Elevated serum retinol-binding protein 4 concentrations are associated with chronic kidney disease but not with the higher carotid intima-media thickness in type 2 diabetic subjects

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Abstract. To examine the association of serum retinol-binding protein 4 (RBP4) concentrations with carotid intima-media thickness (CIMT) in type 2 diabetic subjects with chronic kidney disease (CKD). A total of 239 type 2 diabetic patients (64 ± 13 years, 154 males) were divided into two groups: one with CKD, defined as estimated glomerular filtration rate (eGFR) below 60 mL/min/1.73m² (n = 86), and one without (n = 153). We recorded clinical and biochemical data as well as CIMT. The patients with CKD were older, had had diabetes mellitus longer, and had higher incidence of hypertension, dyslipidemia and microalbuminuria than those without. They also had higher serum concentrations of RBP4 (44.8 ± 6.4 vs 39.5 ± 4.9 μg/mL, p < 0.001), higher mean CIMT (0.75 ± 0.16 vs 0.69 ± 0.14 mm, p = 0.007), and higher incidence of carotid plaques (27.9 vs 11.8%, p = 0.002). The RBP4 were negatively correlated with eGFR (r = -0.514, p < 0.001). However, the RBP4 were not correlated with mean CIMT (r = 0.065, p = 0.318). Moreover, when dividing the patients into two groups by the mean CIMT, those with mean CIMT above 0.71 mm did not have different RBP4 concentrations compared with those below (41.5 ± 5.7 vs 41.3 ± 6.3 μg/mL, p = 0.856). In conclusion, we observed an elevation of serum RBP4 concentrations and CIMT levels in type 2 diabetic subjects with CKD. However, the elevated RBP4 were not associated with the higher CIMT among these patients.

Key words: Retinol-binding protein 4, Carotid intima-media thickness, Estimated glomerular filtration rate, Microalbuminuria

IN PATIENTS with type 2 diabetes mellitus (DM), the presence of nephropathy is associated with poor renal and cardiovascular outcome. Glomerular filtration rate (GFR) is the best measure of overall kidney function in health and disease [1]. An GFR level less than 60 mL/min/1.73m² represents the loss of half or more of the adult level of normal kidney function. Below this level, the prevalence of complications of chronic kidney disease (CKD) increases [1]. Decreased GFR is an independent predictor of adverse outcomes, such as death and cardiovascular disease (CVD) [2-4]. Patients with both DM and CKD have a 2.7-fold risk of cardiac death compared with those without DM and CKD [5]. Carotid intima-media thickness (CIMT) is a surrogate marker of subclinical atherosclerosis; increased CIMT and presence of atherosclerotic plaque of extracranial carotid artery have been identified as important markers for prediction of cardiovascular morbidity and mortality [6-8]. The incidence of cardiovascular events is correlated with measurements of CIMT [6]. The association between CVD and CIMT remains significant after adjustment for traditional risk factors [7]. CIMT has been reported to be higher in diabetic patients than in healthy subjects [9, 10].

Adipose tissue secretes many types of adipokines that contribute to influence the cardiovascular risk coupled with another risk factors. Retinol-binding protein 4 (RBP4) was reported in 2005 as an adipokine that impairs insulin sensitivity [11]. RBP4 is elevated in subjects with insulin resistance, impaired glucose tol-
GFR (eGFR) was calculated using the abbreviated MDRD formula: 175 x \[(\text{Serum Creatinine} \times 0.0113)^{-1.154}\] x (age\textsuperscript{-0.203}) x F, where F = 1 if male, and 0.742 if female \[20\]. The patients were divided into two groups: one with CKD, which defined as eGFR below 60 mL/min/1.73m\textsuperscript{2} (n = 86), and the other without (n = 153). Informed consents were obtained from all after thorough explanation of the procedures. Samples of venous blood were obtained from an antecubital vein after an overnight fast starting at midnight.

**Biochemistry Analyses**

Serum hsCRP concentrations were analyzed by chemiluminescent immunoassay (Immulite 2000; Diagnostic Product Corporation, Los Angeles, CA, USA) with a detection limit of 0.01 mg/dL and a measuring range of 0.01–50 mg/dL. HbA1c was measured by Cation-exchange HPLC method (Tosoh HLC-723 G7; Tosoh Co., Tokyo, Japan). The normal range was 4 – 6%. Serum concentrations of TC, LDL-C, HDL-C, triglyceride, blood urea, and creatinine were measured by enzymatic colorimetry method (Hitachi 7600-110; Hitachi Ltd., Tokyo, Japan). Plasma glucose concentrations were measured by a hexokinase method using an automatic biochemistry analyzer (Hitachi 7170; Hitachi Ltd., Tokyo, Japan). Urine albumin concentrations were analyzed by solid-phase, competitive chemiluminescent enzyme immunoassay (Immulite 2000) with a detection limit of 1.0 μg/mL and a measuring range of 2.5–60 μg/mL. Urine concentrations of creatinine were measured by Johnson & Johnson Vitros 950 analyzer (Johnson & Johnson Clinical Diagnostics Inc., Rochester, NY, USA). Serum RBP4 concentrations were measured by competitive chemiluminescent enzyme immunoassay (Immulex 2000) with a detection limit of 1.0 μg/mL and a measuring range of 2.5–60 μg/mL. Urine concentrations of creatinine were measured by Johnson & Johnson Vitros 950 analyzer (Johnson & Johnson Clinical Diagnostics Inc., Rochester, NY, USA). Serum RBP4 concentrations were measured by quantitative sandwich enzyme immunoassay (Assaypro LLC. St. Charles, MO., USA). The minimum detectable dose is 10 ng/mL.

**Common Carotid Ultrasound**

In all subjects, a high-resolution B-mode ultrasound of the common carotid arteries was performed using the HD11 ultrasound system with a frequency 12-3 MHz (Philips Medical systems, Bothell, WA, USA). All patients were blindly examined and measured by the same experienced operator. The CIMT was defined by two parallel echogenic lines (double line pattern), which corresponded to the lumen-intima and the media-adventitia interfaces. The site of CIMT measurement

**Materials and Methods**

**Subjects**

The study was performed on 239 adult (≥ 18 years) male and female type 2 diabetic patients. This study was approved by the Medical Ethics and Human Clinical Trial Committee of Kaohsiung Veterans General Hospital. The clinical and biochemical evaluations included BMI, blood pressure (BP), serum concentrations of total cholesterol (TC), low density lipoprotein-cholesterol (LDL-C), high density lipoprotein-cholesterol (HDL-C), triglyceride, glucose, blood urea, creatinine, hemoglobin (Hb) A1c, high sensitivity C-reactive protein (hsCRP), and RBP4. BMI was calculated as weight in kilograms divided by the square of the height in meters. Definition of microalbuminuria was made by analysis of a spot urine sample for the albumin-to-creatinine ratio (ACR) ≥ 30 mg/g. Estimated GFR (eGFR) was calculated using the abbreviated MDRD formula: 175 x \[(\text{Serum Creatinine} \times 0.0113)^{-1.154}\] x (age\textsuperscript{-0.203}) x F, where F = 1 if male, and 0.742 if female \[20\]. The patients were divided into two groups: one with CKD, which defined as eGFR below 60 mL/min/1.73m\textsuperscript{2} (n = 86), and the other without (n = 153). Informed consents were obtained from all after thorough explanation of the procedures. Samples of venous blood were obtained from an antecubital vein after an overnight fast starting at midnight.

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was a perpendicular location relative to the transducer beam, 1 cm far from the bifurcation on the far wall of each common carotid artery (CCA) using the longitudinal axis. A minimum of 10 mm length of the CCA was required for CIMT measurement. Only sites free from discrete plaques were considered for measurement. Mean CIMT was calculated as the arithmetic mean of three bilateral values. The carotid plaque was defined as a focal structure encroached the arterial lumen of at least 0.5 mm or 50% of the surrounding CIMT, or an intima-media complex thickness > 1.5 mm [21].

### Statistical Analyses

Values were reported as mean ± SD. Non-normally distributed variables were also expressed as median (1<sup>st</sup>, 3<sup>rd</sup> quartile). Comparisons between the group with CKD and the other without were made by using student t test. Except for sex, which was compared by 2-sample Kolmogorov-Smirnov test, Mann-Whitney U test was used for nonparametric data and non-normally distributed variables. The relations of mean CIMT with other variables were assessed by using Pearson’s correlation or Spearman’s correlation for those non-normally distributed variables. Multivariate linear regression analysis was used to assess the relative independence of predictors for mean CIMT values. A value of $p < 0.05$ was considered as statistically significant.

### Results

The mean age of the study population was 64 years (range 26-88 years). Mean duration of diabetes was 10.8 years. Mean HbA1c was 8.0 %. Mean ± SD of the mean CIMT was 0.71 ± 0.15 mm.

The comparative profiles of patients with CKD and those without are shown in Table. The sex distributions were not different between both groups. Age in the patients with CKD was higher (70 ± 11 vs 60 ± 12 years, $p < 0.001$) and the duration of diabetes was longer (13.9 ± 9.0 vs 9.1 ± 7.5 years, $p < 0.001$) than those

<table>
<thead>
<tr>
<th>Variables</th>
<th>CKD</th>
<th>non-CKD</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>86</td>
<td>153</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>70 ± 11</td>
<td>60 ± 12</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Men (n)</td>
<td>64 (74.4%)</td>
<td>90 (58.8%)</td>
<td>0.137</td>
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<tr>
<td>DM duration (years)</td>
<td>13.9 ± 9.0</td>
<td>9.1 ± 7.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smokers (n)</td>
<td>12 (14.0%)</td>
<td>26 (17.0%)</td>
<td>0.538</td>
</tr>
<tr>
<td>Dyslipidemia (n)</td>
<td>56 (65.1%)</td>
<td>71 (46.4%)</td>
<td>0.005</td>
</tr>
<tr>
<td>Hypertension (n)</td>
<td>67 (77.9%)</td>
<td>83 (54.2%)</td>
<td>&lt;0.001</td>
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<tr>
<td>Microalbuminuria (n)</td>
<td>61 (70.9%)</td>
<td>58 (37.9%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>26.2 ± 6.2</td>
<td>26.3 ± 5.9</td>
<td>0.649</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>139 ± 19</td>
<td>131 ± 20</td>
<td>0.002</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>74 ± 11</td>
<td>76 ± 11</td>
<td>0.163</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.54 ± 0.77</td>
<td>4.53 ± 0.90</td>
<td>0.980</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>2.49 ± 0.69</td>
<td>2.45 ± 0.65</td>
<td>0.616</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.03 ± 0.29</td>
<td>1.12 ± 0.32</td>
<td>0.025</td>
</tr>
<tr>
<td>Triglyceride (mmol/L)</td>
<td>17.1 ± 9.1</td>
<td>16.1 ± 15.0</td>
<td>0.520</td>
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<tr>
<td>Blood urea (mmol/L)</td>
<td>9.39 ± 4.19</td>
<td>5.60 ± 1.45</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Creatinine (μmol/L)</td>
<td>148.8 ± 50.7</td>
<td>85.4 ± 15.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>8.54 ± 2.77</td>
<td>8.3 ± 2.71</td>
<td>0.509</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8.06 ± 1.56</td>
<td>8.04 ± 1.67</td>
<td>0.900</td>
</tr>
<tr>
<td>hsCRP (mg/L)</td>
<td>2.66 ± 4.55</td>
<td>1.73 ± 2.85</td>
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</tr>
<tr>
<td>ACR (mg/g)</td>
<td>0.91 (0.39, 2.94)</td>
<td>0.78 (0.36, 1.98)</td>
<td>0.054</td>
</tr>
<tr>
<td>Mean CIMT (mm)</td>
<td>0.75 ± 0.16</td>
<td>0.69 ± 0.14</td>
<td>0.007</td>
</tr>
<tr>
<td>RBP4 (μg/mL)</td>
<td>44.8 ± 6.4</td>
<td>39.5 ± 4.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Carotid plaque (y)</td>
<td>24 (27.9%)</td>
<td>18 (11.8%)</td>
<td>0.002</td>
</tr>
<tr>
<td>Medications</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Statins (n)</td>
<td>41 (47.7)</td>
<td>65 (42.5%)</td>
<td>0.439</td>
</tr>
<tr>
<td>RAS blockades (n)</td>
<td>58 (67.4%)</td>
<td>69 (45.1%)</td>
<td>0.001</td>
</tr>
<tr>
<td>TZDs (n)</td>
<td>19 (22.1%)</td>
<td>28 (18.3%)</td>
<td>0.480</td>
</tr>
<tr>
<td>Insulin (n)</td>
<td>12 (14.0%)</td>
<td>15 (9.8%)</td>
<td>0.332</td>
</tr>
</tbody>
</table>

Characteristics of the type 2 diabetic patients with and without chronic kidney disease (CKD)

Table
of without CKD. The patients with CKD had higher incidence of hypertension, dyslipidemia, and microalbuminuria. They also had elevated systolic BP, lower HDL-C, with respect to those without CKD. However, levels of glucose, LDL-C, TG, HbA1c, and hsCRP were not different between both groups. In this study, more patients with CKD took medications with rennin-angiotensin system (RAS) blockades. Nevertheless, the patients with CKD did have higher serum concentrations of RBP4 (44.8 ± 6.4 vs 39.5 ± 4.9 μg/mL, \( p < 0.001 \)), higher mean CIMT (0.75 ± 0.16 vs 0.69 ± 0.14 mm, \( p = 0.0070 \)), and higher incidence of carotid plaques (27.9 vs 11.8 %, \( p = 0.002 \)). However, among patients with CKD, those treated with RAS blockades had lower mean CIMT than those without (0.72 ± 0.14 vs 0.80 ± 0.18 mm, \( p = 0.029 \)).

By Pearson’s or Spearman’s correlation, RBP4 concentrations correlated with age (\( r = 0.183, p = 0.005 \)), DM duration (\( r = 0.195, p = 0.002 \)), systolic BP (\( r = 0.184, p = 0.004 \)), TG concentrations (\( r = 0.198, p = 0.002 \)), ACR (\( r = 0.215, p = 0.001 \)), and eGFR levels (\( r = -0.514, p < 0.001 \), Fig. 1). By multivariate linear regression analysis, RBP4 concentrations only correlated with eGFR levels (\( \beta = -0.477, p < 0.001 \)).

By Pearson’s or Spearman’s correlation, mean CIMT correlated with age (\( r = 0.538, p < 0.001 \)), systolic BP (\( r = 0.170, p = 0.008 \)), HDL-C concentrations (\( r = -0.150, p = 0.020 \)), and eGFR levels (\( r = -0.212, p = 0.001 \)). However, the mean CIMT did not correlate with ACR levels (\( r = -0.019, p = 0.770 \)). Moreover, no correlation between CIMT and RBP4 concentrations was found (\( r = 0.065, p = 0.318 \), Fig. 2). The multivariate linear regression analysis showed that only the patient’s age positively correlated with mean CIMT (\( \beta = 0.612, p < 0.001 \)). We also adjusted RBP4 concentrations by eGFR levels, however, the adjusted RBP4/eGFR values were not correlated with mean CIMT (\( r = 0.065, p = 0.318 \)).

Though hsCRP levels negatively correlated with eGFR (\( r = -0.138, p = 0.033 \)). Nevertheless, hsCRP did not correlate with mean CIMT (\( r = 0.079, p = 0.223 \)), or RBP4 levels (\( r = -0.058, p = 0.375 \)).

Dividing the patients into two groups by the mean carotid intima-media thickness (CIMT) levels, those with mean CIMT above 0.71 mm did not have different retinol-binding protein 4 (RBP4) concentrations compared with those below (41.5 ± 5.7 vs 41.3 ± 6.3 μg/mL, \( p = 0.856 \), Fig. 3). The patients with the presence of carotid plaques also had similar serum RBP4 concent-
trations compared with those without (42.38 ± 0.88 vs 41.18 ± 0.43 μg/mL, p = 0.241).

**Discussion**

Elevated RBP4 concentrations and CIMT levels have been reported in subjects with type 2 DM or CKD. Several lines of evidence support a potential role of RBP4 in pathways linking adiposity with atherosclerosis. Also, high RBP4 levels are associated with the risk of coronary artery disease [24] and cerebral infarction [25] in recent studies. Previous study also found higher plasma RBP4 concentrations in patients with moderately renal dysfunction and those with previous clinical arteriosclerosis [26]. Plasma RBP4 concentration might be a biomarker of nephropathy and cardiovascular disease in type 2 diabetic subjects. Thus, we could assume that circulating RBP4 is associated with CIMT in type 2 DM with CKD.

In this present study, type 2 DM with CKD was indeed associated with elevated RBP4 concentrations, levels of CIMT, and the presence of carotid plaques. However, RBP4 could not be the determined factor for elevated CIMT in type 2 DM with CKD. A correlation between RBP4 and CIMT among elderly [18] or hypertensive [17] subjects has recently been described. On the other hand, RBP4 is not associated with CIMT in drug-naïve, newly diagnosed type 2 DM patients without clinical cardiovascular disease [19]. Type 2 DM comprises multiple cardiovascular risk factors. Except for the traditional factors such as smoking, hypertension, dyslipidemia, physical inactivity, and the metabolic syndrome; the underlying insulin resistance, the proinflammatory or prothrombotic factors, and the adipokines which secreted from adipocytes also contribute to the risk for CVD. Additionally, CKD is associated with many risk factors for atherosclerosis. Except for the traditional factors, the altered bone mineral metabolism leading to hyperphosphatemia and increased vascular calcification [27], the increased oxidative stress, cytokines, and endothelial dysfunction [28-30] are also accountable for the increase of atherosclerosis. In view of such complexity of the CVD risk factors on subjects of type 2 DM with CKD; the lack of association between RBP4 concentrations with levels of CIMT is not unreasonable. Moreover, the CKD subjects in the present study were older, had had longer duration of DM and higher incidence of hypertension or dyslipidemia. We believe the traditional risk factors are more associated with the atherosclerotic events than adipokines, such as RBP4. The feature could also be recognized in the present study seeing that mean CIMT were correlated with age, systolic BP, HDL-C concentrations, and eGFR levels.

Our study showed RBP4 itself could not be a determinant factor for CIMT in diabetic CKD subjects. However, there has been reported that retinol/RBP4 ratio is strongly and independently associated with CIMT [31]. Transthyretin, which binds with RBP4 and prevents its glomerular filtration and catabolism in the kidney, has been showed to be significantly decreased in patients with CKD [32], and be a significantly independent risk factor of increased CIMT in maintenance hemodialysis patients [33]. Besides, the RBP4 isoforms including apo-RBP4, RBP4-L and RBP4-LL are all elevated in patients with CKD [32]. Retinol and transthyretin are known to strongly interact with RBP4 in the transport complex of vitamin A and may affect cardiovascular risk [34]. The association of these RBP4 isoforms, the retinol/RBP4 ratio and the transthyretin with CIMT among diabetic CKD patients need further studies to delineate.

Diabetic nephropathy is a major manifestation of microvascular complications that plays a significant role in the prognosis of patients with DM. The type 2 diabetic patients with CKD are also at very high risk of CVD. With the strong links among CKD, diabetes, and cardiovascular mortality, the need for effective risk stratification of diabetic populations assumes unprecedented significance. CIMT is an easy and noninvasive tool to identify subclinical atherosclerosis before the development of CVD. In the present study, we also observed an elevated CIMT in type 2 diabetic patients with CKD. The result underlines the importance of regular measurement of CIMT and early management of the subclinical atherosclerosis among these patients. In addition, RBS blockades could be convincing medications for prevention of atherosclerosis progression.

We demonstrated that serum RBP4 concentrations were elevated in type 2 diabetic patients with CKD. However, RBP4 concentrations were not associated with BMI, HbA1c, or hsCRP levels. The closest association remained with eGFR. Our results are compatible with the previous study revealing that eGFR but not HbA1c influences RBP4 serum levels [35]. These data suggest that elevation of RBP4 serum concentration is more likely to be caused by the presence of impaired kidney function rather than type 2 diabetes. However, there are studies reporting the fact that urinary RBP4.
excretion is increased in early diabetic nephropathy [36, 37], indicating the increased RBP4 serum concentrations are not necessarily related to a decrease of urinary RBP4 excretion. Therefore, how serum RBP4 concentrations influence diabetic patients with CKD requires more investigation in further studies.

A few limitations in our study need to be mentioned. First is that we had no age and sex matched healthy subjects for comparisons. However, higher CIMT in diabetic patients than that in healthy subjects has been documented in a number of studies. We therefore focused on the measurement of CIMT in different stage of type 2 DM. Secondly, the estimation of renal function was based on a single creatinine measurement. Yet, the conditions that would influence renal function had been excluded in the present study. The third limitation is that our study subjects were all Chinese, so these results cannot be generalized as such to populations of other ethnic origins.

In summary, type 2 DM patients with CKD, which defined as eGFR below 60 mL/min/1.73m², are associated with higher serum RBP4 concentrations and CIMT levels. However, the increased CIMT are not related to the elevation of RBP4 concentrations. CIMT measurement could be the easy method for evaluating early atherosclerotic process among these high risk patients.

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Conflict of Interest/Financial Disclosure
None to declare.

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