

# Association between angiotensin-like protein 2 and lectin-like oxidized low-density lipoprotein receptor 1 ligand containing apolipoprotein B in patients with type 2 diabetes

Journal of International Medical Research

2018, Vol. 46(10) 4167–4180

© The Author(s) 2018

Article reuse guidelines:

[sagepub.com/journals-permissions](http://sagepub.com/journals-permissions)

DOI: 10.1177/0300060518791067

[journals.sagepub.com/home/imr](http://journals.sagepub.com/home/imr)



Tatsuhiko Suzuki, Kohzo Takebayashi,  
Kenji Hara, Takafumi Tsuchiya and  
Toshihiko Inukai

## Abstract

**Objective:** This study was performed to evaluate the association of the serum level of angiotensin-like protein 2 (ANGPTL2) with circulating inflammatory markers and oxidized and modified low-density lipoprotein (LDL) cholesterol as evaluated by lectin-like oxidized LDL receptor 1 ligand containing apolipoprotein B (LAB) in patients with type 2 diabetes.

**Methods:** The study included 70 patients with type 2 diabetes hospitalized for glycemic control and 9 control subjects.

**Results:** The serum level of ANGPTL2 was significantly higher in the patients with type 2 diabetes than in the healthy controls. There was a significant positive correlation between ANGPTL2 and the high-sensitivity C-reactive protein, fibrinogen, and LAB levels and a significant negative correlation between ANGPTL2 and the estimated glomerular filtration rate (eGFR).

**Conclusions:** These results suggest that the serum ANGPTL2 level has a close positive association with inflammatory markers, especially fibrinogen and oxidized and modified LDL as evaluated by LAB. The data also suggest that the serum ANGPTL2 level is influenced by renal function as reflected by the eGFR.

---

Department of Internal Medicine, Dokkyo Medical  
University Saitama Medical Center, Koshigaya,  
Saitama, Japan

---

## Corresponding author:

Kohzo Takebayashi, Department of Internal Medicine,  
Dokkyo Medical University Saitama Medical Center, 2-1-  
50, Minamikoshigaya, Koshigaya, Saitama 343-8555, Japan.  
Email: [takebaya@dokkyomed.ac.jp](mailto:takebaya@dokkyomed.ac.jp)



## Keywords

ANGPTL2, LAB, fibrinogen, type 2 diabetes, low-density lipoprotein cholesterol, estimated glomerular filtration rate

Date received: 29 March 2018; accepted: 3 July 2018

## Introduction

Angiopoietin-like proteins (ANGPTLs) are glycosylated proteins with an N-terminal coiled domain and a C-terminal fibrinogen-like domain; the exception to this is ANGPTL8. ANGPTLs are structurally similar to angiopoietins. Eight members of this family (ANGPTL1–8) have been identified to date.<sup>1–3</sup> Although ANGPTLs cannot bind to angiopoietin receptors such as Tie2 or to the related protein Tie1,<sup>1,2</sup> they can regulate angiogenesis similarly to the angiopoietins.<sup>2,3</sup> In addition, some ANGPTLs, including ANGPTL3, 4, 6, and 8, also contribute to lipid, glucose, and/or energy metabolism.<sup>1–7</sup>

ANGPTL2 is abundantly expressed in visceral adipose tissues.<sup>2,3,8</sup> Apart from its angiogenic activity, it causes inflammation of adipose tissue in human patients with obesity, resulting in insulin resistance.<sup>2,3,8</sup> Furthermore, ANGPTL2 can also cause vascular inflammation,<sup>8</sup> endothelial dysfunction,<sup>9</sup> and increased oxidative stress,<sup>10</sup> and it may be associated with tumor metastasis.<sup>11</sup> Although the main sources of circulating ANGPTL2 in humans appear to be adipocytes, endothelial cells, and macrophages that have infiltrated visceral fat tissues,<sup>2,3</sup> it can also be secreted by the heart<sup>1,12</sup> and by both endothelial cells<sup>13</sup> and macrophages<sup>14</sup> in various other tissues. Interestingly, recent studies have demonstrated that the circulating ANGPTL2 level is associated with new-onset type 2 diabetes<sup>15</sup> and with both cardiovascular events and mortality in patients with type

2 diabetes.<sup>16</sup> The association of the circulating ANGPTL2 level with high-sensitivity C-reactive protein (hsCRP) as a circulating inflammation-marker in patients with type 2 diabetes has been previously reported.<sup>8,17</sup> However, the association between the ANGPTL2 level and other inflammatory markers, such as fibrinogen, has not yet been investigated. The association between ANGPTL2 and fibrinogen levels may be particularly interesting because ANGPTLs, including ANGPTL2, have a fibrinogen-like domain at the C terminus, as described above.<sup>1–3</sup> Furthermore, although low-density lipoprotein (LDL) cholesterol is the most important established marker of cardiovascular risk in patients with type 2 diabetes,<sup>18</sup> the importance of oxidation of LDL for the progression of atherosclerosis was recently recognized.<sup>19</sup> Oxidized LDL, but not LDL, binds to the lectin-like oxidized LDL receptor 1 (LOX-1, an oxidized and modified LDL receptor) in vascular endothelial cells<sup>20</sup> and can play an important role in the formation of an atheroma.<sup>19</sup> LOX-1 ligands containing apolipoprotein B (LAB)<sup>21</sup> are a marker of the atherogenicity of oxidized and modified LDL more specifically than one antigenic determinant of oxidized LDL.<sup>22,23</sup> Because ANGPTL2 can enhance both inflammation and oxidative stress,<sup>8,10</sup> we hypothesized that a relationship exists between ANGPTL2 and LAB.

We investigated the association between the circulating ANGPTL2 level and the levels of inflammatory markers such as

hsCRP and fibrinogen, circulating soluble LOX-1 (sLOX-1), and circulating LAB in patients with type 2 diabetes. We also evaluated the association of ANGPTL2 with the various markers related to type 2 diabetes. We hypothesized that the circulating ANGPTL2 level is positively associated with hsCRP and fibrinogen and that this parameter would also be positively associated with the LAB level rather than with the sLOX-1 level.

## Methods

### Patients

This study was registered in the UMIN Clinical Trials Registry (UMIN000025767). Hospitalized patients with type 2 diabetes were prospectively and consecutively enrolled from February 2017 to August 2017.

The detailed key inclusion and exclusion criteria for the patients with type 2 diabetes have been previously described in the UMIN clinical registered system.<sup>24</sup> The baseline characteristics of all patients and healthy control subjects on enrollment are shown in Table 1.

### Methods

The patients with type 2 diabetes underwent blood tests at 9:00 AM after  $\geq 10$ -hour overnight fast the day after hospitalization. The control subjects also underwent blood tests performed under similar conditions, although they were not hospitalized. Blood samples for analyses of ANGPTL2, LOX-1, and LAB were frozen at  $-80^{\circ}\text{C}$  until analysis. Measurements of body weight and blood pressure were also performed at the time of sampling.

### Measurement of serum ANGPTL2 level

The fasting serum ANGPTL2 level was measured in duplicate using a human ANGPTL2 enzyme-linked immunosorbent (ELISA)

assay kit (sandwich ELISA) (Human ANGPTL2 assay Kit-IBL, code no. 27745; Immuno-Biological Laboratories Co., Ltd., Gunma, Japan). The intra-assay and inter-assay coefficients of variation were 3.9% to 5.9% and 6.3% to 10.5%, respectively,<sup>25</sup> based on the manufacturer's information.

### Measurement of serum sLOX-1 and LAB levels

The plasma level of sLOX-1 was measured using a sandwich chemiluminescence enzyme immunoassay (CLEIA) with two different monoclonal antibodies to the extracellular domain of LOX-1 (B017M and a chicken monoclonal anti-human LOX-1 antibody HUC3-48). The plasma level of LAB was measured by a sandwich CLEIA using recombinant sLOX-1 and a monoclonal antibody to the extracellular domain of ApoB (a chicken monoclonal anti-human ApoB antibody HUC20).

### Measurement of carotid intima-media complex thickness

The thickness of the intima-media complex was evaluated in the right carotid artery using echography (aplico i900 and aplicoXV; Toshiba, Tokyo, Japan). The maximum point in three suitable consecutive points at 1-cm intervals was estimated as the intima-media complex thickness in each patient.

### Measurements of fasting plasma glucose, hemoglobin A1c, and insulin levels

Fasting plasma glucose (FPG) was measured immediately after blood collection using an automated glucose oxidase analysis system (Glucose Auto Stat GA1160; Arkray, Kyoto, Japan). Hemoglobin A1c (HbA1c) was evaluated by an enzymatic assay using protease in the first-order reaction and both fructosylpeptide oxidase and

peroxidase in the second-order reaction (Norudia N HbA1c; Sekisui Medical Inc., Tokyo, Japan). The serum insulin level (immune-reactive insulin) was evaluated by a CLEIA using the Lumipulse Presto Insulin Kit (Fujirebio, Tokyo, Japan).

### Homeostasis model assessment-insulin resistance

Homeostasis model assessment-insulin resistance (HOMA-IR) was used as an indicator of insulin resistance and was

**Table 1.** Baseline clinical features of patients with type 2 diabetes

	All patients	Control subjects	P
Sex (male/female)	70 (45/25)	9 (5/4)	
Age (years)	60 (48.3, 72.8)	61 (50.0, 63.0)	0.3503
Mean duration of diabetes (years)	7 (2, 11.8)		
BMI (kg/m <sup>2</sup> )	24.5 ± 4.5	24.8 ± 2.7	0.8438
FPG (mg/dL)	174 (143.3, 257.8)	106 (97, 111)	<0.0001
HbA1c (%)	9.8 (8.8, 11.7)	5.7 (5.6, 5.8)	<0.0001
eGFR (mL/min/1.73 m <sup>2</sup> )	79.7 (64.2, 96.1)	76.1 (65.8, 88.8)	0.6769
SBP (mmHg)	121 (114.3, 134.8)		
DBP (mmHg)	73.0 ± 11.7		
TG (mg/dL) (69)	127 (99, 177)		
HDL-C (mg/dL) (69)	47 (41, 63)		
LDL-C (mg/dL) (69)	123.6 ± 45.0		
Fibrinogen (mg/dL)	349.6 ± 98.2		
AST (U/L)	18 (15, 23)		
ALT (U/L)	18 (13, 26.5)		
GGT (U/L) (69)	30 (20, 39)		
CAVI (66)	8.2 ± 1.4		
IMT (mm) (58)	0.95 (0.8, 1.1)		
UAE (mg/g.Cr) (69)	17 (6.5, 75.5)		
LAB (ng cs/mL)	3.1 (2.6, 3.8)		
<i>Diabetic therapy</i>			
Insulin (–)	35		
Metformin (+)	24		
MD/MDA/MDP/MDSg/MDGn	7/3/2/1/1		
MDPSg/MDPSgGp/MDASg	1/1/2		
MDASu/MGp/MGpPSg	1/2/2		
Metformin (–)	11		
D/DA/DASu/DSu/PAD/PGp/GpGn/N	2/1/1/1/1/1/1/3		
Insulin (+)	35		
Metformin (+)	16		
MD/MDA/MDP/MDSg/MDGn	4/1/2/1/1		
MDPSg/MDPSgGp/MDASg	1/0/0		
MDASu/MGp/MGpPSg	0/0/0		
MDSu/MDPA/M/MA/MASu	1/1/2/1/1		
Metformin (–)	19		
D/DA/DASu/DSu/PAD/PGp/GpGn/N	5/2/0/0/0/0/3		
P/DP/DGn/A	3/4/1/1		

(continued)

**Table 1.** Continued

	All patients	Control subjects	P
Antihypertensive drugs	31		
Ar/C/ArC/ArCB/ArCAB/ArCT/CT/CBAb	4/6/16/1/1/1/1		
Anti-hyperlipidemic drugs			
Statins (+)	20		
S/SE/SCo	18/2/0		
Statins (–)	50		
E/Co/N	2/1/47		

Data are expressed as median with 25<sup>th</sup> and 75<sup>th</sup> interquartile range because of the skewed distribution, excluding BMI, DBP, fibrinogen, and CAVI (these are expressed as mean  $\pm$  standard deviation because of their normal distributions). Measurement of SBP, DBP, TG, HDL-C, LDL-C, fibrinogen, AST, ALT, GGT, CAVI, IMT, UAE, and LAB was performed only in patients with diabetes. For the variables that were not measured in all patients, the number of patients is shown in parentheses. Comparisons of variables between patients with diabetes and healthy subjects were performed by the Mann–Whitney U test, excluding BMI. For BMI, the comparison was confirmed by an unpaired t-test

BMI: body mass index, FPG: fasting plasma glucose, HbA1c: hemoglobin A1c, eGFR: estimated glomerular filtration rate, SBP: systolic blood pressure, DBP: diastolic blood pressure, TG: triglycerides, HDL-C: high-density lipoprotein cholesterol, LDL-C: low-density lipoprotein cholesterol, AST: aspartate aminotransferase, ALT: alanine aminotransferase, GGT: gamma-glutamyl transpeptidase, CAVI: cardio-ankle vascular index, IMT: carotid intima-media complex thickness, UAE: urinary albumin excretion, LAB: lectin-like oxidized LDL receptor 1 ligand containing apolipoprotein B, Diabetic therapy: number of patients treated with respective diabetic therapies, M: metformin, D: dipeptidyl peptidase 4 inhibitor, A:  $\alpha$  glucosidase inhibitor, P: pioglitazone, Sg: sodium glucose cotransporter 2 inhibitor, Gn: glinides, Gp: glucagon-like peptide-1 receptor agonists, Su: sulfonylureas, N: no antidiabetic drugs, Antihypertensive drugs: number of patients treated with respective antihypertensive drugs, Ar: angiotensin-II receptor blockers, C: calcium channel blockers, T: thiazides, B: beta adrenaline receptor blockers, Ab: alpha adrenaline receptor blockers, Anti-lipid drugs: S: statins, E: ezetimibe, Co: colestimide

In therapies, for example, “D” refers to a dipeptidyl peptidase 4 inhibitor alone and “DA” refers to a dipeptidyl peptidase 4 inhibitor + an  $\alpha$  glucosidase inhibitor

calculated as follows:  $\text{HOMA-IR} = \text{FPG (mg/dL)} \times \text{immunoreactive insulin } (\mu\text{U/mL}) / 405.$ <sup>26</sup>

### Estimated glomerular filtration rate

The estimated glomerular filtration rate (eGFR) was calculated as follows: for men,  $\text{eGFR (mL/min/1.73 m}^2) = 194 \times \text{creatinine (mg/dL)}^{-1.094} \times \text{age (years)}^{-0.287}$ , and for women,  $\text{eGFR (mL/min/1.73 m}^2) = 194 \times \text{creatinine (mg/dL)}^{-1.094} \times \text{age (years)}^{-0.287} \times 0.739.$ <sup>27</sup>

### Measurement of serum hsCRP and plasma fibrinogen

The hsCRP level was measured by the latex agglutination method using a kit (CRP-Latex X2; Denka Seiken Co., Ltd.,

Tokyo, Japan), and plasma fibrinogen was measured based on the thrombin time method using a fibrinogen kit (Coagpia; Sekisui Medical Co., Ltd.).

### Evaluation of diabetic retinopathy

Ophthalmologists in our hospital evaluated diabetic retinopathy, including no diabetic retinopathy (NDR), simple diabetic retinopathy (SDR), and proliferative diabetic retinopathy (PDR), according to the criteria established by Davis.<sup>28</sup>

### Ethical considerations

All subjects gave written informed consent for inclusion in this study, and the local ethics committee at our hospital approved the study. This study was performed

according to the guidelines of the Declaration of Helsinki.

### Statistical methods

We expected that the correlation coefficient between ANGPTL2 and other important biomarkers would be  $>0.35$  when these correlations were statistically significant. The required sample sizes were 85 and 62 for mother correlation coefficients of 0.3 and 0.35, respectively, with a two-sided significance level of 0.05 and power of 0.8. The normality of the data in each variable was confirmed by an  $\chi^2$  goodness-of-fit test and/or by the Kolmogorov–Smirnov test. The serum ANGPTL2 level had a normal distribution. Among the variables included in Table 2, the body mass index (BMI), diastolic blood pressure, LDL-C, fibrinogen, and cardio-ankle vascular index (CAVI) had normal distributions. Therefore, the correlation of ANGPTL2 with these variables was confirmed using Pearson's correlation. The remaining variables had skewed distributions. Among the variables with a skewed distribution, FPG, HbA1c, triglycerides, HDL-C, insulin, HOMA-IR, hsCRP, eGFR, and LAB had normal distributions after  $\log_{10}$ -transformation. Therefore, the association of ANGPTL2 with these variables was confirmed using the data after  $\log_{10}$ -transformation by Pearson's correlation. None of the remaining variables had a normal distribution even after  $\log_{10}$ -transformation. Thus, the correlation between ANGPTL2 and these variables was confirmed using Spearman's correlation as a nonparametric coefficient. In the multiple regression analysis, we used two model groups: an inflammatory-related group and a metabolic-related group. With the exception of ANGPTL2, BMI, LDL-C, and fibrinogen, these variables were  $\log_{10}$ -transformed. Comparisons of BMI between patients with type 2 diabetes and healthy subjects were performed using an unpaired

**Table 2.** Correlation of ANGPTL2 with multiple variables

	R	P
Age (years) (70)	-0.0488	0.6885
Duration (years) (70)	-0.1231	0.3099
BMI (kg/m <sup>2</sup> ) (70)	0.0628	0.6056
FPG (mg/dL) (70)	0.1443	0.2335
HbA1c (%) (70)	0.1619	0.1806
SBP (mmHg) (70)	0.0041	0.9730
DBP (mmHg) (70)	0.2177	0.0702
TG (mg/dL) (69)	0.1047	0.3921
HDL-C (mg/dL) (69)	0.1182	0.3334
LDL-C (mg/dL) (69)	0.0665	0.5873
Insulin ( $\mu$ U/mL)(30)	0.3486	0.0590
HOMA-IR (30)	0.3738	0.0419*
hsCRP (mg/L) (70)	0.2397	0.0457*
Fibrinogen (mg/dL) (70)	0.4623	0.0001*
AST (U/L) (70)	0.1756	0.1460
ALT (U/L) (70)	0.0563	0.6453
GGT (U/L) (69)	0.1611	0.1861
eGFR (mL/min/1.73 m <sup>2</sup> ) (70)	-0.2600	0.0298*
CAVI (66)	-0.0120	0.9240
IMT (mm) (58)	0.1260	0.3459
UAE (mg/g.Cr) (69)	-0.2011	0.0975
sLOX (ng/L)(70)	0.1081	0.3729
LAB (ng cs/mL)(70)	0.2906	0.0147*

ANGPTL2, BMI, DBP, LDL-C, fibrinogen, and CAVI followed a normal distribution as confirmed by a  $\chi^2$  goodness-of-fit test and/or the Kolmogorov–Smirnov test. Because of the skewed distribution of FPG, HbA1c, TG, HDL-C, insulin, HOMA-IR, hsCRP, eGFR, and LAB, these variables were  $\log_{10}$ -transformed. After  $\log_{10}$ -transforming, these variables followed a normal distribution. These correlations were evaluated using Pearson's correlation coefficient. Because age, duration, SBP, AST, ALT, GGT, IMT, UAE, and sLOX had a skewed distribution even after  $\log_{10}$ -transforming, these correlations were evaluated using Spearman's correlation coefficient. R indicates Pearson's or Spearman's correlation. The numbers in parentheses indicate the number of patients.

\*Statistically significant ( $P < 0.05$ )

ANGPTL2: angiotensin-like protein 2, BMI: body mass index, FPG: fasting plasma glucose, HbA1c: hemoglobin A1c, SBP: systolic blood pressure, DBP: diastolic blood pressure, TG: triglycerides, HDL-C: high-density lipoprotein cholesterol, LDL-C: low-density lipoprotein cholesterol, HOMA-IR: homeostasis model assessment-insulin resistance, hsCRP: high-sensitivity C-reactive protein, AST: aspartate aminotransferase, ALT: alanine aminotransferase, GGT: gamma-glutamyl transpeptidase, eGFR: estimated glomerular filtration rate, CAVI: cardio-ankle vascular index, IMT: carotid intima-media complex thickness, UAE: urinary albumin excretion, sLOX: soluble lectin-like oxidized LDL receptor I, LAB: LOX-I ligand containing apolipoprotein B

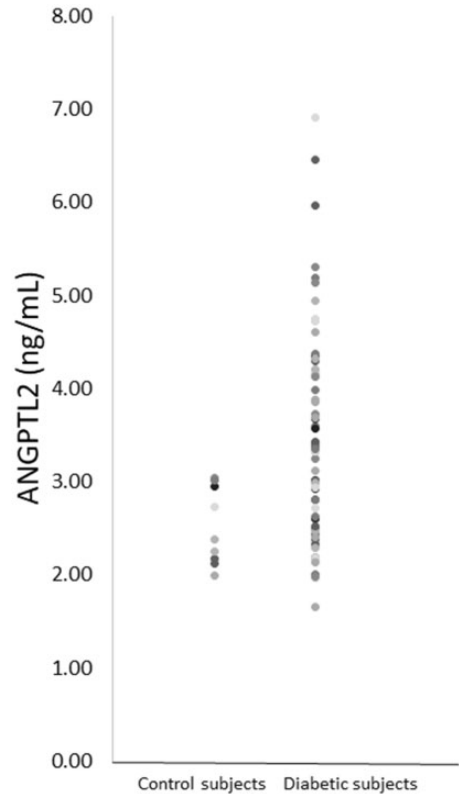


*t*-test. Comparisons of age, FPG, HbA1c, and eGFR between these two groups were performed by the Mann–Whitney U test because of the skewed distribution of these variables. Comparisons of ANGPTL2 between the two groups were performed using an unpaired *t*-test. For comparison of ANGPTL2 among the three groups, homogeneity was confirmed using the Bartlett test. A parametric comparison was done using one-way analysis of variance after confirmation of equal variance. After confirmation of the significance by the analysis of variance, a *post hoc* Holm test was conducted. All statistical analyses were performed using Ekuseru-Toukei 2012 software (Social Survey Research Information Co., Ltd., Tokyo, Japan). A two-sided P value of <0.05 was considered statistically significant.

## Results

In total, 70 patients with type 2 diabetes and 9 healthy control subjects were enrolled in this study. The serum ANGPTL2 level was significantly higher in patients with diabetes ( $n=70$ ) than in healthy controls ( $n=9$ ) [ $3.47 \pm 1.10$  (range, 1.65–6.91) vs.  $2.51 \pm 0.42$  (range, 1.99–3.04) ng/mL, respectively;  $P < 0.0001$ ] (Figure 1).

There were no sex-related differences. The ANGPTL2 levels for men and women were  $3.42 \pm 1.07$  and  $3.57 \pm 1.13$  ng/mL, respectively. When the patients were classified into two groups with statins ( $n=20$ ) and without statins ( $n=50$ ), the serum ANGPTL2 levels were  $3.16 \pm 0.67$  vs.  $3.60 \pm 1.21$  ng/mL, respectively, with a trend toward lower ANGPTL2 levels in the group with than without statins. There was no significant difference in the ANGPTL2 level between patients with insulin therapy ( $n=35$ ) and without insulin therapy ( $n=35$ ) ( $3.45 \pm 1.08$  vs.  $3.50 \pm 1.12$  ng/mL, respectively). In addition, no difference was noted between patients with a



**Figure 1.** Serum levels of ANGPTL2 in healthy subjects without diabetes ( $n=9$ ) and in patients with type 2 diabetes ( $n=70$ ). ANGPTL2: angiotensin-like protein 2

current smoking habit ( $n=29$ ) and those without ( $n=41$ ). Furthermore, when the patients were divided into three groups based on the degree of diabetic retinopathy [NDR ( $n=52$ ), SDR ( $n=5$ ), and PDR ( $n=13$ )], the serum ANGPTL2 levels were  $3.45 \pm 1.14$ ,  $3.61 \pm 1.22$ , and  $3.51 \pm 0.93$  ng/mL, respectively. There were no significant differences in the ANGPTL2 levels among these groups (NDR vs. SDR, NDR vs. PDR, and SDR vs. PDR).

The data obtained from the correlation of the ANGPTL2 level with multiple variables and the data obtained from the multiple regression analysis of the ANGPTL2 level as the dependent variable are

summarized in Tables 2 and 3. A significant positive correlation was found between the ANGPTL2 level and the hsCRP, fibrinogen, and LAB levels ( $P=0.0457$ ,  $P=0.0001$ , and  $P=0.0147$ , respectively), and a significant negative correlation was found between the ANGPTL2 level and the eGFR ( $P=0.0063$ ) (Figure 2). For patients not on insulin therapy whose serum insulin levels were measured ( $n=30$ ), a significant positive correlation was found between the ANGPTL2 and HOMA-IR levels ( $P=0.0419$ ). In the multiple regression analysis, ANGPTL2 had a significant positive association with fibrinogen in all models (Models 2–4), including fibrinogen in the inflammatory-related model group, while ANGPTL2 did not have a significant association with hsCRP, excluding Model 1 in this model group. ANGPTL2 also had a significant positive association with LAB in the models, including LAB (Models 3 and 4) in this model group. In the metabolic-related model, ANGPTL2 did not have a significant association with HbA1c, BMI, triglycerides, or LDL-C, excluding one model with HbA1c (Model 1). ANGPTL2 showed either a significant negative association or a trend toward a negative association with eGFR in all models in both the inflammatory-related and metabolic-related models. We also investigated the correlation between ANGPTL2 and either fibrinogen or LAB in subgroups classified based on the duration of diabetes [Group 1: 0–5 years ( $n=33$ ), Group 2: >5 to 10 years ( $n=16$ ), and Group 3: >10 to 40 years ( $n=21$ )]. The number of patients treated with insulin in each of these three groups was 12 (36%), 8 (50%), and 15 (71%), respectively. There was a significant correlation between ANGPTL2 and fibrinogen in Group 1 ( $R=0.4493$ ,  $P=0.0087$ ) and in Group 3 ( $R=0.6709$ ,  $P=0.0017$ ), but not in Group 2 ( $R=0.3568$ ). ANGPTL2 was correlated with LAB only in Group 3 ( $R=0.4507$ ,

$P=0.0403$ ), while no significant correlation was observed in Group 1 ( $R=0.3347$ ) or Group 2 ( $R=0.1158$ ). There was a significant positive correlation between the duration of diabetes and the urinary albumin excretion (UAE) ( $R=0.4952$ ,  $P<0.0001$ ) and the CAVI ( $R=0.3513$ ,  $P=0.0038$ ).

## Discussion

In this study, the serum ANGPTL2 level was positively associated with hsCRP as an inflammatory marker, and these data are in agreement with previous studies.<sup>8,17</sup> These results appear plausible given the fact that ANGPTL2 can cause inflammation in both adipose tissues and systemic vascular endothelial cells.<sup>2,3,8</sup> On the one hand, this association was lost in the multiple regression analysis with the exception of one model in this study, suggesting that the association may be independent. On the other hand, ANGPTL2 was also positively correlated with fibrinogen, and the association was confirmed by the multiple regression analysis. Therefore, the association between ANGPTL2 and fibrinogen is more dependent than that shown between ANGPTL2 and hsCRP. Although fibrinogen is an important factor associated with the coagulation system, both fibrinogen and hsCRP are also representative inflammatory markers.<sup>29,30</sup> We also previously reported the close positive correlation between these markers.<sup>31</sup> Furthermore, a recent report suggests that baseline and long-term fibrinogen levels are associated with the risk of sudden cardiovascular death.<sup>32</sup> Interestingly, fibrinogen reportedly acts as a ligand for integrins (adhesion factors), including  $\alpha 5\beta 1$ , which are abundantly expressed in monocytes, macrophages, and endothelial cells. Furthermore, this interaction promotes leukocyte adhesion and the secretion of cytokines, resulting in inflammation.<sup>29,30</sup> However, it is likely that ANGPTL2 causes inflammation by



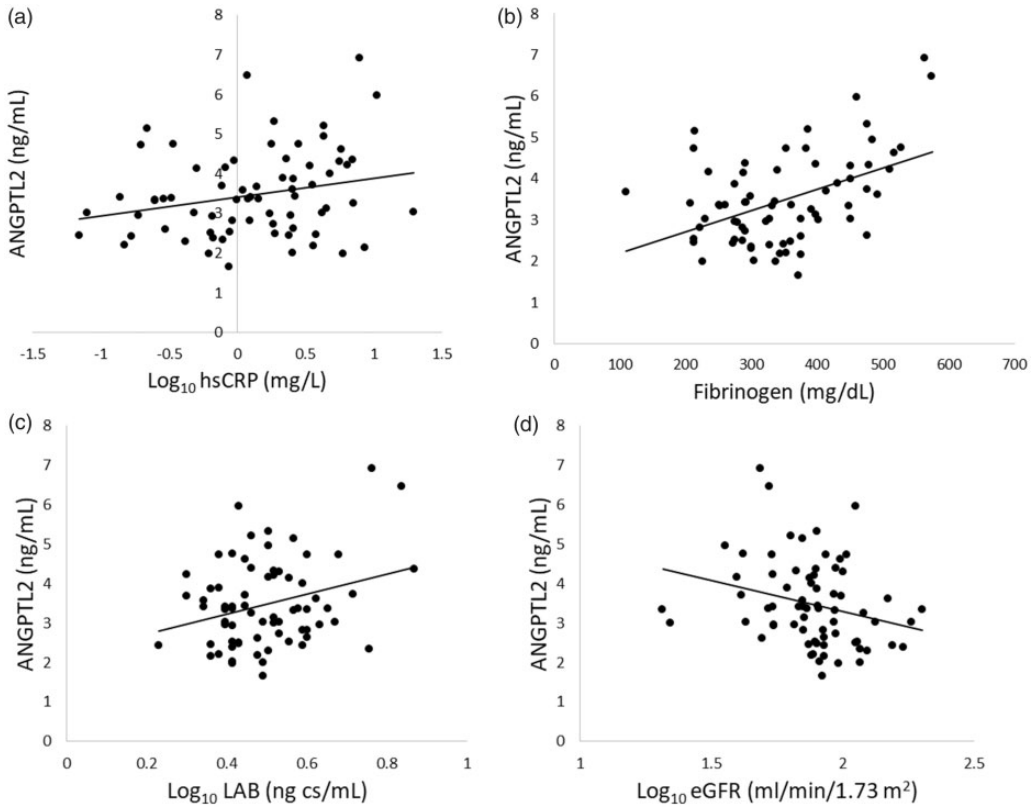
**Table 3.** Multiple regression analysis with ANGPTL2 as the dependent variable

Inflammatory Model			
Model	$\beta$	P	(R <sup>2</sup> )
Model 1			
eGFR (mL/min/1.73 m <sup>2</sup> )	-0.2444	0.0237*	(R <sup>2</sup> = 0.1273)
hsCRP (mg/L)	0.2643	0.0359*	
Model 2			
eGFR (mL/min/1.73 m <sup>2</sup> )	-0.1613	0.1564	(R <sup>2</sup> = 0.2378)
hsCRP (mg/L)	0.0099	0.9400	
Fibrinogen (mg/dL)	0.4186	0.0029*	
Model 3			
eGFR (mL/min/1.73 m <sup>2</sup> )	-0.2068	0.0681	(R <sup>2</sup> = 0.2895)
hsCRP (mg/L)	0.0095	0.9414	
Fibrinogen (mg/dL)	0.3648	0.0083*	
LAB (ng cs/mL)	0.2389	0.0333*	
Model 4			
eGFR (mL/min/1.73 m <sup>2</sup> )	-0.2397	0.0365*	(R <sup>2</sup> = 0.3157)
hsCRP (mg/L)	0.0012	0.9922	
Fibrinogen (mg/dL)	0.3434	0.0122*	
LAB (ng cs/mL)	0.2325	0.0363*	
HbA1c (%)	0.1655	0.1224	
Metabolic Model			
Model 1			
eGFR (mL/min/1.73 m <sup>2</sup> )	-0.2132	0.0727	(R <sup>2</sup> = 0.1117)
HbA1c (%)	0.2968	0.0134*	
Model 2			
eGFR (mL/min/1.73 m <sup>2</sup> )	-0.3023	0.0125*	(R <sup>2</sup> = 0.1177)
HbA1c (%)	0.2114	0.0764	
BMI (kg/m <sup>2</sup> )	0.0777	0.5050	
Model 3			
eGFR (mL/min/1.73 m <sup>2</sup> )	-0.2567	0.0433*	(R <sup>2</sup> = 0.0778)
HbA1c (%)	0.1339	0.2878	
BMI (kg/m <sup>2</sup> )	0.0462	0.7186	
TG (mg/dL)	0.0483	0.7111	
Model 4			
eGFR (mL/min/1.73 m <sup>2</sup> )	-0.2659	0.0399*	(R <sup>2</sup> = 0.0812)
HbA1c (%)	0.1210	0.3499	
BMI (kg/m <sup>2</sup> )	0.0400	0.7578	
TG (mg/dL)	0.0339	0.8011	
LDL-C (mg/dL)	0.0638	0.6303	

ANGPTL2 and fibrinogen followed the normal distribution confirmed by a  $\chi^2$  goodness-of-fit test and/or the Kolmogorov–Smirnov test. All variables except ANGPTL2, BMI, LDL-C, and fibrinogen were log<sub>10</sub>-transformed because of the skewed distribution. After log<sub>10</sub>-transforming, these variables followed the normal distribution.

\*Statistically significant (P < 0.05).

$\beta$ : standard partial regression coefficient, ANGPTL2: angiotensin-like protein 2, eGFR: estimated glomerular filtration rate, hsCRP: high-sensitivity C-reactive protein, LAB: lectin-like oxidized low-density lipoprotein receptor I ligand containing apolipoprotein B, HbA1c: hemoglobin A1c, BMI: body mass index, TG: triglycerides, LDL-C: low-density lipoprotein cholesterol



**Figure 2.** Correlation of ANGPTL2 with hsCRP, fibrinogen, LAB, and eGFR in patients with type 2 diabetes. ANGPTL2: angiopoietin-like protein 2, hsCRP: high-sensitivity C-reactive protein, LAB: lectin-like oxidized LDL receptor 1 ligand containing apolipoprotein B, eGFR: estimated glomerular filtration rate

activating the Ras-related C3 botulinus toxin substrate 1 (Rac1)/nuclear factor kappa-B (NF $\kappa$ B) pathway via  $\alpha$ 5 $\beta$ 1 integrin in vascular endothelial cells.<sup>8</sup> This appears to be initiated by the binding of the C-terminal fibrinogen-like domain of ANGPTL2 to  $\alpha$ 5 $\beta$ 1 integrin. In contrast, ANGPTL2 is highly expressed in both vascular endothelial cells and infiltrated macrophages within atherosclerotic vessels in humans, and this high expression may contribute to the elevation of ANGPTL2 in the circulation.<sup>9</sup> Taken together, these findings lead us to speculate that circulating fibrinogen as well as circulating ANGPTL2 itself can promote ANGPTL2 expression in vascular

endothelial cells by a similar mechanism, resulting in the elevation of circulating ANGPTL2. It is possible that systemic vascular inflammation induced by ANGPTL2 contributes to the up-regulation of fibrinogen expression in the liver, probably by cytokines produced in inflammatory cells, because recombinant ANGPTL2 can reportedly promote the expression of pro-inflammatory cytokines such as tumor necrosis factor  $\alpha$  and interleukin 6.<sup>14</sup> These mechanisms may partially explain the potentially close association between ANGPTL2 and fibrinogen found in this study. Another possible reason for this close association between ANGPTL2 and

fibrinogen levels may be based on the potential cross-reactivity between fibrinogen and the antibodies in the ANGPTL2 kit used in this study because ANGPTL2 has a C-terminal fibrinogen-like domain. However, both antibodies used in the sandwich ELISA have epitopes located in the coiled domain. In contrast, the fibrinogen-like domain does not possess epitopes for these antibodies, and these antibodies therefore do not react with the fibrinogen-like domain in the ANGPTL family. Thus, it is unlikely that the antibodies used in this kit can cross-react with fibrinogen.

This study is the first to reveal a significant positive association between the serum ANGPTL2 and LAB levels, which reflects oxidized and modified LDL.<sup>21</sup> The association was also confirmed by the multiple regression analysis. Although LDL-C is an important risk factor for cardiovascular events in patients with type 2 diabetes,<sup>18</sup> the role of oxidized and modified LDL in the formation of atheroma was recently confirmed.<sup>19</sup> Oxidized and modified LDL, but not LDL, can bind to LOX-1 in vascular endothelial cells<sup>20</sup> and cause local inflammation and consequent atheroma formation.<sup>19</sup> In fact, circulating LAB was positively associated with the CAVI, a marker of arterial stiffness, in men.<sup>33</sup> Furthermore, circulating LAB, but not sLOX-1, is reportedly associated with the risk of cardiovascular disease and ischemic stroke.<sup>34</sup> The reason for the potential positive association between ANGPTL2 and LAB confirmed in this study is not fully apparent. However, the LAB level is associated with lipid sedimentation,<sup>35</sup> inflammation,<sup>36</sup> and foam cell formation.<sup>37</sup> Therefore, we speculate that LAB may be able to promote the expression of ANGPTL2 by this mechanism, although this has not been directly demonstrated to date. Furthermore, because ANGPTL2 can induce oxidative stress,<sup>10</sup> circulating ANGPTL2 or ANGPTL2 expressed in

endothelial cells appears to be partially involved in the oxidization of LDL. Based on these hypotheses regarding the mechanisms involved, it might be important to evaluate whether the possible association between ANGPTL2 and oxidized and modified LDL as reflected by the LAB level is a risk factor for cardiovascular disease in future studies with larger numbers of patients.

Interestingly, the correlation between ANGPTL2 and either fibrinogen or LAB was stronger in the subgroup with the longest duration of diabetes than in patients with a shorter duration of diabetes. As diabetes progresses, patients may develop different complications and use different medications that probably affect the levels of biomarkers. In fact, the duration of diabetes was positively correlated with the UAE and the CAVI, which generally reflect diabetic nephropathy and the degree of arteriosclerosis, respectively; additionally, the number of patients on insulin therapy was highest in the subgroup with the longest duration of diabetes. This may have partially influenced the results.

This study showed a negative correlation between the ANGPTL2 level and the eGFR. A trend toward a positive correlation between the ANGPTL2 level and the UAE was also noted. These findings, which support the results of a previous study,<sup>38</sup> suggest a close association between ANGPTL2 and the progression of diabetic nephropathy. A possible reason for these results is decreased clearance of ANGPTL2 by the kidney. However, the expression of ANGPTL2 in the glomeruli of patients with diabetes is up-regulated.<sup>39</sup> In addition, ANGPTL2 may promote fibrosis in the kidney because of the increased transforming growth factor  $\beta$  via  $\alpha 5 \beta 1$  integrin/extracellular signal-regulated kinase.<sup>40</sup> Therefore, the association between ANGPTL2 and renal dysfunction observed

in this study may be partially independent of the renal clearance of ANGPTL2.

In the current study, the ANGPTL2 level showed a significant positive association with HbA1c in the multiple regression model with eGFR and HbA1c as independent variables. However, because the ANGPTL2 level did not have a significant association with the HbA1c level in either the correlation analysis or the other models of the multiple regression analyses, the ANGPTL2 level does not appear to have been strongly influenced by the HbA1c level. This result is basically in agreement with previous reports,<sup>16,38</sup> although one study showed a significant positive association between the ANGPTL2 and HbA1c levels.<sup>17</sup> However, the ANGPTL2 level was positively correlated with HOMA-IR, which reflects insulin resistance, in patients who did not receive insulin therapy. This result is in agreement with those in previous reports,<sup>8,17</sup> and it appears to be a reasonable finding because ANGPTL2 is produced mainly in visceral adipose tissues and can cause inflammation, resulting in systemic increased insulin resistance.<sup>8</sup>

In the current study, the ANGPTL2 level in patients with type 2 diabetes was significantly higher than that in control subjects without diabetes, which is in agreement with a previous report.<sup>8</sup> It is difficult to explain the detailed reason for this result because basically, ANGPTL2 did not have a significant association with glycemic control, BMI, or age. In addition, there were no differences in BMI, age, or eGFR between these groups. Plausible explanations might include the potential differences in insulin resistance and low-grade inflammation between these groups. However, we did not measure the serum insulin levels or inflammatory markers in the healthy subjects.

This study has some limitations. It was designed as a cross-sectional observation study. The number of patients with diabetes

was relatively small. This may have been especially important in the correlation between ANGPTL2 and either eGFR or LAB because the correlation coefficients were relatively low, although they were statistically significant. Furthermore, the number of control subjects was very small compared with the number of patients with diabetes. This prevented us from investigating the correlation between ANGPTL2 and various markers in control subjects and resulted in difficulty comparing the differences in these correlations between the patient and control groups. In addition, the patients took multiple different drugs for diabetes, which may have influenced the circulating ANGPTL2 level. Finally, cardiovascular events were not assessed in this study; thus, we cannot conclude that ANGPTL2 is a risk factor.

In conclusion, the ANGPTL2 level was significantly higher in patients with type 2 diabetes than in healthy control subjects without diabetes. ANGPTL2 had a significant positive association with inflammatory markers evaluated by hsCRP, fibrinogen, and LAB, which reflects oxidized and modified LDL. There was a significant negative association between the serum ANGPTL2 level and eGFR. Conversely, ANGPTL2 was not basically influenced by glycemic control based on the HbA1c or FPG or influenced by the BMI. Importantly, the clinical significance of the potentially close association between ANGPTL2 and either LAB or fibrinogen should be evaluated in a more detailed analysis.

#### **Declaration of conflicting interest**

The authors declare that there is no conflict of interest.

#### **Funding**

This research received no specific grant from any funding in the public, commercial, or not-for-profit sectors.

## References

1. Kim I, Moon SO, Koh KN, et al. Molecular cloning, expression, and characterization of angiotensin-related protein. angiotensin-related protein induces endothelial cell sprouting. *J Biol Chem* 1999; 274: 26523–26528.
2. Kadomatsu T, Tabata M and Oike Y. Angiotensin-like proteins: emerging targets for treatment of obesity and related metabolic diseases. *FEBS J* 2011 278: 559–564.
3. Thorin-Trescases N and Thorin E. High Circulating Levels of ANGPTL2: Beyond a Clinical Marker of Systemic Inflammation. *Oxid Med Cell Longev* 2017; 2017: 1096385.
4. Ono M, Shimizugawa T, Shimamura M, et al. Protein region important for regulation of lipid metabolism in angiotensin-like 3 (ANGPTL3): ANGPTL3 is cleaved and activated in vivo. *J Biol Chem* 2003; 278: 41804–41809.
5. Ge H, Cha JY, Gopal H, et al. Differential regulation and properties of angiotensin-like proteins 3 and 4. *J Lipid Res* 2005; 46: 1484–1490.
6. Oike Y, Akao M, Yasunaga K, et al. Angiotensin-related growth factor antagonizes obesity and insulin resistance. *Nat Med* 2005; 11: 400–408.
7. Quagliarini F, Wang Y, Kozlitina J, et al. Atypical angiotensin-like protein that regulates ANGPTL3. *Proc Natl Acad Sci USA* 2012; 109: 19751–19756.
8. Tabata M, Kadomatsu T, Fukuhara S, et al. Angiotensin-like protein 2 promotes chronic adipose tissue inflammation and obesity-related systemic insulin resistance. *Cell Metab* 2009; 10: 178–188.
9. Horio E, Kadomatsu T, Miyata K, et al. Role of endothelial cell-derived angptl2 in vascular inflammation leading to endothelial dysfunction and atherosclerosis progression. *Arterioscler Thromb Vasc Biol* 2014; 34: 790–800.
10. Aoi J, Endo M, Kadomatsu T, et al. Angiotensin-like protein 2 accelerates carcinogenesis by activating chronic inflammation and oxidative stress. *Mol Cancer Res* 2014; 12: 239–249.
11. Endo M, Nakano M, Kadomatsu T, et al. Tumor cell-derived angiotensin-like protein ANGPTL2 is a critical driver of metastasis. *Cancer Res* 2012; 72: 1784–1794.
12. Tian Z, Miyata K, Kadomatsu T, et al. ANGPTL2 activity in cardiac pathologies accelerates heart failure by perturbing cardiac function and energy metabolism. *Nat Commun* 2016; 7: 13016.
13. Tazume H, Miyata K, Tian Z, et al. Macrophage-derived angiotensin-like protein 2 accelerates development of abdominal aortic aneurysm. *Arterioscler Thromb Vasc Biol* 2012; 32: 1400–1409.
14. Farhat N, Thorin-Trescases N, Mamarbachi M, et al. Angiotensin-like 2 promotes atherogenesis in mice. *J Am Heart Assoc* 2013; 2: e000201.
15. Doi Y, Ninomiya T, Hirakawa Y, et al. Angiotensin-like protein 2 and risk of type 2 diabetes in a general Japanese population: the Hisayama study. *Diabetes Care* 2013; 36: 98–100.
16. Gellen B, Thorin-Trescases N, Sosner P, et al. ANGPTL2 is associated with an increased risk of cardiovascular events and death in diabetic patients. *Diabetologia* 2016; 59: 2321–2330.
17. Jung CH, Lee WJ, Lee MJ, et al. Association of serum angiotensin-like protein 2 with carotid intima-media thickness in subjects with type 2 diabetes. *Cardiovasc Diabetol* 2015; 14: 35.
18. Turner RC, Millns H, Neil HA, et al. Risk factors for coronary artery disease in non-insulin dependent diabetes mellitus: United Kingdom Prospective Diabetes Study (UKPDS: 23). *BMJ* 1998; 316: 823–828.
19. Stocker R and Kearney JF Jr. Role of oxidative modifications in atherosclerosis. *Physiol Rev* 2004; 84: 1381–1478.
20. Sawamura T, Kume N, Aoyama T, et al. An endothelial receptor for oxidized low-density lipoprotein. *Nature* 1997; 386: 73–77.
21. Sato Y, Nishimichi N, Nakano A, et al. Determination of LOX-1-ligand activity in mouse plasma with a chicken monoclonal antibody for ApoB. *Atherosclerosis* 2008; 200: 303–309.
22. Yamazaki K, Bujo H, Taira K, et al. Increased circulating malondialdehyde-modified LDL in the patients with familial combined hyperlipidemia and its relation

- with the hepatic lipase activity. *Atherosclerosis* 2004; 172: 181–187.
23. Itabe H and Ueda M. Measurement of plasma oxidized low-density lipoprotein and its clinical implications. *J Atheroscler Thromb* 2007; 14: 1–11.
  24. UMIN-CTR clinical trial. [https://upload.umin.ac.jp/cgi-open-bin/ctr\\_e/ctr\\_view.cgi?recptno=R000029642](https://upload.umin.ac.jp/cgi-open-bin/ctr_e/ctr_view.cgi?recptno=R000029642)
  25. Product insert: Immuno-Biological Laboratories, Co, Ltd. #27745 Human ANGPTL2 Assay Kit - IBL.
  26. Matthews DR, Hosker JP, Rudenski AS, et al. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985; 28: 412–419.
  27. Matsuo S, Imai E, Horio M, Yasuda Y, et al. Revised equations for estimated GFR from serum creatinine in Japan. *Am J Kidney Dis* 2009; 53: 982–992.
  28. Davis MD. Natural evolution. In: FA L'Esperance Jr (ed) *Current diagnosis and management of chorioretinal disease*. St Louis, MO: Mosby, 1994, pp 179–184.
  29. Herrick S, Blanc-Brude O, Gray A, et al. Fibrinogen. *Int J Biochem Cell Biol* 1999; 31: 741–746.
  30. Mosesson MW. Fibrinogen and fibrin structure and functions. *J Thromb Haemost* 2005; 3: 1894–1904.
  31. Takebayashi K, Suetsugu M, Matsutomo R, et al. Correlation of high-sensitivity C-reactive protein and plasma fibrinogen with individual complications in patients with type 2 diabetes. *South Med J* 2006; 99: 23–27.
  32. Kunutsor SK, Kurl S, Zaccardi F, et al. Baseline and long-term fibrinogen levels and risk of sudden cardiac death: a new prospective study and meta-analysis. *Atherosclerosis* 2016; 245: 171–180.
  33. Sugiyama D, Higashiyama A, Wakabayashi I, et al. The relationship between Lectin-like oxidized low-density lipoprotein receptor-1 ligands containing apolipoprotein b and the cardio-ankle vascular index in healthy community inhabitants: the KOBE study. *J Atheroscler Thromb* 2015; 22: 499–508.
  34. Inoue N, Okamura T, Kokubo Y, et al. LOX index, a novel predictive biochemical marker for coronary heart disease and stroke. *Clin Chem* 2010; 56: 550–558.
  35. Sawamura T, Kakino A, Fujita Y. LOX-1: a multiligand receptor at the crossroads of response to danger signals. *Curr Opin Lipidol* 2012; 23: 439–445.
  36. Honjo M, Nakamura K, Yamashiro K, et al. Lectin-like oxidized LDL receptor-1 is a cell-adhesion molecule involved in endotoxin-induced inflammation. *Proc Natl Acad Sci U S A* 2003; 100: 1274–1279.
  37. Li L, Sawamura T and Renier G. Glucose enhances human macrophage LOX-1 expression: role for LOX-1 in glucose-induced macrophage foam cell formation. *Circ Res* 2004; 94: 892–901.
  38. Li Q, Gong W, Yang Z, Lu B, et al. Serum Angptl2 levels are independently associated with albuminuria in type 2 diabetes. *Diabetes Res Clin Pract* 2013; 100: 385–390.
  39. Sun H, Zheng JM, Chen S, et al. Enhanced expression of ANGPTL2 in the microvascular lesions of diabetic glomerulopathy. *Nephron Exp Nephrol* 2007; 105:e117–e123.
  40. Morinaga J, Kadomatsu T, Miyata K, et al. Angiopietin-like protein 2 increases renal fibrosis by accelerating transforming growth factor- $\beta$  signaling in chronic kidney disease. *Kidney Int* 2016; 89: 327–341.