Correlations between serum vaspin and type 2 diabetic retinopathy.

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Abstract
This study investigated the role of vaspin in the occurrence and development of type 2 Diabetic Retinopathy (DR). A total of 90 patients clinically diagnosed with type 2 diabetes mellitus (T2DM) were divided into Non-DR (NDR), Non-Proliferation DR (NPDR), and Proliferation DR (PDR) groups, while healthy subjects were included in a control group. Serum Fasting Blood Glucose (FBG), Fasting Insulin (FINS), glycosylated haemoglobin (HbA1c), cholesterol (TC), Triglyceride (TG), High-Density Lipoprotein (HDLC), and Low-Density Lipoprotein (LDLC) levels were measured in each group. The remaining serum was stored at -80˚C for future detection of serum vaspin levels. Vaspin levels were quantified using Enzyme-Linked Immunosorbent Assay (ELISA). The homeostatic model assessment indices of insulin secretion and insulin resistance (HOMA-IS and HOMA-IR, respectively) were also calculated. There were statistically significant differences in serum levels of HbA1c (F=210.06), FGS (F=6.75), FINS (F=22.19), TC (F=5.65), HDLC (F=5.27), LDLC (F=6.90), vaspin (F=94.27), HOMA-IS (F=4.90), and HOMA-IR (F=21.56) between the NDR, NPDR, PDR, and control groups (P<0.05). The severity of DR was significantly correlated with HbA1c, FGS, FINS, LDLC, and HOMA-IR levels, and was also significantly correlated with reduced vaspin, HOMA-IS, and HDLC (P<0.01) levels. Vaspin levels were significantly correlated with HDLC and HOMA-IS (P<0.01), and negatively correlated with HOMA-IR (P<0.01). This study confirmed that patients with DR lesions had significantly reduced serum vaspin levels, which was closely related to abnormal blood glucose and blood lipid metabolism. Low circulating vaspin levels may be a risk factor for DR progression. Further prospective observational studies are required to explain how a decreased circulating vaspin levels may be involved in the progression of DR.

Keywords: Diabetic retinopathy, Vaspin, Cholesterol, Triglycerides, High-density lipoprotein.

Introduction
Diabetic Retinopathy (DR) is a leading cause of blindness worldwide, and seriously threatens the quality of life of diabetic patients. Its pathogenesis is still not entirely clear, although its development is related to blood glucose control and disease duration [1], and has been associated with dyslipidaemia [2,3].

Recent studies have shown that adipose tissue is a major endocrine organ that secretes a variety of cytokines including leptin, resistin, adiponectin, visfatin (a newly identified adipose cytokine that can promote fat accumulation), tumor necrosis factors, plasminogen activator inhibitor, and interleukin-6 [4-6]. Visceral adipose tissue has a more active function in the endocrine secretion of adipokines, and could directly affect insulin sensitivity, endothelial cell function, and inflammation and fibrinolytic activities; therefore, this tissue may be closely related to insulin resistance, type 2 diabetes, and cardiovascular diseases [6,7]. Thus, further studies on the functions and regulation of visceral fat factors could increase our understanding of the mechanisms of obesity and related metabolic abnormalities, thus providing new targets for diagnosis and treatment of metabolic diseases.

Abdominal fat serine protease inhibitor (vaspin) is a newly discovered visceral adipokine that is synthesized and secreted by abdominal fat tissue. Vaspin is a member of the superfamily of serine protease inhibitors. Studies have shown that this protein has a variety of biological functions, including increasing insulin sensitivity, regulating lipid metabolism, and participating in inflammation; therefore, it may be useful as a new target for the treatment of metabolic syndrome [8-10].

Change vaspin levels condition changes
Changes in vaspin levels are reported to be sensitive indicators of metabolic disorders. The powerful insulin-sensitization action of vaspin also makes it a potential target for metabolic syndrome treatment. Therefore, thorough investigation of vaspin levels and its functional regulations could provide new ideas for researching and treating diabetes and related disorders [11].
Although recent studies have shown that changes in vaspin level might be a sensitive indicator of changes in metabolic disorders, the findings remain controversial, and changes in the serum vaspin level and their influence on patients with Type 2 Diabetes Mellitus (T2DM) are not clear. Therefore, this study mainly assessed the correlations between serum vaspin levels at different stages of DR in order to investigate its potential physiological functions and its roles in the pathogenesis of DR.

**Materials and Methods**

**Subjects and grouping**

A total of 90 patients clinically diagnosed with T2DM between January 2014 and June 2015 were consecutively enrolled in this study. They included 49 men and 41 women aged 35 to 71 years (mean age, 51 ± 12.5 years). All patients underwent examinations including visual acuity, intraocular pressure, slit lamp microscopy, indirect ophthalmoscopy, and fundus fluorescein angiography. Participant age, T2DM duration, Height (HI), Weight (WT), and Body Mass Index (BMI=weight/height\(^2\) (kg/m\(^2\))) were also recorded [12]. This study was conducted with approval from the Ethics Committee of the Central Hospital of Yishui. Written informed consent was obtained from all participants. The exclusion criteria included: (1) other types of diabetes or associated acute diabetic complication(s); (2) serious cardio cerebral vascular disease (unstable angina, heart failure, serious arrhythmia, history of myocardial infarction within the previous year, acute cerebral infarction, or cerebral haemorrhage) [13]; (3) hepatonephric dysfunction: more than a 1.2-fold increase of ALT or more than a 2-fold increase of serum creatinine level; (4) no history of cataract, glaucoma, retinal vein, and artery obstruction; and (5) BMI ≥ 24. According to retinoscopic results and DR clinical staging criteria from the American Academy of Ophthalmology [15], the patients were divided into Non-DR (NDR), Non-proliferation DR (NPD), and Proliferation DR (PDR) groups, with 30 cases in each group. Another 30 healthy individuals were selected from the medical center of our hospital as a control group. The control group subjects were age- and sex-matched with the T2DM group subjects. Their mean fasting blood glucose levels and blood glucose levels 2 hours after a meal were <5.6 mmol/L and <7.8 mmol/L, respectively; they had no heart, brain, liver, or kidney disease, and no family history of diabetes, eye disease, or eye surgery. Participants with BMI ≥ 24 were excluded.

**Determination of conventional indicators**

Fasting venous blood samples were obtained from all subjects; the serum was separated and immediately tested for Fasting Blood Glucose (FBG), Fasting Insulin (FINS), glycosylated haemoglobin (HbA1c), cholesterol (TC), Triglyceride (TG), High-Density Lipoprotein (HDLc), and Low-Density Lipoprotein (LDLC) levels. The remaining serum was stored at -80°C for use in the detection of serum vaspin levels. Serum levels of TC, TG, FFA, HDLC, and LDLC were measured using an automated biochemical analyser. Insulin secretion and resistance indices were then calculated as follows: Homeostatic Model Assessment of Insulin Secretion (HOMA-IS) index=(20 × FINS)/(FBG-3.5); Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) index=(FBG × FINS)/22.5 [16].

**Enzyme-linked immunosorbent assay (ELISA)**

Vaspin levels were quantified using a commercial ELISA kit (ALPCO Co., USA) that includes anti-human vaspin monoclonal antiserum. The antiserum exhibits complete cross-reaction with human vaspin and no cross-reaction with rat and mouse vaspin, or other cytokines in human serum, such as leptin, adiponectin, visfatin, tumor necrosis factor-α (TNF-α), and Retinol Binding Protein 4 antibody (RBP4). The linear range was 0.016 to 1 ng/ml, with intra- and inter-assay Coefficients of Variation (CVs) of <5% and <10%, respectively. The measurements were performed in strict accordance with manufacturer’s protocol, using a double-antibody sandwich ELISA assay for detection. Briefly, the microtiter plate was coated with a purified anti-human vaspin-specific monoclonal antibody and a solid-phase antibody was prepared. The standards, unknown samples, biotin-labelled anti-human vaspin-specific polyclonal antibody, or Horseradish Peroxidase (HRP)-labelled avidin were added to the monoclonal antibody-coated wells. The plates were thoroughly washed to remove unbound enzyme, and substrates of the enzyme complex were added, resulting in a blue solution. Finally, the termination solution was added to stop the enzyme-substrate reaction, which converted the solutions to a yellow color. The saturation of the yellow color was positively correlated to the vaspin content. Next, the absorbance (Optical Density (OD)) was measured at 450 nm; the concentrations of the standards were set as the abscissa, with the OD values as the ordinate, in order to produce a standard curve. Based on this standard curve, the corresponding sample concentrations were determined, then either multiplied by the dilution factor or the OD values were used directly to calculate the straight-line regression equation of the standard curve. The OD values were then substituted into the equation in order to determine the sample concentration, which was multiplied by the dilution factor and used to calculate the actual sample concentration.

**Statistical analyses**

SPSS version 13.0 was used for statistical analyses and data processing. The experimental data were expressed as means ± standard deviations (\(\bar{x} \pm s\)) and assessed for normal distribution. The non-normally distributed variables were first log-transformed to give a normal distribution. The intergroup comparisons used Analysis of Variance (ANOVA). Multivariate analysis used partial correlation and linear correlation analyses, with P<0.05 considered statistically significant.

**Results**

The 30 cases in the NDR group included 17 men and 13 women aged 35 to 58 years (41.4 ± 6.4), with a disease
duration of 4 to 7 years (mean 5.8 ± 0.9 years), and no other diabetes-associated complications. The 30 cases in the NPDR group included 16 men and 14 women aged 44 to 66 years (49.3 ± 5.1), with a disease duration of 7 to 12 years (mean 9.5 ± 1.6 years). The 30 cases in the PDR group included 18 men and 12 women aged 42 to 71 years (53.0 ± 8.7), with a disease duration of 13 to 19 years (mean 15.1 ± 3.3 years). The control group included 19 men and 11 women aged 35 to 59 years (45.3 ± 5.4). Age (F=0.581), height (F=0.337), body weight (F=0.341), and BMI (F=0.307) did not differ significantly among the four groups (P>0.05) (Table 1).

Serum levels of HbA1c, FBG, FINS, TC, vaspin, and HOMA-IR levels differed significantly among the four groups (P<0.01); HDLC, LDLC, HOMA-IS levels among the four groups showed highly significant statistical differences (P<0.05), while serum TG levels did not differ significantly (P>0.05) (Table 1).

Pairwise comparisons of serum levels of TC and LDLC between the control and NDR, NPDR, and PDR groups revealed statistically significant differences (P<0.05). Serum levels of HbA1c, FGS, FINS, HDLC, HOMA-IS, HOMA-IR, and vaspin also showed statistically significant differences (P<0.01). There were no significant differences in serum TG levels (Table 2). Comparisons of serum levels of vaspin between the NDR and NPDR revealed no significant differences (t=0.13, P>0.05), however, serum levels of vaspin between the NDR and PDR groups revealed statistically significant differences (t=1.98, P<0.05).

Multivariate analysis showed that the severity of DR was significantly correlated with HbA1c, FGS, FINS, LDLC, and HOMA-IR, and also significantly correlated with reduced vaspin, HOMA-IS, and HDLC levels (P<0.01). After excluding interactions among factors, age, sex, BMI, and DR staging were significantly correlated with the above indicators, while TC was not significantly correlated with DR stage (Table 3).

The results of the linear correlation analysis showed that vaspin was significantly correlated with HDLC and HOMA-IS (P<0.01), and negatively correlated with HOMA-IR (P<0.01).

### Table 1. Comparisons of different indexes among the 4 groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>NDR</th>
<th>NPDR</th>
<th>PDR</th>
<th>Control</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average age (years)</td>
<td>41.4 ± 6.4</td>
<td>49.3 ± 5.1</td>
<td>53.0 ± 8.7</td>
<td>45.3 ± 5.4</td>
<td>0.581</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Average disease duration (years)</td>
<td>5.8 ± 0.9</td>
<td>9.5 ± 1.6</td>
<td>15.1 ± 3.3</td>
<td>0</td>
<td>245.01</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Average BMI</td>
<td>22.8 ± 3.2</td>
<td>23.5 ± 3.7</td>
<td>23.6 ± 3.0</td>
<td>23.2 ± 2.9</td>
<td>0.307</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>5.61 ± 0.41</td>
<td>6.87 ± 0.93</td>
<td>7.55 ± 1.10</td>
<td>4.18 ± 0.56</td>
<td>5.65</td>
<td>0.01</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>2.60 ± 0.54</td>
<td>2.82 ± 0.52</td>
<td>3.03 ± 0.50</td>
<td>1.96 ± 0.62</td>
<td>2.09</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>HDLC (mmol/L)</td>
<td>1.83 ± 0.24</td>
<td>1.75 ± 0.39</td>
<td>1.20 ± 0.22</td>
<td>2.98 ± 0.31</td>
<td>5.27</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>LDLC (mmol/L)</td>
<td>2.76 ± 0.43</td>
<td>3.51 ± 0.55</td>
<td>4.31 ± 0.47</td>
<td>2.62 ± 0.48</td>
<td>6.9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.65 ± 1.06</td>
<td>10.03 ± 0.92</td>
<td>12.25 ± 0.72</td>
<td>4.60 ± 0.94</td>
<td>210.06</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FBS (mmol/L)</td>
<td>6.51 ± 0.4</td>
<td>8.57 ± 0.90</td>
<td>11.45 ± 1.14</td>
<td>5.88 ± 0.46</td>
<td>6.75</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>FINS (mu/l)</td>
<td>9.50 ± 0.55</td>
<td>12.72 ± 0.51</td>
<td>23.23 ± 0.40</td>
<td>8.86 ± 0.42</td>
<td>22.19</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vaspin (ng/ml)</td>
<td>0.53 ± 0.22</td>
<td>0.45 ± 0.13</td>
<td>0.36 ± 0.05</td>
<td>1.31 ± 0.21</td>
<td>94.27</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HOMA-IS</td>
<td>92.70 ± 0.42</td>
<td>83.50 ± 0.59</td>
<td>74.01 ± 0.45</td>
<td>102.60 ± 0.38</td>
<td>4.9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>3.54 ± 0.67</td>
<td>8.11 ± 1.59</td>
<td>11.04 ± 1.6</td>
<td>2.37 ± 0.37</td>
<td>21.56</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

FBS: Fasting Blood Sugar; FINS: Fasting Insulin; HbA1c: Glycosylated Haemoglobin; TC: Cholesterol; TG: Triglyceride; HDLC: High-Density Lipoprotein; LDLC: Low-Density Lipoprotein; HOMA-IS: Homeostatic Model Assessment of Insulin Secretion Index; HOMA-IR: Homeostatic Model Assessment of Insulin Resistance Index.

### Table 2. Pairwise comparisons of the serum index levels among the 4 groups.

<table>
<thead>
<tr>
<th>Detection index</th>
<th>Group control vs. NDR</th>
<th>Group control vs. NPDR</th>
<th>Group control vs. PDR</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA1c</td>
<td>3.42 &lt;0.01</td>
<td>3.77 &lt;0.01</td>
<td>8.12 &lt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FBS</td>
<td>3.52 &lt;0.01</td>
<td>4.11 &lt;0.01</td>
<td>7.93 &lt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FINS</td>
<td>2.99 &lt;0.01</td>
<td>3.76 &lt;0.01</td>
<td>6.98 &lt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TC</td>
<td>0.18 &lt;0.05</td>
<td>0.32 &lt;0.05</td>
<td>0.55 &lt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG</td>
<td>0.05 &gt;0.05</td>
<td>0.07 &gt;0.05</td>
<td>0.09 &gt;0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDLC</td>
<td>0.32 &lt;0.01</td>
<td>0.59 &lt;0.01</td>
<td>0.89 &lt;0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDLC</td>
<td>0.15 &gt;0.05</td>
<td>0.22 &gt;0.05</td>
<td>0.99 &gt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaspin</td>
<td>1.29 &lt;0.01</td>
<td>1.48 &lt;0.01</td>
<td>2.63 &lt;0.01</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
of DR were determined based on retinopathic examination, as after correcting for age, sex, BMI, and other factors. However, included in a control group, and patients with systemic disease results showed significantly lower serum vaspin concentrations and improve abnormalities in glucose tolerance [16,18]. Yang et al. found that vaspin is a fat-derived molecule that may be well as international staging criteria. Healthy individuals were uniformly numbered and measured by laboratory professionals in a blinded fashion, in order to ensure reliable test results. As a specific endocrine organ, adipose tissue plays important roles in the occurrence and development of insulin resistance and T2DM because it participates in energy metabolism. Adipose tissue may also participate in glucose and lipid metabolism [6,7,14,17]. Vaspin is a specific visceral fat protease inhibitor with insulin-sensitizing effects, which may play a role in the regulation of glucose and lipid metabolism, and improve abnormalities in glucose tolerance [16,18]. Yang et al. found that vaspin is a fat-derived molecule that may be involved in the occurrence of insulin resistance and T2DM [19]. These previous studies assessed patients with different stages of DR and analysed the relationships between fasting serum vaspin levels and related metabolic indicators; the results showed significantly lower serum vaspin concentrations in patients with DR compared to those of unaffected individuals; the result remained statistically significant even after correcting for age, sex, BMI, and other factors. However, Akbarzadeh et al. found that there was no significant difference in serum vaspin concentrations between the FDRs and the controls based on a study of Iranian patients with T2DM [20], which is consistent with our study because of differences in detection reagents, methods and research objectives. In the present study, the levels of FBG and HbA1c were significantly higher in the DR patients compared to those in patients from the other two groups, and further correlation analysis showed that these indicators of glucose metabolism were significantly correlated with serum vaspin levels. Therefore, serum vaspin levels were closely related to blood glucose levels [16,21]. Another study [22] reported lower serum vaspin levels among participants with HbA1c>7 compared to the population with HbA1c<7, thus suggesting that increased vaspin levels might indicate a transient protective mechanism of the body against the toxicity of high blood glucose levels. Seeger et al. [16] reported the insulin-sensitizing effects of vaspin when used as an intervention in high-fat and high-glucose-induced diabetic mice, observing improved blood glucose and insulin sensitivity. Another study also found that vaspin was negatively correlated with HOMA-IR [21]. This study also a correlation of vaspin with HOMA-IR and HOMA-IS, further suggesting that vaspin might be an adipokine responsible for increased insulin sensitivity. During clinical blood glucose monitoring, HbA1c is a sensitive indicator of blood glucose control, which was also used to indicate the average blood glucose level of the patient 6 to 8 weeks before examination. A recent 10-year study showed that blood glucose control in diabetic patients was closely related to the occurrence and development of DR [23]. Hypoxia is an important cause of aggravation of DR, and glucose dysmetabolism could deposit a large number of non-enzymatic glycosylated metabolites on the cell basal membrane, thus impeding oxygen diffusion. The non-enzymatic glycosylation of haemoglobin would reduce its ability to carry oxygen, and the combined actions of these two factors could result in retinal hypoxia; therefore, non-enzymatic glycosylated metabolites are significantly correlated with HbA1c levels [7,24]. HbA1c mainly reflects abnormalities in glucose metabolism, and plays an important role in the pathogenesis of diabetic retinopathy; the role of blood lipids in the pathogenesis of diabetic retinopathy has also been studied. Vaspin is a fat secretion adipokine responsible for increased insulin sensitivity. The TC, LDL, and HDL levels in each diabetes group showed statistically significant differences compared to those of the control group, and were correlated with DR stage. In particular, LDLC and HDLC levels (r=0.33, -0.37, P=0.00) were significantly correlated with DR stage, suggesting that dyslipidaemia is involved in DR pathogenesis. Vaspin was significantly correlated with LDLC levels, and negatively correlated with HDLC levels, indicating that TC, LDL, HDLC, and vaspin are jointly involved in DR pathogenesis. Patients with DR in the current study also had serious lipid metabolism disorders, so there is likely to be a relationship between dyslipidaemia and DR occurrence. Increased blood lipid levels could also cause over oxidation of the tissues through direct or indirect activation of the non-enzymatic glycosylated polyol pathway, causing vessel wall damage and endothelial dysfunction [25]. Furthermore, high blood lipid levels could change cellular membrane lipid structures, which would
ultimately cause micro-thrombosis and lead to breakdown of the retinal barrier; therefore, lipid dysmetabolism might further aggravate retinal microcirculation [26].

The results of the linear correlation analysis indicated that vaspin was significantly correlated with HDLC and HOMA-IS (r=0.96, 0.74, P<0.01) and was negatively correlated with HOMA-IR (r=-0.69, P<0.01). Vaspin plays an important role in insulin resistance and fat metabolism, and might be a compensating factor during the pathological processes of IR and metabolism-related diseases, suggesting that vaspin is closely related to IR. Thus, monitoring its levels might be useful for determination of the degree of IR; however, the exact molecular mechanism is still not fully understood. Guclcelik et al. reported that serum vaspin levels in diabetic patients with chronic complications, including neuropathy, retinopathy, and nephropathy, was lower than in patients without these complications [21]. Another study detected lower serum concentrations of vaspin in patients with carotid stenosis who experienced ischemic events compared with asymptomatic patients [27]. These results indicate that diabetic patients with decreased vaspin levels may have poor outcomes and a higher prevalence of micro- or macro-vascular complications over time.

Work Limitation
A limitation of our study is the small sample size; thus, further studies are necessary to reveal the role of vaspin in DR. In addition, the interactions among other angiogenic factors and vaspin also require additional investigation to reveal the potential mechanisms of action of vaspin in the proliferation, migration, and capillary formation of retinal endothelial cells.

Conclusion
In conclusion, our study confirmed that serum vaspin levels were significantly lower in patients with DR, and closely associated with blood glucose and lipid metabolism. Low levels of circulating vaspin may be a risk factor for progression of DR. Further prospective observational studies are necessary to explain the mechanism by which decreased circulating vaspin levels are involved in the progression of DR.

Conflict of Interest
All authors have no conflict of interest regarding this paper.

References


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