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Syventävien opintojen kirjallinen työ

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Sydäntutkimuskeskus

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Abstract

Tobacco smoking is one of the major causes of lifestyle morbidity and mortality by causing cardiovascular disease, lung disease and cancer in particular. Chronic inflammation caused by tobacco smoke plays a significant role in the pathophysiology of chronic diseases. To date, several cytokines have been identified to associate with smoking, but mainly a few inflammatory cytokines have repeatedly been reported. Broader mapping of smoking-related cytokines could help to understand the chronic disease pathogenesis better.

The aim of this study was to elucidate the associations between daily smoking and inflammatory markers such as cytokines, chemokines, and growth factors measured from serum. This study is a part of the ongoing population-based Cardiovascular Risk in Young Finns Study (YFS). The concentrations of 48 cytokines, chemokines, and growth factors were measured with multiplex assay kits. Smoking status was self-reported on a structured questionnaire and a categorized variable was constructed (daily/occasionally/quit/never). Age, BMI, systolic blood pressure, HDL and LDL cholesterol, triglycerides, physical activity level, diet, and alcohol use were used as covariates in the statistical analyses. Analysis of covariance was used to study the differences between the never smoking and other smoking groups. The analyses were performed separately for men and women.

Compared to never smoking, daily smoking associated with six cytokines, including three growth factors (HGF, βNGF and SCF) and three inflammatory cytokines (CXCL10, CXCL9 and IL-18). In men and women, daily smoking was directly associated with HGF (Men: β=0.178, SE=0.038, P<0.001; women: β=0.122, SE=0.036, P<0.001) and indirectly with CXCL10 (Men: β =-0.182, SE=0.050, P<0.001; women: β =-0.245, SE=0.050, P<0.001). In women, daily smoking was indirectly associated with CXCL9 (β=-0.195, SE= 0.055, P<0.001) and with βNGF (β=-0.176, SE= 0.051, P<0.001). Furthermore, in men, daily smoking was indirectly associated with SCF (β=-0.115, SE=0.034, P<0.001) and directly associated with IL-18 (β=0.157, SE=0.039, P<0.001), but these associations diluted after adjusting for the covariates.

My findings support previous studies showing that smoking is associated with acute phase reactions of innate immunity, and with dampening of the adaptive immune response. In addition, I provide novel insight to the study field by showing associations of HGF and βNGF with daily smoking.

Keywords: cytokines, smoking, inflammation, cardiovascular diseases

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1. INTRODUCTION

The production of tobacco products started to develop markedly during the latter half of the 19th century. New methods and machines enhanced the tobacco industry resulting in remarkable growth and higher quality of tobacco products. This started to increase the prevalence of smoking, which peaked during the mid-20th century. Tobacco smoking became a norm in developed countries, since the supply and advertisement of tobacco products was extremely liberate. (Hanafin and Clancy 2015.)

In the 1960s, when the harms of smoking became apparent, some countries already introduced smoking cessation measures (Reitsma et al. 2021). In 2005, the WHO published its "Framework Convention on Tobacco Control", which instructed states to take measures to curb smoking through 5 demand-reduction measures, including tax tightening, bans on smoking in public places and on tobacco advertising, and subsidized smoking cessation programs. In the years following the publication of the guidelines, the demand-reduction measures were being increasingly implemented in most countries, leading to a marked decline in smoking prevalence. (Gravely et al. 2017.)

Despite the decline in smoking prevalence during recent decades, progress has slowed over the past 5 years. In many countries, the decline in smoking prevalence between 1990 and 2019 has not been sufficient to reverse the force of population growth, and the number of smokers will remain the same or grow slowly, leading to increased rates of tobacco-related diseases and deaths in the coming decades. (Reitsma et al. 2021.)

Tobacco smoking is still a significant cause of illness and death worldwide. The major diseases linked with smoking are cardiovascular diseases (Banks et al. 2019), lung diseases (Global Initiative for Chronic Obstructive Lung Disease 2020) and cancer (Weber et al. 2021). Chronic smoking has also been shown to cause complex changes in the immunological processes, which in part lead to broad inflammatory reactions, but also dampen the immunological responses towards pathogens (Gaschler et al. 2008).

Cytokines are small signaling molecules, which are secreted from almost every cell of the human body to plasma and tissues. They convey messages from cell to cell and affect biological processes both locally and systemically. During an infection or when facing other stress factors, like toxins, pro-inflammatory cytokines are released by first-line immune cells,

which leads to rapid activation of the innate immune system and later the adaptive immune system. (Abbas et al. 2017.)

By far, the most human studies on this research field have had an emphasis on just a few acute-stage pro-inflammatory cytokines (Levitzky et al. 2008, Garlichs et al. 2009, Al Rifai et al. 2017, Ugur et al. 2018). The aim of this study was to study associations of tobacco smoking with large set of cytokines and chemokines measured from serum. These associations, if revealed, may be beneficial to further elucidate the pathophysiology of smoking-related diseases.

1.1 Prevalence of smoking

Prevalence of daily tobacco smoking has decreased globally since 1980 (Ng et al. 2014). According to World Health Organization's latest insight to global tobacco use, the estimated global prevalence of tobacco smoking has decreased from 25.7% in 2000 to 19.8% in 2015 and is projected to be 17.1% in 2025 (WHO global report on trends in prevalence of tobacco use 2000-2025, 2019).

The Americas, Europe and the Western Pacific region are experiencing a decrease in the absolute number of smokers. On the other hand, the numbers of smokers are increasing in Africa, Eastern Mediterranean and South-East Asian regions (WHO global report on trends in prevalence of tobacco use 2000-2025, 2019). Increase in the number of smokers can be at least partly explained by population growth (Ng et al. 2014).

Prevalence of smoking varies regionally within a global perspective. In 2015, the highest prevalence of daily smoking was in Europe (27.3%) and Western pacific region (24.6%). According to WHO statistics, the estimated sex-specific prevalence in Europe in 2015 was 35% for men and 19% for women. The largest sex differences were regionally in the Eastern Mediterranean, where an estimated 32% of men and 2.6% of women smoked daily. (WHO global report on trends in prevalence of tobacco use 2000-2025, 2019)

In Finland, the rates of daily smoking have decreased significantly during the past decades. According to the most recent data on tobacco smoking in Finland (Tupakkatilasto, 2020), the proportion of daily smoking adults (aged 20-64 years) has declined from 23% in women and 28% in men in 1996 to 12% in women and 14% in men in 2020. At European level, the difference in the prevalence of smoking between men and women in Finland is small. (Tupakkatilasto, 2020)

1.2 The effect of tobacco smoke components

When smoking a cigarette, its constituents are inhaled into the lungs, and disseminated in blood throughout the body. There are approximately over 6000 chemical components in cigarette smoke alone, of which relatively many are known carcinogens. Polycyclic aromatic hydrocarbons and the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone are considered among the most carcinogenic compounds of tobacco smoke. (Rodgman and Perfetti 2013.)

Free radicals are responsible for the most damage that tobacco smoking causes to cardiovascular system. Also known as reactive oxygen species, free radicals are generally speaking volatile agents creating oxidative stress to tissues. Some of them are as such parts of tobacco smoke, some are end products of host metabolism and some are end products of oxidation reaction of host molecules. In a healthy individual, oxidative burden is handled by antioxidants. Disrupted balance between oxidants and anti-oxidants results in tissue damage. (Kode et al. 2006, Wu et al. 2017.)

Nicotine, the addicting compound of tobacco, affects not only the central, but also peripheral nervous system. It accelerates the release of adrenaline and noradrenaline, which speeds up cardiac function and affects vessel function and lipid metabolism. Carbon monoxide in turn impairs the capacity of oxygen transport via hemoglobin and leads to a compensatory erythrocytosis, increased blood viscosity and reduced peripheral blood circulation (Powell 1998).

Genetic differences in enzymes and susceptibility to diseases between individuals form, together with the great amount of different toxic compounds of cigarette smoke, a very complex network of cause-and-effect relationships. Furthermore, quantity and duration of exposure cause an additional layer to the complex network. Thus, the clinical effects of cigarette smoking are impossible to predict precisely at individual level. (Wang and Wang 2011.)

1.3 Smoking attributable risk of mortality and disease

Today, tobacco smoking is recognized as a major factor increasing the risk of death and a cause for remarkable number of deaths annually. During the 20th century, smoking has led to several hundreds of millions of premature deaths; smokers die approximately 10 years earlier than non-smokers (Luepker 2011, Jha et al. 2013, Banks et al. 2015, Wipfli and Samet 2016).

According to Global Burden of Disease study in the Lancet (Reitsma et al. 2019), tobacco smoking accounted for 13,6% of all deaths, and 80% of the deaths attributed to smoking were among males. Of 87 risk factors, smoking accounted for the greatest number of deaths among males. 72% of deaths attributable to smoking were caused by ischemic heart disease, respiratory cancer, and stroke. All-cause deaths due to tobacco smoking were reported to be significantly increased in 71 countries, of which 66 were low- or middle-income countries. (Reitsma et al. 2021.)

1.3.1 Cardiovascular diseases

For more than half a century there has been evidence that tobacco smoke is causally related to cardiovascular diseases (CVDs) (U.S. Department of Health and Human Services, 2004). It is known that current smokers are at significantly increased risk for a wide spectrum of CVDs, with atherosclerosis and its manifestations being the most abundant (Banks et al. 2019).

Endothelium, the monolayer of cells lining vessels, capable of adapting to altering circumstances by e.g., regulating vascular tone, permeability, and cellular adhesion to the vessel walls (Deanfield et al. 2007). Endothelium normally acts as a homeostatic guardian through its anti-inflammatory and anti-thrombotic effects. Endothelial dysfunction is though as the earliest stage of atherosclerosis and is characterized with excessive amounts of pro-inflammatory mediators, cytokines, chemokines, and adhesion molecules, together with an increased production of reactive oxygen species and oxidized LDL cholesterol, and dampened production of nitric oxide (Wu et al. 2017). It results in increased permeability of vessels, weakened vasodilatory and increased contractile functioning.

Dyslipidemia is a key event together with inflammation preceding atherosclerosis. As apolipoprotein B-containing lipoproteins concentrate in plasma, they eventually retain in

vessel walls, LDL molecules being the most susceptible of lipoproteins. Adhesion to intima layer and retention there triggers an inflammatory response by macrophages and T-cells, which initiates atherosclerotic lesion progression in the subendothelium. (Williams and Tabas 2005, Tabas et al. 2007) Tobacco smokers are prone to have increased levels of serum total cholesterol, LDL cholesterol, and triglycerides. Simultaneously, serum levels of HDL cholesterol have been decreased, showing that smoking modifies serum lipid profile into a more proatherogenic direction. (Gossett et al. 2009, Nakamura et al. 2009, Slagter et al. 2013.)

Long duration and high intensity of smoking are associated with greater risk of CVDs (Pirie et al. 2013), while the risk is observed to decrease gradually after smoking cessation (Ahmed et al. 2015, Ding et al. 2019). Risk for peripheral artery disease (Banks et al. 2019, Ding et al. 2019) and abdominal aortic aneurysm (Pujades-Rodriguez et al. 2015) are five times greater among active smokers than non-smokers. The corresponding risks are approximately two-fold for coronary heart disease and stroke (Ding et al. 2019), myocardial infarction, heart failure (Pujades-Rodriguez et al. 2015, Banks et al. 2019), and cerebrovascular disease (Banks et al. 2019).

The effects of smoking on CVDs appear already at low levels of exposure. This is apparent in the relationship of smoking intensity and CVD risk, which is not linear. The risk of having coronary heart disease or stroke is relatively high already when smoking one cigarette per day: the risk for persons smoking one cigarette daily is approximately half the risk of those smoking 20 cigarettes per day (Hackshaw et al. 2018).

Not only does tobacco smoke make the circumstances beneficial to plaque development, but it also affects already formatted plaques. Smoking seems to increase atherosclerotic plaque instability by accelerating tissue destruction (Kangavari et al. 2004). Vulnerable plaques might include higher amount of lipids, calcification, ulceration, and thinner fibrin caps, being more easily ruptured. Thus, they predict increased occurrence of cardiovascular complications (Picano and Paterni 2015, Jashari et al. 2016).

1.3.2 Lung diseases

Smoking is associated with impaired lung function and diseases in the respiratory system. Smoking accelerates the physiological age-related decline in lung function, measured by forced expiratory volume in one second test. Also, significantly higher number of smokers develop pulmonary obstruction during their lifespan compared to nonsmokers. Smoking has long been held as the predominant cause of chronic obstructive pulmonary disease (COPD). (Kohansal et al. 2009.)

Smokers are prone to have major respiratory symptoms such as coughing, phlegm, wheezing and dyspnea, which are, however, observed to abate already during the first year of smoking cessation (Kanner et al. 1999, Willemse et al. 2004). Asthma is another chronic disease which can be influenced significantly by smoking status. Although there is no robust evidence for smoking being a cause for asthma, it clearly exacerbates symptoms and impairs quality of life among people with asthma (Mims 2015).

Smokers are also more sensitive for acute respiratory infections such as common cold, influenza and pneumonia, due to impaired defense mechanisms of the lungs (Cohen et al. 1993, Willemse et al. 2004, Stämpfli and Anderson 2009, Baskaran et al. 2019). Additionally, active smoking links strongly to both increased incidence and mortality of tuberculosis (Alcaide et al. 1996, Dhamgaye 2008, Huttunen et al. 2011).

1.3.3 Cancer

Lung cancer is the most common cancer caused by smoking, and almost all cases, up to 90% are attributable to smoking (Alberg et al. 2013, Dreyer et al. 1997, Keuhkosyöpä: Käypä hoito -suositus, 2017). However, not any organ system is safe from smoking attributable cancer risk. Current smokers have also an increased risk of cancers of the larynx, head and neck, esophagus, liver, bladder, pancreas and colorectum (Weber et al. 2021). The risk of breast cancer has been linked to current, former and passive smoking (Dossus et al. 2014).

1.3.4 Diabetes

There is strong evidence from clinical and experimental studies indicating that active tobacco smokers are in greater risk of developing type 2 diabetes, and the risk increases in a dose-dependent manner with smoking intensity (Carlsson et al. 2004, Willi et al. 2007, Sliwinska-Mosson and Milnerowicz 2017). Smoking predisposes diabetic patients to premature development of cardiovascular outcomes like coronary heart disease, stroke and myocardial infarction (Rawshani et al. 2018), as well as microvascular complications including neuropathy, nephropathy and retinopathy (Sliwinska-Mosson and Milnerowicz 2017). Risk of death is also significantly increased among smokers with diabetes (Sliwinska-Mosson and Milnerowicz 2017, Rawshani et al. 2018).

1.4 Inflammation

1.4.1 inflammatory response in human body

Inflammation is an adaptive response of living tissue to microbial infection, injury, or otherwise imbalanced homeostasis. Inflammatory response tries to eliminate the original cause of damage and restore homeostasis. Triggers that activate the inflammatory response can be both exo- and endogenous factors, such as surface proteins of microbes, inhaled or ingested toxins, damaged host cells or parts of them, and loss of cellular integrity. Tissue stress and malfunction induced inflammation is present in several chronic diseases. The triggers are quickly detected by host sensing mechanisms, with the result of activation of the immune system. (Medzhitov 2008.)

The human immune system consists of lymphoid tissue in the actual lymphoid organs like spleen and lymph nodes and also scattered in mucosal linings of the body. Immune cells are present all over the body via blood and lymph circulation. Immune cells include all the different types of leukocytes responsible of recognizing foreign and abnormal factors and destroying them. Antigen presenting cells are ones supporting leukocytes' action. Also, many types of non-immune cells are crucial for the normal defense mechanisms. For example, epithelial cells are capable of producing antimicrobial chemicals, and maintaining the physical barrier against invaders. (Medzhitov 2008.)

The immune system is divided into two differently acting compounds; the immediate innate immune system and the slower-acting adaptive immune system, which are constantly

working parallel in the human body. Effective host-defense mechanism consists of the co-operation of both of these immune systems. (Medzhitov 2008.)

1.4.2 Innate immunity

Innate immunity consists of components that an individual already has at birth in the bloodstream, tissues, and surface epithelium. Innate immunity prevents microbes from entering the body at epithelial barriers and initiates an early attack if the infection does begin and thus limits the extent of the infection. Among the main actors of innate immunity response are phagocytes, such as neutrophils and monocytes, which are ready to be activated in the bloodstream and are the first to enter tissues, when foreign structures are being detected. The main advantage of the innate system is speed. Phagocytes have limited recognition of microbes and contaminants, but they distinguish them from the host's own structures quickly, as they are present on all sides of the human body. Phagocytes produce degrading enzymes, as well as various inflammatory mediators such as cytokines and interferons, which attract more immune cells to the site. Recruiting of leukocytes and plasma proteins to the site of infection, and their action to destroy threats is described as inflammation. Enhanced blood circulation and vascular endothelial permeability are required for immune cells to efficiently migrate to the required site. On the other hand, the destructive reaction mediated by innate immunity is gross and simultaneously causes tissue destruction in healthy tissues as well. Therefore, inflammation leads to local redness, swelling and heat. In addition, the function of innate immunity is to activate the more specific adaptive immunity in a way that best serves the type of infection encountered. (Abbas et al. 2017.)

1.4.3 Adaptive immunity

Adaptive immunity evolves and improves during an individual's lifespan, as new pathogens are encountered. Adaptive immunity acts through lymphocytes and can be activated either cell-mediated or directly antigen-antibody mediated. Dendritic cells involved in innate immunity are responsible for activating adaptive cell-mediated immunity. Dendritic cells present foreign antigens in lymph tissues to T-lymphocytes. The humoral side of adaptive immunity acts through B-lymphocytes, instead. The system is activated when a foreign antigen encounters a B-lymphocyte with a specific receptor that begins to divide and

produce an antibody specific for the antigen. Antibodies work in numerous mechanisms, e.g., they prevent microbes from acting normally by binding to them, or antibodies can neutralize microbial toxins. Part of the B-lymphocytes become memory cells, allowing a new response to a new infection of the same cause to be faster in the future. (Abbas et al. 2017.)

1.4.4 Chronic inflammation

The immediate inflammatory response activated by innate immune system is present not only in microbial infections, but also when there are damaged host cells or abnormal accumulation of substances such as toxins. Subsequent destructive but contemporary inflammatory state does not place too much of a burden to healthy tissues. After the threat has been eliminated, an adequate recovery phase is needed, during which the affected tissue is being repaired. If the caused damage cannot be eliminated or the triggering agent remains, the inflammatory response stays active, causing more damage over time. During long-term smoking, there is persistent inflammation in blood vessels due to constant input of toxins from tobacco smoke. That results in destruction of the vessel walls and functional disruption, known as endothelial dysfunction (Messner and Bernhard 2014). Currently, endothelial dysfunction is considered as the key event underlying several chronic diseases, such as atherosclerosis and subsequent cardiovascular diseases, diabetes mellitus, renal disorders and cancer. (Tsoupras et al. 2018.)

1.5 Cytokines

Cytokines are described as small, soluble proteins produced by nearly all cells in human system and affecting most biological processes within. They act as signal transducers between cells, either locally or systemically. Once produced, cytokines act by binding to specific receptors on the surface of the target cell, after which they soon disappear. Some receptors are widely found in many types of cells in the human body, some in only one or a few. (Dinarello 2007.)

Cytokines are often described as “double-edged sword” based on their roles simultaneously in both vital physiological and pathological processes. It is easy to understand that while cytokines e.g., promote growth or inflammation serving the host’s benefit, their persisting effect may eventually become deleterious. For example, IL-1 is an important mediator of

both innate and adaptive immune systems. On the other hand, IL-1 is recognized as a crucial contributor in fibrotic diseases (Borthwick 2016) and cancer development (Garlanda et al. 2013).

1.5.1 Categorization of the cytokines

Over time, cytokines have been categorized based on either due to structural similarities, biological activity or by origin, and thus there is overlapping between the categories. Today, categorical groups include at least interleukins, pro- and anti-inflammatory cytokines, adipokines, interferons, growth factors, and chemokines.

For example, the first class of cytokines called interleukins was founded to group together communication actors between different types of white blood cells. There are 33 interleukins that are divided into three families: IL-1 family, IL-6 family, and IL-10 family. Also families of chemokines and the TNF-family were introduced based on similarities in structure and/or gene locations. (Dinarello 2007, Garlanda et al. 2013.)

While the categorization system seems not to be in line with the actual biological activities, cytokines have also been categorized by their primary properties, for example capability to promote or inhibit inflammation. Lastly, cytokines can be named by their origin, as for example the adipokines family.

It must be emphasized here that even the functional classification is somewhat artificial and simplified. Cytokines possess highly variable natures, as they act differently depending on circumstances. Some properties occur only under certain conditions, such as in the presence of co-stimulatory molecules. Other properties require a certain concentration, and the effect may even be reversed at too low or too high levels of a certain cytokine. (Cavaillon 2001.)

1.5.2 Pro- and anti-inflammatory cytokines

Cytokines are well known for their capabilities to activate immune system and shape immune responses to each situation depending on the type of stress. Cytokines are tightly bound to both innate and adaptive immune systems. The rapid development of acute phase reactions is based on the release of cytokines from neutrophils and other cells at the site of destruction. The later emerging adaptive immunity results from cytokine-mediated communication of immune cells, and is further regulated by cytokines, as well. Cytokines are also the most important mediators of lymphocyte differentiation.

In relation to interleukins, IL-1 family includes mainly pro-inflammatory cytokines, IL-6 family is responsible for hepatic acute phase protein production and IL-10 family members have anti-inflammatory effects (Dinarello 2007, Garlanda et al. 2013). Many interleukins possess features to manipulate leukocyte maturation. For instance, IL-4 is capable of switching the immunoglobulin class in B-lymphocytes and differentiation of T-cells to T helper 2 line. In macrophages the effect of IL-4 is alternative activation. IL-5 activates eosinophils instead. (Abbas et al. 2017.)

Pro-inflammatory cytokines primarily promote innate immune responses. They are produced by immune cells that have detected microbial invaders or damage host cells by their Toll-like receptors. TNF, IL-1, and IL-6, well known pro-inflammatory cytokines, are responsible for e.g. endothelial activation, fever, acute-phase protein synthesis in the liver and activation of neutrophils and T helper cells. (Abbas et al. 2017.)

Anti-inflammatory cytokines act the opposite way to pro-inflammatory cytokines, inhibiting inflammation by shutting down genes promoting the state. They play an important role suppressing autoimmune processes by affecting T regulatory cells. Examples of anti-inflammatory cytokines are IL-10, IL-13 and TGF-β. (Dinarello 2007.)

Adipokines have been divided into a separate group by their origin from white adipose tissue, although many have other origins and belong to other classification groups as well. Adipokine secretion deviates in obesity, which promotes development of metabolic syndrome and other obesity-related complications. Examples of adipokines are resistin which promotes both insulin resistance and inflammation, leptin which is responsible for appetite control, and IL-6 which is also known as a pro-inflammatory cytokine. (Ouchi et al. 2011.)

Interferons are a subgroup which is generally associated with viral infections. Interferons can inhibit viral functions, e.g. replication in host cells, and activate natural killer cells which are the primary immune cells capable of phagocytosis of infected host cells. (Abbas et al. 2017)

1.5.3 Growth factors

Cytokines can regulate cell counts and promote growth in most of the tissues in human body. There are several classes of growth factors, each specific to a target tissue or cell type. Insulin-like growth factor (IGF) is a general anabolic substance. Epidermal, platelet derived, and fibroblast growth factors (EGF, PDGF and FGF, respectively) are essential in wound healing (Park, Hwang and Yoon). Colony stimulating factors (CSF) are responsible for hematopoiesis, e.g. multi-CSF, also known as IL-3, promotes maturation of all hematopoietic cell progenitors. (Barreda et al. 2004.)

1.5.4 Chemokines

The term chemokine refers to “chemotactic cytokine”. This group is unique both in structure and function. Chemokines are divided into four subgroups: CC, CXC, CX3, and XC according to their molecular structures. Chemokines are named by the subgroup at the beginning, followed by “L” referring to “ligand” and then a number referring to the gene encoding the chemokine (Zlotnik and Yoshie 2000).

Chemokines can recruit and traffic cells, mainly immune cells, between the blood, lymph, bone marrow, thymus, spleen, and secondary lymphoid organs, as well as peripheral tissues. The phenomenon is called “chemotaxis” and is crucial for the immune system to work fluently in the right place at the right time. (Sokol and Luster 2015, Hughes and Nibbs 2018.)

An example of chemotaxis is how neutrophils are guided to the right place in the periphery at the right time during an acute microbial invasion: Immune cells are retained in bone marrow by CXCL12. During acute inflammation, the production of CXCL12 is decreased leading to neutrophil release from bone marrow. Then, neutrophils enter tissues from blood by following the gradients of CXCL1, CXCL2, CXCL8, CCL3 and CCL5. (Griffith et al. 2014.)

1.6 Smoking, inflammatory markers and cytokines

1.6.1 Smoking and inflammatory markers

Smoking is associated with multiple biomarkers that are known as inflammatory markers such as C-reactive protein (CRP), fibrinogen, white blood cell count and adhesion molecules. Many of them are related to atherosclerosis and other CVD risk factors apart from smoking, like obesity and high blood pressure. (Lind 2003.)

By far the most studied markers are CRP (Fröhlich et al. 2003, Levitzky et al. 2008, Al Rifai et al. 2017), fibrinogen (Fröhlich et al. 2003, Al Rifai et al. 2017), white blood cell count (Fröhlich et al. 2003, Lavi et al. 2007, Ugur et al. 2018) and plasma viscosity (Fröhlich et al. 2003), all of which have been reported to be elevated in smokers compared to nonsmokers. Conflicting results were given by Lavi et. al (Lavi et al. 2007), who found no difference in either CRP or fibrinogen between smokers and non-smokers. Concentrations of above mentioned inflammatory markers have been observed to correlate with smoking intensity (Wannamethee et al. 2005).

Other circulating markers that are well known to be increased or decreased in smokers are the markers of endothelial function. Concentrations of soluble intracellular adhesion molecule 1 (Bermudez et al. 2002, Lavi et al. 2007, Levitzky et al. 2008, Delgado et al. 2020), E-selectin (Bermudez et al. 2002, Delgado et al. 2020) and P-selectin (Levitzky et al. 2008, Delgado et al. 2020) have shown to be higher, and concentrations of soluble vascular adhesion molecule 1 and L-selectin lower in smokers than non-smokers (Delgado et al. 2020).

1.6.2 Smoking and cytokines

Tobacco smoking is an independent risk factor for multiple diseases, but it also interacts with other risk factors, and these relationships are complex. By studying cytokine expression and their relative proportions among smokers compared to non-smokers might help with elucidating the role of tobacco smoking in pathological processes.

1.6.2.1 Evidence from animal studies

According to animal studies, tobacco smoke appears to inhibit the production of several cytokines, including pro-inflammatory cytokines. In one study, alveolar macrophages isolated from smoke-exposed mice were stimulated with Toll-like receptor ligands to mimic bacterial and viral invasion (Gaschler et al. 2008). Attenuated release of TNF-α, IL-6 and CCL5 was reported from the alveolar macrophages (Gaschler et al. 2008). In another study, cigarette smoke exposure induced a reduction in the release of IL-5, IL-6, IL-12, TNF-α, CXCL10, MCP1, MIP1α, and VEGF from Ana-1 mouse macrophage cells (Zhao et al. 2017).

1.6.2.2 Evidence from *in-vitro* human cell models

Cigarette smoke exposure and its consequences to cytokine release have been studied in different human cell lines, and depending on cell type, pro-inflammatory cytokine production can be stimulated or dampened.

In-vitro studies show, that different types of pulmonary tissue-forming cells react to smoke exposure by increasing pro-inflammatory cytokine production: TNF-α, IL-6 and IL-8 were released in a dose-dependent manner due to cigarette smoke exposure in human bronchial epithelial cells (De Diego Damiá et al. 2011, Li et al. 2016, Xu et al. 2019), primary small airway epithelial cells (Kode et al. 2006), and lung fibroblasts (Li et al. 2007).

In peripheral human macrophages, IL-8 and TNF-α are similarly produced in increased amounts due to cigarette smoke exposure (Demirjian et al. 2006, Yang et al. 2006). However, alveolar macrophages from smokers' lungs possess dampened capability to produce TNF-α, IL-8, and IL-6 as a reaction to bacterial antigen stimulation (Soliman and Twigg 1992, Yamaguchi et al. 1993, Ohta et al. 1998).

Additionally, heightened production of VEGF was reported in human airway smooth muscle cells and lung fibroblasts due to cigarette smoke exposure (Volpi et al. 2011). Individual findings, like increased release of IL-1β (Xu et al. 2019) and decreased release of CXCL10 (Pace et al. 2008) from bronchial epithelial cells have also been reported.

1.6.2.3 Evidence from human studies

Elevated levels of pro-inflammatory cytokines IL-6 and IL-8 have repeatedly been reported among smokers (Levitzky et al. 2008, Garlichs et al. 2009, Al Rifai et al. 2017, Ugur et al. 2018). Elevated concentrations of TNF-α and IL-6 have been reported in epicardial and subcutaneous adipose tissue among smokers (Mach et al. 2016). Shiels et al. (2014) reported heightened levels of inflammatory chemokines CCL17 and CCL11, and decreased levels of inflammatory cytokines IL-1β, IL-15, IL-16, as well as anti-inflammatory cytokine IL-1RA and growth factor SCF, but the study was limited to aged (55-74 years old) participants. In young healthy smokers, significantly decreased level of an inflammatory cytokine IL-18 has been reported (Garlichs et al. 2009). Also slightly decreased levels of inflammatory cytokine TNF-α and inflammatory chemokine CCL2 have been detected, but this study had a very small number of participants (n=27) (Garlichs et al. 2009). Another study, conducted with a small sample of 30 participants of whom the majority (n=21) had COPD, found that smoking associates positively with an inflammatory chemokine CCL11 and negatively with a growth factor HGF (Bade et al. 2014).

2. STUDY AIMS AND HYPOTHESIS

The aim of this study is to determine, whether active or past smoking in healthy adults associates with levels of circulating cytokines compared to healthy never smokers. It is hypothesized that active smoking associates positively with inflammatory cytokines and chemokines and that the association is weaker in occasional smokers and further in former smokers.

3. MATERIALS AND METHODS

3.1 Study population

The Cardiovascular Risk in Young Finns Study (YFS) is a cohort study, that is ongoing in the five university hospital cities and their surrounding communities in Finland. The baseline of the study was conducted in 1980 and included 3596 boys and girls aged 3-18 years. After the baseline, the cohort has been followed in 3–9-year intervals. In the present study, blood samples and data collected in the 27-year follow-up study (in 2007) was used, and it included 2204 participants aged 30-45 years. The participants with missing information on smoking

status, cytokine measurement or any of the covariates were excluded from the study population. Additionally, participants with type 1 or type 2 diabetes, any rheumatic disease, cancer, acute infection, or who were pregnant were excluded. Eventually, a total of 1652 participants, 743 men and 909 women, were included in the analytical sample of the present study. Local ethical committees have approved the study protocol and all study participants have provided written informed consent.

3.2 Assessment of smoking status

Smoking status was assessed by questionnaires, where the subjects were asked to choose the most descriptive alternative of the following: 1) Smoking once a day of more often, 2) smoking at least once a week, but not daily, 3) smoking less often than once a week, 4) has or attempts to quit smoking, and 5) never smoked. In my analyses groups 2) and 3) were merged into one subgroup. In total, I used four subgroups as defining smoking status: 1) Once a day or more often (n=285), 2) Occasionally (n=162), 3) has or attempts to quit smoking (n=381) and 4) Never smoked (n=824).

3.3 Measurement of cytokines, chemokines, and growth factors

After overnight fasting (≥12 hours), the sera were drawn and stored at -70 °C until the measurement of the cytokines. The concentrations of 48 cytokines, chemokines, and growth factors were measured from 2200 serum samples with Bio-Rad's premixed Bio-Plex Pro Human Cytokine 27-plex Assay (catalog no. M500KCAF0Y) and 21-plex Assay (catalog no. MF0005KMII) kits on Bio-Rad's Bio-Plex 200 System. All assays were made according to the manufacturer's instructions except that the number of beads, detection antibodies, and streptavidin-phycoerythrin conjugate were used at half of their recommended concentration; this was determined to be sufficient in preliminary tests. With the exception of IL-12p40 and IL-12p70, and macrophage inflammatory protein-1α and macrophage inflammatory protein-1β, all analytes demonstrated less than 2% cross-reactivity. Eight-point standard curves were generated for each cytokine using recombinant proteins. The values falling outside the standard range were manually extrapolated according to the standard curve of the given plates. After this, all the values that deviated more than 10 SDs from the mean were given the next smallest or largest value. Eight of the analytes (CCL5, GM-CSF, IL-1α, TNF-β, IFN-

α2, LIF, IL-3, IL-12p40) gave values outside the standard range and were thus not included in the analysis. Standard ranges with intra-assay and inter-assay variations of the cytokine measurements are provided within a prior study using the same data as in the present study. (Santalahti et al. 2016.) Names and abbreviations of the cytokines, chemokines and growth factors are listed in a supplemental table.

3.4 Clinical and laboratory measurements

The height (m) and weight (kg) were measured, and BMI (kg/m^2) was calculated. Systolic and diastolic blood pressures (mmHg) were measured with a mercury meter (random zero), and mean values of three separate measurements were calculated. Total cholesterol (mmol/l), HDL cholesterol (mmol/l), LDL cholesterol (mmol/l), triglycerides (mmol/l), fasting plasma glucose (mmol/l), and high-sensitivity CRP (mg/l) were measured. Serum insulin (mU/l) was measured by microparticle enzyme immunoassay kit (Abbott Laboratories, Diagnostic Division, Dainabot) with a detection border of 1.0 mU/l.

3.5 Self-reported clinical and lifestyle data

Self-reported data was used for covariates such as age, sex, BMI, systolic blood pressure, HDL and LDL cholesterol, triglycerides, physical activity level, diet, and alcohol use, and for those variables used as exclusion criteria such as type 1 or type 2 diabetes, any rheumatic disease, cancer, acute infection, or pregnancy.

Physical activity index was calculated by summing up self-reported variables concerning exercise habits (intensity, frequency, durations of weekly exercise and individual performances, attendance in guided training).

The diet of the participants was assessed using a 131-item food frequency questionnaire developed by the Finnish National Institute for Health and Welfare (Paalanen et al. 2006). Quality of food was defined by individual score (0-27) calculated from self-reported portions of healthy (whole grain products, fruits, vegetables excluding potatoes and legumes, fish, and nuts) and unhealthy (red meat and processed meat products, drinks with added sugar, fried potatoes, sweet desserts, pastries and sweets) foods. Average alcohol consumption per day was calculated from self-reported amounts of alcoholic beverages during the previous week.

Socioeconomic position was assessed by educational status (comprehensive school; secondary education, not academic; and academic). The total number of years of education was reported.

3.6 Statistical analyses

The distributions of the cytokine variables were visually inspected. As all cytokine variables were non-normally distributed, they were ln-transformed. Wilcoxon rank-sum test was used for continuous variables and Chi-square test for categorical variables to assess for differences in characteristics between men and women. In line with a previous report leveraging the YFS data, a majority of the cytokines were found to differ in men and women (Santalahti et al. 2016). Thus, all analyses were conducted separately for men and women. Analyses of covariance (ANCOVA) were used to determine differences in serum cytokine concentrations between smoking status groups, adjusting first for age (model 1), and subsequently additionally for BMI, systolic blood pressure, HDL and LDL cholesterol, triglycerides, physical activity level, diet, and alcohol use (model 2). The never smokers were used as a reference group. A Bonferroni corrected 2-sided $P<0.00125$ ($\alpha=0.05/40$; number of cytokines=40) was considered statistically significant to account for multiple testing. All statistical analyses were performed using SAS 9.4.

4. RESULTS

4.1 Characteristics of the study population

The study population included 1652 participants, of which 909 were women and 741 were men. Table 1 presents characteristics of all subjects. Men and women were found to differ in the following characteristics: BMI, blood pressure, blood lipids and glucose were higher in men than in women, while women had higher education, healthier diet and consumed less alcohol than men. Additionally, among women, there were significantly fewer daily smokers (7.87 vs. 9.38%) and more never smokers (30.02 vs. 19.85%) than among men.

Table 1. Characteristics of the study population.

Characteristic	Females (n=909)	Males (n=743)	P-value
Age, years	37.86 (4.94)	37.60 (5.06)	0.30
Education, years	15.83 (3.37)**	14.96 (3.54)**	<.0001
Cardiovascular risk factors			
BMI, kg/m^2	25.11 (4.91)	26.37 (4.06)	<.0001
Systolic BP, mmHg	115.7 (13.60)	125.3 (12.44)	<.0001
Diastolic BP, mmHg	72.85 (10.81)	78.30 (10.79)*	<.0001
CRP, mg/l	1.81 (3.37)	1.49 (4.30)	0.095
Total cholesterol, mmol/l	4.88 (0.79)	5.17 (0.93)	<.0001
LDL cholesterol, mmol/l	2.93 (0.70)	3.28 (0.80)	<.0001
HDL cholesterol, mmol/l	1.45 (0.32)	1.23 (0.29)	<.0001
Triglycerides, mmol/l	1.12 (0.54)	1.47 (0.74)	<.0001
Glucose, mmol/l	5.14 (0.47)	5.42 (0.48)	<.0001
Insulin, mU/l	7.97 (5.85)	8.60 (6.95)*	0.051
Lifestyle factors			
Physical activity index	8.90 (1.70)	8.74 (1.92)	0.086
Diet score	15.08 (3.70)	11.86 (3.74)	<.0001
Alcohol consumption, doses per day	0.57 (0.74)	1.40 (1.92)	<.0001
Smoking status			<.0001
daily	130 (14.30)	155 (20.86)	
occasionally	72 (7.92)	90 (12.11)	
has or attempts to quit smoking	211 (23.21)	170 (22.88)	
never	496 (54.57)	328 (44.15)	
Pack years	2.69 (5.57)	5.10 (8.84)	<.0001

The characteristics are presented for participants with no missing information on smoking status. All values are means and standard deviations for the continuous variables, and number and percentages for the categorical variables.
*Data missing from one participant.
**Data missing from two participants.

4.2 Associations of smoking and cytokines

In the age adjusted analyses, daily smoking was directly associated with HGF in men (β=0.194, SE=0.038, P=0.0000004, model 1 in Table 2) and women (β=0.120, SE=0.036, P=0.0010804, model 1 in Table 3). For both sexes, the HGF associations remained after further adjustments for BMI, systolic blood pressure, HDL and LDL cholesterol, triglycerides, physical activity level, diet, and alcohol use (men: β=0.178, SE=0.038, P=0.0000036, model 2 in Table 2; women: β=0.122, SE=0.036, P=0.0008038, model 2 in Table 3). Daily smoking was indirectly associated with CXCL10 in men and women. In women, the association was significant in age adjusted analyses (β =-0.214, SE=0.048, P=0.0000095, model 1 in Table 3) and remained after full adjustments (β =-0.245, SE=0.050, P=0.000011, model 2 in Table 3), but in men the association was significant only after full adjustments (β =-0.182, SE=0.050, P=0.0002987, model 2 in Table 2).

In men, the age adjusted analyses showed a direct association for IL-18 (β=0.157, SE=0.039, P=0.0000625, model 1 in Table 2), and an indirect association for SCF (β=-0.115, SE=0.034, P=0.0007420, model 1 in Table 2). After full adjustments according to the model 2, the associations diluted for both IL-18 (β=0.126 SE=0.040, P=0.0018626, Table 2) and SCF (β=-0.109, SE=0.036, P=0.0021602, Table 2).

In women, the age adjusted analyses showed an indirect association with CXCL9 (β=-0.185, SE=0.053, P=0.0005137, model 1 in Table 3) and βNGF (β=-0.190, SE=0.050, P=0.0001363, model 1 in Table 3), and the associations remained significant after full adjustments for CXCL9 (β=-0.195, SE= 0.055, P=0.0004296, model 2 in Table 3) and βNGF (β=-0.176, SE= 0.051, P=0.0006161, model 2 in Table 3).

No associations were seen between never smokers and occasional smokers or quit smokers (data not shown).

Table 2. Associations of daily smoking with cytokines in men.

Model 1				**Model 2**			
Cytokine	β-Estimate	Standard Error	P-value	Cytokine	β-Estimate	Standard Error	P-value
βNGF	-0.108	0.050	0.03	βNGF	-0.081	0.052	0.13
CCL27	-0.029	0.028	0.30	CCL27	-0.005	0.028	0.87
CCL11	0.115	0.045	0.01	CCL11	0.122	0.048	0.01
FGF BASIC	2.761 E-6	0.028	1.00	FGF BASIC	0.002	0.030	0.95
GCSF	0.003	0.030	0.93	GCSF	0.008	0.031	0.81
CXCL1	0.019	0.049	0.69	CXCL1	0.032	0.052	0.54
HGF	0.194	0.038	3.70E-7	HGF	0.178	0.038	3.63E-6*
IFNγ	-0.019	0.027	0.48	IFNγ	-0.033	0.029	0.25
IL-10	0.087	0.062	0.16	IL-10	0.086	0.066	0.19
IL-12p70	0.102	0.058	0.078	IL-12p70	0.084	0.061	0.17
IL-13	0.063	0.047	0.18	IL-13	0.043	0.050	0.39
IL-15	0.008	0.242	0.97	IL-15	-0.025	0.263	0.92
IL-16	0.051	0.071	0.47	IL-16	0.099	0.074	0.18
IL-17	-0.015	0.029	0.62	IL-17	-0.008	0.031	0.79
IL-18	0.157	0.039	6.29E-5*	IL-18	0.126	0.040	1.86E-3
IL-1β	-0.036	0.026	0.18	IL-1β	-0.028	0.028	0.31
IL-1RA	-0.058	0.057	0.31	IL-1RA	-0.042	0.061	0.49
IL-2	-0.076	0.046	0.10	IL-2	-0.071	0.049	0.14
IL-2RA	0.147	0.046	1.69E-3	IL-2RA	0.121	0.049	0.02
IL-4	-0.016	0.018	0.38	IL-4	-0.013	0.019	0.49
IL-5	0.001	0.032	0.98	IL-5	0.005	0.034	0.88
IL-6	-0.042	0.042	0.31	IL-6	-0.046	0.044	0.30
IL-7	-0.001	0.041	0.99	IL-7	-0.011	0.043	0.80
IL-8	0.023	0.020	0.24	IL-8	0.029	0.021	0.16
IL-9	-0.007	0.058	0.90	IL-9	-0.026	0.061	0.67
CXCL10	-0.138	0.048	4.15E-3	CXCL10	-0.182	0.050	2.99E-4*
CCL2	0.077	0.034	0.03	CCL2	0.075	0.036	0.04
CCL7	-0.057	0.514	0.91	CCL7	-0.007	0.594	0.99
MCSF	-0.406	0.193	0.04	MCSF	-0.419	0.207	0.04
MIF	0.072	0.058	0.21	MIF	0.078	0.060	0.20
CXCL9	-0.069	0.051	0.18	CXCL9	-0.078	0.054	0.15
CCL3	-0.000	0.022	0.98	CCL3	0.004	0.023	0.87
CCL4	0.052	0.033	0.11	CCL4	0.051	0.034	0.13
PDGFBB	0.025	0.039	0.52	PDGFBB	0.013	0.041	0.75
SCF	-0.115	0.034	7.42E-4*	SCF	-0.109	0.036	2.16E-3
SCGF-β	-0.011	0.037	0.76	SCGF-β	-0.003	0.039	0.93
CXCL12	-0.115	0.049	0.02	CXCL12	-0.109	0.052	0.04
TNF-α	-0.066	0.042	0.12	TNF-α	-0.048	0.045	0.28
TRAIL	0.059	0.047	0.21	TRAIL	0.035	0.048	0.47
VEGF	0.160	0.051	1.65E-3	VEGF	0.138	0.054	0.01

Numbers are beta estimates, standard errors and p-values from ANCOVA models. Due to the non-normal distribution of the cytokines the raw values were log-transformed. The associations are presented first as age adjusted (Model 1) and after that adjusted for age, body mass index, systolic blood pressure, serum LDL and HDL cholesterol, triglycerides, physical activity index, diet score and alcohol consumption (Model 2).

** Association considered as significant, P-value <0.00125*

Table 3. Associations of daily smoking with cytokines in women.

Model 1				**Model 2**			
Cytokine	β-Estimate	Standard Error	P-value	Cytokine	β-Estimate	Standard Error	P-value
βNGF	-0.190	0.050	1.36E-4*	βNGF	-0.176	0.051	6.16E-4*
CCL27	-0.035	0.028	0.22	CCL27	-0.008	0.028	0.78
CCL11	0.036	0.047	0.44	CCL11	0.044	0.050	0.37
FGF BASIC	-0.003	0.032	0.91	FGF BASIC	0.000	0.033	0.99
GCSF	0.020	0.028	0.46	GCSF	0.030	0.029	0.30
CXCL1	-0.117	0.051	0.02	CXCL1	-0.140	0.054	9.01 E-3
HGF	0.120	0.036	1.08E-3*	HGF	0.122	0.036	8.04E-4*
IFNγ	-0.010	0.030	0.73	IFNγ	-0.008	0.031	0.80
IL-10	-0.008	0.051	0.87	IL-10	-0.018	0.053	0.73
IL-12p70	0.024	0.056	0.67	IL-12p70	0.001	0.059	0.99
IL-13	0.014	0.039	0.72	IL-13	-0.004	0.041	0.91
IL-15	0.004	0.211	0.99	IL-15	0.179	0.226	0.43
IL-16	0.091	0.076	0.23	IL-16	0.069	0.080	0.39
IL-17	-0.003	0.034	0.94	IL-17	-0.006	0.036	0.86
IL-18	0.050	0.042	0.24	IL-18	0.024	0.044	0.58
IL-1β	-0.002	0.034	0.96	IL-1β	0.009	0.035	0.79
IL-1RA	-0.027	0.052	0.61	IL-1 RA	-0.026	0.054	0.63
IL-2	-0.008	0.042	0.86	IL-2	0.007	0.044	0.87
IL-2RA	0.137	0.055	0.01	IL-2 RA	0.120	0.057	0.04
IL-4	-0.010	0.019	0.61	IL-4	-0.000	0.020	0.99
IL-5	-0.021	0.029	0.46	IL-5	-0.035	0.030	0.25
IL-6	-0.006	0.043	0.89	IL-6	-0.013	0.045	0.77
IL-7	-0.036	0.037	0.33	IL-7	-0.057	0.039	0.14
IL-8	0.020	0.025	0.43	IL-8	0.026	0.026	0.31
IL-9	-0.082	0.091	0.37	IL-9	-0.148	0.096	0.12
CXCL10	-0.214	0.048	9.50E-6*	CXCL10	-0.245	0.050	1.09E-6*
CCL2	0.057	0.033	0.082	CCL2	0.051	0.034	0.13
CCL7	0.236	0.398	0.56	CCL7	0.264	0.463	0.57
MCSF	-0.068	0.210	0.75	MCSF	-0.175	0.220	0.43
MIF	0.091	0.058	0.12	MIF	0.111	0.061	0.07
CXCL9	-0.185	0.053	5.14E-4*	CXCL9	-0.195	0.055	4.30E-4*
CCL3	-0.007	0.027	0.80	CCL3	-0.006	0.028	0.82
CCL4	-0.005	0.032	0.88	CCL4	-0.024	0.034	0.47
PDGFBB	0.021	0.037	0.57	PDGFBB	0.021	0.038	0.57
SCF	-0.033	0.035	0.34	SCF	-0.034	0.036	0.35
SCGF-β	-0.080	0.040	0.05	SCGF- β	-0.075	0.041	0.07
CXCL12	-0.020	0.047	0.67	CXCL12	-0.023	0.050	0.65
TNF-α	0.002	0.048	0.97	TNF-α	0.001	0.051	0.99
TRAIL	0.009	0.047	0.85	TRAIL	-0.010	0.049	0.85
VEGF	0.063	0.051	0.23	VEGF	0.044	0.053	0.40

Numbers are beta estimates, standard errors, and p-values from ANCOVA models. Due to the non-normal distribution of the cytokines the raw values were log-transformed. The associations are presented first as age adjusted (Model 1) and after that adjusted for age, body mass index, systolic blood pressure, serum LDL and HDL cholesterol, triglycerides, physical activity index, diet score and alcohol consumption (Model 2).

** Association considered as significant, P-value <0.00125*

5. DISCUSSION

In this study, daily smoking was found to associate with six of the assessed 40 cytokines. Of these associations four remained statistically significant after full adjustments for the covariates. This study is currently one of the first studies focusing on the associations between smoking and cytokines in relatively young and healthy population, and thus, the findings may be considered novel. The four cytokines with robust associations included two growth factors (βNGF, HGF) and two inflammatory cytokines (CXCL10, CXCL9). Additionally, daily smoking was observed to be associated with IL-18 and SCF in men, but the associations diluted after full adjustments. No associations were found between occasional or quit smoking and the measured cytokines.

There is very little previous data from human studies for the cytokines which I found to associate with smoking. Associations between smoking and HGF (Bade et al. 2014, Serilmez et al. 2019), CXCL10 (Hahn et al. 2021, Rastogi et al. 2022), CXCL9 (Shiels et al. 2013, Zhang et al. 2022), SCF (Wigren et al. 2016, Björkbacka et al. 2017, Hahn et al. 2021) and IL-18 (Kang et al. 2007, Jefferis et al. 2013) have been reported in humans. However, in these studies the subjects were significantly older and/or had a chronic disease. Furthermore, there is data from *in-vitro* studies showing an indirect association between smoking and CXCL10 (Pace et al. 2008, Zhao et al. 2017) and a direct association between smoking and IL-18 (Kang et al. 2012), which support my findings. In relation to βNGF, no previous studies reporting associations with smoking exist.

Hepatocyte growth factor, HGF, which is a mediator in tissue growth, remodeling, and morphogenesis (Zhang et al. 2003), was directly associated with daily smoking in men and women. My result in healthy, relatively young subjects can be considered as a novel finding. Only two previous studies focusing on the association between HGF, and smoking were found. These studies have been conducted in patients with a chronic disease such as cancer or COPD, and the number of participants was very low in one of the studies (n=30) (Bade et al. 2014, Serilmez et al. 2019). The other study was conducted in cancer patients, and it found no association between HGF expression and smoking habit (Serilmez et al. 2019). However, protein and gene expression levels of HGF were found to be significantly higher in cancer patients compared with healthy controls. Another study conducted in COPD patients and non-COPD controls, reported no difference in HGF levels between non-COPD smokers and non-smokers (Bade et al. 2014). In the same study, an indirect association between smoking and HGF was reported, when both COPD and control groups were

included. To be noted, in that study the number of participants was very low, (n=9 in control group, n=21 in COPD group). In conclusion, there are neither previous studies supporting nor contradicting my finding.

HGF has been studied recently within COPD patients (Kruk et al. 2021) and COPD murine models (Cahill et al. 2016, Kennelly et al. 2016). In these studies, HGF was observed to play a similar protective and repairing role against tissue damage through stem cell function in the lungs as they play in cardiovascular system. The impaired ability of lung mesenchymal stem cells to repair pulmonary damage in COPD seems to be due to deficiency of growth factors produced, including HGF. Conversely, when administering mesenchymal stem cell therapy, HGF was found to protect against lung destruction in an elastase induced model of COPD. More specifically, MSC treatment produced an anti-inflammatory effect by reducing the expression of pro-inflammatory cytokines IL-1B, IL-6 and TNF-α in the lungs.

In COPD, local versus systemic changes in HGF levels seem to reflect the disease in different ways. In COPD patients, HGF is elevated locally in the lungs, while smoking seems to increase especially the systemic HGF concentration, which was found to be highest in smokers with normal lung function compared to COPD patients, regardless of smoking status, and to healthy non-smokers. In COPD patients, smoking did not seem to affect local or systemic HGF concentrations.

Overall, HGF appears to be associated with the late-phase consequences of tobacco smoking, including COPD and cancer, but has also been shown to protect against them. In light of previous and my results it can be hypothesized, that smoking habit might inhibit the body’s attempt to protect the lungs from smoking attributable damage. Although, it is impossible to assess causal relationships of these associations in the light of current knowledge.

In relation to the associations of HGF with cardiovascular diseases, there are interesting results from animal and human studies. In humans, heightened HGF has been suggested to be predictive for atherosclerotic lesions (Tateishi et al. 2002) and all-cause mortality, including CVD and cancer attributable mortality, in general population (Santalahti et al. 2017). In contrast, protective effects of HGF in the treatment of ischemic heart disease have been reported from animal models already from the beginning of 21st century (Nakamura et al. 2000, Wang et al. 2004), and the field has evolved into stem cell therapies (Zhao et al. 2017) and even gene therapies during the last years. There are also ongoing human studies,

in which the HGF gene is introduced into a patient with a viral vector. Successful results have been obtained in phase II studies treating post-infarction heart failure (Meng et al. 2018) and critical limb ischemia (Gu et al. 2019).

HGF is elevated in organ damage such as hepatitis and nephritis, as well as hypertension caused by endothelial damage, as well as in atherosclerotic diseases such as peripheral artery disease. The role of HGF as a systemic regulator is not necessarily enough to induce the needed tissue regeneration locally, such as in the case of smoking, in the lungs. Adequate HGF levels would be required to increase locally, and this is what the latest forms of treatment with stem cells are based on. As Morishita et al. (2002) already discussed over 20 years ago, HGF may work as a general measure of endothelial dysfunction.

Daily smoking was indirectly associated with βNGF in females. NGF is a growth factor secreted by and affecting both peripheral and central nervous systems, as well as epithelial cells and the immune system. In peripheral nervous system, NGF promotes the growth and differentiation of at least somatic sensory and autonomic motor neurons. In central nervous system, NGF accounts for regulation of the hypothalamic-pituitary-adrenal axis and development of cholinergic neurons. In epithelial cells, NGF regulates hair follicles and wound healing, while in the immune system NGF regulates the differentiation of immune cells, e.g., hematopoietic stem cells, granulocytes, lymphocytes, and monocytes. Release of NGF from immune cells, including mast cells, lymphocytes and eosinophils, is increased during inflammation and within inflammatory and autoimmune diseases. (Aloe et al. 2012.)

The properties of NGF in human pathophysiology are uncertain. Some evidence from *in-vitro* studies suggests that βNGF could be a tumorigenic agent (Xu et al. 2010, Yue et al. 2013), while results from an observational human study shows an indirect association between prostate cancer and NGF (Gong et al. 2020). There are no previous studies investigating the relationship between smoking and βNGF, implicating that my finding is novel to the field and sets hypotheses for future studies.

This study showed an indirect association with CXCL10 and daily smoking in men and women. CXCL10, also known as interferon gamma-induced protein 10 (IP-10), acts between innate and adaptive immune reactions, by attracting more leukocytes, e.g., natural killer cells by the site of inflammation and by inducing T helper 1 differentiation of T-lymphocytes (Sokol and Luster 2015). My finding is supported by a recent, relatively large cross-sectional human study that reported that past smoking was associated with lower levels of CXCL10 (Rastogi

et al. 2022). In that study, the mean age of the participants was 55 years and they also included participants with diabetes, hypertension or hyperlipidemia in their study cohort. On the contrary, a cross-sectional study conducted on female nurses (median age 56.8) found no association with current/recent smoking and CXCL10 (Hahn et al. 2021). In this study, only subjects with systemic lupus erythematous were excluded.

My finding is in line with two *in-vitro* studies, that have presented a reduced release of CXCL10 after exposure to cigarette smoke in human bronchial epithelial cells and mouse spleen macrophages (Pace et al. 2008, Zhao et al. 2017). For CXCL10, previous evidence of its association with smoking is weak and still unclear. My finding provides new insight to the field, as I found an indirect association between daily smoking and CXCL10 in relatively young, and healthy population.

CXCL9 was also indirectly associated with daily smoking, but this link was only found in females. CXCL9 possesses the same function in human body as CXCL10 (Sokol and Luster 2015). There were two previous studies reporting a direct association between CXCL9 and smoking, but the studies were conducted in either older population or in cancer patients (Shiels et al. 2013, Zhang et al. 2022), and thus my finding can be considered as novel.

Contrary to my findings, a case-control study focusing on the links between inflammatory markers and lung cancer risk in a general population aged 50-74 years found elevated CXCL9 levels among current and former smokers (Shiels et al. 2013). Similarly, a more recent study conducted in non-small cell lung carcinoma patients found a direct association between active smoking status and elevated CXCL9 (Zhang et al. 2022). Another study linked smoking, CXCL9 and lung cancer risk (Shiels et al. 2015). That study showed that among other markers CXCL9 was directly associated with lung cancer risk in current and former smokers, but not in never smokers.

Both CXCL9 and CXCL10 seem to be involved in the pathogenesis of COPD. They are secreted by lung lymphocytes and induce proteolysis in the lung tissue leading to lung destruction and emphysema. In this context, CXCL9 and CXCL10 appear to be involved in the Th1 immune response, and their effects come through Matrix Metallopeptidase 12 upregulation (Grumelli et al. 2004).

In my study, stem cell factor, SCF, which accounts for hematopoietic stem cell maturation (Baumann, 2017, Zhou et al. 2017), was indirectly associated with daily smoking in males before full adjustments. My finding is in line with a prior, large prospective study, which found

out that smoking was indirectly associated with SCF (Wigren et al. 2016, Björkbacka et al. 2017). They also showed that individuals with higher SCF levels possess a lower risk for cardiovascular events and death. Contrary to my finding, a study conducted in female nurses found no association with SCF and current/recent smoking (Hahn et al. 2021).

Daily smoking was directly associated with IL-18 in men before full adjustments. The association is supported by two previous studies conducted either in an older population (Jefferis et al. 2013) or among COPD patients (Kang et al. 2007). Interleukin-18 is considered as a pro-inflammatory cytokine. IL-18 accounts for activating neutrophils and monocytes and stimulates the release of other early-stage inflammatory cytokines, including GM-CSF, TNF, IL-1β and IFN-gamma. IL-18 possesses pro-inflammatory features, e.g., promotion of cellular adhesion and production of nitric oxide, independently of the synergistic cytokines. Neutralization of IL-18 has been shown to reduce cellular damage, and prevent adherence of malignant cells (Dinarello 2000, Kaplanski 2018). My result in a younger, healthy population thus may be considered to bring supporting evidence to the idea that smoking has pro-inflammatory and cell-damaging properties.

As previously discussed, tobacco smoking is a risk factor for COPD and diabetes. Studies have shown positive associations between IL-18 and these diseases. In humans, increased IL-18 concentrations have been reported in COPD patients compared to healthy controls (Kang et al. 2007). IL-18 has even been suggested to be a link between tobacco smoking and COPD as IL-18 plays a significant role in lung lesion formation in a murine model of COPD. The pathophysiology of COPD shows several different types of inflammatory cascades, which include e.g., type 1, 2 and 17 cytokine responses. IL-18 appears to induce all of these three, contributing to mutually counterregulatory processes. IL-18 could be an explanation that enables the paradox of emphysema and fibrosis to occur simultaneously. (Kang et al. 2012.)

A cross-sectional YFS cohort study has been previously conducted on the associations between cytokines and insulin resistance, which is a major component of type 2 diabetes pathogenesis. Santalahti et al. (2016) reported that IL-18 was one of three independent predictors for insulin resistance in both men and women. Considering these results, IL-18 may be an important link in the complex pathogenesis of both COPD and diabetes, to which smoking is known to contribute significantly.

In summary, the strongest association with daily smoking was with HGF and βNGF. The positive association of smoking and serum HGF may reflect systemic endothelial stress due to smoking. In light of my and recent results from others, βNGF may be a link between smoking-related pathogenesis of cancer which needs further studies. HGF, SCF, IL-18, CXCL9 and CXCL10 have previously been linked to COPD. For HGF, it straightforwardly shows that the positive association fits with previous findings that elevated concentrations of HGF have been observed in COPD patients. However, no causation can be concluded between smoking and the disease. My finding strengthens previous data suggesting that smoking induced heightened IL-18 level might be a central factor in COPD pathogenesis. Regarding SCF, CXCL9 and CXCL10, elevated concentrations have also been observed in COPD patients, but in my study the association between smoking and the cytokines was negative. It would be interesting to know whether the associations reverse when a primarily healthy smoker develops a disease over time.

5.1 Limitations and strengths

As the setting of this study was cross-sectional, causal relationships cannot be drawn between daily smoking and the measured cytokines. Because the study was conducted in Finnish population, the results cannot be generalized directly to other populations. In the cytokine measurements the detection method was non-optimal for some of the cytokines, including CCL5, GM-CSF, IL-1α, TNF-β, IFN-α2, LIF, IL-3, and IL-12p40, which resulted in unsuccessful analytes for these cytokines. Data on the frequency of smoking was self-reported and collected using a questionnaire, and therefore may be unreliable due to human error or if the participant experiences pressure to report quantities incorrectly. On the other hand, the amount of inhaled and absorbed smoke varies depending on the smoking style (Rebagliato 2002). Instead of queried information of smoking, for example serum cotinine measurement would have been a more objective indicator of smoking exposure.

A strength of this study was a broad panel of investigated cytokines, a total of 40 successful analytes, which is advantageous to prior studies in this area. My study sample was relatively large, included men and women equally, and represented healthy, young Finns. The young age of the participants (35-45 years) allowed us to study cytokine levels without the plausible effect of old age and associated cumulative diseases on cytokine levels (Michaud et al.

2013). Making systematic findings from such a young cohort can be considered novel and clinically significant.

5.2 Conclusion

In my study daily tobacco smoking was directly associated with a growth factor HGF and a pro-inflammatory cytokine IL-18. Also, daily smoking was indirectly associated with growth factors βNGF and SCF, and chemokines CXCL9 and CXCL10. These data were obtained from healthy, young adults. The results of my study support the evidence from earlier animal and human studies, suggesting that cigarette smoking associates with alterations in inflammatory response, and innate and adaptive immunity. On the other hand, my findings apply new insight to the field.

The cytokines found in my study may help in clarifying the complex pathogenesis of smoking-related diseases of the cardiovascular and pulmonary system, or some of the cytokines may have potential to function as markers and prognostic factors for endothelial dysfunction. More research is needed to elucidate, how the level of cytokines alters from a healthy baseline, during the early stages of the disease, until a clinical disease.

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SUPPLEMENT

Supplemental table. Names and abbreviations of the chemokines, cytokines and growth factors.

Type	**Name**	**Abbreviation**
INFLAMMATORY CHEMOKINES		
	Monocyte chemotactic protein-1	CCL2/MCP1
	Macrophage inflammatory protein-1α	CCL3/MIP1α
	Macrophage inflammatory protein-1β	CCL4/ MIP1β
	Monocyte specific chemokine 3	CCL7/ MCP3
	Eotaxin	CCL11/ EOTAXIN
	Cutaneous T-cell attracting chemokine	CCL27/ CTACK
	Growth regulated oncogene α	CXCL1/ GROA
	Monokine induced by interferon-gamma	CXCL9/ MIG
	Interferon gamma-induced protein 10	CXCL10/ IP10
	Stromal cell-derived factor-1 α	CXCL12/ SDF1α
INFLAMMATORY CYTOKINES		
	Interferon-γ	IFNγ
	Interleukin-1α	IL-1α
	Interleukin-2	IL-2
	Interleukin-4	IL-4
	Interleukin-5	IL-5
	Interleukin-6	IL-6
	Interleukin-8	IL-8 / CXCL8
	Interleukin-9	IL-9
	Interleukin-12p70	IL-12p70
	Interleukin-13	IL-13
	Interleukin-15	IL-15
	Interleukin-16	IL-16
	Interleukin-17	IL-17
	Interleukin-18	IL-18
	Macrophage migration inhibitory factor	MIF
	Tumor necrosis factor α	TNF-α
	Tumor necrosis factor β	TNF-β
GROWTH FACTORS		
	Granulocyte colony-stimulating factor	GCSF
	Hepatocyte growth factor	HGF
	Interleukin-7	IL-7
	Platetet derived growth factor BB	PDGFBB
	Stem cell factor	SCF
	Stem cell growth factor β	SCGFβ
	Vascular endothelial growth factor	VEGF
	Beta nerve growth factor	βNGF
	Macrophage colony-stimulating factor	MCSF
ANTI-INFLAMMATORY CYTOKINES		
	Interleukin-10	IL-10

	Interleukin-1 receptor antagonist	IL-1RA
	Interleukin-2 receptor antagonist	IL-2RA
GROWTH FACTORS		
	Basic fibroblast growth factor	FGF BASIC
	Granulocyte colony-stimulating factor	GCSF
	Hepatocyte growth factor	HGF
	Interleukin-3	IL-3
	Interleukin-7	IL-7
	Platetet derived growth factor BB	PDGFBB
	Stem cell factor	SCF
	Stem cell growth factor beta	SCGFβ
	Vascular endothelial growth factor	VEGF
	Beta nerve growth factor	βNGF
	Macrophage colony-stimulating factor	MCSF
OTHER CYTOKINES	TNF-related apoptosis inducing ligand	TRAIL