

# Impact of cholesterol on proinflammatory monocyte production by the bone marrow

Lotte C.A. Stiekema<sup>1</sup>, Lisa Willemsen<sup>2</sup>, Yannick Kaiser<sup>1</sup>, Koen H.M. Prange<sup>2</sup>, Nicholas J. Wareham<sup>3</sup>, S. Matthijs Boekholdt<sup>4</sup>, Carlijn Kuijk<sup>5</sup>, Menno P.J. de Winther<sup>2,6</sup>, Carlijn Voermans<sup>5</sup>, Matthias Nahrendorf<sup>7</sup>, Erik S.G. Stroes<sup>1\*</sup>, and Jeffrey Kroon<sup>8\*</sup>

<sup>1</sup>Department of Vascular Medicine, Amsterdam UMC, University of Amsterdam, Amsterdam Cardiovascular Sciences, Meibergdreef 9, Amsterdam 1105 AZ, The Netherlands;

<sup>2</sup>Department of Medical Biochemistry, Amsterdam UMC, University of Amsterdam, Amsterdam Cardiovascular Sciences, Meibergdreef 9, Amsterdam 1105 AZ, The Netherlands; <sup>3</sup>Medical Research Council Epidemiology Unit, University of Cambridge, Cambridge CB2 0QQ, UK; <sup>4</sup>Amsterdam UMC, University of Amsterdam, Department of Cardiology, Amsterdam Cardiovascular Sciences, Meibergdreef 9, Amsterdam 1105 AZ, The Netherlands; <sup>5</sup>Sanquin Research and Landsteiner Laboratory, Department of Hematopoiesis, University of Amsterdam, Plesmanlaan 125, Amsterdam 1066 CX, The Netherlands; <sup>6</sup>Department of Medical Biochemistry, Amsterdam UMC, University of Amsterdam, Amsterdam Infection and Immunity, Meibergdreef 9, Amsterdam 1105 AZ, The Netherlands; <sup>7</sup>Center for Systems Biology, Department of Radiology, Massachusetts General Hospital, Harvard Medical School, 185 Cambridge Street, Boston, MA 02114, USA; and <sup>8</sup>Department of Experimental Vascular Medicine, Amsterdam UMC, University of Amsterdam, Amsterdam Cardiovascular Sciences, Meibergdreef 9, Amsterdam 1105 AZ, The Netherlands

Received 25 October 2020; revised 22 February 2021; editorial decision 24 June 2021; accepted 8 July 2021; online publish-ahead-of-print 3 August 2021

See page 4321 for the editorial comment for this article 'LDL-cholesterol drives reversible myelomonocytic skewing in human bone marrow', by L. Yvan-Charvet and M. Westerterp, <https://doi.org/10.1093/eurheartj/ehab630>.

## Aim

Preclinical work indicates that low-density lipoprotein cholesterol (LDL-C) not only drives atherosclerosis by directing the innate immune response at plaque level but also augments proinflammatory monocyte production in the bone marrow (BM) compartment. In this study, we aim to unravel the impact of LDL-C on monocyte production in the BM compartment in human subjects.

## Methods and results

A multivariable linear regression analysis in 12 304 individuals of the EPIC-Norfolk prospective population study showed that LDL-C is associated with monocyte percentage ( $\beta = 0.131$  [95% CI: 0.036–0.225];  $P = 0.007$ ), at the expense of granulocytes ( $\beta = -0.876$  [95% CI: -1.046 to -0.705];  $P < 0.001$ ). Next, we investigated whether altered haematopoiesis could explain this monocytic skewing by characterizing CD34<sup>+</sup> BM haematopoietic stem and progenitor cells (HSPCs) of patients with familial hypercholesterolaemia (FH) and healthy normocholesterolaemic controls. The HSPC transcriptomic profile of untreated FH patients showed increased gene expression in pathways involved in HSPC migration and, in agreement with our epidemiological findings, myelomonocytic skewing. Twelve weeks of cholesterol-lowering treatment reverted the myelomonocytic skewing, but transcriptomic enrichment of monocyte-associated inflammatory and migratory pathways persisted in HSPCs post-treatment. Lastly, we link hypercholesterolaemia to perturbed lipid homeostasis in HSPCs, characterized by lipid droplet formation and transcriptomic changes compatible with increased intracellular cholesterol availability.

## Conclusions

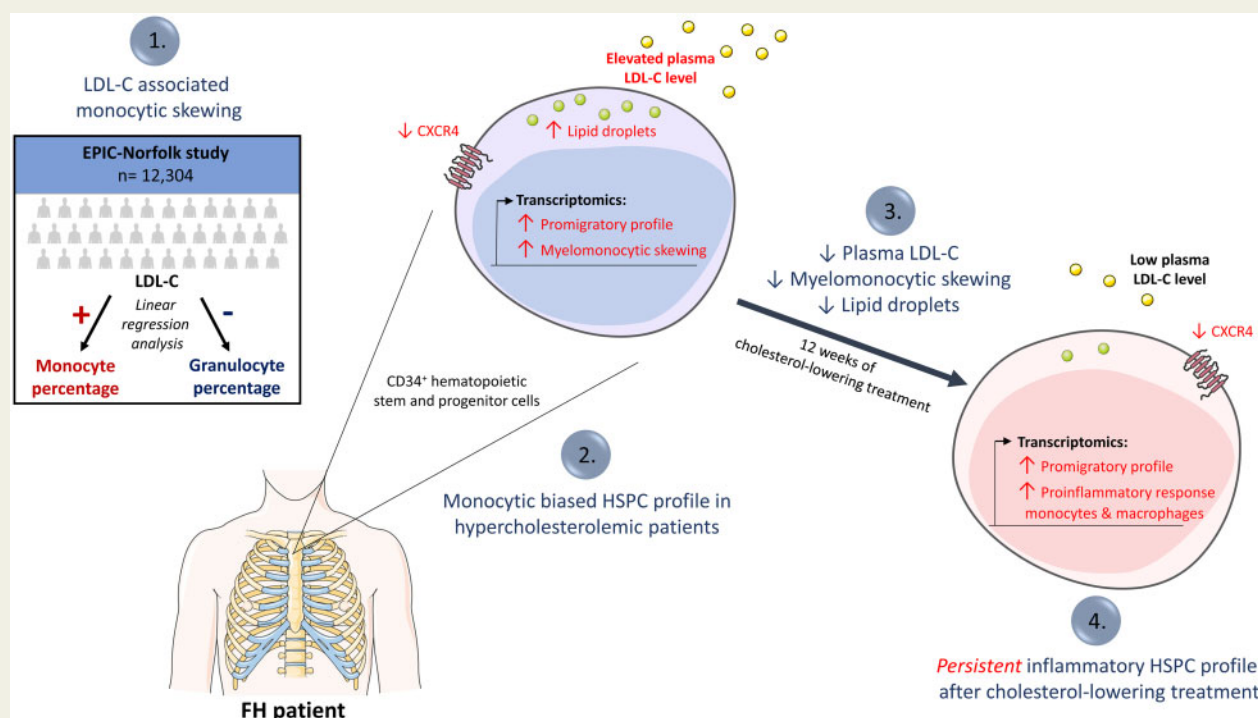
Collectively, these data highlight that LDL-C impacts haematopoiesis, promoting both the number and the proinflammatory activation of circulating monocytes. Furthermore, this study reveals a potential contributory role of HSPC transcriptomic reprogramming to residual inflammatory risk in FH patients despite cholesterol-lowering therapy.

\* Corresponding authors. Tel: +31 (0) 20 5665987, Fax: +31 (0) 20 6968833, Email: [e.stroes@amsterdamumc.nl](mailto:e.stroes@amsterdamumc.nl) (E.S.G. Stroes); Tel: +31 (0) 20 5661150, Email: [j.kroon@amsterdamumc.nl](mailto:j.kroon@amsterdamumc.nl) (J. Kroon)

© The Author(s) 2021. Published by Oxford University Press on behalf of the European Society of Cardiology.

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/4.0/>), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact [journals.permissions@oup.com](mailto:journals.permissions@oup.com)

## Graphical Abstract



LDL-C impacts hematopoiesis, promoting both the number and the proinflammatory activation of circulating monocytes.

## Keywords

Hypercholesterolaemia • Haematopoiesis • Monocytes • Atherosclerosis • Trained immunity • Transcriptomics

## Introduction

Atherosclerotic cardiovascular disease (CVD) is the leading cause of death worldwide, despite improved preventive strategies and survival.<sup>1</sup> Low-density lipoprotein cholesterol (LDL-C) is the key driver of atherosclerotic CVD, making cholesterol-lowering treatment the cornerstone of CVD prevention.<sup>2,3</sup> Although a substantial proportion of cardiovascular (CV) events is prevented by cholesterol-lowering treatment, residual CV risk is considerable, even in patients who reach very low plasma LDL-C levels.<sup>4</sup> One of the contributors to residual CV risk is inflammation, which is suggested to be partly driven by enhanced monocyte activation.<sup>5</sup> Targeting the proinflammatory monocyte response is therefore considered a potential strategy to reduce inflammatory risk with minimal systemic immunosuppression.<sup>6–8</sup>

Mechanistically, atherosclerosis is the result of an unresolved inflammatory response of monocytes and monocyte-derived macrophages to cholesterol retention in the arterial wall.<sup>9</sup> Over the last decade, it has become increasingly clear that hypercholesterolaemia aggravates this inflammatory process by enhancing the production of proinflammatory monocytes in the bone marrow (BM)

compartment.<sup>10,11</sup> This is of clinical interest since epidemiological studies have identified monocyte count as an important CV risk factor,<sup>12,13</sup> whereas translational research has confirmed the association of a proinflammatory monocyte phenotype with increased inflammatory activity in the arterial wall.<sup>14</sup>

In steady state, all types of mature blood cells—including monocytes—are produced in the BM compartment via a process called haematopoiesis. Following specific stimuli, multipotent haematopoietic stem and progenitor cells (HSPCs) undergo lineage commitment steps while proliferating and differentiating into leukocytes, erythrocytes or thrombocytes.<sup>15</sup> Animal studies have shown that hypercholesterolaemia influences this process by promoting HSPC proliferation and myeloid commitment, ultimately leading to monocytosis and accelerated atherosclerosis.<sup>16–18</sup> Also, alterations in lipid metabolism in HSPCs themselves following, for example, activation of cholesterol synthesis<sup>19</sup> or blocking of cholesterol efflux,<sup>20–22</sup> enhances HSPC expansion and myeloid skewing. Together, these studies substantiate that plasma cholesterol levels and intracellular cholesterol homeostasis have impact on HSPC proliferation and differentiation.<sup>23</sup> We therefore hypothesized that the enhanced monocyte response in

hypercholesterolemic patients<sup>5</sup> could be traced back to LDL-C-mediated disruption of cholesterol homeostasis in HSPCs and subsequent altered haematopoiesis.

To examine our hypothesis, we performed two human studies. First, we conducted a mechanistic study in which we performed *ex vivo* unbiased RNA sequencing (RNAseq) and functional analyses of CD34<sup>+</sup> BM HSPCs (hereafter HSPCs) of patients with untreated familial hypercholesterolaemia (FH) before and after cholesterol-lowering treatment, and compared these results to normocholesterolemic healthy control subjects. Next, we used epidemiological data to determine the relationship between LDL-C and leucocyte count and differential in the EPIC-Norfolk study.

## Methods

### Study population and design

#### Study population and design for mechanistic analyses in hypercholesterolemic patients

For mechanistic validation, we conducted a single-centre observational study between July 2017 and May 2019 at the Amsterdam UMC (location AMC), The Netherlands. We included untreated FH patients who had an indication to start lipid-lowering therapy [statin, proprotein convertase subtilisin/kexin type 9 (PCSK9), and/or ezetimibe] according to their treating physician. FH was defined as having a mutation in one of the known FH-causing genes (*LDLR*, *PCSK9*, *APOB*) or, in the absence of such mutation after genetic testing, having a Dutch Lipid Clinic Network score  $\geq 6$  (=probable or definite FH).<sup>24</sup> Exclusion criteria included active smoking, established CVD and recent use (<3 months) of cholesterol-lowering drugs. The healthy controls were age, sex, and body mass index (BMI) matched with the FH patients. After inclusion, FH patients underwent blood withdrawal and a sternal bone marrow aspiration at baseline and after 12 weeks of lipid-lowering therapy. The healthy controls underwent these procedures once. All participants provided written informed consent. The study protocol was approved by the ethics committee of the Amsterdam UMC and was conducted according to the principles of the Declaration of Helsinki.

#### Study population and design for epidemiological analysis in the EPIC-Norfolk cohort

For the assessment of the correlation between LDL-C, apolipoprotein B (ApoB) and leucocyte count and differential, we used data from the European Prospective Investigation into Cancer in Norfolk (EPIC-Norfolk) study.<sup>25</sup> Between 1993 and 1997, 25 639 subjects were recruited from general practices and included in this study. The study protocol was approved by the ethics committee of the Norwich District Health Authority and all study participants gave written informed consent prior to enrolment.

### Bone marrow experiments

All the laboratory experiments and bioinformatics analyses regarding HSPC characterization are available in detail in the Supplementary material online.

### Statistical approach

#### Statistical analysis of the EPIC-Norfolk data

After excluding subjects with C-reactive protein (CRP)  $\geq 10$  g/L [to minimize bias caused by (acute) infections] and missing leucocyte count and

differential values, we performed a univariate regression analysis for LDL-C on leucocyte count, and monocyte, lymphocyte and granulocyte percentage in 12 304 individuals. In addition, we performed multivariable analyses to adjust for age, sex, BMI, smoking, and CRP.

#### Quantification and statistical analyses FH study

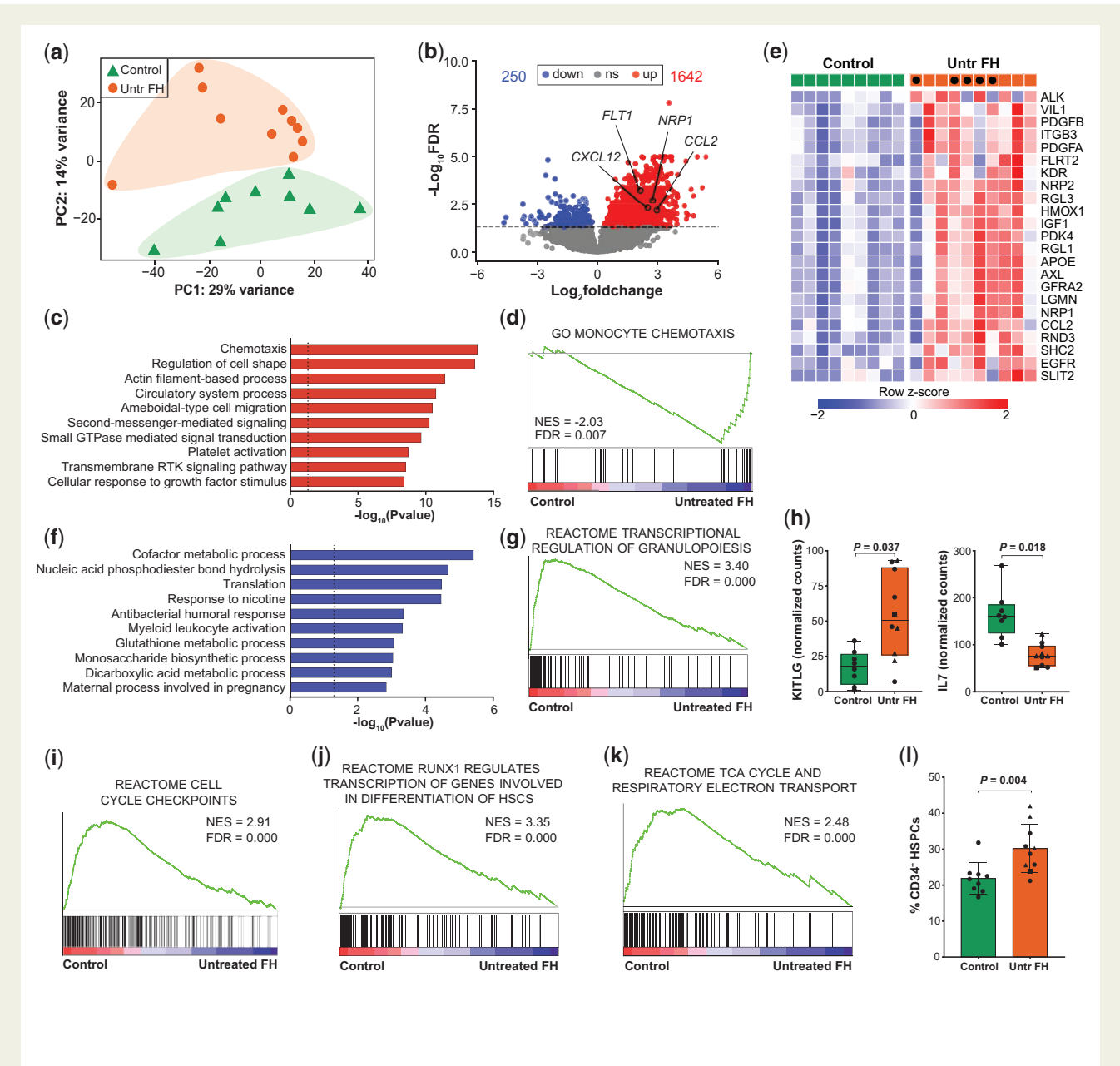
All data were analysed using R version 3.6.3 (R Core Team, Vienna, Austria), SPSS version 25 (SPSS Inc., Chicago, IL, USA), and Graphpad Prism 8 (La Jolla, CA, USA). Data are presented as mean  $\pm$  standard deviation for normally distributed data, median (interquartile range) for non-normally distributed data, or as a number with percentage from total (%) for categorical variables. Changes in biochemical measurements after cholesterol-lowering treatment were assessed using a paired Students *t*-test or Wilcoxon signed-rank test for normally and non-normally distributed data, respectively. Unpaired analyses to compare patients with the healthy control group were performed using an unpaired Student's *t*-test or Mann-Whitney *U*-test for normally and non-normally distributed data, respectively.

## Results

### HSPCs of hypercholesterolaemic patients exhibit myelomonocytic skewing and a promigratory profile

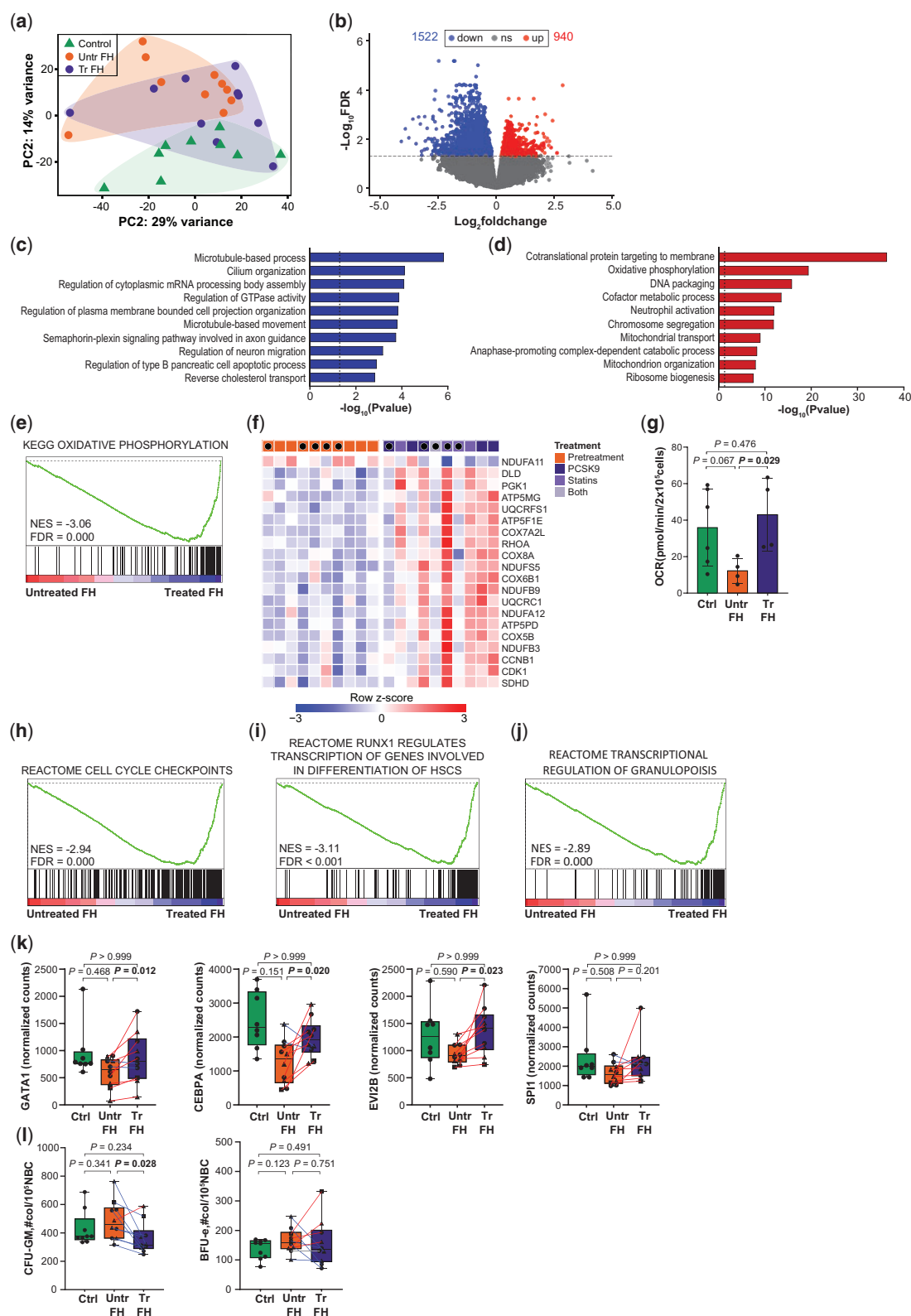
In an *ex vivo* study, we evaluated the impact of hypercholesterolaemia on HSPCs in the BM compartment at a cellular level. We included 10 untreated FH patients (mean baseline LDL-C  $6.0 \pm 2.5$  mmol/L; corresponding with Dutch reference LDL-C  $> 99$ th percentile<sup>26</sup>) and 9 age, sex and BMI matched normocholesterolemic healthy controls (mean LDL-C  $3.3 \pm 0.6$  mmol/L; corresponding with Dutch reference LDL-C  $< 50$ th percentile<sup>26</sup>). Leukocyte (differential) count did not significantly differ between the two groups (additional baseline characteristics are shown in [Supplementary material online, Table S1](#)). In all study participants, a sternal BM aspirate was obtained, from which we isolated and purified CD34<sup>+</sup> HSPCs.

First, principle component analysis of the HSPC RNAseq data showed a separation of the untreated FH patients and healthy controls ([Figure 1A](#)). Differential gene expression analysis revealed 1892 differentially expressed genes (DEGs) with a false discovery rate (FDR) of  $< 0.05$  ([Figure 1B](#)), of which 1642 genes were up- and 250 genes were downregulated in the untreated FH patients vs. healthy controls. Gene ontology (GO) term analysis of the significantly upregulated genes showed predominantly enrichment in pathways related to cell migration ([Figure 1C](#)). Most genes in these upregulated pathways, including *FLT1*, *NRP1*, *CCL2*, and *CXCL12* ([Figure 1B](#)), are members of the vascular endothelial growth factor (VEGF) and chemokine family, respectively. Interestingly, these migratory associated genes promote myeloid progenitor and monocyte mobilization from the BM compartment and increase macrophage and foam cell content in atherosclerotic lesions.<sup>27–29</sup> Gene set enrichment analysis (GSEA) underlined this finding, showing enrichment of the gene set 'monocyte chemotaxis' (FDR = 0.007) in untreated FH patients ([Figure 1D](#)). Of note, VEGF receptors (VEGFRs) belong to the large superfamily of receptor tyrosine kinases (RTKs) that play a central role in fundamental cellular functions including proliferation, differentiation, metabolism and migration.<sup>30</sup> In line, pathway analysis showed



**Figure 1** HSPCs of hypercholesterolemic patients exhibit myelomonocytic skewing and a promigratory profile. (A) Principle component analysis plot of RNAseq data of isolated CD34<sup>+</sup> HSPCs. (B) Volcano plot showing differentially expressed genes. (C) Top 10 most significant enriched pathways using gene ontology (GO) term analysis of significantly upregulated genes in untreated FH patients vs. healthy controls. (D) Enrichment of gene set 'GO monocyte chemotaxis'. (E) Heatmap of most significant upregulated genes in GO RTK signalling and small GTP-ase mediated signalling in untreated FH patients; black dot indicates mutation proven FH. (F) Top 10 most significant enriched pathways using GO term analysis of significantly downregulated genes in untreated FH patients vs. healthy controls. (G) Enrichment of gene set 'REACTOME transcriptional regulation of granulopoiesis'. (H) Normalized gene counts for *KITLG* and *IL7*. P-values are adjusted for multiple testing using Bonferroni–Hochberg correction. Triangle symbol indicates proven *LDLR* mutation, a square indicates proven *APOB* mutation, a dot indicates no FH mutation. (I–K) Enrichment of gene sets 'REACTOME cell cycle checkpoints', 'REACTOME RUNX1 regulates transcription of genes involved in differentiation of HSCs', and 'REACTOME TCA cycle and respiratory electron transport'. (L) Percentage CD34<sup>+</sup> HSPC in bone marrow compartment measured by flow cytometry. Data are mean ± SD. Triangle symbol indicates proven *LDLR* mutation, a square indicates proven *APOB* mutation, and a dot indicates no FH mutation.

enrichment in the RTK signalling pathway (Figure 1C) and the small GTPase mediated signalling pathway (Figure 1C), of which the latter are important downstream effectors for many cell surface receptors including RTKs.<sup>31</sup> The top significantly upregulated genes in these two pathways included *KDR*, a gene encoding VEGFR 2, but also the non-VEGFR RTKs *ALK*, *AXL*, and *EGFR* (Figure 1E). Interestingly, up-regulation of *PDK4*, a gene encoding a key metabolic mitochondrial protein promoting the switch from glucose to fatty acid oxidation,



**Figure 2** Cholesterol-lowering treatment mitigates decreased HSPC differentiation and myelomonocytic skewing. (A) Principle component analysis plot of RNAseq data of isolated CD34<sup>+</sup> HSPCs. (B) Volcano plot showing differentially expressed genes before vs. after cholesterol-lowering treatment in FH patients. (C) Top 10 most significant enriched pathways using gene ontology (GO) term analysis of significantly downregulated genes in treated FH patients vs. untreated FH patients. (D) Top 10 most significant enriched pathways using GO term analysis of significantly upregulated genes in treated FH patients vs. untreated FH patients. (E) Enrichment of gene set 'KEGG oxidative phosphorylation'. (F) Heatmap of most significantly differentially expressed gene in KEGG pathway OXPHOS in treated FH patients, black dot indicates mutation proven FH. (G) Seahorse extracellular flux analysis of Oxygen Consumption Rate (OCR) in CD34<sup>+</sup> HSPCs. (H–J) Enrichment of gene sets relating to HSPC proliferation and differentiation. (K) Normalized read counts for several genes encoding regulators of myeloid HSPC differentiation. P-values are adjusted for multiple testing using Bonferroni–Hochberg correction. Triangle symbol indicates proven *LDLR* mutation, a square indicates proven *APOB* mutation, and a dot indicates no FH mutation. (L) Granulocyte monocyte colony forming unit (CFU-GM) and burst-forming unit-erythroid (BFU-e) assay. Data are mean  $\pm$  SD. Triangle symbol indicates proven *LDLR* mutation, a square indicates proven *APOB* mutation, and a dot indicates no FH mutation.



suggests that fatty acids are the preferred substrates for oxidation in HSPCs of untreated FH patients<sup>32</sup> (Figure 1E).

GO term analysis of the significantly downregulated genes in untreated FH patients demonstrated enrichment of the pathways 'myeloid leucocyte activation' and 'antibacterial humoral response' (Figure 1F). In accordance with our epidemiological data showing a negative association of LDL-C with granulocyte percentage, these two pathways predominantly consisted of downregulated granulocytic associated genes (15 out of 16 genes), which was also reflected by a down-regulation of the gene set 'transcriptional regulation of granulopoiesis' in untreated FH patients (FDR = 0.000) (Figure 1G). In addition to these monocytic-skewed transcriptomic changes in HSPCs of untreated FH patients, an up-regulation of *KITLG*, encoding an important regulator of stem cell survival and myelopoiesis called stem cell factor (SCF)<sup>33</sup> was observed, in addition to down-regulation of lymphoid-associated gene *IL7*<sup>34</sup> (Figure 1H).

HSPCs give rise to mature blood cells through cell proliferation and differentiation.<sup>35</sup> Interestingly, GSEA showed that gene sets related to cell cycle and differentiation were negatively enriched in untreated FH patients (FDR = 0.000 for both) (Figure 1I–J). Metabolically, this was in line with a concordant negative enrichment of the oxidized phosphorylation (OXPHOS) gene set (FDR = 0.000) (Figure 1K), which is a metabolic programme used in more proliferating and mitochondrial active HSPCs.<sup>36,37</sup> The decreased gene expression associated with HSPC differentiation, in addition to the up-regulation of stem cell survival regulator *KITLG*, coincided with a 1.4-fold increase of the percentage CD34<sup>+</sup> HSPCs in the BM compartment ( $P=0.004$ ) (Figure 1L), measured by flow cytometry. Taken together, the HSPC transcriptome of untreated FH patients differs from healthy controls, hallmarked by global up-regulation of promigratory pathways and myelomonocytic skewing.

### Cholesterol-lowering treatment mitigates decreased HSPC differentiation and myelomonocytic skewing in the BM compartment

Following the first BM aspiration, FH patients received maximally tolerated cholesterol-lowering treatment by either a statin, a PCSK9 antibody or a combination, with or without ezetimibe (Supplementary material online, Table S2). After 12 weeks of treatment, a mean 66% reduction in plasma LDL-C levels was achieved ( $P<0.001$ ), resulting in a mean post-treatment plasma LDL-C level of  $1.89 \pm 1.16$  mmol/L (Supplementary material online, Table S2). No significant changes in leucocyte ( $-0.02$  [1.25];  $P=0.961$ ) and monocyte count ( $0.12$  [1.48];  $P=0.803$ ) were observed.

After 12 weeks of treatment, HSPC gene expression demonstrated a trend towards normalization of the transcriptomic profile (Figure 2A). Pairwise comparison of the transcriptomic profile before vs. after treatment showed 2462 significantly DEGs, of which 940 genes were upregulated and 1522 genes were downregulated after treatment (Figure 2B). GO term analysis of the significantly downregulated genes showed predominantly enrichment of pathways involved in cell motility (Figure 2C). In addition, GO term analysis of the significantly upregulated genes and GSEA showed predominantly enrichment of pathways implicated in OXPHOS (Figure 2D–F). To functionally validate these findings, we measured the oxygen

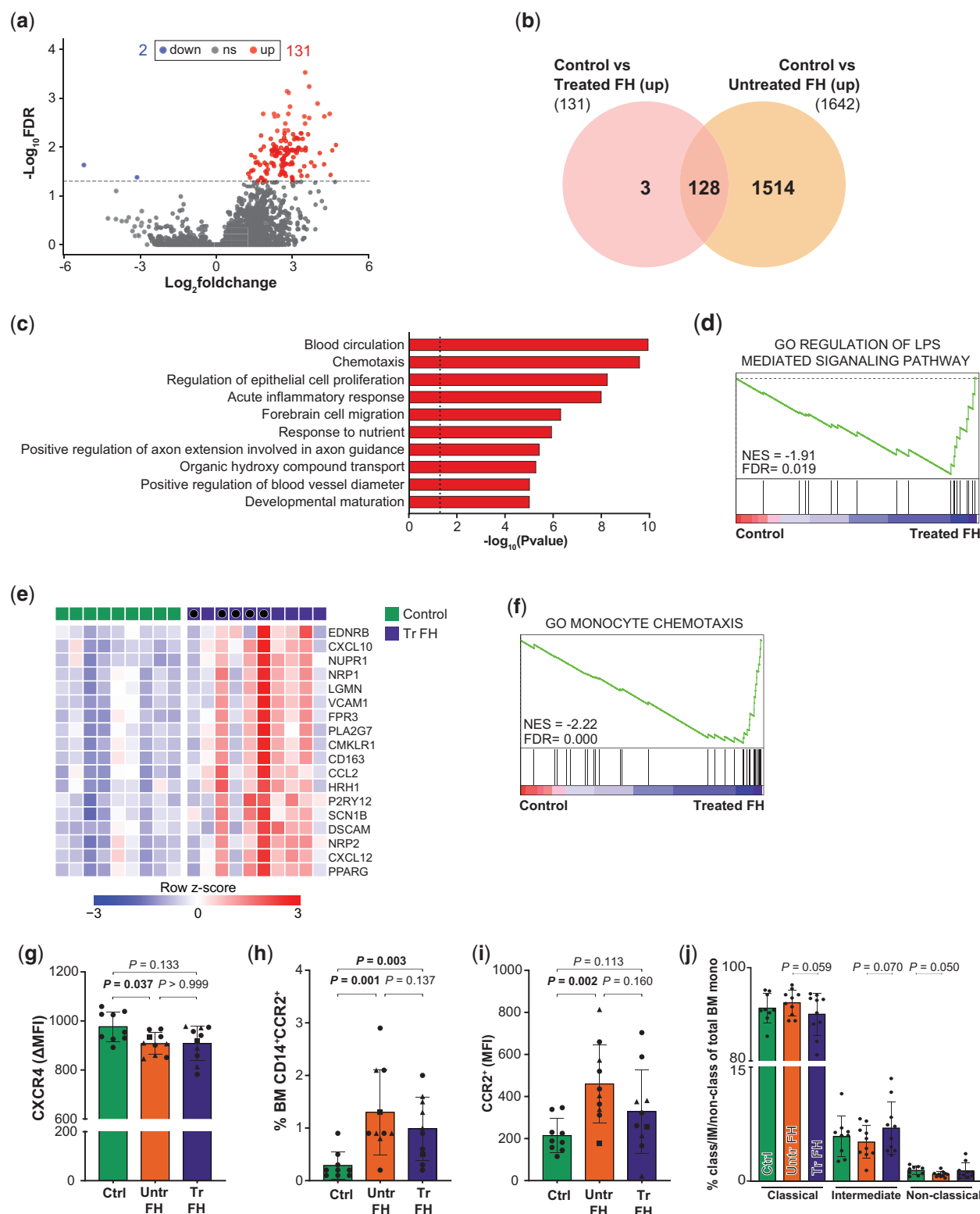
consumption rate in HSPCs by Seahorse Flux Analysis. As expected and while not significant, basal respiration was almost 70% lower ( $P=0.067$ ) in untreated FH patients vs. healthy controls, and was significantly increased after cholesterol-lowering therapy ( $P=0.029$ ) (Figure 2G), following the OXPHOS gene expression pattern seen in the RNAseq.

Since OXPHOS fluxes are higher in more proliferative and differentiated HSPCs,<sup>36,37</sup> we examined whether gene sets involved in proliferation and HSPC differentiation were concomitantly increased after cholesterol-lowering therapy. Indeed, GSEA confirmed enrichment of the cell cycle and RUNX1-mediated differentiation gene sets in FH patients after treatment (FDR = 0.000 and FDR <0.001, respectively) (Figure 2H and I). Earlier we noted that the attenuation of genes involved in granulocyte differentiation was most prominent in untreated FH patients compared to healthy controls. Interestingly, both GO term analysis and GSEA revealed reversibility of this effect, showing significantly increased gene expression associated with neutrophil activation (Figure 2D) and granulocyte differentiation (Figure 2J), respectively. Alleviation of decreased gene expression in pathways involved in HSPC differentiation was further supported by significant enrichment of gene sets involved in lymphopoiesis, erythropoiesis, and megakaryopoiesis, whereas the gene set involved in monocytopoiesis was unaffected (FDR = 0.994) (Supplementary material online, Figure S1). More specifically, expression of key transcriptional determinants of myeloid progenitor commitment *GATA1* (inducing megakaryo-erythroid commitment), and *CEBPA* with its target gene *EVI2B* (inducing granulocytic over monocytic commitment) significantly increased after cholesterol-lowering treatment (Figure 2K).<sup>35,38,39</sup> These data, including the up-regulation of *CEBPA* in the presence of non-significant change in *PU.1* expression, indicate reduced myelomonocytic skewing in HSPCs of treated FH patients (Figure 2K).<sup>39</sup> A significant decrease in functional ex vivo progenitor capacity of the colony-forming unit of granulocytes and monocytes (CFU-GM) ( $-26.2\%$ ;  $P=0.028$ ) in the presence of a significant increase of *CEBPA*, and no effect on progenitor capacity of the burst-forming unit of erythrocytes (BFU-e) ( $-5.3\%$ ,  $P=0.751$ ; Figure 2L), further supports reduced myelomonocytic skewing after cholesterol-lowering treatment, as observed in the gene expression data.

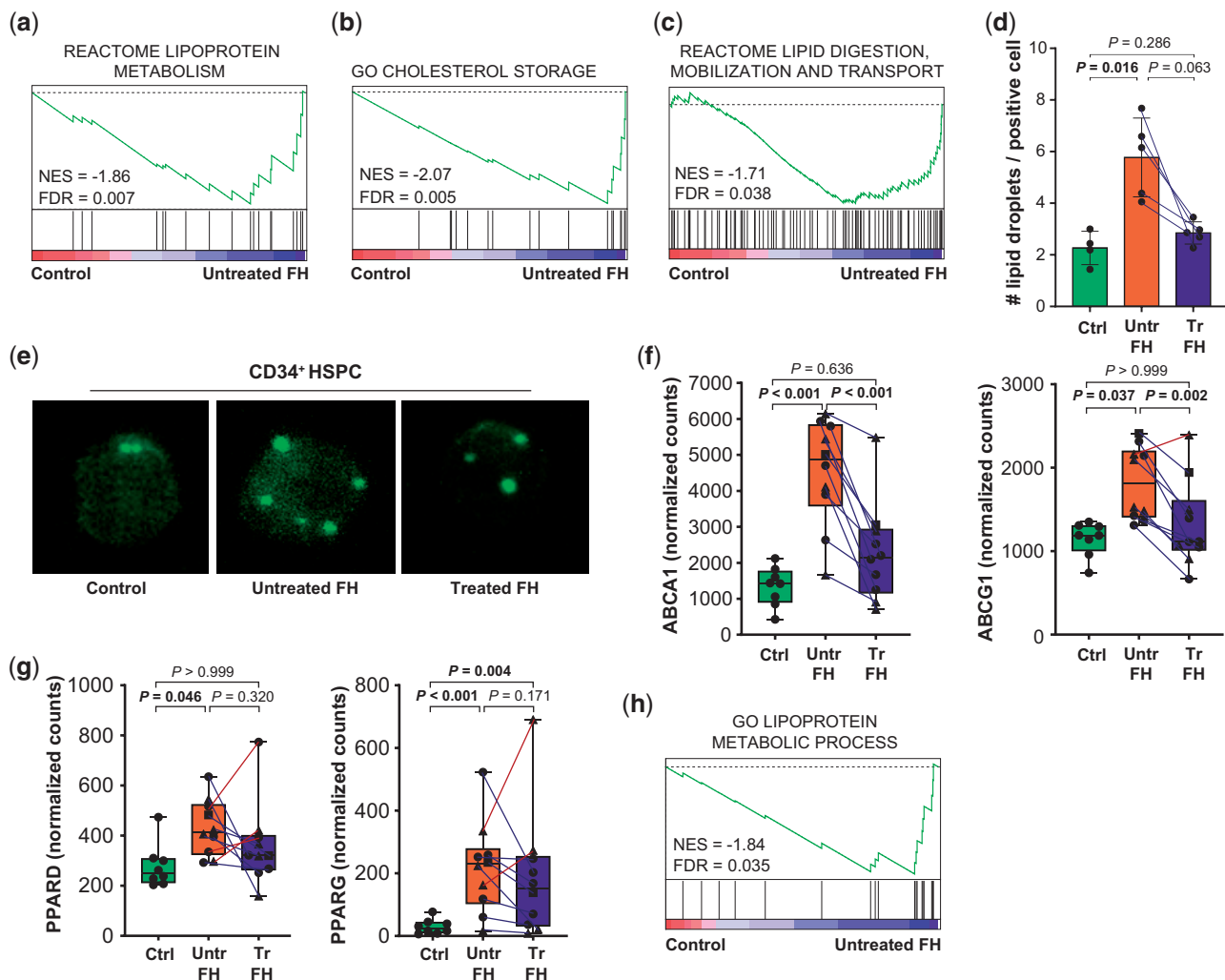
### Persistent proinflammatory and promigratory gene expression in HSPCs after cholesterol-lowering treatment

We previously described a persistent hyper responsiveness of circulating monocytes in treated FH patients termed 'trained immunity'.<sup>5</sup> Since this immune memory persists beyond the short lifespan (hours to days) of circulating monocytes, it has been hypothesized that cholesterol-induced reprogramming of the long-lived progenitors of monocytes maintain this innate immune memory.<sup>5,18</sup> To examine this hypothesis, we compared the HSPC transcriptional profile of the FH patients post-treatment to healthy controls.

Whereas we found 1892 significantly DEGs in untreated FH patients vs. healthy controls (Figure 1B), only 133 genes were differentially expressed after treatment compared to healthy controls (Figure 3A). Interestingly, 128 out of the 131 upregulated genes were also significantly upregulated before treatment (Figure 3B). GO term analysis of these genes showed enrichment of the 'chemotaxis' and 'acute



**Figure 3** Persistent proinflammatory and promigratory gene expression in HSPCs after cholesterol-lowering treatment. (A) Volcano plot showing differentially expressed genes in treated FH patients vs. healthy controls. (B) Venn diagram indicating the number of significantly upregulated genes in untreated FH patients vs. healthy controls, and in treated FH patients vs. healthy controls. (C) Top 10 most significant enriched pathways in treated FH patients vs. healthy controls. (D) Enrichment of gene set 'GO regulation of lipopolysaccharide mediated signalling pathway'. (E) Heatmap of most significant differentially expressed genes in GO chemotaxis and GO acute inflammatory response pathways in treated FH patients; black dot indicates mutation proven FH. (G) Enrichment of gene set 'GO monocyte chemotaxis'. (G–J) CXCR4 expression on  $\text{CD34}^+$  HSPC, CCR2 expression on circulating and bone marrow  $\text{CD14}^+$  monocytes, and monocyte subsets ( $\text{CD14}$ ,  $\text{CD16}$ ) in BM measured by flow cytometry. Data are mean  $\pm$  SD. Triangle symbol indicates proven *LDLR* mutation, a square indicates proven *APOB* mutation, and a dot indicates no FH mutation.



**Figure 4** Both hypercholesterolaemia and cholesterol-lowering treatment affects lipid homeostasis in HSPCs of FH patients. (A–C) Enrichment of gene sets related to lipid homeostasis. (D) Lipid droplet count in lipid droplet positive CD34<sup>+</sup> HSPCs. Data are mean  $\pm$  SD. (E) Representative images of CD34<sup>+</sup> HSPC stained with Nile red. (F and G) Normalized read counts for genes related to lipid homeostasis. P-values are adjusted for multiplicity using Bonferroni correction. Triangle symbol indicates proven *LDLR* mutation, a square indicates proven *APOB* mutation, and a dot indicates no FH mutation. (H) Enrichment of gene set 'GO lipoprotein metabolic process'.

inflammatory response' pathway and GSEA revealed enrichment of the gene set 'regulation of lipopolysaccharide (LPS) mediated signaling pathway' (FDR = 0.019) (Figure 3C and D). This is in line with our previous findings showing that the trained immunity phenotype of circulating monocytes of FH patients is hallmarked by persistent enhanced cytokine production after LPS stimulation *ex vivo*.<sup>5</sup> Among the most upregulated inflammatory genes were plaque macrophage marker *CD163* (Klco et al., 2011)<sup>40</sup> and *PLA2G7*, which encodes lipoprotein-associated phospholipase A2 (Lp-PLA2) (Figure 3E). Lp-PLA2 is an enzyme produced by plaque macrophages, serves as a marker for vulnerable plaques and is a strong predictor of CVD.<sup>41,42</sup> Also, genes involved in chemotaxis were persistently elevated in HSPCs of treated FH patients, including *CCL2*, *CXCL12*, and *VCAM1* (Figure 3E), with concomitant enrichment of the gene set 'monocyte

chemotaxis' (Figure 3F). This gene expression profile coincided with persistent decreased cell surface expression of BM homing receptor *CXCR4* on CD34<sup>+</sup> HSPCs (Figure 3G and Supplementary material online, Figure S2), and sustained increased *CCR2* cell surface expression on both BM (Figure 3H and Supplementary material online, Figure S2) and circulating CD14<sup>+</sup> monocytes (Figure 3I) after treatment. Analysis of the monocyte subset distribution in the BM compartment did not show any significant differences (Figure 3J). Also, we did not find significant correlations between *CCR2* expression on circulating monocytes and *CCR2* expression on bone marrow monocytes, nor with *CXCR4* expression on CD34<sup>+</sup> HSPCs (Supplementary material online, Figure S3). Altogether, these results indicate that transcriptional reprogramming of HSPCs could contribute to the trained immunity phenotype found in circulating monocytes of FH patients.



**Table 1** Relationship between LDL-C and leucocyte differential count ( $n = 12\,304$  individuals, general population of the EPIC-Norfolk cohort)

	Monocytes (%)		Granulocytes (%)		Lymphocytes (%)	
	$\beta$ (95% CI)	P-value	$\beta$ (95% CI)	P-value	$\beta$ (95% CI)	P-value
Unadjusted model	0.197 (0.105–0.289)	<0.001	-0.910 (-1.076 to -0.745)	<0.001	0.742 (0.613–0.870)	<0.001
Model 1	0.126 (0.032–0.220)	0.009	-0.884 (-1.054 to -0.715)	<0.001	0.787 (0.655–0.918)	<0.001
Model 2	0.131 (0.036–0.225)	0.007	-0.876 (-1.046 to -0.705)	<0.001	0.752 (0.622–0.882)	<0.001

Data are standardized coefficient ( $\beta$ ) with 95% CI.

Unadjusted model: univariate linear regression analysis of plasma LDL-C level (mmol/L) and leucocyte number ( $10^9/L$ ), percentage monocytes, granulocytes, or lymphocytes. Model 1: adjusted for age and sex. Model 2: adjusted for Model 1, total white blood cell count, body mass index, smoking, and C-reactive protein. Betas are reported for each standard deviation increase in LDL-C. The variance inflation factors of all predictor variables were <1.3.  $R^2$  monocyte percentage Model 2: 0.0225.  $R^2$  granulocyte percentage Model 2: 0.06384.

$R^2$  lymphocyte percentage Model 2: 0.06557.

CI, confidence interval.

## Both hypercholesterolaemia and cholesterol-lowering treatment affect lipid homeostasis in HSPCs of FH patients

Disrupted lipid homeostasis in HSPCs has major impact on HSPC behaviour.<sup>10,23</sup> Moreover, preclinical studies have linked altered lipid metabolism in myeloid progenitors to trained immunity.<sup>18,43</sup> Indeed, the top 25 of most significantly overexpressed gene sets in untreated FH patients compared to healthy controls included gene sets 'cholesterol storage', 'lipid metabolism', and 'lipid digestion mobilization and transport' (FDR = 0.007, 0.005, and 0.038, respectively) (Figure 4A–C). In parallel, staining of intracellular lipid droplets (LDs) by Nile Red demonstrated an increased number of LDs in HSPCs of untreated FH patients compared to healthy controls (Figure 4D and E). Cells form LDs in reaction to lipid overload to prevent lipotoxicity.<sup>44</sup> In line with GSEA, also other compensatory pathways to lower intracellular cholesterol content were observed in HSPCs of untreated FH patients, including significant up-regulation of cholesterol efflux transporter gene expression of *ABCA1* and *ABCG1* (Figure 4F). In turn, cholesterol-lowering treatment led to normalization of intracellular LD number (Figure 4D and E). This coincided with a significant reduction of *ABCA1* and *ABCG1* (Figure 4F). Lastly, *PPARD* and *PPARG*, encoding lipid sensors peroxisome proliferator-activated receptor delta and gamma, respectively, were significantly upregulated before treatment (Figure 4G). After cholesterol-lowering treatment, *PPARD* expression decreased, whereas *PPARG* remained significantly upregulated (Figure 4G). This persistent up-regulation of *PPARG* post-treatment corresponded with enrichment of the gene set 'lipid metabolism' in treated FH patient vs. healthy controls (Figure 4H).

## LDL-C is positively associated with monocyte percentage but inversely associated with granulocyte percentage

To assess the impact of plasma LDL-C level (mmol/L) on leucocyte count ( $10^9/L$ ) and differential (% and count), we performed a linear regression analysis using data from 12 304 individuals participating in the EPIC-Norfolk prospective population study (Supplementary material online, Table S3). LDL-C was not significantly associated with leucocyte count ( $\beta = -0.017$ , 95% CI (-0.046 to 0.012);  $P = 0.251$ ); whereas we did find a significant positive association between LDL-C and monocyte percentage, also after adjustment for age, sex, BMI, smoking, CRP and leucocyte count [ $\beta = 0.131$ , 95% CI (0.036–0.225);  $P = 0.007$ ]. Conversely, LDL-C was inversely associated with granulocyte percentage [ $\beta = -0.876$ , 95% CI (-1.046 to -0.705);  $P < 0.001$ ] (Table 1). The discrepancy between the positive vs. negative association of monocyte and granulocyte percentage with LDL-C respectively suggests that LDL-C skews haematopoiesis favouring monocyte over granulocyte production, since both immune cells arise from the same bipotential haematopoietic granulocyte–monocyte progenitor (GMP).<sup>35</sup> We found a similar association pattern between LDL-C and monocyte and granulocyte count (Supplementary material online, Tables S4 and S5). Interestingly, ApoB also shows a reciprocal association with monocytes and granulocytes, whereas ApoA1 does not show these associations (Supplementary material online, Table S6).

## Discussion

Here, we report epidemiological and mechanistic evidence for a causal role of LDL-C in driving the production of proinflammatory monocytes at the BM level in hypercholesterolemic patients. Multivariable regression analysis of LDL-C to leucocyte differential count in over 12 000 individuals of the EPIC-Norfolk study showed a

positive association with monocyte percentage, and a negative association with granulocyte percentage. *Ex vivo* BM analyses demonstrated that HSPCs of untreated FH patients are hallmarked by myelomonocytic skewing and a promigratory phenotype, coinciding with perturbed intracellular lipid homeostasis. Twelve weeks of cholesterol-lowering treatment largely reverted these HSPC alterations. However, despite normalization of plasma LDL-C levels, gene expression involved in monocyte and macrophage-mediated inflammation and migration remained upregulated in HSPCs of treated FH patients compared to healthy controls (*Graphical abstract*).

## LDL-C is epidemiologically and mechanistically linked to enhanced monocyte production

Our findings in the EPIC-Norfolk cohort revealed no association of LDL-C with leucocyte count and opposing associations with monocyte and granulocyte percentage, independently of CRP levels. These results suggest that the association of LDL-C with specifically monocyte count is not merely a reflection of the low-grade inflammatory state hallmarked by patients with increased CV risk, but could imply an LDL-C-specific biological effect on leucocyte subset formation. These findings were validated mechanistically, where we showed that the HSPC transcriptomic profile was characterized by myelomonocytic skewing in untreated FH patients. Notably, we demonstrated that the myelomonocytic skewed transcriptomic profile largely normalized after cholesterol-lowering treatment, including up-regulation of master regulator and promotor of granulopoiesis *CEPBA*.<sup>38</sup> Our results are in line with previous preclinical findings demonstrating that hypercholesterolaemia-induced myeloid skewing was in part the result of transcriptional reprogramming of the bipotential GMPs.<sup>18</sup> Combined, our epidemiological and mechanistic results support that LDL-C promotes monocyte production in the BM compartment at least in part via modulated GMP fate, thereby impeding granulocytic differentiation. Of note, in the absence of a lymphoid skewed transcriptomic pattern in BM HSPCs of untreated FH patients, the significant positive association between LDL-C and lymphocyte number in the EPIC-Norfolk study may imply a positive effect of hypercholesterolaemia on extramedullary lymphopoiesis.

## LDL-C promotes promigratory phenotype of monocytes and their progenitors

Besides increased monocyte number, also the promigratory phenotype of monocytes and their progenitors contribute to accelerated atherosclerosis. In this respect, we observed an up-regulation of promigratory genes in HSPCs of untreated FH patients, including *FLT1*. This is of interest, since blocking of Flt1 in a hypercholesterolemic mouse model, abrogated myeloid progenitor egress from the BM compartment into the circulation, which coincided with decreased macrophage content in atherosclerotic lesions.<sup>27</sup> Furthermore, we observed decreased cell surface expression of the BM homing receptor CXCR4 on HSPCs of untreated FH patients of which its down-regulation has been described to promote HSPC mobilization.<sup>45</sup> Indeed, previous human studies have linked hypercholesterolaemia to HSPC migration, evidenced by an association between total cholesterol levels and circulating CD34<sup>+</sup> HSPCs.<sup>46,47</sup> In line, we previously showed that circulating monocytes of FH patients have increased CCR2 cell surface expression,<sup>5,48</sup> facilitating monocyte migration into the atherosclerotic plaque.<sup>49</sup> Interestingly, we

here show that CD14<sup>+</sup> monocytes in the BM compartment of FH patients also have persistent increased CCR2 cell surface expression after cholesterol-lowering treatment. The persistent CCR2 expression on both BM as circulating monocytes and concomitant persistent promigratory HSPC transcriptomic profile in treated FH patients implies hypercholesterolaemia-induced HSPC priming *in vivo*.

## Disrupted lipid homeostasis linked to altered HSPC behaviour

Preclinical work has established that hypercholesterolaemia directly impacts HSPCs and their behaviour.<sup>10</sup> Here, we observed that increased plasma LDL-C levels lead to a profound increase in LD number in HSPCs of untreated FH patients, despite compensatory up-regulation of cholesterol efflux transporter genes *ABCA1* and *ABCG1*. Interestingly, in parallel of the increased LD number in HSPCs, we found at both transcriptional as functional level decreased mitochondrial OXPHOS in HSPCs of untreated FH patients and increased *PD4* expression, marking fatty acid oxidation. Inhibition of OXPHOS has been described in the setting of excess intracellular lipid accumulation<sup>50</sup> and could have driven the observed differentiation impairment of HSPCs in untreated FH patients.<sup>51</sup> Corroborating these findings, normalization of plasma LDL-C levels following cholesterol-lowering treatment coincided with reduced LD number in HSPCs, in addition to an increase in OXPHOS and alleviation of the reduced differentiation. However, the reduction in OXPHOS could also be a passive reflection of the observed increased percentage of upstream HS(P)Cs in untreated FH patients. Future studies on whether these aforementioned metabolic changes drive monocytic biased differentiation of HSPCs, and via which mechanisms the different cholesterol-lowering treatment modalities impact these changes, are therefore warranted.

## Clinical implications

Lessons from the CANTOS trial that targeting inflammation alongside cholesterol-lowering therapy is able to further reduce CVD risk,<sup>52</sup> has fuelled the search for other anti-inflammatory therapies to combat (residual) inflammatory CVD risk. Our study suggests that even after cholesterol-lowering treatment modulated haematopoiesis contributes to the pro-atherogenic monocyte response in hypercholesterolemic patients, highlighting that BM HSPCs could serve as a new therapeutic target. For example, targeting mobilization of monocytes and their precursors has already been suggested to mitigate accelerated atherosclerosis post-myocardial infarction, whereas our findings emphasize a potentially beneficial effect in the chronic inflammatory setting as well.<sup>27,53</sup> However, targeting HSPCs is probably more complex, since several preclinical studies have established that the changes in HSPCs contributing to prolonged monocyte activation are multifaceted, with metabolic, transcriptomic, and epigenetic alterations.<sup>18,43,54</sup> Especially in the context of trained immunity, further research in hypercholesterolemic patients is warranted, to further investigate whether the observed transcriptomic (and metabolic) HSPC reprogramming is accompanied by epigenetic alterations in these cells. In addition, other CV risk factors including sleep deprivation, diabetes mellitus, and lack of exercise contribute to proatherogenic changes in haematopoiesis.<sup>55–57</sup> It would be of interest to further investigate how a combination of these risk factors and the

patient's genetic background impact haematopoiesis and ultimately atherogenesis, both in the acute setting of an ischaemic event and in the setting of chronic inflammation.

## Conclusion

In conclusion, this study provides epidemiological and mechanistic evidence that hypercholesterolaemia modulates HSPC behaviour in the BM compartment, thereby enhancing proinflammatory monocyte production in patients. Moreover, persistent promigratory and proinflammatory gene expression in HSPCs despite normalization of plasma LDL-C levels suggests that prolonged monocyte activation originates in the BM compartment of hypercholesterolemic patients.

## Supplementary material

Supplementary material is available at *European Heart Journal* online.

## Acknowledgements

The authors thank N. Weterings, D. Stalder, C. Homburg, and M. Versloot for their assistance in the lab experiments, R. Hoogveen and J. Schnitzler for their assistance with BM aspirations, L. Reeskamp, D. Collard, and N. Nurmohamed for statistical support, and Servier Medical Art for using their image bank to create the graphical abstract. The authors also wish to thank the participants and staff of the EPIC-Norfolk prospective population study.

## Funding

This work was supported by the European Union's Horizon 2020 research and innovation program (REPROGRAM, grant number 667837) and by the CVON-Dutch Heart Foundation (GENIUS-II, CVON 2017-20). The EPIC-Norfolk Study is funded by Cancer Research UK grant number 14136 and the Medical Research Council grant number G1000143. This work was further supported by the Netherlands Organization for Scientific Research (VENI Grant from ZonMW (Grant number 91619098) of JK). M.P.J.d.W. is supported by The Netherlands Heart Foundation (CVON 2011/B019, CVON 2017-20); Spark-Holding BV (2015B002); the European Union (ITN-grant EPIMAC), and Foundation Leducq (LEAN-Transatlantic Network Grant).

**Conflict of interest:** L.C.A.S., L.W., Y.K., K.H.M.P., N.J.W., S.M.B., C.K., C.V., M.P.J.d.W., and J.K. have nothing to disclose. M.N. received consulting fees from Verseau Therapeutics, Gimv and IFM Therapeutics. E.S.G.S. reports that his institution has received lecturing fees and advisory board fees from Amgen Inc., Regeneron, Sanofi, Akcea, Novartis, and Esperion.

## Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

## References

- Dagenais GR, Leong DP, Rangarajan S, Lanas F, Lopez-Jaramillo P, Gupta R, Diaz R, Avezum A, Oliveira GBF, Wielgosz A, Parambath SR, Mony P, Alhabib KF, Temizhan A, Ismail N, Chifamba J, Yeates K, Khatib R, Rahman O, Zatonska K, Kazmi K, Wei L, Zhu J, Rosengren A, Vijayakumar K, Kaur M, Mohan V, Yusufali A, Kelishadi R, Teo KK, Joseph P, Yusuf S. Variations in common diseases, hospital admissions, and deaths in middle-aged adults in 21 countries from five continents (PURE): a prospective cohort study. *Lancet* 2020;**395**:785–794.
- Mach F, Baigent C, Catapano AL, Koskinas KC, Casula M, Badimon L, Chapman MJ, De Backer GG, Delgado V, Ference BA, Graham IM, Halliday A, Landmesser U, Mihaylova B, Pedersen TR, Riccardi G, Richter DJ, Sabatine MS, Taskinen M-R, Tokgozoglu L, Wiklund O; ESC Scientific Document Group. 2019 ESC/EAS Guidelines for the management of dyslipidaemias: lipid modification to reduce cardiovascular risk: the Task Force for the management of dyslipidaemias of the European Society of Cardiology (ESC) and European Atherosclerosis Society (EAS). *Eur Heart J* 2020;**41**:111–188.
- Grundt SM, Stone NJ, Bailey AL, Beam C, Birtcher KK, Blumenthal RS, Braun LT, de Ferranti S, Faiella-Tommasino J, Forman DE, Goldberg R, Heidenreich PA, Hlatky MA, Jones DW, Lloyd-Jones D, Lopez-Pajares N, Ndumele CE, Orringer CE, Peralta CA, Saseen JJ, Smith SC, Sperling L, Virani SS, Yeboah J. 2018 AHA/ACC/AACVPR/AAPA/ABC/ACPM/ADA/AGS/APHA/ASPC/NLA/PCNA Guideline on the management of blood cholesterol. *J Am Coll Cardiol* 2019;**73**:e285–e350.
- Ridker PM. How common is residual inflammatory risk? *Circ Res* 2017;**120**:617–619.
- Bekkering S, Stiekema LCA, Bernelot Moens S, Verweij SL, Novakovic B, Prange K, Versloot M, Roeters van Lennep JE, Stunnenberg H, de Winther M, Stroes ESG, Joosten LAB, Netea MG, Riksen NP. Treatment with statins does not revert trained immunity in patients with familial hypercholesterolemia. *Cell Metab* 2019;**30**:1–2.
- Hoogveen RM, Stroes ESG, Bekkering S, Nahrendorf M, Netea MG, Riksen NP, de Winther MPJ, Lutgens E, Nordestgaard BG, Neidhart M, Catapano AL. Monocyte and haematopoietic progenitor reprogramming as common mechanism underlying chronic inflammatory and cardiovascular diseases. *Eur Heart J* 2018;**39**:3521–3527.
- Mulder WJM, Ochando J, Joosten LAB, Fayad ZA, Netea MG. Therapeutic targeting of trained immunity. *Nat Rev Drug Discov* 2019;**18**:553–566.
- Schloss MJ, Swirski FK, Nahrendorf M. Modifiable cardiovascular risk, hematopoiesis, and innate immunity. *Circ Res* 2020;**126**:1242–1259.
- Ross R. Atherosclerosis—an inflammatory disease. *N Engl J Med* 1999;**340**:115–126.
- Oguro H. The roles of cholesterol and its metabolites in normal and malignant hematopoiesis. *Front Endocrinol* 2019;**10**:204.
- van der Valk FM, Kuijk C, Verweij SL, Stiekema LCA, Kaiser Y, Zeerleder S, Nahrendorf M, Voermans C, Stroes ESG. Increased haematopoietic activity in patients with atherosclerosis. *Eur Heart J* 2017;**38**:425–432.
- Lassale C, Curtis A, Abete I, van der Schouw YT, Verschuren WMM, Lu Y, Bueno-de-Mesquita HB. Elements of the complete blood count associated with cardiovascular disease incidence: findings from the EPIC-NL cohort study. *Sci Rep* 2018;**8**:3290.
- Olivares R, Ducimetière P, Claude JR. Monocyte count: a risk factor for coronary heart disease? *Am J Epidemiol* 1993;**137**:49–53.
- Verweij SL, van der Valk FM, Stiekema LCA, Nurmohamed NS, Bernelot Moens SJ, Stroes ESG, Duivenvoorden R, Bekkering S, Versloot M, Verberne HJ, Nahrendorf M. CCR2 expression on circulating monocytes is associated with arterial wall inflammation assessed by 18F-FDG PET/CT in patients at risk for cardiovascular disease. *Cardiovasc Res* 2018;**114**:468–475.
- Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. *Cell* 2008;**132**:631–644.
- Swirski FK, Libby P, Aikawa E, Alcaide P, Luscinskas FW, Weissleder R, Pittet MJ. Ly-6Chi monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata. *J Clin Invest* 2007;**117**:195–205.
- Seijkens T, Hoeksema MA, Beckers L, Smeets E, Meiler S, Levels J, Tjwa M, de Winther MPJ, Lutgens E. Hypercholesterolemia-induced priming of hematopoietic stem and progenitor cells aggravates atherosclerosis. *FASEB J* 2014;**28**:2202–2213.
- Christ A, Günther P, Lauterbach MAR, Duestell P, Biswas D, Pelka K, Scholz CJ, Oosting M, Haendler K, Baßler K, Klee K, Schulte-Schrepping J, Ulas T, Moorlag S, Kumar V, Park MH, Joosten LAB, Groh LA, Riksen NP, Espevik T, Schlitzer A, Li Y, Fitzgerald ML, Netea MG, Schultze JL, Latz E. Western diet triggers NLRP3-dependent innate immune reprogramming. *Cell* 2018;**172**:162–175.e14.
- Gu Q, Yang X, Lv J, Zhang J, Xia B, Kim J-D, Wang R, Xiong F, Meng S, Clements TP, Tandon B, Wagner DS, Diaz MF, Wenzel PL, Miller YI, Traver D, Cooke JP, Li W, Zon LI, Chen K, Bai Y, Fang L. AIBP-mediated cholesterol efflux instructs hematopoietic stem and progenitor cell fate. *Science* 2019;**363**:1085–1088.
- Yvan-Charvet L, Pagler T, Gautier EL, Avagyan S, Siry RL, Han S, Welch CL, Wang N, Randolph GJ, Snoeck HW, Tall AR. ATP-binding cassette transporters and HDL suppress hematopoietic stem cell proliferation. *Science* 2010;**328**:1689–1693.
- Wang M, Subramanian M, Abramowicz S, Murphy AJ, Gonen A, Witztum J, Welch C, Tabas I, Westertorp M, Tall AR. Interleukin-3/granulocyte macrophage colony-stimulating factor receptor promotes stem cell expansion, monocytosis, and atheroma macrophage burden in mice with hematopoietic apoE deficiency. *Arterioscler Thromb Vasc Biol* 2014;**34**:976–984.
- Murphy AJ, Akhtari M, Tolani S, Pagler T, Bijl N, Kuo C-L, Wang M, Sanson M, Abramowicz S, Welch C, Bochem AE, Kuivenhoven JA, Yvan-Charvet L, Tall AR.

- ApoE regulates hematopoietic stem cell proliferation, monocytois, and monocyte accumulation in atherosclerotic lesions in mice. *J Clin Invest* 2011;**121**:4138–4149.
23. Pernes G, Flynn MC, Lancaster GI, Murphy AJ. Fat for fuel: lipid metabolism in haematopoiesis. *Clin Transl Immunol* 2019;**8**:e1098.
  24. Nordestgaard BG, Chapman MJ, Humphries SE, Ginsberg HN, Masana L, Descamps OS, Wiklund O, Hegele RA, Raal FJ, Defesche JC, Wiegman A, Santos RD, Watts GF, Parhofer KG, Hovingh GK, Kovanen PT, Boileau C, Aversa M, Boren J, Bruckert E, Catapano AL, Kuivenhoven JA, Pajukanta P, Ray K, Stalenhoef AFH, Stroes E, Taskiran M-R, Tybjaerg-Hansen A; for the European Atherosclerosis Society Consensus Panel. Familial hypercholesterolaemia is underdiagnosed and undertreated in the general population: guidance for clinicians to prevent coronary heart disease: consensus Statement of the European Atherosclerosis Society. *Eur Heart J* 2013;**34**:3478–3490.
  25. Day N, Oakes S, Luben R, Khaw KT, Bingham S, Welch A, Wareham N. EPIC-Norfolk: study design and characteristics of the cohort. European Prospective Investigation of Cancer. *Br J Cancer* 1999;**80**:95–103.
  26. Nurmohamed NS, Collard D, Balder JW, Kuivenhoven JA, Stroes ESG, Reeskamp LF. From evidence to practice: development of web-based Dutch lipid reference values. *Neth Heart J* 2021. doi:10.1007/s12471-021-01562-x.
  27. Luttun A, Tjwa M, Moons L, Wu Y, Angelillo-Scherrer A, Liao F, Nagy JA, Hooper A, Priller J, De Klerck B, Compennolle V, Daci E, Bohlen P, Dewerchin M, Herbert J-M, Fava R, Matthys P, Carmeliet G, Collen D, Dvorak HF, Hicklin DJ, Carmeliet P. Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. *Nat Med* 2002;**8**:831–840.
  28. Aiello RJ, Bourassa P-AK, Lindsey S, Weng W, Natoli E, Rollins BJ, Milos PM. Monocyte chemoattractant protein-1 accelerates atherosclerosis in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* 1999;**19**:1518–1525.
  29. Merckelbach S, van der Vorst EPC, Kallmayer M, Rischpler C, Burgkart R, Döring Y, de Borst G-J, Schwaiger M, Eckstein H-H, Weber C, Pelisek J. Expression and cellular localization of CXCR4 and CXCL12 in human carotid atherosclerotic plaques. *Thromb Haemost* 2018;**118**:195–206.
  30. Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell* 2000;**103**:211–225.
  31. Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell* 2010;**141**:1117–1134.
  32. Takubo K, Nagamatsu G, Kobayashi CI, Nakamura-Ishizu A, Kobayashi H, Ikeda E, Goda N, Rahimi Y, Johnson RS, Soga T, Hirao A, Suematsu M, Suda T. Regulation of glycolysis by Pdk functions as a metabolic checkpoint for cell cycle quiescence in hematopoietic stem cells. *Cell Stem Cell* 2013;**12**:49–61.
  33. Kent D, Copley M, Benz C, Dykstra B, Bowie M, Eaves C. Regulation of hematopoietic stem cells by the steel factor/KIT signaling pathway. *Clin Cancer Res* 2008;**14**:1926–1930.
  34. von Freeden-Jeffery U, Vieira P, Lucian LA, McNeil T, Burdach SE, Murray R. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a non-redundant cytokine. *J Exp Med* 1995;**181**:1519–1526.
  35. Orkin SH. Diversification of haematopoietic stem cells to specific lineages. *Nat Rev Genet* 2000;**1**:57–64.
  36. Daud H, Browne S, Al-Majmaie R, Murphy W, Al-Rubeai M. Metabolic profiling of hematopoietic stem and progenitor cells during proliferation and differentiation into red blood cells. *N Biotechnol* 2016;**33**:179–186.
  37. Simsek T, Kocbas F, Zheng J, DeBerardinis RJ, Mahmoud AI, Olson EN, Schneider JW, Zhang CC, Sadek HA. The distinct metabolic profile of hematopoietic stem cells reflects their location in a hypoxic niche. *Cell Stem Cell* 2010;**7**:380–390.
  38. Radomska HS, Huettner CS, Zhang P, Cheng T, Scadden DT, Tenen DG. CCAAT/enhancer binding protein  $\alpha$  is a regulatory switch sufficient for induction of granulocytic development from bipotential myeloid progenitors. *Mol Cell Biol* 1998;**18**:4301–4314.
  39. Friedman AD. Transcriptional regulation of granulocyte and monocyte development. *Oncogene* 2002;**21**:3377–3390.
  40. Klco JM, Kulkarni S, Kreisel FH, Nguyen T-DT, Hassan A, Frater JL. Immunohistochemical analysis of monocytic leukemias: usefulness of CD14 and Kruppel-like factor 4, a novel monocyte marker. *Am J Clin Pathol* 2011;**135**:720–730.
  41. Kolodgie Frank D, Burke Allen P, Skorija Kristi S, Ladich E, Kutys R, Makuria Addisalem T, Virmani R. Lipoprotein-associated phospholipase A2 protein expression in the natural progression of human coronary atherosclerosis. *Arterioscler Thromb Vasc Biol* 2006;**26**:2523–2529.
  42. Tsimikas S, Willeit J, Knoflach M, Mayr M, Egger G, Notdurfter M, Witztum JL, Wiedermann CJ, Xu Q, Kiechl S. Lipoprotein-associated phospholipase A2 activity, ferritin levels, metabolic syndrome, and 10-year cardiovascular and non-cardiovascular mortality: results from the Bruneck study. *Eur Heart J* 2008;**30**:107–115.
  43. Mitroulis I, Ruppova K, Wang B, Chen L-S, Grzybek M, Grinenko T, Eugster A, Troulinski M, Palladini A, Kourtzelis I, Chatzigeorgiou A, Schlitzer A, Beyer M, Joosten LAB, Isermann B, Lesche M, Petzold A, Simons K, Henry I, Dahl A, Schultze JL, Wielockx B, Zamboni N, Mirtschink P, Coskun Ü, Hajishengallis G, Netea MG, Chavakis T. Modulation of myelopoiesis progenitors is an integral component of trained immunity. *Cell* 2018;**172**:147–161.e12.
  44. Onal G, Kutlu O, Gozuacik D, Dokmeci Emre S. Lipid droplets in health and disease. *Lipids Health Dis* 2017;**16**:128.
  45. Karpova D, Ritchey JK, Holt MS, Abou-Ezzi G, Monlish D, Batoon L, Millard S, Spohn G, Wiercinska E, Chendamarai E, Yang W, Christ S, Gehrs L, Schuettelpelz LG, Dembowski K, Pettit AR, Rettig MP, Bonig H, DiPersio JF. Continuous blockade of CXCR4 results in dramatic mobilization and expansion of hematopoietic stem and progenitor cells. *Blood* 2017;**129**:2939–2949.
  46. Cohen KS, Cheng S, Larson MG, Cupples LA, McCabe EL, Wang YA, Ngwa JS, Martin RP, Klein RJ, Hashmi B, Ge Y, O'Donnell CJ, Vasan RS, Shaw SY, Wang TJ. Circulating CD34(+) progenitor cell frequency is associated with clinical and genetic factors. *Blood* 2013;**121**:e50–e56.
  47. Cimato TR, Palka BA, Lang JK, Young RF. LDL cholesterol modulates human CD34+ HSPCs through effects on proliferation and the IL-17 G-CSF axis. *PLoS One* 2013;**8**:e73861.
  48. Bernelot Moens SJ, Neele AE, Kroon J, van der Valk FM, Van den Bossche J, Hoeksema MA, Hoogeveen RM, Schnitzler JG, Baccara-Dinet MT, Manvelian G, de Winther MPJ, Stroes ESG. PCSK9 monoclonal antibodies reverse the pro-inflammatory profile of monocytes in familial hypercholesterolaemia. *Eur Heart J* 2017;**38**:1584–1593.
  49. Boring L, Gosling J, Cleary M, Charo IF. Decreased lesion formation in CCR2<sup>-/-</sup> mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature* 1998;**394**:894–897.
  50. Aon MA, Bhatt N, Cortassa SC. Mitochondrial and cellular mechanisms for managing lipid excess. *Front Physiol* 2014;**5**:282.
  51. Ito K, Bonora M, Ito K. Metabolism as master of hematopoietic stem cell fate. *Int J Hematol* 2019;**109**:18–27.
  52. Ridker PM, Everett BM, Thuren T, MacFadyen JG, Chang WH, Ballantyne C, Fonseca F, Nicolau J, Koenig W, Anker SD, Kastelein JJP, Cornel JH, Pais P, Pella D, Genest J, Cifkova R, Lorenzatti A, Forster T, Kobalava Z, Vida-Simiti L, Flather M, Shimokawa H, Ogawa H, Dellborg M, Rossi PRF, Troquay RPT, Libby P, Glynn RJ; CANTOS Trial Group. Antiinflammatory therapy with canakinumab for atherosclerotic disease. *N Engl J Med* 2017;**377**:1119–1131.
  53. Dutta P, Sager Hendrik B, Stengel Kristy R, Naxerova K, Courties G, Saez B, Silberstein L, Heidt T, Sebas M, Sun Y, Wojtkiewicz G, Feruglio Paolo F, King K, Baker Joshua N, van der Laan Anja M, Borodovsky A, Fitzgerald K, Hulsmans M, Hoyer F, Iwamoto Y, Vinegoni C, Brown D, Di Carli M, Libby P, Hiebert Scott W, Scadden David T, Swirski Filip K, Weissleder R, Nahrendorf M. Myocardial infarction activates CCR2+ hematopoietic stem and progenitor cells. *Cell Stem Cell* 2015;**16**:477–487.
  54. de Laval B, Maurizio J, Kandalla PK, Brisou G, Simonnet L, Huber C, Gimenez G, Matcovitch-Natan O, Reinhardt S, David E, Mildner A, Leutz A, Nadel B, Bordini C, Amit I, Sarrazin S, Sieweke MH. C/EBP $\beta$ -dependent epigenetic memory induces trained immunity in hematopoietic stem cells. *Cell Stem Cell* 2020;**26**:657–674.e8.
  55. Frodermann V, Rohde D, Courties G, Severe N, Schloss MJ, Amallah H, McAlpine CS, Cremer S, Hoyer FF, Ji F, van Koeveerden ID, Herisson F, Honold L, Masson GS, Zhang S, Grune J, Iwamoto Y, Schmidt SP, Wojtkiewicz GR, Lee IH, Gustafsson K, Pasterkamp G, de Jager SCA, Sadreyev RI, MacFadyen J, Libby P, Ridker P, Scadden DT, Naxerova K, Jeffrey KL, Swirski FK, Nahrendorf M. Exercise reduces inflammatory cell production and cardiovascular inflammation via instruction of hematopoietic progenitor cells. *Nat Med* 2019;**25**:1761–1771.
  56. Hoyer FF, Zhang X, Coppin E, Vasamsetti SB, Modugu G, Schloss MJ, Rohde D, McAlpine CS, Iwamoto Y, Libby P, Naxerova K, Swirski FK, Dutta P, Nahrendorf M. Bone marrow endothelial cells regulate myelopoiesis in diabetes. *Circulation* 2020;**142**:244–258.
  57. McAlpine CS, Kiss MG, Rattik S, He S, Vassalli A, Valet C, Anzai A, Chan CT, Mindur JE, Kahles F, Poller WC, Frodermann V, Fenn AM, Gregory AF, Halle L, Iwamoto Y, Hoyer FF, Binder CJ, Libby P, Taft M, Scammell TE, Nahrendorf M, Swirski FK. Sleep modulates haematopoiesis and protects against atherosclerosis. *Nature* 2019;**566**:383–387.