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CD14++CD16+ Monocytes Independently Predict Cardiovascular Events

A Cohort Study of 951 Patients Referred for Elective Coronary Angiography

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Objectives The aim of this study was to analyze the yet ill-defined relationship of distinct human monocyte subsets with cardiovascular outcomes in a broad patient population at cardiovascular risk.

Background Monocytes, the most abundant immune cell type found in atherosclerotic plaques, are crucial promoters of atherogenesis. Three distinct human monocyte subsets exist: classical CD14++CD16−, intermediate CD14++CD16+, and nonclassical CD14+CD16++ monocytes. Immunomodulation of distinct monocyte subsets has recently been discussed as a new therapeutic avenue in atherosclerosis.

Methods Cardiovascular events in 951 subjects referred for elective coronary angiography were prospectively analyzed. Monocyte subset analysis was performed using flow cytometry, blinded to patients' clinical characteristics, and patients were categorized according to quartiles of total monocyte and monocyte subset counts. The primary endpoint was defined a priori as the first occurrence of cardiovascular death, acute myocardial infarction, or nonhemorrhagic stroke. Endpoint adjudication was done blinded to monocyte subset distribution.

Results During a mean follow-up period of 2.6 ± 1.0 years, 93 patients experienced the primary endpoint. In univariate Kaplan-Meier analysis, counts of total ($p = 0.010$), classical CD14++CD16− ($p = 0.024$), and intermediate CD14++CD16+ ($p < 0.001$) monocytes predicted the primary endpoint, whereas nonclassical monocytes did not ($p = 0.158$). After full adjustment for confounders, CD14++CD16+ monocytes remained the only monocyte subset independently related to cardiovascular events (fourth vs. first quartile: hazard ratio: 3.019; 95% confidence interval: 1.315 to 6.928; $p = 0.009$).

Conclusions CD14++CD16+ monocytes independently predicted cardiovascular events in subjects referred for elective coronary angiography. Future studies will be needed to elucidate whether CD14++CD16+ monocytes may become a target cell population for new therapeutic strategies in atherosclerosis. (J Am Coll Cardiol 2012;60: 1512–20) © 2012 by the American College of Cardiology Foundation

Monocytes are the central drivers of vascular inflammation in atherosclerosis. They contribute to atherogenesis from the formation of the earliest asymptomatic atherosclerotic lesions, namely fatty streaks, to final plaque rupture with potentially fatal outcomes (1,2). Although experimental studies have proven a causative role of monocytes in atherogenesis (3), epidemiological analyses have failed to unequivocally demonstrate an association between circulating monocyte counts and cardiovascular disease (4).

Importantly, monocytes display substantial heterogeneity, which is reflected by the differential surface expression of the lipopolysaccharide receptor (CD14) and the low-affinity Fcγ-III receptor (CD16). Although a subset-specific contribution of monocytes has been proposed in recent years on the basis of laboratory data (3), monocyte heterogeneity has not been analyzed thoroughly in the context of human atherosclerosis.

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The existence of 3 distinct monocyte subsets is acknowledged by recent consensus (5), namely, classical CD14++CD16− monocytes, intermediate CD14++CD16+ monocytes, and nonclassical CD14+CD16++ monocytes. However, most previous studies did not distinguish between intermediate CD14++CD16+ and nonclassical CD14+CD16++ monocytes but subsumed these 2 subsets as CD16-positive monocytes. Consequently, the intermediate monocyte subset was by far the most poorly characterized monocyte subset until recently (6). Although numerically the smallest monocyte subpopulation and seemingly displaying just an intermediate immunophenotype, CD14++CD16+ monocytes are a clearly distinguishable subset, as evidenced by the distinct gene expression profile recently been reported by Wong et al. (7) as well as our group (6), independently of each other.

We previously reported a predictive role of intermediate CD14++CD16+ monocyte counts in patients with chronic kidney disease (CKD) (8), a selected patient group at highest cardiovascular risk. Of note, CKD-associated immune dysfunction, which is characterized by profound shifts in monocyte subsets (9,10), and the CKD-specific pattern of accelerated atherosclerosis both preclude an uncritical generalization of our previous findings to the general population.

Against this background, we initiated the HOM SWEET HOMe (Heterogeneity of Monocytes in Subjects Who Undergo Elective Coronary Angiography—The Homburg Evaluation) study to test the hypothesis that counts of intermediate CD14++CD16+ monocytes predict cardiovascular events in subjects at cardiovascular risk referred for elective coronary angiography.

Methods

Between May 2007 and June 2010, subjects who were admitted to Saarland University Medical Center for elective coronary angiography were invited to participate in the HOM SWEET HOMe study. The study was approved by the local ethics committee, and all participants provided written informed consent.

At study inclusion, a history of smoking, diabetes, current drug intake, and cardiovascular comorbidities were recorded using a standardized questionnaire. Patient-reported comorbidities were confirmed by chart review. Prevalent cardiovascular disease was diagnosed in subjects with coronary artery disease (a history of myocardial infarction or coronary artery angioplasty, stent implantation, or bypass surgery), cerebrovascular disease (a history of major stroke [defined as acute onset of neurological symptoms persisting for >24 h] or carotid endarterectomy or stent implantation) or peripheral artery disease (a history of nontraumatic lower extremity amputation or lower limb artery bypass surgery, angioplasty, or stent implantation).

Subjects were categorized as active smokers if they were current smokers or had stopped smoking <1 month before study entry. Subjects with self-reported or physician-reported diabetes mellitus, with nonfasting blood glucose levels ≥200 mg/dl, with fasting blood glucose levels ≥126 mg/dl, or with current use of hypoglycemic medication were categorized as having diabetes. Body mass index was calculated as weight in kilograms divided by the square of height in meters.

Abbreviations and Acronyms

CKD = chronic kidney disease

CRP = C-reactive protein

Coronary angiography was performed on the day of hospital admission. Coronary artery disease was defined in subjects who had stenoses ≥ 50% in a major coronary artery on current coronary angiography and/or who had a history of coronary revascularization for coronary artery stenosis.

Because of their potential interference with monocyte subset counts, intake of systemic immunosuppressive agents was considered an exclusion criterion for the present analysis.

Laboratory measurements. Blood samples were obtained under standardized conditions. Blood levels of creatinine, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol and C-reactive protein (CRP) were measured using standard methods. Estimated glomerular filtration rate was calculated using the 4-variable Modification of Diet in Renal Disease study equation.

Leukocyte and monocyte counts were measured with automated cell counters using standard techniques. Using flow cytometry, monocyte subpopulations were analyzed in a whole-blood assay using 100 μl of whole blood, as described and validated previously (6). Cells were stained by monoclonal antibodies (CD86 PE [HA5.2B7; Beckman-Coulter, Krefeld, Germany], CD16 PeCy7 [3G8; BD Biosciences, Heidelberg, Germany], CD14 PerCP [Mφ9; BD Biosciences]) and analyzed using flow cytometry (FACSCalibur and FACS Canto II; BD Biosciences) using Cell Quest and FACSDiva software (BD Biosciences).

In brief, monocytes were gated in an SSC/CD86+ dot plot, identifying monocytes as CD86+ cells with monocyte scatter properties. Subsets of CD14++CD16−, CD14++CD16+, and CD14+CD16++ monocytes were defined according to the surface expression pattern of the lipopolysaccharide receptor CD14 and the Fcγ-III receptor CD16 (compare Fig. 1 for a representative example). Nomenclature of monocyte subsets followed the recommendations of the Nomenclature Committee of the International Union of Immunological Societies (5).

Outcome analysis. After study inclusion, all study participants, or their next of kin, were contacted annually until death or until September 30, 2011, for outcome analysis.

We obtained medical records from the treating physicians for verification of all events reported by study participants or their next of kin. All events were adjudicated by the same investigators, who were blinded to monocyte data, according to the following definitions.

The composite primary endpoint was defined a priori as the first occurrence of cardiovascular death, acute myocardial infarction, or nonhemorrhagic stroke. Definition of

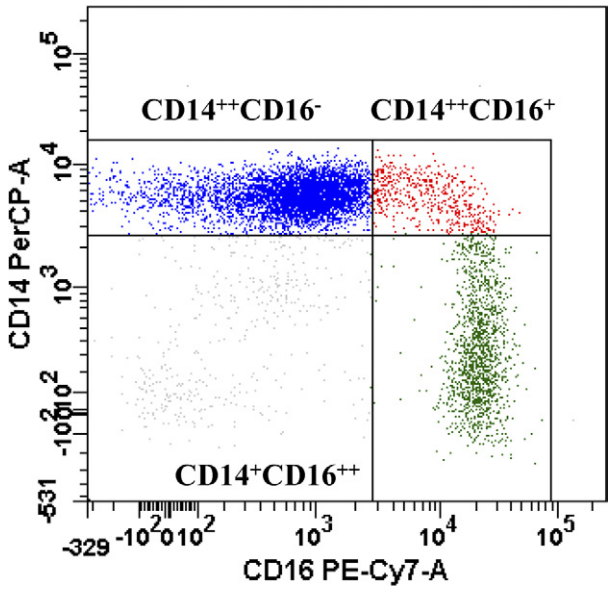

Figure 1 **Flow Cytometric Analysis of Monocyte Subpopulations**

Depicted are classical CD14++CD16− monocytes, intermediate CD14++CD16+ monocytes, and nonclassical CD14+CD16++ monocytes (representative example).

acute myocardial infarction followed the joint European Society of Cardiology, American College of Cardiology Foundation, American Heart Association, and World Heart Federation task force consensus (11). Stroke was defined as "rapidly developing clinical symptoms and/or signs of focal (or at times global) disturbance of cerebral function lasting > 24 hours (unless interrupted by surgery) or leading to death, with no apparent cause other than of vascular origin," in accordance with the World Health Organization (12). Those subjects without evidence of cerebral hemorrhage on cerebral imaging were defined to have nonhemorrhagic stroke. Deaths due to cardiovascular causes included sudden cardiac death, death from acute myocardial infarction, death from congestive heart failure, fatal stroke, fatal arrhythmia, and other fatal vascular events.

As a secondary endpoint, we defined the first occurrence of any cardiovascular event (defined as myocardial infarction; coronary artery angioplasty, stent implantation, or bypass surgery; stroke [with acute onset of neurological symptoms persisting for >24 h]; carotid endarterectomy or stent implantation; nontraumatic lower extremity amputation; or lower limb artery bypass surgery, angioplasty, or stent implantation) or death.

Statistical analysis. Data management and statistical analysis were performed using PASW Statistics 18 (SPSS, Inc., Chicago, Illinois) and the software environment for statistical computing R. Two-sided p values <0.05 were considered significant. Categorical variables are presented as percents of patients and were compared using chi-square tests. Continuous data are expressed as mean ± SD (or, in case of skewed distributions, as median [interquartile range]) and were compared using Mann-Whitney *U* tests. The associations between continuous variables were assessed using Spearman rank correlation testing.

Subjects were divided into 4 equally sized groups (quartiles) according to their total monocyte counts and monocyte subset counts. Kaplan-Meier survival curves were used to compare event-free survival (i.e., time until first occurrence of the primary or secondary endpoint) between groups. Participants with noncardiovascular death were censored at the time of death. The log-rank test was used to test the hypothesis that at least 1 of the survival curves differs from the others.

Cox proportional-hazards models were calculated to analyze the relationship of total monocyte and monocyte subset cell counts with event-free survival after adjustment for age and sex (model 1); further adjustment for systolic blood pressure, plasma cholesterol, diabetes, smoking, and body mass index (model 2); and finally further adjustment for estimated glomerular filtration rate, CRP, prevalent cardiovascular disease, and total leukocyte count (model 3).

To assess the predictive discrimination of each Cox model, we calculated the C-statistic (13), which provides the proportion of evaluable patient pairs that can be correctly classified by a model.

Results

Baseline characteristics. Monocyte subset analysis was intended in 999 HOM SWEET HOMe participants. Of these, 36 subjects were on systemic immunosuppressive medication, and 12 did not undergo planned coronary angiography after admission, leaving 951 subjects in the present analysis.

Baseline characteristics of these 951 patients are presented in Table 1. As expected, study participants had a high burden of risk factors and cardiovascular disease. Consequently, cardioprotective medication use was highly prevalent.

Study participants were followed for a mean of 2.6 ± 1.0 years; 3 patients were lost to follow-up. The predefined composite primary endpoint (cardiovascular death, acute myocardial infarction, or nonhemorrhagic stroke) occurred in 93 subjects, of whom 29 subjects had nonfatal acute myocardial infarctions, 24 had nonfatal nonhemorrhagic strokes, and 45 subjects died of cardiovascular causes. Study participants who experienced the primary endpoint were older, had more comorbidities, and had higher markers of systemic inflammation (Table 1).

Monocyte subset counts and cardiovascular risk factors. At study initiation, the mean monocyte count was 583 ± 211 cells/μl, of which 470 ± 179 cells were CD14++CD16− monocytes, 42 ± 24 cells were CD14++CD16+ monocytes, and 71 ± 37 cells were CD14+CD16++ mono-

Table 1 Baseline Characteristics of Study Participants

Variable	Total Cohort (n = 951)	No Primary EndPoint (n = 858)	Primary Endpoint (n = 93)	p Value
Age (yrs)	65.1 ± 10.3	64.7 ± 10.3	68.8 ± 9.1	<0.001
Body mass index (kg/m^2)	28.5 ± 4.6	28.4 ± 4.6	29.0 ± 5.0	0.396
Systolic BP (mm Hg)	147 ± 22	147 ± 22	150 ± 23	0.547
Diastolic BP (mm Hg)	82 ± 11	82 ± 10	81 ± 11	0.192
Hip circumference (cm)	103.5 ± 10.0	103.4 ± 9.7	104.2 ± 12.3	0.990
Waist circumference (cm)	101.5 ± 12.5	101.1 ± 12.1	105.6 ± 15.0	0.039
Creatinine (mg/dl)	1.0 ± 0.3	1.0 ± 0.3	1.1 ± 0.5	<0.001
eGFR (ml/min/1.73 m^2)	75 ± 20	76 ± 19	69 ± 22	<0.001
CRP (mg/l)	1.9 (0.9–4.0)	1.9 (0.9–3.8)	3.7 (1.6–6.4)	<0.001
Total cholesterol (mg/dl)	187 ± 47	187 ± 47	181 ± 42	0.184
LDL cholesterol (mg/dl)	113 ± 41	114 ± 41	111 ± 37	0.561
HDL cholesterol (mg/dl)	49 ± 15	49 ± 15	48 ± 13	0.032
Triglycerides (mg/dl)	129 (93–182)	129 (92–182)	124 (96–182)	0.866
Leukocytes (1/μl)	6,709 ± 1,957	6,640 ± 1,931	7,344 ± 2,094	0.001
Total monocytes (1/μl)	583 ± 211	577 ± 211	633 ± 209	0.005
Women	305 (32.1%)	283 (33.0%)	22 (23.7%)	0.079
Smoking	122 (12.8%)	113 (13.2%)	9 (9.7%)	0.415
Diabetes mellitus	363 (38.2%)	312 (36.4%)	51 (54.8%)	0.001
Prevalent CVD	568 (59.7%)	496 (57.8%)	72 (77.4%)	<0.001
Prevalent CAD	513 (53.9%)	449 (52.3%)	64 (68.8%)	0.003
Cerebrovascular artery disease	94 (9.9%)	72 (8.4%)	22 (23.7%)	<0.001
Peripheral artery disease	67 (7.0%)	52 (6.1%)	15 (16.1%)	0.002
Beta-blockers	710 (74.7%)	639 (74.5%)	71 (76.3%)	0.906
ACE inhibitors	559 (58.8%)	494 (57.6%)	65 (69.9%)	0.067
Angiotensin receptor blockers	193 (20.3%)	169 (19.7%)	24 (25.8%)	0.372
Calcium-channel blockers	191 (20.1%)	169 (19.7%)	22 (23.7%)	0.653
Antiplatelet agents (%)	714 (75.1%)	645 (75.2%)	69 (74.2%)	0.912
Statins (%)	562 (59.1%)	501 (58.4%)	61 (65.6%)	0.399

Values are mean ± SD, median (interquartile range), or n (%).
ACE = angiotensin-converting enzyme; BP = blood pressure; CAD = coronary artery disease; CRP = C-reactive protein; CVD = cardiovascular disease; eGFR = estimated glomerular filtration rate; HDL = high-density lipoprotein; LDL = low-density lipoprotein.

cytes. Associations of monocyte (subset) counts with traditional and nontraditional cardiovascular risk factors are specified in Table 2. Cell counts of all 3 monocyte subsets were correlated with traditional markers of inflammation (CRP and leukocyte counts). Additionally, CD14++CD16+ and CD14+CD16++ monocyte

Table 2 Correlation Coefficients of Monocyte (Subset) Counts With Traditional and Nontraditional Cardiovascular Risk Factors

	Total Monocytes		CD14++CD16− Monocytes		CD14++CD16+ Monocytes		CD14+CD16++ Monocytes	
Variable	r	p Value	r	p Value	r	p Value	r	p Value
Age	−0.019	0.564	−0.048	0.138	0.066	0.040	0.100	0.002
Body mass index	0.047	0.150	0.011	0.728	0.064	0.049	0.164	<0.001
Systolic BP	0.006	0.853	−0.019	0.563	0.044	0.174	0.137	<0.001
Diastolic BP	−0.042	0.196	−0.055	0.091	−0.013	0.682	0.057	0.078
Hip circumference	0.064	0.054	0.024	0.461	0.104	0.002	0.185	<0.001
Waist circumference	0.122	<0.001	0.086	0.010	0.128	<0.001	0.206	<0.001
Creatinine	0.084	0.010	0.058	0.075	0.152	<0.001	0.099	0.002
eGFR	−0.020	0.537	0.012	0.709	−0.145	<0.001	−0.089	0.006
CRP	0.213	<0.001	0.192	<0.001	0.233	<0.001	0.102	0.002
Total cholesterol	−0.048	0.136	−0.051	0.116	−0.045	0.163	−0.007	0.838
LDL cholesterol	−0.024	0.461	−0.025	0.451	−0.019	0.554	−0.007	0.819
HDL cholesterol	−0.150	<0.001	−0.148	<0.001	−0.131	<0.001	−0.052	0.112
Triglycerides	0.078	0.016	0.057	0.077	0.069	0.035	0.104	0.001
Leukocytes	0.629	<0.001	0.632	<0.001	0.443	<0.001	0.242	<0.001

Abbreviations as in Table 1.

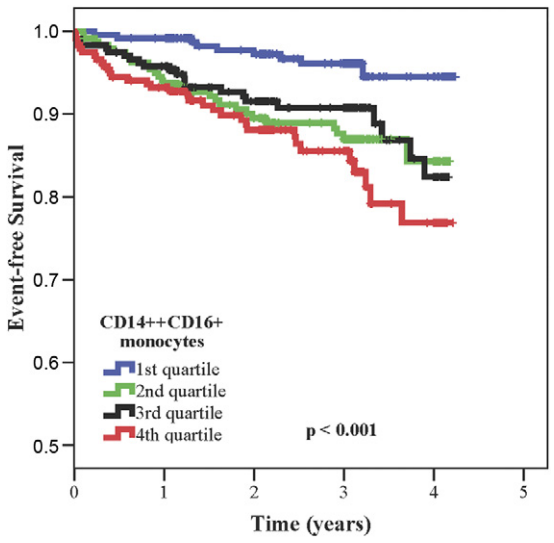

Figure 2 Relationship Between Quartiles of CD14++CD16+ Monocyte Counts and Event-Free Survival

Primary endpoint: cardiovascular death, acute myocardial infarction, or nonhemorrhagic stroke; Kaplan-Meier analysis with log-rank test.

counts showed weak, albeit significant, correlations with body mass index, renal dysfunction, age, and serum triglycerides.

Total monocyte and CD14++CD16− monocyte counts were higher in men than in women. In patients with diabetes, all monocyte subset counts were elevated (Online Table S1). Interestingly, prevalent cardiovascular disease was not associated with a shift in monocyte (subset) counts. Further associations between monocyte (subset) counts and intake of cardioprotective medication are listed in Online Table S1.

Total monocyte counts, monocyte subset counts, and cardiovascular outcomes. Patients who experienced events had a mean of 508 ± 178 CD14++CD16− monocytes/μl, 47 ± 22 CD14++CD16+ monocytes/μl, and 77 ± 43 CD14+CD16++ monocytes/μl. In contrast, patients who had uneventful follow-up had a mean of 466 ± 178 CD14++CD16− monocytes/μl ($p = 0.011$ compared with patients with events), 41 ± 24 CD14++CD16+ monocytes/μl ($p = 0.001$), and 70 ± 37 CD14+CD16++ monocytes/μl ($p = 0.105$).

After stratifying the study cohort by monocyte (subset) cell counts into quartiles, higher counts of total monocytes (log-rank test, $p = 0.010$), CD14++CD16− monocytes ($p = 0.024$), and CD14++CD16+ monocytes ($p < 0.001$), but not of CD14+CD16++ monocytes ($p = 0.158$), were univariately associated with the primary endpoint of cardiovascular death, acute myocardial infarction, or nonhemorrhagic stroke in Kaplan-Meier survival analysis (Fig. 2, Online Figs. S1A to S1C).

Similarly, higher CD14++CD16+ monocyte counts significantly predicted the occurrence of any cardiovascular event, pre-defined as a secondary endpoint, in Kaplan-Meier analysis ($p = 0.028$) (Fig. 3), whereas counts of total monocytes ($p = 0.098$), CD14++CD16− monocytes ($p = 0.066$), and CD14+CD16++ monocytes ($p = 0.062$) only tended to predict adverse cardiovascular outcomes.

In multivariate regression analysis, subjects with higher cell counts of CD14++CD16+ monocytes remained at higher risk for adverse outcomes after adjustment for age and sex (model 1); further adjustment for systolic blood pressure, plasma cholesterol, smoking, diabetes mellitus, and body mass index (model 2); and additional adjustment for renal function, CRP, prevalent cardiovascular disease, and total leukocyte counts (model 3), compared with individuals with the lowest counts of CD14++CD16+ monocytes (quartile 1) (Table 3).

In contrast to CD14++CD16+ cells, neither total monocyte counts nor counts of CD14++CD16− or CD14+CD16++ monocytes predicted adverse outcomes in fully adjusted models (Table 3).

In addition, Table 4 provides the C-statistics corresponding to the Cox analyses reported in Table 3. Discrimination overall improved for covariate adjustment. Regardless of adjustment, a specific benefit in terms of prediction performance was seen for CD14++CD16+ monocytes. Thus, the C-statistic analysis supports our conclusion that only CD14++CD16+ monocyte measurements improve risk prediction over the total monocyte count. To further illustrate that CD14++CD16+ monocytes might render prognostic information for cardiovascular outcome in addition to more conventional markers of inflammation, we cross-stratified the study cohort by the median of CD14++CD16+ monocyte counts and CRP higher than

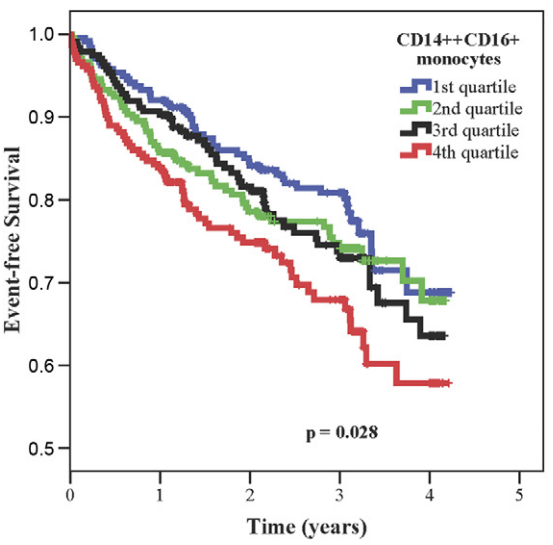

Figure 3 Relationship Between Quartiles of CD14++CD16+ Monocyte Counts and Event-Free Survival

Secondary endpoint: cardiovascular event; Kaplan-Meier analysis with log-rank test.

Table 3 Adjusted Cox Regression Analysis (Different Models) for Cardiovascular Events

Monocyte Subset	n	Events (n)	Model 1 HR	Model 1 95% CI	Model 1 p Value	Model 2 HR	Model 2 95% CI	Model 2 p Value	Model 3 HR	Model 3 95% CI	Model 3 p Value
Total monocytes											
Quartile 1	242	17	1.000			1.000			1.000		
Quartile 2	235	19	1.263	(0.656–2.432)	0.485	1.228	(0.636–2.368)	0.541	1.009	(0.515–1.976)	0.979
Quartile 3	236	24	1.528	(0.820–2.848)	0.182	1.487	(0.796–2.779)	0.213	1.229	(0.644–2.348)	0.532
Quartile 4	238	33	2.308	(1.280–4.162)	0.005	2.180	(1.195–3.977)	0.011	1.381	(0.681–2.801)	0.371
CD14++CD16− monocytes											
Quartile 1	238	18	1.000			1.000			1.000		
Quartile 2	238	21	1.186	(0.631–2.229)	0.597	1.182	(0.627–2.227)	0.605	1.051	(0.551–2.006)	0.880
Quartile 3	237	21	1.261	(0.671–2.369)	0.471	1.251	(0.665–2.352)	0.487	1.036	(0.540–1.988)	0.915
Quartile 4	238	33	2.144	(1.198–3.836)	0.010	2.039	(1.124–3.701)	0.019	1.259	(0.625–2.536)	0.520
CD14++CD16+ monocytes											
Quartile 1	238	9	1.000			1.000			1.000		
Quartile 2	238	28	3.191	(1.505–6.766)	0.002	3.121	(1.470–6.629)	0.003	3.218	(1.459–7.095)	0.004
Quartile 3	237	23	2.74	(1.267–5.926)	0.010	2.508	(1.154–5.454)	0.02	2.444	(1.074–5.563)	0.033
Quartile 4	238	33	3.899	(1.861–8.168)	<0.001	3.722	(1.770–7.829)	0.001	3.019	(1.315–6.928)	0.009
CD14+CD16++ monocytes											
Quartile 1	238	21	1.000			1.000			1.000		
Quartile 2	238	18	0.796	(0.424–1.495)	0.479	0.793	(0.421–1.494)	0.473	0.867	(0.456–1.649)	0.663
Quartile 3	237	25	1.225	(0.685–2.190)	0.494	1.193	(0.661–2.156)	0.558	1.138	(0.622–2.082)	0.675
Quartile 4	238	29	1.348	(0.767–2.372)	0.300	1.251	(0.705–2.220)	0.444	1.037	(0.571–1.883)	0.906

Model 1 includes monocyte (subset) counts, age, and sex; model 2 is further adjusted for systolic blood pressure, plasma cholesterol, diabetes, smoking, and body mass index; and model 3 is further adjusted for estimated glomerular filtration rate, C-reactive protein, prevalent cardiovascular disease, and total leukocyte count.
CI = confidence interval; HR = hazard ratio.

and lower than 2 mg/l, which corresponds to the CRP inclusion criterion of Justification for the Use of Statins in Prevention: An Intervention Trial Evaluating Rosuvastatin (14). Those subjects who had CD14++CD16+ monocyte counts higher than the median in conjunction with higher CRP levels had the worst outcomes (Online Fig. S1D) in Kaplan-Meier analysis ($p < 0.001$).

Discussion

The identification of a subset-specific involvement of monocytes in murine models of atherosclerosis was a breakthrough in cardiovascular research (15,16). Lately, specific immunomodulation of a distinct monocyte subset has been discussed as a promising therapeutic avenue in atherosclerosis (17). This notion is backed by the successful application of cell-targeted therapy in other fields of medicine, such as the introduction of rituximab in patients with lymphoma (18).

Unfortunately, advances in understanding of murine atherosclerosis are not paralleled by a better grasp of human pathology, given that the potential monocyte target subpopulation in atherosclerosis has not been convincingly identified yet. So far, most information derives from few cross-sectional studies: in a heterogenous cohort of 308 subjects, a shift toward CD16-positive monocytes was associated with the presence of coronary artery disease (19). Later, small cross-sectional studies linked high counts of CD16-positive monocytes with the presence of vulnerable atherosclerotic plaques in patients with stable angina pectoris (20) and with fibrous cap thickness in patients with unstable angina pectoris (21). Of note, none of these studies distinguished intermediate CD14++CD16+ monocytes from nonclassical CD14+CD16++ monocytes (as discussed in detail previously [22,23]). Consequently, so far, the respective roles of CD14++CD16+ and CD14+CD16++ monocytes in human atherosclerosis have not been fully discerned. Against this background, large prospective studies of monocyte subsets and cardiovascular outcomes have been demanded recently (17).

We previously set out to analyze the relationship between subset-specific monocyte counts and adverse cardiovascular outcomes in selected patient groups. Initially, we focused our analysis on patients with CKD receiving dialysis treatment (10,24,25), reasoning that this highest cardiovascular risk group would allow us to gather information in a limited sample size because of the high event rate. This allowed us to demonstrate CD14++CD16+ monocytes to be independent predictors of cardiovascular events in dialysis patients (10). However, a drawback of this approach was the observed strong shift in monocyte subsets toward CD16-

Table 4 C-Statistics Corresponding to the Cox Regression Analyses Reported in Table 3

Monocyte Subset	Model 1	Model 2	Model 3
Total monocytes	0.666	0.705	0.734
CD14++CD16− monocytes	0.661	0.701	0.732
CD14++CD16+ monocytes	0.699	0.723	0.748
CD14+CD16++ monocytes	0.666	0.698	0.731

positive cells compared with healthy controls (10), precluding a translation of these results to non-CKD populations.

Next we prospectively analyzed monocyte heterogeneity in subjects with earlier stages of CKD not requiring dialysis. At baseline, these patients had CD14++CD16+ cell counts between those of subjects with preserved renal function and those of patients with CKD receiving dialysis. Nonetheless, higher CD14++CD16+ monocyte counts again predicted cardiovascular outcomes in these patients (8).

To test the validity of these findings in a broad patient group not affected by the CKD-associated monocyte subset shift, we recruited subjects from the general population at cardiovascular risk, who were referred for elective coronary angiography. Our HOM SWEET HOMe study, the largest prospective cohort study on monocyte heterogeneity so far, thereby comprised a representative sample of subjects at cardiovascular risk with either prevalent coronary artery disease or a high burden of risk factors. At baseline, cell counts of all 3 monocyte subsets were correlated with traditional as well as nontraditional cardiovascular risk factors. Interestingly, CD14++CD16+ monocytes seem particularly to integrate cardiovascular risk burden, a notion that is supported by earlier data (26). Of most interest, CD14++CD16+ monocytes were the only independent predictors of adverse cardiovascular outcomes after full adjustment for traditional and nontraditional cardiovascular risk factors in the HOM SWEET HOMe study.

With regard to our fully adjusted Cox regression analysis, one might suspect a threshold effect of CD14++CD16+ monocyte counts, because in this model, quartiles 2 to 4 predicted adverse outcomes to a similar extent. A comparable conclusion could be also drawn from our previous study on CD14++CD16+ monocytes and survival in non-dialysis-dependent patients with CKD; here, the Kaplan-Meier curve of the second tertile clearly separated from the first tertile but ran close and parallel to the third tertile (8).

However, this concept is merely a hypothesis originating a posteriori from the present dataset. Future studies with predefined cell count cutoff values should therefore prospectively test this idea.

Circumstantial evidence suggesting a role of CD16-positive monocytes in atherosclerosis supports our present findings. Mechanistically, the significance of CD16-positive monocytes in atherosclerosis is emphasized by their proinflammatory capacity (6,27) along with their endothelial affinity (28). Their preferential adherence to activated endothelial cells (29) together with their potential to secrete interleukin-6, matrix metalloproteinase-9, and chemokine (C-C motif) ligand 2 and to attract T-lymphocytes and further monocytes (30) should be considered proatherosclerotic features.

Furthermore, the role of CD16-positive monocytes in atherosclerotic vascular disease is underscored by their association with subclinical atherosclerosis (31).

Of note, among CD16-positive monocytes, intermediate CD14++CD16+ monocytes can be viewed as particularly proatherogenic, as they selectively express C-C chemokine receptor type 5 (6,8,29), which has been associated with atherosclerosis in experimental (1) and large epidemiological (32–34) studies. Moreover, subset-specific high reactive oxygen species production and CD74 expression predispose CD14++CD16+ monocytes to propagate atherogenesis (6). These proatherogenic virtues are further extended by the proangiogenic capacity of intermediate CD14++CD16+ monocytes (6,35), linking them to potential plaque neovascularization as an important component in advanced stages of atherosclerosis (36).

Our study has several strengths that support the significance of CD14++CD16+ monocytes in cardiovascular disease.

First, the large cohort size provides firm ground for our results. Second, our monocyte analysis protocol, which has been validated (6) against a suggested reference method (37), reliably distinguishes between intermediate CD14++CD16+ and nonclassical CD14+CD16++ monocytes (6). Third, analysis of monocyte subsets was performed blinded to baseline characteristics, and endpoints were adjudicated blinded to the distribution of monocyte subpopulations.

As the major limitation, our study cannot prove causality, because of its nature as a cohort study.

However, our study provides an extensive dataset on human monocyte heterogeneity in atherosclerosis, a field in which a plethora of murine data on monocyte heterogeneity contrast with the paucity of human data. Considering the limitations of murine models (1,2,38), clinical studies have been demanded to fill this knowledge gap (17).

One might argue that our present findings are in contradiction to results from murine studies that favor a proatherosclerotic role of $Ly6C^{high}$ monocytes, conventionally regarded as counterpart of human CD14++CD16− monocytes, over $Ly6C^{low}$ monocytes, which are conventionally viewed as homologues of both human CD14++CD16+ and CD14+CD16++ monocytes (39).

However, this concept has recently been refined by Cros et al. (40), who reported that human CD14++CD16− and CD14++CD16+ monocytes both cluster together with murine $Ly6C^{high}$ monocytes, whereas CD14+CD16++ monocytes cluster together with $Ly6C^{low}$ monocytes. This finding is of great importance, because it could help reconcile murine studies with our present findings as well as with previous human studies that favored CD16-positive monocytes in cardiovascular disease.

In addition, 2 aspects deserve special consideration when interpreting results from murine studies. First, most murine studies on monocyte heterogeneity in atherosclerosis analyzed only 2 murine monocyte subsets: $Ly6C^{high}$ and $Ly6C^{low}$ cells (16). Second, many murine studies are performed in apolipoprotein $E^{-/-}$ mice on an atherogenic diet, which accordingly show massively elevated cholesterol levels

and a very profound rise of $Ly6C^{high}$ cells (up to 14-fold in individual studies [15]) without concomitant changes in $Ly6C^{low}$ monocyte counts (15). In humans, such extreme alterations are restricted to rare disease states such as familial hypercholesterolemia, in which a significant increase of CD14++CD16− monocytes has been reported (41). In contrast, wild-type mice on an atherogenic diet display significant increases neither of $Ly6C^{high}$ nor of $Ly6C^{low}$ cell counts and no atherosclerotic lesions despite persisting hypercholesterolemia (15). Accordingly, in a previous study in healthy volunteers, we did not observe associations between CD14++CD16− monocytes and cholesterol levels or subclinical atherosclerosis (31).

Taken together, we believe that our present results do not conflict with findings from earlier murine studies, if critical attention is paid to the respective experimental setting.

Ideally, studies on monocyte heterogeneity in other experimental animals, such as pigs, whose immune system is evolutionary closer to that of humans and which show a comparable monocyte subset distribution (42), will complement our existing knowledge in the future.

Conclusions

We report an association of CD14++CD16+ monocytes with cardiovascular events in subjects from the general population referred for elective coronary angiography. It remains to be proven whether the CD14++CD16+ monocyte subset represents a target cell population for new therapeutic strategies in atherosclerosis.

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Key Words: atherosclerosis ■ cardiovascular disease ■ CD14 ■ CD16 ■ monocytes.

APPENDIX

For a supplementary table and a figure and its legend, please see the online version of this article.