Correlation between albuminuria and spontaneous platelet micro-aggregate formation in type 2 diabetic patients

Running title: Activated platelet profile and albuminuria

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**Objective.** Albuminuria in type 2 diabetic patients is a risk factor for cardiovascular disease. We investigated the correlation between albuminuria and spontaneous micro-aggregation of platelets (SMAP) formed under shear stress.

**Research Design and Methods.** The study subjects were 401 type 2 diabetics (252 with normoalbuminuria, 149 with albuminuria) who were examined for SMAP under conditions of shear stress only (no agonist stimulation) and the reversibility of platelet micro-aggregation after stimulation with 1 µM adenosine diphosphate (ADP), measured by a laser-light scattering method. Active GPIIb/IIIa and P-selectin expression levels on platelets as index of platelet activation were measured by whole-blood flow cytometry.

**Results.** SMAP formation was noted in 53% of diabetic patients. All patients with SMAP showed irreversible pattern of platelet micro-aggregates by a low dose of ADP. SMAP was observed in 75% of diabetics with albuminuria, and in 39% of those with normoalbuminuria. Multivariate logistic regression analysis identified urinary albumin excretion rate and brachial-ankle pulse wave velocity as independent factors associated with SMAP. The degree of SMAP correlated with active GPIIb/IIIa (γ=0.59, P<0.001) and P-selectin (γ=0.55, P<0.001) expression levels. These early-activated platelet profiles were significantly inhibited in albuminuric patients with aspirin intake, although the effect was incomplete.

**Conclusions.** Our study demonstrated independent association between albuminuria and early changes in activated platelet profiles of type 2 diabetic patients. Further follow-up and intervention studies are needed to establish whether the inhibition of SMAP affects the course of cardiovascular disease in type 2 diabetic patients.
There is growing evidence that increased urinary albumin excretion rate (AER) in type 2 diabetic patients is both a predictor of progression to chronic renal failure and an independent risk factor for cardiovascular disease (1). The United Kingdom Prospective Diabetes Study (UKPDS) group demonstrated that type 2 diabetic patients with microalbuminuria or overt proteinuria have a 2-3-fold greater risk of cardiovascular death, compared to those patients with normoalbuminuria (2). Furthermore, our group and others recently reported that a reduction of AER in type 2 diabetic patients was associated with a decreased occurrence of cardiovascular complications (3, 4). Thus, albuminuria is considered as an important therapeutic target for cardiovascular protection. However, the pathophysiological factors underlying this cardio-renal interaction remain unknown. Identifying such factors and developing tools to identify patients at higher risk of both conditions might allow the design of new therapeutic strategies to improve outcomes in type 2 diabetic patients.

Platelet activation and aggregation are fundamental processes in the development of atherosclerosis and thrombosis, both of which contribute to cardiovascular risk (5, 6). Platelets aggregate when activated, to form micro-aggregates of only a few cells as the initial response to various stimuli but large, tighter platelet aggregates appear with prolonged stimulation (7). In diabetic patients, platelets tend to hyperaggregate (8) although previous platelet aggregation measurements by optical density (9) or impedance methods (10) generally reflect the formation of large platelet aggregates in response to exogenous stimulation with various agonists. These conventional methods do not measure platelet micro-aggregate formation, which occurs at the initial process of platelet activation. Recently, a particle-counting method based on laser light scattering was developed to effectively detect platelet micro-aggregate formation (11). This new method also measures spontaneous micro-aggregation of platelets (SMAP), which occurs with a stirring force only and has no requirement for exogenous agonist stimulation. Using this new method, Matsuno et al. (12) reported SMAP as the predominant platelet aggregation event in type 2 diabetic patients. However, the above study did not address the association between SMAP and albuminuria.

We therefore investigated the association between changes in early-activated platelet profiles and increased albuminuria in type 2 diabetic patients. The incidence of SMAP was investigated in relation to albuminuria status. To further evaluate the active state of SMAP, we simultaneously measured the cell surface-expression levels of two markers of platelet activation: active fibrinogen receptor glycoprotein IIb/IIIa (GPIIb/IIIa), an important player in platelet adhesion, and P-selectin (CD62p), a transmembrane protein present in the α- or dense granules.

METHODS

Subject recruitment. The diabetic subjects were recruited from type 2 diabetic patients who regularly visited the outpatient clinic of the Department of Medicine, Shiga University of Medical Science, in 2006-2007. Patients were clinically diagnosed with type 2 diabetes mellitus in accordance with the criteria of the World Health Organization. Patients were excluded based on the following criteria: complicating cancer, liver disease, infectious disease, collagen disease, non-diabetic kidney disease confirmed by renal biopsy, intake of non-steroidal anti-inflammatory drugs within the previous 2 weeks, and intake of anti-platelet drugs with the exception of aspirin. Eligible patients were informed of the study protocol in oral
and written forms. A total of 401 patients were finally enrolled in this study. Thirty healthy volunteers were also enrolled as the healthy control group to confirm the results of our previous study [male/female: 17/13, age: 60±7 years, body mass index (BMI): 23.1±2.9 kg/m², systolic blood pressure (BP): 131±9 mmHg, total cholesterol: 199±20 mg/dl, triglycerides: 109.8±26.4 mg/dl (mean ± SD), no medication]. Each individual provided a blood sample for biochemical analysis and detection of platelet micro-aggregation under fasting conditions, underwent standard physical examinations, and brachial-ankle pulse wave velocity (baPWV) was measured. AER was determined by immunoturbidimetry assay (HITACHI 7070E; Hitachi High-Technologies Co, Tokyo, Japan) in a single 24-hour urine sample collected on the same day as taking a blood sample. An AER <20 µg/min was considered normoalbuminuria, while that exceeding 20 was classed as albuminuria. The group with albuminuria comprised 120 patients with microalbuminuria (20 µg/min ≤ AER < 200 µg/min) and 29 patients with overt proteinuria (200 µg/min ≤ AER). baPWV was measured by an automatic device (BP-203RPE; Colin, Komaki, Japan). The estimated glomerular filtration rate (eGFR) was calculated using the simplified prediction equation proposed by the Japanese Society of Nephrology: eGFR (ml/min/1.73 m²) = 194 × [age (years)]^{-0.287} × [serum creatinine (mg/dl)]^{-1.094} × 0.739 (if female).

The study protocol and informed consent procedure were approved by the Ethics Committee of Shiga University of Medical Science. All participants provided written informed consent.

Detection of platelet micro-aggregation. Platelet-rich plasma (PRP) was obtained from blood collected into sodium citrate (14 µM) by immediate centrifugation at 1,750 x g for 10 min at room temperature in the presence of prostaglandin I₂ (20 µM). The resultant platelet was washed with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-Tyrode’s buffer, and resuspended to 4 × 10⁸ cells/ml. In this platelet suspension, platelets did not form the micro-aggregates without stirring force or any exogenous agonists. Platelet micro-aggregation was determined by measuring the light scattering (LS) intensity (13) on a PA-200 aggregometer (Kowa Co, Tokyo). SMAP was observed under low shear-stress conditions at a stirring speed of 1,000 rpm (26 dyn/cm²) without stimulation with an exogenous agonist. This degree of low-shear stress is considered as the degree same as shear stress occurring by arterial blood stream, suggesting that this system provides the condition which mimicked the state of platelets in the artery. The reversibility of platelet micro-aggregation in response to a low dose of adenosine diphosphate (ADP) (1 µM) was also investigated. The data were recorded as a two-dimensional graph showing the change in total light intensity over time, expressed as a cumulative summation at 10-second intervals of scattered light intensity (Ii) and the number of particles corresponding to that intensity (Ni) in terms of particle size (intensity) (ΣIiNi) (volt x count per sec). Particles with an intensity of 25 to 400 mV represented small aggregates (9-25 µm). The degree of SMAP was described by measurement of the area under curve (AUC) of each detection line for 5 minutes. AUC data were expressed as x10⁵ particles. Each measurement was performed twice. All individuals showed identical results as to whether SMAP was formed. The mean values of two AUC data as the degree of SMAP in each individual were used for the analysis to minimize the variability.
Quantification of GPIIb/IIIa and P-selectin expression levels. The platelet surface expression levels of GPIIb/IIIa and P-selectin were assessed by whole-blood flow cytometry using a fluorescein isothiocyanate (FITC)-conjugated PAC-1 antibody and a phycoerythrin (PE)-conjugated CD62p antibody, as described previously (14, 15). In brief, a saturating antibody concentration in 50 µl was added to 50 µl of whole blood. Negative control samples contained prepared IgG and IgM. The samples were incubated for 15 min at room temperature and then analyzed within 2 hours by flow cytometry. The platelets were identified based on particle size and complexity using a peridinin-chlorophyll (PerCP)-conjugated CD61 antibody. Fluorescence data from 10,000 platelet events were collected. The expression levels were expressed as a percentage of the positively identified platelets. All antibodies were purchased from Becton Dickinson (San Jose, CA).

Statistical analysis. Categorical variables were compared between two groups using χ² tests, while unpaired Student’s t test was used for normally distributed variables, and the Mann-Whitney U test for non-normally distributed variables. For the non-parametric comparison among four groups, Kruskal Wallis test was first performed to find a measure of the aggregate degree to which the group means differed and then the Mann-Whitney U test with Bonferroni correction were used to compare the differences between groups. Spearman’s correlation coefficient was used to analyze the association between two variables. A multivariate logistic regression model was applied to evaluate the independency of factors that were significantly different between the groups, using logarithmic transformed values of non-normally distributed variables. All data were analyzed using SPSS software package (version 11; SPSS Inc., Chicago, IL). A P value <0.05 was considered statistically significant.

RESULTS
Platelet micro-aggregation in diabetic patients. SMAP was detected in 211 (52.6%) out of 401 patients with type 2 diabetes mellitus (Fig. 1A), but not in any of the 30 healthy volunteers tested here (Fig. 1B) as well as our previous study (12). The diabetic patients with SMAP showed an abnormal and irreversible pattern of platelet micro-aggregation even after stimulation by a low dose of ADP (Fig. 1C), while a normal reversible pattern was observed in patients without SMAP and in all healthy volunteers (Fig. 1D). Table 1 provides details of the clinical characteristics of the diabetic subgroups according to the formation of SMAP. Waist to hip ratios, the frequency of taking RAS inhibitors, urinary AER, eGFR, and baPWV were significantly different between diabetic patients with SMAP and those without such events. Multiple logistic regression analysis including these factors identified the levels of AER [OR = 3.4 (95% CI: 2.2-5.3), P<0.001] and baPWV [OR = 15.3 (95% CI: 1.1-227.5), P<0.05] as independent factors associated with SMAP detection.

Correlation between platelet micro-aggregation and albuminuria. Next, the frequency and degree of SMAP were analyzed according to albuminuria status (>20 µg/min). The frequency of the formation of SMAP in diabetic patients with albuminuria was significantly higher than those with normoalbuminuria (75% of 149 patients with albuminuria vs. 39% of 252 patients with normoalbuminuria, P<0.001). Similarly, the degree of SMAP evaluated by AUC was significantly higher in those with albuminuria than those with normoalbuminuria (0.52 [interquartile range: 0.01-1.46] vs. 0.00 [0.00-0.16], P<0.001). Also, the degree of SMAP was significantly correlated with the
levels of urinary AER as continuous variables (Spearman’s $\gamma = 0.43$, $P<0.001$). Regardless the status of albuminuria, the platelet micro-aggregates in all patients with SMAP showed the irreversible pattern after stimulation by a low dose of ADP and those in the patients without SMAP were reversible.

**Effect of aspirin medication on platelet micro-aggregation.** We evaluated the effect of aspirin medication on the frequency and degree of SMAP. Of the diabetic patients, 163 (41%) were prescribed 100 mg/day aspirin. Patients with albuminuria and on aspirin intake had a significantly lower frequency of SMAP than those without it (50 of 76 patients taking aspirin vs. 62 of 73 patients not given aspirin), while the SMAP rates were similar in the two subgroups in patients with normoalbuminuria (37 of 87 patients taking aspirin vs. 62 of 165 patients without aspirin intake). The degree of SMAP among the patients with albuminuria was similarly significantly lower with than without aspirin (0.33 [0.00-1.27] vs. 0.88 [0.17-1.76]), while patients with normoalbuminuria showed no such difference according to aspirin intake (0.00 [0.00-0.14] vs. 0.00 [0.00-0.21]; Fig. 1). In addition, the strong correlation between the degree of SMAP and the levels of urinary AER as continuous variables was investigated in the patients without aspirin intake ($\gamma = 0.53$, $P<0.001$), while the weak association between them was found in those with aspirin intake ($\gamma = 0.27$, $P<0.001$).

**Correlation between platelet micro-aggregation and GPIIb/IIIa and P-selectin expression.** To further evaluate the active state of SMAP, we quantitated the expression levels of active GPIIb/IIIa and P-selectin on the platelets. The expression levels of active GPIIb/IIIa were higher in diabetic patients with SMAP than in those without SMAP [29.9% (interquartile range: 19.3-42.2) vs. 15.5% (11.7-21.9)]. Similarly, the expression levels of P-selectin were higher in diabetic patients with detectable SMAP than those without it [8.1% (5.1-16.0) vs. 4.4% (3.2-5.6)]. The expression levels of both markers correlated significantly with the degree of SMAP (Spearman’s $\gamma = 0.59$ for active GPIIb/IIIa and $\gamma = 0.55$ for P-selectin).

Finally, we investigated the relationship between the expression levels of these surface markers and urinary AER in the subgroups based on aspirin intake. In patients not taking aspirin, the expression level of each surface marker correlated significantly with urinary AER (Spearman’s $\gamma = 0.60$ for active GPIIb/IIIa and $\gamma = 0.57$ for P-selectin, Fig. 2A and 2C). Similar correlations were also observed in patients taking aspirin, albeit with a slightly weaker coefficient of correlation (Spearman’s $\gamma = 0.51$, for active GPIIb/IIIa and $\gamma = 0.34$ for P-selectin, Fig. 2B and 2D).

**DISCUSSION**

The present study provided new evidence that both increased AER and baPWV are independent factors associated with the abnormal formation of SMAP in type 2 diabetic patients. Furthermore, the patients with SMAP showed an irreversible pattern of platelet micro-aggregation by ADP and the degree of SMAP correlated with the enhanced expression of active GP IIb/IIIa and P-selectin, indicating that SMAP is pathophysiologically active. These early-activated platelet profiles were significantly inhibited in albuminuric patients with aspirin intake, although the effect was incomplete.

Assessment of inappropriate platelet activation is one way to risk-stratify patients who are at high risk of atherosclerosis and atherothrombosis. The micro-aggregates produced in the early phase of platelet activation are now considered to potentially aggravate thrombus formation (7). Under normal conditions, these platelet micro-aggregates dissolve within a few
minutes, as shown in this study (Fig. 1D). In the present study, 53% of type 2 diabetic patients showed SMAP and abnormal reversibility of the micro-aggregation following low-dose ADP. This high proportion of diabetic patients with abnormal platelet hyperaggregability is in agreement with a previously studied cohort (12). The SMAP formation was observed under a low-shear stress which mimicked the state of arterial blood stream without the stimulation of exogenous agonists. Also, the degree of SMAP was associated with enhanced expression of active GPIIb/IIIa and P-selectin. Thus, the formation of SMAP is considered to show the abnormal activated state of platelets in the diabetic patients, which is enhanced according to the increase of albuminuria.

The present study associated SMAP occurrence with both albuminuria and baPWV. In agreement with these results, other investigators reported that high levels of platelet micro-aggregation were associated with adverse outcomes in patients with cardiovascular disease (16) and ankle brachial index in patients with peripheral arterial disease (17). In addition, diabetic patients with SMAP showed overexpression of platelet surface markers, active GPIIb/IIIa and P-selectin. Such upregulation of these molecules has also been associated with the development of atherogenesis (18). Taken together, the early platelet activation in type 2 diabetic patients may be a risk factor for cardiovascular disease.

A recent guideline of the American Diabetes Association recommends prophylactic use of antithrombotic agents, with low-dose aspirin, for diabetics, especially those with albuminuria (19). However, Di Minno et al. (20) indicated that aspirin alone is insufficient to prevent thrombosis in diabetic patients with angiopathy. In the present study, the SMAP formation was inhibited in albuminuric patients taking aspirin compared to those not taking it. However, the SMAP formation was still associated with albuminuria, even in patients taking aspirin, suggesting that the inhibitory effect of aspirin may be insufficient in diabetic patients with albuminuria. Further follow-up and intervention studies are required to investigate whether the incomplete inhibition of early-activated platelet profiles is a cardiovascular risk.

Several plausible mechanisms underlying the cardio-renal interaction have been proposed. Deckert et al. (21) proposed in the Steno hypothesis that excess leakage of albumin into urine reflects widespread vascular (endothelial) damage. This endothelial dysfunction is considered a common feature of cardio-renal interactions (22); it leads to platelet activation, adhesion, and subsequent platelet aggregate formation. Platelets are also a rich source of chemokines and cytokines, released within seconds of platelet activation (23). Thus, the formation of SMAP in diabetic patients with albuminuria might either cause or reflect systemic vascular endothelial dysfunction. An alternative explanation is that any pathophysiological alteration in diabetic kidney could directly affect platelet activation. Glomerular hypertension, which induces mechanical shear stress (24), and excess renal production of type IV collagen in the kidney (25), a powerful activator of platelets, might induce activation of platelets that circulate into the kidney.

The present study had some limitations. It was not possible to ascertain whether the early-activated platelet profile was a cause or consequence of the increased urinary albumin excretion due to the nature of the cross-sectional study. The present study also could not address whether the incidence of future cardiovascular disease is higher in patients with SMAP and whether the inhibitory effect of aspirin on the degree of SMAP would be sufficient to prevent the development of cardiovascular disease.
In conclusion, the majority of type 2 diabetic patients with albuminuria showed an altered profile of early platelet activation including SMAP events. Given the growing concern over cardiovascular consequences in type 2 diabetic patients, further follow-up and intervention studies are needed to establish whether the inhibition of SMAP is a therapeutic target to prevent cardiovascular complications in type 2 diabetic patients.

Acknowledgments
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Disclosure: The authors have no conflict of interest to declare.
References

Table 1. Clinical characteristics of the study subjects according to the formation of SMAP.

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<th>Diabetic patients without SMAP</th>
<th>Diabetic patients with SMAP</th>
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<tbody>
<tr>
<td>Number</td>
<td>190</td>
<td>211</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>110/80</td>
<td>119/92</td>
</tr>
<tr>
<td>Age (year)</td>
<td>64 ± 11</td>
<td>65 ± 9</td>
</tr>
<tr>
<td>Duration of diabetes (year)</td>
<td>17 ± 11</td>
<td>16 ± 9</td>
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<tr>
<td>Body mass index (kg/m²)</td>
<td>24.6 ± 4.3</td>
<td>24.7 ± 3.8</td>
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<tr>
<td>Waist to hip ratio</td>
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<td>0.95 ± 0.08 *</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.2 ± 0.8</td>
<td>7.2 ± 1.0</td>
</tr>
<tr>
<td>Diabetes treatment (%) (diet/oral agents/insulin)</td>
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<td>7/49/44</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>133 ± 17</td>
<td>134 ± 14</td>
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<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>73 ± 10</td>
<td>72 ± 10</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>66</td>
<td>74</td>
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<tr>
<td>Taking RAS inhibitors (%)</td>
<td>41</td>
<td>59 *</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
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<td>197 ± 29</td>
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<tr>
<td>Triglycerides (mg/dl)</td>
<td>86 (61-116)</td>
<td>92 (62-134)</td>
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<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>54 (46-65)</td>
<td>52 (45-62)</td>
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<tr>
<td>Taking statins (%)</td>
<td>52</td>
<td>48</td>
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<tr>
<td>Urinary albumin excretion rate (µg/min)</td>
<td>9 (6-16)</td>
<td>23 (8-87) †</td>
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<tr>
<td>Estimated GFR (ml/min/1.73m²)</td>
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<td>70 ± 20 *</td>
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<tr>
<td>baPWV (cm/sec)</td>
<td>1606 (1416-1800)</td>
<td>1726 (1521-1987) †</td>
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Data are expressed as mean ± SD for normally distributed continuous variables or median (25th-75th interquartiles) for skewed continuous variables.

*P<0.05, †P<0.01 vs. diabetic subjects without spontaneous micro-aggregation of platelets (SMAP).

RAS: renin-angiotensin system, GFR: glomerular filtration rate, baPWV: brachial-ankle pulse wave velocity
Activated platelet profile and albuminuria

Figure legends

Figure 1: Typical patterns of SMAP and reversibility of platelet micro-aggregation by low-dose ADP, measured by a laser-scattering system. The spontaneous formation of micro-aggregated platelets under low shear stress alone without any exogenous agonists was observed in 52.6% of diabetic patients (A), while it was not observed in 47.4% of diabetic patients and all healthy volunteers (B). Diabetic patients with SMAP showed irreversible platelet micro-aggregation in response to ADP (1 µM) (C), while the others showed the typical reversible pattern of platelet micro-aggregation within five minutes (D).

Figure 2: Box-and-whisker plots of SMAP assessed by AUC in diabetic patients with normoalbuminuria and albuminuria, treated with or without aspirin. In these plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively; and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. Kruskal Wallis test: $P<0.001$, *$P<0.001$ vs. normoalbuminuric patients treated with and without aspirin, †$P=0.01$ vs. albuminuric patients without aspirin (Mann-Whitney U test).

Figure 3: Correlation between urinary albumin excretion rate and expression of platelet surface markers, active GPIIb/IIIa and P-selectin. The patients were divided into two subgroups based on aspirin intake: for active GPIIb/IIIa, those not taking aspirin (A) ($\gamma=0.60$, $P<0.001$) and those taking aspirin (B) ($\gamma=0.51$, $P<0.001$), and for P-selectin, those not taking aspirin (C) ($\gamma=0.57$, $P<0.001$) and those administered aspirin (D) ($\gamma=0.34$, $P<0.001$). Log-transformed values were plotted on each figure.
Activated platelet profile and albuminuria

Figure 2.

![Box plots showing AUC of the formation of SMAP](image)

<table>
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<tr>
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<th>Normoalbuminuria</th>
<th>Albuminuria</th>
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<tbody>
<tr>
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<td>-</td>
<td>+</td>
</tr>
<tr>
<td>-</td>
<td>165</td>
<td>73</td>
</tr>
<tr>
<td>+</td>
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Figure 3.

![Scatter plots](image)