.27)

HRs and 95%Cis are from Cox regression models adjusted for age, sex, region of residence, mass spectrometry plate, BMI, family history of diabetes, systolic blood pressure, use of antihypertensive medication, triglycerides, leisure-time physical activity, prevalent CVD and use of lipid treatment for the FINRISK and DILGOM cohorts; for the FHS Offspring cohort the model was adjusted for the same variables except use of lipid treatment and family history of diabetes.

The positive association between plasma 8-iso-PGA1 at baseline with the risk of incident type 2 diabetes found in the FINRISK cohort ($p<0.0001$) was replicated in the FHS Offspring ($p<0.001$) and the DILGOM 2007 cohort ($p=0.025$) (table 5). The other two results were also directionally consistent, although the associations were not statistically significant.

We determined an eicosanoid risk score out of the three eicosanoids: the risk score at baseline was statistically significantly associated with the risk of incident type 2 diabetes in a random effects meta-analysis (Figure 6).

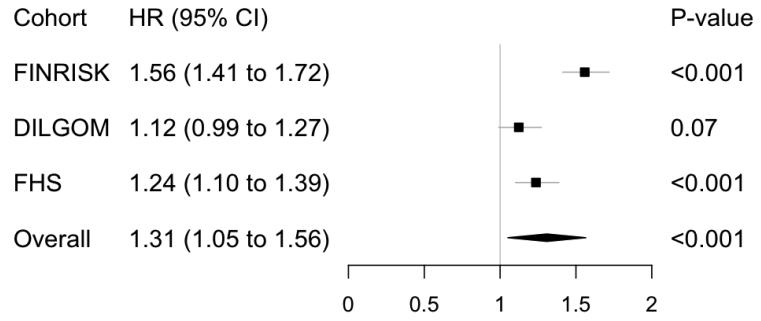

Figure 6 Hazard ratios (HR) and their 95% confidence intervals (95% CI) for the eicosanoid risk score in the FINRISK, DILGOM and FHS cohorts and meta-analysis.

Survival analysis comparing the participants in the top risk quartile of the eicosanoid risk score with those in the lowest quartile of risk score produced a HR (95%CI) of 2.74 (2.09-3.61) in the FINRISK cohort, 1.37 (1.00-1.74) in the DILGOM cohort and 1.74 (1.31-2.31) in the FHS cohort.

In the FINRISK 2002 discovery cohort, we also found that the medians of fasting plasma glucose and insulin as well as HbA1c and HOMA-IR increased across the eicosanoid risk score quartiles after adjusting for age, sex and BMI at baseline ($p<0.0001$ for linear trend). The increase in the median of HOMA-B across the quartiles was weaker but statistically significant ($p=0.014$).

Furthermore, we explored correlations between the three plasma eicosanoids included in the risk score, the measures of glucose metabolism and a panel of peptide markers of inflammation. 12-HHTrE had strong positive correlations with the indicators of glucose metabolism and serum CRP. The correlations of the other two eicosanoids with the same variables were weaker. None of the eicosanoids correlated with plasma IL-1Ra, IL-6 and TNF-alpha.

Finally, Cox regression analysis further adjusting for the peptide markers of inflammation remained statistically significant for both the continuous eicosanoid risk score and the 3rd and 4th risk quartiles of the score.

5.3 INFLAMMATION, CVD AND TOTAL MORTALITY (PAPER III)

We explored the risk of CHD and CVD events as well as deaths from any cause in 836 men and women from the FINRISK 1992 cohort using a case-cohort design.

We compared participants with or without baseline CVD and we found out that both men and women with CVD had significantly lower serum HDL cholesterol, and lower systolic and diastolic blood pressure at baseline and were more likely to have history of diabetes at baseline than those without CVD. Women with CVD also had higher BMI and WHR than women without CVD. We did not observe differences in serum inflammation marker levels between women with CVD and those without CVD. However, men with CVD had significantly higher IL-6 and TNF-alpha levels than those without CVD.

In the stratified random sample of 313 individuals from the original FINRISK cohort we observed a strong positive correlation between serum CRP and IL-6, but a weaker positive correlation between CRP and TNF-alpha. IL-6 and TNF-alpha were moderately and positively correlated.

Out of the 692 participants free of CVD at baseline, 205 developed CVD during the 10-year follow-up. Serum CRP ($p=0.004$) and TNF-alpha ($p<0.001$) levels at baseline were higher in men who had a CVD event during follow-up. We observed no differences in IL-6 levels at baseline between participants with a CVD event and those without it during follow-up.

There were no differences in levels of inflammation markers at baseline between women who had a CVD event and those without it during follow-up. In fact, among women, the Cox proportional hazard regression models did not reveal any significant associations between inflammation marker quartiles and the studied endpoints, even after further adjusting for hormone replacement therapy.

Among men, the risk of CHD events, CVD events and all-cause mortality increased with increasing quartiles of CRP at baseline adjusted for all established risk factors (Table 6, models 1 and 2). The risk of these events also increased with increasing quartiles of IL-6 at baseline in men after adjustment for age and sex (Table 6, model 1). However, only the association with all-cause mortality remained statistically significant after further adjustment for all established risk factors (Table 6, Model 1). The associations of TNF-alpha with CHD events, CVD events and all-cause mortality in men were non-linear (Table 6). We found a cut-off point for the risk of these events between the first and second quartiles of TNF-alpha, which was consistent with the detection limit.

Table 6. *Hazard ratios (95% confidence intervals) for coronary and cardiovascular events and all-cause mortality across quartiles of inflammation markers in 426 men aged 25-65 years, without cardiovascular disease at baseline.*

	1st quartile	2nd quartile	3rd quartile	4th quartile	p-value
CRP					
CHD event					
model 1	1.00	1.47 (0.67-3.23)	2.30 (1.10-4.78)	3.40 (1.67-6.91)	0.002
model 2	1.00	1.17 (0.50-2.72)	1.67 (0.74-3.76)	2.39 (1.08-5.28)	0.031
CVD event					
model 1	1.00	1.44 (0.69-3.01)	2.33 (1.18-4.63)	3.47 (1.78-6.75)	<0.001
model 2	1.00	1.15 (0.52-2.55)	1.75 (0.82-3.72)	2.42 (1.15-5.12)	0.026
All-cause mortality					
model 1	1.00	1.62 (0.75-3.54)	2.02 (0.96-4.25)	4.05 (2.02-8.14)	<0.001
model 2	1.00	1.43 (0.61-3.34)	1.66 (0.71-3.92)	3.48 (1.51-8.05)	0.001
IL-6					
CHD event					
model 1	1.00	1.19 (0.58-2.43)	1.34 (0.68-2.67)	2.00 (1.03-3.87)	0.032
model 2	1.00	0.97 (0.43-2.19)	0.77 (0.34-1.76)	1.25 (0.59-2.66)	0.211
CVD event					
model 1	1.00	1.10 (0.55-2.18)	1.55 (0.81-2.94)	2.24 (1.20-4.17)	0.008
model 2	1.00	0.91 (0.42-1.96)	0.92 (0.43-1.97)	1.48 (0.73-3.00)	0.091
All-cause mortality					
model 1	1.00	0.78 (0.35-1.76)	1.66 (0.82-3.36)	3.44 (1.79-6.60)	<0.001
model 2	1.00	0.66 (0.27-1.59)	1.20 (0.55-2.61)	2.77 (1.36-5.68)	<0.001
TNF-alpha					
CHD event					
model 1	1.00	3.14 (1.58-6.25)	2.26 (1.15-4.44)	2.11 (1.05-4.25)	0.792
model 2	1.00	2.84 (1.34-6.01)	2.23 (1.08-4.63)	1.65 (0.75-3.65)	0.600
CVD event					
model 1	1.00	3.18 (1.65-6.17)	2.17 (1.13-4.18)	2.61 (1.36-5.03)	0.237
model 2	1.00	2.78 (1.35-5.71)	2.14 (1.06-4.34)	2.13 (1.03-4.43)	0.721
All-cause mortality					
model 1	1.00	2.22 (1.16-4.26)	1.21 (0.63-2.35)	1.95 (1.04-3.66)	0.165
model 2	1.00	2.27 (1.11-4.64)	1.36 (0.66-2.78)	1.93 (0.94-3.96)	0.324

The hazard ratios and their 95% confidence intervals are from Cox regression models adjusted for age and gender (model 1) and further adjusted for total to HDL cholesterol ratio, systolic blood pressure, smoking, waist and hip circumference ratio and diabetes (model 2). P-values are for linear trends across quartiles of inflammatory markers.

Among men, event-free survival was significantly worse for the risk of coronary event during the 10-year follow-up with increasing quartiles of CRP

(p=0.03) and between the lowest and the upper three quartiles of TNF-alpha (p=0.01).

We ran additional analyses to understand whether the associations of CRP and TNF-alpha in men were affected by each other. When CRP was added as a covariate for the association of TNF-alpha with the end-points, the HRs were but slightly attenuated, however the association with total mortality became non-significant (Figure 7). Moreover, men who were in the highest quartiles of both CRP and TNF-alpha had no higher risk of coronary events compared to men in the lowest quartiles of both (HR 1.51, 95%CI 0.78-2.91).

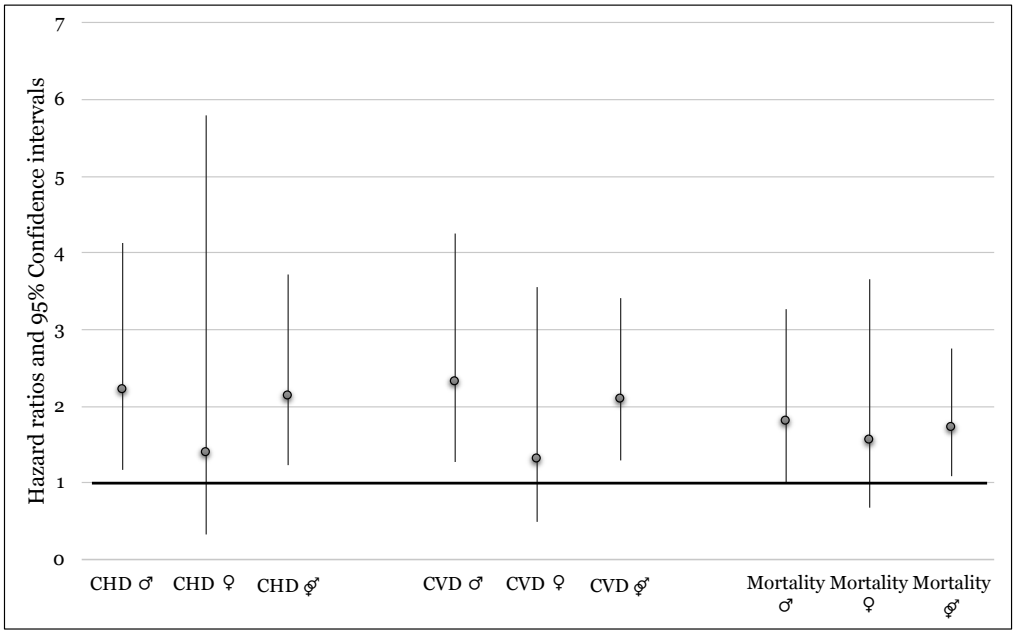

Figure 7 Hazard ratios and 95% Confidence intervals, for coronary and cardiovascular events and all-cause mortality for three highest quartiles of TNF-alpha compared with the lowest quartile, adjusted for established cardiovascular risk factors in men, women and both sexes combined. ♂ men, ♀ women, ⚥ both sexes

5.4 PERIODONTITIS, INFLAMMATION AND CARDIOVASCULAR DISEASE (PAPER IV)

We explored the risk for CVD events among 784 individuals from the FINRISK 1992 cohort using a case-cohort design.

Participants who experienced an incident CVD event during the 10-year follow-up, had lower levels of serum HDL cholesterol, and higher serum total cholesterol, BMI, systolic and diastolic blood pressure, and were more likely to be smokers than those who remained free of CVD. Furthermore,

participants who had an incident CVD event during follow-up also had higher serum endotoxin concentrations than those without a CVD event, but there were no differences in the levels of antibodies to periodontal pathogens between these groups.

Using weighted and adjusted Cox proportional hazards models to compare the highest quartiles of levels antibodies to periodontal pathogens to the lowest three quartiles, we observed that these species-specific antibody levels were not associated with incident CVD (Figure 8). However, the association between the combined antibody response and incident CVD was statistically significant, the HR (95% CI) for incident CVD in the highest quartile of the combined antibody response being 1.87 (1.13-3.08). Moreover, the joint occurrence of high combined antibody response and high CRP or IL-6 increased the risk of CVD markedly (Figure 9). Using individuals with low levels of these antibodies as the reference, the multivariate models resulted in HRs (95% CIs) for CVD of 3.01 (1.27-7.09) and 3.11 (1.42-6.83).

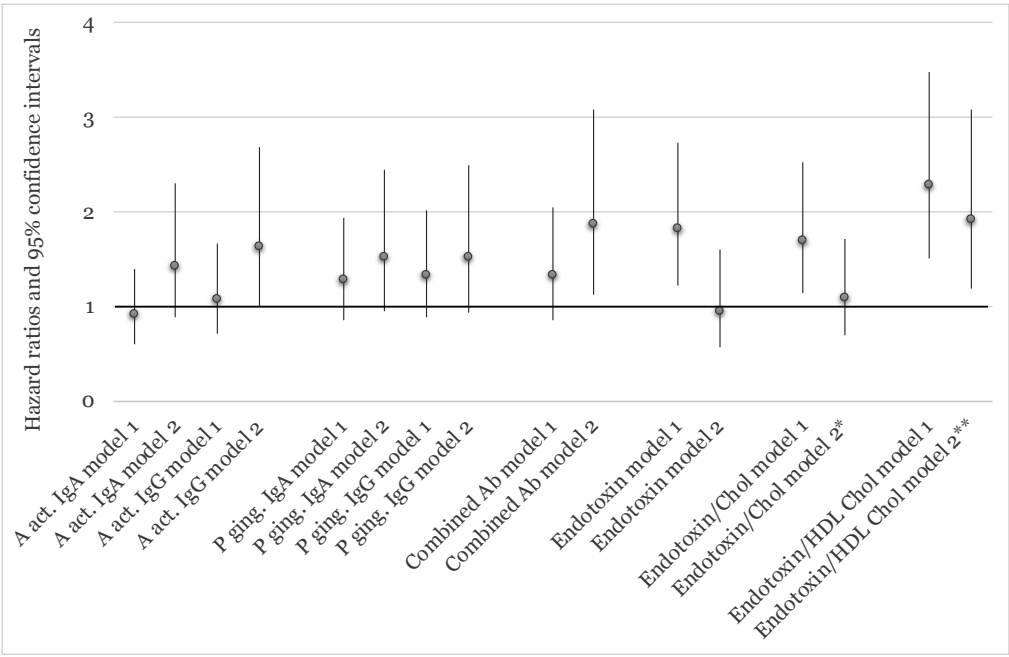

Figure 8 Hazard ratios (95% confidence intervals) for incident CVD in the lowest three quartiles of serum levels of antibodies for periodontal pathogens in 481 individuals free of CVD at baseline. A act. = *A actinomycetemcomitans*; P ging = *P gingivalis*; Combined Ab=Combined antibody response; Model 1 is adjusted for age and gender, and model 2 further adjusted for total cholesterol to HDL cholesterol ratio, systolic blood pressure, waist-to-hip ratio, diabetes, smoking status, and years of education; *Adjusted for the same covariates as the previous model but HDL cholesterol is used instead of total cholesterol to HDL cholesterol ratio; **Adjusted for the same covariates as model 2, but total cholesterol concentration is used instead of total cholesterol to HDL cholesterol ratio.

Hazard ratios (95% confidence intervals) for incident CVD in the highest quartile of serum endotoxin concentrations were 1.82 (1.22-2.73) after adjustment for age and sex, however after further adjustment for systolic blood pressure, waist-to-hip ratio, diabetes, smoking status, and years of education, as well as for total cholesterol to HDL cholesterol ratio, the association disappeared.

Endotoxin to total cholesterol ratio was not associated with incident CVD. However, endotoxin to HDL cholesterol ratio was statistically significantly associated with incident CVD even after full adjustment (p=0.007), HR (95% CI) for incident CVD in the highest quartile of endotoxin to HDL cholesterol ratio being 1.92 (1.10-3.08).

Age-and-sex-adjusted HRs for CVD were significant for simultaneous occurrence of high concentrations of endotoxin and inflammation markers, however after adjusting for established CVD risk factors, the HRs were not significant (Figure 9).

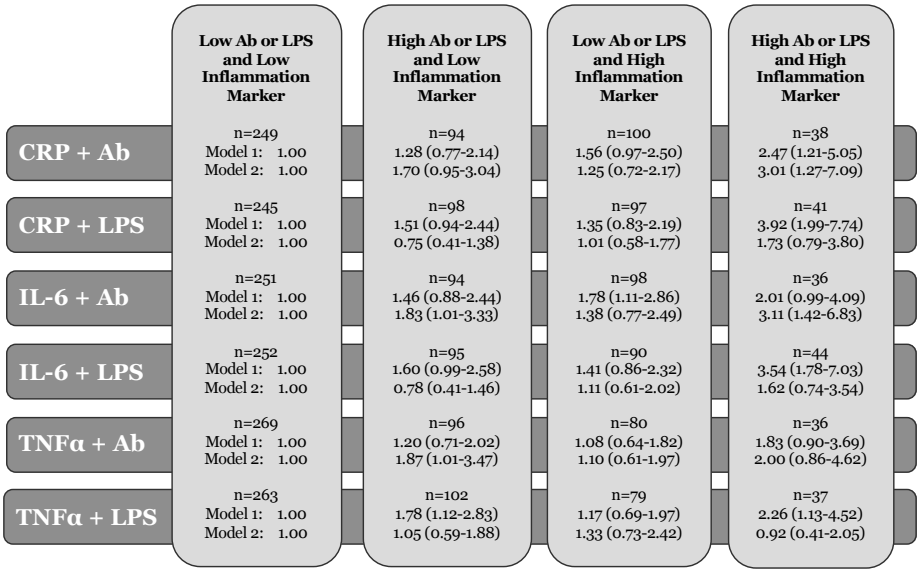

Figure 9 Hazard ratios (95% confidence intervals) of the joint effect of combined antibody response (Ab) or endotoxin concentrations (LPS), and CRP, IL-6, and TNF-alpha, for incident CVD in 481 individuals free of CVD at baseline. Hazard ratios (95% confidence intervals) are from Cox regression models adjusted for age and sex (model 1) and further adjusted for cholesterol to HDL cholesterol ratio, systolic blood pressure, waist-to-hip ratio, diabetes, smoking status, and years of education (model 2).

6 DISCUSSION

6.1 SUMMARY OF MAIN FINDINGS

In paper I, we found that serum levels of peptide-derived markers of inflammation such as CRP, IL-1Ra and adiponectin were associated with changes in obesity indicators. However, after adjusting the models for baseline BMI, the associations were no longer significant for any of our outcomes (changes in weight, waist circumference, % body fat or BMI), implying that subclinical inflammation may not be responsible for weight gain or accumulation of adipose tissue. The findings of paper II showed that a three-eicosanoid risk score was associated with incident type 2 diabetes in three independent cohorts, suggesting an independent role for lipid-derived mediators of inflammation in the prediction of incident type 2 diabetes.

In papers III and IV we identified markers of inflammation as independent predictors of CVD and total mortality. Namely, serum CRP and TNF-alpha predicted incident CHD and CVD events as well as total mortality among men. Mortality was also predicted by high levels of serum IL-6 in men. Furthermore, increased serum levels of IgG class antibodies to periodontal pathogens *A actinomycetemcomitans* and *P gingivalis*, especially when accompanied by elevated levels of serum inflammation markers CRP and IL-6, predicted incident CVD events. Increased levels of serum endotoxin were also identified as a risk factor for CVD, however the association was not independent of serum cholesterol levels.

6.2 METHODOLOGICAL CONSIDERATIONS

6.2.1 STUDY POPULATIONS AND STUDY DESIGNS

We used three population-based samples of Finnish adults, namely FINRISK 1992, FINRISK 2002 and DILGOM 2007, the participants of the latter being invitees of the FINRISK 2007 cohort, in papers I-IV. The FINRISK samples are representative, independent and random. They have been drawn every five years using stratification by age and sex since 1972, with a varied selection and number of geographical areas included (Borodulin et al 2018). Eastern and Southern geographical areas of Finland have been slightly better represented in the earlier FINRISK cohorts, however since 2002 Northern Finland has been better covered in the cohorts. Participation rates of the independent studies have been at the very least moderate. This allows for a decent

generalisation in the Finnish population, as geographical differences do not affect the associations of inflammation markers with the varied outcomes.

The different geographical areas have had similar participation rates; however, attendance has been better in smaller towns and municipalities than in larger cities. The participants of the surveys have been slightly more likely to be women, older, better educated and have had better health status than non-participants.

There are no straight-forward participation rates for the fourth sample used as a replication cohort in our study, the FHS Offspring cohort (paper II). The original FHS sample was drawn from adults living in Framingham, Massachusetts, USA, after stratification for family size and precinct of residence, with a participation rate of 68.8% (Kannel et al 1979). The FHS Offspring cohort includes the children and spouses of the first-generation cohort participants. The retention rate for the various examination cycles of both FHS and FHS Offspring studies has been reported to be around 99% and the ancestry of participants to be European American (Tsao & Vasan 2015). Results of the study presented in paper II could therefore be considered generalisable to Caucasian white Americans.

6.2.2 MEASUREMENTS AND LABORATORY METHODS

In 1992 and 2002, the anthropometric and other measurements were taken at the examinations carried out by nurses specifically trained for the purpose of the FINRISK studies. The examinations took place in the first three months of each study year.

The additional measurements such as body fat % in DILGOM took place between April and May in 2007, and the follow-up study measurements were performed between April and June 2014. The follow-up examinations were only conducted for participants living in the cities of Helsinki and Vantaa as well as the areas of Turku and Loimaa, whereas those living in the three other study areas, received a mail-back questionnaire and reported their weight, height and waist circumference by themselves. The self-reported measurements have been validated against the measurements performed by trained nurses (Kanerva et al 2018), which has allowed us to use the follow-up measurements in our study (paper I). As not all study participants were invited for the follow-up examinations, % body fat was available only for those who attended these examinations.

Serum samples were collected for each of the FINRISK cohorts at baseline after at least a 4-hour fasting period. In 1992 and 2002, samples were transported fresh to the central lab at the Finnish Institute for Health and Welfare and analysed fresh in the following days. Some parameters (e.g. lipids)

were analysed from a fresh sample, while most of the samples were frozen immediately. In later studies, samples were transported to the Finnish Institute for Health and Welfare already frozen. In DILGOM, the serum samples were collected after a minimum 8-hour fasting period. Furthermore, in FINRISK 2002, the 2-hour OGTTs, following at least an 8-hour fast as instructed in the WHO protocol for OGTT, were conducted in a sub-cohort of the study population, quantified and calculated according to the WHO recommendations.

Back in 1992, the serum samples were stored at -20°C whereas in later cohorts they were stored at much colder temperatures of -70°C, allowing for better preservation. Furthermore, the quantification of the inflammation markers was carried out a decade later for the 1992 cohort. Thus, for papers III and IV using FINRISK 1992 data, frozen serum samples were no longer sufficient in volume for about 20% of the study sample. However, our analyses showed, that a missing serum sample did not significantly predict CVD events, one of the outcomes of the thesis.

The assay used to identify serum endotoxin levels from the FINRISK 1992 samples was not specific to the periodontal pathogens but to a wider range of Gram-negative bacteria. Some Gram-negative bacteria such as Chlamydia pneumoniae and Helicobacter pylori also cause chronic infections and have been associated with incident CVD. However, one of the most common infections in Finnish adults is periodontitis (Knuuttila 2004). Therefore, it is safe to assume that although technically mirroring the burden of Gram-negative bacteria endotoxin, the bulk of the burden detected in our study population is derived from periodontal pathogens.

The LC-MS analysis of plasma metabolites for all three cohorts used in paper II (FINRISK 2002, DILGOM 2007, FHS Offspring) was conducted at our collaborator's laboratory at the University of California San Diego, USA. The LC-MS approach was used in conjunction with computational chemical networking of spectral fragmentation patterns to allow for putative classification of some of the unknown molecules as oxylipins (Watrous et al 2019). Although the methodologies used at the laboratory could not differentiate between enantiomers of the different metabolite molecules, the substances were identified accurately.

6.2.3 STUDY DESIGN AND STATISTICAL METHODS

For paper I, the choice of study design and statistical methods was straightforward. As we explored continuous outcome variables at a single follow-up in a reasonably large cohort, it made sense to choose linear regression as the statistical method. We created some categorical variables as

well, from changes in weight and BMI, and therefore also used logistic regression.

In papers II-IV, we were able to use methods of time-to-event analysis, namely Cox regression, as we had exact dates for the diagnoses or events for the outcome variables. However, for papers III and IV, we used a case-cohort design to allow for examination of several different endpoints as well as to account for the relatively small number of events. In our case-cohort design, we selected the sub-cohort for the most common outcome of CVD, thus providing flexibility to include additional outcomes such as CHD and all-cause death (Kulathinal et al 2007).

Furthermore, for paper III, we opted to present results not only for sexes combined but also for men and women separately despite the low number of events in women. There were indications of differences in the risk profiles for CVD between the sexes, and the literature at the time included very few studies on the role of subclinical inflammation and the risk of CVD in women specifically. In general, despite efforts to tackle the gender reporting bias, women keep being underrepresented and underreported in medical studies (Liu & Mager 2016). For paper I, we ran the analyses for men and women separately. However, as the results did not differ notably, we decided to report the results for the sexes combined. In the analysis phase of paper II, we tested for interaction between sex and the eicosanoid risk score, and thus opted to report the results for the whole study population. The analyses of paper IV were performed for sexes combined and adjusted for sex, as the total number of events for each sex would have been low.

In paper II, we chose to use stepwise Cox regression with forward selection in order to identify which plasma eicosanoids would remain in the model. It has been argued that this methodology is relatively rough and more sophisticated methods using penalisation, such as Least Absolute Shrinkage and Selection Operator (LASSO) or the adaptive elastic net method may have added some predictive accuracy to the design (Tibshirani 1997; Zou & Hastie 2005). However, the predictors did not seem to be highly correlated, suggesting that stepwise regression would be a suitable method.

6.3 ASSOCIATIONS OF SUBCLINICAL INFLAMMATION WITH THE OBESITY-TYPE 2 DIABETES-CVD TRIFECTA

6.3.1 OBESITY MAY NOT BE CAUSED BY SUBCLINICAL INFLAMMATION

There exists a clear association between subclinical inflammation, overweight and obesity, however few studies have been able to show that inflammation has any power to predict the development of overweight or obesity. And those that have done so, have reported such results mainly among smoking quitters. Our findings do not support those of the ARIC and MONICA/KORA studies, which reported that higher levels of serum inflammation markers preceded weight gain in smoking quitters (Duncan et al 2000, Holz et al 2012), as we also examined these associations in different smoker categories. Moreover, although the Malmö Preventive Study found that inflammation-sensitive plasma proteins such as fibrinogen predicted weight gain in middle-aged men (Engström et al 2003), examinations of participants at baseline or follow-up did not include measurements relating to the accumulation of adipose tissue.

Adipose tissue has been previously identified as a source of subclinical inflammation, perhaps as a response to overnutrition and an attempt to adapt to obesity (Hotamisligil et al 1993; Weisberg et al 2003; Hotamisligil 2017; Reilly & Saltiel 2017). Studies examining the association between inflammation and obesity using the Mendelian randomization (MR) design seem to support our findings and shed further light on the causality between inflammation markers and obesity. Using data from a Danish adult population, Timpson and co-workers concluded that CRP was rather a marker of elevated adiposity and not a driver of BMI (Timpson et al 2011). A more recent work using the UK biobank data supported the notion of subclinical inflammation being a consequence rather than a cause of obesity (van Zuydam et al 2018).

Moreover, in a study using data from the China Health and Nutrition Survey, it was found that the steeper rates of weight gain were associated with an higher inflammation risk, even after controlling for baseline body weight, whereas weight loss was associated with lower inflammation risk (Thompson et al 2016). These observations suggest that trajectories of weight gain reflect on the risk status independent of body weight. In our study using linear regression analysis, we saw all associations of inflammation markers with changes in obesity indicators disappear after adjusting for baseline BMI, faintly suggesting that the effect of the initial association could have been explained by existing adipose tissue or a tendency to overnutrition.

Although not within the scope this thesis, the link between gut microbiota and subclinical inflammation may be relevant in relation to obesity. A recent review proposed that whereas genetic variants have been associated with susceptibility to obesity and type 2 diabetes, gut microbiota may be a key environmental factor driving these metabolic diseases via stimulation of subclinical inflammation (Boulangé et al 2016). Another review suggested that intestinal microbiota modulate intestinal permeability, especially of dietary fats, resulting in immune responses leading to subclinical inflammation (Cox et al 2015). Thus, promoting a more balanced gut microbiota may have some beneficial effects on subclinical inflammation.

The initial trigger of inflammation especially in the adipose tissue remains an unanswered question. Even with our modest findings, the debate on whether subclinical inflammation is a consequence of weight gain and the accumulation of adipose tissue, especially around the waist, continues. Further prospective follow-up studies using well-defined and professionally measured indicators of obesity and inflammation with appropriate linkages to health registers will be needed to confirm the role of subclinical inflammation in the development of obesity.

6.3.2 PLASMA EICOSANOIDS ASSOCIATE WITH INCIDENT TYPE 2 DIABETES

Inflammation markers such as CRP and cytokines have been studied abundantly in relation to type 2 diabetes, and several of these peptide-derived mediators of inflammation have been suggested to serve as markers predicting type 2 diabetes. Results from an MR study in the participants of the Whitehall II study suggested non-causal associations between CRP, insulin resistance, glycaemia and type 2 diabetes and proposed that markers situated more upstream were more likely to play a causal role in the development of type 2 diabetes (Brunner et al 2008).

Although not confirming causality for upstream mediators of inflammation, our findings on lipid-derived markers of inflammation, namely plasma eicosanoids, support the hypothesis that they play a role in the development of type 2 diabetes. Interestingly. We found that increased levels of CRP and a number of serum cytokines, namely IL-6, IL-1Ra and TNF-alpha, and of the three plasma eicosanoids were associated with incident type 2 diabetes. Interestingly however, these cytokines, and eicosanoids were not really correlated with each other. Moreover, the associations of the continuous three-eicosanoid risk score with incident type 2 diabetes remained statistically significant after further adjustment for the cytokines among individuals in the FINRISK cohort.

This further suggests that cytokines may not comprehensively reflect all aspects of subclinical inflammation associated with type 2 diabetes, thus implying for some of the effect to be situated further up in the inflammation pathways. Another possibility is that the eicosanoid risk score could increase the risk of type 2 diabetes through other than traditional inflammatory mechanisms. It has been proposed that eicosanoids metabolised from arachidonic acid by the different enzymatic and non-enzymatic pathways actively contribute to beta-cell function (Luo & Wang 2011) and more recently to insulin sensitivity (Yang et al 2018).

Two of the individual eicosanoids identified for the three-eicosanoid risk score, 8-iso-PGA1 and 12-HHTrE, are metabolites with known bioactivity. Isoprostanes such as 8-iso-PGA1, are stereoisomers of prostaglandins that are formed through peroxidation of arachidonic acid, independent of COX enzymes. They have been linked with oxidative stress, which in turn may lead to subclinical inflammation, and it has been hypothesised that isoprostanes have a causal role in the development of type 2 diabetes and its complications, such as nephropathy and neuropathy (Roberts et al 2000; Park et al 2009; Vincent et al 2004; Elmarakby & Sullivan 2012; Odegaard et al 2016).

Further, different enantiomers of 12-HHTrE are metabolites of both non-enzymatic pathway as well as COX and LOX pathways. For example, 12(S)-HHTrE is a COX-pathway-metabolite that induces chemotaxis of mast cells and accelerates wound closure (Okuno et al 2008, Liu et al 2014), whereas 12(L)-HHTrE is a LOX-pathway metabolite known to be a stimulator of endothelial prostacyclin formation (Mezei et al 1997). Its enantiomers have recently been implicated in a number of conditions including breast cancer (Chocholoušková et al 2019), responses to acute exercise and high carbohydrate intake (Nieman et al 2019) and hypertension (Palmu et al 2019). In the last of these studies, our colleagues used the same mass spectrometry platform and found plasma 12-HHTrE to be robustly associated with blood pressure (Palmu et al 2019).

The association between the eicosanoid risk score and incident type 2 diabetes that we found replicated in the FHS cohort but was not statistically significant in the DILGOM cohort. However, the finding in DILGOM was directionally consistent with those of FINRISK and FHS, and the direct association between the eicosanoid risk score and incident type 2 diabetes observed in the meta-analysis further supports our hypothesis. Moreover, one of the three eicosanoids remaining in the model after our stepwise regression analysis showed an independent inverse association with incident type 2 diabetes in the FINRISK cohort. Although this association did not reach statistical significance in the other two cohorts, the finding does suggest that some eicosanoids may play a protective role in the development of type 2 diabetes.

One of the many questions that remain open is about the pathogenetic mechanisms for the effects of these eicosanoids on the development of type 2 diabetes. It has been speculated that metabolites of LOX pathways may contribute to the development of insulin resistance via an early response to high-fat feeding in white adipose tissue (Cole et al 2013). LOX and COX pathway metabolites have also been linked with beta-cell dysfunction in pancreatic islets (Luo & Wang 2011). We found that plasma 12-HHTrE had modest associations with HOMA-IR and other indicators of insulin resistance, whereas plasma 8-iso-PGA1 did not correlate with fasting plasma glucose levels and only weakly with indicators of insulin resistance.

More understanding is needed on the biology of eicosanoids and the pathogenesis of type 2 diabetes. With further improvements in mass spectrometry and metabolomics methodologies, alongside with computer networking methods and use of artificial intelligence, it should be possible to explore plasma metabolites at larger scope and scale in the future.

6.3.3 SUBCLINICAL INFLAMMATION IS A SIGNIFICANT RISK FACTOR FOR INCIDENT CARDIOVASCULAR DISEASE

Our study demonstrated that CRP was associated in a fairly linear way with incident coronary and cardiovascular events among men, supporting the role of subclinical inflammation in the pathogenesis of CVD. Our findings support the results of several studies in European, North American and East Asian populations, using incident CHD, stroke or CVD events in general as the main outcomes (Ridker et al 1997; Koenig et al 1999; Lowe et al 2004; Lin et al 2016; Penson et al 2018; Karim et al 2020). Furthermore, we observed stronger associations of CRP with cardiovascular outcomes than the Framingham Heart Study and the Reykjavik study (Wilson et al 2005; Danesh 2004). Our findings are also in line with the results of a study using MR in four independent Danish cohorts (Zacho et al 2008). This study demonstrated that serum CRP was associated with an increased risk of CVD and that genetic variation in the CRP gene was associated with serum CRP levels but not with cardiovascular risk.

Since our study, CRP's role in atherosclerosis has been defined as non-causal and suggested to merely be a marker of either the extent of the disease or of inflammatory activity of atherosclerotic plaques (Nordestgaard & Zacho 2009; CCGC 2011; Zhuang et al 2019). However, subclinical inflammation has been constantly believed to contribute to the development CVD, and this belief has gradually obtained just about definitive support from large randomised controlled trials (RCTs), namely the JUPITER (Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin) and CANTOS (Canakinumab Anti-inflammatory Thrombosis Outcomes Study) studies testing the effect of various anti-inflammatory agents on

cardiovascular risk (Ridker et al 2008; Ridker et al 2017). The trials and the significance of their results will be discussed in more detail in chapter 6.5.

In our study, serum TNF-alpha was also an independent predictor of coronary and cardiovascular events, which supports the findings from a number of studies (Cesari et al 2003; Jefferis et al 2009; Cui et al 2012; Kaptoge et al 2014). There was a threshold for the associations of serum TNF-alpha with CHD and CVD events. A 2.5-fold increase in CHD and CVD risk was observed for any increase above the detection limit of TNF-alpha levels. It is interesting to note, that the Health ABC Study, which also found an association between TNF-alpha and CHD risk did not report any threshold phenomenon (Cesari et al 2003).

Curiously, neither the findings for TNF-alpha nor for CRP in our study were affected by adjustment for each other, suggesting that these inflammation markers reflect different features of subclinical inflammation and could be complemental in the assessment of CVD risk. However, a British study that found a modest association between elevated levels of TNF-alpha and MI risk but not stroke reported that the predictive value of TNF-alpha for MI all but disappeared after adjustment for CRP and IL-6 (Jefferis et al 2009). Finally, in our study, elevated levels of both CRP and TNF-alpha did not lead to an even higher risk in our study.

There is some evidence to suggest that there are race and gender differences in CRP levels at population level (Khera et al 2005). Our findings among women for CRP and TNF-alpha were not very different from those of men, however they did not reach statistical significance and adjusting for hormone replacement therapy did not alter the results in a substantial way. Many large studies have not examined women separately and those that have, have not found many if any associations of these inflammation markers with incident CHD or CVD after controlling for other risk factors in women (Pai et al 2004; Wilson et al 2004).

However, very recent results from the Women's Health Study suggest that elevated levels of CRP in women are a strong and independent predictor of CHD (Dugani et al 2021). These findings also suggested that subclinical inflammation accompanied by states of excess adiposity such as insulin resistance and type 2 diabetes, could be an even more important predictor of premature CHD. Earlier results from the Women's Health Study proposed that CRP was useful in cardiovascular risk prediction at both very low and very high levels (Ridker & Cook 2004). On the other hand, results from the Cardiovascular Health Study in older adults suggest that CRP provides information for the prediction of CVD beyond conventional risk factors only in women at high risk of CVD (Cushman et al 2005).

Among men, IL-6 was a significant predictor of incident CHD and CVD events in age-adjusted models, but this association was largely explained by traditional CHD risk factors. In women only, IL-6 was not associated with CHD or CVD incidence, in contrast with results from the Women's Health Initiative Study that found IL-6 to be an independent predictor of CHD in women (Pradhan et al 2002). Results from the Nurses' Health Study and from the Health Professionals Follow-up Study on the other hand found that the association between IL-6 and incident CHD was not independent of other cardiovascular risk factors (Pai et al 2004).

Later meta-analyses of a number of larger studies (including paper III) found that IL-6 was an independent predictor of CHD in healthy individuals (Kaptoge et al 2014) and that it was an independent predictor of CVD (Zhang et al 2018), suggesting that IL-6 does indeed play a role in CVD prediction. The role of IL-6 pathways was discussed in detail in a comment published in the Lancet by Boekholdt & Stroes (2012). They proposed, based on a large meta-analysis and an MR study (IL6R Genetics Consortium Emerging Risk Factors Collaboration 2012; IL-6R MR Consortium 2012) that IL-6 pathways may even have a causal role in determining risk of CHD (Boekholdt & Stroes 2012). However, as IL-6 and its receptor have a short half-life and diurnal variation (Dugue & Leppänen 1998), their use as biomarkers or indeed in epidemiological and other studies without a genetic component may be questionable.

Our findings support the important role of subclinical inflammation in the pathogenesis of CVD events (Ross 1999; Ridker et al 2017). It is important to note that we took smoking, waist-to-hip ratio, total to HDL-cholesterol ratio and diabetes, all of which are associated with subclinical inflammation and could be considered as parts of the same biological pathway, into account in our analyses. For instance, LDL cholesterol has been suggested to be a moderator of the contribution of CRP to incident CHD and stroke (Lin et al 2016; Penson et al 2018). The associations of TNF-alpha with incident CHD and CVD were less affected by controlling for other cardiovascular risk factors than those of CRP and IL-6. TNF-alpha's correlation with obesity, smoking and blood pressure was weaker than the other markers, supporting the idea that a larger part of TNF-alpha's effect may be mediated through other than the traditional risk factors.

Since our findings were published, the use of CRP, IL-6 and TNF-alpha as biomarkers of subclinical inflammation, in risk prediction and as targets of anti-inflammatory therapy has increased substantially. This will be further discussed in chapter 6.5.

6.3.4 PERIODONTITIS IS ASSOCIATED WITH AN INCREASED RISK OF INCIDENT CVD

The chronic nature of periodontitis is reflected by elevated levels of IgG antibodies. The high levels persist in patients who have periodontitis, displaying its recurrence or episodes of systemic exposure to periodontal pathogens, but not necessarily the current disease status (Papapanou et al 2004). Furthermore, a previous study showed that serum levels of IgG antibodies against *A actinomycetemcomitan*s or *P gingivalis* were higher in patients with stable periodontitis than in those with active periodontitis (Rams et al 2006).

Our study demonstrated that elevated levels of serum IgG antibodies to the two above-mentioned major periodontal pathogens were risk factors for future CVD events. A large recent study found that an IgG antibody cluster of pathogens including *P gingivalis* was not associated with CVD mortality (Qi et al 2020), although being grouped with a number of other pathogens may complicate the interpretation of these results. A smaller clinical study by de Boer and co-workers (2014) also found no significant association between levels of plasma IgA and IgG antibodies to periodontal pathogens with coronary atherosclerosis, except for IgG antibodies for *P gingivalis* in a subgroup of patients with diabetes (de Boer et al 2014). However, a number of epidemiological and clinical studies have been in line with our findings especially with regards to *P gingivalis* (Damgaard et al 2017; Bohnstedt et al 2010; Bizzarro et al 2010; Spahr et al 2006). Moreover, *A actinomycetemcomitan*s and *P gingivalis* have both been found in the thrombi removed from patients with an acute transmural MI (Ohki et al 2012).

We found that serum TNF-alpha levels increased with increasing quartiles of serum levels of IgG antibodies to *P gingivalis* after controlling for age and sex. However, we observed no such association for CRP. It has been reported that higher serum concentrations of CRP are present in patients with clinical or advanced periodontitis compared with periodontally healthy individuals (Buhlin et al 2003; Linden et al 2008; Paraskevas et al 2008; Torrungruang et al 2019; Meisel et al 2021) and periodontal therapy has been shown to be associated with decreases in CRP concentrations together with improvements in cardiovascular health (Demmer et al 2013). However, rare reports have linked periodontal bacteria or antibodies to these pathogens directly with inflammation markers (Pussinen et al 2003; Desvarieux et al 2005; Dye et al 2005; Nibali et al 2008; Miyashita et al 2012). The lack of such associations may be due to the fact that periodontal pathogens are also present in normal flora and thus frequently found in persons without periodontitis.

Another potential confounding factor in our study design is socioeconomic status. Low socioeconomic status is known to be associated both with periodontitis and the risk of CVD (Stringhini et al 2017; Borrell & Crawford

2012; Kaplan & Keil 1993). Socioeconomic status is nowadays almost invariably used as a covariate in epidemiological studies, including the current thesis. However, measuring socioeconomic status is challenging and not very precise. We opted to use years of education that is an accepted measure of socioeconomic status and is especially recommended for studies on CVD (Tousoulis et al 2020; Hamad et al 2019). By using just one measure of socioeconomic status, however, it is difficult to completely rule out residual confounding that may lead to overestimation of the true association between periodontitis and the risk of CVD.

Our study showed that an increased serum endotoxin concentration was associated with incident CVD. We also found that a serum endotoxin concentration correlated positively with serum IgG antibodies to *P gingivali*s and *A actinomycetemcomitans*. However, endotoxin is a short-lived compound in circulation. Therefore, our results do not distinguish between long-term endotoxemia caused by chronic infections such as periodontitis and transient bacterial translocation at baseline. Yet, it has previously been demonstrated that the severity of periodontitis is relative to the degree of endotoxemia and the area of inflamed periodontal tissue (Geerts et al 2002; Pussinen et al 2004; Paju et al 2006). Patients with periodontitis are known to have higher serum endotoxin concentration than periodontally healthy people or patients after treatment of periodontitis (Geerts et al 2002; Pussinen et al 2004).

Serum total cholesterol and HDL cholesterol were negatively correlated with serum endotoxin concentrations in our study, which suggested that any association between serum endotoxin and CVD was not statistically independent of blood lipids. This is especially interesting in the light of an in vitro study proposing that endotoxin derived from *A actinomycetemcomitans* is involved in the induction of atherogenesis mediated by LDL (Morishita et al 2013). Endotoxin is cleared by all classes of lipoproteins; however the binding and neutralizing properties of HDL have been found to be particularly effective (Levine et al 1993; Levels et al 2001). Moreover, endotoxin-induced inflammation processes are known to decrease HDL cholesterol levels, indicating that the increased serum endotoxin levels and decreased blood HDL levels associated with incident CVD in our study are in line with the findings of other studies (Lakio et al 2006; Pussinen et al 2001; Pussinen et al 2004).

In contrast to our findings regarding levels of serum IgG antibodies to *A actinomycetemcomitan*s and *P gingivalis*, we observed only weak associations of serum endotoxin quartiles and serum inflammation markers. This is interesting in the light of the direct and indirect contribution of endotoxin to the release of cytokines such as TNF-alpha and IL-6 (Cavaillon 2018). Nevertheless, the lack of correlations among these substances is not surprising due to the short-lived nature of especially endotoxins and interleukins in the circulation.

The pathogenic mechanism underlying the links between infection and atherosclerosis has long been thought to be the induction of subclinical inflammation. Our study supports this hypothesis, as we found that an increased combined response of serum antibodies *A actinomycetemcomitan*s or *P gingivalis* and increased serum endotoxin levels were clearly associated with incident CVD when accompanied by simultaneous subclinical inflammation. Those who respond to these potentially atherogenic infectious agents with systemic inflammation thus seem to be at the highest risk of developing CVD. This could be explained by genetic factors or other chronic infections (Kiechl et al 2001; D'Aiuto et al 2004; Sanz et al 2020).

Periodontitis has been established as a risk factor for CVD by consensus based on systematic reviews (Sanz et al 2020; Larvin et al 2020; Dietrich et al 2013). Periodontitis has also been linked with obesity (Meisel et al 2021; Shungin et al 2015), hyperglycaemia (Merchant et al 2014), insulin resistance (Demmer et al 2012), diabetes (Pussinen & Salomaa 2018; Polak & Shapira 2018) and most recently with metabolic syndrome (Määttä et al 2021). In their study examining periodontitis, inflammation and obesity, Meisel and co-workers (2021) proposed that subclinical inflammation may have a mediating function between adiposity and periodontitis (Meisel et al 2021). However, two earlier MR studies did not support a causal relationship between abdominal adiposity and periodontitis (Shungin et al 2015; Winning & Linden 2017).

The major question that still remains unanswered is whether the association of periodontitis with incident CVD is from pathogen exposure or periodontitis itself. A recent review by Winning & Linden (2017) has proposed that it may actually be the pathogen exposure that is key in the association (Winning & Linden 2017). Moreover, causality in general is yet to be confirmed or disputed (d'Aiuto et al 2013; Qi et al 2020), and the relationship between periodontitis and systemic diseases is very much considered bi-directional. These different aspects of periodontitis, subclinical inflammation and CVD need to be further studied to gain more in-depth understanding on their associations as well as to plan relevant interventions for the primary and secondary prevention of CVD.

6.3.5 SUBCLINICAL INFLAMMATION IS LINKED WITH ALL-CAUSE MORTALITY

In addition to increasing the risk of CVD, our study found elevated serum TNF-alpha to be associated with increased all-cause mortality. As with CVD, the risk increased of death from any cause increased from the second quartile of serum TNF-alpha upwards and was therefore increased in three quarters of the population. Other studies have reported that elevated levels of serum TNF-alpha are predictive of a recurrent event after a MI and coronary mortality (Ridker et al 2000; Koukkunen et al 2001). TNF-alpha has also been identified

as a predictor of congestive heart failure (Vasan et al 2003). These reports combined with the pleiotropic nature of TNF-alpha may in part explain its strong association with all-cause mortality in our study.

The usual suspects of all-cause mortality prediction CRP and IL-6 showed such evidence only among men in our study. Increased levels of these inflammation markers have been linked with all-cause mortality in other studies (Singh-Manoux et al 2017; Ahmadi-Abhari et al 2013). A recent study using the National Health and Nutrition Examination Survey (NHANES) Linked Mortality cohort also found the associations of CRP with all-cause and CVD mortality to be more pronounced in men than in women. Moreover, that diet quality modified the association between CRP and CVD mortality (Liu et al 2020). A recent systematic review and meta-analysis concluded that dose-response associations of CRP with all-cause and CVD mortality existed in men and were curvilinear (Ni et al 2020).

It is clear that subclinical inflammation is linked with all-cause mortality. Using nuclear magnetic resonance, an alternative high throughput metabolic biomarker platform to LC-MS, certain metabolite profiles have been identified to be associated with all-cause mortality (Deelen et al 2019), and one of the potential mechanisms for this association is inflammation. In our study, serum TNF-alpha showed the most promise in predicting death from any cause among both sexes combined. However, especially among men, serum CRP and IL-6 were also predictors of death. Better understanding on the usefulness of inflammation markers in death prediction and prevention is needed to further establish the likely multi-faceted links between subclinical inflammation and death.

6.4 STRENGTHS AND LIMITATIONS

The major strengths of the FINRISK and DILGOM studies include their high participation rates and their prospective, population-based design. Moreover, in the studies using data from FINRISK, follow-up was complete. Due to linkages to national health information registers, we were also able exclude certain diseases from the analyses and control for important co-morbidities. In all the studies, we controlled for traditional known risk factors of the disease outcomes for which information had been obtained in the broad surveys or through blood samples.

We carried out simultaneous measurements of several inflammation markers, eicosanoids and related oxylipins (paper II) as well as antibodies to periodontal pathogens and endotoxin concentrations (paper IV) from serum and plasma samples. The eicosanoid profiling, for all the three cohorts used in paper II, was done in the same laboratory with the same methodology. That methodology was sophisticated, directed, non-targeted LC-MS combined with

computational chemical networking, allowing us to identify hundreds of known and putative eicosanoids from the majority of the study participants.

All the cohorts used in the analyses included both men and women, and in paper III, we demonstrated differences in the findings between the two sexes. Finally, the partial replication of our results in three different cohorts presented in paper II is an important strength of the study. The eicosanoid known as 8-iso-PGA1 replicated robustly in the FHS cohort and the other two were directionally consistent.

Nevertheless, our studies did include a number of limitations as well. A common limitation to all our studies is their ethnic profile: our studies included mainly white participants of European ancestry. Therefore, it may not be feasible to generalise our findings to all ethnic groups.

Although in DILGOM the participation rate for the follow-up was high, it was not complete. Furthermore, those who dropped out from follow-up differed from the final study population by being younger, heavier and having higher serum levels of inflammation markers. However, we deemed it unlikely that there would be a substantial difference between the participants and the non-participants in the associations between changes in obesity measures and inflammation markers.

Additionally, in DILGOM, we could not invite all participants for the physical re-examination. Despite the validation of the self-measurements against measurements performed by the study nurses, reporting bias may still exist. And although we measured hs-CRP from serum samples of participants who attended the re-examination, there was no data available on the other inflammation markers.

There may be reporting bias due to self-reporting in all of our studies, namely and where relevant, self-reported smoking status, leisure-time physical activity, alcohol use and total energy intake. For paper III, we did not have data on anti-inflammatory medicines, except acetylsalicylic acid, angiotensin-converting enzyme inhibitors, or vaccinations, all of which may have had an effect on inflammation markers. We also had no information on the actual oral health status of the study participants for paper IV, apart from the levels of serum antibodies to periodontal pathogens. Moreover, some of these participants may actually have been edentulous, potentially confounding the associations studied and contributing to the negative findings on IgA antibodies. And finally, in paper III, the only consideration of the possible contribution of genetic risks to the associations studies was in the form of controlling for reported family history of type 2 diabetes.

There was some concern about limited statistical power in the analyses of all the papers. For papers III and IV, there were a number of participants for

whom stored serum was no longer available at the time of the study. Although this was a random phenomenon and was evaluated to have no effect on the results, it weakened statistical power in the analyses. Furthermore, in paper III, the number of incident cases of CVD for women was low, which could explain our negative findings among women. Similarly, the lack of an additional effect in persons with a combination of elevated CRP and elevated TNF-alpha should be interpreted with caution. Lastly, for paper II, the peptide markers of inflammation, as well as the measures of glucose and insulin metabolism, were available only for a subgroup of the study population.

Replication of our results in paper II lacked statistical significance for 12-HHTrE and the unknown eicosanoid. This may also be due to the limited statistical power in both DILGOM and FHS Offspring, and perhaps the participants of the latter being on average 20 years older than those of FINRISK participants. Finally, a relevant limitation in paper II is the fact that the identity of several eicosanoids remains unknown, including the eicosanoid with an inverse association with incident type 2 diabetes. As the laboratory methods used were not able to distinguish between different enantiomers of the molecules, some identified substances may still represent a number of eicosanoids derived through different pathways and acting in different ways. Further scaled-up advancements in chemistry could lead to the identification of different isomers of the metabolites and thus allow for improved accuracy of eicosanoid enantiomers.

6.5 CLINICAL AND PUBLIC HEALTH SIGNIFICANCE OF FINDINGS AND FURTHER RESEARCH NEEDS

Our findings discussed together with recent literature paint a picture of subclinical inflammation as an important player in the obesity-type 2 diabetes-CVD trifecta and suggest the following progression (Figure 10):

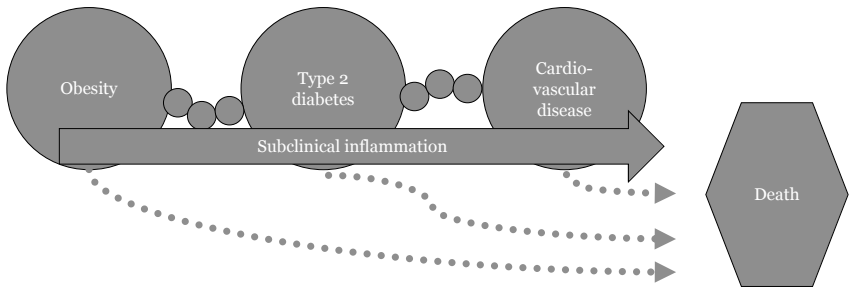

Figure 10 Proposed sequence of subclinical inflammation and the obesity-type 2 diabetes-CVD trifecta

However, establishing causality is tricky, especially when based mostly on observational studies (Franks 2006). It is important to note that in Figure 10, there are only a few parts for which we can confidently assign some level of causality. These are namely that cardiovascular events may be fatal and that subclinical inflammation may indeed lead to CVD, as demonstrated by the recent RCTs on anti-inflammatory medicines' effects on cardiovascular disease outcomes (Ridker et al 2008; Ridker et al 2017; Bäck et al 2019). We can furthermore affirm, that adipose tissue may act as a source of subclinical inflammation (Hotamisligil et al 1993; Weisberg et al 2003; Reilly & Saltiel 2017). Yet, we cannot say for sure what triggers subclinical inflammation.

Interesting recent findings from genome-wide associations studies (GWAS), MR studies and RCTs provide us with further thoughts with regards to the interplay between subclinical inflammation and the obesity-type 2 diabetes-CVD trifecta. For example, Ligthart and co-workers (2018) concluded based on their GWAS examining causality between CRP and CHD that genetic regulation of subclinical inflammation seemed to be largely independent of BMI (Ligthart et al 2018).

All in all, we know that subclinical inflammation is linked with metabolic and atherothrombotic aspects of obesity, type 2 diabetes, CVD and death. The case for why we should prevent and detect subclinical inflammation has been made as it will allow us to improve the prevention and detection of these diseases. In the following sections, I discuss how subclinical inflammation can be prevented, detected, and finally, why and how we should treat it with therapeutic measures.

6.5.1 PREVENTION AND REDUCTION OF SUBCLINICAL INFLAMMATION VIA CONTROL OF RISK FACTORS

As set out in section 2.2.1, there are a number of factors driving or increasing subclinical inflammation. Obvious steps to decrease the risk of subclinical inflammation would be by preventing atherosclerosis, periodontitis, weight gain or accumulation of adipose tissue. This could be achieved by eating a healthy and balanced diet, leading an active lifestyle throughout the lifespan, breathing clean air, not taking up smoking and regularly brushing one's teeth and flossing. There are however also preventive measures as well as treatment options available for decreasing on-going subclinical inflammation.

Smoking cessation is known to reduce inflammation marker levels in circulation (McElroy et al 2019; Reichert et al 2009). Currently, there are several smoking cessation therapies available, including support groups, psychotherapy and medicines. Smoking cessation is known to reduce the risk of CVD and may bring the smoking-related risk down to the level of never-smokers within a few years (Caldwell et al 2019). However, it is important to

remember that smoking cessation is often accompanied by weight gain, thus undermining some of the benefits of quitting smoking (Yoon et al 2010).

For people who are overweight or obese, losing weight may be an important way to both decrease subclinical inflammation. Therapeutic options for overweight and obesity include support groups, psychotherapy, eating diaries, mobile apps, medicines and surgery, often as a combination of two or more. There is evidence of bariatric surgery substantially decreasing subclinical inflammation, although this may not happen in everyone (Askarpour 2019; Schmitz et al 2016). Furthermore, a recent UK biobank study demonstrated that decreased BMI was consistently associated with reduced risk of type 2 diabetes across categories of other risk factors (Wainberg et al 2019). This finding suggests that the risk of type 2 diabetes could be decreased irrespective of an individual's diabetes risk through weight loss.

Each of the weight loss therapies mentioned above aim in one way or another to improve diet and increase physical exercise. The Finnish Diabetes Prevention study found that increasing physical activity and increasing fibre intake were strongly associated with reduction in circulating CRP and IL-6 levels in individuals at increased risk of CVD (Herder et al 2009). A review by Pedersen (2017) further concluded that physical activity is a good anti-inflammatory strategy through inhibiting TNF-alpha and stimulating IL-1Ra with minor side effects (Pedersen 2017). Furthermore, a long-term increase in physical activity has indirect anti-inflammatory effects via improved body composition.

It is currently suggested that a balanced diet is important for both human health and gut microbiota, and although probiotic therapies may improve gut microbiota composition, preference over unsaturated fats versus saturated ones should be considered to regulate gut microbiota as well as inflammation (Candido et al 2018). Another interesting finding from a recent study is that individuals with genetic susceptibility to type 2 diabetes are more likely to have oxidative stress and inflammation following a high-carbohydrate meal (Baig et al 2020). This observation suggests that an improved carbohydrate composition of meals may play a role in reducing subclinical inflammation.

As subclinical inflammation is associated with a number of consequences of obesity, it is important to reflect on whether reducing the inflammatory impact of obesity through any kind of losing weight is effective. As mentioned above, a decrease in subclinical inflammation is not a given after weight loss, and not in all tissues. Phillips & Grayson (2020) have recently concluded that the degree of reduction in the load of inflammation markers is dependent on the method of weight loss. For instance, specific surgical procedures followed by weight stabilisation may lead to positive benefits, whereas lifestyle changes only may not (Phillips & Grayson 2020).

Some antidiabetic medicines also come with added advantages of losing weight and reducing subclinical inflammation. In addition to its effects on insulin and glucagon secretion, treatment with glucagon-like peptide-1 (GLP-1) receptor agonists has been found to slow down gastric emptying, reduce food intake and decrease body weight, as well as to reduce levels of circulating inflammation markers such as CRP, IL-1beta, IL-6 and TNF-alpha (Nauck et al 2017). Furthermore, sodium-dependent glucose cotransporter-2 (SGLT-2) inhibitors, which induce urinary glucose excretion and reduce fasting and postprandial blood glucose levels, also lower body weight (Cai et al 2018) and reduce levels of circulating inflammation markers such as CRP and TNF-alpha (Bonnet & Sheen 2018).

There is evidence that promoting better oral health reduces existing subclinical inflammation. A large study from Scotland showed that poor oral health behaviours, such as irregular tooth brushing, was associated with increased levels of serum CRP (de Oliveira et al 2010). Moreover, systematic reviews by Lam and co-workers (2011) and D'Aiuto and co-workers (2013) concluded that oral health promotion activities and periodontal treatments improve periodontal health and reduce systemic inflammation (Lam et al 2011; D'Aiuto et al 2013).

However, these systematic reviews were not able to confirm whether reduced subclinical inflammation decreases cardiovascular risk in the long term. A review on the treatment of periodontitis concluded that non-surgical therapy of periodontitis reduced systemic inflammation at least in the short-term (D'Isidoro et al 2019). Whether such actions reduce the risk of CVD remains under debate. Even so, results of a recent systematic review and meta-analysis suggest that prevention and management of periodontitis may prevent or improve hypertension (Munoz Aguilera et al 2020).

A study on the systemic effects of treating periodontitis in patients with type 2 diabetes found that intensive periodontitis treatment improved metabolic control compared with usual care (d'Aiuto et al 2018). Furthermore, the reductions in blood HbA1c and fasting plasma glucose concentrations were mirrored by reduced levels of circulating inflammation markers such as CRP and TNF-alpha, suggesting a causal relationship between periodontitis and type 2 diabetes.

Of interest is also the recent debate concerning the causality of the links between the oral microbiome, systemic inflammation and chronic disease. A critical review by Kleinstein and co-workers (2020) identified the still open question as to whether inflammation and disease are caused by the oral microbiome, their metabolites or the host-response (Kleinstein et al 2020). Host-response has been suggested to play a major role in inflammation, and active resolvers of inflammation may be promising in the treatment of periodontitis and other inflammatory disease (van Dyke 2017). Indeed, a shift

in paradigm over the last years has been moving research interests towards resolvers rather than inhibitors of inflammation in the treatment of periodontitis and by extension systemic inflammation (Van Dyke et al 2020).

A healthier lifestyle exemplified by a combination of smoking cessation, increased physical activity and weight loss was found to decrease levels of plasma CRP in patients with stable CVD in a recent study from the Netherlands (van't Klooster et al 2020). To summarise, lifestyle changes accompanied by treatment options such as medical assistance for smoking cessation, bariatric surgery for weight loss or medicines such as GLP-1 receptor agonists or SGLT-2 inhibitors for type 2 diabetes, may play an important role in the reduction of subclinical inflammation. Primary and secondary prevention activities alike are likely to reduce the burden of systemic inflammation and potentially that of inflammation-associated diseases.

6.5.2 CLINICAL DETECTION OF SUBCLINICAL INFLAMMATION TO ESTABLISH DISEASE RISK

Although major improvements have been made in reducing cardiovascular mortality over the years by advancements in acute management or MI and stroke, the incidence of non-fatal cardiovascular events has not experienced the same rates of improvement. Indeed, the trends in the incidence CHD, especially among younger individuals in the Global North, are worrisome (Salomaa 2020), calling for more emphasis on primary prevention of CVD.

Ever since Pearson and colleagues (2003) published their statement on the use of inflammation markers for the benefit of preventing CVD, CRP has been variably used as an adjunct risk factor to the major risk factors to improve the assessment of CHD risk (Pearson et al 2003). Perhaps due to the recommendations by Pearson and co-workers (2003), hs-CRP is by far the most, if not the only, inflammation marker test used in conventional clinical practice in conjunction with other risk factors to determine an individual's cardiovascular risk.

In current clinical practice, an individual's cardiovascular risk is often assessed using one of the many developed risk scores. Most commonly used risk scores include the Systematic COronary Risk Evaluation (SCORE) and its recently upgraded version SCORE2, the WHO risk score, the American College of Cardiology/American Heart Association 2013 Pooled Cohort risk equations (PCE) and the QRISK3 (Conroy et al 2003; SCORE2 WG & ESC CRC 2021; WHO CVD Risk Chart Working Group 2019; Goff et al 2014; Hippisley-Cox et al 2017). In North America, Framingham Risk Scores (FHS-CHD and FHS-CVD) as well as the Reynolds Risk Score (d'Agostino et al 2008; Ridker et al

2007) are also in use. Out of these scores, only the Reynolds Risk Score (RRS) includes hs-CRP in the risk calculation.

A recent meta-analysis by the Emerging Risk Factors Collaboration (2019) comparing four of the risk algorithms before and after a recalibration, considering differences in population risk characteristics, found that FRS, SCORE and PCE overpredicted risk, whereas RRS modestly underpredicted it (Pennells et al 2019). However, after recalibration all scores performed nearly equally well, improving targeting of prevention (Pennells et al 2019). This suggests that in clinical practice, the use of any risk score, once recalibrated to account for population risk characteristics and clinical need, would be beneficial to the prevention of CVD. However, the usefulness and cost-effectiveness of hs-CRP testing as part of risk assessment for CVD still remains under debate.

Although not in routine use in cardiovascular risk assessment of symptomless, low-risk people, hs-CRP has been proposed to be useful in predicting CVD or death in patients with familial hypercholesterolemia and those who have experienced a MI (Rahman et al 2017; Swiatkiewicz et al 2012; Carrero et al 2019; Mani et al 2019). Rahman et al (2017) found that both patients with familial hypercholesterolemia and their related unaffected family members had higher levels of inflammation markers (Rahman et al 2017). It is therefore possible that inflammatory markers are useful in establishing their CHD risk. And since it was especially hs-CRP that was elevated, the test could be used for risk screening purposes in both patients with familial hypercholesterolemia and their unaffected relatives.

Carrero and co-workers (2019) concluded that using hs-CRP tests may help to assess the risk of death or plan secondary prevention of acute coronary events in stable patients with MI (Carrero et al 2019). Furthermore, Mani and co-workers (2019) proposed that serial measurements of hs-CRP during clinical follow-up after an acute coronary event could be helpful in identifying patients at higher risk for recurrent coronary events as well as cardiovascular and all-cause death (Mani et al 2019).

Given the commercial availability and relatively low cost of hs-CRP tests, this type of use may well also be cost-effective after a MI and therefore research focused on their economic evaluation research is warranted. However, the existing guidelines of the European Society of Cardiology and Finnish Current Care guidelines, both of which are currently under review, suggest that the contribution of measuring hs-CRP to existing methods of cardiovascular risk assessment is likely to be small (Piepoli et al 2016; Porela et al 2015).

A report from the UK cohort of the European Study on Cardiovascular Risk Prevention and Management in Usual Daily Practice (EURIKA) revealed that a large proportion of patients with a low 10-year QRISK2 score and a ‘heart

age' greater than their chronological age according to the recommendation on the prevention of CVD of the Joint British Society had elevated levels of plasma hs-CRP (Johns et al 2018). The authors of this report concluded that measuring hs-CRP levels in conjunction with lifetime risk considerations, such as heart age, may help identify those at higher risk amongst the low-risk populations.

Mirroring these results with the reports from the JUPITER and CANTOS trials, there is a potential for earlier intervention in terms of prevention measures for CVD in this kind of otherwise low risk population. Another study in a Chinese population examined cumulative exposure to high levels of hs-CRP and found it to reflect CVD risk, and especially MI risk (Wang et al 2017). The authors suggested that serial measurement of hs-CRP may be better than a single measurement, as repeated measurements are less prone to misclassification. Furthermore, they found that the length of exposure to elevated cumulative hs-CRP levels affected future risk of CVD in a dose-responsive manner. These findings are in line with the now near-proved theory of atherosclerosis being a progressive inflammatory disease leading to coronary and other clinical consequences of atherosclerosis.

Finally, in relation to CRP, it is important to address the fact that although often treated as a purely pro-inflammatory marker in observational and clinical studies, like IL-6, it actually possesses both anti- and proinflammatory characteristics. Whereas the multifunctionality of IL-6 is explained by its actions through two distinct signalling pathways, the bilateral role of CRP is due to its existence in two different isoforms (Del Giudice 2018). Therefore, the clinical use of CRP as well as the potential use of IL-6 as a biomarker in risk prediction and particularly in clinical practice should be considered with caution.

The findings of the present study relative to an eicosanoid risk score predicting type 2 diabetes (paper II) suggest potential windows of opportunity for intervention in terms of prevention of type 2 diabetes. The two eicosanoids 8-iso-PGA1 and 12-HHTrE could be used as personalised biomarkers for early the identification of people at increased risk of type 2 diabetes. Adding strength to our speculation, 12-HHTrE was also associated with incident hypertension (Palmu et al 2020). However, the role of theses eicosanoids in the early development of insulin resistance and type 2 diabetes should be first examined in closer detail.

Other novel biomarkers of inflammation are being identified and linked with the risk of cardiovascular disease (Krintus et al 2014; Thomas & Lip 2017; Dugani et al 2021). Some fairly recent reports propose glycoprotein acetylation (GlycA) as a novel biomarker of subclinical inflammation and increased risk of CVD, and especially premature CHD, being also associated with type 2 diabetes (Connelly et al 2017; Kettunen et al 2018; Ballout & Remaley 2020;

Dugani et al 2021). Connelly and co-workers (2017) argued that GlycA could be a useful marker of CVD risk especially in patients with autoimmune diseases (Connelly et 2017), whereas Ballout & Remaley (2020) proposed that it was a better test than hs-CRP, being a more stable compound with less variability between sexes (Ballout & Remaley 2020). Further research on this biomarker closely linked to CRP is called for, as well as continuing the hunt for other, better biomarkers.

6.5.3 THERAPEUTIC TARGETING OF SUBCLINICAL INFLAMMATION TO REDUCE CARDIOVASCULAR RISK

The past two decades has been an important phase in targeting subclinical inflammation to prevent CVD. Section 6.5.1 summarised activities to reduce subclinical inflammation through its risk factors, whereas in this section therapeutics targeting subclinical inflammation directly will be explored. RCTs examining the effects of anti-inflammatory medicines such as statins, IL-1beta inhibitors and methotrexate have received the most interest and have provided us with near-certainty of the causality between subclinical inflammation, atherosclerosis and its complications (Ridker et al 2008; Ridker et al 2017; Ridker et al 2019).

The JUPITER trial demonstrated the benefits of statin therapy in people with high CRP levels and low LDL levels who would not normally be considered for pharmacotherapy (Ridker et al 2008). In their study population, rosuvastatin reduced the rates of the first CVD event and death from any cause. Although not able to prove causality between subclinical inflammation and CVD, the JUPITER trial did give a boost for interest in further research on anti-inflammatory medicines.

Studies conducted after the JUPITER trial using Mendelian randomization have increasingly concluded that CRP does not play a causal role in the development of atherosclerosis (Elliott et al 2009; Nordestgaard & Zacho 2009; CCGC 2011). Moreover, the results of consortia examining IL-6 receptor pathways suggested that these pathways, which are slightly more upstream from CRP, may play some of the causal role in the development of CHD (IL6R MR Consortium 2012; IL-6R Genetics Consortium Emerging Risk Factors Collaboration 2012). The judgement drawn from the results of these consortia was that the development of therapeutic strategies targeting reductions in CRP levels was unlikely to be the panacea of treating atherosclerosis and its complications, but perhaps therapeutic targets could be identified more upstream.

As a result, the CANTOS trial focused on targeting IL-1beta, a cytokine more upstream from CRP and the driver of the IL-6 signalling pathway (Ridker et al 2017). The trial was designed to test the inflammatory hypothesis of

atherothrombosis. In CANTOS, patients with a history of MI were treated with canakinumab, an antibody against IL-1beta, which was found to reduce CRP and IL-6 levels from baseline, compared to placebo. Furthermore, unlike in JUPITER, lipid levels did not reduce in response to the canakinumab treatment in CANTOS. And finally, those who were treated with canakinumab had a lower incidence of recurrent cardiovascular events compared to those receiving placebo (Ridker et al 2017).

The CANTOS results were modest in terms of all-cause mortality, as patients treated with canakinumab were more likely to die from infection, probably due to the host defence-compromising potential of a purely anti-inflammatory therapy (Tabas & Glass 2013). However, it did show that those with most reduction in CRP levels also saw a more dramatic reduction in cardiovascular and all-cause mortality (Swirski 2017), thus finally confirming the inflammation hypothesis in the development of atherosclerosis.

A similar RCT going by the name of CIRT targeted lowering IL-1beta, IL-6 and CRP levels in patients with a previous MI or otherwise at high risk of a coronary event. The research group found that treatment with methotrexate did not reduce levels of these inflammation markers nor did it reduce the incidence of CVD, compared to placebo (Ridker et al 2019). Furthermore, preclinical and clinical data from trials on the inhibition of TNF-alpha, although mostly not originally planned with cardiovascular risk in mind, has produced inconsistent results in terms of cardiovascular outcomes (Ait-Oufella et al 2019).

Colchicine, an anti-inflammatory medicine affecting amongst others cytokine secretion and used predominantly in the prevention of acute inflammatory episodes such as those in gout, has recently been brought into the limelight as a potential new medicine for the prevention of CVD (Crea 2021). A meta-analysis based on a systematic review of RCTs has concluded that low-dose colchicine seems to reduce the risk of cardiovascular events such as MI and stroke, as well as need for coronary revascularisation, but not all-cause mortality (Fiolet et al 2021). These results support the conclusions from CANTOS and further encourage the concept of targeting subclinical inflammation as a way of preventing and treating atherosclerosis and its complications.

Other interesting findings from recent years, have opened further new horizons in targeting subclinical inflammation to prevent cardiovascular events. Inhibition of clonal hemotopoiesis (Jaiswal et al 2017) leaning towards precision medicine and pro-resolving mediator therapy (Bäck et al 2019) that would entail less compromising effect on host defence are both new possibilities in the treatment of atherosclerosis and its complications (Boland & Long 2021; Libby & Everett 2019). Both of these therapies that are linked to the immune response, they may well prove to have potential in reducing the

risk of CVD. Further research into clonal hematopoiesis and to potential pro-resolving therapies as well as clinical trials with anti-inflammatory and anti-cytokine agents is called for.

Based on this thesis it can be concluded that subclinical inflammation is strongly involved in major public health diseases, namely obesity, type 2 diabetes and cardiovascular disease. The results of the CANTOS trial provide evidence that anti-inflammatory treatment may reduce CVD incidence, however this beneficial effect appears to come at a cost of potential serious infectious disease complications.

It seems to be possible to reduce subclinical inflammation with lifestyle changes and medical treatment, however further research is warranted in this area. Improving one's diet, losing weight and increasing physical activity have some anti-inflammatory effects (Herder et al 2009; Pedersen et al 2017; Phillips & Grayson 2020), yet confirmation on the extent of their role in reducing subclinical inflammation is still needed. As for medical targeting of subclinical inflammation, the identification of potential therapeutic targets within the inflammatory pathways remains a challenge.

Regarding targeting subclinical inflammation to prevent or treat diabetes, some antidiabetic agents and other medicines used in the treatment of type 2 diabetes and prevention of its complications have both direct and indirect anti-inflammatory properties (Goldfine & Shoelson 2017; Donath 2016; Santilli et al 2015). This strongly suggests that prevention and treatment of type 2 diabetes and its complications through targeting subclinical inflammation presents possibilities. However, it is good to keep in mind, that e.g. glucocorticoids, which have strong anti-inflammatory properties, are known to promote insulin resistance and worsen glucose homeostasis. So far though, other immunomodulatory therapies are not known to alter glucose control in a substantial way.

Both positive and negative effects of e.g. the inhibition of the various COX receptors on cardiovascular risk have been extensively studied, although further understanding of eicosanoid signalling would allow us to get a better picture of atherothrombotic development (Gleim et al 2012). Based on our findings, it may be worthwhile to investigate further eicosanoids such as 8-iso-PGA1 and 12-HHTrE that are associated with incident type 2 diabetes, as potential targets for treating subclinical inflammation in order to prevent or treat type 2 diabetes and its cardiovascular complications.

The hypothesis, supported by our findings and the literature presented, that subclinical inflammation is what links obesity, type 2 diabetes and CVD together, allows for speculation that there may well be a common treatment target within the inflammatory pathways for both type 2 diabetes and CVD to prevent associated morbidity and mortality. Although a single approach

treatment may not be optimal (Goldfine & Shoelson 2017), we need to have a better understanding of the underlying biological processes in order to be able to develop safe and cost-effective therapies which would consider the metabolic and atherothrombotic characteristics of the obesity-type 2 diabetes-CVD trifecta.

7 CONCLUSIONS

Based on the observations made in this study, a number of conclusions can be drawn. Subclinical inflammation is linked with both metabolic and atherothrombotic aspects of common public health problems. It seems to be at least one of the links between obesity, type 2 diabetes, cardiovascular disease and death. Clinical detection of subclinical inflammation may help in establishing disease risk and allow for early intervention. Moreover, subclinical inflammation can be reduced by addressing its risk factors, and if necessary, with medications.

Therapeutic targeting of subclinical inflammation to reduce the risk of cardiovascular disease has been shown to be possible. We need a better understanding of the biological processes underlying subclinical inflammation and the obesity-type 2 diabetes-CVD trifecta, in order to be able to develop safe and cost-effective therapies, which would consider both the metabolic and atherothrombotic characteristics of these diseases.

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