



## Original Article

Proinflammatory CD14<sup>+</sup>CD16<sup>+</sup> monocytes are associated with vascular stiffness in predialysis patients with chronic kidney disease

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**Background:** Chronic inflammation is frequently noted in patients with chronic kidney disease (CKD) and contributes to the development and progression of cardiovascular diseases. Monocytes are heterogeneous populations of cells, and they can be divided into subtypes with different phenotypes and functions based on CD14 and CD16 positivity. This study examined whether the proinflammatory CD14<sup>+</sup>CD16<sup>+</sup> monocyte subset expands in predialysis CKD patients, and also whether the expansion of these cells is closely associated with systemic inflammation and cardiovascular risk factors.

**Methods:** The percentages of proinflammatory CD14<sup>+</sup>CD16<sup>+</sup> monocytes were analyzed in 111 predialysis CKD patients using a flow cytometer, and they were compared with brachial–ankle pulse wave velocity as well as the cytokine plasma levels and other clinical parameters.

**Results:** The proportion of CD14<sup>+</sup>CD16<sup>+</sup> monocytes was significantly higher in patients with advanced stages of CKD than in patients with the early stages. Interleukin-6 levels were also high in patients with advanced stages of CKD. The expansion of CD14<sup>+</sup>CD16<sup>+</sup> monocytes showed significant positive correlations with the high-sensitive C-reactive protein levels, and negative correlations with the levels of serum albumin, hemoglobin, and 25(OH)-vitamin D. In addition, the expansion of CD14<sup>+</sup>CD16<sup>+</sup> monocytes was an independent factor correlated with brachial–ankle pulse wave velocity in diabetic CKD patients.

**Conclusion:** Expansion of the proinflammatory CD14<sup>+</sup>CD16<sup>+</sup> monocyte subset partially accounts for chronic inflammation, malnutrition, and atherosclerosis in CKD.

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## Introduction

Chronic low-grade inflammation is prevalent in chronic kidney disease (CKD) patients and is known to play an important role in

the development and progression of cardiovascular (CV) diseases [1–3]. In particular, inflammation in CKD is frequently associated with malnutrition, and atherosclerosis, which are known as MIA syndrome [4].

As a predictor of CV mortality, vascular stiffness is also increased in CKD patients. Indeed, the Framingham Heart Study showed a positive correlation between arterial stiffness and albuminuria, thereby suggesting that arterial stiffness in CKD patients may be involved in the observed increased CV morbidity and mortality [5].

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Monocytes are a heterogeneous population of cells that can be divided into several subtypes with different phenotypes, and they function based on the CD14 and CD16 positivity [6]. Some monocytes are Fc-receptor-positive, and these CD16<sup>+</sup> monocytes can be further grouped into CD14<sup>+</sup>CD16<sup>+</sup> or CD14<sup>++</sup>CD16<sup>+</sup> cells. Among these cells, CD14<sup>+</sup>CD16<sup>+</sup> cells have been recently reported to be proinflammatory due to the efficient production of proinflammatory cytokines [7]. Merino et al [8] revealed that senescent CD14<sup>+</sup>CD16<sup>+</sup> monocytes exhibit proinflammatory and proatherosclerotic activity, and Kim et al [9] recently demonstrated that the number of these cells was significantly high in patients undergoing hemodialysis (HD). However, little is known about the role of CD14<sup>+</sup>CD16<sup>+</sup> cells in CV disease (CVD) of predialysis CKD patients.

In this study, we examined whether this proinflammatory monocyte subset expands in predialysis CKD patients, and also whether the expansion of these cells was closely associated with systemic inflammation and CV risk factors. In particular, its correlation with pulse wave velocity (PWV), which is a noninvasive and widely used method for measuring arterial stiffness [10] was examined.

## Methods

### Study population

One hundred and eleven stable patients diagnosed to have CKD stage 1–5 based on the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (K/DOQI) and not receiving renal replacement therapy were enrolled in the study. We recruited 11 healthy volunteers for comparison of the levels of proinflammatory cytokines with CKD patients. The comorbidity and medication history of all the patients were determined by standardized interviews and an assessment of their medical records. None of the patients had symptomatic infections in the past 3 months. Patients with a history of collagen vascular disease, malignancy, or those using immunosuppressive agents were excluded. The study protocol was approved by the Institutional Review Board of the Korea University Anam hospital. Informed consent was obtained from all patients.

### Measurement of PWV

The brachial–ankle PWV (baPWV) was measured using a Colin noninvasive vascular screening device (Colin, Co., Ltd., Courbevoie, France). The device simultaneously records the bilateral arm and ankle blood pressure, the pulse volume of the brachial and posterior tibial arteries, the heart sounds, and an electrocardiogram.

### Laboratory methods

Complete blood counts with differential counts of the white blood cell, high-sensitive C-reactive protein (hs-CRP) were measured. In addition, the levels of albumin, calcium, phosphorus, total cholesterol, triglyceride, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, intact parathyroid hormone (iPTH), and 25(OH)-vitamin D were also determined. iPTH and 25(OH)-vitamin D were measured by immunochemiluminescence assay method and the estimated glomerular filtration rate (eGFR) was assessed by creatinine clearance calculated by the modification of diet in renal disease (MDRD) GFR equation.

### Flow cytometric determination of the proinflammatory monocyte subset

The heparinized blood samples (100  $\mu$ L) were stained with an anti-human CD14 antibody conjugated with allophycocyanin (CD14-APC) and an anti-human CD16 antibody conjugated with phycoerythrin (CD16-PE; BD Biosciences, San Diego, CA, USA) for 15 minutes at room temperature. Following lysis and washing, the monocyte subsets were analyzed using flow cytometric detection (FACSCaliber; BD Biosciences, San Diego, CA, USA). One million cells were analyzed from each sample, and the percentage and number of cells out of the total monocytes were compared.

### Quantification of plasma cytokines

Whole blood samples (2.5 mL) were collected in a heparinized tube, and the plasma was obtained to measure the cytokine concentrations. Quantification of plasma cytokines was performed using a cytometric bead array. A human inflammation kit (BD Biosciences) was used, according to the manufacturer's instructions, to simultaneously detect the levels of human proinflammatory [tumor necrosis factor- $\alpha$ , interleukin (IL)-1 $\beta$ , IL-6, and IL-8] and anti-inflammatory (IL-10) cytokines.

### Statistical analysis

All the analyses and calculations were performed using SPSS software, version 20.0 (IBM Corporation, Armonk, NY, USA). Data are expressed as mean  $\pm$  standard deviation or median [interquartile range] according to the distribution. Categorical variables were compared with the Chi-square test or Fisher's exact test and continuous variables were compared using Student t test or Mann–Whitney test between two groups, and analysis of variance (ANOVA) or Kruskal–Wallis test among three or four groups. Pearson correlation or Spearman rank correlation analysis were used to assess the correlations between CD14<sup>+</sup>CD16<sup>+</sup> monocytes and other variables. Multiple linear regression analysis was used to identify factors associated with baPWV. A  $P < 0.05$  was considered statistically significant.

## Results

### Baseline characteristics

The patients were divided into four groups according to the CKD stages; 39 patients were assigned to the early stage CKD group (CKD Stages 1–2) and 28 patients, 27 patients, and 17 patients to the CKD Stage 3, Stage 4, and Stage 5 groups, respectively. The baseline characteristics for each group are shown in Table 1. The patients in the advanced stage CKD group had a higher prevalence of diabetes mellitus, lower levels of serum calcium, albumin, hemoglobin, and 25(OH)-vitamin D, and higher levels of serum phosphorus, hs-CRP, and iPTH. However, there were no significant differences in the lipid profiles and the percentage of statin users.

### CD14<sup>+</sup>CD16<sup>+</sup> proinflammatory monocytes and cytokine production in predialysis CKD patients

Three different monocyte subpopulations were readily identified according to the CD14 and CD16 positivity using flow cytometry (Fig. 1). When we regarded CKD stage 3 to 5 groups as advanced stage group, the percent of CD14<sup>+</sup>CD16<sup>+</sup> monocytes

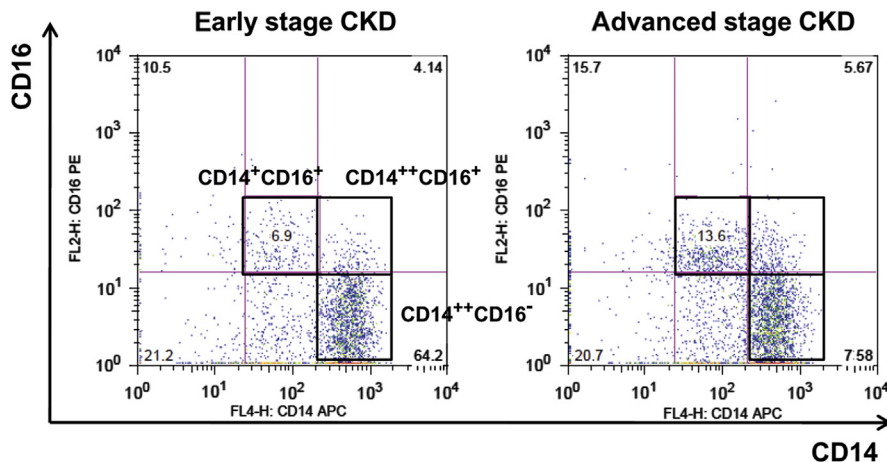
**Table 1. Baseline characteristics**

	CKD Stages 1–2 (n=39)	CKD Stage 3 (n=28)	CKD Stage 4 (n=27)	CKD Stage 5 (n=17)	P
Age (y)	48.6 ± 16.3	62.4 ± 11.6*	62.6 ± 15.7*	57.3 ± 14.0	< 0.001
Women	18 (46.2)	9 (32.1)	11 (40.7)	11 (64.7)	0.197
eGFR	80.2 ± 18.3	46.4 ± 7.6*	22.3 ± 3.7*	9.8 ± 2.2*	< 0.001
BMI (kg/m <sup>2</sup> )	23.3 [21.7, 25.6]	27.3 [22.5, 29.7]	22.4 [20.4, 24.7]	24.7 [21.8, 26.7]	0.127
DM	1 (2.5)	8 (28.6)*	10 (37.0)*	8 (47.1)*	< 0.001
Statins	16 (41.0)	20 (71.4)*	10 (37.0)	5 (29.4)	0.019
baPWV (cm/s)	1331 [1252,1447]	1556 [1366,1621]	1302 [1113,1650]	1443 [1313,1561]	0.430
WBC (/μL)	6610 ± 1570	6350 ± 1470	6570 ± 1520	7220 ± 1930	0.367
Hb (g/dL)	14.1 ± 1.4	13.3 ± 1.8	11.2 ± 1.7*	11.2 ± 1.5*	< 0.001
hs-CRP (mg/L)	1.2 ± 2.3	1.2 ± 2.3	1.3 ± 1.8	4.2 ± 7.6*	0.042
Albumin (g/dL)	4.1 ± 0.4	4.1 ± 0.2	4.0 ± 0.3	3.7 ± 0.5*	0.003
Ca (mg/dL)	9.2 ± 0.5	9.4 ± 0.4	8.9 ± 0.6	8.2 ± 1.0*	< 0.001
P (mg/dL)	3.4 ± 0.6	3.4 ± 0.5	3.5 ± 0.9	4.7 ± 1.2*	< 0.001
T.chol (mg/dL)	158.7 ± 35.5	154.7 ± 32.7	149.6 ± 44.8	158.2 ± 31.2	0.803
TG (mg/dL)	102 [67,161]	143 [77,224]	103 [77,190]	150 [120,183]	0.396
iPTH (pg/mL)	28.2 ± 12.7	32.4 ± 16.9	89.1 ± 55.4*	196.7 ± 119.3*	< 0.001
25(OH)D (ng/mL)	15.5 [10.4, 24.7]	23.3 [14.3, 40.9]	11.8 [7.4, 15.6]*	10.8 [4.8, 13.3]*	< 0.001

\*  $P < 0.05$  vs. CKD Stages 1–2.

Data are presented as mean ± standard deviation, n (%) or median [25%, 75%].

25(OH)D, 25(OH)-vitamin D; BMI, body mass index; Ca, calcium; CKD, chronic kidney disease; DM, diabetes mellitus; eGFR, estimated glomerular filtration rate; Hb, hemoglobin; hs-CRP, high-sensitive C-reactive protein; iPTH, intact parathyroid hormone; P, phosphorus; T.chol, total cholesterol; TG, triglyceride; WBC, white blood cells.



**Figure 1. Flow cytometric detection of CD14<sup>+</sup>CD16<sup>+</sup> proinflammatory monocyte subsets.** After gating the monocytes using the forward scatter channel and side scatter channel, the monocytes were divided into three groups according to the CD14 and CD16 positivity. The proinflammatory monocytes were determined by CD14<sup>+</sup> and CD16<sup>+</sup>. CKD, chronic kidney disease.

**Table 2. Percentage of monocyte subsets and plasma cytokine levels**

	Healthy Control	CKD Stages 1–2	CKD Stages 3–5	P
CD14 <sup>+</sup> CD16 <sup>+</sup> /total monocytes (%)		7.92 ± 0.74	11.60 ± 0.86*	0.026
TNF-α (pg/mL)	0.39 ± 0.39	0.43 ± 0.17	0.37 ± 0.10	0.619
IL-6 (pg/mL)	19.47 ± 17.07	17.64 ± 9.72	24.09 ± 7.99*	0.029
IL-1 (pg/mL)	6.61 ± 5.83	8.63 ± 5.35	5.26 ± 1.62	0.693
IL-8 (pg/mL)	455.77 ± 379.89	522.07 ± 203.11	854.45 ± 193.81	0.362
IL-10 (pg/mL)	0.35 ± 0.15	0.26 ± 0.09	0.40 ± 0.10	0.578

\*  $P < 0.05$  vs. CKD Stages 1–2.

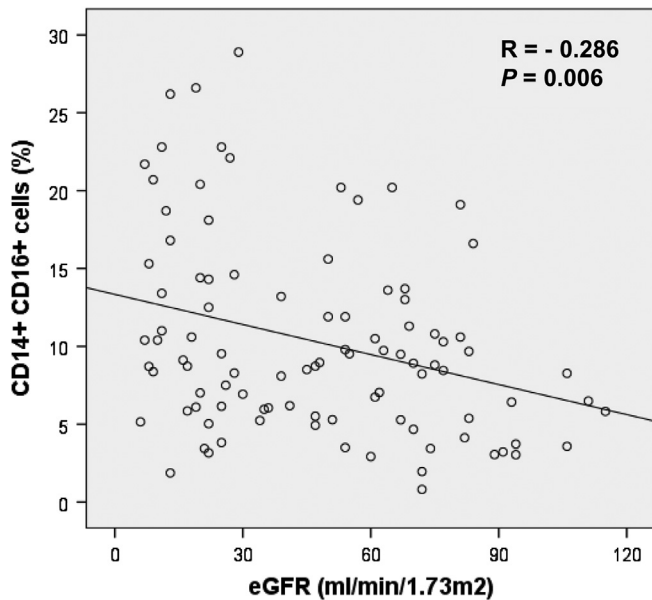
Data are presented as mean ± standard error of the mean.

CKD, chronic kidney disease; IL, interleukin; TNF, tumor necrosis factor.

was significantly higher in the advanced CKD group than in the early group (Table 2), whereas CD14<sup>++</sup>CD16<sup>+</sup> monocyte population was not significantly different between them (data not shown). In addition, the percentage of CD14<sup>+</sup>CD16<sup>+</sup> cells is

negatively correlated with the eGFR (Spearman's  $R = -0.286$ ,  $P = 0.006$ , Fig. 2).

In CKD patients, malnutrition and vascular calcification are known to comprise chronic inflammation driven by



**Figure 2.** Relationship between estimated glomerular filtration rate and the percentage of CD14<sup>+</sup>CD16<sup>+</sup> cells. eGFR, estimated glomerular filtration rate.

proinflammatory cytokines (IL-1, IL-6, tumor necrosis factor- $\alpha$ , interferon- $\gamma$ , and others). Therefore, we also examined the plasma levels of the proinflammatory cytokines and found that IL-6 was markedly increased in the advanced CKD patients in addition to an increased percentage of proinflammatory monocytes (Table 2).

#### Correlation between the percentage of CD14<sup>+</sup>CD16<sup>+</sup> proinflammatory monocytes and CV parameters

It is well known that a strong interaction exists between CVD and inflammation as well as nutritional status in patients with CKD (malnutrition, inflammation, and atherosclerosis MIA syndrome). Therefore, to evaluate their association with inflammatory monocytes, we examined hs-CRP, albumin, hemoglobin, 25(OH)-vitamin D, and baPWV in CKD patients.

The percentage of CD14<sup>+</sup>CD16<sup>+</sup> cells showed a significant positive correlation with hs-CRP levels (Spearman's  $R=0.270$ ,  $P=0.011$ , Fig. 3A) and baPWV (Spearman's  $R=0.280$ ,  $P=0.006$ , Fig. 3B), whereas CD14<sup>++</sup>CD16<sup>+</sup> cell population did not showed significant association with them (data not shown). It also negatively correlated with the level of serum albumin (Spearman's  $R=-0.235$ ,  $P=0.027$ , Fig. 3C), hemoglobin (Spearman's  $R=-0.287$ ,  $P=0.004$ , Fig. 3D), and 25(OH)-vitamin D (Spearman's  $R=-0.271$ ,  $P=0.028$ , Fig. 3E). In multivariate analysis, the expansion of CD14<sup>+</sup>CD16<sup>+</sup> monocytes showed an independent positive correlation with baPWV in the diabetic CKD patients; however, in the nondiabetic CKD patients, there was no significant association (Table 3).

## Discussion

We have previously reported that microinflammation in HD patients is associated with the expansion of CD14<sup>+</sup>CD16<sup>+</sup> proinflammatory monocytes and also possible modification by online hemodiafiltration (HDF) [9]. We extended these previous

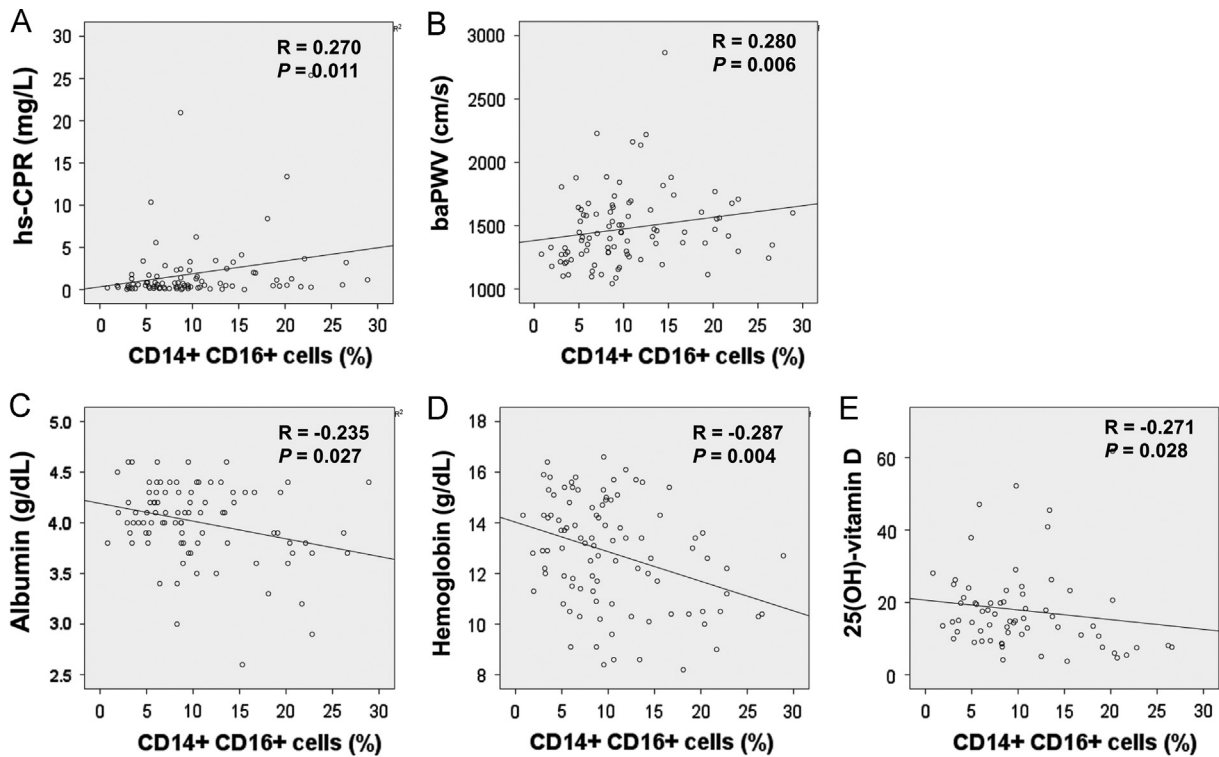
observations in HD patients to predialysis CKD patients and tested the possible important contribution of this proinflammatory monocyte subset to low-grade systemic inflammation and increased CVD risk.

Monocyte heterogeneity is widely acknowledged. Cell-surface expression of CD14 and CD16 defines functionally and phenotypically distinct subsets of monocytes: classical (CD14<sup>++</sup>CD16<sup>-</sup>), intermediate (CD14<sup>++</sup>CD16<sup>+</sup>), and nonclassical (CD14<sup>+</sup>CD16<sup>+</sup>) monocytes [11]. The latter two are often denoted as proinflammatory CD16<sup>+</sup> monocytes and these monocytes were first reported in 2001 and are considered to be the main culprit in patients suffering from chronic inflammation, such as rheumatoid arthritis, systemic lupus erythematosus, or HD [12]. Merino et al [8] demonstrated that nonclassical CD14<sup>+</sup>CD16<sup>+</sup> cells are senescent monocytes with shortened telomere lengths that express increased levels of chemokine receptors and subsequently more readily adhere to the endothelial cells. In addition, a growing body of evidence suggests that these proinflammatory monocytes contribute to the development of atherosclerosis [13,14]. Although several studies show the significant correlation between CD14<sup>++</sup>CD16<sup>+</sup> monocytes and CVD in CKD patients [15,16], little is known about the nonclassical proinflammatory CD14<sup>+</sup>CD16<sup>+</sup> monocyte subset especially in nondialysis CKD patients; moreover, its correlation with vascular stiffness as an important predictor of cardiovascular mortality, has not been assessed in previous studies.

In this study, we observed that the CD14<sup>+</sup>CD16<sup>+</sup> monocyte subset also expands in predialysis CKD patients, similar to that observed in HD patients. The expansion was greater in the advanced CKD patients (CKD Stages 3–5) than in those in the early stage of CKD, thereby suggesting that retained uremic toxins might be key factors in the expansion of this monocyte subset and the subsequent systemic inflammatory burden. In addition, the plasma levels of IL-6 were significantly high only in the advanced CKD patients, thereby suggesting that the production of IL-6 from this monocyte subset contributes to hypercytokinemia and systemic inflammation. Although impaired excretion might also contribute to hypercytokinemia, preferential production of inflammatory cytokine from CD16<sup>+</sup> monocytes has been demonstrated in our previous study.

The proinflammatory cytokines produced from the expanded proinflammatory monocytes may induce endothelial damage and subsequently promote accelerated atherosclerosis and increased CVD risk. This is a probable suggestion because the critical role of lipid laden macrophages in the development and progression of atherosclerosis is well known [11].

Next, we tested whether the expansion of CD14<sup>+</sup>CD16<sup>+</sup> monocytes is also closely associated with several other parameters of increased CVD risk. The indicator of vascular stiffness, baPWV showed a positive correlation with the percentage of CD14<sup>+</sup>CD16<sup>+</sup> monocytes, moreover, in the multivariate analysis, the percentage of CD14<sup>+</sup>CD16<sup>+</sup> monocytes was an independent factor associated with baPWV in the diabetic patients, but not in nondiabetic patients. In our analysis, the patients with diabetes had more advanced renal failure (CKD Stages 3–5) and were older than the patients without diabetes ( $67.19 \pm 8.58$  years vs.  $54.72 \pm 15.92$  years); therefore it could be possible that the expansion of CD14<sup>+</sup>CD16<sup>+</sup> monocytes is associated with vascular stiffness especially in diabetic or elderly and advanced CKD patients. Considering that strong correlations between PWV and CV events and all-causes of mortality have already been demonstrated in the general population as well as CKD patients



**Figure 3.** The relationship between percentage of CD14<sup>+</sup>CD16<sup>+</sup> cells and (A) hs-CRP, (B) baPWV, (C) serum albumin, (D) hemoglobin, and (E) 25(OH) vitamin D. baPWV, brachial-ankle pulse wave velocity; hs-CRP, high-sensitive C-reactive protein.

**Table 3.** Multiple linear regression analysis of risk factors associated with brachial-ankle pulse wave velocity

Subgroup*	Variables	Coefficient value	P
1. Diabetic patients	Age	2.625	0.086
	CD14 <sup>+</sup> CD16 <sup>+</sup> /total monocytes (%)	2.058	0.033
2. Nondiabetic patients	Age	1.572	< 0.001

\* Excluded variables: *Subgroup 1* C-reactive protein, estimated glomerular filtration rate, and triglyceride; *Subgroup 2* C-reactive protein, estimated glomerular filtration rate, triglyceride, and CD14<sup>+</sup>CD16<sup>+</sup> population.

[17–19], the expansion of proinflammatory monocytes could serve as a possible target to suppress systemic inflammation and decrease CV risks.

In addition, the percentage of CD14<sup>+</sup>CD16<sup>+</sup> monocytes also positively correlated with CRP levels, which are a well-known indicator of increased CVD risk. This observation, together with the finding that the percentage of proinflammatory monocytes had a negative correlation with the serum albumin levels, strengthens the proposal that proinflammatory monocytes play a critical role in premature CV death in CKD patients.

Several recent studies have suggested the important link between 25(OH)-vitamin D levels and CV events in general populations [20] and showed the inverse correlation of 25(OH)-vitamin D levels with CRP and IL-6 levels [21]. Interestingly, we observed that the 25(OH)-vitamin D levels were inversely correlated with the percentage of proinflammatory CD14<sup>+</sup>CD16<sup>+</sup> monocytes in CKD patients. Although the exact mechanisms linking vitamin D and inflammation or CV events are not clear, one of the plausible mechanisms by which vitamin D modifies the risk for CVD outcomes is that vitamin D modulates the inflammatory response, including the monocyte-macrophage activity, via the nuclear vitamin D receptor [22,23]. In addition, vascular smooth muscle cells

are also known to express vitamin D receptors and relax if they bind to vitamin D [24]. London et al [25] showed the relationship between arterial alterations and circulating levels of vitamin D. Vitamin D deficiency is a common condition in patients with CKD and, therefore, it is possible that vitamin D deficiency is directly responsible for the increased proinflammatory monocyte subset and subsequent systemic inflammation and increased CVD risks.

Despite several meaningful findings, there are a number of limitations in our study. First, this was a cross-sectional study involving a relatively limited number of patients from a single center. Second, analyses of the exact doses of calcium-containing salts or active vitamin D treatments that might affect bone status, iPTH, or plasma calcium and phosphorous levels were not performed. Third, all parameters including the proinflammatory monocyte subset, plasma cytokines, and the levels of hs-CRP, albumin, and 25(OH)-vitamin D were analyzed once without a follow-up of their changes over the time of measurement.

In conclusion, the results of our study suggest that the expansion of the proinflammatory CD14<sup>+</sup>CD16<sup>+</sup> monocyte subset partially accounts for chronic inflammation, malnutrition, and atherosclerosis in predialysis CKD patients. In addition, a better understanding of the mechanisms of chronic inflammation will help develop treatment strategies in CKD patients.

## Conflict of interest

The authors report no conflicts of interest.

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