

Chemokines in Children With Heterozygous Familiar Hypercholesterolemia

Selective Upregulation of RANTES

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Objective—Increasing data support the involvement of chemokines in atherogenesis. However, although several studies have shown increased chemokine levels in adult patients, the literature is virtually devoid of data on chemokines in children with hypercholesterolemia.

Methods and Results—We examined the gene expression of chemokines in peripheral blood mononuclear cells (PBMCs) from clinically healthy children with and without heterozygous familial hypercholesterolemia (FH). Our main findings were: (1) compared with healthy controls, PBMCs from FH children showed significantly higher mRNA levels of RANTES, but not of the other examined chemokines; (2) an opposite pattern was seen in adult FH subjects, with markedly enhanced expression of macrophage inflammatory peptide-1 α , but not of RANTES; (3) this increased gene expression of RANTES in PBMCs from FH children seemed to reflect enhanced RANTES expression in monocytes but not in T cells; (4) FH children also had raised serum levels of neopterin, additionally suggesting monocyte/macrophage activation in these children; and (5) PBMCs from both FH children and controls showed enhanced release of interleukin 8 on RANTES stimulation in vitro.

Conclusions—Our findings support a role of inflammation also in the early stages of atherogenesis possibly involving monocyte-derived RANTES as an important mediator. (*Arterioscler Thromb Vasc Biol*. 2006;26:200-205.)

Key Words: atherosclerosis ■ hypercholesterolemia ■ inflammation ■ RANTES ■ leukocytes

Familial hypercholesterolemia (FH) is caused by a mutation in the gene encoding for low-density lipoprotein (LDL) receptor, resulting in 2-fold to 3-fold elevated plasma levels of LDL. It is believed that the atherosclerotic process starts in childhood.^{1,2} Indeed, children with heterozygous FH that are born with increased LDL levels show premature atherosclerosis and cardiovascular disease (CVD) in both genders.¹ Moreover, FH children seem also to have greater intima media thickness than sex- and age-matched healthy controls.³ Furthermore, autopsy material from the general population shows atherosclerotic lesions and fatty streaks at a young age.^{4,5}

Increasing amounts of data support the involvement of inflammation in atherogenesis, and several lines of evidence suggest that chemokines are important mediators in this process by activating and directing leukocytes into the atherosclerotic lesions.^{2,6,7} Thus, enhanced expression of interleukin (IL) 8 and monocyte chemoattractant protein (MCP) 1 is found within human atherosclerotic plaques.^{8,9} Moreover, recent in vivo studies have shown that targeted disruption of the genes for CCR2 (ie, MCP-1 receptor) and CXCR2 (ie, IL-8 receptor)

decrease atherosclerotic lesion formation in mice prone to develop atherosclerosis.¹⁰⁻¹² However, whereas several studies have shown increased chemokine levels in adult patients with hypercholesterolemia, with and without CVD,¹³⁻¹⁷ the literature is virtually devoid of data on chemokines in children with hypercholesterolemia. Such data could be of importance to clarify the role and nature of inflammation in the early stages of atherosclerosis. To elucidate these issues, we examined the expression of several chemokines in peripheral blood mononuclear cells (PBMCs) from clinically healthy children with and without heterozygous FH.

Methods

Subjects

Children

Thirty-three children with heterozygous FH were recruited at the Lipid Clinic, Rikshospitalet, Oslo, Norway (Table). All of the subjects were diagnosed with definite heterozygous FH by DNA testing. Twenty healthy subjects of the same sex and age, without hypercholesterolemia, recruited among friends of the FH children, were asked to take part in the study as a control group (Table). All

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Characteristics of the Participants

Characteristic	FH Children (n=33)	Control Children (n=20)	Adult FH (n=14)	Adult Controls (n=10)
Age, y	14 (12 to 16)	14 (13 to 17)	47 (39 to 58)	42 (38 to 50)
Female, %	61	60	50	50
Total cholesterol, mmol/L	7.7 (6.9 to 8.5)†	4.1 (3.6 to 4.6)	9.1 (7.9 to 10.7)†	4.6 (4.2 to 5.3)
LDL cholesterol, mmol/L	6.3 (5.2 to 6.7)†	2.6 (2.1 to 2.9)	7.8 (6.6 to 8.7)†	3.0 (2.5 to 3.5)
HDL cholesterol, mmol/L	1.3 (1.1 to 1.5)*	1.5 (1.3 to 1.8)	1.2 (1.0 to 1.6)	1.3 (0.9 to 1.6)
CRP, mg/L	0.58 (0.26 to 0.84)	0.36 (0.21 to 0.62)	1.44 (0.91 to 3.87)	1.77 (0.63 to 3.29)

Data are presented as median (25th to 75th percentile).

* $P<0.05$ vs control subjects.

† $P<0.001$ vs control subjects.

of the FH children were clinically healthy without any diagnosis of CVD, and none were using statins. Not all of the analyses were performed in all of the children because of the limited amount of blood sample available from each individual.

Adults

For comparison, blood samples were also collected from 14 adults with heterozygous FH (7 males and 7 females) with a definite FH diagnosis as based on DNA testing. Three of the FH patients had diagnosed CVD, but all of these had been free of symptoms for >6 months. Before blood sampling, all of the adult FH patients had a 4-week washout period without statin treatment. Ten healthy sex- and age-matched adult volunteers (5 females and 5 males) with no history of hypertension, diabetes, CVD, or other acute or chronic illness were recruited as controls for the adult FH patients (Table).

None of the FH individuals (children and adults) had any concomitant inflammatory disease, such as infection and autoimmune disorders, or liver or kidney disease. Neither the FH children nor the FH adults or any of the controls were taking anti-inflammatory drugs including statins. Plasma and serum samples from both children and adults were divided into aliquots and stored at -80°C as described previously.¹⁸ Written informed consent was obtained from all of the participants or from 1 of their parents when the children were <18 years of age. The study was conducted according to the ethical guidelines at our hospital according to the Helsinki declaration and was approved by the hospital's authorized representative.

Isolation of Cells

PBMCs were obtained from heparinized blood by isopaque-Ficoll (Lymphoprep, Nycomed Pharma AS) gradient centrifugation within 45 minutes. Additional separation of monocytes (CD14-labeled magnetic beads; MACS, Miltenyi Biotec) and CD3⁺ T cells (negative selection by monodisperse immunomagnetic beads, Dynal) was performed as described elsewhere.^{19,20} After isolation, PBMCs, monocytes, and T cells were immediately stored at -80°C (mRNA analyses) or used for cell culturing (PBMCs, see below). The selected T cells consisted of >90% CD3⁺ cells and the isolated monocytes of >95% CD14⁺ cells (flow cytometry).

Cell Culturing

Freshly isolated PBMCs were incubated in flat-bottomed 96-well trays (Costar, Corning Inc; 2×10^6 /mL; 100 μL /well) in medium alone [RPMI-1640 with 2 mmol/L L-glutamine and 25 mmol/L HEPES buffer (Gibco) supplemented with 5% FCS], or with different concentrations of regulated on activation normally T-cell expressed and secreted (RANTES). In some experiments, the cells were stimulated with oxidized LDL (oxLDL, final concentration 20 $\mu\text{g}/\text{mL}$) and recombinant human heat shock protein 60 (hsp60; Low Endotoxin, StressGen Biotechnologies Corp; final concentration 1 $\mu\text{g}/\text{mL}$). LDL was isolated from human endotoxin-free heparin plasma²¹ and oxidatively modified by Cu²⁺-ions.²² Cell-free supernatants and cell pellets were harvested after culturing for 24 hours and stored at -80°C until analysis. The endotoxin levels of all of the

stimulants and culture media were <10 pg/mL (Limulus Amebocyte Assay, BioWhittaker).

RNase Protection Assay

Total RNA was extracted from PBMCs using RNeasy columns (Qiagen) and stored in RNA storage solution (Ambion) at -80°C . RNase protection assay was performed with the chemokine (hCK5) multiprobe (Pharmingen).²³ The mRNA signal was normalized to the signal from the housekeeping genes rpL32 and GAPDH.

Real-Time Quantitative RT-PCR

Total RNA was extracted from PBMCs in adult individuals with the RNA Isolation kit I using the MagNa Pure LC Instrument (Roche Applied Science) and from T cells and monocytes in children as described above (RNase protection assay), subjected to DNase I treatment (RQI DNase; Promega) and stored at -80°C . Primers for RANTES [forward primer (FP): 5'-CCAGCAGTCGTCTTG-TCA-3' and reverse primer (RP): 5'-TCCGAACCCATTCTTCT-CT-3'] and macrophage inflammatory peptide (MIP) 1 α (FP: CTGCATCACTTGCTGACA-3' and RP: 5'-CACTGGCTGC-TCGTCTCAAAG) were designed using the Primer Express software version 1.5 (Applied Biosystems). Quantification of mRNA was performed using the ABI Prism7000 (Applied Biosystems) and the qPCR Master Mix for SyBr Green I (Eurogenetec).²⁴ The gene expression of the housekeeping gene β -actin (FP: 5'-AGGCACCAGGGCGTGAT-3' and RP: 5'-TCGTCCCAGTT-GGTGACGAT-3'; Applied Biosystems) was used for normalization.

Enzyme Immunoassays

IL-8, RANTES, and MCP-1 levels in cell-free supernatants were measured by enzyme immunoassays from R&D Systems. Plasma levels of neopterin were measured by an enzyme immunoassay provided by IBL Hamburg. The intra-assay and interassay coefficients of variations were as follows: RANTES, 2.9% and 6.7%; MCP-1, 5.9% and 5.9%; IL-8, 4.7% and 8.1%; neopterin, 6.8% and 10.3% for the intra-assay and interassay, respectively.

Miscellaneous

Standard blood chemistry and lipid parameters were measured in serum/plasma using routine laboratory methods at our hospital.²⁵ Serum level of C-reactive protein (CRP) was measured by a high-sensitivity immunoturbidimetric assay (Roche Diagnostic).

Statistical Analysis

Nonparametric tests were used for all of the statistical calculations. Data from FH and control subjects were compared by using the Mann-Whitney *U* test. Friedman and Wilcoxon matched-pairs tests were used for repeated measures. The present study was designed to detect rather large differences ($>30\%$), and with 15 patients and 10 healthy controls, we had a 95% power to detect 30% difference with an α of 0.05. *P* values (2-sided) were considered statistically significant at a value of $P<0.05$.

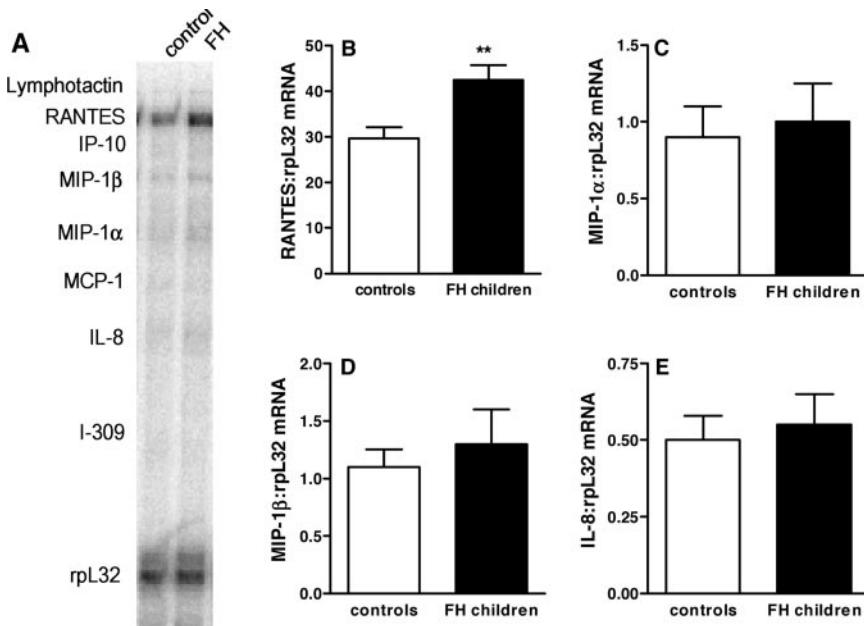


Figure 1. Expression of chemokines in freshly isolated PBMCs from FH children ($n=20$) and sex- and age-matched healthy controls ($n=11$). A, RNase protection assay from 1 representative FH child and 1 representative healthy control. The other panels show RANTES (B), MIP-1 α (C), MIP-1 β (D), and IL-8 (E) mRNA levels in the FH children and the healthy controls given as mean \pm SEM. The gene expression is normalized to the housekeeping gene rpL32. Similar results were obtained when GAPDH was used as control gene. IP-10, IFN- γ inducible protein 10; I-309, inducible 309.

** $P<0.01$ vs controls.

Results

Characterization of the Subjects

Characterization of the participants is shown in the Table. Compared with healthy control children, FH children had significantly higher plasma concentrations of total and LDL cholesterol, and significantly lower levels of HDL cholesterol. Compared with healthy adults, adult FH subjects had significantly higher levels of total and LDL cholesterol. Moreover, either FH children or FH adults had raised plasma levels of CRP as compared with their respective controls (Table).

The Expression of Chemokines in PBMCs

Blood samples for isolation of PBMCs were available in 20 of the FH children, and in 11 of the healthy control children, and as shown in Figure 1, cells from the FH children had significantly enhanced gene expression of RANTES. In contrast, there was no difference in the mRNA levels of MIP-1 α , MIP-1 β , and IL-8 between these 2 groups of children (Figure 1). The other examined chemokines [ie, MCP-1, interferon (IFN) γ inducible protein 10, and inducible 309] were nondetectable in both FH and control children (Figure 1A). In contrast to the enhanced RANTES expression in PBMCs, RANTES levels in plasma were not upregulated in the FH children [41.7 ng/mL (17.2 to 49.7 ng/mL) and 61.5 ng/mL (39.9 to 85.6 ng/mL); median (25th to 75th percentile), FH and control children, respectively]. Because platelets are thought to be the major contributor to plasma levels of RANTES,²⁶ this finding could reflect that platelet activation is not an important feature in FH children, as well as in the early stage of atherosclerosis.

We and others have shown previously a different chemokine pattern in PBMCs from adult patients with CVD with raised levels of MIP-1 α , MIP-1 β , and IL-8, but not of RANTES, comparing healthy adult controls,^{8,13} and, notably, such a pattern was also seen in the adult FH subjects. Thus, whereas there was a very modest and nonsignificant increase in RANTES expression in PBMCs from adult FH subjects,

MIP-1 α expression was markedly upregulated in the adult FH group (Figure 2). This pattern was in marked contrast to the expression pattern in PBMCs from FH children showing a significant upregulation of RANTES accompanied by no or very modest changes in MIP-1 α and MIP-1 β expression (Figure 1 as compared with Figure 2).

Expression of RANTES in Monocytes and T Cells

To study the relative contribution to the enhanced RANTES expression of the various subsets within the PBMC population, we examined the gene expression of RANTES in freshly isolated T cells and monocytes from 10 of the FH children and 9 of the age- and sex-matched healthy controls. Although there was no difference in RANTES expression in T cells between these 2 groups of children, monocytes from FH children showed markedly enhanced RANTES expression comparing healthy controls (≈ 2.5 -fold increase, Figure 3).

Plasma Neopterin Levels in FH and Controls

Our findings so far suggest enhanced monocyte activation in FH children compared with healthy control children, and to additionally elucidate this issue, we measured plasma neopterin levels in these 2 groups of individuals. Neopterin is regarded as a reliable and stable marker of monocyte activa-

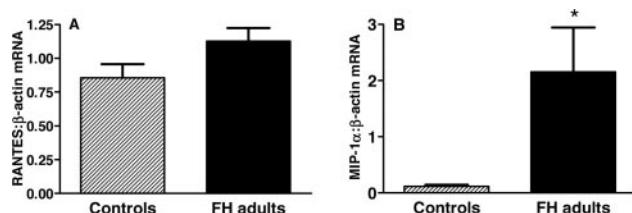


Figure 2. Gene expression of RANTES (A) and MIP-1 α (B) in freshly isolated PBMCs from adult FH subjects ($n=14$) and sex- and age-matched healthy controls ($n=10$). Data are mean \pm SEM. mRNA levels were quantified by real-time RT-PCR, and data are presented relative to the gene expression of the housekeeping gene β -actin. * $P<0.05$ vs controls.

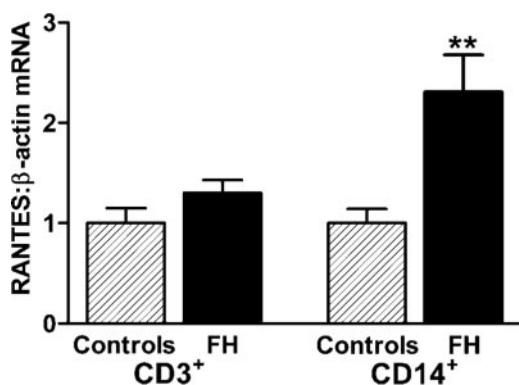


Figure 3. Gene expression of RANTES in T cells ($CD3^+$) and monocytes ($CD14^+$) from 10 FH children and 9 sex- and age-matched healthy controls. Data are mean \pm SEM. mRNA levels were quantified by real-time RT-PCR, and data are presented relative to the gene expression of the housekeeping gene β -actin. ** $P < 0.01$ vs controls.

tion *in vivo*,²⁷ and, notably, we found that FH children had significantly raised neopterin levels compared with healthy controls (Figure I, available online at <http://atvb.ahajournals.org>). In contrast, we found no differences in neopterin levels between adult FH subjects and adult controls [6.16 (5.02 to 8.10) nmol/L versus 5.20 (4.53 to 7.94) nmol/L, median (25th to 75th percentile), respectively]. Whereas this finding may at least partly reflect that relatively few adult individuals were examined, it may also additionally suggest a different pattern of monocyte activation in adult and young FH individuals. Although IFN- γ -activated pathways have been shown to enhance neopterin levels, we found no differences in T-cell mRNA expression of IFN- γ between FH ($n=5$) and control children ($n=5$; data not shown).

OxLDL Promotes RANTES Expression in PBMCs From FH Children

FH children have been reported to have raised oxLDL levels in the circulation,²⁸ and several lines of evidence support a role for hsp60 in the initial stages of atherosclerosis.²⁹ To elucidate the possible mechanisms for the enhanced RANTES expression in PBMCs from FH children, we next examined RANTES levels in PBMCs after stimulation with oxLDL and hsp60 for 24 hours in 8 FH children. Whereas oxLDL markedly enhanced RANTES levels in these cells, as shown both at the mRNA and protein levels, the hsp60-mediated RANTES increase did not reach statistical significance (Figure II, available online at <http://atvb.ahajournals.org>). Thus, although additional studies are needed, these

findings may suggest that the enhanced RANTES expression in PBMCs/monocytes from FH children primarily reflect lipid-dependent mechanisms.

RANTES Promotes Chemokine Release in PBMCs

To elucidate any possible consequences of the enhanced RANTES expression in FH children, we next examined the *in vitro* effect of RANTES on the release of IL-8 and MCP-1 in PBMCs from 9 of the FH children and 7 of the healthy control children. As shown in Figure 4A, RANTES dose-dependently increased the release of IL-8 in both groups of children, with a trend toward a stronger RANTES response in PBMCs from FH children, in particular, at lower RANTES concentrations. The RANTES-stimulated release of MCP-1 was more modest, reaching statistical significance only at the highest RANTES concentration in cells from healthy children (Figure 4B).

Discussion

We and others have previously shown markedly enhanced expression of IL-8, MIP-1 α , and MIP-1 β in PBMCs from CVD patients, with and without hypercholesterolemia, with no difference in the expression of RANTES,^{14,17,30} and, herein, we show a similar chemokine pattern in adult FH patients. Surprisingly, an “opposite” pattern was seen in PBMCs from FH children with markedly enhanced RANTES expression comparing mRNA levels in healthy control children, accompanied by low IL-8, MIP-1 α , and MIP-1 β expression in both groups of children. These results, together with our findings of increased neopterin levels in FH children, confirm a role of inflammation also in children with hypercholesterolemia. However, the selective upregulation of RANTES in FH children suggests that the relative contribution of the various inflammatory mediators may be different in the early as compared with the late stage of atherosclerosis.

It has been reported previously that platelet-derived RANTES could promote monocyte recruitment and arrest to atherosclerotic arteries and accelerate the formation of atherosclerotic lesions, as well as thrombus formation.^{31,32} Moreover, enhanced expression of RANTES and CCR5 has been reported in LDL receptor^{-/-} mice fed with a high-cholesterol diet,³³ as well as in human atherosclerotic plaques.^{34,35} Furthermore, Veillard et al³³ have shown recently that the CC chemokine antagonist Met-RANTES reduces the progression of atherosclerosis in a hypercholesterolemic mouse model accompanied by decreased expression of several chemokines and chemokine receptors, a reduction in leukocyte infiltration, and an increase of collagen-rich atheroma, features associated with plaque stabilization. Herein, for the first time,

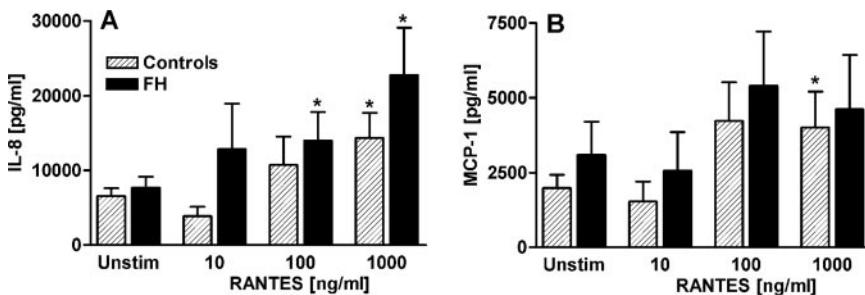


Figure 4. The effect of different concentrations of RANTES on the release of IL-8 (A) and MCP-1 (B) in PBMCs from 9 FH children and 7 healthy control children after culturing for 24 hours. The protein levels of IL-8 and MCP-1 were measured in cell-free supernatants by enzyme immunoassays. Data are mean \pm SEM. * $P < 0.05$ vs unstimulated (unstim) cells.

we report enhanced RANTES expression in PBMCs from FH children suggesting the involvement of this chemokine also in the early stages of atherosclerosis. Our findings showing that oxLDL is a potent inducer of RANTES in PBMCs from FH children, suggesting that this enhanced RANTES expression may at least partly be lipid driven.

Several reports suggest that the immune response in monocytes/macrophages may change during aging, including the response to oxLDL,³⁶ and such mechanisms could potentially contribute to the different chemokine expression in PBMCs/monocytes from young and adult FH individuals. Moreover, an early upregulation of RANTES within the atherosclerotic lesion, as opposed to a late upregulation of other chemokines, such as MIP-1 α and MIP-1 β , was recently demonstrated during atherogenesis in apolipoprotein E^{-/-} mice, and, notably, this late upregulation of MIP-1 α and MIP-1 β was accompanied by a downregulation of RANTES.³⁷ This report may additionally support our findings in the present study with a different ratio between RANTES and MIP-1 α in young and adult FH individuals. However, the present study lacks mechanistic data on this issue, and the reasons for the different chemokine profile in young and adult FH individuals is at present unclear and will have to be clarified in forthcoming studies.

The immunopathogenesis of atherosclerosis involves a complex interaction between various leukocyte subsets, platelets, endothelial cells, and vascular smooth muscle cells.^{2,6} Arterial recruitment of monocytes leading to foam cell formation within the vessel wall is an early step in atherosclerosis,² and in the present study we show enhanced expression of RANTES in monocytes, but not in T cells, from FH children. These FH children also had raised serum levels of neopterin, additionally supporting monocyte activation as an important feature in these individuals. Although IFN- γ -activated pathways have been shown to enhance neopterin levels in various disorders, we found no differences in T-cell expression of IFN- γ between FH and control children. However, it has been questioned whether raised neopterin levels are exclusively mediated by enhanced release of IFN- γ . Thus, several monocyte-derived cytokines, such as IL-1, IL-6, and tumor necrosis factor α , have been found to upregulate neopterin levels,^{38,39} and some of these could potentially be operating in FH children.

The lack of systemic T-cell activation, however, does not exclude T-cell activation within the vessel wall. Actually, the enhanced RANTES expression in monocytes, as shown in the present study, could in itself contribute to inflammation involving T cells and other leukocyte subsets. An inflammatory response to RANTES was also suggested by our in vitro studies showing an enhanced RANTES-mediated release of IL-8 in PBMCs from both FH children and healthy controls. Thus, it is tempting to hypothesize that whereas the RANTES levels in circulating monocytes may be too low to induce any systemic inflammatory responses, the concentration of RANTES within the atherosclerotic plaque may be much higher, particularly in the monocyte/macrophage-rich area, leading to activation of adjacent leukocytes and other cellular components of the atherosclerotic lesion.^{31,32} Nevertheless, our findings in the present study showing enhanced expres-

sion of RANTES in monocytes but not in T cells from FH children, as well as increased plasma levels of neopterin but not of CRP in these FH children, suggest that systemic monocyte activation is an early feature of the atherosclerotic disorder. In fact, whereas the lack of increased CRP levels in FH children may reflect that relatively few individuals were studied, it also suggests that other inflammatory markers, such as neopterin, should be more thoroughly examined as potential stable markers of inflammation in these individuals.

Millonig et al⁴⁰ have previously reported the presence of inflammatory cells in atherosclerotic plaques in 15- to 34-year-old subjects, arguing in favor of an initiating role of the immune system in atherogenesis. In the present study, we show that FH children at an even lower age are characterized by an enhanced systemic inflammation involving a selective upregulation of the CC-chemokine RANTES in circulating monocytes, as well as raised levels of neopterin being a stable marker of monocyte/macrophage activation. Although relatively few subjects were examined, these findings additionally support a role for inflammation and, in particular, monocyte/macrophage-mediated inflammation also in the early stages of atherogenesis. However, whereas these findings indicate low-grade inflammation in the early stages of the atherosclerotic process, they also suggest that the character of inflammation may be different in FH children compared with adult FH patients with and without accompanying CVD.

Acknowledgments

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Chemokines in Children With Heterozygous Familiar Hypercholesterolemia: Selective Upregulation of RANTES

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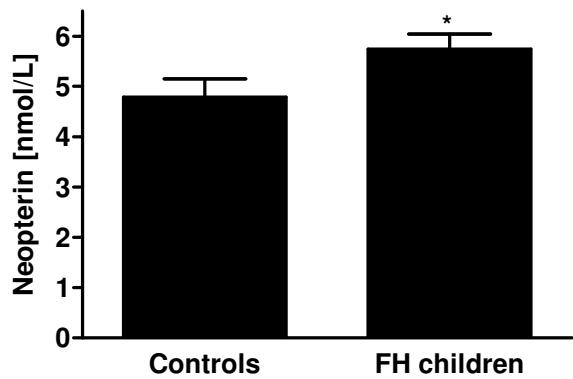
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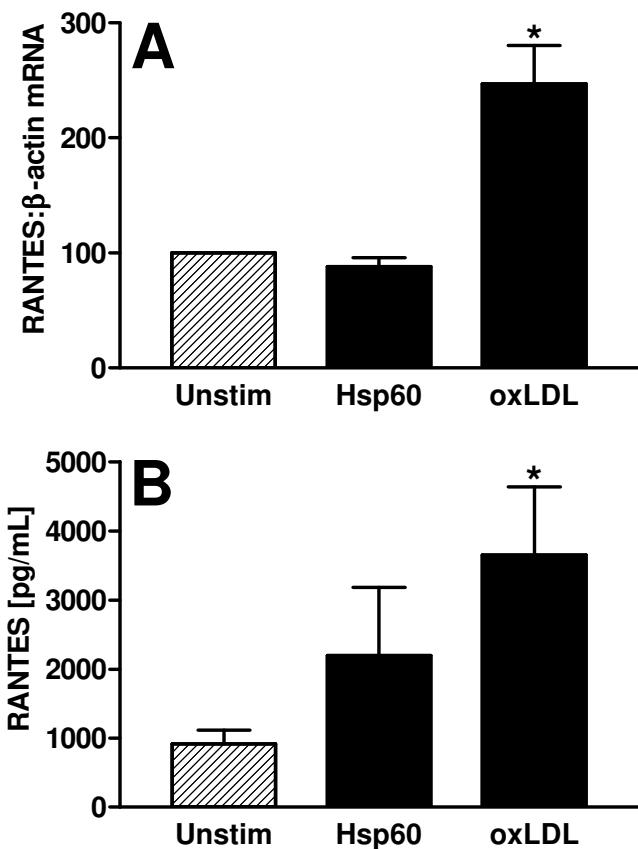
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Feature I



Feature I. Plasma neopterin levels in 33 FH children and 20 sex- and age-matched healthy controls. Data are given as mean \pm SEM. * $P<0.05$ versus controls.

Feature II



Feature II. The effect of oxidized LDL (oxLDL, 20 µg/mL) and recombinant human heat shock protein 60 (hsp60, 1 µg/mL) on the gene expression of RANTES (panel A) and the release of RANTES (panel B) in PBMC from eight FH children after culturing for 24 hours. mRNA levels were quantified by real-time RT-PCR and data are presented relative to the gene expression of the house-keeping gene β-actin. The protein levels of RANTES were measured in cell-free supernatants by EIA. Data are given as mean±SEM.

* $P<0.05$ versus unstimulated (unstim) cells.