Methods

Study population

The CARE FOR HOMe study is a prospective cohort study in stable CKD patients aiming to characterize cardio-
renal interactions.1,2 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.1 100 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.4 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-Diazas-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


