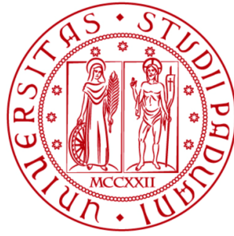

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The monocyte continuum and cardiovascular disease: Evaluation of the prognostic cardiovascular meaning of monocyte displacement along their continuum.

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INDEX

INDEX **1**

ABSTRACT **3**

RIASSUNTO **5**

ABBREVIATIONS **7**

INTRODUCTION **9**

1. INFLAMMATION AND ATHEROSCLEROSIS **9**

1.1 The atherosclerotic process 10

1.1.1 Fatty streak formation 10

1.1.2 Low-density lipoproteins 12

1.1.3 Endothelial disfunction 13

1.2 Innate and adaptive immunity in ATH 14

1.3 Conventional risk factors for atherosclerotic disease 16

1.3.1 Non modifiable risk factors 17

1.3.2 Modifiable risk factors 17

1.4 Clinical results of atherosclerosis 19

2. MONONUCLEAR PHAGOCITIC SYSTEM **20**

2.1 Monocytes 21

2.1.1 Monocytes subsets 22

2.1.2 Functional characteristics of monocyte subpopulations 25

Classical monocytes 25

Intermediate monocytes 26

Non-classical monocytes 27

2.1.3 The monocyte population: discrete entities or a functional continuum? 29

2.2 Macrophages 31

3. ROLE OF MONOCYTES AND MACROPHAGES IN ATHEROSCLEROSIS AND CARDIOVASCULAR DISEASES 33

3.1 Monocyte subsets, risk factors and cardiovascular diseases 34

3.2 Prognostic value of alterations in monocyte subsets in cardiovascular disease 36

AIM OF THE STUDY 38

MATERIALS AND METHODS 39

1. Patients' characteristics 39

2. Follow up and definition of MACE 40

3. Flow cytometry 42

3.1 Flow cytometry protocol 44

4. Statistical analysis 45

5. "Clock Rule" Analysis 46

RESULTS 47

1. Patients' characteristics 47

2. Identifycation of monocyte subtypes at baseline 48

3. Monocyte subsets and traditional cardiovascular risk factors 50

4. Monocyte subsets and prevalent cardiovascular disease 54

5. Monocytes subsets and cardiovascular outcomes 56

6. Monocyte subpopulations and mace prediction 59

7. Analysis according to the "Clock Rule" 61

DISCUSSION AND CONCLUSIONS 63

REFERENCES 68

ABSTRACT

Introduction. Monocytes are cells of the innate immunity system with high heterogeneity and plasticity and are involved in acute and chronic inflammatory states. Monocytes are traditionally distinguished in three subsets, based on CD14 (LPS co-receptor) and CD16 (FcγIII receptor with low IgG affinity) expression: classical, intermediate and non-classical. Monocyte subsets have a developmental relationship and differ in phenotypic and functional characteristics. Distribution of monocyte subsets has been shown to predict cardiovascular outcomes. Nevertheless, monocytes have now been redefined as a continuum of subsets with dynamic changes of their characteristics and classification into different subtypes may be an oversimplification. Monocytes have been studied in cardiovascular diseases because they are involved in inflammatory processes linked with these pathological states: they have a central role in the development of atherosclerotic plaques, that represent the major cause of cardiovascular events. Changes within different monocyte subsets are reported in several studies in relation with cardiovascular risk factors and cardiovascular diseases.

Aim of the study. The aim of this study is to establish whether distribution of monocytes based on CD14 and CD16 fluorescence intensity provides incremental and complementary information in relation to cardiovascular risk factors, prevalent cardiovascular diseases and cardiovascular outcomes beyond enumeration of traditional subsets.

Materials and methods. A cohort of 227 patients with high cardiovascular risk (patients with at least two classical cardiovascular risk factors or with establish cardiovascular disease) were recruited for this study and followed up for a median of 4 years. Monocyte subsets were quantified and characterized at baseline using polychromatic flow cytometry, based on the CD14 e CD16 expression; for each monocyte subset frequency and mean fluorescence intensity (MFI) of CD14 and CD16 were determined, evaluating the continuous distribution. These monocyte characteristics were studied in patients in relation to cardiovascular risk factors, prevalence of coronary artery disease (CAD) and occurrence of major adverse cardiovascular events (MACE) during follow-up.

Results. In relation to cardiovascular risk factors, every monocyte subset of patients with type 2 diabetes showed a consistent shift toward higher CD16 fluorescence intensity, despite no changes in their frequencies. Patients with coronary artery disease (CAD) at baseline displayed a doubled amount 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 displacement similar to those of patients with CAD at baseline. Using a Cox proportional hazard regression models, among monocytes

parameters, only the higher CD16 expression on classical monocytes, independently predicted adverse cardiovascular outcomes, but not the level of intermediate monocytes or other subsets.

Discussion and conclusion. Changes within monocyte subsets in patients with CAD and in patients with incident MACE during follow-up suggested a shift of classical and non-classical monocytes towards intermediate monocytes, showing phenotypic changes within the monocyte continuum. The predictive role of CD16 MFI on classical monocytes highlights how the concept of monocyte continuum can be used to shape the cardiovascular risk more than frequencies of monocyte subsets can do.

RIASSUNTO

Introduzione. I monociti sono cellule del sistema dell'immunità innata con elevata eterogeneità e plasticità e sono coinvolti in stati infiammatori acuti e cronici. I monociti sono tradizionalmente distinti in tre sottopopolazioni, in base all'espressione del CD14 (co-recettore dell'LPS) e CD16 (recettore FcγIII con bassa affinità per IgG): classici, intermedi e non classici. Questi sottogruppi monocitari hanno una relazione evolutiva e differiscono per caratteristiche fenotipiche e funzionali. La distribuzione dei sottoinsiemi monocitari ha dimostrato di prevedere gli esiti cardiovascolari. Tuttavia, i monociti sono recentemente stati ridefiniti come un continuum di sottoinsiemi con cambiamenti dinamici delle loro caratteristiche e la categorizzazione in sottoinsiemi discreti può essere considerata come un'eccessiva semplificazione. Nelle malattie cardiovascolari i monociti sono stati studiati in quanto coinvolti in processi infiammatori legati a questi stati patologici: hanno un ruolo centrale nello sviluppo delle placche aterosclerotiche, che rappresentano la principale causa per gli eventi cardiovascolari. Diversi studi hanno dimostrato cambiamenti all'interno dei sottoinsiemi monocitari in relazione ai tradizionali fattori di rischio cardiovascolare e alle patologie cardiovascolari.

Scopo dello studio. Lo scopo di questo studio è stabilire se la distribuzione dei monociti basata sull'intensità di fluorescenza del CD14 e del CD16 fornisce informazioni incrementali e complementari in relazione ai fattori di rischio cardiovascolare, alle patologie cardiovascolari prevalenti e agli esiti cardiovascolari rispetto alla quantificazione della frequenza dei sottogruppi tradizionali. L'obiettivo dello studio è anche quello di verificare se questi cambiamenti predicono esiti cardiovascolari.

Materiali e metodi. 227 pazienti ad alto rischio cardiovascolare (pazienti con almeno due classici fattori di rischio cardiovascolare o con malattia cardiovascolare stabilita) sono stati reclutati per questo studio e seguiti per una mediana di 4 anni. Le sottopopolazioni monocitarie sono state quantificate e caratterizzate al basale utilizzando la citometria a flusso policromatica, in base all'espressione di CD14 e CD16; per ciascun sottogruppo sono stati determinati la frequenza e l' intensità media di fluorescenza (MFI) di CD14 e CD16, valutando la loro distribuzione lungo il continuum monocitario. Queste caratteristiche dei monociti sono state studiate nei pazienti correlandole ai fattori di rischio cardiovascolare, alla prevalenza di malattia coronarica (CAD) e alla comparsa di eventi avversi cardiovascolari maggiori (MACE) durante il follow-up.

Risultati. In relazione ai fattori di rischio cardiovascolare, nei pazienti con diabete di tipo 2 è stato osservato un aumento consistente dell' intensità di fluorescenza del CD16 all'interno di ciascun gruppo di monociti, nonostante non si sia rilevato nessun cambiamento nelle loro frequenze. I

pazienti con malattia coronarica (CAD) al basale hanno mostrato un raddoppio nella frequenza dei monociti intermedi $CD14^{++}$ $CD16^{+}$ e uno spostamento di monociti classici e non classici verso quelli intermedi. Durante il follow-up, la morte cardiovascolare o eventi cardiovascolari si sono verificati in 26 pazienti, che hanno mostrato uno spostamento dei monociti simile a quelli dei pazienti con CAD al basale. Utilizzando il modello di Cox di regressione di rischio proporzionale, tra i parametri dei monociti, solo l'espressione del CD16, più elevata sui monociti classici, ma non il livello di monociti intermedi o di altri sottogruppi, predice indipendentemente gli eventi cardiovascolari avversi.

Discussione e conclusione. I cambiamenti nei sottogruppi monocitari in pazienti con CAD e in pazienti evoluti in MACE durante il follow-up hanno suggerito uno "shift" dei monociti classici e non classici verso gli intermedi, mostrando cambiamenti fenotipici all'interno del continuum monocitario. Il ruolo predittivo dell'MFI del CD16 sui monociti classici evidenzia come il concetto di continuum monocitario possa essere utilizzato per modellare il rischio cardiovascolare più della frequenza delle diverse sottopopolazioni monocitarie.

ABBREVIATIONS

ADA	American Diabetes Association
AGEs	Advanced Glycation Products
ATH	Atherosclerosis
BMI	Body Mass Index
CAD	Coronary Artery Disease
CCR2	CC-receptor-2
CD	Cluster of Differentiation
CerVD	Cerebrovascular Disease
CKD	Chronic Kidney Disease
CVD	Cardiovascular Disease
CX3CR1	fractalkine chemokine receptor
EMPs	Erythro-Myeloid Progenitors
FITC	Fluorescein Isothiocyanate
FMO	Fluorescence Minus One
FSC	Forward Scatter
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HDL	High Density Lipoprotein
HSC	Hematopoietic Stem Cell
ICAM-1	Intracellular Adhesion Molecule-1
INF- γ	Interferon-γ
LDL	Low Density Lipoproteins
LPS	lipopolysaccharides
mAb	monoclonal Antibody
MACE	Major Adverse Cardiovascular Events
MCP-1	Monocyte Chemotactic Protein-1
MCSF	Macrophage Stimulating Growth Factor
MFI	Mean Fluorescence Intensity
MHC	Major Hstocompatibility Complex
MP	Myeloid Precursor
NO	Nitric Oxide

NOS	Nitric Oxide Syntase
PAD	Peripheral Artery Disease
PE	Phycoerythrin
ROS	Reactive Oxygen Species
SD	Standard Deviation
SMC	Smooth Muscle Cells
SR	Scavenger Receptors
TIA	Transient Ischemic Attack
TNF-α	Tumor Necrosis Factor-α
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
VCAM-1	Vascular Cell Adhesion Molecule-1

INTRODUCTION

1. INFLAMMATION AND ATHEROSCLEROSIS

Atherosclerosis (ATH) is an inflammatory disease of the large arteries; it is a chronic process that causes thickening of the large and medium calibre arteries and a loss of elasticity of the arterial walls, which appear with the progress of age and can reduce or prevent blood flow. Clinically, ATH may be asymptomatic or manifest, with ischemic events on cerebral arteries, myocardial infarction and other effects such as renal failure, hypertension and aneurysms.[1] It tends to manifest where the blood flow is not laminar, at bifurcations or curvature of the vessels. The traditional risk factors for atherosclerosis that stimulate the initial lesion have been known for a long time such as hyperlipidemia, hypertension, smoking, diabetes mellitus, infections and genetic abnormalities.[2] It is now clear that atherosclerosis pathogenesis and complications are caused by inflammatory, thrombotic, metabolic and other mechanisms.[3]
The critical endpoints of atherosclerosis are cardiovascular diseases (CVD) which are the main cause of death in developed countries.[4] CVD include a lot of adverse events and vascular diseases, including coronary artery disease (CAD), stroke, infarction, peripheral arterial disease (PAD), which account for most cardiovascular morbidity and mortality.[5]

In 1970, the Ross's theory "Response to injury" was the first to question the "lipid theory" according to which atherosclerotic plaque was caused by an accumulation of cholesterol inside the vessel.[6] But already in 1958, Poole and Florey claimed that following the deposition of cholesterol, the monocytes adhered to the endothelium and migrated through the endothelium rabbit aorta.[7] Several years later, Gimbrone proposed for the first time the concept of endothelial dysfunction highlighting the main role of healthy endothelium safekeeping against atherosclerosis: alteration in its normal functionality was at the base of atherosclerotic disease.[8] In 1986 Ross revised his "Response to injury" theory claiming that an endothelial damage is the first major event leading to ATH[9] and in 1999 published an important review in which he supports the idea that ATH is a chronic inflammatory disease in which cells of the immune system play a role of significant importance.[2] Afterwards, several works sustain this hypothesis, considering that the adhesion of monocytes and lymphocytes to the activated endothelium represents the first important phase of atherosclerotic pathology.

1.1 The atherosclerotic process

At the beginning of the atherosclerotic process, there are two prominent pathological mechanisms: lipid accumulation in arteries and inflammation. Through these processes arteries lose their normal function, the lumen is constricted and an inadequate blood flow may provoke CVD including coronary artery disease and stroke.[10] The events of ATH have been greatly clarified by studies in animal models.[1]

1.1.1 Fatty streak formation

The wall of normal arteries consists of three layers where the inner layer, or *intima,* consists of the endothelium and the basal lamina that borders the *media*, the middle layer is build up by smooth muscle cells and connective tissue, collagen and elastin. The outer layer, the *adventitia*, is composed of elastin fibers; it contains small blood vessels, the *vasa vasorum*, lymph vessels and nerves.[11]

The first visible manifestation of ATH involves the formation of a lesion at *intima* level, defined by the name of "fatty streak", which begins with activation of the endothelium, oxidation of low-density lipoproteins (LDL), recall of macrophages at the inflammation site that incorporate the oxidized low density lipoproteins (oxLDLs) and subsequently they turn into "foam cells".[12,13]

At this stage, fatty streaks are constituted only by foam cells and lipids while T-cells and extracellular lipids are still present in reduced number. A classification of the American Heart Association divides atherosclerotic lesions into six types starting by isolated foam cells (called "lipid point"), through the fatty streak stage, atheroma, fibroatheroma, up to the most complicated lesion [Fig. 1].[10]

Nomenclature and main histology	Sequences in progression	Main growth mechanism	Earliest onset	Clinical correlation
Type I (initial) lesion Isolated macrophage foam cells	I	Growth mainly by lipid accumulation	From first decade	Clinically silent
Type II (fatty streak) lesion Mainly intracellular lipid accumulation	II			
Type III (intermediate) lesion Type II changes and small extracellular lipid pools	III		From third decade	
Type IV (atheroma) lesion Type II changes and core of extracellular lipid	IV			Clinically silent or overt
Type V (fibroatheroma) lesion Lipid core and fibrotic layer, or multiple lipid cores and fibrotic layers, or mainly calcific, or mainly fibrotic	V	Accelerated smooth muscle and collagen increase	From fourth decade	
Type VI (complicated) lesion Surface defect, hematoma-hemorrhage, thrombus	VI	Thrombosis, hematoma		

Fig. 1. American Heart Association classification of human atherosclerotic lesions from the fatty streak (type I) to the more complicated type VI lesion. The diagram also includes growth mechanisms and clinical correlations.[14]

Not all fatty streaks are clinically relevant but they are precursors of more advanced lesions characterized by a necrotic core rich in lipids, debris and smooth muscle cells (SMCs). Calcification, ulceration at the luminal surface, and small vessels bleeding can increase the complexity of the plaques.[1] The continuous intima's thickening leads to the formation of the mature lesion, defined atherosclerotic plaque or atheroma, that is characterized by a great inflammatory condition and is rich in lipids, dead cells, immune cells (especially macrophages and T lymphocytes), endothelial vascular cells, SMCs and extracellular matrix.[15]

Atheroma have a more complex structure than the fatty streak, invading often the artery's lumen, consists of a central part, called core, which contains foam cells, lipids, dead cells and cell debris, surrounded by a cap of smooth muscle cells and collagen.[15] Other cell types present at plaque level and described in literature are dendritic cells,[16] Mast cell,[17] a small number of B lymphocytes, natural killer T lymphocytes. The interface between core and fibrous cap is instead rich in T lymphocytes and macrophages [Fig.2].[15]

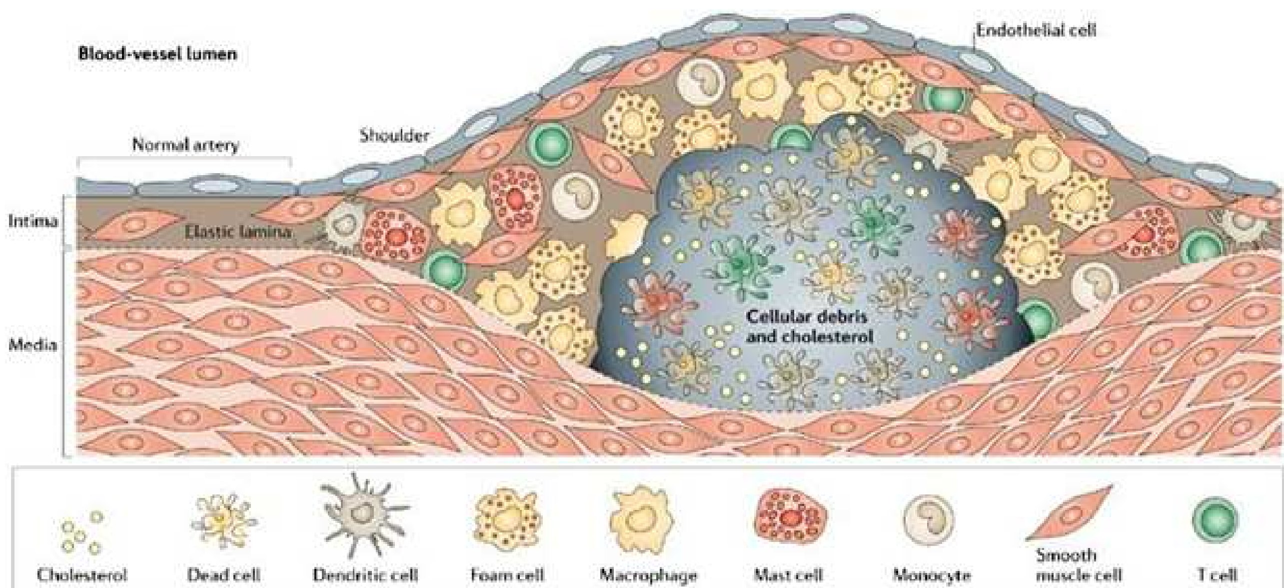

Fig. 2. Cell composition of the atherosclerotic plaque. The atherosclerotic plaque has a core containing lipids and debris originated from dead cells. It also contains smooth muscle cells and collagen fibers, which have the task of stabilizing the plaque itself, immune cells, macrophages, T cells and mast cell.[15]

Most of the immune cells located at plaque level are activated and produce pro-inflammatory cytokines such as interferon-γ (INF- γ) or tumor necrosis factor-α (TNF-α).[12]
Plaque can develop to a more complex form: extracellular matrix secretes proteases and cells infiltrated in the plaque secretes cytokines, aggregation of cholesterol and formation of crystals leads to the formation of a "fibrous cap" that defines the plaque and prevents contact between the blood circulation and the prothrombotic material. Finally, the fibrous cap can break, causing the release of prothrombotic material in the bloodstream; this represents the most severe and damaging clinical event that can cause occlusion of arteries at the rupture site. At heart level, atherosclerosis can lead to myocardial infarction and cardiac arrest, while at cerebral arteries level it can cause ischemia or stroke. If the ATH also affects other branches of arteries, it can lead to renal dysfunction, hypertension and aortic aneurism.[18]

1.1.2 Low-density lipoproteins

Oxidation of low density lipoproteins (oxLDLs) plays a fundamental role in the intima's chronic inflammatory reaction; the increased vascular permeability trapped LDL in the subendothelial space's extracellular matrix.[19] Reactive oxygen species (ROS) induce changes that triggers the oxidation of lipids.[20] These oxLDLs are no longer recognized by LDL-receptors (LDL-R), but they bind "scavenger receptors" (SR): SR-A, CD36 and CD68. They introduce active macromolecules in the cells that engulf them and cause intracellular accumulation of cholesterol esters, responsible for the transformation in foam cells.
Interaction with the corresponding LDL-R and SR (and the consequent generation of intracellular messengers, in particular ROS) and the introduction into the cell of oxidized products are the biochemical basis of the pathogenic action of LDL. The OxLDL activate transcription factors (NFk-B) in the cells (endothelial cells, macrophages, smooth muscle cells), which induce the expression of genes coding for cytokines, adhesion molecules, growth factors and rise the inflammatory response [Fig.3].[21]

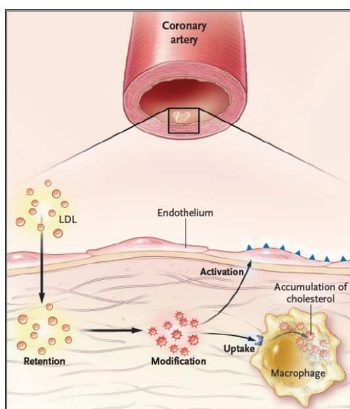

Fig. 3. Effects of activation and infiltration of LDL in inflamed arteries. An excess of LDL infiltrates arteries in hypercholesterolemic patients. Oxidative and enzymatic modifications lead to the release of inflammatory cytokines that induce endothelial cells to introduce active macromolecules in the cells that engulf them and cause intracellular accumulation of cholesterol esters, responsible for the transformation in foam cells.

1.1.3 Endothelial disfunction

Over the years, animal models have been widely used in order to better understand and clarify the mechanisms of initiation of ATH. Hansson and Libby in 2006[15] and later Weber[22] allowed to clarify the different phases of the inflammatory process during the formation of the atherosclerotic plaque. In particular, it is possible to highlight several sequential phases in the formation of lesions [Fig. 4]:

- activation of the endothelium and increased expression of adhesion molecules, as vascular cell adhesion molecules-1 (VCAM-1) and intracellular adhesion molecules-1 (ICAM-1);
- adhesion of monocytes to the endothelium and infiltration of other immune system's cells in the intima;
- accumulation and recall of additional cells by chemokines, for example the monocyte chemotactic factor (MCP-1);
- transformation of monocytes into foam cells in response to cytokine production, such as macrophage stimulating growth factor (MCSF);
- alteration of the stability of the plaque caused by infiltrated cells' production of cytokines and enzymes, modification of the fibrous cap, which, can lead to plaque rupture and thrombosis, resulting in stroke or myocardial infarction.[15]

Over the last 15 years, it has been established that endothelium is not a simple coating of cells on the inner wall of the arteries. Endothelial cells secrete a large variety of active molecules[3]. Healthy endothelium represents an important selective barrier for free passage of molecules and cells through the gap junctions; it is an endocrine and dynamic organ, which not only mediates endothelium-dependent vasodilation, but also actively inhibits leukocytes adhesion and their migration to the intima, as well as adhesion and platelet aggregation, proliferation of vascular smooth muscle cells and their migration. Inhibits coagulation, fibrinolysis and actively promotes and participates in immune and inflammatory reactions.[23]

A great variety of stimuli, such as oxLDL, presence of free radicals caused by smoking, hypertension, diabetes, genetic alterations, high concentration of plasma homocysteine and infections, can cause endothelial dysfunction or activation. All these conditions lead to alterations in endothelial homeostasis and influence endothelium permeability, vasoconstriction, coagulation and triggers inflammatory and immunological reactions. Endothelial dysfunction has been shown to be one of the first signs in ATH, even in absence of angiographic evidence of disease. In the past, the

reduced nitric oxide (NO) activity was one of the most significant markers also in detecting endothelial dysfunction.[24]

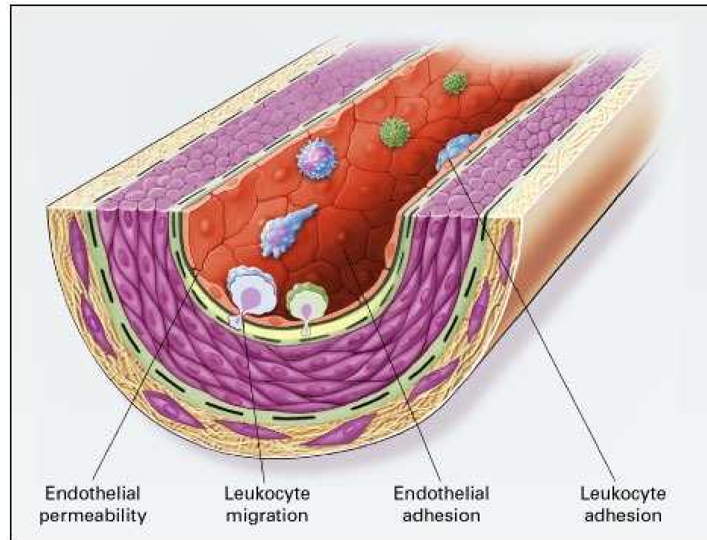

Fig. 4. Endothelial dysfunction: increased endothelial permeability provokes an upregulation of endothelial adhesion molecules: leukocytes adhere to the endothelial cells and migrate into the artery wall.

1.2 Innate and adaptive immunity in ATH

As previously described, inflammation and immune response are key components of the atherosclerosis pathophysiology.

ATH is characterized by the accumulation and modification of lipids in the vascular wall followed by infiltration of inflammatory cells. The cells involved in the initial phases and in the progression of atherosclerotic plaque, are both from the innate and the acquired immunity.[25]

Elements involved in the innate immunity are monocytes, dendritic cells, mast cells and platelets and they all have a role in the pathogenesis of atherosclerosis.[26]

The first step of the inflammatory process in atherosclerosis is the activation of monocytes from the arterial lumen to intima. Monocytes adhere to activated endothelial cells by leukocyte adhesion molecules.[27] There are a lot of subtypes of adhesion molecules, for example VCAM-1, ICAM-1 and selectines [Fig. 5].[15]

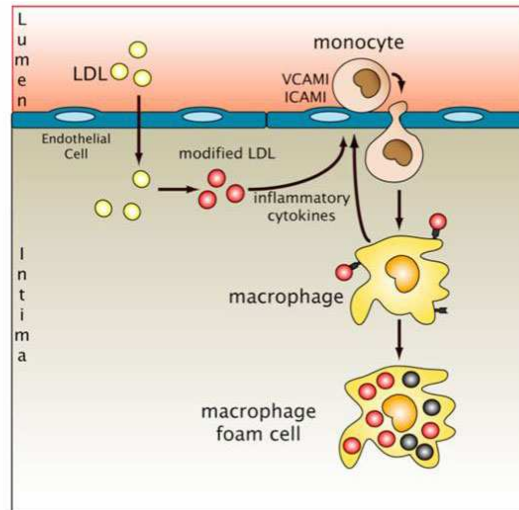

Fig. 5. Recruitment, activation, and development of monocyte and macrophages in response to modified LDL in atherosclerotic lesions.[28]

It is partly unclear which mediators stimulate the expression of these adhesion molecules. Monocytes are also recruited to the intima by specialized cytokines and chemokines, which attract attached monocytes to migrate into the intima. The most studied is the monocyte chemoattractant protein 1 (MCP-1) and its receptor CC-receptor-2 (CCR2). In experimental animal models it is proven that deficiency of MCP-1 and CCR2 reduce atherosclerosis.[29]

In the intima, monocytes are activated and become macrophages with an increase of scavenger receptors and toll-like receptors (TLR).[30] These receptors recognize a large amount of molecules including particles of ox-LDL, bacterial endotoxins, and fragments of apoptotic cells. These fragments are recognized and then phagocyte, especially LDL particles. If macrophages internalize enough lipid they become lipid rich foam cells.

All these cells through the production of cytokines, ROS, proteinases, lipid mediators, factors of growth, promote the proliferation of smooth muscle cells, the deposition of the matrix extracellular, vessel remodeling, angiogenesis and further inflammation.[25]

Among the cells of the acquired immunity, we find the Th1 lymphocytes which, by the production of cytokines such as INF-γ and TNF-α, promote the plaque development, exacerbating endothelial activation; and Th2 lymphocytes that produce anti-inflammatory cytokines such as IL-4 and IL-10 with an anti-inflammatory action and reduction of endothelial activation and foam cell formation.[31,32]

Finally, there are Treg lymphocytes with positive action on the plaque stabilization by production of anti-inflammatory cytokines and B cells.

However, endothelial activation and the continuous recall of immune cells, both from innate or acquired immunity, leads to the progression of the lesion, to the migration of smooth muscle cells from the vascular medial layer and circulating progenitor cells contribute to the formation of the fibrous cap and to the stabilization of the plaque.

Over the last 10 years, the role of T and B lymphocytes has always been recognized even if they are not necessary for atherogenesis they are able to modulate the progression of this disease, despite their relatively low number in the plaque.[32]

In recent years, attention has focused on a specific subpopulation of T lymphocytes $CD4^+$ $CD25^+$ defined Tregs which contribute to the suppression of the immune response addressed either against the self that against the non-self.[33] A reduction in the number of Tregs was observed in various autoimmune diseases;[34] a study conducted on patients with coronary artery disease, suggested how an alteration of their function could represent a symptom of plaque instability.

While the role of monocytes, macrophages, T and B lymphocytes, and platelets is well recognized in the context of the ATH, only recently emergent studies have provided evidence that poly-morphonuclear cells (PMNs) have been overlooked in the pathogenesis of cardiovascular diseases. Migration and adhesion of PMNs to endothelial cells are critical events during inflammation.[35] PMNs, platelets and monocytes adhere to the activated endothelial cells and interact each other through aggregates, resulting in a greater adhesion of leukocytes to the endothelium.[26] PMN, monocytes and endothelial cells create ROS in response to activation.[32] ROSs have been implicated in the promotion of inflammation and proliferation of smooth muscle cells leading to increased development of atherosclerotic lesions.[36] ROSs are responsible for the oxidation of LDL, contributing to the development of atherosclerosis.[37]

1.3 Conventional risk factors for atherosclerotic disease

A risk factor is something increasing the chance of developing cardiovascular disease. There is a vast amount of evidence that links “conventional” risk factors to atherosclerotic vascular disease, but only half of the cases of clinical atherosclerotic disease can be explained by them.[38] However, certain conditions, traits or habits increases the chance of atherosclerosis development. Most risk factors including high cholesterol and LDL, low level of high density lipoprotein (HDL) in the blood, hypertension, smoking, diabetes mellitus, obesity, inactive lifestyle, age can be controlled and atherosclerosis can be delayed or prevented.

1.3.1 Non modifiable risk factors

In the pathogenesis of atherosclerotic disease, non-modifiable risk factors can be identified, such as genetic predisposition, age and gender.

Even in the absence of atherosclerotic pathology, aging produce a thickening of the vessel wall affecting the intima and the medium tunic with consequent increase in the vessel stiffness. With regard to the formation of atherosclerotic plaques, the phenomenon of cellular senescence in endothelial cells typical of advancing age leads to increase the expression of pro-inflammatory molecules and decrease eNOS, contributing to the endothelial dysfunction and the passage of monocytes into the vessel wall. In addition there is a greater production of chemokines by smooth muscle cells that promotes the migration of inflammatory cells.[39] Regarding sex, estrogens have a protective role against inflammation and atherosclerosis at endothelial level and in post-menopausal women there is an increased cardiovascular risk.[40]

1.3.2 Modifiable risk factors

Smoking is with no doubt recognized as one of the most important life style risk factors correlated to atherosclerotic lesions.

Smoking causes damage and endothelial activation by inactivating NO through increased ROS production; increases the oxidation of pro-atherogenic lipids and interferes with the lipid profile leading to a decrease in HDL lipoproteins. It also induces a shift towards greater coagulability at the endothelial level: due to the alteration of platelet function and leads to an imbalance between anti and pro-coagulant factors. Smoking also leads to a systemic inflammatory response with an increase in leukocyte counts and an increase in PCR.[41]

In contrast to smoking, hypertension is a silent cardiovascular risk factor.

Hypertension acts as a risk factor through increased hemodynamic stress. The mechanism which can accelerate atherosclerosis is still unknown but in animal models, with high fat diet, is proven that hypertension accumulate fatty substances in the arterial walls. Perturbations of blood flow and hemodynamic forces contribute to endothelial dysfunction, as evidenced by the fact that atherosclerotic plaques are preferentially located in the bifurcations of vessel where blood flow is more turbulent.[42] Increased production of adhesion molecules, altered production of extracellular matrix, haemostatic dysregulation are the results of these stimuli on endothelial cells. Furthermore, to support the role of hypertension, it has been shown that angiotensin II (a vasoconstrictive hormone) can cause endothelial dysfunction stimulating the production of superoxide anion by

endothelial cells and vascular smooth muscle cells, and the expression of adhesion molecules (such as VCAM-1) on endothelial cells.[42] Hypertension is related to an increased risk of myocardial infarction.

Plasma cholesterol levels and particularly the accumulation of LDL represent the key events of the initial phases of atherosclerotic plaque development. In conditions of dyslipidemia there's an increase of the plasma LDL that leads to a greater passage through the endothelial cells and their localization and retention at intima's level.[13]

The reduction of plasma cholesterol levels has proven effective in reducing the incidence of cardiovascular events in diabetic patients.[43]

The hyperglycemia associated with type 1 (T1D) and type 2 diabetes (T2D) is another factor contributing to endothelial dysfunction. In type 2 diabetes, insulin resistance and dyslipidemia also contribute to endothelial dysfunction: hyperglycemia leads to advanced glycation end-products (AGEs) formation by endothelial cells (both intra and extracellular); these products interact with their receptors present on endothelial cells (RAGE), triggering the production of adhesion molecules, pro-inflammatory cytokines and a haemostatic imbalance with greater production of pro-coagulant factors and reduction of NO production.[44]

Diabetes also induces an inflammatory state through nuclear activation and translocation of transcription factors such as NF-kB and "activator protein 1" (AP-1). These factors determine the expression of genes responsible for the production of chemokines, pro-inflammatory cytokines and leukocyte adhesion molecules proving that diabetic patients have, on average, more severe atherosclerosis than not diabetic.[45]

Obesity predisposes to insulin resistance and diabetes and also causes a condition of dyslipidemia, all risk factors of atherosclerosis.[27]

Metabolic syndrome, defined as the presence of at least three factors including obesity, insulin resistance, reduced glucose tolerance, triglyceride increase, reduction of HDL lipoproteins and hypertension, is a set of risk factors for the development of atherosclerotic plaques.[46]

1.4 Clinical results of atherosclerosis

The atherosclerotic plaque instability can cause chronic or acute complications in the vessel and lead to cardiovascular disease.

Stable atherosclerotic plaques, characterized by a relevant fibrous component, increase during time and determine arterial stenosis with reduction of the blood flow. This should cause stable angina at coronary level or peripheral artery disease at lower limbs level, usually manifests with claudication.

However, the plaques complicate frequently with their rupture and the formation of thrombi, leading to the vessel occlusion. This situation occurs when the plaque is unstable and the inflammation state in the microenvironment damage the fibrous cap leading to its break, in addition to the erosion of the endothelial layer. Unstable plaques contain generally a high concentration of cholesterol; the rupture is preceded by the thinning of the fibrous, also promoted by a condition of hemodynamic stress.[47] This fracture exposes the blood in contact with the lipid core, tissue factor, collagen and other elements that induce the formation of a thrombus.[48] This situation predisposes to acute myocardial infarction (AMI) and ischemic stroke. At coronary level the atherosclerotic disease, plaque rupture and subsequent thrombus formation is the basic event in most cases of acute coronary syndrome, unstable angina, myocardial infarction and sudden death.[49]

The progression of stenosis caused by atherosclerotic plaque can cause unstable angina.[50] Plaque rupture can result in a geometric alteration which increases the degree of stenosis, regardless of the possible formation of the thrombus. Unstable angina can also be caused by transient thrombosis due to more limited plaque ruptures. The formation of the thrombus can also involve vasoconstriction, which contributes to the symptomatology, following serotonin and prostanoids released from the platelets.

In myocardial infarction, however, plaque rupture is more relevant and leads to the formation of a stable thrombus that prevents blood flow. Sudden death is characterized by a fatal electrical instability resulting from ischemia.[50]

Atherosclerotic plaque can in general also be complicated by the formation of an aneurysm: this depends on the destruction of the extracellular matrix as a result of increased activity of enzymes such as collagenases and elastase, which make the wall less resistant. This is also associated with a genetic predisposition and a situation of increased hemodynamic stress such as hypertension.[49]

2. MONONUCLEAR PHAGOCITIC SYSTEM

Monocytes and macrophages are essential components of innate immunity; they play a pivotal role at tissue level both in the maintenance of steady state homeostasis and during inflammation, providing the starting process and extinguishing the immune response.[51]

The mononuclear phagocytic system has historically been categorized by Ellie Metchnikoff into monocytes, macrophages and dendritic cells that exhibit their phagocytic ability during immune responses.[52] All these cells are characterized by high heterogeneity and originate from a unique hematopoietic stem cell (HSC) from which derives a progenitor for monocytes and macrophages and a progenitor for dendritic cells [Fig. 6].[53,54] According to a more remote view of the dynamics of the monocyte-macrophage system, monocytes egress from the bone marrow, circulate in the blood for few days and then migrate into the tissues, where they turn into macrophages: this occurs in steady state conditions and especially during inflammation.[55]

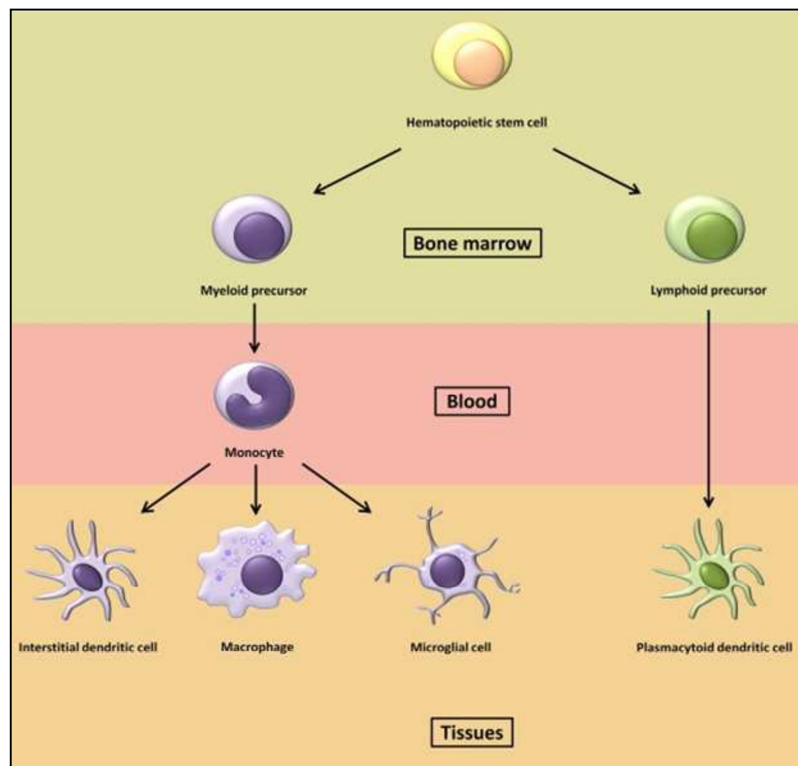

Fig. 6. The monocyte-macrophage lineage. The hematopoietic stem cell is a unique multipotent progenitor cell, located in the bone marrow, which may differentiate either into a myeloid or a lymphoid precursor. This can divide either into the myeloid (blue) or plasmocytoid (green) lineage. The myeloid precursor has the possibility to migrate into the bloodstream and to develop into a monocyte. Monocytes migration to specific tissues and their differentiation takes place upon a stimulation of different cytokines, interleukins and other factors. Based on the localisation, monocytes become either interstitial dendritic cells, macrophages or microglial cells. Lymphoid precursor runs parallel with the myeloid one, but can directly differentiate into another type of dendritic cell, the plasmocytoid dendritic cell.[56]

This vision of the monocyte-macrophage system has changed over the years; in steady state condition, a macrophage component exists in tissues resulting from local tissue proliferation, independently from circulating monocytes; this cellular component derives from macrophages localized in tissues during embryogenesis that are maintained by self-renewal.[57–60]

The fact that macrophages can have different origins (blood versus tissue monocyte) and exhibit differing phenotypes in some settings provided evidence of heterogeneity among phagocytic cells, but there is no lineage divergence. Recent data utilizing specific fate mapping technologies has now provided evidence for an embryonic origin of some tissue macrophages.[61] These macrophages derive from two cell precursors during embryonic development: the erythro-myeloid progenitors (EMPs) of the yolk sac, which give rise to microglial cells; the EMPs progenitors resident in the fetal liver, which give rise to the residual macrophages of many other tissues.[62] The contribution of circulating monocytes in steady state is actually limited to few tissues, for example intestine and dermis, with a rapid cell turnover that require the continuous migration of circulating monocytes to constitute the tissue macrophage population.[63]

Tissue monocytes can differentiate into macrophages or dendritic cells:[64] it has been shown that monocytes recruited at site of inflammation can also differentiate into inflammatory dendritic cells;[65] in particular conditions monocytes can also differentiate into splenic dendritic cells, Langherhans cells and cutaneous dendritic cells.[66] Overall, cells of the phagocytic-mononuclear system are interrelated and all these cells also share various markers. In humans, monocytes were initially defined on the basis of morphology and cytochemistry and later by polychromatic flow cytometry, based on light scatter properties and on cell-surface markers.[67]

2.1 Monocytes

Human monocytes represent the 3% to 8% of peripheral blood leukocytes. They represent an important role in innate immunity, inflammation, and tissue remodeling. This system is not antigen-specific but it depends on pattern recognition receptors for conserved components of various pathogens, including their membrane, for example, lipopolysaccharides (LPS).[30] They are characterized by high heterogeneity and plasticity, differing by phenotype, function and transcriptional profile:[68] they phagocyte pathogens but they also promote the process of pathogen neutralization and elimination, producing cytokines, antigen processing and presentation.[69] Monocytes have a remarkable developmental plasticity and following their migration to tissues they can lose their characteristics and differentiate into various types of macrophages, but also give rise to cells of other lineages at appropriate culture conditions. Monocytes isolated from different

anatomical sites show various phenotypes and functions in accordance with local tissues demand.[70]

2.1.1 Monocytes subsets

The various and distinct roles attributed to monocytes in homeostasis, inflammation and repair lead to the concept of monocyte heterogeneity and the hypothesis that monocytes may commit to specific functions in the bloodstream,[71] proposing the identification of different phenotypic subsets in humans and mice.[72,73]

In the past, blood monocytes were analyzed using microscopy of stained slides or their light scatter properties in haematology analysers,[55] but to analyze different monocyte subsets these approaches are no longer suitable. Flow cytometry has become the standard method for the analysis of monocyte subsets because it provides the capacity of simultaneous analysis of several surface markers combined with rapidity and observer independent collection of data from a large number of cells.

Following the discovery of several monocyte subsets the CD14, part of the LPS co-receptor and the CD16, the FcγIII receptor with low IgG affinity, have emerged as standard markers for definition of monocyte subpopulations.[73]

The current nomenclature of monocytes, defined by Ziegler-Heitbrock and colleagues in 2010,[74] distinguish three different subpopulations identified on the basis of the cell surface expression levels of these two markers (CD14 and CD16), detected with the use of monoclonal antibodies conjugated with fluorochromes by polychromatic flow cytometry [Tab. I].

Older definition		2010 Nomenclature definition		Suggested numerical assignment
CD14^{+}CD16^{-} CD14^{++}CD16^{-} CD14highCD16^{-}	Classical	CD14^{++}CD16^{-}	Classical	Mon1
CD14^{+}CD16^{+} (can be related to both or either CD16^{+} subset) CD14lowCD16^{+} CD14^{-}CD16^{+}	Nonclassical	CD14^{++}CD16^{+} CD14^{++}CD16^{+}CCR2^{+} CD14^{++}CD16^{+}SLAN^{+}	Intermediate	Mon2
		CD14^{+}CD16^{++}	Nonclassical	Mon3

Tab. I. Definition of monocytes subsets in humans.[74]

They are distinguished as follows:

- Classical monocytes ($CD14^{++}$ $CD16^{-}$) (also called Mon1), representing up the 85% of circulating monocytes.
- Intermediate monocytes ($CD14^{++}$ $CD16^{+}$) (also called Mon2), 0-5% of circulating monocytes.
- Non-classical monocytes ($CD14^{+}$ $CD16^{++}$) (also called Mon3), 10% of circulating monocytes [Fig. 7].[68,75,76]

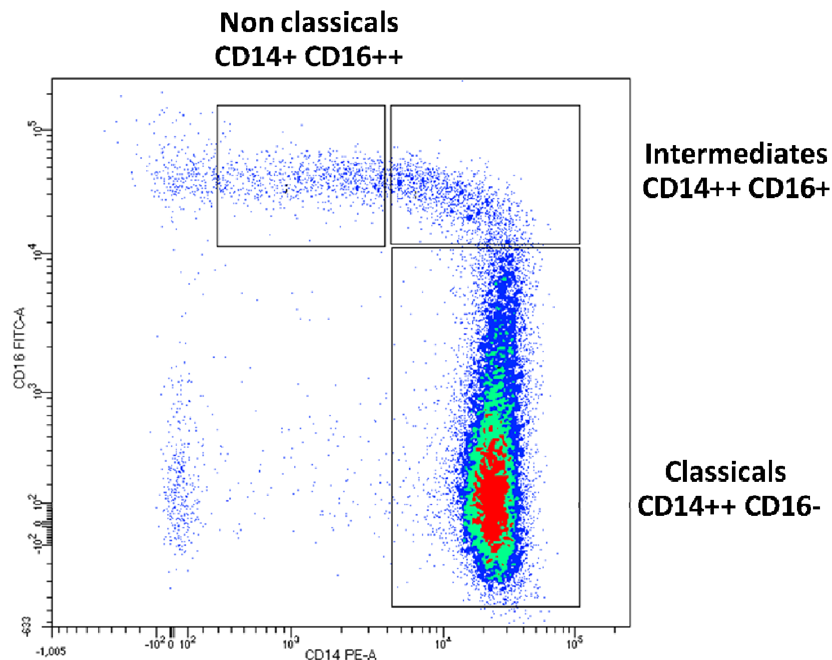

Fig. 7. Plot CD16 versus CD14. Distinction of monocyte subsets (classical, intermediate and non-classical, based on the CD16 and CD14 intensity).

The subdivision of monocytes based on the expression of CD14 and CD16 through the use of flow cytometry requires first of all a morphological distinction of monocytes from other white blood cells using their dimension, physical and morphological characteristics (FSC/SSC plot).[77]

Immune cells are usually characterized by the presence of cell surface marker, but a more sophisticated classification approach relies on gene expression profiling. Using mass cytometry by time-of-flight, Thomas and colleagues identified CCR2 (the receptor for MCP-1), CD36, HLA-DR, CD11c or LRP-1 as additional cell surface markers that provide better resolution of intermediate and non-classical monocyte subsets and also classical monocytes are identified with higher precision than using only CD14 and CD16.[76,78,79] The additional use of a pan-monocytic marker in the quantification of the subtypes of circulating monocytes was recognized in the most recent consensus statements and was not available at the date of the analysis performed in the present study [Fig. 8].

Moreover, the use of CD45 allows the exclusion of potentially overlapping $CD45^{neg}$ platelets, dead cells and debris.[80]

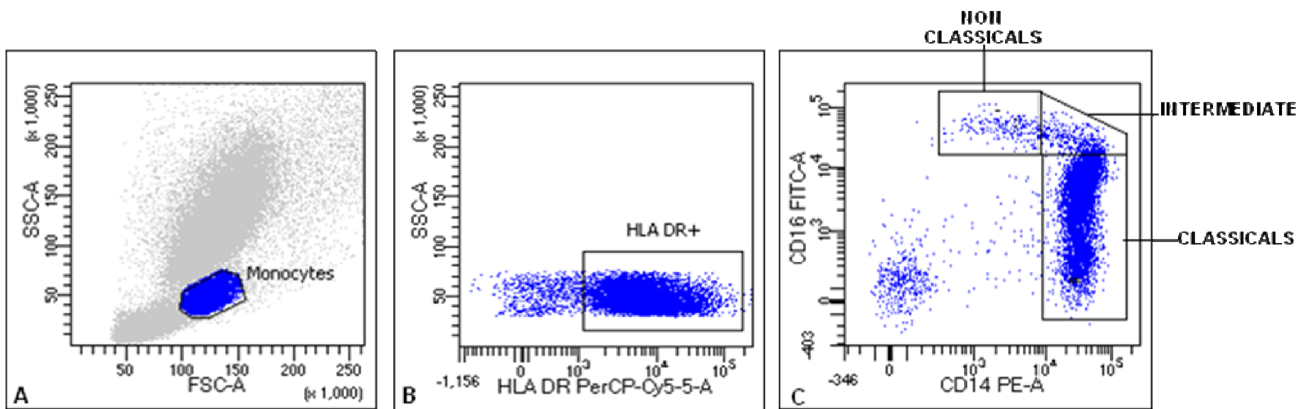

Fig. 8. Gating strategy for monocytes analysis using a pan-monocytic marker: A) Distinction of monocytes based on physical and morphological characteristics in the FSC-A versus SSC-A plot. B) Use of a pan-monocytic marker, distinction of monocytes HLA-DR+. C) Distinction of monocyte subsets based on the surface expression of CD14 and CD16.

However, most studies about monocyte subsets in atherogenesis have been performed using mice, the monocyte surface markers can't be compared to humans and other species, so it is difficult to extrapolate comparable data.

Parallel researches in mouse models have defined mouse subsets by the differential expression of CD62L (L-Selectin), CCR2 (CC-Receptor-2), fractalkine chemokine receptor (CX3CR1) and Ly6C (part of the epitope of the GR1) and distinguished two mouse monocyte subsets: $CD62L^{+}$ $CCR2^{high}$ $CX3CR1^{low}$ Ly-$6C^{high}$ monocytes (called Ly-$6C^{high}$ monocytes) and $CD62L^{-}CCR2^{low}$ $CX3CR1^{high}$ Ly-$6C^{low}$ (called Ly-$6C^{low}$ monocytes).[81,82]

The same differentiation in classical, intermediate and non-classical monocytes can also be applied to mice using the CD43 as a further marker and the three subpopulations are then identified:

- classical monocytes: Ly-$C6^{high}$ $CD43^{+}$ $CX3CR1^{neg}$;
- intermediates: Ly-$C6^{high}$ $CD43^{++}$ $CX3CR1^{pos}$;
- non-classicals: Ly-$C6^{low}$ $CD43^{++}$ $CX3CR1^{high}$.[83]

Various studies have investigated the homology between human and mouse regarding the monocyte subpopulations: gene expression seems to reveal that the classical and intermediate human monocytes would look more similar to the Ly-$6C^{high}$ murine monocyte population, and there would be homology between the human non-classical population and the murine Ly-$6C^{low}$ subtype.[84]

2.1.2 Functional characteristics of monocyte subpopulations

Several studies have been performed at molecular level to better characterize the three subpopulations. The identification of a peculiar gene expression profile within the individual subpopulations highlights their different functional capacities and phenotypic characteristics.[85]

Monocyte subsets function has been more extensively studied in mouse models than in humans. In mice Ly6C^{high}, inflammatory monocytes, circulate in the blood and following infection migrate into tissues, where they differentiate into macrophages or DCs, producing inflammatory cytokines and ROS, stimulating effector T-cell proliferation and mediating tissue repair. These cells also contribute to a population of monocyte-derived suppressor cells that inhibit T-cell function in cancer and autoimmune models. Ly6C^{low} monocytes adopt a patrolling phenotype, that allows them to monitor tissue damage.[86–88]

In humans classical monocytes normally release pro-inflammatory cytokines like IL-6, are phagocytic and mediate a wide range of responses to pathogens.[77,89,90] In contrast, the intermediate monocytes seem to release anti-inflammatory cytokines such as IL-10 and pro-inflammatory cytokines such as TNF-α and IL-1β.[68,77,91,92] Finally, the non-classical subset seems to be involved in tissue repair [Tab.II].[93]

Classical monocytes

Represent approximately the 85% of total monocytes in the circulation, typically defined as CD14^{pos}, CD16^{neg}, CCR2^{high}, CX3CR1^{low}, CD62L^{high} in human and Ly6C^{pos}, CCR2^{high}, CX3CR1^{low}, CD62L^{high} in mouse. Classical monocytes are commonly viewed as pro-inflammatory and highly phagocytic.[92] They are characterized by a greater expression of genes involved in cell adhesion and migration, genes encoding pathogen recognition receptors and genes involved in phagocytosis.[76] In steady state conditions they supply the organs resident macrophage population with a rapid cell turnover such as the intestine[63] and the skin[94] and during inflammation they are recalled in the place of damage and infection by bacterial products (LPS) and cytokines or chemokines produced by the damaged tissue.[59]

Monocytes CD16^{neg} express high level of CCR2 and are more sensitive to CCL2 (also called MCP-1), chemokine that recalls monocytes in the sites of inflammation. Stimulating with LPS, classical monocytes express various chemokines and cytokines, such as IL-6, IL-8, CCL2, CCL3.[77,95]

Intermediate monocytes

Intermediate monocytes are cells of more recent discovery. The term "intermediate" is due to the CD16 expression. It seems to be a developmental relationship between these cells (from classical through intermediate to non-classical) during an infection or with macrophage colony stimulating factor (M-CSF) treatment, there is an increasing in the intermediate cells followed by an increase of the non-classical monocytes. Accumulating evidence demonstrates differences between intermediate and non-classical population in term of phenotype, function, gene expression and responses to disease. Intermediates are bigger, more granular and have higher surface expression of receptors involved in inflammation and immunity than non-classical: CD14, Toll like receptor-4, CCR2, CCR5, HLA-DR, and they are defined as $CD14^{pos}$, $CD16^{pos}$, $CCR2^{dim}$, $CX3CR1^{dim}$, $CD62L^{dim}$.

Functional studies demonstrated that intermediates have a higher antigen presenting capacity, a higher phagocytic activity, a higher production of TNF-α, IL-6 and IL-1β in response to LPS and a higher rate of aggregation with platelets.[76,96] Zawada and colleagues identified 258 genes up-regulated in intermediate monocytes and 301 genes in non-classical. Intermediate monocaytes have a significantly higher expression of genes involved in the defense against pathogens (CD14) and MHC II–restricted antigen processing and presentation (HLA-DRA, CD74). In contrast, the non-classical subset express higher levels of genes connected to MHC I–restricted processes (HLA-B, B2M), trans-endothelial mobility (LSP1) and cell-cycle progression (CDKN1C, STK10).[68,97]

A large number of studies have examined blood monocyte subset phenotype and proportions in inflammatory diseases such as Crohn's disease,[98] sepsis,[99] chronic kidney disease,[100] obesity[101] and diabetes mellitus and have revealed an expansion of the intermediate monocyte subset. In other studies, in vitro stimulation of intermediate monocytes with LPS resulted in a strong pro-inflammatory cytokine profile. In a study of Cros and colleagues, intermediate monocytes produced the highest amounts of TNF-α and IL-1β, while additionally producing IL-6 and CCL3 at high levels similar to classical monocytes.[92] In another study in vitro, intermediates and classical produce similar high levels of TNF-α while classical monocytes produce highest levels of IL-1β, and non-classical monocytes produced little of either cytokine.[102]

However, the pro-inflammatory profile of intermediates has not been consistently observed. For example, a study of Wong reported that intermediate monocytes were the lowest producers of TNF-α, and IL-1β (highest production by non-classical monocytes).[68]

Cros discovered a hierarchical cluster of gene expression profiles of monocyte subsets and indicated that intermediates are more closely associated with classical rather than non-classical, suggesting

that they may derived directly from classical monocytes.[92] However, other two studies which also use a gene profiling approach with hierarchical clustering reported the opposite, and interpreted their results as indicating that intermediate and non-classical subsets are more closely related.[68,97] A great number of the studies quote that they represent a highly phagocytic cell type associated with pro-inflammatory cytokines production.

Non-classical monocytes

Defined as $CD14^{dim}$, $CD16^{high}$, $CCR2^{low}$, $CX3CR1^{high}$, $CD62L^{low}$ are characterized by a "patrolling" endothelial function,[98] both in homeostatic and inflammatory conditions, as demonstrated by the wide expression of genes involved in the cytoskeletal rearrangement necessary for cellular movement.[68] This "patrolling" function, due to the expression of the chemokine receptor CX3CR1, determines the retention of monocytes at the endothelial surface level and allows them to quickly arrive to the inflammation site:[86] non-classical monocytes express high level of CX3CR1 and are more sensitive to CX3CL1 (fractalkine, chemokine linked to the membrane of activated endothelial cells).[96] They have poor expression of genes involved in phagocytosis,[98] a poor response to the stimulation with LPS, but a high expression of pro-inflammatory cytokines such as TNF-α and IL-6 after activation in response to viruses and immune complexes.[92]

All these considerations may conclude that these last two subsets should not be analyzed as a single population of $CD16^{pos}$ monocytes, as was intended a few years ago. There is still not enough understanding about the primary biological roles of these last two minor subsets of monocytes in contrast to classical monocytes, but the differences in their dynamics and function have been well documented. These dynamic changes of their characteristics suggest that their categorisation into discrete subsets may be an oversimplification and we support the idea that the whole monocyte population should now be redefined as a continuum of subsets.

Monocyte subset Definition markers	CLASSICAL $CD14^{++}CD16^{-}$	INTERMEDIATE $CD14^{++}CD16^{+}$	NON CLASSICAL $CD14^{+}$ $CD16^{++}$	References
Proportion of overall monocytes	85%	5%	10%	[72,96]
Corresponding mouse subsets	Ly-$C6^{high}$ $CD43^{+}$	Ly-$C6^{high}$ $CD43^{++}$	Ly-$C6^{low}$ $CD43^{++}$	[83]
Size and granularity	++	+++	+	[96]
Functions	Rapid recruitment to sites of inflammation High phagocytosis and subsequent production of ROS Contribute to macrophage populations in the intestine and skin in steady state Tissue specific surveillance in steady state for antigen with subsequent draining to lymph nodes with minimal differentiation	Highly phagocytic and associated production of inflammatory cytokines Lipid scavenging with decreased cholesterol efflux. High basal level of ROS	Low phagocytic activity Anti-inflammatory activity Healing Patrol endothelium	[48,86,92,96]
Gene profiles	Pro-inflammatory mediators Wound healing Plastic response to stimuli	MHCII antigen processing and presentation Pro-angiogenic	Cytoskeletal mobility Complement components Phagocytosis Oxidative pathway components	[76]

Tab. II. Monocyte subsets properties.

2.1.3 The monocyte population: discrete entities or a functional continuum?

Studies performed in order to characterize every monocyte subpopulations have demonstrated diversity but also overlap in gene expression among subtypes (especially between intermediate and non-classical monocytes).[97] The gene profile of the monocyte subpopulations seems to detect a progressive increased expression of genes associated with maturation from classical monocytes to intermediate and non-classical monocytes.[68,97,103]

Further studies, trying to understand the kinetic and correlation between different monocyte subpopulations, have shown a developmental relationship between them both in steady state condition and during inflammation, delineating these subpopulations as a continuum of differential stages.

Recently, by in vivo labelling of human monocytes with deuterium, Patel and colleagues showed that monocytogenesis proceeds through the subsequent evolution of classical monocytes into intermediate and non-classical ones. After being released into the blood flow, classical monocytes leave the circulation by death or migration, while a minority of them differentiate into intermediate monocytes within 3 days. Intermediate monocytes, instead, circulate for 3 more days before transiting to non-classical monocytes. Finally, non-classical monocytes have the longest lifespan (7 days), and they ultimately leave the bloodstream.[104]

The same kinetic was observed and demonstrated in mice.[105] Spleen represents a reservoir of monocytes outside the bone marrow, which mirror the circulating monocytes that can be mobilized when is necessary.[51,106]

Regarding the expression of CD14 and CD16, markers considered for the subdivision into monocyte subpopulations, there is a progressive decrease of the CD14 expression and an increase in the expression of the CD16, starting from the classical monocytes and passing through the intermediate and non-classical state.[68]

Many other markers characterize the monocyte surface. Some markers have shown their gradual increase/decrease depending on the developmental relationship between the subpopulations,[68] for example it was observed that the intermediate monocytes express some surface markers at an intermediate level and intensity between classical and non-classical.[85] Other markers have instead shown a greater expression in the intermediate monocytes than the other subpopulations conferring to these cells certain phenotypic and functional characteristics.[103,107]

In line with this consideration, Hijdra suggested that the monocyte population can be hypothetically divided into a greater number of sub-populations than the traditional division, representing the monocyte subpopulations as a continuum [Fig. 9].

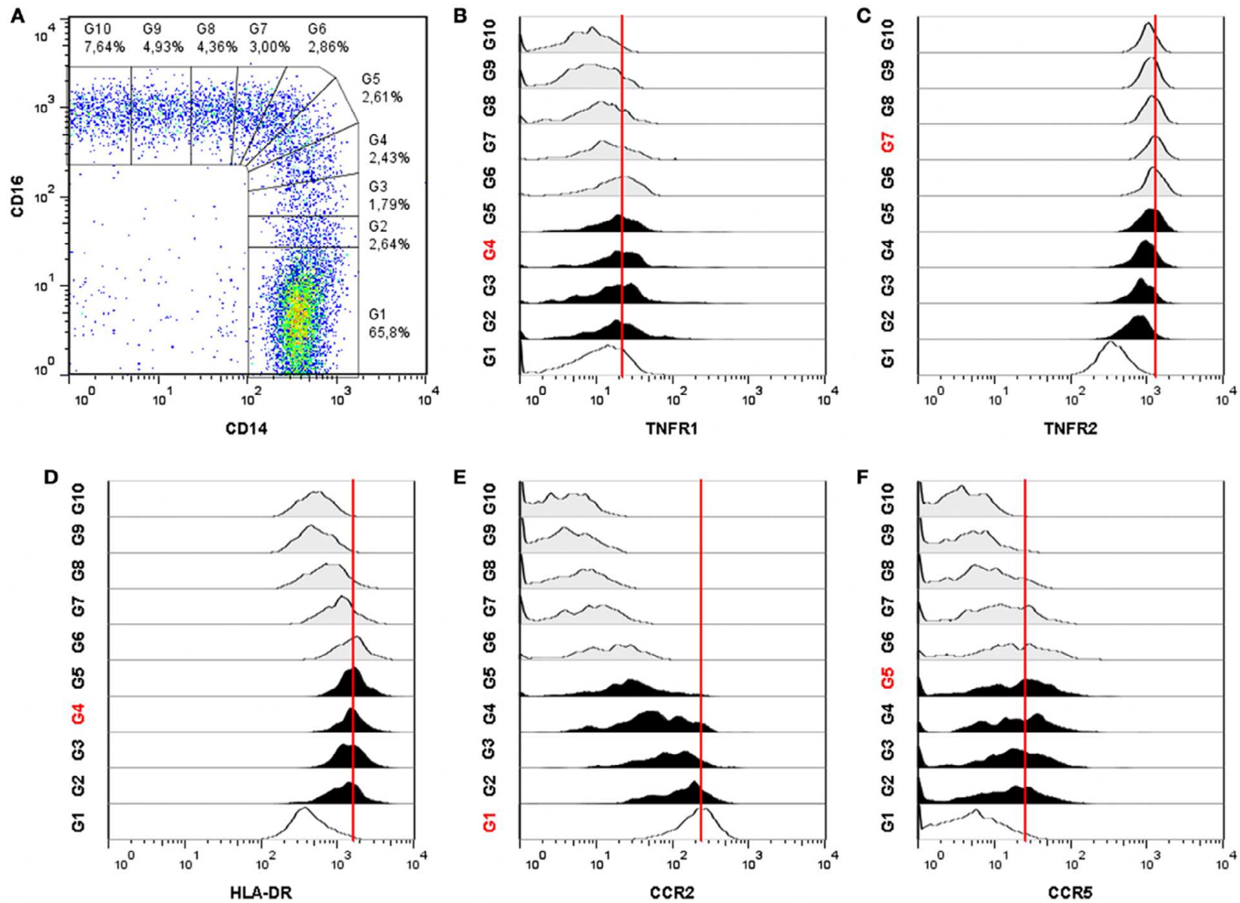

Fig. 9. Example of the subdivision of the monocyte population into a larger number of subpopulations suggested by Hijdra and colleagues, based on the expression of CD14 and CD16 (the subpopulations are indicated with G1-G10) , and respective changes in the different surface markers expression.[103]

Dynamic changes of monocytes subsets may occur during the course of disease, such as systemic inflammation[104], and following pharmacologic treatment.

Monocytes have been variously investigated in the cardiovascular pathology and frequency and phenotype alterations have been identified and suggest the ability of such cells to change along their continuum in relation to the circumstances.[108] On the contrary, some studies have highlighted the distinctive features of the individual sub-populations, representing them as discrete entities with their own functional and phenotypic characteristics.[76,109]

For this reason, the quantification of different subsets in peripheral blood may become useful for diagnosis and follow-up in human diseases, and in particular a baseline assessment might be used as a predictive parameter or as a biomarker for future cardiovascular events.[110]

2.2 Macrophages

Macrophages are cells with great variability and plasticity, which can respond to environmental signals by changing their phenotype and functional properties. They are in fact a heterogeneous cellular population, consisting of specialized macrophages depending on the environment where they are found.[82]

They have a central role in the maintenance of tissue homeostasis, removal of cellular debris, apoptotic cells and tissue remodeling products; this process does not provide for an immune response and does not involve, if not in a small amount, the production of immune mediators by macrophages. They act in case of necrosis, stress, trauma, tissue inflammation: phagocytosis of necrotic debris leads the activation of signal pathways, which cause alterations in the expression of surface proteins and production of cytokines and pro-inflammatory mediators, defining them activated macrophages.[111]

Activated macrophages are "paradigmatically" divided into two groups:

- M1 macrophages, with a pro-inflammatory phenotype;
- M2 macrophages, correlated with an anti-inflammatory function and tissue repair.[112,113]

This subdivision represents the two extremes of a continuous distribution of the same population environment and cytokine equilibrium dependent.[114]

M1 macrophages, characterized by high activity against bacterial and tumor cells, are also called classically activated macrophages, and are the result of a macrophage differentiation that occurs in the immune response cell-induced.

For the M1 differentiation is necessary a stimulation with INF-γ and TNF-α. In particular, a constant stimulation with INF-γ, produced by NK cells in a transient manner and by Th1 in a stable manner, is necessary; TNF-α, on the other hand, is produced by macrophages themselves following the stimulation of the TLR and acts in an autocrine way, produce INF-β, which acts similarly to INF-γ.[115]

Also the macrophage colony stimulating factor (GM-CSF) produced by a lot of cells, including macrophages and parenchymal cells, can lead to a differentiation versus M1 direction, through a signal pathways stimulated by INF-γ and TNFα.[113] M1 macrophages produce pro-inflammatory cytokines such as IL-1, IL-6, IL-12, IL-18, IL-23, IL-26 and promote Th1 and Th17 responses.[111]

More precisely, INF-γ leads to the transcription of genes for cytokine receptors, markers of cell activation, cell adhesion molecules, pro-inflammatory cytokines, chemokines (e.g. CCL2), antigen presentation molecules (for example Major Histocompatibility Complex, MHC).[113]

M1 macrophages are characterized by the expression of CD68 and CCR2.[116]

Signals from cells of innate and acquired immunity can also lead the differentiation in M2.

Tissue damage produce anti-inflammatory cytokines, such as IL-4, which directs a macrophage differentiation in the M2 direction; IL-13 also directs differentiation in the M2 direction. These macrophages produce extracellular matrix components, very few pro-inflammatory cytokines and have a poor ability to present the antigen to T cells.[115] They are characterized by the expression of CX3CR1, CD163, CD206.[117] Within M2 macrophages we can distinguish an M2-like category, which is activated by IL-10, glucocorticoids (released in stress situations), or molecules released by apoptotic cells. This kind of macrophages shares part of the characteristics of M2 but differs from these, especially as regards the cytokines production and phagocytic capacity.[114]

Similarly to the concept of monocyte continuum the traditionally M1/M2 macrophage polarization paradigm assume incremental differences in the expression of cell surface markers and cytokines confirming that they must be considered as a continuum rather than as distinct phenotypes and this is confirmed by gene expression studies.[115,118–120]

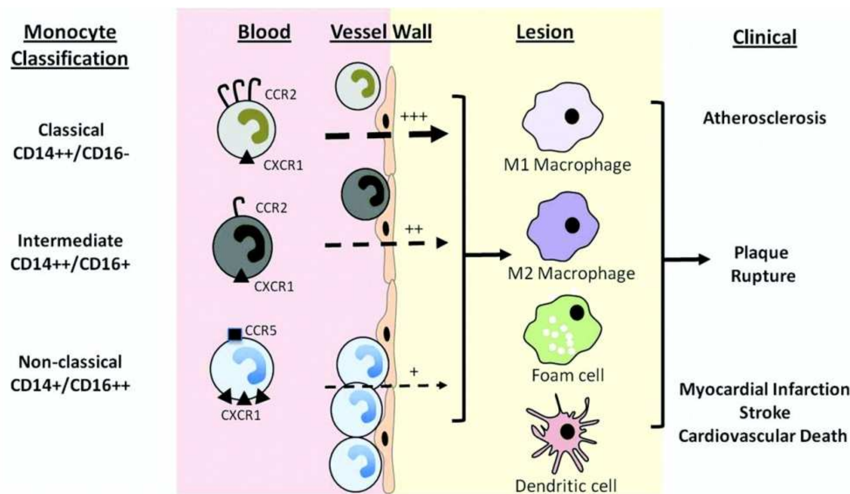

Fig. 10. A summary of the monocyte macrophage system in ATH and CVD.[115]

3. ROLE OF MONOCYTES AND MACROPHAGES IN ATHEROSCLEROSIS AND CARDIOVASCULAR DISEASES

Monocytes, macrophages and dendritic cells are essential to the development of atherosclerosis.[15] They play a fundamental role in atherogenesis both in the early stages of lesion formation and in the subsequent phases when atherosclerotic plaque is already formed. In the early stages circulating monocytes, recalled by chemokines, adhere to activated endothelial cells expressing adhesion molecules; then, following the chemokine gradient, monocytes migrate into the intima layer of the vessel.[26]

A great amount of works underlines the monocyte heterogeneity at the atherosclerotic plaque level. Studies on mice show how the chemokine receptors expressed by monocytes are involved in their migration to the plaque: monocytes Ly-6c^{high} use both CCR2 (chemokine receptor of CCL2) and CX3CR1 (receptor of CX3CL1, expressed at low level by monocytes Ly-6c^{high}), while the Ly-6c^{low} monocytes seem to use more CCR5 (which binds chemokine CCL5); anyway, CCR2, CCR5 and CX3CR1 are essential for the extravasation of monocytes at plaque level.[105]

In humans, while classical monocytes express previously CCR2 and non-classical monocytes CX3CR1, intermediate monocytes are characterized by the higher expression of CCR5 and co-expression of the other two receptors.[100] Intermediate monocytes are the most involved in the development of atherosclerosis:[121] once migrated to the vessel wall, monocytes become proliferating macrophages, macrophages interact with the oxLDL and then trigger the macrophages signaling pathways which evolve towards the inflammatory type, M1.[122]

Macrophages contribute to the formation of the necrotic core of plaque and to the production of inflammatory signals; they are also involved in the neo-angiogenesis of atherosclerotic plaque, which is stimulated by inflammation and hypoxia and contributed to the plaque growth; they are also related to the instability of the plaque itself.[123]

In conditions of cardiac damage (for example AMI) or haemodynamic stress, there’s a great recruitment of circulating monocytes to restore the lower level of resident tissue macrophages caused by ischemia;[124] in these conditions a large amount of macrophages, at cardiac tissue level, originate from circulating monocytes, and not from proliferating resident macrophages, which then differ in pro-inflammatory macrophages.[60]

After cardiac cells death, granulocytes, especially neuthrophils, and then monocytes are recalled by inflammatory signals from the damaged area. Studies on mice demonstrated that the damaged heart cells modify their chemokine expression over time: CCR2 shows a spike during the first phase;

CX3CR1 is most expressed in the second phase. So at first time (1-4 days after AMI) Ly-6c^{high} monocytes are recruited, with their pro-inflammatory and phagocytic function and later (after 5 days), Ly-6c^{low} monocytes migrate with a reparative function. Subsequently, monocytes can differentiate into macrophages, depending on the cytokine tissue environment.[125]

3.1 Monocyte subsets, risk factors and cardiovascular diseases

Monocytosis was found to predict cardiovascular events in some studies[126] but not in others,[127] and lymphocyte counts seem to be inversely correlated with coronary disease and its complications.[128] While differences in study design may account for some discrepancies regarding the correlation of monocyte counts and adverse cardiac events in epidemiological studies, flow cytometry has allowed for more sophisticated risk stratification.

Alterations in frequency or in surface markers expression of the various monocyte subpopulations are related to inflammatory and pathological conditions, typical of cardiovascular diseases. Patients with one or more traditional cardiovascular risk factors or patients with cardiovascular disease were recruited in a large amount of study in order to evaluate the correlation with alterations in the frequency of traditional monocyte subsets (classical, intermediate, and non-classical).

The identification of changes in the frequency or phenotype of mononuclear cells in inflammatory conditions could be a valid prognostic marker in the field of risk factors and cardiovascular diseases[108] and support the idea of a continuum of the monocyte population with changes related to different conditions.

A weak but positive correlation of CD14^{+} CD16^{+} monocytes with total plasma cholesterol and triglyceride levels was first described in 1999 in hypercholesterolemic patients with a positive stress ECG indicative of coronary heart disease.[129]

The number of CD16^{+} monocytes but not overall monocyte counts positively correlate with Body Mass Index (BMI), insulin resistance/diabetes and intima-media-thickness. Weight loss after gastric bypass surgery in severely obese patients is associated with a significant reduction of CD16^{+}monocytes.[100,130] Likewise, exercise training reduced the number of CD16^{+} monocytes in a physically inactive study population.[131]

Regarding obesity, an increased percentage of intermediate monocytes was found in obese patients with insulin resistance, but not in obese without insulin resistance, and in both the categories a reduction in the proportion of intermediate monocytes was shown after one hour of aerobic exercise, demonstrating that changes within the monocyte continuum can take place even in the short term.[132]

In addition, a study of 569 patients shows that $CD16^+$ monocytes are increased in patients with high BMI, and the increase in frequency of $CD16^+$ monocytes is also correlated with subclinical atherosclerosis.[80]

An alteration in all monocyte subpopulations can be observed in a non-optimal lipid profile context, traditional risk factor for atherosclerotic disease; although intermediate monocytes are by definition more inflammatory (they have a higher capacity to produce inflammatory cytokines), a transition to an inflammatory state (with increased cytokine production) of all monocyte subpopulations has been observed.[133]

Intermediate monocytes are generally implicated in cardiovascular disease,[134] and their participation in the atherosclerotic plaque formation process is well known.[100,135] In this context, a study shows that patients with CAD have higher percentages of $CD16^+$ monocytes than controls.[136]

In addition, an increase in the frequency of intermediates and a decrease in non-classical, the day after the event, was observed in most patients hospitalized for AMI compared to controls, with a return to pre-event values after about 30 days.[137] Similar alterations were observed in stroke patients: an increase in intermediate monocytes and a simultaneous reduction of non-classical the days following the event was demonstrated.[138,139] On the other hand, considering other surface markers, phenotypic alterations in all monocytes have been observed in patients with coronary disease: an increase in IL-6R expression on classical and intermediate (highlighting the role of inflammatory cytokines in atherosclerosis); an increase of CXCR4 chemokine receptor on non-classical and intermediate (favoring their recruitment at vascular level); and an increase in the CD34 expression in all monocytes.[140]

However, in studies conducted by our research group was detected no significant effect of traditional risk factors on the levels of traditional classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$), and non-classical ($CD14^{+}CD16^{+}$) monocyte subsets.[116,117,141]

In a study carried out measuring the frequency of monocyte subpopulations in pre-diabetic subjects there wasn't significant change in their frequency compared to controls[117]. Even in subjects with type 2 diabetes, no significant difference in the frequency of monocyte subpopulations has been demonstrated.[116] However, in patients with type 2 diabetes, a state of increased activation of innate immunity was observed, with a greater expression of inflammatory cytokines by neutrophils and monocytes;[142] another study demonstrates an increase in CD11b and CD66b (integrins involved in cell adhesion and migration) on both granulocytes and monocytes, and also in the CD16 expression on monocytes.[143] It has been shown that even in patients with hypercholesterolemia there are no changes in the frequency of monocyte subpopulations compared to controls.[141]

Similarly, another small study in 26 patients referred for elective coronary angiography doesn't point out considerable differences in the percentages of classical, intermediate, non-classical and the whole monocyte population between patients with and without hypercholesterolemia.[144]

3.2 Prognostic value of alterations in monocyte subsets in cardiovascular disease

Some studies have also focused on the predictive role of the alterations of the monocyte subpopulations found in the patients being studied.

In this regard, intermediate monocytes appear to have a prognostic relevance.[134] This is demonstrated, in a large prospective cohort of 951 patients undergoing elective coronary angiography, increased numbers of intermediate $CD14^{++}$ $CD16^{+}$ monocytes independently predicted cardiovascular death, myocardial infarction and stroke over a period of 2 and a half years.[145]

This association can also be applied in patients on dialysis with chronic kidney failure in patients, in addition to the detection of an increase in $CD16^{+}$ monocytes, the association between the increase in intermediate monocytes and the incidence of cardiovascular events has been demonstrated.[100,146]

$CD14^{+}$ $CD16^{+}$ monocytes but not total monocyte counts predict cardiovascular events in patients with chronic kidney disease and end stage of renal disease on dialysis, a patient population at increased risk for atherosclerotic complications.[147]

Interestingly however, among a study population with stable coronary artery disease, those patients with more than 5 cardiovascular risk factors and especially with a positive family history for coronary artery disease presented relatively higher percentages of $CD14^{high}$ $CD16^{-}$ monocytes.[100,148].

In a general population (n=659) with no known cardiovascular disease, increased numbers of circulating classical $CD14^{++}$ $CD16^{-}$ monocytes predicted cardiovascular events within a mean of 15-year follow-up independently of sex, age, and classical cardiovascular risk factors.[149] This is the first large cohort study identifying increased numbers of classical $CD14^{high}$ $CD16^{-}$ monocytes as an independent risk factor for ischemic cardiovascular events in a general population.

An increase in intermediates, 2 days after the initial event, was found in stroke patients and this correlated with mortality inversely.[139]

A cross-sectional study in 53 patients with stable CAD and 50 age- and sex-matched healthy controls highlights significant differences in monocyte phenotype in cardiovascular disease, which were differentially attributable to the 3 monocyte subsets defined according to contemporary nomenclature, but not in monocyte subsets proper.[96]

During AMI, the number of circulating classical and intermediate monocytes increased acutely over

3 days and were associated with impaired left ventricular function and larger infarct size.[150]
By contrast, it has been observed in patients with more advanced stages of peripheral artery occlusive disease, an increase in $CD14^{++}$ $CD16^{+}$ intermediate monocytes, suggesting that they have a pro-inflammatory function and can represent a promising biomarker for disease progression.[151]
However, it has been acknowledged that other conditions, a hypertension and coronary artery disease, may be related to significant changes.[152]

AIM OF THE STUDY

The identification of new prognostic markers able to predict the incidence of cardiovascular events is an important topic of the last years. Many studies have been performed on the monocyte population involved in pathological conditions related to cardiovascular disease.
It is well established that alteration in the frequency of monocyte subpopulations is associated with cardiovascular disease and can provide information about the incidence of cardiovascular events.
Considering the monocyte population as a continuum of different subpopulations capable of continuous phenotypic adaptations, we tried to investigate the possible variations in the frequency and their phenotypic and functional alterations within this continuum, and we tried to determine the correlations between these alterations, traditional risk factors and cardiovascular diseases.
The aim of this study was to investigate whether any cellular changes within the monocyte continuum, in the context of risk factors and cardiovascular diseases, can be better appreciated by analyzing the mean fluorescence intensity of CD14 and CD16, monocyte cell surface markers, rather than the frequency of the three traditional subpopulations, and whether these changes may aid additional information and should be a more useful predictive value for the incidence of cardiovascular events.
Finally, their distribution along the CD14/CD16 continuum could be represented by a 90° curve. The position of each cell on the curve could be analyzed using a “clock rule” and described by an angle that could capture CD16 and CD14 expression as a single number, the angular coefficient. We applied this concept to evaluate whether this more objective approach should give better information associated to cardiovascular risk or best predictive of incident MACE.

MATERIALS AND METHODS

1. Patients' characteristics

The study was approved by the Ethical Committee of the Province of Padua, was conducted in accordance with the Declaration of Helsinki and all patients provide a written informed consent.

Patients were recruited from the Outpatient clinic of the Division of Metabolic Disease of the University Hospital of Padua from March 2011 to June 2015.

The inclusion criteria in this study were:

- age 18-80 years;
- presence of at least 2 classical cardiovascular risk factors (diabetes, obesity, hypertension, smoking) or established cardiovascular disease.

The exclusion criteria in this study were:

- acute infections or inflammatory conditions;
- recent trauma or surgery;
- autoimmunity;
- organ transplantation;
- pregnancy;
- lactation;
- inability to provide informed consent.

For all patients we collected the following data:

- Anthropometric data: age, sex and Body Mass Index (BMI).
- Presence of traditional cardiovascular risk factors: pre-diabetes and type 2 diabetes, hypertension, obesity, dyslipidemia, active smoking, chronic kidney disease (CKD).
- Presence of atherosclerotic CVD: CAD (coronary stenosis at coronary angiography, symptomatic or asymptomatic); PAD (claudication, rest pain, ischemic wound ulcers with evidence of leg ischemia at ultrasound examination or angiography); CerVD, a past history of stroke or transient ischemic attack, or the presence of ≥30% stenosis of extracranial carotid arteries).
- Data collected on medication.

For each patient, starting from weight and height, was calculated the BMI (Kg/m^2).

Active smoking was considered as one or more cigarettes for a day.

Pre-diabetic patients and patients with T2D were defined according to the ADA (American Diabetes Association) guidelines.[153] According to these guidelines for the diagnosis of diabetes, one of the following conditions is required: HbA1c ≥ 6.5%; fasting plasma glucose ≥126 mg/dl (7 mmol/L); blood glucose ≥200 mg/dl 2 hours after OGTT; plasma glucose random ≥200 mg/dl (11.1 mmol/L) in a patient with classic symptoms of hyperglycemia or hyperglycemic crisis. For the diagnosis of pre-diabetes, one of the following conditions must be present: fasting plasma glucose between 100 mg/dl (5.6 mmol/L) and 125 mg/dl (6.9 mmol/L) (IFG); glycemia between 140 mg/dl (7.8 mmol/L) and 199 mg/dl (11.0 mmol/L) after 2 hours from OGTT (IGT); Hb1Ac 5.7-6.4%.[153]
The presence of hypertension has been defined as PA ≥140/90 mmHg, or as an antihypertensive therapy.
Obesity has been defined as BMI ≥30 kg/m².
The condition of dyslipidemia was considered as a non-optimal lipid profile: total cholesterol >200 mg/dl, LDL cholesterol >100 mg/dl, HDL cholesterol <40 mg/dl or triglycerides >150 mg/dl.
The glomerular filtration rate was defined according to the CDK-EPI formula[154] and the condition of chronic renal failure was identified as a glomerular filtration rate estimated with this formula infer to 60 ml/min/1.73 m² of body surface area.
The presence of CAD was defined by the presence of significant coronary stenosis (symptomatic or asymptomatic) on coronary angiography confirmation, or as a history of a previous acute coronary syndrome.
PAD was defined as the presence of symptoms/signs related to reduced blood flow, such as claudication, pain at rest, ischemic ulcers, together with demonstration by ultrasound or angiography of effective ischemia in the lower limbs.
CerVD has been described as a history of stroke or transient ischemic attack (TIA), or as a presence of a ≥30% stenosis of the extracranial carotid arteries. Finally, atherosclerotic cardiovascular disease was defined by the presence of one of the three previous arterial pathologies, or a combination of the three.
On the other hand, have been considered the therapy in progress: ACE inhibitors and aldosterone receptor antagonists, antihypertensive drugs, antiplatelet agents, statins, hypoglycemic agents and insulin.

2. Follow up and definition of MACE

Patients were followed with a 4.2 years median follow-up (IQR 3.1-4.8), by routine visits, telephone contacts and access to health electronic chart records and death registry.

During follow-up, in these high-risk patients was considered the incidence of major adverse cardiovascular events (MACE).

The cause of death was considered to be cardiovascular in case of:

- sudden death;
- death occurred within 14 days after an AMI;
- death occurred after worsening symptoms and/or signs of heart failure;
- death occurred within 30 days after a stroke;
- death due to another documented cardiovascular cause (e.g. dysrythmia, pulmonary embolism or intervention, non-fatal myocardial infarction, non-fatal stroke, unstable angina, heart failure and death for cardiovascular reasons);
- death not attributed to a not-cardiovascular cause were presumed to be cardiovascular.

Non-fatal myocardial infarction was defined by the presence of at least 2 of the following 3 criteria:

1. increasing of cardiac biomarkers;
2. ECG changes consistent with new ischemia;
3. imaging evidence of new non-viable myocardial or new wall motion abnormalities.

Non-fatal stroke was defined as the sudden appearance of signs/symptoms referred to a neurological deficit (for example emergence of hemiplegia, hemiparesis, loss of sensibility of one side of the body, alterations of the state of consciousness, dysphasia/aphasia) with a duration ≥24 hours (or with a duration <24 hours if the event has been associated with pharmacological treatment, or in the presence of available brain imaging showing a new hemorrhage or ischemia, or resulting in death), later confirmed by a neurology specialist or by brain imaging.

Unstable angina has been defined as resting, new onset or as an aggravation of a pre-existing angina in absence of cardiac biomarkers elevation, but in the presence of a new or a worsening ST-T on ECG, or evidence of ischemia by cardiac imaging, or evidence of ≥70% stenosis in an epicardial coronary artery.

Heart failure was defined by the presence of typical symptoms/signs or their worsening (dyspnoea, orthopnea, paroxysmal nocturnal dyspnea, pulmonary edema, pulmonary basilar crackles, jugular venous distension, third heart sound or gallop rhythm, radiologic evidence of worsening heart failure), requiring the initiation of new therapy or an increase in doses in case of therapy already in place (diuretics, ionotropes, vasodilators), supported by an increase in biomarkers such as brain natriuretic peptide (BNP).

3. Flow cytometry

Flow cytometry is a powerful tool to evaluate the characteristic of cells including particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity. These characteristics are determined using an optical-to-electronic coupling system that records how the cell scatters incident laser light and emits fluorescence. Fluorescence emission might be associated to dyes or conjugated monoclonal antibodies (mAb), which specifically bind molecules on the cell surface or intracellular components of the cell, allowing the identification of different cell types within a heterogeneous population.

A flow cytometer consists of three main systems:

• a fluidic system, which transports particles in a suspension to the laser beam for interrogation;

• an optic system, which consists of lasers to illuminate the particles in the sample stream and optical filters to direct the resulting light signals to the appropriate detectors;

• an electronic system, which converts the detected light signals into electronic signals that can be processed by the computer [Fig.11].

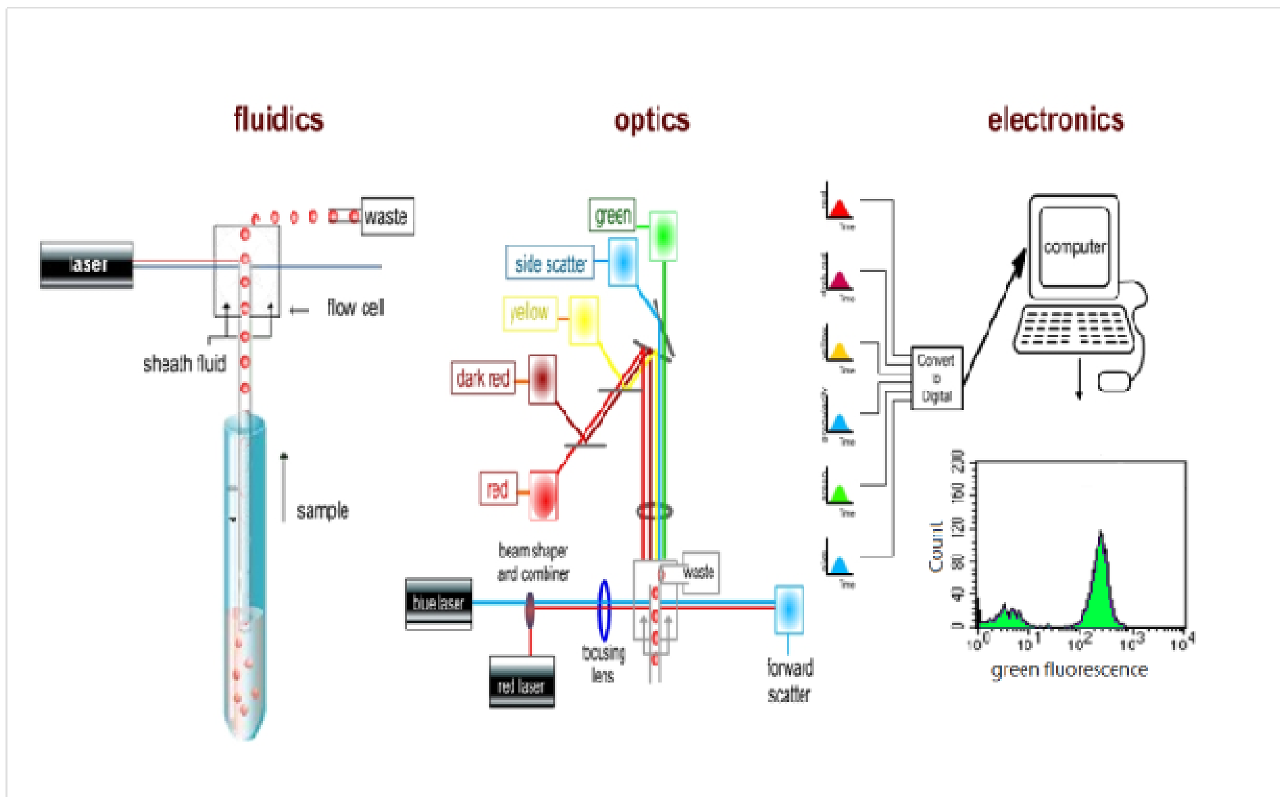

Fig. 11. Schematic organization of a flow cytometer: fluidic, optic and electronic systems.

For optimal illumination, when a cell suspension is run through the cytometer, sheath fluid is used to hidrodynamically focus the cell suspension through a small nozzle, causing cells to pass through a laser beam one cell at a time.

When particles pass through the laser intercept, they scatter laser light. Moreover, any fluorescent molecules present on the particle fluoresce. The scattered and fluorescent light is collected by appropriately positioned lenses. Light scattering properties of each cell are analyzed by different detectors. Detectors aimed directly in line with the laser beam (forward scatter, FSC) determine the cells size; while detectors that are addressed perpendicular to the laser beam (side scatter, SSC) assess the granularity within the cytoplasm and the complexity of cells. Additional detectors are used to determine the amount of fluorescent intensity emitted by individual cells, which corresponds to the number of receptors expressed by the cells. The fluorescent data is translated into information and plotted into a single-dimension histogram that demonstrates size, granularity or expression intensity of an antibody. Multiple parameters can also be displayed using a dot plot, which shows expression of up to two fluorochrome-labeled receptors, size versus granularity, or a combination of these factors.

Fluorochrome	**λ excitation**	**λ emission**
Fluorescein Isothiocyanate (FITC)	488 nm	530 nm
Phycoerythrin (PE)	488 nm	585 nm
Peridinin-Chlorophyll Protein/Cyianine (PE-Cy5.5)	488 nm	690 nm
Alexa Fluor 647	633 nm	668 nm

Tab. III. Examples of fluorochromes specification used for a polychromatic flow cytometric analysis.

The emission spectra of different fluorochromes [Tab. III] might overlap with the emission spectra each other. Whenever the fluorescence emission of one fluorochrome is detected in a detector designed to measure signal from another fluorochrome, spillover occurs. To correct the spectral overlap, a process of fluorescence compensation is used. Compensation aims to remove the signal from a given fluorochrome from all neighboring channels where it is also detected, in order to ensure that the fluorescence detected by a particular detector derives exclusively from the fluorochrome that is being measured. For this reason, fluorescence minus one (FMO) controls, containing all the antibodies to be tested in the experiment except for one, were considered as negative control: FMO controls are required to understand how other fluorochromes of the panel affect the left out parameter.

3.1 Flow cytometry protocol

Identification and quantification of monocytes subtypes was performed at baseline on fresh blood samples, within three hours after collection, using polychromatic flow cytometry.
For analysis of the cell surface expression patterns, peripheral blood of every patients was stained with a cocktail of monoclonal antibody (mAb) specific for surface antigens:

- 10µL of mAb anti-CD16, FITC conjugated (Beckman-Coulter, clone 3G8);
- 10µL of mAb anti-CD14, PE conjugated (Beckman-Coulter, clone RM052).

100µL of whole blood was added to each tube, vortexed gently and incubated for 15 minutes in the dark at room temperature. Then 4 ml of 1X lysing solution of ammonium chloride (NH_4Cl) were added, inverted to mix and incubated for 10 minutes at room temperature. After lysis was completed, indicated by the clearness of the sample, each sample was centrifuged at 1600 rpm at room temperature for 5 minutes and supernatants were decanted. Pellets were then resuspended in 200µL of PBS and analyzed by flow cytometry. A total amount of 100.000 cells was recorded for each tube.
All measurements were performed on a BD FACS CantoII flow cytometer (BD Biosciences; San Jose, CA) equipped with a blue laser (488 nm), a red laser (633 nm) and a violet laser (405 nm) and analyzed by BD FACSDiva® software (Becton Dickinson, San Jose, CA).
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".[76]
We first performed a stringent monocytic *"morphological gate"* in the SSC-A versus FSC-A plot, then based on the combination of the CD14 and CD16 expression intensity have been described and characterized three discrete subpopulations of monocytes in the plot CD14 versus CD16:

- classical monocytes $CD14^{++}CD16^{-}$ cells: defined as $CD14^{high}$ (about 10^4) $CD16^{low/neg}$;
- intermediate monocytes $CD14^{++}$ $CD16^{+}$ cells: $CD14^{high}$ $CD16^{pos}$ (about 10^4);
- non-classical monocytes $CD14^{+}CD16^{+}$ cells: $CD14^{low}$ (about 10^2-10^3) $CD16^{pos}$.[74]

However, definition of $CD16^{neg}$, $CD16^{dim}$ and $CD16^{high}$ cells should be subjective and show inter-individual variability.
In addition, within each of these gates and for the total monocyte population we recorded the mean fluorescence intensity (MFI) of the CD14 and the CD16 expression and the respective standard deviations (SD) to precisely position monocytes and the different subsets along the continuum of the CD14/CD16 plot.

4. Statistical analysis

Continuous variables are expressed as mean ± standard error, whereas categorical data are expressed as percentage.

Non-normal variables were log transformed before statistical analysis.

Comparison between two or more groups were performed with unpaired Student's t-test.

Owing (caused by) the very large number of comparisons, the Bonferroni correction was used to adjust for multiple testing.

Event-free survival was analyzed by Cox proportional hazard regression models.

Model 1 included age and sex as covariates.

Model 2 was fully adjusted for covariates that displayed significant Bonferroni-corrected imbalance at baseline between patients with and those without MACE.

Reclassification metrics (C-statistics and net reclassification improvement (NRI) were calculated as previously described.[155]

Statistical significance was accepted at $p < 0.05$.

Microsoft Excel 2010 with embedded macros were used.

5. "Clock Rule" Analysis

We have also re-analyze the CD14/CD16 plot of all the considered patients in a more objective way, using the "Clock Rule" analysis.[110]

Monocytes were selected among all leukocytes on the FSC versus SSC plot and set on the CD16 versus CD14 plot. From this plot, coordinates of each monocyte were exported using FlowJo® software, obtaining an X value corresponding to the FITC intensity and a Y value corresponding to the PE intensity.

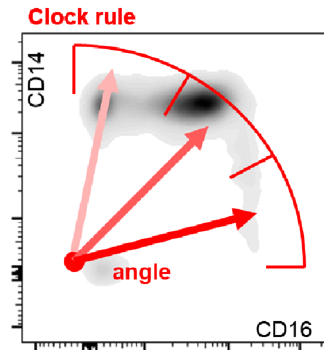

Fig. 12. Distribution of monocytes as a 90° angle; example of the clock rule.

For each patient, firstly the double negative cells were gated to determine the coordinates of the point to be considered as the vertex (V) of the 90° angle [Fig. 12]. Secondly, for each monocyte to be analyzed, the angular coefficient has been calculated using the following formula:

$$m = \frac{y_A - y_V}{x_A - x_V}$$

The value of the angle of each monocyte has been calculated using the arc-tangent function on the previously determined angular coefficient. As a result, lower angular values correspond to higher CD14 expression and lower CD16 expression, while higher angular value correspond to higher CD16 expression and lower CD 14 expression. Finally, the 90° angle was divided into 9 subsets of 10° to quantify the distribution of monocytes on the curve.

RESULTS

1. Patients' characteristics

The study included 227 patients. All patients had a high prevalence of cardiovascular risk factors, most showed a non optimal lipid profile and about the 50% had symptomatic or asymptomatic atherosclerotic cardiovascular disease at baseline [Tab. IV].[156]

Demographics	All patients	Without MACE	With MACE
Number	227	221	26
Age, years	61.0±0.7	60.4±0.7	66.0±1.6*
Sex male, n (%)	139 (61.2)	122 (60.7)	17 (65.4)
Body mass index, kg/m^2	27.6±0.3	27.5 (0.3)	28.8 (0.7)
Risk factors			
Active smoking, n (%)	49 (21.9)	42 (20.9)	7 (26.9)
Hypertension, n (%)	146 (65.2)	125 (62.2)	21 (80.7)
Obesity, n (%)	59 (26.0)	50 (24.9)	9 (34.6)
Pre-diabetes, n (%)	38 (16.7)	37 (18.4)	1 (3.8)
Type 2 diabetes, n (%)	100 (44.1)	83 (41.3)	17 (65.4)
Non-optimal lipid profile, n (%)	165 (72.7)	146 (72.6)	19 (73.1)
Laboratory exams			
Fasting plasma glucose, mg/dl (mmol/l)	100.5±1.6 (5.6±0.1)	100.3±1.6 (5.6±0.1)	106.3±6.4 (5.9±0.4)
Total cholesterol, mg/dl (mmol/l)	201.7±5.0 (5.2±0.1)	203.6±5.3 (5.2±0.1)	187.3±14.9 (4.8±0.4)
HDL cholesterol, mg/dl (mmol/l)	52.0±1.1 (1.33±0.03)	53.3±1.2 (1.37±0.03)	42.4±1.8* (1.09±0.05)
LDL cholesterol, mg/dl (mmol/l)	125.2±4.6 (3.2±0.1)	125.8±4.9 (3.2±0.1)	120.1±14.0 (3.1±0.4)
Triglycerides, mg/dl (mmol/l)	131.0±7.3 (1.5±0.1)	129.3±8.1 (1.5±0.1)	143.8±13.8 (1.6±0.2)
eGFR, ml/min/1.73 m^2	83.2±2.4	85.7±2.5	69.2±6.4*
CKD, n (% of total)	28 (12.3)	21 (10.4)	7 (26.9)
Cardiovascular disease			
Coronary artery disease, n (%)	56 (24.7)	41 (20.4)	15 (57.7)*
Peripheral arterial disease, n (%)	15 (6.6)	8 (4.0)	7 (26.7)*
Cerebrovascular disease, n (%)	42 (18.5)	35 (17.4)	7 (26.9)
Atherosclerotic CVD, n (%)	112 (49.3)	94 (46.7)	18 (69.3)
Medications			
ACE inhibitors / ARBs, n (%)	111 (42.9)	91 (45.3)	17 (65.4)
Other anti-hypertensive agents, n (%)	68 (26.3)	54 (26.9)	11 (42.3)
Anti-platelet agents, n (%)	85 (32.8)	67 (33.3)	16 (61.5)
Statins, n (%)	178 (68.7)	146 (72.6)	23 (88.4)
Glucose-lowering medications, n (%)	106 (46.6)	91 (45.2)	15 (57.7)
Insulin, n (%)	36 (15.9)	27 (13.4)	9 (34.6)

Tab. IV. Patients' clinical characteristics. Comparison of patients without and with MACE after Bonferroni correction. Data are presented as mean ± standard error or as number or percentage. (*value significantly different in the comparison).[156]

2. Identifycation of monocyte subtypes at baseline

We determined the frequency of the various subpopulations (mean ± standard error) using polychromatic flow cytometry in the patients' blood samples collected at baseline.

According to the traditional gating strategy we first performed a stringent monocytic *"morphological gate"* in the SSC-A versus FSC-A plot, then based on the combination of the CD14 and CD16 expression intensity have been described and characterized the discrete subpopulations of monocytes in the plot CD14 versus CD16 [Fig.13].

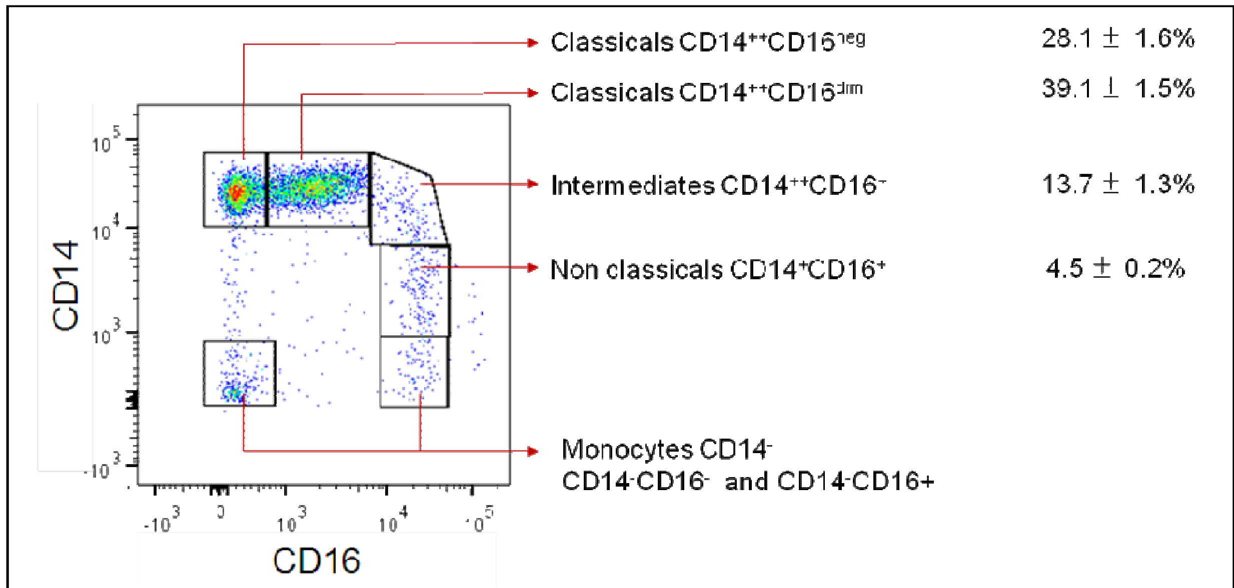

Fig. 13. Our gating strategy for the definition of the monocyte subtypes.

- Classical monocytes CD14^{high} CD16$^{dim/neg}$: 67,1±1,4%.

The classical monocyte population was split in two because was always detected a bivariate distribution of the CD16 expression on CD14^{high} monocytes with various predominance of the two.

A) Classical monocytes CD14^{++}CD16^{neg}: 28.1±1.6% [Fig.14 Example1].

B) Classical monocytes CD14^{++} CD16^{dim}: 39.1± 1.5% [Fig. 14 Example2].

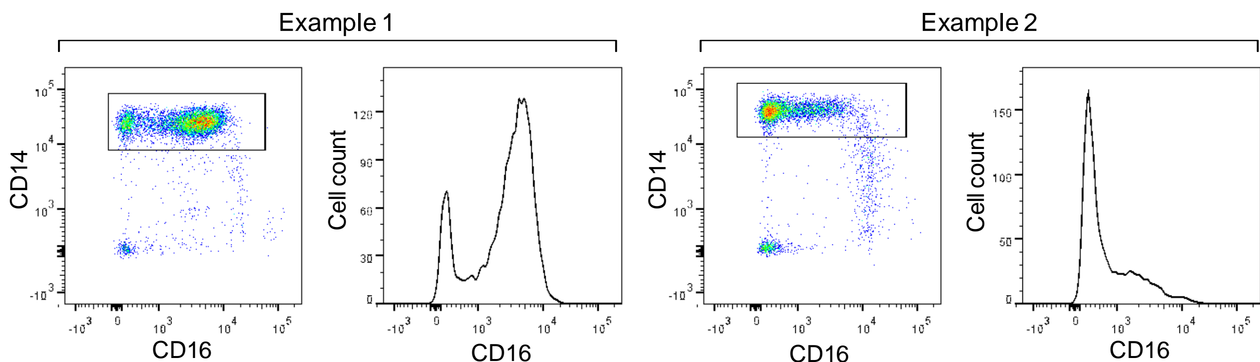

Fig. 14. CD14 versus CD16 plot and distribution of the classical population CD16^{neg} (A) and CD16^{dim} (B).

- Intermediate monocytes $CD14^{++}CD16^{+}$: 13.7±1.3%.
- Non-classical monocytes ($CD14^{+}CD16^{+}$): 4.5±0.2%.
- Two $CD14^{neg}$ cell subpopulations were identified:
  1) $CD14^{neg}CD16^{pos}$ subpopulation: 2.6 ± 0.1%;
  2) $CD14^{neg}CD16^{neg}$ subpopulation: 7.1 ± 0.4% of total monocytes.

  To determine the nature of these two subpopulations, an immunophenotipic flow cytometric analysis was performed using various surface markers: most of these cells resulted negative for CD3 (T lymphocyte marker), CD19 (B lymphocyte marker), CD56 (NK cell marker), CD10 (granulocyte marker), but positive for CD33 (myeloid line marker) [Fig. 15].

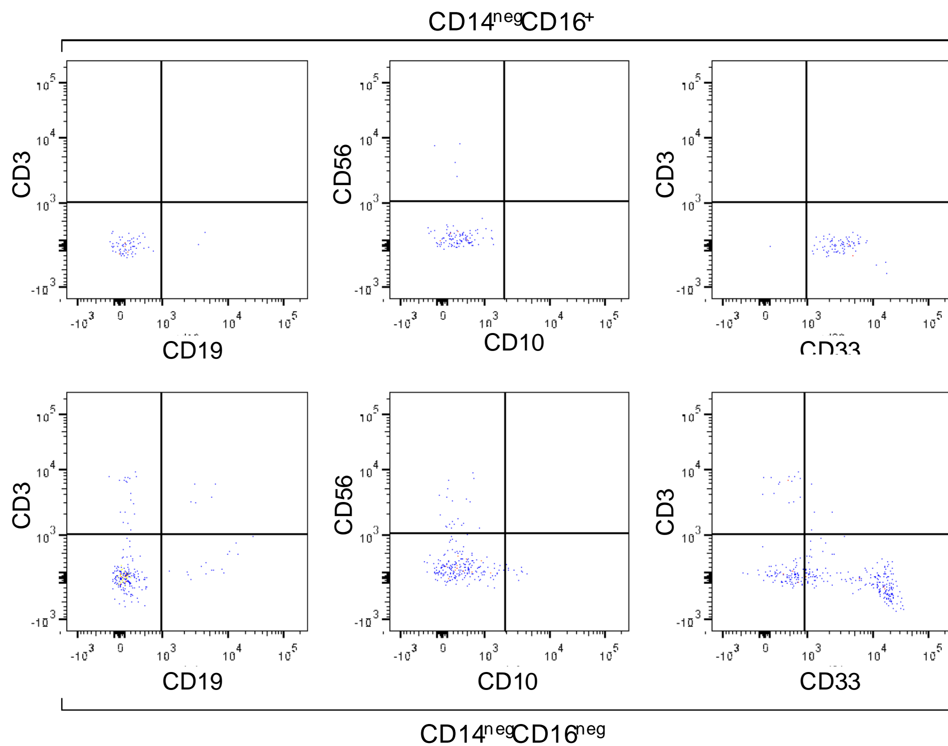

Fig. 15. Plots describing the phenotype of the two subpopulation of monocytes $CD14^{neg}$ respectively $CD16^{+}$ and $CD16^{neg}$.

These $CD14^{neg}$ subpopulation aren't routinely considered so we did not further analyze them.[156]
When summing the frequencies of the different subpopulations you don't reach a 100% of monocytes because we did not include monocytes $CD14^{neg}$ in our analysis. Compared to the literature, our data show higher level of intermediate monocytes and lower of non-classical ones [Tab. II].
In addition to the absolute number and the percentage frequency, we recorded also the mean fluorescence intensity (MFI) of CD14 and CD16.

3. Monocyte subsets and traditional cardiovascular risk factors

All these information have been studied in relation to the cardiovascular risk factors considered in our research (type 2 diabetes, hypertension, active smoking, non-optimal lipid profile, obesity), and in relation to age (<or> age 65) and sex [Tab. V].

	Age		Sex		Type 2 diabetes		Hypertension		Smoke		Lipid profile		Obesity	
	<65 years	**≥65 years**	**Females**	**Males**	**No**	**Yes**	**No**	**Yes**	**No**	**Yes**	**No**	**Yes**	**No**	**Yes**
Number	136	91	88	139	127	100	79	146	178	49	165	62	168	59
WBC, 10^3 / ml	7.55±0.22	7.68±0.27	7.40±0.26	7.72±0.22	7.74±0.22	7.43±0.26	7.49±0.28	7.66±0.22	7.60±0.20	7.59±0.36	7.74±0.19	7.17±0.35	7.48±0.19	7.96±0.36
Granulocytes, %	57.2±0.7	54.8±0.9*	56.2±0.9	56.1±0.7	56.8±0.8	55.6±0.8	58.2±1.0	55.1±0.7*	56.4±0.6	55.6±1.1	56.6±0.6	55.3±1.1	55.8±0.7	57.6±1.0
10^3/ml	4.34±0.14	4.21±0.16	4.17±0.16	4.34±0.14	4.41±0.14	4.14±0.16	4.34±0.17	4.25±0.14	4.29±0.12	4.25±0.22	4.38±0.12	3.99±0.22	4.18±0.12	4.58±0.21
Lymphocytes, %	33.2±0.7	36.3±1.0*	34.3±1.0	34.7±0.8	33.8±0.8	35.4±0.9	32.2±1.1	35.7±0.7*	34.3±0.7	34.9±1.2	34.2±0.7	35.4±1.3	35.0±0.7	33.1±1.1
10^3/ml	2.49±2.79	2.79±0.12*	2.54±2.66	2.66±0.09	2.61±2.62	2.62±0.11	2.43±2.71	2.71±0.09	2.61±2.62	2.62±0.15	2.65±2.50	2.50±0.14	2.60±2.64	2.64±0.15
Monocytes, %	9.6±0.3	8.9±0.3	9.4±0.4	9.2±0.3	9.5±0.3	9.1±0.3	9.6±0.4	9.2±0.3	9.3±0.3	9.4±0.5	9.2±0.3	9.4±0.4	9.3±0.3	9.3±0.4
10^3/ml	0.72±0.03	0.68±0.04	0.69±0.03	0.71±0.03	0.72±0.03	0.68±0.04	0.72±0.04	0.70±0.03	0.70±0.03	0.71±0.05	0.71±0.03	0.67±0.05	0.69±0.03	0.74±0.04
Classical $CD16^{neg}$														
10^3/ml	0.21±0.02	0.17±0.02	0.24±0.03	0.17±0.02*	0.22±0.02	0.17±0.02	0.20±0.02	0.20±0.02	0.19±0.02	0.22±0.03	0.21±0.02	0.17±0.02	0.20±0.02	0.20±0.02
%	29.9±2.1	25.4±2.3	32.8±2.6	24.6±1.9*	30.1±2.2	25.5±2.2	27.6±2.7	27.7±1.9	26.1±1.7	33.1±3.9	28.6±1.9	26.4±3.0	28.5±1.9	26.8±2.8
CD14 MFI	11701±665	11782±730	11070±746	12226±655	10985±701	12683±672	11508±919	11822±579	11806±581	11379±898	11693±611	11855±728	11231±555	13163±1037
CD16 MFI	155±7	168±8	154±9	166±7	121±6	210±6#	145±10	171±6*	158±6	175±10	155±6	176±10	152±6	184±10*
Classical $CD16^{dim}$														
10^3/ml	0.26±0.02	0.29±0.02	0.24±0.02	0.29±0.02*	0.28±0.02	0.26±0.02	0.29±0.03	0.26±0.02	0.28±0.02	0.24±0.03	0.27±0.02	0.27±0.03	0.27±0.02	0.28±0.02
%	36.9±2.0	42.4±2.3	36.1±2.3	41.3±1.9	38.9±2.1	39.3±2.1	39.3±2.5	39.4±1.9	40.6±1.6	34.9±3.5	38.8±1.7	39.8±2.8	39.1±1.8	39.0±2.6
CD14 MFI	12237±683	12105±757	11524±726	12675±694	11484±713	13073±708	11839±894	12343±621	12208±593	12020±987	12136±630	12332±746	11580±554	13905±1133*
CD16 MFI	2075±145	2071±126	2140±197	2042±106	1705±125	2541±151#	2238±193	2008±116	2052±113	2216±227	2051±121	2142±172	2042±121	2163±172
Intermediate														
/ml	107.38±17.0	88.69±14.52	86.1±18.6	109.5±15.2	87.6±14.6	115.5±19.1	84.00±17.39	110.2±15.64	89.4±11.07	142.77±36.8	101.0±14.25	96.46±19.3	93.2±12.82	118.9±26.54
%	14.0±1.7	13.2±2.1	12.0±2.0	14.9±1.8	12.1±1.7	15.7±2.0	12.1±2.0	14.8±1.8	13.1±1.4	16.6±3.6	13.5±1.5	14.2±2.6	13.6±1.6	13.9±2.3
CD14 MFI	11861±673	13041±1352	12618±1428	12222±626	11155±709	13830±1228*	11692±934	12680±916	12482±837	11815±844	12480±862	11886±748	11288±553	15312±2028
CD16 MFI	9243±691	8923±529	9452±966	8950±444	7270±555	11457±720#	9307±843	9133±561	9057±550	9682±848	8967±559	9564±797	8959±577	9558±702
Non-classical														
/ml	33.38±2.28	30.75±2.25	32.11±2.63	32.57±2.10	33.67±2.24	30.62±2.38	36.71±3.23	30.15±1.84	34.11±1.91	26.46±3.15	33.40±1.98	29.04±2.66	31.20±1.90	35.55±3.17
%	4.6±0.2	4.5±0.3	4.6±0.3	4.5±0.2	4.6±0.2	4.5±0.2	5.0±0.3	4.3±0.2*	4.8±0.2	3.6±0.3#	4.6±0.2	4.5±0.3	4.4±0.2	4.8±0.3
CD14 MFI	1501±85	1616±236	1413±104	1642±164	1400±88	1733±215	1340±105	1660±156	1534±136	1603±108	1598±140	1391±82	1560±141	1511±97
CD16 MFI	15161±1213	15127±981	15680±1673	14893±828	11694±999	19533±1251#	14563±1416	15665±1030	15017±996	16225±1351	14838±990	16092±1432	14723±1000	16356±1401

Tab. V. Leukocyte counts (absolute and relative) and characterization of monocyte cells, in particular frequency (absolute and relative) and CD14 and CD16 MFI, in relation to age, sex and the presence of type 2 diabetes, hypertension, smoking, dyslipidemia and obesity. * Indicates a significant change in group comparison with $p < 0.05$; # indicates a significant difference after Bonferroni correction for multiple tests.

We first examined monocyte subsets in relation to the traditional cardiovascular risk factors, such as age, sex, type 2 diabetes, hypertension, active smoking, dyslipidemia and obesity.

Comparing the different groups of patients, there were no significant differences in the subpopulations of monocytes associated with obesity, type 2 diabetes and lipid profile.

In men versus women, we detected a reduction in the frequency of classical monocytes $CD16^{neg}$ and an increase in the frequency of classical monocytes $CD16^{dim}$.

In hypertensive patients (compared to those without hypertension) an increased in CD16 MFI on $CD16^{neg}$ and a reduction of non-classical monocytes was found. However, these variations are not significant after applying the Bonferroni correction for multiple tests.

Instead, in smokers (compared to non-smokers), there was a robust decrease in non-classical monocytes ($3.6 \pm 0.3\%$ vs. $4.8 \pm 0.2\%$), which remained significant also after the correction for multiple tests.

None of the examined risk factors was associated with changes in total white blood cell count and frequency of monocytes.

Then, by analyzing the CD14 and CD16 MFI in each monocyte subpopulations, patients with type 2 diabetes showed a strong and consistent up-regulation of CD16 compared to those without type 2 diabetes [Fig. 16]:

- 210 ± 6 vs 121 ± 6 on classical $CD16^{neg}$;
- 2541 ± 151 vs 1705 ± 125 on classical $CD16^{dim}$;
- 11457 ± 720 vs 7270 ± 555 on intermediate;
- 19533 ± 1251 vs 11694 ± 999 on non-classical;

despite we didn't observe significant changes in the frequency of every subsets.

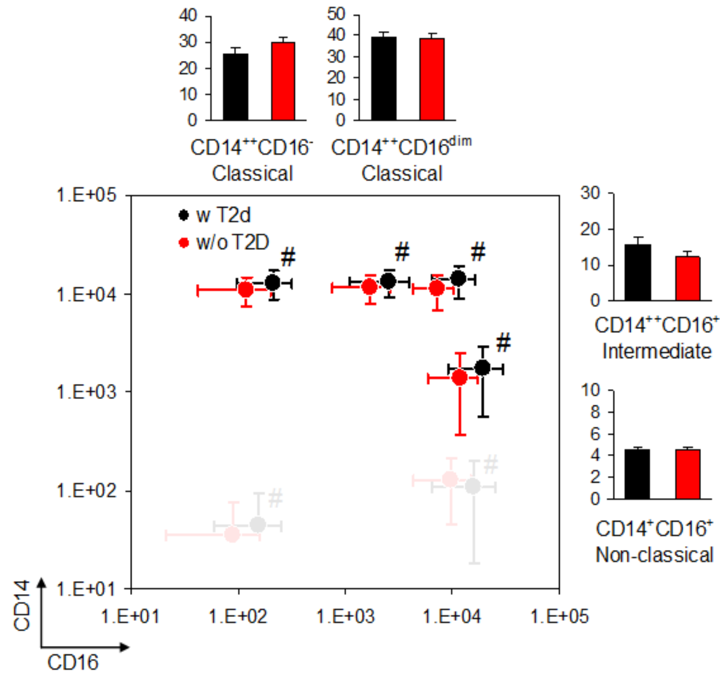

Fig. 16. The plot shows the variations (and their respective error bars) of CD16 MFI (indicated with #) detected in patients with type 2 diabetes (in black) compared to non-diabetic patients (in red) in the four monocytic subpopulations considered in the patients of the study (in transparency the subpopulations $CD14^{neg}$ $CD16^{neg}$ and $CD14^{neg}$ $CD16^{+}$ not further investigated). The histograms around show the average percentages of the monocytic subpopulations of diabetic patients (in black) and non-diabetics (in red), which show no significant differences.

In hypertensive patients, an increase of CD16 MFI was noted in classical monocytes $CD16^{neg}$, while in obese subjects an increase of MFI CD16 in classical $CD16^{neg}$ and an increase of CD14 MFI in classical $CD16^{dim}$ was observed.

However, all these variations are not significant after the correction for multiple tests.

No variation of the monocytic subpopulations related to age and the non-optimal lipid profile has emerged.

4. Monocyte subsets and prevalent cardiovascular disease

Then patients were divided according to the presence of CAD at baseline [Tab.VI].

	Prevalent CAD	
	No	**Yes**
Number	171	56
WBC, 10^3 / ml	7.68±0.22	7.51±0.33
Granulocytes, %	56.3±0.7	55.0±1.1
10^3/ml	4.34±0.14	4.15±0.20
Lymphocytes, %	34.5±0.8	35.6±1.2
10^3/ml	2.64±2.67	2.67±0.15
Monocytes, %	9.2±0.3	9.4±0.4
10^3/ml	0.71±0.03	0.69±0.04
Classical CD16^{neg}		
10^3/ml	0.20±0.02	0.18±0.03
%	29.7±2.1	25.2±3.0
CD14 MFI	11683±644	11199±894
CD16 MFI	140±7	195±9$^{\#}$
Classical CD16^{dim}		
10^3/ml	0.28±0.02	0.27±0.03
%	38.9±1.9	40.9±3.4
CD14 MFI	12105±664	11914±1019
CD16 MFI	1765±113	2039±140
Intermediate		
/ml	75.42±11.37	157.6±32.6*
%	10.1±1.3	20.9±3.7$^{\#}$
CD14 MFI	12251±1014	11997±845
CD16 MFI	7493±499	9784±618*
Non-classical		
/ml	33.29±2.11	26.25±2.83
%	4.6±0.2	3.7±0.3*
CD14 MFI	1260±57	2086±372$^{\#}$
CD16 MFI	12026±897	17202±1008$^{\#}$

Tab. VI. White blood cells (WBC), relative and absolute counts of leukocytes, frequency and CD14/CD16 mean fluorescence intensity for each monocyte subsets in patients with and without baseline coronary artery disease (CAD).*p<0.05; #significant after Bonferroni correction.

In patients with prevalence of CAD, compared to subjects without, a significant increase in the frequency of intermediate monocytes emerged (20.9 ± 3.7% versus 10.1 ± 1.3%).

We analyzed the mean fluorescence intensity of CD14 and CD16: there was an increase in

CD16MFI in classical monocytes CD16^{neg} (195 ± 9 versus 140 ± 7); an increase of CD14 MFI (2086 ± 372 versus 1260 ± 57) and CD16 MFI (17202 ± 1008 versus 12026 ± 897) on non-classical. These increases remained significant after the correction for multiple tests.

There is also a significant reduction in non-classical monocytes (3.7 ± 0.3% versus 4.6 ± 0.2%, with $p < 0.01$), but this variation was not significant after the correction for multiple tests. Similarly, the increase of CD16 MFI on intermediate monocytes also loses its meaning (9784 ± 618 versus 7493 ± 499) [Fig. 17].

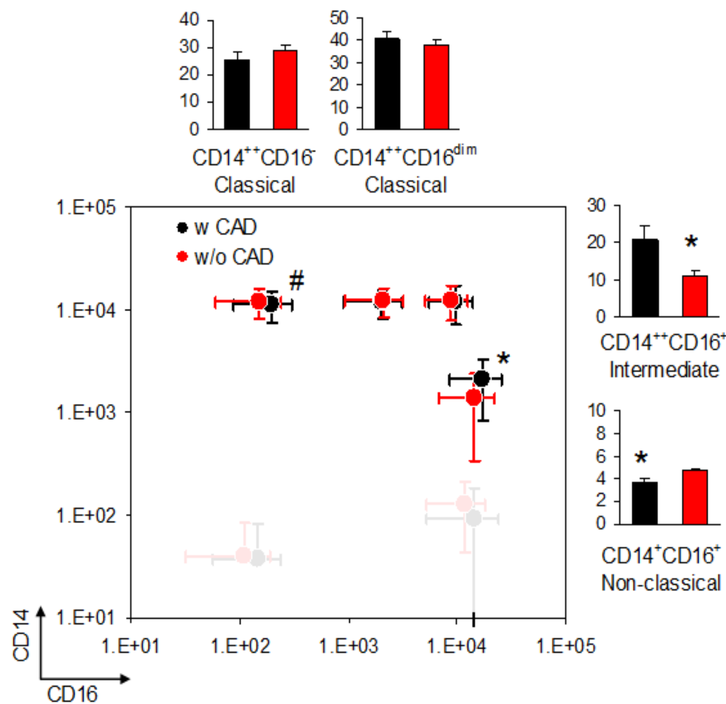

Fig. 17. The plot shows the significant variations (and their respective error bars) of CD16MFI (indicated with #), highlighted at the level of classical monocytes CD16^{neg}, and CD14 (indicated with *) at the level of non-classical monocytes, detected in CAD patients (in black) compared to patients without CAD (in red) (in transparency the monocytic subpopulations CD14^{-}CD16^{-} and CD14^{-}CD16^{+} not further investigated in this study).

The histograms represent the average percentage frequencies of monocytic subpopulations in CAD patients (in black) and in non-CAD patients (in red). The * in the histogram indicates a significant difference ($p < 0.05$).

5. Monocytes subsets and cardiovascular outcomes

Patients were followed-up for a median of 4.2 (3.1-4.8) years. During the follow up:

• 7 patients died: 4 for cardiovascular death and 3 for cancer related causes;

• 22 patients had cardiovascular events of which: 9 AMI, 1 stroke, 5 hospitalizations for unstable angina, 7 hospitalizations for heart failure. Not considering the 3 deaths for non-cardiovascular reasons.

The combined MACE endpoint (composed by cardiovascular events and cardiovascular death) occurred in 26 patients of the 227 (equal to an annual rate 2.9%).

	Incident MACE	
	No	**Yes**
Number	201	26
WBC, 10^3 / ml	7.60±0.18	7.59±0.52
Granulocytes, %	56.5±0.6	54.1±1.8
10^3/ml	4.31±0.11	4.13±0.32
Lymphocytes, %	34.2±0.6	36.7±1.9
10^3/ml	2.59±2.77	2.77±0.22
Monocytes, %	9.3±0.2	9.2±0.7
10^3/ml	0.70±0.02	0.69±0.07
Classical CD16^{neg}		
10^3/ml	0.20±0.02	0.17±0.04
%	28.4±1.7	25.5±4.2
CD14 MFI	11652±528	12364±1404
CD16 MFI	154±6	209±15$^{\#}$
Classical CD16^{dim}		
10^3/ml	0.27±0.02	0.23±0.04
%	39.6±1.6	35.4±4.2
CD14 MFI	12094±539	12885±1551
CD16 MFI	2037±108	2356±265
Intermediate		
/ml	90.95±11.66	169.0±47.2*
%	12.6±1.3	21.8±4.6*
CD14 MFI	12213±733	13269±1643
CD16 MFI	8837±488	11262±1433
Non-classical		
/ml	32.58±1.77	30.38±4.00
%	4.6±0.2	4.5±0.4
CD14 MFI	1419±56	2540±816$^{\#}$

Tab. VII. White blood cells (WBC), relative and absolute counts of leukocyte, frequency and CD14/CD16 mean fluorescence intensity (MFI) for each monocyte subsets are presented in patients with or without incident MACE. *p<0.05; #significant after Bonferroni correction.

The most significant clinical observations in patients with MACE during follow-up, compared to those without, after adjusting for multiple testing, are summarized in Tab. VII:

- were significantly older (66.0 ± 1.6 vs 60.4 ± 0.7);
- had lower level of HDL-cholesterol (mg/dl) (42.4 ± 1.8 vs 53.3 ± 1.2);
- had a minor EGFR (ml/min/1.73 m2) (69.2 ± 6.4 vs at 85.7 ± 2.5);
- had a higher prevalence of coronary artery disease (57.7% vs 20.4%) and peripheral arterial disease (26.7% vs 4.0%).

Similar monocytic alterations to those observed in CAD patients have occurred in patients with MACE during follow-up, compared to those without [Tab.VII].

They displayed a higher baseline frequency of intermediate monocytes ($21.8 \pm 4.6\%$ versus $12.6 \pm 1.3\%$, with $p < 0,026$, but not significant after the correction for multiple tests).

According to the analysis of monocytes distribution, patients with incident MACE compared to those without showed a shift of non-classicals towards intermediates, as evidenced by the increase in both CD16 MFI (20569 ± 2804 versus 14446 ± 848, with $P < 0,017$) and CD14 MFI (2539 ± 815 versus 1418 ± 56; $p < 0.0001$) on non-classicals.

A significant shift of $CD14^{++}$ $CD16^{neg}$ towards $CD14^{++}$ $CD16^{dim}$ monocytes was noted based on CD16 MFI (208 ± 15 versus 154 ± 5; $p = 0.001$) [Fig. 18].

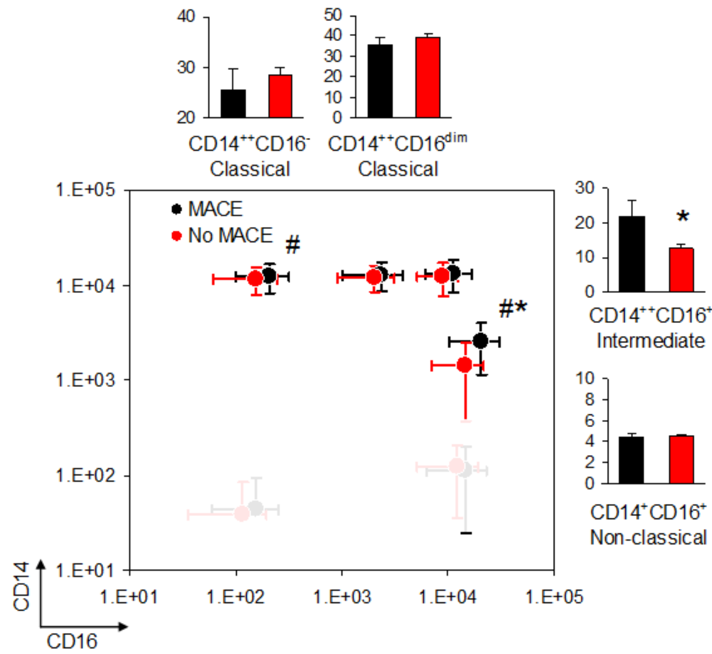

Fig. 18. The plot represents the variations (and their respective error bars) of CD16 MFI (indicated with #) in the classical monocytes CD16neg and the increase of CD14 MFI (indicated with*) and CD16 MFI (indicated with #) on non-classical monocytes, in patients with MACE (in black) compared to patients without MACE (in red) during follow-up (in transparency the monocytic populations CD14-CD16- and $CD14^{-}CD16^{+}$ not further investigated in this study). Histograms represent the average percentage frequencies of the monocyte subpopulations in patients with MACE (in black) compared to those without (in red). The * in the histogram of intermediate monocytes shows the difference in the two groups ($p < 0.05$).

6. Monocyte subpopulations and mace prediction

The Cox proportional hazard regression model was applied to assess whether the significant alterations of the monocytic cells highlighted in patients with MACE (Table III), i.e. the increase in the percentage frequency of intermediates, the increase in CD14 MFI and CD16 MFI on non-classical, and the increase in CD16 MFI on classical $CD16^{neg}$ monocytes, can independently predict the incidence of MACE.

In the first regression model (Model 1) the adjustment was made by age and sex; in the second model (Model 2) in addition to age and sex, adjustment has been made for different clinical characteristics significantly at baseline between patients who had MACE compared to those who hadn't MACE: Cholesterol HDL, chronic renal failure, coronary artery disease [Tab VIII].

Variables	**Model 1**		**Model 2**	
	HR for 1 SD	***p***	**HR for 1 SD**	***p***
Intermediate monocytes, %	1.35 (1.06-1.71)	0.014	1.19 (0.93-1.51)	0.167
Non-classical CD14 MFI	1.18 (1.04-1.35)	0.011	1.10 (0.95-1.27)	0.214
Non-classical CD16 MFI	1.37 (1.07-1.74)	0.011	1.23 (0.93-1.64)	0.142
$CD14^{++}CD16^{neg}$ CD16 MFI	1.87 (1.30-2.69)	0.001	1.63 (1.07-2.49)	0.023

Tab VIII. Analysis of survival free from MACE. Application of Cox regression model correction. Model 1 is adjusted by age and gender; Model 2 is adjusted by age, sex, HDL-cholesterol, chronic kidney failure, coronary artery disease, variables that were significantly different between patients with and without MACE during follow-up.

In Model 1 all variables, intermediate monocytes, CD16 MFI on classical, CD16 and CD14 MFI on non-classical have been significantly predicting the incidence of MACE ($p < 0.05$ for each variable).

However, in Model 2, only the CD16 MFI on classical monocytes $CD16^{neg}$ remained significantly associated with the incidence of MACE ($p < 0.05$), showing a 63% increased risk for one SD increased in CD16 MFI.

The Kaplan Meier curves derived from Model 2 for CD16 MFI on classical monocytes, categorized as high or low based on the median value, shows that patients with higher CD16 MFI on classical CD16^{neg} have less event-free survival [Fig. 19].

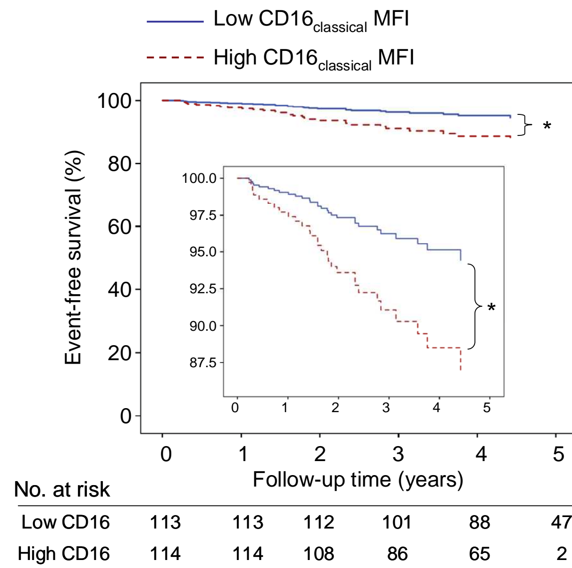

Fig. 19. Kaplan-Meier curve derived from Model 2 highlights lower events free-survival in patients with higher CD16MFI on classic CD16^{neg} monocytes. The * indicates a significant difference between the two curves. The table below shows the number of patients at risk over time. For the reclassification criteria, the addition of the variable "CD16MFI in classical monocytes CD14^{++} CD16^{neg}" in Model 2 led to a significant increase in C statistics and to a significantly increased NRI (34.4%, with $p < 0.02$), especially because it allows a better reclassification of patients without events.

7. Analysis according to the "Clock Rule"

We have also re-analyze the CD14 versus CD16 plot of all the patients in a more objective way, using the "Clock Rule" analysis.[110]

Monocytes were selected among all leukocytes on the FSC versus SSC plot, and set on the CD16 versus CD14 plot. From this plot, coordinates of each monocyte were exported using FlowJo® software, obtaining an X value corresponding to the FITC intensity and a Y value corresponding to the PE intensity.

For each patient, the double negative cells were first gated to determine the coordinates of the point to be considered as the vertex (V) of the 90° angle. Secondly, for each monocyte to be analyzed, the angular coefficient has been calculated.

The value of the angle of each monocyte has been calculated using the arc-tangent function on the previously determined angular coefficient.

As a result, lower angular values correspond to higher CD14 expression and lower CD16 expression, while higher angular value correspond to higher CD16 expression and lower CD 14 expression. Finally, the 90° angle was divided into 9 subsets of 10° to quantify the distribution of monocytes on the curve.

First, monocyte distribution was examined in relation to the presence or absence of baseline CAD [Fig. 20].

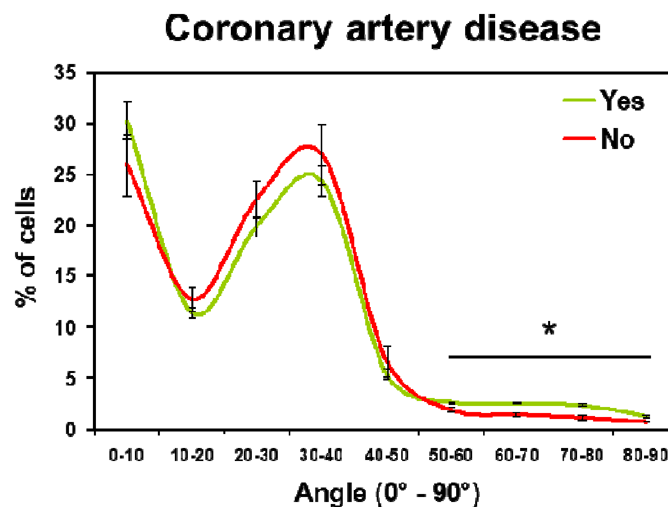

Fig. 20. Figure Clock Rule analysis for monocytes distribution in patients with (green line) and without (red line) baseline CAD. * Significant difference between the two curves.

The only significant difference observed between these two groups was in the higher angular value part of the histogram, which correspond to an higher CD16 expression and a lower CD14 expression, characteristic of the intermediate and non-classical populations.

Instead, when comparing monocyte distribution of patients with and without MACE, no significant differences were noticed [Fig. 21].

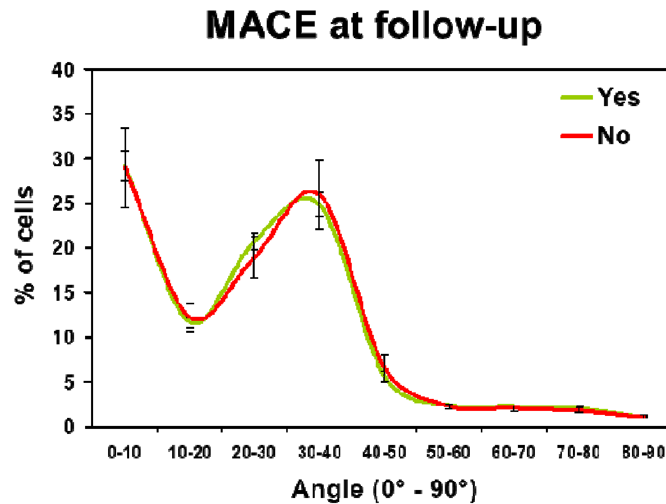

Fig. 21. Clock Rule analysis for monocytes distribution in patients with (green line) and without (red line) MACE at follow-up. There isn't a significant difference between the two curves.

DISCUSSION AND CONCLUSIONS

Previous studies of our research group on prediabetic, diabetic, and hypercolesterolemic patients did not show any significant effect of these traditional risk factors on the levels of traditional monocyte subsets.[116,117,141] With the present work, we confirmed that cardiovascular risk factors caused marginal effects on the frequency of classical, intermediate and non-classical monocytes. The most important finding of this study was that, analyzing the monocyte positioning along the continuum of the CD14 versus CD16 plot, it is possible to provide incremental information on cardiovascular risk and outcomes over the traditional assessment of monocyte subsets.[156]
During the course of disease, such as systemic inflammation[104] and also following pharmacologic treatment may occur dynamic changes in monocyte subsets. For this reason, it can be useful for diagnosis and follow-up in human diseases to quantify these functionally different subsets in peripheral blood and a baseline assessment might be used as a predictive parameter or as a biomarker for future cardiovascular events.[110]

Based on the recent kinetic work of Patel and colleagues, by in vivo labelling of human monocytes with deuterium, we can summarize the physiological cascade of monocyte subsets in the following way: monocytogenesis proceeds through the subsequent evolution of classical monocytes into intermediate and non-classical ones [Fig. 22].

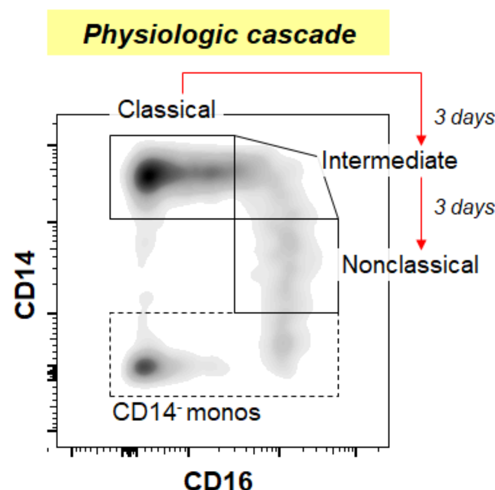

Fig. 22. Schematic representation of monocytes frequency in a healthy subject.[110]

After being released into the circulation, classical monocytes dead or migrate and leave the bloodstream, while a minority of them differentiate into intermediates within 3 days. Intermediates, instead, circulate for other 3 days before transiting to the non-classical phenotype. Finally, non-classical have the longest lifespan (7 days), and they ultimately leave the bloodstream.[104]
By analyzing monocyte position along their continuum in the CD16/CD14 plot, we provided incremental information on cardiovascular risk and outcomes, complementing the traditional assessment of monocyte subsets. In fact, patients with a condition of high cardiovascular risk are characterized by a shift of monocytes from classical to intermediates and a reduction of non-classical ones.[156] This kind of altered distribution be result of an enhanced differentiation of classical to intermediates, along with a reduced differentiation of intermediates into classical ones [Fig. 23].

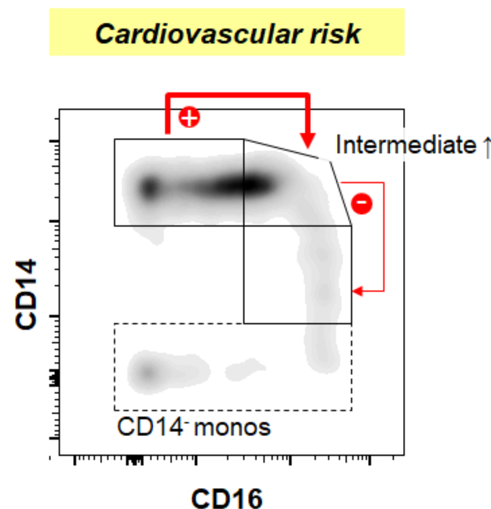

Fig. 23. Schematic representation of changes in monocytes frequency in a patient with cardiovascular risk.[110]

The meaning of the two additional small populations of monocytes $CD14^{neg}$ ($CD14^{-}CD16^{+}$ and $CD14^{-}CD16^{-}$) remains still unclear and needs further investigation;[156] we suggested they should represent phenotypes in-transition towards DCs or "aged" cells.
By scoring hundreds of plots, we observed that, differently from the traditional gating of monocyte subsets, the classical monocyte compartment, $CD14^{high}$ $CD16^{-/low}$ contained a bivariate distribution of $CD16^{neg}$ and $CD16^{dim}$ cells, with remarkable differences among patients. We thus speculated that significant heterogeneity within each monocyte subset may convey clinical information.[156]
Regarding the monocyte population as a continuum of subsets[103,123] we hypothesized that displacement of monocytes in the CD14 versus CD16 plot was better detectable by analyzing the

distribution, mean and standard deviation, of CD14 and CD16 fluorescence intensity rather than cell frequency in each discrete gate. For example, type 2 diabetic patients, despite having no changes in the monocyte subsets frequency, showed a consistent and highly significant shift of all subsets towards a higher CD16 expression.[156] We speculated that a generalized induction of CD16 may be related to cardiovascular risk in diabetes, because $CD16^+$ monocytes correlated with subclinical atherosclerosis in a large sample of healthy volunteers,[101] while CD16 deletion in murine models shows a reduction in the atherosclerosis development.[157]

Substantially, in patients with prevalent coronary artery disease, in addition to a doubled frequency of intermediate monocytes, the analysis of distribution revealed a shift of classical and non-classical towards intermediate monocytes. In CAD patients, an expansion of $CD16^{pos}$ monocytes has previously been noticed and is linked with levels of the pro-inflammatory cytokine TNF-α.[136]

Previously there has been several efforts to describe monocyte heterogeneity in a simplified way compared to than the traditional subdivision,[134] while the functional implications remained unknown.

We carried out a longitudinal patient evaluation with a follow-up of 4 years to establish whether an irregular monocyte distribution was predictive of future cardiovascular disease. Patients who occurred a MACE compared to those who did not, had baseline alterations in monocytes similar to those of CAD patients, characterized by higher levels of intermediate monocytes and a shift of classical and non-classical monocytes toward intermediate ones.[156] Because it was possible to confound the association with baseline CAD, a Multivariate Cox proportional hazard regression analyses has been used. In fully-adjusted models including baseline CAD, only higher CD16 expression on classical monocytes remained significantly associated with incident MACE: showing a 63%.

As already reported in literature, levels of either intermediate[104,158] or classical[149] monocytes predicted cardiovascular events. Our findings, by showing that distribution within specific monocyte subsets predict cardiovascular outcomes more than their frequencies, provide possible explanations for inconsistencies in our previous studies.[156]

This study has some limitations. First, the relatively small number of patients who experienced MACE, despite a reasonably long period of follow-up.

In addition, in this study monocytes were only defined based on CD14 and CD16 expression, while more antigenic specification has been recently performed to improve definition of monocyte phenotypes that may aid a better stratification of cardiovascular risk.[78] It should also be noted that, besides antigenic definition, additional work is required to profile these subsets in terms of function

and gene expression.[97,98] In fact, these subpopulations may be associated with differences in pro-inflammatory gene expression as previously shown e.g. in CAD patients.[136]
Finally, monocytes were only analyzed in the beginning of the study, while a time-course analysis may better clarify how stable are the baseline alterations observed and whether there are temporal trend preceding the MACE.

Certainly, a polychromatic flow cytometry analysis has also some methodological limitations. First of all the lack of consensus in the monocyte subtypes phenotype. Scoring the literature there are a lot of different immune-phenotype panels used to identify the subpopulations of monocytes.
In terms of technical performance, new consensus statements recommend the use of an additional pan-monocytic marker (such as CD86 or HLA-DR) but in this work we have only use the CD14 and the CD16.[76] The use these two markers alone might be limited by different gating strategies that may generate various phenotypic profile patterns.[107] Discrimination among the monocyte subpopulations is subjective and depends on the operator: may be affected by the selection of different gate, for example "rectangular" versus "trapezoidal" or more sophisticated gates.[159,160] Scoring CD14 versus CD16 plots in the literature, sometimes it seems that $CD14^{++} CD16^{low}$ subsets may be part of $CD14^{++} CD16^{+}$ subsets.[110] It depends on how CD16 negativity is defined. However, showing the entire population as an ideally infinite number of monocyte subsets described by the expression of CD14 and CD16 on monocytes, we conclude that distribution of key markers within specific monocyte subsets predicts cardiovascular outcomes more than the frequency of monocyte subsets.[156]
Another matter is providing percentages as opposed to absolute counts. Thus, also absolute counts of monocytes, neutrophils and lymphocytes based on white blood cell count may be useful in detecting significant differences in the monocyte population recorded at baseline. The confounding effects of an enhanced white blood cell count are often viewable in patients with high cardiovascular risk.[161] Absolute counts would be more relevant unless it were found that one subset provides to high levels of a particular risk factor and other subsets to lower levels of the same risk factor. In this regard, the absolute count of intermediate monocytes predict cardiovascular events in patients with kidney disease patients.[145,146]
On the other hand, during our research the use of the more objective "Clock Rule" analysis didn't show the expected prognostic information in term of cardiovascular risks and outcomes. A great amount of data were exported for this kind of analysis. The only significant difference observed was between the groups of patients with and without CAD only for the distribution of monocytes

displaying a higher CD16 expression and a lower CD14 expression, characteristic of the intermediate and non-classical populations. Instead, when comparing monocyte distribution of patients with and without MACE, no significant difference was observed. So, we can conclude that our first analysis based on CD14 and CD16 MFI provides a better prognostic information rather than the 'Clock Rule" analysis.

Our aim is not to propose a new way of monocytes analysis and this sophisticated flow cytometry approach should not be applied to define an individual patient's cardiovascular risk. On the other hand, our data straightforwardly demonstrate how the concept of monocyte continuum provides further information in cardiovascular disease and in the prediction of cardiovascular risk and should be used to study several pathological conditions in which monocytes are involved.

REFERENCES

1. Lusis A. Atherosclerosis. *Nature*. 2000;

2. Ross R. Atherosclerosis--an inflammatory disease. *N. Engl. J. Med.* 1999;

3. Hansson GK, Robertson A-KL, Söderberg-Nauclér C. INFLAMMATION AND ATHEROSCLEROSIS. *Annu. Rev. Pathol. Mech. Dis.* 2006;

4. Hansson GK. Inflammation, atherosclerosis and coronary artery disease. *N. Engl. J. Med.* 2005;

5. Herrington W, Lacey B, Sherliker P, Armitage J, Lewington S. Epidemiology of Atherosclerosis and the Potential to Reduce the Global Burden of Atherothrombotic Disease. *Circ. Res.* 2016;

6. Ross R, Glomset J, Harker L. Response to injury and atherogenesis. *Am J Pathol*. 1977;

7. POOLE JC, FLOREY HW. Changes in the endothelium of the aorta and the behaviour of macrophages in experimental atheroma of rabbits. *J. Pathol. Bacteriol.* 1958;

8. GIMBRONE MA. Endothelial Dysfunction and Atherosclerosis. *J. Card. Surg.* 1989;

9. Ross R. Cell biology of atherosclerosis. *Annu. Rev. Physiol.* 1995;

10. Ward MR, Pasterkamp G, Yeung AC, Borst C. Arterial Remodeling : Mechanisms and Clinical Implications. *Circulation*. 2000;

11. Caro GG. Discovery of the role of wall shear in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 2009;

12. Tedgui A. Cytokines in Atherosclerosis: Pathogenic and Regulatory Pathways. *Physiol. Rev.* 2006;

13. Rafieian-Kopaei M, Setorki M, Doudi M, Baradaran A, Nasri H. Atherosclerosis: Process, indicators, risk factors and new hopes. *Int. J. Prev. Med.* 2014;

14. Stary HC, Chandler a B, Dinsmore RE, et al. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 1995;

15. Hansson GK, Libby P. The immune response in atherosclerosis: a double-edged sword. *Nat. Rev. Immunol.* 2006;

16. Niessner A, Weyand CM. Dendritic cells in atherosclerotic disease. *Clin. Immunol.* 2010;

17. Kovanen PT. Mast cells: Multipotent local effector cells in atherothrombosis. *Immunol. Rev.* 2007;

18. Bentzon JF, Otsuka F, Virmani R, Falk E. Mechanisms of plaque formation and rupture. *Circ. Res.* 2014;

19. Badimon L, Vilahur G. Thrombosis formation on atherosclerotic lesions and plaque rupture. *J. Intern. Med.* 2014;

20. Li H, Horke S, Förstermann U. Vascular oxidative stress, nitric oxide and atherosclerosis. *Atherosclerosis*. 2014;

21. Linton MF, Yancey PG, Davies SS, et al. The Role of Lipids and Lipoproteins in Atherosclerosis. 2000;

22. Weber C, Zernecke A, Libby P. The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. *Nat. Rev. Immunol.* 2008;

23. Galley HF, Webster NR. Physiology of the endothelium. *Br. J. Anaesth.* 2004;

24. Davignon J, Ganz P. Role of endothelial dysfunction in atherosclerosis. *Circulation*. 2004;

25. Packard RRS, Lichtman AH, Libby P. Innate and adaptive immunity in atherosclerosis. *Semin. Immunopathol.* 2009;

26. Libby P, Ridker PM, Hansson GK. Inflammation in AtherosclerosisFrom Pathophysiology to Practice. *J. Am. Coll. Cardiol.* 2009;

27. Rocha VZ, Libby P. Obesity, inflammation, and atherosclerosis. *Nat. Rev. Cardiol.* 2009;

28. Ougrinovskaia A, Thompson RS, Myerscough MR. An ODE model of early stages of atherosclerosis: Mechanisms of the inflammatory response. *Bull. Math. Biol.* 2010;

29. Boring L, Gosling J, Cleary M, Charo IF. Decreased lesion formation in CCR2(-/-) mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature*. 1998;

30. Janeway C a, Medzhitov R. Innate immune recognition. *Annu. Rev. Immunol.* 2002;

31. Taleb S. L'inflammation dans l'athérosclérose. *Arch. Cardiovasc. Dis.* 2016;

32. Miteva K, Madonna R, De Caterina R, Van Linthout S. Innate and adaptive immunity in atherosclerosis. *Vascul. Pharmacol.* 2018;

33. Meng X, Yang J, Dong M, et al. Regulatory T cells in cardiovascular diseases. *Nat. Rev. Cardiol.* 2015;

34. Sherer Y, Shoenfeld Y. Mechanisms of disease: Atherosclerosis in autoimmune diseases. *Nat. Clin. Pract. Rheumatol.* 2006;

35. Baetta R, Corsini A. Role of polymorphonuclear neutrophils in atherosclerosis: Current state and future perspectives. *Atherosclerosis*. 2010;

36. Pashkow FJ. Oxidative Stress and Inflammation in Heart Disease: Do Antioxidants Have a Role in Treatment and/or Prevention? *Int. J. Inflam.* 2011;

37. Stocker R. Role of Oxidative Modifications in Atherosclerosis. *Physiol. Rev.* 2004;

38. Libby P, Libby P. Inflammation in atherosclerosis. *Nature*. 2002;

39. Wang JC, Bennett M. Aging and atherosclerosis: Mechanisms, functional consequences, and potential therapeutics for cellular senescence. *Circ. Res.* 2012;

40. Mendelsohn ME. Protective effects of estrogen on the cardiovascular system. *Am. J. Cardiol.* 2002;

41. Chen Z, Boreham J. Smoking and Cardiovascular Disease. *Semin. Vasc. Med.* 2002;

42. Gimbrone MA. Endothelial dysfunction, hemodynamic forces, and atherosclerosis. *Thromb. Haemost.* 1999;

43. American Diabetes Association. Standards of medical care in diabetes--2011. *Diabetes Care*. 2011;

44. Hadi HAR, Suwaidi J Al. Endothelial dysfunction in diabetes mellitus. *Vasc. Health Risk Manag.* 2007;

45. Morigi M, Angioletti S, Imberti B, et al. Leukocyte-endothelial interaction is augmented by high glucose concentrations and hyperglycemia in a NF-kB-dependent fashion. *J. Clin. Invest.* 1998;

46. Paoletti R, Bolego C, Poli A, Cignarella A. Metabolic syndrome, inflammation and atherosclerosis. *Vasc. Health Risk Manag.* 2006;

47. Fuster V, Badimon L, Cohen M, et al. Insights into the pathogenesis of acute ischemic syndromes. *Circulation*. 1988;

48. Libby P. The molecular mechanisms of the thrombotic complications of atherosclerosis. *J. Intern. Med.* 2008;

49. Bui QT, Prempeh M, Wilensky RL. Atherosclerotic plaque development. *Int. J. Biochem. Cell Biol.* 2009;

50. Virmani R, Burke AP, Willerson JT, et al. The Pathology of Vulnerable Plaque. *Vulnerable Atheroscler. Plaque Strateg. Diagnosis Manag.* 2007;

51. Shi C, Pamer EG. Monocyte Recruitment Suring Infection and Inflammation. *Nat Rev Immunol*. 2014;

52. van Furth R, Cohn ZA, Hirsch JG, et al. The mononuclear phagocyte system: a new classification of macrophages, monocytes, and their precursor cells. *Bull. World Health Organ.* 1972;

53. Geissmann F, Manz MG, Jung S, et al. Development of monocytes, macrophages, and dendritic cells. *Science (80-.).* 2010;

54. Hettinger J, Richards DM, Hansson J, et al. Origin of monocytes and macrophages in a committed progenitor. *Nat. Immunol.* 2013;

55. van Furth R, Cohn ZA. The origin and kinetics of mononuclear phagocytes. *J. Exp. Med.* 1968;

56. Le Douce V, Herbein G, Rohr O, Schwartz C. Molecular mechanisms of HIV-1 persistence in the monocyte-macrophage lineage. *Retrovirology*. 2010;

57. Ginhoux F, Jung S. Monocytes and macrophages: developmental pathways and tissue homeostasis TL - 14. *Nat. Rev. Immunol.* 2014;

58. Yona S, Kim KW, Wolf Y, et al. Fate Mapping Reveals Origins and Dynamics of Monocytes and Tissue Macrophages under Homeostasis. *Immunity*. 2013;

59. Hashimoto D, Chow A, Noizat C, et al. Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity*. 2013;

60. Hulsmans M, Sam F, Nahrendorf M. Monocyte and macrophage contributions to cardiac remodeling. *J. Mol. Cell. Cardiol.* 2016;

61. Hoeffel G, Ginhoux F. Ontogeny of tissue-resident macrophages. *Front. Immunol.* 2015;

62. Davies LC, Jenkins SJ, Allen JE, Taylor PR. Tissue-resident macrophages. *Nat. Immunol.* 2013;

63. Bain CC, Mowat AMI. The monocyte-macrophage axis in the intestine. *Cell. Immunol.* 2014;

64. Wynn T, Chawla A, Pollard J. Macrophage biology in development, homeostasis and disease. *Nature*. 2013;

65. Segura E, Amigorena S. Inflammatory dendritic cells in mice and humans. *Trends Immunol.* 2013;

66. Collin M, Mcgovern N, Haniffa M. Human dendritic cell subsets. *Immunology*. 2013;

67. Ziegler-Heitbrock HW. Definition of human blood monocytes. *J. Leukoc. Biol.* 2000;

68. Wong KL, Tai JJ-Y, Wong W-C, et al. Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. *Blood.* 2011;

69. Kantari C, Pederzoli-Ribeil M, Witko-Sarsat V. The role of neutrophils and monocytes in innate immunity. *Contrib. Microbiol.* 2008;

70. Zhao Y, Glesne D, Huberman E. A human peripheral blood monocyte-derived subset acts as pluripotent stem cells. *Proc. Natl. Acad. Sci.* 2003;

71. Woollard KJ, Geissmann F. Monocytes in atherosclerosis: Subsets and functions. *Nat. Rev. Cardiol.* 2010;

72. Ziegler-Heitbroc HWL, Thiel E, Futterer A, et al. Establishment of a human cell line (mono mac 6) with characteristics of mature monocytes. *Int. J. Cancer*. 1988;

73. Passlick B, Flieger D, Ziegler-Heitbrock HW. Identification and characterization of a novel monocyte subpopulation in human peripheral blood. *Blood*. 1989;

74. Ziegler-Heitbrock L, Ancuta P, Crowe S, et al. Nomenclature of monocytes and dendritic cells in blood. *Blood*. 2010;

75. Ziegler-heitbrock L, Ancuta P, Crowe S, et al. Nomenclature of monocytes and dendritic cells in blood Nomenclature of monocytes and dendritic cells in blood. 2014;

76. Weber C, Shantsila E, Hristov M, et al. Role and analysis of monocyte subsets in cardiovascular disease. *Thromb. Haemost.* 2016;

77. Boyette LB, MacEdo C, Hadi K, et al. Phenotype, function, and differentiation potential of human monocyte subsets. *PLoS One*. 2017;

78. Thomas GD, Hamers AAJ, Nakao C, et al. Human Blood Monocyte Subsets: A New Gating

Strategy Defined Using Cell Surface Markers Identified by Mass Cytometry. *Arterioscler. Thromb. Vasc. Biol.* 2017;

79. Ferrer DG, Jaldín-Fincati JR, Amigone JL, et al. Standardized flow cytometry assay for identification of human monocytic heterogeneity and LRP1 expression in monocyte subpopulations: Decreased expression of this receptor in nonclassical monocytes. *Cytom. Part A*. 2014;

80. Zawada AM, Rogacev KS, Schirmer SH, et al. Monocyte heterogeneity in human cardiovascular disease. *Immunobiology*. 2012;

81. Sunderkotter C, Nikolic T, Dillon MJ, et al. Subpopulations of Mouse Blood Monocytes Differ in Maturation Stage and Inflammatory Response. *J. Immunol.* 2004;

82. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. *Nat. Rev. Immunol.* 2005;

83. Ziegler-Heitbrock L, Hofer TPJ. Toward a refined definition of monocyte subsets. *Front. Immunol.* 2013;

84. Ziegler-Heitbrock L. Reprint of: Monocyte subsets in man and other species. *Cell. Immunol.* 2014;

85. Ancuta P, Liu KY, Misra V, et al. Transcriptional profiling reveals developmental relationship and distinct biological functions of CD16+ and CD16- monocyte subsets. *BMC Genomics*. 2009;

86. Auffray C, Fogg D, Garfa M, et al. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science (80-.)*. 2007;

87. Carlin LM, Stamatiades EG, Auffray C, et al. Nr4a1-dependent Ly6Clow monocytes monitor endothelial cells and orchestrate their disposal. *Cell*. 2013;

88. Saha P, Geissmann F. Toward a functional characterization of blood monocytes. *Immunol. Cell Biol.* 2011;

89. Skrzeczy??ska-Moncznik J, Bzowska M, Loseke S, et al. Peripheral blood CD14high CD16+ monocytes are main producers of IL-10. *Scand. J. Immunol.* 2008;

90. Mukherjee R, Kanti Barman P, Kumar Thatoi P, et al. Non-Classical monocytes display inflammatory features: Validation in Sepsis and Systemic Lupus Erythematous. *Sci. Rep.* 2015;

91. França CN, Izar MCO, Hortêncio MNS, et al. Monocyte subtypes and the CCR2 chemokine receptor in cardiovascular disease. *Clin. Sci.* 2017;

92. Cros J, Cagnard N, Woollard K, et al. Human CD14dimMonocytes Patrol and Sense Nucleic Acids and Viruses via TLR7 and TLR8 Receptors. *Immunity*. 2010;

93. Finak G, Langweiler M, Jaimes M, et al. Standardizing Flow Cytometry Immunophenotyping Analysis from the Human ImmunoPhenotyping Consortium. *Sci. Rep.* 2016;

94. Tamoutounour S, Guilliams M, Sanchis FM, et al. Origins and Functional Specialization of Macrophages and of Conventional and {Monocyte-Derived} Dendritic Cells in Mouse Skin.

Immunity. 2013;

95. Mantovani A, Martinez FO, Gordon S, Locati M. Gene Expression Polarization: New Molecules and Patterns of and Monocyte-to-Macrophage Differentiation Transcriptional Profiling of the Human Transcriptional Profiling of the Human Monocyte-to-Macrophage Differentiation and Polarization: New Molecules and. *J Immunol Mater. Suppl. J. Immunol.* 2018;

96. Shantsila E, Wrigley B, Tapp L, et al. Immunophenotypic characterization of human monocyte subsets: possible implications for cardiovascular disease pathophysiology. *J Thromb Haemost*. 2011;

97. Zawada AM, Rogacev KS, Rotter B, et al. SuperSAGE evidence for CD14 ++CD16 + monocytes as a third monocyte subset. *Blood*. 2011;

98. Gren ST, Rasmussen TB, Janciauskiene S, et al. A single-cell gene-expression profile reveals inter-cellular heterogeneity within human monocyte subsets. *PLoS One*. 2015;

99. Poehlmann H, Schefold JC, Zuckermann-Becker H, Volk H-D, Meisel C. Phenotype changes and impaired function of dendritic cell subsets in patients with sepsis: a prospective observational analysis. *Crit. Care*. 2009;

100. Rogacev KS, Seiler S, Zawada AM, et al. CD14++CD16+ monocytes and cardiovascular outcome in patients with chronic kidney disease. *Eur. Heart J.* 2011;

101. Rogacev KS, Ulrich C, Blömer L, et al. Monocyte heterogeneity in obesity and subclinical atherosclerosis. *Eur. Heart J.* 2010;

102. Thiesen S, Janciauskiene S, Uronen-Hansson H, et al. CD14hiHLA-DRdim macrophages, with a resemblance to classical blood monocytes, dominate inflamed mucosa in Crohn's disease. *J. Leukoc. Biol.* 2014;

103. Hijdra D, Vorselaars ADM, Grutters JC, Claessen AME, Rijkers GT. Phenotypic Characterization of Human Intermediate Monocytes. *Front. Immunol.* 2013;

104. Patel AA, Zhang Y, Fullerton JN, et al. The fate and lifespan of human monocyte subsets in steady state and systemic inflammation. *J. Exp. Med.* 2017;

105. Tacke F, Alvarez D, Kaplan TJ, et al. Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. *J. Clin. Invest.* 2007;

106. Swirski FK, Nahrendorf M, Etzrodt M, et al. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science* 2009;

107. Appleby LJ, Nausch N, Midzi N, et al. Sources of heterogeneity in human monocyte subsets. *Immunol. Lett.* 2013;

108. Gratchev A, Sobenin I, Orekhov A, Kzhyshkowska J. Monocytes as a diagnostic marker of cardiovascular diseases. *Immunobiology*. 2012;

109. Stansfield BK, Ingram DA. Clinical significance of monocyte heterogeneity. *Clin. Transl. Med.* 2015;

110. Cignarella A, Tedesco S, Cappellari R, Fadini GP. The continuum of monocyte phenotypes:

Experimental evidence and prognostic utility in assessing cardiovascular risk. *J. Leukoc. Biol.* 2018;

111. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* 2008;

112. Italiani P, Boraschi D. From monocytes to M1/M2 macrophages: Phenotypical vs. functional differentiation. *Front. Immunol.* 2014;

113. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep.* 2014;

114. Sica A, Mantovani A. Macrophage plasticity and polarization: In vivo veritas. *J. Clin. Invest.* 2012;

115. Murray PJ, Allen JE, Biswas SK, et al. Macrophage Activation and Polarization: Nomenclature and Experimental Guidelines. *Immunity*. 2014;

116. Fadini GP, De Kreutzenberg SV, Boscaro E, et al. An unbalanced monocyte polarisation in peripheral blood and bone marrow of patients with type 2 diabetes has an impact on microangiopathy. *Diabetologia*. 2013;

117. Fadini GP, Cappellari R, Mazzucato M, et al. Monocyte-macrophage polarization balance in pre-diabetic individuals. *Acta Diabetol.* 2013;

118. Xue J, Schmidt S V, Sander J, et al. Transcriptome-based network analysis reveals a spectrum model of human macrophage activation. *Immunity*. 2014;

119. Nahrendorf M, Swirski FK. Abandoning M1/M2 for a network model of macrophage function. *Circ. Res.* 2016;

120. Piccolo V, Curina A, Genua M, et al. Opposing macrophage polarization programs show extensive epigenomic and transcriptional cross-talk. *Nat. Immunol.* 2017;

121. Hristov M, Heine GH. Monocyte subsets in atherosclerosis. *Hamostaseologie*. 2015;

122. Moore KJ, Sheedy FJ, Fisher EA. Macrophages in atherosclerosis: A dynamic balance. *Nat. Rev. Immunol.* 2013;

123. Ammirati E, Moroni F, Magnoni M, et al. Circulating CD14+ and CD14highCD16– classical monocytes are reduced in patients with signs of plaque neovascularization in the carotid artery. *Atherosclerosis*. 2016;

124. Heidt T, Courties G, Dutta P, et al. Differential contribution of monocytes to heart macrophages in steady-state and after myocardial infarction. *Circ. Res.* 2014;

125. Schloss MJ, Hilby M, Nitz K, et al. Ly6Chigh Monocytes Oscillate in the Heart During Homeostasis and After Myocardial Infarction-Brief Report. *Arterioscler. Thromb. Vasc. Biol.* 2017;

126. Horne BDB, Anderson JLJ, John JJM, et al. Which white blood cell subtypes predict increased cardiovascular risk? *J Am Coll Cardiol.* 2005;

127. Gurm HS, Bhatt DL, Lincoff AM, et al. Impact of preprocedural white blood cell count on

long term mortality after percutaneous coronary intervention: insights from the EPIC, EPILOG, and EPISTENT trials. *Heart*. 2003;

128. Coller BS. Leukocytosis and ischemic vascular disease morbidity and mortality: is it time to intervene? *Arter. Thromb Vasc Biol*. 2005;

129. Rothe G, Herr AS, Stöhr J, et al. A more mature phenotype of blood mononuclear phagocytes is induced by fluvastatin treatment in hypercholesterolemic patients with coronary heart disease. *Atherosclerosis*. 1999;

130. Poitou C, Dalmas E, Renovato M, et al. $CD14^{dim}CD16^{+}$ and $CD14^{+}CD16^{+}$ monocytes in obesity and during weight loss: Relationships with fat mass and subclinical atherosclerosis. *Arter. Thromb Vasc Biol*. 2011;

131. Timmerman KL, Flynn MG, Coen PM, Markofski MM, Pence BD. Exercise training-induced lowering of inflammatory (CD14+CD16+) monocytes: a role in the anti-inflammatory influence of exercise? *J. Leukoc. Biol.* 2008;

132. de Matos MA, Duarte TC, Ottone V de O, et al. The effect of insulin resistance and exercise on the percentage of CD16+ monocyte subset in obese individuals. *Cell Biochem. Funct.* 2016;

133. Patel VK, Williams H, Li SCH, Fletcher JP, Medbury HJ. Monocyte inflammatory profile is specific for individuals and associated with altered blood lipid levels. *Atherosclerosis*. 2017;

134. Ziegler-Heitbrock L. Blood monocytes and their subsets: Established features and open questions. *Front. Immunol.* 2015;

135. Ulrich C, Heine GH, Garcia P, et al. Increased expression of monocytic angiotensin-converting enzyme in dialysis patients with cardiovascular disease. *Nephrol. Dial. Transplant*. 2006;

136. Schlitt A, Heine GH, Blankenberg S, et al. CD14+CD16+ monocytes in coronary artery disease and their relationship to serum TNF-α levels. *Thromb. Haemost.* 2004;

137. Tapp LD, Shantsila E, Wrigley BJ, Pamukcu B, Lip GYH. The CD14++CD16+ monocyte subset and monocyte-platelet interactions in patients with ST-elevation myocardial infarction. *J. Thromb. Haemost.* 2012;

138. Kaito M, Araya SI, Gondo Y, et al. Relevance of Distinct Monocyte Subsets to Clinical Course of Ischemic Stroke Patients. *PLoS One*. 2013;

139. Urra X, Villamor N, Amaro S, et al. Monocyte subtypes predict clinical course and prognosis in human stroke. *J. Cereb. Blood Flow Metab.* 2009;

140. Shantsila E, Tapp LD, Wrigley BJ, et al. Monocyte subsets in coronary artery disease and their associations with markers of inflammation and fibrinolysis. *Atherosclerosis*. 2014;

141. Fadini GP, Simoni F, Cappellari R, et al. Pro-inflammatory monocyte-macrophage polarization imbalance in human hypercholesterolemia and atherosclerosis. *Atherosclerosis*. 2014;

142. Hatanaka E, Monteagudo PT, Marrocos MSM, Campa A. Neutrophils and monocytes as potentially important sources of proinflammatory cytokines in diabetes. *Clin. Exp. Immunol.*

2006;

143. Horvath P, Oliver SR, Ganesan G, et al. Fasting glucose level modulates cell surface expression of CD11b and CD66b in granulocytes and monocytes of patients with type 2 diabetes. *J. Investig. Med.* 2013;

144. Latet SC, Van Craenenbroeck AH, Van Herck PL, et al. A critical view of monocyte subpopulations in human hypercholesterolemia. *Atherosclerosis*. 2016;

145. Rogacev KS, Cremers B, Zawada AM, et al. CD14++CD16+ monocytes independently predict cardiovascular events: A cohort study of 951 patients referred for elective coronary angiography. *J. Am. Coll. Cardiol.* 2012;

146. Heine GH, Fliser The Fundación Jiménez Díaz D, Institutet K, et al. Monocyte subpopulations and cardiovascular risk in chronic kidney disease. *Nat. Rev. Nephrol.* 2012;

147. Heine GH, Ulrich C, Seibert E, et al. CD14++CD16+monocytes but not total monocyte numbers predict cardiovascular events in dialysis patients. *Kidney Int.* 2008;

148. M. H, T. L, C. S, et al. Monocyte heterogeneity and cardiovascular risk factors in coronary artery disease. *Eur. Heart J.* 2010;

149. Berg KE, Ljungcrantz I, Andersson L, et al. Elevated CD14++CD16-monocytes predict cardiovascular events. *Circ. Cardiovasc. Genet.* 2012;

150. Van Der Laan AM, Hirsch A, Robbers LFHJ, et al. A proinflammatory monocyte response is associated with myocardial injury and impaired functional outcome in patients with ST-segment elevation myocardial infarction: Monocytes and myocardial infarction. *Am. Heart J.* 2012;

151. Wildgruber M, Czubba M, Aschenbrenner T, et al. Increased intermediate CD14++CD16++monocyte subset levels associate with restenosis after peripheral percutaneous transluminal angioplasty. *Atherosclerosis*. 2016;

152. Fingerle-Rowson, Angstwurm, Andreesen, Ziegler-Heitbrock. Selective depletion of CD14+ CD16+ monocytes by glucocorticoid therapy. *Clin. Exp. Immunol.* 1998;

153. American Diabetes Association. Diagnosing Diabetes and Learning About Prediabetes. *Www.Diabetes.Org/Diabetes-Basics/Diagnosis*. 2015;

154. Xie P, Huang JM, Lin HY, Wu WJ, Pan LP. CDK-EPI equation may be the most proper formula based on creatinine in determining glomerular filtration rate in Chinese patients with chronic kidney disease. *Int. Urol. Nephrol.* 2013;

155. Pencina MJ, D'Agostino RB, Demler O V. Novel metrics for evaluating improvement in discrimination: Net reclassification and integrated discrimination improvement for normal variables and nested models. *Stat. Med.* 2012;

156. Cappellari R, D'Anna M, Bonora BM, et al. Shift of monocyte subsets along their continuum predicts cardiovascular outcomes. *Atherosclerosis*. 2017;

157. Kelly JA, Griffin ME, Fava RA, et al. Inhibition of arterial lesion progression in CD16-deficient mice: Evidence for altered immunity and the role of IL-10. *Cardiovasc. Res.* 2010;

158. Wildgruber M, Aschenbrenner T, Wendorff H, et al. The ‘intermediate’ $CD14^{++}$ $CD16^{+}$ monocyte subset increases in severe peripheral artery disease in humans. *Sci. Rep.* 2016;

159. Lambert C, Preijers FWMB, Yanikkaya Demirel G, Sack U. Monocytes and macrophages in flow: an ESCCA initiative on advanced analyses of monocyte lineage using flow cytometry. *Cytom. Part B - Clin. Cytom.* 2017;

160. Zawada AM, Fell LH, Untersteller K, et al. Comparison of two different strategies for human monocyte subsets gating within the large-scale prospective CARE FOR HOMe Study. *Cytometry. A*. 2015;

161. Yarnell JW, Baker I a, Sweetnam PM, et al. Fibrinogen, viscosity, and white blood cell count are major risk factors for ischemic heart disease. The Caerphilly and Speedwell collaborative heart disease studies. *Circulation*. 1991.