Taking One Step Back in Familial Hypercholesterolemia

STAP1 Does Not Alter Plasma LDL (Low-Density Lipoprotein) Cholesterol in Mice and Humans


OBJECTIVE: STAP1, encoding for STAP1 (signal transducing adaptor family member 1), has been reported as a candidate gene associated with familial hypercholesterolemia. Unlike established familial hypercholesterolemia genes, expression of STAP1 is absent in liver but mainly observed in immune cells. In this study, we set out to validate STAP1 as a familial hypercholesterolemia gene.

APPROACH AND RESULTS: A whole-body Stap1 knockout mouse model (Stap1−/−) was generated and characterized, without showing changes in plasma lipid levels compared with controls. In follow-up studies, bone marrow from Stap1−/− mice was transplanted to Ldlr−/− mice, which did not show significant changes in plasma lipid levels or atherosclerotic lesions. To functionally assess whether STAP1 expression in B cells can affect hepatic function, HepG2 cells were cocultured with peripheral blood mononuclear cells isolated from STAP1 variant carriers and controls. The peripheral blood mononuclear cells from STAP1 variant carriers and controls showed similar LDLR mRNA and protein levels. Also, LDL (low-density lipoprotein) uptake by HepG2 cells did not differ upon coculturing with peripheral blood mononuclear cells isolated from either STAP1 variant carriers or controls. In addition, plasma lipid profiles of 39 carriers and 71 family controls showed no differences in plasma LDL cholesterol, HDL (high-density lipoprotein) cholesterol, triglycerides, and lipoprotein(a) levels. Similarly, B-cell populations did not differ in a group of 10 STAP1 variant carriers and 10 age- and sex-matched controls. Furthermore, recent data from the UK Biobank do not show association between STAP1 rare gene variants and LDL cholesterol.

CONCLUSIONS: Our combined studies in mouse models and carriers of STAP1 variants indicate that STAP1 is not a familial hypercholesterolemia gene.

VISUAL OVERVIEW: An online visual overview is available for this article.

Key Words: atherosclerosis ■ cholesterol ■ genetics ■ hyperlipoproteinemia type II ■ mice

Familial hypercholesterolemia (FH) is a common genetic disorder characterized by lifelong elevated levels of LDL (low-density lipoprotein) cholesterol (LDL-c) and increased risk for premature atherosclerotic cardiovascular disease. In ≈30% of patients with extreme LDL-c (LDL >4.9 according to DLCN [Dutch Lipid Clinic Network])
Nonstandard Abbreviations and Acronyms

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<tr>
<td>γGT</td>
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<td>APOB</td>
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<td>BMT</td>
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<td>CRISPR/Cas9</td>
<td>clustered regularly interspaced short palindromic repeats/clusters of regularly interspaced short palindromic repeat–associated</td>
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<td>STAP1</td>
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<td>VLDL</td>
<td>very-low-density lipoprotein</td>
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Highlights

- Whole-body or bone marrow–specific STAP1 deficiency does not influence plasma cholesterol levels in mice.
- STAP1 variant carriers do not present higher LDL (low-density lipoprotein) cholesterol levels or alteration in B-cell populations, compared with age- and sex-matched family controls.
- STAP1 is not a familial hypercholesterolemia gene.

Since its discovery, several investigators have studied STAP1 as a gene responsible for FH: an incomplete association was found between the STAP1 p.Pro176Ser variant and an FH phenotype while a p.Glu97Asp variant was discovered in only 1 Spanish FH patient who experienced an acute myocardial infarction. A pThr47Ala variant was furthermore found in 2 family members with a myocardial infarction and elevated plasma LDL-c. In all these studies, the relatively small number of carriers of STAP1 variants have precluded firm conclusions about a possible causal relationship with hypercholesterolemia, especially because no clear damaging genetic variants or homozygous for loss-of-function variants have yet been described. In addition, in a recent study, investigators reported being unable to find an association between STAP1 gene variants and lipid traits in the Berlin FH cohort.

STAP1 (signal transducing adaptor family member 1) protein is mainly expressed in immune tissues including thymus, spleen, lymph nodes, and bone marrow (BM) and particularly in B cells. The protein is also detected in ovary, kidney, and colon but current data show that STAP1 is not expressed in hepatocytes. This remarkable, since the liver plays a crucial role in regulating LDL-c plasma levels by virtue of hepatic VLDL (very-low-density lipoprotein) production, a precursor of LDL, and LDLR-mediated LDL uptake. This led us to hypothesize that STAP1 expression in B cells may affect hepatocyte function.

To study the mechanisms potentially underlying the association between STAP1 and cholesterol homeostasis, we developed and characterized 2 mouse models and investigated possible effects of peripheral blood mononuclear cells (PBMCs) from STAP1 variant carriers on LDL metabolism in a hepatocarcinoma cell line. We also investigated the characteristics of the B cells of these carriers. The findings of these studies motivated us to readdress the association of STAP1 gene variants with plasma lipid and lipoproteins in 4 families. These combined results indicate that STAP1 is not an FH or LDL-c–modulating gene and should not be considered as such for FH genetic screening.

MATERIALS AND METHODS

All data, analytic methods, and materials included in this study are available to other researchers on reasonable request to the corresponding authors.
**Animals Experiments**

All animal experiments were approved by the Institutional Animal Care and Use Committee from the University of Groningen (Groningen, the Netherlands). Animals were housed under standard laboratory conditions with a light cycle of 12 hours and ad libitum food and water.

**Generation and General Characterization of Whole-Body Stap1−/− Mice**

Two mouse lines of whole-body Stap1−/− were generated using CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/clustered regularly interspaced short palindromic repeat–associated 9) technology as described previously (technical details provided in Materials and Methods in the Data Supplement). Male and female Stap1−/− and wild-type littermates (mixed background 50% FvB and 50% C57BL/6J) were grouped and fed a standard laboratory diet (RMH-B; AB Diets, the Netherlands) until 13 weeks of age. Next, the mice were fed a high-fat–high-cholesterol diet (cholesterol, 0.25%; Research Diets, Denmark) for 4 weeks. Blood was taken by orbital punctures under anesthesia with isoflurane, after 4 hours of fasting in the morning, before the start of the high-fat–high-cholesterol diet and after 2 weeks on the high-fat–high-cholesterol diet. Termination was performed by heart puncture under isoflurane anesthesia. Blood was collected in tubes with EDTA-K+, and plasma was separated by centrifugation at 2000 rpm for 10 minutes at 4°C. Organs and plasma were snap-frozen in liquid nitrogen and stored at −80°C. The processing and analysis of mouse tissues was performed as indicated below.

**BM Transplantation and Diet-Induced Atherosclerosis**

Stap1−/− mice were backcrossed to C57BL/6J mice for 8 generations. BM transplants were performed as described elsewhere. In brief, 5 × 10^6 whole BM cells were isolated from either Stap1−/− or wild-type littermate control donors and transplanted into lethally irradiated (9 Gy) Ldlr−/− female recipient mice, which are prone to develop a more severe hyperlipidemic phenotype, as well as extensive atherosclerosis than male Ldlr−/− mice (for more details, see Materials and Methods in the Data Supplement). After a recovery period of 5 weeks, transplanted animals were fed a Western type diet (WTD; 0.15% cholesterol; Research Diets; D14010701) for 12 weeks. Blood samples for plasma lipid measurements were obtained by orbital puncture under isoflurane anesthesia from 4-hour fasted mice before the initiation of the WTD and after 8 weeks of WTD. Blood samples for flow cytometry analysis of cell populations were taken by tail bleeds at the indicated time points (Figure 2A). The animals were overnight fasted and then sacrificed by heart puncture under isoflurane anesthesia, after which heart, aorta, liver, spleen, thymus, and blood were collected for further analyses. The technical details of the flow cytometry analysis for mice are described in Materials and Methods in the Data Supplement.

**Atherosclerotic Lesion Analysis**

Atherosclerotic lesion analysis in the Ldlr−/− BM transplanted mice was performed according to the guidelines from the American Heart Association. The heart was isolated and fixed using formaldehyde 4% solution in phosphate buffer (Klinpath BV, the Netherlands). The hearts were dehydrated and embedded in paraffin and cut into 4-μm cross sections throughout the aortic root area. Hematoxylin–eosin staining was performed on the sections, and the average from 6 sections (with 40 μm of separation between them) for each animal was used to determine lesion size. Lesion size was quantified in a blinded fashion, by morphometric analysis of the valves using Aperio ImageScope Software, version 12.4.0.5043 (Leica Biosystems Pathology).

**Protein Analyses by Targeted Quantitative Proteomics**

Tissue homogenates were prepared at 10% v/v in NP-40 buffer supplemented with Roche cOmplete Protease Inhibitor Cocktail and phosphatase inhibitors 2 and 3 (Sigma-Aldrich), for posterior protein analysis by mass spectrometry.

Murine STAP1 protein was quantified in various tissues using known concentrations of isotopically labeled peptide standards (13C-labeled lysines and arginines), derived from synthetic protein concatamers (PolyQuant GmbH, Germany) using the targeted proteomics workflow as described previously for other targets. Briefly, homogenized tissues (~50 μg protein) were subjected to in-gel digestion, where the proteins were digested by trypsin (1:100 μg/g; Promega) after reduction with 10 mmol/L dithiothreitol and alkylation with 55 mmol/L iodoacetamide, followed by solid-phase extraction (SPE C18-Aq 50 mg/1 mL, Gracepure; ThermoFisher Scientific) for sample cleanup. Liquid chromatography on a nano-ultra-high-performance liquid chromatography system (Ultimate UHPLC focused; Dionex, ThermoFisher Scientific) was performed to separate the peptides. The target peptide (amino acid sequence NYSITIR for murine STAP1) was analyzed by a triple quadrupole mass spectrometer equipped with a nano-electrospray ion source (TSQ Vantage; ThermoFisher Scientific), and the data were analyzed using Skyline. For the liquid chromatography–mass spectrometer measurements, an amount of the digested peptides equivalent to a total protein amount of 1 μg total protein starting material was injected together with ≤0.64 fmol of isotopically labeled concatamer–derived standard peptides for STAP1 (OconCAT technology; PolyQuant GmbH, Germany). The concentrations of the endogenous peptides were calculated from the known concentrations of the standards and expressed in fmol/μg of total protein.

**Lipid Measurements**

Total cholesterol (TC) levels were measured with a colorimetric assay (11489232; Roche Molecular Biochemicals) with cholesterol standard FS (DiaSys Diagnostic Systems) as reference. Triglyceride levels were measured using Trig/GB kit (Roche Molecular Biochemicals) with Roche Precimat Glycerol standard (Roche Molecular Biochemicals) as reference.

**Fast-Performance Liquid Chromatography in Mice**

As part of the initial characterization of the whole-body Stap1−/− FvB mice, cholesterol in the main lipoprotein classes was determined using fast-performance liquid chromatography. The system contained a PU-980 ternary pump with an LG-980-02
linear degasser, FP-920 fluorescence, and UV-975 UV/VIS detectors (Jasco). An extra PU-2080i Plus pump (Jasco) was used for in-line cholesterol PAP or triglyceride enzymatic reagent (Roche, Basel, Switzerland) addition at a flow rate of 0.1 mL/min. The plasma from individual mice was run over a Superose 6 HR 10/30 column (GE Healthcare Hoevelaken, the Netherlands) using TBS pH 7.4, as eluent at a flow rate of 0.31 mL/min. Quantitative analysis of the chromatograms was performed with ChromNav chromatographic software, version 1.0 (Jasco). The plots for individual fast-performance liquid chromatography profiles were generated with R, version 3.6.1 (2019-07-05), and RStudio, using ggplot2_3.2.1, RCColorBrewer_1.1-2, dplyr_0.8.3, and tidyrr_0.8.3.

For the BM transplantation (BMT) study, fast-performance liquid chromatography profiles were obtained using pooled plasma samples (350 μL) from 12 animals of the corresponding genotype, collected before starting WTD diet and after 8 weeks. These fast-performance liquid chromatography profiles were run using 2 Superose6 columns (Pharmacia LKB Biotechnology), after which individual fractions (n=50) were analyzed for cholesterol using the aforementioned colorimetric kit.

Selection of STAP1 Variant Carriers
We contacted and invited all carriers of STAP1 gene variants (p.Glu97Asp, p.Leu69Ser, p.Ile71Thr, or p.Asp207Asn) originally described by Fouchier et al11 to participate. As described previously, these individuals did not carry mutations in LDLR, APOB, or PCSK9 as assessed by Sanger sequencing and multiplex ligation-dependent probe amplification for LDLR11 as controls, we used age- and sex-matched unaffected family controls. The study was approved by the Institutional Review Board at the Academic Medical Center in Amsterdam, and all subjects gave written informed consent before participation in this study. Pathogenicity of the STAP1 variants was assessed with Polymorphism Phenotyping v226 and SIFT36 (Sorting Intolerant From Tolerant; https://sift.bii.a-star.edu.sg/).

Plasma Lipid and Immune Cell Profiling in Patients
Blood was sampled after an overnight fast, and plasma was isolated as described.11 Plasma levels of TC, LDL-c, HDL (high-density lipoprotein) cholesterol, triglycerides, and lipoprotein(a) (Lp(a)) were measured using commercially available assays (Wako Chemicals, Neuss, Germany; DiaSys Diagnostic Systems, Holzheim, Germany; Roche Diagnostics, Almere, the Netherlands), on a Vitalab Selectra E analyzer (Vital Scientific, The Netherlands), at −80°C for RNA isolation and gene expression analysis. Three million PBMCs were stored in TriPure Isolation Reagent (Roche Applied Sciences, Almere, the Netherlands) at −80°C for RNA isolation and gene expression analysis. Three million PBMCs were incubated for 30 minutes at 4°C protected from light with antibodies against CD3 (cluster of differentiation 3), CD19, CD24, CD27, IgD, and CD43 with or without an antibody against CD38 (see Major Resources in the Data Supplement for information about the antibodies). Subsequently, the PBMCs were washed twice with cold PBS +1% BSA and centrifuged at 281g for 5 minutes at 4°C. The final pellet was resuspended in 200 μL PBS +1% BSA and subjected to flow cytometry analysis on the BD LSRFortessa flow cytometer and analyzed with FlowJo (FlowJo, LLC). The selection of the different B-cell subtypes is adapted from Meeuwsen et al60 (Figure VI in the Data Supplement).

Cell Lines
The human hepatoma cell line HepG2 was purchased from American Type Culture Collection (Manassas, VA) and maintained in DMEM with 4.5 g/L glucose, GlutaMAX, and pyruvate (Gibco; Invitrogen, Breda, the Netherlands) supplemented with 10% fetal bovine serum (Gibco), 100 IU/mL penicillin (Gibco), and 100 μg/mL streptomycin (Gibco). The human B-cell precursor leukemia cell lines Kasumi-2 and Nalm6 were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Leibniz, Germany) and maintained in RPMI 1640 with GlutaMAX and HEPES (Gibco) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin.

Coculture Experiments
For cocultures, 125 000 HepG2 cells per well were plated in 24-well plates, allowed to proliferate for ≈70 hours, washed with PBS, and subsequently cultured in coculture medium (DMEM with 4.5 g/L glucose, GlutaMAX, and pyruvate [Gibco] supplemented with 10% lipoprotein-depleted human serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, 5 μM simvastatin [Sigma-Aldrich, Zwijndrecht, the Netherlands], and 10 μM mevalonic acid [Sigma-Aldrich]). PBMCs were isolated from whole blood and resuspended in the coculture medium at a concentration of 1.7 million cells/mL. Of this suspension, 350 μL was added to a 6.5-mm diameter transwell insert with a 0.4-μm pore size (Corning, Corning, NY) that were placed on top of the HepG2 cells in the 24-well plate. After 24 hours of coculture, HepG2 cells were either collected for gene expression analysis, used for LDL uptake studies, or analyzed for LDLR protein expression. Using a similar setup, HepG2 cells were

Immunologic Profiling in Patients
White blood cell counts and blood cell types were determined using flow cytometry (Sysmex, Göttingen, Germany) in a subgroup of 10 STAP1 variant carriers and 10 age- and sex-matched controls. IgM and IgG were measured using immunoturbidimetry (Roche Diagnostics).

PBMCs were isolated from whole blood, sampled in EDTA-coated tubes. This blood was diluted 1:1 with PBS +2 mmol/L EDTA after which 30 mL of this mixture was layered upon 15 mL Lymphoprep (STEMCELL Technologies, Inc, Vancouver, Canada), centrifuged at 944g for 20 minutes at RT with slow acceleration and no brake. The PBMC-containing interphases was collected, washed 3x with cold PBS +2 mmol/L EDTA and centrifuged at 563g for 10 minutes at 4°C. Cells were counted and sample volume was adjusted with cold PBS +1% BSA to 1 million PBMCs per 100 μL. A proportion of the PBMCs was stored in TriPure Isolation Reagent (Roche Applied Sciences, Almere, the Netherlands) at −80°C for RNA isolation and gene expression analysis. Three million PBMCs were incubated for 30 minutes at 4°C protected from light with antibodies against CD3 (cluster of differentiation 3), CD19, CD24, CD27, IgD, and CD43 with or without an antibody against CD38 (see Major Resources in the Data Supplement for information about the antibodies). Subsequently, the PBMCs were washed twice with cold PBS +1% BSA and centrifuged at 281g for 5 minutes at 4°C. The final pellet was resuspended in 200 μL PBS +1% BSA and subjected to flow cytometry analysis on the BD LSRFortessa flow cytometer and analyzed with FlowJo (FlowJo, LLC). The selection of the different B-cell subtypes is adapted from Meeuwsen et al60 (Figure VI in the Data Supplement).

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cocultured with B-cell precursor acute lymphoblastic leukemia cells Kasumi-2 and Nalm6 instead of isolated human PBMCs.

**Gene Expression Analysis**

Total RNA from HepG2 after 24 hours of coculture and isolated PBMCs was isolated using Tripure Isolation Reagent (Roche) according to manufacturer’s instructions. Reverse transcription was performed using a cDNA synthesis kit (SensiFAST cDNA synthesis kit; Bioline, London, United Kingdom) according to the manufacturer’s instructions. Quantitative RT-PCR was performed using SensiFAST SYBRgreen (Bioline) with a CFX384 Real-Time PCR System (Bio-Rad Laboratories, Hercules, CA). Sequences of the used primers are listed in Table IV in the Data Supplement. The expression of each gene was expressed in arbitrary units after normalization to the average expression level of the housekeeping genes RN18S, HPRT1, and RPLP0 using the 2−ΔΔCt method.41

**LDLR Flow Cytometry Analysis**

After 24 hours of coculture, HepG2 cells were washed with PBS, detached from the plates with Accutase (Sigma-Aldrich), and washed twice with ice-cold PBS with 1% BSA and centrifuged at 12,000 rpm for 4 minutes at 4°C. Next, the cells were incubated for 30 minutes on ice with 50 μL 40-fold diluted APC-conjugated anti-human LDLR (catalog No. FAB2148A; R&D Biosciences, Minneapolis, MN), washed twice with ice-cold PBS with 1% BSA, and centrifuged at 12,000 rpm for 4 minutes at 4°C; resuspended in ice-cold PBS with 1% BSA, and measured on a BD FACSCANTO II (BD Biosciences) and analyzed using FlowJo (BD Life Sciences).

**LDL Uptake Studies**

LDL with a density of 1.019 to 1.063 g/mL was isolated from plasma of a healthy, normolipidemic donor through gradient ultracentrifugation after which it was fluorescently labeled with DyLight apoB-labeled LDL per well was added. Thirty minutes later, HepG2 cells were washed twice with ice-cold PBS +0.2% BSA after which they were lysed on ice for 30 minutes with ice-cold RIPA buffer (Pierce, Rockford, IL) supplemented with protease inhibitors (Complete; Roche). The lysates were centrifuged at 13,523 g for 15 minutes at 4°C. The fluorescence at 488 nm in the supernatant was determined and compared with cells that were not incubated with labeled LDL.

**Statistical Analysis**

Statistical analyses were performed with GraphPad Prism (version 8; GraphPad Software, Inc) or R (version 3.6.1 2019-07-05) and R studio (2018 version 1.2.1335). An unpaired parametric Student t test for normally distributed data or a Mann-Whitney U test for not-normally distributed data was performed when 2 different groups were compared. When >2 groups were compared, Kruskal-Wallis test or 2-way ANOVA was performed with Tukey post hoc test or Sidaks correction for multiple comparisons. \( P < 0.05 \) was considered significant.

**RESULTS**

**Generation, Validation, and Initial Characterization of Stap1−/− Mice**

Two Stap1 knockout (Stap1−/−) mouse lines were generated by CRISPR/Cas9-mediated editing of exon 3 (Figure IA and IB in the Data Supplement). Mouse line A has a deletion of 5 base pairs (Del15bp), and mouse line B carries a 14-bp deletion (Del14bp). Both defects introduced premature stop codons as illustrated in Figure IC in the Data Supplement. Stap1−/− mice were born at the expected Mendelian ratios without any overt phenotype. Both lines were characterized, but only data from mouse line A is shown and discussed here. Confirmatory data from mouse line B is shown in Figure II in the Data Supplement.

Using targeted proteomics, we confirmed that in wild-type mice, Stap1 is mainly expressed in spleen, thymus, and lymph nodes while it is below the detection limit in the liver (Figure 1A and 1B). Protein expression of Stap1 was not detected in Stap1−/− mice confirming that a premature stop codon at positions Ser81X (due to Leu76fs) and Gly78X (due to Cys75fs) results in a loss of protein in our mouse lines (Figure 1B; Figure I in the Data Supplement).

**Stap1−/− Mice Present No Alterations in Plasma Lipid Levels**

Compared with wild-type littermates, Stap1−/− male and female mice did not show differences in TC or triglyceride plasma levels on a standard laboratory diet and after 4 weeks on a high-fat–high-cholesterol diet (Figure 1C through 1F; similar data for line B in Figure IIA through IID in the Data Supplement). In addition, plasma lipoprotein profiles of Stap1−/− mice did not show significant differences compared with wild-type littermates (Figure 1E and 1F).

**Irradiated Female Ldlr−/− Mice Transplanted With BM of Stap1−/− Donors Do Not Show Changes in Plasma Lipid Levels or Atherosclerosis Compared With Controls**

In contrast to humans, wild-type mice carry plasma cholesterol mainly in HDL while presenting low levels of LDL-c. Since STAP1 is mainly expressed in immune cells (B cells),25 we used BMT to evaluate the effect of STAP1 deficiency, specifically in hematopoietic cells, on plasma lipids and atherosclerosis. The BMT study was performed in Ldlr−/− mice that carry cholesterol.
Figure 1. Characterization of whole-body Stap1<sup>−/−</sup> (mouse line A) on a standard laboratory diet and after 2 and 4 wk on high-fat–high-cholesterol diet (HFCD).

A. Quantification of STAP1 protein in spleen using a mass spectrometry–based targeted proteomics assay. The black peak indicates the stable (heavy) isotope-labeled standard, and the gray peak represents the endogenous peptide. B, STAP1 protein expression profile per tissue for Stap1<sup>+/+</sup> and Stap1<sup>−/−</sup> mice determined by targeted proteomics (n=3 per genotype). All tissues of Stap1<sup>−/−</sup> mice present STAP1 peptide levels below the detection limit (BD). C and D, Total cholesterol plasma levels in male (C) and female (D) Stap1<sup>+/+</sup> and Stap1<sup>−/−</sup> mice on a standard laboratory diet and after 2 and 4 wk on HFCD. E and F, Triglyceride plasma levels for Stap1<sup>+/+</sup> and Stap1<sup>−/−</sup> male (E) and female (F) mice on a standard laboratory diet and after 2 and 4 wk on HFCD. C–F, Two-way ANOVA with Sidak multiple comparisons test; *P<0.05, **P<0.01; n=8 animals per genotype. G and H, Fast-performance liquid chromatography profiles for plasma cholesterol of individual mice for Stap1<sup>+/+</sup> and Stap1<sup>−/−</sup> males (G) and females (H) at termination after 4 wk on HFCD. The dark line indicates the mean, and the light shades indicate SEM; n=7 to 8 per genotype. Data shown as mean±SEM.
mainly in (V)LDL and better resemble the human lipoprotein phenotype. This study allowed to experimentally test the hypothesis proposed by Fouchier et al,11 that STAP1 expression in B cells can affect plasma cholesterol levels in a mouse model with a human-like lipoprotein profile.

A BMT study into \( \text{Ldlr}^{-/-} \) recipients was performed as illustrated in Figure 2A. Transplantation of BM from

**Figure 2. Bone marrow (BM) deficiency of\( \text{Stap1} \) in \( \text{Ldlr}^{-/-} \) female mice does not induce changes in plasma lipids and does not affect the development of atherosclerosis plaques.**

A. Experimental design to evaluate BM \( \text{Stap1} \) deficiency on lipid metabolism and atherosclerosis in \( \text{Ldlr}^{-/-} \) mice. Samples for flow cytometry analysis and plasma lipids were taken on separate days. B. Relative number of copies of \( \text{Stap1} \) WT gene in total blood after BM transplantation assessed by qPCR. C. Plasma cholesterol and (D) triglyceride levels of \( \text{Ldlr}^{-/-} \) transplanted with BM from \( \text{Stap1}^{-/-} \) compared with those that received \( \text{Stap1}^{+/+} \) BM. C and D. Two-way ANOVA with Sidak correction for multiple comparisons test; \( n=13\) to 16 animals per genotype. E. Fast-performance liquid chromatography (FPLC) profile of pool plasma samples of \( \text{Ldlr}^{-/-}\text{BM}^{\text{Stap1}^{-/-}} \) and \( \text{Ldlr}^{-/-}\text{BM}^{\text{Stap1}^{+/+}} \) on a standard laboratory diet. F. FPLC profile of pooled plasma samples from \( \text{Ldlr}^{+/+}\text{BM}^{\text{Stap1}^{-/-}} \) and \( \text{Ldlr}^{+/+}\text{BM}^{\text{Stap1}^{+/+}} \) animals after 8 wk on Western type diet (WTD). G. Representative example for hematoxylin-eosin staining of hearts showing cardiac valves with atherosclerosis for \( \text{Ldlr}^{-/-}\text{BM}^{\text{Stap1}^{-/-}} \) and \( \text{Ldlr}^{-/-}\text{BM}^{\text{Stap1}^{+/+}} \). H. Quantification of atherosclerotic lesion area in \( \text{Ldlr}^{-/-}\text{BM}^{\text{Stap1}^{-/-}} \) and \( \text{Ldlr}^{-/-}\text{BM}^{\text{Stap1}^{+/+}} \) (H; Student t test). Data shown as mean±SEM. HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; ns, nonsignificant; and VLDL, very-low-density lipoprotein.
Stap1−/− into Ldlr−/− mice (Ldlr−/−BMStap1−/−) nearly annihilated the presence of Stap1 wild-type sequence in blood, resulting in 92% of BM reconstitution. The absence of Stap1 protein in spleen was confirmed by mass spectrometry after sacrifice, indicating long-term downregulation of Stap1 (Figure IIA in the Data Supplement). As in the whole-body Stap1−/− mice, no differences in plasma cholesterol or triglyceride concentrations were observed on a standard laboratory diet or after 8 or 12 weeks of WTD (Figure 2C and 2D). The absence of changes in blood lipids and lipoproteins was corroborated by unchanged lipoprotein profiles (Figure 2E and 2F). No difference in atherosclerotic lesion area was observed in the aortic root of these mice (Figure 2G and 2H), indicating that ablation of Stap1 in the hematopoietic system does not affect atherosclerotic lesion size. Also, no differences in body weight were observed in these animals (Figure IIB in the Data Supplement).

Stap1 Depletion in BM Causes Minor Changes in Lymphocytes and Monocytes in Mice

As BMT induces stress and inflammation, possibly triggering phenotypic differences in the immune system, we also assessed the main immune cell populations in peripheral blood during the BMT study. On a standard laboratory diet, as well as after starting WTD, we observed a small increase in lymphocytes and B cells in the Ldlr−/−BMStap−/− mice compared with Ldlr−/−BMStap+/+/ (Figure VA through VC in the Data Supplement). For monocytes, no differences were observed on a standard laboratory diet, but WTD induced a 30% decrease of the percentage of monocytes in the Ldlr−/−BMStap+/− mice compared with controls (Figure VD in the Data Supplement). This difference appeared to specifically involve the Ly6Clow subpopulation (Figure VE and VF in the Data Supplement). We do not have explanations for the changes in immune cell populations. We assume, however, that their biological relevance for the phenotypes of interest in this study is negligible since no differences were observed in terms of plasma lipid levels or atherosclerosis development. Therefore, we did not further investigate these differences.

Variants in STAP1 Are Not Associated With Changes in Blood-Derived Human (B) Cell Populations

Since our mouse studies did not show an effect of Stap1 deficiency on plasma LDL-c concentrations, we decided to more closely study the effects of Stap1 variants in humans. As Stap1 is predominantly expressed in B cells,26 we first studied B-cell populations in 10 carriers of STAP1 variants (4 p.Leu69Ser, 5 p.Glu97Asp, and 1 p.Asp207Asn carriers) and 10 age- and sex-matched family controls. Table 1 shows that plasma lipids, liver enzymes, IgM and IgG concentrations, as well as white blood cell counts did not differ between the groups. GT (gamma-glutamyltranspeptidase) was the only blood parameter in which a significant difference was observed between STAP1 variant carriers and controls. Although this might signal differences in liver function, the lack of correlation with other hepatic enzymes and the absence of a clear plasma cholesterol phenotype suggest a limited biological relevance of this observation. Subsequent fluorescence-activated cell sorting analyses did not reveal differences among these groups (Figure 3A through 3E). STAP1 mRNA expression appeared lower in PBMCs from carriers compared with controls, but this difference did not reach significance (Figure 3F).

Hematopoietic Cells of Carriers of STAP1 Gene Variants Do Not Affect LDL Metabolism Ex Vivo

STAP1 is not expressed in the main organ controlling LDL homeostasis, the liver, but is abundantly expressed in B cells. We, therefore, investigated whether B cells from carriers of a STAP1 variant can affect hepatic LDL homeostasis by coculturing hematopoietic cells collected from STAP1 variant carriers and controls with HepG2 cells. We used hematopoietic cells from STAP1 p.Leu69Ser or p.Glu97Asp variant carriers since these 2 variants are predicted to negatively affect STAP1 protein function, based on 2 predictive algorithms (Table I in the Data Supplement). Hematopoietic cells of STAP1 variant carriers did not affect mRNA expression of genes encoding for proteins controlling VLDL secretion, such as APOB and MTTP (Figure 3I). Moreover, no differences in LDLR, PCSK9, and SREBP2 mRNA expression were found (Figure 3I). In line, cell surface LDLR expression and LDL uptake by HepG2 cells were not different between cocultures of hematopoietic cells from carriers of STAP1 gene variant and controls (Figure 3G and 3H). Finally, HepG2 cells were cocultured with 2 different B-cell precursor leukemia cell lines Kasumi-2 and Nalm6, which have previously been reported to have low and high STAP1 mRNA expression, respectively.33 We could confirm this (Figure VIIA in the Data Supplement) but did not observe significant changes in the expression of APOB, LDLR, MTTP, PCSK9, and SREBP2 mRNA in HepG2 cells upon coculturing with these 2 cell lines (Figure VIIB in the Data Supplement). In line, there was no effect on cell surface LDLR protein or LDL uptake (Figure VIIC and VIID in the Data Supplement).

Variants in STAP1 Are Not Associated With Elevated Plasma Lipids in Humans

The lack of any effect of the STAP1 variants studied on B-cell population and ex vivo LDL homeostasis prompted us to reassess plasma lipid levels in carriers of STAP1
Figure 3. Characterization of blood-derived cells from 10 selected carriers of STAP1 gene variants and age- and sex-matched family controls (Table 1).

A–E, Relative amount of different B-cell subtypes in STAP1 variant carriers and controls: plasmablasts (A), class-switched B cells (B), non-class-switched B cells (C), naive B cells (D), and transitional (Trans) and regulatory (Reg) B cells (E), depicted as percentage of the total CD19+ (cluster of differentiation) cells. Data shown as mean±SEM, n=10 per group. F, Relative STAP1 mRNA expression in peripheral blood mononuclear cells (PBMCs) from STAP1 variant carriers and family controls, normalized to RN18S, HPRT1, and RPLP0 with data from controls set to 1. Mann-Whitney U test was used in A–F. G–I, PBMCs isolated from either STAP1 variant carriers or controls were cocultured for 24 h with HepG2 cells. G, Relative uptake of DyLight-labeled LDL (low-density lipoprotein) by the HepG2 cells after coculturing. Uptake is corrected for cellular protein content and data from HepG2 cells cocultured with control PBMCs set at 100% (n=12–15). H, Relative LDLR (LDL receptor) protein on the surface of the HepG2 cells after coculturing as determined by fluorescence-activated cell sorting analysis. Data are corrected for the amount of cells, and data from HepG2 cells cocultured with control PBMCs were set at 100% (n=12–15); Mann-Whitney U test was used in A–E. I, Relative mRNA expression in the HepG2 cells after coculturing. Expression is normalized to RN18S, HPRT1, and RPLP0 with data from HepG2 cells cocultured with control PBMCs defined as 1 (n=12–15). J, Comparison of plasma lipoprotein(a) (Lp[a]) concentrations between STAP1 variant carriers and their control family members in 4 different families (2 families in which a p.Glu97Asp variant was found, 1 family with a p.Ile71Thr variant, and 1 family with a p.Leu69Ser variant). Values shown as mean±SEM; 1-way ANOVA and Kruskal-Wallis test were used in G–J. *P<0.05.
gene variants and controls. For this, we compared lipid profiles in newly collected plasma of 39 carriers of STAP1 gene variant carriers with those of 71 family controls. Levels of TC and LDL-c were not different between groups, which was also true for HDL cholesterol and triglyceride levels (Table 2). Also, when stratifying for the 3 different STAP1 gene variants and controls, no differences were observed. Finally, we found overall higher mean Lp(a) levels in pooled carriers versus controls, largely due to increased Lp(a) levels in the family carrying the p.Ile71Thr STAP1 variant (Table 2). However, this was not statistically different when Lp(a) levels were compared within the respective family, suggesting genetic susceptibility for elevated Lp(a) in this specific family (Figure 3J).

DISCUSSION
In 2014, STAP1 was reported as a novel FH candidate gene.11 This finding was intriguing especially because STAP1 is mainly expressed in immune tissues and absent in liver—the main organ involved in lipoprotein metabolism.26,27 Thus far, functional validation studies have not been reported, and possible mechanisms by which STAP1 could influence plasma lipid levels are not known. In experimental mouse studies and studies with PBMCs of carriers of STAP1 gene variants, we were unable to find a role for STAP1 in controlling plasma LDL-c concentrations. Following these negative findings, our combined studies exclude STAP1 as an FH gene.

In line with our current findings, supportive evidence for STAP1 as an FH gene has not grown in the 5 years following its identification in 2 FH families by Fouchier et al, despite the inclusion of the gene in sequencing panels for the screening of patients with hypercholesterolemia. Three additional studies reported STAP1 variants in individual FH patients.20–22 However, none of the STAP1 gene variants published thus far rendered clear-cut loss-of-function effects (eg, out-frame deletions/insertions and nonsense variants leading to premature protein truncation) and did not show clear segregation with high LDL-c levels in small families, hindering the interpretation of these limited findings. Moreover, recent large genome-wide association studies have not provided support for STAP1 as a lipid gene.44,45 Finally, a recent study reported no association between lipid traits in carriers

Table 1. Characteristics of 10 Carriers of STAP1 Gene Variants and 10 Age- and Sex-Matched Family Controls

<table>
<thead>
<tr>
<th></th>
<th>STAP1 Controls</th>
<th>STAP1 Variant Carriers</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males, n</td>
<td>6</td>
<td>6</td>
<td>1.000</td>
</tr>
<tr>
<td>Age, y</td>
<td>58±14</td>
<td>60±15</td>
<td>0.835</td>
</tr>
<tr>
<td>Subjects on lipid-lowering therapy, n</td>
<td>4</td>
<td>6</td>
<td>0.178</td>
</tr>
<tr>
<td>TC, mmol/L</td>
<td>4.8±1.2</td>
<td>5.1±0.9</td>
<td>0.557</td>
</tr>
<tr>
<td>LDL-c, mmol/L</td>
<td>3.1±0.9</td>
<td>3.1±0.9</td>
<td>0.962</td>
</tr>
<tr>
<td>HDL-c, mmol/L</td>
<td>1.3±0.2</td>
<td>1.4±0.3</td>
<td>0.614</td>
</tr>
<tr>
<td>TG, mmol/L</td>
<td>0.9±0.4</td>
<td>1.4±1.0</td>
<td>0.210</td>
</tr>
<tr>
<td>Lp(a), mg/dL</td>
<td>143±226</td>
<td>232±167</td>
<td>0.066</td>
</tr>
<tr>
<td>Bilirubin, μmol/L</td>
<td>9.6±3.1</td>
<td>12.8±8.5</td>
<td>0.280</td>
</tr>
<tr>
<td>ASAT, U/L</td>
<td>25±6</td>
<td>28±7</td>
<td>0.332</td>
</tr>
<tr>
<td>ALAT, U/L</td>
<td>23±7</td>
<td>28±11</td>
<td>0.206</td>
</tr>
<tr>
<td>AF, U/L</td>
<td>69.5±12.7</td>
<td>68.9±22.1</td>
<td>0.941</td>
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<tr>
<td>γGT, U/L</td>
<td>23±12</td>
<td>52±39</td>
<td>0.035*</td>
</tr>
<tr>
<td>IgG, g/L</td>
<td>10.8±2.4</td>
<td>9.9±3.0</td>
<td>0.487</td>
</tr>
<tr>
<td>IgM, g/L</td>
<td>0.8±0.4</td>
<td>0.8±0.6</td>
<td>0.966</td>
</tr>
<tr>
<td>Leucocytes, 10⁹/L</td>
<td>5.7±0.9</td>
<td>6.2±2.2</td>
<td>0.445</td>
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<tr>
<td>Neutrophils, 10⁹/L</td>
<td>3.1±0.7</td>
<td>3.8±1.7</td>
<td>0.251</td>
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<tr>
<td>Lymphocytes, 10⁹/L</td>
<td>1.8±0.2</td>
<td>1.7±0.5</td>
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<tr>
<td>Monocytes, 10⁹/L</td>
<td>0.5±0.1</td>
<td>0.5±0.3</td>
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<tr>
<td>Eosinophils, 10⁹/L</td>
<td>0.15±0.08</td>
<td>0.16±0.08</td>
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<td>Basophils, 10⁹/L</td>
<td>0.05±0.02</td>
<td>0.05±0.02</td>
<td>1.000</td>
</tr>
</tbody>
</table>

LDL-c concentrations were calculated by the Friedewald formula.37 Values are mean±SD or median with interquartile range (TG and Lp[a]). γGT indicates gamma-glutamyltransferase; AF, alkaline phosphatase; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; Lp(a), lipoprotein(a); TC, total cholesterol; and TG, triglycerides.

*P<0.05.
and noncarriers of STAP1 gene variants in a Berlin FH cohort and a population-based cohort from South Tyrol.\textsuperscript{23}

A recent genome-wide rare variant analysis, based on exome sequencing data from >50,000 UK Biobank participants, also aligned with our findings.\textsuperscript{46} In this data set, 150 rare variants (minor allele frequency, <0.1%) affecting coding regions of STAP1 were found in 37,889 individuals. Carriers of these variants did not present with statistically significant changes in LDL-c values (summary statistics: \( p=0.049193; \ SE=0.080952; P=0.054 \)). Of the variants included in our present study, only STAP1 p.Ile71Thr and p.Pro176Ser were found in the UK Biobank data set and did not show association with LDL-c levels (Table II in the Data Supplement).

Retrospectively, LDL-c levels in carriers of STAP1 gene variants in the original publication were only 11% higher compared with controls\textsuperscript{11}—an effect considerably smaller than observed in carriers of causal mutations in \( \text{LDLR} \), \( \text{APOB} \), and \( \text{PCSK9} \). To detect a statistically significant difference of 11% in LDL-c levels, power calculations reveal that one needs around 100 subjects per group. Forty STAP1 variant carriers were studied in the original report by Fouchier et al,\textsuperscript{11} suggesting that the statistically significant association that was initially identified was likely a spurious finding.

In addition, the used FH classification was not stringent: FH was defined as TC or LDL-c levels above the 95th percentile for age- and sex, leaving room for polygenic contributions or elevated Lp(a) levels.\textsuperscript{27} This is particularly important for the field of lipoprotein metabolism where so many novel genes have been proposed as novel FH candidate genes. Clearly, our findings emphasize the importance of in-depth validation studies, which is particularly important for the field of lipoprotein metabolism where so many novel genes have been proposed as novel candidate genes for plasma lipid regulation without functional follow-up.

Our findings have practical implications for the molecular diagnosis of FH as STAP1 is currently included in targeted sequencing panels for FH: we propose to exclude STAP1 from these panels. Furthermore, our findings are relevant to patients in whom STAP1 gene variants have been identified with respect to screening family members, as well as for studies aiming to find novel FH candidate genes. Clearly, our findings emphasize the importance of in-depth validation studies, which is particularly important for the field of lipoprotein metabolism where so many novel genes have been proposed as novel candidate genes for plasma lipid regulation without functional follow-up.

One of the limitations of reassessing the association between STAP1 gene variation and plasma lipid levels is that we were unable to include carriers of all known STAP1 variants. On the other hand, our observations in 10 carriers of STAP1 variants predicted to be damaging were all negative, as well as ex vivo studies into a possible role of immune cells in controlling LDL homeostasis. Thus, our study also highlights that in silico predictions of the effect of gene variation at the protein level should be interpreted with care.

Our findings have practical implications for the molecular diagnosis of FH as STAP1 is currently included in targeted sequencing panels for FH: we propose to exclude STAP1 from these panels. Furthermore, our findings are relevant to patients in whom STAP1 gene variants have been identified with respect to screening family members, as well as for studies aiming to find novel FH candidate genes. Clearly, our findings emphasize the importance of in-depth validation studies, which is particularly important for the field of lipoprotein metabolism where so many novel genes have been proposed as novel candidate genes for plasma lipid regulation without functional follow-up.

**ARTICLE INFORMATION**

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