Children with familial hypercholesterolemia are characterized by an inflammatory imbalance between the tumor necrosis factor α system and interleukin-10

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Objective: Familial hypercholesterolemia (FH) is associated with increased risk of premature atherosclerosis. Increasing evidence supports involvement of inflammation in atherogenesis. The inflammatory cytokine tumor necrosis factor (TNF)α has been regarded as a key mediator in the development of atherosclerosis due to its involvement in several stages in this process. We hypothesized that children with FH, as a model of early atherosclerosis, have different serum levels of inflammation markers than healthy control children.

Methods: We measured serum levels of TNFα, as well as its endogenous inhibitors (i.e., soluble TNF receptors [sTNFR] 1 and 2) and the anti-inflammatory cytokine interleukin (IL)-10 in healthy children (7–20 years) with (n = 102) and without (n = 48) heterozygote FH as well as adult FH subjects (n = 20) and healthy adult controls (n = 16).

Results: The main findings were: Compared to control children, FH children had higher serum levels of TNFα, accompanied by lower sTNFRs levels, resulting in an increased TNFα/sTNFRs ratio (P < 0.05), potentially reflecting enhanced TNFα activity. In contrast to the increased TNFα levels, FH children had decreased serum levels of IL-10 (P < 0.01) resulting in an increased TNFα/IL-10 ratio (P < 0.01). We did not observe any difference in the same parameters between adult subjects with and without FH.

Conclusions: FH children are characterized by an inflammatory imbalance between TNFα and IL-10, potentially contributing to the accelerated atherosclerotic process in these individuals.

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1. Introduction

Familial hypercholesterolemia (FH) is a monogenic disorder usually caused by mutations in the low density lipoprotein (LDL)-receptor gene. Heterozygote FH patients are characterized by markedly elevated serum concentration of cholesterol from birth, and show premature atherosclerosis and cardiovascular disease (CVD) in both genders [1–3].

In general, atherosclerosis is a progressive process in which lipids, extracellular matrix and activated vascular smooth muscle cells accumulate in the arterial wall resulting in growth of an atherosclerotic plaque. Several lines of evidence support a major role of inflammation in these processes, involving up-regulation of inflammatory cytokines, activation of monocytes and other leukocyte subsets as well as endothelial cell activation [4,5]. In fact, one of the initial key events in atherogenesis is the bidirectional inflammatory interaction between lipids and monocytes/macrophages, leading to foam cell formation, endothelial cell activation and a state of non-resolving inflammation within the vessel wall [4,6].

While numerous studies show enhanced inflammation in adult patients with hypercholesterolemia or CVD [7–9], few studies have elucidated the inflammatory arm of early atherosclerosis. While the phenotype in FH adults is heavily influenced by confounding fac-
tors such as lifestyle and dietary habits, children with FH are less prone to be influenced by such confounding factors and may therefore serve as a model for studies on the interplay between lipids and inflammation. Recently, we have shown that the inflammatory profile may differ between FH children and FH adults [10,11], but these issues are far from clear.

The inflammatory response does not only depend on the levels of inflammatory cytokines, but also on the ability of endogenous inhibitors (e.g., soluble cytokine receptors and antagonists) and anti-inflammatory cytokines to counteract these inflammatory mediators [12]. Tumor necrosis factor (TNFα) has been regarded as a key mediator in the development of atherosclerosis at least partly due to its involvement in the recruitment of inflammatory cells into the lesion and its ability to promote foam cell formation and macrophage activation [5]. To further investigate the inflammatory pathway in children with FH, we therefore measured serum levels of TNFα as well as the soluble TNF receptors (sTNFRs) and interleukin (IL)-10, a prototypical anti-inflammatory cytokine [13] in clinically healthy children with and without heterozygous FH.

2. Methods

2.1. Subjects

2.1.1. Children

One hundred-and-two children with heterozygous FH were consecutively recruited at the Lipid Clinic, Oslo University Hospital Rikshospitalet, Oslo, Norway (Table 1). All the FH children included in the study, except for two, had definite FH as diagnosed by genetic testing. Two children had a definite FH diagnosis based on clinical criteria. The diagnostic criteria for FH was based on the Dutch Lipid Clinic network classification (World Health Organization publication no WHO/HCN/FH/CONS/99.2) where definite (certain) FH is defined with a score of 8 or more. Forty-eight healthy children of the same sex and age, without hypercholesterolemia, recruited among friends of the FH children or children of the hospital staff, were included as a control group. All FH children were clinically healthy without diagnosis of CVD and none were using statins or any other cholesterol lowering or anti-inflammatory drugs at the time of the blood sampling.

2.1.2. Adults

For comparison, blood samples were also collected from twenty adults with heterozygous FH (seven females and thirteen males) with a definite FH diagnosis as based on DNA testing (Table 2). Prior to blood sampling all adult FH patients had a four week wash-out period without statin treatment. Sixteen healthy sex- and age-
matched adult volunteers (seven females and nine males) with no history of hypertension, diabetes, CVD or other acute or chronic illness were recruited as controls for the adult FH patients. None of the FH individuals (children or adults) had any concomitant inflammatory disease such as infection and autoimmune disorders or liver or kidney disease. Not all analyses were performed in all the subjects due to the limited amount of blood sample available from each individual. Serum samples from both children and adults were collected and stored as previously described [14]. The study was approved by the Regional Committee of Medical Ethics and it complies with the Declaration of Helsinki. Written informed consent was obtained from all participants or from one of their parents when the children were <16 years of age.

2.2. Enzyme immunoassay

Concentration of sTNFR type 1 (sTNFR1), sTNFR type 2 (sTNFR2), TNFα, IL-10 and high sensitivity C-reactive protein (hs-CRP) were measured by enzyme immunoassays from R&D Systems (Minneapolis, MN). The intra- and inter-assay coefficient of variation was <10% for all assays.

2.3. Miscellaneous

Standard blood chemistry and lipid parameters were measured in plasma using in house routine laboratory methods [15]. Z-score was calculated using the formula \[ z = x - \mu / \sigma \], where \( x \) is the raw score to be standardized; \( \mu \) is the mean of the population and \( \sigma \) is the standard deviation of the population.

2.4. Statistical analysis

Data are given as median (minimum–maximum) if not otherwise stated. Comparisons between two groups of individuals were made by using the Mann–Whitney U-test. Coefficients of correlation were calculated by the Spearman rank test. Probability values (2-sided) were considered statistically significant at a value of \( P < 0.05 \). Calculations were performed by PASW statistics 18. Based on standard deviations from the manufacturer of the immunoassays and previous studies by ourselves (standard deviation = 0.45 ng/ml for sTNFR2, standard deviation = 1.2 ng/ml for CRP) and a ratio of controls to patients of 1:4, the required sample size with 80% power and alpha of 0.05 to detect a 30% difference was 15 and 60, controls and patients respectively.

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**Table 1** Characteristics of participants.

<table>
<thead>
<tr>
<th></th>
<th>Control children (n = 48)</th>
<th>Children with FH (n = 102)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>14 (8–19)</td>
<td>14 (9–20)</td>
</tr>
<tr>
<td>Male (%)</td>
<td>48</td>
<td>54</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>4.2 (2.4–6.3)</td>
<td>7.1 (4.0–10.5)</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>2.3 (1.2–3.6)</td>
<td>5.3 (2.4–8.7)</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.5 (0.8–2.7)</td>
<td>1.3 (0.8–2.4)</td>
</tr>
<tr>
<td>ApoA-I (g/l)</td>
<td>1.4 (0.7–2.4)</td>
<td>1.3 (0.9–2.1)</td>
</tr>
<tr>
<td>ApoB (g/l)</td>
<td>0.6 (0.2–0.9)</td>
<td>1.3 (0.6–1.8)</td>
</tr>
<tr>
<td>hs-CRP (mg/l)</td>
<td>0.17 (0.05–0.90)</td>
<td>0.11 (0.05–3.15)</td>
</tr>
</tbody>
</table>

Data are presented as median (range). LDL were only available in control n = 46. CRP were only available in FH n = 97 and control n = 39. ApoA-I and ApoB were only available in FH n = 86 and control n = 45. Apo (A and B) = apolipoprotein, FH = familial hypercholesterolemia, HDL = high density lipoprotein, hs-CRP = high sensitivity C-reactive protein, LDL = low density lipoprotein.

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**Table 2** Characteristics of participants.

<table>
<thead>
<tr>
<th></th>
<th>Control adults (n = 16)</th>
<th>Adults with FH (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>41 (20–69)</td>
<td>41 (19–74)</td>
</tr>
<tr>
<td>Male (%)</td>
<td>56</td>
<td>65</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>4.8 (2.5–6.3)</td>
<td>8.5 (6.7–11.1)</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>3.1 (1.4–4.6)</td>
<td>6.7 (3.1–9.5)</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.3 (0.8–1.8)</td>
<td>1.1 (0.7–1.8)</td>
</tr>
<tr>
<td>ApoA-I (g/l)</td>
<td>1.4 (1.0–1.9)</td>
<td>1.2 (1.0–1.8)</td>
</tr>
<tr>
<td>ApoB (g/l)</td>
<td>1.0 (0.5–1.2)</td>
<td>1.8 (1.4–2.6)</td>
</tr>
<tr>
<td>hs-CRP (mg/l)</td>
<td>1.44 (0.20–5.90)</td>
<td>1.20 (0.60–13.40)</td>
</tr>
</tbody>
</table>

Data are presented as median (range). Apo (A and B) = apolipoprotein, FH = familial hypercholesterolemia, HDL = high density lipoprotein, hs-CRP = high sensitivity C-reactive protein, LDL = low density lipoprotein. * P < 0.05 vs. control subjects.
3. Results

3.1. Characterization of the subjects

Compared to healthy control children, FH children had significantly higher plasma concentrations of total- and LDL-cholesterol and apolipoprotein (Apo)B (Table 1) and significantly lower levels of HDL and apoA-1. However, no significant difference was observed in the levels of hs-CRP (Table 1). Body mass index (BMI) (19.9 [13.9–30.3] kg/m², n = 95), Z-score (−0.18 [−1.94 to 2.87], n = 95), and plasma triglycerides (0.8 [0.4–3.6] mmol/l, n = 69), median [min–max], respectively, were available only for the FH population. Compared to healthy control adults, FH adults had significantly higher plasma concentrations of total- and LDL-cholesterol and ApoB as well as significantly lower plasma concentrations of ApoA-1 (Table 2). There was no significant difference in hs-CRP levels between the two groups (Table 2).

3.2. Circulating levels of TNFα and the sTNFRs

As shown in Fig. 1(A)–(C), FH children were characterized by higher serum levels of TNFα (P = 0.075) accompanied by significant lower serum levels of the sTNFR2, whereas there was no significant difference in the level of sTNFR1. The ratio between TNFα and sTNFRs may provide an estimate of the molar balance in serum between TNFα and its soluble receptors, potentially being a parameter of TNF activity[16]. In molar terms, this ratio was defined as TNFα (pmol/l)/(sTNFR1 + sTNFR2) (pmol/l), assuming a molecular mass of (17 × 3) kDa for TNFα (trimer) and 30 kDa for both types of sTNFRs [16]. As can be seen in Fig. 1(D), FH children had significantly raised TNFα/sTNFRs ratio as compared with healthy control children suggesting enhanced TNFα activity in these individuals.

3.3. Circulating levels of IL-10

In contrast to serum levels of TNFα, FH children had significantly lower serum levels of the anti-inflammatory cytokine IL-10 (Fig. 2(A)). TNFα is a potent stimulus for IL-10, potentially reflecting a physiological counter mechanism [12], and notably FH children had markedly increased TNFα/IL-10 ratio (molecular mass of [17 × 3] kDa for TNFα [trimer] and 18.6 kDa for IL-10) as compared with control children, potentially reflecting an inflammatory imbalance in the FH group (Fig. 2(B)). Within the FH group, IL-10
levels were significantly correlated with levels of sTNFR1 ($r = 0.43$, $P < 0.01$) and sTNFR2 ($r = 0.37$, $P < 0.01$). In contrast, there were no significant correlations (either positive or negative) between TNFα and IL-10 or between TNFα and its soluble receptors (data not shown).

### 3.6. The association between LDL receptor mutation type and plasma lipids and inflammatory markers

In our group of FH children carriers of 24 different LDL receptor mutations were present, with the majority of the mutations represented by the three Norwegian founder mutations FH-Elverum, FH-Svartor and FH-C210G. Two of these founder mutations (FH-Elverum and FH-Svartor) are predicted to be null-allele mutations whereas FH-C210G is predicted to disrupt the ligand-binding domain [17]. We therefore examined if there were any difference in circulating lipid and inflammatory levels in the FH children carrying the null-allele mutations (FH-Elverum and FH-Svartor, $n = 40$) compared to the FH children carrying the other mutation types ($n = 60$). FH children carrying the null-allele LDL receptor mutation type had significant higher plasma total cholesterol ($P < 0.01$), LDL cholesterol ($P < 0.01$) and ApoB levels ($P < 0.05$) as well as higher sTNFR2 levels ($P < 0.05$). However, none of the other inflammatory markers did differ between the two LDL receptor mutation groups (data not shown).

### 4. Discussion

In the present study we have analyzed serum levels of TNFα parameters and IL-10 in a relatively large cohort of children with and without FH. We found that apparently healthy FH children were characterized by higher serum levels of TNFα, concomitantly with lower sTNFRs levels leading to an increased TNFα/sTNFRs ratio, potentially reflecting enhanced activity in the TNFα system. In addition, FH children had significantly lower IL-10 levels as compared with the controls, resulting in an increased TNFα/IL-10 ratio. In contrast to the FH children, none of these parameters differed between adult FH patients and adult healthy controls. Although we can not exclude some anti-inflammatory effects of statins even four weeks after stop of therapy in the adult FH group, these findings may suggest some differences in the inflammatory phenotype between children and adults with FH, implicating that inflammation is an early characteristic of atherogenesis in these pre-disposed individuals.
TNFα has been regarded as a key mediator in the development of atherosclerosis due to its involvement in the recruitment of inflammatory cells to the lesion, its ability to promote foam cell formation and macrophage activation as well as its feature as a potent inducer of matrix degradation and development of a pro-thrombotic phenotype [5]. TNFα is expressed in human atherosclerotic plaques, co-localized with foam cells, vascular smooth muscle cells and mast cells [18]. In experimental studies TNFα-deficient hypercholesterolemic mice develop less atherosclerosis [19], and with relevance to FH, TNFα deficiency seems to retard fatty-streak lesion formation [20]. TNFα levels have been associated with increased intima-media thickness [21] and to be an independent predictor of future cardiovascular events in patients with unstable angina [22] and healthy individuals [23]. Previous data show lower normal [24] and increased [25] TNFα levels have been reported in FH adolescents and adults, in small studies (n < 15). In the present study, examining 102 FH children and 48 sex- and age-matched controls, we show that the FH group had higher TNFα levels accompanied by lower sTNFRs levels, resulting in a marked increase in the molar ratio between TNFα and sTNFRs. The role of sTNFRs has been debated, potentially mediating antagonizing or stabilizing effects on TNFα activity [26]. Serum/plasma levels of these soluble receptors have also been suggested to reflect circulating TNFα level [27]. However, simultaneous measurement of TNFα and its soluble receptors, with calculation of a TNFα/sTNFRs ratio, seems to be a more reliable parameter of TNFα activity, significantly correlated with TNFα bioactivity [28].

IL-10 seems to attenuate atherogenesis. Studies in IL-10 transgenic and IL-10-deficient mice models [29] suggest an important protective role for this cytokine in both the formation and the stabilization of atherosclerotic lesions [29,30]. We have shown that IL-10 inhibits the release of inflammatory cytokines, tissue factor, and matrix metalloproteinases from peripheral blood mononuclear cells (PBMCs) in patients with acute coronary syndrome, potentially promoting plaque stabilization [12]. Individuals with high cholesterol levels and normal coronary angiogram are characterized by raised IL-10 levels and low ratios of TNFα/IL-10, suggesting anti-inflammatory net effect in this “high-risk” group without coronary artery disease [31]. In fact, IL-10 has been proposed as an “immunological scalpel” in the atherosclerotic process [32]. Here, we demonstrate significantly decreased serum levels of IL-10 and increased TNFα/IL-10 ratio in apparently healthy FH children as compared with healthy controls, further underscoring an inflammatory phenotype in FH children. IL-10 has been found to stimulate the release of soluble TNF receptors in PBMCs [33], and we found a significant correlation between IL-10 and serum levels of both soluble TNFRs within the FH group. Thus, the decreased levels of IL-10 and sTNFRs in FH children may reflect an impaired interrelated anti-inflammatory potential in these individuals contributing to a state of non-resolving inflammation in this early stage of atherogenesis. TNFα is a potent stimulus for IL-10, and the combination of raised TNFα levels and decreased IL-10 levels may perhaps seem somewhat surprising. However, an attracting hypothesis could be that monocytes and other IL-10 secreting cells from FH patients may have an attenuated IL-10 response on TNFα activation, which could be of importance for a state of persistent inflammation in these individuals. However, although attracting, this hypothesis will have to be investigated in forthcoming studies.

There was no correlation between LDL cholesterol and the inflammatory markers in our FH children population, which may have several not-mutually exclusive explanations. First, the various plasma cholesterol levels observed in untreated FH subject seems to be less associated with cardiovascular risk than in subject without FH. In FH, it seems as if the most important risk factor in patients who do not use statins, is whether there is a mutation or not [34–36] as also supported by our data. Therefore, within a FH cohort, LDL cholesterol levels and HDL cholesterol levels may not be expected to correlate with inflammatory markers in the same manner as in non-FH subjects. Second, it is well established that children during puberty experience a reduction in circulating cholesterol levels [17], at least partly, reflecting the increased production of sex hormones. The median age of our FH children are 14 years, and it is therefore conceivable that a correlation between cholesterol levels and inflammatory markers may be masked. Finally, lack of correlation does not necessarily mean lack of causal relationship, and correlation data should in general be interpreted with some caution.

Although we have no data on the cellular origin of the inflammatory cytokines in FH patients, it is tempting to hypothesize that the early vascular lesion that may be seen in these individuals may contribute to a low-grade systemic inflammation. While not necessarily correlated to the level of LDL, this lipid component may promote inflammatory responses within early vascular lesions. These responses could again activate additional inflammation pathways leading to persistent inflammation that are not dependent on LDL. In particular, the low levels of IL-10 may further contribute to such a state of non-resolving inflammation that characterizes the atherosclerotic process.

In contrast to parameters of TNF activation, we found no difference in serum levels of hs-CRP between children with and without FH. The leptherole of CRP as an inflammatory biomarker in CVD is primarily based on its ability to reflect upstream inflammation such as activity in the IL-6 system. However, it is unlikely that one particular marker could reflect the entire activity of the rather complicated inflammatory network that is operating in atherogenesis. The relatively importance of these different pathways may also differ between the different stages of the atherosclerotic process. Although further studies are needed, our finding in the current study may suggest that markers of TNF activity may be more relevant than CRP to reflect systemic inflammation in FH children.

The present study has some limitations as lack of longitudinal data, the relative small number of healthy control subjects, lack of data on sub-clinical atherosclerosis (e.g., intima-media thickness), lack of data on fasting glucose and insulin levels and lack of data on effect of statin treatment. Nonetheless, our findings suggest an inflammatory phenotype in apparently healthy FH children. Although we have no firm data on this issue, we believe that combination of raised levels of inflammatory mediator (i.e., TNFα) and decreased levels of anti-inflammatory or resolving mediators (i.e., IL-10) may be of importance for the accelerated atherogenesis in FH children. However, further studies are needed to elucidate the role of TNFα and IL-10 in early atherosclerosis.

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References


