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$CD16^+$ monocyte subsets are increased in large abdominal aortic aneurysms and are differentially related with circulating and cell-associated biochemical and inflammatory biomarkers

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Abstract. Proinflammatory components are present in abdominal aortic aneurysm (AAA). Circulating monocytes display heterogeneity, and three subsets have been identified, based on the differential expression for CD14 and CD16 receptors: $CD14^+CD16^-$, classical, $CD14^+CD16^+$, intermediate and $CD14^{dim}CD16^+$, non-classical monocytes. Increased proinflammatory $CD16^+$ monocytes with high expression of CD143 are present in CKD patients. D-dimer is increased in AAA patients, and might contribute to the pro-inflammatory response associated to circulating monocytes. We aimed to investigate the frequency of $CD14^+CD16^+$, $CD14^{dim}CD16^+$ monocytes and monocyte CD143 expression in AAA patients, and their relationship with D-dimer, eGFR and other inflammatory parameters. Blood from 74 AAA patients and 30 healthy controls was analyzed to determine the frequency of $CD14^+CD16^+$, $CD14^{dim}CD16^+$ monocytes and the monocyte CD143 expression by means of flow-cytometry. AAA patients had expanded $CD16^+$ subsets ($CD14^+CD16^+$: $7.66 \pm 0.31\%$ vs $5.42 \pm 0.27\%$; $CD14^{dim}CD16^+$: $7.43 \pm 0.48\%$ vs $5.54 \pm 0.38\%$, AAA vs controls, mean $\pm$ SE, both $p < 0.05$). $CD14^+CD16^+$ cells were associated to D-dimer and age, and to reduced eGFR. $CD14^{dim}CD16^+$ cells were associated to uric acid, surface CD143, and reduced count of total leukocytes and neutrophils. Within AAA patients, the two $CD16^+$ subsets and the monocyte CD143 expression display different relationships with D-dimer, parameters of renal function and circulating biochemical and inflammatory biomarkers.

Keywords: Monocytes, CD14 antigen, D-dimer, immunoglobulin G receptor, chronic renal failure, angiotensin converting enzyme

1. Introduction

Current research suggests that atherosclerosis, genetic [1], environmental [2] and immunological [3] factors contribute to the onset and the development of abdominal aortic aneurysms (AAA). Important histological features of the chronic inflammatory response

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seen in AAA include extreme medial thinning associated to vascular smooth muscle cells (SMC) apoptosis, adventitial inflammatory cell infiltration, elastin fragmentation and degeneration, that are rarely seen in atherosclerotic lesions of other districts, such as carotid and coronary atherosclerosis [4–6].

Chronic inflammation participates to the pathogenesis of AAA. In addition to neutrophils [7] and lymphocytes [8], monocytes may play a role for the progression of damage in AAA by migrating through the endothelium and differentiating into tissue macrophages.

Male apoE(-/-) mice infused with Angiotensin II (AngII) to induce AAA showed presence of monocyte-derived macrophages in the media, adventitia and perivascular adipose tissue of new formed AAA; after long-term infusion (3 months) of AngII, macrophages became the predominant leukocyte population within AAA [9]. Similar pathologic features have been demonstrated in human AAAs, suggesting that local vascular inflammation contributes to AAAs [10].

Monocytes and monocyte-derived macrophages are heterogeneous for phenotype and function and different subsets raise in response to local cytokine environment [11]. Monocyte subsets exert different effects on immunity, vascular homeostasis, atherogenesis and reparative processes.

In humans, monocyte subpopulations can be distinguished on the basis of the expression of surface markers CD14 (co-receptor for LPS) and CD16 (receptor for FcγRIII). Most of monocytes from healthy subjects are CD14$^+$CD16$^-$; CD16$^+$ monocytes increase their frequency in response to chronic inflammatory conditions, such as chronic kidney disease (CKD). Nockher reported that the subset of monocytes expressing CD16 together with low levels of CD14 was expanded in dialysis patients [12]. More recently, it has been shown that CD16$^+$ monocytes are further differentiated by phenotype into CD14$^+$CD16$^+$ and CD14^{dim}CD16$^+$ monocytes [13]. Monocytes have been thus classified as "classical monocytes", CD14$^+$CD16$^-$, representing about the 85% of the monocyte population in healthy subjects, "intermediate monocytes", CD14$^+$CD16$^+$, and "non-classical", CD14^{dim}CD16$^+$ [14].

Angiotensin converting enzyme (ACE), detected as CD143, is present at high levels on monocytes [15]. Monocytic ACE expression was found highest among CD14$^+$CD16$^+$ monocytes; monocyte CD143 expression was increased in dialysis patients with prevalent cardiovascular disease and in patients with congestive heart failure (CHF) [16,17]. Alterations in monocyte CD143 expression might be related to the process of vascular damage during development of atherosclerotic and of AAA lesions [18,19], since the product of CD143, angiotensin II, promotes pro-oxidant bursts, endothelial release of proinflammatory cytokines and leukocyte adhesion to the vessel wall [20].

ACE-stained areas attributable to macrophages are also increased in coronary atherosclerotic plaques retrieved with directional coronary atherectomy derived from patients with unstable angina, when compared to plaques retrieved from patients with stable angina [21].

AAA patients are characterized by a chronic low grade inflammation and by increased levels of D-dimer, a biomarker of haemostatic activation [22]. *In vitro*, D-dimer and other fibrin degradation products promote multiple proinflammatory effects [23]. Fibrin turnover at the level of AAA covered with a mural thrombus might contribute to the systemic inflammatory response associated to circulating monocytes. Ulrich et al. in a previous study established that out of CD16$^+$ monocytes, only high levels of CD14$^+$CD16$^+$ monocyte subset in CKD patients were associated with reduced survival at 35 months [24]. We hypothesized that an altered pattern of CD16$^+$ monocyte subsets might be present in patients with AAA, representing a functional profile favorable to the progression vascular damage. In the present work, we determined the circulating levels of monocyte subsets and of monocyte CD143 expression in AAA patients and in a group of healthy adults, and investigated the relationship among these monocyte subsets, D-dimer levels, estimated Glomerular Filtration Rate (eGFR) as a measure of renal function, and other laboratory parameters of inflammation.

2. Materials and methods

The investigation conforms to the principles outlined in the Declaration of Helsinki (Code of Ethics of the World Medical Association). The study was approved by the Ethics Committee at our institution, and informed consent from the patients and the controls in the study was obtained. Seventy-four consecutive Caucasian male AAA patients undergoing elective open repair for an asymptomatic infrarenal AAA (aneurysm diameter of at least 5.5 cm) between September 2009 and October 2011 at the Unit of Vascular and Endovascular Surgery of the University of Genova (Italy) were included in our study. All AAA patients demonstrated the presence of intraluminal thrombus- covered aneurismal walls at a pre-operative aortic imaging carried out by computed tomography angiography (CTA) scans. Thir-

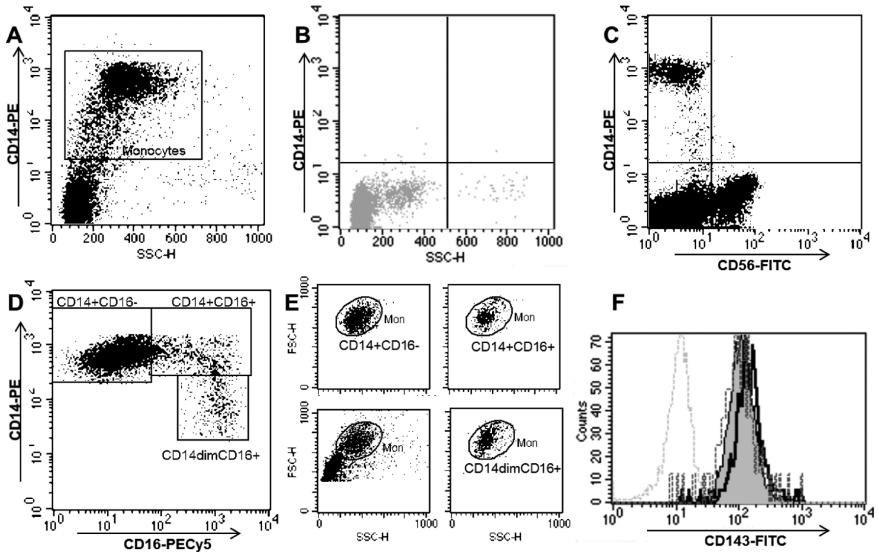

Fig. 1. Representative example of the monocyte subsets analysis procedure. A) dot plot of CD14 gating on monocytes, and B) isotypic negative control staining; C) dot plot showing CD56 staining of mononuclear cells to exclude the presence of CD56 positive NK cells; D) representative dot plot of the three monocyte subsets CD14$^+$CD16$^-$, CD14$^+$CD16$^+$, CD14^{dim} CD16$^+$; E) morphological plots illustrating the back gating of the three monocyte subsets to evidence size and granularity heterogeneity: total mononuclear cells with a reference monocyte gate and CD14$^+$CD16$^-$, CD14$^+$CD16$^+$, CD14^{dim}CD16$^+$ monocyte subsets are shown; F) representative histograms of monocyte CD143 fluorescence; CD14$^+$CD16$^-$monocytes (grey filled line), CD14$^+$CD16$^+$ subset (black solid line), CD14^{dim}CD16$^+$ subset (dotted line) and isotypic control (dashed light grey line) are illustrated. FSC–H: forward scatter; SSC-H: side scatter.

ty Caucasian male control subjects (Controls) with non-dilated infrarenal aortas ($<$ 30 mm, confirmed with abdominal ultrasound), and with one or more major modifiable cardiovascular risk factors were recruited from our outpatient cardiac service. Study subjects with concomitant major diseases such as infections, osteoarthritis, cancer, autoimmune disease, chronic liver disease or eGFR below 30 ml/min/1.73 m^2 body surface area were excluded.

2.1. Laboratory methods

Fasting blood samples were collected from all study subjects in the morning after resting in the supine position for 20 minutes. D-dimer was assayed using the BC D-dimer PLUS turbidimetric assay from Dade Behring, Marburg, Germany [25]. Serum fibrinogen, white blood cell count (WBC), plasma glucose, lipid and renal profile, total protein, albumin, C-reactive protein (CRP) were obtained using standard techniques. Baseline eGFR was estimated using the four-variable Modification of Diet in Renal Disease formula [26]. Blood was withdrawn from an antecubital vein and then centrifuged at 1710 g for 15 minutes at 4°C. Immediately after centrifugation, serum and plasma samples were stored at -80°C until they were assayed.

Monocyte analysis: mononuclear cells were isolated from blood by density gradient centrifugation over Ficoll, and were immunostained with the following monoclonal antibodies: anti-CD143-FITC antibody (clone 9B9, GeneTex, Inc.), anti-CD14 PE antibody (clone RMO52; Beckman Coulter), anti-CD16 PE-Cy5 (clone 3G8; Biolegend). After centrifugation, cells were washed twice and resuspended in phosphate-buffered saline containing 0.1% bovine serum albumin, 1 mM $CaCl_2$ and $MgCl_2$.

Monocyte subpopulations were analysed by flow-cytometry (FACS calibur and CellQuest software from Becton Dickinson). Monocytes were distinguished on the basis of morphology and of SSC/CD14 dotplot where a gate was set, based on isotypic control staining, on all CD14 positive cells. Moreover, the presence of natural killer (NK) cells, most of which are CD16 positive and could interfere with CD16$^+$ monocytes count, was checked by CD56 antibody. To this purpose, PBMCs were stained for CD14-PE/CD16-PECy5/CD56-FITC to check whether CD56 positive NK cells are excluded from CD14 monocyte gate.

Monocyte subsets CD14$^+$CD16$^-$, CD14$^+$CD16$^+$ and CD14^{dim}CD16$^+$ were defined according to the surface expression of the lipopolysaccharide receptor CD14 and of the Fc-gamma receptor CD16 (Fig. 1).

Table 1
Nomenclature of CD143 Ratios within monocyte subsets

R1:	$\frac{\text{CD143 on CD14}^+\text{CD16}^+\text{cells}}{\text{CD143 on CD14}^+\text{CD16}^-\text{cells}}$
R2:	$\frac{\text{CD143 on CD14}^{dim}\text{CD16}^+\text{cells}}{\text{CD143 on CD14}^+\text{CD16}^-\text{cells}}$
R3:	$\frac{\text{CD143 on CD14}^+\text{CD16}^+\text{cells}}{\text{CD143 on CD14}^{dim}\text{CD16}^+\text{cells}}$

Monocyte surface expression of CD143 was reported both as total amount, and within the three monocyte subsets. In addition, the variations in CD143 expression between monocyte subsets were calculated as ratios, R. Three ratios (R1, R2, R3) are reported in Table 1.

2.2. Statistical analysis

Data were assessed for normality using a D'Agostino -Pearson test. Normally distributed data are expressed as mean ± standard error (SE). Variables with a skewed distribution were presented as median (including the first and third quartiles), and were log transformed to normalise the distribution before performing statistical analysis. Categorical variables are presented as a proportion (%) and compared by Fisher's exact test. Two-sided t tests were used in the comparison of normally distributed continuous variables, while Mann Whitney test was used for non-Gaussian data. The association of CD14$^+$CD16$^-$, of CD14$^+$CD16$^+$ and of CD14^{dim}CD16$^+$ measurements with other biochemical parameters was assessed by the Pearson correlation coefficient (Pearson correlation coefficient r, univariate analysis). All variables with a p-value < 0.1 in the univariate analysis were included in multivariate linear regression analysis to determine independent predictors of CD14$^+$CD16$^+$ and of CD14^{dim}CD16$^+$ cell measurements in the study subjects.

3. Results

3.1. Demographics

Relevant characteristics of the two study groups are shown in Table 2. Age range for study subjects was 56–83 years. AAA patients had more frequently history of previous smoking habit and, as expected, had more elevated blood D-dimer, fibrinogen and CRP levels when compared to Controls. A trend for a statistically significant difference between the two study groups was also present for a personal history of hypercholesterolemia (p:0.15), for the levels of systolic blood pressure (p:0.10) and for neutrophil count (p:0.08). Finally, stage of renal damage (CKD) revealed significant differences between AAA patients and Controls, with a worse renal function for AAA patients (Table 2).

3.2. Monocyte subset concentrations in the two study groups

CD14$^+$CD16$^-$ monocytes levels were lower in AAA patients than Controls (mean ± SE: 79.06 ± 0.82 vs 85.53 ± 0.7, $p < 0.0001$ AAA vs Controls).

Both frequencies of CD14$^+$CD16$^+$ and of CD14^{dim} CD16$^+$ monocytes were significantly increased in AAA patients compared to Controls (mean ± SE: 7.66 ± 0.31 vs 5.42 ± 0.27, $p < 0.0001$ and 7.43 ± 0.48 vs 5.54 ± 0.38, p: 0.02, respectively, AAA vs Controls).

3.3. Atherothrombotic risk factors, CD14$^+$CD16$^-$, CD14$^+$CD16$^+$ and CD14^{dim} CD16$^+$ subset concentrations in the study groups

Separate analysis regarding the association between plasma CD14$^+$CD16$^-$, CD14$^+$CD16$^+$ and CD14^{dim}CD16$^+$ subset concentrations and previous smoking habit showed no significant differences between the two study groups. Similarly, personal history of hypercholesterolemia and levels of systolic blood pressure were not associated to significant differences for the three monocyte subset concentrations between the two study groups (data not shown). ACE inhibitors, lipid lowering drugs and beta-blockers are known to possess anti-inflammatory effects. Nonetheless, we observed no differences in monocyte subset levels within the two study groups, irrespective whether they were taking these drugs or not. All AAA patients had evidence of intraluminal thrombus at CTA scan. Monocyte subsets were not correlated with either the morphology of aneurysm (sacciform aneurysm vs non-sacciform aneurysm, p: NS) or to aneurysm size, evaluated as anteroposterior diameter (p: NS).

3.4. Associations of circulating laboratory parameters with CD14$^+$CD16$^-$, CD14$^+$CD16$^+$ and CD14^{dim}CD16$^+$ subset concentrations in AAA patients

CD14$^+$CD16$^-$ levels showed a direct association with eGFR (Table 3). CD14$^+$CD16$^+$ subset frequency was associated inversely with eGFR and positively with age and D-dimer values (Fig. 2 and Table 3).

Table 2
Demographics and laboratory tests in the two study groups

	AAA patients (74)	Controls (30)	P
Age, (years)	72.1 ± 0.7	69.4 ± 1.4	NS
Heart rate, (bpm)	65.4 ± 1.6	72.4 ± 3.8	NS
Ejection Fraction, (%)	56.8 ± 1.9	59.5 ± 0.7	NS
BMI, (Kgm^{-2})	26.2 ± 0.5	26.8 ± 0.9	NS
Systolic blood Pressure, (mm Hg)	141.9 ± 1.9	135.9 ± 2.8	NS
Diastolic blood Pressure, (mm Hg)	84.9 ± 0.9	84.1 ± 1.8	NS
Famil Hx of IHD, (%)	18%	14%	NS
Previous myocardial Infarction, (%)	21%	0%	–
Hx of chronic atrial fibrillation, (%)	14%	0%	–
Current Smoker, (%)	38%	22%	NS
Hx of Smoking, (%)	89%	60%	0.003
Hx of Hypertension, (%)	83%	79%	NS
Hx of Hypercholesterolaemia, (%)	85%	70%	NS
Presence of CKD			
Normal-Stage 1	11%	20%	0.002
Stage 2	57%	73%	
Stage 3	32%	2%	
Concomitant treatment			
Calcium-blockers, (%)	12%	19%	NS
ACE-inhibitors, (%)	37%	55%	NS
Diuretics, (%)	21%	33%	NS
B-blockers, (%)	29%	54%	0.04
Aspirin, (%)	38%	29%	NS
Oral or subcutaneous anticoagulant, (%)	7%	0%	–
Lipid lowering drugs, (%)	46%	29%	NS
Laboratory tests			
CD 16- cells, (%)	79.06 ± 0.82	85.53 ± 0.7	0.0001
CD16$^+$ cells, (%)	15.05 ± 0.60	10.96 ± 0.55	0.0001
CD14$^+$CD16$^+$, (%)	7.66 ± 0.31	5.42 ± 0.27	0.0001
CD14^{dim}CD16$^+$, (%)	7.43 ± 0.48	5.54 ± 0.38	0.02
Uric acid, (mg dL^{-1})	6.2 ± 0.2	5.9 ± 0.3	NS
Sodium, (mEq L^{-1})	142.2 ± 0.3	141.7 ± 0.4	NS
Potassium, (mEq L^{-1})	4.1 ± 0.1	4.0 ± 0.1	NS
Creatinine, (mg dL^{-1})	1.13 ± 0.03	1.00 ± 0.03	0.023
eGFR, (ml/min/1.73 m^2)	67.5 ± 2.0	76.8 ± 2.3	0.0093
Cholesterol, (mg dL^{-1})	208 ± 5.3	200 ± 7.7	NS
Triglycerides, (mg dL^{-1})	125 (96–186)	93 (74–172)	NS
HDL-cholesterol, (mg dL^{-1})	48.2 ± 1.6	51.6 ± 2.9	NS
Glycemia, (mg dL^{-1})	101 ± 2.2	104 ± 3.1	NS
Serum fibrinogen, (mg dL^{-1})	356 (302–432)	316 (284–368)	0.04
D-dimer, (ng mL^{-1})	370.9 ± 41.4	186.2 ± 17.3	0.0049
C-reactive protein, (mg L^{-1})	7.1 ± 0.8	3.8 ± 0.3	0.03
White blood cell count, (mm^{-3})	7076 ± 201	6798 ± 240	NS
Neutrophil Count, (mm^{-3})	4685 ± 155	4184 ± 211	NS
Monocyte Count, (mm^{-3})	484 ± 16	498 ± 29	NS
Homocysteine, (microMol/L)	17.1 ± 1.0	14.2 ± 0.9	NS

Values are expressed as mean (SE), median (interquartile range) or as percentage (%); BMI body mass index; Hx history; IHD ischemic heart disease; CKD, chronic kidney disease; ACE angiotensin converting enzyme; eGFR estimated Glomerular Filtration Rate; HDL high density lipoprotein. – not determined.

Multiple linear regression analysis revealed that D-dimer levels only were independently correlated with CD14$^+$CD16$^+$ subset concentrations (p:0.002). Because declining renal function and age are important predictors of CD14$^+$CD16$^+$ subset levels, we also analysed data from our two study groups on the basis of either similar stage of renal damage or similar arbitrarily selected age intervals (Table 4). Counts of CD14$^+$CD16$^+$cells significantly differed between AAA and Controls with CKD stage 2, and showed a trend (p:0.11) for a statistically significant difference between the two study groups with normal-stage 1 CKD. The two study groups belonging to the same CKD stage did not differ for mean age. In relation to age, counts of CD14$^+$CD16$^+$ monocytes significantly differed between AAA patients and Controls in the age

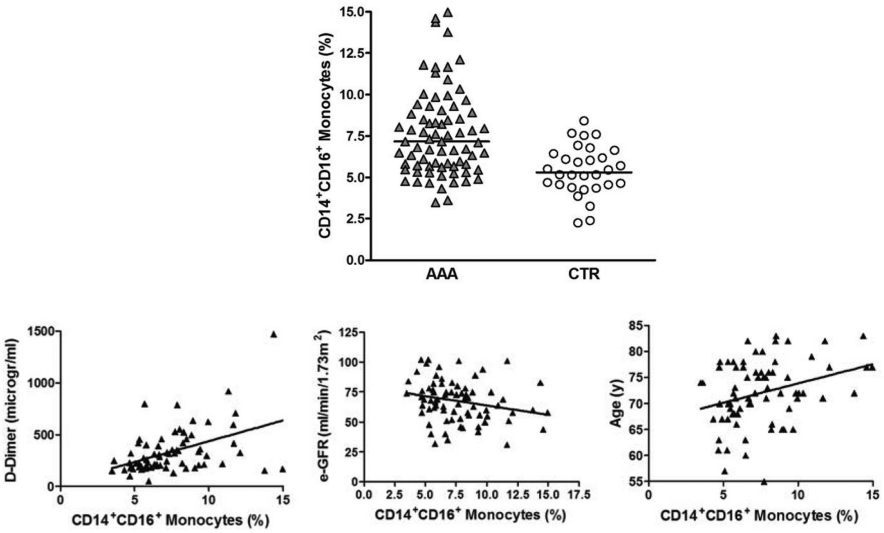

Fig. 2. $CD14^+CD16^+$ monocyte results. Top panel: Scattergram of $CD14^+CD16^+$ monocyte percentages in AAA patients (filled triangles) and in Controls (circles). Median lines are represented. Results were compared by the two-sided t test (p: 0.0001, AAA patients vs Controls). Bottom panels: Relationships of $CD14^+CD16^+$ monocyte percentages with D-Dimer, eGFR and Age in AAA patients: scattergrams and regression lines are illustrated.

intervals of 65–74 yrs and of 75–83 yrs, while no differences were shown between the two study groups of 56–64 yrs.

Significant increases of $CD14^{dim}CD16^+$ subset were predicted by increased levels of uric acid, of monocyte CD143 expression and by reduced counts of total leukocytes and neutrophils (Fig. 3 and Table 3). Multiple linear regression analysis revealed that uric acid levels only were independently correlated with $CD14^{dim}CD16^+$ subset concentrations (p:0.01).

3.5. *Monocyte surface CD143 expression in the two study groups, CD143 differences between subsets, and associations with circulating laboratory parameters in AAA patients*

Monocyte surface CD143 showed no significant changes between AAA patients and Controls, both when considering its expression on total monocytes and on monocyte subsets (Table 5). CD143 was differentially expressed among monocyte subsets: the $CD14^+CD16^+$ subset displayed the highest CD143 surface expression density ($p < 0.0001$). The difference in CD143 expression observed among subsets varied in different subjects, both AAA and Controls (Fig. 4a). According to the ratios (R) of expression density of ACE between the different monocyte subpopulations reported in the section of Material and Methods, we observed that R3, which expresses the ratio between CD143 in $CD14^+CD16^+$ and in $CD14^{dim}CD16^+$ monocytes, was significantly higher in AAA patients than Control subjects (Fig. 4b and Table 5). Within AAA patients, R3 was significantly correlated with uric acid (r:0.25), ferritin (r:0.29) and $CD14^{dim}CD16^+$(r:0.35) circulating levels.

Table 3
Pearson r showing relationships between $CD14^+CD16^-$, $CD14^+CD16^+$ and $CD14^{dim}CD16^+$ subset levels with laboratory variables

Variable	r	P-value
$CD14^+CD16^-$ monocytes		
eGFR	0.233	0.0485
$CD14^+CD16^+$ monocytes		
D-dimer	0.439	0.0002
Age	0.310	0.0077
eGFR	−0.236	0.0428
$CD14^{dim}CD16^+$ monocytes		
WBC count	−0.305	0.0087
Neutrophil count	−0.294	0.0116
CD143	0.277	0.0185
Uric Acid	0.250	0.0377

4. Discussion

It has been recognized that inflammation has a central role in the pathophysiology of atherosclerosis and of thrombosis in many arterial districts [27]. Both circulating monocytes and macrophages are considered important components for the inflammatory response as-

Table 4
Percentage of **CD14$^+$CD16$^+$** monocytes in AAA and CTR separated by either similar stage of renal damage or age intervals

CKD stage								
I			II			III		
AAA (n=8)	**CTR** (n=6)	P	**AAA** (n=42)	**CTR** (n=22)	P	**AAA** (n=24)	**CTR** (n=2)	P
6.8 ± 0.9	5.1 ± 0.6	**0.11**	7.2 ± 0.4	5.5 ± 0.3	**0.004**	8.7 ± 0.6	5.1 ± 1.7	–

Age intervals (*years*)								
56–64			65–74			75–83		
AAA (n=7)	**CTR** (n=9)	P	**AAA** (n=37)	**CTR** (n=14)	P	**AAA** (n=30)	**CTR** (n=7)	P
5.8 ± 0.4	5.8 ± 0.5	**ns**	7.3 ± 0.4	5.2 ± 0.4	**0.003**	8.5 ± 0.5	5.3 ± 0.6	**0.007**

Table 5
CD143 surface expression (as units of fluorescence) in monocyte subsets and CD143 ratios between monocyte subsets

	AAA Median and IQ range	CTR Median and IQ range	p
CD143 in Total Monocytes	45.32 (31.34–75.67)	49.03 (36.46–97.15)	NS
CD143 in CD14$^+$CD16$^-$ cells	44.40 (31.14–75.67)	45.32 (33.70–103.70)	NS
CD143 in CD14$^+$CD16$^+$ cells	58.10 (39.95–96.47)	62.08 (42.13–119.30)	NS
CD143 in CD14^{dim}CD16$^-$ cells	39.60 (27.63–69.16)	54.25 (28.70–103.50)	NS
R1	1.25 (1.14–1.35)	1.23 (1.13–1.31)	NS
R2	0.93 (0.77–1.08)	0.96 (0.86–1.24)	NS
R3	1.39 (1.21–1.63)	1.21 (1.08–1.49)	0.045

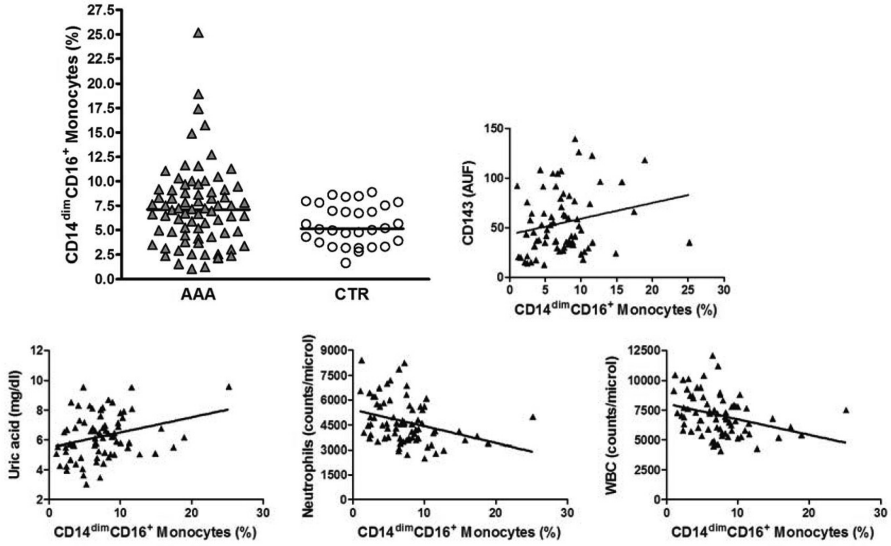

Fig. 3. CD14^{dim}CD16$^+$ monocyte results. Left Top panel: Scattergram of CD14$^+$CD16$^+$ monocyte percentages in AAA patients (filled triangles) and in Controls (circles). Median lines are represented. Results were compared by the two-sided t test (p: 0.02, AAA patients vs Controls). Top right panel and bottom panels: Relationships of CD14$^+$CD16$^+$ monocyte percentages with CD143, uric acid, Neutrophil Count and WBC counts in AAA patients: scattergrams and regression lines are illustrated.

sociated to the atherosclerotic damage [28]. Monocytes display heterogeneity in humans [29] and mice [30] for phenotype and function, and selective recruitment of distinct monocyte subsets into the atherosclerotic lesion has been described [31]. Developmental origins and functions of monocyte subsets are not defined yet: although the three monocyte subsets here described have been found also in bone marrow [32], it is still matters of debate whether circulating monocyte subsets derive from different myeloid precursors or, rather, represent the same cell at different maturation stages. The trafficking of monocyte subsets has been described in several inflammatory settings [33]. In a mouse model of myocardial infarction, levels of Ly-6C^{hi} murine monocytes, which appear concordant with human classical CD14$^+$CD16$^-$ subset, initially increase in the periph-

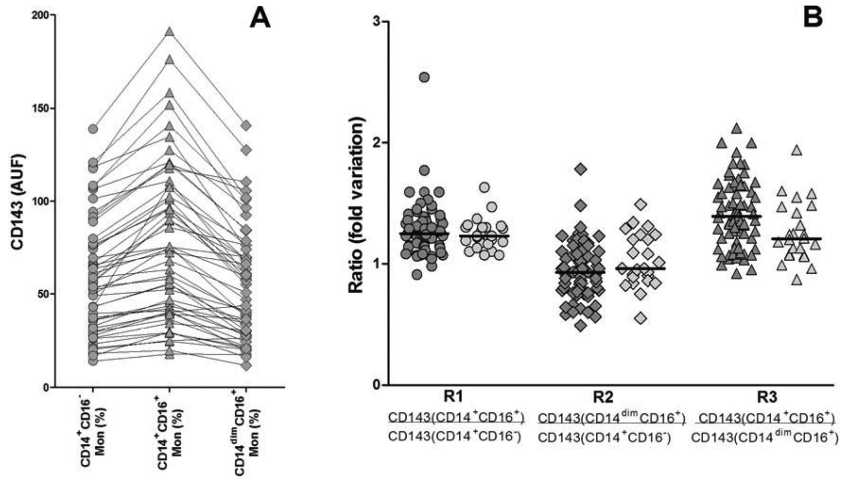

Fig. 4. representative CD143 values in subsets and their ratios. A) differential CD143 expression among monocyte subsets: while the CD14$^+$CD16$^+$ subset displays the highest CD143 surface expression density, the difference in CD143 expression observed among subsets varies in different subjects. The CD143 values were compared by repeated measures ANOVA. B) scatterplot with medians of R1, R2 and R3 ratios of CD143 in AAA and Controls. Medians were compared by the Mann Whitney test.

eral blood and first seed the infarcted heart and exert proinflammatory functions, while the non-classical mouse subset is recruited later, expresses high amounts of VEGF and promotes tissue healing [34]. Although the contribution of non-classical monocytes is "site specific", and does not occur universally in all the inflamed tissue and organs, it is generally accepted that classical monocytes dominate early in acute inflammatory responses. Intermediate CD14$^+$CD16$^+$ monocytes are found at low frequency in healthy adults, but possess unique features [14], expand with cytokine treatment and in many inflammatory conditions, such as renal dialysis [16] and Crohn's disease [35]. Recently, Zawada et al. analyzed the monocyte subset transcriptomes, providing biological and genetic evidence that this monocyte subset is linked to high activation and has an important inflammatory potential within the three monocyte subsets [11].

Circulating monocyte subsets have been recognized to possess different migratory rate and functions in inflammatory tissue damage [34], and to differentially express, among others, the membrane-bound ACE enzyme (CD143), the higher levels being expressed on CD14$^+$CD16$^+$cells. CD143 is differentially expressed in different stages of maturation or aging in many cell types. In monocytes, CD143 expression increases during differentiation toward macrophages and dendritic cells [36]. Previous studies demonstrated the presence of infiltrating monocytes and a diffuse expression of monocyte/macrophage cell markers in the damaged aortic wall of AAA patients [37], but no information is available about the pattern of monocyte subsets and tissue macrophages in AAA patients. We initially set this study to define the circulating frequency of CD14$^+$CD16$^+$ and of CD14^{dim}CD16$^+$ subsets and the CD143 cell surface expression in patients with AAA.

Our findings are the following: 1) patients with advanced AAA had higher percentage of circulating CD14$^+$CD16$^+$ and CD14^{dim}CD16$^+$ monocytes, when compared to Controls; 2) frequency of CD14$^+$CD16$^+$ subset in AAA patients was directly associated to D-dimer and age, and inversely related to eGFR; 3) frequency of CD14^{dim}CD16$^+$ subset in AAA patients was directly associated to total monocytic CD143 surface expression and uric acid levels, and was inversely related to total white cell and neutrophil counts; 4) total monocyte CD143 expression did not differ between the two study groups, and was higher within the CD14$^+$CD16$^+$ cells, compared to the other two monocyte subsets; R3, expressing the ratio of CD143 levels between CD14$^+$CD16$^+$ and CD14^{dim}CD16$^+$cells, was significantly different between AAA and Controls.

It is well known that D-dimer is elevated in AAA patients and reflects ongoing fibrin turnover at the level of aortic mural thrombus. In our population, D-dimer levels were independently correlated with CD14$^+$CD16$^+$ monocyte concentrations after the use of multiple linear regression. We speculated that the increased fibrin turnover in AAA patients might contribute to the systemic inflammatory response associated to circulat-

ing monocytes. D-dimer contribute to the risk of coronary disease and of ischemic stroke independently of conventional atherothrombotic risk factors [38].

A significant association has been observed between D-dimer concentration and AAA, and Takagi in a recent systematic review concluded that D-dimer concentrations were higher in AAA patients than in subjects without AAA [39]. Mural thrombus is a common event in AAA patients, and represents an interface with circulating monocytes [40]. Similarly to elastin degradation peptides that possess monocyte chemotactic activity in human AAA [41], also D-dimer in AAA patients is released locally and spread systemically, and is chemotactic for several cell types, including monocytes. Previous authors demonstrated *in vitro* that human monocytes increase the secretion of proinflammatory cytokines when treated with D-dimer [42]; THP-1 cells treated with 1,25-$(OH)_2$ vitamin D3 to mimic activated monocytes, when incubated in the presence of a fibrin clot, release proinflammatory cytokines, such as IL-1 [43].

Circulating inflammatory biomarkers at large are associated also with kidney function in patients with cardiovascular diseases [44]. Our current data confirm and extend the previously described association between increased CD14$^+$CD16$^+$ levels and renal damage observed in dialysis-CKD patients [16]. Our AAA patients showed changes in renal function at a more subtle level in comparison to dialysis-CKD patients. To this regard, it has been shown that there is a gradual rise in serum of uraemic toxins, such as indoxyl sulphate, from the very earliest stages of CKD [45]. Similarly to D-dimer, also low levels of indoxyl sulphate enhanced *in vitro* pro-inflammatory cytokine production in human THP-1 cells [46]. Accordingly, raised CD14$^+$CD16$^+$subset levels in these patients may be due to the presence of moderate renal dysfunction and of raised D-dimer levels.

Increased uric acid is a clinical indicator of excessive oxidative stress, and results from increased xanthine oxidase activity. Because circulating levels of CD14^{dim}CD16$^+$ monocytes are related to pro-oxidant indicators (high levels of uric acid and of monocytic CD143 surface expression) we believe that expansion of this monocyte subset is observed in patients with increased oxidative stress. Within AAA patients, decreased counts of CD14^{dim}CD16$^+$monocytes were observed in patients with the highest circulating counts of neutrophils and of total white blood cell counts. We previously made this observation in a cohort of CHF patients with a reduced ejection fraction of the left ventricle [17]. We then attributed this finding to a distinct pattern of transendothelial migration between CD16$^+$ and CD16$^-$ monocytes. Ancuta reported that CD14^{dim}CD16$^+$ monocytes express the highest CX3CR1 levels, making them prone to adhere to activated endothelium and to migrate within the vessel wall in response to the fractalkine pathway [47]. Increased counts of total white cells and of neutrophils are responsible for active ongoing nonspecific inflammation and are often associated to endothelial perturbation. Accordingly, CD14^{dim}CD16$^+$ monocytes of AAA patients with this profile of white blood cell counts may undergo increased transendothelial migration and appear reduced in the circulating compartment.

We confirmed a high CD14$^+$CD16$^+$subset-associated CD143 expression, as Ulrich previously demonstrated [16]. This finding is probably important at local level, because recruitment of monocytes with increased RAS activity may lead to tissue damage [48]. CD143 in the circulating blood and within the aortic vessel wall is known to promote oxidative damage [49], and inflamed monocytes may contribute to the introduction of this enzyme into the vessel wall lesion [48]. ACE/CD143 influence the type of macrophage polarization probably via intracellular signaling, and this influence is independent from circulating levels of enzyme activity [50]. Experimental and observational studies suggest that monocyte CD143 is strongly linked to subsets and may be a useful marker of cell function or differentiation [36]. At the moment, CD143 expression on monocytes may be regarded as the consequence of an activation/deactivation pathway induced by milieu (e.g. cytokines), as well as a marker of cell subset (since CD14$^+$CD16$^+$ cells have higher levels of surface CD143, while CD14^{dim}CD16$^+$ cells show the lowest expression), and/or a marker of the cell status within a specific subset. Because we observed that differences in CD143 expression between monocyte subsets are not constant, we hypothesized that fluctuations of the CD143 expression may be linked to cell status. We found of particular interest the analysis of the Ratio for CD143 expression between the CD14$^+$CD16$^+$ and the CD14^{dim}CD16$^+$ subset (R3). As a working hypothesis, the differences we found in the ratio R3 between AAA patients and Controls and the relationship displayed between R3 and CD14^{dim}CD16$^+$ monocytes indicates different profiles of differentiation, migration or survival. We believe that, while the increased CD143 expression in CD14$^+$CD16$^+$ monocytes may potentially be linked to high inflammatory activity, the CD143 expression on CD14^{dim}CD16$^+$ subset is more

linked to different maturation stage of the cell, potentially indicating/influencing its fate.

In summary: Our study described for the first time that expanded levels of CD14$^+$CD16$^+$ and of CD14^{dim}CD16$^+$ monocyte subsets cluster in patients with large AAA ($>$ 55 mm) respectively with moderate renal dysfunction and raised D-dimer (CD14$^+$CD16$^+$ cells) and with levels of uric acid, of surface CD143 and reduced count of total leukocytes and neutrophils (CD14^{dim}CD16$^+$ cells). We were thus able to attribute specific associations between monocyte subsets frequency and circulating and cell-associated (CD143) biochemical and inflammatory biomarkers. A clinical implication of our findings is that a few low-cost blood measurements (plasma D-dimer, creatinine and age to derive eGFR, uric acid, total white blood and neutrophil counts) might be used to discriminate AAA patients with different monocyte-dependent inflammatory profiles. In AAA patients there is a definite need for circulating biomarkers that could provide additional predictive value than the size of AAA. For the individual patient, the diameter threshold of 5.5 cm is a poor predictor of risk, because some AAA undergo rupture at diameter $\leqslant$ 5.5 cm and others progress to over 7 cm without rupture occurring. Prospective observational studies in patients with both small and large AAA as well as intervention studies modulating CD16$^+$ function via the CCR5-CCL5 axis [51,52] could establish whether specific monocyte subsets play a role for the expansion of aortic damage or for future cardiovascular events in selected AAA patients with mild impairment of renal function.

5. Limitation of the study

Cross-sectional design and relatively small number of patients are the major limitations of our study. The selection of an A-P aortic diameter $>$ 55 mm did not allow us to provide information on the relationship between size of AAA and values of circulating monocyte subsets in patients at different stages of expansion of aortic damage.

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