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# Shift of monocyte subsets along their continuum predicts cardiovascular outcomes

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ABSTRACT

*Background and aims:* Distribution of monocyte subsets has been shown to predict cardiovascular outcomes. However, monocytes form a continuum and categorization into discrete subsets may be an oversimplification. We herein aimed at establishing whether distribution of monocytes based on CD14 and CD16 fluorescence intensity provides incremental and complementary information on cardiovascular outcomes beyond enumeration of traditional subsets.
*Methods:* A cohort of 227 patients at high cardiovascular risk was characterized at baseline and followed for a median of 4 years. We quantified monocytes subsets by flow cytometry based on CD14 and CD16 expression and evaluated the continuous distribution of CD14 and CD16 fluorescence within each subset.
*Results:* A consistent shift toward higher CD16 fluorescence intensity within each monocyte subset was observed in patients with type 2 diabetes, despite no change in their frequencies. Patients with coronary artery disease (CAD) at baseline showed a doubling of $CD14^{++}CD16^{+}$ intermediate monocytes and a shift of non-classical and classical monocytes towards intermediates ones. During follow-up, cardiovascular death or cardiovascular events occurred in 26 patients, who showed monocyte skewing similar to those of patients with baseline CAD. In fully adjusted Cox proportional hazard regression models, higher CD16 expression on classical monocytes, but not the level of intermediate monocytes or other subsets, independently predicted adverse cardiovascular outcomes.
*Conclusions:* Shift of monocyte subsets along the CD14/CD16 continuum, more than their frequencies, predicted adverse cardiovascular outcomes. This finding illustrates how the concept of monocyte continuum can be used to model the cardiovascular risk.



## 1. Introduction

Chronic inflammation is a hallmark feature of atherosclerosis and cardiovascular diseases. Traditional cardiovascular risk factors are associated with various changes in the activation state of innate immunity [1]. Monocyte-macrophages are key components of the innate immune system, and are involved in several pathologic processes linked to cardiovascular diseases, such as adipose tissue expansion [2], atherosclerotic plaque growth [3], and myocardial remodelling [4]. Notably, monocytes and macrophages display considerable heterogeneity, and can exert either protective or harmful effects in the cardiovascular system [5]. This is paradigmatically represented by the Yin/Yang distinction of macrophages into pro-inflammatory pro-atherosclerotic M1 *versus* anti-inflammatory anti-atherosclerotic M2 [6]. It is now clear that the M1/M2 polarization dichotomy is an oversimplification and that the spectrum of macrophage diversity is a continuum [7]. Although monocytes generate macrophages during inflammation, in steady-state conditions, tissue resident macrophages originate from local proliferation rather than differentiation of monocytes recruited from the circulation [8]. For this reason, monocytes and macrophages are today conceived as distinct entities.

Monocytes are traditionally distinguished in 3 different subsets based on expression of the LPS co-receptor CD14 and the scavenger receptor CD16 (FcγRIII), which are important for cellular function [9]. While the majority of monocytes in basal conditions express

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high levels of CD14 and low levels of CD16 (classical $CD14^{++}CD16^{neg/dim}$ monocytes), inflammation triggers the appearance of monocytes expressing low levels of CD14 and high levels of CD16 (non-classical $CD14^{+}CD16^{++}$ monocytes), possibly through an intermediate state ($CD14^{++}CD16^{+}$) [10]. A kinetic study in humans has recently clarified that monocytes undergo sequential transition from the classical to intermediate and non-classical form in steady state and during inflammation [11].

Alterations in the frequency of monocyte subsets have been associated with vascular disease, such as unstable plaque features [12] and restenosis [13]. Remarkably, an expansion of intermediate monocytes has been associated with adverse cardiovascular outcomes [14]. Although intermediate monocytes display a distinct gene expression signature from classical and non-classical ones [15], new data on sequential transition now clearly suggest that monocytes form a continuum [12]. To model this concept, flow cytometry approaches have evolved to divide the monocyte population within the CD14/CD16 plot in a far higher number of subsets than traditionally done, confirming the existence of a continuous transition, rather than distinct subsets [16].

Our previous studies on pre-diabetes, diabetes, and hypercholesterolemia detected no change in traditional monocyte subsets defined by CD14/CD16 expression [17–19]. We herein hypothesize that identification of monocyte subsets based on discrete gating and fluorescence thresholds may be relatively insensitive to disease states. Single cell transcriptomic analyses support the idea that monocyte phenotypes represent a continuum, revealing significant heterogeneity within individual monocyte subsets [20], To account for this continuum, in addition to determining the frequency of monocyte subsets by traditional CD14 and CD16 discrete gates, in the present study, we also analysed the continuous distribution of CD14 and CD16 fluorescence intensity in the whole monocyte population and within each subset. Such analysis has been carried out in a cohort of patients with high cardiovascular risk to verify whether distribution of monocyte subsets along the CD14/CD16 continuum provides complementary information with respect to their frequencies, and is differentially associated with prevalent and incident cardiovascular disease. Intriguingly, we found that significant displacement of CD14/CD16 expression can occur even in the absence of changes in the frequency of monocyte subsets, and that distribution of CD16 expression predicts adverse cardiovascular outcomes more than the levels of intermediate monocytes.

## 2. Materials and methods

### 2.1. Patients

The study was approved by the Ethical Committee of the Province of Padova, was conducted in accordance with the Declaration of Helsinki, and all patients provided written informed consent. Patients were recruited from the Outpatient clinic of the Division of Metabolic Disease, University Hospital of Padova from March 2011 to June 2015. Inclusion criteria were: age 18–80 years, presence of at least 2 classical cardiovascular risk factors (diabetes, obesity, hypertension, smoking) or established cardiovascular disease (see below for definitions). Exclusion criteria were: acute infections or inflammatory conditions, recent trauma or surgery, autoimmunity, organ transplantation, pregnancy, lactation, or inability to provide informed consent. Pre-diabetes and type 2 diabetes were defined according to the American Diabetes Association (ADA) guidelines [21]. For all patients, we collected the following data: age, sex, body mass index, active smoking (of one or more cigarettes per day), fasting plasma glucose, quantitative lipid profile, prevalence of hypertension, coronary artery disease (CAD, defined as a history of past acute coronary syndrome and/or the presence of significant coronary stenosis at coronary angiography, either symptomatic or asymptomatic), peripheral arterial disease (PAD, defined as claudication, rest pain, or ischemic wound ulcers with evidence of leg ischemia at ultrasound examination or angiography), cerebrovascular disease (CerVD, defined as a past history of stroke or transient ischemic attack, or the presence of ≥30% stenosis of extracranial carotid arteries). Atherosclerotic CVD was defined as either CAD, PAD, CerVD, or a combination thereof. Optimal lipid profile was defined as total cholesterol <200 mg/dl, LDL cholesterol <100 mg/dl, HDL cholesterol >40 mg/dl and triglycerides <150 mg/dl. Chronic kidney disease (CKD) was defined as an CDK-EPI estimated glomerular filtration rate (eGFR) of less than 60 ml/min/1.73 $m^2$ [22]. Finally, we collected data on medications.

### 2.2. Follow-up

Patients were prospectively followed-up by routine visits, telephone contacts, and by accessing the electronic chart records and death registry. The cause of death was considered to be cardiovascular in case of: sudden death; death occurring up to 14 days after an acute myocardial infarction; death after worsening symptoms and/or signs of heart failure; death occurring up to 30 days after a stroke; death due to another documented cardiovascular cause (e.g. dysrythmia, pulmonary embolism, or intervention). Any deaths not attributed to a non-cardiovascular cause were presumed to be cardiovascular. Nonfatal myocardial infarction was defined in the presence of at least 2 of the following 3 criteria: cardiac biomarker elevation; ECG changes consistent with new ischemia; imaging evidence of new non-viable myocardium or new wall motion abnormalities. Non-fatal stroke was defined as the rapid onset of a focal/global neurological deficit (change in level of consciousness, hemiplegia, hemiparesis, numbness or sensory loss affecting one side of the body; dysphasia/aphasia; hemianopia, other new neurological sign/symptom), with a duration of ≥24 h (<24 h if the event was associated with pharmacologic treatment, or in the presence of available brain imaging showing new haemorrhage or infarct, or resulting in death), and confirmed by a neurology specialist or by brain imaging. Unstable angina was defined as resting, new onset, or worsening angina, in the absence of elevation in cardiac biomarkers, and in the presence of new or worsening ST-T changes on ECG, or evidence of ischemia by cardiac imaging, or angiographic evidence of ≥70% stenosis in an epicardial coronary artery. Hospitalization for heart failure was defined based on review of hospital discharge diagnoses and codes. Heart failure was defined in the presence of typical clinical manifestations or their worsening (dyspnea, orthopnea, paroxysmal nocturnal dyspnea, edema, pulmonary basilar crackles, jugular venous distension, third heart sound or gallop rhythm, radiologic evidence of worsening heart failure), requiring new therapy or uptitration of doses (diuretics, inotropes, vasodilators), eventually supported by changes in biomarkers (e.g. brain natriuretic peptides). Left ventricular ejection fraction was not available for all patients.

### 2.3. Flow cytometry

Identification and quantification of monocyte subtypes was performed on baseline fresh blood samples, within 3 h after collection, using polychromatic flow cytometry, as previously described [18]. Cells were stained with PE-conjugated anti-CD14 (Becton-Dickinson, clone RMO52) and FITC-conjugated anti-CD16 mAbs (Beckman-Coulter, clone 3G8). The analysis was essentially conducted according to the minimal requirement suggested by the joint consensus document of the European Society of Cardiology (ESC) Working Groups “Atherosclerosis & Vascular Biology” and “Thrombosis” [23], except that non pan-monocyte marker was