Elevated CD14++CD16− Monocytes Predict Cardiovascular Events

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**Background**—Although monocytes in peripheral blood are no longer considered to be a homogeneous population, associations between distinct monocyte subsets and cardiovascular disease have not been highlighted in large epidemiological studies.

**Methods and Results**—The study included 700 randomly selected subjects from the cardiovascular arm of the Malmö Diet and Cancer study. Among these, 123 subjects experienced ischemic cardiovascular events during the follow-up until December 2008. Mononuclear leukocytes frozen at the baseline investigation in 1991 to 1994 were thawed and analyzed with flow cytometry to enumerate monocyte subsets, based on CD14 and CD16 expression. The percentage and number of classical CD14++CD16− monocytes were increased in the cardiovascular-event group compared with the event-free subjects (median, 69% [interquartile range, 62% to 76%] versus 67% [59% to 72%], P=-0.017; 344 [251 to 419] cells/μL versus 297 [212 to 384] cells/μL, P=0.003). The hazard ratio was 1.66 for suffering a cardiovascular event in the highest tertile of the number of CD14++CD16− monocytes compared with the lowest tertile, even after adjustment for common risk factors (HR, 1.66; 95% CI: 1.02 to 2.72). CD14++CD16− monocytes did not, however, associate with the extent of atherosclerosis at baseline. In contrast, the percentage of monocytes expressing CD16 was negatively associated to the extent of carotid atherosclerosis measured as intima-media thickness at baseline. The chemokine receptors CCR2, CX3CR1, and CCR5 were not differentially expressed between cases and controls on any of the monocyte subsets, but CCR5 expression on CD14++CD16− monocytes was negatively associated to carotid intima-media thickness.

**Conclusions**—This study shows that classical CD14++CD16− monocytes can predict future cardiovascular risk independently of other risk factors in a randomly selected population. (Circ Cardiovasc Genet. 2012;5:122-131.)

**Key Words:** monocytes ■ leukocytes ■ cardiovascular disease ■ myocardial infarction ■ ischemic stroke

Monocytes and macrophages are considered indisputable players in the etiology of atherosclerosis. Although monocyte count is a predictor of subclinical carotid atherosclerosis and plaque formation, associations between monocyte counts and cardiovascular disease (CVD) risk, have not been highlighted in large epidemiological studies. In contrast, both white blood cell and neutrophil counts are independent risk factors for cardiovascular disease. The lack of association between monocyte count and CVD may be explained by the fact that human monocytes were, in the past, considered to be a homogeneous population. At the present time, at least 3 human monocyte populations can be defined by the expression of CD14, a part of the lipopolysaccharide receptor, and the FcγIII receptor CD16. The dominant classical monocyte species are the CD14++CD16− monocytes that constitute 65% to 85% of all monocytes. CD14++CD16− monocytes express CCR2, which is the receptor for monocyte chemo-attractant protein-1 and are believed to actively be recruited to sites of inflammation. Monocytes expressing CD16 are a heterogeneous population that can be divided into intermediate CD14++CD16− and nonclassical CD14++CD16− monocytes. The CD16-positive monocytes express higher levels of the fractalkine receptor CX3CR1 than the CD14++CD16− monocytes. The CD16-positive monocytes have been shown to produce more TNFα and less IL-10 than the CD14++CD16− monocytes and have accordingly been coined pro-inflammatory monocytes. An increase in CD14++CD16− monocytes have been reported for a number of inflammatory and infectious diseases in humans.

**Clinical Perspective on p 131**

In mice, monocytes are divided into a Ly-6ChighCX3CR1lowCCR2+ subset that is actively recruited into inflamed tissue and a Ly-6ChighCX3CR1highCCR2− subset that home into...
noninflamed tissues in a CX3CR1-dependent process.\(^6\) Notably, the expression pattern of CX3CR1 and CCR2 on mouse monocyte subsets can also be used to identify human monocyte subsets, but, although considerable additional overlap in expression profile exists, it remains to be confirmed if these subsets can be functionally translated across species.\(^6\) The Ly-6\(^{C\text{high}}\) monocytes are increased in hypercholesterolemic apolipoprotein E-deficient mice consuming a high-fat diet, and Ly-6\(^{C\text{high}}\) monocytes are recruited to atherosclerotic plaques.\(^7\) Ly-6\(^{C\text{low}}\) monocytes have been shown to enter plaques less frequently than Ly-6\(^{C\text{high}}\) monocytes but are more prone to develop into plaque-resident cells expressing CD11c.\(^2\) Recruitment of monocytes into plaques in mice has been shown to depend on expression of chemokine receptors CCR2, CCR5 (the receptor for RANTES, MIP-1\(\alpha\) and MIP-1\(\beta\)), and CX3CR1, and these receptors have been attributed nonredundant and independent roles in experimental atherosclerosis.\(^2\)–\(^4\) Interestingly, disruption of CCL2 (monocyte chemo-attractant protein-1)/CCR2 or CX3CL1 (fractalkine)/CX3CR1 interactions in mice reduces the numbers of circulating monocytes, which may, at least in part, contribute to the reduced atherosclerosis observed in mice lacking these chemokine/chemokine receptors.\(^3\),\(^4\)

Although evidence for a role of monocyte subsets in atherosclerosis is accumulating from animal studies, there are few clinical studies investigating the role of monocyte subsets in CVD in humans. Still, studies in populations enriched in prevalent CVD patients and in populations with increased CVD risk show that certain monocyte subsets can show stronger associations with CVD than others. A high CD14\(^+\)/CD16\(^-\) monocyte count, but not total monocyte or CD16-negative monocyte count, has been shown to be associated with higher rates of cardiovascular events in a prospective cohort of 94 patients on dialysis, followed for close to 3 years, and in 119 patients not on dialysis, but with chronic kidney disease, followed up to 5 years.\(^5\)–\(^7\) Monocytes expressing CD16 have also been shown to be associated with prevalence of coronary artery disease in a study population of 247 patients, with stable or unstable angina pectoris or acute coronary syndrome, and 61 healthy controls.\(^8\) In patients with stable coronary artery disease, the percentage of CD14\(^+\)/CD16\(^+\) monocytes was elevated in patients with 5 or more risk factors compared with low- to medium-risk patients.\(^8\) Here we show that monocyte subsets measured at baseline can predict incident CVD, also, in a randomly selected population cohort over a median follow-up of 15 years. We also investigate if monocyte subsets are associated with extent of atherosclerosis at baseline by correlating monocyte subsets to intima media thickness in the carotid artery measured by ultrasound.

Methods

Study Population

The study population consists of a cohort from the cardiovascular arm of the Malmö Diet and Cancer study.\(^1\) In the Malmö Diet and Cancer study, complete birth cohorts, born between 1921 and 1949, were invited from the population in the city of Malmö, Sweden. A health examination of the participants included a physical examination, a panel of laboratory tests, and a self-administered questionnaire, with items relevant for the occurrence of CVC. Between October 1991 and February 1994, every other participant was invited to take part in a substudy of the epidemiology of carotid artery disease.\(^2\) From these, a subcohort of 700 randomly selected individuals was included in this study (Figure 1). Individuals with prevalent CVD (24) were excluded from further analysis. For 17 samples, the cell number in the thawed samples was too low to be analyzed, or the flow cytometry data were disqualified, because of technical errors. The health service authority of Malmö approved and funded the screening program. All participants gave written informed consent. The study was approved by the Ethics Committee of Lund University and was conducted in accordance with the Helsinki Declaration.

Baseline Characteristics

Information on baseline characteristics was collected from the self-administered questionnaire and clinical examination. Body mass index was calculated as weight/height\(^2\) (kg/m\(^2\)) and categorized into normal weight (body mass index <25) and overweight/obese (body mass index ≥25). Smoking habits were categorized into never or former (smokers who had quit smoking at least 1 year before the examination) and current smokers. Diabetes mellitus was defined as fasting whole blood glucose >6.1 mmol/L or self-reported diabetes, according to the questionnaire. By using a mercury sphygmomanometer, blood pressure (mm Hg) was measured twice in the right arm after a 10-minute rest. The average of the 2 measurements was used. Hypertension was defined as systolic blood pressure ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg or the use of blood pressure lowering medication.

Laboratory Tests and Measurement of Carotid Intima-Media Thickness

Blood samples were drawn after overnight fasting. Fasting venous blood glucose, serum cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, C-reactive protein, and triglycerides were analyzed with standard methods at the laboratory of the Malmö University Hospital. Hypercholesterolemia was defined as total cholesterol >6.2 mmol/L (>240 mg/dL). The intima-media thickness (IMT) of the right common carotid artery was measured according to a standardized protocol by trained, certified sonographers. The carotid IMT was determined off-line as the mean wall thickness 1 cm proximal to the bifurcation, and each image was analyzed by a blinded observer. Methods of quality control have been published previously.\(^9\)

Follow-Up Period

All subjects were followed from the baseline examination until first hospitalization because of incident myocardial infarction, stroke, death, emigration, or December 31, 2008. Incident cases experiencing cardiovascular events or ischemic strokes were retrieved by data linkage to the Swedish Hospital Discharge Register, the Malmö Myocardial Register, the Stroke register of Malmö (STROMA), and Cause of Death Registry of Sweden. Ascertainment of cases and validity of the registries used have been proven to be high.\(^2\)–\(^2\) The median follow-up period was 15.2 (interquartile range [IQR] 11.3 to 16.2) years. Throughout the follow-up period, 123 incident cardiovascular disease events were identified. A cardiovascular disease event was defined as a fatal or nonfatal myocardial infarction (ICD-9: 410), ischemic stroke (ICD-9: 434), or death attributable to underlying coronary heart disease (ICD-9: 410 to 414), using the Swedish Hospital Discharge Register, the Malmö Myocardial Register, the Stroke register of Malmö (STROMA), and Cause of Death Registry of Sweden. Individuals experiencing subarachnoid and intracerebral bleedings (ICD-9: 430 or 431) were excluded from the CVD case group and included in the control group, because the etiology differs from ischemic disease.

Isolation of Mononuclear Cells

Blood samples for cell isolation were obtained in heparin tubes, put on top of a Ficoll-Paque cushion, and centrifuged at 1350 g for 12 minutes at 20°C.\(^2\) Fractions of platelet-poor plasma, mononuclear cells, and red blood cells were collected by centrifugation.
leukocytes, granulocytes, and erythrocytes were isolated. The mononuclear cells were washed with saline and RPMI medium, suspended in autologous serum, and counted. An equal volume of RPMI medium with 20% dimethyl sulfoxide was added to the mononuclear cells, and the cells were frozen slowly by placing them in a Styrofoam box at $-80^\circ$C overnight. Frozen mononuclear cells were stored at $-140^\circ$C.

Flow Cytometry
Prior to thawing, cells were transferred from $-140^\circ$C freezers to liquid nitrogen. Cells were then thawed to room temperature within 2 minutes, followed by continuous addition of 4 mL 37°C PBS (containing 1% human serum [HS]) during 1 to 2 minutes. An additional 4 mL of 37°C PBS (containing 1% HS) was added but at a faster pace (duration of approximately 30 seconds). Cells were centrifuged and resuspended in complete medium (RPMI 1640), supplemented with 10% HS (Invitrogen), 1% sodium pyruvate, 1% hepes, 1% penicillin/streptomycin, 1% L-glutamine, and 0.1% β-mercaptoethanol (Gibco) at a concentration of $2 \times 10^6$ cells/mL. Cells (4 x 10^5 per sample) were stained with anti-CD14-Pacific Blue (clone M5E2), anti-CD16-PE/Cy7 (3G8), anti-CCR2-PerCP/Cy5.5 (TG5/CCR2), anti-CCR5-Alexa Fluor 700 (HEK/1/85a), and anti-CX3CR1-FITC (2A9-1) for 30 minutes at 4°C. All antibodies were from BioLegend, except for anti-CX3CR1, which was from MBL International. Stained cells were washed in PBS with 5% BSA (wt/vol) and 2 mMol/L EDTA. Flow cytometry data were acquired on an ADP-CyAn flow cytometer (Beckman Coulter), and analysis was performed using FlowJo 7.5.5 (Treestar Inc.). CompBeads (BD) were used to correct for fluorescence spillover in multicolor analyses, and gate boundaries were set by fluorescence-minus-one (FMO) controls. Cell numbers in blood were calculated by multiplying percentages of gated monocyte populations with counts obtained from a blood cell count analysis, using a Sysmex K-1000 with data unit DA 1000 (TOA Medical Electronics Co.).

Statistics
Differences in baseline characteristics and monocyte populations between the case and control groups were evaluated with Mann-Whitney nonparametric tests, and differences in categorical data were calculated with $\chi^2$ tests. The $\chi^2$ test was used to evaluate linear trends in case-control proportions across tertiles of monocyte subsets. The Kaplan-Meier method was used to evaluate rates of cardiovascular event-free survival corresponding to tertiles of monocyte subsets. Cox proportional hazard regression models were used to compare incidence of cardiovascular events between tertiles of monocyte subsets and to calculate linear trends and risk factor-adjusted hazard ratios (95% confidence interval [CI]).
characteristics that differed between monocyte tertiles (ANOVA or χ² test for linear trend). Natural-logarithmic transformation was used on monocyte parameters, with distributions deviating from normality before analyzing bivariate Pearson correlations to carotid IMT and laboratory parameters. Laboratory parameters that also displayed significant Pearson correlations to carotid IMT were included as covariates in linear regression models evaluating multivariable adjusted correlations between monocytes and carotid IMT. P<0.05 was considered significant. Statistical analyses were performed using PASW Statistics 18 (SPSS) and GraphPad 5 software.

Figure 2. Gating strategy and phenotyping of monocyte subsets. A, Gating of monocyte subsets, based on expression of CD14 and CD16 on SSChigh MNLs, determined by flow cytometry. The dashed line in the second panel encircles CD16 NK cells (also expressing CD56 but not HLA-DR). B, Expression of phenotypic markers on classical CD14+CD16− (—, red), intermediate CD14+CD16+ (—, turquoise), and nonclassical CD14−CD16++ (—, blue) monocytes, as well as CD14dimCD16dim (—, green) cells. Gate boundaries were set by fluorescence-minus-one (FMO) controls (—, gray). C, Monocyte subsets, analyzed by flow cytometry in freshly drawn blood and in thawed mononuclear cells from the same blood donor. D, Monocyte subset counts in freshly drawn blood and in thawed mononuclear cells (mean±SEM; n=4; ns=not significant by paired t test).
Table 1. Baseline Clinical Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Case (n = 123)*</th>
<th>Control (n = 536)*</th>
<th>P†</th>
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<tbody>
<tr>
<td>Age at screening</td>
<td>65.7 ± 1.2</td>
<td>65.6 ± 1.1</td>
<td>n.s.</td>
</tr>
<tr>
<td>Male gender (% male)</td>
<td>65 (52.8%)</td>
<td>202 (37.7%)</td>
<td>0.002</td>
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<tr>
<td>BMI ≥25 (%)‡</td>
<td>77 (62.6%)</td>
<td>323 (60.4%)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Current smoker (%)</td>
<td>30 (26.3%)</td>
<td>105 (20.4%)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Diabetes (%)§</td>
<td>30 (24.4%)</td>
<td>58 (10.8%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Hypertension (%)∥</td>
<td>105 (85.4%)</td>
<td>425 (79.3%)</td>
<td>n.s.</td>
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<tr>
<td>High cholesterol (%)**</td>
<td>61 (54.5%)</td>
<td>313 (62.0%)</td>
<td>n.s.</td>
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Laboratory parameters

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<table>
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<tr>
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<tbody>
<tr>
<td>Blood pressure lowering (%)</td>
<td>41 (33.3%)</td>
<td>106 (19.8%)</td>
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<table>
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<tbody>
<tr>
<td>Fasting venous blood glucose (mmol/L)</td>
<td>5.9 ± 2.1</td>
<td>5.3 ± 1.3</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>6.4 ± 1.2</td>
<td>6.5 ± 1.2</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.6 ± 0.8</td>
<td>1.4 ± 0.8</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.3 ± 0.4</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>4.3 ± 1.2</td>
<td>4.4 ± 1.1</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>155 ± 19</td>
<td>150 ± 19</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>90 ± 9</td>
<td>88 ± 9</td>
</tr>
<tr>
<td>hsCRP (mg/L)</td>
<td>4.5 ± 7.7</td>
<td>2.8 ± 4.6</td>
</tr>
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</table>

n.s. indicates not significant data; BMI, body mass index.

*Data presented as n (% of cases/controls) for categorical data and mean ± SD for continuous measures.
†Mann-Whitney U test or χ² test for categorical data.
‡Body mass index (BMI) was calculated as weight/height² (kg/m²) and categorized as normal weight (BMI < 25) and overweight/obese (BMI ≥ 25).
§Positive questionnaire, medication or glucose ≥ 6.1 mmol/L.
‖Blood pressure ≥ 140/90 mm Hg or treatment.
**Total plasma cholesterol > 6.2 mmol/L (≥ 240 mg/dL).

Results

During the follow-up period, 39 men and 36 women experienced coronary events, and 26 men and 22 women experienced ischemic strokes, making up a total of 123 in the cardiovascular disease cases group. The median time from baseline to occurrence of an event in the case group was 9.0 (IQR, 5.3 to 12.6) years. The case group contained more male and diabetic individuals than the control group (Table 1). The number of individuals on anti-diabetic or blood pressure lowering medication was also higher in the case group, compared with the controls. The number of individuals with high cholesterol (> 6.2 mmol/L), however, was not significantly different between the case and control group. Laboratory parameters were in agreement with the clinical makeup of the case and control groups.

Three monocyte populations were identified as CD14++CD16− classical monocytes, CD14+CD16++ nonclassical monocytes, and CD14++CD16+ intermediate monocytes, in accordance with the proposed nomenclature (Figure 2A). To confirm that these were indeed discrete monocyte subsets, we analyzed the expression of several markers, including CCR2, CX3CR1, and CCR5 on these subsets (Figure 2B). CD14++CD16− monocytes expressed CCR2, and CD14+CD16++ monocytes expressed >3-fold more CX3CR1 than CD14++CD16− monocytes, as has been described previously. whereas only the CD16-positive monocyte subsets expressed CCR5. The CD16-positive monocytes also expressed more CD40 and HLA-DR than CD14++CD16− monocytes, whereas CD86 expression was similar between the subsets. None of the subsets were CD3-, CD19-, or CD56-positive (data not shown). The identity of the CD14++CD16− population is unclear, but it is not an artifact arising from freezing and thawing of cells, as we have observed this population in fresh samples. The CD14++CD16− population was not associated with events or extent of atherosclerosis. To test if the monocyte subsets are sensitive to freezing and thawing, we compared monocyte counts in freshly drawn blood to counts in thawed mononuclear cells from the same donor, frozen using the same protocol as during the baseline examination (Figure 2C). No significant difference between monocyte counts in fresh and frozen samples was observed (Figure 2D). Although cells with compromised plasma membrane integrity (7-AAD positive) were found among the isolated mononuclear cells (median, 6.6%; IQR, 5.2 to 9.9%), no significant difference in cell viability was found between fresh and frozen CD45+ mononuclear cells (data not shown). Also, no differences in cell viability were observed between monocyte subsets, indicating that the monocyte subsets were equally sensitive to freeze-thawing (data not shown).

The case group displayed significantly elevated percentages, as well as numbers of CD14++CD16− monocytes, compared with the controls (Table 2). The number of cases increased from the lowest to the highest tertile of both percentage and numbers of CD14++CD16− (χ² test P for positive linear trend = 0.029 for %CD14++CD16− and χ² test P for positive linear trend = 0.002 for the number of CD14++
CD16− monocytes; Table 3). Also, during a median of 15.2 (IQR, 11.3 to 16.2) years’ follow-up, survival curves displayed a reduced event-free survival in the highest tertile of the number of CD14+CD16− monocytes (Log rank [Mantel Cox] test \(P=0.002\); Figure 3). Cox proportional hazard regression revealed that the hazard ratio (HR) for suffering a cardiovascular event in the highest tertile of number of CD14+CD16− monocytes was 1.95 (95% CI: 1.25 to 3.04; Table 3) compared with the lowest tertile. The hazard ratio remained significant after adjustment of the model for risk factors, including age, gender, current smoking, HDL cholesterol, and presence of diabetes and hypertension (HR, 1.66; 95% CI: 1.02 to 2.72). The number of cases increased from the lowest to the highest tertile of numbers of CD16-positive monocytes (\(\chi^2\) test \(P=0.029\); Table 3), but the positive linear trend did not remain significant in the covariate-adjusted Cox regression model.

Given that only the number of classical CD14+CD16− monocytes was associated with increased risk (comparing the highest tertile to the lowest), after adjustment of covariates and that most monocytes are classical monocytes, we compared automated mixed cell (monocyte) counts between the case and the control groups. The case group displayed significantly elevated mixed cell counts compared with the controls (Table 2), and the number of cases increased from the lowest to the highest tertile of mixed cells (\(\chi^2\) test \(P=0.013\); Table 3). The positive linear trend did not, however, remain significant in the covariate-adjusted Cox regression model.

Notably, there was no correlation between CD14+CD16− monocytes and extent of atherosclerosis evaluated by ultrasound of the carotid artery at baseline (Table 4). There was,

<table>
<thead>
<tr>
<th>Table 3. CD14+CD16− Monocytes and CVD Risk</th>
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<tbody>
<tr>
<td>Monocyte Subset Tertiles§</td>
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<tr>
<td></td>
</tr>
<tr>
<td>%CD14+CD16−</td>
</tr>
<tr>
<td>No. of cases (%)</td>
</tr>
<tr>
<td>HR (95% CI)</td>
</tr>
<tr>
<td>Covariate adjusted†</td>
</tr>
<tr>
<td>No. of CD14+CD16−</td>
</tr>
<tr>
<td>No. of cases (%)</td>
</tr>
<tr>
<td>HR (95% CI)</td>
</tr>
<tr>
<td>Covariate adjusted†</td>
</tr>
<tr>
<td>No. of CD16 positive</td>
</tr>
<tr>
<td>No. of cases (%)</td>
</tr>
<tr>
<td>HR (95% CI)</td>
</tr>
<tr>
<td>Covariate adjusted†</td>
</tr>
<tr>
<td>No. of mixed cells</td>
</tr>
<tr>
<td>No. of cases (%)</td>
</tr>
<tr>
<td>HR (95% CI)</td>
</tr>
<tr>
<td>Covariate adjusted†</td>
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</table>

\(\chi^2\) test for linear trend.

§\%CD14+CD16− tertile 1 =62.9%, tertile 3 =71.0%; No. of CD14+CD16− tertile 1 =244 cells/μl whole blood, tertile 3 =361 cells/μl whole blood; No. of CD16-positive tertile 1 =41 cells/μl whole blood, tertile 3 =63 cells/μl whole blood; No. of mixed cells tertile 1 =403 cells/μl whole blood, tertile 3 =547 cells/μl whole blood.

Figure 3. Cardiovascular event-free survival in relation to number of CD14+CD16− monocytes (in tertiles) during a median of 15.2 (IQR, 11.3 to 16.2) years’ follow-up (Log rank [Mantel Cox] test \(P=0.002\)).
were there any significant linear trends in presence of plaques counts between individuals with and without plaques; nor There were no significant differences in monocyte subset
4). These associations remained significant when controlling media thickness (IMT) in the common carotid artery (Table 5). Interestingly, except for a weak negative correlation to the levels, HDL levels, and diastolic blood pressure but not to whole blood‡

Monocyte subsets displayed correlations to cholesterol levels, HDL levels, and diastolic blood pressure but not to any other laboratory parameters measured at baseline (Table 5). Interestingly, except for a weak negative correlation to the percentage of CD14⁺⁺CD16⁻ monocytes (data not shown), C-reactive protein levels did not display correlations to monocyte subsets, indicating that variations in the monocyte subsets do not merely reflect general inflammatory status.

The chemokine receptors CCR2, CX3CR1, or CCR5 have been shown to be important for the recruitment of monocytes into plaques in mice. The normalized median fluorescence intensity (MFI) of these chemokine receptors was not significantly different between cases and controls on any of the monocyte subsets (data not shown). Expression of CCR5 on CD14⁺⁻CD16⁺ monocytes, however, displayed a significant negative bivariate correlation to IMT (Pearson correlation coefficient, −0.115; P=0.003). The correlation was significant, also, when controlling for fasting plasma glucose, HDL cholestrol, LDL cholesterol, and systolic blood pressure in a linear regression model (multivariable adjusted correlation coefficient, −0.099; P=0.011).

Discussion
Here we show that elevated CD14⁺⁺CD16⁻ monocytes predict cardiovascular events. Elevated CD14⁺⁺CD16⁻ monocytes predicted CVD risk independently of gender, age, current smoking, HDL cholesterol, and presence of diabetes and hypertension. CD14⁺⁺CD16⁻ monocytes did not, however, associate with the extent of atherosclerosis at baseline. In contrast, the percentages of monocytes expressing CD16 were negatively associated to carotid IMT at baseline. This seems contradictory but might indicate that different monocyte subsets have different biological functions. CD14⁺⁺CD16⁻ monocytes might cause inflammation that weakens the fibrous cap covering plaques and thus be associated with increased risk of clinical events, whereas CD16-expressing monocytes might play a greater role in determining the size of the plaque, perhaps even having a protective, or reparative, rather than plaque-promoting function. The chemokine receptors CCR2, CX3CR1, and CCR5 were not differentially expressed between cases and controls on any of the monocyte subsets, but CCR5 expression on CD14⁺⁺CD16⁺ monocytes was negatively associated to carotid IMT. To the best of our knowledge, this is the first study to show that classical CD14⁺⁺CD16⁻ monocytes can predict future cardiovascular risk independently of other traditional risk factors in a general population.

In mice, the Ly-6C⁻ low subset can be considered to be equivalent to the CD14⁺⁺CD16⁻ monocytes in humans, mainly because both populations lack expression of CCR2. Ly-6C⁻ low subset has been termed resident in mice because of their longer half-life in vivo and their localization to both inflamed and noninflamed tissues. The Ly-6C⁻ low monocytes in mice have also been proposed to promote healing in ischemic myocardium, and transcriptional profiling of these monocytes have indicated that they initiate a differentiation program that resembles the one described for macrophages of the M2-type, which are thought to be involved in tissue repair.28,29 Interestingly, we found a negative correlation between the percentage of CD16-positive monocytes and carotid IMT at baseline that might reflect a more protective, and perhaps reparative, rather than plaque-promoting function of the CD16-positive monocytes, in line with the proposed function of Ly-6C⁻ low monocytes in mice. In support of this idea, the number of CD16-expressing monocytes has been shown to be higher in patients with stable angina pectoris compared with patients with acute myocardial infarction.30 Also, CD14⁺⁺CD16⁻ CX3CR1⁺ monocyte counts have been shown to be higher in patients with restenosis than in

### Table 4. Association Between CD16-Positive Monocytes and Carotid Inter-Media Thickness

<table>
<thead>
<tr>
<th>Carotid IMT</th>
<th>Bivariate Correlation</th>
<th>Multivariable Linear Regression</th>
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<tbody>
<tr>
<td></td>
<td>Correlation Coefficient*</td>
<td>P</td>
</tr>
<tr>
<td>%CD14⁺⁺CD16⁻</td>
<td>−0.002</td>
<td>n.s.</td>
</tr>
<tr>
<td>%CD14⁺⁺CD16⁻</td>
<td>−0.093</td>
<td>0.017</td>
</tr>
<tr>
<td>%CD14⁺⁺CD16⁻</td>
<td>−0.114</td>
<td>0.003</td>
</tr>
<tr>
<td>%CD16 positive‡</td>
<td>−0.109</td>
<td>0.005</td>
</tr>
<tr>
<td>CD14⁺⁺ cells/µl whole blood</td>
<td>0.083</td>
<td>0.033</td>
</tr>
<tr>
<td>CD14⁺⁺CD16⁻ cells/µl whole blood</td>
<td>−0.025</td>
<td>n.s.</td>
</tr>
<tr>
<td>CD16 positive cells/µl whole blood</td>
<td>−0.032</td>
<td>n.s.</td>
</tr>
<tr>
<td>CD16 positive cells/µl whole blood</td>
<td>−0.028</td>
<td>n.s.</td>
</tr>
<tr>
<td>Mixed cells/µl whole blood</td>
<td>0.103</td>
<td>0.008</td>
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</tbody>
</table>

n.s. indicates not significant.
*Pearson correlation coefficient.
†Multivariable adjusted correlation coefficient, adjusted for fasting plasma glucose, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and systolic blood pressure.
‡CD16⁻ monocytes is the sum of CD14⁺⁺CD16⁻ and CD14⁺⁺CD16⁻.

### Table 5. Pearson Correlation Coefficients Between Monocyte Subsets and Laboratory Parameters at Baseline

<table>
<thead>
<tr>
<th></th>
<th>CD14⁺⁺CD16⁻</th>
<th>CD14⁺⁺CD16⁻</th>
<th>CD14⁺⁺CD16⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>−0.062</td>
<td>−0.086*</td>
<td>−0.089*</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>−0.190†</td>
<td>−0.126†</td>
<td>−0.178†</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>0.138†</td>
<td>0.086*</td>
<td>0.128†</td>
</tr>
</tbody>
</table>

*P<0.05.
†P<0.01.
patients without restenosis 9 months after bare-metal stent implantation following acute myocardial infarction. In stroke patients, CD16-positive monocytes have been found to have beneficial effect on clinical outcome, whereas CD14\textsuperscript{++} CD16\textsuperscript{−} monocytes have been associated with poor outcome and higher mortality.\textsuperscript{32} In 1 study, however, the number of CD16-positive monocytes has been shown to correlate positively to carotid IMT in 622 healthy volunteers, but the association was not significant when adjusting for age, gender, and body mass index or Framingham risk score.\textsuperscript{33} In contrast, in our study population, which was, on average, more than 20 years older, the negative association between CD16-positive monocytes and carotid IMT remained significant, even after adjustment for traditional risk factors.

It should be noted, however, that we only found a negative association between the percentage of CD16-positive monocytes and carotid IMT, whereas we did not find any association between the number of CD16-positive monocytes and carotid IMT. Instead, an increased number of CD16-positive monocytes were associated with increased risk, even if the significant association was lost when adjusting for traditional risk factors. A high CD14\textsuperscript{++} CD16\textsuperscript{−} monocyte count has been associated with higher rates of cardiovascular events in a prospective cohort of 94 patients on dialysis, followed for close to 3 years.\textsuperscript{15} CD14\textsuperscript{++} CD16\textsuperscript{+} monocytes have also been independently associated with cardiovascular events in 119 nondialysis patients, with chronic kidney disease followed up to 5 years.\textsuperscript{16} Both dialysis patients and patients with chronic kidney disease have an elevated risk of cardiovascular disease, and traditional risk factors only account for a fraction of the excess risk of CVD in these patients, whereas nontraditional risk factors, such as the immune system, have a dominant role.\textsuperscript{34} Furthermore, CD14\textsuperscript{++} CD16\textsuperscript{+} CX3CR1\textsuperscript{+} monocyte counts have been shown to be higher in patients with stable angina pectoris, with vulnerable coronary plaques, identified by multidetector computer tomography, than in patients without vulnerable plaques.\textsuperscript{35} The number of CD14\textsuperscript{++} CD16\textsuperscript{+} CX3CR1\textsuperscript{+} monocytes have also been shown to be elevated in patients with unstable angina pectoris, with ruptured plaques, identified by optical coherence tomography, in comparison with patients with unstable angina pectoris, with nonruptured plaques and patients with stable angina pectoris.\textsuperscript{36} Our findings that the percentage of CD16-positive monocytes are negatively associated with carotid IMT, whereas CD16-positive monocyte counts might be associated with increased risk of cardiovascular events, reflect the disease-promoting and protective roles proposed in the various studies above. Clearly, more clinical studies and studies of the biological function of the CD16-positive monocytes are needed to fully understand their role in CVD.

The Ly-6\textsuperscript{low} subset in mice, thought to correspond to human CD14\textsuperscript{−} CD16\textsuperscript{+} monocytes, has been shown to express CCR5 and, interestingly, anti-CCR5 antibody treatment has been shown to inhibit Ly-6\textsuperscript{low} monocyte entry into plaques in Apoe\textsuperscript{−/−} mice.\textsuperscript{12,25} In this study, CCR5 expression on CD14\textsuperscript{+} CD16\textsuperscript{−} monocytes was negatively associated with carotid IMT at baseline. It is tempting to speculate that CD14\textsuperscript{+} CD16\textsuperscript{+} monocyte recruitment to plaques is dependent on CCR5 expression and that recruitment of CD14\textsuperscript{+} CD16\textsuperscript{+} monocytes, ascribed protective/reparative functions, reduces the extent of atherosclerosis; however, CCR5 expression is not restricted to monocytes, and there is overwhelming evidence from animal studies for an atherosclerosis-promoting role for CCR5.\textsuperscript{37} Also, in human populations, a naturally occurring deletion mutation of the CCR5 gene, which results in a truncated nonfunctional CCR5, has been associated with lower carotid IMT in the Bruneck study cohort.\textsuperscript{39} Consequently, it has been speculated that CCR5 antagonists approved for HIV therapy could also limit plaque development.\textsuperscript{39} Still, the plaque-promoting contribution of various immune cells expressing CCR5, such as T cells and monocytes, remains to be fully defined. In addition, it should be noted that we did not observe the selective expression of CCR5 on intermediate CD14\textsuperscript{+} CD16\textsuperscript{+} monocytes, which has been reported by others (Figure 2B).\textsuperscript{16,26}

In the present study, several monocyte subtypes displayed a positive correlation to diastolic blood pressure and negative correlations to HDL and cholesterol. Previous studies have reported similar correlations to blood pressure and HDL, whereas the correlation to cholesterol is unclear.\textsuperscript{2,33} One possible explanation to the blood pressure and HDL correlations is that they could reflect the general inflammatory activation associated with diabetes; however, the lack of association between monocyte subsets and C-reactive protein, as well as a lack of difference in monocyte subsets between subjects with or without diabetes (data not shown), argue against this possibility.

This study was performed on thawed cells that were stored frozen at −140°C for up to 15 years. Unfortunately, we have not been able to control how the cells have changed from fresh cells over this time period. For instance, we do not know the long-term effects of dimethyl sulfoxide, a known immunomodulator, used in the cryopreservation.\textsuperscript{40} Our own (data not shown), and studies by others, on how cells and markers on cells withstand freezing and thawing show that cryopreserved cells generally compare well to fresh cells, demonstrating that cryopreserved cells could be used in studies aimed at identifying risk markers.\textsuperscript{41,42} Still, direct incubation of freshly drawn blood, with antibodies followed by red cell lysis, is preferred as ficoll centrifugation, and, perhaps also, freeze-thawing could alter monocyte activation status.\textsuperscript{43} Our data suggest, however, that the number of monocytes is not greatly affected by freeze thawing (Figure 2C–D).

Although we show that CD14\textsuperscript{+} CD16\textsuperscript{−} monocytes are associated with a considerable increase in cardiovascular risk, more studies are needed before monocyte analysis will be used in predicting cardiovascular risk in the clinic. Even if considering monocytes as a useful risk marker is premature, clinical studies in larger and more specialized cohorts, with more phenotypic markers, might help to elucidate the biological role of monocyte subsets in the various stages of the human disease. Animal models also show great potential to elucidate the function of monocyte subsets in experimental atherosclerosis, even though care must be taken in extrapolating these results to humans because of the differences that exists in atherosclerotic disease and monocyte subsets. For these reasons, and in the light of our data presented here, more studies of monocytes in humans are crucial for future
risk stratification, the development of novel targeted therapies, and disease-monitoring, based on knowledge of human monocyte biology.

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CLINICAL PERSPECTIVE

Macrophage foam cells are the archetypical cells of the atherosclerotic lesion, and recruitment of monocytes is thought to be a prerequisite for lesion formation. Although monocyte count is a predictor of subclinical carotid atherosclerosis and plaque formation, associations between monocyte counts and cardiovascular disease risk have not been highlighted in large epidemiological studies. In the present study, mononuclear leukocytes, frozen at the baseline investigation in 1991 to 1994, from 700 randomly selected individuals, were thawed, and monocyte subsets were enumerated with flow cytometry based on their expression of CD14 and CD16. Classical CD14+/CD16− monocytes were increased in the group of individuals experiencing cardiovascular events compared with the event-free group during follow up, until 2008. Elevated classical monocytes predicted cardiovascular disease risk, independently of common risk factors. Classical monocytes did not, however, associate with the extent of carotid atherosclerosis measured as intima-media thickness at baseline. In contrast, the percentage of monocytes expressing CD16 was negatively associated to the extent of atherosclerosis. These findings might indicate that different monocyte subsets have different biological functions and thus contribute to lesion size and clinical events in different ways. Clinical studies in larger and more specialized cohorts with more phenotypic markers might help to elucidate the biological role of monocyte subsets in the various stages of human atherosclerosis and cardiovascular disease. The relation between circulating monocyte subsets and macrophage phenotypes found within the atherosclerotic lesion and the mechanisms of monocyte recruitment also needs to be clarified before we fully understand the role of monocytes in cardiovascular disease.
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