Early Atherosclerotic Markers in Children with Familial Hypercholesterolemia

Master Thesis by

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Ingunn Narverud

Summary

Patients with familial hypercholesterolemia (FH) have an increased risk of premature atherosclerosis and coronary artery disease (CAD). Recently, inflammation has been suggested to play a major role in the development of atherosclerosis, and indeed children with FH have previously been suggested to have increased levels of selective inflammation markers. The aim of this study was to increase the knowledge about inflammation in the early steps of atherosclerosis.

Sixty-two FH children (aged 7-20 years) and twenty-two sex- and age-matched control children were included. Quantification of the circulating proteins soluble (s)E-selectin, vascular cell adhesion molecule-1 (sVCAM-1), intercellular adhesion molecule-1 (sICAM-1), adiponectin and leptin was performed in serum samples from each subject. Furthermore, gene expression levels of tumor necrosis factor α (TNFα), ICAM-1 and leptin receptor in peripheral blood mononuclear cells (PBMCs) were determined by quantitative reverse transcription polymerase chain reaction (Q-RT-PCR).

The main results showed: i) FH children have increased TNFα gene expression levels and a tendency to increased sE-selectin levels compared to control children; ii) FH boys have enhanced sE-selectin and sVCAM-1 levels compared to FH girls; iii) FH boys above 15 years have increased levels of sE-selectin compared to age-matched FH girls; iv) FH boys below 15 years have enhanced levels of sVCAM-1 compared to FH girls in the same age group; v) FH girls have enhanced leptin levels compared to FH boys; vi) there was a tendency to enhanced leptin levels, whereas adiponectin levels were decreased in FH children above 15 years compared to FH children below 15 years.

In conclusion, our results may support the notion of increased inflammation in FH children. Furthermore, the results may also indicate that the gender difference in the levels of early atherosclerotic markers may be established already in childhood and may thus partly explain the gender difference in the risk of CVD.

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List of Abbreviations

AHA	American Heart Association
Apo	Apolipoprotein
BSA	Bovine serum albumin
CAD	Coronary artery disease
cDNA	Complementary deoxyribonucleic acid
CPT	Cell preparation tube
CVD	Cardiovascular disease
DEPC	Diethylpyrocarbonate
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ELISA	Enzyme-linked immunosorbent assay
FAI	Free androgen index
FAM	6-carboxyfluorescein
FH	Familial hypercholesterolemia
gDNA	Genomic deoxyribonucleic acid
GUSB	β-glucuronidase
HDL-C	High density lipoprotein-cholesterol
HMGCoA reductase	Hydroxymethylglutaryl coenzyme A reductase
HRP	Horseradish-peroxidase
hs-CRP	High sensitive-C-reactive protein
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
IDL	Intermediate density lipoprotein
IL-1β	Interleukin-1β
IMT	Intima-media thickness
IFNγ	Interferon γ
LDL-C	Low density lipoprotein cholesterol
LDL-R	Low density lipoprotein receptor
MI	Myocardial infarction
Min	Minute
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NCEP	National Cholesterol Education Program
NF-κB	Nuclear factor κ B
NO	Nitric oxide
OD	Optical density
oxLDL	Oxidised low density lipoprotein

PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PDAY	Pathobiological Determinants of Atherosclerosis in Youth
Q-RT-PCR	Quantitative reverse transcription polymerase chain reaction
RANTES	Regulated on activation normally T-cell expressed and secreted
RCTs	Randomized double-blind placebo-controlled trials
RIN	Ribonucleic acid integrity number
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
ROS	Reactive oxygen species
ROX	6-Carboxyl-X-Rhodamine
RT	Reverse transcriptase
rt	Room temperature
sE-selectin	soluble E-selectin
SHBG	Sex hormone binding globulin
sICAM-1	soluble intercellular adhesion molecule-1
SMC	smooth muscle cells
sVCAM-1	soluble vascular adhesion molecule-1
TBP	TATA box binding protein
TNFα	Tumor necrosis factor α
UNG	Uracil-N-glucosylase
VCAM-1	Vascular cell adhesion molecule-1
VLDL-C	Very low density lipoprotein-cholesterol

1. Introduction

1.1 Familial Hypercholesterolemia

Late in the 19th century, the Norwegian pathologist Francis Harbitz described the first cases of xanthomatosis (1). The pioneering discovery was followed by several observations of xanthomatosis associated with hypercholesterolemia and cardiovascular disease (CVD) led by the physician Carl Müller. He subsequently claimed that hypercholesterolemia was a strong predictor in the development of CVD (1). Although the mechanism behind the disease was unknown, Müller regarded the disease to be an inherited metabolic disorder, and called it the Müller-Harbitz disease. These observations and statements were the first steps in the discovery of the autosomal dominant disease known today as Familial Hypercholesterolemia (FH). The disease is characterised by an elevated level of both total cholesterol and low density lipoprotein cholesterol (LDL-C) which give rise to xanthomatosis, deposits of cholesterol in peripheral tissues and accelerated atherosclerosis thereby increasing the risk of premature coronary artery disease (CAD) (2).

In 1985 Brown and Goldstein were awarded the Nobel price for their discovery of the low density lipoprotein receptor (LDL-R) in which gene mutations are the pathogenic cause for FH (3). Its function is crucial in regulating the cholesterol homeostasis and thus mutations in the gene will result in enhanced cholesterol levels seen in FH patients.

1.1.1 Genetics and Prevalence

The high affinity LDL-R binds apolipoprotein (apo) E- and apoB-containing lipoprotein particles, including very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), LDL and a subclass of high density lipoprotein (HDL), and is mainly expressed on liver cells (3). Hence, the LDL particle is taken up by the cells through a receptor-mediated endocytosis (Figure 1). This pathway enables the LDL-C to be released in the cell and the LDL-R to recycle multiple times. The gene encoding the LDL-R is located on chromosome 19 and consists of 18 exons separated by 17 introns.

FH is caused by mutations in the LDL-R gene (3). Worldwide, more than 800 different mutations in the LDL-R gene have been found (4). The majority of these results in one of five phenotypes of receptors: 1) none-detectable, 2) transport defective, 3) ligand defective, 4) internalization defective and 5) recycling defective (2). Approximately 1 of 500 people is estimated to be affected by heterozygous FH, which is calculated to approximately 10 million people worldwide (5). In certain populations like the Icelandic, the French Canadians and the Norwegians, an increased prevalence is estimated. The estimated prevalence is set to approximately 1/300 in Norway (6), and FH is thus affecting approximately 16000 people which makes it a common inherited disease in Norway. Homozygous FH patients are rare (1/1000000) (2), and herein the term FH will further refer to heterozygous FH patients.

In Norway, approximately 130 different mutations have been identified in the LDL-R gene among FH patients (6). However, three founder mutations account for approximately 40 % of mutations found in the FH patients in the Norwegian population. These are FH Elverum, FH Svartor and FH C210G, accounting for 25%, 8% and 8%, respectively (7). Both FH Elverum and Svartor predict none-detectable LDL-R, while FH C210G results in a ligand defective receptor. The increased prevalence in Norway can hence be explained by a founder effect. This founder effect is reflected in a population established of a few original founders carrying the

mutations mentioned above (5), due to the Norwegian geography, landscape and climate (8).

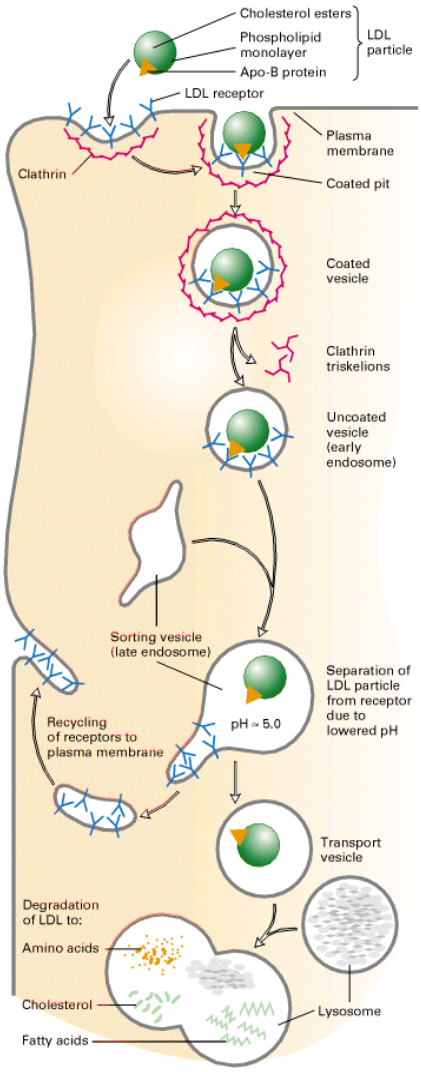


Figure 1. Fate of an LDL particle and its receptor after endocytosis. After an LDL particle binds to an LDL receptor on the plasma membrane, the receptor-ligand complex is internalized in a clathrin-coated pit that pinches off to become a coated vesicle. The clathrin coat then depolymerizes to triskelions, resulting in an early endosome. This endosome fuses with a sorting vesicle, known as a late endosome, where the low pH (≈5) causes the LDL particles to dissociate from the LDL receptors. A receptor-rich region buds off to form a separate vesicle that recycles the LDL receptors back to the plasma membrane. A vesicle containing an LDL particle may fuse with another late endosome but ultimately fuses with a lysosome to form a larger lysosome. There, the apolipoprotein B of the LDL particle is degraded to amino acids and the cholesterol esters are hydrolyzed to fatty acids and cholesterol. Abundant imported cholesterol inhibits synthesis by the cell of both cholesterol and LDL receptor protein. LDL= low density lipoprotein. (9)

1.1.2 Characteristics and Diagnosis of FH

Because of the reduced amount of or non-functional LDL-R in FH patients, clearance of LDL-C is impaired and results in two- to three-fold elevated plasma levels of LDL-C (3;10). The elevated LDL-C levels may in turn result in cholesterol accumulations in extravascular tissues forming premature atherosclerosis as well as the typical characteristics of FH: Achilles tendon xanthomas and corneal arcus (11).

Previously, FH patients were diagnosed clinically by observations of inherited hypercholesterolemia and xanthomatosis (12). Today, FH patients are primarily diagnosed by an identification of a mutation in the LDL-R gene using a deoxyribonucleic acid (DNA) test. The most cost-effective approach to diagnose FH is screening family members of an already diagnosed patient (12;13), and the DNA test is the most specific method doing this (12). Detection of inherited hypercholesterolemia is still a diagnostic criterion in families without an identified mutation by DNA-test. Children and young adults from these families can be diagnosed by a total and LDL cholesterol level above 5.5 mmol/l and 3.5 mmol/l, respectively (6).

In Norway, approximately 3900 patients from approximately 1000 different families have a diagnosis verified by a DNA test (6).

1.1.3 Treatment

The risk of premature onset of atherosclerosis in patients with FH require an early initiation of lifelong cholesterol reducing therapy among patients with this disorder (14). Treatment goal for adult FH patients, according to the European Atherosclerosis Society[1], is level of total cholesterol below 4.5 mmol/l and LDL-C below 2.5 mmol/l (15), which may be implemented by a treatment combined of drug and diet. Children

[1] Adult FH patients are considered as high risk subjects.

with FH are treated towards a target LDL-C below 3.35 mmol/l (minimal) or 2.85 mmol/l (ideal) (16).

Pharmacological Treatment

Statins or Hydroxymethylglutaryl Coenzyme A (HMG CoA) reductase inhibitors is the most preferred drug used in adult FH patients (14). They act by inhibiting the enzyme, HMG CoA reductase, which catalyzes the rate limiting step of cholesterol biosynthesis (17). As an effect the intracellular concentration of cholesterol is lowered and to compensate the cell's need for cholesterol, expression of LDL-R is up-regulated. The cholesterol-reducing effect of statins is about 25-45% depending on the dosage and drug (17;18). A new approach to reduce LDL-C is combining statins with ezetimibe, a selective cholesterol inhibiting drug which reduces the absorption of dietary and biliary cholesterol by preventing its transport through the intestinal wall (5). In co-administration with a statin, ezetimibe may result in an 18% incremental decrease in LDL-C. LDL-apheresis is mostly used in the LDL-C lowering therapy of homozygous FH patients, but is also used in heterozygous FH patients not responding to conventional drug therapy.

Until recently, optimal treatment in children with FH have been resins and consumption of a diet low in saturated fat and cholesterol (19). Resins act by binding bile salts in the intestine, a major pool of cholesterol, preventing their reabsorption and promoting their excretion without being absorbed in the intestine (16). As a result, an increased number of LDL-R is expressed on the liver cell surface and the clearance of LDL-C from the circulation is increased. However, their reducing effect of total and LDL cholesterol is modest (13% to 20%), and the compliance is poor due to their gastrointestinal side effects and poor palatability. Adverse effects as increased triglycerides levels and interaction with the absorption of some medications and fat-soluble vitamins may occur.

Numerous studies have shown that short-term use of statins in FH children is safely and efficient (20-23). Statin treatment in FH children (aged 8 to 18 years) have been evaluated in a meta-analysis of six randomized, double-blind, placebo-controlled

trials (RCTs) (24). Together the RCTs, gave a comparison of 798 children with a treatment duration ranging from 12 to 104 weeks. The results showed significantly reduced levels of total cholesterol, LDL-C and apoB, whereas HDL-C and apoA1 were significantly increased after statin treatment. Moreover, comparing the statin-treated children with the placebo-treated children, no significant differences in the occurrence of adverse events, sexual development, muscle toxicity or liver toxicity were observed. These results support the notion that statin treatment of FH children is efficient and safe. Another finding which emphasise statin treatment in children, is the observation of improved endothelial function in FH children after using statins in a short period compared to FH children receiving placebo (22).

American Heart Association (AHA) has announced a scientific statement on drug therapy of high-risk lipid abnormalities in children and adolescents (16). Current modifications of the National Cholesterol Education Program (NCEP) guidelines, concerning FH children, include:

- For children meeting criteria for starting lipid-lowering drug therapy[2], a statin is recommended as first line treatment
- For children with high risk lipid abnormalities, the presence of additional risk factors or high-risk conditions may also lower the recommended cut-point LDL-C level for initiation of drug therapy, lower the desired target LDL-C levels, and in selected cases, may prompt considerations for initiation below the age of 10 years. These risk factors and high-risk conditions are listed in Table 1.

[2] Drug therapy are considered after 6- to 12-months trial of fat- and cholesterol-restricted dietary management by these conditions: 1) LDL-C remains above 4.9 mmol/l or 2) LDL-C remains above 4.1 mmol/l, and there is a positive family history of premature CVD and presence of 2 or more risk factors in the child or adolescent after vigorous attempt to control these risk factors (16).

Table 1. Additional risk factors or high-risk conditions for children with high-risk lipid abnormalities.

Male gender
Strong family history of premature cardiovascular disease or events
Presence of associated low HDL-C, high triglycerides, small dense LDL particles
Presence of overweight or obesity and aspects of the metabolic syndrome[3]
Presence of other medical conditions associated with an increased atherosclerotic risk e.g diabetes
Presence of hypertension
Current smoking and passive smoking exposure
Presence of novel and emerging risk factors and markers e.g. elevated lipoprotein(a), homocystein, C-reactive protein

Adapted from McCrindle et al (16).

Lifestyle Recommendations

Together with drug therapy, all FH patients are advised to follow certain lifestyle recommendations, in children under the age of statin treatment this is the first line of treatment to lower the cholesterol level (5). Lifelong diet recommendations include low intake of cholesterol (200 mg/day) and fat, especially saturated fat, which should not account for more than 30% and 10% of the total calories per day, respectively. However, this diet should not begin before the age of two because of growth and neurological development in young children. According to AHA Dietary Guidelines, a healthy fatty acid composition rich in unsaturated fatty acids have beneficial effects on HDL-C, LDL-C and triglycerides and hence should substitute saturated fat in the diet (25). In addition, a Mediterranean diet rich in vegetables, fruits, legumes, whole grains, fish and vegetable oils is reported to have beneficial effects on a dyslipidemic

[3] Children meeting three of these five criteria are defined to have metabolic syndrome: 1) Body mass index (BMI) above the 97th percentile; 2) Triglycerides above the 95th percentile; 3) HDL-C below the 5th percentile; 4) systolic or diastolic blood pressure above the 95th percentile; 5) Impaired glucose tolerance as defined by the American Diabetes Association.

profile and thus in the prevention of CVD (26-28). The beneficial effect of this diet is probably due to the synergy effect of dietary fibre, antioxidants and unsaturated fatty acids (29). Certain soluble fibres (oat products, pectin, psyllium and guar gum) acts by reducing LDL-C (25), and dietary antioxidants is hypothesized to reduce the oxidative stress (30) which creates oxidised LDL (oxLDL), both shown to be involved in the atherosclerosis process (31). Another documented cholesterol-reducing agent is plant sterols, which is found naturally in several vegetable oils and fats (25;32). Plant sterols can be isolated and used as fortification of margarines (32). Consumption of 2 to 3 g plant sterols per day has been shown to decrease total cholesterol and LDL-C levels by 9% to 20% (25). Omega-3-fatty acids derived from fish (eicosapentaenoic acid and docosahexaenoic acid [DHA]) or vegetable products (α-linoleic acid) have been shown to have a cardioprotective effect (25;33). In a cholesterol-reducing diet, at least 2 servings of fish per week, 5 servings of fruits and vegetables per day and >25 g fibres a day, included soluble fibres, are recommended (25).

Physical activity is highly associated with prevention of CVD because of its favourable effect on blood pressure, triglyceride levels, LDL-C and HDL-C (28). A general recommendation, concerning FH patients, is engaging in at least 30 min moderate-intensity physical activity per day.

The numerous evidences for adverse effects of cigarette smoking on CVD have generated general recommendations against smoking (25).

1.1.4 None Modifiable Risk Factors for CAD

The gender difference in the risk of developing CAD is well documented (34;35). In pre-menopausal women the risk of developing CAD is significantly lower than in age-matched men, however this protection disappear after menopause, as the risk is similar to or even higher in women than in men (34). Several studies have been conducted to understand the mechanism behind this pre-menopausal protection, suggesting sex hormones playing a role (35;36). Oestrogens are shown to mediate several beneficial effects related to the pre-menopausal protection. First, endogenous

oestrogens are shown to enhance release of endothelial-derived nitric oxide (NO) which increases expression of reactive oxygen species (ROS) eliminating enzymes (34;35;37). In atherogenesis, ROS are pivotal, contributing to endothelial dysfunction and generating oxLDL, modifications involved in the development of atherosclerosis. Moreover, oestrogens have been shown to decrease expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase an enzyme generating ROS. Other reported effects of oestrogens are promoting vascular smooth muscle cell (SMC) relaxation, inhibiting vascular SMC proliferation/migration, suppressing vascular inflammation and exerting direct antioxidant effects in vascular cells (35). In addition to its effects on oxidative stress, oestrogens favourably affect circulating levels of lipoprotein. Thus, oestrogens increase HDL-C and apoA1 and decrease LDL-C and apoB levels, probably by enhancing the LDL-R activity (38), hence causing a beneficial lipid profile. As levels of oestrogens are up to sevenfold higher in pre-menopausal women compared to men and post-menopausal women, these sex hormones are hypothesised to play an important role in the pre-menopausal protection (35).

The risk factors age and gender are closely related in the development of CAD (39). This association is established already in puberty, where HDL-C in boys decreases markedly, while LDL-C firstly decreases and then rises in late puberty (40). In girls minor fluctuations in HDL-C and a markedly decrease in LDL-C is seen during puberty (40). These gender differences in lipid levels are not abolished until after menopause as total cholesterol and triglycerides has been reported to increase (36). Due to this sex divergence, men are exposed to an atherogenic lipid profile for many years longer compared to women (40). In addition, other risk factors for CAD as abdominal obesity and metabolic syndrome are more pronounced in men, which all together may result in an onset of CAD 10-15 years earlier in men compared to women (39). Thus, a major fraction of the CAD in men occurs in the middle-age, while most CAD cases in women occur after age 65 years.

1.2 Atherosclerosis

1.2.1 Mechanisms Behind Atherosclerosis

Atherosclerosis is a multi-step, progressive disease which may eventually result in blocking of blood supply to the tissues causing e.g. myocardial infarction (MI) or stroke (41). Inflammation plays a key role in this process and is present from the initiation, during the development of atherosclerotic plaques, and to the endpoint of the disease. Dysfunction of the endothelial cells in arteries is supposed to be the initial step, which can occur from several factors such as elevated and modified LDL-C (e.g. oxLDL); free radicals caused by cigarette smoking, hypertension and diabetes mellitus, and genetic mutations (31). Due to dysfunctional endothelium, enhanced levels of cell adhesion molecules are expressed on the surface and thereby adhere increasingly number of monocytes and T lymphocytes (31;41) (Figure 2). In addition, an attenuated permeability of the endothelium is established. Fatty streaks, the initial lesion of atherosclerosis, are developed by monocytes which migrate through the dysfunctional endothelium into the intima and mature to macrophages (41) (Figure 2). Within the intima, expression of scavenger receptors on the macrophage's surface enables the cells to engulf oxLDL and thus form foam cells by accumulating cholesterol esters, seen as fatty streaks in the artery. Sustaining and progression of the process is enabled by monocytes, T lymphocytes, SMC, endothelial cells, oxLDL and numerous chemokines and cytokines released from the involving cells, all together working in concert (31). Formation of an advanced lesion or an atherosclerotic plaque occurs as a necrotic core of leukocytes and lipids and a fibrous cap produced of SMC and collagen, evolve in the fatty streaks (31;41) (Figure 3). Finally, an inflammatory process involving T lymphocytes and several cytokines promote a physical disruption of the atherosclerotic plaque, and the thrombus formed is responsible for most of the acute cardiovascular events following atherosclerosis (41).

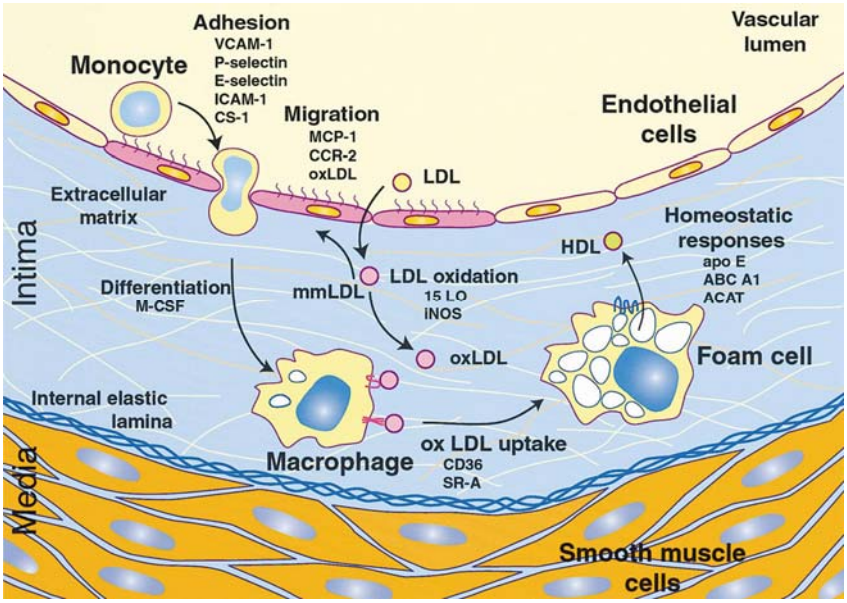


Figure 2. Initiating events in the development of a fatty streak lesion. 15-LO= 15 lipoxigenase, ABC-A1= adenosine triphosphate-binding cassette A1, ACAT= acyl coenzyme A cholesterol acyltransferase-1, ApoE= apolipoprotein E, CCR-1= chemokine receptor-1, CD36= cluster designation 36, CS-1= connecting segment 1, HDL= high density lipoprotein, ICAM= intercellular adhesion molecule, INOS= inducible nitric oxide synthase, LDL= low density lipoprotein, MCP-1= monocyte chemotactic protein 1, M-CSF= macrophage colony-stimulating factor, SR-A= scavenger receptor A, VCAM= vascular cell adhesion molecule. (42)

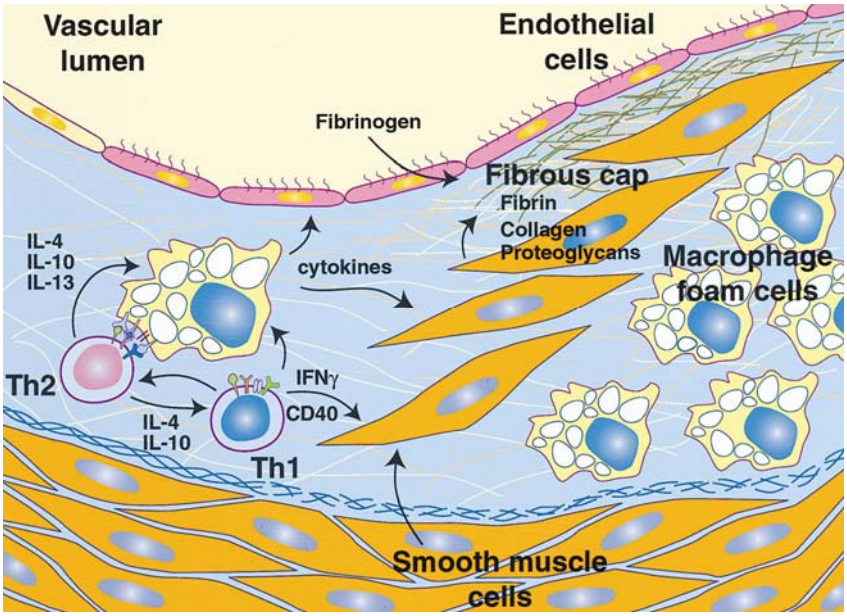


Figure 3. Lesion progression. IFNγ= interferon γ, IL= interleukin, Th= T helper cell. (42)

1.2.2 Adhesion Molecules

Adhesion molecules are key mediators responsible for the atherosclerotic initiation; the recruitment of the leukocytes from the circulation and their transendothelial migration, which is also one of the first signs of inflammation in the atherosclerotic process (43;44) (Figure 4). Selectins, immunoglobulin-like molecules and integrins are categorised into this group of molecules which are expressed on endothelial cells and on circulating leukocytes in response to inflammatory stimuli. As a protective mechanism to limit or stop inflammation, adhesion molecules is either cleaved by proteases or shed by the cells, resulting in soluble forms (43). Associations between certain adhesion molecules and CAD risk are emphasized in several studies (43-45).

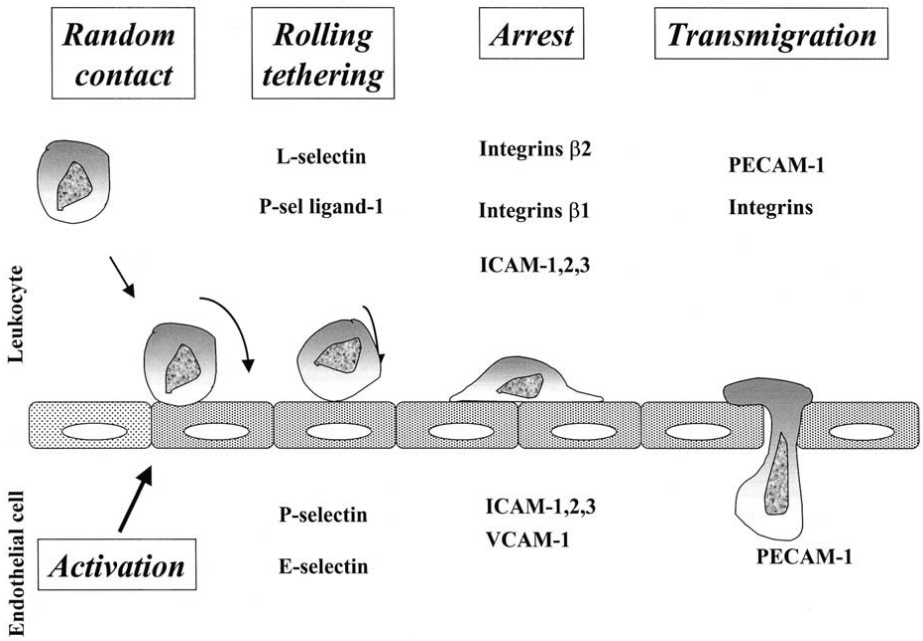


Figure 4. Schematic representation of the leukocyte-endothelial cell interaction during the initial steps of atherosclerosis, and the role of the different adhesion molecules in this process. ICAM= intercellular adhesion molecule, PECAM-1= platelet endothelial cellular adhesion molecule-1, P-sel ligand-1= P-selectin ligand-1, VCAM-1= vascular cell adhesion molecule-1. (43)

Selectins

This family of adhesion molecules include L-selectin, P-selectin and E-selectin, which are all involved in the early leukocyte recruitment by mediating rolling and tethering of the leukocytes to the endothelium (43). The selectins interact with their ligands and create weak bonds between activated endothelial cells and leukocytes. Amplification of the recruitment process is dependent of selectins by their interaction between platelets and leukocytes or platelets; or between leukocytes, and hence they are contributors to progress the atherosclerosis. L-selectin is constitutively expressed on leukocytes (T cells, B cells and natural killer cells), but the degree of expression is regulated upon activation of the cells. On the contrary, P-selectin is stored in resting cells and is mainly expressed on platelets upon activation. After activation P-selectin is expressed within minutes. E-selectin is in detail described beneath.

E-selectin

E-selectin is primarily expressed on the surface of activated endothelial cells in response to inflammatory cytokines (43). *In vitro* the cytokines tumor necrosis factor α (TNFα) and interleukin (IL)-1β have been shown to induce endothelial expression of E-selectin (46;47). However, E-selectin is not stored in the endothelial cells and its expression is dependent of cytokine-mediated nuclear factor-κB (NF- κB) induced gene transcription, a mechanism involving a cascade of molecules (47;48). This induction takes a few hours before E-selectin is expressed on the surface of endothelial cells. The disappearance rate of the adhesion molecule from the membrane, occurring as internalization or proteolytic cleavage/shedding to the extracellular space, is an additional factor important in the regulation of E-selectin (47). Soluble E-selectin (sE-selectin) is formed by this proteolytic cleavage/shedding.

Knock-out mice deficient in E-selectin have been shown to develop fewer arterial lesions than normal mice (49;50). Moreover, expression of E-selectin have been observed in atherosclerotic plaques (51;52). An observation indicate that sE-selectin may serve as a molecular marker for atherosclerosis and the development of CAD, since the CAD risk were observed to be two times greater in cases with elevated

plasma levels of sE-selectin (53). However, a meta-analysis revealed no significant, increased risk for CAD in cases with elevated sE-selectin (44). Thus, this finding confirms the need for further investigation of associations between sE-selectin and CAD risk.

Immunoglobulin-like Molecules

Intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are members of the immunoglobulin superfamily (54).

The basal expression level of ICAM-1 in endothelial cells and leukocytes, has been shown to increase *in vitro* at atherosclerotic-prone areas in the aorta in response to pro-inflammatory stimuli such as TNFα, oxLDL and LDL (43;45;55). By creating strong bonds to integrins on the surface of leukocytes, ICAM-1 has been shown to mediate several leukocyte processes; adhesion to activated endothelial cells, arresting on the vascular surface and endothelial transmigration (43;54) (Figure 4). A soluble type of ICAM-1 (sICAM-1) is formed by shedding (43).

Deficiency of ICAM-1 in knock-out mice have been suggested to protect against atherosclerosis (50;56). Reinforced evidence of associations between ICAM-1 and CAD risk have been emphasized as elevated expression of this molecule has been observed in atherosclerotic plaques (51;57), and has been suggested to be a predictor of cardiovascular disease among healthy individuals (54).

VCAM-1 is mainly expressed on endothelial cells, but also on macrophages and other inflammatory cells (43). Induction of VCAM-1 *in vitro* is similar to the induction of both E-selectin and ICAM-1; TNFα-, IL-1β- and oxLDL-induced up-regulation through NF-κB activation (45;47). The expression of VCAM-1 has been observed to be increased at atherosclerotic-prone areas of the endothelium. In interaction with integrin α4β1, VCAM-1 has been shown to induce signals in endothelial cells that trigger changes in shape and allow leukocyte transmigration, and adhere leukocytes to activated endothelium (43) (Figure 4). Soluble VCAM- 1 (sVCAM-1) is created by proteolytic cleavage (43).

Both sICAM-1 and sVCAM-1 are correlated to lipid levels (43), obesity and other CAD risks, which suggest that these factors influence the development of CAD (54). sVCAM-1 has been observed to be up-regulated at an advanced stage in atherosclerosis, suggesting the molecule to be a predictor of mortality among patients with existing CAD (54).

1.2.3 Cytokines

Another sign of atherosclerosis as an inflammatory disease, is the involvement of cytokines from the initiation to the endpoint of the process (55). Cytokines are pro- and anti-inflammatory mediators released from cells involved in inflammation, e.g. monocytes/macrophages, T cells and endothelial cells. Stimulation of cytokine release from these cells is carried out by oxLDL, free radicals, hemodynamic stress, hypertension or infectious organisms. They mediate cross-talk between the cells resulting in cell activation, differentiation, chemotaxis and proliferation.

TNFα

TNFα is a classical pro-inflammatory cytokine, mediating pro-atherogenic processes (55). *In vitro*, TNFα together with other pro-atherogenic cytokines, enhance the surface expression of adhesion molecules on endothelial cells, SMC or macrophages. T_1 lymphocytes mediate increased secretion of TNFα and IL-1β from activated macrophages, which is associated with progression of atherosclerosis. Together with other pro-inflammatory cytokines, e.g. IL-1α, β and interferon γ (IFNγ), TNFα is involved in several inflammatory and atherogenic processes, for instance; foam cell formation through e.g. enchanced expression of scavenger receptor on macrophages; chemokine release from endothelial cells, SMC and macrophages; activation and proliferation of monocytes; apoptosis (programmed cell death) and thrombus formation. In these processes TNFα acts by increasing the expression of other cytokines, chemokines and growth factors which in turn carry out the performance, therefore TNFα seems to be a central mediator in the atherosclerotic process.

Gene expression levels of TNFα have been shown to be significantly higher in adult FH patients compared to healthy controls, and were also positively correlated to plasma total and LDL cholesterol (58).

1.2.4 Other Inflammatory Markers

Adiponectin

Recently, the endocrine function of the adipose tissue has revealed the organ to be more than an energy depot (59;60). Adiponectin is a adipokine abundantly and exclusively expressed in adipose tissue, in addition the protein is abundant in the circulation (61;62). The gene expression of adiponectin is modulated by other cytokines secreted from the adipose tissue, such as TNFα (59). Adiponectin levels have been observed to be inversely associated with TNFα and C-reactive protein (CRP), a strong inflammation marker, in numerous human and mice studies (63). In obese children and adolescents decreased levels of adiponectin were also found to be associated with higher levels of hs-CRP, low levels of HDL-C and a high triglyceride-HDL-C ratio, the two latter are features of metabolic syndrome. These findings support a role of adiponectin in obesity, but also in the development of CVD (63) probably through their common denominator, inflammation (64).

Observations that adiponectin suppresses the attachment of monocytes to activated endothelial cells and stimulates NO production in vascular cells, which improves endothelial function, have suggested that adiponectin have anti-atherogenic properties (62). Serum adiponectin has been shown to be closely related to several factors important in the progression of CAD in dyslipidemic patients; positively associated with HDL-C and negatively associated with high-sensitive CRP (hs-CRP) (65). In adolescents and young adults with FH, serum levels of adiponectin have been shown to be significantly lower than in healthy controls (61), which may exacerbate the risk of premature CAD in adult FH patients (66). However, the association between adiponectin and the risk of CAD is still controversial (67).

Leptin and Leptin Receptor

The most abundant expression of leptin, a cytokine-like hormone, is in adipocytes (64). Both gene expression and circulating levels of leptin are stimulated by inflammatory cytokines such as IL-1β and TNFα (68). Six different leptin receptors are known, of which one is soluble and one is widely expressed.

Although leptin is primarily a regulator of the body's energy balance (64), its function also comprises immunomodulatory effects (68). Mechanisms connected to inflammation, involving leptin include e.g. T lymphocyte proliferation, pro-inflammatory cytokine secretion from T lymphocytes and promoting phagocytic function of macrophages. Recently, the hormone has been observed to have free radical generating ability, a feature important in the development of atheroslcerosis (69). Some types of leptin receptors are involved in the activation of NADPH oxidase, and leptin itself have been reported to induce superoxid anion more intensely in patients with hypercholesterolemia compared to healthy controls. This free radical generating ability is widely accepted to be involved in the foam cell formation through the modification of LDL-C forming oxLDL. Moreover, leptin has been shown to be able to increase endogenous cholesterol synthesis in human monocytes, an effect more pronounced in monocytes from hypercholesterolemic patients, which might participate in the progression of an advanced atherosclerotic lesion (69).

The effect of leptin and leptin receptor deficiency in atherosclerosis is still not clear. According to Wu et al. mice lacking both leptin receptor and apoE are more prone to develop larger lesions of atherosclerosis compared to apoE deficient mice with functional leptin receptor (70). Furthermore, Taleb et al have reported up to 6-fold reduction in atherosclerotic lesion development in both leptin and LDL-R deficient mice compared to LDL-R deficient mice with similar cholesterol level (64).

1.3 Atherosclerosis in Children and Young Adults

Initiation of atherosclerosis in young adults was described over 50 years ago (71). In this study coronary atherosclerosis was found in approximately 77% of the hearts dissected from 300 soldiers (average age 22 years) who were killed in war.

More recently, the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study, a multi-institutional study of atherosclerosis in 15 to 34-year-old black and white males and females who died of causes not related to CVD, has amplified the findings 50 years ago (72). This study revealed intimal lesions of atherosclerosis in all the aortas and in more than half of the right coronary arteries in the youngest age group (15-19 years). In the group aged 30-34 years, advanced lesions were more prevalent and in larger extent compared to the other age groups in both the aorta and the right coronary artery. Risk factors observed to be associated with the fatty streaks and the advanced lesions, included VLDL- and LDL-C. HDL-C however was inversely associated with the two stages of atherosclerosis. Of the trauma victims in the PDAY study, men and women with a favourable lipid profile participated in observations of non-lipid risk factors in atherosclerosis (73). Known atherosclerotic risk factors such as smoking, hypertension, obesity and impaired glucose intolerance were observed to be associated with more extensive lesions in persons exposed to these factors.

The relationship between hypercholesterolemia and premature CAD is well established (1-3;31). Children with FH have hence participated in studies to understand pathological mechanisms involved in the onset of atherosclerosis (10;74). Measurements of the intima-media thickness (IMT) in carotid arteries have been used to assess the development of atherosclerosis in children and young adults with FH (74;75), since IMT in these arteries has been shown to be associated with MI (74). The mean IMT was observed to be significantly greater in children with FH compared to age- and sex-matched control subjects (74;75). In 10% of the FH children carotid artery plaque was found (74). This was however not seen in the

control subjects. Factors related to mean IMT were; gender, LDL-C (74;75), age (75), apoB and fibrinogen (74).

Endothelial dysfunction is crucial in the initiation of atherosclerosis (31), as mentioned earlier, and has been reported as a predictor in the future risk of CVD (76). OxLDL have been suggested to mediate endothelial dysfunction (31), and indeed markers for oxLDL have been shown to be increased in children with FH compared to unaffected siblings (77). Furthermore, measurements in FH children have shown that endothelial function is impaired in comparison with matched healthy controls, and this dysfunction was even more pronounced in FH children with a positive family history of premature CVD (76). Strikingly, antioxidant therapy with vitamins C (500mg/d) and E (400 IU/d) for 6 weeks in addition to a fat- and cholesterol-restricted diet for 6 months have been shown to restore endothelial function in hyperlipidemic (included FH) children (78). Similar results have been reported after supplementation with DHA (79).

Lipid levels in FH children are in majority determined by the type of mutation causing FH, but other genetic and modifiable environmental factors have also been shown to contribute determining the levels, such as serum cholesterol levels in both the parents, percent body fat, pubertal stage, sugar intake and apoE genotype (80). In fact, a particular genotype, apoE4, has been associated with lower HDL-C levels in children with FH, and may thus exacerbate their risk for future CAD (81).

Recently, regulated on activation normally T-cell expressed and secreted (RANTES) and neopterin levels derived from monocytes were shown to be significantly higher among children with FH compared to control subjects (10). Adult FH patients did not show this elevated level of RANTES. These findings have been amplified by other results showing significantly enhanced serum levels of neopterin and hs-CRP in children with FH compared to healthy siblings (82). Together, the latter results indicate a role of inflammation also in the early stages of atherosclerosis (10;82), and suggest that a different inflammatory pattern in children compared to adults might exist (10).

In conclusion, FH children seem to have a different inflammation pattern. However, little is known about early atherosclerotic markers in these children.

2. Aims of the Study

Children with FH are of high interest to study in order to detect pathological mechanisms in the early stages of atherosclerosis.

Few studies have however been conducted to investigate inflammation and markers involved in the early atherosclerotic process. We hypothesise that FH children have increased levels of early atherosclerotic markers. Therefore, to increase the knowledge in this field, more specifically the aims of the following study were:

1. To compare age- and sex-matched children with and without FH with respect to:

    a. circulating levels of early atherosclerotic markers: sE-selectin, sVCAM-1 and sICAM-1

    b. gene expression levels of early atherosclerotic markers in circulating peripheral blood mononuclear cells (PBMCs): TNFα and ICAM-1

    c. circulating levels of adipokines (leptin and adiponectin) and gene expression of leptin receptor in circulating PBMCs

2. To investigate if there are differences in the same markers in subgroups of the FH population according to gender and age.

3. List of Materials

Chemicals	**Distributors**
2 N H_2SO_4 (stop solution)	Merck
BSA	PAA the cell culture company
DEPC-water	Sigma-Aldrich
Ethanol	Arcus Kjemi
PBS, sterile	Sigma-Aldrich
PBS tablets	Gibco
RNase away	Fluka
Substrate solution	Zymed Laboratories
TaqMan gene expression assay GUSB	Applied Biosystems
TaqMan gene expression assay ICAM-1	Applied Biosystems
TaqMan gene expression assay Leptin receptor	Applied Biosystems
TaqMan gene expression master mix	Applied Biosystems
TaqMan gene expression assay TBP	Applied Biosystems
TaqMan gene expression assay TNFα	Applied Biosystems
Testoterone	Sigma-Aldrich
TNFα	R&D Systems
Triton X-100	Sigma-Aldrich
TRIzol reagent	Invitrogen
Tumor necrosis factor alpha (TNFα)	R&D Systems
Tween	Sigma-Aldrich
Kits	
Human Adiponectin Duoset	R&D Systems
Human E-selectin Duoset	R&D Systems
Human ICAM-1 Duoset	R&D Systems
Human Leptin Duoset	R&D Systems
Human VCAM-1 Duoset	R&D Systems
RNA 6000 Nano Kit	Agilent
RNase-free DNase set	Qiagen
RNeasy mini kit	Qiagen
Superscript™ First-Strand Synthesis System for RT-PCR	Invitrogen
Software	
Genesis Version 3.05	Life Sciences Ltd.
GraphPad Prism 4	GraphPad Software Inc.
Microsoft Office	Microsoft Inc.
Reference Manager 11	ISI Research Soft

SDS 2.3	Applied Biosystems
SPSS for Windows	SPSS Inc.

Equipment

24-wells cell culture plates	Corning Inc.
96-wells microplate	Costar
Cell preparation tubes (CPT)	BD
Cell scraper	Costar
MicroAmpTH Optical 96-well Reaction Plate with barcode	Applied Biosystems
Pipette boy	IBS Integra Biosciences
Pipettes	Thermo Scientific
Pipette tips	Thermo Scientific

Instruments

ABI 7900 HT	Applied Biosystems
Agilent 2100 Bioanalyzer	Agilent
Biofuge, Primo R	Heraeus
Multiskan Ex	Thermo Electro Corporation
Nanodrop ND-1000	Thermo Scientific
Wellwash 4 MK2	Thermo Electro Corporation

4. Subjects and Methods

4.1 Subjects

Sixty-two children 7-20 years of age with heterozygous FH were recruited consecutive from the nationwide competence centre for children with FH at the Lipid Clinic, Rikshospitalet University Hospital, Oslo, Norway. All the children were diagnosed with definite FH by DNA test, but were clinically healthy without any diagnosis of CAD, and none were on current statin treatment. Twenty-two healthy, age- and sex-matched children without hypercholesterolemia, recruited among friends of the FH children or children of the staff at the hospital were asked to take part in the study as a control group. Written informed consent was obtained from all of the participants ≥18 years or from 1 parent when the child was <18 years. The study was approved by the Regional Committee of Medical Ethics.

4.2 Blood Samples

Venous blood samples were collected from all the participants of the study from year 2003-2007 by Holven et al (10). Serum samples were immersed in melting ice and allowed to clot for 1 hour before centrifugation (1000 g, 10 minute [min], room temperature [rt]) (83). The samples were divided into multiple aliquots and stored at -80ºC. Limited blood samples from each individual made it impossible to perform all the analyses from all of the children. PBMCs were available in 34 of the FH subjects and 12 of the control subjects. Baseline parameters such as total cholesterol, LDL-C, HDL-C, apoB and apoA1 were measured by the Department of Medical Biochemistry, Rikshospitalet University Hospital, Oslo, Norway.

4.3 Isolation of PBMCs

Background

PBMCs include monocytes and lymphocytes, cells which are central in inflammation and hence in the atherosclerosis process. At the latter point of view, initial activation of these cells is carried out by endothelial dysfunction, enable them to increase the expression of and release of cytokines, chemokines and receptors for adhesion molecules (31). Thus, alterations in gene expression levels in these cells are demonstrable early in the process before signs of inflammation can be seen *in vivo*. Further activation retains the PBMCs participating during the whole atherogenesis and in concert with cytokines, adhesion molecules, chemokines and endothelial cells they finally form an atherosclerotic lesion (31). However, their high availability and the fact that they are exposed to many of the same environmental factors as the intima, where the atherogenesis takes place, make them suitable for studying gene expression levels of mediators involved in the development of CAD (84). Contrary to red blood cells, PBMCs have a nucleus which is crucial to synthesize ribonucleic acid (RNA).

Procedure

Cell preparation tubes (CPT) (Catalogue # 362761, BD) were used for the isolation of PBMCs to be used for the quantitative reverse transcription polymerase chain reaction (Q-RT-PCR), and the method used is based on the same principles as the method described by Schlenke et al (85).

CPT were centrifuged at 1600 g, rt for 20 min after approximately 45 min on the bench. Red blood cells and granulocytes penetrated through the gel-layer, while PBMCs formed a whitish layer just beneath the plasma layer on top, see Figure 5. The PBMCs were then transferred into a new tube and washed twice with phosphate buffered saline (PBS) (Catalogue # P3813, Sigma). After centrifugation with PBS (300 g, rt, 10 min), the pellet from the PBMCs was stored at -80 ºC before TRIzol treatment and isolation of RNA.

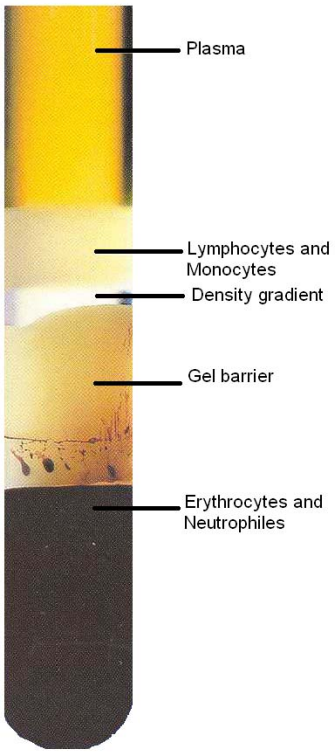


Figure 5. Cell preparation tube.

4.4 Isolation of RNA

Background

Isolation of RNA was performed through a combination of TRIzol Reagent (Catalogue # 15596-018, Invitrogen) and RNeasy mini kit (Cataloge # 74104, Qiagen). Use of the TRIzol Reagent is an improvement of the single-step RNA isolation method developed by Chomeczynski et al (86) which allows RNA to separate from DNA after extraction under acidic conditions using guanidinium thiocyanate, phenol and chloroform (87). The TRIzol Reagent (containing phenol and guanidine isothiocyanate) is a denaturant which lyses the cell, dissolves the cell components and maintains intact RNA by denaturing endogenous RNase (88). Addition of chloroform and following centrifugation performs two phases, an organic and an aqueous one. Extraction of RNA by phenol/chloroform leaves RNA exclusively in the aqueous phase. Ethanol will then precipitate RNA from the aqueous phase and leave proteins and disposal behind in the supernatant. The RNeasy

spin columns collect RNA and purification can be combined with RNase-free DNase set (Catalogue # 79254, Qiagen) (89) which digests genomic DNA (gDNA) in the sample and hence avoids spurious results in Q-RT-PCR.

Procedure

The method used is described by de Vries et al (90). In order to avoid degradation of RNA by RNAse, isolation hood and all equipment were cleaned with RNase away and subsequently diethylpyrocarbonate (DEPC) water (Catalogue # W4502, Sigma). The pellet from the PBMCs was resuspended in TRIzol reagent immediately after transferring from the freezer and incubated for 5 min. Chloroform was added and the sample was subsequently centrifuged (12000 g, 4ºC, 15 min). The aqueous phase was transferred into a new tube and added 70% ethanol (diluted in DEPC water and made fresh every time) (Catalogue # 60068, Arcus Kjemi). Further, the solution was transferred to an RNeasy column, washed with several buffers and purified with a DNase mix (10:80 DNase stock 1 solution and RDD buffer). Several washing steps with two different buffers were carried out, before RNA was eluted in RNase free water (30µl). Distribution of the sample into several tubes avoids several cycles of freezing and thawing which decreases degradation of RNA later in the process and was done as a final step. Finally, the samples were stored at -80ºC.

4.5 Quantification and Qualification of RNA

4.5.1 Quantification

Background

The Nanodrop ND-1000 spectrophotometer (Thermo Scientific) enables RNA quantification by sending a light through the sample and analyzing the intensity (91).

Procedure

Prior to quantification, the samples were thawed on ice and centrifuged briefly. The instrument requires 1 µl, but to ensure enough volume, 1.2 µl were added onto the end of the fibre optic cable (91).

4.5.2 Qualification

Background

RNA integrity is of high importance for application in Q-RT-PCR, as short fragments in a degraded RNA can cause unreliable results in the relative quantification (92;93). The Agilent 2100 Bioanalyzer (Agilent Technologies) in combination with the RNA 6000 Nano Kit (Part # 5067-1511, Agilent Technologies) enables a qualification of RNA by utilizing the capillary forces in an electrophoresis provided by the Lab on a Chip technique (Agilent Technologies) (94). Voltage-induced size separation of RNA subunits in gel-filled channels, is described in a curve of an electropherogram (Figure 6) (93). The curve shows peaks of RNA subunits, and two peaks are essential for achieving a high integrity: the 18S and 28S ribosomal RNA (rRNA) subunits (95). A well established algorithm of RNA integrity, the RNA integrity number (RIN) counting from 1 to 10 (best), is based on several features of RNA integrity. One is the total RNA ratio of areas under the 18S and the 28S peaks compared to the area under the whole curve (93).

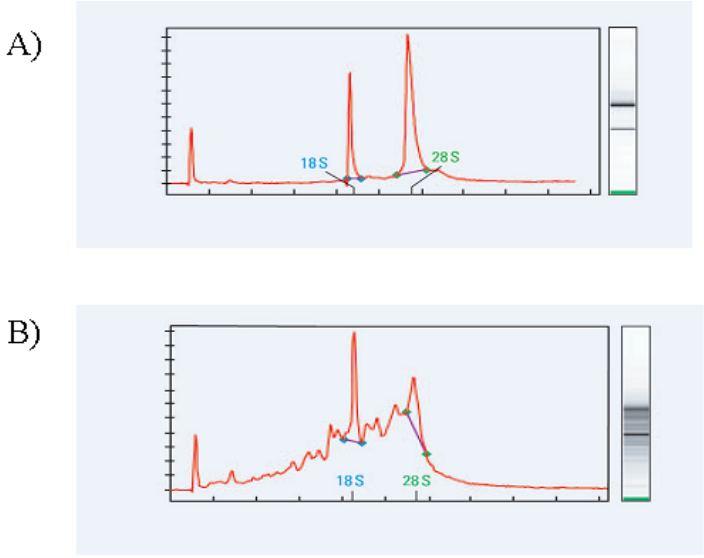


Figure 6. Electropherogram of intact total RNA from a sample with a RIN 9.2 (A) and of partially degraded total RNA from a sample with a RIN 5.8 (B). 18S= subunit of ribosomal ribonucleic acid, 28S= subunit of ribosomal ribonucleic acid, RNA = ribonucleic acid, RIN= ribonucleic acid integrity number. (95)

Procedure

The method used is described in the user's manual on Agilent's homepage (96). Agilent RNA 6000 Nano Kit (Part # 5067-1511, Agilent) was equilibrated to rt 30 min before use.

Gel matrix was centrifuged (1500 g, 10 min, rt) and subsequently 65µl were portioned into a new tube. Preparing the gel-dye mix involved mixing dye concentrate in the gel mix and centrifuge (13000 g, 10 min, rt). A RNA 6000 Nano chip were put in the chip priming station and loaded with the gel-dye mix in the well marked G in a circle. By means of air pressure the plunger distributed the gel-dye mix all over the chip. Another two amounts of gel-dye mix were loaded into the wells marked G. RNA 6000 Nano marker were added in all the 12 sample wells and the ladder well. Both samples and ladder were added into the chip, in one well each and the well marked with symbol ladder, respectively. Ladder and samples were denatured (2 min, 70 ºC), before loading on the chip.

The chip was vortexed horizontally in the adapter for 1 min and ran in the Agilent 2100 Bio Analyzer immediately. To avoid any RNase contamination of analysis, the

electrodes were decontaminated with both “RNase away” (Catalogue # 83931, Fluka) and DEPC-water before inserting the chip.

Samples reaching a RIN above 6 (n=34 FH and n=12 control subjects) were further synthesised to cDNA and determined by Q-RT-PCR.

4.6 First-strand cDNA Synthesis

Background

The first-strand cDNA synthesis provides a transcript (cDNA) of gene expression (messenger RNA [mRNA]) which is further used for gene quantification in Q-RT-PCR (97). This reaction requires a short single stranded oligonucleotide (primer) for the enzyme which catalyzes the cDNA synthesis reaction. Primers are complementary to a known sequence initially of the transcription area. The Oligo dT primer is used for its specification to hybridize to poly-A-tails, which are particularly found on the vast majority of eukaryotic mRNA (97). Catalyzation of the cDNA synthesis reaction is performed by the reverse transcriptase (RT), the Superscript™ II, because of its reducing effect on RNase H activity. This both improves the synthesis of full-length cDNA and gives higher yields of first-strand cDNA compared to other RTs. Deoxyribonucleotide triphosphate (dNTP) mix contains the nucleotides of which the cDNA is synthesised of and thus is consumed by the Superscript™ II RT in the reaction. Removal of RNA template in the cDNA sample can increase the sensitivity of PCR from cDNA and is done by RNase H in the final step of first-strand synthesis of cDNA.

Procedure

Both the procedure (97) and the kit, Superscript™ First-Strand Synthesis System for RT-PCR (Catalogue # 11904-018, Invitrogen), were from Invitrogen. Amounts equivalent 500 ng total RNA were transferred to a new tube to perform cDNA, and added oligo dT, dNTP mix and DEPC-water. The components annealed at 65 ºC, 5 min, before cooling on ice ≥ 1 min. Reaction mix (10x RT buffer, 25mM $MgCl_2$, 0,1

M DTT and RNase Out) were added before incubation (42 ºC, 2 min). Immediately after incubation, the samples were added RT while still staying on the heat block. The samples were further incubated at same temperature in 50 min. To terminate the reactions, the samples were transferred to 70 ºC for 15 minutes before cooling on ice. As a final step, RNase H was added to the samples, and incubated (37 ºC, 20 min). The samples were placed on ice and then stored at -80ºC.

4.7 Q-RT-PCR

Background

In basic research, the Q-RT-PCR is a widely used tool to determine, characterise and quantify gene expression (98). Quantification by this method is based on the equation: $N = N_0 (1+E)^n$, where N is the number of amplified sequences after n cycles of amplification, N_0 is the initial number of target sequences and E is the efficiency of amplification per cycle (99).

Single-stranded cDNA performed in the first strand cDNA synthesis is applied as a template for amplification in the Q-RT-PCR (100). Table 2 shows the course in the PCR using TaqMan gene expression assay. The AmpErase Uracil-N-Glycosylase (UNG) activation avoids PCR contamination as it destroys both single- and double-stranded dU-containing DNA (101). Two steps in PCR are temperature sensitive (100). Initially, denaturation of template DNA is ensured by a temperature at 95ºC, this enables the primers to anneal to their target sequence in the second step, which is set to a temperature at 60 ºC. This step is critical in the PCR as too high temperature results in a reduced number of amplification products because of poorly annealed primers (102). If the temperature is too low, primers may anneal to non-specific sequences and cause spurious amplification products. After primer annealing, extension of the target sequence is carried out by the polymerase. Forty cycles of denaturation, annealing and DNA synthesis is then repeated.

Compared to the traditional PCR, using agarose gel to detect PCR amplification in the plateau phase of the reaction, Q-RT-PCR method measures the cycles of

amplification in the exponential phase of the reaction (103). The TaqMan chemistry assay for Q-RT-PCR (Applied Biosystems) consists of two primers and a fluorogenic probe which is sequence-specific to the target sequence (100). An intact probe has a reporter dye, 6-carboxyfluorescein (FAM^{TM}) bound to the 5'-end and a quencher on the 3'-end, which highly reduces the fluorescence emitted by the reporter by receiving the energy by the fluorescence resonance energy transfer. However, if the target sequence is present the probe anneals between the forward primer and the reverse primer. Q-RT-PCR starts and the probe is cleaved by the polymerase during extension, causing fluorescence (100). Reliable results are achieved by normalization of the fluorescent emission using a passive reference, 6-Carboxyl-X-Rhodamine (ROX^{TM}), calculating a ratio between the FAM^{TM} dye emission and the ROX^{TM} emission, the R_n value (100). Further, the R_n value is used to determine ΔR_n which is defined as: R_n – baseline, where baseline is the initial cycles of Q-RT-PCR where there is little change in the fluorescent signal. The point in time when the ΔR_n crosses a fixed level (threshold) is defined as the threshold cycle (C_T). An amplification plot graphically displays the C_T and the fluorescence detected over the number of cycles that were performed (Figure 7) (100).

Manually calculation of the final results also requires quantification of an endogenous control from every experimental sample, which means a gene with a stable expression in all the samples.

Table 2. Conditions in the PCR.

2. PCR Step Using TaqMan 2× Universal PCR Master Mix	Initial Steps		PCR (40 Cycles)	
	AmpErase UNG Activation	AmpliTaq Gold DNA Polymerase Activation	Melt	Anneal/ Extend
	HOLD	HOLD	CYCLE	
	2 min @ 50 °C	10 min @ 95 °C	15 sec @ 95 °C	1 min @ 60 °C

Adapted from Applied Biosystems (100). DNA= deoxyribonucleic acid, PCR= polymerase chain reaction, UNG= uracil-N-glycosylase.

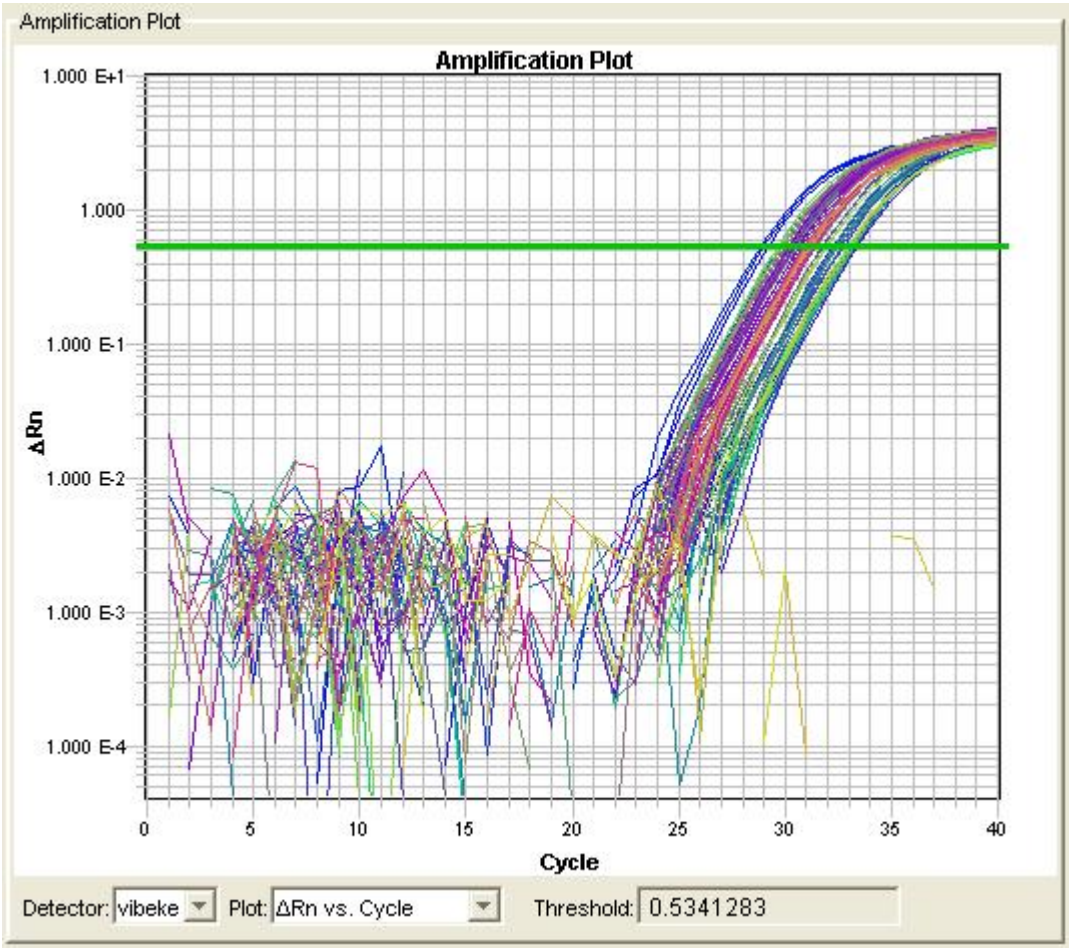


Figure 7. Amplification plot of the endogenous control TBP consisting of 27 individual samples showing ΔR_n vs cycle. ΔR_n = (fluorescence emission of reporter dye/fluorescence emission of reference dye)-baseline. Cycle shows how many amplification cycles the PCR has performed. The green line represents threshold. PCR= polymerase chain reaction, TBP= TATA box binding protein.

Procedure

The standard curve method was chosen in this procedure and it was made of an equal amount cDNA from all the samples (n= 49) and diluted 1:2 in DEPC-water to a four point curve with the highest concentration of 50 ng. Every cDNA sample was diluted in DEPC-water to a working concentration of 5 ng/µl. Q-RT-PCR was performed by using TaqMan Gene Expression assay (Applied Biosystems). The TaqMan gene expression master mix (Part # 4369514, Applied Biosystems), containing AmpliTaq Gold® DNA polymerase, Uracil-DNA Glycosylase (similar to AmpErase UNG), dNTP, ROX™ and buffers (104), was blended with the primer solution (consisting of primers [900 nM] and the TaqMan probe [200 nM], both specific to the target sequence) and DEPC-water, and added to every single well on a 96-well standard plate. Both standards and samples were added in triplicates on the plate in accordance with a specific layout. DEPC-water was used as a blank control. The plate (MicroAmp™ Optical 96-well Reaction Plate with barcode, part # 4314320, Applied Biosystems) was centrifuged (1200 rpm, 1 min, rt) and ran in the ABI 7900 HT (Applied Biosystems) immediately for one and a half hour on the 96-well standard block. Testing genes are shown in Table 3. The principles of the PCR amplification method is shown in Figure 8.

Table 3. Genes tested in Q-RT-PCR.

Target	Gene type	Assay number from Applied Biosystem
ICAM-1	testing	Hs00164932_m1
Leptin receptor	testing	Hs00174497_m1
TNFα	testing	Hs00174128_m1
TBP	housekeeping	Hs00427620_m1
GUSB	housekeeping	Hs99999908_m1

GUSB = β-glucuronidase, ICAM-1= intercellular adhesion molecule 1, TBP = TATA box binding protein, TNFα= tumor necrosis factor α.

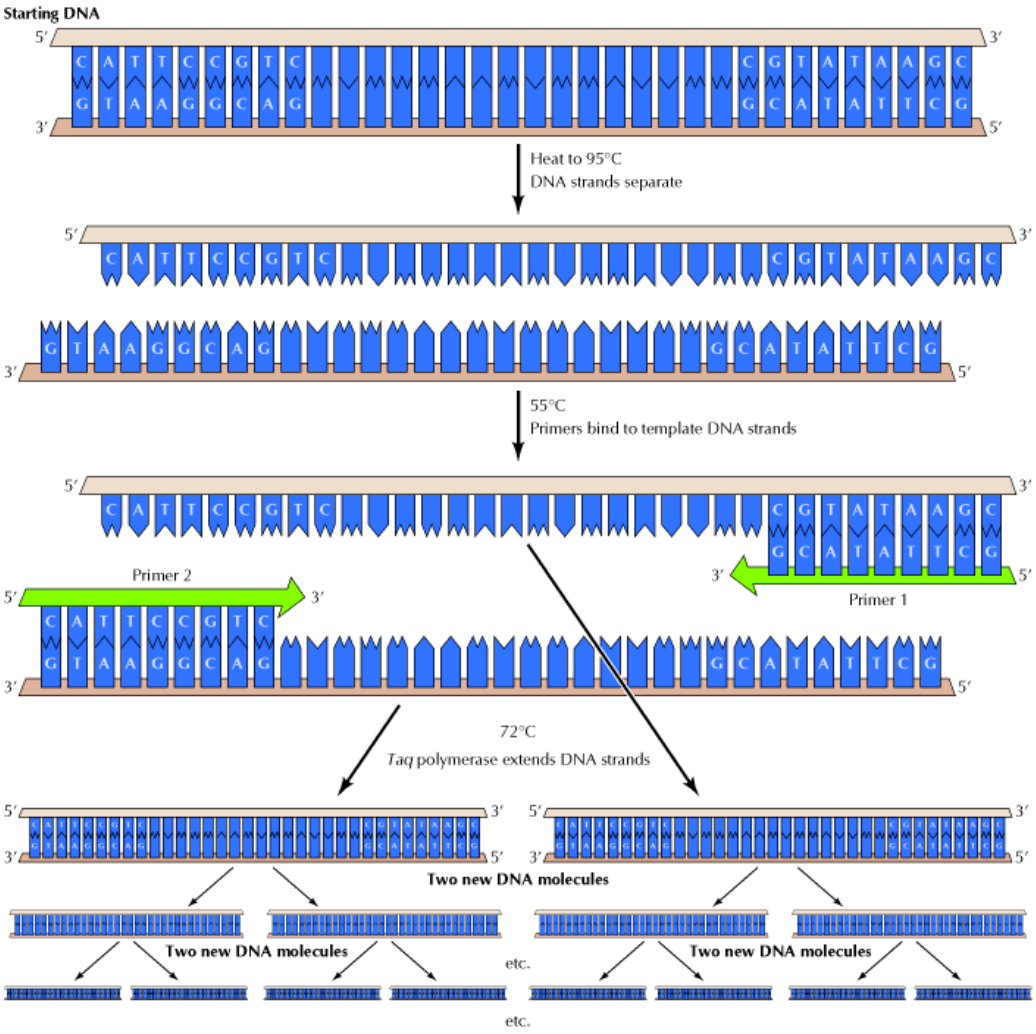


Figure 8. Amplification of DNA by PCR the region of DNA to be amplified is flanked by two sequences used to prime DNA synthesis. The starting double-stranded DNA is heated to separate the strands and then cooled to allow primers (usually oligonucleotides of 15 to 20 bases) to bind to each strand of DNA. DNA polymerase from Thermus aquaticus (Taq polymerase) is used to synthesize new DNA strands starting from the primers, resulting in the formation of two new DNA molecules. The process can be repeated for multiple cycles, each resulting in a twofold amplification of DNA. DNA= deoxyribonucleic acid, PCR= polymerase chain reaction. (105)

4.8 Sandwich Enzyme Linked ImmunoSorbant Assay (ELISA)

Background

The sandwich ELISA technique is based on the analyte-binding capacity of antibodies (106). Initially, all wells in a microtiter plate are coated with an analyte-specific capture antibody which will capture the molecules to measure e.g leptin. Before the samples/standards are incubated with the capture antibodies, a blocking

reagent is added to avoid unspecific binding of the samples/standards. This reagent is ousted by the samples/standards in the incubation. A detection antibody is added and binds to a different epitope of the molecule which is measured. This aggregate is detected by a reagent, streptavidin-horseradish-peroxidase (HRP), an enzyme which emits colour (blue) when it converts a substrate. Finally, a stop solution (2 N H_2SO_4) is terminating the enzyme reaction and changing the colour from blue to yellow. High colour intensity reflects high concentration of the molecule which is measured. The optical density (OD) of the colour can be measured by a microtiter plate reader. Between every step in the procedure, except the last one, washing steps are carried out to remove unbound antibodies/analytes.

Procedure

The method used is based on the user's manual on R&D's homepage (107). All wells in a microtiter plate was incubated with capture antibody (diluted in PBS [working concentration differs from the molecule to be measured]) in rt over night before washing with wash buffer (0.05% Tween 20 [Catalogue # 90005-64-5, Sigma-Aldrich] in PBS). Blocking the whole plate with Reagent Diluent (1% Bovine Serum Albumine [BSA] [Catalogue # K41-001, PAA the cell culture company] in PBS) was carried out and incubated for 1 hour. Washing step was repeated. Both samples (diluted in Reagent Diluent in an appropriate concentration) and standards, a seven point standard curve using a 2-fold serial dilutions in Reagent Diluent (highest concentration differs from the molecule to be measured), were added to their respective wells and incubated for 2 hours. Washing step was repeated. Detection antibody (diluted in Reagent Diluent [working concentration differs from the molecule to be measured]) were subsequently added in all wells and incubated for 2 hours. A new washing step was carried out. Streptavidin-HRP (working dilution: 1:200 in Reagent Diluent) were then added in all wells and incubated in room temperature for 20 minutes in a dark place. Washing step was repeated. A substrate solution (Catalogue # 00-2023, Zymed Laboratories) was added to all the wells and the plate was incubated in room temperature for 20 minutes in a dark place. Terminating the procedure was done by adding stop solution (2 N H_2SO_4) (Catalogue

3.0731.1, Merck) to all the wells and immediately measuring the OD at a wavelength of 450 nm. The procedure was carried out in accordance with manufacturer's instructions. Testing molecules are listed in Table 4.

Table 4. Molecules tested in sandwich ELISA.

Molecules	Catalogue # from R&D systems
Leptin	DY398
sICAM-1	DY720
sVCAM	DY809
sE-selectin	DY724
Adiponectin	DY1065

ELISA= enzyme linked immunosorbant assay, sICAM= soluble intercellular adhesion molecule-1, sVCAM= soluble vascular cell adhesion molecule, sE-selectin= soluble E-selectin.

4.9 Hormone Levels

The serum levels of the sex hormones, testosterone and sex hormone binding globuline (SHBG) were determined at the Hormone laboratory, Aker University Hospital, Oslo. Free androgen index (FAI) was calculated as the ratio between testosterone and SHBG, and accounts for the fraction of testosterone which is bound to SHBG (108).

4.10 Statistical Analysis

Data are given in median values (range) unless otherwise stated. The non-parametric Mann-Whitney test was used to determine significant differences between FH children and control subject, and between subgroups in the FH population. Correlations in the FH population, the control subjects and within FH subgroups were calculated by the non-parametric Spearman coefficient. Data in the pilot small study

of statin treatment were tested with Wilcoxon signed rank test. Pearson's Chi Square test was used when data were given as < or > than certain values, such as in the variables testosterone, SHBG and FAI. Statistical significance was determined by a p-value <0.05.

5. Results

5.1 Characterisation of the Subjects

Characterisation of the subjects is shown in Table 5. Serum concentrations of total cholesterol, LDL-C and apoB were significantly higher in the FH population compared to healthy control subjects. However, HDL-C and apoA1 were not significantly different between the two groups.

Table 5. Baseline characteristics of the participants.

	FH (n=62)	Controls (n= 22)
Age, years	14.5 (7-20)	16 (11-19)
Male gender, %	54	41
Total cholesterol, mmol/L	7.1 (4.4-10.6) *	4.1 (2.4-4.9)
LDL cholesterol, mmol/L	5.3 (2.8-8.7) *	2.6 (1.2-3.4)
HDL cholesterol, mmol/L	1.3 (0.8-2.1)	1.5 (1.0-2.1)
ApoA1, g/L	1.2 (0.9-2.1)	1.3 (0.7-1.7)
ApoB, g/L	1.2 (0.6-1.8) *	0.6 (0.2-0.7)

Data are presented in median values (range). ApoA1/B were available in n=44 FH children and n=20 control subjects. *p<0.01 vs control subjects. ApoA1= apolipoprotein A1, apoB= apolipoprotein B, FH= familial hypercholesterolemia, HDL= high density lipoprotein, LDL= low density lipoprotein.

5.2 FH Children versus Control Subjects

5.2.1 Early Atherosclerotic Markers

First we analysed whether there was a difference in the circulating levels of the early atherosclerotic markers sE-selectin, sVCAM-1 and sICAM-1 in children with and without FH. We found that there was a trend towards increased levels of sE-selectin (Figure 9A) in the FH population compared to the control subjects, p=0.082. There were no significant differences in the circulating levels of sVCAM-1 (Figure 9B) and sICAM-1 (Figure 9C). Levels of the measured atherosclerotic markers in the FH group compared to the control subjects are listed in Table 6.

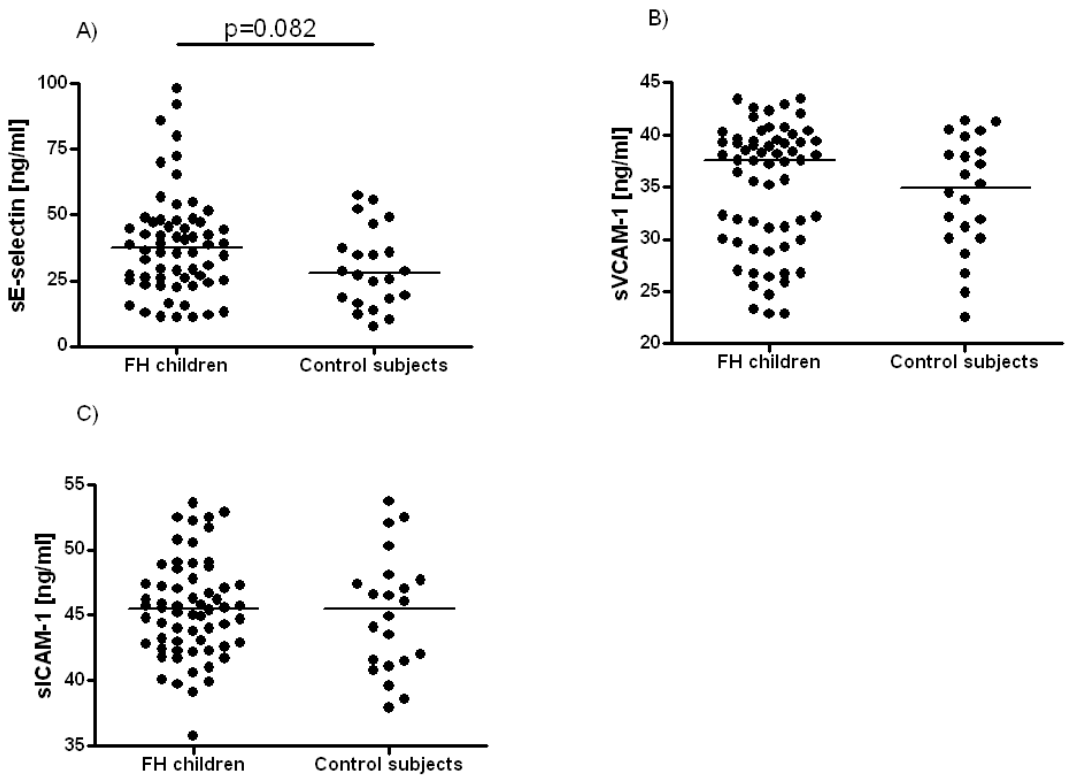


Figure 9. Circulating levels of sE-selectin (A), sVCAM (B) and sICAM-1 (C) in FH children (n=62) and in age- and sex-matched control subjects (n=22). Horizontal bars represent median values. FH= familial hypercholesterolemia, sE-selectin= soluble E-selectin, sICAM-1= soluble intercellular adhesion molecule-1, sVCAM-1= soluble vascular cell adhesion molecule-1.

Table 6. Levels of the circulating inflammation markers in the FH population and the control subjects.

	FH population (n=62)	Control subjects (n=22)
sE-selectin, ng/ml	38 (11-98)	28 (7-58)
sICAM-1, ng/ml	46 (36-54)	46 (38-54)
sVCAM-1, ng/ml	38 (23-44)	35 (23-41)
Leptin, ng/ml	6 (1-81)	7 (1-71)
Adiponectin, ng/ml	677 (110-1360)	522 (209-1430)

Data are presented in median values (range). FH= familial hypercholesterolemia, sE-selectin= soluble E-selectin, sICAM-1= soluble intercellular adhesion molecule-1, sVCAM-1= soluble vascular cell adhesion molecule-1.

In order to investigate if there were differences in the gene expression of early atherosclerotic markers between the FH children and the control children gene expression of TNFα and ICAM-1 from isolated PBMCs were analysed. Significant increased levels of TNFα gene expression (Figure 10A) was observed in the FH population compared to the control subjects, p=0.019. There was however no significant difference in ICAM-1 levels (Figure 10B).

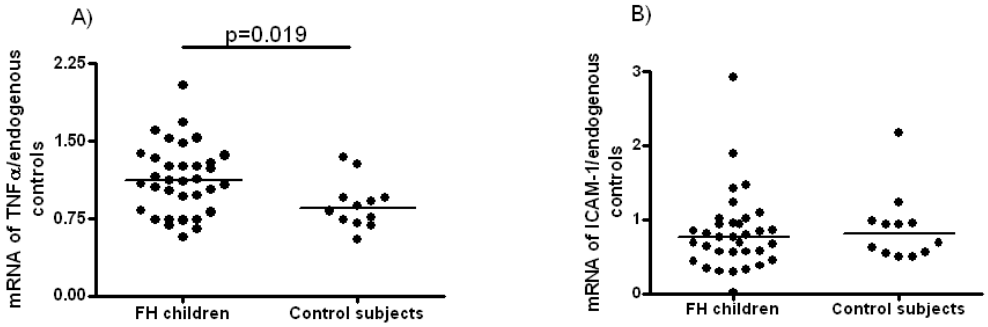


Figure 10. Gene expression levels of TNFα (A) and ICAM-1 (B) in FH children (n=34) and control subjects (n=12). Endogenous controls are defined by the mean gene expression of TBP and GUSB. Horizontal bars represent median values. FH= familial hypercholesterolemia, GUSB= β-glucuronidase, ICAM-1= intercellular adhesion molecule, TBP= TATA box binding protein, TNFα= tumor necrosis factor α.

5.2.2 Adipokines and Leptin Receptor

Numerous studies suggest an association between leptin and serum cholesterol levels (69;109-112), and in addition adiponectin has been shown to be related to hyperlipidemia (61;62;67). FH children are characterised by increased levels of total cholesterol and LDL-C compared to healthy controls. We therefore investigated whether there were differences in the levels of leptin, adiponectin and the gene expression of the leptin receptor in PBMCs from children with and without FH. No significant differences however, were found in the levels of leptin (Figure 11A), adiponectin (Figure 11B) or in the gene expression of the leptin receptor (Figure 11C) between the two groups. Levels of the measured adipokines in the FH group compared to the control subjects are listed in Table 6.

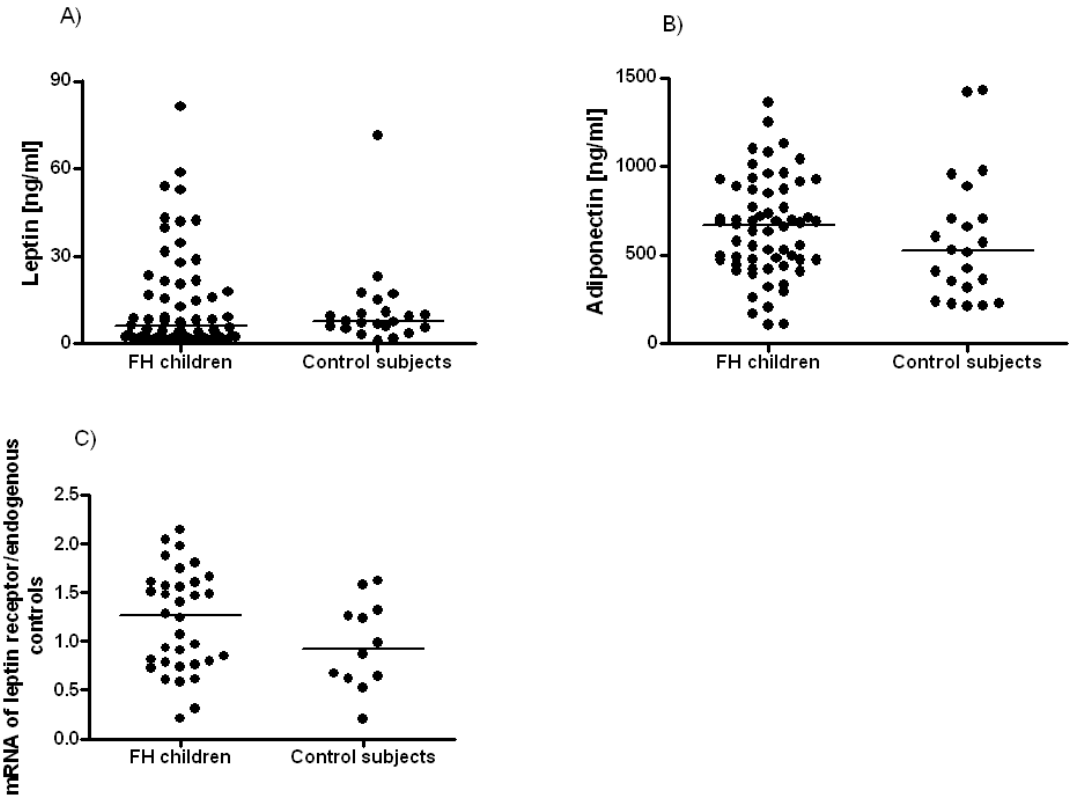


Figure 11. Serum levels of leptin (A) and adiponectin (B) in FH children (n=62) and control subjects (n=22), and gene expression levels of leptin receptor (C) in FH children (n=34) and control subjects (n=12). Endogenous controls are defined by the mean gene expression of TBP and GUSB. Horizontal bars represent median values. FH= familial hypercholesterolemia, GUSB= β-glucuronidase, TBP= TATA box binding protein.

5.2.3 Correlations

We next analysed whether there was correlations between the baseline characteristics and the tested parameters in all the participants. Several correlations were found between baseline characteristics and testing parameters in the study population (Table 7). The main findings include positive correlations between some of the early circulating atherosclerotic markers, sE-selectin, sICAM-1, sVCAM-1 and the gene expression of TNFα (Table 7). Adiponectin was inversely correlated to age, whereas leptin was positively correlated to age. A similar pattern was found when the correlations were performed in the FH group and the control group, separately (see Appendix, Table I and II, respectively). In addition inverse correlations were observed between the early atherosclerotic markers sICAM-1, sVCAM-1 and TNFα gene expression, and LDL-C, total cholesterol and apoB in the FH population (see Appendix, Table I). This was however not seen in the whole study population, neither in the control group separately. The inverse correlation between age and adiponectin, and a positive correlation between age and leptin were only observed in the FH population and not in the control group separately.

When puberty starts, the circulating levels of cholesterol (both for FH children and control children) are reduced (113), probably due to an increased synthesis of the steroid sex hormones, a mechanism which is probably more pronounced among children with FH compared to healthy control children. This cholesterol decrement in adolescence should be regarded by clinicians, particularly in diagnosis of children with FH. Since we found an inverse correlation between total cholesterol and LDL-C, and sICAM-1 and VCAM-1 in the FH population, we correlated the circulating markers and the lipid parameters in the oldest age group of the whole study population. When looking at the correlations only in children above 15 years (n=43) we found significant, positive correlations between sE-selectin, sICAM-1 and sVCAM-1, and apoB, $r=0.383$, $p=0.031$; $r=0.525$, $p=0.02$ and $r=0.395$, $p=0.025$ respectively.

Table 7. Correlations in the study population (n=84).

		Adiponectin	sE-selectin	sICAM-1	Leptin	sVCAM-1	mRNA TNFα	mRNA Leptin receptor
Total cholesterol	Correlation coefficient	0.226						
	p-value	0.039						
LDL-C	Correlation coefficient	0.257						
	p-value	0.021						
ApoB	Correlation coefficient	0.257						
	p-value	0.038						
sE-selectin	Correlation coefficient			0.329	-0.248			
	p-value			0.002	0.023			
sICAM-1	Correlation coefficient		0.329			0.698	0.551	
	p-value		0.002			0.000	0.000	
Leptin	Correlation coefficient		-0.248					
	p-value		0.023					
sVCAM-1	Correlation coefficient			0.698			0.553	
	p-value			0.000			0.000	
mRNA ICAM-1	Correlation coefficient							-0.330
	p-value							0.025
Age	Correlation coefficient	-0.381			0.246			
	p-value	0.000			0.024			

Not all parameters were available in all participants. ApoA1= apolipoprotein A1, apoB= apolipoprotein B, ICAM-1= intercellular adhesion molecule-1, LDL-C= low density lipoprotein cholesterol, mRNA= messenger ribonucleic acid, sE-selectin= soluble E-selectin, sICAM-1= soluble intercellular adhesion molecule-1, sVCAM-1= soluble vascular cell adhesion molecule, TNFα= tumor necrosis factor α.

5.3 Gender and Age

Gender has been shown to be a risk factor in CAD in the adult population (34;38;114). In addition, some studies have shown a gender difference of higher sE-selectin levels in men compared to women (114;115) and a significant positive correlation between sVCAM-1 and age was found by Blann et al (115).

5.3.1 Characterisation of the FH Population Subdivided According to Gender

Characterisation of the FH children separated in boys and girls is shown in Table 8. No significant differences in the baseline parameters were found between the two groups.

Table 8. Baseline characteristics of the FH children separated in boys and girls.

	FH boys (n=33)	FH girls (n=29)
Age, years	14 (7-20)	15 (9-18)
Total cholesterol, mmol/L	7.0 (4.4-10.3)	7.3 (4.4-10.6)
LDL cholesterol, mmol/L	5.3 (3.3-8.0)	5.5 (2.8-8.7)
HDL cholesterol, mmol/L	1.3 (0.9-2.0)	1.3 (0.8-2.1)
ApoA1, g/L	1.2 (0.9-1.7)	1.1(0.9-2.1)
ApoB, g/L	1.2 (0.6-1.8)	1.3 (0.6-1.7)

Data are presented in median values (range). ApoA1/B were not available in all the participants. ApoA1= apolipoprotein A1, apoB= apolipoprotein B, FH= familial hypercholesterolemia, HDL= high density lipoprotein, LDL= low density lipoprotein.

5.3.2 Gender and Early Atherosclerotic Markers

We next analysed whether there was a gender difference in the levels of sE-selectin, sVCAM-1 and sICAM-1 in the FH population. Increased levels of sE-selectin (Figure 12A) and sVCAM-1 (Figure 12B) were observed in FH boys compared to FH girls, p=0.006 and p=0.051 respectively. However, no significant difference was found in sICAM-1 (Figure 12C). Levels of the early circulating atherosclerotic markers among boys and girls in the FH population are listed in Table 9. There were no significant differences in the levels of sE-selectin, sVCAM-1 and sICAM-1 between FH boys and control boys or between FH girls and matched controls (data not shown).

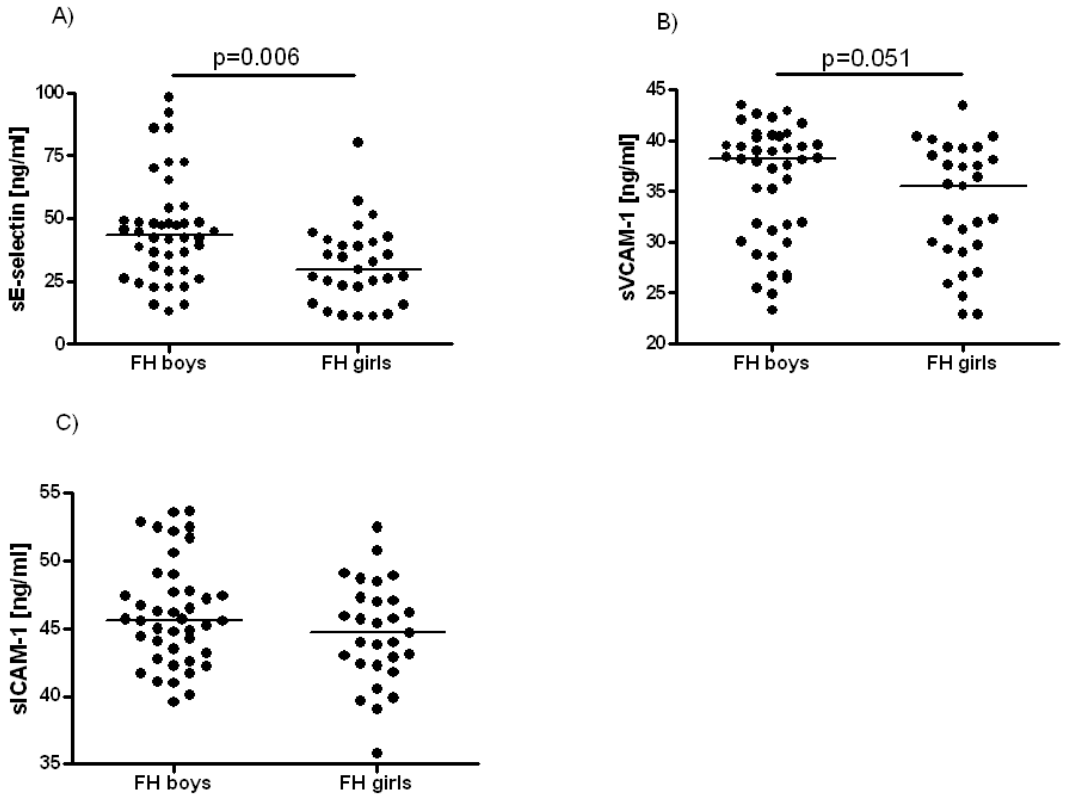


Figure 12. Circulating levels of sE-selectin (A), sVCAM (B) and sICAM-1 (C) among boys (n=33) and girls (n=29) in the FH children. Horizontal bars represent median values. FH= familial hypercholesterolemia, sE-selectin= soluble E-selectin, sICAM-1= soluble intercellular adhesion molecule-1, sVCAM-1= soluble vascular cell adhesion molecule-1.

Table 9. Levels of the circulating inflammation markers in boys and girls with FH.

	FH boys (n=33)	FH girls (n=29)
sE-selectin, ng/ml	42 (13-98)**	30 (11-80)
sICAM-1, ng/ml	46 (40-54)	45 (36-53)
sVCAM-1, ng/ml	38 (23-44)*	36 (23-43)
Leptin, ng/ml	3 (1-32)**	18 (2-81)
Adiponectin, ng/ml	577 (110-1360)	687 (107-1130)

Data are presented in median values (range). *p=0.051, **p<0.01 vs FH girls. FH= familial hypercholesterolemia, sE-selectin= soluble E-selectin, sICAM-1= soluble intercellular adhesion molecule-1, sVCAM-1= soluble vascular cell adhesion molecule.

At gene expression level, we analysed whether there was a difference in the inflammation markers TNFα and ICAM-1 in FH boys and girls. There were however no significant differences between the genders in either TNFα or ICAM-1 (Figure 13A and B respectively). We also wanted to investigate if there were differences between FH girls and boys and their control counterparts. There was a trend towards higher gene expression levels of TNFα in FH girls compared to control girls, p=0.08 (data not shown). This was however not observed between FH boys and control boys (data not shown). There were no significant differences between either FH boys and control boys or FH girls and matched controls in the gene expression of ICAM-1 (data not shown).

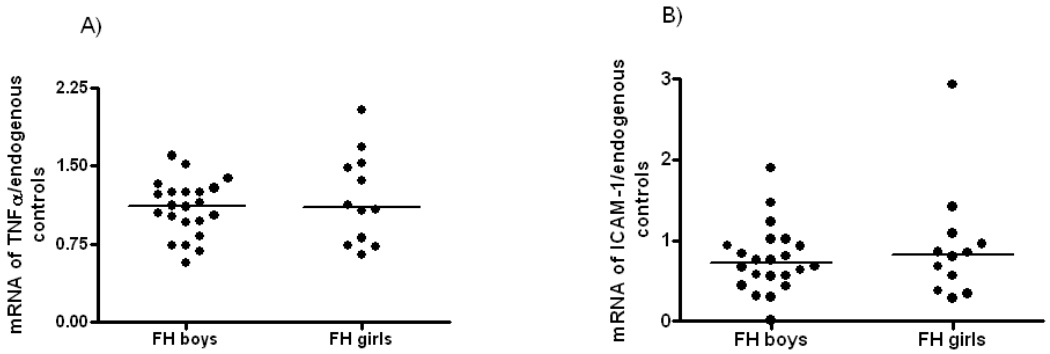


Figure 13. Gene expression levels of TNFα (A) and ICAM-1 (B) among boys (n=22) and girls (n=12) in the FH population. Endogenous controls are defined by the mean gene expression of TBP and GUSB. Horizontal bars represent median values. FH= familial hypercholesterolemia, GUSB= β-glucuronidase, ICAM-1= intercellular adhesion molecule-1, TBP= TATA box binding protein, TNFα= tumor necrosis factor α.

5.3.3 Gender, Adipokines and Leptin Receptor

Leptin, adiponectin, and the gene expression of leptin receptor were analysed among boys and girls with FH. As expected, we found significant increased level of leptin in FH girls compared to FH boys (Figure 14A), p=0.000. There were however no significant differences either in adiponectin (Figure 14B) or in the gene expression of the leptin receptor (Figure 14C). Levels of the adipokines are listed in Table 9. We also investigated if FH girls and boys had differences in the levels of adipokines and leptin receptor than their control counterparts. FH girls tended to have enhanced leptin levels (18 [2-81] ng/ml) than control girls (10 [3-71] ng/ml), p=0.09. This was not observed between FH boys and control boys. There were no significant differences in the levels of adiponectin or the gene expression levels of leptin receptor between either FH girls and control girls, or FH boys and matched controls (data not shown).

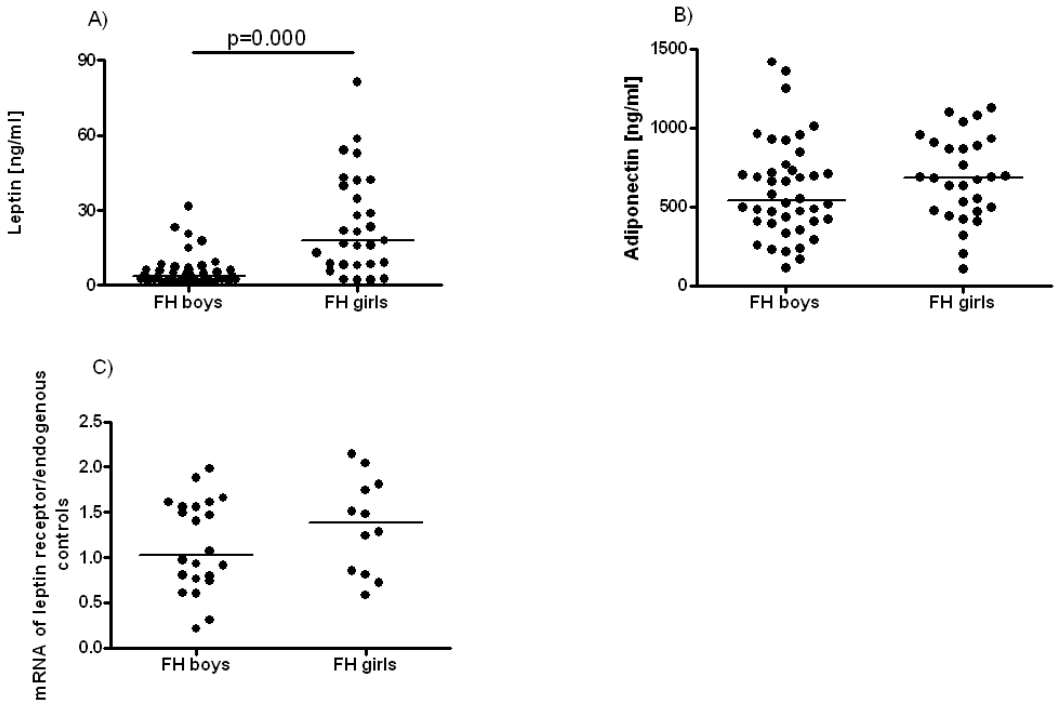


Figure 14. Levels of adiponectin (A) and leptin (B) among boys (n=33) and girls (n=29) in the FH population, and the gene expression of leptin receptor (C) among boys (n=22) and girls (n=12) in the FH population. Endogenous controls are defined by the mean gene expression of TBP and GUSB. Horizontal bars represent median values. FH= familial hypercholesterolemia, GUSB= β-glucuronidase, TBP= TATA box binding protein.

5.3.4 Age

The gender differences in the levels of sE-selectin (Figure 12A), sVCAM-1 (Figure 12B) and leptin (Figure 14A) led us to hypothesise differences in age groups as well. Therefore, we also wanted to see if there were differences in the measured markers when subdividing the FH children in age groups, above and below 15 years (median age). There were no significant differences either in the levels of sE-selectin (Figure 15A), sVCAM-1 (Figure 15B) or sICAM-1 (data not shown) in FH children above and below the age of 15. However, there was a trend towards increased levels of leptin in FH children above 15 years compared to FH children below 15 years, p=0.075 (Figure 15C). Moreover, the levels of adiponectin were significantly decreased in FH children above 15 years compared to FH children below 15 years, p=0.000 (Figure 15D). Levels of the circulating inflammation markers are listed in Table 10.

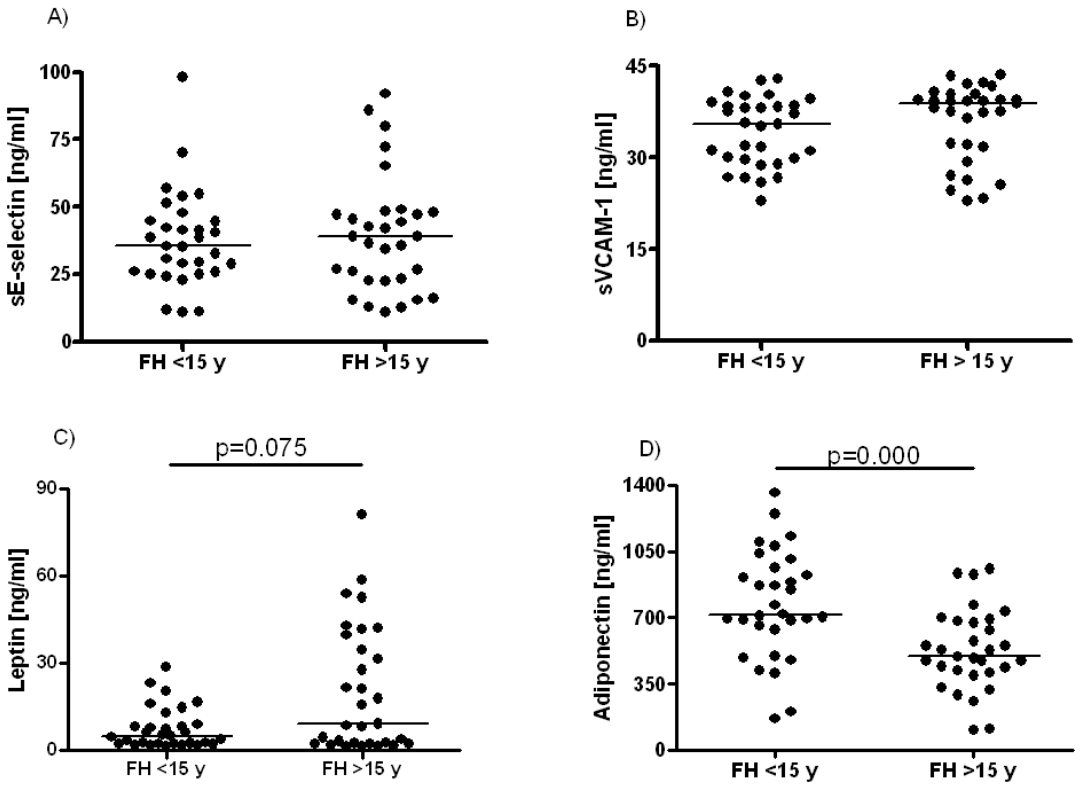


Figure 15. Levels of sE-selectin (A), sVCAM-1 (B), leptin (C) and adiponectin (D) in the FH subgroups below (n=31) and above 15 years (n=31). Horizontal bars represent median values. FH= familial hypercholesterolemia, sE-selectin= soluble E-selectin, sVCAM-1= soluble vascular cell adhesion molecule-1, y= years.

Table 10. Levels of the circulating inflammation markers in the FH population subdivided in age groups above and below 15 years.

	FH children <15 y	FH children >15 y
sE-selectin, ng/ml	36 (11-98)	39 (11-92)
sVCAM-1, ng/ml	36 (23-43)	39 (23-44)
Leptin, ng/ml	5 (2-29)	9 (1-81)
Adipoenctin, ng/ml	717 (167-1360)*	494 (107-958)

Data are presented in median values (range). *p<0.01 vs FH children >15 y. FH= familial hypercholesterolemia, sE-selectin= soluble E-selectin, sVCAM-1= soluble vascular cell adhesion molecule-1, y= years.

5.3.5 Gender and Age

When we subdivided the FH children according to both gender and age, we found that there were significantly enhanced levels of sE-selectin in boys above 15 years (Figure 16A) compared to FH girls in the same age group, p=0.019. For sVCAM-1 a trend towards increased levels in boys below 15 years (Figure 16D) was observed compared to FH girls in the same age group, p=0.068. As expected, there were significantly enhanced levels of leptin in girls above 15 years (Figure 16 E) compared to FH boys in the same age group, p=0.000. Furthermore, a trend towards enhanced levels of leptin in FH girls below 15 years (Figure 16F) compared to FH boys in the same age group was also observed, p=0.068. Levels of the circulating inflammation makers are listed in Table 11.

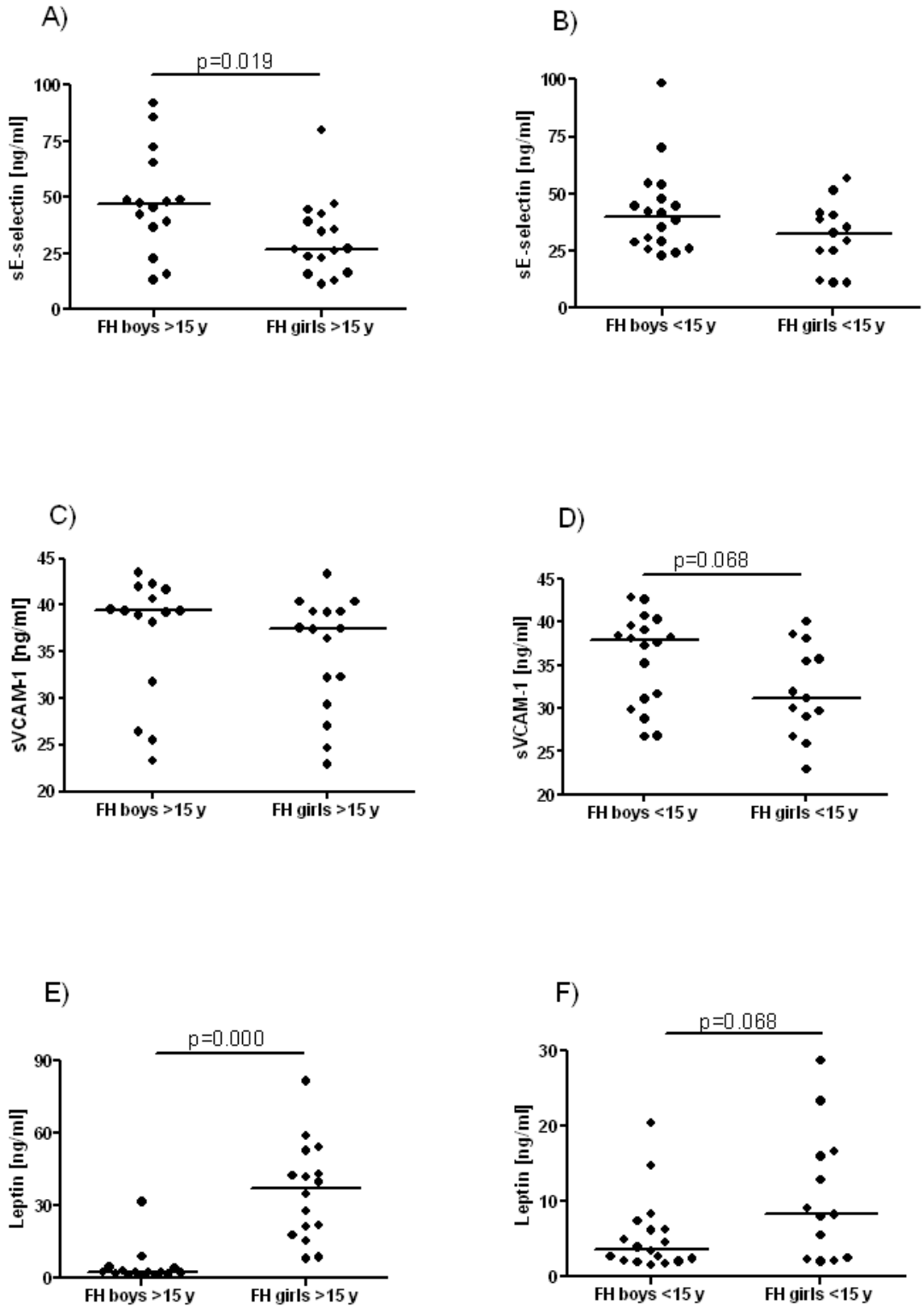


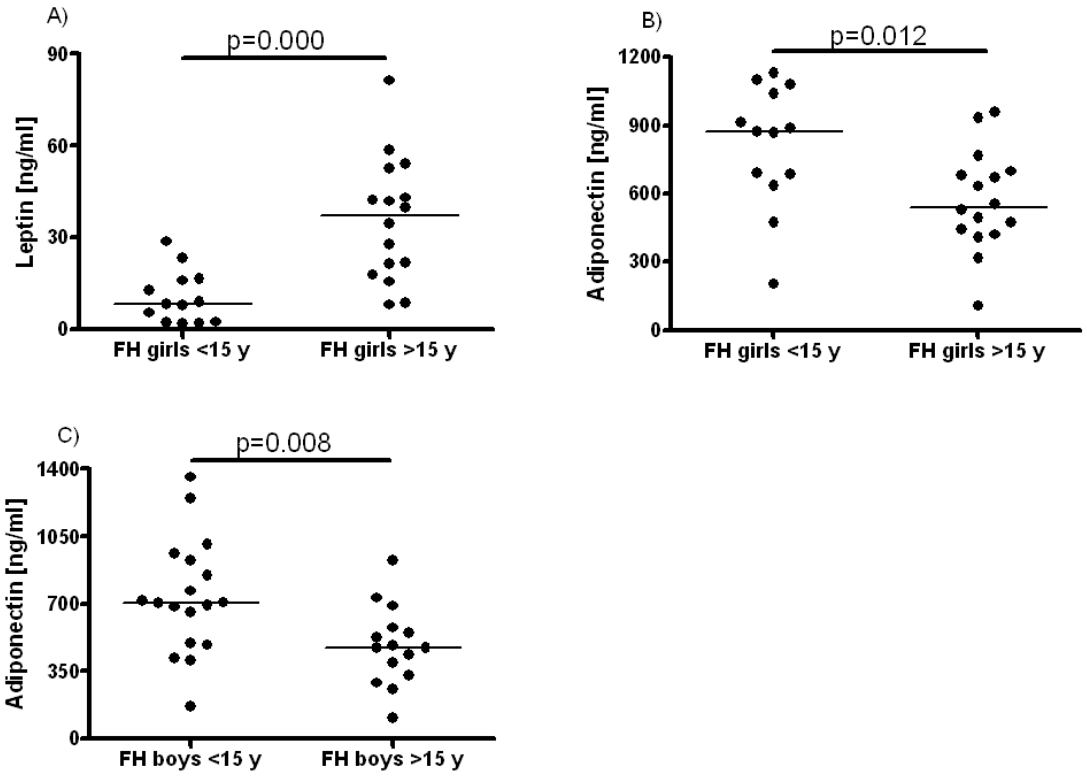
Figure 16. The FH population subdivided in boys above 15 years (n=15) and girls above 15 years (n=16) and boys below 15 years (n=18) and girls below 15 years (n=13) showing levels of sE-selectin (A and B), sVCAM-1 (C and D) and Leptin (E and F), respectively. Horizontal bars represent median values. FH= familial hypercholesterolemia, sE-selectin= soluble E-selectin, sVCAM-1= soluble vascular cell adhesion molecule-1, y= years.

Table 11. Levels of the circulating inflammation markers in the FH population subdivided according to gender and age.

	FH boys >15 y (n=15)	FH girls >15 y (n=16)	FH boys < 15 y (n=18)	FH girls <15 y (n=13)
sE-selectin, ng/ml	47.3 (13.0-92.0)*	27.0 (11.2-80-0)	40.0 (22.9-98.3)	32.9 (11.1-56.9)
sVCAM-1, ng/ml	39.4 (23.3-43.5)	37.5 (22.9-43.4)	37.9 (26.7-42.9)#	31.2 (22.9-40.1)
Leptin, ng/ml	2.4 (1.3-31.6)**	37.2 (8.1-81.3)§	3.7 (1.6-20.5)#	8.3 (2.0-28.8)
Adiponectin, ng/ml	473 (110-928)¤	541 (107-958)ıı	707 (167-1360)	871 (203-130)

Data are presented in median values (range). *p<0.05, **p<0.01 vs FH girls >15 y. ¤p<0.01 vs FH boys <15 y. §p<0.01 vs FH girls <15 y. ııp<0.05 vs FH girls <15 y. #p=0.068 vs FH girls <15 y. FH= familial hypercholesterolemia, sE-selectin= soluble E-selectin, sVCAM-1= soluble vascular cell adhesion molecule-1, y= years.

We also investigated whether FH girls below 15 years differed from FH girls above 15 years, and likewise in FH boys. FH girls above 15 years have increased leptin levels compared to FH girls below 15 years (Figure 17A). This was not seen in FH boys above and below 15 years. Furthermore, FH girls above 15 years (Figure 17B) have significantly decreased adiponectin levels compared to FH girls below 15 years, the same result was seen in FH boys (Figure 17C), p=0.008 and p=0.012 respectively. When subdividing the control subjects likewise, no differences were observed (data not shown). Levels of the circulating inflammation markers are listed in Table 11.



Figur 17. Levels of leptin (A) and adiponectin (B) in FH girls below (n=13) and above (n=16) 15 years, and levels of adiponectin (C) in FH boys below (n=18) and above (n=15) years. FH= familial hypercholesterolemia, y= years.

5.4 Sex Hormones

Some studies emphasise that sex hormones is involved in the induction of adhesion molecule expression on endothelial cells (46;116). The observation that elevated sE-selectin levels only were present in FH boys above 15 years compared to age-matched FH girls and not in the gender groups below 15 years, led us to hypothesise that sex hormones were involved in this mechanism. We subsequently analysed the levels of testosterone and SHBG and calculated the FAI. Serum samples available were n=58 from FH children and n=19 from control subjects. The levels of testosterone, SHBG and FAI in the FH children and the control subjects subdivided in boys and girls are shown in Table 12. We found as expected increased testosterone levels ($p<0.01$) and FAI ($p<0.01$) among FH boys compared to FH girls, however there were no differences observed between FH boys and control boys. In Table 13 the levels of these parameters in the FH children subdivided in both age and gender

are listed. We then analysed whether the SHBG, testosterone and FAI were correlated to the circulating early atherosclerotic markers and serum cholesterol. We found an inverse correlation between total cholesterol, testosterone and FAI (Table 14). In addition testosterone was positively correlated to all the circulating early atherosclerotic markers, sE-selectin, sICAM-1 and sVCAM-1 (Table 14) whereas FAI was positively correlated to sE-selectin and sICAM-1.

Table 12. Levels of testosterone, SHBG and FAI in the FH children (n=58) and the control subjects (n=19) subdivided in boys and girls.

	FH boys (n=31)	FH girls (n=27)	Control boys (n=9)	Control girls (n=10)
Testosterone, nmol/l	10.0 (<0.5-28.7)	1.0 (<0.5-4.3)	13.3 (<0.5-23.8)	1.0 (<0.5-1.9)
SHBG, nmol/l	51 (15-175)	67 (8->180)	39 (14-103)	58 (37->180)
FAI	0.26 (0-1.41)	0.02 (0-0.13)	0.61 (0-1.05)	0.02 (0-0.03)

Data are presented in median values (range). FAI= free androgen index, FH= familial hypercholesterolemia, SHBG= sex hormone binding globuline.

Table 13. Levels of testosterone, SHBG and FAI in the FH children (n=58) subdivided in age and gender.

	FH boys >15 y (n=13)	FH girls >15 y (n=16)	FH boys <15 y (n=18)	FH girls <15 y (n=11)
Testosterone, nmol/l	17.8 (7.6-28.7)	1.4 (0.7-4.3)**	6.7 (<0.5-17.9)	<0.5 (<0.5-1.2)
SHBG, nmol/l	34 (15-75)	57 (8->180)	66 (19-175)	79 (30-120)
FAI	0.57 (0.24-1.41)#	0.03 (0-0.13)*	0.68 (0-0.84)	0.01 (0-0.03)

Data are presented in median values (range). #p=0.069 vs FH boys <15 y. **p<0.01 vs FH girls <15 y. *p<0.01 vs FH girls <15 y. FAI= free androgen index, FH= familial hypercholesterolemia, SHBG= sex hormone binding globuline.

Table 14. Correlations between hormones, serum cholesterol and early atherosclerotic markers in the FH population (n=58).

		SHBG	Testosterone	FAI
Total cholesterol	Correlation coefficient		-0.416	-0.345
	p-value		0.001	0.008
LDL-C	Correlation coefficient		-0.405	-0.321
	p-value		0.002	0.014
HDL-C	Correlation coefficient	0.404		-0.286
	p-value	0.002		0.014
ApoB	Correlation coefficient		-0.334	-0.315
	p-value		0.029	0.040
sE-selectin	Correlation coefficient		0.301	0.285
	p-value		0.022	0.030
sVCAM-1	Correlation coefficient		0.404	0.330
	p-value		0.002	0.012
sICAM-1	Correlation coefficient		0.363	0.310
	p-value		0.005	0.018

FAI= free androgen index, FH= familial hypercholesterolemia, HDL-C= high density lipoprotein cholesterol, LDL-C= low density lipoprotein cholesterol, SHBG= sex hormone binding globulin, sE-selectin= soluble E-selectin, sICAM-1= soluble intercellular adhesion molecule-1, sVCAM-1= soluble vascular cell adhesion molecule.

5.5 Effects of Statin Treatment – A Pilot Study

In five of the FH children, samples before and after statin treatment (with treatment duration of 4-80 weeks) were available. These samples were used as a small preliminary pilot study to see whether statin treatment could have an effect on any of the markers measured. We found a trend towards a reduction of sE-selectin levels after treatment with statins compared to baseline measurements, p=0.08 (Table 15). This finding indicates a need to further explore the effect of statin on early atherosclerotic markers.

Table 15. Levels of circulating inflammation markers before and after statin treatment in the FH children (n=5).

	Before	After
sE-selectin, ng/ml	35.6 (16.3-65.3)	26.8 (8.6-55.0)
sICAM-1, ng/ml	41.7 (39.7-44.0)	43 (40.0-45.4)
sVCAM-1, ng/ml	26.4 (23.3-28.8)	27.1 (20.1-31.9)
Adiponectin, ng/ml	472 (258-695)	623 (336-764)
Leptin, ng/ml	4.6 (3.3-27.8)	7.2 (1.8-19.5)

Data are presented in median values (range). FH= familial hypercholesterolemia, sE-selectin= soluble E-selectin, sICAM-1= soluble intercellular adhesion molecule-1, sVCAM-1= soluble vascular cell adhesion molecule-1.

6. Discussion

6.1 Discussion of Methods

6.1.1 Methods for Isolating PBMCs and RNA

Use of gene expression in PBMCs has several advantages in determining possible risk markers in CVD (117). Since plasma/serum levels of molecules may not reflect their production by specific tissues, measurements of gene expression in the specific tissue involved in the process is a better approach. Thus, gene expression in PBMCs may be used in the understanding of cardiovascular conditions (117), as PBMCs are exposed to many of the same environmental factors as the intima, where the atherogenesis takes place (84). One crucial factor when consider them as a model is their ability to synthesise RNA, in addition their high availability is very advantageous. When investigating genes in the lipid metabolism, PBMCs may not be the most suitable model, however they are very suitable when studying other atherosclerotic markers. However, immediately after leaving their environment, the gene expression in the PBMCs may be altered due to stress (99). Therefore it is important to use a rapid method when isolating PBMCs.

CPT is an easy and to our knowledge the most rapid method for isolation of PBMCs (118). The method should hence be performed continuously and performance-time should be shortened where this is possible, as this performance is a critical step for further analysis of PBMCs. Although use of the TRIzol reagent inhibits RNase degradation of the samples and delivers high yields of RNA (87), caution must be taken as the reagent constitute health risk (119). In combination with the RNeasy columns, RNAse-free DNase treatment of the samples avoids gDNA contamination and thus increases the fidelity in further analysis (98).

6.1.2 Q-RT- PCR

Q-RT-PCR was used to quantify mRNA levels in PBMCs due to its simplicity, specificity and demand for small amounts of mRNA (120). In the Q-RT-PCR target genes are detected in the exponential phase of the reaction (103). This contributes to a very precise measurement since none of the reaction components are consumed or none of the Q-RT-PCR products are degraded in the exponential phase. This enables maximal reaction efficiency and an exact doubling of products in this phase.

However, there are several pitfalls in the steps prior to and during the Q-RT-PCR method (92). Since quantitative detection of a target is dependent of the template abundance (120), both quality and quantity of RNA should be assessed prior to cDNA synthesis (92;121). We determined the quality of all the samples in the Agilent 2100 Bioanalyzer, which has been shown to be one of the most accurate qualifying methods (121). To ensure complete cDNA synthesis, only RNA samples with a RIN above 6 and a gel free of smear were chosen in further analysis. Measurements of RNA quantity were performed by the Nanodrop ND-1000. For normalisation of sample size, all samples synthesised to cDNA had amounts of 500 ng total RNA, which is within the range of use in Q-RT-PCR (100 pg -1 μg) (99). Template abundance may also be poor if cDNA synthesis is inadequate. Oligo dT was used as primer in the cDNA synthesis due to its specificity targeting poly-A-tails present in the vast majority of mRNA (97). However, drawbacks of using oligo dT is incomplete cDNA when targeting RNA with low integrity (99). Normalisation to endogenous controls which are constantly expressed in all cells independent of experimental conditions controls for internally errors in Q-RT-PCR (121;122). However, choice of endogenous controls remains as a major problem in Q-RT-PCR (98;121;122). Two of the classically used endogenous controls, glyceraldehyd-3-phosphate dehydrogenase and β-actin, have been reported to be inappropriate for normalisation in the Q-RT-PCR (121). Therefore, we used GUSB and TBP which have to our previous experience shown to be constantly expressed in PBMCs.

6.1.3 Sandwich ELISA

Sandwich ELISA is a rapid, simple, specific and sensitive method for measuring protein quantity (123). In addition, the protein binding capacity of the plates is high and thus the method requires low sample volume. Awareness should be taken as plates made of different material bind protein different e.g. some material types have high binding to protein, but increase "noise" (124). In the Sandwich ELISA method all samples are related to a standard curve, and hence a poor standard curve may give spurious results (125). Inaccurate results may also occur if: incubation times are not kept; incubation temperatures vary; the content of the wells evaporate.

6.1.4 Subjects and Statistics

Although the number of participants is acceptable compared to other published studies similar to the present one (10;82), limitations are still connected to the number of participants in several of our statistical analysis. In analysis where the FH population and the control subjects have been subdivided according to gender and/or age, the number of participants is too small to be representative. Particularly this is pronounced among the control subjects whose number is below the half of the number of participants in the FH population. Thus, we assume that in some statistical analysis significant results will not appear due to the small number of participants. On the other hand, even in some of the small test groups significant results appear, which may suggest strong associations between the tested markers and the subjects. Since normal distribution was mainly not obtained, statistically non-parametric methods were used throughout.

6.2 Discussion of Results

The main findings of the present study are: i) FH children have increased TNFα gene expression levels and a tendency to increased sE-selectin levels compared to control children; ii) FH boys have enhanced sE-selectin and sVCAM-1 levels compared to

FH girls; iii) FH boys above 15 years have increased levels of sE-selectin compared to age-matched FH girls; iv) FH boys below 15 years have enhanced levels of sVCAM-1 compared to FH girls in the same age group; v) FH girls have enhanced leptin levels compared to FH boys; vi) there was a tendency to enhanced leptin levels, whereas adiponectin levels were decreased in FH children above 15 years compared to FH children below 15 years. Taken together these results may support and confirm the observation of increased inflammation and further extends the knowledge of early inflammation markers in FH children. This in turn may contribute to understand the early atherosclerotic processes.

6.2.1 Inflammatory Markers

TNFα can be seen as a key mediator in the development of atherosclerosis due to its involvement in the recruitment of inflammation cells, the formation of foam cells and fatty streaks, the cellular activation and proliferation, and finally in the thrombus formation (55). Enhanced secretion of TNFα from T cells, macrophages and foam cells due to inflammatory response, leads to further activation of these cells and a positive feedback on the secretion of TNFα. The increased gene expression levels of TNFα seen in the present study may suggest enhanced inflammation in FH children. In fact, these increased levels in PBMCs may also contribute locally to progress the inflammation and hence the atherosclerotic process in the intima after the transendothelial migration of the PBMCs.

E-selectin is primarily expressed by endothelial cells in response to inflammatory stimuli such as TNFα, but is almost absent under normal conditions (43). In order to limit or stop an inflammatory process, E-selectin is either internalised and degraded in lysosomes or shed/proteolytically cleaved by the endothelial cells, the latters resulting in circulating levels of this molecule. It is thus conceivable that the tendency towards increased levels of sE-selectin seen in FH children may be due to increased surface expression of E-selectin as a response to inflammatory mediators, a process

resulting in dysfunction of endothelial cells which in fact is previously shown to be significantly more pronounced in FH children compared to control subjects (22).

Previously, FH children have been shown to have a significant increased gene expression of RANTES and elevated circulating levels of neopterin compared to healthy control children, indicating the presence of a proinflammatory monocyte (10). Furthermore, others have also reported elevated circulating levels of neopterin, and increased circulating levels of hs-CRP in children with FH compared to control children (82). Our findings are consistent with these observations, and together they suggest an enhanced inflammation already present in children with FH. TNFα have been found to up-regulate neopterin levels (10). Thus, the increased gene expression levels of TNFα in PBMCs from FH children may potentially increase serum neopterin levels and may thus partly explain the previous findings.

Studies have shown that circulating levels of TNFα may predict cardiovascular events in humans (126-128). In these studies circulating levels of TNFα were suggested to be an independent predictor of risk for cardiovascular events in patients with unstable angina (126), and in healthy individuals (127;128). Moreover, circulating levels of TNFα were correlated to several established CVD risk factors e.g. LDL-C (127). Observation of increased gene expression levels of TNFα in adult FH patients, which have an increased risk of premature CAD, compared to healthy adult controls (58), may support the findings above. However to our knowledge, increased gene expression of TNFα already in children with FH has not previously been reported.

In vitro, TNFα acts by increasing the surface expression of the early atherosclerotic markers, ICAM-1, VCAM-1 and E-selectin on endothelial cells, SMC and macrophages (55). Indeed we found a significant correlation between gene expression levels of TNFα and sICAM-1 and sVCAM-1. The two circulating adhesion molecules, sICAM-1 and sVCAM-1 have previously been reported to predict CVD in different manners (53;129-132). sICAM-1 have been suggested to be a predictor of CVD in healthy adult individuals (129;130;133), whereas sVCAM-1 levels are

enhanced in adult patients with risks of CVD, and may hence predict future cardiovascular events or cardiovascular mortality (131;132;134).

In fact, TNFα may also mediate an indirect effect on the adhesion molecules through the up-regulation of CD40 and CD40 ligand (CD40L), cell-associated members of the TNF family. Subsequent binding of CD40 and CD40L may in turn enhance the surface expression levels of ICAM-1, VCAM-1 and E-selectin on the same cells mentioned above. However, serum levels of soluble CD40L (sCD40L) were not different between FH children and unaffected siblings (82). Thus, the increasing effect of TNFα on sCD40L is probably a pathway activated later in the atherogenesis as enhanced levels of sCD40L are regarded as a reliable marker of enhanced platelet activation (82).

An interesting finding in our study is the gender difference in the levels of circulating early atherosclerotic markers in the FH population. Here we show that FH boys are characterised by elevated levels of sE-selectin and sVCAM-1.

In the adult population gender difference for developing CAD is well established, where pre-menopausal women have a significantly decreased risk of developing CAD compared to age-matched men (34). Indeed, boys with FH have significantly thicker mean IMT of the carotid artery than FH girls (75), and a correlation between male gender and greater IMT has also been observed (74). When it comes to early atherosclerotic markers, increased sE-selectin and soluble P-selectin (sP-selectin) levels have been observed in men compared to age-matched women (114). Our study is to our knowledge, the first report showing that gender differences in the levels of sE-selectin and sVCAM-1 are present already in children with FH.

The gender difference in the levels of sE-selectin was only apparent in the FH population among the children above 15 years, whereas levels of sVCAM-1 showed a tendency to be higher in FH boys below the age of 15 compared to FH girls in the same age group. Together with sICAM-1, sE-selectin and sVCAM-1 were significantly correlated to the levels of testosterone in the FH population.

Regarding inflammation in atherosclerosis, effects of testosterone *in vitro* are reported to increase both mRNA and protein expression of E-selectin and VCAM-1 in human umbilical vein endothelial cells (HUVEC) stimulated with TNFα (46). Additionally, testosterone has been associated with increased total and LDL cholesterol and has been shown to increase atherosclerosis in primates and rabbits (135). Thus, the previously mentioned functions of testosterone may explain the increased risk of developing CAD in men compared to women, a gender difference in the risk of CAD which exists already when the boys enter puberty and lasts until the women pass by menopause (34). On the other hand, testosterone administration has been shown to decrease atherosclerosis in castrated male rabbits, and androgens seem in fact to have an antiatherogenic effect in men (135). Hence, the effect of testosterone in atherosclerosis is still unclear.

Surprisingly, in the FH population, we found an inverse correlation between total cholesterol and LDL-C and the early atherosclerotic markers (Appendix, Table I). Total and LDL cholesterol have been shown to decrease in the first period of puberty (from 9-15 years of age) (113), probably caused by increased production of sex hormones. Because of the enhanced cholesterol levels it is likely that this feature is more pronounced in an FH population. In our study a positive correlation was found between apoB and the early atherosclerotic markers in the oldest age group (above 15 years). ApoB and/or LDL-C levels have previously been shown to be associated with early atherosclerotic changes in the carotid arteries, and in addition apoB has been significantly associated with carotid artery IMT in children with and without FH (74). In summary, it seems that increased levels of testosterone (maybe in combination with the enhanced cholesterol level) may lead to increased levels of sE-selectin which again over time will aggravate the FH boys' risk for developing CAD compared to FH girls.

Since the increased levels of sVCAM-1 and sE-selectin differed in FH boys below 15 and above 15 years, sVCAM-1 may be differently regulated compared to sE-selectin. Below the age of 15 years FH boys have significantly decreased levels of testosterone

compared to FH boys above 15 years. Hence the tendency towards a higher level of sVCAM-1 may be influenced by other factors. Children with FH have been shown to have higher levels of oxLDL antibodies compared to control subjects (77). Since elevated LDL-C and/or oxLDL are suggested to cause endothelial dysfunction (31), these factors may potentially result in the tendency towards higher sVCAM-1 levels in FH boys below 15 years.

In five of the FH children serum samples before and after a period of statin treatment were available. We did a very small pilot study to see whether there were differences in the levels of the early atherosclerotic markers. Even in this small group of FH children we found a trend towards a reduction in the levels of sE-selectin ($p=0.08$). This is consistent with the finding showing that early statin treatment has a beneficial effect on endothelial function (22). In fact, decreased gene expression levels of E-selectin and VCAM-1 have been reported in HUVEC stimulated with TNFα in the presence of statin (47). The protein expression levels of E-selectin were however increased, whereas the levels of VCAM-1 were decreased after statin stimulation. This may be explained by inhibited disappearance rate of E-selectin from the endothelial surface after statin stimulation (47), and different time-regulation compared to expression of VCAM-1. Thus, it is not impossible that these effects of statins also operate in FH children, and may be responsible for the improvement of the endothelium seen in FH children after statin treatment (22). In addition, a recent study has shown that early initiation of statin treatment in FH children (8-18 years) was associated with a smaller IMT (14). Taken together these findings support early initiation of statin treatment in children with FH.

According to the NCEP guidelines for drug therapy recommendations in children, male gender is a risk factor in which drug therapy in children below 10 years with high-risk lipid abnormalities is considered (16). Our results support and strengthen this recommendation for initiation of statin therapy in boys before puberty as an attempt to abolish the adverse effects of testosterone on the early atherosclerotic markers later in puberty.

6.2.2 Adipokines and Leptin Receptor

There were no significant differences in the levels of adiponectin, leptin or leptin receptor in the FH population compared to the control subjects.

Adiponectin have recently been inversely correlated to markers of endothelial dysfunction and systemic inflammation, and hence suggested to be an important mediator in the development of CVD (63). Previously, decreased levels of adiponectin have been observed in both adolescent and middle-aged hyperlipidemic patients (61;62). Our results are inconsistent with these observations and suggest that the levels of adiponectin do not diverge in children with and without FH. Regarding the age of the adolescent participants in the previous study all the participants were below the age of 30 years and the mean age was approximately 20 years (61). In our study median age was 14.5 years in FH children and 16 years in control subjects. This difference in age may explain the opposing results between the two studies and may suggest that the decrement in serum levels of adiponectin occurs later in the atherosclerotic process. In fact, we have shown that serum levels of adiponectin are significantly decreased in FH children above 15 years compared to FH children below 15 years. This age difference was not seen when subdividing the control subjects likewise. In addition, inverse correlations were found between adiponectin levels and age only in the FH group. Although the number of control subjects were fewer, these results may suggest that adiponectin levels decrease with increasing age in FH children while the levels of adiponectin may stay constant in control children. However, more studies are needed to determine whether adiponectin levels decrease in adolescents with a risk of premature CVD.

Leptin has been linked to atherosclerosis through its pro-inflammatory effects (68). In dyslipidemic patients enhanced leptin levels have been observed compared to control subjects (111). The elevated levels of leptin in these patients correlated with higher BMI (111), a relation which is well established (136). However, children with FH are not charaterised by having higher BMI than control subjects (82), which might

explain why leptin levels in children with and without FH do not differ in the present study.

Not surprisingly, levels of leptin were significantly increased in FH girls compared to FH boys, and the difference was most pronounced in FH girls above 15 years. Gender divergence in serum levels of leptin where women have significantly enhanced levels of leptin is well established (136). This is probably caused by different levels of sex hormones and different amount of body fat mass in men and women. Our finding is consistent with this knowledge. A trend towards increased leptin levels in FH girls compared to control girls was additionally observed. However, an important finding is that FH girls above 15 years have significantly increased leptin levels compared to FH girls below 15 years. This finding was not present in control girls when subdividing likewise. Again, the small number of control girls might explain why we can not observe any age difference. Regarding the levels of leptin it is still tempting to suggest that FH girls may have increased levels of leptin compared to healthy girls. A possible explanation may be that LDL-C (which is significantly higher among FH girls) in combination with oestrogens may exacerbate this leptin profile in FH girls. Further investigation is needed to detect whether leptin levels really differs in girls with FH compared to control girls.

7. Conclusion and Clinical Implications

In the present study we have shown that:

1. FH children have an increased inflammatory profile compared to healthy control children, shown by:
    a. tendency towards increased sE-selectin levels
    b. significantly increased levels of TNFα gene expression
2. There are subgroup differences in atherosclerotic markers within the group of FH children:
    a. boys have significantly increased levels of sE-selectin and sVCAM-1 compared to girls
    b. boys above 15 years have significantly enhanced levels of sE-selectin compared to girls in the same age group
    c. boys below the age of 15 years have enhanced sVCAM-1 levels compared to girls in the same age group
    d. girls above 15 years have significantly enhanced levels of leptin compared to boys with in the same age group
    e. children above 15 years have significantly decreased levels of adiponectin compared to children below 15 years

In conclusion, our results may support the notion of increased inflammation in FH children. Furthermore, the results may also indicate that the gender difference in the levels of early atherosclerotic markers may be established already in childhood and may thus partly explain the gender difference in the risk of CVD. Based on these findings it may be suggested that initiation of statin treatment in FH children should start early in an attempt to reduce the levels of early atherosclerotic markers.

Initiation of statin treatment before puberty in boys may be of particularly importance to abolish the assumed adverse effect of testosterone on these markers.

8. Future Perspective

The present study has generated new questions and hypothesis.

8.1 Early Atherosclerotic Markers

Since we found significantly increased gene expression levels of TNFα in PBMCs from FH children compared to control subjects, we speculate that serum levels of TNFα may be enhanced in the same population as well. This will be investigated as one of the next steps. According to the *in vitro* effects of TNFα on the adhesion molecules (55), it is conceivable that serum levels of TNFα may be connected to these molecules. A link between TNFα and the trend towards an increased level of sE-selectin seen in FH children might be found when correlating these markers.

Furthermore, we want to investigate other early atherosclerotic markers, such as sP-selectin and fractalkine. P-selectin is another adhesion molecule involved in the recruitment and adhesion of leukocytes to the endothelium (43), and thus may be increased in the early steps of atherosclerosis. Additionally, gender differences in levels of sP-selectin have been observed in adults (114), and we wonder whether this gender difference is present already in FH children. Another adhesion molecule, fractalkine, which is expressed on endothelial cells upon stimulation of e.g. TNFα is also of interest (137). Together with its chemotactic effect, its adhesive properties support a presence of the molecule early in the atherosclerotic process.

8.2 Adipokines

The observed trend in increased leptin levels in FH girls compared to control girls needs further investigation. Therefore we want to increase the number of subjects and perform further analysis regarding leptin levels in girls. Visfatin, another adipokine, have been associated with cardiovascular conditions (138), and we want to

investigate if FH children have a different level of this marker. CD36, or the scavenger receptor expressed on macrophages, is responsible for the engulfing of modified LDL by macrophages in the intima (31). Thus, CD36 is involved in the early steps of atherosclerosis by contributing to formation of foam cells and fatty streaks. Furthermore, Rodenburg et al have shown that FH children have enhanced levels of some oxLDL antibodies compared to control subjects (77). Therefore, we want to investigate whether gene expression levels of CD36 is upregulated in FH children compared to control children.

8.3 Effects of Statin Treatment

Since the levels of sE-selectin had a tendency to be decreased by statins in the small preliminary pilot study, we want to collect a larger number of FH children before and after statin treatment in order to confirm whether statins really reduce sE-selectin and other early atherosclerotic markers. This collection of samples has already started and will be continued and analysed during the autumn 2008.

8.4 Screening of New Genes Involved in Early Atherosclerosis

In order to detect more genes which may be modified in FH children compared to control children, a microarray will be conducted. FH children with extremely high levels of LDL-C and severe, pathological family history of CAD, and age- and sex-matched control children will participate in this analysis.

8.5 *In Vitro* Effects of OxLDL and Testosterone

Previously, testosterone has been shown to increase expression of E-selectin and VCAM-1 in TNFα stimulated HUVEC (46). We speculate that testosterone and/or in combination with oxLDL may mediate increased expression of E-selectin and

VCAM-1 therefore we want to investigate the expression of these atherosclerotic markers on the surface of HUVEC after stimulation in presence or absence of testosterone, oxLDL and TNFα.

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10. Appendix

Table I. Correlations in the FH population (n=62).

		Adiponectin	sE-selectin	sICAM-1	Leptin	sVCAM-1	mRNA TNFα
Total cholesterol	Correlation coefficient	0.252		-0.407		-0.413	-0.551
	p-value	0.048		0.001		0.001	0.001
LDL-C	Correlation coefficient			-0.508		-0.459	-0.632
	p-value			0.000		0.000	0.000
ApoB	Correlation coefficient			-0.360		-0.386	-0.561
	p-value			0.015		0.009	0.004
Age	Correlation coefficient	-0.466			0.294		
	p-value	0.000			0.021		
sE-selectin	Correlation coefficient			0.331		0.278	
	p-value			0.009		0.029	
sICAM-1	Correlation coefficient		0.331			0.746	0.513
	p-value		0.009			0.000	0.002
Leptin	Correlation coefficient		-0.330				
	p-value		0.009				
sVCAM-1	Correlation coefficient		0.278	0.746			0.442
	p-value		0.029	0.000			0.009
mRNA ICAM-1	Correlation coefficient						0.375
	p-value						0.029

Not all the parameters were available from all the participants. ApoB= apolipoprotein B, FH= familial hypercholesterolemia, LDL-C= low density lipoprotein cholesterol, sE-selectin= soluble E-selectin, sICAM-1= soluble intercellular adhesion molecule-1, sVCAM-1= soluble vascular cell adhesion molecule-1, TNFα= tumor necrosis factor α.

Table II. Correlations among the control subjects (n=22).

		sE-selectin	sVCAM-1	mRNA leptin receptor	mRNA TNFα	mRNA ICAM-1
LDL-C	Correlation coefficient					-0.746
	p-value					0.013
ApoB	Correlation coefficient	-0.502	0.455			
	p-value	0.024	0.044			
sICAM-1	Correlation coefficient		0.582		0.713	
	p-value		0.005		0.009	
sVCAM-1	Correlation coefficient			0.580	0.615	-0.853
	p-value			0.048	0.033	0.000
mRNA leptin receptor	Correlation coefficient					-0.587
	p-value					0.045
mRNA ICAM-1	Correlation coefficient		-0.853	-0.587		
	p-value		0.000	0.045		

Not all the parameters were available from all the participants. CRP= C-reactive protein, ICAM-1= intercellular adhesion molecule-1, LDL-C= low density lipoprotein cholesterol, mRNA= messenger ribonucleic acid, sICAM-1= soluble intercellular adhesion molecule-1, sVCAM-1= soluble vascular cell adhesion molecule, TNFα= tumor necrosis factor α.