Hepcidin as well as TNF-α are significant predictors of arterial stiffness in patients on maintenance hemodialysis

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Abstract

Background. Dysregulated iron metabolism has been suspected to be linked to anemia of chronic disease and to cardiovascular disease (CVD). For the purpose of clarifying the factors affecting arterial stiffness, we evaluated the relationship between iron metabolism, brachial-ankle (ba)-pulse wave velocity (PWV) and several risk factors for CVD in maintenance hemodialysis (MHD) patients.

Methods. A total of 168 MHD patients were recruited, and the levels of iron parameters, hepcidin, CVD risk factors and ba-PWV were evaluated. The level of serum hepcidin-25 was specifically measured by liquid chromatography–tandem mass spectrometry.

Results. Serum levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and hepcidin were higher in MHD patients, which was consistent with results from our previous study. ba-PWV significantly correlated with age (P < 0.01, R = 0.34), total cholesterol (T-CHO; P = 0.02, R = 0.21), TNF-α (P < 0.01, R = 0.24) and hepcidin (P < 0.01, R = 0.25) but not with other iron parameters and CVD risk factors. According to multiple regression analysis, age (β = 0.30), T-CHO (β = 0.24) TNF-α (β = 0.19) and hepcidin (β = 0.23) were selected as the significant predictors of ba-PWV in MHD patients.

Conclusion. Serum levels of both hepcidin and TNF-α are independently associated with arterial stiffness in MHD patients, suggesting that microinflammation and iron metabolism might affect the integrity of arterial walls.

Keywords: hemodialysis; hepcidin; iron metabolism; TNF-α; vascular stiffness

Introduction

High mortality and morbidity associated with cardiovascu- lar disease (CVD) in maintenance hemodialysis (MHD) patients is well documented [1]. Although several factors have been proposed to explain the risk of CVD in MHD patients, they remain controversial.

A recently discovered iron regulatory peptide, hepcidin, has been suspected to be one of the culprits for endothelial dysfunction via iron sequestration [2]. Hepcidin binds to ferroportin-1, a cellular iron export channel protein, causing it to be internalized and degraded in lysosomes [3], thereby preventing the efflux of iron from iron-exporting tissues into the plasma. Hepcidin thus promotes the de-struction of ferroportin-1 and inhibits iron export from cells, leading to ‘iron sequestration’ [4]. Consequently, we suspect that high serum levels of hepcidin in MHD patients might contribute to iron sequestration in vascular endothelial cells and plaque macrophages, which may cause CVD via oxidative stress.

To estimate future CVD risks, the structure and function of arterial walls must be examined since the integrity of arterial walls may be altered in response to atherosclerotic factors, as well as changes in the hemodynamic burden [5]. Pulse wave velocity (PWV) measurement is a method for determining the degree of arterial stiffness and has been well demonstrated as a powerful predictor of future CVD risks. In the meta-analysis of 17 longitudinal studies, aortic stiffness expressed as aortic PWV was shown to be a strong predictor of future CV events and all-cause mortality [6].
intima-media thickness of the carotid artery, as well as aortic PWV [7]. Patients undergoing MHD, who are within the highest quartile of ba-PWV, have been shown to have significantly increased hazard ratios of all-cause and cardiovascular mortality [8]. Thus, ba-PWV has been demonstrated a significant surrogate of the arterial disease, although it is an indirect measurement of PWV and arterial stiffness as compared to direct determination of pulse wave profile recorded by applanation tonometry.

We recently demonstrated that in 198 MHD patients treated with recombinant human erythropoietin, serum hepcidin-25 levels were higher than in healthy volunteers as determined by measuring the levels of transferrin and ferritin [9]. Among them, ba-PWV was measured simultaneously in 168 patients. We examined the factors affecting ba-PWV in MHD patients, including iron-related factors as well as several other risk factors associated with chronic kidney disease (CKD).

Materials and methods

Subjects

The patients included in this study were part of a previous clinical trial designed to evaluate the factors affecting serum hepcidin and included examination of indices of anemia, iron metabolism, inflammation and the erythropoietin dose, as previously reported [9]. We evaluated ba-PWV in 168 of the 198 MHD patients, enrolled in the previous study. Patients who had been on MHD for <1 year; patients >75 years old; patients with chronic inflammation, malignancy, hematological disorders or severe liver dysfunction and patients receiving anti-inflammatory drugs or immunosuppressants were excluded from the study reported [9]. All subjects gave informed consent in accordance with the requirements of the institutional ethics committee (Hyogo College of Medicine No. 293).

Sample collection and measurement of parameters

Blood samples were collected at the time of starting the first hemodialysis (HD) session of the week. The levels of hemoglobin (Hb), urea nitrogen (UN), creatinine (Cr), total protein, albumin, total cholesterol (T-CHO), triglycerides (TG), iron, calcium (Ca) and phosphorus (P) were measured by standard laboratory methods using an autoanalyzer. Serum β2-microglobulin (MG) was measured by latex immunoassay and intact-parathyroid hormone (int-PTH) was determined by immunoradiometric assay. Transferin was measured by the Nitro-so-PSAP test, and transferrin saturation (TSAT) was calculated as serum iron/total iron-binding capacity. The Kt/V was calculated by formal urea kinetic modeling. Serum levels of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were measured by enzyme-linked immunosorbent assay (Human IL-6 and Human TNF-α immunoassay kit; Biosource International Inc., CA). Serum levels of hepcidin were determined by liquid chromatography–tandem mass spectrometry, as previously described [10].

Measurements of ba-PWV

ba-PWV was measured using a volume-plethysmographic apparatus (form PWV/ABI; Colin Co., Komaki, Japan). This device records the phonocardiogram, electrocardiogram, volume pulse form and arterial blood pressure at both the left and right brachia and ankles. In all patients, ba-PWV was measured after at least 5 min of rest [7].

Statistical analysis

Values are presented as the mean ± SD. Differences between two groups were analyzed by the unpaired Student’s t-test. Analysis of variance was used for comparison between low- and high-TNF-α and hepcidin groups. A P-value < 0.05 was taken as an indication of statistical significance. Potential associations between ba-PWV and iron parameters or inflammatory parameters were assessed by linear regression analysis. Multiple regression analysis was used to determine which factors had an independent influence on ba-PWV in MHD patients. Statistical analyses were performed with Statview software ver.5.0 for Macintosh.

Results

Clinical characteristics of subjects

The mean age of MHD patients was 58 ± 10 years, and the study included 108 males and 60 females. The mean time on HD was 9.0 ± 0.7 years, and the mean Kt/V was 1.3 ± 0.2. The nutritional status, hemodialysis parameters, levels of inflammatory cytokines and iron parameters of the patients in this study were similar to those of patients described in our previous report [9] (Table 1).

All patients were treated by epoetin-β. Mean dose of epoetin-β was 4898.4 ± 2513.4 IU/week. Furthermore, 31.8% of patients were treated with iron (226.4 ± 189.9 mg/month) in this study.

Correlation between CVD risk factors and ba-PWV

Among traditional risk factors, T-CHO (R = 0.214, P = 0.021) and age (R = 0.349, P = 0.001) had a significant correlation with ba-PWV in MHD patients (Table 2). However, ba-PWV did not show any significant correlation with other traditional risk factors (body mass index, blood pressure, pulse pressure, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, TG and Hb) and CKD risk factors (Ca × P, int-PTH, UN, β2MG, IL-6, Kt/V and time on HD) (Table 2).

Among the CVD risk factors, we found a significant correlation between ba-PWV and TNF-α (R = 0.24, P = 0.018) (Figure 1). On the other hand, there was no correlation between ba-PWV and IL-6, Ca × P, int-PTH, UN, β2MG, albumin, Kt/V or time on HD in MHD patients (Table 2). While ba-PWV was not correlated with iron parameters, such as serum iron, unsaturated iron-binding capacity, TSAT, or ferritin in MHD patients (Table 2), it did show a positive correlation with hepcidin (R = 0.28, P = 0.0064) (Figure 1). There was no significant correlation between serum TNF-α or IL-6 and hepcidin levels.

Stepwise regression analysis

Multivariate analysis was performed to investigate the predictors of ba-PWV in MHD patients among the variables listed in Table 1. The results are shown in Table 2.
that correlated (P < 0.1) with ba-PWV in simple regression analysis (age, time on HD, serum level of TNF-α, hepcidin, transferrin, ferritin and T-CHO). Among these variables, age (β = 0.30), serum levels of T-CHO (β = 0.24), TNF-α (β = 0.19) and hepcidin (β = 0.23) were found to be significant independent determinants of ba-PWV (Table 3).

**ba-PWV in low- and high-TNF-α and hepcidin groups**

MHD patients were divided into four groups according to the median level of serum TNF-α (27 pg/mL) and hepcidin (45 ng/mL), as follows: (i) high-hepcidin (>45 ng/mL) and high-TNF-α (>27 pg/mL) groups (n = 30), (ii) low-hepcidin (≤45 ng/mL) and high-TNF-α groups (n = 41), (iii) high-hepcidin and low-TNF-α (≤27 pg/mL) groups (n = 36) and (iv) low-hepcidin and low TNF-α groups (n = 54). The ba-PWV in the high-hepcidin and high-TNF-α groups (2062 ± 594) was significantly (P < 0.05) higher than that of the low-hepcidin and low-TNF-α groups (1791 ± 424) (Figure 2).

**Discussion**

The significant observation in the present study was that T-CHO, hepcidin and TNF-α as well as age were selected as the significant determinants of ba-PWV in MHD patients by using multivariate analysis, which could be recognized as an index of arterial dysfunction and future CVD risks. It has already been reported that age and T-CHO are associated with ba-PWV [11, 12].

**Hepcidin**

With respect to the relationship between hepcidin and ba-PWV in MHD patients, we suspected that iron sequestration in vascular cells might be linked to arterial stiffness. Hepcidin plays an important role in iron accumulation in several types of cells, binding to and degrading the iron export protein ferroportin-1, potentially leading to iron sequestration in various tissues and organs, including vascular cells. We have already demonstrated that ferroportin-1 is expressed in human umbilical endothelial cells [13]. The exact mechanism by which hepcidin promotes arterial stiffness has not been clarified. However, several reports have demonstrated a relationship between iron accumulation and arterial alteration [14], including generation of oxidized low-density lipoprotein [15], endothelial cell dysfunction [16] and arterial smooth muscle proliferation [17].

Drücke et al. [18] reported that the thickness of the internal carotid artery wall (cIMT) has a positive correlation with the serum levels of ferritin and advanced oxidation protein products, as well as with the total load of intravenous iron administration. Furthermore, Reis et al. [19] found the significant correlations between cIMT and serum ferritin or intravenous iron administration doses in 60 MHD patients, suggesting that excessive administration or accumulation of iron may account for accelerated

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**Table 2.** Simple correlation coefficients between traditional risk factors, CKD risk factors for CVD, iron parameters and ba-PWV in MHD patients

<table>
<thead>
<tr>
<th>R</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
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</tr>
<tr>
<td>s-BP</td>
<td>0.03</td>
</tr>
<tr>
<td>d-BP</td>
<td>0.13</td>
</tr>
<tr>
<td>Pulse pressure</td>
<td>0.08</td>
</tr>
<tr>
<td>T-CHO</td>
<td>0.21</td>
</tr>
<tr>
<td>LDL-C</td>
<td>0.18</td>
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<tr>
<td>HDL-C</td>
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</tr>
<tr>
<td>TG</td>
<td>0.1</td>
</tr>
<tr>
<td>Hb</td>
<td>0.11</td>
</tr>
<tr>
<td>Age</td>
<td>0.34</td>
</tr>
<tr>
<td>Ca × P</td>
<td>0.03</td>
</tr>
<tr>
<td>int-PTH</td>
<td>0.08</td>
</tr>
<tr>
<td>UN</td>
<td>0.1</td>
</tr>
<tr>
<td>β2 MG</td>
<td>0.03</td>
</tr>
<tr>
<td>Albumin</td>
<td>0.13</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.15</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.24</td>
</tr>
<tr>
<td>K/VV</td>
<td>0.13</td>
</tr>
<tr>
<td>Time on HD</td>
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</table>

<table>
<thead>
<tr>
<th>R</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron</td>
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</tr>
<tr>
<td>UIBC</td>
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<tr>
<td>TSAT</td>
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</tr>
<tr>
<td>Transferrin</td>
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<td>Ferritin</td>
<td>0.16</td>
</tr>
<tr>
<td>Hepcidin</td>
<td>0.25</td>
</tr>
</tbody>
</table>

β2 MG, β2 microglobulin; BMI, body mass index; d-BP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; s-BP, systolic blood pressure; s-UN, serum urea nitrogen; UIBC, unsaturated iron-binding capacity.

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**Fig. 1.** Correlation between ba-PWV and levels of serum hepcidin (a) TNF-α (b) in MHD patients. ba-PWV significantly correlated with levels of serum hepcidin (R = 0.251, P = 0.0064) and TNF-α (R = 0.229, P = 0.018).
arteriosclerosis in MHD patients. Moreover, using electron paramagnetic resonance spectroscopy and inductively coupled plasma–mass spectroscopy, Stadler et al. [20] confirmed that there is a significantly greater accumulation of iron in arteriosclerotic lesions than in healthy vessels in non-CKD patients. These reports suggest that iron accumulation might be associated with the development and/or progression of arteriosclerosis.

**Tumor necrosis factor-α**

In the present study, the level of serum TNF-α was also identified as an independent predictor of ba-PWV in MHD patients. It had already been demonstrated that TNF-α induced vascular calcification. Tintu et al. [21] reported that TNF-α enhances in vitro vascular calcification by promoting osteoblastic differentiation of vascular cells through the cyclic adenosine monophosphate pathway. Factors including RANKL (receptor activator of nuclear factor κB ligand), its receptor RANK and osteoprotegerin modulate osteoclastogenesis. Proinflammatory cytokines (e.g. TNF-α, IL-1) or adherence of activated lymphocytes to the vascular wall at the site of atherosclerosis have been shown to induce release of RANKL from endothelial cells [22]. From these observations, we speculate that increased ba-PWV in MHD patients may be caused by high serum TNF-α, resulting from vascular calcification.

**Table 3.** Stepwise multiple linear regression analysis of factors influencing ba-PWV in MHD patients

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>Unstandardized β-coefficient</th>
<th>Standardized β-coefficient</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>16.13</td>
<td>0.30</td>
<td>4.82</td>
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</tr>
<tr>
<td>T-CHO</td>
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</tr>
<tr>
<td>TNF-α</td>
<td>12.46</td>
<td>0.19</td>
<td>5.90</td>
<td>0.0001</td>
</tr>
<tr>
<td>Heparin</td>
<td>2.67</td>
<td>0.23</td>
<td>1.02</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

*R = 0.42

*Age and levels of serum TNF-α and hepcidin were found to be significant determinants of ba-PWV in MHD patients.*

![Fig. 2](https://example.com/image.png)

**Fig. 2.** Comparison of ba-PWV between low-and high-TNF-α and hepcidin groups. (i) High-hepcidin (>45 ng/mL) and high-TNF-α (>27 pg/mL) groups (*n* = 30), (ii) low-hepcidin (<45 ng/mL) and high-TNF-α (<27 pg/mL) groups (*n* = 41), (iii) high-hepcidin (>45 ng/mL) and low-TNF-α (<27 pg/mL) groups (*n* = 36), (iv) low-hepcidin (<45 ng/mL) and low-TNF-α (<27 pg/mL) groups (*n* = 34), ba-PWV in the high-hepcidin and high-TNF-α groups (i) was significantly higher (P < 0.05) than that of the low-hepcidin and low-TNF-α groups (iv).

With regard to iron metabolism, TNF-α may also accelerate iron sequestration in vascular endothelial cells. We previously reported that iron is sequestered by TNF-α in human umbilical endothelial cells (HUVECs) in connection with the up-regulation of iron import proteins, such as transferrin receptor and divalent metal transporter-1, and down-regulation of iron export proteins, such as ferroportin-1 [12]. Thus, we could not rule out the possibility that iron accumulation in vascular endothelial cells, which is caused by TNF-α, may also affect arterial stiffening in MHD patients.

Regarding the relation between hypercytokinemia and hepcidin, we have previously reported that serum IL-6 and TNF-α levels were significantly higher in MHD patient without apparent inflammation than in healthy volunteers but were not correlated with hepcidin [9]. Thus, subclinical inflammation as reflected by elevated serum cytokine levels is not necessarily involved in the increase in serum hepcidin level observed in patients with CKD.

Limitation of this study is the lack of data on the administration of antihypertensive drugs and statins in our patients, although rennin angiotensin aldosterone system blockers, statins, phosphate binder and vitamin D may affect the arterial stiffness.

In conclusion, our results suggest that elevated levels of serum TNF-α and/or hepcidin may be closely associated with arterial stiffness or possibly the development of arteriosclerosis in MHD patients. Heparin and TNF-α synergistically increase ba-PWV in MHD patients (Figure 2).

Serum levels of both TNF-α and hepcidin are usually higher in MHD patients. Therefore, controlling these two risk factors may provide the significant benefits in helping prevent CVD in MHD patients. The level of serum hepcidin depends exclusively on ferritin levels and iron administration [9], and these factors may be controlled by maneuver applied for the therapy of renal anemia.

**Conflict of interest statement.** None declared.

**References**


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