



# Profiling of immune-related gene expression in children with familial hypercholesterolaemia

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**Abstract.** Narverud I, Christensen JJ, Bakke SS, Ulven SM, Rundblad A, Aukrust P, Espevik T, Bogsrud MP, Retterstøl K, Ueland T, Halvorsen B, Holven KB (Oslo University Hospital; University of Oslo, Oslo; Norwegian University of Science and Technology, Trondheim; Oslo University Hospital; University of Oslo; Oslo University Hospital, Oslo; The Arctic University of Tromsø, Tromsø, Norway). Profiling of immune-related gene expression in children with familial hypercholesterolaemia. *J Intern Med* 2019; <https://doi.org/10.1111/joim.13001>

**Background.** Innate and adaptive immune responses are pivotal in atherosclerosis, but their association with early-stage atherosclerosis in humans is incompletely understood. In this regard, untreated children with familial hypercholesterolaemia may serve as a human model to investigate the effect of elevated low-density lipoprotein (LDL)-cholesterol.

**Objectives.** We aimed to study the immunological and inflammatory pathways involved in early atherosclerosis by examining mRNA molecules in peripheral blood mononuclear cells (PBMCs) from children with FH.

**Methods.** We analysed the level of 587 immune-related mRNA molecules using state-of-the-art Nanostring technology in PBMCs from children

with ( $n = 30$ ) and without ( $n = 21$ ) FH, and from FH children before and after statin therapy ( $n = 10$ ).

**Results.** 176 genes (30%) were differentially expressed between the FH and healthy children at  $P < 0.05$ . Compared to healthy children, the dysregulated pathways in FH children included the following: T cells (18/19); B cells (5/6); tumour necrosis factor super family (TNFSF) (6/8); cell growth, proliferation and differentiation (5/7); interleukins (5/9); toll-like receptors (2/5); apoptosis (3/7) and antigen presentation (1/7), where the ratio denotes higher expressed genes to total number of genes. Statin therapy reversed expression of thirteen of these mRNAs in FH children.

**Conclusion.** FH children display higher PBMC expression of immune-related genes mapped to several pathways, including T and B cells, and TNFSF than healthy children. Our results suggest that LDL-C plays an important role in modulating expression of different immune-related genes, and novel data on the involvement of these pathways in the early atherosclerosis may represent future therapeutic targets for prevention of atherosclerotic progression.

**Keywords:** familial hypercholesterolaemia, children, gene expression, inflammation, statins.

## Introduction

Coronary heart disease (CHD) is a main contributor to the global burden of disease [1]. It is primarily caused by atherosclerosis where a

complex interaction between lipids and inflammation is the pathological hallmark [2]. Innate immune responses are pivotal through for example the formation of foam cells from lipid-exposed macrophages, which in turn activate cells in the adaptive immune system, such as T and B cells. These cells may again activate innate immunity

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through for example release of various inflammatory cytokines. All these subsets are important cell types in atherosclerosis [2]. Peripheral blood mononuclear cells (PBMCs), which comprise lymphocytes and monocytes, may thus be considered a unique niche since they are primed by atherogenic factors and directly contribute to the atherosclerosis process. Furthermore, previous studies have shown that alterations in PBMC gene expression level may reflect systemic health [3] and have suggested PBMCs to be a good model system to elucidate the cardiovascular system in relation to inflammation [4].

Due to the nature of CHD, most studies on inflammation and immunity in atherosclerosis are performed in adult patients with established atherosclerosis [5–7]. However, these studies may be confounded by co-occurring disease or risk factors, or environmental exposures. Thus, findings in these studies may reflect secondary phenomena and not the molecules that promote the development of atherosclerosis and its complications. It is therefore of major interest for future prevention of CHD to identify and map molecules that could play a pathogenic role in the *early* stages of this disorder, using study populations free from severe confounding. To this, subjects with familial hypercholesterolaemia (FH) may represent an optimal population.

FH is an inherited disorder caused primarily by a mutation in the gene encoding the low-density lipoprotein (LDL) receptor [8]. Phenotypically, FH individuals have higher total- and LDL-cholesterol (LDL-C) compared to the general population, leading to accelerated atherosclerosis and premature CHD [9]. Accordingly, nonstatin-treated children with FH may serve as a human model to investigate the isolated effect of LDL-C on early development of atherosclerosis caused by a mutation rather than secondary hypercholesterolaemia induced by, for example lifestyle. Indeed, it has been shown that FH children have increased carotid intima-media thickness (IMT) [10] and display signs of inflammation [11] at both protein and mRNA level, including activation of certain pathways such as members of the tumour necrosis factor super family (TNFSF) [10, 12]. However, these issues are far from clear and to this end; to the best of our knowledge, no studies have shown comprehensive mapping of the multitude of inflammatory and immune-related pathways that

could be involved in the early stages of atherosclerosis in children with FH.

Herein, we aimed to profile the immunological and inflammatory pathways involved in early atherosclerosis in PBMCs from children with FH compared to healthy children.

## Methods

### Subjects

The subjects have been described previously [12]. Briefly, children with FH were recruited from the Lipid Clinic, Oslo University Hospital, Norway. Age- and sex-matched (by median and ranges) healthy control children were recruited through colleagues, employees and friends at the Lipid Clinic and the Department of Nutrition, University of Oslo, Norway. Common inclusion criterion for the two groups was age 18 years or younger. Common exclusion criteria for the two groups were any chronic disease (including inflammatory or metabolic disorders), or use of anti-inflammatory drugs or lipid-lowering therapy (LLT) (at first visit). A nonfasting blood sample was obtained from each individual. Additionally, nonfasting blood samples were available before and after statin treatment in ten FH children, also previously described [12]. The study was approved by the Regional Committee of Medical Ethics and was conducted according to the Declaration of Helsinki. Written informed consent was obtained from all participants, or from one of their parents if the child was below the age of 16 years.

### Gene expression analyses

PBMCs were isolated, and RNA quantity and quality were analysed as previously described [12]. We ran RNA expression analysis on the nCounter® analysis system (Nanostring Technologies), running 12 samples at a time (one 'strip'). The procedure was performed according to the manufacturer's instructions, applying about 100 ng mRNA. The kit used was a fixed code set for mRNA analysis that comprised genes involved in human immunology: nCounter GX Human Immunology Kit v2 (Nanostring Technologies). The number of mRNA molecules per gene was accounted for detection level  $average(negativecontrol) + 2SD(negativecontrols) = 10$  mRNA molecules, normalized against instrument variations (positive controls) and endogenous control genes found to be stable (*ABCF1*, *EEF1G*,

*HPRT1*, *RPL19*, *TUBB*, *GUSB* and *POLR2A*) using nSolver analysis software 2.5.34 (NanoString Technologies).

#### Miscellaneous

Oxidized LDL (oxLDL) was measured in serum using an enzyme-linked immunosorbent assay as previously described [12]. Standard blood biochemistry analyses were measured in plasma or serum by standard methods at the accredited medical laboratory, Oslo University Hospital, Rikshospitalet, Oslo, Norway (NS-EN ISO 15189:2007). Not all analyses were performed in all subjects because of the limited volume of blood sample available from each individual.

#### Statistical analyses

We imported the raw gene expression data (594 genes) into Partek Genomics Suite 6.6, and batch-corrected for strip, age and sex. In principal component analysis (PCA), one individual was a clear outlier and was thus excluded from the statistical analyses. Statistical power analysis based on the entire dataset showed that the minimum sample size for this study was 17 at significance level 0.01 and power 0.90. After exclusion of the used endogenous controls ( $n = 7$ , see gene expression analysis section), we performed a 4-way analysis of variance (ANOVA) model using log<sub>2</sub>-transformed data to test for statistical significance between FH children and healthy children and adjusted for multiple testing using false discovery rate (FDR). FDR-adjusted  $P$ -value  $< 0.05$  was considered significantly different between the groups. Additionally, based on data from the 4-way ANOVA as input, we ran standard linear regression models for each gene using batch-corrected raw data in R version 3.5.0 [13]. We ran three models per gene, with 1) no covariates, 2) HDL-C and CRP as covariates, or 3) LDL-C as covariate. Also, to further examine associations between gene expression and clinical and biochemical variables, we used Spearman's rank correlation.

For the main cohort, we allocated differentially expressed genes (DEGs) into different immunological pathways using two strategies. First, we manually allocated FDR-adjusted genes to pathways based on their function; *main pathways* were defined as having  $\geq 5$  DEGs. Information about gene function was obtained from the following

databases NCBI (<https://www.ncbi.nlm.nih.gov/pubmed/>, main resource), STRING (<https://string-db.org>), GeneCards (<https://www.genecards.org>), UniProt (<https://www.uniprot.org>), OMIM (<http://omim.org/>) and Ensembl (<http://www.ensembl.org/>). Secondly, we created clusters of genes (not FDR-adjusted) in an unsupervised manner; we calculated the Euclidean distance of the correlation matrix for all genes, performed hierarchical clustering (complete linkage) and cut the dendrogram into eight clusters.

We estimated the relative proportion of blood cell types in each sample with the CIBERSORT algorithm [14]. The algorithm takes a whole-genome gene expression matrix as input and returns the relative proportion of 22 leucocyte subtypes by use of feature selection and linear support vector regression (SVR). Because we supplied the algorithm with a limited number of genes ( $n = 587$ ), the sensitivity of this analysis was expected to be lower than by use of whole-genome datasets. Despite this, all 51 samples had adequate goodness of fit ( $P < 0.05$ ). Since the PBMC pool should be completely absent neutrophils, we used the estimated proportion of neutrophils as an indicator to remove low-proportion leucocyte variables; hence, we included mainly estimated monocyte and lymphocyte subtypes.

In a subsequent analysis in ten FH children that were not part of the main FH cohort, we analysed gene expression before and after starting statin treatment. The analysis pipeline (gene expression and statistical analyses) was similar as for the main cohort, described above. As for the main cohort, we estimated the change in gene expression (for all genes) corrected for age, sex and strip upon statin therapy. Then, we investigated the association between the two sets of coefficient estimates (for each of the 587 genes): (i) the association between FH and healthy children (exposure variable) and (ii) the change upon statin therapy (outcome variable). We interpreted this association as whether or not statins completely or partially reversed the gene expression differences observed in FH children compared to control children.

Baseline characteristics are given as median (25th–75th percentile), mean (standard deviation [SD]) or frequency (%). Mann–Whitney U test or Independent sample t-test was used to test for statistically significant differences in baseline characteristics between the two groups. We used

Wilcoxon test to test characteristics before and after statin initiation.

## Results

### Characteristics

The characteristics of the participants are shown in Table 1. Thirty children with and twenty-one without FH were included in this substudy. All the children with FH had a genetically verified FH diagnosis. Total cholesterol, LDL-C, apolipoprotein (apo) B, oxLDL and C-reactive protein (CRP) were higher ( $P < 0.001$ ), whereas high-density lipoprotein cholesterol (HDL-C) was lower (nonsignificant,  $P = 0.059$ ), in the FH children compared to control children.

### DEGs between FH and healthy children

Of the 587 examined genes, 176 were differentially expressed between the FH and healthy children after adjustment for strip, age and sex at  $P < 0.05$  (unadjusted, Fig. 1a). The most DEGs sorted by significance level are shown in Fig. 1b. After adjustment for 5% FDR, ninety-three genes were differentially expressed between FH and healthy children, and these were allocated into 21 related pathways. In FH versus healthy children, the main pathways (defined as  $\geq 5$  allocated genes) related to these genes were T cells (18/19); B cells (5/6); TNFSF (6/8); cell growth, proliferation and differentiation (5/7); interleukins (5/9); toll-like

receptors (TLR) (2/5); apoptosis (3/7); antigen presentation (1/7), where the ratio denotes higher expressed genes to total DEGs (Fig. 2).

In order to explore the leucocyte distribution, we predicted the different leucocyte subtypes using a CIBERSORT analysis based on all the mRNA expressions from the nCounter analysis. Children with FH had higher levels of predicted naïve B cells ( $P < 0.01$ ), but lower levels of predicted monocytes ( $P < 0.05$ ) and resting mast cells ( $P < 0.01$ ) compared to healthy children (Fig. S1).

### Regulation of immune-related genes after statin therapy

In order to investigate the effect of statin therapy on immune-related genes, we analysed PBMC gene expression before and after statin therapy in ten children with FH. The decrease in total- and LDL-C were 38.5 (34.5–41.4) % and 46.3 (40.6–48.9) % ( $P < 0.001$  for both), median (25th–75th percentile), respectively [12]. In total, 62 genes (FDR  $< 0.05$ ) were modified by statin therapy (Fig. S2). Figure 3 shows the relationship between differences in gene expression in FH compared to control children and change after statin therapy in FH children. Of the sixty-two genes, ten genes that were higher in FH vs controls at baseline, were reduced after statin initiation *IRAK4*, *ATG16L1*, *CUL9*, *IKBKB*, *CRADD*, *TRAF5*, *ENTPD1*, *C1R*, *IL1RL1* and *CTLA4* (Fig. 3). In contrast, *CTSS*, *PYCARD* and *C14orf166* were lower in FH vs controls at baseline and increased

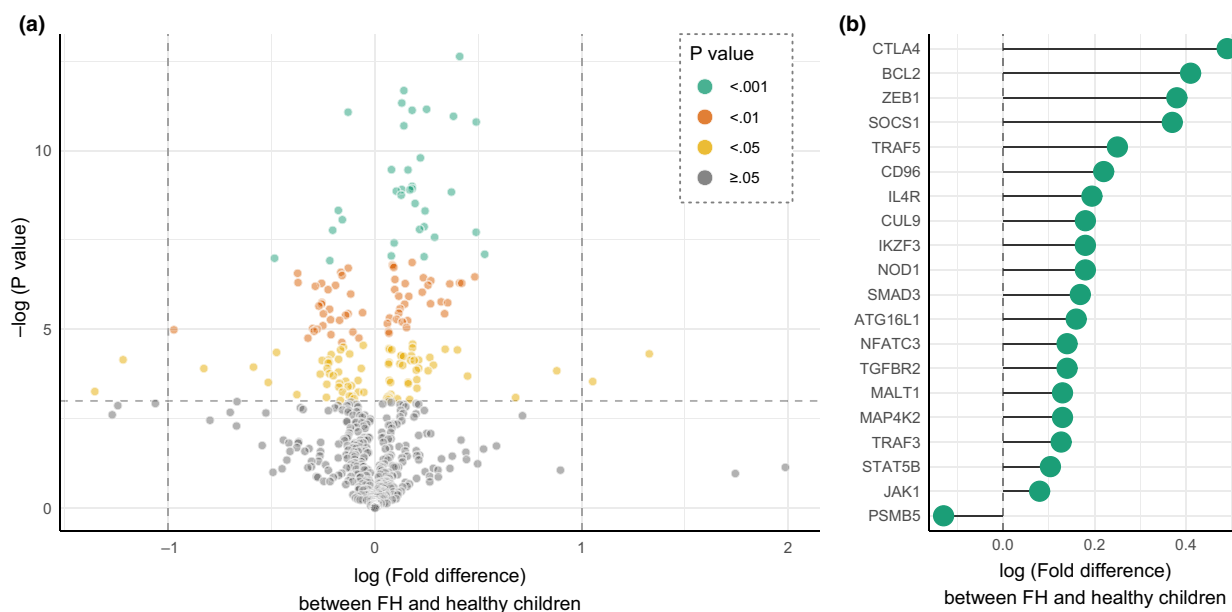
**Table 1.** Characteristics of the participants

	<i>n</i>	FH children	<i>n</i>	Control children	<i>P</i> *
Age, year	30	12 (11–14)	21	14 (11–14)	0.23
Sex, female	30	12 (40)	21	9 (43)	0.83
Triglycerides, mmol L <sup>-1</sup>	25	0.7 (0.6–0.9)	16	0.6 (0.5–1.2)	0.75
Total-C, mmol L <sup>-1</sup>	30	6.9 (6.5–7.7)	21	4.4 (4–4.7)	<b>&lt;0.001</b>
HDL-C, mmol L <sup>-1</sup>	30	1.4 (1.2–1.5)	21	1.5 (1.3–1.7)	0.059
LDL-C, mmol L <sup>-1</sup>	30	5 (4.4–5.4)	21	2.4 (2.2–2.8)	<b>&lt;0.001</b>
ApoA1, g L <sup>-1</sup>	28	1.4 (1.3–1.5)	21	1.4 (1.4–1.6)	0.28
ApoB, g L <sup>-1</sup>	29	1.2 (1.1–1.4)	21	0.6 (0.5–0.8)	<b>&lt;0.001</b>
oxLDL, U L <sup>-1</sup>	25	105.5 (81.5–142.1)	16	53.8 (42.2–56.4)	<b>&lt;0.001</b>
CRP, mg L <sup>-1</sup>	28	1.0 (1.0–1.0)	21	0.6 (0.6–1.0)	<b>&lt;0.001</b>
Glucose, mmol L <sup>-1</sup>	29	4.7 (0.4)	19	4.7 (0.7)	1.00

Data are given as median (IQR), mean (SD) or frequency (%). Bold and italic *P*-values indicate significance and borderline significance, respectively.

Apo, apolipoprotein; CRP, C-reactive protein; FH, familial hypercholesterolaemia; g/L, grams per litre; HDL, high-density lipoprotein; LDL, low-density lipoprotein; mmol/L, millimoles per litre; oxLDL, oxidized low-density lipoprotein.

\*Mann–Whitney U test or Independent sample t-test between the groups.



**Fig. 1** Differentially expressed genes in PBMCs from FH children ( $n = 30$ ) compared with healthy children ( $n = 21$ ). (a) Association between fold difference and nominal P-value for all genes. (b) Top 20 genes with lowest nominal P-value, here sorted by fold difference. This analysis is based on a 4-way ANOVA using  $\log_2$ -transformed data as input, with strip, age and sex as factors. Positive fold differences translate to higher expression in FH children, compared with healthy children, and opposite for negative fold differences. Note that results are presented on log scale. ANOVA, analysis of variance; FH, familial hypercholesterolaemia. Abbreviations of gene names are found in supplemental material.

after statin therapy (Fig. 3). Overall, statins thus seem to partially reverse the DEGs observed in FH children in line with the expression observed in healthy children.

#### Associations between gene expression and clinical data

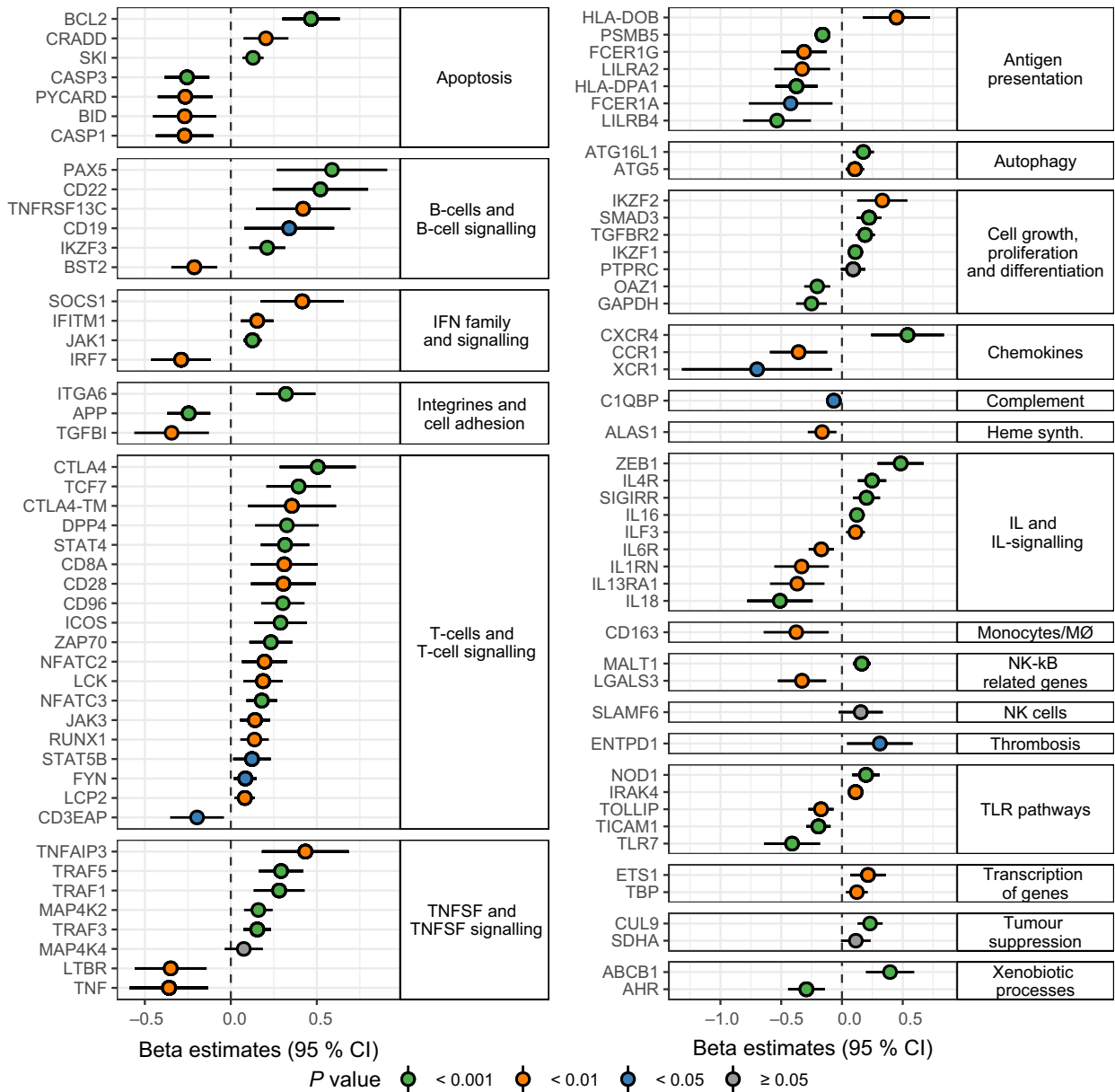
To further investigate associations between DEGs in the main pathways and clinical data, we calculated the inter-variable correlation coefficients (Fig. 4). The apoB-containing lipoprotein parameters and CRP were mainly correlated to the immune-related gene expressions. Whereas we found mostly positive correlations between total cholesterol, LDL-C, oxLDL, apoB and CRP, and DEGs related to the pathways: T-cell signalling, B-cell signalling, TNFSF signalling, and cell growth, proliferation and differentiation (Fig. 4), the DEGs related to antigen presentation, apoptosis and TLR pathways were mostly negatively correlated to apoB-containing lipoprotein parameters and CRP (Fig. 4). There were only few correlations between the DEGs and age, HDL-C, apoA1, triglycerides and glucose (Fig. 4).

In addition to LDL-C, CRP and HDL-C were different between the two groups of children (Table 1), thus to investigate the impact of these biochemical variables, we performed a linear regression adjusting for either HDL-C and CRP combined, or LDL-C alone, for all DEGs ( $n = 176$ ) (Fig. S3). The significance of the DEGs was attenuated or neutralized only when adjusting for LDL-C, but not when adjusting for HDL-C and CRP, suggesting that LDL-C is the main driver for the observed alterations (Fig. S3).

In order to show a direct correlation between LDL-C and mRNA levels, we show a strong correlation in mRNA levels when correlating the difference per mmol/L difference in LDL-C and difference in FH children compared to healthy children (Fig. 5).

#### Discussion

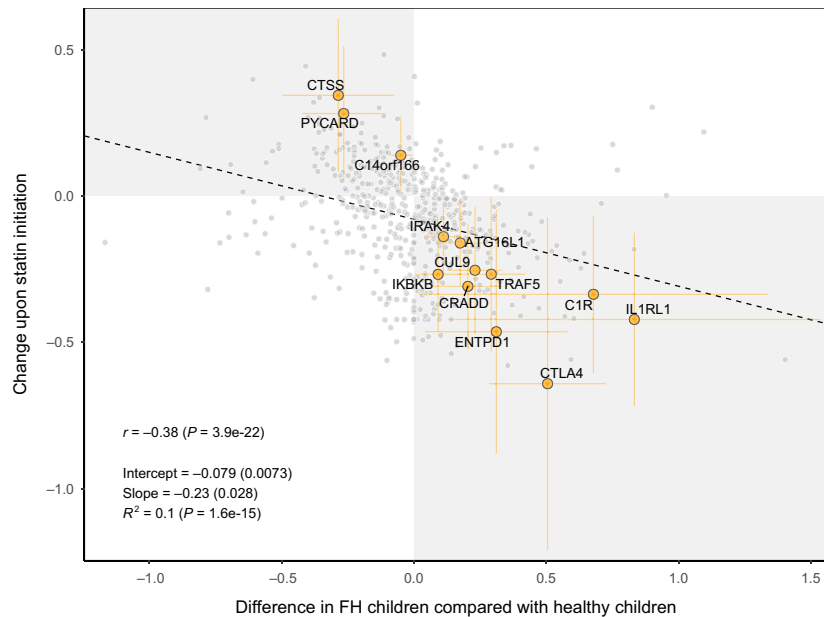
In the present study, we found that FH children display several changes in mRNA molecules related to pathways including, for example T- and B-cell signalling, TNFSF, interleukin-signalling and cell



**Fig. 2** Differentially expressed genes at  $FDR < 0.05$  between FH and healthy children. Data are based on a linear regression with input from a 4-way ANOVA using log<sub>2</sub>-transformed data as input, with strip, age and sex as factors. The x-axis shows the beta-estimate and 95% confidence interval. ANOVA, analysis of variance; CI, confidence interval; FDR, false discovery rate; FH, familial hypercholesterolaemia; IFN, interferon; IL, interleukin; NF- $\kappa$ B, nuclear factor  $\kappa$  beta; NK, natural killer; TLR, toll-like receptor; TNFSF, tumour necrosis factor super family. Abbreviations of gene names are found in supplemental material.

growth, proliferation and differentiation, compared to healthy control children. Interestingly, the DEGs reflected both anti-inflammatory (e.g. transforming growth factor- $\beta$ -related molecules) and pro-

inflammatory (e.g. TNFSF and various chemokines) molecules. Furthermore, LDL-C, as expected, seems to be the main driver of these alterations further underscoring the role of LDL-C as a trigger



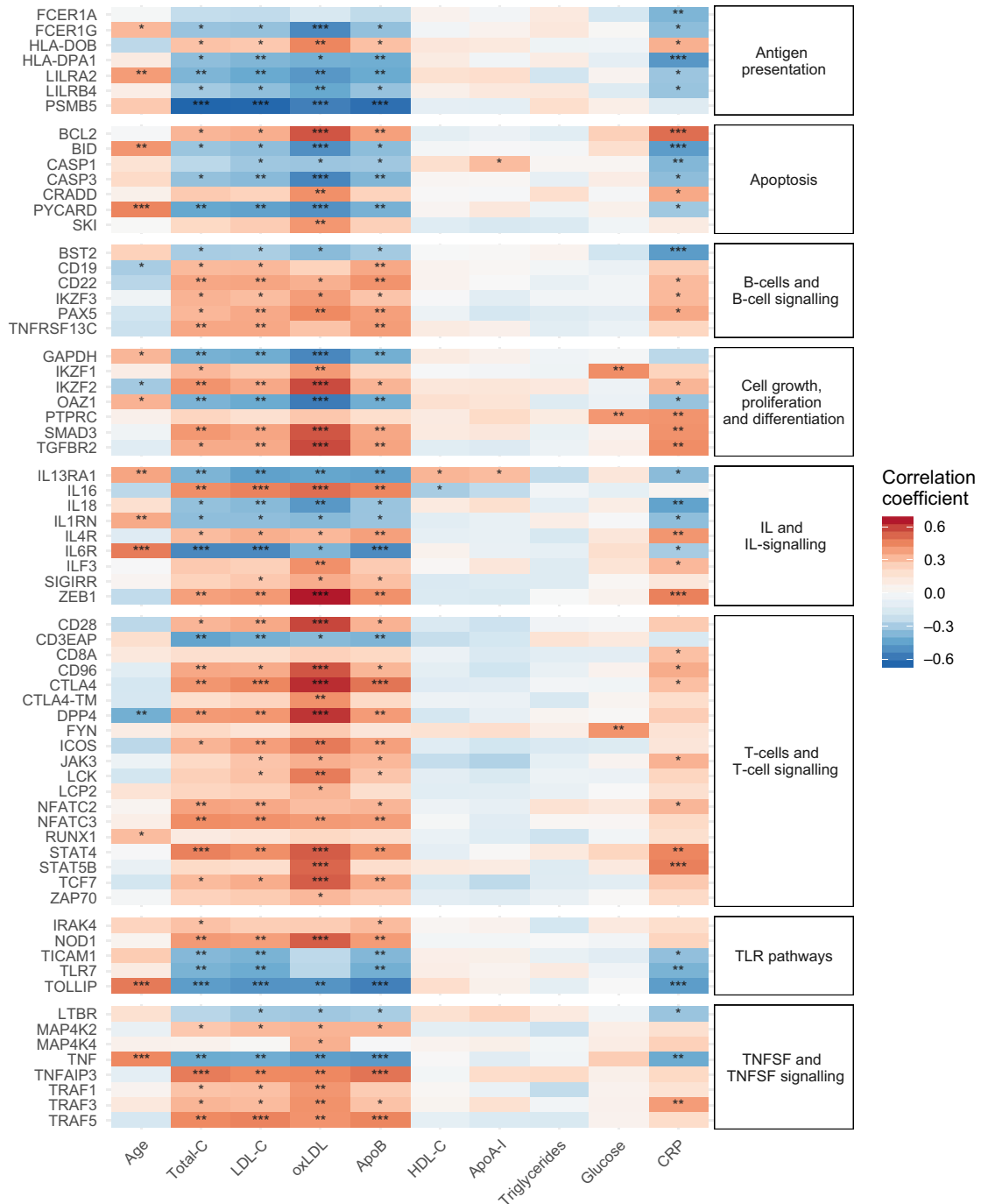
**Fig. 3** Association between difference in PBMC gene expression in FH children ( $n = 30$ ) compared with healthy children ( $n = 21$ ) and change in PBMC gene expression in the same genes upon statin treatment in FH children ( $n = 10$ ). Interpretation: each grey or orange point represents a combination of two linear regression estimates; the lower right grey-shaded area covers genes that were higher expressed in FH children compared with healthy children, but were reduced upon statin treatment; the upper left grey-shaded area covers genes that were lower in FH children compared with healthy children, but were increased upon statin treatment; orange-coloured points are significant at  $P < 0.05$  in both settings (with 95% confidence intervals). The correlation coefficient is Spearman's rho. The intercept is where the regression line crosses x, and the slope is the beta-coefficient.  $R^2$  is explained variance for this association. Note that the children subjected to statin treatment (y-axis) were not the same as in the cross-sectional analysis (x-axis). FH, familial hypercholesterolaemia; PBMC, peripheral blood mononuclear cells. Abbreviations of gene names are found in supplemental material.

of inflammation also in the early stage of atherosclerosis, before any overt disease.

Human atherosclerotic plaques contain many immune cells, such as monocytes/macrophages, antigen-presenting cells, and T and B lymphocytes, of which monocytes and macrophages are the most common cell type [15]. Recently we showed that children with FH were characterized by monocyto-sis with a shift towards more circulating pro-inflammatory/nonclassical monocytes when compared to healthy controls, supporting a central role of monocytes/macrophages in early atherosclerosis [11]. Furthermore, in the same study, we found a tendency to difference in distribution of certain T-cell subsets [11]. Herein we further extend this finding by showing an upregulation of genes related to T- and B-cell signalling in PBMCs from children with FH compared to control children, suggesting an involvement of these cells in an early stage of atherogenesis. Moreover, whereas mRNA

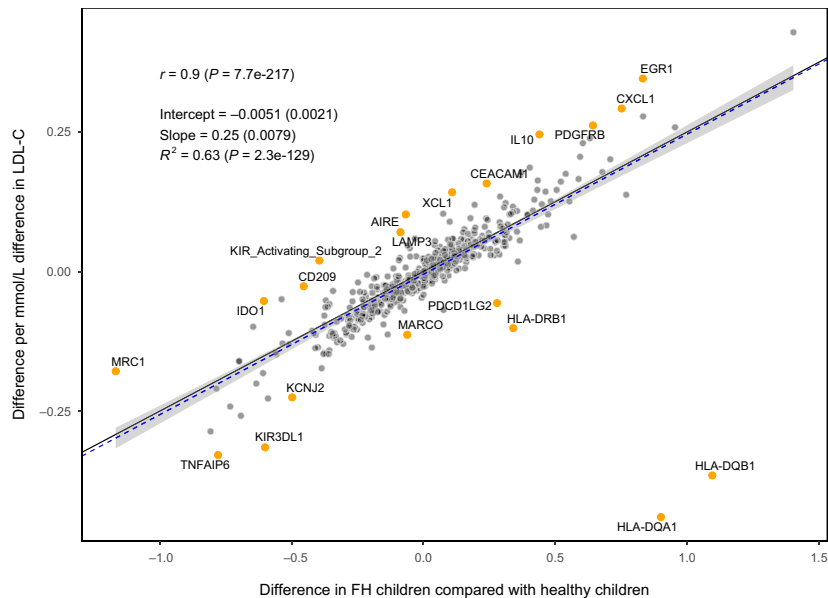
molecules related to antigen presentation, apoptosis and TLR pathway were mostly negatively correlated with lipid parameters and CRP, an opposite pattern was seen for genes related to T- and B-cell signalling suggesting that these pathways are differently related to lipid parameters and systemic inflammation.

Several genes related to T-cell activation (e.g. *CTLA4*, *CD28*) and T- and B-cell interaction (*ICOS*) were differently regulated in FH versus healthy control children. Members of the B7-CD28 superfamily exert immune-regulatory effects [16]. First, activation, expansion, differentiation and survival of T cells are precisely regulated through co-stimulatory and co-inhibitory signals after CD80/CD86 binding to CD28 and CTLA4, respectively [17]. In experimental studies, hypercholesterolaemia has been associated with CD28 expression on T cells in the atherosclerotic plaque [18, 19], and blocking of CTLA4 has been shown to prevent intimal



**Fig. 4** Correlation heatmap of clinical variables and significant genes at  $FDR < 0.05$  within main gene groups from both FH ( $n = 30$ ) and healthy children ( $n = 21$ ). Method is Spearman's rank correlation. Data are based on a 4-way ANOVA using log<sub>2</sub>-transformed data as input, with strip, age and sex as factors. ANOVA, analysis of variance; Apo, apolipoprotein; CRP, C-reactive protein; FDR, false discovery rate; FH, familial hypercholesterolaemia; HDL-C, high-density lipoprotein cholesterol; IL, interleukin; LDL-C, low-density lipoprotein cholesterol; oxLDL, oxidized low-density lipoprotein; TLR, toll-like receptor; TNFSF, tumour necrosis factor super family; total-C, total cholesterol. Abbreviations of gene names are found in supplemental material.





**Fig. 5** Changes in gene expression are similar using either FH status or LDL-C as the exposure variable. The figure shows the strong association between difference in PBMC gene expression in FH children ( $n = 30$ ) compared with healthy children ( $n = 21$ ) (x-axis) and difference in PBMC gene expression in the same genes per mmol/L difference in LDL-C (all FH and healthy children,  $n = 51$ ) (y-axis). Note that the figure shows two regression lines: one solid black reference line (intercept = 0 and slope = 0.25), and one in dashed blue (specifics highlighted at plot). Note the high similarity between the reference line and the actual regression line. Interpretation: each grey or orange point represents a combination of two linear regression estimates; orange-coloured points are the top 20 genes with highest absolute value of residuals (i.e. the 20 points that deviate the most from the blue linear regression line).  $R^2$  for the association is 0.63.

thickening [20]. We observed higher expression of *CTLA4*, *CTLA4-TM* and *CD28* in FH children compared to control children; in particular, *CTLA4* was the most significant DEG. Secondly, the ICOS: ICOSL axis is involved in T-cell proliferation into T helper 2-cells and is also of major importance for B- and T-cell interaction [16]. Moreover, ICOS has been shown to be present in human and murine atherosclerotic plaques [21, 22] and may be induced by oxLDL [21]. Expression of ICOS was higher in FH children compared to control children. Thus, our data may suggest an active regulation of T-cell responses mediated by the B7-CD28 superfamily as well as T- and B-cell interaction mediated by ICOS:ICOSL in children with FH. Interestingly, *CTLA4* in addition to the JAK pathway were up-regulated in FH children, and importantly, these pathways can be therapeutically targeted by medications used in various autoimmune disorders [23, 24]. If these pathways are up-regulated in the early stages of atherosclerosis, they could potentially represent interesting future therapeutic targets for prevention of atherosclerosis progression.

The intracellular signal transducers of the TNF receptor-associated factor (TRAF) family regulate cell survival, proliferation, differentiation and activation in addition to cytokine production and autophagy [25]. Pathways activated through CD40 or BAFFR via TRAF1/2/3/5/6 may subsequently activate mitogen-activated protein kinases or trigger BCL2 transcription, respectively, in B cells [25]. Regarding prevention of atherosclerosis, targeting TRAF6 through CD40 has drawn attention [26], but enhanced expression of TRAF1/2/3/5 in human atherosclerotic plaques also indicate a role of these TRAF members in atherosclerosis [27]. We found higher expression of BAFFR (TNFRSF13C), TRAF1/3/5, MAP4K2 and BCL2 in children with FH, possibly suggesting LDL-C-induced activation of several TRAF members and pathways in PBMCs, and again, some of these could be targeted therapeutically [26].

Previously, we and others have shown increased systemic inflammation in children with FH, in particular, an imbalance between TNFSF members and IL-10 [28], CRP [29, 30], P-selectin [29] and

neopterin [30, 31]. Indeed, using a single-gene approach, we have previously observed altered gene expression of certain TNFSF-related genes in the same children with FH compared to control children [12]. However, a holistic approach to map not only single genes but a wide range of candidate genes is important to expand our understanding of atherosclerosis and is of clinical relevance in the prevention of CHD. The present study is to the best of our knowledge the first study to perform a comprehensive analysis of immune-related gene expression in young FH children, representing an early stage of atherosclerosis without any overt symptoms, compared to healthy children, contributing to characterize the early inflammatory profile in this disorder.

Our multivariate analyses suggest that LDL-C is the main driver of the different expression of immune-related genes in FH children compared to control children. Indeed, ten genes were reduced and three were increased after statin initiation, indicating normalization towards the expression level in healthy controls. This further supports a crucial impact of LDL-C on these immune genes. In line with others, we have, in the same FH children, shown changes in inflammatory molecules at gene expression and circulating level after statins [12, 30] or apheresis [32] in children with FH. In contrast, we have previously shown a lasting presence of low-grade inflammation in adults with FH despite long-term LLT [33], which questions whether statins or other LLT completely can reverse the detrimental effects of elevated LDL-C on the immune system for many years in childhood. Nonetheless, our findings further support a role for LDL-C as a trigger of inflammation and show that statins could modulate several pathways that are dysregulated in PBMC from FH children. Most data suggest that the anti-inflammatory effect of statins is mediated through the lipid-lowering effect [34–36]; however, anti-inflammatory effects of statins cannot be ruled out. Hence, if these effects in FH children are secondary to down-regulation of LDL-C, direct immunomodulatory effects or a combination thereof is at present not clear.

The major strength of this study is the comprehensive analysis of about 600 immune-related mRNA molecules in PBMCs by using state-of-the-art Nanostring technology, and subsequent statistical and bioinformatic analyses. Secondly, genetically verified FH children unbiased by LLT are suitable

for studying the pathophysiological role of isolated, elevated levels of LDL-C. The main limitations are the relatively low number of subjects, which increases the probability of type 1 and 2 errors, and lack of measurement of subclinical atherosclerosis, for example IMT. Furthermore, a previous study has shown that neither leucocyte, lymphocyte nor monocyte count did differ between FH subjects and healthy controls [37]; however, we have recently shown altered distribution of leucocytes in FH children [11]. Moreover, our CIBERSORT analysis shows a slightly different distribution in some predicted subtypes; thus, another limitation is the lack of leucocyte count as we cannot account for confounding association with leucocyte subset. Since PBMCs are exposed to atherogenic factors and are pivotal in the atherosclerotic process, we have used these cells as a model system to investigate inflammation in atherosclerosis. However, this model does not necessarily reflect the *in vivo* situation and other methods could have been used, such as macrophage stimulation *in vitro*.

In conclusion, FH children display higher PBMC expression of immune-related genes mapped to several pathways, including T and B cells, TNFSF and cell growth, proliferation and differentiation than healthy children. These novel data support the notion that several immune cells and related pathways play a role in the early stage of atherosclerosis before any disease manifestation. Our results suggest that LDL-C plays a key role in modulating expression of different immune-related genes. Some of the genes that were characterized in PBMC from FH children could potentially also represent future therapeutic targets for prevention of atherosclerosis progression.

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#### Authors' contribution

Authors' contribution: IN, JJC, SSB, BH and K.B.H. conceived and designed research; I.N., J.J.C., S.S.B., A.R. and K.B.H. conducted research; I.N., J.J.C., S.S.B. and A.R. performed statistical analyses; I.N., J.J.C., S.S.B., S.M.U., A.R., P.A., T.E., M.P.B., K.R., T.U., B.H. and K.B.H. interpreted results; I.N., J.J.C. and K.B.H. were responsible for drafting the manuscript; I.N., J.J.C. and K.B.H. were responsible for final content; all authors read, critically revised and approved the final manuscript.

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#### Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Predicted leucocyte subtypes using CIBERSORT analysis based on all the mRNA expressions from the nCounter analysis in children with ( $n = 30$ ) and without ( $n = 21$ ) FH.

**Figure S2.** Differential gene expression before and after statin initiation in FH children ( $n = 10$ ) at  $P < 0.05$ .

**Figure S3.** Association between fold difference and nominal P-value for all genes in FH ( $n = 30$ ) and healthy children ( $n = 21$ ). ■