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Oxidized LDL level is related to gene expression of tumour necrosis factor super family members in children and young adults with familial hypercholesterolaemia

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Abstract. Narverud I, Halvorsen B, Nenseter MS, Retterstøl K, Yndestad A, Dahl TB, Ulven SM, Olstad OK, Ose L, Holven KB, Aukrust P (Institute for Basic Medical Sciences, University of Oslo, Oslo; Faculty of Health Sciences, Oslo and Akershus University College of Applied Sciences, Oslo; The Lipid Clinic Oslo University Hospital Rikshospitalet, Oslo; Research Institute for Internal Medicine, Oslo University Hospital Rikshospitalet, Oslo; Faculty of Medicine, University of Oslo, Oslo; Oslo University Hospital Ullevål, Oslo; and Oslo University Hospital Rikshospitalet, Oslo, Norway). Oxidized LDL level is related to gene expression of tumour necrosis factor super family members in children and young adults with familial hypercholesterolaemia. *J Intern Med* 2013; **273**: 69–78.

Objective. Familial hypercholesterolaemia (FH) is associated with increased risk of premature atherosclerosis. Inflammation is a key event in atherogenesis, and we have previously reported an inflammatory imbalance between tumour necrosis factor (TNF)α and interleukin-10 in children with FH. Based on the potential role of TNF-related molecules in inflammation, we investigated the regulation of other members of the TNF superfamily (TNFSF)/TNF receptor superfamily (TNFRSF) in children and young adults with FH and matched healthy controls.

Methods. Expression of *TNFSF/TNFRSF* genes in peripheral blood mononuclear cells (PBMCs) was quantified in children and young adults with FH prior to (n = 42) and after statin treatment (n = 10) and in controls (n = 25) by quantitative real-time polymerase chain reaction.

Results. First we found that, compared with controls, the mRNA levels of *OX40L, BAFFR* and *TRAILR1* were significantly higher, whereas *TRAIL* and *TRAILR3* were significantly lower in children and young adults with FH. Secondly, levels of oxidized low-density lipoprotein (oxLDL) were significantly raised in the FH group, and correlated with the expression of *OX40L*, *BAFFR* and *TRAILR1*. Thirdly, oxLDL increased mRNA levels of *BAFFR, TRAILR1* and *TRAILR4* in PBMCs *ex vivo* from individuals with FH. Fourthly, OX40, acting through OX40L, enhanced the oxLDL-induced expression of matrix metalloproteinase-9 in THP-1 monocytes *in vitro*. Finally, after statin treatment in children with FH (n = 10), mRNA levels of *OX40L* and *TRAILR1* decreased, whereas levels *BAFF, TRAIL* and *TRAILR3* increased.

Conclusion. Our findings suggest the involvement of some TNFSF/TNFRSF members and oxLDL in the early stages of atherogenesis; this may potentially contribute to the accelerated rate of atherosclerosis observed in individuals with FH.

Keywords: atherosclerosis, cardiovascular risk factors, children, familial hypercholesterolaemia, inflammation.

Introduction

Familial hypercholesterolaemia (FH) is an autosomal dominant disorder mainly caused by a mutation in the gene encoding the low-density lipoprotein (LDL) receptor. FH patients are characterized by increased serum levels of total and LDL cholesterol from birth, with increased risk of premature

development of atherosclerosis and cardiovascular disease (CVD) [1–3].

The pathophysiological hallmark of atherosclerosis is a combination of lipid accumulation and enhanced inflammation within the vessel wall, involving the interaction of lipids with various inflammatory cells such as monocytes/macrophages and T cells [4, 5]. Several inflammatory mediators are involved in this process including tumour necrosis factor α (TNFα) and other members of the TNF superfamily (TNFSF)/TNF receptor superfamily (TNFRSF). Thus, both clinical and experimental studies of atherosclerosis have shown the involvement of TNFα as well as OX40/TNFRSF4 and OX40 ligand (OX40L)/TNFSF4, receptor activator of nuclear factor κB (RANK)/TNFRSF11A and RANK ligand (RANKL)/TNFSF11A, and TNF-related apoptosis-inducing ligand (TRAIL)/TNFSF10 in atherogenesis, with both enhancing (e.g. TNFα, OX40/OX40L and RANK/RANKL) and inhibitory (e.g. TRAIL) effects on this process [6–11].

Although the role of inflammation in adult patients with hypercholesterolaemia and established CVD is well documented [12, 13], few studies have examined the regulation of inflammatory mediators in early atherosclerosis. In adults, the atherosclerotic process has been influenced by confounding factors, such as lifestyle and dietary habits, for decades. Therefore, investigating inflammatory markers in children and young adults with FH provides a suitable approach to study inflammation in early atherosclerosis. We have previously shown an inflammatory imbalance between TNFα and interleukin (IL)-10 in children and young adults with FH [14]. Because of the potential role of several TNF-related molecules in atherogenesis, in the present study we have investigated the gene expression of members of the TNFSF/TNFRSF in peripheral blood mononuclear cells (PBMCs) in children and young adults with FH and matched healthy controls.

Materials and methods

Subjects

Forty-two children and young adults with heterozygous FH were consecutively recruited to the study at the Lipid Clinic, Oslo University Hospital Rikshospitalet, Oslo, Norway. All 42 children and young adults included in the study had definite FH as diagnosed by genetic testing [2], were clinically healthy without CVD and were not using anti-inflammatory drugs or statins at the time of blood sampling (nonfasting). None of the subjects with FH had any concomitant inflammatory disease, including infection and autoimmune disorders, or liver or kidney disease. For comparison, nonfasting blood samples were also collected from 25 healthy children and young adults without hypercholesterolaemia, matched with the FH group according to age (median and ranges) and gender distribution, recruited among children of colleagues, friends and employees at the University of Oslo. Not all analyses were performed in all subjects because of the limited volume of blood sample available from each individual. The study was approved by the Regional Committee of Medical Ethics, and was conducted in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from all participants or from one of their parents for children under 16 years of age.

Isolation of PBMCs

After blood collection, PBMCs were isolated using BD Vacutainer Cell Preparation tubes according to the manufacturer's instructions (Becton Dickinson, San Jose, CA, USA) and stored as pellets at −80 °C, until required for mRNA isolation. Alternatively, PBMCs were obtained from heparinized blood by Isopaque-Ficoll (Lymphoprep, Nycomed, Oslo, Norway) gradient centrifugation within 45 min of collection and used for *ex vivo* experiments within 2 h.

Cell Experiments

For *ex vivo* experiments, freshly isolated PBMCs were incubated in flat-bottomed 24-well trays (Costar, Corning Inc., Cambridge, MA, USA; 8×10^6 cells mL^{-1}; 250 μL per well) in culture medium [RPMI-1640 with 2 mmol L^{-1} L-glutamine and 25 mmol L^{-1} HEPES buffer (Gibco, Grand Island, NY, USA)] supplemented with 5% fetal calf serum (Sigma, St Louis, MO, USA) with or without oxidized (ox)LDL (20 μg mL^{-1}). THP-1 cells (a human monocytic cell line; American Type Culture Collection, Rockville, MD, USA) were pre-incubated with recombinant human (rh) TNFα (5 ng mL^{-1}; R&D Systems, Minneapolis, MN, USA) for 96 h as a model of the inflammatory microenvironment [15], before further incubation in culture medium with rhOX40 (40 ng mL^{-1}; R&D Systems), rhOX40L (20 μg mL^{-1}; R&D Systems) or oxLDL (20 μg mL^{-1}) alone or in combination. In all experiments, cell pellets were

harvested after 24 h and stored at −80 °C until required for mRNA analyses. LDL was isolated from human endotoxin-free heparin-plasma [16] and oxidized by Cu^{2+} [17]. In all control experiments, the vehicle of the stimulus was added in place of the stimulus. Lactate dehydrogenase leakage was routinely determined as a measure of toxicity in the cell cultures using a cytotoxicity detection kit (Roche Applied Science, Indianapolis, IN, USA).

mRNA analysis

Total RNA was isolated from all cell pellets using the RNeasy mini kit (Qiagen, Hilden, Germany) with lysis buffer containing β-mercaptoethanol (Sigma) according to the manufacturer's instructions, or the RNA Isolation kit I using the MagNa Pure LC Instrument (Roche Applied Science, Oslo, Norway), and stored at −80 °C. Measurements of RNA quantity and quality were performed using the ND 1000 Spectrophotometer (Saveen Werner Carlson Circle Tampa, FL, USA) and Agilent Bioanalyser (Agilent Technology, Santa Clara, CA, USA), respectively. Quantitative real-time polymerase chain reaction (QRT-PCR) was performed using an ABI PRISM 7300, 7500 or 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using SYBR Green technology (Sigma or Eurogentec, Seraing, Belgium) or custom-designed TaqMan Low Density Array (TLDA) cards (Applied Biosystems). Sequence-specific QRT-PCR primers for *OX40*, *OX40L*, *B cell-activating factor* (*BAFF/TNFSF13B*), *BAFF receptor* (*BAFFR*)/*TNFRSF13C*, *TRAIL*, *TRAIL receptor 1* (*TRAILR1*)/*TNFRSF10A*, *TRAILR3*/*TNFRSF10C*, *TRAILR4*/*TNFRSF10D* and *matrix metalloproteinase 9* (*MMP-9*) were designed by the Primer Express software, version 2.0 (Applied Biosystems). *RANK* and *RANKL* were measured with inventoried TaqMan gene expression assays using TLDA cards. Primer sequences can be provided upon request. The relative mRNA level for each transcript was calculated by the ΔΔ cycle threshold method [18]. *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*), *glucuronidase β* (*GUSβ*) and *TATA box-binding protein* (*TBP*) (all designed by the Primer Express software, version 2.0, from Sigma or Eurogentec) were used as endogenous controls for normalization.

Biochemical analyses

The concentration of oxLDL in serum was measured by enzyme-linked immunosorbent assay (Mercodia, Uppsala, Sweden). The inter- and intra-assay coefficients of variation were <10%. Standard blood chemistry and lipid parameters were measured in plasma using routine in-house laboratory methods [19].

Statistical analysis

Data are given as the median (25–75th percentile) if not otherwise stated. In a study population of relatively small size, as in the present study, the use of nonparametric tests even for apparently normally distributed data is recommended. Accordingly, comparisons between two groups of individuals were made using the Mann–Whitney *U*-test for continuous data or chi-square test for categorical data, and coefficients of correlation were calculated by the Spearman rank test. For related samples, we used the Wilcoxon signed rank test. In *in vitro* experiments in cell lines, rather than in cells from different individuals, parametric tests are preferred; independent samples *t*-test was therefore used to detect significant differences in THP-1 cell experiments. Probability values (two-sided) were considered statistically significant at $P < 0.05$, but because of multiple testing, particular attention is drawn against $P \leq 0.01$. Calculations were performed using IBM SPSS Statistics v19 (Armonk, NY, USA) or GRAPHPAD PRISM v5 (La Jolla, CA, USA). Based on our previous studies in children with FH [20], a sample size of 15 patients and 10 healthy controls was determined to be sufficient to detect differences of >30%.

Results

Study participants

The FH group consisted of children and young adults ($n = 42$) ranging from 6 to 19 years of age (median 12 years); the age range in the control group ($n = 25$) was 8–18 years (median 14 years). Moreover, there was no significant differences in the percentage of subjects aged ≥ 15 years [young adults; accounting for 17% of the FH subjects, median age 17 (16–19) years, and accounting for 36% of the controls, median age 16 (15–17) years, $P = 0.091$], or those <15 years [children; accounting for 83% of the FH subjects, median age 12 (11–13) years and 64% of the controls, median age 13 (10–14) years, $P = 0.386$] between the FH and the control group. As shown in Table 1, children and young adults with FH had significantly higher serum levels of total cholesterol, LDL cholesterol and apolipoprotein

Table 1 *Subject characteristics*

	Total population			Statin-treated population		
	Controls (n = 25)	FH (n = 42)	P-value	Before (n = 10)	After (n = 10)	P-value
Age, years	14 (12–15)	12 (11–14)	0.096	12 (11–14)		
Siblings,%	44	38	0.634	40		
Male,%	52	55	0.826	40		
Glukose, mmol L^{-1}	4.7 (4.2–5.0)	4.6 (4.4–4.9)	0.783[a]	4.4 (4.2–4.7)	4.4 (4.2–4.8)	0.833
Triglycerides, mmol L^{-1}	0.6 (0.5–1.3)	0.8 (0.6–1.0)	0.746[b]	0.7 (0.4–0.9)	0.6 (0.4–0.7)	0.241
Total cholesterol, mmol L^{-1}	4.4 (3.9–4.7)	7.3 (6.6–8.0)	<0.0001	7.2 (6.1–8.7)	4.7 (4.4–5.3)	0.005
LDL-C, mmol L^{-1}	2.4 (2.2–2.8)	5.1 (4.6–5.8)	<0.0001	6.0 (4.6–7.3)	3.3 (3.0–4.0)	0.005
HDL-C, mmol L^{-1}	1.5 (1.3–1.7)	1.3 (1.1–1.5)	0.069	1.2 (1.1–1.4)	1.3 (1.1–1.6)	0.192
Lp(a), mmol L^{-1}	124 (60–350)	269 (183–671)	0.135[c]			
ApoA-1, g L^{-1}	1.4 (1.3–1.6)	1.4 (1.3–1.5)	0.192[d]			
ApoB, g L^{-1}	0.6 (0.5–0.8)	1.3 (1.1–1.5)	<0.0001[e]			
hsCRP, mg L^{-1}	0.1 (0.1–0.2)	0.1 (0.1–0.4)	0.596[f]			

Data are presented as median (25–75th percentile).
[a]Controls, n = 23.
[b]Controls, n = 17; FH, n = 35.
[c]Controls, n = 7; FH, n = 20.
[d]FH, n = 40.
[e]FH, n = 41.
[f]controls, n = 21; FH, n = 40.
FH, familial hypercholestaerolemia; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; Lp(a), lipoprotein (a); Apo (A-1/B), apolipoprotein (A-1/B); hsCRP, high sensitivity C-reactive protein.

B, and tended to have lower levels of high-density lipoprotein (HDL) cholesterol compared with gender- and age-matched healthy controls. By contrast, we observed no significant differences in the serum levels of nonfasting glucose, triglycerides, lipoprotein (a) and high-sensitivity C-reactive protein (hsCRP) between the two groups of subjects (Table 1). Body mass index (BMI) data were only available for the FH group (19.4 [17.1–22.1] kg m^{-2}, n = 39).

mRNA levels of ligand and receptor pairs in the TNFSF/TNFRSF in PBMCs from children and young adults with FH and controls

As shown in Fig. 1a–d, children and young adults with FH (n = 42) had significantly higher mRNA levels of *OX40L* (P < 0.0001) and *BAFFR* (P = 0.001), with no significant changes in the corresponding receptor (*OX40*) and ligand (*BAFF*), compared with gender- and age-matched healthy controls (n = 24). A more complex pattern was seen for *TRAIL*-related genes, with enhanced expression of *TRAILR1* (P < 0.0001), decreased expression of *TRAIL* (P = 0.013) and *TRAILR3* (P = 0.019) and no changes in *TRAILR4* in the FH group, compared with healthy controls (Fig. 1e–h). By contrast, we did not observe any differences in mRNA levels of *RANKL* and *RANK* between children and young adults with and without FH (Fig. 1i,j).

Association between serum levels of oxLDL and expression of TNFSF/TNFRSF members in PBMCs

The presence of oxLDL in the arterial intima is regarded as one of the initial key events in atherogenesis, and is a major contributor to the progression of atherosclerosis [5]. Therefore, next we measured serum levels of oxLDL in children and young adults with and without FH. As shown in Fig. 2, the FH group had significantly higher serum levels of oxLDL, compared with the group of gender- and age-matched healthy controls. Moreover, within the study population as a whole, oxLDL levels were significantly positively correlated with mRNA levels of *OX40L* (r = 0.358, P = 0.007), *BAFFR* (r = 0.438, P = 0.001) and *TRAILR1* (r = 0.366, P = 0.006), and significantly inversely correlated with *TRAIL* (r = −0.239, P = 0.039) and *TRAILR3* (r = −0.300, P = 0.034) mRNA levels in PBMCs. By contrast, we found no correlation

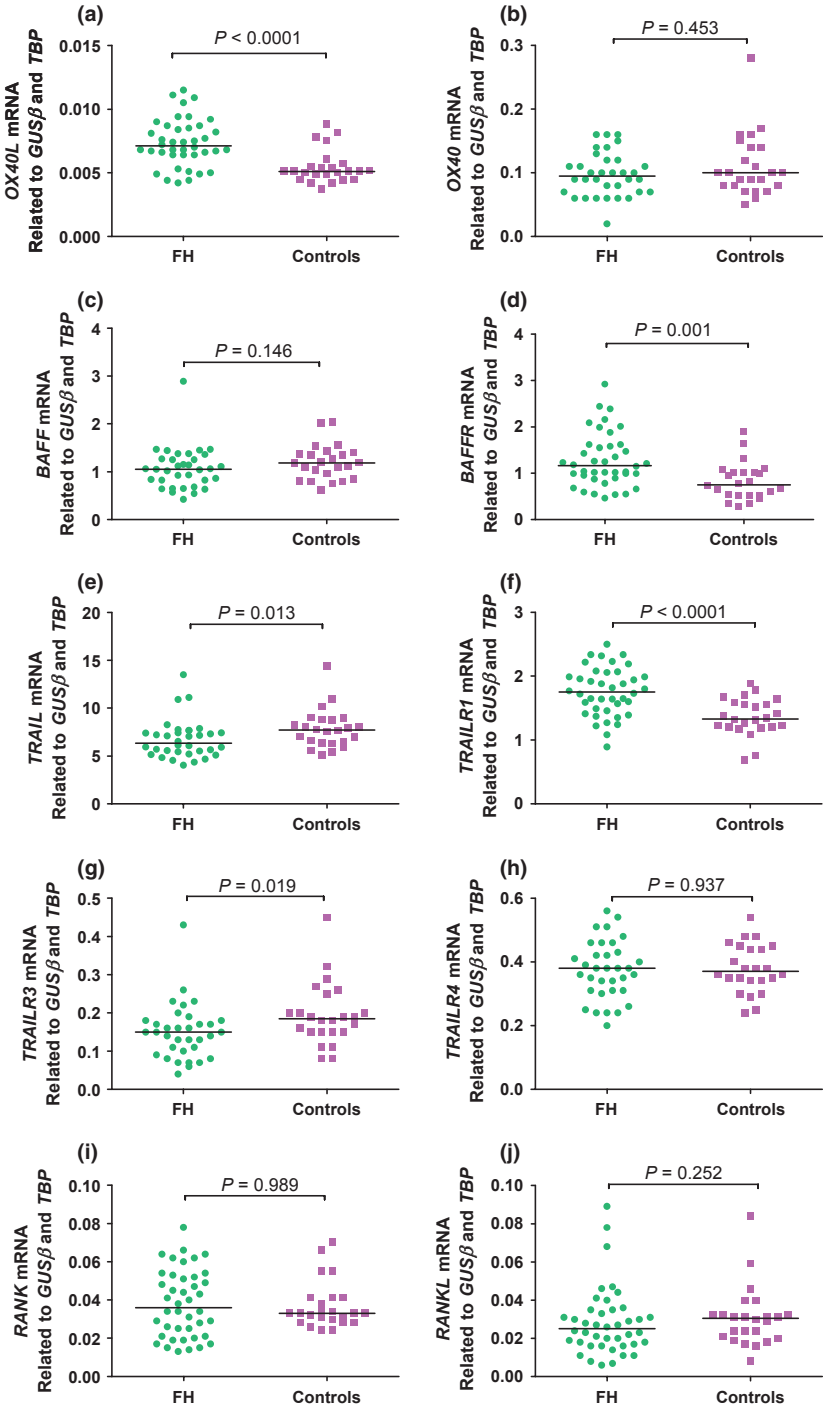

Fig. 1 *mRNA levels of tumour necrosis factor (TNF)-related molecules in peripheral blood mononuclear cells (PBMCs) from children and young adults with familial hypercholesterolaemia (FH) and controls. The expression of TNF-related molecules in individuals with FH (a, d and f, n* = 40; *b, c, e, g and h, n* = 34; *i and j, n* = 42) *and controls (all, n* = 24) *were analysed by QRT-PCR relative to the endogenous control genes GUSβ* and *TATA box-binding protein (TBP). Data are shown as individual values and the median.*

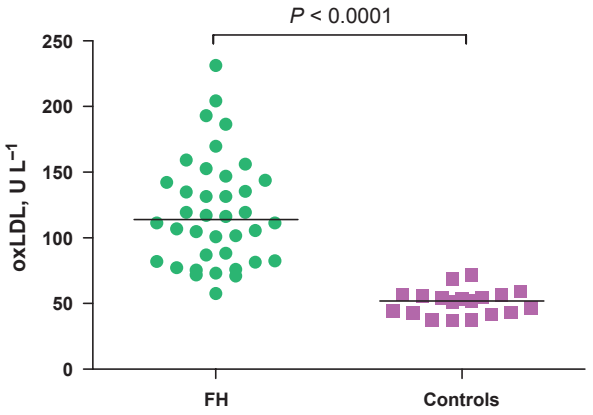

Fig. 2 *Serum levels of oxidized low-density lipoprotein (oxLDL) in children and young adults with familial hypercholesterolaemia (FH) (n = 38) and controls (n = 19). Data are shown as individual values and the median.*

between any of the TNFSF/TNFRSF members and BMI (BMI data only available for FH patients) or nonfasting glucose (data not shown).

Effects of oxLDL on mRNA levels of TNFSF/TNFRSF members in PBMCs

The above-mentioned findings suggest a link between oxLDL and altered expression of some TNF-related molecules in subjects with FH. To further explore this possibility, we cultured PBMCs from children and young adults with FH ($n = 7$) and healthy controls ($n = 7$) in the presence and absence of oxLDL (20 μg mL^{-1}) to determine the impact of oxLDL on mRNA levels of the TNFSF/TNFRSF-related genes. As shown in Fig. 3a, oxLDL increased the expression of all TNF-related molecules examined in PBMCs from subjects with FH, but the effect was only statistically significant for *TRAILR1* ($P = 0.043$) and *TRAILR4* ($P = 0.018$) or borderline significant for *BAFFR* ($P = 0.063$; Fig. 3a). By contrast, oxLDL had no effect on these TNF-related molecules in PBMCs from healthy controls (Fig. 3b); possible explanations for this are that receptors for modified LDL may be up-regulated in cells from individuals with FH or that PBMCs from these individuals may be pre-activated *in vivo* and are therefore 'primed' for further exposure to activation *in vitro*.

Regulation of MMP-9 by OX40, OX40L and oxLDL in THP-1 monocytes

Emerging evidence supports a role of OX40–OX40L in atherosclerosis [21]. Furthermore, in the present study, OX40L was found to be up-regulated in PBMCs from children and young adults with FH. Monocyte-related MMP-9 is an important mediator of plaque development [22]. To determine any potential pathogenic consequences of enhanced OX40L expression in individuals with FH, we examined the effect of recombinant OX40L and OX40, with and without co-stimulation with oxLDL (20μ 5 ng mL^{-1}) for 96 h (see Methods) (20 μg mL^{-1}), on

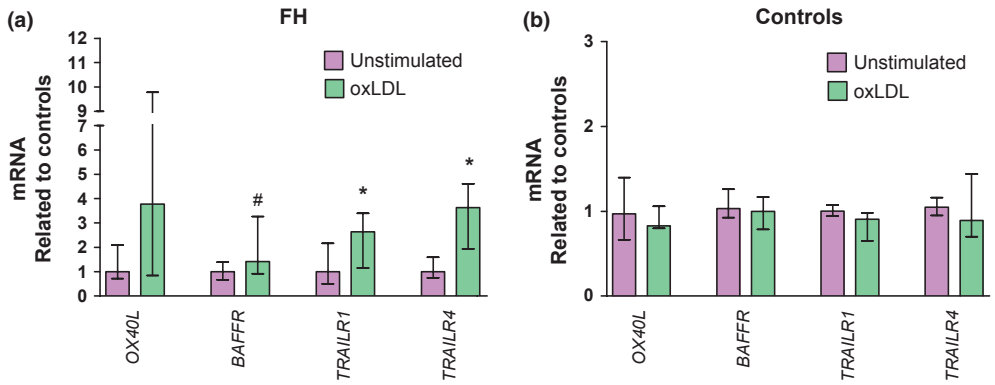

Fig. 3 *Effect of oxidized low-density lipoprotein (oxLDL) on mRNA levels of tumour necrosis factor (TNF)-related molecules in peripheral blood mononuclear cells (PBMCs) from children and young adults with familial hypercholesterolaemia (FH) and controls. Expression of OX40L (n = 6), BAFFR (n = 7), TRAILR1 (n = 7) and TRAILR4 (n = 7) in PBMCs after 24 h in culture with and without oxLDL (20 μg mL^{-1}) from individuals with FH (a) and from healthy controls (n = 7) (b). mRNA levels of TNF-related molecules were analysed by QRT-PCR relative to the endogenous control genes GAPDH or GUSβ and TATA box-binding protein (TBP). Data are shown as median and interquartile range relative to the unstimulated level for each individual. #P = 0.063 and *P < 0.05 versus unstimulated.*

the expression of *MMP-9* in THP-1 monocytes pre-activated by TNFα. As for some of the other TNF-related molecules (such as CD137L/CD137 and glucocorticoid-induced tumor necrosis factor receptor [GITR]/GITR ligand), there is a bidirectional interaction between OX40L and OX40 with both able to function as either receptor or ligand. While OX40 and OX40L had no effect on their own, OX40, but not OX40L, enhanced the oxLDL-mediated increase in mRNA *MMP-9* expression in THP-1 monocytes ($P = 0.0006$; Fig. 4).

Effects of statin treatment on mRNA levels of TNFSF/TNFRSF members in PBMCs from children and young adults with FH

In 10 patients with FH, PBMCs were available before and after treatment with statins [treatment duration 12 (4–35) weeks]. Most of these 10 individuals were treated with rosuvastatin (5 mg; $n = 7$), whereas two received simvastatin (10 and 20 mg) and one took atorvastatin (10 mg). As expected, statins markedly decreased levels of total and LDL cholesterol ($P = 0.005$), with no changes in glucose, HDL cholesterol and triglyceride levels (Table 1). Concomitantly, serum oxLDL levels also significantly decreased after statin treatment [from 94.6 (69.2–117.2) to 68.2 (51.7–76.4) U L^{-1}, $P < 0.008$, $n = 9$]. As seen in Fig. 5, this decrease in oxLDL was accompanied by a decrease in mRNA levels of *OX40L* ($P = 0.059$) and *TRAILR1* ($P = 0.017$) and an increase in mRNA levels of *BAFF* ($P = 0.005$), *TRAIL* ($P = 0.074$) and *TRAILR3* ($P = 0.059$), although not all changes reached statistical significance. By contrast, no changes were seen in mRNA levels of *OX40, BAFFR* and *TRAILR4* (Fig. 5). Subjects with FH who used statins for a duration greater than or equal to the median treatment time (12 weeks, $n = 6$) had a significantly more pronounced decrease in mRNA levels of *OX40L* compared with those receiving statins for less than the median treatment duration ($P = 0.038$, $n = 4$). There were no other significant differences in the changes in mRNA level between the two groups of FH subjects with different statin treatment durations.

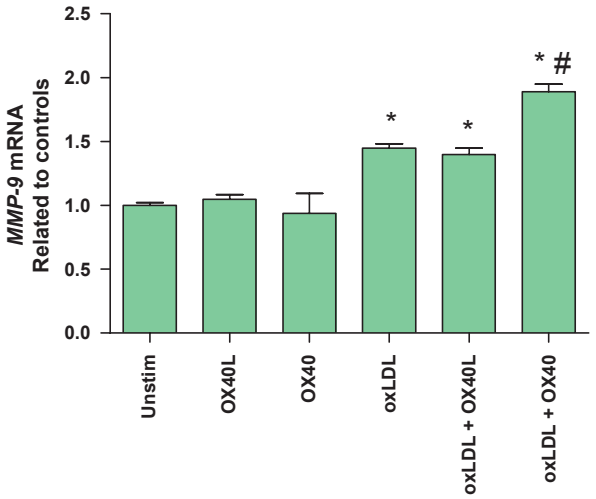

Fig. 4 *Effect of OX40, OX40L and oxidized low-density lipoprotein (oxLDL) on matrix metalloproteinase 9 (MMP-9) levels in THP-1 monocytes. The effects of OX40 (40 ng mL^{-1}), OX40L (20 μg mL^{-1}) and oxLDL (20 μg mL^{-1}) either alone or the combination thereof on the mRNA level of MMP-9 in THP-1 cells pre-activated with tumour necrosis factor (TNF)α (5 ng mL^{-1}) for 96 h before the start of the experiment. mRNA levels of MMP-9 were analysed by QRT-PCR relative to the endogenous control genes GUSβ and TATA box-binding protein (TBP). Data are presented as mean ± SEM of quadruplicates, relative to the mean level in unstimulated (Unstim) cells. *$P \leq 0.0003$ versus Unstim, #$P = 0.0006$ versus oxLDL-stimulated values.*

Association between mutation type and mRNA levels of TNFSF/TNFRSF members

The children and young adults with FH ($n = 42$) in the present study carried 12 different LDL receptor mutations ($n = 41$) and 1 apolipoprotein B mutation ($n = 1$); the three Norwegian founder mutations FH-Elverum, FH-Svartor and FH-C210G accounted for the majority of the mutations present and were observed in 48% of the children. With class 1 mutations (including FH-Elverum and FH-Svartor), the LDL receptor is not produced, whereas dysfunctional LDL receptors are caused by class 2–5 mutations (including FH-C210G) [23]. However, we found no differences in mRNA levels of TNFSF/TNFRSF members in PBMCs between FH subjects carrying the class 1 mutations ($n = 23$) and those with other mutation types ($n = 19$) (data not shown).

Discussion

In the present study we have shown that the mRNA levels of some members of the TNFSF/TNFRSF (i.e. OX40L, BAFFR and TRAIL-related molecules) differ significantly in PBMCs from apparently healthy children and young adults with FH compared with those from gender- and age-matched healthy controls. Moreover, serum levels of oxLDL were significantly correlated with several of these TNF-related molecules, and our *ex vivo* findings in oxLDL-exposed PBMCs may further suggest a link

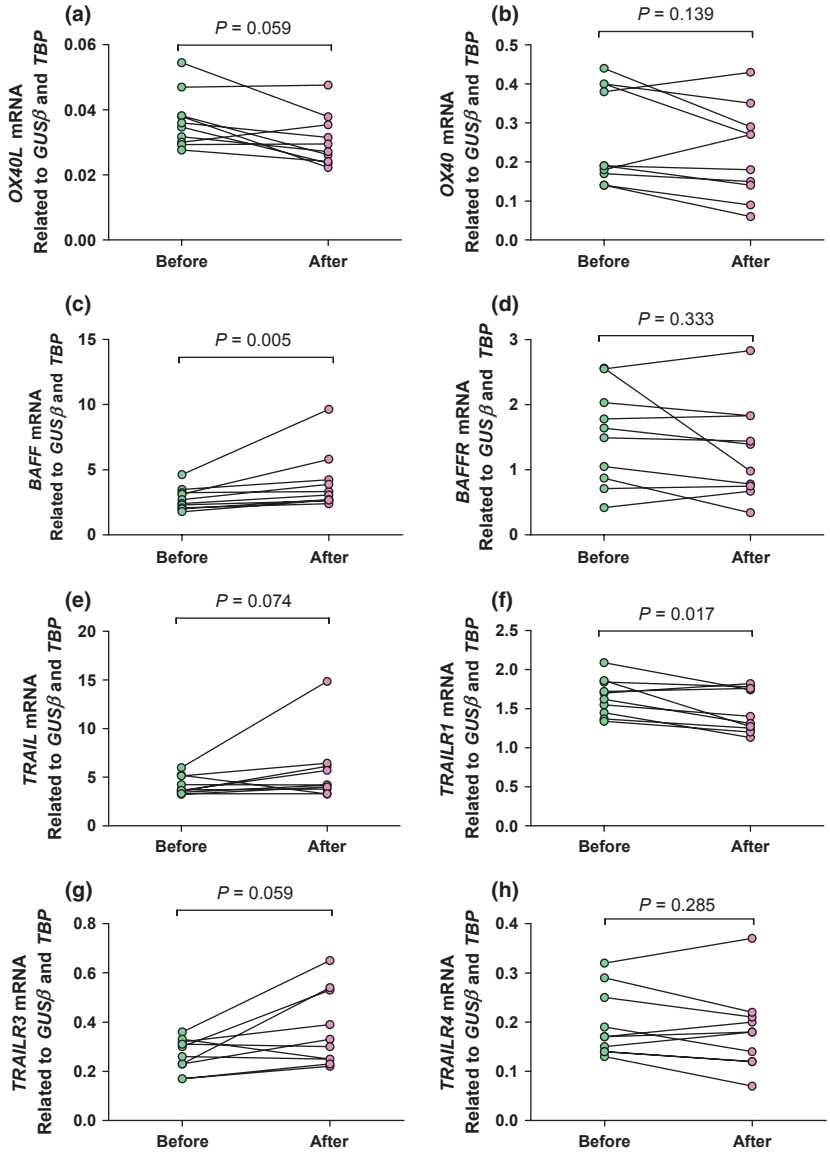

Fig. 5 *mRNA levels of tumour necrosis factor (TNF)-related molecules in peripheral blood mononuclear cells (PBMCs) from children and young adults with familial hypercholesterolaemia (FH) before and after statin treatment. mRNA levels were analysed by QRT-PCR relative to the endogenous control genes GUSβ* and *TATA box-binding protein (TBP)* (*n* = 10).

between oxLDL and the altered expression of these TNFSF/TNFRSF members in PBMCs from children and young adults with FH. We have previously shown an inflammatory imbalance between TNFα and IL-10 in children and young adults with FH [14]. The present findings suggest that the inflammation in atherogenesis in children and young adults with FH, representing an early stage of the atherosclerotic process, may involve not only TNFα but also other TNF-related molecules.

TNFSF/TNFRSF members regulate immune and inflammatory responses, and are associated with various autoimmune and inflammatory disorders [24, 25], including atherosclerosis [11, 21]. However, to the best of our knowledge, this is the first report to show altered expression of several TNFSF/TNFRSF members in PBMCs from children and young adults with FH. Thus, although we found no differences in hsCRP levels between the FH and the control group, children and young

adults with FH had higher levels of *OX40L* and *BAFFR* and altered expression of several TRAIL-related molecules in PBMCs. Whereas studies of inflammation in patients with established CVD may be hampered by secondary phenomena not related to the primary atherosclerotic process, studies in healthy children and young adults with FH, but without established CVD could provide insight into the early stages of atherogenesis. These findings in the present study suggest that members of the TNFSF/TNFRSF may be involved in these early atherogenic processes.

The gene encoding OX40L has been found to influence the development of atherosclerosis in both mice and humans [21]; in the present study we have shown that PBMCs from children and young adults with FH have enhanced expression of *OX40L* compared with those from matched healthy controls. Moreover, OX40, acting through OX40L, enhanced the oxLDL-induced expression of *MMP-9* in THP-1 monocytes. Although the OX40–OX40L interaction in atherogenesis and other immune-mediated disorders has been linked primarily to T cell activation [26], increased expression of OX40L has previously been reported in activated monocytes in humans [27]. Although we have no *in vivo* data, it is tempting to hypothesize an interaction between the OX40L-OX40 system and oxLDL within the early atherosclerotic lesion, which may contribute to matrix remodelling through enhanced expression of MMP-9 in monocytes/macrophages. Furthermore, statins have previously been shown to decrease the expression of TNF-related molecules such as OX40L/OX40 and CD40L(TNFSF5)/CD40(TNFRSF5) in patients with late atherosclerosis [28, 29]. Herein, we have shown that statin treatment decreases *OX40L* expression in PBMCs from children and young adults with FH, suggesting a possible anti-inflammatory effect of statins on OX40L in early atherosclerosis. However, our statin data were not based on a controlled study, and should be confirmed in larger placebo-controlled trials.

In addition to increased expression of *OX40L*, PBMCs from children and young adults with FH had enhanced expression of *BAFFR*, and our *ex vivo* findings suggest that this may reflect a direct effect of oxLDL on these cells. At present, the role of the BAFF–BAFFR interaction in atherogenesis is unclear. The results of a very recent experimental study suggest that deficiency of BAFFR markedly attenuates the pro-atherogenic effects of B cell activation, without inhibiting the atheroprotective effects of these cells [30]; however, these findings need to be confirmed. In line with this, although our findings of enhanced expression of *BAFF* in PBMCs from children and young adults with FH receiving statin therapy could reflect inflammatory and potential harmful side effects of these medications, the influence of BAFF activation on the B cell phenotypes during atherogenesis is still unclear. TRAIL is a major inducer of apoptosis, and enhanced apoptosis could have a beneficial effect in the early phase of atherogenesis by limiting the growth of the atherosclerotic lesion [31]. In the present study, we revealed a complex pattern of *TRAIL*-related genes in PBMCs from children and young adults with FH, with decreased expression of *TRAIL* combined with enhanced expression of the pro-apoptotic *TRAILR1* and reduced expression of the inhibitory decoy receptor *TRAILR3*. Thus, although FH clearly seems to influence the expression of *TRAIL*-related genes in PBMCs from children and young adults, the net effect of these changes remains unclear.

The present study has some limitations including: (i) a relatively small sample size, particularly in the *ex vivo* experiments in which not all analyses were performed in all individuals; (ii) an uneven number of subjects with FH and controls without one to one matching; (iii) lack of fasting glucose/insulin measurements and data on waist circumference in the total population or BMI in the healthy controls; and (iv) multiple comparisons in a relatively small sample size. Moreover, some of the findings showed only borderline significance. Although our results should be interpreted with caution, they suggest the involvement of some TNFSF/TNFRSF members, in particular the OX40–OX40L interaction, in the early stages of atherogenesis, which may contribute to the accelerated atherosclerotic process observed in individuals with FH. Furthermore, our data emphasize a possible link between oxLDL and TNFSF/TNFRSF members.

Conflict of interest statement

No conflict of interest to declare.

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