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FATTY LIVER AND CYTOKINES

Syventävien opintojen kirjallinen työ

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Non-alcoholic fatty liver is the most common liver disease worldwide with the prevalence of around 25% in adult population. Comorbidities of non-alcoholic fatty liver include type 2 diabetes, metabolic syndrome, cardiovascular diseases, and chronic liver diseases. In addition, fatty liver can in some cases develop into non-alcoholic steatohepatitis or cirrhosis, later of which predisposes a patient to hepatocellular carcinoma. Cytokines, small proteins acting on communications and interactions between cells, are known to predict the development of insulin resistance and play a crucial role in many metabolic diseases. Under normal physiologic conditions hepatic cytokine production is minimal or absent whereas hepatic cells produce inflammatory cytokines as a result to pathological stimuli. There is not yet a clinical serum marker for the prediction or diagnosis of non-alcoholic fatty liver and most of the previous studies and publications have concentrated more on cytokines' associations to non-alcoholic steatohepatitis than simple steatosis. This thesis includes a review of literature on the subject which cytokines are associated to liver steatosis and an analysis based on Young Finns Study (YFS) data on cytokines' association to later fatty liver.

YFS is a large population-based follow-up study about the determinants of cardiovascular risk factors from childhood into adulthood. Regarding of the subject of this thesis, a study population of 2130 participants were measured for 48 cytokine, chemokine, and growth factor concentrations and four years later 2042 of the subjects were examined for liver steatosis using liver ultrasound imaging. The association between cytokine concentrations and liver steatosis four years later was studied on a statistical analysis using Spearman's correlation test and logistic regression analysis with confounding factors including age, body mass index, smoking status, alanine-aminotransferase (ALT), LDL cholesterol and total cholesterol. Men and women were analyzed separately.

The results of the study indicate that higher serum interleukin-18 and hepatocyte growth factor (HGF) serum levels predict fatty liver in women. Higher serum ALT concentrations were also associated to later fatty liver in men. In the review of literature part of the thesis several cytokines, including TNF-α, IL-1, IL-6, IL-10, IL-17, and members of the CC and CXC family were found to be associated to non-alcoholic fatty liver disease progression. Interleukin-18 and hepatocyte growth factor were not included in these previous findings. Hepatocyte growth factor has been earlier studied only in rodent models of fatty liver and the association has been protective against liver steatosis, making this a novel finding. Further research is needed to determine if either of these cytokines could have clinical relevance in prediction of fatty liver in the future.

Key words: fatty liver, non-alcoholic fatty liver, hepatic steatosis, cytokine, interleukin, Cardiovascular Risk in Young Finns Study

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1 INTRODUCTION

Fatty liver without excessive alcohol consumption, drug-related or viral causes is the most common liver disease worldwide with a prevalence of around 25% in adult population and 70-90% in the diabetics and obese (Targher et al. 2010, Younossi et al. 2016). In The Cardiovascular Risk in Young Finns Study (YFS) the prevalence of fatty liver was 19% (n=385) on patients aged 39-49 years (Suomela et al. 2016). Fatty liver is the result of triacylglycerol accumulation in the hepatocytes and by definition includes the presence of 5% or more hepatic steatosis (Paschos & Paletas 2009, Brown & Kleiner 2016). Fatty liver is often associated with impaired insulin activity and other metabolic comorbidities such as type 2 diabetes mellitus and metabolic syndrome as well as with cardiovascular diseases and chronic kidney disease (Brown & Kleiner 2016, Kwak & Kim 2018). Fatty liver can also in some cases develop into more severe liver disease, inflammatory non-alcoholic steatohepatitis (NASH) or cirrhosis which can in turn predispose the patient to hepatocellular carcinoma (HCC) (Cohen et al. 2011). Non-alcoholic fatty liver disease (NAFLD) is based on the disease severity divided into fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH), with the latter associated with faster fibrosis progression (Singh et al. 2015, Wong et al. 2018).

Cytokines are small proteins that act on communications and interactions between cells and that are secreted by several different cell types of the immune system, including T-cells and macrophages (Zhang & An 2007). Circulating cytokines have been shown to predict the development of insulin resistance in population cohort studies (Santalahti et al. 2016)

1.1 Fatty liver

1.1.1 Diagnosis and epidemiology

NAFL is a clinical diagnosis that includes the presence of 5% or more hepatic steatosis, by visual estimate, with the secondary causes of lipid accumulation such as excessive alcohol consumption ($\leq$20g in women and $\leq$30g in men daily), viral infections or medication excluded. Hepatic steatosis has to be determined by liver imaging or biopsy (Bellentani et al. 2000, Chalasani et al. 2012). In histology, NAFL is referred to as predominantly macrovesicular steatosis with or without nonspecific inflammation. Even though the steatosis in NAFL is typically macrovesicular it may be composed of a mixture of large and small vacuoles (Brown & Kleiner 2016).

NAFLD is the most common liver disease worldwide and the leading underlying cause of chronic liver disease (Kotronen et al. 2010, Younossi et al. 2016). According to a meta-analysis by Younossi et al., the global prevalence is 25% in adult population, and the incidence is continuously increasing (Younossi et al. 2016).

In the YFS fatty liver was shown in total 19 % of the 1998 participants determined by ultrasound imaging. The subjects were aged 34-49 years. In men, 28.0 % showed evidence of fatty liver, compared to 10.8% of women (Kaikkonen et al. 2017).

1.1.2 Pathogenesis

The pathogenesis of NAFLD is multifactorial and it involves metabolical, environmental and genetic factors. NAFL is most often associated with conditions that include impaired insulin activity such as obesity, metabolic syndrome and diabetes mellitus as well as chronic kidney disease and cardiovascular diseases (Brown & Kleiner 2016, Kwak & Kim 2018). There are longitudinal studies using aminotransferase levels suggesting a chronological association between the progression of metabolic syndrome and the occurrence of NALFD (Hamaguchi et al. 2005, Suzuki et al. 2005). The findings suggest that rather than being a separate disease, NAFLD may be a hepatic manifestation of fat-related epidemic diseases such as metabolic syndrome (Chen et al. 2011). The close relationship between metabolic dysfunction and NAFLD has also further led to proposal of name-change to metabolic-associated fatty liver disease, MAFLD (Eslam, Newsome et al. 2020a, Eslam, Sanyal et al. 2020b).

Consistent supporting evidence exists that both NAFLD and metabolic syndrome have common mechanisms, which most often involve insulin resistance and visceral obesity (Paschos & Paletas 2009, Kim et al. 2016, Kwon et al. 2017). Insulin resistance and hyperinsulinemia are key factors in NAFL pathophysiology (Fu et al. 2012). In adipose tissue insulin acts by inhibiting lipolysis and promoting esterification of free fatty acids and further storage in lipid droplets. Decreased insulin activity correspondingly results in increased adipocyte lipolysis and high circulating free fatty acids that are available to subsequent hepatic uptake. In hepatic cells insulin resistance leads to reduced hepatic glycogen storage and increased gluconeogenesis. Insulin also activates key regulators of de novo lipogenesis in liver. (McGarry 1992.) Triglyceride production from fatty acids can originate also from non-lipid sources, such as monosaccharides or amino acids converted into saturated fatty acids through de novo lipogenesis when consumed in excess (Aarsland & Wolfe 1998, Charidemou

et al. 2019). As a response to systemic insulin resistance, hyperinsulinemia occurs, augmenting hepatic de novo lipogenesis. The net effect of all these events is accumulation of lipids in hepatocytes: steatosis. The very-low density lipoprotein secretion, containing triglycerides, from liver also increases, compounding adipocytes already reduced ability to store these lipids. (McGarry 1992.)

1.1.3 Progression

Simple steatosis, NAFL, is the most benign condition on the NAFLD spectrum. NASH is defined as inflammatory reaction and hepatocyte ballooning degeneration in addition to steatosis. Fibrosis may or may not be present (Mazzolini et al. 2020). In a long-term clinical follow-up fatty liver without other accompanying histological changes had a remarkably benign course with only few patient dying from liver disease or progressing to cirrhosis. The leading causes of death in NAFLD spectrum patients are cancers and cardiovascular events. (Teli et al. 1995, Dam-Larsen et al. 2009.) It is important to note that patients with fatty liver are more susceptible to the toxic effects of drugs, alcohol, viral infections and other insults to the liver (Mazzolini et al. 2020). NAFLD patients are known to be more likely to be injured by the same drug and dose compared to healthy individuals (Michaut et al. 2014). In FINRISK cross-sectional population-based study the incidence of liver-related outcomes (fatal and non-fatal advanced liver disease requiring admission to hospital, liver cancer or liver-related death by non-alcoholic mechanisms) were determined from individuals with baseline fatty liver index FLI ≥60. The incidence rate was 0.97 per 1000 person years. The cumulative risk increased with age, and at age of 80, after a 20-year follow-up, it was 2.4% in men and 1.5% in women. (Männistö et al. 2021.) A meta-analysis based on small paired-biopsy studies indicates that hepatic fibrosis progresses on average by one stage in 14 years on patients with NAFL (Singh et al. 2015).

The non-progressivity of NAFL to NASH, is nevertheless controversial with some studies showing that NAFL may clearly progress, with one quarter of patients developing bridging fibrosis over a relatively short time period (Pais et al. 2013, McPherson et al. 2015). The prevalence of NASH is estimated to be between 1.5% to 6.5% in general population (Younossi et al. 2016). In a study by McPherson et al. (2015), 108 patients underwent paired liver biopsies and 44% of patients with baseline NAFL had progressed to NASH over the median follow-up period of 8 years and consecutively 37% of these subjects showed evidence of fibrosis progression.

1.1.4 Genetics and fatty liver

One of NAFLD's distinctive features is substantial inter-patient variation in disease progression. The reasons aren't completely understood but can be at least partially attributed to genetic factors. Genetic risk variants for NAFLD have been identified. The one associated with the highest risk is the patatin-like phospholipase domain containing protein 3 (*PNPLA3*) gene *I148M* variant carried by approximately 30% of the people in Western countries (Romeo et al. 2008). Variants in transmembrane 6 superfamily member 2 (*TM6SF2)*, membrane bound O-acyltransferase domain-containing 7 (*MBOAT7)* and glucokinase regulator *(GCKR)* genes have moderate effect size but are shown to have a significant contribution (Eslam et al. 2018). In family studies the first-degree relatives of patients are at much higher risk of the disease compared to general population. The association is independent of hepatic adiposity. These observations led to estimates that between 38% and 100% of fat content in the liver and NAFLD variability are caused by genetic factors. (Willner et al. 2001, Schwimmer et al. 2009.)

The scientific evidence supporting the role *PNPLA3* single nucleotide polymorphism (isoleucine to methionine substitution at position 148) in liver diseases is robust and the topic has been widely researched (Romeo et al. 2008). The I148M substitution results in loss-of-function in PNPLA3 hydrolase activity causing the retinyl esters and triglycerides to stay entrapped as lipid droplets in hepatocytes and hepatic stellate cells and further increasing the accumulation of hepatic fat (Huang et al. 2011, Pingitore et al. 2014). Homozygosity of the *PNPLA3* variant has been shown to increase the risk of NAFLD progression to hepatocellular carcinoma 10-fold compared to the risk of general European population (Liu et al. 2014).

TM6SF2 rs58542926 C>T polymorphism results in a loss-of-function of the protein that is involved in the enrichment of triglycerides to apolipoprotein B100 in the pathway of hepatocyte very low-density lipoprotein secretion. This variant further reduces the level of circulating lipoproteins and increases the liver triglyceride levels. (Kozlitina et al. 2014.) *MBOAT7* rs641738 C>T variant has also been associated with the increased NAFLD risk, fibrosis and inflammation through downregulation of *MBOAT7* leading to reduced levels of phosphatidyl-inositol containing arachidonic acid both in circulation and hepatic cells (Luukkonen et al. 2016, Mancina et al. 2016). Another variant associated with increased risk of NAFLD is a common missense loss-of-function (rs1260326) in *GCKR*. CGKR regulates de-novo lipogenesis by monitoring the influx of glucose into

hepatocytes and the variant has been shown to be associated with accumulation of fat in the liver. (Beer et al. 2009.)

1.1.5 Nutrition and fatty liver

The diet has been described to account for 15% of the liver triglycerides in NAFLD spectrum patients (Donnelly et al. 2005). Preclinical studies show that diets high with sucrose and fructose are steatogenic, possibly by dysregulation of key lipid metabolic pathways and hormones and promotion of gut dysbiosis (Festi et al. 2014). An increase in fibre intake has been found to reduce NAFLD risk (Noureddin et al. 2020). NAFL does not only concern overweight or obese as the reported prevalence of non-obese patients varies between 10% and 30% (D. Kim & Kim 2017). In non-obese patients, the consumption of fructose and cholesterol-rich diet has been connected with NAFLD development (Enjoji et al. 2012).

In a cross-sectional and prospective analysis of the YFS several lipids and metabolites were examined to find out if they were associated with the presence of fatty liver. The strongest direct association was observed for extremely large very-low-density lipoprotein triglycerides, other very-low-density lipoprotein measures and chained amino acids. The strongest inverse association in turn was observed for high-density lipoprotein measures and several fatty acids, omega-6 included. Independently of routine metabolic risk factors, circulating amino acids, lipids and fatty acids reflect hepatic steatosis. Metabolic aberrations in young adults appear to precede the development of fatty liver. (Kaikkonen et al. 2017.) Multiple cohort-studies with different-size, -age and -ethnicity cohorts proved that decreased consumption of fish and respectively increased consumption of meat, especially processed meat and red meat, and sugar-sweetened beverages were associated with NAFLD (Musso et al. 2003, Toshimitsu et al. 2007, Kim et al. 2010, Zelber-Sagi et al. 2018, Noureddin et al. 2020).

1.1.6 Physical activity and fatty liver

Increasing physical activity as a lifestyle modification in NAFLD patients reduces hepatic triglyceride levels and markers of hepatocellular injury independent of weight loss (Keating et al. 2012, Thoma et al. 2012, Oh et al. 2015, Orci et al. 2016, Katsagoni et al. 2017). Cross-sectional studies have demonstrated an inverse correlation between NAFLD and physical activity (Perseghin et al. 2007, Keating et al. 2012). It was found that in a study conducted in high fat diet-induced rodent models of

NAFLD exercise reduced insulin, triacylglycerides, fasting glycemia and liver damage but had no effect on intrahepatic triacylglyceride levels and the number of lipid droplets but reduced the size of them (la Fuente et al. 2019).

1.2 Cytokines

Cytokines are small proteins, polypeptides of around 150 amino acids, secreted by several different cell types in the immune system such as lymphocytes, macrophages, neutrophils, eosinophils, basophils, and mast cells, that have a specific effect on the communications and interactions between cells. The most remarkable producers are Th-cells and macrophages. Cytokine is a generic name, and the group includes for example interleukins (leukocyte-secreted cytokines that influence other leukocytes), chemokines (cytokines with chemotactic action), lymphokines (secreted by lymphocytes) and monokines (secreted by monocytes) and interferons. (Kelso 2000, Zhang & An 2007, Ramadori & Armbrust 2001.)

Cytokines may have autocrine or paracrine action and, in some instances, also endocrine action. Most cytokines have pleiotropic function: one cytokine can affect several different cell types, and many cell types can secrete the same cytokine, respectively. Similar functions can also be stimulated by different cytokines. (Ramadori & Armbrust 2001, Zhang & An 2007.) Cytokines bind on specific cell-surface receptors triggering further intracellular signaling cascades that possibly alter cell functions such as migration, proliferation, adhesion, and apoptosis. They are involved in essentially all important biological processes including inflammation, immunity, fibrosis, angiogenesis, repair and embryogenesis. (Oppenheim 2001, Vilček & Feldmann 2004.) Many of them act synergistically or antagonistically on target cells (Ramadori & Armbrust 2001).

1.2.1 Proinflammatory cytokines

Proinflammatory cytokines up-regulate inflammatory responses and are predominantly produced by activated macrophages. These include tumour necrosis factor alpha (TNF-α) and tumour necrosis factor beta (TNF-β) and several interleukins (IL), most importantly IL-1 and IL-6 but also IL-8, IL-12, IL-15, IL-18, and IL-23. Antigen-presenting cells in innate immunity produce these cytokines through the activation of pattern recognition receptors when coming across typical pathogen

components. An alternate way leading to production is antigen presenting cells taking up the antigen, processing it and presenting it to T-helper lymphocytes. (Zhang & An 2007.)

1.2.1.1 TNF

TNF-α is primarily derived from mononuclear phagocytes but neutrophils, natural killer cells, activated lymphocytes, endothelial cells and mast cells can also secrete it, and TNF-β is produced by lymphocytes (Beutler & Cerami 1989). In liver TNF-α is secreted by Kuppfer cells, hepatocytes and other cell types, as a response to chronic inflammatory activity (Montecucco & Mach 2008). Lipopolysaccharide is the strongest inducer of TNF production by monocytes, acting through Toll-like receptors TRL2 and TLR4 (Borish & Steinke 2003). TNFs have direct cytotoxic effect on tumour cells as well as the ability to stimulate antitumour immune responses in cancer cells. In addition to cytotoxicity TNF is a powerful activator of neutrophils, chemotaxis, cell adherence mediator amongst other functions. (Beutler & Cerami 1989.) TNF-α is also secreted indirectly in abdominal adipose tissue (Montecucco & Mach 2008). Macrophages infiltrated in adipose tissue of obesity models are responsible for the indirect secretion (Stojsavljević et al. 2014).

1.2.1.2 Family of IL-1 cytokines

The IL-1 family and IL-1 receptor members are mostly proinflammatory but can also be anti-inflammatory. These include cytokines IL-1α, IL-1β, IL-1R antagonist, IL-18, IL-33, IL-36, IL-37 and IL-38. Cytokines IL-1α and IL-1β are synthesized as precursors mainly by phagocytic mononuclear cells but also by endothelial cells, neutrophils, osteoblasts, and several other cells. The effects of IL-1 family members are transmitted through IL-1 receptors 1-10 and are involved in almost all inflammatory conditions, regulating the acute phase response and both differentiation and function of several innate and lymphoid cells, such as enhancement of B-cell proliferation, as well as directing immunoglobulin synthesis and many other complex inflammatory processes. (Garlanda et al. 2013.)

1.2.1.3 IL-6

IL-6 is secreted by neutrophils, macrophages, and monocytes through Toll-like receptor binding, and it activates several different cell types, including hepatocytes, immune cells, hematopoietic stem cells and osteoclasts. IL-6's functions include induction of inflammation, for instance it is a strong inducer

of acute phase proteins in the liver such as C-reactive protein, haptoglobin and fibrinogen regulating immune responses and supporting hematopoiesis. (Kishimoto 2010, Schmidt-Arras & Rose-John 2016.)

1.2.2 Anti-inflammatory cytokines

Anti-inflammatory cytokines include interleukin-1 receptor antagonist, IL-4, IL-10, IL-11 and IL-13 and they control the proinflammatory cytokine response. Interferon α, IL-6 and TGF-β are categorized either anti-inflammatory or proinflammatory depending on the situation on which they function.
IL-10 is a strong anti-inflammatory cytokine with the ability to repress the expression of several inflammatory cytokines like TNF-α, IL-1 and IL-6 by activated macrophages, down-regulate proinflammatory cytokine receptors and up-regulate endogenous anti-cytokines (Zhang & An 2007).

1.2.3 Chemokines

Chemokines are a family of low molecular-weight heparin-binding proteins of which main effect is to induce chemotaxis, the activation and migration of leukocytes to inflammation site. Chemokines are divided to four different groups by conserved cysteine residues they contain: C-C (RANTES, monocyte chemoattractant protein MCP-1, monocyte inflammatory protein or MIP-1α, and MIP-1 β), C-X-C (IL-8), C (lymphotactin) and CXXXC (fractalkine) chemokines. (Zhang & An 2007.)

1.3 Fatty liver and cytokines

Under normal physiological conditions hepatic cytokine production is minimal or absent whereas hepatic cells produce inflammatory cytokines as a result to pathological stimuli such as excessive lipid accumulation in NAFL (Braunersreuther et al 2012). The connection between liver steatosis and inflammatory processes is two-directional. The activation of inflammatory pathways, including proinflammatory cytokine production, is involved in inducing steatosis and respectively adipose tissue is able to activate inflammation. (Valenti et al. 2009.) There is increasing evidence supporting the idea that both classical cytokines and adipokines released from the adipose tissue of obese patients play a central role in NAFLD (Moschen et al. 2010, Moschen et al. 2011, Anstee et al. 2013, Yki-Järvinen 2014).

Both innate immunity and adaptive immunity are involved in the fatty liver pathophysiology. Innate immunity acts through TLR4-signalling dependent Kupffer cell activation, complement cascade activation, balancing the cytokine network towards pro-inflammatory state, alternation in natural killer and natural killer T cell activity and number and activation of the adaptive immunity. (Valenti et al. 2009.) The contribution of adaptive immunity is under research and further clarification, the evidence suggests that it participates in fatty liver maintenance as well as disease progression and comorbidities of it (Valenti et al. 2009, Sutti et al. 2016).

1.3.1 Insulin resistance and cytokines

In obesity, which is often associated to NAFL as the prevalence of NAFLD increases linearly with BMI, the excessive accumulation of triglycerides in adipose tissue leads to adipocyte dysfunction in which the adipocytes release adipokines (cytokine-like hormonal mediators) and proinflammatory cytokines including TNF-α, IL-6, monocyte chemoattractant protein 1 (MCP-1), plasminogen activator inhibitor 1 and resistin, all of which impair adipocyte insulin sensitivity (Baranova et al. 2006, Hardy et al. 2016, Marchisello et al. 2019). Cytokines, particularly IL-6 and TNF-α which are produced by both dysfunctional visceral adipose tissue and Kuppfer cells contribute to the emergence of liver insulin resistance. Under hepatic insulin resistance conditions, the precense of insulin does not inhibit hepatic glucose synthesis and glycogenolysis or activate glycogen synthesis. (Michael et al. 2000.)

Gut endotoxins have a significant role in activating Kuppfer cells cytokine production in liver. Diets containing excessive amounts of fat and fructose alter the motility of the intestine and further lead to dysbiosis and increased intestinal wall permeability (Spruss & Bergheim 2009). These alterations induce the increased release of pro-inflammatory dietary components e.g. trans FFAs and fructose into blood (Tilg & Moschen 2010). Certain fructose and fat-enriched diets enhance the profusion of Gram-negative bacteria in gut microbiota. The wall of Gram-negative bacteria such as Enterobacteriaceae has the potential to produce pro-inflammatory endotoxin (LPS) which activates Toll-like receptor subtype 4 (TLR4) that are set particularly in cell membrane of Kuppfer cells but also in hepatocytes and hepatic stellate cells. In NAFLD the expression of TLR4 is enhanced (Cani et al. 2007, Amar et al. 2008). LPS attachment to TLR4 activates different signalling cascades inside the cell including MAP kinases, leading to the activation of pro-inflammatory transcription factors like NF-κB and adaptor protein-1 (AP-1) and further cytokine production, TGF-β and IL-8

particularly (Tilg & Diehl 2000, Seki & Brenner 2008, Pradere et al. 2010). These cytokines have chemoattractant features that promote the recruitment of immune effector cells such as neutrophils, with subsequent hepatocyte injury through oxidative stress mediated mechanisms in (Carter-Kent et al. 2008, Baffy 2009, Pradere et al. 2010).

Lipid accumulation in hepatocytes is the primary reason for inflammatory response and cytokine production rather than TLR4 activation through LPS (Csak et al. 2011).

1.3.2 TNF-α

Several studies suggest that TNF-α plays a key role in the development of NAFLD in animals and humans. In diverse animal models of obesity, type 2 diabetes mellitus and insulin resistance TNF-α expression in adipose tissue was increased, and mice lacking TNF-α had improved insulin activity (Lang et al. 1992, Hotamisligil et al. 1993, Uysal et al. 1997). Accordingly, in humans, TNF-α levels were measured to be higher in obese compared to lean individuals (Hotamisligil et al. 1995, Dandona et al. 1998). Not all studies support the importance of TNF-α in NAFL pathogenesis since some studies did not show correlation between TNF-α levels and insulin resistance (Müller et al. 2002, Bruun et al. 2003) and two clinical studies did not find any improvement in insulin sensitivity after use of an antagonist and anti-TNF-α antibody (Ofei et al. 1996, Bernstein et al. 2006). In a study by (Lucero et al. 2011) no difference in serum levels of TNF-α were observed between patients with NALFD and control group of patients without NAFLD.

Extensive evidence supports the key role of TNF-α and other proinflammatory cytokines in obesity-related insulin resistance and liver steatosis (Carter-Kent et al. 2008). TNF-α levels have a correlation with the degree of insulin resistance in humans which is also supported by evidence that certain TNF-α polymorphisms modify the susceptibility to insulin resistance between different genotypes (Valenti et al. 2002, Tokushige et al. 2007).

1.3.3 IL-1

IL-1α is a precursor molecule present also in a healthy liver (Chen et al. 2007), responsible for the initiation of the acute phase response. IL-1β is in turn produced following inflammasome activation. Both IL-1α and IL-1β bind in the same IL-1R type 1 receptor and have similar biological functions,

most of them proinflammatory. (Hoffman et al. 2001, Tilg et al. 2016.) IL-1α precursor, a prototypic alarmin, is released in necrotic cell death and induces a cascade of proinflammatory cytokines an chemokines, resulting in sterile inflammation, a feature of NAFLD (Rider et al. 2011, Kubes & Mehal 2012, Tilg et al. 2016).

Studies on rodent models of NAFLD have shown an important role of IL-1F members. The role of IL-1α and IL-1β in steatosis/steatohepatitis was examined using diet-induced rodent models. A remarkable increase in hepatic IL-1α/β was measured. Even in lower levels of inflammation IL-1a$^{-/-}$ mice had increased plasma and hepatic cholesterol levels, suggesting that hepatic steatosis and inflammation might develop in a diverse manner. Respectively, deficiency in IL-1α/β protected mice with diet-induced steatosis from inflammation. (Kamari et al. 2011, Tilg et al. 2016.)

1.3.4 IL-6

IL-6 has complex participation in liver pathology and its effect on NAFLD development progression is unclear (Braunersreuther et al. 2012). Leukocytes, endothelial cells, adipocytes and also hepatocytes produce IL-6 and secretion can be in response to other cytokines such as TNF-α (Carter-Kent et al. 2008). In liver IL-6 inhibits insulin signalling leading to increased gluconeogenesis and further hyperglycemia (Klover et al. 2003). In a study by Klover et al. (2005) diet-induced obese mice treated with IL-6 antibodies had improved insulin sensitivity. Decreased IL-6 concentrations were associated with insulin sensitivity improvements and weight loss in patients undergoing bariatric surgery (Kopp et al. 2003). A study conducted in mice with methionine choline deficient diet-induced NASH showed that IL-6 pathway neutralization treatment with tocilizumab, an antibody against IL-6 receptor, enhanced liver steatosis but improved liver damage (Yamaguchi et al. 2010).

Serum IL-6 levels and expression in hepatocytes and Kuppfer cells are elevated in NAFLD patients (Haukeland et al. 2006). In a study by Kumar et al. (2012) it was demonstrated that IL-6 levels were elevated selectively to NAFLD patients (n=34) in comparison to control groups of chronic hepatitis B patients and healthy subjects. IL-6 levels also positively correlate with the stage of liver inflammatory activity and fibrosis (Wieckowska et al. 2008). Acute IL-6 secretion has hepatoprotective effect as it improves hepatic regeneration and repair, but chronic excessive exposure may have an adverse effect as it may abolish the protective effect and even sensitize the liver to injury and apoptotic cell death (Jin et al. 2006). IL-6 mediates the synthesis of several acute phase proteins

like C reactive protein and thus it is possible that IL-6 has an unidentified indirect harmful effect on NAFLD pathogenesis (Braunersreuther et al. 2012).

1.3.5 IL-10

IL-10 is an anti-inflammatory cytokine that regulates pathological and physiological inflammatory reactions in several different tissues and organs (Moore et al. 2001). In liver IL-10 has been detected in hepatocytes, Kuppfer cells and stellate cells but few studies have been performed to clarify the role of endogenous IL-10 in the progression of NAFLD (Braunersreuther et al. 2012). A study using IL-10 deficient mice fed on high fat diet suggested that IL-10 protects liver from steatosis but not from concomitant insulin resistance (Den Boer et al. 2006). Another study by Cintra et al. (2008) observed that the inhibition of IL-10 (by either an anti-IL-10 antibody or an IL-10 antisense oligonucleotide) resulted in impaired insulin activity and hepatic steatosis, and increased production of proinflammatory markers such as TNF-α, IL-6, IL-1β and F4/80 in mice that had developed NAFLD and diabetes in response to high-fat diet. In humans, an inverse correlation between IL-10 levels and metabolic syndrome in obese women was observed, suggesting that IL-10 may have beneficial in metabolic syndrome patients, also affected by NAFLD (Esposito et al. 2003). However, this association was not confirmed in obese children and adolescents (Calcaterra et al. 2009).

1.3.6 IL-17

A study by Tang et al. (2011) indicates that IL-17 plays a critical role in hepatic steatosis. IL-17 disturbs the insulin-signaling pathway and aggravates steatosis in HepG2 cells. IL-17 and free fatty acids also further induced IL-6 production.

1.3.7 Chemokines

The members of CC and CXC family chemokines have been detected in the liver repeatedly regarding acute and chronic liver damage, but the knowledge about their specific role in fatty liver development is limited (Charo & Ransohoff 2006, Berres et al. 2010). In patients with features of NASH, mRNA levels of different CC and CXC chemokines have been proven to be elevated compared to healthy subjects (Berres et al. 2010).

2 AIMS OF THE STUDY

Most of the previous studies and publications have concentrated more on cytokines' associations to NASH than simple steatosis. There are also few early fatty liver cohort studies. The purpose of this thesis is to introduce existing literature findings about cytokines association with the incidence of fatty liver and examine whether circulating cytokine levels correlate with later steatosis in YFS population.

3 METHODS

3.1 Study cohort

In The YFS, in 1980 baseline 3596 boys and girls aged 3, 6, 9, 12, 15 and 18 participated in the first cross-sectional survey. The subjects were randomly chosen from the national registry, from five different study districts. 4320 subjects were invited and 83.2% accepted the invitation. The whole study population has been followed up several times since, in 1983, 1986, 2001, 2007 and 2018. In addition to the original cohort, the study was extended to include three generations by inviting the participants' parents and children. The local ethical committees approved protocol of the study and the participants were proved written informed consents. (Suomela et al. 2016.)

Participants were determined for body mass index (BMI) (kg/m^2) by height (m) and weight (kg) measurements, systolic blood pressure (mmHg) in supine position, using automatic manometer, and high-density lipoprotein (HDL) (mmol/l), fasting plasma glucose (mmol/l), triglycerides (mmol/l), insulin (mU/l), alanine aminotransferase (ALT) (U/l), and high sensitivity C-reactive protein (CRP) (mg/l) were measured. (Raitakari et al. 2008.)

Clinical characteristics were self-reported with structured questionnaires. These included age, type 1 and type 2 diabetes, pregnancy, smoking and level of physical activity (Santalahti et al. 2016).

3.2 Fatty liver examination

In 2011 the 2042 participants, aged 39-49 years, were examined for liver steatosis by ultrasound imaging using a validated protocol, Sequoia 512 ultrasound mainframes and 4.0 MHz adult abdominal transducers. Liver steatosis was defined and evaluated according to criteria of hepatic

parenchymal brightness, liver to kidney contrast, deep beam attenuation and bright vessel walls. The presence of hepatic steatosis was determined visually by a trained ultrasonographer. Participants were then divided to normal liver and fatty liver groups. (Suomela et al. 2016.)

3.3 Cytokine, chemokine, and growth factor measurement

In 2007 a study population of 2130 individuals, diabetics and pregnant women excluded, were measured for 48 cytokine, chemokine, and growth factor concentrations. Serum samples were collected from participants after overnight (≥12 hour) fasting and then stored at -70 °C. The concentrations were determined using Bio-Rad's premixed Bio-Plex Pro Human Cytokine 27-plex Assay and 21-plex Assay kits on Bio-Rad's Bio-Plex 200 system. (Santalahti et al. 2016.) A table of all the measured cytokines, chemokines and growth factors is on the supplements (Supplemental table 1).

3.4 Statistical analysis

Analysis of collected data was done using SAS version 9.1. The original data included cytokine levels, age, and ALT levels in 2007 and liver status in 2011. Participants with type 1 or type 2 diabetes and participants whose cytokine levels were not measured were excluded from statistical analysis. Data from total of 1776 participants were analyzed. The data was sorted according to study number, and smoking data, CRP, LDL cholesterol, total cholesterol and BMI values were combined with the original data. Outlayer observations were excluded from the data. Men and women were analyzed separately. P-value was determined by Chi-square test for categorical variables and by Mann-Whitney's U-test for continuous variables. The normality of distribution of variables was assessed by visual inspection. Study data was first analyzed using Spearman correlation test, for which hepatic steatosis was divided into three categories: normal liver, mild liver steatosis and distinct liver steatosis. Cytokine variables were logarithmically transformed for the use of logistic regression test and hepatic steatosis was divided into two categories: normal liver or liver steatosis. Age, BMI, smoking status, ALT, LDL, and total cholesterol values were considered as confounding factors in logistic regression test.

4 RESULTS

At baseline in 2007, the subjects mean age was 38.0 years for women and 37.9 years for men. BMI values were 25.3 for women and 26.7 for men. Higher proportion of men were smokers: 20% versus 14% of women. Total cholesterol, LDL cholesterol and ALT concentrations were higher in men but CRP concentrations higher in women. Liver status was determined by ultrasound in 2011 and subjects were divided into three groups according to fatty liver status. Mild liver steatosis was significantly more common in men: 22.6% compared to 7.8% in women. Distinct liver steatosis occurred in 5.7% of men and 2.7% of women. Characteristics of the study sample are reported in Table 1.

Table 1. Characteristics of the study sample in the 2007 baseline and liver status in 2011[1].

	Women	Men	P-value[2]
Age	38.0 (4.94)	37.9 (5.06)	0.6103
BMI	25.33 (5.08)	26.71 (4.18)	<.0001
Smoking (%)	13.66	20.40	0.0002
Type 1 diabetes (%)	0.51	0.51	0.9873
Type 2 diabetes (%)	0.51	1.02	0.2195
Total cholesterol	4.93 (0.85)	5.20 (0.94)	<.0001
LDL cholesterol	2.95 (0.72)	3.29 (0.82)	<.0001
ALT	13.36 (10.34)	24.11 (15.50)	<.0001
CRP	2.02 (3.36)	1.60 (4.27)	0.0010
Liver status in 2011			<.0001
Normal liver (%)	89.58	71.77	
Mild liver steatosis (%)	7.76	22.58	
Distinct liver steatosis (%)	2.66	5.65	

[1]Data are presented as mean (standard deviation) except for smoking, type 1 diabetes, type 2 diabetes, and liver status, which are presented as number (%).
[2]P- value < 0.05 was presented as statistically significant.
BMI body mass index, LDL low density lipoprotein, ALT alanine aminotransferase, CRP C-reactive protein.

Spearman's correlation test indicated that proinflammatory cytokines IL-18 and IP-10, allergy marker IL-5 and growth factor HGF in women had statistically significant positive correlation to the occurrence of liver steatosis 4 years later. Of these cytokines IL-18 and HGF were correspondingly positively correlated to liver steatosis in men. Inflammatory chemokine MIP-1β was found statistically significant for positive correlation in men, but similar finding did not occur in women. Inflammatory chemokine CTACK appeared to have weak negative correlation to fatty liver in both men and women. Statistically significant Spearman's correlation test results are listed in Table 2 and all results in Supplemental table 2.

Table 2. Statistically significant Spearman's correlation test results for the associations between cytokines and fatty liver status[1].

	Women			Men		
Proinflammatory Cytokines	Correlation Coefficient	P-value[2]	Number of Observations	Correlation Coefficient	P-value[2]	Number of Observations
IL-18	**0.179**	**<.0001**	979	**0.159**	**<.0001**	797
IP-10	**0.117**	**0.0002**	979	0.086	0.0152	797
Allergy markers						
IL-5	**0.105**	**0.0010**	979	0.038	0.2852	796
Growth factors						
HGF	**0.185**	**<.0001**	979	**0.171**	**<.0001**	797
Inflammatory chemokines						
MIP-1β	0.058	0.0698	979	**0.116**	**0.0011**	796
CTACK	**-0.127**	**<.0001**	979	**-0.155**	**<.0001**	797

[1]Statistically significant values are indicated by bolded text.
[2]Statistically significant P-value limit is determined by Bonferroni-correction: 0.05/37 (number of cytokines) = 0.00135.

In 2007 measured ALT and CRP values were examined as potential markers for liver inflammation and steatosis. For both ALT and CRP there were a statistically significant correlation with liver steatosis. The correlation was significant for both women and men, mut was stronger in men: correlation coefficient for ALT and liver steatosis was 0.37 for men and 0.18 in women with the corresponding values in CRP 0.26 in men and 0.23 in women. Spearman's correlation test results are reported in Table 3.

Table 3. Spearman's correlation test results for the associations between ALT and CRP values in 2007 and fatty liver status in 2011.

	Women			Men		
	Correlation Coefficient	P-value[1]	Number of Observations	Correlation Coefficient	P-value[1]	Number of Observations
ALT	0.180	<.0001	979	0.368	<.0001	797
CRP	0.232	<.0001	979	0.259	<.0001	797

[1]P- value < 0.05 was presented as statistically significant.

Logistic regression analysis was performed, and several confounding factors were considered in the analysis. Statistically significant association was found between two cytokines, IL-18 (OR 3.0) and HGF (OR 2.36), and liver steatosis, but this finding only occurred in women. Statistically significant logistic regression analysis test results are listed in Table 4 and all results in Supplemental table 3.

Table 4. Statistically significant logistic regression analysis test results for the associations between cytokines and fatty liver status[1,2].

Proinflammatory cytokines	Women				Men			
	Odds Ratio [3]	Lower 95 % Confidence Limit	Upper 95 % Confidence Limit	P-value	Odds Ratio [3]	Lower 95 % Confidence Limit	Upper 95 % Confidence Limit	P-value
IL-18	**3.00**	**1.70**	**5.29**	0.0001	1.20	0.75	1.92	0.44
Growth factors								
HGF	**2.36**	**1.22**	**4.57**	0.01	1.28	0.75	2.18	0.37

[1]Statistically significant values are indicated by bolded text.
[2]Age, BMI, smoking status, ALT, LDL cholesterol and total cholesterol were considered as confounding factors in the analysis.
[3]Odds ratio values are determined for logarithmically transformed variables.

Associations between ALT and CRP in 2007 and later liver steatosis were examined by logistic regression analysis. CRP values showed no evidence of association with liver steatosis in women or men. ALT value was weakly associated with liver steatosis in men with OR 1.03. Logistic regression analysis test results are listed in Table 5.

Table 5. Logistic regression analysis test results for the associations between ALT and CRP in 2007 and fatty liver status in 2011[1].

	Women				Men			
	Odds Ratio	Lower 95 % Confidence Limit	Upper 95 % Confidence Limit	P-value[2]	Odds Ratio	Lower 95 % Confidence Limit	Upper 95 % Confidence Limit	P-value[2]
ALT	1.02	1.00	1.05	0.06	1.03	1.02	1.04	**<.0001**
CRP	0.98	0.90	1.06	0.56	0.98	0.95	1.02	0.38

[1]Statistically significant values are indicated by bolded text.
[2]P- value < 0.05 was presented as statistically significant.

5 DISCUSSION

A total of 2130 YFS participants were measured for 48 cytokine, chemokine, and growth factor concentrations and ALT and CRP values. 2042 of these individuals were examined for liver steatosis 4 years later using liver ultrasound imaging. One of the aims of this thesis was to determine whether some of the measured cytokines predicted the progression of liver steatosis 4 years later.

Analyzed using Spearman's correlation test, in women cytokines IL-18 and IP-10, allergy marker IL-5 and HGF were found to have positive correlation to the occurrence of liver steatosis. In men positive correlation between IL-18, HGF, inflammatory chemokine MIP-1β and later hepatic steatosis was found. Inflammatory cytokine CTACK had weak negative correlation to fatty liver in both women and men. The results of correlation analysis, although interesting, did not take confounding factors into account. Using logistic regression analysis with several confounding factors considered, statistically significant association was found between IL-18, HGF and later hepatic steatosis, but only in women.

ALT and CRP values were examined as potential markers for liver inflammation and steatosis. Both ALT and CRP values had statistically significant correlation with liver steatosis and the correlation was stronger in men. In logistic regression analysis CRP values showed no evidence of association with later liver steatosis in neither men nor women. ALT values were weakly associated with liver steatosis in men.

In the study population both cytokines statistically significant to later liver steatosis, IL-18 and HGF, were found to predispose an individual to liver steatosis progression, cytokines protecting against fatty liver were not found. Interestingly, even though fatty liver is more common in men, statistically significant findings in association between cytokine concentrations and liver steatosis only occurred in women. ALT however was found to weakly predict hepatic steatosis in men.

Interleukin-18 is a proinflammatory cytokine involved in differentiation and activation of several T-cell populations. For instance, IL-18 induces IFNγ production by CD4 T cells which in turn causes macrophages to release inflammatory cytokines (Nakanishi et al. 2001). IL-18 also functions as a direct macrophage activator and acts as a part of Th1 paradigm together with IL-12 (Munder et al. 1998, Nakanishi et al. 2001). In rodent models Fas-ligand mediated IL-18 production from macrophages resulted in acute liver injury and interleukin-18 -gene deficiency in rodents resulted in dyslipidemia and NAFLD (Tsutsui et al. 1999, Yamanishi et al. 2016). A more recent study by Sim et al. (2021) demonstrated that in HIV monoinfection patients IL-18 was associated with elevated liver enzymes and hepatic steatosis. There seems to be a lack of research results on the connection between interleukin-18 and liver steatosis in healthy individuals.

Hepatocyte growth factor HGF is an antifibrotic and antiapoptotic hepatic factor and an adipokine that has many functions such as acute and chronic liver regeneration and embryonic development

(Rahimi et al. 1994, Balaban et al. 2006). Study findings in this YFS population and studies in rodent models were found to be contradictory. No previous findings about the association of HGF and hepatic steatosis in humans exist. In rodents elevated HGF expression significantly alleviated high-fat diet-induced and alcohol-induced fatty liver (Tahara et al. 1999, Kosone et al. 2007, Jing et al. 2019, Du et al. 2023). In addition, HGF has an antioxidant effect in high-cholesterol-fed mice hepatocytes, reducing reactive oxygen species (Domínguez-Pérez et al. 2016).

5.1 Study strengths and limitations

One of the strengths of the YFS in the setting of this thesis is a large population-based study group invited randomly from the national registry from five different regions in Finland. In total 48 different cytokine, chemokine, and growth factor markers could be assessed, and the data also included several confounding factors. Limitations in this study include a relatively short follow-up time of four years between the cytokine measurements and liver ultrasound assessment, homogenous ethnic group, and loss of participants during follow-up. Participants continuing in the study were more often female and older in age. Other limitations are the visual determination of the presence of hepatic steatosis, although conducted by a trained ultrasonographer, and the low sensitivity of ultrasound imaging (AlShaalan et al. 2015).

6 CONCLUSIONS

The results indicate that higher interleukin-18 and hepatocyte growth factor serum levels predict fatty liver in women. Cytokine markers for fatty liver found in previous studies have not included these two cytokines. Hepatocyte growth factor has been earlier studied only in rodent models of fatty liver and the association has been protective against liver steatosis, making this an exciting new finding. Further research is needed to determine if either of these cytokines could have clinical relevance in prediction of fatty liver in the future.

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SUPPLEMENTS

Supplemental table 1. Cytokine abbreviations.

IL-1β	Interleukin-1-beta
IL-1Ra	Interleukin-1 receptor antagonist
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-7	Interleukin-7
IL-8	Interleukin-8 (CXCL8)
IL-9	Interleukin-9
IL-10	Interleukin-10
IL-12p70	Interleukin-12p70
IL-13	Interleukin-13
IL-17	Interleukin-17
Eotaxin	Eotaxin (CCL11)
bFGF	Basic fibroblast growth factor
G-CSF	Granulocyte colony-stimulating factor
IFN-γ	Interferon-gamma
IP-10	Interferon gamma-induced protein 10 (CXCL10)
MCP-1	Monocyte chemotactic protein-1 (CCL2)
MIP-1α	Macrophage inflammatory protein-1α (CCL3)
PDGF-BB	Platelet derived growth factor BB
MIP-1β	Macrophage inflammatory protein-1β (CCL4)
TNF-α	Tumor necrosis factor-alpha

VEGF	Vascular endothelial growth factor
IL-2Rα	Interleukin-2 receptor, alpha subunit
IL-16	Interleukin-16
IL-18	Interleukin-18
CTACK	Cutaneous T-cell attracting (CCL27)
GRO-α	Growth regulated oncogene-α (CXCL1)
HGF	Hepatocyte growth factor
MIF	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)
MIG	Monokine induced by interferon-gamma (CXCL9)
β-NGF	Beta nerve growth factor
SCF	Stem cell factor
SCGF-β	Stem cell growth factor beta
SDF-1α	Stromal cell-derived factor-1 alpha (CXCL12)
TRAIL	TNF-related apoptosis inducing ligand

Supplemental table 2. Spearman's correlation test results for the associations between cytokines and fatty liver status[1].

	Correlation Coefficient	P-value[2]	Number of Observations	Correlation Coefficient	P-value[2]	Number of Observations
Proinflammatory Cytokines						
IL-1β	0.101	0.0015	979	-0.026	0.4672	796
IL-2	0.059	0.0644	979	0.032	0.3680	796
IL-6	0.072	0.0248	979	0.001	0.9739	796
IL-8	0.096	0.0027	979	0.055	0.1227	797
IL-12p70	0.070	0.0276	979	0.096	0.0069	797
IL-16	0.036	0.2813	887	0.008	0.8220	738
IL-17	0.023	0.4809	979	0.007	0.8416	797
IL-18	**0.179**	**<.0001**	979	**0.159**	**<.0001**	797
MIF	0.036	0.2548	979	0.089	0.0116	796
IFN-γ	0.081	0.0109	979	0.005	0.8830	797
IP-10	**0.117**	**0.0002**	979	0.086	0.0152	797
TNF-α	0.067	0.0367	979	0.061	0.0873	797
Allergy markers						
Eotaxin	0.059	0.0642	978	-0.026	0.4707	795

IL-4	0.060	0.0590	979	0.007	0.8479	797	
IL-5	**0.105**	**0.0010**	979	0.038	0.2852	796	
IL-9	0.098	0.0021	978	0.070	0.0487	796	
IL-13	0.093	0.0034	979	0.106	0.0028	797	
Apoptosis factors							
TRAIL	0.097	0.0025	976	0.081	0.0224	795	
Anti-inflammatory cytokines							
IL-10	0.100	0.0018	978	0.078	0.0278	796	
IL-1Ra	0.094	0.0032	979	0.087	0.0138	797	
IL-2Rα	0.054	0.0898	976	0.064	0.0727	795	
Growth factors							
bFGF	0.082	0.0104	979	0.035	0.3290	796	
PDGF-BB	0.051	0.1141	979	0.033	0.3506	797	
VEGF	0.074	0.0206	979	0.098	0.0054	797	
HGF	**0.185**	**<.0001**	979	**0.171**	**<.0001**	797	
SCF	0.054	0.0884	979	0.019	0.5848	795	
SCGF-β	-0.0005	0.9885	978	0.004	0.9080	796	
β-NGF	0.080	0.0133	959	0.076	0.0347	765	
G-CSF	0.076	0.0172	979	0.046	0.1967	795	
IL-7	0.097	0.0024	979	0.056	0.1165	797	
Inflammatory chemokines							
MCP-1	0.098	0.0021	979	0.014	0.6958	797	
MIP-1α	0.093	0.0038	978	0.023	0.5128	797	
MIP-1β	0.058	0.0698	979	**0.116**	**0.0011**	796	
GRO-α	0.040	0.2077	973	0.057	0.1121	786	
MIG	0.077	0.0160	979	0.043	0.2235	797	
SDF-1α	-0.039	0.2450	899	-0.059	0.1186	701	
CTACK	**-0.127**	**<.0001**	979	**-0.155**	**<.0001**	797	

[1]Statistically significant values are indicated by bolded text.
[2]Statistically significant P-value limit was determined by Bonferroni-correction: 0.05/37 (number of cytokines) = 0.00135.

Supplemental table 3. Logistic regression analysis test results for the associations between cytokines and fatty liver status[1,2].

Cytokine	Women				Men			
	Odds Ratio [3]	Lower 95 % Confidence Limit	Upper 95 % Confidence Limit	P-value	Odds ratio [3]	Lower 95 % Confidence Limit	Upper 95 % Confidence Limit	P-value
Proinflammatory Cytokines								
IL-1β	1.76	0.84	3.71	0.14	0.88	0.47	1.63	0.67
IL-2	1.38	0.77	2.46	0.27	1.06	0.68	1.65	0.80

IL-6	1.03	0.54	1.96	0.93	0.68	0.39	1.20	0.18
IL-8	2.02	0.79	5.14	0.14	1.20	0.51	2.85	0.68
IL-12p70	1.17	0.77	1.80	0.46	1.19	0.87	1.62	0.29
IL-16	1.16	0.83	1.62	0.39	0.98	0.74	1.30	0.88
IL-17	0.90	0.41	2.02	0.81	0.85	0.47	1.53	0.58
IL-18	**3.00**	**1.70**	**5.29**	0.0001	1.20	0.75	1.92	0.44
MIF	1.40	0.94	2.09	0.10	1.24	0.90	1.70	0.19
IFN-γ	1.29	0.56	2.97	0.55	0.86	0.46	1.61	0.65
IP-10	1.32	0.80	2.17	0.27	1.10	0.77	1.58	0.59
TNF-α	1.17	0.67	2.05	0.59	1.01	0.64	1.59	0.96
Allergy markers								
Eotaxin	1.39	0.84	2.31	0.20	1.21	0.82	1.77	0.34
IL-4	1.63	0.42	6.27	0.48	0.94	0.37	2.38	0.90
IL-5	1.42	0.64	3.15	0.39	0.98	0.56	1.69	0.93
IL-9	0.97	0.75	1.26	0.81	1.08	0.80	1.48	0.61
IL-13	1.23	0.69	2.17	0.48	1.16	0.81	1.68	0.41
Apoptosis factors								
TRAIL	1.22	0.72	2.05	0.46	0.83	0.56	1.24	0.36
Anti-inflammatory cytokines								
IL-10	1.23	0.81	1.86	0.33	1.10	0.82	1.49	0.52
IL-1Ra	1.23	0.76	1.98	0.40	0.97	0.67	1.41	0.88
IL-2Rα	0.93	0.62	1.39	0.71	1.29	0.88	1.91	0.20
Growth factors								
bFGF	1.61	0.75	3.42	0.22	1.29	0.70	2.39	0.42
PDGF-BB	0.81	0.41	1.58	0.54	0.95	0.59	1.52	0.83
VEGF	1.20	0.77	1.89	0.42	1.22	0.86	1.73	0.26
HGF	**2.36**	**1.22**	**4.57**	0.01	1.28	0.75	2.18	0.37
SCF	0.91	0.45	1.84	0.79	1.33	0.73	2.39	0.35
SCGF-β	0.86	0.47	1.55	0.61	0.99	0.61	1.59	0.96
β-NGF	1.29	0.75	2.21	0.36	1.00	0.69	1.45	0.99
G-CSF	1.30	0.53	3.18	0.56	1.62	0.80	3.31	0.18
IL-7	1.16	0.63	2.12	0.64	0.94	0.59	1.50	0.80
Inflammatory chemokines								
MCP-1	1.58	0.83	3.03	0.17	0.93	0.55	1.56	0.77
MIP-1α	1.81	0.75	4.36	0.19	1.16	0.54	2.48	0.70
MIP-1β	1.08	0.52	2.26	0.84	0.94	0.50	1.76	0.84
GRO-α	1.03	0.65	1.64	0.90	1.48	0.98	2.22	0.06
MIG	1.11	0.71	1.74	0.64	0.99	0.70	1.41	0.96
SDF-1α	0.73	0.43	1.24	0.25	0.79	0.53	1.18	0.25
CTACK	0.57	0.24	1.37	0.21	0.80	0.42	1.53	0.51

[1]Statistically significant values are indicated by bolded text.

[2]Age, BMI, smoking status, ALT, LDL cholesterol, total cholesterol were considered as confounding factors in the analysis.
[3]Odds ratio values are determined for logarithmically transformed variables.