Elevated CD14\textsuperscript{++}CD16\textsuperscript{−} Monocytes Predict Cardiovascular Events

Running title: Berg et al.; CD14\textsuperscript{++}CD16\textsuperscript{−} monocytes predict CVD

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Abstract:

**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 suffered ischemic cardiovascular events during the follow-up until December 2008. Mononuclear leukocytes frozen at the baseline investigation in 1991-94 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 to the event-free subjects (median 69% (inter-quartile range 62-76)% vs. 67 (59-72)%, p=0.017; 344 (251-419) cells/$\mu$L vs. 297 (212-384) cells/$\mu$L, 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 to the lowest tertile even after adjustment for common risk factors (HR 1.66, 95%CI 1.02-2.72). 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 (IMT) 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 IMT.

**Conclusions** - This study shows that classical CD14$^{++}$CD16$^{-}$ monocytes can predict future cardiovascular risk independently of other risk factors in a randomly selected population.

**Key words:** monocytes, leukocytes, cardiovascular disease, myocardial infarction, ischemic stroke
Introduction

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. Presently, at least three 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 65-85% of all monocytes. CD14⁺CD16⁻ monocytes express CCR2, which is the receptor for monocyte chemoattractant protein-1 (MCP-1), and are believed to actively be recruited to sites of inflammation. Monocytes expressing CD16 are a heterogenous population that can be divided into intermediate CD14⁺CD16⁺ and non-classical 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 proinflammatory monocytes. An increase in CD14⁺CD16⁺ monocytes have been reported for a number of inflammatory and infectious diseases in humans.

In mice, monocytes are divided into a Ly-6C<sup>high</sup> CX3CR1<sup>low</sup> CCR2<sup>+</sup> subset that is actively recruited into inflamed tissue and a Ly-6C<sup>low</sup> CX3CR1<sup>high</sup> CCR2<sup>+</sup> subset that home into noninflamed tissues in a CX3CR1-dependent process. 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. The Ly-6C$^{\text{high}}$ monocytes are increased in hypercholesterolemic apolipoprotein E-deficient mice consuming a high-fat diet and Ly-6C$^{\text{high}}$ monocytes are recruited to atherosclerotic plaques. Ly-6C$^{\text{low}}$ monocytes have been shown to enter plaques less frequently than Ly-6C$^{\text{high}}$ monocytes, but are more prone to develop into plaque resident cells expressing CD11c. Recruitment of monocytes into plaques in mice has been shown to depend on expression of chemokine receptors CCR2, CCR5 (the receptor for RANTES, MIP-1α and MIP-1β) and CX3CR1 and these receptors have been attributed non-redundant and independent roles in experimental atherosclerosis. Interestingly, disruption of CCL2 (MCP-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.

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 other. 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 dialysis patients followed for close to three years, and in 119 non-dialysis patients with chronic kidney disease followed up to five years. 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. In patients with stable coronary artery disease, the percentage of CD14$^{++}$CD16$^{-}$ monocytes was elevated in patients with five or more risk factors compared to low-to-medium risk patients. 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 (MDC) study. In the MDC 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 cardiovascular disease. Between October 1991 and February 1994, every other participant was invited to take part in a substudy of the epidemiology of carotid artery disease. From these a subcohort of 700 randomly selected individuals was included in this study (Fig 1). Individuals with prevalent cardiovascular disease (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 was disqualified due to 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 (BMI) was calculated as weight/height² (kg/m²) and categorized into normal weight (BMI <25) and overweight/obese (BMI ≥ 25). Smoking habits were categorized into never or former (smokers who had quit smoking at least one 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 (mmHg) was measured twice in the right arm after a 10-minute rest. The average of the two measurements was used. Hypertension was defined as systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg or the use of blood pressure lowering medication.

**Laboratory tests and measurement of carotid IMT**

Blood samples were drawn after overnight fasting. Fasting venous blood glucose, serum cholesterol, LDL cholesterol, HDL cholesterol, CRP 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.21

Follow-up period

All subjects were followed from the baseline examination until first hospitalization due to incident myocardial infarction, stroke, death, emigration or December 31, 2008. Incident cases suffering 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.22,23 The median follow-up period was 15.2 (Inter-quartile range, IQR 11.3-16.2) years. Throughout the follow-up period 123 incident cardiovascular disease events were identified. A cardiovascular disease event was defined as a fatal or non-fatal myocardial infarction (ICD-9:410) or ischemic stroke (ICD-9:434) or death attributable to underlying coronary heart disease (ICD-9:410-414) using the Swedish Hospital Discharge Register, the Malmö Myocardial Register, the Stroke register of Malmö (STROMA) and Cause of Death Registry of Sweden. Individuals suffering 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 x g for 12 minutes at 20°C. Fractions of platelet-poor plasma, mononuclear 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% DMSO was added to the mononuclear cells and the cells were frozen slowly by placing them in a Styrofoam box at -80°C over night. Frozen mononuclear cells were stored at -140°C.

**Flow cytometry**

Prior to thawing, cells were transferred from -140°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 1-2 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 2×10⁵ cells/mL. Cells (4×10⁵ 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 (San Diego, CA, USA), except for anti CX3CR1 that was from MBL International (Woburn, MA, USA). Stained cells were washed in PBS with 5% BSA (w/v) and 2 mM EDTA. Flow cytometry data was 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., Japan). Median fluorescence intensities (MFI) were normalized to the fluorescence of AccuCount particles (Spherotech Inc., Lake Forest, IL, USA) included as an internal reference in each sample. Monocytes subsets were identified by their scatter properties and level of expression of CD14 and CD16 (Fig 2 A). The FSC-SSC gate was positioned to include CD14⁺CD16⁺ monocytes, but exclude CD16⁺ NK cells (expressing CD56, but not HLA-DR) (Fig 2 A and data not shown). In some analyses CD14⁺⁺CD16⁺ and CD14⁺CD16⁺⁺ monocytes were considered as a single group of CD16-positive monocytes to allow comparison to previous studies.

Statistics

Differences in baseline characteristics and monocyte populations between the case and control groups were evaluated with Mann-Whitney non-parametric tests and differences in categorical data were calculated with χ² tests. The χ² 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). The adjusted model included potential confounders among the baseline 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.

Results

During the follow-up period, 39 men and 36 women suffered coronary events and 26 men and 22 women suffered 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 5.3-12.6) 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 to 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++ non-classical monocytes and CD14++CD16⁻ intermediate monocytes in accordance with the proposed nomenclature (Fig 2 A)⁷. To confirm that these were indeed discrete monocyte subsets we analyzed the expression of several markers including CCR2, CX3CR1 and CCR5 on these subsets (Fig 2 B). CD14++CD16⁻ monocytes expressed CCR2 and CD14⁻CD16++ monocytes expressed >3 fold more CX3CR1 than CD14++CD16⁻ monocytes as has been described previously ²⁵,²⁶, 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^{dim}\text{CD16}^{dim} 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^{dim}\text{CD16}^{dim} population was not associated with events or extent of atherosclerosis. To test if the monocyte subsets are sensitive to freezing and thawing we compared monocytes 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 (Fig. 2C). No significant difference between monocyte counts in fresh and frozen samples was observed (Fig. 2D). Although cells with compromised plasma membrane integrity (7-AAD positive) were found among the isolated mononuclear cells (median 6.6% IQR 5.2-9.9%), 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^{++}\text{CD16}^{-} monocytes compared to the controls (Table 2). The number of cases increased from the lowest to the highest tertile of both percentage and numbers of CD14^{++}\text{CD16}^{-} (\chi^2 test p for positive linear trend ~0.029 for %CD14^{++}\text{CD16}^{-} and \chi^2 test p for positive linear trend ~0.002 for the number of CD14^{++}\text{CD16}^{-} monocytes; Table 3). Also, during a median of 15.2 (IQR 11.3-16.2) years follow-up, survival curves displayed a reduced event-free survival in the highest tertile of the number of CD14^{++}\text{CD16}^{-} monocytes (Log rank (Mantel Cox) test p=0.002; Fig. 3) \cite{27}. Cox proportional hazards regression revealed that the hazard ratio (HR) for suffering a cardiovascular event in the highest tertile of number of CD14^{++}\text{CD16}^{-} monocytes was 1.95 (95% Confidence interval [CI]: 1.25-3.04; Table 3) compared to 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: 1.02-2.72). The number of cases increased from the lowest to the highest tertile of numbers of CD16-positive monocytes ($\chi^2$ test p for positive linear trend $=0.029$; 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 to the controls (Table 2) and the number of cases increased from the lowest to the highest tertile of mixed cells ($\chi^2$ test p for positive linear trend $\approx 0.013$; 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, however, a significant negative correlation between the percentage, but not number, of CD14$^{++}$CD16$^+$, CD14$^+$CD16$^{++}$ and CD16-positive monocytes and the mean intima-media thickness (IMT) in the common carotid artery (Table 4). These associations remained significant when controlling for fasting plasma glucose, HDL cholesterol, LDL cholesterol and systolic blood pressure, in a linear regression model. There were no significant differences in monocyte subset counts between individuals with and without plaques, nor were there any significant linear trends in presence of plaques between tertiles of monocyte subset counts (data not shown).

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), CRP 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 cholesterol, 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\textsuperscript{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\textsuperscript{low} subset has been termed *resident* in mice because of their longer half-life *in vivo* and their localization to both inflamed and non-inflamed tissues. The Ly-6C\textsuperscript{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, that are thought to be involved in tissue repair \textsuperscript{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\textsuperscript{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 to acute myocardial infarction patients \textsuperscript{30}. Also, CD14+CD16+CX3CR1+ monocyte counts have been shown to be higher in patients with restenosis than in patients without restenosis 9 months after bare-metal stent implantation following acute myocardial infarction \textsuperscript{31}. 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 one 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 BMI 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 was 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 dialysis patients followed for close to three years\textsuperscript{15}. CD14\textsuperscript{++}CD16\textsuperscript{+} monocytes have also been independently associated with cardiovascular events in 119 non-dialysis patients with chronic kidney disease followed up to five 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 non-traditional 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 stable angina pectoris patients 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 has also been shown to be elevated in unstable angina pectoris patients with ruptured
plaques, identified by optical coherence tomography, in comparison to unstable angina pectoris patients with non-ruptured plaques and stable angina pectoris patients\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-6C\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-6C\textsuperscript{low} monocyte entry into plaques in Apoe\textsuperscript{-/-} mice\textsuperscript{12,25,26}. 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 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{38}. 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 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 on by others (Fig 2 B)\textsuperscript{16,26}. 

\[\text{Image 171x366 to 458x453}\]
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. 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 CRP, 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 for how the cells have changed from fresh cells over this time period. For instance, we do not know the long term effects of DMSO, a known immunomodulator, used in the cryopreservation. 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. 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. Our data suggests, however, that the number of monocytes is not greatly affected by freeze thawing (Fig 2 C-D).

Although we show that CD14+CD16− 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 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.


**Conflict of Interest Disclosures:** None.

**References:**


**Table 1:** Baseline clinical characteristics.

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<th></th>
<th>Case (n=123) *</th>
<th>Control (n=536) *</th>
<th>P†</th>
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<td>Age at screening</td>
<td>65.7 ± 1.2</td>
<td>65.6 ± 1.1</td>
<td>n.s.</td>
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<tr>
<td>Male gender (% Male)</td>
<td>65 (52.8%)</td>
<td>202 (37.7%)</td>
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<td>BMI ≥ 25 (%)‡</td>
<td>77 (62.6%)</td>
<td>323 (60.4%)</td>
<td>n.s.</td>
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<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>105 (20.4%)</td>
<td>n.s.</td>
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<tr>
<td>Hypertension (%)‖</td>
<td>105 (85.4%)</td>
<td>425 (79.3%)</td>
<td>n.s.</td>
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<td>High cholesterol (%)#</td>
<td>61 (54.5%)</td>
<td>313 (62.0%)</td>
<td>n.s.</td>
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<tr>
<td>Medication</td>
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<tr>
<td>Anti-diabetic (%)</td>
<td>11 (8.9%)</td>
<td>12 (2.2%)</td>
<td>P&lt;0.001</td>
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<tr>
<td>Lipid lowering (%)</td>
<td>5 (4.1%)</td>
<td>14 (2.6%)</td>
<td>n.s.</td>
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<tr>
<td>Statins (%)</td>
<td>4 (3.3%)</td>
<td>8 (1.5%)</td>
<td>n.s.</td>
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<tr>
<td>Blood pressure lowering (%)</td>
<td>41 (33.3%)</td>
<td>106 (19.8%)</td>
<td>P=0.001</td>
</tr>
<tr>
<td>Laboratory parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting venous blood glucose (mmol/L)</td>
<td>5.9 ± 2.1</td>
<td>5.3 ± 1.3</td>
<td>P=0.004</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>6.4 ± 1.2</td>
<td>6.5 ± 1.2</td>
<td>n.s.</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.6 ± 0.8</td>
<td>1.4 ± 0.8</td>
<td>n.s.</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.3 ± 0.4</td>
<td>1.4 ± 0.3</td>
<td>P=0.007</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>4.3 ± 1.2</td>
<td>4.4 ± 1.0</td>
<td>n.s.</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>155 ± 19</td>
<td>150 ± 19</td>
<td>P=0.022</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>90 ± 9</td>
<td>88 ± 9</td>
<td>n.s.</td>
</tr>
<tr>
<td>hsCRP (mg/L)</td>
<td>4.5 ± 7.7</td>
<td>2.8 ± 4.6</td>
<td>P=0.041</td>
</tr>
</tbody>
</table>

* Data presented as n (% of cases/controls) for categorical data and mean ± sd for continuous measures.
† Mann-Whitney test or χ² test for categorical data. Not significant (n.s.).
‡ 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 mmHg or treatment
# Total plasma cholesterol > 6.2 mmol/L (>240 mg/dL)
Table 2: CD14++CD16− monocytes are increased at baseline in individuals that later suffered cardiovascular events.

<table>
<thead>
<tr>
<th>Monocyte subset tertiles</th>
<th>Tertile 1</th>
<th>Tertile 2</th>
<th>Tertile 3</th>
<th>P for linear trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>%CD14++CD16−</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of cases (%)</td>
<td>34 (27.6%)</td>
<td>37 (30.1%)</td>
<td>52 (42.3%)</td>
<td>P=0.029 ‡</td>
</tr>
<tr>
<td>HR (95% CI)</td>
<td>1.0</td>
<td>1.00 (0.74-1.95)</td>
<td>1.95 (1.25-3.04)*</td>
<td>P=0.002 ‡</td>
</tr>
<tr>
<td>Covariate adjusted †</td>
<td>1.0</td>
<td>0.98 (0.57-1.67)</td>
<td>1.66 (1.02-2.72)*</td>
<td>P=0.026 ‡</td>
</tr>
<tr>
<td>No. of CD14++CD16−</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of cases (%)</td>
<td>30 (24.4%)</td>
<td>37 (30.1%)</td>
<td>56 (45.5%)</td>
<td>P=0.002 ‡</td>
</tr>
<tr>
<td>HR (95% CI)</td>
<td>1.0</td>
<td>1.64 (1.03-2.60)*</td>
<td>1.74 (1.09-2.76)*</td>
<td>P=0.022 ‡</td>
</tr>
<tr>
<td>Covariate adjusted †</td>
<td>1.0</td>
<td>1.39 (0.84-2.31)</td>
<td>1.44 (0.87-2.39) n.s.</td>
<td></td>
</tr>
<tr>
<td>No. of CD16 positive</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of cases (%)</td>
<td>29 (23.6%)</td>
<td>47 (38.2%)</td>
<td>47 (38.2%)</td>
<td>P=0.029 ‡</td>
</tr>
<tr>
<td>HR (95% CI)</td>
<td>1.0</td>
<td>1.44 (0.90-2.30)</td>
<td>1.84 (1.17-2.89)*</td>
<td>P=0.008 ‡</td>
</tr>
<tr>
<td>Covariate adjusted †</td>
<td>1.0</td>
<td>1.29 (0.77-2.16)</td>
<td>1.61 (0.98-2.66) P=0.060</td>
<td></td>
</tr>
</tbody>
</table>

* p<0.05 vs. tertile 1.
† Adjusted for age, gender, smoking status (current/occasional smokers versus current non-smokers), HDL cholesterol, and presence of diabetes and hypertension.
‡ χ² 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
Table 4: Association between CD16-positive monocytes and carotid IMT.

<table>
<thead>
<tr>
<th>Carotid IMT</th>
<th>Bivariate correlation</th>
<th>Multivariable linear regression</th>
</tr>
</thead>
<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>CD14++CD16+ 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>
</tr>
</tbody>
</table>

* Pearson correlation coefficient † Multivariable adjusted correlation coefficient adjusted for fasting plasma glucose, HDL cholesterol, LDL cholesterol and systolic blood pressure ‡ CD16-positive monocytes is the sum of CD14++CD16− and CD14+CD16++.
Not significant (n.s.)

Table 5: Pearson correlation coefficients between monocytes subsets and laboratory parameters at baseline.

<table>
<thead>
<tr>
<th></th>
<th>CD14++CD16− cells/μl whole blood</th>
<th>CD14++CD16+ cells/μl whole blood</th>
<th>CD14+CD16++ cells/μl whole blood</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 (mmHg)</td>
<td>0.138 †</td>
<td>0.086 *</td>
<td>0.128 †</td>
</tr>
</tbody>
</table>

* p<0.05 † p<0.01
**Figure Legends:**

**Figure 1:** Study design chart.

**Figure 2:** Gating strategy and phenotyping of monocyte subsets. **A.** Gating of monocyte subsets based on expression of CD14 and CD16 on SSC$_{\text{high}}$ 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 *non-classical* CD14$^+$CD16$^{++}$ (—, blue) monocytes as well as CD14$_{\text{dim}}$CD16$_{\text{dim}}$ (—, green) cells. Gate boundaries were set by fluorescence-minus-one (FMO) controls (—, grey). **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).

**Figure 3:** Cardiovascular event-free survival in relation to number of CD14$^{++}$CD16$^-$ monocytes (in tertiles) during a median of 15.2 (IQR 11.3-16.2) years follow-up (Log rank (Mantel Cox) test p =0.002).
Elevated CD14\(^{++}\)CD16\(^{-}\) Monocytes Predict Cardiovascular Events
Katarina E. Berg, Irena Ljungcrantz, Linda Andersson, Carl Bryngelsson, Bo Hedblad, Gunilla N. Fredrikson, Jan Nilsson and Harry Björkbacka

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