

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^{lo}CD16^{hi}) and all CD16⁺ 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⁺) 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⁺ monocytes and M2 macrophages marked CAD severity. CAD was also found to
37 be more tightly associated with CD16⁺ 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⁺ monocytes and inversely
41 with M2 (CD206⁺) 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^{hi}CD16⁻) are involved with phagocytosis and cytokine
54 production. On the other hand, non-classical monocytes (CD14^{lo}CD16^{hi}) can be pro-
55 inflammatory or potentially pro-angiogenic depending on the context [5,6], and the
56 intermediate monocyte (CD14^{hi}CD16^{lo}) phenotype partly overlaps with that of non-
57 classical cells [6]. These CD16⁺ 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 α) 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 μ 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^{hi}/CD16⁻, intermediate as
143 CD14^{hi}/CD16^{lo}, and non-classical as CD14^{lo}/CD16^{hi} [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 ± 1.1 vs. $1.5 \pm 0.2\%$; $p < 0.01$) and MVD (6.8 ± 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⁺ (combined non-classical and intermediate) monocytes were similarly
179 elevated in SVD (8.6 ± 1.8 vs. $2.8 \pm 0.6\%$; $p < 0.05$) and MVD (10.5 ± 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⁺/CD206⁻) were detected as a function of CAD
189 burden (**Fig 2A**), but M2 macrophages (CD86⁻/CD206⁺) were decreased in patients with
190 SVD (0.3 ± 0.1 vs. $1.4 \pm 0.4\%$; $p < 0.05$) and MVD (0.3 ± 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 ± 92 vs. 125 ± 45 ; $p<0.05$) and MVD (434 ± 73 vs. 125 ± 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⁺ 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⁺ 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⁺ 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⁺ 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⁺ 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⁺ 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⁺ 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⁺ 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.

271

272

273 **FINANCIAL SUPPORT**

274

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410

	No CAD (n=7)	SVD (n=7)	MVD (n=16)
Age (years)*	52.0 ± 10.8	65.3 ± 10.0	71.0 ± 10.0
Male sex	4 (57%)	2 (29%)	11 (69%)
Race	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%)
BMI (kg/m²)	30.3 ± 10.8	25.9 ± 7.4	29.0 ± 4.7
SBP (mm Hg)	127.9 ± 16.7	142.7 ± 24.2	138.9 ± 25.1
DBP (mm Hg)	78.3 ± 17.5	80.7 ± 12.7	76.0 ± 17.4
EF (%)	42.3 ± 15.4	37.9 ± 21.6	42.5 ± 15.0
Total cholesterol (mg/dl)	149.3 ± 23.6	179.5 ± 34.1	151.9 ± 45.5
HDL cholesterol (mg/dl)	44.0 ± 14.7	52.3 ± 17.0	42.4 ± 7.4
Triglycerides (mg/dl)	115.0 ± 38.3	126.8 ± 24.4	131.8 ± 51.8
LDL cholesterol (mg/dl)	82.5 ± 19.8	102.3 ± 44.3	83.0 ± 38.1
Leukocytes (1/μl)	8.2 ± 3.0	8.5 ± 2.4	6.4 ± 1.8
Total monocytes (1/μl)	0.54 ± 0.17	0.62 ± 0.36	0.65 ± 0.30
% monocytes	5.8 ± 2.5	7.4 ± 3.0	10.3 ± 3.9
Creatinine (mg/dl)	1.0 ± 0.4	1.3 ± 0.9	1.5 ± 1.4
Smoking history	3 (43%)	3 (43%)	9 (56%)
Diabetes Mellitus	2 (29%)	3 (43%)	4 (25%)
Hypertension	5 (71%)	6 (86%)	14 (88%)
Hyperlipidemia	3 (43%)	6 (86%)	11 (69%)
Beta-blockers	4 (57%)	4 (57%)	15 (94%)
ACE inhibitors	3 (43%)	0 (0%)	7 (44%)
ARBs	0 (0%)	3 (43%)	2 (13%)
CCBs	0 (0%)	3 (43%)	3 (19%)
Antiplatelet drugs	7 (100%)	7 (100%)	16 (100%)
Statins*	4 (57%)	6 (86%)	16 (100%)

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.

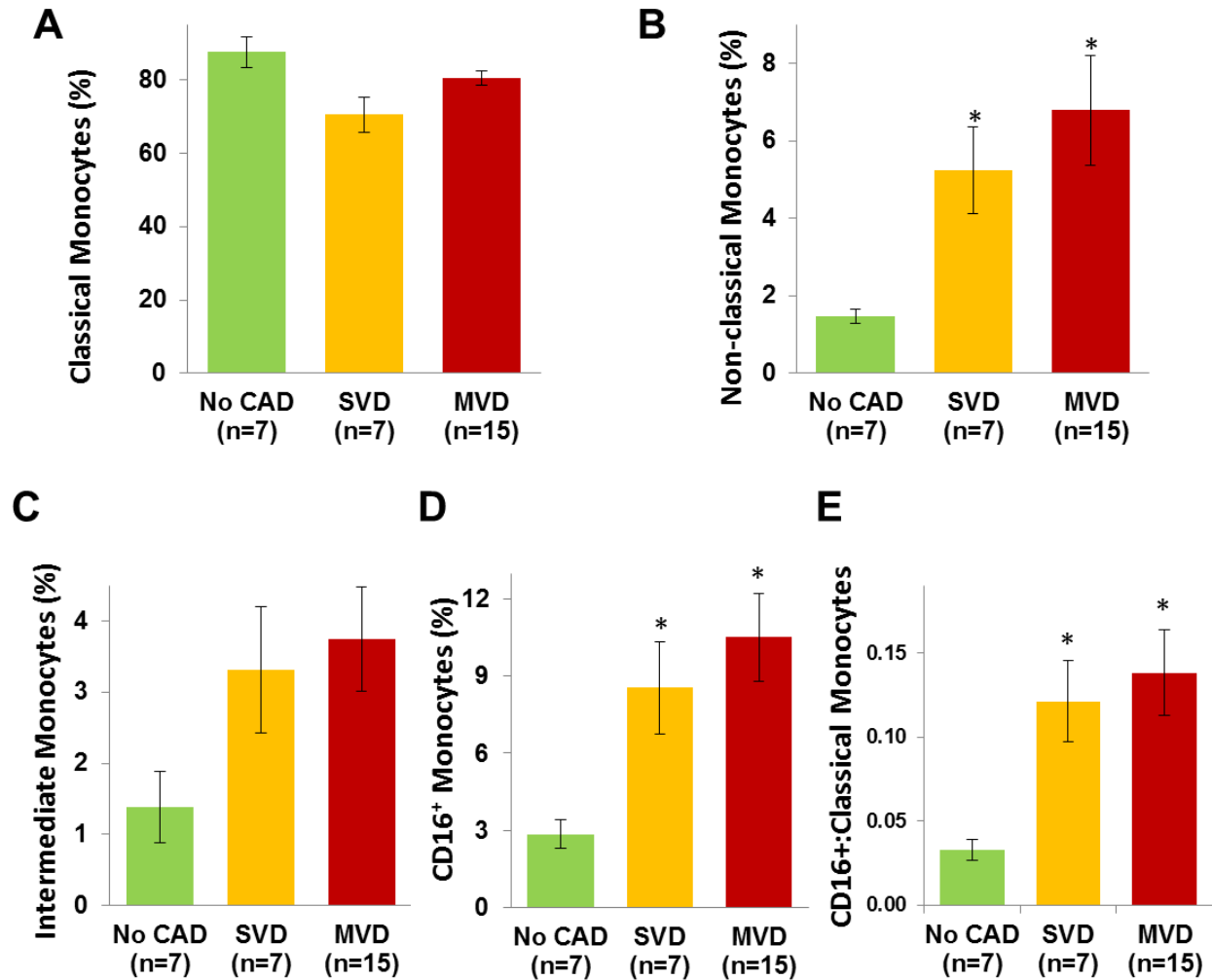


Figure 1: Monocytes subtypes as a function of CAD burden. (A) Classical monocytes are the majority of circulating monocytes and show no consistent pattern as a function of CAD severity. (B) Although still a minority population, non-classical monocytes were relatively enriched in patients with CAD. (C)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

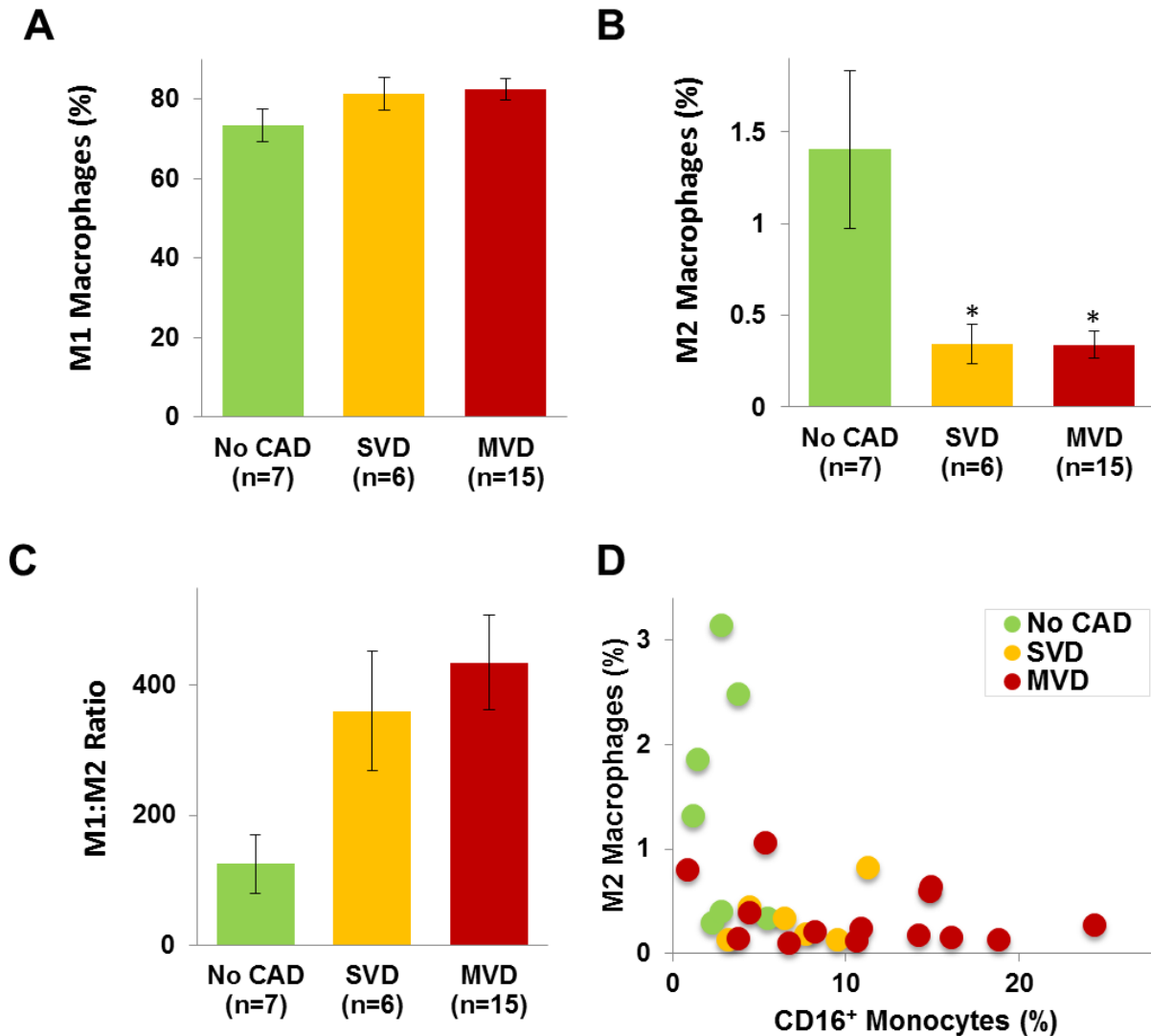


Figure 2: Profiles of cultured macrophage from patients with CAD. (A) Of the macrophages cultured from all patients with or without CAD, most had the M1 surface marked (CD86), without significant differences between clinical presentations. (B) The minority population of M2 macrophages (CD206⁺) was suppressed 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⁺ monocytes show an inverse relationship that tracks with CAD burden.

	Univariate		Multivariate	
	β	p	β	p
CD16⁺ Monocytes	0.64	0.0003	0.43	0.01
Age	0.59	0.001	0.26	0.14
Risk Factors	0.5	0.01	0.23	0.18
M2 Macrophages	-0.43	0.03	-0.11	0.48
WBC	-0.34	0.08		
Creatinine	0.32	0.11		
MAP	-0.16	0.42		
BMI	-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 (β) are shown. The variables significantly associated ($p < 0.05$) with CAD severity were CD16⁺ monocytes, age, cumulative risk factors, and M2 macrophages (inversely). These were included in multiple linear regression analysis, in which CD16⁺ count was the most significant association.