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Original article

Circulating $CD14^+CD16^-$ classical monocytes do not associate with a vulnerable plaque phenotype, and do not predict secondary events in severe atherosclerotic patients

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ABSTRACT

Aims: Mouse studies have established distinct monocyte subtypes that participate in the process of atherosclerotic lesion formation. The pro-inflammatory $Ly6C^{high}$ monocyte subtype actively contributes to murine plaque progression and destabilization. Also in humans, different peripheral monocyte subtypes have been identified, of which the $CD14^+CD16^-$ classical monocyte is suggested to display similar pro-atherosclerotic properties as the murine $Ly6C^{high}$ subtype. We aimed to investigate if circulating $CD14^+CD16^-$ classical monocytes associate with characteristics of a vulnerable carotid atherosclerotic plaque and if they associate with the risk of secondary adverse manifestations of atherosclerotic disease.

Methods and results: We enrolled 175 carotid endarterectomy patients of the Athero-Express biobank in our study. Just prior to surgical procedure, blood was collected and peripheral blood mononuclear cells were isolated. Characterization of monocyte subsets was performed by flow cytometry. Plaque characteristics were semi-quantitatively scored for the presence of fat, collagen, intraplaque hemorrhage and calcification. Vessel density, smooth muscle cells and macrophages were assessed quantitatively on a continuous scale. All features of a vulnerable plaque phenotype, including low amounts of collagen and smooth muscle cells, and increased fat content, vessel density, intraplaque hemorrhage and plaque macrophages were not significantly associated with differential levels of peripheral classical $CD14^+CD16^-$ monocytes or other monocyte subsets. Using Cox regression models to evaluate the prognostic value of circulating monocyte subtypes, we found that total counts of peripheral monocytes, as well as $CD14^+CD16^-$ classical and other monocyte subtypes were not associated with the risk of secondary cardiovascular events during 3 years follow-up.

Conclusion: Circulating classical $CD14^+CD16^-$ monocytes do not associate with specific vulnerable plaque characteristics. In addition, they do not predict secondary adverse manifestations. This suggests that in patients with established carotid artery disease, the circulating monocytes do not reflect plaque characteristics and have no value in identifying patients at risk for future cardiovascular events.

1. Introduction

The link between monocytes and atherosclerosis is well established. Plaque macrophages primarily derive from infiltrating circulating monocytes in early atherosclerosis, while they may also arise from local macrophage proliferation during plaque progression [1]. Macrophages play a pivotal role in plaque initiation, development and progression by phagocytosis of (oxidized) LDL and the secretion of inflammatory

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cytokines [2–4]. In addition, dying foam cells form a major part of the necrotic core, which is known to further enhance the inflammatory response.

Experimental studies in mice show that circulating inflammatory Ly6C^{high} monocytes, the suggested equivalent of human classical CD14$^+$CD16$^-$ monocytes [4,5], contribute to the process of atherosclerotic lesion formation. Ly6C^{high} monocytes attach to the damaged endothelium, and upon arrest, they migrate through the endothelial layer into the plaque, primarily involving the chemokine receptor CCR2 [6–8]. The Ly6C^{high} monocytes can subsequently differentiate into inflammatory M1 plaque macrophages, which are associated with a vulnerable plaque phenotype [6,9–11]. Ly6C^{low} monocytes patrol the vascular endothelium, and upon plaque infiltration may be involved in scavenging of lipids and dead cells [9,12]. The association between Ly6C^{low} monocytes and atherosclerotic plaque development remains inconclusive. On the one hand, low levels of Ly6C^{low} monocytes were associated with increased atherosclerotic lesion size [13,14], but showed no effect on atherosclerosis development on the other hand [15].

Since classical CD14$^+$CD16$^-$ monocytes contribute to plaque progression and vulnerability they may hold value in predicting patients at risk for CVD. However, existing data regarding the prognostic value of circulating monocytes remain inconclusive. Some studies indicate that monocytes have no prognostic value [16–19], whereas other studies suggest that high monocyte counts associate with increased risk for recurrent CVD events or mortality [20–23]. This may partly be explained by the fact that different monocyte subsets exist with distinct plaque modifying properties [3,4,24,25]. Indeed, high levels of classical and intermediate monocytes have been associated with increased risk for adverse manifestations in CVD patients [26–28]. Moreover, classical monocytes are linked to the presence and severity of atherosclerosis [29].

Thus, although it is evident that monocyte subsets play a divergent role in CVD, the question whether circulating monocyte subsets in humans do relate to plaque characteristics has to the best of our knowledge not been comprehensively addressed before. Moreover, prospective cohort studies examining the prognostic value of monocyte subsets in CVD patients are limited. We took advantage of our unique biobank enclosing elaborate information of both plaque composition and occurrence of secondary adverse manifestations during three years follow-up. We investigated whether classical monocytes associate with atherosclerotic plaque characteristics and if they associate with the risk of secondary CVD events during three years follow-up.

2. Materials and methods

2.1. Patient selection

The current study comprises 175 patients in a subset from the Athero-Express biobank [30]. All patients undergoing carotid endarterectomy between 2009 and 2011 were included based on availability of PBMCs. In addition to the standard procedure, including an extensive patient questionnaire and detailed histological plaque characterization, peripheral blood mononuclear cells (PBMCs) were isolated from blood that was drawn preoperatively. Isolated PBMCs were stored in liquid nitrogen until further analyses were performed. Patient follow-up was executed for three years or until the occurrence of a secondary cardiovascular event (cardiovascular death, stroke, myocardial infarction, coronary intervention, peripheral intervention (including amputation)). All events were validated using health records kept by general practitioners. The study protocol conforms to the Declaration of Helsinki and has been approved by the Institution's ethics committee on research on humans. All patients provided written informed consent.

2.2. PBMC isolation

Twenty ml blood was collected in Li-Heparin tubes, and a complete blood cell profile was determined by a general hematology cell counter (Cell Dyn 1800 Abbott, Minesota, USA). PBMCs were isolated by Ficoll gradient fractionation and stored in liquid nitrogen, as described previously [31], until further analyses were performed.

2.3. Flow cytometry

After gentle thawing from liquid nitrogen, the PBMCs were washed with RPMI 1640 ((61,870,010, Gibco Carlsbad, CA, USA) supplemented with GlutaMax (room temperature (RT)) containing 25 nM HEPES, 1% penicillin/streptomycin and 2% Fetal Bovine Serum (FBS) (10270–106, Gibco, Carlsbad, CA, USA)). Cells were kept on ice during the whole procedure, unless stated otherwise. To obtain single cell suspensions, PBMCs were gently filtered over a 40 μm cell strainer (542,040, Greiner bio-one), washed with RPMI again and centrifuged at 350 *g* for 5 min at 4 °C. Subsequently, the cells were washed with cold PBS supplemented with 2% FBS and 20 mM EDTA, centrifuged at 350 *g* for 5 min at 4 °C and resuspended in cold PBS with 1% BSA. Then, the cells were incubated with fluorescent antibodies (Supplemental table 1) for 30 min at RT in the dark, washed with cold PBS and centrifuged at 350 *g* for 5 min at 4 °C. Isotype controls are shown in Supplemantal Fig. 2. To be able to exclude dead cells, the cells were incubated with fixable viability, eFluor506 (eBioscience) for 30 min at RT in the dark. Then, cells were washed with cold PBS with 1% BSA and measured on the flow cytometer (Gallios, Beckman Coulter, Fullerton, CA, USA). Gating analyses of the flow cytometry data were performed using Kaluza 1.3 software. We selected viable CD11B$^+$CD115$^+$ monocytes, and based on the combination of forward - sideward scatter and CD14 and CD16 expression, we identified classical (CD14$^+$CD16$^-$), intermediate (CD14$^+$CD16$^+$) and non-classical (CD14$^-$CD16$^+$) monocytes (Supplemental fig. 1). In addition, we measured the surface expression of CCR2, CXC3CR1 and CD11B and CD31 at the different monocyte subsets.

2.4. Atherosclerotic plaque characterization

We performed a comprehensive analysis of the atherosclerotic plaque composition, as described previously [30,32,33]. This analysis includes a semi-quantitative assessment of fat deposition, collagen deposition, intraplaque hemorrhage and calcification. Vessel density, smooth muscle cells (SMC's), and plaque macrophages were quantitatively determined on a continuous scale. In addition, we stained sections for M1 macrophages.

2.5. Immunostaining for M1 macrophages (iNOS)

For immunostaining, a subset of 50 athero express paraffin sections were deparaffinized before endogenous peroxidase quenching and heat-induced epitope retrieval (citrate buffer for Antigen Retrieval, Vector Laboratories; H-3300). After blocking, slides were incubated with anti-iNOS (Abcam; 15,323; 1:75) antibody binding followed by incubation with anti-rabbit secondary antibody (klinipath; DPVR-HRP). Thereafter visualization was performed with ImmPact Amec red (Vector laboratories; SK-4285) followed by a counterstain with hematoxylin.

2.6. Analysis of plaque composition

All stained sections are examined microscopically and digitally stored. Two observers independently semi-quantitatively scored all different stainings. The relative lipid core size was estimated visually as a percentage of total plaque area with the use of H&E and picrosirius red stains, and divided into (1) ≤10% and (2) > 10% of the total plaque area. Collagen deposition (picrosirius red) was divided into no,

minor, moderate or heavy staining along the entire luminal border. Intraplaque hemorrhage (H&E and fibrin staining) was defined as the composite of plaque bleeding at the luminal side of the plaque as a result of plaque disruption and intraplaque hemorrhage, and scored as (1) absent or (2) present. Calcification (hematoxylin and eosin, H&E) was scored as no, minor, moderate or heavy staining along the luminal border or evident parts within the lesion. More specified definitions for these categories have been described earlier (30) and for macrophages these were defined as 1) no/minor = CD68 staining with negative or clusters with < 10 cell present and 2) moderate/heavy = cell clusters with > 10 cells present or abundance of positive cells. The alfa actin (smooth muscle cell), CD68 (macrophage) and iNOS (M1 macrophage) stainings are also analyzed quantitatively by computerized analyses. For this, colour thresholds are set and adjusted until the computerized detection meets the visual interpretation. The stainings are scored quantitatively as percentage of stained area to total plaque area. (AnalySiS version 3.2, Soft Imaging GmbH, Munster, Germany) as percentage of plaque area [30,32–34]. Plaque microvessels were stained with CD34 and quantified in 3 hotspots per plaque and scored as an average amount of vessels per hotspot.

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2.7. Statistical analyses

Normally distributed continuous variables were indicated as means ± standard deviations and compared by Student's *t*-tests or one-way ANOVA. Non-normal distributed data were presented as medians [inter-quartile ranges] and compared by Mann-Whitney *U* tests or Kruskal-Wallis tests. Categorical variables were indicated as percentages and compared by Chi-square or Fisher's exact-tests. Univariable Cox proportional hazard models were used to study the association between monocyte subsets and the occurrence of adverse cardiovascular events over time. To obtain a normal distribution, we performed a log transformation on the values of smooth muscle cells and plaque macrophages. To analyze the correlation between peripheral monocytes and vessel density, smooth muscle cells or plaque macrophages, linear regression models were used. Data management and statistical analyses were performed with RStudio [35] and the R software package [36] (version 3.2.0., Vienna, Austria). *P* values < .05 were considered as significant. GraphPad Prism 7.02 software (La Jolla, CA, USA) was used to produce the graphs.

3. Results

3.1. Patient characteristics

The current subcohort of the Athero-Express biobank comprised 175 patients with severe carotid atherosclerosis, undergoing carotid endarterectomy. During three years of follow-up, 55 of the 175 patients (31%) experienced a secondary adverse cardiovascular event. The median age of the patients was 71 years and 63.4% was male. Other risk factors, clinical manifestations, medication use and laboratory parameters are depicted in Table 1 for all patients, and also stratified by occurrence of secondary adverse CVD events. Since the clinical manifestations (asymptomatic and symptomatic) were comparable among patients regarding circulating monocyte subset counts (Table 2), and also between patients with and without secondary adverse events, we included all patients undergoing carotid endarterectomy in the current analyses. In addition, because the circulating monocyte subtypes did not differ between males and females (Table 2), we chose to evaluate their association with plaque characteristics and secondary CVD events in males and females together.

3.2. Circulating monocyte subsets

In the current study, patients have median monocyte values of 10.8% [inter-quartile range (IQR) 9.3–12.3]. Absolute monocytes counts were approximately 800 cells/μl (IQR 600–1000 cells/μl). On average, classical $CD14^+CD16^-$ monocytes represented 89.2%, intermediate $CD14^+CD16^+$ monocytes 4.8%, and nonclassical $CD14^-CD16^+$ monocytes 5.9% of the total monocyte pool (Supplemental fig. 3), which corroborates previous studies [24,25].

To explore which patient characteristics associated with the number of circulating monocytes, monocyte counts were presented for the presence or absence of different patient characteristics (Table 2). The classical monocyte counts negatively correlated with HDL levels (p = 0.03; Table 2), and positively correlated with hsCRP levels (p = 0.02). In addition, classical monocyte counts were higher in patients taking calcium antagonists (p = 0.04). Peripheral nonclassical monocytes were lower in current smokers than nonsmokers (p = 0.003), higher in patients on beta blockers (p = 0.008), and increased upon increasing BMI (p = 0.02). No statistically significant associations with patient characteristics were identified for circulating

Table 1
Patients characteristics are depicted for all patients and stratified by occurrence of secondary adverse CVD events during follow-up.

	Overall (n = 175)	No events (*n* = 120)	Events (*n* = 55)	*P*
Risk factors				
Age (years)	71.0 [63.0–78.0]	71.0 [63.0–78.0]	73.0 [63.5–79.0]	0.50
Sex (% male)	111 (63.4)	74 (61.7)	37 (67.3)	0.59
BMI	26.0 [23.6–29.1]	26.1 [23.5–29.3]	25.7 [23.6–28.6]	0.74
Current smoker (%)	62 (35.6)	42 (35.3)	20 (36.4)	1
Contralateral stenosis (%)	75 (48.4)	47 (44.8)	28 (56.0)	0.25
Diabetes mellitus (%)	45 (25.7)	32 (26.7)	13 (23.6)	0.81
Hypertension (%)	152 (86.9)	102 (85.0)	50 (90.9)	0.41
Hypercholesterolemia (%)	107 (71.8)	74 (70.5)	33 (75.0)	0.72
CAD history (%)	63 (36.0)	42 (35.0)	21 (38.2)	0.81
PAOD history (%)	32 (18.3)	16 (13.3)	16 (29.1)	0.02
Clinical manifestations				
Asymptomatic	27 (15.4)	15 (12.5)	12 (21.8)	
Amaurosis fugax	29 (16.6)	19 (15.8)	10 (18.2)	
Stroke	42 (24.0)	31 (25.8)	11 (20.0)	
TIA	77 (44.0)	55 (45.8)	22 (40.0)	
Medication				
Statins (%)	142 (81.1)	96 (80.0)	46 (83.6)	0.72
Diuretics (%)	79 (45.1)	52 (43.3)	27 (49.1)	0.58
Beta blockers (%)	85 (48.6)	56 (46.7)	29 (52.7)	0.56
Calcium antagonists (%)	43 (24.6)	26 (21.7)	17 (30.9)	0.26
ACE-inhibitors (%)	58 (33.1)	37 (30.8)	21 (38.2)	0.43
Angiotensin-2 antagonists (%)	46 (26.4)	30 (25.2)	16 (29.1)	0.72
Anticoagulants (%)	11 (6.3)	5 (4.2)	6 (10.9)	0.10
Aspirin (%)	142 (81.6)	100 (84.0)	42 (76.4)	0.32
Antiplatelets (%)	153 (87.9)	106 (89.1)	47 (85.5)	0.67
Laboratory parameters				
Cholesterol (mmol/L)	4.0 [3.3–4.8]	4.0 [3.3–4.8]	4.0 [3.3–4.7]	0.67
HDL (mmol/L)	1.0 [0.9–1.2]	1.0 [0.9–1.3]	1.0 [0.9–1.2]	0.14
LDL (mmol/L)	2.1 [1.6–2.8]	2.0 [1.6–2.7]	2.2 [1.8–2.9]	0.16
hsCRP (μg/mL)	1.7 [0.7–3.6]	1.7 [0.7–3.1]	2.0 [0.7–4.8]	0.49
GFR MDRD (ml/min)	69.1 (18.4)	70.1 (17.6)	66.9 (20.1)	0.30

Values are presented as mean ± standard deviation for normal distributions, number of patients (frequency in percentage) for categorical variables, and median [inter-quartile range] for non-normal distributions. *P* values are calculated using Students' *t*-tests, Chi-square tests, and Mann-Whitney U tests, respectively. BMI indicates body-mass index; Contralateral stenosis, 50–100% stenosis of the contralateral carotid artery; CAD history, history of Coronary Artery Disease; PAOD, peripheral artery occlusive disease; Amaurosis fugax, temporary loss of vision; ACE-inhibitors, angiotensin-converting-enzyme inhibitor; HDL, high-density lipoprotein; LDL, low-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; GFR MDRD, Glomerular filtration rate according to 'Modification of Diet in Renal Disease' formula.

Table 2
The monocyte subset counts are depicted in relation to the presence or absence of patient characteristics.

	Classical monocytes		Intermediate monocytes		Nonclassical monocytes	
	(cells/uL)		(cells/uL)		(cells/uL)	
Categorial variables	Risk factor present		Risk factor present		Risk factor present	
Risk factors	Yes	No	Yes	No	Yes	No
Male sex	688 [497, 837]	656 [537, 770]	33 [18, 45]	31 [19, 50]	42 [25, 61]	38 [27, 58]
Current smoker	708 [554, 929]	644 [481, 810]	26 [18, 42]	33 [18, 49]	35 [19, 46]	43 [28, 65] **
Contralateral stenosis	663 [491, 828]	656 [481, 802]	28 [19, 46]	32 [17, 47]	41 [26, 56]	39 [24, 63]
Diabetes mellitus	722 [602, 1030]	656 [494, 799]	35 [24, 55]	30 [17, 46]	45 [32, 62]	39 [25, 60]
Hypertension	669 [505, 821]	631 [552, 848]	31 [17, 47]	35 [25, 47]	42 [25, 61]	32 [26, 45]
Hypercholesterolemia	672 [537, 825]	647 [475, 808]	32 [17, 45]	37 [22, 50]	43 [27, 60]	37 [21, 63]
CAD history	698 [557, 820]	656 [473, 832]	36 [17, 50]	28 [19, 44]	47 [27, 61]	37 [25, 60]
PAOD history	702 [554, 810]	659 [505, 830]	34 [17, 57]	32 [19, 47]	53 [28, 61]	39 [25, 60]
Medication use	Yes	No	Yes	No	Yes	No
Statins	679 [537, 839]	625 [434, 765]	33 [19, 48]	29 [15, 42]	42 [26, 62]	31 [23, 44]
Diuretics	663 [472, 772]	669 [543, 931]	29 [18, 44]	33 [19, 50]	40 [26, 60]	42 [24, 61]
Beta blockers	657 [503, 826]	700 [536, 826]	31 [18, 46]	32 [18, 48]	46 [31, 63]	36 [22, 51] **
Calcium antagonists	733 [605, 940]	644 [471, 812] *	36 [16, 68]	31 [19, 44]	42 [34, 63]	40 [25, 60]
ACE inhibitors	663 [475, 760]	670 [520, 836]	33 [22, 50]	31 [17, 46]	41 [25, 64]	39 [25, 60]
Angiotensin-2 antagonists	719 [530, 883]	649 [508, 820]	30 [16, 47]	32 [18, 48]	42 [31, 60]	39 [24, 61]
Anticoagulants	755 [640, 839]	663 [494, 822]	24 [19, 37]	32 [18, 48]	45 [40, 70]	39 [25, 60]
Aspirins	659 [487, 805]	756 [556, 867]	32 [17, 48]	31 [19, 42]	39 [25, 60]	45 [29, 78]
Antiplatelets	666 [497, 825]	684 [589, 832]	32 [18, 47]	28 [19, 59]	39 [25, 60]	56 [37, 86]
Clinical manifestations						
asymptomatic	677 [472, 909]		30 [21, 62]		47 [27, 60]	
occular	625 [463, 774]		31 [21, 39]		39 [24, 64]	
stroke	657 [530, 919]		34 [19, 51]		43 [31, 67]	
TIA	672 [549, 799]		30 [14, 44]		40 [23, 57]	
Continuous variables						
Risk factors						
Age (per 1 year increase)	−2.16 [−6.47, 2.16]		0.16 [−0.26, 0.58]		0.36 [−0.1, 0.81]	
BMI (per unit increase)	2.25 [−6.74, 11.24]		0.21 [−0.67, 1.09]		1.12 [0.17, 2.06] *	
Laboratory parameters						
Cholesterol (per mmol/L increase)	−38.28 [−78.35, 1.79]		1.01 [−3.15, 5.17]		−3.98 [−8.42, 0.47]	
HDL (per mmol/L increase)	−167.82 [−320.22, −15.42] *		1.25 [−14.64, 17.15]		−15.07 [−32.03, 1.89]	
LDL (per mmol/L increase)	−24.01 [−72.61, 24.59]		3.99 [−1.17, 9.15]		−1.67 [−7.23, 3.9]	
hsCRP (per µg/mL increase)	0.65 [0.09, 1.2] *		0.05 [−0.01, 0.1]		0.03 [−0.03, 0.09]	
GFR MDRD (per ml/min increase)	2.06 [−0.32, 4.44]		−0.1 [−0.33, 0.13]		−0.04 [−0.29, 0.22]	

Values are presented as median [inter-quartile range] for categorical variables, and beta [95% confidence interval] for continuous variables. BMI indicates body-mass index; CAD history, history of Coronary Artery Disease; PAOD, peripheral artery occlusive disease; Amaurosis fugax, temporary loss of vision; ACE-inhibitors, angiotensin-converting-enzyme inhibitor; Contralateral stenosis, 50–100% stenosis of the contralateral carotid artery; HDL, high-density lipoprotein; LDL, low-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; GFR MDRD, Glomerular filtration rate according to 'Modification of Diet in Renal Disease' formula. *P*-values were calculated using a one-way ANOVA or Kruskal-Wallis tests for categorical variables where appropriate, and linear regression models for continuous variables. * indicates $p < 0.05$ and ** $p < 0.01$.

intermediate monocyte counts (Table 2).

3.3. Circulating classical monocytes do not associate with plaque characteristics

To address the question if classical monocytes associate with a more vulnerable plaque phenotype, we studied the number of monocytes in relation to atherosclerotic plaque characteristics (A representative overview of pictures for each plaque characteristic category is shown in Fig. 1). However, we observed no differences in the numbers of classical $CD14^+CD16^-$ monocytes, nor intermediate $CD14^+CD16^+$ or non-classical $CD14^-CD16^+$ monocytes in relation to fat deposition (< 10% vs. > 10%), collagen deposition (no/minor vs. moderate/heavy), intraplaque hemorrhage (yes/no) or calcification (no/minor vs. moderate/heavy) (Fig. 2). Next, we further specified the categories again showing no differences in the numbers of classical $CD14^+CD16^-$ monocytes, nor intermediate $CD14^+CD16^+$ or nonclassical $CD14^-CD16^+$ monocytes in relation to fat deposition (3 categories: < 10%, 10–40% and ≥ 40%, Supplemental fig. 4)), collagen (4 categories: no, minor, moderate and heavy, Supplemental fig. 5)) and calcification (categories: no, minor, moderate and heavy, Supplemental fig. 6). In addition, we found no association between circulating monocytes and high plaque vessel density (Fig. 3) or decreased numbers of smooth muscle cells (Fig. 4), which have been linked to a more vulnerable plaque phenotype.

Since plaque macrophages are thought to derive –at least partly– from circulating monocytes, we studied the association between circulating monocytes and plaque macrophages. Absolute counts of total monocytes or classical monocytes were not associated with macrophage plaque content ($R^2 = 0.01$, $p = 0.24$) and ($R^2 = 0.00$, $p = 0.46$), respectively (Fig. 5A-B). On the other hand, statistically significant, though weak negative correlations were observed for absolute counts of intermediate ($R^2 = 0.04$, $p = 0.02$) and nonclassical ($R^2 = 0.06$, $p < 0.01$) monocytes with plaque macrophages (Fig. 5C-D). Next, we assessed if an association with plaque iNOS content, a specific marker for M1 macrohages. Plaque M1 macrophage content showed a weak, non significant correlation ($R^2 =$, $p = 0.18$) with plaque iNOS area (Fig. 6A). When assessing the circulation monocytes, neither the total pool nor the specific subsets associate to plaque M1 macrophages (Fig. 6B-E).

Because monocyte migration, activation and plaque infiltration has been described to be crucially dependent on the presence of surface receptors like CCR2, CD11B and CXCR3 in mouse studies [6–8], we also analyzed the association between monocyte surface expression of these

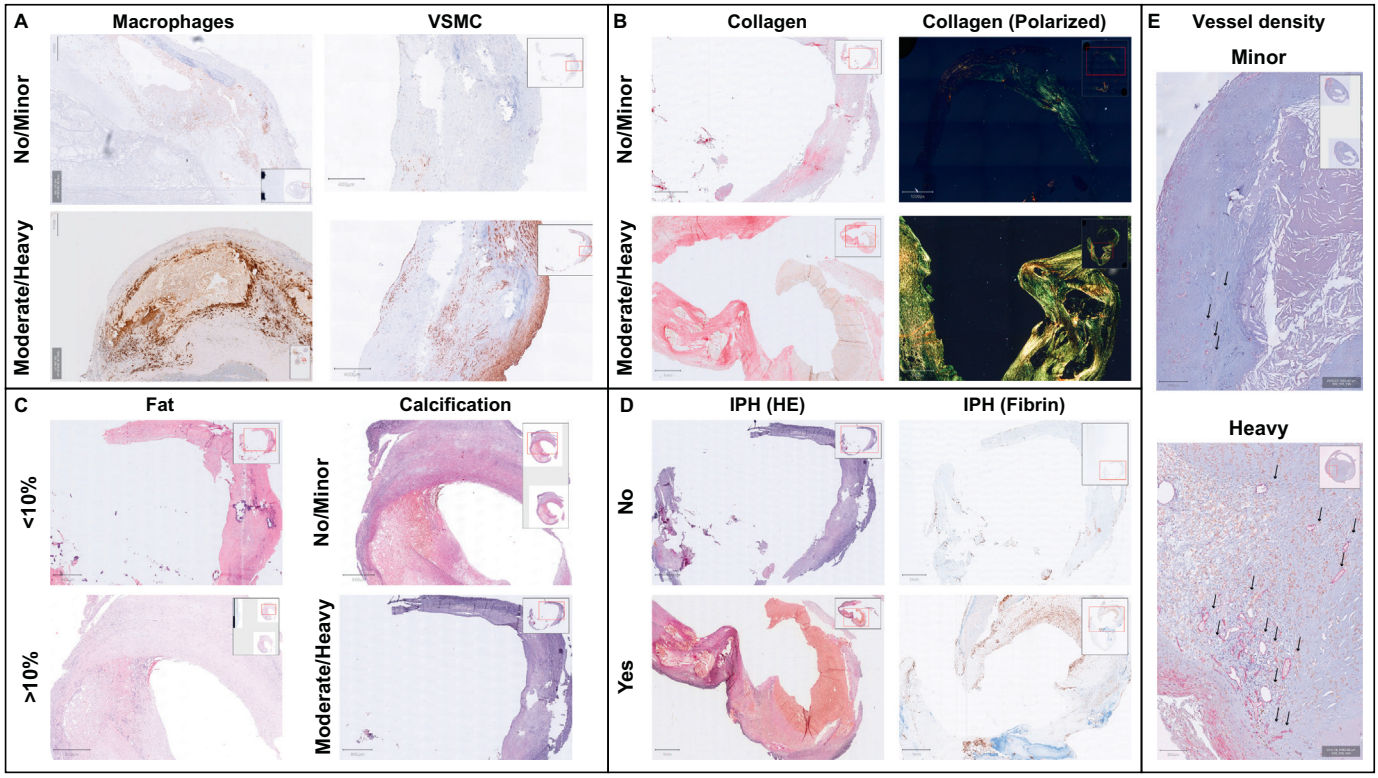

Fig. 1. Representative pictures of the different plaque characteristics of Athero Express Plaques. Plaque characteristics include (A) Macrophages Vascular Smooth Muscle Cells (VSMC), (B) Collagen, displayed as Sirius Red staining under bright and polarized light and categorized on no/minor and moderate/heavy staining, (C). Fat content, categorized on < 10% or ≥ 10% fat and Calcification categorized on no/minor and moderate/heavy staining. (D) Intra plaque hemorrhage, displayed on H&E and fibrin stain and categorized on No and Yes. (E) Vessel density displayed by hotspots of CD34 staining and categorized on no/minor and moderate/heavy stain.

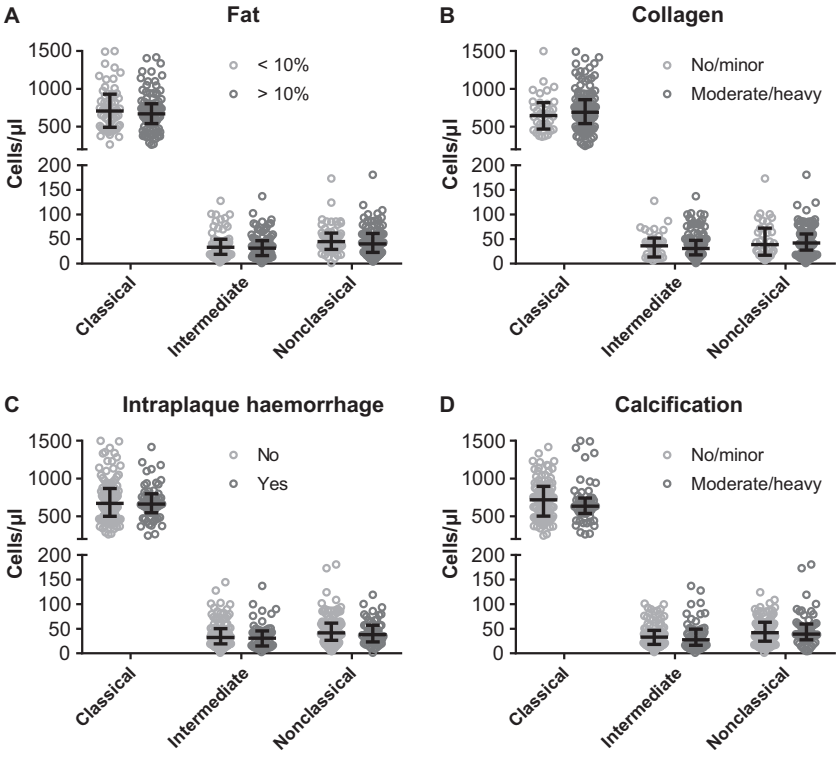

Fig. 2. Classical monocyte subsets are not associated with vulnerable plaque characteristics. Absolute cell numbers of classical, intermediate and nonclassical monocytes are not associated with fat deposition (A) presence of collagen (B), intraplaque hemorrhage (C) and calcification (D). For each monocyte subtype, median and inter-quartile range is indicated. Statistical significance was considered as $p < 0.05$, calculated with the Mann-Whitney U test ($n = 166$).

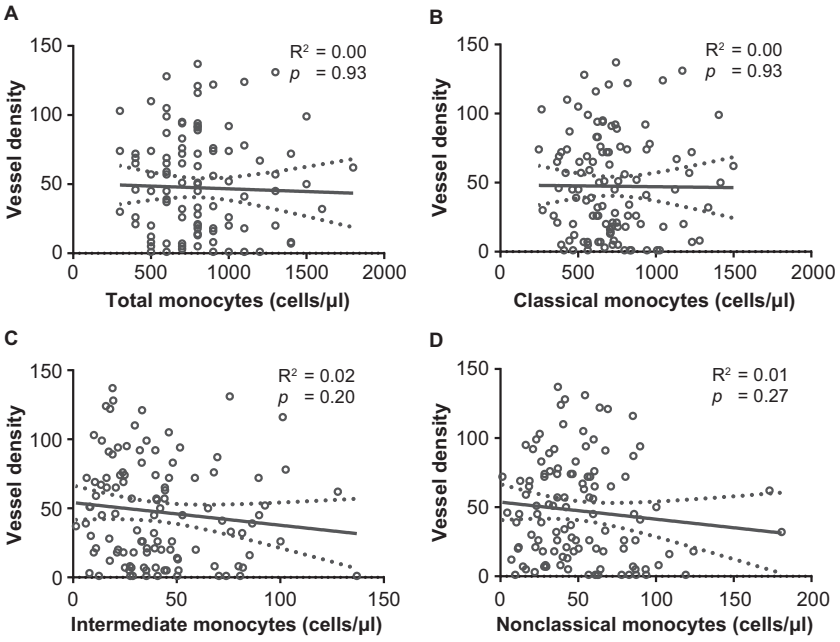

Fig. 3. Classical monocyte subsets are not associated with intraplaque vessel density. Total (A), classical (B), intermediate (C), and nonclassical monocytes (D) show no significant association with intraplaque vessel density. Solid line indicates the slope and dashed lines indicate the 95% confidence interval of the slope. Statistical significance was considered as $p < 0.05$, calculated using linear regression analyses ($n = 118$).

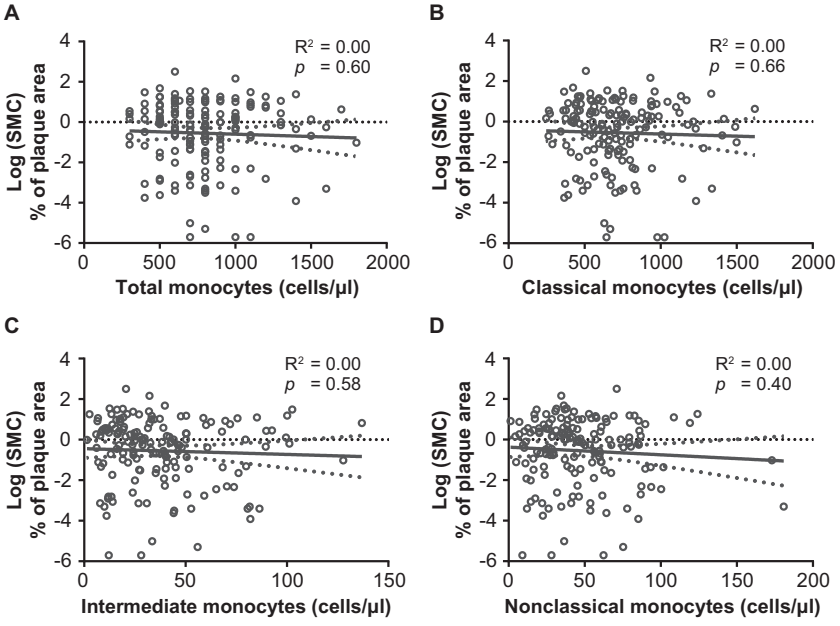

Fig. 4. Classical monocyte subsets are not associated with the amount of smooth muscle cells in atherosclerotic plaques. Total (A), classical (B), intermediate (C), and nonclassical monocytes (D) reveal no significant association with relative area of smooth muscle cells in the plaque. Solid line indicates the slope and dashed lines indicate the 95% confidence interval of the slope. Statistical significance was considered as $p < 0.05$, calculated using linear regression analyses ($n = 166$).

receptors and the number of plaque macrophages. However, we found no association between monocyte surface expression of CCR2, CD11B, and CXCR3 and plaque macrophages (Supplemental table 2).

3.4. Circulating monocytes do not associate with secondary cardiovascular endpoints

We then investigated whether the sheer number of circulating monocyte subsets differed between patients who experienced a secondary cardiovascular event during follow-up as compared to patients who did not. Classical monocyte numbers were comparable among patients with secondary events as compared to patients without (median 670 [IQR 475–805] vs. 668 [536–864] cells/µL, p = 0.59) as were the numbers of intermediate (28 [19–46] vs. 33 [17–47], p = 0.62) and nonclassical (39 [25–60] vs. 40 [25–61], p = 0.74) monocytes (Fig. 7).

To investigate if monocyte subsets associated with the risk of secondary adverse manifestations over time, we performed univariable Cox proportional hazard regression analyses. Circulating monocytes were not associated with the risk of secondary events during follow-up in univariable Cox regression analyses (Supplemental table 3).

4. Discussion

In the current study, we investigated the role of monocyte subsets in association to the plaque phenotype, and in relation to recurrent adverse CVD events in a patient cohort with advanced atherosclerotic disease. We observed no association between classical $CD14^+CD16^-$ monocytes and plaque characteristics. In addition, we found that monocyte subsets could not prognosticate secondary CVD events during three years of follow-up.

First, we addressed the question if circulating classical monocytes

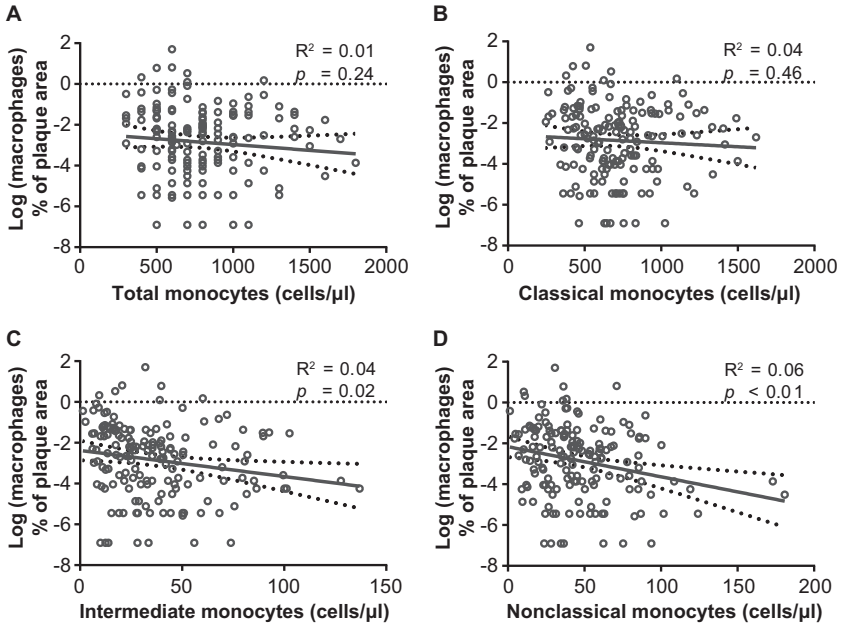

Fig. 5. Circulating intermediate and nonclassical monocytes weakly associate with the number of plaque macrophages. The relation between peripheral blood monocyte counts and relative area of plaque CD68$^+$ staining is shown. No correlation was observed between total monocyte count and plaque macrophages (A). Within the monocyte subsets, classical monocytes were not associated (B), whereas intermediate and nonclassical monocytes displayed a weak inverse correlation with plaque macrophages (C-D). Solid line indicates the slope and dashed lines indicate the 95% confidence interval of the slope. Statistical significance was considered as $p < 0.05$, calculated using linear regression analyses ($n = 166$).

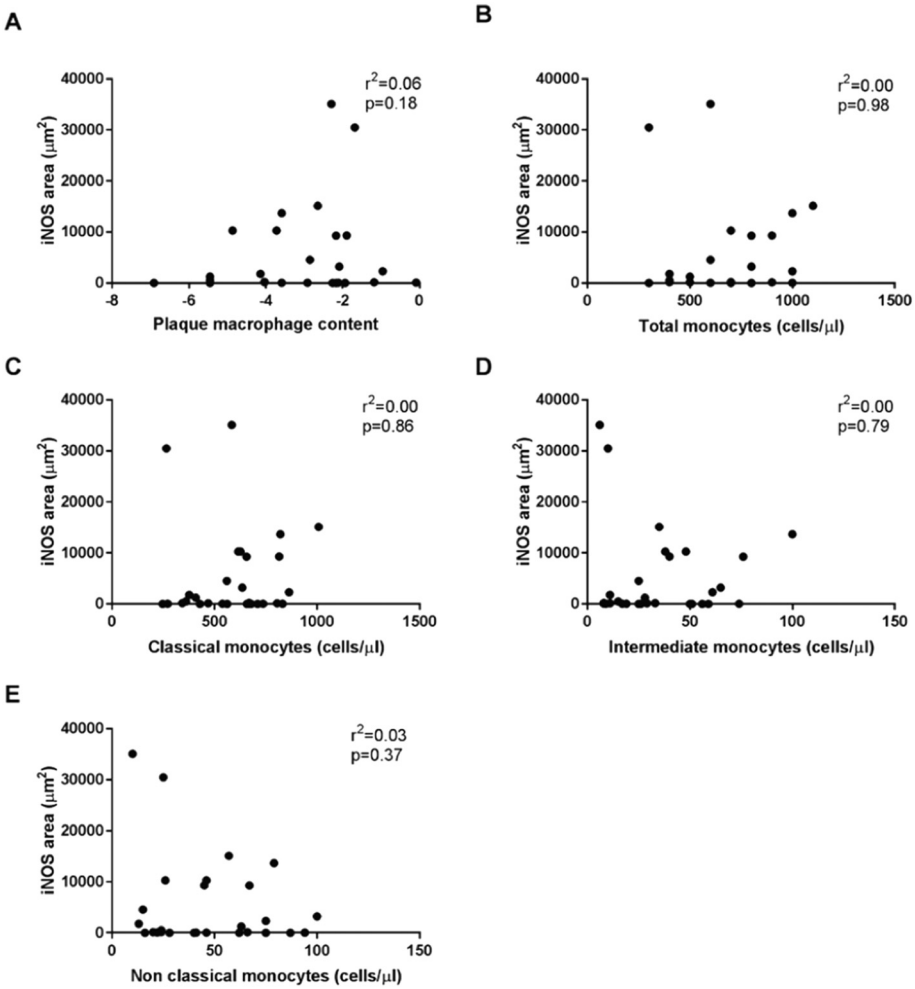

Fig. 6. Plaque M1 macrophage content weakly associates with the number of plaque macrophages, but not to circulating monocytes. A weak correlation between plaque M1 macrophages and CD68 plaque macrophage content was observed (A). No correlation was observed between total monocyte count (B), classical (C) intermediate (D) and nonclassical monocytes (E) and plaque M1 macrophages.

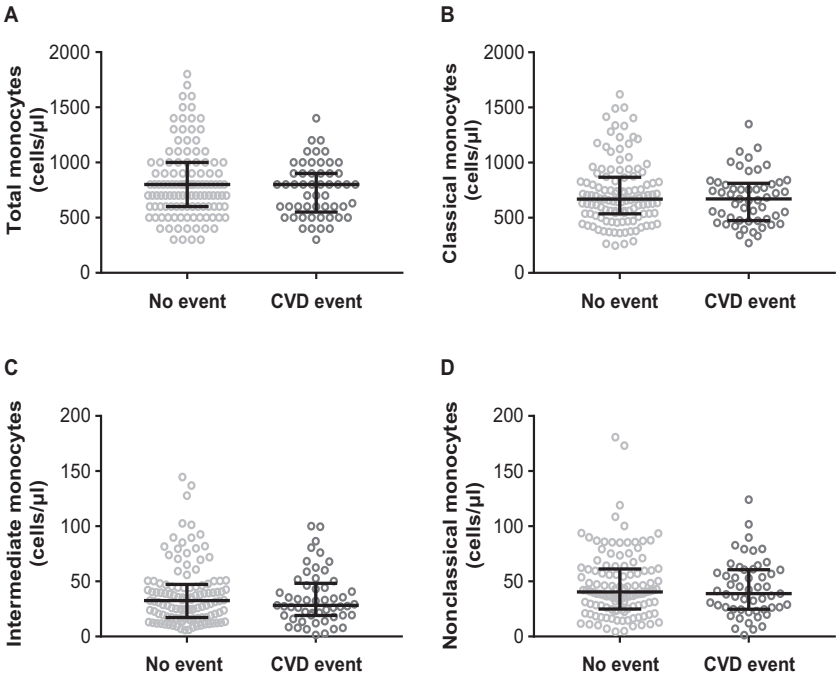

Fig. 7. Circulating monocyte subsets do not associate with secondary CVD events. Absolute counts of total monocytes (A) and monocyte subtypes (B-D) are depicted for patients without and with secondary adverse CVD manifestations as mean and SD. For each monocyte subtype, median and interquartile range are indicated. Statistical significance was considered as $p < 0.05$, calculated with the Mann-Whitney U test ($n = 175$).

were related to atherosclerotic plaque phenotype. In contrast to their role in experimental mice studies, in the current study in patients with an advanced stage of atherosclerotic disease, classical monocytes were not associated with characteristics of a vulnerable plaque phenotype. In agreement with our study, a recent study using optical coherence tomography also showed no association between classical monocytes and vulnerable plaque characteristics, including thickness of the fibrous cap, arc of lipid core and plaque calcification in UAP and STEMI patients [37]. However, absolute numbers of intermediate monocytes were inversely correlated with thickness of the fibrous cap and positively correlated with arc of lipid core [37]. In another study in patients with carotid and systemic atherosclerosis, researchers used ultrasonography to determine plaque neovascularization. They observed that patients with grade 1 or grade 2 neovascularization had higher levels of classical monocytes (464 or 482 cells/µl, respectively) as compared to patients without intraplaque neovascularization (406 cells/µl) [29]. However, contrasting results were obtained in a study where asymptomatic patient with carotid plaque neovascularization had lower levels of classical monocytes (255 cells/µl) than patients without (310 cells/µl). The discrepancy between these studies and our current study might be explained by the difference in study populations, and the different techniques used for intraplaque vessels. Thus, the exact association between classical monocytes and plaque neovascularization remains to be further investigated [38].

In addition, we questioned if monocyte subsets reflect the presence of plaque macrophages. We observed no association between circulating classical $CD14^+CD16^-$ monocytes and plaque macrophages, and a statistically significant, but weak correlation between peripheral intermediate $CD14^+CD16^+$ and nonclassical $CD14^+CD16^-$ monocytes and plaque macrophages, which might indicate that in advanced atherosclerosis the peripheral monocytes only marginally contribute to the intraplaque macrophage pool. As plaque macrophages encompass different subsets as well we performed immunohistochemistry staining for inflammatory M1 macrophages and immune regulatory M2 macrophages. Unfortunately, in our hands we were unable to reliably stain for M2 macrophages in our plaque material. Despite the fact that M1 macrophages have been described as a marker for vulnerable plaques, we observe a weak correlation between plaque macrophages (CD68 and iNOS) and we were not able to associate plaque iNOS content to a more vulnerable plaque phenotype (data not shown). M1 and M2 macrophages are overall equally present during plaque progression, but it has been established that M1 macrophages are more abundantly present in the shoulder region of the plaque (Stöger et al. Atherosclerosis. 2012 Dec; 225(2):461–8.). Unfortunately, given the fact that we use carotid endarterectomy specimens, it is very difficult to assess regional differences in iNOS content and as such we have not been able to assess M1 content in the shoulder region. Besides, murine studies have shown that plaque macrophages display signs of plasticity [39,40] and local proliferation [1]. Strikingly, local proliferation accounted for approximately 90% of the plaque macrophages in advanced murine plaques [1]. Thus, although monocyte influx is a crucial process in atherogenesis (as displayed by the fact that atherosclerosis is diminished in absence of chemokine receptors that facilitate monocyte trafficking [6,7,10,11,41,42]), monocyte recruitment may be less important in more advanced atherosclerotic plaques [43–46]. In line, our patient population presents with advanced atherosclerotic plaques, where monocyte influx might be less pronounced.

We observed no prognostic value for total monocytes, nor for monocyte subsets for the identification of high risk patients. In patients with coronary artery disease (CAD), different studies observed diverse associations regarding the prognostic value of total monocytes, i.e. no risk [16–19] or increased risk [20–23] of CVD events and mortality. Cross-sectional studies have indicated that mainly intermediate monocytes are associated with CVD and plaque progression [24]. However, because of the cross-sectional design of these studies, no straightforward conclusions can be drawn about the prognostic value for the occurrence of adverse cardiovascular events. Cohort studies assessing the prognostic value of specific monocyte subsets are limited, but suggest classical and intermediate monocytes to be predictive of CVD events during follow-up [26–28]. In the current study, we found no differences in monocyte subsets between patients with and without recurrent adverse events. This discrepancy might be explained by the fact that patients in the current study are older, receive pharmaceutical treatment and have more severe atherosclerosis than random aged Swedish subjects [28] or patients undergoing elective angiography [26]. In addition, differences in clinical manifestations (i.e. patients undergoing elective angiography [26], STEMI patients [27], or random older Swedish subjects [28]), and follow-up time (2 years in the STEMI

patients, up to 9 years in random Swedish subjects), may contribute to the inconsistency between the studies.

4.1. Future perspectives

As we investigated the correlation between classical $CD14^+CD16^-$ monocytes and plaque vulnerability in a cross-sectional manner, it would be interesting to see whether monocyte numbers can predict plaque composition over time. A marker of plaque progression could for example be a useful indicator for intervention in asymptomatic patients. In addition, since monocyte influx is crucially involved in developing plaques, the association between peripheral monocyte subsets and plaque phenotype merits further investigation in developing plaques, rather than advanced atherosclerotic plaques. Recently, technetium-99 m labeled PBMCs were tracked in vivo, in CVD patients and healthy controls. Interestingly, PBMCs accumulation could be visualized in atherosclerotic plaques [47]. This approach might be used to study monocyte trafficking in CVD patients in order to gain mechanistic insights of monocyte plaque infiltration, to support the identification of vulnerable plaques, or to validate therapeutic interventions that target inflammatory cells in CVD. Regarding the prognostic value of monocyte subsets, it is important that the findings from the current study will be replicated in large standardized cohort studies of CVD patients. Meanwhile, easily available risk markers, such as the Neutrophil To Lymphocyte ratio (NLR) [19,48–51], may be considered for short term implementation, in order to enhance risk prediction and to prevent hospitalization, secondary events and mortality.

In conclusion, classical monocytes do not associate with a vulnerable plaque phenotype. In addition, we show that circulating monocyte subsets have no prognostic value for secondary CVD manifestations in a population of severe atherosclerotic patients. These results suggest that circulating monocytes do not reflect plaque phenotype and have only limited value in identifying patients at risk for future cardiovascular events.

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Conflict of interest

None declared.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yjmcc.2019.01.002.

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