Correlates of endothelial function and their relationship with inflammation in patients with familial hypercholesterolaemia.


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Atherosclerosis is characterized by a low-grade systemic inflammatory response and endothelial dysfunction. The aim of the present study was to investigate a possible relationship between systemic markers of inflammation, serum markers of endothelial activation and endotheliodependent vasodilatation in a group of high-risk patients, and to evaluate the effects of intervention with high doses of simvastatin on these parameters. In patients with heterozygous familial hypercholesterolaemia (FH), without atherosclerotic events, flow-mediated vasodilatation (FMD) of the brachial artery was measured after a wash-out period for lipid-lowering drugs (baseline) and after 6 weeks of treatment with simvastatin 80 mg daily. Levels of C-reactive protein (CRP), soluble intercellular adhesion molecule-1 (s-ICAM) and soluble E-selectin (s-E-selectin) were determined at baseline and again after 6 weeks and 12 months of therapy. A total of 35 patients participated in the study (age 42 years, 60% male). When divided into tertiles according to FMD (<3.9%, 3.9-9.0%, >9.0%), no differences in levels of CRP, s-ICAM-1 and/or s-E-selectin were detected between the groups. Moreover, no changes in FMD, levels of CRP or levels of s-ICAM-1 and/or s-E-selectin were found during treatment with simvastatin. We conclude that endothelial function, as reflected by FMD, does not seem to be related to markers of inflammation in FH patients at high risk of, but without clinically overt signs of, atherosclerosis. Moreover, aggressive lipid-lowering therapy with simvastatin does not result in improved endothelial function or in a reduction of markers of inflammation in these patients.

INTRODUCTION

The atherosclerotic process is characterized by a low-grade inflammatory response affecting the endothelium of large arteries [1]. Markers of inflammation such as C-reactive protein, and levels of soluble adhesion molecules E-selectin and intercellular adhesion molecule-1 are independent indicators for cardiovascular disease [2;3].

Endothelial dysfunction is a collective term that incorporates a number of changes that the endothelium undergoes during atherogenesis, i.e. loss of anticoagulant properties, increased expression of cellular adhesion molecules and increased vascular tone by loss of bioavailability of vasodilatory endothelial nitric oxide (NO) [4]. NO-dependent vasodilatation is thought to reflect endothelial function and, if impaired, is predictive for future cardiovascular disease [5]. Flow-mediated vasodilatation (FMD) of the brachial artery is a non-invasive technique used to assess endothelial function. FMD has been shown to be mediated by endothelial NO release [6]. Several risk factors for cardiovascular diseases are associated with an impaired flow mediated vasodilatation [7;8]. Modification of cardiovascular risk factors such as hypercholesterolaemia [9] and hypertension [10] has been shown to improve endothelial function.

Although both inflammation and endothelial dysfunction have been investigated extensively as hallmarks of atherosclerosis, the extent to which these entities are related is unclear. The present study aimed to investigate a possible relationship between endothelial dysfunction, as measured by FMD, and the inflammatory response. Furthermore, we studied whether correction of hyperlipidaemia with high doses of simvastatin resulted in improvement of endothelial dysfunction and/or markers of inflammation in high-risk patients without clinically overt atherosclerosis.

MATERIALS AND METHODS

Study population

Patients were participants in an open-label incremental-dose multicenter study, designed to evaluate the safety and tolerability of simvastatin 80 mg in
Inflammation and endothelial dysfunction

patients with heterozygous familial hypercholesterolaemia (FH). Patients were included in the study if they met at least one of the following criteria for heterozygous FH: a proven mutation in the LDL receptor gene or a clinical diagnosis according to standardised, well-established criteria [11]. Patients had to be at least 18 years of age.

Exclusion criteria were: homozygous FH, a documented history of myocardial infarction, coronary artery bypass grafting or percutaneous coronary angioplasty, diabetes mellitus, a history of cancer, rheumatoid arthritis, vasculitis, idiopathic lung fibrosis, inflammatory bowel disease or auto-immune inflammatory disorders, hypersensitivity to simvastatin, patients with hypercholesterolaemia type I, IV or V, pregnant or nursing women, or fertile women who were not on contraception, persistent elevations of serum transaminases (> 1.2 x upper limit of normal), renal in-sufficiency (serum creatinin > 2.0 mg/dl), creatin kinase > 3x ULN, hypothyroidism, nephrotic syndrome, alcohol or drug abuse, unstable angina within the past 3 months, concurrent use of niacin or fibrates, concurrent use of erythromycin or systemic antifungal agents, and treatment with an investigational drug.

Ethics
All patients gave written informed consent. The research has been carried out in accordance with the Declaration of Helsinki (1989) of the World Medical Association, and has been approved by the local medical ethical committee.

Study design
After a washout period of 8 weeks for fibrates and 6 weeks for all other lipid-lowering drugs, baseline measurements were performed. Next, all patients were given simvastatin 80 mg once daily in an open-label fashion. Follow-up laboratory measurements were performed after 6 weeks and 12 months of taking the study drug. FMD was measured at baseline and after 6 weeks.

Data collection
Baseline screening included a medical history, physical examination and laboratory investigations (described in further detail below). Directly afterwards FMD was assessed using a wall-track system (further details below).

Laboratory measurements
Overnight fasting venous blood was obtained following non-traumatic venipuncture. Total cholesterol, HDL-cholesterol and triglycerides were determined according to routine laboratory techniques. LDL-cholesterol was calculated using the Friedewald formula. For other measurements, blood was collected in EDTA tubes, centrifuged immediately at 2500g during 5 min at 4°C and plasma samples were stored at −20°C until further analysis. Levels of CRP [12] and of s-ICAM [13] were determined using an in-house ELISA as described previously. Levels of s-Es-electin were measured using a commercially available ELISA kit (Bender MedSystems, Vienna, Austria). All measurements were done using the same batch and in duplicate.

Measurement of flow-mediated brachial-artery reactivity
An ultrasonographic method was used to assess flow-mediated brachial-artery reactivity was used [14]. The measurement system consisted of an ultrasound scanner (Scanner 200, Pie Medical, Maastricht, the Netherlands) and a PC with a high-speed data acquisition board, sample frequency 21.5 MHz. Dedicated software (Wall Track system 2.0, Pie Medical) was used to measure and analyse the changes in vessel diameter. Using a 7.5 MHz transducer the brachial artery was visualized. A two-dimensional longitudinal B-mode image of the brachial artery was obtained. The radio frequency (RF) signals from the M-mode output were relayed to the wall-tracking system and stored digitally.

Measurements were conducted with the patient in supine position at a constant room temperature. A custom-built holder
was used to stabilize the probe during the measurements. Procedures were as follows: (1) two baseline measurements of brachial-artery diameter; (2) inflation of a pneumatic tourniquet placed around the forearm distal to the segment of artery scanned; (3) deflation of the tourniquet after 4 min, which increases blood flow to the distal part of the forearm, inducing an endothelium-dependent brachial-artery reaction; (4) measurement of the brachial-artery diameter after deflation continuously for six cycles of 22 s; (5) measurement of the brachial artery 2.5 and 5 min after giving nitroglycerine (NTG) 0.4 mg sublingually, resulting in endothelium independent brachial-artery reaction. Using the RF signal, the anterior and posterior vessel wall were identified and marked. Vessel wall movements were tracked using off-line analysis. This enabled measurement of end-diastolic diameter for each beat. Each measurement consisted of data acquisition for 22 s, and the average end-diastolic diameter of these 22 s was used.

The intra- and inter-observer variability of this system at our institution is 2.5 % and 5.0 % for end-diastolic brachial-artery diameter, respectively. The off-line data analysts were blinded to the clinical and time point parameters of the patients. Flow-mediated brachial-artery reactivity was calculated as the maximal percentage increase in arterial diameter during hyperaemia compared to the average of two baseline diameters, and NTG-mediated brachial-artery reactivity was calculated the same way to obtain the maximal post-NTG diameter.

Statistics
Continuous data are presented as median (interquartile range). Categorical data are presented by percentage or count of each category, as indicated.

Baseline comparisons between the biohumoral parameters and FMD were performed using an F-test or the Kruskal-Wallis test. Paired group comparisons were performed using Friedman's test for more than two groups. In case of a two-group comparison a paired Student's t-test or, if the distribution was skewed, the Wilcoxon signed rank test was used. A Shapiro-Wilk test was used to assess normality.

In the correlation analysis, Pearson's (for normally distributed variables) or Spearman’s (in case of skewed distribution) correlation coefficients were calculated and tested under Ho:Rho = 0. A P-value of < 0.05 (two tailed) was considered statistically significant. All analyses were performed using commercially available computer software (Statistical Analysis System version 6.12; SAS Institute, Cary, NC, USA).

RESULTS
Study population
Baseline FMD data were obtained in 35 patients. Patient data were stratified into tertiles, according to percentage FMD. The lower tertile had a % FMD ranging from -9.7 % to 3.6 %, the second tertile had a % FMD ranging from 3.6 % to 9.0 % and the highest tertile had a % FMD ranging from 9.0 % to 32.8 %. The baseline characteristics of these patients are provided in table 1. Study drugs were well tolerated by all subjects; none of the patients changed medication during the study period.

<table>
<thead>
<tr>
<th>Table 1.</th>
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<tbody>
<tr>
<td><strong>Baseline characteristics (n=35)</strong></td>
</tr>
<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>Sex (M/F)</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
</tr>
<tr>
<td>Risk factors</td>
</tr>
<tr>
<td>Hypertension (%)</td>
</tr>
<tr>
<td>Current smoking (%)</td>
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<tr>
<td>Familiar predisposition for cardiovascular disease (%)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
</tr>
<tr>
<td>Co-medication</td>
</tr>
<tr>
<td>Angiotensin converting enzyme-inhibitors (%)</td>
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<td>Angiotensin II-antagonists (%)</td>
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<tr>
<td>Diuretics (%)</td>
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</tbody>
</table>

Data are presented as median (interquartile range)
Flow mediated dilatation and post-NTG responses at baseline
At baseline the median (interquartile range, IQR) diameter of the brachial artery was 4.9 (4.4-5.2 mm). The median post-ischemia diameter was 5.1 (4.6-5.7 mm). \( P < 0.001 \) compared with baseline, and the median diameter after nitroglycerine was 5.3 (4.7-6.0 mm). Median FMD was 5.7 % (12.1-13.6 %), whereas the median NTG-mediated brachial-artery response, as compared with baseline, was 9.1 % (6.0-16.2 %).

Levels of serum lipids
Mean (±SD) levels of serum total cholesterol were 10.3 ± 2.6 mmol/L at baseline, 6.3 ± 1.7 mmol/L after 6 weeks of treatment, and 5.8 ± 1.0 mmol/L after 12 months of treatment \( P < 0.001 \). Levels of LDL-cholesterol were respectively 8.3 ± 2.6 mmol/L, 4.4 ± 1.6 mmol/L and 3.8 ± 0.9 mmol/L \( P < 0.001 \). HDL-cholesterol increased from 1.22 ± 0.39 to 1.39 ± 0.48 and 1.35 ± 0.44 mmol/L respectively \( P < 0.001 \). Triglyceride levels were 1.80 ± 0.94 mmol/L at baseline, 1.16 ± 0.44 mmol/L after 6 weeks and 1.28 ± 0.60 mmol/L at 12 months \( P < 0.01 \).

Markers of inflammation and endothelial cell activation graded for brachial artery response at baseline.
Levels of CRP, s-ICAM and s-E-selectin were calculated according to tertiles FMD. None of these markers differed statistically between groups (figure 1).

Table 2. Markers of inflammation and endothelial function during follow-up

<table>
<thead>
<tr>
<th>Marker</th>
<th>baseline</th>
<th>6 weeks</th>
<th>1 year</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP (mg/L)</td>
<td>1.7</td>
<td>1.0</td>
<td>1.3</td>
</tr>
<tr>
<td>(1.1-3.0)</td>
<td>(0.5-2.5)</td>
<td>(0.6-1.9)</td>
<td></td>
</tr>
<tr>
<td>ICAM (ng/mL)</td>
<td>138</td>
<td>129</td>
<td>143</td>
</tr>
<tr>
<td>(106-181)</td>
<td>(99-185)</td>
<td>(101-192)</td>
<td></td>
</tr>
<tr>
<td>E-selectin (ng/mL)</td>
<td>66</td>
<td>63</td>
<td>57</td>
</tr>
<tr>
<td>(52-88)</td>
<td>(49-87)</td>
<td>(46-83)</td>
<td></td>
</tr>
<tr>
<td>FMD (%)</td>
<td>5.7</td>
<td>6.2</td>
<td>n.a.</td>
</tr>
<tr>
<td>(1.2-13.7)</td>
<td>(1.3-12.5)</td>
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</table>

Data are presented as median (interquartile range). No significant differences are apparent. n.a., not applicable

Correlation coefficients
Spearman’s Rho for the relationship between FMD and levels of CRP at baseline was –0.14 \( P = 0.43 \). For baseline FMD and levels of s-ICAM, the correlation coefficient was –0.21 \( P = 0.27 \), and for baseline FMD and levels of s-E-selectin it was 0.15 \( P = 0.38 \). Pearson’s correlation coefficient between baseline FMD and post-NTG dilatation was 0.83 \( P < 0.001 \).

Markers of inflammation and endothelial function during follow-up in all patients.
Data for levels of s-ICAM, s-E-selectin and CRP during follow-up as well as data on FMD during follow-up, are provided in table 2.

DISCUSSION
The key finding of our present study is the absence of a relationship between endothelial dysfunction, as defined by FMD, and the low-grade inflammatory response, as reflected by levels of CRP, s-ICAM-1 and s-E-selectin in high-risk patients without clinically overt atherosclerosis. Moreover, no changes in endothelial function after 6 weeks or in inflammatory response after 1 year of aggressive lipid-lowering therapy were found.

At first sight, our results may seem to contradict earlier reports. However, reports in the literature regarding the existence of impaired FMD in patients with hypercholesterolaemia are not consistent. Using intra-arterial infusions of acetylcholine in the lower arm, Stroes et al. [9] reported endothelial dysfunction in hypercholesterolemic persons off treatment without overt atherosclerotic disease. Short-term lipid lowering normalized the response. Our young, clinically unaffected persons with FH had FMD values comparable with those measured in persons with microalbuminuria, which is often considered as a marker of endothelial dysfunction [15]. The FMD values in our young FH patients are considerably lower than those reported recently by Hoffman et al. [16] in FH.
patients (mean 10.7%), but are not different from those reported by Spieker et al. [17]. Hayoz et al. [18] found no difference in FMD between patients newly diagnosed with hypercholesterolaemia and control persons. We conclude that some degree of endothelial dysfunction, as defined by FMD measurements, may indeed have been present in our patients.

Signs of low-grade inflammation, as reflected by CRP levels, were also present in our group with early atherosclerosis. Hwang et al. [3] found higher levels of endothelial adhesion molecules in patients with manifest cardiovascular disease. The possible difference in levels of ICAM and E-selectin in the present study as compared with that of Hwang et al. [3] may be related to the degree of progression of atherosclerosis. Ridker et al. [2] found that higher CRP levels in apparently healthy people were associated with an increased risk of the later development of cardiovascular disease. The CRP levels in our study were even higher than those reported by Ridker et al. [2].

Few studies have addressed the relationship between markers of inflammation and the occurrence of endothelial dysfunction. Recently, important evidence has been brought forward linking these two major hallmarks of atherosclerotic disease. A reduced FMD was found in patients with active systemic necrotizing vasculitis, and FMD returned towards normal after the inflammatory disease diminished [19]. Healthy volunteers who had an acute inflammatory response after Salmonella Typhi vaccination exhibited transient endothelial dysfunction [20]. It can, therefore, be postulated that more fulminant inflammation is required to induce endothelial cell dysfunction, since these previous studies investigated endothelial dysfunction in acute inflammatory disease, whereas hypercholesterolaemia is merely a chronic condition. A direct relationship between levels of CRP and the occurrence of endothelial dysfunction was seen in a clinical study, although it comprised only

patients with proven coronary artery disease [21]. Moreover, recombinant CRP in the presence of human serum induces the expression of cell adhesion molecules on human endothelial cells in vitro [22]. Johns et al. [23], on the other hand, could not demonstrate a relationship between endothelial function and levels of soluble cell adhesion molecules in a patient group quite similar to ours. We feel that endothelial dysfunction and inflammation may be related in more severe and/or acute disease entities than can be found in hypercholesterolaemic patients without evidence for cardiovascular disease.
Possibly less surprising was the lack of correlation between FMD and levels of the soluble cell adhesion molecules s-ICAM-1 and s-E-selectin. Although both adhesion molecules are known to be elevated in patients at risk of atherosclerosis [3;24], their value as markers of cardiovascular disease remains controversial [23;25].

The second study objective was to evaluate the effects of simvastatin on markers of inflammation and flow-mediated vasodilation. Perhaps our most remarkable finding is the lack of effect on levels of CRP after instituting aggressive lipid-lowering therapy. It has been elegantly demonstrated that statins exert anti-inflammatory effects on endothelial cells as well as monocytes/macrophages [26]. The effects of lipid-lowering therapy on both endothelial function and markers of inflammation have been investigated extensively in different populations and with different compounds. Most reports that have been published support an effect [27;28], although the majority of these studies have been performed in patients with established atherosclerosis. It should be emphasized that the population that we studied did not have any sign of clinical atherosclerotic vascular disease. A previous study [29] also did not detect an effect of simvastatin on levels of CRP, again in patients with FH, most of them free of cardiovascular disease. Another possible explanation for our results is that various statins seem to have different pharmacological effects [30]. One objection might be that the duration of the washout period in our study was too short. However, comparable time periods have been used in other studies [9]. The dosage of simvastatin and the treatment duration should also be considered sufficient.

With regard to the effects of simvastatin on endothelial function testing, our findings are consistent with those of Vita et al. [31], who were the first to report a placebo-controlled study on the effects of simvastatin on coronary endothelial function, and in this setting they found no effects of the statin. Notably, blinded analysis of the FMD end-points in a group of patients who otherwise exhibit the expected response to simvastatin (i.e., drastic lowering of serum lipids) supported the notion that the lack of correlations and the lack of a response to simvastatin are real. A type II error may also have been the cause of the lack of outcomes, but in another report [15] no relationship between FMD and microalbuminuria was demonstrated in a large group of 770 patients. Moreover, it is nowadays ethically unacceptable to leave patients with FH untreated, and thus a placebo-controlled design was not an option.

Last but not least, the methodology of FMD measurements must be a matter of concern. The outcomes of different endothelial function tests should not be generalized [32]. The longitudinal reproducibility of FMD testing has been disputed [33], but this would not explain the total lack of correlation at baseline in our study.

In conclusion, our data do not support the hypothesis that low-grade inflammation and endothelial dysfunction (as defined by FMD) co-occur in clinically non-atherosclerotic patients with heterozygous FH. Moreover, we were not able to confirm that instituting aggressive lipid-lowering therapy with simvastatin in these patients reduces these early risk markers of atherosclerotic disease.

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