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Circulating CD14$^+$ monocytes in patients with aortic stenosis

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Abstract

Background Calcific aortic stenosis (AS) is an active process sharing similarities with atherosclerosis and chronic inflammation. The pathophysiology of AS is notable for three cardinal components: inflammation, fibrosis and calcification. Monocytes play a role in each of these processes. The role of circulating monocytes in AS is not clear. The aim of the present study was to study an association between circulating apoptotic and non apoptotic CD14$^+$ monocytes and AS features. **Methods** We assessed the number of CD14$^+$ monocytes and apoptotic monocytes in 54 patients with significant AS (aortic valve area 0.74 ± 0.27 cm^2) and compared them to 33 patients with similar risk factors and no valvular disease. The level of CD14$^+$ monocytes and apoptotic monocytes was assessed by flow cytometry. **Results** There was no difference in the risk factor profile and known coronary or peripheral vascular diseases between patients with AS and controls. Patients with AS exhibited increased numbers of CD14$^+$ monocytes as compared to controls (9.9% ± 4.9% *vs.* 7.7% ± 3.9%, P = 0.03). CD14$^+$ monocyte number was related to age and the presence and severity of AS. In patients with AS, both CD14$^+$ monocytes and apoptotic monocytes were inversely related to aortic valve area. **Conclusions** Patients with significant AS have increased number of circulating CD14$^+$ monocytes and there is an inverse correlation between monocyte count and aortic valve area. These findings may suggest that inflammation is operative not only in early valve injury phase, but also at later developed stages such as calcification when AS is severe.

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1 Introduction

Degenerative aortic stenosis (AS) is the most common valvular disease and increases in prevalence with age.[1] Severe AS accounts for considerable morbidity and death, especially in older patients. Aortic valve stenosis is the primary indication for valve replacement in Western countries, and the number continues to increase as the population grows older. The treatment of elderly patients with severe AS is difficult. Compared to younger groups, elderly patients have increased risk. Calcific AS is an active process sharing similarities with atherosclerosis with lipoprotein deposition, chronic inflammation, and active leaflet calcification. As in atherosclerosis, the histological findings seen at different stages of the disease include presence of macrophages, foam cells, T lymphocytes, lipoproteins, heterotrophic calcification, and bone tissue.[2–4] The monocyte is considered a key cellular player in atherogenesis and its transition to macrophage is a requisite to the initial and subsequent stages of the process.[5] Moreover, monocytes are the most abundant immune cell type in atherosclerotic plaques and are the central drivers of vascular inflammation in atherosclerosis.[6,7] Circulating CD14$^+$CD16$^+$ monocytes were shown to be related to atherosclerosis and coronary artery disease severity, as well as progression.[8,9] Although, monocyte apoptosis is suggestive to be protective in atheromas, monocyte derived microparticles promote atherogenesis.[10] Inflammatory cells are the predominant cell type in early aortic valve lesions. Inflammatory infiltration of activated macrophages and T-cells, as well as cytokine release has been reported in stenotic aortic valves,[4,11,12] and enhanced levels of mRNA of inflammatory leukotriene pathway enzymes are found in thickened stenotic aortic valves and correlate with stenosis severity.[13] Monocytes infiltrate endothelial layer via adhesion molecules and differentiate into macrophages. Macrophages express osteopontin, which is related to valvular calcification.[2,11,12,14–16] The role of circulating CD14$^+$ monocytes in AS is not clear. We designed our study to assess an association between circulating CD14$^+$ monocyte levels, subsets of apoptotic monocytes and features of AS.

2 Methods

Fifty four consecutive patients with significant AS (10

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with moderate and 44 with severe AS) and a control group of 33 patients were enrolled in study. Patients who had significant valvular disease other than AS or those who underwent aortic valve replacement or percutaneous balloon valvuloplasty were excluded. Functional class was assessed according to NYHA classification. The study was approved by the institutional ethics committee and all patients provided written informed consent

2.1 Assessment of risk factors

Risk factors were assessed in all patients based on medical records. We defined diabetes mellitus as hyperglycemia requiring pharmacologic therapy; hypertension as either a systolic or diastolic increase in blood pressure (> 140/90 mmHg) or use of antihypertensive therapy; hypercholesterolemia as a total cholesterol level of greater than 200 mg/dL or use of lipid-lowering agents; and cigarette smoking as being an active smoker or having a smoking history of at least 10 pack-years. Coronary artery disease (CAD) was defined as a history of myocardial infarction or presence of CAD on coronary angiography. Peripheral vascular disease (PVD) was defined as history of intermittent claudication or presence of PVD on peripheral angiography.

2.2 Transthoracic echocardiography

Transthoracic echocardiography including assessment of the aortic valve was performed according to established guidelines.[17] Standard M-mode, 2-dimensional, continuous wave Doppler of the aortic, mitral and tricuspid flow as well as pulsed-wave Doppler examination of left ventricular outflow tract and mitral inflow were performed in each subject. Left ventricle dimensions, left atria area, aortic root and ascending aorta diameter were assessed. Grade of diastolic dysfunction, pulmonary pressure and left ventricular mass were calculated. Left ventricular ejection fraction (LVEF) was visually estimated by experienced independent operators. Aortic valve was assessed in short and long-axis echocardiographic views. Maximal velocity, maximal and mean pressure gradient were assessed. Aortic valve area (AVA) was calculated by the continuity equation.

2.3 Quantification of $CD14^+$ monocytes by flow cytometry

Peripheral blood was drawn from patients with AS and control donors. The number of monocytes (defined as $CD14^+$ cells) was quantified by flow cytometry. 7.5×10^6 mononuclear cells (MNCs) were isolated from peripheral blood by density gradient centrifugation, and were incubated at 4℃ in the dark for 30 min with mouse anti-human CD14-PE and its isotope control (bioscience, San Diego, USA). After incubation, cells were washed with phosphate buffered saline (PBS), and analyzed by FACS (LSRII, Becton Dickinson). For a clear analysis, at least 50,000 total events or 1000 $CD14^+$ events were collected by flow cytometry.

2.4 Flow cytometry evaluation of early and late apoptotic $CD14^+$ monocytes

Apoptosis of $CD14^+$ monocyte was assessed using Southern Biotech Apo Screen Annexin V apoptosis detection kit (Annexin V-FITC, 7-AAD solution and Annexin V binding buffer). This assay involves staining peripheral blood mononuclear cells with Annexin V-FITC (a phospholipid-binding protein binding to disrupted cell membranes) in combination with 7-AAD (a vital dye binding to DNA penetrating into apoptotic cells). Fluorescence-activated cell sorting (FACS) analysis of $CD14^+$ monocytes that are in early (annexin V^+/7-AAD^-) or late (annexin V^+/7-AAD^+) apoptotic phase was performed. The percentage of apoptotic $CD14^+$ monocytes (out of total circulating $CD14^+$ cells) was assessed by staining peripheral blood mononuclear cells for 3 color FACS analysis employing anti-CD14-PE (eBioscience), Annexin V-FITC and 7-AAD (SouthernBiotech). The cells were then washed again with PBS and re-suspended in 100 μL Annexin V-FITC binding buffer and incubated with 5 μL of Annexin V- FITC for 15 min at room temperature. Another 200 μL of binding buffer and 5 μL of 7-AAD solution were added without washing, and 100,000 cells were acquired by flow cytometry (FACS Calibur, Becton Dickinson) and analyzed by CellQuest software (BD Bioscience). All analyses and readings were made by researchers who were blinded to the study questions.

2.5 Statistical analysis

Continuous data are presented as medians and interquartile ranges (25^{th}–75^{th} percentiles) for skewed distributed variables or as mean ± SD when normally distributed, and categorical data are presented as absolute numbers and respective percentages. Chi-square tests were used for categorical variables and Student's *t* test for continuous variables. Student's *t* test was used to compare $CD14^+$ monocytes and apoptotic monocytes in patients with AS and control group. Regression analysis and analysis of variance for monocytes by independent variables was performed in all cases. All above analyses were considered significant at $P \leq 0.05$.

3 Results

The study included 54 consecutive patients with moder-

ate and severe AS who met the inclusion criteria and a control group of 33 patients with no significant valvular disease. The mean AVA was 0.74 ± 0.27 cm^2. The etiology of AS in all patients was degenerative disease. A control group included 33 stable patients with no valvular disease who were referred to coronary angiography for chest pain evaluation or patients with atrial arrhythmia referred to echocardiography.

Baseline characteristics and laboratory results of the patients are shown in Table 1. The patients with AS were older and predominantly female, features that have not been known to associate with circulating $CD14^+$ cell levels. There was no difference in the risk factors and known coronary or peripheral vascular diseases between patients with AS and controls. The hemoglobin level was lower in patients with AS compared to patients with no AS.

There was more significant left ventricular hypertrophy in patients with AS, however there was no change in left ventricular chamber systolic and diastolic diameter and LVEF. Patients with AS had higher pulmonary artery pressure, however this difference was not statistically significant (Table 2).

As shown in Figure 1, patients with severe AS have increased number of monocytes as compared to controls (9.9% ± 4.9% *vs*. 7.7% ± 3.9%, $P = 0.03$). The absolute monocyte number was also higher in patients with AS compared to the control group (0.77 ± 0.06 × 10^9/L *vs*. 0.55 ± 0.06 × 10^9/L, $P = 0.0256$). There is no difference in the number of early apoptotic monocytes among the groups (*P*

Table 1. Baseline characteristics of patients with AS and control group.

	AS, *n* = 54	Control, *n* = 33	*P*
Age, yrs	76 ± 10	63 ± 12	< 0.001
Male	36 (67%)	12 (36%)	0.02
Hypertension	38 (70%)	16 (48%)	0.07
Diabetes mellitus	19 (35%)	10 (30%)	0.43
Hypercholesterolemia	37 (69%)	24 (72%)	0.86
Smoking	5 (9%)	3 (9%)	0.9
CAD/PVD	25 (46%)	10 (30%)	0.2
Aspirin therapy	37 (68%)	14 (42%)	0.32
Statin therapy	34 (72%)	17 (50%)	0.6
β-lockers	25 (46%)	12 (35%)	0.98
ACEI	29 (64%)	11 (33%)	0.68
HG, g/dL	11.4 ± 2.4	13.8 ± 1.5	0.006
WBC × 10^3/μL	7.4 ± 2.4	7.5 ± 1.5	0.19
Platelets × 10^3/μL	246 ± 144	208 ± 59	0.52
Creatinine, mg/dL	0.94 ± 0.4	0.87 ± 0.2	0.18
Total cholesterol, mg/dL	166 ± 40	179 ± 40	0.26
LDL cholesterol, mg/dL	88 ± 36	104 ± 36	0.32
Triglycerides, mg/dL	123 ± 59	126 ± 52	0.91
HDL cholesterol, mg/dL	47 ± 15	52 ± 11	0.57

Data are presented as *n* (%) or mean ± SD. ACEI: angiotensin converting enzyme inhibitor; AS: aortic stenosis; CAD: coronary artery disease; HDL: high density cholesterol; HG: haemoglobin; LDL: low density cholesterol; PVD: peripheral artery disease; WBC: white blood cells.

Table 2. Echocardiographic data in patients with moderate and severe AS and control group.

	AS, *n* = 54	Control, *n* = 33	*P*
LVDD, mm	45.1 ± 6	46.5 ± 6	0.4
LVSD, mm	28.1 ± 7	27 ± 6	0.75
Septal thickness, mm	13.9 ± 1.8	12.5 ± 2.7	0.04
Posterior wall thickness, mm	12.5 ± 1.9	11.3 ± 1.5	0.02
LVEF, %	56.2 ± 6.7	56.9 ± 5.6	0.68
Aortic root diameter, mm	30 ± 4	32.7 ± 3.1	0.19
Ascending aorta diameter, mm	33 ± 6	32.8 ± 2	0.6
Left atrial area, cm^2	24.9 ± 6	22.3 ± 4	0.18
AVA, cm^2	0.74 ± 0.27		
Peak AV gradient, mmHg	75.8 ± 34		
Mean AV gradient, mmHg	49.2 ± 22		
Pulmonary artery pressure, mmHg	43.9 ± 13	35 ± 4	0.073

Data are presented as mean ± SD. AS: aortic stenosis; AV: aortic valve; AVA: aortic valve area; LVDD: left ventricular end diastolic diameter; LVEF: left ventricular ejection fraction; LVSD: left ventricular end systolic diameter.

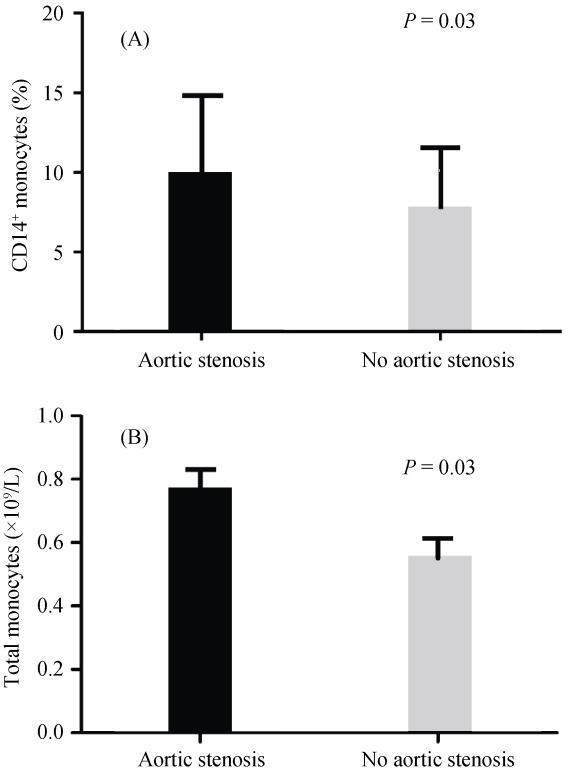

Figure 1. The percent (A) and absolute number (B) of monocytes in patients with moderate and severe aortic stenosis, and controls.

= 0.24, Figure 2). A representative flow cytometric evaluation of $CD14^+$ monocytes and relative number of apoptotic $CD14^+$ cells in a patient with severe AS and without AS is shown in Figure 3.

We tested the association between monocytes and various clinical and echocardiographic parameters in patients with AS and controls. $CD14^+$ monocyte number was related to age, the morphology of the aortic valve and the presence of AS only. There was no correlation between NYHA class and monocyte levels (P = 0.86). The number of monocytes was similar in patients with and without coronary artery disease (P = 0.77). Using backward stepwise regression analysis, AS was found to be the only predictor for $CD14^+$ monocytes levels in the entire study population (P = 0.03). $CD14^+$ early apoptotic monocytes, however, were associated with age only (P = 0.02).

We also tested the correlation between $CD14^+$ monocytes and apoptotic monocytes and various clinical and echocardiographic parameters in patients with AS only. There was no correlation between monocytes or apoptotic monocytes and LVEF (P = 0.61 and P = 0.57, respectively). In patients with AS both $CD14^+$ monocytes and apoptotic monocytes correlated negatively with AVA only (Figure 4).

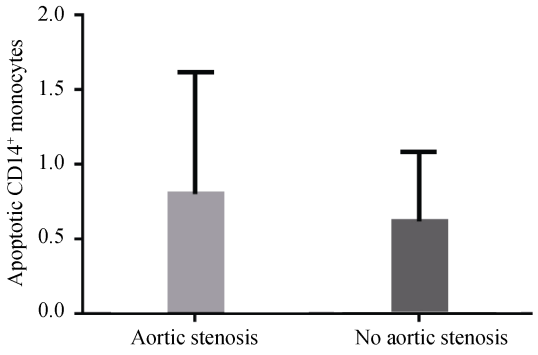

Figure 2. The number of apoptotic monocytes in patients with moderate and severe aortic stenosis and controls.

4 Discussion

In the present study, we have shown an increased number of circulating $CD14^+$ monocytes in patients with significant senile AS. Also, we have found an inverse correlation between monocyte count and aortic valve area. There was no difference in the number of apoptotic $CD14^+$ monocytes between patients with AS and controls; however apoptotic monocytes correlated with aortic valve area.

An inflammatory basis for AS is supported by studies demonstrating increased C-reactive protein concentrations in patients with AS,[18] and increased temperature in stenotic aortic valve cusps.[19] Recently, 18F fluorodeoxyglucose uptake has been shown to be increased in patients with AS compared with controls, displaying a progressive rise in activity with increasing valve severity.[20,21] AS pathophysi-

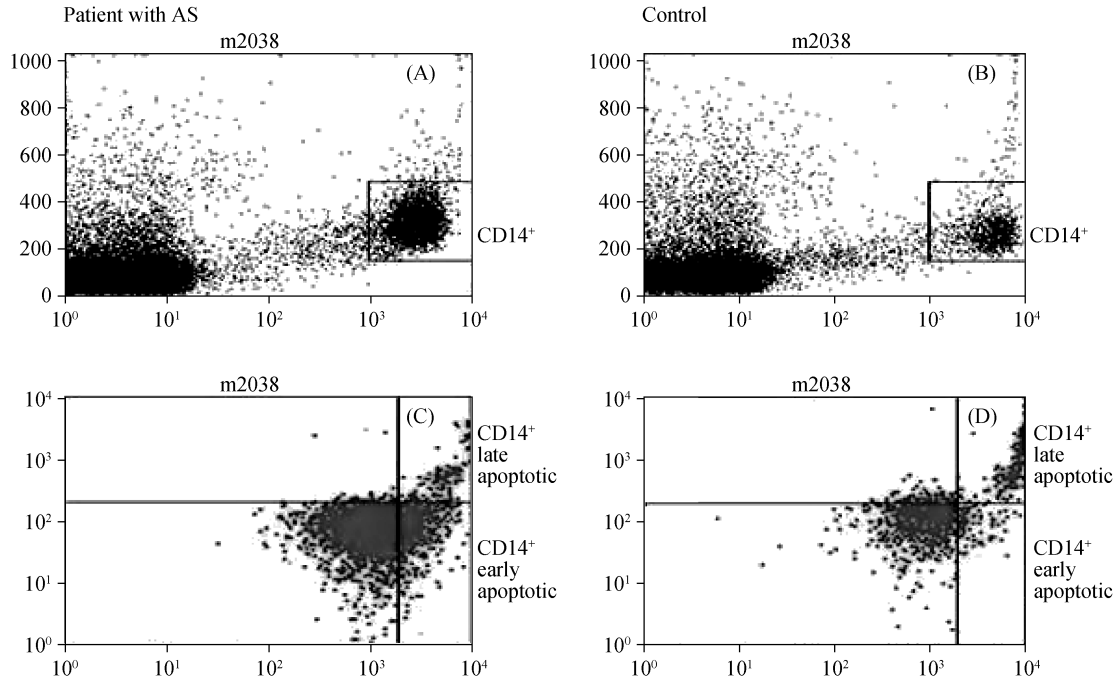

Figure 3. Representative flow cytometric evaluation of $CD14^+$ and apoptotic $CD14^+$ in a patient with aortic stenosis and control. (A & B): Cell percentage of $CD14^+$ in the two groups. The gated $CD14^+$ cells were further analyzed for $CD14^+$ early apoptotic and $CD14^+$ late apoptotic sub-populations, in a patient with AS (C) and control (D). AS: aortic stenosis.

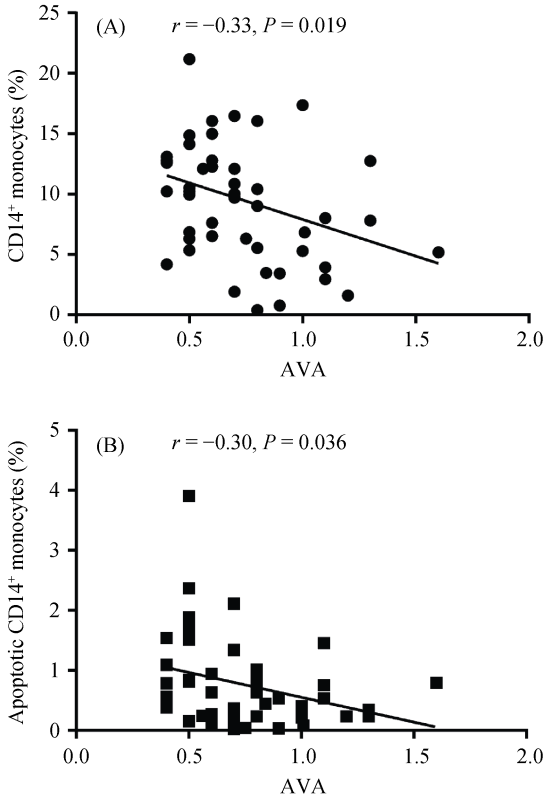

Figure 4. The correlation between CD14$^+$ (A) and apoptotic CD14$^+$ (B) monocytes and AVA in patients with aortic stenosis. AVA: aortic valve area.

ology is notable for three cardinal components: inflammation, fibrosis and calcification.[22] Monocytes play a role in each of these components. Valve injury with endothelial disruption allows lipids to penetrate the valvular endothelium and accumulate in areas of inflammation.[23,24] Oxidized lipoproteins are highly cytotoxic and capable of stimulating intense inflammatory activity and subsequent mineralization.[25] The expression of adhesion molecules allows infiltration of the endothelial layer by monocytes that differentiate into macrophages,[26] and T cells that play crucial role releasing pro-inflammatory cytokines, including transforming growth factor-beta-1,[27] interleukin-1-beta[28] and tumor necrosis factor. Monocytes are also related to tissue fibrosis regulation in general and in valve tissue as well. A subpopulation of fibroblast-like cells, that were found in all three layers of the valve become activated by the inflammatory activity within the valve and differentiate into myofibroblasts, which are believed to be responsible for the accelerated fibrosis observed in this condition.[29] In addition, matrix metalloproteinase's are secreted by myofibroblasts and inflammatory cells and have an important and complex role in restructuring of the valve leaflet matrix. As for calcification, elevated levels of adhesion molecules, T lymphocytes, and monocytes are present in calcified valves, suggesting that an inflammatory infiltrate is necessary for lesion formation and progression. Animal models of heterotopic bone formation demonstrate that inflammatory signals are necessary to trigger distant ossification in a bone morphogenetic proteins conducive environment.[30] In one of these models, cells of the monocyte lineage were required for initiating extra skeletal bone following injury.[31] Fadini and colleagues reported mineralization potential in cells of hematopoietic origin: circulating myeloid cells that produce calcium mineral when cultured in a solubilized basement membrane matrix derived from mouse sarcoma cells.[32] These circulating cells also express monocyte/macrophage lineage markers (CD45, CD14, and CD68), as well as the osteoblastic markers alkaline phosphatase (BAP, also known as tissue-nonspecific alkaline phosphatase), osteocalcin and the osteochondrogenic transcription factors Runx2 and osterix. Egan, *et al*.[33] found that circulating osteogenic cells home to sites of valvular injury and are intimately associated with bone formation. Circulating osteogenic precursors (COP) cells, as well as monocyte-derived mesenchymal progenitors, are derived from CD14 precursors and can be induced to differentiate into osteoblast-like cells. Diehl, *et al*.[34] found that patients with severe AS had increased numbers of CD11b$^+$ activated monocyte cells in their blood compared with controls and also had higher numbers of platelet microparticles conjugated to monocytes compared to controls.

The presence of large number of monocyte/macrophages in the stenotic valves suggests that circulating monocytes contribute to aortic valve calcification and stenosis. The primary function of circulating monocytes is non-specific host protection against foreign pathogens via their prompt elimination (innate immunity). However, the 'dark' side of the process is the damage of the host's own tissues. Circulating monocytes derive from hematopoietic precursor cells in the bone-marrow and are mobilized into the circulation and thence to the vascular wall by biological stimulants abundantly produced by damaged tissues. Since the vascular and valvular walls are located in direct proximity to the circulation, they are especially vulnerable to the effect of the circulating monocytes.

In our previous study, we evaluated the association between the averages of repeated white blood cells differential counts sampled during the previous three years and subsequent echocardiographic AS indices.[35] We found that severe AS was associated with decreased total monocyte count during the previous three years. The present findings do not support this observation. On the contrary, we found

increased number of $CD14^+$ monocytes in patients with significant AS. However, we believe that there is no true contradiction in results since the monocyte count assessment in our previous study was done by automatic blood analyzers that have disadvantages of relating to a highly heterogeneous population of mononuclear cells.

Activated monocytes appear to be resistant to apoptosis and apoptotic monocytes have been shown to be related to better prognosis in sepsis. However, micro-particles of apoptotic monocytes were shown to have procoagulative and proatherosclerotic effect. We did not find difference in the number of $CD14^+$ apoptotic monocytes in patients with AS as compared to controls. However in patients with AS, a negative correlation was found between apoptotic $CD14^+$ monocytes and AVA. We cannot infer whether this finding suggest causality or that the higher number of apoptotic monocytes and more severe AS is due to the hemodynamic changes seen in these patients.

Our study is limited by a relatively small study population. AS is a relatively common valvular disease however the number of patients is significantly smaller than those with coronary atheroscleortic disease. The control group was patients with no AS. A larger scale study is needed to compare patients with various stages of AS and aortic sclerosis. We looked on $CD14^+$ and did not assess monocyte sub populations. Future studies may need to identify cellular subsets and role in AS. The patients with AS were older compared to the control group and there were more female in this group. However, the difference in the monocyte levels between patients with AS and the control group remained significant after correcting for this factors. Circulating $CD14^+$ monocytes do not change significantly with age.[36] In elderly patients with chronic disease, the number of $CD14^+$ monocytes may even decrease, supporting our results that the increase in circulating $CD14^+$ is related to AS.[37]

We found, similar to the reports on circulating monocytes in atherosclerosis, a correlation between circulating monocytes and AS severity. The significance and relevance of measuring circulation monocytes and apoptotic monocytes in patients with AS is not clear and needs to be further evaluated.

Higher percent of $CD14^+$ monocytes in patients with significant AS may represent a marker of disease severity, reflecting increased mobilization of leukocytes. However, it is possible that circulating monocytes are involved in the inflammatory process of AS. Further studies are needed to assess the involvement of monocytes in AS progression; this may also focus attention to novel measures to treat this disease by modulating monocytes.

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