Lower Apo A-I and Lower HDL-C Levels Are Associated With Higher Intermediate CD14^{++}CD16^{+} Monocyte Counts That Predict Cardiovascular Events in Chronic Kidney Disease

Kyrill S. Rogacev, Adam M. Zawada, Insa Emrich, Sarah Seiler, Michael Böhm, Danilo Fliser, Kevin J. Woollard,* Gunnar H. Heine*

Objective—Patients with chronic kidney disease (CKD) display impaired cholesterol efflux capacity and elevated CD14^{+}CD16^{+} monocyte counts. In mice, dysfunctional cholesterol efflux causes monocytopoiesis. It is unknown whether cholesterol efflux capacity and monocyte subsets are associated in CKD.

Approach and Results—In 438 patients with CKD, mediators of cholesterol efflux capacity (high-density lipoprotein cholesterol/apolipoprotein A-I) and monocyte subsets were analyzed as predictors of cardiovascular events. Monocyte subset-specific intracellular lipid content, CD36, CD68, and ABCA1 were measured in a subgroup. Experimentally, we analyzed subset-specific cholesterol efflux capacity and response to oxidized low-density lipoprotein cholesterol stimulation in CKD. Epidemiologically, both low Apo-I and low high-density lipoprotein cholesterol were associated with high CD14^{++}CD16^{+} monocyte counts in linear regression analyses (apolipoprotein A-I: β=−0.171; P<0.001; high-density lipoprotein cholesterol: β=−0.138; P=0.005), but not with counts of other monocyte subsets. In contrast to apolipoprotein A-I or high-density lipoprotein cholesterol, higher CD14^{++}CD16^{+} monocyte counts independently predicted cardiovascular events (hazard ratio per increase of 1 cell/μL: 1.011 [1.003–1.020]; P=0.007). Experimentally, CD14^{++}CD16^{+} monocytes demonstrated preferential lipid accumulation, high CD36, CD68, and low ABCA1 expression and, consequently, displayed low cholesterol efflux capacity, avid oxidized low-density lipoprotein cholesterol uptake, and potent intracellular interleukin-6, interleukin-1β, and tumor necrosis factor-α production.

Conclusions—Taken together, mediators of cholesterol efflux are associated with CD14^{++}CD16^{+} monocyte counts, which independently predict adverse outcome in CKD. (Arterioscler Thromb Vasc Biol. 2014;34:2120-2127.)

Key Words: apolipoproteins  ■  cardiorenal syndrome  ■  CD14  ■  CD16  ■  cholesterol  ■  monocytes

Recent experimental evidence demonstrated that impaired cholesterol efflux pathways and low high-density lipoprotein cholesterol (HDL-C) cause hematopoietic stem cell proliferation, especially monocytopoiesis, and thereby drive atherosclerotic plaque progression in mice. However, it is currently unknown whether these findings can be translated to humans. In this respect, chronic kidney disease (CKD) is an interesting condition to study because patients with CKD show an altered lipid metabolism, with reduced cholesterol efflux capacity owing to low and dysfunctional HDL-C at the same time, CKD is characterized by immunoinflammatory and, specifically, changes in monocyte biology have been found to mediate inflammation in CKD.

Up to 3 monocyte subsets can be distinguished by flow cytometry according to differential surface expression of CD14 and CD16 in humans and of Ly6c in mice. Both species exhibit differences in chemokine receptor expression of CCR2, CCR5, and CXCR1. In humans, monocyte subsets are defined as classical CD14^{++}CD16^{−}, intermediate CD14^{+}CD16^{−}, and nonclassical CD14^{+}CD16^{+} monocytes; the latter have been often summarized as CD16-positive monocytes. In mice, Ly6c^{+}CCR2^{−}CXCR1^{−} monocytes are the homologues of human classical CD14^{++}CD16^{−} monocytes and Ly6c^{−}CCR2^{−}CXCR1^{+} monocytes are homologues of human nonclassical CD14^{+}CD16^{−} monocytes; furthermore, Ly6c^{−}CCR2^{+}CXCR1^{+} monocytes have been found to cluster with intermediate CD14^{+}CD16^{+} monocytes. Murine monocyte subsets differentially contribute to atherogenesis, and only recently, an outstanding role of a CCR5^{+} murine monocyte subset has been demonstrated in atherosclerosis. A deeper discussion of monocyte heterogeneity has recently been provided in excellent reviews.
In line with these experimental findings in rodents, epidemiological evidence suggests that CD16-positive monocytes are associated with cardiovascular risk in healthy volunteers, and specifically, intermediate CD14++CD16+ monocytes predict cardiovascular events in subjects at elevated cardiovascular risk.

Because of unknown mechanisms, declining renal function is associated with a shift toward CD16-positive monocytes, and CKD patients with highest counts of intermediate monocytes have worse cardiovascular outcomes.

Against this background, we now hypothesized that cholesterol handling, encompassing cellular uptake, efflux, and accumulation of cholesterol, could be associated with shifts in monocyte subsets in patients with CKD, in whom cholesterol efflux capacity is impaired due to low apolipoprotein A-I (Apo A-I) and low HDL-C.

To date, there have been no reports examining the relationship between lipid metabolism, human monocyte subsets, and cardiovascular outcomes in a CKD population.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Mediators of Cholesterol Efflux, Monocyte Subsets, and Cardiovascular Outcomes in 438 CARE FOR HOME Participants
Monocyte subset analysis is exemplified in Figure I in the online-only Data Supplement. Baseline characteristics of the participants according to kidney function are summarized in Table 1.

Mediators of cholesterol efflux (lower Apo A-I and lower HDL-C) were significantly associated with higher counts of intermediate monocytes, but not with counts of total or classical monocytes (Table 2). To further analyze determinants of monocyte subsets, we computed linear regression analyses with estimated glomerular filtration rate (eGFR), lipid parameters, and other traditional cardiovascular risk factors as independent variables. Active smoking predicted total and classical monocyte counts, whereas body mass index predicted nonclassical (and again total) monocyte counts. Interestingly, these multivariate regression analyses identified neither lipid parameters nor eGFR as predictors of total, classical, or nonclassical monocyte counts. By contrast, more advanced CKD and mediators of cholesterol efflux (lower Apo A-I [Table 3] and lower HDL-C [Table 4]) both predicted intermediate monocyte counts, as did old age in the model including HDL-C.

The inter-relationship between mediators of cholesterol efflux, eGFR, and intermediate monocyte counts is further illustrated in Figure 1A and 1B, demonstrating higher intermediate monocyte counts in patients with more advanced eGFR categories and with levels of cholesterol efflux mediators below the median. Interestingly, an almost linear increase in intermediate monocyte counts with declining renal function was seen only in patients with low levels of cholesterol efflux mediators. Of note, patients in eGFR category 4 (15–29 mL/min per 1.73 m²) with Apo A-I and HDL-C below median had 35% and 41% higher intermediate monocyte counts, respectively, compared with subjects with HDL cholesterol levels above median. No interaction between mediators of cholesterol efflux and eGFR on classical and nonclassical monocyte counts was found (Figures IIa and IIb and IIIa and IIIb in the online-only Data Supplement).

To analyze the potential clinical significance of low levels of cholesterol efflux mediators and cellular mediators of inflammation, we recorded the subsequent occurrence of a composite cardiovascular end point (first occurrence of acute myocardial infarction; surgical or interventional coronary/cerebrovascular/peripheral-arterial revascularization; stroke with symptoms ≥24 hours; amputation above the ankle; or death of any cause). After a mean follow-up of 2.5±1.3 years, 81 patients experienced this end point. Those experiencing a subsequent event had lower plasma lipoproteins and higher levels of inflammatory markers (monocyte subsets and high-sensitivity C-reactive protein). Strikingly, patients with a subsequent event had a 41% increase in intermediate monocyte counts but only a 10% higher classical monocyte count at baseline evaluation compared with patients without events. Further details of study participants stratified by event status are presented in Table I in the online-only Data Supplement.

To illustrate the prognostic implications of lipid metabolism and inflammation, participants were stratified into tertiles of levels of cholesterol efflux mediators (Apo A-I and HDL-C) and intermediate monocyte counts, and Kaplan–Meier survival curves were calculated. Higher intermediate monocyte counts (Figure 2A) and lower concentrations of Apo A-I and HDL-C (Figure 2B and 2C) were significant outcome predictors after categorization. In Kaplan–Meier analysis, neither classical nor nonclassical monocytes predicted outcome (Figure 1Va and 1Vb in the online-only Data Supplement).

Having found that lower levels of cholesterol efflux mediators and higher intermediate monocyte counts were associated with each other and that they predicted the end point, we finally performed Cox regression analyses that included well-known predictors of adverse outcome—prevalent diabetes mellitus, log-transformed albuminuria, age, cholesterol efflux mediators (either Apo A-I or HDL-C), low-density lipoprotein cholesterol, prevalent cardiovascular disease, eGFR, mean blood pressure, and sex—and intermediate monocyte counts. In this multivariate model, intermediate monocytes remained significant outcome predictors, whereas eGFR and plasma lipoproteins did not predict outcome. Of note, every increase of 10 intermediate monocytes/μL of blood conferred a 10% and 11% higher risk of reaching the end point in Cox regression models with Apo A-I and HDL-C, respectively (Table 5; P=0.019 [model including Apo A-I] and P=0.007 [model including HDL-C]).

Experimental Analyses of Cholesterol Handling in Monocyte Subsets
To better understand the inter-relationship between lipid metabolism and intermediate monocytes, we analyzed in more detail cellular cholesterol content and mediators of cholesterol uptake...
In a randomly selected subgroup of 48 CARE FOR HOMe participants who consecutively presented at the outpatient department. Finally, 2 functional assays of cellular cholesterol efflux and cytokine production were performed in a further subgroup of 6 and 8 patients with CKD, respectively, randomly selected from renal clinics (for inclusion and exclusion criteria for all patients used in this study, please see Methods in the online-only Data Supplement). These functional assays could not be performed on all 48 CARE FOR HOMe participants due to assay design (ie, not high throughput).

### Intracellular Lipid Content in Monocyte Subsets From Patients With CKD

We reasoned that if an association between lipid metabolism and intermediate monocytes existed, intermediate monocytes should have highest intracellular lipid content of all monocyte subsets in patients with CKD. To investigate this, we initially performed intracellular staining using the lipid fluorophore BODIPY. As demonstrated in Figure 3A, intermediate monocytes had highest intracellular BODIPY-median fluorescence intensity, indicating lipid enrichment in intermediate monocytes ($P<0.05$) in patients with CKD, from the 48-patient subgroup of CARE FOR HOMe.

### Distribution of Scavenger Receptors (CD36, CD68) and of ABCA1 in Monocyte Subsets

We next asked whether monocyte subsets show different expression of crucial mediators of cellular cholesterol uptake, such as scavenger receptors CD36 and CD68, and...
the cholesterol efflux pump ABCA1 in our subgroup of 48 patients with CKD. Interestingly, intermediate monocytes had highest expression of CD68 (P<0.001; Figure 3B). CD36 expression on intermediate monocytes was similar to classical monocytes, but significantly higher than on nonclassical monocytes (P<0.001; Figure 3C). Furthermore, ABCA1 expression differed between monocyte subsets (P<0.001), with lower ABCA1 expression by intermediate monocytes than by classical monocytes (P<0.001; Figure 3D).

Cholesterol Efflux Capacity in Purified Monocyte Subsets
To further investigate the relationship between monocyte subsets and intracellular cholesterol content, we performed functional analyses of H3-labeled cholesterol efflux capacity in sorted monocyte subsets of 6 donors with CKD. Of note, cholesterol efflux capacity was lowest in intermediate monocytes compared with the other 2 subsets (P<0.001; Figure 4), providing a mechanism for preferential cholesterol accumulation in this subset.

Intracellular Cholesterol Content and Intracellular Cytokine Production in Monocyte Subsets After Oxidized Low-Density Lipoprotein Cholesterol Stimulation
Finally, we determined the monocyte subsets’ response to oxidized low-density lipoprotein cholesterol, which is an established driver of atherosclerosis in blood obtained from 8 donors with CKD. After stimulation with oxidized low-density lipoprotein cholesterol, intermediate monocytes displayed highest intracellular BODIPY content (P<0.01; Figure 5A). Additionally, we observed a commensurate increase in intracellular proinflammatory cytokines interleukin (IL)-6, tumor necrosis factor-α, and IL-1β, which was most pronounced in intermediate monocytes (IL-6, P<0.05; tumor necrosis factor-α, P<0.001; IL-1β, P<0.05; Figure 5B–5D).

Taken together, our experimental results demonstrate relatively selective lipid enrichment, increased expression of cholesterol receptors, and impaired cholesterol efflux in intermediate monocytes compared with the other 2 subsets and suggest activation of intermediate monocytes by intracellular cholesterol.

Discussion
Investigating monocyte heterogeneity in a large cohort of patients with CKD, we discovered an inter-relationship between low concentrations of cholesterol efflux mediators (low Apo A-I and low HDL-C), higher intermediate monocyte counts, and cardiovascular outcomes. Of note, our Cox regression analyses suggest that intermediate monocytes provide a significant insight over traditional risk factors on the prediction of cardiovascular events in patients with CKD.

When further probing the inter-relationship between cholesterol metabolism and intermediate monocytes, we found rather selective lipid enrichment in intermediate monocytes. This finding may be explained by the differential endowment of monocyte subsets with scavenger receptors and efflux pumps. Intermediate monocytes had higher CD68, similar CD36, and significantly lower ABCA1 expression than

<table>
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<tr>
<th>Table 2. Correlation Coefficients Between Monocyte (Subset) Counts and Lipid Parameters (n=438 Patients)</th>
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<tr>
<td>Total Cholesterol, mg/dL</td>
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<tr>
<td>Correlation coefficients</td>
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<tr>
<td>Total monocytes</td>
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<td>Classical monocytes</td>
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<td>Intermediate monocytes</td>
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Intracellular cholesterol content and intracellular cytokine production in monocyte subsets after oxidized low-density lipoprotein cholesterol stimulation.

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<th>Table 4. Linear Regression Analysis With Monocyte (Subset) Counts as Dependent Variable (Model With HDL-C as Cholesterol Efflux Mediator; n=438 Patients)</th>
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BMI indicates body mass index; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; HDL-C, high-density lipoprotein cholesterol; and LDL-C, low-density lipoprotein cholesterol.

ApoA-I, HDL, and Monocyte Subsets in CKD

Total monocytes had highest expression of CD68 (P<0.001; Figure 3B). CD36 expression on intermediate monocytes was similar to classical monocytes, but significantly higher than on nonclassical monocytes (P<0.001; Figure 3C). Furthermore, ABCA1 expression differed between monocyte subsets (P<0.001), with lower ABCA1 expression by intermediate monocytes than by classical monocytes (P<0.001; Figure 3D).

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classical monocytes in our CKD cohort. In functional analyses, intermediate monocytes had the lowest capacity to efflux cholesterol. Moreover, intermediate monocytes avidly took up oxidized low-density lipoprotein cholesterol and subsequently had highest intracellular proinflammatory cytokine production of all 3 monocyte subsets.

Impaired cholesterol efflux capacity, owing to low and dysfunctional HDL-C, could be an important mediator of immunoactivation and resulting cardiovascular disease in CKD because cholesterol efflux was shown to regulate monocytopoiesis in mice. In keeping with these experimental results and animal data, a recent analysis of the Malmo Diet and Cancer Study in the general population described an association between HDL-C levels and cells of the myeloid lineage, that is, monocytes, basophils, and eosinophils, which was dependent on renal function. However, neither murine studies nor the Malmo Diet and Cancer study addressed a potential relationship between mediators of cholesterol efflux (Apo A-I and HDL-C) and monocyte heterogeneity, which comprises 3 human monocyte subsets: classical CD14++CD16−, intermediate CD14++CD16+, and nonclassical CD14++CD16− monocytes.

Specific characteristics of intermediate monocytes probably account for their relation with cardiovascular outcomes. First, intermediate monocytes have highest expression of CCR5 alongside high expression of CCR2 and fractalkine receptor (CX3CR1), comprising a chemokine receptor triad of well-established relevance in atherosclerosis. Specifically, the outstanding role of CCR5 has been established by Braunersreuther et al who demonstrated that although CCR1 and CCR5 both mediate monocyte interaction with CCL5, which is expressed in atherosclerotic plaques, genetic deletion specifically of CCR5 but not of CCR1 protects against experimental atherogenesis. Specifically, the outstanding role of CCR5 has been established by Braunersreuther et al who demonstrated that although CCR1 and CCR5 both mediate monocyte interaction with CCL5, which is expressed in atherosclerotic plaques, genetic deletion specifically of CCR5 but not of CCR1 protects against experimental atherogenesis. In an elegant experimental study, the role of CCR5 has been recently further underscored; additional support derives from single nucleotide polymorphism studies demonstrating the CCR5 Δ32 variant to be protective in cardiovascular disease and by the efficacy of maraviroc, a

Figure 1. A, Inter-relationship between estimated glomerular filtration rate (eGFR) strata, apolipoprotein A-I (Apo A-I) below and above median (161 [142–184] mg/dL), respectively, (B) high-density lipoprotein cholesterol (HDL-C) below and above median (48 [39–61] mg/dL), and intermediate monocyte counts (given as mean±SEM; comparison between GFR strata by 1-way ANOVA) in 438 patients with CKD (entire CARE FOR HOMe cohort).

Figure 2. Kaplan–Meier analysis for tertiles of intermediate monocyte counts (A), tertiles of apolipoprotein A-I (Apo A-I; B), and tertiles of HDL-C (C) and event-free survival, followed by log-rank test; n=438 patients with CKD.
CCR5 antagonist, for preventing atherosclerotic plaque development in an apoE mouse model.31

Second, intermediate monocytes have the highest inflammatory capacity among monocyte subsets as evidenced by rather selective tumor necrosis factor-α and IL-1β secretion upon stimulation10 and highest reactive oxygen species production,26 further accompanied by high angiotensin-converting enzyme expression, the up-regulation of which has been associated with poor outcome in patients with CKD.32

Third, although virtually all studies in mice analyzed only 2 major monocyte subsets, clustering analyses link human intermediate monocytes to murine proinflammatory Gr1+ monocytes10 that have been identified as the dominant monocyte subset in murine models of atherosclerosis.11–13

Finally, our finding of lipid accumulation in intermediate monocytes may be of great importance because experimental studies found intracellular cholesterol accumulation to trigger inflammation by activating the inflammasome33

Table 5. Cox Proportional Hazard Models (Mod1: Including Apo A-I; Mod2: Including HDL-C) With Composite End Point as Dependent Variable (n=438 Patients With CKD)

<table>
<thead>
<tr>
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<th>HR Mod1 95% CI</th>
<th>P Value</th>
<th>HR Mod2 95% CI</th>
<th>P Value</th>
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</thead>
<tbody>
<tr>
<td>Diabetes mellitus</td>
<td>1.368 0.852–2.198</td>
<td>0.195</td>
<td>1.425 0.880–2.307</td>
<td>0.150</td>
</tr>
<tr>
<td>Log UAE</td>
<td>1.472 1.097–1.976</td>
<td>0.010</td>
<td>1.490 1.110–2.000</td>
<td>0.008</td>
</tr>
<tr>
<td>Age, y</td>
<td>1.029 1.002–1.056</td>
<td>0.032</td>
<td>1.026 1.000–1.053</td>
<td>0.050</td>
</tr>
<tr>
<td>Apolipoprotein A-I, mg/dL</td>
<td>0.999 0.990–1.009</td>
<td>0.910</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>NA</td>
<td>NA</td>
<td>1.005 0.989–1.021</td>
<td>0.553</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>0.995 0.987–1.002</td>
<td>0.135</td>
<td>0.996 0.989–1.003</td>
<td>0.261</td>
</tr>
<tr>
<td>Prevalent CVD</td>
<td>3.372 2.214–6.293</td>
<td>&lt;0.001</td>
<td>4.144 2.476–6.929</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>eGFR, mL/min per 1.73 m²</td>
<td>0.985 0.969–1.002</td>
<td>0.092</td>
<td>0.986 0.969–1.003</td>
<td>0.098</td>
</tr>
<tr>
<td>Mean BP, mmHg</td>
<td>0.986 0.970–1.003</td>
<td>0.098</td>
<td>0.984 0.969–1.001</td>
<td>0.060</td>
</tr>
<tr>
<td>Sex (male)</td>
<td>1.318 0.773–2.247</td>
<td>0.310</td>
<td>1.373 0.812–2.323</td>
<td>0.237</td>
</tr>
<tr>
<td>Intermediate monocytes, cells/μL</td>
<td>1.010 1.002–1.019</td>
<td>0.019</td>
<td>1.011 1.003–1.020</td>
<td>0.007</td>
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Apo A-I indicates apolipoprotein A-I; BP, blood pressure; CI, confidence interval; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; HR, hazard ratio; LDL, low-density lipoprotein; log UAE, log-transformed urinary albumin excretion; and NA, not applicable.

Figure 3. A, Intracellular lipid content in the 3 monocyte subsets in 48 patients with CKD (flow cytometry using BODIPY 493/503). Statistical analysis was performed with 1-way ANOVA for comparison between 3 monocyte subsets. B–D, Surface expression of scavenger receptor CD68 (mediating cholesterol uptake [B]), CD36 (mediating cholesterol uptake [C]), and ABCA1 (mediating cholesterol efflux [D]) in 48 patients with CKD (flow cytometry). Statistical analysis was performed with the Kruskal-Wallis test for comparison of 3 monocyte subsets. MFI indicates median fluorescence intensity.
and to promote experimental atherosclerosis by inducing monocytopoiesis.\textsuperscript{34}

\textbf{Study Strengths and Limitations}

The rationale for our study is well founded by elegant animal models. We now translate these findings to human pathophysiology and provide evidence for their clinical relevance. Of note, the present study is the largest study to date that has evaluated monocyte heterogeneity in patients with CKD, providing a solid base for our findings. In addition, monocyte subset analysis has been done blinded to patients’ clinical characteristics and vice versa outcome adjudication was blinded to baseline monocyte subset distribution status.

As a limitation, lipid parameters have to be interpreted as on-treatment lipid profiles because lipid-modifying medication was commonly used. Furthermore, we had to limit analyses of monocyte subset-specific lipid handling to a subgroup of patients due to technically challenging, time-consuming, and costly methods.

\textbf{Conclusions}

Our data demonstrate an association between low levels of cholesterol efflux mediators and high intermediate monocyte counts, which in turn predicted subsequent cardiovascular events in CKD. Together, our experimental and clinical findings might suggest that increasing Apo A-I concentrations or restoring HDL-C functionality could be an attractive means to modulate skewed monocyte subset distribution and influence monocyte subset counts in CKD.

\textbf{Acknowledgments}

We are grateful to Martina Wager for her excellent technical assistance and to Marie-Theres Blinn, Dagmar Kolb, Annette Offenhäuser, and Fabio Lizzi for organizational help. We thank Dr Frederick Tam for his assistance in donor blood collection for efflux assays.

\textbf{Figure 4.} Cholesterol efflux capacity in monocyte subsets. Monocyte subsets were isolated from 6 patients with CKD; cholesterol efflux capacity was measured with $\text{1}_{\alpha},\text{2}_\alpha(n)-\text{H}]$ cholesterol (0.1 MBq/mL). Statistical analysis was performed with 1-way ANOVA for comparison between 3 monocyte subsets.

\textbf{Figure 5.} Stimulation of monocyte subsets with oxidized low-density lipoprotein (oxLDL). \textbf{A}, Intracellular lipid content in the 3 monocyte subsets after oxLDL stimulation (flow cytometry using BODIPY 493/503). Intracellular cytokines interleukin (IL)-6 (\textbf{B}), tumor necrosis factor (TNF)-$\alpha$ (\textbf{C}), and IL-1$\beta$ (\textbf{D}) in 3 monocyte subsets after oxLDL stimulation (flow cytometry with intracellular staining). Blood was obtained from 8 patients with CKD. Statistical analysis was performed with 1-way ANOVA for comparison of the 3 monocyte subsets.
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Disclosures
None.

References
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Methods

Study population

The CARE FOR HOMe study is a prospective cohort study in stable CKD patients aiming to characterize cardio-
renal interactions. The local Ethics Committee approved the study and all participants gave their written informed consent. The study was conducted in accordance to the principles stated in the Declaration of Helsinki.

Patients with CKD GFR categories 2 – 4, corresponding to an estimated glomerular filtration rate (eGFR) between 90 and 15 ml/min/1.73 m², were invited to participate. Unstable clinical status (active malignancy, systemic infection), acute kidney injury and intake of immunosuppressants were defined as exclusion criteria.

Information on co-morbidity was gathered by a standardized questionnaire and chart review. Prevalent diabetes mellitus was defined as self- or physician-reported diabetes mellitus, a fasting glucose > 126 mg/dl or intake of glucose-lowering medication. A history of cardiovascular disease was defined as previous myocardial infarction, coronary artery angioplasty / stenting / bypass grafting, major stroke, carotid endarterectomy / stenting, nontraumatic lower extremity amputation, or lower limb artery bypass surgery / angioplasty / stenting. Active smoking status was assigned to those individuals having quit smoking < 1 month before study enrolment and to current smokers. Blood pressure was measured after 5 mins of rest with an automated blood pressure recording apparatus (GE Carescape DINAMAP V100; GE Healthcare). Body mass index (BMI) was calculated as weight (kg)/[height (m)]².

Outcome Analysis

Patients were invited for annual follow-up visits. The composite cardiovascular end-point was defined as the first occurrence of any of the following: acute myocardial infarction; surgical or interventional coronary / cerebrovascular / peripheral-arterial revascularization; stroke with symptoms ≥ 24 hours, amputation above the ankle; or death of any cause. Outcome adjudication was performed blinded to baseline monocyte subset distribution status. For all outcomes, confirmatory medical documentation was obtained.

Laboratory analyses

Standard laboratory parameters were analyzed in the Central Laboratory at Saarland University Medical Centre. Classification of CKD followed K/DIGO GFR categories (Cat. 2: eGFR 60 - 90 ml/min/1.73 m²; Cat. 3a eGFR 45 - 59 ml/min/1.73 m²; Cat. 3b: eGFR 30 - 44 ml/min/1.73 m²; Cat. 4: eGFR 15 – 29 ml/min/1.73 m³), glomerular filtration rate at enrolment was estimated by the MDRD 4 variable equation.

Flow-cytometry was performed in the nephrological-immunological laboratory at the Department of Internal Medicine IV, Saarland University Medical Centre, according to our validated standard protocol. 100 µl EDTA anticoagulated whole blood was stained with antibodies against CD86 (CD86-PE, HA5.2B7, Beckman-Coulter, Krefeld, Germany), CD14 (CD14-PerCP, MΦ9, BD Biosciences, Heidelberg, Germany) and CD16 (CD16-PeCy7, 3G8, BD Biosciences) and analysed flow-cytometrically using the FACS Canto II with CellQuest Software (BD Biosciences). Monocytes were first gated in the SSC/CD86 dot plot to identify CD86+ cells and subsequently in the SSC/FSC dot plot to further gate for cells with monocyte scatter properties. Three monocyte subsets – classical CD14++CD16- monocytes, intermediate CD14+CD16- monocytes and nonclassical CD14-CD16++ monocytes – were subsequently defined according to the surface expression pattern of CD14 (lipopolysaccharide receptor) and CD16 (Fcy-III receptor; Figure 1). Nomenclature of monocyte subsets follows the recommendations of the Nomenclature Committee of the International Union of Immunological Societies. Monocyte subset quantification was done in 438 CKD patients who participated in the CARE FOR HOMe study. In a subgroup of 48 CKD patients, an in depth analysis of intracellular lipid content and of proteins involved in cholesterol uptake and efflux was performed, as described below. These flow-cytometry analyses were performed by technicians blinded to patient baseline clinical characteristics.
Measurement of intracellular lipid content

Intracellular lipid was measured in a whole-blood assay with 100 µl lithium heparin anticoagulated blood. Whole-blood was incubated with BODIPY® 493/503 (4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-s-Indacene, Life Technologies) and antibodies against CD14, CD16 and CD86 for 30 min. Intracellular lipid levels within the monocyte subsets were determined as MFI by flow-cytometric analysis.

Surface protein expression and intracellular staining

Expression of surface and intracellular antigens was determined using a whole-blood assay with 100 µl lithium heparin anticoagulated blood. Protein expression was quantified flow-cytometrically as median fluorescence intensity (MFI) and standardized against coated fluorescent particles (SPHEROTM; BD Biosciences). The following antibodies were used: CD36 APC (5-271) and CD68 FITC (Y1/82A), both from BioLegend and ABCA1 FITC (HJ1) from Abcam.

Determination of cholesterol efflux in purified monocyte subsets

Monocyte subsets from six donors with CKD were isolated as previously described. In brief, NK cells and neutrophils were first depleted from PBMCs using CD56 and CD15 MicroBeads (Non-Monocyte Depletion Cocktail, CD16 Monocyte Isolation Kit; MiltenyiBiotec). Afterwards, cells were incubated with anti-CD14 FITC (MiltenyiBiotec) and subsequently with anti-FITC MultiSortMicroBeads (Anti-FITC MultiSort Kit; MiltenyiBiotec) to separate CD14++ from CD14+- cells. Finally, both CD14++ and CD14+- cells were incubated with CD16 MicroBeads (MiltenyiBiotec) and separated into 3 monocyte subsets. Cholesterol efflux experiments were performed as similar to previously described. Briefly, cellular cholesterol was labelled in purified monocyte subsets (0.1x10⁶) by incubation in serum-free medium with [1α,2α(n)-3H]cholesterol (GE Health, 0.1MBq/ml) for 3 hrs. Cells were washed three times and HDL isolated from plasma of healthy human donor (100 μg/ml; EMDMillapore) added in fresh media for 1 hr at 37°C. After incubation supernatant media were harvested and cells washed thrice before cell lysis and harvesting. 3H was analysed on a PerkinElmer scintillation counter (counts/minute). Efflux was calculated as % of 3H remaining in media from total cholesterol loading in cells.

oxLDL stimulation and intracellular cholesterol content and cytokine production

Low Density Lipoprotein was purchased from Biomol (Hamburg, Germany) and oxidized with CuSO4 (10 µM) for 24 h at 37°C. The reaction was stopped with EDTA (0.5 mM) and dialysed over night at 4°C. Lithium heparin anticoagulated whole blood from eight donors with CKD was incubated with 50 µg/ml oxLDL for 1 h and afterwards stained for intracellular cholesterol using BODIPY® 493/503. Additionally, intracellular cytokines were determined flow-cytometrically after intercellular staining. The following antibodies were used: IL-6 FITC (MQ2-13A5) and IL-1β Alexa Fluor® 647 (JK1B-1) from Biolegend and TNF FITC (MAb11) from BD Biosciences.

Statistics

Data management and statistical analysis were performed using PASW Statistics 21 (SPSS, Inc., Chicago, Illinois) and GraphPad Prism4 (GraphPad, San Diego, California). Two-sided p values < 0.05 were considered significant.

For clinical data, categorical variables are presented as percentages of patients and were compared using chi-square or Fisher’s exact tests, as appropriate. Continuous data are expressed as mean ± standard deviation and compared using T-Test for two independent samples or one-way analysis of variances (ANOVA) for more than two independent samples, partitioning the between-groups sums of squares into trend components, as appropriate. In case of skewed distributions, median [interquartile range] are given, and Mann-Whitney U test or Kruskal-Wallis test were used. The associations between continuous variables were assessed using Pearson correlation testing. Linear
regression analyses were computed with age, HDL and LDL cholesterol, estimated glomerular filtration rate, body mass index and smoking status as independent variables and monocyte (subset) counts as dependent variable.

Subjects were divided into 3 equally sized groups (tertiles) according to their levels of cholesterol efflux mediators (i.e. Apo A-I or HDL-C) and monocyte (subset) counts. Kaplan-Meier survival curves were used to compare event-free survival (i.e., time until first occurrence of the composite endpoint) between groups. The log-rank test was used to test the hypothesis that at least 1 of the survival curves differs from the others. Cox proportional hazard models were calculated to analyze the relationship of inflammation indicators – log high-sensitivity C-reactive protein or monocyte (subset) cell counts, respectively – with event-free survival after adjustment for diabetes mellitus, log urinary albumin excretion, age, mediators of cholesterol efflux (Apo A-I or HDL-C) and LDL-C, prevalent cardiovascular disease, estimated glomerular filtration rate, mean blood pressure and gender.

Experimental study data are presented as mean ± SD and compared using one-way analysis of variances (ANOVA) for normally distributed variables and the Kruskal-Wallis tests for non-normally distributed variables, as appropriate. Testing for normality was performed with the D’Agostino-Pearson normality test (omnibus K² test) for n ≥ 8 and with the Kolmogorov-Smirnov test for n < 8.

Supplemental Figure Legends

**Figure I**: Flow-cytometric gating strategy for identification of monocyte subsets after surface staining (a) and after additional intracellular staining (b). Indicated are classical CD14++CD16- (blue), intermediate CD14++CD16+ (red) and nonclassical CD14+CD16++ (green) monocytes (representative dot plot).

**Figure II**: Interrelationship between eGFR strata, (a) Apo A-I below and above median (161 [142-184] mg/dl), respectively (b) HDL-C below and above median (48 [39-61] mg/dl), and classical monocyte counts (given as mean ± SEM; comparison between GFR strata by one-way ANOVA); n=438 CKD patients.

**Figure III**: Interrelationship between eGFR strata, (a) Apo A-I below and above median (161 [142-184] mg/dl), respectively (b) HDL-C below and above median (48 [39-61] mg/dl), and nonclassical monocyte counts (given as mean ± SEM; comparison between GFR strata by one-way ANOVA); n=438 CKD patients.

**Figure IV**: Kaplan-Meier analysis for (a) tertiles of classical, (b) tertiles of nonclassical monocyte counts and event-free survival, followed by log-rank test; n=438 CKD patients.
References


**Table I: Baseline characteristics after stratifying patients by event Status**

<table>
<thead>
<tr>
<th></th>
<th>Total Cohort</th>
<th>No Event</th>
<th>Event</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 438</td>
<td>N = 357</td>
<td>N = 81</td>
<td></td>
</tr>
<tr>
<td>Gender (men)</td>
<td>263 (60 %)</td>
<td>205 (57 %)</td>
<td>58 (72 %)</td>
<td>0.023</td>
</tr>
<tr>
<td>Prevalent CVD (yes)</td>
<td>134 (31 %)</td>
<td>81 (23 %)</td>
<td>53 (65 %)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smoking (yes)</td>
<td>43 (10 %)</td>
<td>32 (9 %)</td>
<td>11 (14 %)</td>
<td>0.216</td>
</tr>
<tr>
<td>Diabetes (yes)</td>
<td>167 (38 %)</td>
<td>124 (35 %)</td>
<td>43 (53 %)</td>
<td>0.003</td>
</tr>
<tr>
<td>Age (years)</td>
<td>65.1 ± 12.1</td>
<td>63.8 ± 12.9</td>
<td>70.9 ± 8.1</td>
<td>&lt;0.001</td>
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<tr>
<td>BMI (kg/m$^2$)</td>
<td>30.1 ± 5.4</td>
<td>30.3 ± 5.5</td>
<td>29.7 ± 5.3</td>
<td>0.353</td>
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<tr>
<td>Systolic BP (mmHg)</td>
<td>154 ± 24</td>
<td>154 ± 24</td>
<td>153 ± 27</td>
<td>0.885</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>89 ± 13</td>
<td>88 ± 12</td>
<td>81 ± 13</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean BP (mmHg)</td>
<td>109 ± 15</td>
<td>110 ± 14</td>
<td>105 ± 16</td>
<td>0.012</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73 m$^2$)</td>
<td>45.3 ± 16.0</td>
<td>47.1 ± 15.9</td>
<td>37.2 ± 13.9</td>
<td>&lt;0.001</td>
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<tr>
<td>UAE (mg/g creatinine)</td>
<td>36 [8 – 189]</td>
<td>29 [7 - 151]</td>
<td>93 [24 - 317]</td>
<td>0.001</td>
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<tr>
<td>Total Cholesterol (mg/dl)</td>
<td>193 ± 42</td>
<td>196 ± 41</td>
<td>178 ± 43</td>
<td>&lt;0.001</td>
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<tr>
<td>HDL Cholesterol (mg/dl)</td>
<td>51 ± 17</td>
<td>52 ± 17</td>
<td>47 ± 17</td>
<td>0.006</td>
</tr>
<tr>
<td>LDL Cholesterol (mg/dl)</td>
<td>116 ± 35</td>
<td>118 ± 35</td>
<td>105 ± 33</td>
<td>0.003</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>135 [96 - 191]</td>
<td>136 [97 - 194]</td>
<td>131 [96 - 187]</td>
<td>0.623</td>
</tr>
<tr>
<td>Parameter</td>
<td>Baseline</td>
<td>10 Years</td>
<td>20 Years</td>
<td>p-value</td>
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<tr>
<td>---------------------------------</td>
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<td>----------</td>
</tr>
<tr>
<td>Apo A-I (mg/dl)</td>
<td>161 [142-184]</td>
<td>164 [145-188]</td>
<td>148 [134-167]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>hsCRP (mg/l)</td>
<td>2.7 [1.2 – 5.4]</td>
<td>2.7 [1.1 - 5.0]</td>
<td>4.0 [1.7 - 9.3]</td>
<td>0.004</td>
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<tr>
<td>Hb (g/dl)</td>
<td>13.4 ± 1.6</td>
<td>13.5 ± 1.6</td>
<td>13.0 ± 1.7</td>
<td>0.004</td>
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<tr>
<td>Leukocytes / μl</td>
<td>6856 ± 1998</td>
<td>6749 ± 1987</td>
<td>7331 ± 1878</td>
<td>0.017</td>
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<tr>
<td>Total Monocytes / μl</td>
<td>561 ± 203</td>
<td>548 ± 200</td>
<td>615 ± 206</td>
<td>0.007</td>
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<tr>
<td>Classical monocytes / μl</td>
<td>463 ± 174</td>
<td>454 ± 174</td>
<td>500 ± 171</td>
<td>0.034</td>
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<tr>
<td>Intermediate monocytes / μl</td>
<td>34 ± 21</td>
<td>32 ± 18</td>
<td>45 ± 29</td>
<td>&lt;0.001</td>
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<td>Nonclassical monocytes / μl</td>
<td>64 ± 32</td>
<td>62 ± 30</td>
<td>71 ± 38</td>
<td>0.064</td>
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<tr>
<td>ARB (yes)</td>
<td>220 (50 %)</td>
<td>185 (52 %)</td>
<td>35 (43 %)</td>
<td>0.177</td>
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<tr>
<td>ACE-Inhibitor (yes)</td>
<td>164 (37 %)</td>
<td>127 (36 %)</td>
<td>37 (46 %)</td>
<td>0.099</td>
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<tr>
<td>MRA (yes)</td>
<td>99 (23 %)</td>
<td>77 (22 %)</td>
<td>22 (27 %)</td>
<td>0.306</td>
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<td>Statin (yes)</td>
<td>213 (49 %)</td>
<td>162 (45 %)</td>
<td>51 (63 %)</td>
<td>0.005</td>
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<tr>
<td>Non-statin cholesterol lowering</td>
<td>43 (10 %)</td>
<td>31 (9 %)</td>
<td>12 (15 %)</td>
<td>0.100</td>
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<tr>
<td>drugs (yes)</td>
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<td></td>
<td></td>
<td>0.061</td>
</tr>
<tr>
<td>VDR-A (yes)</td>
<td>32 (7 %)</td>
<td>22 (6 %)</td>
<td>10 (12 %)</td>
<td></td>
</tr>
</tbody>
</table>

CVD: cardiovascular disease; BMI: body mass index; BP: blood pressure; eGFR: estimated glomerular filtration rate; UAE: urinary albumin excretion; hsCRP: high-sensitivity C-reactive protein; Hb: hemoglobin; ARB: angiotensin receptor blocker; ACE inhibitor: angiotensin converting enzyme inhibitor; MRA: mineralocorticoid receptor antagonist; VDR-A: vitamin D receptor agonist. Data are presented as numbers (percentages) or means ± standard deviation as appropriate. Values for UAE, HDL-C, Apo A-I, hsCRP and triglycerides are given as median and interquartile range.
Figure 1
IIa

Classical CD14++CD16- monocytes (cells/μl)

- Apo A-I below median p (for trend) = 0.172
- Apo A-I above median p (for trend) = 0.321

IIb

Classical CD14++CD16- monocytes (cells/μl)

- HDL below median p (for trend) = 0.040
- HDL above median p (for trend) = 0.737

IIa

eGFR categories (ml/min/1.73 m²)

IIb

eGFR categories (ml/min/1.73 m²)
IIIa

Nonclassical CD14+CD16++ monocytes (cells/μl)

Apo A-I below median p (for trend) = 0.253
Apo A-I above median p (for trend) = 0.511

eGFR categories (ml/min/1.73 m²)

15-29  30-44  45-59  60-90

IIIb

Nonclassical CD14+CD16++ monocytes (cells/μl)

HDL below median p (for trend) = 0.117
HDL above median p (for trend) = 0.603

eGFR categories (ml/min/1.73 m²)

15-29  30-44  45-59  60-90