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Haemodialysis-induced transient CD16$^+$ monocytopenia and cardiovascular outcome

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Abstract

Background. Haemodialysis with bioincompatible membranes led to transient leukocyte activation and intradialytic leukopenia due to endothelial adherence. After the introduction of biocompatible membranes, only CD16$^+$ (i.e. CD14^{++}CD16$^+$ and CD14$^{(+)}$CD16$^+$) monocytes showed an impressive transient intra-dialytic decrease. Presently, it is unclear whether this CD16$^+$ monocyte drop is detrimental. We investigated whether a prominent intradialytic decrease of CD16$^+$ monocytes predicts future cardiovascular (CV) events.

Methods. We measured leukocyte and monocyte subpopulations in 70 patients before and 10 min after haemodialysis initiation. Patients were stratified by their intra-dialytic CD14^{++}CD16$^+$ monocyte drop (pre-defined major drop: decline of cell counts at 10 min to $<$50% of pre-dialytic values; pre-defined minor drop: decline to values $>$50% of pre-dialytic counts). Patients were followed up for 42 $\pm$ 2 months; endpoints were CV events and death.

Results. Patients with a minor CD14^{++}CD16$^+$ monocyte drop had more CV events than patients with a major drop. In multivariate analysis, a minor CD14^{++}CD16$^+$ monocyte drop was the strongest independent predictor of future CV events [hazard ratio 2.405 (95% CI 1.192–4.854)].

Conclusions. These data refute the assumption that a prominent intra-dialytic decrease of CD14^{++}CD16$^+$ monocytes is detrimental. Instead, a minor cell drop could mirror CD14^{++}CD16$^+$ monocyte dysfunction, with inadequate migratory reaction towards an immunologic stimulus posed by membrane and tubing contact.

Keywords: Biocompatibility; cardiovascular disease; epidemiologic; haemodialysis; immunologic

Introduction

Persistent microinflammation and alterations in immune function, which are characterized by an increase in pro-inflammatory cytokines such as TNF-α and IL-6 [1,2], a shift in monocyte subsets [3] and monocytic dysfunction [4], are thought to contribute to the devastating prognosis of haemodialysis patients [5].

In humans, differential expression of the LPS receptor CD14 and the FcγIII receptor CD16 distinguishes three monocyte subsets: CD14^{++}CD16$^-$ cells, CD14^{++}CD16$^+$ cells and CD14$^{(+)}$CD16$^+$ cells. The latter two subsets are summarized as CD16$^+$ monocytes, which make up 10–20% of all circulating monocytes [6]. CD16$^+$ monocytes have traditionally been considered to represent pro-inflammatory monocytes with a high endothelial affinity [6,7].

Patients with chronic kidney disease (CKD) show a profound expansion of CD16$^+$ monocytes [3]. CD16$^+$ monocytes have been discussed as central players in atherogenesis [6,8]. Thus the increase of these cells in CKD might contribute to the enormous burden of cardiovascular morbidity and mortality in dialysis patients [9].

Since the early days of haemodialysis treatment, activation of leukocytes and a transient decline in circulating leukocyte numbers during haemodialysis have been known [10]. This fall in total leukocyte numbers is most pronounced with bioincompatible membranes, which induce an activation of the alternative pathway of the complement system [11] with following transient adherence, mainly of activated granulocytes to the pulmonary vasculature [12]. After the introduction of biocompatible membranes, a specific subset of human monocytes—i.e. CD16$^+$ monocytes—remains the only leukocyte subset to show a profound intra-dialytic decrease. About one-half of all CD16$^+$ monocytes transiently disappear from the circulation within 10 min after initiation of a dialysis treatment [13,14].

The intra-dialytic fate of CD16$^+$ monocytes during dialysis treatment with biocompatible membranes remains enigmatic. In analogy to granulocytes during haemodialysis with bioincompatible membranes, a proportion of CD16$^+$ monocytes presumably attach to the endothelium of pulmonary vasculature. Another proportion of CD16$^+$ monocytes might adhere to nascent atherosclerotic lesions and atherosclerotic plaques of the systemic vasculature, where they might contribute to plaque progression through local production of inflammatory cytokines. If this holds true, patients with a more pronounced intra-dialytic CD16$^+$ monocyte drop should be at an increased cardiovascular risk.

To the best of our knowledge, the relationship between the extent of the dialysis-induced drop in CD16$^+$ monocyte counts and the prevalence and progression of cardiovascular disease has not been assessed so far. We therefore analysed whether haemodialysis patients with a marked intra-dialytic decline in CD16$^+$ monocyte counts are at increased risk for future cardiovascular events.

Subjects and methods

Study population

Between March and September 2005, 70 patients with prevalent CKD stage V on haemodialysis treatment were recruited for a prospective cohort study. Patients were excluded if they had any kind of medical complication requiring hospital admission. Underlying causes for CKD stage V were diabetic nephropathy ($N = 26$), glomerulonephritis ($N = 13$), nephrosclerosis ($N = 8$), interstitial nephritis ($N = 8$), autosomal dominant polycystic kidney disease ($N = 5$), other primary renal diseases ($N = 4$) and unknown conditions ($N = 6$). The mean duration of renal replacement therapy was 3.4 ± 3.9 years (range 0.1–18.2 years). Haemodialysis was performed using bicarbonate dialysate and polyamide or polysulfone dialysers.

Patients with a history of diabetes mellitus, with a spontaneous plasma glucose level of >200 mg/dl, with hypoglycaemic treatment and/or with self-reported diabetes mellitus were categorized as diabetic.

Patients were defined as active smokers if they were current smokers or had stopped smoking <1 month before entry into the study. The body mass index (BMI) was calculated as body weight (kg)/height (m)^2.

Prior to a haemodialysis session, systolic and diastolic blood pressure (BP sys and BP dia) was measured. The mean blood pressure was defined as BP dia + (BP sys − BP dia)/3.

In all patients, comorbidity was assessed by chart review and by standardized interviews. Prevalent cardiovascular disease was diagnosed in patients with a history of a cardiovascular event, as defined below.

Informed consent was obtained from all patients, and the study design was approved by the local ethics committee.

All participants were followed from the baseline examination until death or until 31 December 2008. Patients who received a renal allograft were censored at the time of kidney transplantation. No patient was lost to follow-up. The pre-specified combined clinical endpoint was the first occurrence of a cardiovascular event (defined as myocardial infarction, coronary artery angioplasty/stenting/bypass surgery, stroke with symptoms lasting >24 h, carotid endarterectomy/stenting, non-traumatic lower extremity amputation or lower limb artery bypass surgery/angioplasty/stenting) or death. We additionally assessed time until first admission for any infectious complication.

Laboratory methods

Before the start of the haemodialysis session (pre-dialytic sample) and after 10 min (intra-dialytic sample), blood was drawn from all patients. Plasma glucose, total cholesterol, high-density lipoprotein cholesterol (HDL-C), calcium, phosphorus, albumin and C-reactive protein were analysed from pre-dialytic blood samples, using standard techniques.

Leukocyte and monocyte counts were obtained from pre- and intra-dialytic blood samples with automated cell counters. Monocyte subsets were determined via flow cytometry in a whole blood assay using 100 μl of heparin anti-coagulated blood. The cells were stained with monoclonal antibodies—anti-CD86 (HA5.2B7, Beckman-Coulter, Krefeld, Germany), anti−CD16 (3G8, Invitrogen, Hamburg, Germany) and anti-CD14 (Mϕ9, BD Biosciences, Heidelberg, Germany)—as described before [15], and analysed by flow cytometry (FACSCalibur, BD Biosciences) using the CellQuest software.

Monocytes were gated in a SSC/CD86$^+$ dot plot, identifying monocytes as CD86$^+$ cells with monocyte scatter properties. Subpopulations of CD14^{++}CD16$^-$, CD14^{++}CD16$^+$ and CD14$^{(+)}$CD16$^+$ monocytes were distinguished by their surface expression pattern of the LPS receptor CD14 and the FcγIII receptor CD16 (cf. Figure 1). For lymphocyte characterization, the following antibodies were used: anti-CD3 (SK7), anti-CD8 (SK1), anti-CD4 (RPA-T4), anti-CD28 (10F3), anti-CD19 (SJ25C1) and anti-CD3/anti-CD56/anti-CD16 mix (SK7/B73.1/MY31, all from Becton Dickinson, Heidelberg, Germany).

Statistical analysis

Categorical variables were presented as percentage of patients, and compared using Fisher's exact test.

Continuous data were expressed as means ± standard deviation and compared using the Mann–Whitney test (for two independent samples) or the Wilcoxon test (for comparison of pre-dialytic and intra-dialytic cell counts), as appropriate.

In order to analyse the impact of the dialysis-induced cell count drop on event-free survival, we arbitrarily defined a decline in cell counts to <50% from the baseline as a major drop, and a decline in cell counts to >50% from the baseline as a minor drop. Kaplan–Meier survival curves were calculated, and event-free survival was compared using the log-rank test.

Cox proportional hazards models were calculated to study relationships of the intra-dialytic drop in monocyte subset cell counts with event-free survival after adjustment for those risk factors that were predictors of the combined endpoint of death or cardiovascular events in univariate analysis.

Finally, to understand the relative predictive value of pre-dialytic CD14^{++}CD16$^+$ monocyte counts versus the intra-dialytic drop in CD14^{++}CD16$^+$ monocytes, we assessed the percentage of patients suffering from cardiovascular events and/or death by tertiles of pre-dialytic

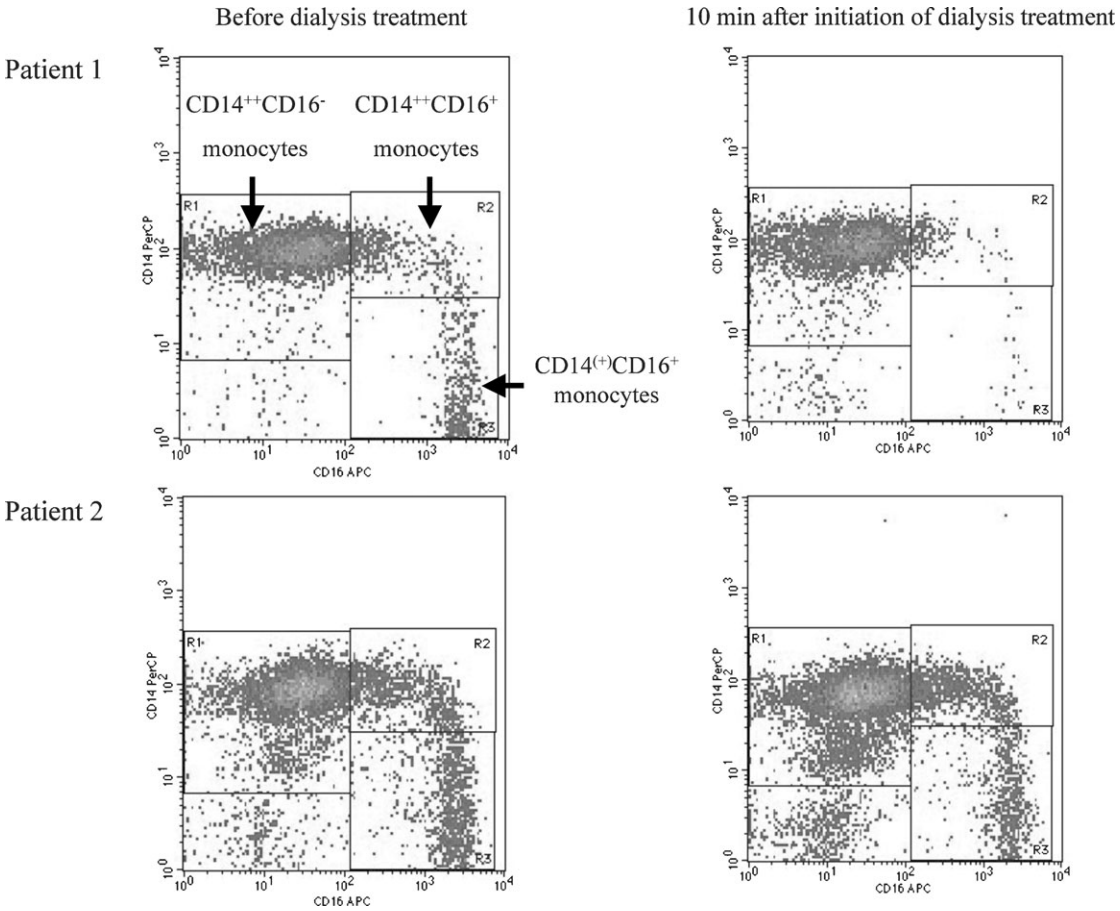

Fig. 1. Monocyte subsets: $CD86^+$ cells with monocyte scatter properties were gated, and monocyte subpopulations were defined according to their surface expression pattern of the LPS receptor CD14 and the FcγIII receptor CD16 [representative example of two age- and gender-matched haemodialysis patients; monocyte subset measurements at baseline (left column) and 10 min after dialysis initiation (right column). Patient 1 (upper row) did not experience a cardiovascular event during the follow-up; patient 2 (lower row) had a myocardial infarction 7 months after study initiation].

$CD14^{++}CD16^+$ monocyte counts cross-classified by the level of the intra-dialytic drop in $CD14^{++}CD16^+$ monocytes (major versus minor drop). Data management and statistical analysis were performed with SPSS 13.0. The level of significance was set at $P \leq 0.05$.

Results

The baseline characteristics of all 70 study participants are shown in Table 1.

A cardiovascular event had occurred in 42 patients (60%) by the time of the last follow-up. Six patients were censored after receiving a renal allograft without prior cardiovascular event. The remaining 22 patients have been followed up for 42 ± 2 months. Patients who experienced an event were older, had a higher prevalence of cardiovascular disease and diabetes mellitus at baseline, had a higher baseline C-reactive protein, higher baseline total monocyte counts and lower serum albumin (Table 1).

Total leukocyte and leukocyte subpopulation counts were obtained at the beginning and 10 min after the beginning of the haemodialysis session. The patients had a mean total leukocyte count of 7501 ± 2142/μl at the initiation of the dialysis session. Within 10 min after the beginning of haemodialysis treatment, leukocyte values decreased to 5703 ± 2296/μl, which corresponds to a 24.5 ± 18.8% decline.

The most pronounced decline in cell numbers was observed in $CD14^{++}CD16^+$ monocytes and $CD14^{(+)}CD16^+$ monocytes (Figure 2). Within lymphocyte subsets, only NK cells showed a marked intra-dialytic decrease, even though the comparison of pre- and intra-dialytic cell counts achieved formal statistical significance for all leukocyte subsets.

Patients with a major drop of $CD14^{++}CD16^+$ monocyte (i.e. cell counts at 10 min of haemodialysis to <50% of baseline) did not differ in their baseline characteristics from patients with a minor drop (Table 2). The same holds true when stratifying patients by their drop in $CD14^{(+)}CD16^+$ monocyte counts (data not shown), as the intra-dialytic drop in $CD14^{++}CD16^+$ monocytes and the drop in $CD14^{(+)}CD16^+$ monocytes (both expressed as percentage of baseline) were very strongly correlated ($r = 0.918$, $P < 0.001$). Accordingly, 41 patients had a major drop in both monocyte subsets, and 20 patients had a minor drop in both subsets, leaving only 9 patients who had a minor drop in CD14 $^{++}CD16^+$ monocytes, but a major drop in $CD14^{(+)}CD16^+$ monocytes.

Table 1. Baseline characteristics of the study participants

	Overall ($N = 70$)	No event ($N = 28$)	Event ($N = 42$)	P-value
Age (years)	64.5 ± 15.6	55.5 ± 15.8	70.5 ± 12.3	<0.001
Women (%)	31 (44%)	15 (54%)	16 (38%)	0.228
Smokers (%)	13 (19%)	7 (25%)	6 (14%)	0.349
Diabetes mellitus (%)	35 (50%)	8 (29%)	27 (64%)	0.007
History of CVD (%)	29 (41%)	5 (18%)	24 (57%)	<0.001
Vintage of renal replacement therapy (years)	3.4 ± 3.9	3.4 ± 4.0	3.4 ± 3.9	0.947
Mean blood pressure (mmHg)	97 ± 17	102 ± 17	94 ± 16	0.097
C-reactive protein (mg/l)	14.3 ± 18.0	10.0 ± 20.0	17.1 ± 16.3	0.013
Total cholesterol (mg/dl)	166 ± 44	174 ± 44	161 ± 47	0.106
HDL cholesterol (mg/dl)	49 ± 17	50 ± 15	48 ± 18	0.401
Body mass index (kg/m^2)	27.1 ± 4.8	26.8 ± 4.6	27.2 ± 5.0	0.737
Plasma calcium (mmol/l)	2.3 ± 0.2	2.3 ± 0.2	2.4 ± 0.2	0.770
Plasma phosphorus (mg/dl)	6.1 ± 1.8	6.5 ± 2.0	5.8 ± 1.6	0.143
Albumin (g/l)	39 ± 4	40 ± 3	38 ± 4	0.042
Leukocytes/μl	7501 ± 2142	6979 ± 1541	7850 ± 2419	0.226
Monocytes/μl	639 ± 219	563 ± 203	689 ± 216	0.016
CD14^{++}CD16$^-$ monocytes/μl before HD	463 ± 181	417 ± 185	494 ± 175	0.047
CD14^{++}CD16$^+$ monocytes/μl before HD	33 ± 19	27 ± 17	37 ± 19	0.011
CD14$^{(+)}$CD16$^+$ monocytes/μl before HD	143 ± 83	119 ± 49	158 ± 97	0.089

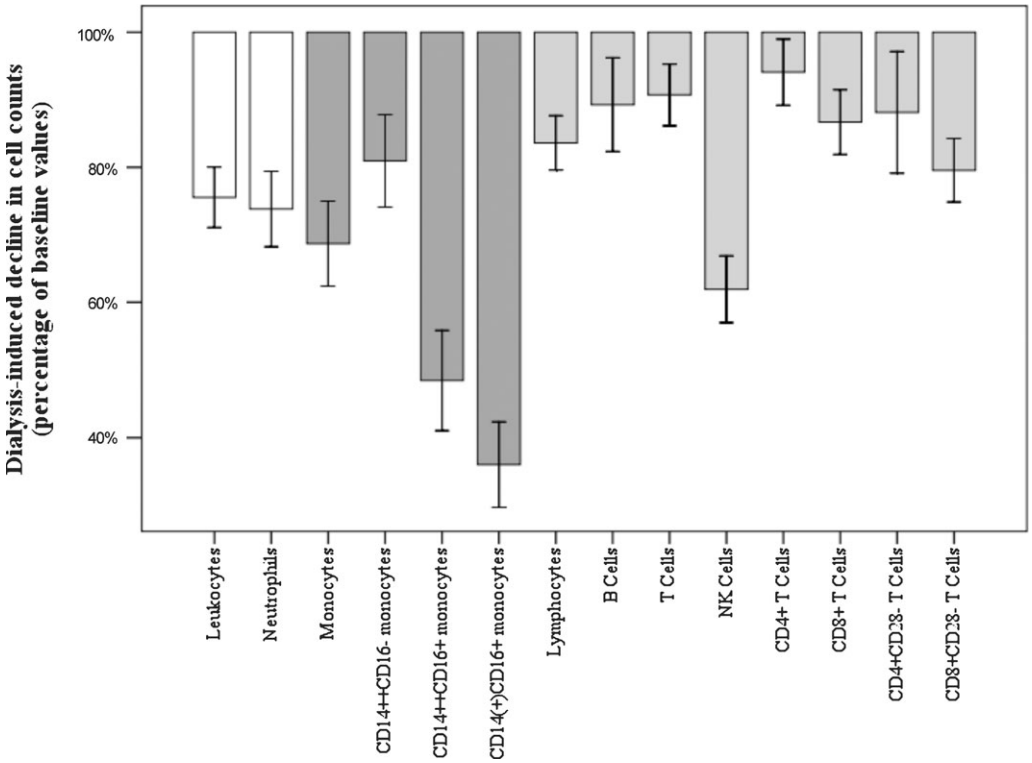

Fig. 2. Dialysis-induced drop in circulating white cell counts by leukocyte subpopulations. Means and their 95% confidence intervals are indicated. For all leukocyte subpopulations, intra-dialytic cell counts were significantly lower than pre-dialytic cell counts.

When analysing the dialysis-induced cell drop with regard to event-free survival in univariate analysis, patients with a major drop of CD14^{++}CD16$^+$ monocytes had significantly longer event-free survival compared to patients with a minor CD14^{++}CD16$^+$ monocyte drop (Figure 3; cf. Figure 1 for a representative example). Similarly, event-free survival tended to be longer in patients with a major dialysis-induced decline in CD14$^{(+)}$CD16$^+$ monocytes (Figure 4). The same holds true when considering non-fatal cardiovascular events and cardiovascular death, rather than cardiovascular events and total mortality, as combined clinical endpoint (data not shown).

A minor drop of CD14^{++}CD16$^+$ monocytes (i.e. decline of cell counts at 10 min to >50% of pre-dialytic values) remained an significant predictor of cardiovascular events after adjustment for those risk factors that were predictors in univariate analysis, namely

Table 2. Baseline characteristics of the study participants with a major versus a minor intra-dialytic decline in $CD14^{++}CD16^{+}$ monocyte counts

	Major decline ($N = 41$)	Minor decline ($N = 29$)	*P*-value
Age (years)	62.6 ± 16.0	67.0 ± 14.9	0.478
Women (%)	19 (46%)	12 (41%)	0.808
Smokers (%)	8 (19%)	5 (17%)	1.000
Diabetes mellitus (%)	18 (44%)	17 (59%)	0.332
History of CVD (%)	17 (42%)	12 (41%)	1.000
Mean blood pressure (mmHg)	99 ± 18	94 ± 14	0.442
C-reactive protein (mg/l)	14.4 ± 19.1	14.0 ± 16.4	0.909
Plasma cholesterol (mg/dl)	170 ± 47	161 ± 44	0.417
HDL cholesterol (mg/dl)	45 ± 13	53 ± 20	0.128
Body mass index (kg/m^2)	27.3 ± 4.2	26.7 ± 5.6	0.387
Plasma calcium (mmol/l)	2.3 ± 0.2	2.4 ± 0.2	0.180
Plasma phosphorus (mg/dl)	5.8 ± 1.7	6.4 ± 1.9	0.217
Albumin (g/l)	38.3 ± 2.7	38.8 ± 4.6	0.541
Leukocytes/μl	7234 ± 1832	7879 ± 2503	0.414
Monocytes/μl	618 ± 205	670 ± 236	0.493
$CD14^{++}CD16^{-}$ monocytes/μl before HD	452 ± 176	479 ± 191	0.681
$CD14^{++}CD16^{+}$ monocytes/μl before HD	31 ± 17	37 ± 21	0.176
$CD14^{(+)}CD16^{+}$ monocytes/μl before HD	135 ± 67	153 ± 102	0.844

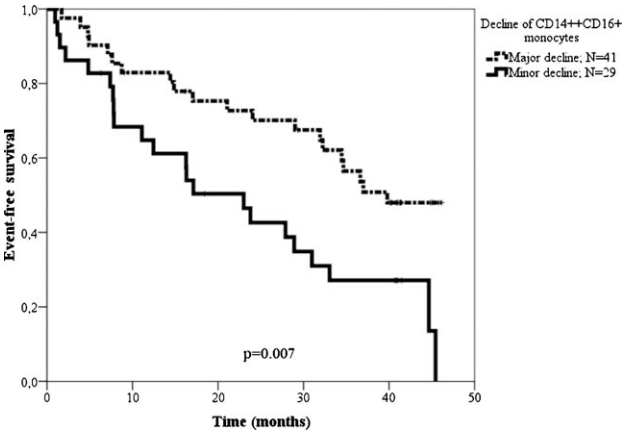

Fig. 3. Event-free survival in haemodialysis patients stratified by the intra-dialytic decline of $CD14^{++}CD16^{+}$ monocytes (Kaplan–Meier analysis with the log-rank test).

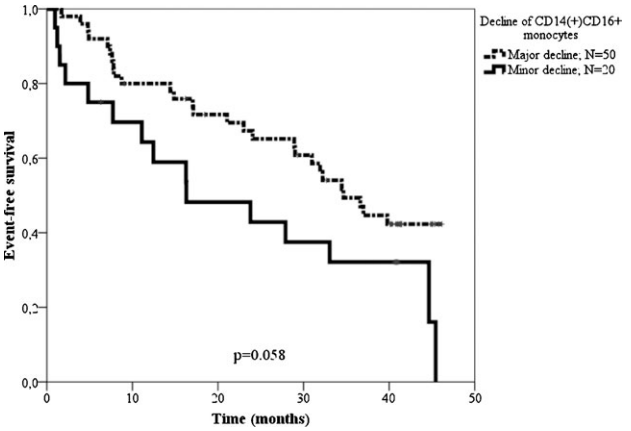

Fig. 4. Event-free survival in haemodialysis patients stratified by the intra-dialytic decline of $CD14^{(+)}CD16^{+}$ monocytes (Kaplan–Meier analysis with the log-rank test).

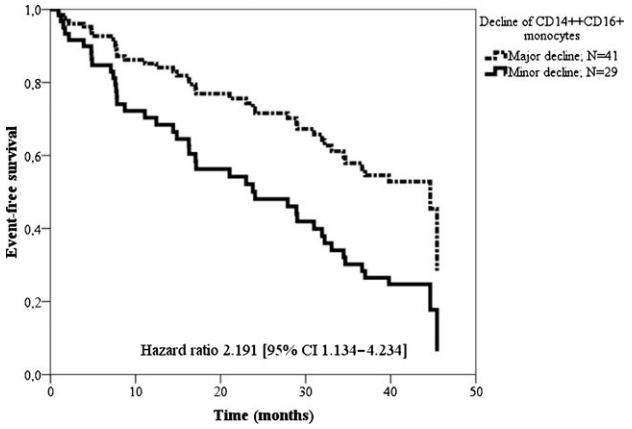

Fig. 5. Event-free survival in haemodialysis patients stratified by the intra-dialytic decline of $CD14^{++}CD16^{+}$ monocytes [Cox regression survival plot with separate lines for patients with intra-dialytic decrease of $CD14^{++}CD16^{+}$ monocytes to values <50% of pre-dialytic cell counts (major decline), and patients with intra-dialytic decrease of $CD14^{++}CD16^{+}$ monocytes to values >50% of pre-dialytic cell counts (minor decline), respectively].

age, prevalence of cardiovascular disease, diabetes mellitus, C-reactive protein, total number of monocytes and albumin [hazard ratio 2.91 (95% CI 1.134–4.234) Figure 5].

Grouping patients with regard to their pre-dialysis $CD14^{++}CD16^{+}$ monocyte counts as well as to the dialysis-induced decline of $CD14^{++}CD16^{+}$ monocyte counts revealed the highest cardiovascular event rate among those patients who had the highest pre-dialysis levels of $CD14^{++}CD16^{+}$ monocytes and a minor intra-dialytic drop in $CD14^{++}CD16^{+}$ monocytes (Figure 6).

Time until first hospitalization for infectious diseases did not differ between patients with a major versus a minor drop in $CD14^{++}CD16^{-}$ ($P = 0.735$), $CD14^{++}CD16^{+}$ ($P = 0.054$) and $CD14^{+}CD16^{+}$ monocytes ($P = 0.346$; the log-rank test).

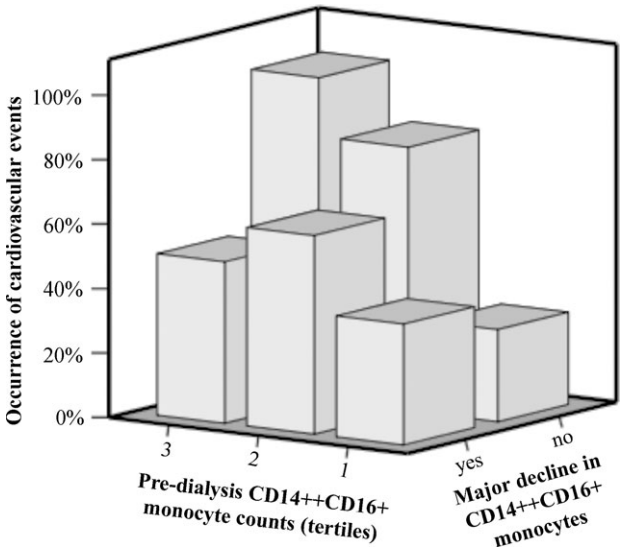

Fig. 6. Percentage of patients suffering cardiovascular events and/or death by tertiles of pre-dialytic CD14^{++}CD16$^+$ monocyte counts cross-classified by the level of the intra-dialytic drop in CD14^{++}CD16$^+$ monocytes (major versus minor drop).

Discussion

Patients with CKD stage V have a markedly reduced life expectancy, comparable to that of patients with certain types of malignant diseases [16]. Epidemiologic data show that cardiovascular events due to an accelerated atherogenesis are the single biggest cause of mortality in patients with CKD [17]. The underlying reasons for this increased atherosclerotic burden have not been completely elucidated. Among a plethora of pathologic changes due to CKD—such as disturbances in calcium, phosphate and vitamin D homeostasis, volume overload, arterial hypertension and oxidative stress—a persistent microinflammatory state and alterations in the immune system are well-established mechanisms [18].

These mechanisms comprise a shift in monocyte subpopulations leading to an increase in CD16$^+$ monocyte counts [3]. Given the potential role of CD16$^+$ monocytes in atherogenesis [6,19], our group recently reported that CD14^{++}CD16$^+$ monocyte counts were independent predictors of cardiovascular events in dialysis patients after adjustment for established cardiovascular risk factors [9].

CD16$^+$ monocytes are superior producers of the pro-inflammatory cytokine TNF-α in comparison to the CD14^{++}CD16$^-$ monocyte subset [20]. In addition, CD16$^+$ monocytes constitutively express high levels of the fractalkine receptor CX3CR1 [21], a pivotal chemokine receptor in atherogenesis [22]. Furthermore, compared to CD14^{++}CD16$^-$ monocytes, CD16$^+$ monocytes display higher expression of certain adhesions molecules [7].

In line with their high endothelial affinity [7], CD16$^+$ monocytes remain the only leukocyte subset that experiences a relevant transient cell count decline in haemodialysis with biocompatible membranes [13,14]. Our group has previously shown that CD16$^+$ monocytes return roughly to baseline values at the end of a haemodialysis session. As these reappearing CD16$^+$ monocytes do not express markers of immature monocytes, such as CD34, transient sequestration and not apoptosis is the most likely mechanism of this cell count decline [13]. Extrapolating data from seminal studies on bioincompatibility and leukocytopenia leads to the assumption that a majority of CD16$^+$ monocytes adhere to pulmonary vasculature during dialysis. Nonetheless, given their proatherosclerotic chemokine receptor pattern, some dialysis-activated CD16$^+$ monocytes might adhere to dysfunctional endothelium overlaying atherosclerotic lesions, causing subsequent local propagation of an inflammatory *milieu*. Thus, each dialysis-induced drop of CD16$^+$ monocytes could potentially contribute to atherosclerosis. If this hypothesis holds true, patients with a marked intra-dialytic decline in CD16$^+$ monocyte counts should be at an increased risk for future cardiovascular events.

Surprisingly, instead of a major drop of CD16$^+$ monocytes, a minor decline was actually associated with a worse cardiovascular prognosis. After adjustment for all factors that were predictors of cardiovascular events in univariate analysis, a minor dialysis-induced CD16$^+$ monocyte depletion—i.e. a decrease to values >50% of pre-dialytic cell counts–was identified as the strongest independent predictor of the occurrence of cardiovascular events in this study.

Determining both the absolute pre-dialytic CD16$^+$ monocyte count and the relative extent of the dialysis-induced CD16$^+$ monocyte count drop helped to identify those patients at the highest cardiovascular risk: patients in the highest tertile of pre-dialytic CD16$^+$ monocyte counts, displaying at the same time a minor intra-dialytic drop, had by far the highest cardiovascular event rates.

We can only speculate which mechanisms might underlie our results. However, an elegant study in a murine model of atherosclerosis [23] provides some data regarding monocyte biology and atherosclerosis that might give us valuable hints for the interpretation of our data: Llodra and co-workers found that in a pro-atherosclerotic *milieu,* monocytes migrated into atherosclerotic plaque; reversing the pro-atherosclerotic *milieu* led to plaque regression, characterized by monocyte emigration, which in turn is dependent on preserved monocytic cell trafficking.

We propose the hypothesis that a minor dialysis-induced CD16$^+$ monocytopenia indicates an inadequate migratory reaction of CD16$^+$ monocytes towards an adequate immunologic stimulus posed by membrane and tubing contact during dialysis, which mirrors a dysfunctional state of CD16$^+$ monocytes in high-risk patients. One aspect of this dysfunctional state might be disturbed monocytic cell trafficking, which itself could be an explanation for the higher cardiovascular event rate according to the experimental data of Llodra and co-workers.

There are several limitations of our study that have to be taken into account.

Firstly, due to the nature of our study, we cannot provide mechanistic data on dialysis-induced CD16$^+$ monocytopenia and their relation to cardiovascular outcome; therefore, the interpretation of our results needs further confirmation. Secondly, because of the rather small number of patients included, the association between dialysis-induced monocytopenia and cardiovascular outcome should be reassessed

in larger cohort studies. Thirdly, as we did not primarily aim to assess the relationship between monocyte behaviour and infections, the association between intra-dialytic monocytopenia and infectious complications should be examined in future studies that primarily assess infectious disease with respect to specific classes of pathogens.

Nonetheless, this study adds valuable clinical data to the rapidly expanding research area of monocyte heterogeneity that is currently dominated by data from murine models, and generates hypotheses about the functional state of human monocytes in uraemia.

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Conflict of interest statement. None declared.

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