

Endothelial Activation Markers in Anemic Non-Dialysis Chronic Kidney Disease Patients

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Key Words

Anemia · Chronic kidney disease · Endothelial markers · Fibrinogen · Vascular cell adhesion molecule-1 · Von Willebrand factor

Abstract

Background/Aims: Anemia in chronic kidney disease is an independent predictor of cardiovascular disease (CVD). We explored the relationship between anemia and markers of inflammation and endothelial activation in non-dialysis chronic kidney disease (ND-CKD) patients to understand this mechanism. **Methods:** Cross-sectional analysis was performed on 30 adult ND-CKD patients for markers of inflammation and endothelial activation using a multiplexed immunoassay. Data were analyzed according to the anemic status defined by the modified World Health Organization criteria. **Results:** Seventeen patients were classified as anemic. Baseline characteristics by anemic status were similar except that anemic patients were older ($p = 0.006$), had lower estimated glomerular filtration rate (eGFR; $p = 0.01$) and higher prevalence of CVD ($p = 0.02$). Compared to non-anemic patients, log-transformed values of fibrinogen ($p = 0.012$); von Willebrand factor (vWF, $p = 0.008$), vascular cell adhesion molecule-1 (VCAM-1, $p = 0.025$) and C-reactive protein ($p = 0.043$) were elevated in anemic patients. Serum ferritin ($p = 0.93$) and serum albumin ($p = 0.06$) were not different. Age and eGFR-adjusted logistic regression anal-

ysis showed that anemic patients had increased odds for a composite of higher median values of fibrinogen, vWF and VCAM-1 ($p = 0.01$, odds ratio 8.1, 95% CI 1.08–111.0). **Conclusion:** We report the association of anemia with elevated markers of endothelial activation in ND-CKD patients. Longitudinal studies are needed to confirm our findings.

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Introduction

In chronic kidney disease, the overall cardiovascular disease (CVD) mortality rate is up to 30-fold higher than the age-adjusted CVD mortality in the general population [1]. Traditional biomarkers appear insufficient to predict CVD risk and novel non-traditional biomarkers are being scrutinized to better predict adverse CVD events [2–6]. These novel markers are detectable even among patients without evidence of abnormalities in traditional biomarkers [6, 7]. Anemia is a common complication among patients with chronic kidney disease (CKD) [8], and is an independent risk factor for adverse cardiovascular outcome in CKD [9–12]. However, the mechanism remains unclear. Identifying mechanisms beyond the traditional risk factors may help to better explain CVD in anemic non-dialysis CKD (ND-CKD) and end-stage renal disease patients.

Markers of inflammation have received attention as predictors of CVD outcome in the general population and patients with CKD [2, 13–15]. However, markers of endothelial activation have not been explored in anemic ND-CKD patients. We sought to explore the relationship between biomarkers of endothelial activation and inflammation and anemia in ND-CKD patients. We analyzed ferritin, albumin, C-reactive protein (CRP), fibrinogen, von Willebrand factor (vWF) and vascular adhesion molecule-1 (VCAM-1). These markers have been associated with increased mortality in different population groups [6, 16–18]. In addition to being previously validated, they are all available in commercial assays allowing replication of our study.

Materials and Methods

We utilized data from a prospective observational ND-CKD cohort that we established in 2004 at the Brigham and Women's Hospital. This cohort is designed to study the influence of specific modifiable factors on the risk of progression of CKD and the risk of CVD in patients with CKD. For inclusion in the cohort, subjects were required to be aged >18 years and have CKD as defined by Levey et al. [19]. The estimated glomerular filtration rate (eGFR) was calculated by the Modification of Diet in Renal Disease (MDRD) II equation ($eGFR [ml/min \text{ per } 1.73 \text{ m}^2] = 186 \times [Cr (mg/dl)]^{-1.154} \times [age]^{-0.203} \times [1.21 \text{ if the subject is black}] \times [0.74 \text{ if the subject is a female}]$). Exclusion criteria included: unwillingness to provide informed consent; patients who were hospitalized or had a thrombotic or CVD event 1 month prior to enrollment; institutionalized subjects; those with a life expectancy of <6 months; known HIV patients; patients with an active malignant neoplastic disease other than localized non-melanoma skin cancer; patients on immunosuppressive treatment; patients with multiple myeloma; patients enrolled in an intervention study, and patients on dialysis. The institutional review board approved the study and informed consent was obtained from each patient. Anemia was defined by the modified World Health Organization (WHO) criteria: Hgb <120 g/l in women, Hgb <130 g/l in men or treatment with epoetin. Treatment with epoetin and the dose utilized was ascertained by review of the medical records, direct contact with the patient, and reviewing the pharmacy record. Iron deficiency was defined by a total iron-binding capacity (TSAT) of <15% or serum ferritin of <100 pmol/l.

Analysis of the Serum Specimens

Blood was collected from 30 patients after overnight fasting using venipuncture and collection tubes from Becton-Dickinson. Samples were aliquotted and frozen at -80°C . The samples were shipped on dry ice to Rules-Based Medicine, Inc. (Austin, Tex., USA) for tests. Markers of inflammation and endothelial activation were analyzed by Luminex with internal controls: CRP, albumin, ferritin, fibrinogen, vWF, and VCAM-1.

Statistical Analysis

Baseline data were analyzed using the following statistical methods: continuous data were analyzed with an independent sample non-parametric test and categorical data were analyzed with Fisher's exact test in the analysis of demographic characteristics. Biomarkers that reached significance were further analyzed. The levels of serum ferritin, albumin, CRP, fibrinogen, vWF, and VCAM-1 levels deviated from the normal distribution and log-transformation reduced skew deviation. We used the Mann-Whitney U test to compare anemic and non-anemic patients. Spearman correlation coefficients were calculated for pairs of continuous variables. Logistic regression was used to calculate the odds ratio for reaching a composite end point of a higher median of fibrinogen, vWF and VCAM-1. SPSS version 15 (SPSS Inc., Chicago, Ill., USA) was used to analyze the data.

Results

Patient Characteristics by CKD-Associated Anemia

The baseline characteristics of the subjects are presented in table 1. Anemia was defined by the modified WHO criteria as <120 g/l in women and <130 g/l in men or treatment with epoetin. Of the 30 patients analyzed, 17 patients were anemic by the modified WHO criteria. As expected, the mean Hgb level differed significantly among the groups (106 ± 16 vs. 138 ± 12 g/l, $p < 0.001$, anemic vs. non-anemic). Anemic patients were older (68.18 ± 14.17 vs. 49.23 ± 20.83 years, $p = 0.006$, anemic vs. non-anemic). Serum creatinine was not different between the groups (274.04 ± 97.24 vs. 212.16 ± 132.6 $\mu\text{mol/l}$, $p = 0.13$, anemic vs. non-anemic); however, the eGFR reached statistical significance (22.4 ± 13.5 vs. 47.0 ± 35.0 ml/min, $p = 0.01$, anemic vs. non-anemic). Of note, anemic patients had a higher prevalence of CVD ($p = 0.02$). Serum ferritin, low-density lipoprotein, smoking status, presence of proteinuria, serum albumin, complement C3, and parathyroid hormone were not significantly different between the 2 groups. Erythropoiesis-stimulating agents (ESAs) were prescribed in 8 anemic patients at a mean dose of 85 ± 42 units/kg/week.

Biomarkers by Anemic Status

In this study, we focused on endothelial markers. The levels of ferritin, albumin, CRP, fibrinogen, vWF and VCAM-1 were examined with respect to anemic status. Since the markers were not normally distributed, values were log-transformed. Non-parametric test showed elevated levels of log-transformed CRP in patients with anemia and reached significance ($p = 0.043$). However, log-

Table 1. Baseline characteristics of CKD patients by anemia status¹

Characteristic	Anemic CKD (n = 17)	Non-anemic CKD (n = 13)	p value ²
Age, years	68.18 ± 14.17	49.23 ± 20.83	0.006
Female gender	10 (58%)	5 (38%)	0.28
Race			0.46
Caucasian	7 (41%)	5 (53%)	
African American	8 (53%)	3 (23%)	
Hispanic	1 (5%)	2 (15%)	
Others	1 (5%)	1 (7%)	
Etiology of chronic kidney disease			0.69
Diabetic nephropathy	6 (35%)	3 (23%)	
Non-diabetic	11 (64%)	10 (76%)	
Hypertension	8 (47%)	2 (15%)	
Chronic glomerulonephritis	2 (11%)	6 (53%)	
Others	1 (5%)	1 (5%)	
PTH, ng/l	240 ± 183.7	146.4 ± 127.5	0.14
Serum creatinine, μmol/l	274.04 ± 97.24	212.16 ± 132.6	0.13
MDRD GFR, ml/min/1.73 m ²	22.4 ± 13.5	47.0 ± 35.0	0.01
Hemoglobin, g/l	106 ± 16	138 ± 12	<0.001
Transferrin saturation, %	22 ± 15	29 ± 15	0.11
Ferritin, pmol/l	544.89 ± 865.3	321.3 ± 173	0.26
Low-density lipoprotein cholesterol, mmol/l	2.58 ± 1.3	2.85 ± 1.1	0.65
Proteinuria of >300 mg/g present, n	11 (64%)	5 (38%)	0.28
Proteinuria, mg/g	987.5 ± 810.6	447.9 ± 689	0.08
Albumin, g/l	41 ± 3.0	43 ± 3.0	0.1
Smoking, n	3	2	0.36
Complement 3, g/l	0.76 ± 0.15	0.74 ± 0.19	0.63
Coronary artery disease, n	6	2	0.02
Congestive heart failure, n	4	2	0.27
Blood group A-positive, n	3	4	0.23

All continuous data are expressed as mean ± SD and categorical data are expressed in percentages.

¹ The modified WHO classification of anemia is used where anemia is defined as the presence of Hgb <120 g/l in women or Hgb <130 g/l in men or treatment with erythropoiesis-stimulating agents.

² Significant if p value is <0.05.

transformed levels of serum albumin ($p = 0.06$) and serum ferritin ($p = 0.93$) were not different in anemic and non-anemic CKD patients. Log-transformed values of the markers of endothelial activation were significantly elevated in the anemic patients: fibrinogen ($p = 0.012$), vWF ($p = 0.008$), and VCAM1 ($p = 0.025$).

Sensitivity Analysis

Analysis of Biomarkers by Various Anemia Definitions

We performed sensitivity analyses using different classifications of anemia to evaluate the robustness of the associations between various biomarkers and anemia status (table 2). The first sensitivity analysis strictly clas-

sified patients based on the WHO criteria regardless of epoetin treatment as a cause of anemia (WHO definition of anemia is <130 g/l for adult males and <120 g/l an adult females): 16 patients were classified as anemic. Log CRP ($p = 0.06$) was higher in the anemic patients but did not reach significance. Log albumin was lower in anemic patients ($p = 0.03$). In contrast, markers of endothelial activation were significantly elevated in anemic patients: log fibrinogen ($p = 0.025$), log vWF ($p = 0.004$), and log VCAM-1 ($p = 0.042$). Log serum ferritin was not significant between the groups ($p = 0.82$). A second sensitivity analysis was performed based on the National Kidney Foundation (NKF) criteria for anemia (anemia is <135 g/l for an adult male and <120 g/l for an adult female): 19

Table 2. Sensitivity analysis of inflammatory and endothelial markers by anemia status in non-dialysis CKD patients

Marker	p value by		
	NKF criteria anemic (n = 19)	Modified WHO criteria anemic (n = 17)	WHO criteria anemic (n = 16)
CRP	0.023	0.043	0.06
Ferritin	0.611	0.93	0.82
Serum albumin	0.03	0.06	0.031
VCAM-1	0.005	0.025	0.042
Von Willebrand factor	0.006	0.008	0.004
Fibrinogen	0.005	0.012	0.025

Mann-Whitney U test was performed for analysis with log-transformed values as dependent variables.

patients were classified as anemic. The log CRP was significantly higher ($p = 0.02$) and serum albumin was significantly lower ($p = 0.03$) in anemic patients compared to the non-anemic patients. Serum ferritin levels were again not significant ($p = 0.61$). The endothelial markers were again significantly elevated: log fibrinogen ($p = 0.005$), log vWF ($p = 0.006$) and log VCAM-1 ($p = 0.005$). Eight subjects were receiving ESAs. These markers did not differ significantly by ESA status among the anemic subjects (i.e., anemic subjects exposed to ESA vs. anemic subjects not exposed to ESA). Serum fibrinogen (4.9 $\mu\text{mol/l}$ in anemic patients on ESA vs. 5.2 $\mu\text{mol/l}$ in anemic patients not on ESA, $p = 0.77$), VCAM-1 (647 ng/l in anemic patients on ESA vs. 717 ng/l in anemic patients not on ESA, $p = 0.33$) and vWF (70.12 ng/ml in anemic patients on ESA vs. 70.33 ng/ml in anemic patients not on ESA, $p = 0.98$). Two subjects in each group met the criteria for iron deficiency. Two subjects in the anemic and 1 in the non-anemic group were receiving oral iron. None of the patients were on intravenous iron treatment.

Correlation between Markers of Endothelial Activation

A positive Spearman rank correlation was noted among the markers of endothelial activation: log fibrinogen levels positively correlated with log vWF levels ($r = 0.767$; $p < 0.001$); log fibrinogen and log VCAM-1 ($r = 0.26$; $p = 0.023$), and log VCAM-1 and log vWF ($r = 0.42$, $p = 0.019$). We analyzed the correlation by using median levels: there was a significant correlation between VCAM-1 and fibrinogen levels ($r = 0.40$, $p = 0.023$), VCAM-1 and

vWF ($r = 0.21$, $p = 0.03$), and vWF and fibrinogen levels ($r = 0.46$, $p = 0.009$).

Analysis of Anemia Status by Median Values of Biomarkers

In addition to the analysis of log-transformed values of the biomarkers, different cutoff values were used to further analyze the association of these biomarkers with anemic status as defined by the modified WHO criteria. Data were analyzed by median values as cutoff. CRP (median 2.5 g/l, $p = 0.15$), ferritin (308.9 pmol/l, $p = 1.0$) and albumin (42 g/l, $p = 0.07$) were not significantly different between anemic and non-anemic patients. However, serum fibrinogen (median 12.9 $\mu\text{mol/l}$, $p = 0.02$, $r = 0.47$), vWF (median 56 ng/ml, $p = 0.02$, $r = 0.47$) and VCAM-1 (median 615.5 ng/l, $p = 0.02$, $r = 0.39$) were significantly elevated in anemic compared to non-anemic patients. Median eGFR (23 ml/min) approached statistical significance with respect to anemia by the modified WHO criteria ($p = 0.07$). Anemia was analyzed by median age of 65 years. Age influenced the anemic status of significantly ($p = 0.025$). A multivariate model was constructed with a composite of medians of the three-endothelial markers and median age and median eGFR. After adjusting for age and eGFR, the composite endpoint of reaching higher median values of VCAM-1, vWF and fibrinogen was significant in anemic compared to non-anemic patients ($p = 0.01$, OR 8.1, 95% CI 1.08–111.0, $r = 0.61$).

Endothelial Markers by Renal Function and Age

The interaction of serum fibrinogen, vWF and VCAM-1 with age and renal function was analyzed. Log serum fibrinogen ($p = 0.10$), log vWF ($p = 0.08$) and log VCAM-1 ($p = 0.06$) showed a trend for statistical significance. Renal function by eGFR showed that patients with lower eGFR had higher VCAM-1 ($p = 0.06$) and higher vWF ($p = 0.07$), but this did not reach significance. Serum log fibrinogen was significantly elevated ($p = 0.04$) by renal function status.

Discussion

In this study our main finding was an association of higher fibrinogen, vWF and VCAM-1 with anemia in ND-CKD patients. Log-transformed values of CRP, fibrinogen, vWF and VCAM-1 were significantly elevated in anemic compared to non-anemic CKD patients. Serum albumin was significantly lower in anemic CKD patients. In a multivariate analysis model, median fibrino-

gen, vWF and VCAM-1 were significantly elevated in anemic CKD patients. Sensitivity analyses using different cutoff values for anemia and the biomarkers confirmed the robustness of these observations. Other markers of inflammation did not differ by anemia status.

Studies have demonstrated that anemia in CKD patients is an independent risk factor for CVD events and mortality. Walker et al. [12] found that patients with anemia (defined as Hgb <120 g/l) had a fivefold increase in hospitalization for myocardial infarction. Another study found a threefold higher risk of CVD in anemic patients (defined as Hgb <130 g/l for men and <120 g/l for women) regardless of the degree of renal impairment [9]. In the present study, most of the traditional risk factors did not differ between anemic and non-anemic patients, namely gender, etiology of CKD, ferritin, LDL cholesterol or smoking status. In contrast, endothelial activation markers were elevated in anemic patients.

Fibrinogen, an acute-phase reactant and marker of endothelial activation [20–22], is an independent risk factor for CVD disease, left ventricular hypertrophy and mortality in patients without kidney disease [12, 22, 23]. Recently, it has been associated with cardiovascular morbidity, left ventricular hypertrophy and clotting of the vascular access in hemodialysis patients [6, 23, 24]. In our study, the fibrinogen levels in anemic patients were comparable to the high-risk general population for CVD [25] but lower than those found in hemodialysis patients at risk of CVD [26]. Age influences the fibrinogen level [27]. Thus, we adjusted for age in a multivariate model. Furthermore, there was no difference between groups in the known factors that may influence fibrinogen levels – gender, smoking status, and low-density lipoprotein cholesterol, and proteinuria status. Molecular mechanisms underlying the pathogenic role of fibrinogen are still being uncovered. Integrin-dependent signal transduction pathway leading to induction to chemotactic activity by fibrinogen may contribute to induction of chemokines in early atherosclerosis. Fibrinogen also increases monocyte chemoattracting protein-1, platelet-derived growth factor and interleukin-8 [22]. Thus, fibrinogen may activate various pathways implicated in CVD pathogenesis.

In our study, we found higher vWF levels in a high-risk population not previously reported. vWF, a glycoprotein secreted by endothelial cells, has been shown to be involved in platelet aggregation and adhesion [28]. An animal model showed that inactivation of vWF resulted in stopping endothelial cell activation and delayed formation of fatty streaks [29]. vWF is a weak predictor of CVD events in the general population, but it strongly predicts

CVD events and mortality in high-risk patients (e.g. diabetes, vascular disease). The significance persisted after adjusting for multiple risk factors including CRP levels [17, 30]. Since blood group type may influence vWF levels [31], we looked at the prevalence of blood group A by anemia status. We did not find any difference between the 2 groups. In summary, vWF may play an important role in CVD events in ND-CKD patients.

In our study, VCAM-1 was significantly elevated in anemic as opposed to non-anemic patients. VCAM-1, an endothelial activator and mediator of lymphocyte adhesion and migration, has also been associated with adverse CVD events [32–35]. The mechanism of endothelial cell activation by VCAM-1 may be by generating reactive oxygen species via NADPH oxidase, activation of matrix metalloproteinase and cross-talk with very-late antigen 4 [35–38]. In a diabetic population, VCAM-1 along with other adhesion molecules predicted the development of macrovascular disease [39]. Another study reported that elevated VCAM-1 levels were associated with CVD events in a hemodialysis population [26]. Thus, VCAM-1 may also play an important role in CVD events in ND-CKD patients.

Our study had several strengths and limitations. The major limitation was that baseline covariates might have confounded the association between anemia and the markers of endothelial activation. Although both groups were similar at baseline, sample size was small. We also excluded patients who were hospitalized or had history of infection in the past 30 days. We did not include patients on dialysis as the dialysis membrane may itself activate inflammatory and endothelial activation markers [26, 40–42]. The assays were performed using defined validated techniques with limited intra-assay variability. Although we performed sensitivity analyses of anemia and biomarkers thereby increasing the robustness of the study, we believe that this study is hypothesis generating.

In conclusion, we report an association between anemia and increased levels of fibrinogen, vWF and VCAM-1 in ND-CKD patients. Elevated levels of these endothelial markers may be germane to define heightened CVD risk among anemic CKD patients. Prospective studies are needed to confirm these findings.

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Conflict-of-Interest Disclosure

Dr. Singh reports receiving consulting fees from Ortho Biotech Clinical Affairs/Johnson & Johnson, Amgen, Roche, Merck, Abbott, Watson, and lecture fees from Ortho Biotech Clinical Affairs/Johnson & Johnson, Roche, Amgen, Abbott, Watson, and Genzyme; serving on advisory boards for Ortho Biotech Clinical Affairs, Roche, Watson, AMAG Inc., and Amgen, and receiving grant support from Ortho Biotech Clinical Affairs, Dialysis Clinic Inc., Roche, Baxter, Johnson & Johnson, Amgen, and Watson. Dr. Singh is a Medical Director of Dialysis Clinics Inc.

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