

1 **Monocyte and Macrophage Subtypes as Paired Cell Biomarkers for Coronary**  
2 **Artery Disease**

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15 **ABSTRACT**

16

17 **Background:** Monocytes and macrophages are central to atherosclerosis, but how they  
18 mark progression of human coronary artery disease (CAD) is unclear. We tested  
19 whether patients' monocyte subtypes paired with their derived macrophage profiles  
20 correlate with extent of CAD.

21

22 **Methods:** Peripheral blood was collected from 30 patients undergoing cardiac  
23 catheterization, and patients were categorized as having no significant CAD, single  
24 vessel disease, or multivessel disease according to the number of affected coronary  
25 arteries. Mononuclear cells were measured for monocyte markers CD14 and CD16 by  
26 flow cytometry, and separate monocytes were cultured into macrophages over 7 days  
27 and measured for polarization markers CD86 and CD206.

28

29 **Results:** At baseline, patients with greater CAD burden were older with higher rates of  
30 statin use, whereas all other characteristics were similar across the spectrum of  
31 coronary disease. Non-classical (CD14<sup>lo</sup>CD16<sup>hi</sup>) and all CD16<sup>+</sup> monocytes were  
32 elevated in patients with single vessel and multivessel disease compared to those  
33 without significant CAD (8.6% and 10.5% vs. 2.8%,  $p < 0.05$ ), whereas regulatory M2  
34 macrophages (CD206<sup>+</sup>) were decreased in patients with single vessel and multivessel  
35 disease (0.34% and 0.34% vs. 1.4%,  $p < 0.05$ ). An inverse relationship between paired  
36 CD16<sup>+</sup> monocytes and M2 macrophages marked CAD severity. CAD was also found to  
37 be more tightly associated with CD16<sup>+</sup> cells than age or traditional cardiovascular risk  
38 factors on multiple regression analysis of these patients.

39

40 **Conclusions:** CAD extent is correlated directly with CD16<sup>+</sup> monocytes and inversely  
41 with M2 (CD206<sup>+</sup>) macrophages, suggesting circulating monocytes may influence  
42 downstream polarization of lesional macrophages. These measures of monocyte and  
43 macrophage subtypes hold potential as biomarkers in CAD.

## 44 INTRODUCTION

45  
46 Atherosclerosis has a significant inflammatory component characterized by an  
47 imbalance between pro-inflammatory and regulatory influences [1]. Monocytes and their  
48 descendent cells, macrophages, play significant roles in the initiation and progression of  
49 the disease [2]. The cells' involvement in this complex pathophysiology is best  
50 understood in the context of cellular subsets, as various classes of monocytes and  
51 macrophages serve distinct, characteristic functions [3,4]. Monocytes can be classified  
52 as classical, intermediate, or non-classical according to CD14 and CD16 expression.  
53 Classical monocytes (CD14<sup>hi</sup>CD16<sup>-</sup>) are involved with phagocytosis and cytokine  
54 production. On the other hand, non-classical monocytes (CD14<sup>lo</sup>CD16<sup>hi</sup>) can be pro-  
55 inflammatory or potentially pro-angiogenic depending on the context [5,6], and the  
56 intermediate monocyte (CD14<sup>hi</sup>CD16<sup>lo</sup>) phenotype partly overlaps with that of non-  
57 classical cells [6]. These CD16<sup>+</sup> cells (i.e. the overlap of intermediate and non-classical  
58 monocytes) can be elevated in CAD patients [7,8], and the presence of these cells may  
59 associate with plaque vulnerability and rupture [9]. Moreover, intermediate monocytes  
60 have been shown to correlate with traditional cardiovascular risk factors and may be  
61 predictive of cardiovascular events [10].

62  
63 Macrophages, derived from all these monocyte subtypes, become the foam cells that  
64 comprise the core of the atheroma. They are induced by the local microenvironment to  
65 various heterogeneous polarization states along a multidimensional continuum [11,12],  
66 but represented at its extremes by so-called "M1" and "M2" macrophages, which can be  
67 identified by established cell surface markers and functional phenotype. Classically  
68 activated, or M1, macrophages are characterized by expression of pro-inflammatory  
69 mediators such as tumor necrosis factor alpha (TNF $\alpha$ ) and proteases, which may  
70 contribute to plaque destabilization. M2 macrophages, conversely, are anti-inflammatory  
71 cells involved in immunoregulation and tissue repair [13]. M1 macrophages are thought  
72 to be more prevalent in rupture-prone, unstable regions of atherosclerotic plaques,  
73 whereas M2 cells are more prominent in stable plaque regions [14].

74

75 Because specific monocyte and macrophage subsets may associate with atherosclerotic  
76 burden, characterization of monocyte surface markers and macrophage polarity in CAD  
77 may help identify patients with advanced disease who could benefit from more intensive  
78 cardiovascular risk reduction. An important step in understanding this relationship is to  
79 measure paired monocyte and macrophage phenotypes from patients with differing CAD  
80 burden. Comparing matched monocyte and cultured macrophage phenotypes from the  
81 same patient would be a novel approach to approximating the fate of circulating  
82 monocytes in clinically significant atheroma. Further, if circulating monocyte phenotypes  
83 associate with their descendent macrophage polarization states as well as disease  
84 burden, these cells could serve as more focused anti-inflammatory therapeutic targets or  
85 surrogates than many of those currently under investigation [15]. Therefore, our aim was  
86 to characterize the relationship between CAD burden and the matched phenotypes of  
87 monocytes and macrophages derived from peripheral blood of patients whose CAD is  
88 defined by coronary angiography.

## 89 **MATERIALS AND METHODS**

90

### 91 *Subjects and study design*

92 Patients scheduled for cardiac catheterization at the University of Chicago were  
93 screened for participation in this study. Patients were included if they underwent left  
94 heart catheterization and excluded if they had prior heart transplantation. Upon  
95 completion of the angiogram, participants were classified according to coronary artery  
96 disease (CAD) severity as having no significant CAD, single vessel disease (SVD), or  
97 multivessel disease (MVD). The *no CAD* group had no lesions beyond mild or minimal  
98 luminal irregularities, whereas the SVD and MVD were classified according to the  
99 number of major coronary arteries with either a stent or an atherosclerotic lesion greater  
100 than 75% of vessel diameter [16]. The study was approved by the Institutional Review  
101 Board of the University of Chicago and conforms to the ethical guidelines of the 1975  
102 Declaration of Helsinki. All participants provided written informed consent.

103

### 104 *Monocyte isolation*

105 2.5 mL of fresh peripheral blood, collected into an anticoagulating citrate tube from the  
106 arterial sheath of each patient upon obtaining access, was mixed with an equal volume  
107 of phosphate-buffered saline, gently layered over 2.5 mL of Lymphoprep (Axis-Shield,  
108 Oslo, Norway), and centrifuged for 20 minutes at 800 x g without brakes. The peripheral  
109 blood mononuclear cell (PBMC) layer was collected and washed twice in 3 mL  
110 phosphate-buffered saline, centrifuged for 5 minutes at 1500 x g to recover cells after  
111 each washing. PBMCs for flow cytometry analysis were harvested after these steps and  
112 fixed in 2% paraformaldehyde for 15 minutes, and stored at 4°C. Remaining PBMCs  
113 were resuspended in 4 mL RPMI 1640 (Thermo Fisher Scientific, Indianapolis, IN),  
114 plated on a 60 mm tissue culture dish for two hours, and allowed to adhere. Media was  
115 then aspirated and the dish washed with phosphate-buffered saline to remove non-  
116 adherent cells [17].

117 *Differentiation of monocytes to macrophages and macrophage detachment*

118 Adherent cells were incubated in 4 mL RPMI media with 10% macrophage colony  
119 stimulating factor-containing CMG-conditioned media and 1% penicillin/streptomycin  
120 [18,19]. Then, 500  $\mu$ l RPMI supplemented with 10% CMG media, 10% fetal bovine  
121 serum (Life Technologies, Carlsbad, CA) and 1% penicillin/streptomycin were added to  
122 the media daily for four days, after which the media was replaced with fresh 4 mL RPMI  
123 media containing 10% CMG media, 4% fetal bovine serum and 1%  
124 penicillin/streptomycin. Macrophages were detached from the dish after 7 days by 15  
125 minute incubation in 2 mL TrypLE (Thermo Fisher Scientific) followed by trituration with  
126 RPMI media containing 10% fetal bovine serum. Cells were fixed in 2%  
127 paraformaldehyde solution for 15 minutes and stored at 4°C.

128

129 *Flow cytometry*

130 Fixed monocytes and macrophages were stained with conjugated monoclonal  
131 antibodies (Alexa Fluor 488 CD14 and Alexa Fluor 647 CD16 for monocytes; PE/Cy7  
132 CD86 and Alexa Fluor 488 CD206 for macrophages) (BioLegend, San Diego, CA).  
133 Corresponding conjugated isotype antibodies were used in all experiments as negative  
134 controls (Alexa Fluor 488 Mouse IgG, clone MOPC-21; Alexa Fluor 647 Mouse IgG,  
135 clone MOPC-21; PE/Cy7 Mouse IgG, clone MOPC-11) (BioLegend). A 1:200 dilution  
136 was used for both monoclonal and isotype antibodies. Cells were analyzed by flow  
137 cytometry (LSR II; BD Biosciences) using FACSDiva software (BD Biosciences). Flow  
138 cytometry data were analyzed using Flowing software (University of Turku, Finland).

139

140 Monocytes and macrophages were gated in a forward scatter/side scatter plot as  
141 described previously [20]. Monocytes were divided into subsets according to CD14 and  
142 CD16 expression, with classical monocytes defined as CD14<sup>hi</sup>/CD16<sup>-</sup>, intermediate as  
143 CD14<sup>hi</sup>/CD16<sup>lo</sup>, and non-classical as CD14<sup>lo</sup>/CD16<sup>hi</sup> [21]. Each subset was quantified as  
144 a percentage of the total monocytes. Macrophages were classified as M1 or M2  
145 according to CD86 and CD206 positivity, respectively [22]. Each polarization state was  
146 quantified as a percentage of the total macrophages.

147 *Statistical analysis*

148 One-way ANOVA and  $X^2$  testing was used to compare baseline characteristics.

149 Unpaired, two-tailed Student's t-tests were used to compare FACS data between

150 groups. Data are presented as mean  $\pm$  SD. Calculations were performed using

151 GraphPad Prism software (San Diego, CA).

152

153 Univariate linear regression was performed using 8 independent variables: Age, WBC

154 count, mean arterial pressure (MAP) just prior to angiogram, body mass index (BMI),

155 serum creatinine, CD16+ monocyte count, M2 macrophage count, and number (ranging

156 from 0 to 5) of cardiovascular risk factors [hypertension, hyperlipidemia, diabetes,

157 smoking, African-American race]. The dependent variable was a validated CAD severity

158 score [23], a simplified alternative to the SYNTAX score [24,25]. The variables

159 significantly associated ( $p < 0.05$ ) with CAD severity on univariate analysis were included

160 in multiple linear regression analysis. Analysis was performed with R.

161 **RESULTS**

162

163 Samples were obtained from 30 patients, of whom 7 had no significant CAD, 7 had  
164 SVD, and 16 had MVD. Baseline characteristics of study participants are shown in  
165 **Table 1**. Most measures were similar across groups, but differences were observed in  
166 age and statin use which were both greater, as expected, in patients with more severe  
167 disease.

168

169 To assess circulating monocyte phenotypes, each patient's PBMCs were isolated from  
170 fresh blood and stained for monocyte markers, then measured by flow cytometry with  
171 gating on the monocyte population and quantification of CD14 and CD16 expression.  
172 Classical monocytes were the majority of circulating monocytes for all patients and did  
173 not show a consistent pattern as a function of CAD severity (**Fig 1A**). Although they  
174 remained a minority population, non-classical monocytes were increased in patients with  
175 SVD ( $5.2\pm 1.1$  vs.  $1.5\pm 0.2\%$ ;  $p < 0.01$ ) and MVD ( $6.8\pm 1.4$  vs.  $1.5\pm 0.2\%$ ;  $p < 0.05$ )  
176 compared to those without significant CAD (**Fig 1B**). Intermediate monocytes also  
177 showed this trend (compared to *no CAD*,  $p = 0.08$  for SVD and  $0.05$  for MVD, **Fig 1C**).  
178 Total CD16<sup>+</sup> (combined non-classical and intermediate) monocytes were similarly  
179 elevated in SVD ( $8.6\pm 1.8$  vs.  $2.8\pm 0.6\%$ ;  $p < 0.05$ ) and MVD ( $10.5\pm 1.7$  vs.  $2.8\pm 0.6\%$ ;  $p <$   
180  $0.01$ ), both in absolute percentages (**Fig 1D**) and when expressed relative to classical  
181 monocytes (**Fig 1E**).

182

183 To determine whether differences in circulating monocyte phenotype translate into  
184 differences in macrophage polarization, each patient's monocytes, cultured in conditions  
185 that drive macrophage differentiation [18,19], were differentiated over seven days into  
186 adherent peripheral blood-derived macrophages. These cells were assessed for  
187 macrophage polarization markers CD86 and CD206 by flow cytometry. No significant  
188 differences in M1 macrophages (CD86<sup>+</sup>/CD206<sup>-</sup>) were detected as a function of CAD  
189 burden (**Fig 2A**), but M2 macrophages (CD86<sup>-</sup>/CD206<sup>+</sup>) were decreased in patients with  
190 SVD ( $0.3\pm 0.1$  vs.  $1.4\pm 0.4\%$ ;  $p < 0.05$ ) and MVD ( $0.3\pm 0.1$  vs.  $1.4\pm 0.4\%$ ;  $p < 0.01$ )  
191 compared to those without significant CAD (**Fig 2B**). The ratio of M1:M2 macrophages



192 was elevated in SVD ( $360\pm 92$  vs.  $125\pm 45$ ;  $p<0.05$ ) and MVD ( $434\pm 73$  vs.  $125\pm 45$ ;  
193  $p<0.05$ ) (**Fig 2C**). Regarding a potential relationship between distribution of monocyte  
194 subsets matched to their descendent macrophage polarization, CAD severity roughly fell  
195 on an inverse relationship between CD16<sup>+</sup> monocytes and differentiation into M2  
196 macrophages (**Fig 2D**).

197  
198 Univariate linear regression was performed using several documented clinical variables,  
199 in addition to CD16<sup>+</sup> and M2 counts, to determine association with CAD severity based  
200 on an established score that serves as a simplified alternative to the SYNTAX score  
201 [23]. CD16<sup>+</sup> monocyte count, age, and total number of cardiovascular risk factors  
202 [hypertension, hyperlipidemia, diabetes, smoking, African-American race] were  
203 significantly and directly associated with CAD severity, whereas M2 macrophage count  
204 was inversely associated with CAD severity (**Table 2**). When these four variables were  
205 analyzed through multiple regression, CD16<sup>+</sup> monocytes remained significantly  
206 associated with CAD severity (**Table 2**).

## 207 **DISCUSSION**

208  
209 A major limitation in understanding the pathogenesis of CAD is the inability to study  
210 living cells from the coronary plaques of living patients. Surrogate biomarkers abound,  
211 but a cell biomarker based on monocytes and macrophages, main participants in plaque  
212 initiation and growth, may help to clarify CAD progression and risk further. In this study,  
213 we characterized monocyte subsets matched with their *ex vivo* macrophage profiles  
214 from peripheral blood samples of patients with varying CAD severity. Non-classical and  
215 total CD16<sup>+</sup> monocytes were elevated in CAD patients, and these patients' monocytes,  
216 once differentiated into macrophages, demonstrated impaired expression of markers  
217 associated with a regulatory, anti-inflammatory state. CD16<sup>+</sup> monocytes and M2  
218 macrophages thus showed inverse correlation with each other, suggesting that  
219 circulating monocytes influence polarization of their descendant macrophages in target  
220 tissues, such as the coronary atheroma. This is a new approach to systematically  
221 culture peripheral blood-derived macrophages for the purpose of comparing monocyte  
222 and monocyte-derived macrophage phenotypes from the same patient and correlating to  
223 his or her CAD burden.

224  
225 Our finding that non-classical monocytes are increased in stable CAD is consistent with  
226 previous studies. While all three monocyte subsets play a role in the initiation and  
227 progression of the atheroma, non-classical monocytes are distinguished by their  
228 capacity for recruitment to inflamed tissues [6]. In addition to CD16, these cells express  
229 CX3CR1, the receptor for fractalkine, an adhesion molecule expressed on activated  
230 endothelium [26]. Thus, an increase in the proportion of non-classical monocytes may  
231 be associated with progression of stable CAD through recruitment of these cells to the  
232 intima [27]. Additionally, the decrease in M2 macrophages and the imbalance between  
233 M1 and M2 phenotypes we observe is consistent with findings in mouse models of  
234 atherosclerosis. These murine studies suggest that M2 macrophages dominate during  
235 the initiation of the atherosclerotic lesion, whereas a shift to the M1 phenotype is  
236 observed with disease progression [28]. The shift away from regulatory M2  
237 macrophages that we measure could reflect a transition from early to established

238 disease, consistent with these patients' clinical status. Further, the concept of a skewed  
239 ratio of pro-inflammatory and regulatory macrophages predisposing to atherosclerosis  
240 has been backed by murine and human studies [13,29,30].

241  
242 Several limitations have bearing on the interpretation of our data. Exclusion criteria were  
243 kept to a minimum to allow for a range of patients with multiple comorbidities, which  
244 likely contributed to the variability in data, yet this better reflects real clinical practice.  
245 The small sample size also limits our statistical power. Finally, differences in other  
246 clinical factors between the groups may have an impact on the expression of these  
247 biomarkers, although many, including age and traditional risk factors, were taken into  
248 account for the multivariable analysis.

249  
250 In sum, non-classical and CD16<sup>+</sup> monocytes are increased and monocyte-derived M2  
251 regulatory macrophages are decreased in patients with CAD compared to those without.  
252 That the differences in macrophage polarization persist after a week of *ex vivo* culture  
253 demonstrates a durability in the impact of monocyte phenotype on eventual macrophage  
254 polarization, a hypothesis supported by studies of monocyte epigenetic memory [31];  
255 this suggests that both circulating monocytes and tissue macrophages may be useful  
256 therapeutic targets for preventing initiation and progression of disease. Indeed,  
257 translational studies examining the pleiotropic effects of cardiovascular medications on  
258 monocytes and macrophages have demonstrated changes in these cells' phenotype  
259 and function in response to treatment [32–38]. Further, distributions of pro-inflammatory  
260 monocytes and macrophages hold potential as markers of stable CAD burden. In our  
261 data, CD16<sup>+</sup> monocytes in particular were more closely associated with quantified CAD  
262 burden than traditional cardiovascular risk factors. As alterations in monocyte and  
263 macrophage profiles have been identified in human studies of carotid and femoral  
264 plaque rupture [39–41], our methods may allow for future replication of these findings in  
265 surgically excised coronary plaques. Finally, future studies in patients with unstable CAD  
266 could help determine whether pro-inflammatory monocytes and macrophages can better  
267 predict acute coronary syndromes.

268 **CONFLICT OF INTEREST**

269

270 No conflicts of interest to declare.

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272

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274

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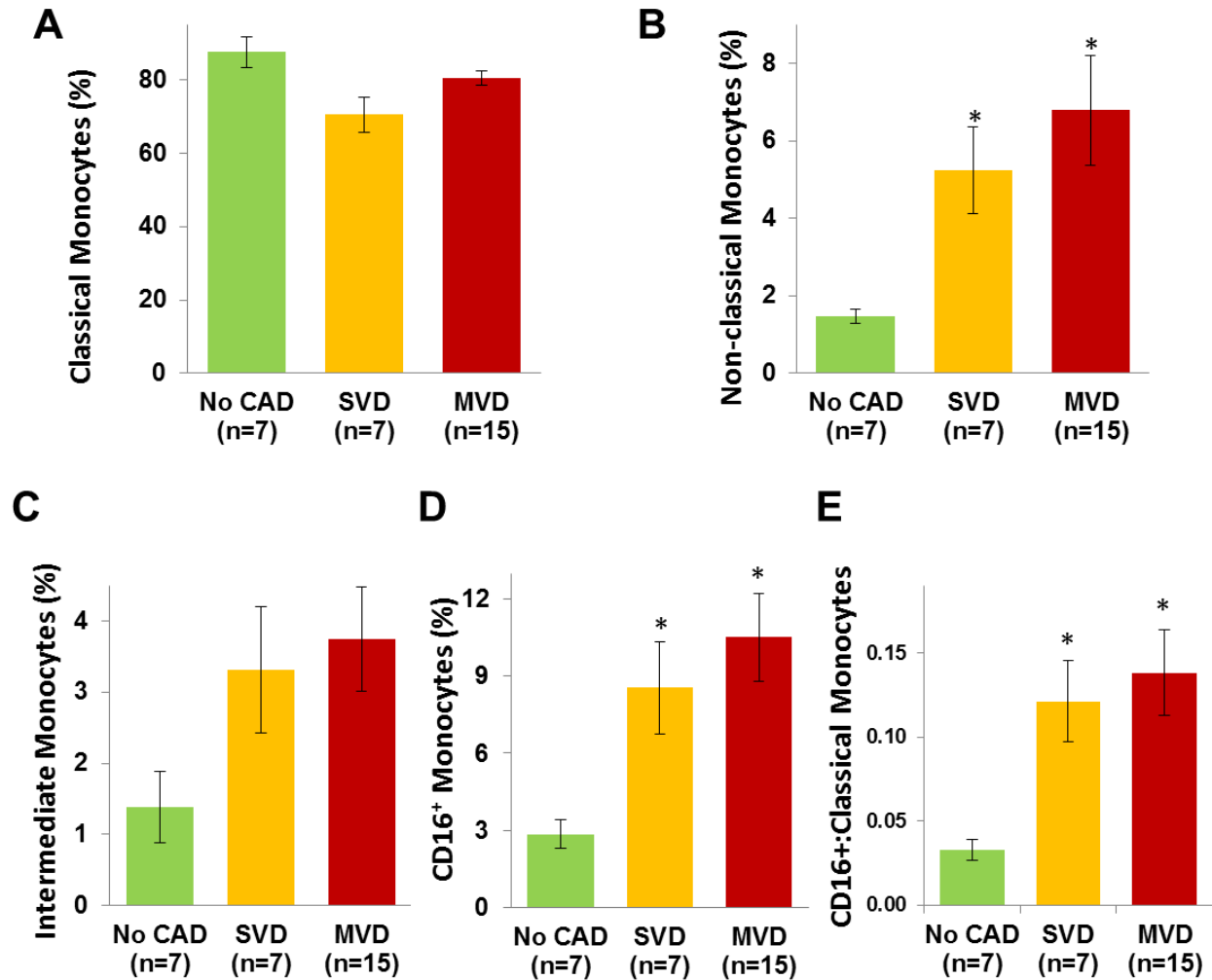


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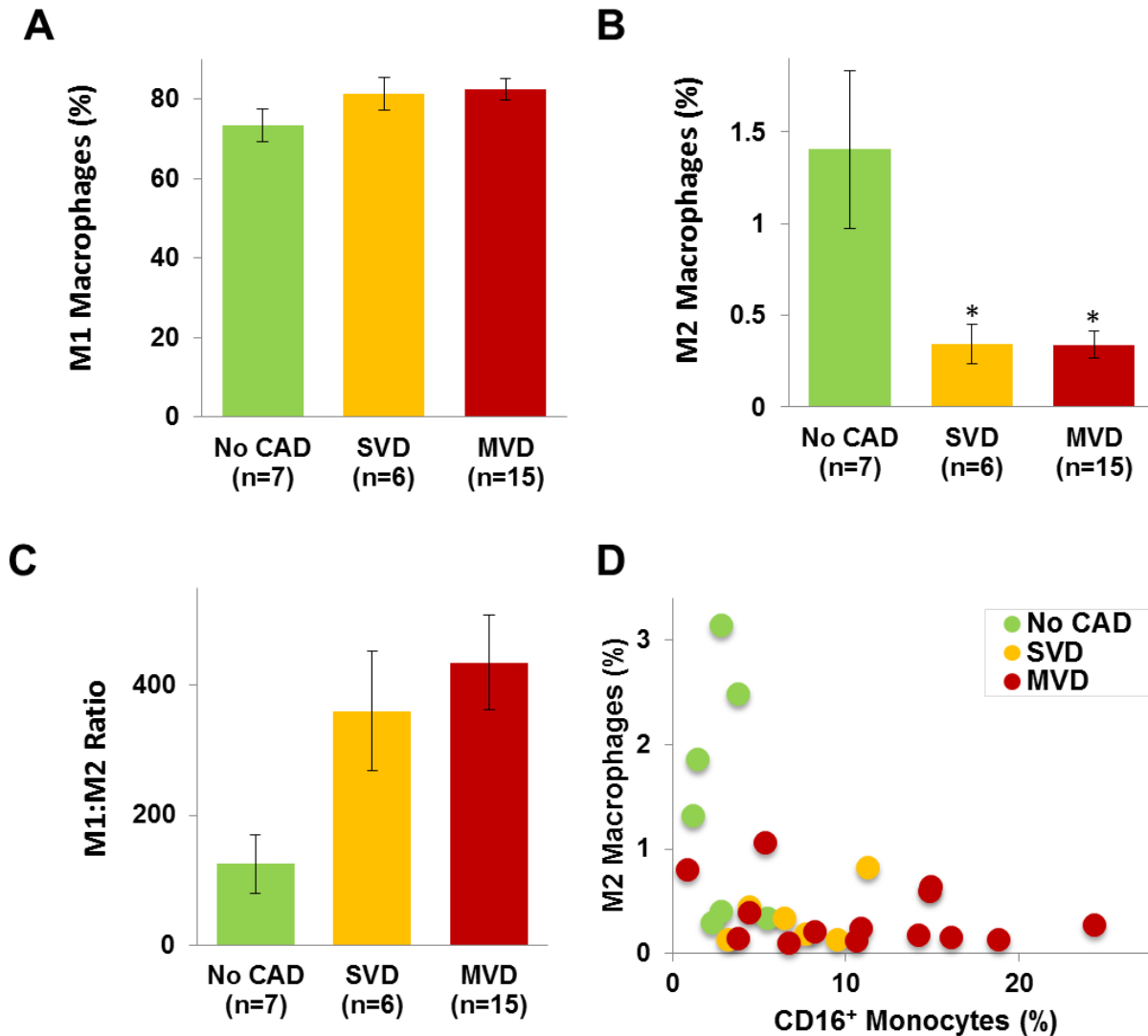
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	No CAD (n=7)	SVD (n=7)	MVD (n=16)
<b>Age (years)*</b>	<b>52.0 ± 10.8</b>	<b>65.3 ± 10.0</b>	<b>71.0 ± 10.0</b>
<b>Male sex</b>	4 (57%)	2 (29%)	11 (69%)
<b>Race</b>	AA 3 (43%) WH 3 (43%) OT 1 (14%)	AA 5 (71%) WH 2 (29%) OT 0 (0%)	AA 7 (44%) WH 9 (56%) OT 0 (0%)
<b>BMI (kg/m<sup>2</sup>)</b>	30.3 ± 10.8	25.9 ± 7.4	29.0 ± 4.7
<b>SBP (mm Hg)</b>	127.9 ± 16.7	142.7 ± 24.2	138.9 ± 25.1
<b>DBP (mm Hg)</b>	78.3 ± 17.5	80.7 ± 12.7	76.0 ± 17.4
<b>EF (%)</b>	42.3 ± 15.4	37.9 ± 21.6	42.5 ± 15.0
<b>Total cholesterol (mg/dl)</b>	149.3 ± 23.6	179.5 ± 34.1	151.9 ± 45.5
<b>HDL cholesterol (mg/dl)</b>	44.0 ± 14.7	52.3 ± 17.0	42.4 ± 7.4
<b>Triglycerides (mg/dl)</b>	115.0 ± 38.3	126.8 ± 24.4	131.8 ± 51.8
<b>LDL cholesterol (mg/dl)</b>	82.5 ± 19.8	102.3 ± 44.3	83.0 ± 38.1
<b>Leukocytes (1/μl)</b>	8.2 ± 3.0	8.5 ± 2.4	6.4 ± 1.8
<b>Total monocytes (1/μl)</b>	0.54 ± 0.17	0.62 ± 0.36	0.65 ± 0.30
<b>% monocytes</b>	5.8 ± 2.5	7.4 ± 3.0	10.3 ± 3.9
<b>Creatinine (mg/dl)</b>	1.0 ± 0.4	1.3 ± 0.9	1.5 ± 1.4
<b>Smoking history</b>	3 (43%)	3 (43%)	9 (56%)
<b>Diabetes Mellitus</b>	2 (29%)	3 (43%)	4 (25%)
<b>Hypertension</b>	5 (71%)	6 (86%)	14 (88%)
<b>Hyperlipidemia</b>	3 (43%)	6 (86%)	11 (69%)
<b>Beta-blockers</b>	4 (57%)	4 (57%)	15 (94%)
<b>ACE inhibitors</b>	3 (43%)	0 (0%)	7 (44%)
<b>ARBs</b>	0 (0%)	3 (43%)	2 (13%)
<b>CCBs</b>	0 (0%)	3 (43%)	3 (19%)
<b>Antiplatelet drugs</b>	7 (100%)	7 (100%)	16 (100%)
<b>Statins*</b>	<b>4 (57%)</b>	<b>6 (86%)</b>	<b>16 (100%)</b>

**Table 1: Baseline characteristics of study participants.** Data are presented as mean ± SD or count (%). AA, African American; ACE, angiotensin converting enzyme; ARB, angiotensin receptor blocker; BMI, Body Mass Index; CCB, calcium channel blocker; DBP, diastolic blood pressure; EF, ejection fraction; HDL, high-density lipoprotein; LDL, low-density lipoprotein; OT, Other (Pacific Islander, more than one race); SBP, systolic blood pressure; WH, White. \*p < 0.05, highlighted in red.



**Figure 1: Monocytes subtypes as a function of CAD burden.** (A) Classical monocytes are the majority of circulating monocytes and show no consistent pattern as a function of CAD severity. (B) Although still a minority population, non-classical monocytes were relatively enriched in patients with CAD. (C) Intermediate monocytes also showed this trend (compared to *no CAD*,  $p = 0.08$  for SVD and  $0.05$  for MVD). (D) Total CD16<sup>+</sup> monocytes (intermediate and non-classical combined) are shown, as is the ratio of CD16<sup>+</sup> to classical monocytes (E), all as a function of CAD burden. Data are presented as mean  $\pm$  SEM. \*  $p < 0.05$  in all panels.



**Figure 2: Profiles of cultured macrophage from patients with CAD.** (A) Of the macrophages cultured from all patients with or without CAD, most had the M1 surface marked (CD86), without significant differences between clinical presentations. (B) The minority population of M2 macrophages (CD206<sup>+</sup>) was suppressed in patients with CAD. (C) The M1:M2 macrophage ratio rises according to CAD severity. Data are presented as mean  $\pm$  SEM. \* $p < 0.05$ . (D) M2 macrophages plotted against CD16<sup>+</sup> monocytes show an inverse relationship that tracks with CAD burden.

	Univariate		Multivariate	
	$\beta$	p	$\beta$	p
<b>CD16<sup>+</sup> Monocytes</b>	<b>0.64</b>	<b>0.0003</b>	<b>0.43</b>	<b>0.01</b>
<b>Age</b>	<b>0.59</b>	<b>0.001</b>	0.26	0.14
<b>Risk Factors</b>	<b>0.5</b>	<b>0.01</b>	0.23	0.18
<b>M2 Macrophages</b>	<b>-0.43</b>	<b>0.03</b>	-0.11	0.48
<b>WBC</b>	-0.34	0.08		
<b>Creatinine</b>	0.32	0.11		
<b>MAP</b>	-0.16	0.42		
<b>BMI</b>	-0.08	0.68		

**Table 2: Clinical metrics and monocyte/macrophage markers associated with CAD severity.** Univariate linear regression was performed using 8 independent variables in association with each patient's CAD severity score. Standardized correlation coefficients ( $\beta$ ) are shown. The variables significantly associated ( $p < 0.05$ ) with CAD severity were CD16<sup>+</sup> monocytes, age, cumulative risk factors, and M2 macrophages (inversely). These were included in multiple linear regression analysis, in which CD16<sup>+</sup> count was the most significant association.