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# Large HDL particles negatively associate with leukocyte counts independent of cholesterol efflux capacity: A cross sectional study in the population-based LifeLines DEEP cohort

Anouk G. Groenen [a,1], Venetia Bazioti [a,1], Isabelle A. van Zeventer [b], Lianmin Chen [a,c], Hilde E. Groot [d], Jan-Willem Balder [e], Alexandra Zhernakova [c], Pim van der Harst [d,e], Antoine Rimbert [a,f], Jan Albert Kuivenhoven [a], Jingyuan Fu [a,c], Marit Westerterp [a,*]

[a] Department of Pediatrics, University Medical Center Groningen, University of Groningen, Groningen, 9713AV, the Netherlands
[b] Department of Hematology, University Medical Center Groningen, University of Groningen, Groningen, 9713AV, the Netherlands
[c] Department of Genetics, University Medical Center Groningen, University of Groningen, Groningen, 9713AV, the Netherlands
[d] Department of Cardiology, University Medical Center Groningen, University of Groningen, Groningen, 9713AV, the Netherlands
[e] Department of Cardiology, Division of Heart and Lungs, University Medical Center Utrecht, Utrecht University, Utrecht, 3584CX, the Netherlands
[f] Université de Nantes, CHU Nantes, CNRS, INSERM, l'Institut du Thorax, F-44000, Nantes, France

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ABSTRACT

*Background and aims:* Leukocytosis, the expansion of white blood cells, is associated with increased cardiovascular risk. Studies in animal models have shown that high-density lipoprotein cholesterol (HDL-c) suppresses leukocytosis by mediating cholesterol efflux from hematopoietic stem and progenitor cells. HDL-c showed a moderate negative association with leukocyte numbers in the UK Biobank and Multi-Ethnic Study of Atherosclerosis. Cholesterol efflux capacity of HDL (HDL-CEC) or HDL particle (HDL-P) number has been proposed as improved inverse predictor of CVD compared to plasma HDL-c. In the LifeLines DEEP (LLD) cohort (n = 962), a sub-cohort representing the prospective population-based LL cohort from the North of The Netherlands, we tested the hypothesis that HDL-CEC and HDL-P were associated with lower leukocyte counts.

*Methods:* We carried out multivariable regression and causal mediation analyses (CMA) to test associations between HDL-c, HDL-CEC, or HDL-P and leukocyte counts. We measured HDL-CEC in THP-1 macrophages and HDL-P and composition using nuclear magnetic resonance.

*Results:* HDL-c associated negatively with leukocyte counts, as did extra-large and large HDL-P, while HDL-CEC showed no association. Each one-standard deviation (SD) increase in extra-large HDL-P was associated with 3.0% and 4.8% lower leukocytes and neutrophils, respectively ($q < 0.001$). In contrast, plasma concentration of small HDL-P associated positively with leukocyte and neutrophil counts, as did small HDL-P triglycerides (TG) and total plasma TG. CMA showed that the association between S-HDL-P and leukocytes was mediated by S-HDL-TG.

*Conclusions:* The association between HDL-P and leukocyte counts in the general population is dependent on HDL-P size and composition, but not HDL-CEC.

## 1. Introduction

Leukocytosis, the expansion of white blood cells, has been linked to an increased cardiovascular risk in humans. The association between leukocytosis and cardiovascular disease (CVD) has mainly been attributed to the expansion of neutrophil and monocyte populations [1–3]. Several cardiovascular risk factors, including inflammation, diabetes, smoking, and metabolic syndrome, show a positive association with monocytosis and neutrophilia [4].

Hematopoietic stem and progenitor cells (HSPCs) drive monocyte and neutrophil production. Studies in animal models have shown that cholesterol accumulation in HSPCs due to defective cholesterol efflux mediated by the cholesterol transporters ATP Binding Cassette A1 and G1 (ABCA1 and ABCG1), or apolipoprotein (apo)E, enhances

* Corresponding author. University Medical Center Groningen, ERIBA Building 3226 room 04.14, Antonius Deusinglaan 1, 9713AV, Groningen, the Netherlands.
*E-mail address:* m.westerterp@umcg.nl (M. Westerterp).
[1] These authors contributed equally to this work.

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monocytosis, neutrophilia, and atherosclerosis [5,6]. In addition, cholesterol efflux pathways in macrophages also control blood myeloid cell counts, although to a lesser extent than in HSPCs [7]. Overexpression of human *APOA1*, which increases plasma HDL-c, or injections of reconstituted HDL (rHDL), reversed these effects [5–7], suggesting a major role for HDL and cholesterol efflux pathways in suppressing leukocytosis, with athero-protective effects.

The association between HDL-c and leukocyte levels has been assessed in several large population cohorts. HDL-c showed a modest negative association with leukocyte counts in the Multi-Ethnic Study of Atherosclerosis (MESA) study [8], and a strong negative association with monocytes in children with familial hypercholesterolemia (n = 49) [9]. Mendelian randomization (MR) studies in the Danish general population and the UK Biobank using 9 genetic variants associated with low plasma HDL-c levels found a negative association between HDL-c and total leukocytes, monocytes, and lymphocytes in blood, after correction for age and gender [10], suggesting a causal relationship between low HDL-c and high leukocytes. A later study in the UK Biobank cohort could not replicate the initial observations [11], because of adjustment for total plasma triglycerides (TG). Without this adjustment, HDL-c and leukocytes showed a significant negative association [11]. Since VLDL and HDL exchange TG and cholesteryl esters via the action of cholesteryl ester transfer protein (CETP) [12], adjusting for total TG may indirectly affect the contribution of HDL-c to the association with leukocytes. HDL-c thus negatively associates with leukocyte numbers in several large population cohorts. The original studies in animal models suggest that these effects of HDL-c may be attributed to its cholesterol efflux capacity (CEC), *i.e.* its ability to act as an acceptor for cholesterol efflux from macrophages, the first step in reverse cholesterol transport. However, this has not been tested directly. In addition, whether HDL particle (HDL-P) size or number affects its association with leukocyte counts is unknown.

We here tested the hypothesis that HDL-CEC and HDL-P were associated with lower leukocyte counts in the LifeLines DEEP (LLD) cohort [13], a sub-cohort representing the prospective population-based LL cohort from the North of The Netherlands [14]. We additionally hypothesized, supported by studies in animal models [5–7], that HDL-CEC and HDL-P would be improved inverse predictors of leukocyte counts compared to plasma HDL-c.

## 2. Materials and methods

### 2.1. Data availability

The authors declare that all supporting data are available within the article and its Supplemental files.

An extensive version of the Materials and methods section can be found in the Supplemental material.

### 2.2. Cohort description

#### 2.2.1. LLD cohort

The LLD cohort is a sub-cohort of the large, prospective, population-based LL cohort (167 729 subjects) from the North of the Netherlands [14]. From April until August 2013, 1520 participants (age: 17–81 years) were included in the LLD cohort at baseline (Fig. 1) [13]. Participants completed extensive questionnaires on demographics, medical status, lifestyle, and psychosocial aspects. Fasting blood was collected and plasma lipoprotein markers have been characterized, including lipoprotein particle number, size, and composition.

Among the 1520 participants, 1273 subjects had valid NMR data. From these participants, 1191 had valid data on other variables of interest including covariates, lipid levels, and leukocyte counts. We excluded 21 participants based on self-reported history of diabetes, 118 participants based on self-reported history of CVD, 83 participants who were taking lipid lowering medicine or drugs for hypertension, and 7 non-fasting subjects. As a result, 962 participants were included in the analyses.

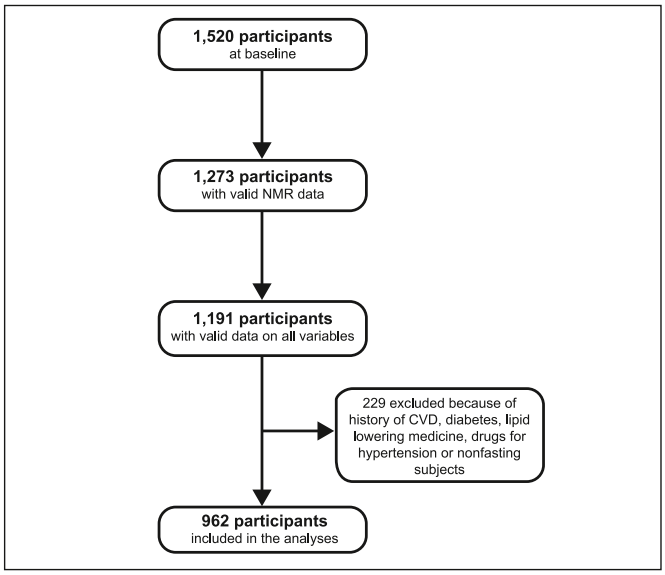


**Fig. 1.** Flow chart of participants included from the LifeLines DEEP population. Of the 1520 participants at baseline, 962had valid data on all variables of interest and matched the inclusion criteria.

The LLD study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the medical ethical committee of the University Medical Centre Groningen, the Netherlands. All participants signed an informed consent form prior to enrollment.

### 2.3. Statistical analyses

Data were analyzed using R, version 4.1.2. Variables with a skewed distribution including immune cell counts and multiple covariates, were subjected to natural logarithm (ln) transformation prior to analysis. A constant of 1 was added to high sensitivity C-reactive protein (hsCRP) before ln-transformation because of zero values among the participants included in the analyses. To deal with NMR measurements, including lipoprotein number, size, and composition under detectable limit (reported as zero values), we added half of the minimum detectable value of the corresponding measurement prior to ln-transformation, as described previously [15].

Spearman coefficients were calculated to determine the magnitude of correlation between HDL-CEC, lipoprotein number, size, and composition. Multivariable linear regression analysis was performed with lipids, lipoprotein number, size, and composition as predictor variables, and leukocyte and leukocyte subpopulations counts as outcome variables. Predictor variables of interest were scaled for mutual comparison. As a result, the regression coefficient (B) was estimated as the ln-transformed cell counts per one-standard deviation (SD) increase in lipoprotein levels, number, size, or composition. The regression coefficient was converted to a percentage change in cell counts using the following formula: % change = (exp(B)-1)*100.

Model 1 is presented as the univariate model. In model 2, data were adjusted for the covariates age and gender, and in model 2a data were adjusted for ln-transformed BMI. In model 3, data were adjusted for age, gender, smoking status, alcohol consumption, ln-transformed BMI, ln-transformed systolic blood pressure, ln-transformed serum creatinine, ln-transformed hsCRP, and ln-transformed HbA1c, as described previously [11]. In model 4, model 3 was further adjusted for (a) HDL-cholesterol efflux capacity, (b) ln-transformed plasma TG level, or (c) ln-transformed HDL-TG within their corresponding subclass.

The correlation between each pair of predictor variables was

assessed to check for collinearity (Supplemental Table I). To control for collinearity in our analyses, we only adjusted for multiple predictor variables in the same multivariable regression model if each pair of predictor variables correlated at a low degree ($r > -0.8$ or $r < 0.8$). To control for multiple testing, *p*-values for each model were subjected to false-discovery rate (FDR) correction. A *q*-value <0.05 was considered statistically significant.

Causal mediation analysis (CMA) was performed to assess potential mediation of the causality between the predictor and outcome variables by an intermediate variable [16]. The mediator model was a multivariate linear regression analysis with HDL-P and all covariates including age, gender, smoking status, alcohol consumption, BMI, systolic blood pressure, serum creatinine, hsCRP, and HbA1c as predictors, and HDL-TG or HDL-C as outcome variables. The outcome model was a multivariate linear regression on leukocytes or leukocyte subpopulations with HDL-P, all covariates, and HDL-TG or HDL-C as predictor variables. Using the "mediate" function in R, the beta coefficients (B) from the total effect, the direct effect (Average Direct Effect; ADE), and the indirect effect (Average Causal Mediation Effect; ACME) as well as the proportion of the total effect mediated by the intermediate variable were calculated.

## 3. Results

### 3.1. Baseline characteristics

Supplemental Table II shows baseline characteristics of the participants of the LLD cohort. The LLD cohort is representative of the whole LL population [13]. From the 1520 participants at baseline, 962 were included in the analyses (Fig. 1). Participants were excluded because of non-valid data with regard to NMR analysis, self-reported history of CVD or diabetes, usage of lipid lowering medicine or drugs for hypertension, or non-fasting measurements, as described in the Materials and methods section.

The mean age of the 1520 subjects at baseline was 43.1 years and 58.7% were female. From the 962 participants included in analyses, the mean age was 41.5 years and 58.3% were female (Supplemental Table II). The median plasma HDL-c, LDL-c, and TG were 1.50, 3.05, and 0.92 mmol/L, respectively. The median leukocyte count was 5.80 x $10^9$ cells/L.

### 3.2. Associations between lipids, lipoproteins, and leukocyte counts in LLD

We first assessed the associations between HDL-c, LDL-c, or total plasma TG with leukocytes, neutrophils, monocytes, and lymphocytes, in a univariate model (model 1), then adjusted for age and gender

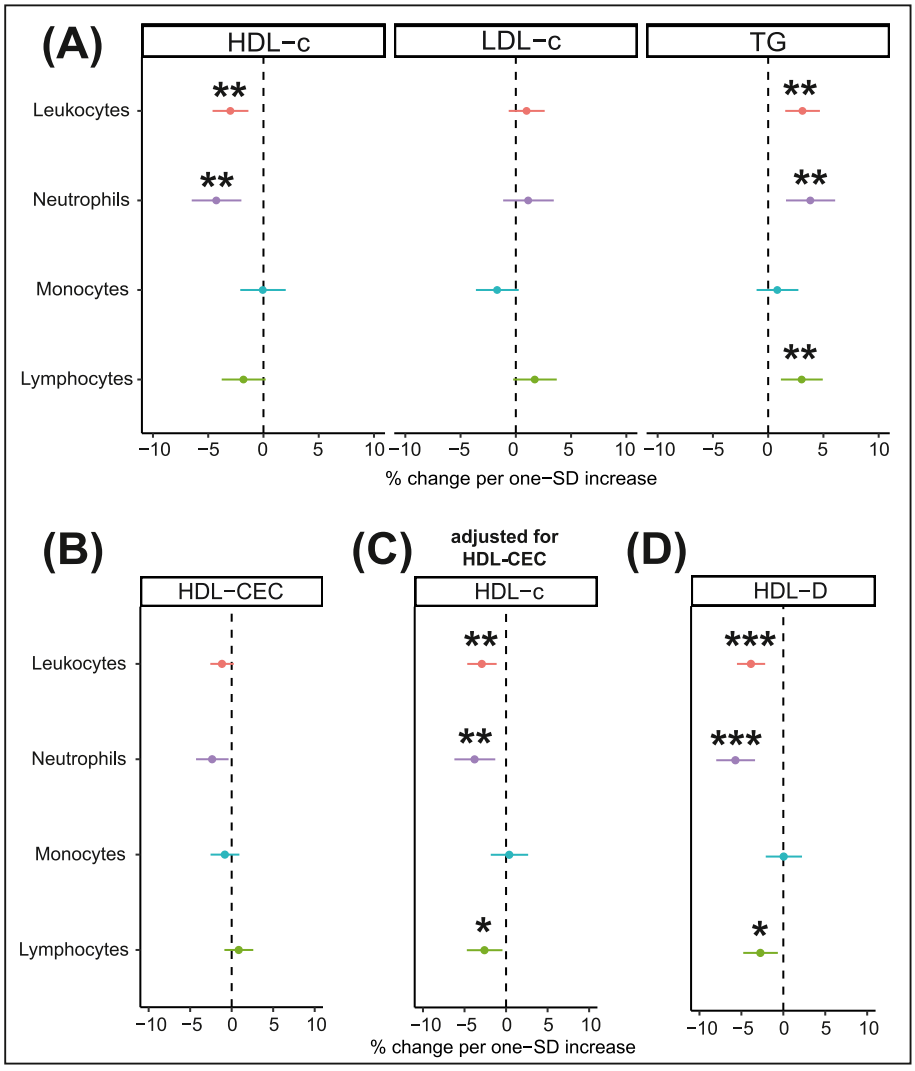


**Fig. 2.** Relationships of plasma lipoprotein levels, HDL-cholesterol efflux capacity, and HDL-diameter with leukocytes and leukocyte subpopulations in the LLD cohort.
Regression beta-coefficient is presented as percentage change of ln-transformed cell counts (x $10^9$ cells/L) per one-SD increase in HDL-cholesterol (HDL-c), LDL-cholesterol (LDL-c), triglycerides (TG) level, HDL-cholesterol efflux capacity (HDL-CEC), or HDL-diameter (HDL-D). (A, B, D) The models were adjusted for age, gender, smoking status, alcohol consumption, ln-transformed BMI, ln-systolic blood pressure, ln-transformed serum creatinine, ln-transformed CRP, and ln-transformed HbA1c (model 3). (C) Model 3 was further adjusted for HDL-CEC (model 4a). Asterisks (*) indicate FDR corrected *p*-values within each panel, *$q < 0.05$, **$q < 0.01$, ***$q < 0.001$. BMI, body mass index; CRP; C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

(model 2) or BMI (model 2a), and then adjusted for age, gender, smoking status, alcohol consumption, BMI, systolic blood pressure, serum creatinine, CRP, and HbA1c (model 3) (Supplemental Table III). Plasma HDL-c levels negatively associated with leukocyte counts in all models, with BMI showing a major contribution to this relationship. Each one-SD increase in HDL-c level (0.40 mmol/L) was associated with 4.6%, 5.5%, 3.9%, and 3.0% lower leukocytes in model 1, 2, 2a, and 3, respectively (B = −0.047, B = −0.056, B = −0.039, B = −0.030, respectively; all $q <$ 0.001) (Fig. 2A and Supplemental Table III). Each one-SD increase in HDL-c level (0.40 mmol/L) was associated with 5.9%, 7.6%, 4.5%, and 4.3% lower neutrophils in model 1, 2, 2a, and 3, respectively ($q < 0.001$) (Fig. 2A and Supplemental Table III). Plasma HDL-c levels negatively associated with monocytes or lymphocytes in model 1, 2, and 2a, but not in model 3 (Supplemental Table III). Relationships between LDL-c levels and leukocytes or leukocyte subpopulations did not reach statistical significance. Similar to findings in the UK Biobank and MESA cohorts [8, 11], plasma TG was associated with higher leukocyte, neutrophil, and lymphocyte counts (Fig. 2A and Supplemental Table III; model 3).

### 3.3. Associations between HDL-CEC and leukocyte counts in LLD

We then measured HDL-CEC in participants of the LLD cohort using THP-1 monocytes that we differentiated into macrophages with PMA. By incubation with the liver X receptor ligand T0901317, ABCA1 and ABCG1 expression was upregulated, similar to previous cholesterol efflux assays [17–19]. Subsequently, we measured cholesterol efflux to apoB-depleted serum from LLD participants. We then assessed whether HDL-CEC was associated with leukocyte counts. In the adjusted model for the covariates (model 3), we found no associations between HDL-CEC and leukocytes, neutrophils, or monocytes (Fig. 2B and Supplemental Table IV). This was unexpected given that HDL-c levels showed a strong positive correlation with HDL-CEC (Supplemental Table V). However, even though HDL-c and HDL-CEC generally correlate strongly in radioactive cholesterol efflux assays [20–23], this does not necessarily imply that the association of HDL-CEC with outcome variables depends on HDL-c. In one major study that introduced the concept of HDL-CEC as an inverse predictor for CVD [21], HDL-CEC strongly correlated with HDL-c (r = 0.58), but HDL-CEC, and not plasma HDL-c, was inversely associated with carotid intima media thickness (cIMT) or coronary artery disease (CAD), also after correction for HDL-c, suggesting that the correlation of HDL-CEC with these outcome variables was independent of HDL-c [21]. In our cohort, the relationship between plasma HDL-c and leukocytes or leukocyte subpopulations was not affected by adjustment for HDL-CEC (Fig. 2C and Supplemental Table V). Together, these data suggest that the negative association between HDL-c and leukocytes in the general population does not depend on HDL-CEC, at least when based on this HDL-CEC assay in THP-1 macrophages.

### 3.4. Associations between HDL particle number, size, or lipoprotein composition and leukocyte counts in LLD

In addition to HDL-CEC, HDL-P, rather than HDL-c has been suggested as an improved predictor of CVD risk [24]. We therefore assessed relationships between HDL-P subclasses, previously measured by NMR in the LLD cohort [25], and leukocyte counts. Four HDL-P subclasses were distinguished according to size: XL-HDL-P, L-HDL-P, M-HDL-P, and S-HDL-P (Supplemental Table VI) [13]. Using NMR, also apoA-I, the main apolipoprotein on HDL, was measured (Supplemental Table VI). In model 1, each one-SD increase in apoA-I (0.17 g/L) was associated with 2.0%, 3.0%, and 3.5% lower leukocytes, neutrophils, and monocytes, respectively (Supplemental Table VII). After adjustment for age and gender, we found a minimal negative association between apoA-I and leukocytes, while the negative association with neutrophils remained. Each one-SD increase in apoA-I (0.17 g/L) was associated with 4.2% lower neutrophils (Supplemental Table VII; model 2). In the adjusted analyses of model 3, each one-SD increase in apoA-I (0.17 g/L) was associated with a 2.7% lower neutrophil count, which was borderline significant (B = −0.028, $q = 0.039$) (Supplemental Table VII).

HDL diameter (HDL-D), which reflects HDL size, was measured by NMR analysis as well (Supplemental Table VI). We found that HDL-D showed a negative association with leukocyte and neutrophil counts in the adjusted analyses (model 3) (Fig. 2D and Supplemental Table VII). A one-SD higher HDL-D (0.24 nm) was associated with 3.9% and 5.7% lower leukocytes and neutrophils, respectively ($q < 0.001$).

In line with this observation, in the adjusted analyses (model 3), each one-SD increase in XL-HDL-P (0.19 μmol/L) was associated with 3.0% and 4.8% lower leukocyte and neutrophil counts, respectively ($q <$ 0.001) (Fig. 3A and Supplemental Table VIII). Similarly, each one-SD increase in L-HDL-P (0.47 μmol/L) was associated with 2.7% and 4.7% lower leukocyte and neutrophil counts, respectively (Fig. 3A and Supplemental Table VIII). XL-HDL-P or L-HDL-P did not associate with monocyte or lymphocyte counts. In contrast to XL-HDL-P and L-HDL-P, S-HDL-P showed a positive association with leukocytes and neutrophils (Fig. 3A and Supplemental Table VIII). Each one-SD increase in S-HDL-P (0.41 μmol/L) was associated with 4.2% and 4.3% higher leukocyte or neutrophil counts, respectively ($q < 0.001$).

We then asked whether HDL-P subclasses correlated with HDL-CEC. As expected, all four HDL-P subclasses correlated positively with HDL-CEC, but this correlation was strongest for XL-HDL-P, L-HDL-P, and M-HDL-P (Supplemental Table IX). In line with our earlier observation showing no associations between HDL-CEC and leukocytes (Supplemental Table IV), adjustment for HDL-CEC did not affect associations between HDL-P with leukocytes (Supplemental Table X; model 4a).

Subsequently, we investigated whether the lipoprotein composition of HDL was associated with leukocyte counts, focusing on HDL phospholipids (HDL-PL), HDL total cholesterol (HDL-C), and HDL-TG (Supplemental Table VI). In the adjusted model (model 3), we observed similar relationships for HDL-PL compared to HDL-P (Fig. 3A and Supplemental Table VIII). Interestingly, also associations between HDL-C, leukocytes, and leukocyte subpopulations were similar to HDL-P; except for S-HDL-C that did not associate with any leukocyte population (Fig. 3A and Supplemental Table VIII). For HDL-TG, we only found positive associations of the smaller HDL particles (M-HDL-TG and S-HDL-TG) with leukocyte, neutrophil, and lymphocyte counts, while L-HDL-TG and XL-HDL-TG did not associate with any leukocyte population (Fig. 3A and Supplemental Table VIII).

HDL-TG is cleared rapidly from plasma and therefore may not have major biological effects [26]. HDL-TG as measured by NMR has been suggested to rather reflect plasma TG [27], especially for S-HDL-TG [28]. Similarly, in LLD, plasma TG showed a strong positive correlation with S-HDL-TG and M-HDL-TG and to a lesser extent with XL-HDL-TG, but not L-HDL-TG (Supplemental Table XI).

A previous study in the UK Biobank cohort has shown that after adjustment for plasma TG, the negative association between HDL-c and leukocyte counts lost statistical significance [11]. When we adjusted the relationship between XL-HDL-P or L-HDL-P and total leukocytes or leukocyte subpopulations for plasma TG, all associations lost statistical significance except for the association of XL-HDL-P with neutrophils (Supplemental Fig. 1 and Supplemental Table XII; model 4b). For M-HDL-P or S-HDL-P, adjustment for plasma TG decreased statistical significance of the associations with leukocytes or leukocyte subpopulations, and the positive association between S-HDL-P and neutrophils lost significance.

However, when we adjusted the associations between HDL-P and leukocytes or leukocyte subpopulations for HDL-TG, we found that the associations between XL-HDL-P or L-HDL-P and leukocytes or neutrophils were independent of this parameter (Fig. 3B and Supplemental Table XII; model 4c). For M-HDL-P and S-HDL-P, relationships with leukocytes were similarly affected compared to adjustment for plasma TG (Fig. 3B and Supplemental Table XII; model 4b, c), reflecting the strong correlation between M-HDL-TG or S-HDL-TG and plasma TG

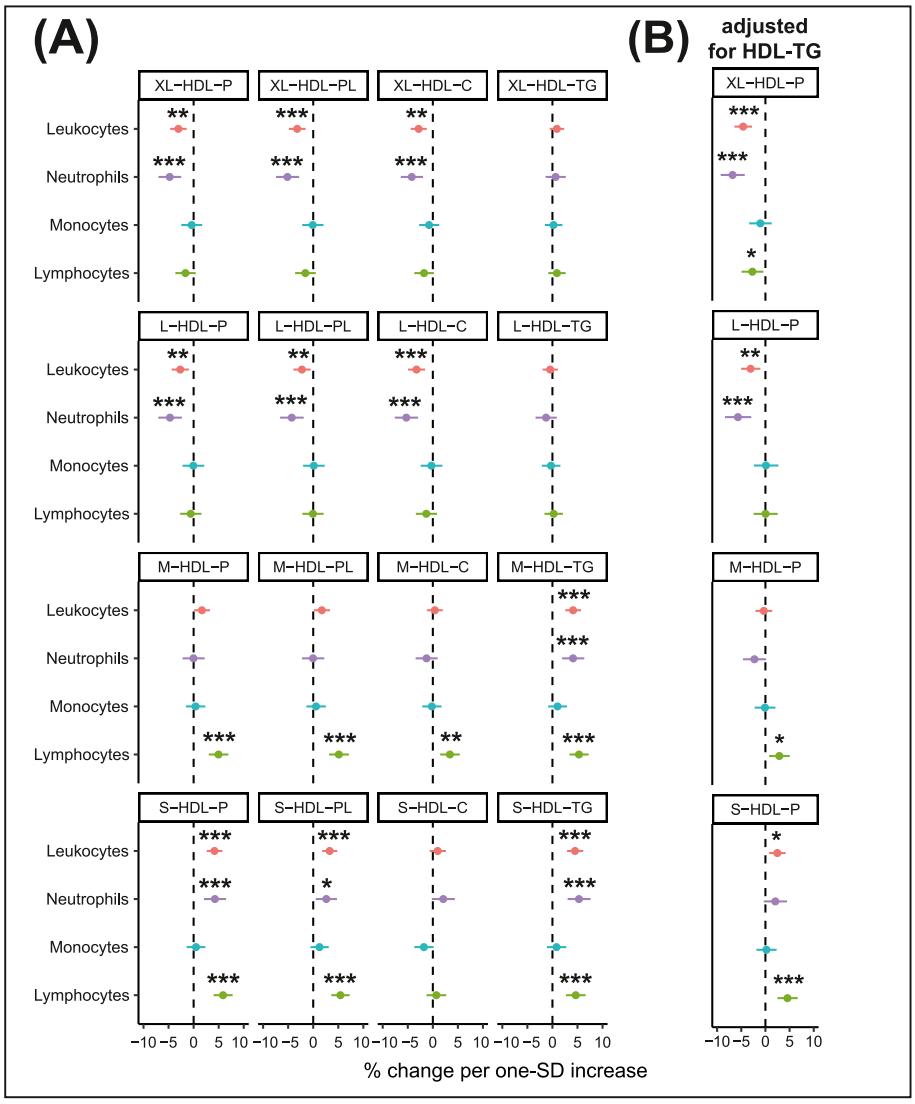


**Fig. 3.** Relationship of HDL particle concentration, size, and lipid composition with leukocytes and leukocyte subpopulations in the LLD cohort.
Results are presented as percentage change in cell counts (x $10^9$ cells/L) per one-SD increase in HDL particle (HDL-P) concentration, HDL phospholipids (HDL-PL), HDL total cholesterol (HDL-C), or HDL triglycerides (HDL-TG). Four HDL particle subclasses were distinguished according to size: XL-HDL, L-HDL, M-HDL, and S-HDL. (A) The models were adjusted for age, gender, smoking status, alcohol consumption, ln-transformed BMI, ln-transformed systolic blood pressure, ln-transformed serum creatinine, ln-transformed CRP, and ln-transformed HbA1c (model 3). (B) Model 3 was further adjusted for ln-transformed HDL-TG within their corresponding subclass (model 4c). Asterisks (*) indicate FDR corrected *p*-values within each panel, $*q < 0.05$, $**q < 0.01$, $***q < 0.001$. BMI, body mass index; CRP, C-reactive protein.

(Supplemental Table XI).

To examine whether HDL-TG determines the relationship between HDL-P and leukocytes, we performed causal mediation analysis (CMA) (Supplemental Table XIII). We observed a direct effect of S-HDL-P, L-HDL-P, and XL-HDL-P on leukocytes ($B_{direct} = 0.025$, $p = 0.002$, S-HDL-P; $B_{direct} = -0.031$, $p < 0.001$, L-HDL-P; $B_{direct} = -0.047$, $p < 0.001$, XL-HDL-P), with a mediation effect of HDL-TG for S-HDL-P and XL-HDL-P ($B_{indirect} = 0.017$, S-HDL-TG; $B_{indirect} = 0.016$, XL-HDL-TG; both $p < 0.001$). In line with findings in Fig. 3A, the direct effect of S-HDL-P on leukocytes was positive, and for L-HDL-P and XL-HDL-P this was negative. For both S-HDL-P and XL-HDL-P, HDL-TG contributed positively to the relationship between HDL-P and leukocytes (Supplemental Table XIII), even though XL-HDL-P showed a negative association with leukocytes (Fig. 3A). 41% of the relationship between S-HDL-P and leukocytes was mediated by HDL-TG ($p_{mediation}<0.001$) (Supplemental Table XIII, Fig. 4); for XL-HDL-P this was 53% ($p_{mediation}<0.001$) (Supplemental Table XIII). HDL-TG also mediated the effect of S-HDL-P or XL-HDL-P on neutrophils (52%, $p_{mediation}<0.001$, S-HDL-P; 44%, $p_{mediation} = 0.002$, XL-HDL-P) (Supplemental Table XIII). We did not observe that HDL-C causally mediated relationships between HDL-P and leukocyte or neutrophil counts (Supplemental Table XIV). Together, CMA suggests that HDL-TG mediates associations between XL-HDL-P or S-HDL-P and leukocytes or neutrophils.

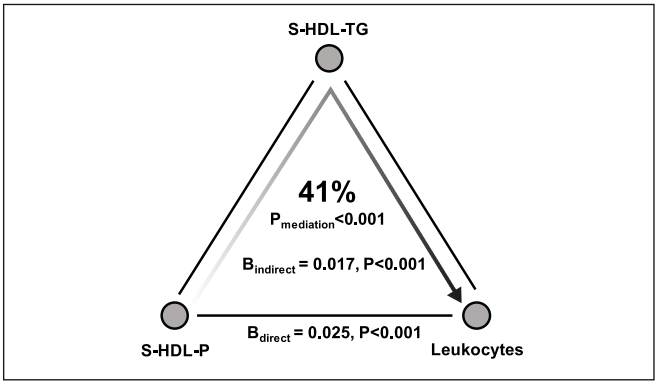


**Fig. 4.** Triglycerides mediate the association between the small HDL particle concentration and leukocytes in the LLD cohort.
Causal mediation analysis suggests that 41% of the relationship between small HDL particle (S-HDL-P) concentration and leukocytes can be explained by triglycerides on these particles (S-HDL-TG). The models were adjusted for age, gender, smoking status, alcohol consumption, ln-transformed BMI, ln-transformed systolic blood pressure, ln-transformed serum creatinine, ln-transformed CRP, and ln-transformed HbA1c (model 3). BMI, body mass index; CRP, C-reactive protein.

### 3.5. Association of VLDL particles with leukocyte counts

We then further assessed the contribution of TG rich lipoproteins to leukocyte counts and mainly focused on the VLDL particles. Six subclasses of VLDL particles were distinguished: XXL-VLDL-P, XL-VLDL-P, L-VLDL-P, M-VLDL-P, S-VLDL-P, and XS-VLDL-P (Supplemental Table VI) [25]. All subclasses of VLDL-P, except for XS-VLDL-P, were positively associated with leukocyte, neutrophil, and lymphocyte counts (Fig. 5 and Supplemental Table XV). Associations between VLDL-TG, leukocyte, neutrophil, and lymphocyte counts were positive for VLDL particles of all sizes, while for VLDL-C this was only the case for the five largest VLDL particles (Fig. 5 and Supplemental Table XV) that are relatively more enriched in TG compared to cholesterol than XS-VLDL-P, reflected by a higher TG/cholesterol ratio in these particles (Supplemental Table VI). These data substantiate the earlier findings on positive associations between plasma TG, leukocytes and leukocyte

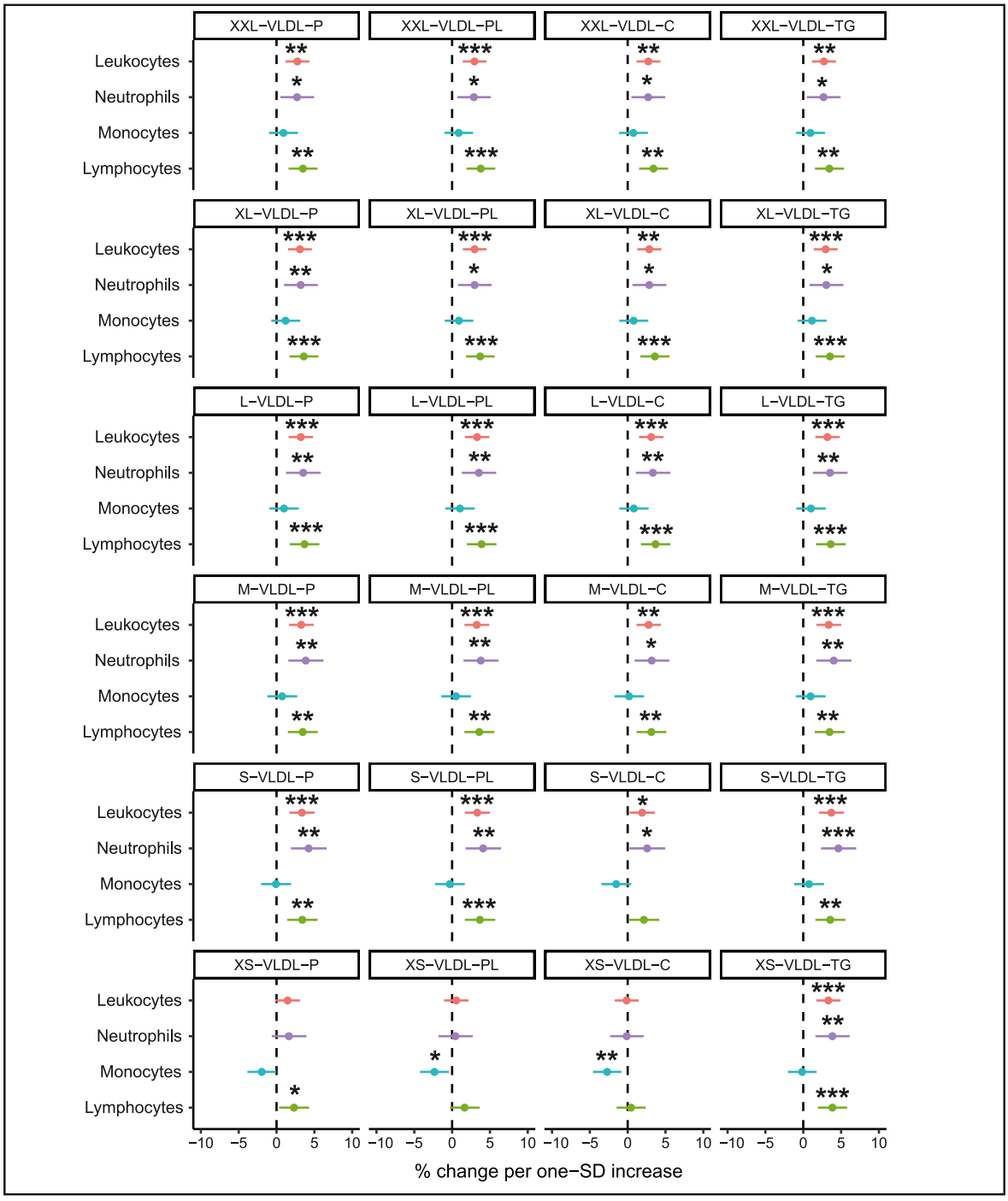


**Fig. 5.** Relationship of VLDL particle concentration, size, and lipid composition with leukocytes and leukocyte subpopulations in the LLD cohort.
Results are presented as percentage change in cell counts (x $10^9$ cells/L) per one-SD increase in the VLDL particle (VLDL-P) concentration, VLDL phospholipids (VLDL-PL), VLDL total cholesterol (VLDL-C), or VLDL triglycerides (VLDL-TG). Six VLDL particle subclasses were distinguished according to size: XXL-VLDL, XL-VLDL, L-VLDL, M-VLDL, S-VLDL, and XS-VLDL. The models were adjusted for age, gender, smoking status, alcohol consumption, ln-transformed BMI, ln-transformed systolic blood pressure, ln-transformed serum creatinine, ln-transformed CRP, and ln-transformed HbA1c (model 3). Asterisks (*) indicate FDR corrected *p*-values, *$q < 0.05$, **$q < 0.01$, ***$q < 0.001$. BMI, body mass index; CRP, C-reactive protein.

subpopulations as shown in the UK Biobank and MESA cohorts [8,11], and suggest that mainly the large VLDL particles account for this positive association.

## 4. Discussion

Studies in the UK Biobank and MESA cohorts have shown a negative relationship between HDL-c and blood leukocytes [8,11]. These studies were largely inspired by findings in animal models showing that HDL and cholesterol efflux pathways suppress leukocytosis [5,6], but it was unclear whether HDL-CEC contributed to these effects. We found that HDL-c, but not HDL-CEC, associates negatively with leukocytes and neutrophils in the LLD cohort, despite a strong correlation between HDL-c and HDL-CEC. Since HDL-P have also been proposed as improved inverse predictors of CVD compared to HDL-c [19,21,22,24,28,29], we then evaluated correlations between HDL-P and leukocytes. The XL-HDL-P and L-HDL-P showed a negative association with leukocytes and neutrophils, which was, as expected, also true for HDL diameter. Unexpectedly, S-HDL-P showed a positive association with blood leukocytes and neutrophils. CMA showed that this was mediated by S-HDL-TG. The positive association with leukocyte counts may reflect the strong correlation of S-HDL-TG with plasma TG, as shown previously [27,28]. Indeed, we found that plasma TG and VLDL-TG also showed a positive correlation with leukocyte counts. These studies thus indicate that HDL particle size, and HDL-c, but not HDL-CEC, determines the association of HDL with leukocyte counts.

Although all HDL-P showed a strong association with HDL-CEC, adjustment for HDL-CEC did not affect their relationship with any leukocyte population. Because of the data in animal models showing a crucial role for cholesterol efflux pathways in HSPCs and macrophages in leukocytosis [5,6], we had expected a major contribution of HDL-CEC to the association with leukocyte counts. Nonetheless, these animal models exhibit complete deficiency for the cholesterol transporters ABCA1 and G1 in either HSPCs or macrophages, and in our study, HDL-CEC was not completely abrogated.

Patients with rheumatoid arthritis (RA) show low expression of *ABC* transporters in peripheral blood mononuclear cells, which was accompanied by elevated monocyte counts [30]. Based on findings in an RA mouse model it was suggested that the decrease in ABC transporter expression as well as decreased HDL-CEC in RA accounted for the monocytosis [30]. HDL also showed an inverse relationship with monocytes in a cohort of children with high LDL-c levels due to familial hypercholesterolemia (FH) ($n = 49$) [9]. These data suggest that perhaps under extreme conditions, such as in RA, a highly pro-inflammatory condition, or FH, which is associated with extreme hypercholesterolemia, decreased HDL-CEC may affect leukocyte counts, while in the general population, these extreme conditions are rare and therefore HDL-CEC may not be a limiting factor in affecting white blood counts.

CMA showed that the positive relationship between S-HDL-P and leukocyte counts was mediated by S-HDL-TG. Recent studies have shown a positive relationship between S-HDL-TG and myocardial infarction or coronary artery disease in the UK Biobank and CARDIoGRAMplusC4D cohorts [28]. Because of a strong positive correlation of total plasma TG with S-HDL-TG, this was attributed to an overall increase in plasma TG, and not a specific HDL effect [28]. Our studies also showed a strong association between S-HDL-TG and plasma TG in LLD. It has been suggested that S-HDL-P rather behaves like an apoB particle instead of an HDL particle [27]. The association between S-HDL-TG and leukocytes may simply reflect the positive association of plasma TG with leukocytes. The latter was also found in the UK Biobank and MESA cohorts [8,11].

In sum, our data show that HDL-c, and especially the large HDL particles, negatively associate with leukocyte counts, independent of HDL-CEC. Further, our data suggest that pharmaceutical approaches to lower plasma TG, as currently being evaluated in the Pemafibrate to Reduce Cardiovascular OutcoMes by Reducing Triglycerides IN patiENts With diabetes (PROMINENT) trial [31], may have beneficial effects on CVD by decreasing leukocyte counts. Indeed diabetes is associated with increased leukocyte counts [4]. If TG lowering in the PROMINENT trial also results in lower HDL-TG, this could add to the beneficial effects of permafibrate in CVD. Similarly, other approaches to lower plasma TG such as antisense oligonucleotides for APOC3 (Volanesorsen) [32] or angiopoietin-like protein 3 (Angptl3; Vupanorsen) [33] may decrease CVD risk by lowering leukocyte counts.

### 4.1. Limitations

Limitations of our study include the relatively small sample size of the LLD population that may not have allowed for observing a relationship of HDL-CEC with leukocyte counts. Further, the design of the HDL-CEC assay may have its limitations. While studies in animal models have shown that macrophage cholesterol efflux pathways control myeloid cell counts, cholesterol efflux pathways in HSPCs control myeloid cell counts to a greater extent [5,7], and our HDL-CEC assay does not directly reflect cholesterol efflux from HSPCs. In addition, there is bidirectional exchange of cholesterol between cells and HDL in radioactive cholesterol efflux assays. Even though these assays are often used as a method to measure HDL-CEC [20–23], the efflux of labeled cholesterol from macrophages may be partly or wholly counter-balanced by the uptake of nonlabeled cholesterol from HDL, and may not reflect net movement (efflux minus influx) of cholesterol between cells and HDL, which can only be assessed in a cholesterol mass efflux capacity assay [18,19]. Further, for NMR analyses, the lipid content of lipoprotein particles has been calculated based on NMR data and not measured by enzymatic assays. Nonetheless, the TG content of HDL does show a strong correlation with plasma TG that has been enzymatically measured.

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## CRediT authorship contribution statement

**Anouk G. Groenen:** Cholesterol efflux experiments, Formal analysis, Writing – original draft. **Venetia Bazioti:** Cholesterol efflux experiments, Formal analysis, Writing – original draft. **Isabelle A. van Zeventer:** Validation, Writing – review & editing. **Lianmin Chen:** Formal analysis, Writing – review & editing. **Hilde E. Groot:** Formal analysis, Writing – review & editing. **Jan-Willem Balder:** Formal analysis, Writing – review & editing. **Alexandra Zhernakova:** Resources, Supervision, Writing – review & editing. **Pim van der Harst:** Supervision, Writing – review & editing. **Antoine Rimbert:** Formal analysis, Writing – review & editing. **Jan Albert Kuivenhoven:** Supervision, Writing – review & editing. **Jingyuan Fu:** Resources,

Supervision, Writing – review & editing. **Marit Westerterp:** Resources, Supervision, Writing – review & editing.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2022.01.008.

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